# BIOACTIVE XYLOOLIGOSACCHARIDES FROM CORNCOB: ENZYMATIC PRODUCTION AND APPLICATIONS

A thesis submitted to the
UNIVERSITY OF MYSORE


In fulfillment of the requirements for the award of
DOCTOR OF PHILOSOPHY in BIOTECHNOLOGY by

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Under the supervision of
Dr. (Mrs.) S. G. Prapulla
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Mysore- 570 020, India
APRIL 2009

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## DECLARATION

I hereby declare that the thesis entitled "**BIOACTIVE XYLOOLIGOSACCHARIDES FROM CORNCOB: ENZYMATIC PRODUCTION AND APPLICATIONS"** submitted to the **University of Mysore,** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY,** is the result of the research work carried out by me under the guidance of **Dr. (Mrs.) S. G. Prapulla**, Scientist F, Department of Fermentation Technology & Bioengineering, Central Food Technological Research Institute, Mysore- 570 020, India, during the period 2004-2009.

I further declare that the results presented in this thesis have not been submitted for the award of any other degree.



Date: Ayyappan Appukuttan Aachary

Place: Mysore (Candidate)

## ABSTRACT

The use of foods that promote a state of wellbeing, better health and reduction of the risk of diseases have become popular as the consumer is becoming more and more health conscious. In this regard, a lot of attention has been paid to specific types of non-digestible oligosaccharides termed as prebiotics. Xylooligosaccharides (XOS) represent one such category and are generally produced by the enzymatic hydrolysis of xylan.

The interest in the microbial utilization of food processing waste into value-added products has increased. Corncob is an important by-product of the corn industry which is used either as animal feed or returned to the harvested fields. The xylan content of corncob is about 35%. Enough scope exists for value addition to corncob and its utilization for food applications such as production of XOS, xylose, xylitol and xylanase. The present research encompasses on the evaluation of microbial endoxylanases for XOS production, followed by the selection of *Aspergillus oryzae* MTCC 5154 as a potent endoxylanase source, production and characterization of XOS from pretreated corncob, evaluation of efficacy of XOS in the reduction of DMH induced colon cancer in rodent model and the use of XOS to improve fermentation characteristics of *idli* batter and quality attributes of *idli.*

The results indicated that *Aspergillus oryzae* MTCC 5154 is a potent producer of cellulase-free endoxylanase under submerged fermentation conditions using corncob as an inducer. The results of XOS production from birch wood xylan or pretreated corncob using corncob-induced endoxylanase indicated that the major product formed is xylobiose which can be further used for food applications. *In vivo* evaluation of the effect of XOS on DMH induced colon cancer in rats showed that XOS supplementation can significantly reduce the number of aberrant crypt foci and can retard the multiple crypt foci development. The observations of the study intended to evaluate the effects of XOS on the quality attributes of *idli* indicated that *idli* made from batter containing 0.4% XOS has supreme quality attributes, within a very short period of 6 h of batter fermentation.

**Dr. (Mrs.) S. G. Prapulla** Fermentation Technology & Bioengineering

Scientist- F April, 2009



# CERTIFICATE

This is to certify that the thesis entitled "**BIOACTIVE XYLOOLIGOSACCHARIDES FROM CORNCOB: ENZYMATIC PRODUCTION AND APPLICATIONS"** submitted by **Mr. Ayyappan Appukuttan Aachary** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** to the **UNIVERSITY OF MYSORE** is the result of research work carried out by him under my guidance in Department of Fermentation Technology & Bioengineering, Central Food Technological Research Institute, Mysore- 570 020, India during the period 2004-2009.

**S. G. Prapulla**

(**Guide)**

Come to the edge, they said. He said: I am afraid.
Come to the edge, they said again.
He came.
They pushed him and he flew.



For my mother and father

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The wind beneath my wings

***Acknowledgements***

*A very special word must be given to my supervisor, Dr. (Mrs.). S. G. Prapulla, whose expert guidance permitted me to achieve this final result. Her willingness to nurture, mentor, discuss and critique is highly appreciated. Thanks for unstinting enthusiasm, uncompromising and unconditional faith in my abilities. I will ever be grateful to her for giving me this wonderful opportunity and a very relevant, yet challenging topic. I would also like to cherish forever memories of Late. Dr. M. N. Ramesh for his guidance, collaboration and encouragement during the initial stages of this work.*

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*I acknowledge and thank all the scientists and researchers who made valuable contributions to the field of prebiotics in general and to the research of xylooligosaccharides in particular, many of whose names appear in the reference section. Thanks to FOSTIS, CFTRI for providing the unlimited resources of scientific literature. I would like to acknowledge Council of Scientific and Industrial Research, India, for the award of Junior and Senior research fellowship to carry out this research work and for their continuous contribution to the development of science, technology and higher education in India.*

*CFTRI is home to many supportive people. Colleagues in FTBE and many other departments helped me in numerous ways. Most importantly, I would like to thank all the scientists, staff and fellow colleagues who made my research comfortable: Dr. K. Srinivasan for supporting the animal studies on cancer, Dr. Arun Chandrasekhar for helping me to decipher xylanase sequence, Mr. A. Srinivas, Mr. B.A. Umesh Kumar and Mr. Girish Ghiwari for milling the corncob, Mrs. Rita Das for dissecting the rats, Mr. S.G. Jayaprakash for concentrating the XOS syrup, my friends Anilettan, Lincy and Shibin for teaching me the molecular techniques and Gopinath, my lab mate, for being with me through out the animal work and product development.*

*It was heartwarming to be a part of community where knowledge is shared and education is valued. I thank all the staff and students of FTBE department for the support offered. My sincere thanks to Dr. M. S. Thakur, Dr. A. P. Sattur, Dr. H. K. Manonmani, Dr. K. Yogish and Dr. D. Somashekar for all the supports offered. I also thank those who said 'no' which challenged and made me to learn many things.*

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*I would like to share my happiness and thank a few important people who joined me in the discovery of what is life about and how to make the best of it: Sreekanth CN for his mystic presence in my life which turned my fears into desires to succeed, Sujeeth Neerakkal for the strong bond of friendship and Rakhesh KC for his unmatched and unconditional fondness.*

*Thanks to my family, especially my mother and father, who have endured my many absences during my research period. My parents always supported and encouraged me to do my best in all matters of life. To them I dedicate this thesis. My everlasting gratitude to my sister, brother-in-law and nephew: Rani, Ramesh and Ampadi, for their love, care and support and also for their encouragement to embrace the moments and try everything life has to offer. I am forever indebted to my brothers, Kannan and Aneesh, for being my thoughts, feelings, colours and shadows. They inspire me and I owe them profound admiration and love for they are my heroes. A very special word must be given to my fiancée, Dr. Divya Sukumar who has endured the many holidays during which thesis writing took precedence. Thanks for caring and providing a different angle to my life.*

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*Ayyappan Appukuttan Aachary*

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# ACRONYMS AND ABBREVIATIONS

# *Acronyms and abbreviations*

### *Units, measures and symbols*

| | |
|---|---|
| % | percentage |
| ~ | approximately |
| < | less than |
| ≤ | less than or equal |
| > | greater than |
| ≥ | greater than or equal |
| °C | degree Celsius |
| µl | microlitre |
| µM | micromolar |
| µm | micrometer |
| µmol | micromoles |
| µs | microseconds |
| bar | unit of pressure |
| cfu | colony forming units |
| cm | centimeter |
| g | gram |
| g/l | gram/litre |
| h | hour |
| Hz | Hertz |
| INR | Indian Rupee |
| kb | kilo base pairs |
| kDa | kilo Dalton |
| kg | Kilogram |
| $K_M$ | Michaelis–Menten constant |
| l | litre |
| l/h | litre/hour |
| log | logorithm |
| M | Molarity |
| m/z | mass/charge |
| Mb | mega base pairs |
| mg | milligram |
| mg/ml | milligram/millilitre |
| MHz | mega hertz |
| million | $1\times10^6$ |
| min | minutes |
| ml | millilitre |
| ml/h | millilitre/hour |
| ml/min | milliliter/minute |
| mm | millimeter |
| mM | millimolar |
| mm/ min | millimeter/minute |
| N | normality or Newton |
| ng | nanogram |
| nm | nanometer |
| nmol | nanomoles |

| | |
|---|---|
| $OD_{600}$ | optical density at 600nm |
| P | probability value in statistics |
| ppm | parts per million |
| rpm | rotation per minutes |
| RT | retention time |
| s | seconds |
| tonne | 1000 kg |
| U | enzyme units |
| U/ml/min | unit activity of enzyme/millilitre/minute |
| USD | US dollar |
| v/v | volume/volume |
| w/v | weight/volume |
| w/w | weight/weight |
| $W_0$ | initial weight |
| wk | week |
| Wt | weight at time 't' |
| x g | relative centrifuge force in terms of gravity (times gravity) |
| yen | Japanese currency |
| $\delta_{ppm}$ | chemical shifts in ppm |

***Carbohydrates and their structures***

| | |
|---|---|
| $[M+ Na]^+$ | sodium adduct of carbohydrate 'M' |
| $[M+H]^+$ | hydrogen adduct of carbohydrate 'M' |
| AGX | arabino (glucurono) xylan |
| *Araf* | arabinofuranosyl residue |
| AX | neutral arabinoxylans |
| BWX | birch wood xylan |
| C1-C6 | carbon in sugar molecules, according to their position in the chain |
| CCX | arabinoglucuronoxylan from corncobs |
| CMC | carboxy methyl cellulose |
| DeXO | de-acetylated XOS from almond shells |
| GA | 2-*O*-linked α-D-glucopyranosyl uronic acid unit |
| *Glup* | glucopyranosyl residue |
| GX | 4-*O*-methylglucuronoxylan |
| MeGA | 4-*O*-methyl-α-D-glucopyranosyl uronic acid units |
| OSX | oat spelt xylan |
| WU-AX | water-unextractable arabinoxylan |
| X | xylose |
| $X_2$ | xylobiose |
| $X_3$ | xylotriose |
| $X_4$ | xylotetraose |
| $X_5$ | xylopentaose |
| $X_6$ | xylohexaose |
| Xm | homoxylans with mixed β-(1→3) and β-(1→4) glycosidic linkages |
| Xn | homoxylans |
| XOS | xylooligosaccharides |
| Xyl | xylose |
| *Xylp* | xylopyranosyl residue |

***Techniques and protocols***

| | |
|---|---|
| $^{13}$C-NMR | Carbon-13 nuclear magnetic resonance spectroscopy |
| 1D $^{13}$C-NMR | 1D Carbon-13 nuclear magnetic resonance spectroscopy |
| $^{1}$H-NMR | Proton nuclear magnetic resonance spectroscopy |
| 2D-HSQC | two dimensional heteronuclear single quantum coherence |
| ANOVA | Analysis of Variance |
| CID | collision induced dissociation |
| ESI | electrospray ionization |
| ESI-MS/MS | electrospray tandem mass spectrometry |
| FT-ICR | Fourier transform ion cyclotron resonance mass spectrometry |
| GPC | gel permeation chromatography |
| HPLC | high performance liquid chromatography |
| Hunter L, a, b | Hunter values representing brightness, redness and yellowness respectively |
| IR | Infrared |
| MALDI-MS | matrix-assisted laser desorption/ionization mass spectrometry |
| MALDI-TOF | matrix-assisted laser desorption/ionization-time of flight |
| MS/MS | tandem mass spectra |
| PCR | polymerase chain reaction |
| QIT | quadrupole ion trap |
| RCT | randomized, controlled human trial |
| RID | refractive index detector |
| SD | standard deviation |
| SEC | size-exclusion chromatography |
| SEM | scanning electron microscopy |
| SHIME | simulated human intestinal microbial ecosystem |

***Molecular genetics***

| | |
|---|---|
| ABC | ATP-binding cassette |
| ATP | adenosine tri phosphate |
| *abfB* | genes encoding α-arabinofuranosidase |
| *aguA* | genes encoding α-glucuronidase |
| ALL | acute lymphoblastic leukemia |
| AoXlnR | transcriptional activator for genes encoding xylanases in *Aspergillus oryzae* |
| *axeA* | genes encoding acetylxylan esterase |
| *axhA* | genes encoding arabinoxylan arabinofuranohydrolase |
| *bglA* | genes encoding β-glucosidase |
| bp | base pair |
| cDNA | complementary DNA |
| CDNB | 1-chloro-2,4-dinitrobenzene |
| dATP | deoxyadenosine triphosphate |
| DH5α | commonly used E.coli strain in transformation experiments |
| DNA | deoxyribo nucleic acid |
| dNTP | deoxyribonucleotide triphosphate |
| *eglA* and *eglB* | genes encoding endoglucanase |
| *faeA* | genes encoding feruloyl esterase |
| IPTG | isopropyl-beta-thio galactopyranoside |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| PAX1 | clone of exon 1 of *Aspergillus oryzae* MTCC 5154 |
| PAX2 | clone of exon 2 of *Aspergillus oryzae* MTCC 5154 |

| | |
|---|---|
| *p*TZ57R/T | a ready-to-use cloning vector |
| TAE buffer | tris-acetate-EDTA buffer |
| *Taq* polymerase | a thermostable DNA polymerase from *Thermus aquaticus* |
| TCSs | two-component systems |
| TE buffer | tris-EDTA buffer |
| *xlnB* and *xlnC* | genes encoding endoxylanases |
| *xlnD* | genes encoding β-xylosidase |
| XlnR | a gene encoding a transcriptional activator in *Aspergillus niger* |
| XYLF1 | forward primer for first exon of *Aspergillus oryzae* MTCC 5154 |
| XYLF2 | forward primer for second exon of *Aspergillus oryzae* MTCC 5154 |
| XYLR1 | reverse primer for first exon of *Aspergillus oryzae* MTCC 5154 |
| XYLR2 | reverse primer for second exon of *Aspergillus oryzae* MTCC 5154 |
| *xynF1* | gene encoding the major xylanase in *Aspergillus oryzae* |
| *xynG1* | endoxylanase-encoding gene from *Aspergillus oryzae* |

***Enzymes***

| | |
|---|---|
| ALT | alanine transaminase |
| AST | aspartate aminotransferase |
| BlxA | xylanase A from *Bacillus licheniformis* |
| BxlA | intracellular β-D-xylosidase from *Streptomyces thermoviolaceus* |
| CFXcd | endo-1,4-β-xylanase from *Cellulomonas fimi* |
| CMCase | carboxy methyl cellulase |
| *Eco RI* | an endonuclease enzyme commonly used for restriction digestion |
| GH10 | glycosyl hydrolase family 10 |
| GH52 | glycosyl hydrolase family 52 |
| GH8 | glycosyl hydrolase family 8 |
| GST | glutathione *S*-transferase |
| *Pst I* | a restriction endonuclease used for restriction digestion |
| reBlxA | xylanase A of *Bacillus licheniformis* expressed in *Pichia pastoris* |
| StxI-StxIV) | extracellular enzymes of *Streptomyces thermoviolaceus* |
| XYL A | family 11 endoxylanases of *Sporotrichum thermophile* |
| XYL I | family 10 endoxylanases of *Thermoascus aurantiacus* |
| XynB | recombinant xylanase B |

***Latin words***

| | |
|---|---|
| A.D. | *Anno Domini* |
| *ad libitum* | Latin expression for “free access to water and food” |
| *de novo* | Latin expression for "from the beginning," "afresh," or "anew”. |
| *e.g.* | *exempli gratia*: Latin expression meaning “for the sake of example” |
| *et al.* | Latin expression for “and others” |
| *ex vivo* | Latin expression for “which takes place outside an organism” |
| *in situ* | Latin phrase for “in the place” |
| *in vitro* | Latin for “within the glass” |
| *in vivo* | Latin for "within the living" |

***Organizations and Institutions***

| | |
|---|---|
| AOAC | The Association of Official Analytical Chemists |
| ATCC | American Type Culture Collection |
| CFTRI | Central Food Technological Research Institute |
| EC | Enzyme Commission |

| | |
|---|---|
| FAO | Food and Agricultural Organization |
| FDA | Food and Drug Administration |
| ICR | Institute of Cancer Research |
| IMTECH | Institute of Microbial Technology |
| ISI | Indian Standards Institution |
| IUBMB | International Union of Biochemistry and Molecular Biology |
| IUPAC | International Union of Pure and Applied Chemistry |
| JCBN | Joint Commission on Biochemical Nomenclature |
| MTCC | Microbial Type Culture Collection and Gene Bank |
| NCBI | National Center for Biotechnology Information |
| WHO | World Health Organization |

***Media chemicals, biochemical reagents and metabolites***

| | |
|---|---|
| BIM | *Bifidobacterium* iodoacetate medium |
| $CaCl_2$ | calcium chloride |
| $CO_2$ | carbon dioxide |
| CYEA | Czepadox yeast extract agar |
| $D_2O$ | heavy water |
| DMH | 1,2-dimethyl hydrazine |
| DNS | 3,5-dinitrosalicylic acid |
| FA | ferulic acid |
| $FeSO_4.7H_2O$ | ferrous sulphate (hepta hydrate) |
| GSH | reduced glutathione |
| $H_2$ | hydrogen |
| $H_2O_2$ | hydrogen peroxide |
| $H_2SO_4$ | sulfuric acid |
| HCl | hydrochloric acid |
| HMF | hydroxyl methyl furfural |
| $KH_2PO_4$ | potassium dihydrogen phosphate |
| KOH | potassium hydroxide |
| LB | Luria-Bertani Medium |
| MDA | malonaldehyde |
| $MgCl_2$ | magnesium chloride |
| $MgSO_4.7H_2O$ | magnesium sulphate (hepta hydrate) |
| $MnSO_4.2H_2O$ | manganese sulphate (di hydrate) |
| MRS | de Man, Rogos and Sharpe |
| $N_2$ | nitrogen |
| NA | nutrient agar |
| NaCl | sodium chloride |
| $NaNO_3$ | sodium nitrate |
| NaOH | sodium hydroxide |
| PDA | potato dextrose agar |
| PDB | potato dextrose broth |
| PEG | polyethylene glycol |
| SCFA | short chain fatty acids |
| SOC | super optimal broth with catabolite repression |
| TAG | triacylglycerol |
| TBARS | thiobarbituricacid reactive substances |
| TSC | tryptose-sulfite-cycloserine |
| X-Gal | 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside |

***Cell biology***

| | |
|---|---|
| ACF | aberrant crypt foci |
| apoE-deficient | apolipoprotein-E-deficient |
| CRC | colorectal cancer |
| GI | gastrointestinal |
| IBD | inflammatory bowel disease |
| IFNγ | interferon gamma |
| IgA | immunoglobulin A |
| IL 10 | inter leukin 10 |
| ML-2 | myelogenous leukemia |
| NF-KB | nuclear factor kappa-light chain enhancer of B cells |
| NK | natural killer cell |
| TGF-β | transforming growth factor beta |
| Th2 | T Helper cells 2 |
| Th1 | T Helper cells 1 |

***Miscellaneous***

| | |
|---|---|
| CAGR | compound annual growth rate |
| CF | culture fluids |
| Co. | company |
| EXL | endoxylanase activity |
| FOSHU | Foods for Specific Health Uses |
| FPA | filter paper activity |
| GRAS | Generally Regarded As Safe |
| HCW | hot-compressed-water |
| L. | Linnaeus ( in nomenclature) |
| LAB | lactic acid bacteria |
| LCMs | lignocellulosic materials |
| Ltd | limited |
| ND | Not detected |
| PER | protein efficiency ratio |
| spp. | species |
| UV | ultra violet |

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## KEY WORDS & BRIEF DESCRIPTIONS

**Colonic nutrients:** They are neither digested nor absorbed as such or after hydrolysis, in upper intestinal tract but serve as metabolic substrates, biosynthesis precursors or cofactors for colonic microorganisms. However, metabolic end products of fermentation and/or specific signaling molecules that are released by these microorganisms can become absorbed to feed the intestinal cell wall and then be distributed, *via* blood or lymph, amongst the tissues and organs to serve, indirectly, as metabolic substrates, biosynthetic precursors or cofactors for eukaryotic cells.

**Prebiotics:** They are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve host health. Prebiotics represent one category of specific colonic nutrients and are therefore nutrients that have the potential to considerably influence the physiology of whole body and consequently health and well-being.

**Xylooligosaccharides (XOS):** They are sugar oligomers made up of xylose units, which appear naturally in bamboo shoots, fruits vegetables, milk and honey and they represent one important category of prebiotics. Depend upon the various xylan sources used for XOS production, the structure of XOS vary in its degree of polymerization, monomeric units and type of linkages. Generally XOS is a mixture of oligosaccharides with β-(1,4)-linkages. The number of xylose residues involved in the XOS formation can vary from 2-10 and they are named as xylobiose, xylotriose and so on.

**Xylan:** It is a β-1,4-linked polymer of xylose and is the most abundant hemicellulose in the majority of plants. The β-(1,4)-linked D-xylosyl backbone of xylan is decorated through an α-1,2- linkage with glucuronic acid and 4-O-methyl-α-D-glucuronic acid, whereas α-L-arabinofuranosyl residues are linked through α-1,3 to the polymer. Xylan represents an immense resource of biopolymers for practical applications, accounting for 25-35% of the dry biomass of woody tissues of dicots and lignified tissues of monocots, and occurs up to 50% in some tissues of cereal grains.

**Endo-β-D-xylanases:** They are generally known as endoxylanases (EC 3.2.1.8) and are the key enzyme components of microbial xylanolytic systems which hydrolyze β-(1, 4)-xylosidic linkages of the xylan backbone to XOS of various chain lengths. A number of microorganisms, including bacteria, yeasts and filamentous fungi have been reported to produce xylanases. The microbial xylanases have been used commercially in the pulp and paper industry for the past several years.

***Aspergillus oryzae* MTCC 5154:** *A. oryzae* has traditionally been used as koji molds for fermented food and beverage production in East Asian countries. This filamentous fungus is GRAS cleared and has safely been used for the industrial production of enzymes. The strain *A.oryzae* MTCC 5154 have been reported for the production of fructosyl transferase and fructooligosaccharides.

**Corncob:** It is the central core of a maize (*Zea mays* ssp mays L.) ear. Corncobs contain approximately 35% xylan and are important by-product of the corn industry that is used either as animal feed or returned to the harvested fields. Enough scope exists for value addition to corncob and its utilization for food applications such as production of XOS, xylose, xylitol and xylanase.

**Pretreatment:** The availability of xylan component of the cell wall matrix for the enzymatic reaction is restricted by the surrounding lignin network as well as ester and ether lignin-carbohydrate linkages. Thus before enzyme hydrolysis, the xylan-lignin bonds needs to be broken. The process of making xylan available for enzymatic reaction without the complete extraction is known as pretreatment. Generally the pretreatments are carried out with the aid of steam, acids and alkali.

**Colorectal cancer (CRC):** Considered globally, CRC is second in terms of cancer-related deaths. There are nearly one million new cases of CRC diagnosed world-wide each year and half a million deaths and is largely attributable to environmental, especially dietary, factors. It has been estimated that as many as 70% of the CRC may be due to

dietary and lifestyle factors and that an optimal dietary approach might prevent the occurrence and development of this disease. CRC arises as the cumulative effect of multiple mutations within the cell, allowing it to escape growth and regulatory control mechanisms. This step-wise progression of mutations facilitates the histological transition from normal mucosa to adenoma to carcinoma.

**1, 2-dimethyl hydrazine (DMH):** It is a powerful colon carcinogen which induces colorectal tumors in experimental animals. DMH, an alkylating agent when injected subcutaneously, is transported to the liver and where it undergoes dehydrogenation gets converted to an active carbonium ion through several processes, and is excreted in the bile, where it mediates its carcinogenic activities on the colonic mucosa while passing through the digestive tract.

**Aberrant crypt foci (ACF):** They are readily discernible 'preadenomatous' morphological putative lesions within the colonic mucosa of rodents and even in cancer patients that may contribute to the stepwise progression to colon cancer. The formation and growth of ACF are associated with the induction of colon tumors in rats and are influenced by exposure to chemopreventive agents.

***Idli:*** Among several of the Indian traditional foods, *idli*, a fermented steamed product with a soft and spongy texture is highly popular and very widely consumed throughout India. It is also becoming popular in other countries. From the nutritional and health status point, *idli* appears to be an ideal human food for all ages and at all times. Being a cereal-legume based fermented product, it has an improved nutritive value as evident from its higher protein efficiency ratio and increased essential amino acid and vitamin contents.

# SYNOPSIS

# SYNOPSIS:

**Title: Bioactive xylooligosaccharides from corncob: Enzymatic production and applications**

**Summary and highlights of the investigation:**

The interest in nutraceuticals and functional foods continues to grow, powered by progressive research efforts to identify properties and potential applications of nutraceutical substances and coupled with public interest and consumer demand. The use of foods that promote a state of wellbeing, better health and reduction of the risk of diseases have become popular as the consumer is becoming more and more health conscious. In this regard, a lot of attention has been paid to specific types of non-digestible oligosaccharides termed as prebiotics. By definition, a prebiotic, classified as a specific colonic nutrient, is a selectively fermented ingredient that allows specific changes, both in the composition and/ or activity in the gastrointestinal microflora that confers benefits. The potential of plant cell wall polysaccharides as sources of bioactive prebiotics has received considerable attention.

Xylan, a β-1,4-linked polymer of D-xylose, is the most abundant hemicellulose in the majority of plants. The β-(1,4)-linked D-xylosyl backbone of xylan is decorated through an α-1,2- linkage with glucuronic acid and 4-O-methyl-α-D-glucuronic acid, whereas α-L-arabinofuranosyl residues are linked through α-1,3 to the polymer. 1,4-β-D-xylan xylanohydrolase (EC 3.2.1.8) generally known as endoxylanase hydrolyze β-1,4-xylosidic bonds within these polysaccharide backbone, producing β-anomeric xylooligosaccharides (XOS).

XOS present important physicochemical and physiological properties that are beneficial to the health of consumers, and for this reason, their use as food ingredients has increased rapidly. XOS are also associated with a lower risk of infections and diarrhea, and an improvement of the immune system response. By virtue of a decrease in the intestinal pH due to their fermentation by beneficial bacteria in the colon, XOS provoke a reduction of

pathogenic microflora, an increase in bifidobacterial population, and an increase in the availability of minerals. In the food industry, XOS have great potential to improve the quality of many foods, providing modifications to food flavor and improving its physicochemical characteristics.

XOS are water soluble and typically 0.3 times as sweet as sucrose. The sweetness depends on chemical structure, degree of polymerization of the oligosaccharides present and levels of mono- and disaccharides in the mixture. The relatively low sweetness makes these oligosaccharides useful in the preparation of food products where a bulking agent with reduced sweetness is desirable. In addition, when compared with monosaccharides, the higher molecular weight of XOS provides an increased viscosity, leading to improved body and mouth feel.

The interest in the microbial utilization of food processing waste into value-added products has increased. Corncob is an important by-product of the corn industry which is used either as animal feed or returned to the harvested fields. The xylan content of corncob is about 35%. Xylans represent an immense resource of biopolymers for practical applications and can be used as a carbon source for various microorganisms for their growth and production of food grade enzymes. Enough scope exists for value addition to corncob and its utilization for food applications such as production of XOS, xylose, xylitol and xylanase.

The work presented in the thesis describes the possibilities of value addition to corncob for xylanase and XOS production. The effects of XOS on the reduction of colorectal cancer in a rodent model and use of XOS in *Idli*, an Indian traditional food for the reduction of fermentation time and improvement of quality attributes are also discussed.

**The objectives of the thesis are:**

I. Evaluation of microbial xylanases for the production of XOS
II. Enzymatic production of XOS from corncob
III. Studies on bioactivity of XOS
IV. Studies on application of XOS

The contents of the thesis have been organized and presented in 7 different chapters

**Chapter: 1**

**Introduction**

Concept, criteria, classification, functional properties, beneficial health effects, applications, global and Indian scenario and regulatory aspects of prebiotics and scope of the investigation are presented.

**Chapter: 2**

**Review of literature**

A thorough review of relevant literature cited on the topic of investigation for the past decade is presented in this chapter. Recent developments in the manufacture, purification and biological effects of XOS are reviewed. The chapter focuses mainly on the enzymatic methods for production of XOS from a variety of xylan-containing raw materials. As food ingredients, XOS impart favorable technological properties and exhibit prebiotic property to modulate the intestinal function. Besides their beneficial effects in the large bowel, a range of additional biological activities have been also reviewed. Other topics discussed include the utilization of XOS in synbiotic preparations, their technological properties and market perspectives.

**Chapter: 3**

**Evaluation of microbial xylanases for the production of xylooligosaccharides**

The first section of this chapter is titled as **'Production of xylooligosaccharides from glucurono-xylan using corncob-induced endo-1,4-β-D-xylanase'.** This section details the cultivation of eight different fungi in peptone-yeast extract medium containing 1% oat spelt xylan (OSX) to evaluate the endoxylanase secretion for XOS production. *A. oryzae* MTCC 5154, *A. flavus*, *A. niger* and *A. ochraceus* showed significant titers of endoxylanases, which were further used for the production of XOS from birch wood xylan (BWX). *A. oryzae* produced 89.5±1.13% XOS in the hydrolysate at 24 h of reaction. The effect of OSX, BWX and raw corncob on the induction of endoxylanase in

*A. oryzae* was studied and the xylanase activity was maximum at 96 h of cultivation in 3% corncob containing medium. XOS produced at 36 h of reaction from 1% BWX was 5.87±0.53 mg/ml (12±2% xylose, 48±2.43% xylobiose and 40±3.6% higher oligomers). The HPLC/RID and ESI/MS analysis of fractions obtained by gel permeation chromatography corresponded to neutral and 4-O-methyl-α-D-glucuronic acid substituted acidic oligosaccharides. The major fraction β-D-xylopyranosyl-(1→4)-D-xylanopyranose was characterized using $^{13}$C NMR.

The second section of the chapter titled **"Molecular cloning and sequencing of endoxylanase gene of *Aspergillus oryzae* MTCC 5154"** elaborates the molecular cloning and sequencing of xylanase gene of *A. oryzae.* Using PCR based genomic cloning approach, the partial genomic clones for endoxylanase (XYL) gene from *A. oryzae* were obtained. Primers for PCR amplification of XYL gene were designed based on available cDNA sequences for XYL gene of *Aspergillus* genus. Different sets of primers were designed for the two exons of XYL gene and were used to obtain amplicons for partial XYL gene. The PCR conditions were optimized. Transformation and cloning experiments were performed with *E.coli* DH5α using pTZ vector. Various PCR products were cloned and sequenced. Sequence analysis of the clones PAX1 and PAX2 showed high similarity in the nucleotide sequence of exon regions of XYL gene of other *Aspergillus* species reported.

**Chapter: 4**

**Enzymatic production of xylooligosaccharides from corncob**

In the current trend of a complex and more effective utilization of biomass and developments of eco-friendly industrial processes, increasing research activities have been directed to the exploitation of xylan component of economically important plants and plant residues as biopolymer resources. The present chapter details the value addition to corncob for XOS production through biotechnological routes.

Enzymatic production of XOS is preferred in food industry because of the problems associated with the other strategies of XOS production. The availability of xylan

component of the cell wall matrix for the enzymatic reaction is restricted by surrounding lignin network as well as ester and ether lignin-carbohydrate linkages. For an efficient production of XOS from agro-residues, the xylan should be exposed to endoxylanase action. Since the complete extraction of xylan is time consuming and is often related with environmental issues, a mild pretreatment method is suggested by various authors for making the xylan available for enzymatic reaction. Most of the hemicellulose preparations are soluble in water after alkaline extraction or treatment. Their isolation actually involves alkaline hydrolysis of ester linkages to liberate them from ligno-cellulosic matrix followed by extraction with aqueous media. The first section of the chapter titled "**Evaluation of pretreatment of corncob for enhanced availability of xylan for xylooligosaccharides production"** deals with the comparison of various pretreatments such as mild alkali / acid treatments and pressure cooking of corncob to expose its lignin-saccharide complex to enhance enzymatic hydrolysis of xylan to XOS. It also focuses on the changes in super molecular structure of lignin-saccharide complex during pretreatment. Scanning electron micrographs of lignin-saccharide complex of native and pretreated corncob powder showed that the complex was greatly altered during alkali pretreatment. Hydrolysis of alkali pretreated corncob powder using a commercial endoxylanase produced 81±1.5% of XOS in the hydrolyzate equivalent to 5.8±0.14 mg/ ml of XOS.

The second section of the chapter titled **"Bioconversion of pretreated corncob to xylooligosaccharides using endoxylanase from** *Aspergillus oryzae* **MTCC 5154: Optimization, purification and characterization"** focuses on production of XOS from alkali pretreated corncob powder. Reaction parameters for the production of XOS from corncob using endoxylanase from *A. oryza*e were optimized. An XOS yield of 10.2±0.14 mg/ ml corresponding to 81±3.9% was obtained and the present study has a distinct advantage in terms of the lesser period of reaction (14 h). At 14 h of reaction, the percentage xylobiose was about 73.5 which is considerably higher in comparison other published literature reports. The production of high content xylobiose is generally a time consuming and expensive process. In this context, the leads obtained from the present investigation can further be explored for large scale production of XOS with a high

content of xylobiose. HPLC/RID and ESI/MS analysis of XOS mixture and purified fractions showed that XOS was a mixture of neutral oligosaccharides of DP, 2-7. Major fraction of disaccharide (β-D-xylopyranosyl-(1→4)-D-xylanopyranose) was characterized using $^{13}$C NMR and 2D-HSQC NMR.

**Chapter: 5**

***In-vivo* investigations on the effect of xylooligosaccharides on 1,2-dimethylhydrazine induced colon cancer in rats**

Colorectal cancer (CRC) is one of the leading causes of cancer morbidity and mortality world-wide. Human metabolic and laboratory animal model studies indicated that beneficial effects of prebiotics in relation to colon cancer development depend on the composition and physico-chemical properties of the oligosaccharides. Based on current knowledge of the pathogenesis of colon cancer, it is reasonable to conclude that consumption of a fiber-rich diet is associated with reduced risk of colon cancer. The present chapter describes the studies on efficacy of XOS in the reduction of colon cancer progression in 1,2-dimethylhydrazine (DMH, an alkylating agent specifically induce colon cancer) induced colon carcinogenesis in a Wistar rat model. The rats were randomly assigned to 5 groups: normal control, XOS control (100g/ kg diet), treatment with 1,2-dimethylhydrazine (DMH) [20 mg/ kg body wt/ week for 3 week], treatment with DMH + 50 g XOS/ kg diet, and treatment with DMH+100 g XOS/ kg diet. Rats were fed with experimental diets for 45 days, beginning 1week after the third dose of DMH injection. XOS intake markedly increased the total cecal weight, decreased the cecal pH, and increased the population of *Bifidobacteria*.

The study also focused on the effect of XOS supplementation on the reduction of number of Aberrant Crypt Foci (ACF) in colon induced by DMH. ACF were distinguished from normal crypts by their thicker, darker-stained and raised walls, with elongated, slit-like lumens. The results showed that XOS supplementation (5% and 10%) significantly reduced ($p \leq 0.05$) the number of ACF and retarded the multiple crypt foci development. The results also showed increased levels of liver lipid peroxidation products such as tissue thiobarbituricacid substances (TBARS) in DMH treated rats, which were

significantly ($p \leq 0.05$) reversed on XOS supplementation. Moreover colonic TBARS and glutathione *S*-transferase were significantly diminished in DMH treated rats, which were significantly ($p \leq 0.05$) elevated on simultaneous XOS supplementation. These results indicate that XOS probably plays a significant role in the reduction of colon carcinogenesis induced by DMH.

**Chapter: 6**

**Effects of xylooligosaccharides on quality attributes of *Idli*, a cereal-legume based Indian traditional food**

The food industry is constantly looking for economic ways of generating products with even more desirable textural and organoleptic properties than are currently provided by available functional food ingredients. Prebiotics show both important technological characteristics and interesting nutritional properties. In food formulations, they can significantly improve organoleptic characteristics, upgrading both taste and mouth-feel. The present chapter explores the utility of hydrocolloid like behavior of XOS obtained from corncob xylan to improve the textural properties of *idli*. The study also focuses on the prebiotic effect of XOS on the quality attributes of *idli*. The *idli* batter has been prepared by the conventional method with different concentrations of XOS (0, 0.2, 0.4 and 0.6% w/v) and allowed to ferment for 4, 6, 12, and 18 h. The results suggested that addition of XOS, due to its prebiotic effects, significantly increased ($p \leq 0.05$) the number of lactic acid bacteria, thereby a rapid reduction in pH (4.61± 0.03), an increase in percent total acidity (0.846± 0.033) and specific gravity (0.693± 0.015) has been reached even at 6 h of fermentation with 0.4% XOS. The conventional *idli* batter took 18 h to reach comparable values of pH (4.57± 0.08), percent total acidity (0.882± 0.021) and specific gravity (0.632± 0.015).

The log cfu values of lactic acid bacteria/ g of *idli* batter were significantly higher ($p \leq 0.05$) in batter fermented for 6 h with 0.4% XOS (9.88± 0.08) compared to conventional *idli* batter fermented for 18 h (9.40± 0.06). However, 0.6% XOS has decreased the overall acceptability of *idli* mainly because of the woody flavor and more yellowish colour. *Idlis* with XOS have higher moisture content, compared to those

without XOS which insights into the hydration properties of these short chain oligosaccharides and provides a softer texture to the products. Instrumental (colour and texture) and sensory (appearance, colour, texture, flavor, taste and overall quality) analysis showed that *idli* made from batter with 0.4% XOS fermented for 6 h have a better overall quality which is significantly higher ($p \leq 0.05$) than *idlis* made by the conventional way.

**Chapter: 7**

**Summary and conclusions**

Summary, conclusions and recommendations are given in this chapter. The thesis ends with a list of references arranged in alphabetical order.

Overall, an investigation is made on the evaluation of microbial endoxylanases for XOS production, followed by the selection of *A. oryzae* MTCC 5154 as a potent endoxylanase source, production and characterization of XOS from pretreated corncob, evaluation of efficacy of XOS in the reduction of DMH induced colon cancer in rodent model and the use of XOS to improve the fermentation characteristics of *idli* batter and the quality attributes of *idli*.

# CHAPTER: 1

## INTRODUCTION
## PREBIOTICS: SPECIFIC COLONIC NUTRIENTS

**Overview**

In recent decades, researchers have made considerable progress in our understanding of possible association between diets and health problems. This chapter provides an overview of the prebiotics field. The aim of the chapter is to provide a clear, comprehensive, rigorous and balanced introduction to prebiotic functional foods. This chapter enables the basic ideas to be integrated in the rest of the thesis. Included in this chapter are the basic concepts of prebiotics: definition, criteria, types and production strategies. The chapter also includes a discussion of the health benefits of various prebiotics as evident from animal and human experimental data. It also designed to summaries the information regarding various food and non food applications of prebiotics and their market potential as a functional food ingredient. Lastly, the chapter describes the scope of the present research work.

## 1.1. Introduction

Diet is an important determinant of disease risk in all age of population and there is emerging evidence that functional food ingredients can have an impact on a number of gut-related diseases and dysfunctions associated with changing lifestyle and age. The words of the renowned scientist Thomas Alva Edison ***"The doctor of the future will give no medicine, but will interest in the care of the human frame, in diet and in the cause and prevention of disease"*** are relevant in the present scenario where each man desires to live long in a healthy manner. The importance of the colonic microbiota in human health and well being is a major breakthrough in both medical and nutrition research, even if this still remains to be fully accepted, especially in medicine. The symbiosis between the complex community of prokaryotes and the colon is increasingly recognized as a major player in health and well-being. In order to achieve this, colonic microflora needs to be fed adequately. Accordingly, it is proposed that a balanced nutrition should provide both systemic and colonic nutrients *(Roberfroid, 2008).*

Systemic nutrients or digestible nutrients that are hydrolyzed in the gastrointestinal (GI) tract to provide monomers (monosaccharides, amino acids, fatty acids) or small oligomers that are absorbed *via* blood and/or lymph, and distributed among the various tissues and organs to serve as metabolic substrates, biosynthetic precursors, or cofactors for their eukaryotic cells. On the other hand colonic nutrients or non-digestible nutrients are neither digested nor absorbed as such or after hydrolysis, in upper intestinal tract but serve as metabolic substrates (fermentation process), biosynthesis precursors or cofactors for colonic microorganisms. However, metabolic end products of fermentation and/or specific signaling molecules that are released by these microorganisms can be absorbed to feed the intestinal cell wall, and then be distributed, *via* blood or lymph, amongst the tissues and organs to serve, indirectly, as metabolic substrates (e.g., butyrate for the colonocytes), biosynthetic precursors (e.g., acetate) or cofactors for eukaryotic cells *(Roberfroid, 2008).*

Further, the colonic nutrients are of two types. General colonic nutrients are those nutrients that function for most types of prokaryotic cells in the microbiota. On the other hand, specific colonic nutrients that function for one or a limited number of specific prokaryotic cells in the colonic microbiota thus provide these with a proliferation advantage. Prebiotics represent one such category of specific colonic nutrients and it is through such modification that health effects of prebiotics are mediated. Prebiotics are nutrients that have the potential to considerably influence whole body's physiology and consequently health and well-being. However, because prebiotics affect specifically and selectively the gut microflora, the importance of which is likely to become greater and greater as biomedical research progresses, it is proposed to go further and to classify a 'prebiotic' as an essential, specific colonic nutrient.

### 1.2. Prebiotics: concept and definition

A prebiotic is defined as a 'non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health' *(Gibson and Roberfroid, 1995).* Later, a more refined definition for prebiotics was suggested. According to the new definition, a prebiotic is a 'selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits' *(Gibson et al., 2004).* These definitions have attracted, and still continue to attract, a great deal of interest in the field of nutrition both in scientific research and in food applications.

### 1.3. Criteria for classification of a food ingredient as a prebiotic

It is necessary to establish clear criteria for classifying a food ingredient as a prebiotic. Indeed such classification requires a scientific demonstration that the ingredient (i) resists gastric acidity, (ii) is not hydrolyzed by mammalian enzymes, (iii) is not absorbed in the upper GI tract, (iv) is fermented by intestinal microflora, and (v) selective stimulation of growth and/or activity of intestinal bacteria potentially associated with health and well-being. These requirements have been classified as the three prebiotic criteria *(Gibson and Roberfroid, 1995).*

1. Resistance to digestive processes in the upper part of the GI tract.
2. Fermentation by intestinal microbiota.
3. Selective stimulation of growth and/or activity of a limited number of the health-promoting bacteria in that microbiota.

### 1.4. Foods for Specific Health Uses (FOSHU) and prebiotics

The concept of 'functional foods' started in Japan where the world's first health-claim approval system popularly known as FOSHU and the market for functional foods were developed in the last decade. A specific study on the 'development and systematic analysis of functional foods' was initiated in Japan in 1984 with support from the Ministry of Education, Science and Culture. The study report states that food has three functions, (i) the nutrient function to maintain life or growth of the body, (ii) the taste function by some components interacting with the sensory system, and (iii) the body-enhancing function related to body defense or modification of body conditions contributing to health maintenance and prevention of diseases. The study proposed the new concept 'functional foods' which focuses on the third function of food and the term functional foods immediately took root internationally *(Arai et al., 2001).*

The first FOSHU product was approved in 1993. Since the Pharmaceutical Affairs Law stipulates that a product aiming at influencing function or body structure should be categorized as a drug, the legal name of functional foods changed to FOSHU, and the concept 'foods with functionality' was subsequently modified to 'foods with approved label'. Among various prebiotics fructooligosaccharides, galactooligosaccharides, xylooligosaccharides (XOS), lactulose, raffinose, lactosucrsoe etc have been used as the major functional component in many FOSHU products with certain health claim. These FOSHU products include chocolates, candy, vinegar, pudding, table sugar, soft drinks etc *(Fukushima and Lino, 2006).* The *g*uidelines for evaluation of FOSHU application for products with oligosaccharides are given in table 1.1.

**Table 1:** Guidelines for evaluation of FOSHU application for products with oligosaccharides.

| Test items | Requirements |
|---|---|
| ***Efficacy confirmation*** | |
| ***In vitro and animal study on oligosaccharides*** | |
| 1. *In vitro* digestive study | Indigestibility in the intestinal conditions with digestive juice |
| 2. *In vitro* utilization test by intestinal bacteria | Selective utilization by bifidobacteria or lactic acid bacteria |
| 3. Animal study | Capability to reach the colon without digestion |
| ***Human study using final product*** | |
| 1. Intestinal flora analysis | Improvement of intestinal flora to show with increase in beneficial bacterial (%) like bifidobacteria and decrease in harmful bacteria (%) like *Clostridium perfringens* |
| 2. Defecation frequency | Increase in subject with mild constipation |
| 3. Fecal conditions or intestinal environment | Improvement of the quantity, hardness, color or shape of fecal sample, or decrease in pH or putrefactive compounds like ammonia |
| ***Safety confirmation*** | |
| 1. Acute, sub-acute, sub-chronic toxicity tests* | No side effect in rodent |
| 2. *In vitro* mutagenesis test* | Negative |
| 3. Maximum ineffective dose test in human | No temporary diarrhoea |
| 4. Excess dose study on final product in human | No side effect on general health status |

* Toxicity test can be excluded when the related functional component has adequate dietary history in humans.

*Source: Modified based on Fukushima and Lino, 2006*

### 1.5. Natural sources of prebiotics

Prebiotics of various types can be found as natural components in milk, honey, fruits and vegetables such as onion, Jerusalem artichoke, chicory, leek, garlic, artichoke, banana, rye, barley, yacon and salsify. In most of these sources, concentrations of prebiotics range between 0.3% and 6% of fresh weight. Asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato and rye are special sources of fructooligosaccharides *(Sangeetha et al., 2005, Ziemer and Gibson, 1998, Yun, 1996).* Isomaltulose naturally occurs in honey, sugarcane juice, and products derived thereof such as treacles or food-grade molasses *(Lina et al., 2002).* XOS appear naturally in bamboo shoots, fruits, vegetables, milk and honey *(Vazquez et al., 2000).* Galactooligosaccharides are found naturally in human milk and to a smaller extent in cow's milk *(Alander et al., 2001).* Seeds of legumes, lentils, peas, beans, chickpeas and mustard are rich in raffinose oligosaccharides *(Sanchez-Mata et al., 1998, Johansen et al., 1996).*

### 1.6. Candidate prebiotics and their commercial production

#### 1.6.1. Transgalactooligosaccharides

Enzymatic transglycosylation of lactose produces a mixture of oligosaccharides known as transgalactooligosaccharides *(Crittenden, 1996).* They generally consists of oligosaccharides from trisaccharides to pentasaccharides with β-(1→6), β-(1→3) and β-(1→4) linkages *(Matsumoto et al., 1993).* In the industrial production of transgalactooligosaccharides, a highly concentrated solution of lactose is used as a substrate and β-galactosidase is used as the enzyme.

#### 1.6.2. Fructooligosaccharides and oligofructose

Fructooligosaccharides are easily understood as inulin-type oligosaccharides of D-fructose attached by β-(2→1) linkages that carry a D-glycosyl residue at the end of the chain *(Yun, 1996).* The industrial processes for fructooligosaccharides production can be divided into two classes. In the first strategy, they are produced from the disaccharide sucrose using the transfructosylation activity of β-fructofuranosidase or fructosyl

transferase enzymes. Similar to the production of transgalactooligosaccharides, a high concentration of the starting material is required for efficient transglycosylation *(Park and Almeida, 1991).* According to Yun (1996) it is recommended that a sucrose concentration ranging from 600 to 850 g/l should be used as a substrate in order to save evaporation cost for final processing. The second method, a controlled enzymatic hydrolysis of the polysaccharide inulin which is extracted from chicory roots *(Crittenden and Playne, 1996)* results in oligofructose.

### 1.6.3. Xylooligosaccharides (XOS)

XOS are oligosaccharides formed of D-xylose linked through β-(1→4) bonds. XOS production at an industrial scale is carried out from the polysaccharide xylan, which is extracted from lignocellulosic materials. Typical raw materials for XOS production are hardwoods, corn cobs, straws, bagasses, hulls, malt cakes and bran. Three different approaches have been used for XOS production from these feedstocks: (i) enzyme treatments of native xylan-containing lignocellulosic material, (ii) chemical fractionation of a suitable lignocellulosic material to isolate (or to solubilize) xylan, with further enzymatic hydrolysis of this polymer to XOS and (iii) hydrolytic degradation of xylan to XOS by steam, water or dilute solutions of mineral acids. Xylanolytic enzyme complexes with low β-xylosidase activity and high endo-1,4-β-xylanase activity are desired for XOS production *(Vazquez et al., 2000).*

### 1.6.4. Maltooligosaccharides and isomaltooligosaccharides

Maltooligosaccharides contain α-D-glucose residues linked by α-(1→4) glycosidic linkages. They are produced commercially from starch by the action of debranching enzymes such as pullulanase and isoamylase, combined with hydrolysis by various α-amylases *(Crittenden and Playne, 1996).* Isomaltooligosaccharides are produced from starch as the raw material, but unlike maltooligosaccharides their production requires a combination of immobilized enzymes in a two-stage reactor. In the first stage, starch is liquefied using α-amylase. The liquefied starch is then processed in a second- stage that involves reactions catalyzed by both β-amylase and α-glucosidase. The β-amylase first hydrolyses the liquefied starch to maltose and α-glucosidase converts α-(1→4) linked

maltooligosaccharides into α-(1→6) linked isomaltooligosaccharides with different molecular weights *(Kaneko et al., 1994).*

### 1.6.5. Gentiooligosaccharides and soybean oligosaccharides

Gentiooligosaccharides consist of several glucose residues linked by β-(1→6) glycosidic bonds. They are produced from acid or enzymatic hydrolysis of starch and subsequent transglycosylation action of the obtained glucose syrup catalyzed by β-glycosidase enzyme *(Crittenden and Playne, 1996).* Soybean oligosaccharides are extracted directly from the raw material without the use of enzymes. Soybean whey, a by-product from the production of soy protein isolates and concentrates, contains the oligosaccharides raffinose, stachyose, and verbascose, which consist of 1, 2, or 3 α-(1→6) linked units of galactose linked to a terminal sucrose through α-(1→3) bonds. The oligosaccharide found in the highest concentration is stachyose, followed by raffinose, followed by verbascose *(Karr- Lilienthal et al., 2005).*

### 1.6.6. Lactulose

Lactulose is manufactured by the isomerization of lactose to generate the disaccharide galactosyl β-(1→4) fructose. It is widely prescribed as a laxative *(Tamura, 1993).* Lactulose can be classified as a prebiotic because of significant data in human studies. However, up to now, this compound has not been used as a food ingredient or as a food supplement.

### 1.6.7. Lactosucrose

Lactosucrose is produced from a mixture of lactose and sucrose using the enzyme β-fructofuranosidase *(Playne and Crittenden, 1996).* The fructosyl residue is transferred from sucrose to the $C_1$-position of the glucose moiety in the lactose, producing a non-reducing oligosaccharide *(Hara et al., 1994).*

### 1.6.8. Glucooligosaccharides

Glucooligosaccharides are synthesized by the action of the enzyme dextran sucrase on sucrose in the presence of maltose. The resulting oligosaccharides contain α-(1→2)

linkages. They can also be produced *via* fermentation in presence of *Leuconostoc mesenteroides.*

### 1.6.9. Other carbohydrates with prebiotic potential

The prebiotic potential of several other compounds has also been investigated. However, evidences pointing towards any prebiotic effect are too sparse to justify a detailed review and a classification as prebiotic at the present time. These carbohydrates and their derivatives include *N*-acetylchitooligosaccharides *(Chen et al., 2002),* lactose *(Szilagyi, 2002),* gluconic acid *(Tsukahara et al., 2002),* mannan oligosaccharides *(White et al., 2002),* polydextrose *(Murphy, 2001),* oligodextrans *(Olano-Martin et al., 2000),* pectic-oligosaccharides *(Olano-Martin et al., 2000),* resistant starch *(Silvi et al., 1999)* and a few sugar alcohols *(Piva et al., 1996).*

## 1.7. Structure–function relationships of prebiotics

The carbohydrates can be classified according to their molecular size or degree of polymerization (DP) into monosaccharides, oligosaccharides or polysaccharides. According to the nomenclature proposed by the Joint Commission on Biochemical Nomenclature (JCBN) of the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry and Molecular Biology (IUBMB), oligosaccharides are defined as saccharides containing between 3 and 10 sugar moieties. However, there is not a rational physiological or chemical reason for setting these limits *(Voragen, 1998).* Consequently, oligosaccharides are low molecular weight carbohydrates. At the same time, based on the physiological properties, the carbohydrates can be classified as digestible or non-digestible. The concept of non-digestible oligosaccharides originated from the observation that the anomeric C atom ($C_1$ or $C_2$) of the monosaccharide units of some dietary oligosaccharides has a configuration that makes their osidic bonds non-digestible to the hydrolytic activity of the human digestive enzymes *(Roberfroid and Slavin, 2000).*

The prebiotic properties of carbohydrates are likely to be influenced by their monosaccharide composition, glycosidic linkages and molecular weight *(Manning and*

*Gibson, 2004).* The most commonly available prebiotics consist mainly of glucose, galactose, xylose and fructose as building units. The prebiotic properties of oligosaccharides formed by other monosaccharides are not much known at present. The linkage between the monosaccharide residues is a crucial factor in determining both selectivity of fermentation and digestibility in the small intestine. Fermentation of fructooligosaccharides is selective because of a cell-associated β-fructofuranosidase in the bifidobacteria. Polysaccharides generally do not exhibit prebiotic properties but most of the oligosaccharides are prebiotics in nature *(Wang and Gibson, 1993).* The effect of molecular weight on prebiotic properties can be seen from the fact that xylan is not selective whereas XOS are prebiotic in nature *(Jaskari et al., 1998, Okazaki et al., 1990)* and similar effects were found in pectin also *(Olano-Martin et al., 2001, Olano-Martin et al., 2000).*

### 1.8. Ease of use of prebiotics

Unlike probiotics, prebiotics are non-viable and reach the colon intact and these properties make prebiotics more attractive. Their food manufacturing applications in the form of thickening agents or sweeteners make them more amenable to industrial processes. Prebiotics have a longer shelf-life and can be incorporated into a large variety of food, such as infant formulae, weaning food, cereals, confectionery, beverages, dairy products, dietary supplements etc.

### 1.9. Limitations of prebiotics

Possible side effects may be encountered when using prebiotics in excess and it results in an increased volume of fermentation end products. An increase in stool frequency and stool weight is often reported in human feeding trials *(Chen et al., 2001, Bouhnik et al., 1996, Ito et al., 1995).* Consumption of a large dose (>20 g/day) of prebiotics such as inulin or lactulose may lead to a laxative effect *(Bouhnik et al., 1999).* It is critical that newly developed products are selective towards non-gas producer bacteria, as gas distension may discourage the intake of prebiotics.

### 1.10. Dose effects of prebiotics

Optimum doses of prebiotics have been determined for common prebiotics such as fructooligosaccharides and transgalactooligosaccharides in various populations. Doses of fructooligosaccharides administered in feeding and clinical trials range from 3-20 g/day in adults and 0.4-3.0 g/day in infants *(Moore et al., 2003, Moro et al., 2002, Bouhnik et al., 1999).* These doses were fixed based on the amount of oligosaccharides present in diet rich in vegetables *(van Loo et al., 1995).* A minimal intake of 4-10 g/day for induction of a bifidogenic effect is often suggested for prebiotics, but there is no recommended intake available.

### 1.11. Purity and safety of prebiotics

Due to limitations in the manufacturing process, current prebiotic preparations are generally mixtures of oligosaccharides of various chain lengths. The presence of mono- and disaccharides may hinder the specificity of the prebiotic. Chemical extraction of oligosaccharides from food may also result in undesirable color or flavor. To overcome these drawbacks, new enzymatic processes providing higher oligosaccharide selectivity and more palatable properties are developed *(Rastall and Gibson, 2002).* The risk of bacteremia associated with prebiotics is almost negligible.

### 1.12. GRAS status and persistent effect of prebiotics

The recognition of GRAS status of existing prebiotics and/or their history of safe use is sufficient to guarantee their safety for public authorities, the users and the consumers *(Pascal, 2008).* The persistence of prebiotic effects when their intake is stopped has not been well established. In many feeding studies, colonic microbial changes are observed after treatment with prebiotics, but the effect generally ceased with the interruption of treatment *(Tuohy et al., 2002, Gionchetti et al., 2000).* Long-term daily intake of prebiotics seems to be necessary to achieve optimum efficiency.

### 1.13. Guidelines for the evaluation and substantiation of prebiotics

The following guidelines (figure 1.1) have been given by FAO *(FAO, 2007)* for the evaluation and substantiation of prebiotics.

### 1.13.1. Product specification/characteristics of the prebiotic

The component, to which the claim of being prebiotic is attributed, must be characterized for any given product. This includes: (i) source, origin, (ii) purity, (iii) chemical composition and structure, and (iv) vehicle, concentration and amount in which it is to be delivered to the host.

### 1.13.2. Functionality

At a minimum, there needs to be evidence of a correlation between the measurable physiological outcome and modulation of the microbiota at a specific site (primarily the gastrointestinal tract, but potentially also other sites such as vagina and skin). There is a need to correlate a specific function at a specific site with the physiological effect and its associated timeframe. Within a study, the target variable should change in a statistically significant way and the change should be biologically meaningful for the target group consistent with the claim to be supported. Substantiation of a claim should be based on studies with the final product type, tested in the target host. A suitably sized randomized control trial (compared to placebo or a standard control substance) is required, preferably with a second independent study.

### 1.13.3. Qualifications

Bifidogenic effects are not sufficient without demonstrated physiological health benefits. It is recognized that at this time, determining events that take place within compartments of the intestine are often difficult. Until such times as specific site sampling or more sophisticated methods can reliably link microbiota modulation with health benefits, faecal analysis will be deemed suitable, with limitations.

### 1.13.4. Safety

As with any food component, safety parameters are established by all national regulations. It is recommended that the following issues need to be covered in any safety assessment of a prebiotic final product formulation: (i) if, according to local legislation, the product has a history of safe use in the target host, such as GRAS or its equivalents, then it is suggested that further animal and human toxicological studies may not be

necessary, (ii) the safe consumption levels with minimal symptoms and side effects should be established, (iii) the product must not contain contaminants and impurities, and (iv) based upon current knowledge, the prebiotic should not alter the microbiota in such a way as to have long term detrimental effects on the host.

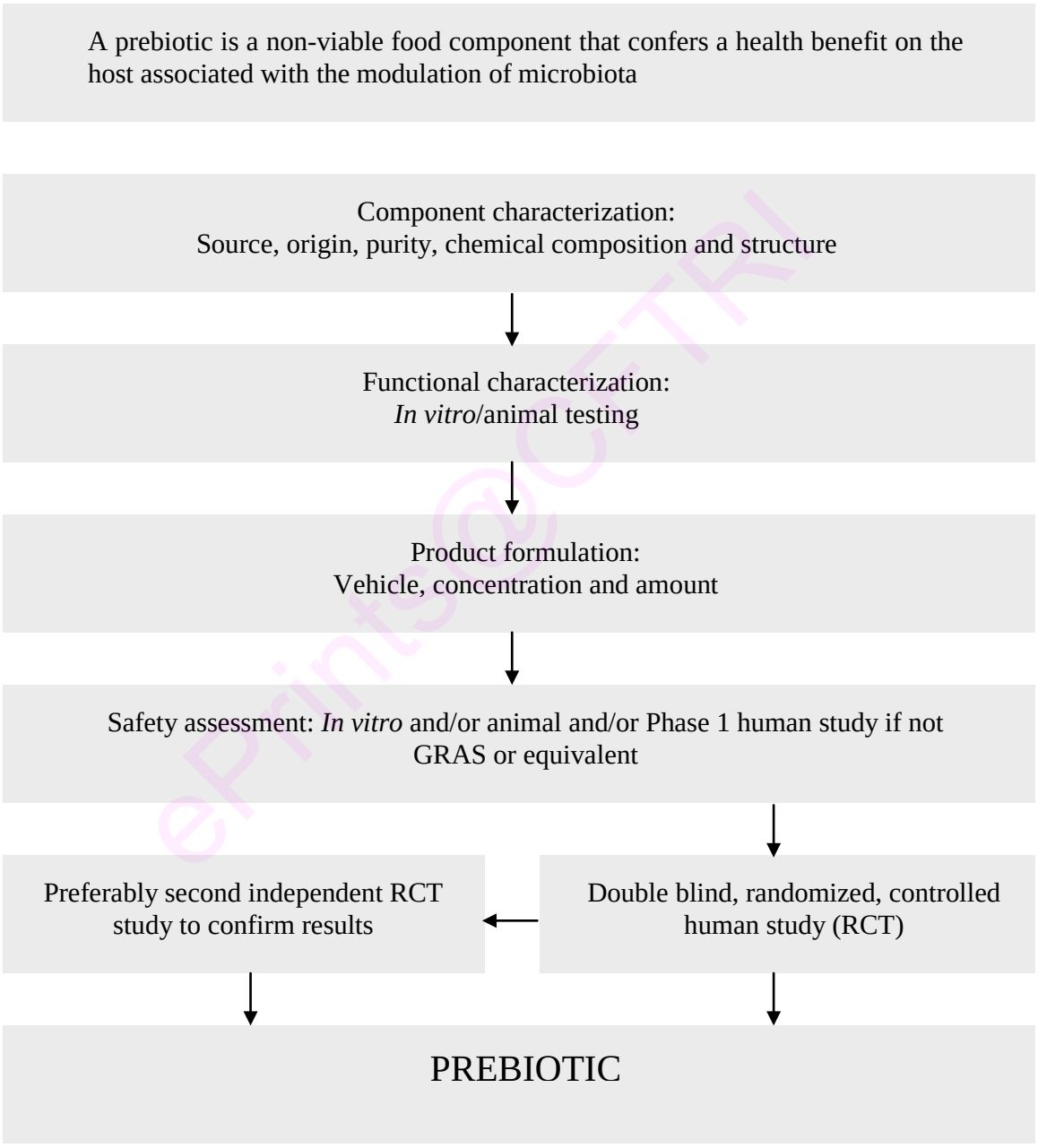



**Figure 1:** FAO guidelines for the evaluation and substantiation of prebiotics

*Source: Modified based on FAO, 2007*

**1.14. Prebiotics in human milk**

The bifidogenic effect of human milk is not related to a single growth-promoting substance, but rather to a complex of interacting factors. In particular, the prebiotic effect has been ascribed to the low concentration of proteins and phosphates, the presence of lactoferrin, lactose, nucleotides and oligosaccharides. The definite role of prebiotics is not yet clearly defined, with the exception of oligosaccharides which undoubtedly promote a bifidobacteria-dominant microflora *(Coppa et al., 2006).* In 1926 Schonfeld first demonstrated that the bifidogenic effect of mother's milk was the result of a 'non-protein fraction'. Later, Gyorgy *et al.* (1954) confirmed that the prebiotic effect was linked with an oligosaccharide mixture and Kuhn (1958) further specified that *N*-acetyl-glucosamine containing oligosaccharides were the most active. The oligosaccharides are the third major component of human milk from a quantitative point of view, after lactose and lipids. They achieve maximum concentration in the colostrums (above 20 g/l) and, after a couple of weeks, they reach stability, *i.e.* about 12−14 g/l in mature milk *(Coppa et al., 1999, Coppa et al., 1993).*

Several studies on the metabolic fate of the oligosaccharides in human milk have outlined that these substances resist the digestion *(Chaturvedi et al., 2001, Coppa et al., 2001, Engfer et al., 2000)* and reach the colon where they stimulate the development of the bifidus-predominant flora, thus representing the paradigm of prebiotics *(Coppa et al., 2004).* The oligosaccharides which are not fermented in colon are excreted in the faeces and act as dietary fibres *(Coppa et al., 2000).*

No natural substance has the same biochemical composition as human milk oligosaccharides, and these cannot be synthesized in large quantities and at acceptable cost. For this reason the industry has been focusing on the production of carbohydrates which, though have a different composition, can stimulate the growth of bifidobacteria and lactobacilli in the colon, thus reproducing the prebiotic effect of human milk oligosaccharides. In particular, galactooligosaccharides, fructooligosaccharides and inulin have been used as non-digestible oligosaccharides in the supplementation of infant formulae.

**1.15. Prebiotics in animal nutrition**

Prebiotics seem to exert their nutritional benefits in various animal species, which by definition have an intestinal tract populated by a complex bacterial intestinal ecosystem. Incorporations of prebiotics into pig feeds have resulted in mixed but generally non-significant effects regarding beneficial modulation of microbial populations in various intestinal segments and feces of swine *(Loh et al., 2006, Mountzouris et al., 2006, Flickinger et al., 2003a, Mikkelsen et al., 2003).* However some authors have demonstrated a significant increase in bifidobacteria, lactobacilli and enterococci population in the presence of prebiotics *(Shim et al., 2005, Tzortzis et al., 2005, Smiricky-Tjardes et al., 2003).* Use of prebiotics as antimicrobial agent was reported by Naughton *et al.* and demonstrated in an *in vitro* porcine intestinal tissue model that 2.5% oligofructose was able to reduce the numbers of *Escherichia coli* and *Salmonella (Naughton et al., 2001).*

Prebiotics in broiler diets have been shown to increase lactobacilli counts in the GI tract *(Yusrizal and Chen, 2003).* It also improved weight gain, feed conversion and carcass weight. The addition of oligofructose to broiler diets may also reduce the volatile ammonia contents of feces *(Yusrizal and Chen, 2003).* Chen *et al.* (2005) demonstrated an elongation of both small and large intestine in laying hens receiving fructans supplementation. This was associated with concomitant egg production and improved feed efficiency *(Chen et al., 2005).* Moreover, fructans supplementation increased skeletal and plasma calcium levels, resulting in increased egg shell strength *(Chen and Chen, 2004).*

The effect of prebiotics, predominantly fructans on canine intestinal microbiota has been demonstrated by several authors *(Vanhoutte et al., 2005, Grieshop et al., 2004, Beynen et al., 2003, Flickinger et al., 2003b, Howard et al., 2000, Willard et al., 2000).* Supplementing canine diets with oligofructose or lactulose may lead to a raised magnesium and calcium absorption *(Beynen et al., 2002, Beynen et al., 2001).* Inulin or oligofructose fed to hyperlipidemic dogs was shown to cause a transient decrease in

circulating cholesterol *(Jeusette et al., 2004).* The prebiotic effect of oligofructose in cats has been described by Sparkes *et al.* (1998), who found increased fecal counts of lactobacilli and bacteroides and decreased fecal numbers of *Escherichia coli* and *Clostridium perfringens.* Although prebiotics are often being incorporated in commercial horse feeds or additives, there is a paucity regarding scientific literature on this subject. An excessive cecal lactate production and a decrease in pH in the gut have been reported in horses fed with an excess amount of fructans *(Al Jassim et al., 2005).*

### 1.16. Functional properties of prebiotics

A number of benefits can be ascribed to prebiotic intake and a few of the widely addressed areas of high relevance to human health are described below.

#### 1.16.1. Prebiotics and mineral absorption

There is good evidence that prebiotics increase calcium absorption in animal models *(Coudray et al., 2005, Beynen et al., 2002, Ohta et al., 1998a, Ohta et al., 1998b, Delzenne et al., 1995),* and in a few human subjects *(Abrams et al., 2005, Griffin et al., 2002, van den Heuvel et al., 1999).* Most mechanistic studies have been carried out in animal models. Under normal conditions very little calcium is absorbed in the large intestine. When a prebiotic is given, calcium absorption in the intestine increases (figure 1.2). This may be related to fermentation of prebiotics to short chain fatty acids (SCFA) that lower the intra-luminal pH, which increases the solubility of calcium and thus increase calcium absorption. The most compelling data from humans where studies have demonstrated that regular consumption of inulin-type fructans lead to increased calcium absorption in some, but not all, subjects *(Abrams et al., 2005, Griffin et al., 2003, Griffin et al., 2002)* and lead to improvements in clinically relevant outcomes including bone mineral density *(Abrams et al., 2005).* A study on subjects with ileostomies because of ulcerative colitis, almost 90% of orally administered inulin or oligofructose were recovered intact in ileostomy fluid *(Ellegard et al., 1997).* This lead to the theory that fermentation of prebiotics in the large intestine was required for them to affect mineral absorption, especially as no changes in mineral absorption were noted in subjects with ileostomies *(Ellegard et al., 1997).*

Many animal studies have shown that prebiotics can increase magnesium absorption *(Yasuda et al., 2006 Coudray et al., 2005b, Coudray et al., 2003, Beynen et al., 2002, Delzenne et al., 1995, Ohta et al., 1995, Ohta et al., 1994)* although results in humans are more equivocal *(Tahiri et al., 2001, Coudray et al., 1997).* In contrast to calcium, the rat cecum is probably an important source of magnesium absorption. Prebiotics increase magnesium absorption in the large intestine, but a simple theory of the effects of SCFA and pH on mineral solubility is probably inadequate to explain the effects of prebiotics on magnesium absorption. Some animal studies have shown beneficial effects of prebiotics on absorption of other minerals, such as iron *(Yasuda et al., 2006, Ohta et al., 1998b, Delzenne et al., 1995),* zinc *(Coudray et al., 2006, Delzenne et al., 1995)* and copper *(Coudray et al., 2006, Delzenne et al., 1995)* although human data are limited *(Ellegard et al., 1997, Coudray et al., 1997).*

*Griffin et al.* (2002) reported that a supplementation of inulin-type fructans at a dose of 40 g/day enhanced calcium absorption in young men who consumed modest amounts (approximately 0.8 g/day) of calcium. A randomized, double-blind crossover study evaluated the effect of a 9-day intervention of a 20 g/day transgalactooligosaccharides on calcium absorption using dual-method stable isotope techniques *(Griffin et al., 2003)* and the intervention showed a significant increase of calcium absorption by 16% in postmenopausal women. A most recent study on postmenopausal women who received 10 g/day of a 1:1 mixture of oligofructose and long-chain inulin fructans showed a significant increase of 8.4% in calcium and 9.5% in magnesium absorption relative to the placebo *(Ohta et al., 1998a).*

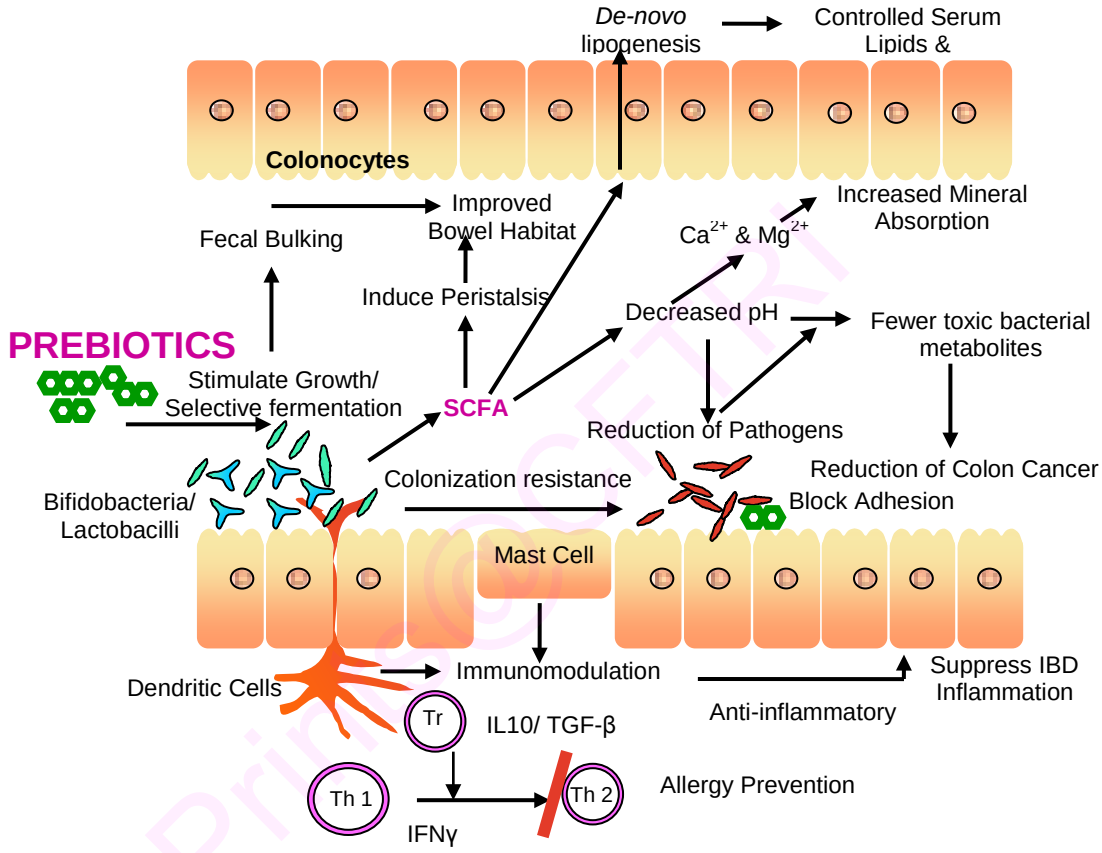



**Figure 2:** Health benefits of prebiotics

*Source: Based on Liong et al., 2007, Fava et al., 2006, Abrams et al., 2005, Beylot, 2005, Coudray et al., 2005, Daubioul et al., 2005, Beynen et al., 2002, Griffin et al., 2002, Qiao et al., 2002, Williams and Jackson, 2002, Daubioul et al., 2000, Delzenne and Williams, 1999, Fukushima et al., 1999, Murosaki et al., 1999, van den Heuvel et al., 1999, Ohta et al., 1998a, Roberfroid and Delzenne, 1998, Takahashi et al., 1998, and Mackey and Gibson, 1997.*

### 1.16.2. Prebiotics and immune function

Human intervention and animal studies indicate that prebiotics modulate immune functions. The underlying mechanisms of prebiotic-induced alterations are not yet known. Substantial experimental data suggest that prebiotics induce their immunological effects by several ways. Selective increase/decrease in specific bacteria that modulate cytokine and antibody production is found to be one such mechanism. Ingestion of bifidobacteria is associated with increased Immunoglobulin A (IgA) levels in the small intestine and feces and *ex vivo* IgA production by Peyer's patches B lymphocytes *(Qiao et al., 2002, Fukushima et al., 1999, Takahashi et al., 1998).*

Prebiotic intake enhances the production of SCFA, which are known to regulate proliferation and apoptosis of lymphocytes and monocytes and to inhibit the activity of nuclear factor kappa-light chain enhancer of B cells (NF-κB) in colonic epithelial cells *(Kurita- Ochiai et al., 2003, Millard et al., 2002, Inan et al., 2000).* In addition to NF-κB inhibition, colonic infusion of butyrate or a combination of SCFA resulted in enhanced epithelial proliferation in distant intestinal segments *(Ichikawa et al., 2002, Kripke et al., 1989)* suggesting that the production of SCFA in the colon induces physiological changes throughout the intestinal tract. *In vitro*, butyrate is known to suppress lymphocyte proliferation, to inhibit cytokine production of T helper-1 lymphocytes, to induce T lymphocyte apoptosis and to up-regulate Interleukin-10 production of dendritic cells *(Cavaglieri et al., 2003, Kurita- Ochiai et al., 2003, Millard et al., 2002, Saemann et al., 2000).* Intravenous application of pharmacological doses of acetate further enhanced natural killer (NK) cell cytotoxicity *(Ishizaka et al., 1993).* These data suggest that SCFA as fermentation products of prebiotics may affect immune cells within the gut-associated lymphoid tissue. But the mechanisms by which intraluminal SCFA are sensed by leukocytes are not completely known. Another mechanism points to interactions of prebiotic carbohydrates with carbohydrate receptors on immune cells. *In vitro*, the non-digestible oligosaccharides stimulated NK cell cytotoxicity pointing to a direct effect of these oligosaccharides on NK cells *via* specific lectin-type receptors *(Murosaki et al., 1999).*

Recently, two clinical trials reported the therapeutic outcome of a prebiotic and symbiotic treatment in subjects with ulcerative colitis and Crohn's disease. Among these, the studies by Furrie *et al.* showed that a supplementation of *Bifidobacterium longum* and oligofructose enriched inulin resulted in an improvement of the full clinical appearance of chronic inflammation in subjects with ulcerative colitis *(Furrie et al., 2006).* Lindsay *et al.* (2006) demonstrated that a daily intake of 15 g of oligofructose (70%)/inulin (30%) significantly reduced the disease activity in subjects with Crohn's disease. Several studies have looked at the effect of the combined application of probiotics with galactooligosaccharides *(Kukkonen et al., 2007, Sheih et al., 2001, Chiang et al., 2000).* The combination of four probiotics with 0.8 g/day of galactooligosaccharides reduced eczema incidences in infants *(Kukkonen et al., 2007).* The currently available human study data suggest that oral intake of prebiotics can modulate the human immune system.

**1.16.3. Prebiotics and lipid metabolism**

There are several prebiotics which exhibit interesting effects on lipid metabolism. Changes in intestinal microflora composition or fermentation activity could be implicated in modulation of fatty acid and cholesterol metabolism. There is not a single biochemical locus through which prebiotics modulates serum, hepatic and whole-body lipid content in animals. In rats fed a lipid rich diet containing 10% fructans, a decrease in triglyceridemia occurred without any protective effect on hepatic triacylglycerol (TAG) accumulation and lipogenesis, suggesting a possible peripheral mode of action *(Roberfroid and Delzenne, 1998).* A TAG lowering effect was shown in beagle dogs receiving 5% oligofructose, associated with 10% sugar beet fiber *(Diez et al., 1997).* Interestingly a TAG lowering effect of inulin-type fructans was shown in apolipoprotein-E-deficient (apoE-deficient) mice with a pronounced inhibition of plaque formation *(Rault-Nania et al., 2006).*

Several studies have also reported a decrease in total serum cholesterol after dietary supplementation with inulin (10%) in mice or rats *(Fava et al., 2006, Rault-Nania et al., 2006, Delzenne et al., 2002, Mortensen et al., 2002, Fiordaliso et al., 1995, Levrat et al.,*

*1991).* It is suggested that a decrease in serum cholesterol could reflect a decrease in TAG-rich lipoproteins which are also rich in cholesterol in apoE-deficient animals *(Rault-Nania et al., 2006).* Other interesting effects of prebiotics could be in the context of cardiovascular diseases. Busserolles *et al.* (2003) showed that oligofructose is protective against the pro-oxidative effects of fructose rich diet in rats and this could contribute to lower heart lipid peroxidation and thus could contribute to the cardiovascular effects of prebiotics. The effects of prebiotics against obesity have been studied in various animals *(Daubioul et al., 2002, Delzenne and Williams, 2002, Lopez et al., 1999)* and results showed that a supplementation of prebiotics reduced both subcutaneous and visceral fat mass.

The effects of prebiotics on blood lipids report some positive outcomes obtained from a small number of well-designed human studies *(Fava et al., 2006, Beylot 2005, Daubioul et al., 2005, Williams and Jackson, 2002, Daubioul et al., 2000, Delzenne and Williams, 1999).* No clear conclusion can be drawn concerning the influence of the duration of the treatment and the efficacy of prebiotics to lower blood lipids *(Delzenne and Williams, 2002, Delzenne and Williams, 1999).* Some studies show that the dietary supplementation with 15 or 20 g/day fructooligosaccharides for 4 weeks had no effect on serum cholesterol or triglycerides in type 2 diabetic patients *(Luo et al., 2000, Alles et al., 1999).*

### 1.16.4. Prebiotics and colon cancer

Most of the protective effects of prebiotics on colon cancer have emphasized on the oligofructose based prebiotics such as fructooligosaccharides and inulin. In one of the animal trial conducted (trial of 31 weeks), it was reported that the protective effect of probiotics on azoxymethane-induced carcinogenesis was less compared to the effects of prebiotics *(Femia et al., 2002).* Although the authors found that probiotics could reduce malignant tumors in the colon of rats, their effect was insignificant statistically, while colonic proliferation was lower when rats were fed the prebiotics. The authors subsequently conducted further studies on the expression of genes encoding enzymes involved in colon carcinogenesis processes. Glutathione *S*-transferase (GST) and GST placental enzyme pi type were found to be expressed lower when rats were fed the

prebiotic individually or fed in combination with *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12. In addition, the inducible nitric oxide synthase, found to play an important role in colon tumor growth and progression *(Ahn and Ohshsima, 2001),* was also depressed in the tumors from rats in the prebiotic group. The authors also evaluated the levels of cyclooxygenase-2, an enzyme found to be up-regulated in cancers *(DuBois et al., 1996)* and cyclooxygenase-2 inhibitors which are often associated with chemopreventive activities. Cycloxygenase-2 expression was found to be increased in the tumors of the control rats but not in those fed prebiotics. Although the exact mechanisms remain unknown, Femia *et al.* (2002) postulated that prebiotic reduced carcinogenesis *via* modification of gene-expressions.

The fermentation of prebiotics in the colon often generates SCFA. Considering that butyrate is not a common end product from the fermentation of lactobacilli and bifidobacteria, its production would originate from the fermentation by other intestinal flora. It has been found that butyrate is produced in the colon at varying concentrations depending on the type of prebiotics *(Liong et al., 2007).* Although the production of butyrate is approximately 5% of total SCFA, it is of particular interest because butyrate has been found to induce differentiation of colorectal tumor cells *(Reddy, 1999).* The reduction of colonic cell proliferation and induction of differentiation in colonic epithelial cells have now led to increased clinical trials of butyrate in the treatment of ulcerative colitis *(Reddy et al., 1997).* In addition, sodium butyrate was revealed as a powerful inhibitor of growth and inducer of phenotype differentiation and apoptosis and is considered to exert beneficial effects in reducing risk factors involved in the etiology of colon cancer and adenoma development *(Kotunia et al., 2004).* Treptow-van Lishault *et al.* (1999) found that the fermentation of gut bacteria on a retrograded, high amylose starch had produced butyrate that may increase the detoxification of both electrophilic products and compounds associated with oxidative stress. The enzyme induction by butyrate or by the microflora and increased activity by prebiotics may be an important mechanism of protection against carcinogen-enhanced colon cancer *(Wollowski et al., 2001).*

The combination of suitable prebiotics with probiotics has been found to enhance the survival and activity of the organism, both in *in vitro* and *in vivo* experiments, for example fructooligosaccharides in conjunction with a bifidobacterium strain or lactitol in conjunction with a lactobacillus *(Gibson and Roberfroid, 1995).* The combination of prebiotic and probiotic has synergistic effects because in addition to promoting growth of existing strains of beneficial bacteria in the colon, synbiotics also act to improve the survival, implantation and growth of newly added probiotic strains. The combination of bifidobacterium and oligofructose synergistically retarded colon carcinogenesis in rats compared to when both were given individually *(Gallaher and Khil, 1999).*

In a randomized, double-blind and placebo-controlled trial, Rafter *et al*. ( 2007) evaluated the effect of synbiotics on reducing cancer risk factors in 37 colon cancer patients and 43 polypectomized patients. The synbiotic contained *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 as the probiotics and oligofructose-enriched inulin as the prebiotic. The authors found that certain colorectal cancer intermediate biomarkers can be altered *via* synbiotic intervention, where colorectal proliferation and the capacity of fecal water to induce necrosis in colonic cells were reduced. In addition, polypectomized patients showed improved epithelial barrier functions. The authors conducted genotoxicity assays using the colonic biopsy samples, and found that the exposure to genotoxins in polypectomized patients decreased at the end of the intervention period. Although the exact mechanisms of these remain unknown, the authors postulated that the synbiotic intervention had contributed to the alterations in the composition of the colonic bacterial ecosystem, and subsequently the metabolic activity of the colon.

### 1.16.5. Prebiotics and effects on pathogens

Good evidence for the success of prebiotics lies in their ability to improve resistance to pathogens by increasing bifidobacteria and lactobacilli. Lactic acid producing microorganisms are known for their inhibitory properties *(Fuller, 1997).* In humans, viruses, protozoa, fungi and bacteria can all cause acute gastroenteritis. Metabolic end products, such as acids excreted by these microorganisms, may lower the gut pH to levels below those at which pathogens are unable to compete. Also, many bifidobacteria and

lactobacilli species are able to excrete natural antibiotics, which can have a broad spectrum of activity. For the bifidobacteria, some species are able to exert antimicrobial effects on various Gram-positive and Gram-negative intestinal pathogens *(Mackey and Gibson, 1997).* A recent study in mice has shown that fructooligosaccharides and inulin protected against enteric and systemic pathogens and tumor inducers *(Buddington et al., 2002).* This includes the verocytotoxin strain of *Escherichia coli* O157:H7 and campylobacters.

A rational way to reduce the food-poisoning burden may be to fortify certain components of the intestinal flora such that it becomes much more resistant to invasion. This is achievable through the use of prebiotics that target bifidobacteria and/or lactobacilli. Taking this further, some other gut-related conditions more chronic than acute gastroenteritis, additionally labeled microbiological pathogens, may also be susceptible to prevention or treatment by altering the gut flora. Examples would include ulcerative colitis, bowel cancer, peptic ulcers, pseudo-membranous colitis and Candida-induced conditions.

### 1.17. Potential food applications of prebiotics

Due to the difficulties of characterizing the colonic microflora at the species level, virtually all of the data on prebiotic properties of oligosaccharides are on microflora changes at the genus level. It may, however, be desirable to develop prebiotics which are targeted at particular species of bifidobacterium and lactobacillus. As prebiotics exploit the use as non-viable dietary components to improve gut health, the range of foods into which they can be added is much wider than that for probiotics, where culture viability needs to be maintained.

Potential applications for prebiotics as food ingredients to improve the gastrointestinal health of the consumer are beverages and fermented milks, health drinks, bakery products, table spreads, sauces, infant formulae and weaning foods, cereals, biscuits, confectionery, cakes, desserts, snack bars, soups, salad dressings and dairy products.

### 1.17.1. Use of prebiotics in infant formulae

It has long been thought that the gut flora of the breast-fed infant is dominated by bifidobacteria and that this is not the case for formula-fed infants *(Benno et al., 1984, Cooperstock and Zedd, 1983).* This is seen to be one reason for the improved resistance of the breast-fed infants to infections. If prebiotics could be developed with particular selectivity towards those bifidobacteria that are present in the guts of breast-fed infants, a new range of synbiotic formula foods could be envisaged.

### 1.17.2. Prebiotics as functional foods for the elderly

Above the age of about 55-60 years, faecal bifidobacterial counts have been shown to markedly decrease compared to those of younger people *(Kleessen et al., 1997, Mitsuoka, 1990).* This decrease in bifidobacteria is a cause for concern as the natural elderly gut flora may have become compromised through reduced bifidobacterial numbers, resulting in a diminished ability to resist colonization with invading pathogens. Prebiotics may be potentially utilized as a dietary intervention in the attempt to restore the microflora balance of the gut in the elderly population concurrently with indirectly providing antipathogenic protection.

### 1.17.3. Synbiotics with defined health benefits

A very promising area in the development of enhanced functional food ingredients is the development of synbiotics. These are defined as a combination of a probiotic and a prebiotic *(Gibson and Roberfroid, 1995)* and there has been a lot of recent interest in the concept.

There seems little doubt that synbiotics will result in elevated levels of bifidobacteria due to the prebiotic component. A comparative *in vitro* study *(Bielecka et al., 2002)* of several strains of bifidobacterium and fructooligosaccharides and inulin concluded that strains of *Bifidobacterium longum, Bifidobacterium catenulatum* and *Bifidobacterium animalis* grew best on fructooligosaccharides with much lower growth rates seen on inulin. The selected synbiotics were then fed to rats and faces analyzed for bifidobacteria, coliforms and total cell counts. Rats that were fed fructooligosaccharides and the

synbiotics displayed higher levels of bifidobacteria and lower coliform levels. The synbiotic preparation was, however, no more effective than the prebiotic. The ability of fructooligosaccharides to promote survival of the particular species of bifidobacterium was not evaluated; however, fructooligosaccharides has been reported to increase bile resistance in bifidobacteria *(Perrin et al., 2000).* *Lactobacillus reuteri* has been investigated as a component in a synbiotic with soygerm powder *(de Boever et al., 2001).* Soygerm powder contains a few prebiotic oligosaccharides studied by others *(Rycroft et al., 2001),* but it is also a source of isoflavones, which are believed to be protective against some forms of estrogen-related cancer, osteoporosis and cardiovascular conditions. Soygerm powder (4 g/l) increased resistance of *Lactobacillus reuteri* to bile salts. In addition, the lactobacilli cleaved the isoflavone glycosides to liberate the aglycone isoflavone, increasing its bioavailability *(Izumi et al., 2000).*

One of the principal benefits of synbiotics is believed to be increased persistence of the probiotics in the GI tract. A synbiotic preparation of *Lactobacillus acidophilus* and fructooligosaccharides has been studied in an *in vitro* model of the human gut *(Gmeiner et al., 2000).* The model used was the simulated human intestinal microbial ecosystem popularly known as SHIME reactor and the synbiotics resulted in higher levels of lactobacilli (an increase of 0.89 log) in the vessel corresponding to the ascending colon. An increase in bifidobacteria was seen in the vessels corresponding to the ascending (1.27 log), transverse (0.9 log) and descending (0.47 log) colon, presumably due to the prebiotic component of the synbiotics. Increases were also seen in levels of propionate and butyrate and in β-galactosidase.

Persistence of a particular ingested strain of probiotic, *Bifidobacterium lactis* Bb-12, as a synbiotic preparation with galactooligosaccharides has been investigated using randomly amplified polymorphic DNA genotyping *(Alander et al., 2001).* A total of 30 healthy volunteers consumed prebiotics, probiotics or synbiotics in a yoghurt medium for two weeks. Volunteers consuming each supplement showed increase in lactic acid bacteria (LAB) and all of the volunteers taking the probiotic (or synbiotic) had showed the presence of *Bifidobacterium lactis* in the faecal flora. No increase in survival of the

*Bifidobacterium lactis* due to galactooligosaccharides was seen; however, as pointed out by Alander *et al., Bifidobacterium lactis* had very good survival properties to begin with. The development of synbiotics might be more important for strains of probiotic with poorer survival properties.

The ability of a synbiotic preparation with bifidobacterium with galactooligosaccharides to protect against Salmonella infection in mice has been investigated *(Asahara et al., 2001).* Mice were treated with streptomycin to compromise the gut flora by selective removal to undetectable levels of bifidobacteria, lactobacilli and enterobacteria. Feeding with *Bifidobacterium breve* at $10^8$ cfu/mouse/day or the synbiotic preparation, which additionally contained galactooligosaccharides at a concentration of 2-50 mg/mouse/day, resulted in re-colonisation of the gastrointestinal tract with *Bifidobacterium breve*. Mice fed the probiotic and synbiotic displayed reduced faecal excretion of *Salmonella enterica* serovar Typhimurium after pathogen challenge. In addition the synbiotic blocked extra-intestinal translocation of the pathogen, whereas galactooligosaccharides alone did not.

### 1.18. Global scenario of functional foods and prebiotics

The market for prebiotics is growing rapidly from a small base. Prebiotics are mainly associated with breakfast cereals, baked goods, cereal bars and baby foods, as well as some dairy products. The probiotics market has become better established, based primarily on the launch of special yogurt and fermented milk drinks

Whatever their respective merits, the European consumer currently seems keen to consume products that promise to aid digestive health. Although these markets are relatively small, both pre and probiotic foods have been showing substantial value growth rates of between 10% and 20% per year. This has to be compared to a total food market growth of only 1% to 2%. Sales of functional food and beverage in Europe have experienced considerable growth, with a doubling of sales between 2000 and 2005, at prevailing prices. RTS puts the value of European sales of functional foods at €8.0 billion, currently, and is destined to reach in excess of €10.0 billion by 2010 *(RTS Resource Ltd, 2008).* Whilst functional drinks still occupy the largest market share (at

around 50% of all sales) probiotics (mainly dairy products) and prebiotics (comprising mainly dairy products, cereals and baked goods) are the next largest sectors.

Across Europe, the probiotic industry accounts for more than €1.4 billion at consumer prices, whilst the prebiotics sector is valued at €0.9 billion *(RTS Resource Ltd, 2008).* RTS calculates that the strict market for added prebiotic ingredients in functional foods, as defined, in the EU, USA and Asia, totals some 25,000 tones, which is forecast to rise in volume by more than 6% per year. However, the European market remains relatively small at €880 million in terms of finished product sales values. Market potential though is considered high. Growth could occur at an even higher rate than the predictions if the industry develops more new products and markets them successfully, given legislative restrictions *(RTS Resource Ltd, 2008).*

**1.19. Indian scenario of functional foods and prebiotics**

Large global food companies, which are always on the lookout for ways to diversify their product line, have set up functional food or nutraceutical divisions. Pharmaceutical companies are now adopting the nutraceuticals and the recent trend is convergence of food manufacturing companies with pharmaceuticals to implement the research necessary for drug discovery; the move into the less expensive and time consuming nutraceuticals research process. It is thus becoming a logical progression for many food companies to enter into nutraceuticals market.

Along with the growing healthcare industry there is an emerging trend in 'Fast Moving Healthcare Goods' in India; worldwide known as nutraceuticals, which are by definition, ingredients with human health benefits beyond basic nutrition. According to Cygnus estimates *(Cygnus, 2008),* nutraceuticals market in 2007 was INR18.75 billion and expected a Compound Annual Growth Rate (CAGR) of 20% to achieve a market size of INR27 billion in 2009. Global nutraceuticals market is estimated at USD120 billion in 2007 with a CAGR of 7%. The US has been the major market for nutraceuticals with India and China becoming fastest growing market. Nutraceuticals are gaining acceptance for their ability to address several diseases. Vitamins, minerals and nutrients constitute

about 85% of the market while antioxidants and anti-agents account for 10% other segments such as herbal extracts occupy 5% of the market, globally. Cygnus has considered nutraceuticals along with functional foods to estimate the total market of nutraceuticals, both global and Indian market. The market of probiotics and prebiotics in India is still at its emerging phase. A few fructooligosaccharides based products are available in Indian consumer markets and are getting more attention by the health conscious individuals day by day.

**1.20. Scope of investigation**

The research program on 'Bioactive xylooligosaccharides from corncob: enzymatic production and application' is based on the following facts.

1. Prebiotics have great potential as agents to improve or maintain a balanced intestinal microflora to enhance health and well being. They can be incorporated into many food stuffs. There are, however, several aspects that still need to be studied including novel economic processes for their production, their use in the existing foods to improve quality attributes etc.
2. The evidence for prebiotic related effects of XOS is still not sufficient. As an emerging food ingredient, more studies are required to ascertain XOS, the status of a prebiotic. Detailed experiments are required to establish their functional properties *in vivo*.
3. Even though, there are a variety of processes available for the production of XOS, enzymatic production of XOS from an abundantly available xylan rich agricultural by-product is advisable in food industry, because it eliminates the traditional problems associated with production of XOS based on chemical methods.
4. In India the production of maize is at an increasing phase and 15.1 million tones of maize have been produced in 2007-2008. It is estimated that for every 100 kg of corn grain, approximately 18 kg of corncob is produced as the major by-product and the production of maize will rise to 26.2 million tones in the next 12 years, which will result in the accumulation of a large quantity of corncob. New strategies are envisaged in various directions for the value addition of corncob.
5. Corncob is one of the richest source of xylan and can be used an inducer for endoxylanase production by microorganisms and also as a substrate for the enzymatic production of XOS.

The work presented in the thesis describes the possibilities of value addition of corncob for xylanase and XOS production. The effects of XOS on the reduction of

colorectal cancer models and use of XOS in *idli,* an Indian traditional food for the reduction of fermentation time and improvement of quality attributes are also discussed. These specific aspects are covered in the thesis under the following headings.

**Chapter 1:** Introduction

Prebiotics: Specific colonic nutrients

**Chapter 2:** Review of literature

Xylooligosaccharides: An emerging prebiotic

**Chapter 3:** Evaluation of microbial xylanases for the production of xylooligosaccharides

**Section 1:** Production of xylooligosaccharides from glucurono-xylan using corncob-induced endo-1, 4-β-D-xylanase

**Section 2:** Molecular cloning and sequencing of endoxylanase gene of *Aspergillus oryzae* MTCC 5154

**Chapter 4:** Enzymatic production of xylooligosaccharides from corncob

**Section 1:** Evaluation of pretreatment of corncob for enhanced availability of xylan for xylooligosaccharides production

**Section 2:** Bioconversion of pretreated corncob to xylooligosaccharides using endoxylanase from *Aspergillus oryzae* MTCC 5154: Optimization, purification and characterization

**Chapter 5:** *In vivo* investigations on the effect of xylooligosaccharides in the reduction of DMH induced colon cancer

**Chapter 6:** Effects of xylooligosaccharides on the quality attributes of *Idli*, a cereal-legume based Indian traditional food

**Chapter 7:** Summary and conclusions

Bibliography

# CHAPTER: 2

## REVIEW OF LITERATURE
## XYLOOLIGOSACCHARIDES: AN EMERGING PREBIOTIC

**Overview**

The aim of the chapter is to provide a clear, comprehensive, rigorous and balanced introduction to xylooligosaccharides (XOS). This chapter includes a brief look at structural aspects of xylan, various xylan rich lignocellulosic materials and potential of corncob as a xylan source. Yet the focus of this chapter is on XOS as an emerging prebiotics. This chapter is framed to compile the information on XOS production with a special interest on their microbial production and utilization. The chapter also covers a detailed search on the health benefits of XOS and their food and non-food applications. It also enables the basic ideas to be integrated in the rest of the thesis. The chapter also covers the structural characterization of XOS and discusses their market scenario as a functional food ingredient.

**2.1. Xylan: Structural features**

The cell wall of woody tissues of higher plants, particularly of hardwoods and softwoods, consists of cellulose and other plant polysaccharides (so-called hemicelluloses) as well as lignin *(Klemm et al., 1998).* While for cellulose, the main cell wall constituent is a highly uniform β-1→4-linked polyglucan, hemicelluloses represent polysaccharides of different structure containing glucose, xylose, mannose, galactose, arabinose, fucose, glucuronic acid and galacturonic acid in various amounts or traces depending upon the natural source. Xylans are the most common hemicelluloses and they are considered to be the second most abundant biopolymer in the plant kingdom. The xylan-type polysaccharides are known to occur in several structural varieties in terrestrial plants *(Wilkie, 1979)*, algae *(Painter, 1983)* and even in different plant tissues within one plant. The structural diversity of xylans is related to their functionality in plants and may explain the distribution of certain xylan types in the plant kingdom. The occurrence of xylans can be traced up to the botanically oldest plant families.

Homoxylans (X) with β-(1→3) glycosidic linkages, are known to substitute cellulose in the cell wall architecture of green algae (*Caulerpa* sp.), whereas homoxylans with mixed β-(1→3) and β-(1→4) glycosidic linkages (Xm) are known cell wall components of red seaweeds of the *Palmariales* and *Nemaliales (Painter, 1983).* Xylans of all higher plants possess β-(1→4) linked *Xylp* units as the backbone, usually substituted with sugar units and *O*-acetyl groups *(Stephen, 1983)*. In the wood of dicots, plants of the highest evolutionary level, only the 4-*O*-methylglucuronoxylan (GX) type (figure 2.1) was found to be present which contains single side chains of 2-linked 4-*O*-methyl-α-D-glucopyranosyl uronic acid units (MeGA). Arabino (glucurono) xylan (AGX) types containing single side chains of 2-*O*-linked α-D-glucopyranosyl uronic acid unit (GA) and/or its 4-*O*-methyl derivative (MeGA) and 3-linked α-L-arabinofuranosyl units (figure 2.2) are typical of softwoods and the lignified tissues of grasses and annual plants.

Neutral arabinoxylans (AX) with *Xylp* residues substituted at position 3 and/or at both positions 2 and 3 of *Xylp* by α-L-*Araf* units represent the main xylan component of cereal grains. Highly branched water soluble AX (figure 2.3) differing in frequency and distribution of mono- and di-substituted *Xylp* residues, are present in the endospermic as well as pericarp tissues *(Vinkx, 1996, Izydorczyk, 1995).* In the last decade, many detailed structural characteristics of water soluble- and water insoluble-AX of various cereal grains of commerce (wheat, rye, barley and oat) have been reported *(Izydorczyk et al., 1998, Debyser et al., 1997, Vinkx, 1996, Izydorczyk, 1995, Westerlund et al., 1993).* In particular, attention has been paid to the water-unextractable AX *(Harkonen et al., 1997, Nilsson et al., 1996),* which has similar but stronger bread improving properties than their water extractable counterparts. They exhibit relatively low contents of *Araf* residues which are positioned mainly on mono-substituted *Xylp* residues and are of the water insoluble-AX type (figure 2.4).

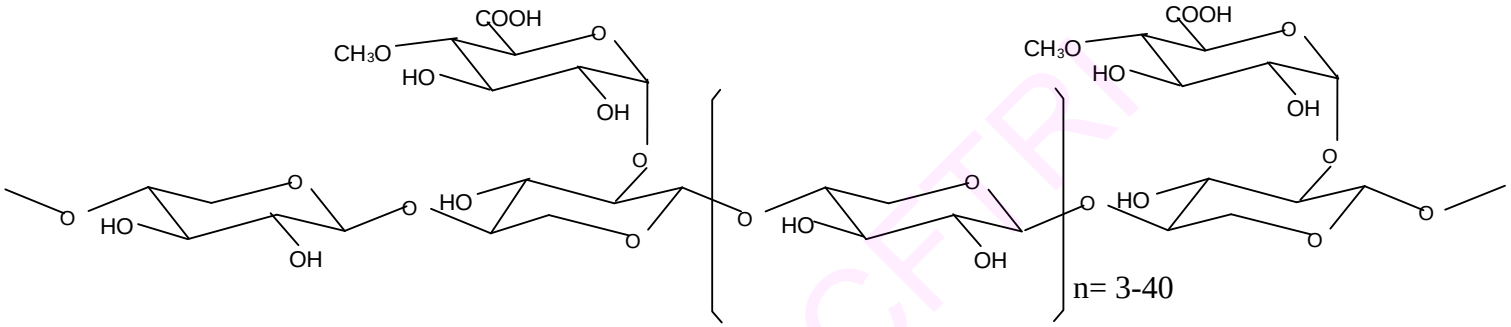



**Figure 1.1:** 4-*O*-methylglucuronoxylan (GX)

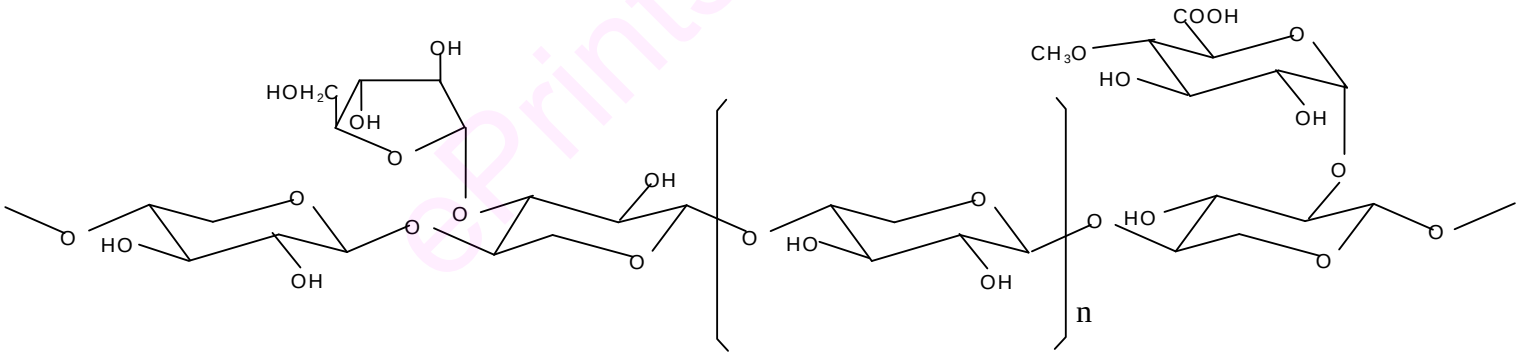



**Figure 2.2:** Arabino (glucurono) xylan (AGX)

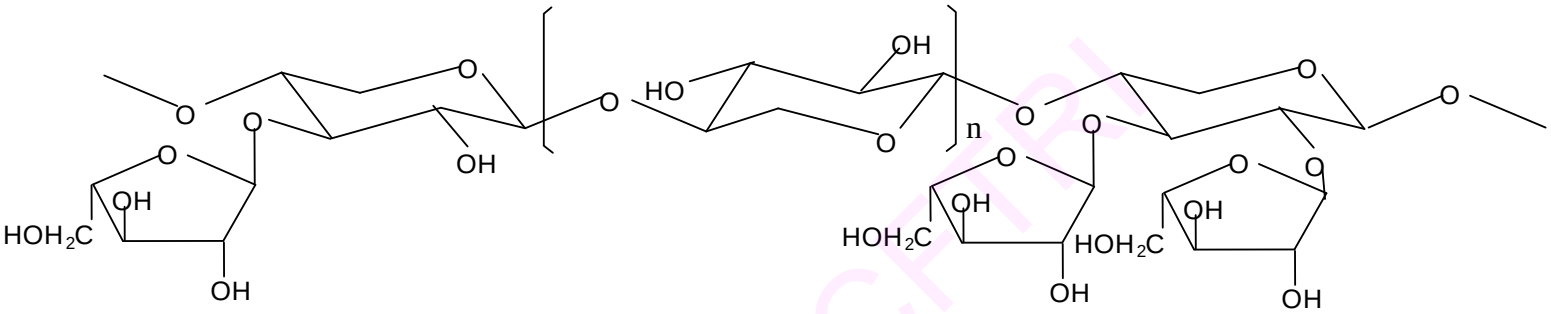



**Figure 2.3:** Branched water-soluble arabinoxylan

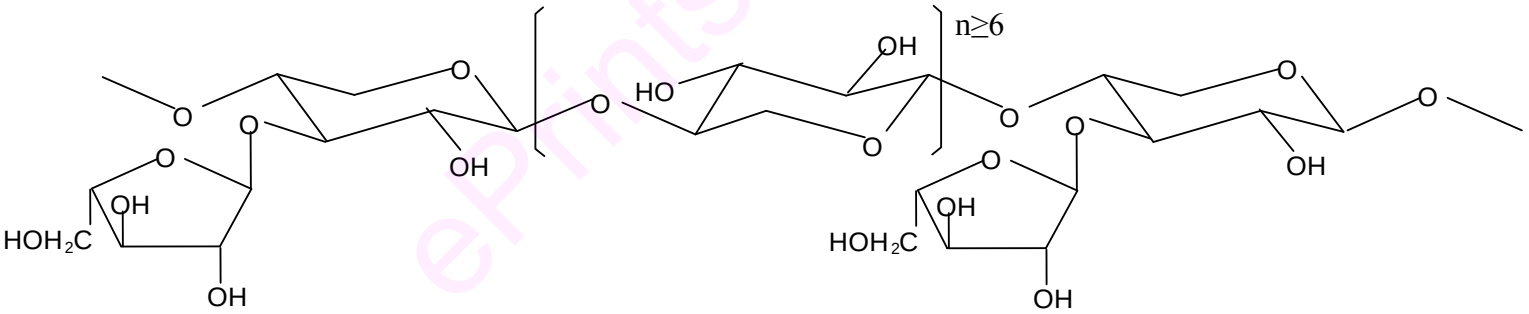



**Figure 2.4:** Water-unextractable arabinoxylan

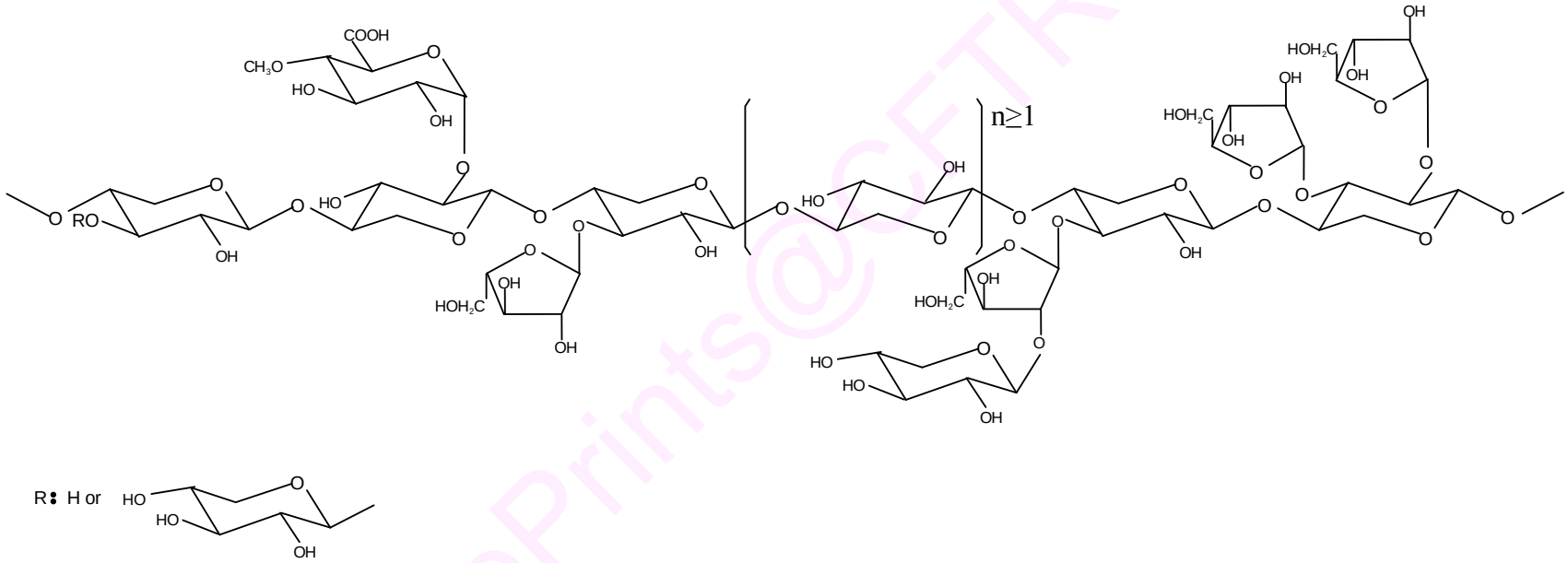



**Figure 2.5:** Water-soluble corncob xylan

**2.2. Lignocellulosic materials (LCMs) as potential sources of xylan**

Various LCMs such as wood meal and shavings, forest chips and annual plant crops (straw, stalks, husks, bran, hulls, etc.) are potential sources of xylans. In principal, xylans can be prepared by extraction from plant materials or they appear as by-products of various technologies of wood and annual plants processing. Typical examples of the latter are the hemicelluloses from the viscous process of the rayon fiber technology *(Lenz et al., 1984)* and from xylitol production *(Paronen et al., 1985).*

The richest sources of xylans are represented by the woody tissues of dicots and non-graminaceous monocots (grasses), where heteroxylans of the GX and AGX types comprise 25-35% of the biomass *(Thomas, 1977),* as well as seeds and cereal grains which contain 30-50% of the AX, AGX and HX types *(Wilkie, 1979).* Several fiber crops of agricultural plants available in huge amounts have been reported as xylan sources such as wheat straw *(Sun et al., 1998),* corn cobs *(Silva et al., 1998, Ebringerova et al., 1992),* sweet sorghum stalks *(Billa et al., 1997),* sunflower hulls *(Bazus et al., 1993),* red gram *(Swamy and Salimath, 1990),* bagasse *(Saavedra et al., 1986)* and husks of guar seeds *(Sajjan and Salimath, 1986).* Xylans have been isolated from sisal *(Stewart et al., 1997),* fibers of kenaf *(Neto et al., 1996),* flax *(van Hazendonk et al., 1996),* steamed bamboo grass *(Aoyama et al., 1995),* ramie fibers *(Bhaduri et al., 1995),* olive pulp *(Coimbra et al., 1994)* and jute bark *(Begum et al., 1993).* Corn fibers contain more than 50% of a highly branched, viscous heteroxylan, based on the dry, starch-free fiber *(Doner and Hicks, 1997).* It is commonly referred to as corn fiber gum, applicable as replacement for currently used food gums, thickeners and adhesives *(Whistler, 1993).*

**2.3. Corncob as a potential source of xylan**

Corncob is the central core of a maize (*Zea mays* ssp. mays L.) ear (figure 2.5). As the plant matures, the cob becomes tougher until only kernels are edible and when harvesting corn, the cob is collected as a part of ear. Corncobs contain approximately 35% xylan *(Tan et al., 2008, Yang et al., 2005)* and are important by-product of the corn industry that is used either as animal feed or returned to the harvested fields. Corncobs are reported as an excellent substrate for the growth of various industrially important bacteria

and fungi for the production of pharmaceutically and nutraceutically important enzymes. Alkali pretreated corncobs are used as a carbohydrate source for bacterial protein production *(Pece et al., 1994).* Corncobs are relatively new candidate for water decontamination *(Billon et al., 2006)* and studies were carried out for the removal of selected metal ions from aqueous solution using modified corncobs *(Vaughan et al., 2001).* Enough scope exists for value addition to corncob and its utilization for food applications such as production of oligosaccharides, xylose, xylitol and xylanase. The efficiency of corncob in the maximum induction of xylanase production over other complex carbon sources such as sawdust, wheat bran, rice bran and bagasse has also been reported. There are a few reports on the production of oligosaccharides from corncob using different strategies *(Tan et al., 2008, Parajo et al., 2004, Garrote et al., 2002).*

A peculiarity of the water-soluble corncob xylan (water soluble- AGX, figure 2.6) is the presence of terminal β-D-xylopyranose residues and disaccharide side chains composed of 2-*O*-β-D-xylopyranosyl-α-L-arabinofuranose next to the single *Araf* and MeGA side chains *(Ebringerova et al., 1998, Ebringerova et al., 1992, Kusakabe et al., 1983, Stephen, 1983, Wilkie, 1979).* This disaccharide, usually esterified by ferulic acid (FA) at position *O*-5 of the *Araf* unit, is a widespread component of grass cell walls *(Ishii, 1997, Wende, 1997).* The FA-containing water soluble-AGX was isolated by ultrasonically assisted extraction of corncobs with water and diluted alkali hydroxide solution *(Hromadkova et al., 1999).*

**Figure 2.6:** Corncobs

**2.4. Xylooligosaccharides (XOS): Natural sources and diversity**

XOS are sugar oligomers made up of xylose units, which appear naturally in bamboo shoots, fruits, vegetables, milk and honey. However, there is no report available on the exact quantity of XOS present in these sources. Depending upon the various xylan sources used for XOS production, the structure of XOS vary in its degree of polymerization (DP), monomeric units and type of linkages (figure 2.7). Generally XOS are mixtures of oligosaccharides formed by xylose residues linked through β-(1→4)-linkages. The number of xylose residues involved in the XOS formation can vary from 2-10 and they are named as xylobiose, xylotriose and so on. For food applications, xylobiose (DP=2) is considered to be an XOS, even if for other purposes the concept 'oligo' is associated with higher DP. In addition to xylose residues, the xylan is usually decorated with other side groups such as α-D-glucopyranosyl uronic acids or its 4-O-methyl derivative, acetyl groups or arabinofuranosyl residues. The presence of these side groups results in branched XOS with diverse biological properties.

**2.5. XOS production strategies**

XOS are produced from xylan containing LCMs by chemical methods, direct enzymatic hydrolysis of a susceptible substrate *(Katapodis and Christakopoulos, 2005, Izumi et al., 2004, Vardakou et al., 2004, Christakopoulos et al., 2003, Izumi et al., 2002, Katapodis et al., 2002)* or a combination of chemical and enzymatic treatments *(Yang et al., 2005, Izumi et al., 2004, Kokubo et al., 2004, Yuan et al., 2004, Ikemizu and Azumi, 2002, Izumi and Azumi, 2001).* The production of XOS with chemical methods can be accomplished by steam, diluted solutions of mineral acids or alkaline solutions. Extraction of xylan with steam or acid produces large amounts of monosaccharides and their dehydration products *(Nabarlatz et al., 2006, Yang et al., 2005, Yuan et al., 2004).*

Steam or hydrolytic degradation of xylan, known as autohydrolysis, involves the deacetylation of xylans to produce acetic acid, which hydrolyzes the hemicellulose *(Garrote et al., 2002, Kabel et al., 2002, Garrote et al., 1999).* This method eliminates the use of corrosive chemicals for the extraction of xylan. However, it requires special

equipment that can be operated at high temperatures. The production of XOS with direct enzymatic treatment of xylan-containing materials is suitable only for susceptible materials such as citrus peels *(Alonso et al., 2003).*

### 2.5.1. Chemical methods for the production of XOS from LCMs

When the aqueous processing of xylan-containing LCMs (autohydrolysis or hydrothermal treatment) is carried out under suitable operational conditions, the hemicellulosic chains are progressively broken down by the hydrolytic action of hydronium ions (generated from water autoionization and from *in situ* generated organic acids), yielding soluble products (mainly oligosaccharides) and leaving both cellulose and lignin in solid phase with little chemical alteration. XOS have been manufactured by autohydrolysis of a variety of feedstocks including hardwoods *(Vazquez et al., 2005, Garrote and Parajo 2002),* softwoods *(Palm and Zacchi, 2003),* corncobs *(Nabarlatz et al., 2004, Garrote et al., 2002),* barley hulls and barley spent grains *(Vegas et al., 2005, Garrote et al., 2004),* brewery spent grains *(Carvalheiro et al., 2005, Carvalheiro et al., 2004),* almond shells *(Nabarlatz et al., 2005),* corn fiber *(Kim et al., 2005, Mosier et al., 2005)* and rice hulls (*Kumagai et al., 2004, Vegas et al., 2004, Vila et al., 2002).*

In autohydrolysis treatments, XOS behave as typical reaction intermediates and their maximum concentration is achieved under medium-severity conditions. The molecular weight distribution depends on both the substrate employed and the reaction conditions. Treatments of increased severity lead to decreased DP, but also to increased decomposition of XOS into xylose. Kinetic studies dealing with XOS productions from a variety of substrates have been recently reported *(Nabarlatz et al., 2005, Carvalheiro et al., 2004, Garrote et al., 2004, Nabarlatz et al., 2004, Garrote and Parajo, 2002, Vila et al., 2002).*

Production of XOS from *Eucalyptus globulus* wood samples using hydrothermal treatments under mild operational conditions has been reported *(Garrote et al., 1999).* Kinetic models were developed which describe the hydrolysis of hemicelluloses. Xylan degradation, XOS and xylose generation and xylose dehydration to furfural were

accurately described by models based on pseudo-homogeneous, first-order kinetics with Arrhenius-type temperature dependence. These models are useful for a technical evaluation of this environmentally friendly technology.

Water-soluble hemicelluloses were extracted from milled aspen wood (*Populus tremula*) employing microwave oven treatment at 180 °C for 10 min and from this extract oligo- and polysaccharides were isolated and subsequently fractionated by size-exclusion chromatography *(Teleman et al., 2000).* The polysaccharides present in the first two fractions eluted were *O*-acetyl-(4-*O*-methylglucurono) xylans. The third fraction was an oligosaccharide fraction contained acetylated XOS that might be a hydrolysis product of acetylated 4-*O*-methylglucuronoxylan.

Jacobsen and Wyman (2002) studied the effects of varying sugarcane bagasse concentrations on xylose and XOS yields in a batch reactor without adding acids or other chemicals at 200 °C. A greater drop in pH was observed at higher solids concentrations. Furthermore, only about 7-13% of the total xylose recovered in solution was as monomers at the maximum total xylose yield point, with the rest being oligomers and although monomer yields could be increased at longer hold times, overall yields declined. These results and the general yield versus time profiles are consistent with the predictions of first-order models. However, a possible trend toward greater yields was observed at lower solids concentrations, but a paired difference test showed that these yield differences were only statistically significant between the extremes in biomass concentrations.

In another study, brewery's spent grain was treated with water in a process oriented towards the production of XOS *(Carvalheiro et al., 2004).* A wide range of temperatures and reaction times were tested and the effects of these operational variables on hemicellulose solubilization and reaction products were investigated. The maximal XOS yield (61% of the feedstock xylan) was obtained at 190 °C after 5 min of reaction. Several oligosaccharide mixtures with different molecular weight distributions were obtained depending on temperature and reaction time. Longer reaction times led to

decreased oligosaccharide production and enhanced concentrations of monosaccharides, sugar decomposition products and acetic acid. From the composition of processed solids, it was calculated that 63-77% of the initial xylan was selectively solubilized in autohydrolysis treatments.

Xylan isolated from the steeping-lye was subjected to hydrothermal degradation for production of XOS *(Griebl et al., 2006).* The experiments were carried out at 120, 150 and 180 °C. This hydrothermal treatment led to a soluble fraction, consisting of neutral and acidic XOS and an insoluble residue predominantly made up of highly crystalline cellulose. A mass balance was established to calculate the activation energy for hydrothermal xylan degradation from weight loss kinetics. The results suggested that the DP of the neutral product fraction could be influenced in a wide range by the reaction conditions applied.

In a recent study, samples of *Arundo donax* were subjected to isothermal autohydrolysis for the production of XOS *(Caparros et al., 2007).* The effects of operational variables on the yield and composition of both liquid and solid phases obtained after the treatments have been studied. The oligomers concentration and composition have been determined. In the conditions leading to maximum oligomers concentration, it can produce up to 17.7 g oligomers/100 g raw material and four acetyl groups/10 xylose monomers. These oligomers are the mean of 50% of non-volatile compounds. In these conditions, cellulose is almost quantitatively retained in the solid phase, whereas lignin is solubilized at 9%.

Partially *O*-acetylated XOS isolated from almond shells by autohydrolysis as well as their de-acetylated form were subjected to chemical, molecular and structural analysis *(Nabarlatz et al., 2007).* They represent a mixture of neutral and acidic oligomers and low molecular weight polymers related to (4-*O*-methyl-D-glucurono)-D-xylan. Later, Nabarlatz *et al.* have studied the production of XOS from almond shells by autohydrolysis at 150-190 °C *(Nabarlatz et al., 2008).* The yield, composition and molar mass distribution of XOS were dependent on temperature and time. The results showed that the maximum yield of XOS increased from 42% at 150 °C and 300 min to 63% at

190 °C and 19 min, while their anhydroarabinose-to-anhydroxylose and acetyl-to-anhydroxylose mass ratios were 0.039 and 0.076 at 150 °C and 0.129 and 0.125 at 190 °C, respectively.

### 2.5.2. Enzymatic methods for the production of XOS

#### 2.5.2.1. Endoxylanase-based production of XOS

To produce XOS with chemical and enzymatic methods, xylan is generally extracted with an alkali such as KOH or NaOH from suitable LCMs and extracted xylan is converted to XOS by xylanase enzyme having low exo-xylanase and/or β-xylosidase activity. In contrast to autohydrolysis, this method is more desirable because it does not produce undesirable byproducts or high amount of monosaccharides and does not require special equipments. Therefore, there are many papers that describe production of XOS by enzymatic hydrolysis of xylan from oat spelt *(Chen et al., 1997),* beech wood *(Freixo and Pinho, 2002),* corncob *(Yoon et al., 2006, Ai et al., 1991, Pellerin et al., 1991),* wheat straw (*Swennen et al., 2005, Zilliox and Debeire, 1998)* and hardwood *(Nishimura et al., 1998).*

Acidic XOS were obtained from birch wood xylan by treatment with family 10 endoxylanases from *Thermoascus aurantiacus* and family 11 endoxylanase from *Sporotrichum thermophile (Christakopoulos et al., 2003).* The main difference between the products liberated by these xylanases concerned the length of the products containing 4-*O*-methyl-D-glucuronic acid. The xylanase from *Thermoascus aurantiacus* liberated an aldotetrauronic acid from glucuronoxylan as the shortest acidic fragment in contrast with the enzyme from *Sporotrichum thermophile*, which liberated an aldopentauronic acid.

The recombinant xylanase B (XynB) from a hyperthermophilic Eubacterium, *Thermotoga maritima* is not only an extremely thermostable enzyme but also stable in the neutral to alkaline region. Jiang *et al.* (2004) demonstrated that XynB exhibited the highest activity towards the beech wood xylan and a low activity towards carboxy methyl cellulose. XynB hydrolyzed XOS and xylans to yield predominantly xylobiose as end product, suggesting it was an endoxylanase. Therefore, the enzyme could be used for the

large-scale production of xylobiose from xylans. Jiang *et al.* concluded that the recombinant XynB from *Thermotoga maritima* could be of commercial interest in the near future for the large scale production of xylobiose.

A 47 kDa xylanase from *Streptomyces olivaceoviridis* E-86 was immobilized on Eudragit S-100 for the production of XOS and an immobilization efficiency of 90% was obtained *(Ai et al., 2005).* The immobilized xylanase was used for hydrolyzing the corncob powder pretreated with 2% NaOH solution for XOS production. The final extent of xylan hydrolysis using pretreated corncob powder was 84% for the immobilized enzyme after 24 h of incubation at 55 °C. The immobilized xylanase retained 81% of its initial hydrolysis activity even after being recycled four times. Therefore, Ai *et al.* concluded that the immobilized xylanase is suitable to produce XOS from corncob powder pretreated with dilute alkaline solution.

In another study, three commercial xylanase preparations (Rapidase Pomaliq from Gist-brocades, Clarex ML from Genenor and Validase from Valley Research) were evaluated as a sole enzyme source for the enzymatic production of pentoses from the hemicellulose fraction of corn husks and corncobs *(Yoon et al., 2005).* The hemicellulose fraction was obtained by extracting corn husks or corncobs with 1.25 mol/l NaOH and the alkaline extract was used as a substrate for enzymatic hydrolysis. Rapidase Pomaliq was found to yield significantly more pentoses from corn husks and corncob than Clarex ML or Validase. Rapidase Pomaliq was capable of increasing the concentration of pentoses from an initial value of 106.5 to 210.6 g/kg dry matter of corn husks or 8.6 to 141.6 g/kg dry matter of corncobs, respectively under favorable conditions (480 min of reaction at pH 5.0 and 50 °C). Products of the enzymatic reaction were identified as arabinose, xylose, xylobiose and xylotriose. The results indicated that Rapidase Pomaliq, an enzyme preparation derived from *Aspergillus niger* and *Trichoderma reesei*, could serve as a sole enzyme source for the production of pentoses and XOS from corn residues.

Enzymatic production of XOS from cotton stalks has been reported *(Akpinar et al., 2007).* Akpinar *et al.* demonstrated that cotton stalk, which had no economical value,

could be converted by enzymatic hydrolysis to a more valuable XOS product without production of significant quantities of xylose after extraction of its xylan. A two-step ultrafiltration process, using membranes of 10 and 3 or 1 kDa cutoff, fractionated XOS syrup without much loss.

Yang *et al.* (2007) reported the production of XOS from xylans by extracellular xylanases from *Thermobifida fusca* NTU22. In this process, lignocellulosic agricultural waste was used to induce *Thermobifida fusca* for production of the xylanolytic enzymes such as xylanase, β-xylosidase and acetyl esterase. A heat treatment of the crude enzymes at 70 °C for 30 min selectively inactivated 90% of the β-xylosidase activity and this heat-treated crude xylanase preparation was successfully used for the production of XOS.

The mature peptide of *Bacillus licheniformis* xylanase A (BlxA) was successfully expressed in *Pichia pastoris* under the control of AOX1 promoter *(Liu and Liu, 2008).* This is the first report on the expression of reBlxA in yeast and on determining and quantifying the hydrolysis products released from xylans by reBlxA. Xylotriose was the major product from birch wood xylan and wheat bran insoluble xylan by reBlxA, which made it potentially suitable for production of XOS. $X_2$-$X_6$ could be further hydrolyzed by reBlxA and only trace amount xylose was detected among hydrolysis products of $X_2$-$X_6$ by reBlxA. The main products of hydrolysis of $X_4$, $X_5$ and $X_6$ by reBlxA were $X_2$, $X_3$, and $X_3$, respectively. These results revealed that reBlxA preferentially cleaved the internal glycosidic bonds of XOS and it was an endoxylanase *(Liu and Liu, 2008).* Endo-mode enzyme shows low susceptibility of substrate of DP=2, such as xylobiose, chitobiose and maltose *(Biely et al., 1981).* Jiang *et al.* reported that the xylobiose degradation might proceed by a transglycosylation reaction *(Jiang et al., 2004).* No XOS with DP>2 was detected in the hydrolysis product mixture of $X_2$ by reBlxA. The results suggest that reBlxA might directly hydrolyze the $X_2$, which is similar to those of xylanases from *Thermomonospora fusca* and *Aspergillus sojae (Sun et al., 2007, Kimura and Tajima, 1998).* Production of acidic XOS by a family 10 endoxylanase from *Thermoascus aurantiacus* has also been reported *(Katapodis et al., 2002).*

**2.5.2.2. β-xylosidase-based production of XOS**

A few studies have reported the enzymatic synthesis of various alkyl-β-xylosides by application of the transxylosylation of β-xylosidase enzymes *(Shinoyama et al., 1988, Shinoyama and Yasui, 1988).* The β-xylosidase from *Aspergillus niger* IFO 6662 has a strong transxylosyl activity and has been reported to produce a novel non-reducing disaccharide *(Yasui et al., 1989).* Synthesis of XOS from β-(1→4)-xylobiose in the presence of D-mannose by transxylosylation with β-xylosidase from *Aspergillus niger* IFO 6662 has also been reported *(Kizawa et al., 1991).* In this study, transxylosylation with β-xylosidase has resulted in two xylosylmannoses and non-reducing XOS. This XOS has been identified by Proton-nuclear magnetic resonance spectrometry ($^{1}$H-NMR) as *O*-β-D-xylopyranosyl-(1→1')-β-D-xylopyranose, which is a novel xylobiose.

**2.5.2.3. Glycosynthase-based production of XOS**

Glycosynthases are synthetic enzymes derived from retaining glycosidases in which the catalytic nucleophile has been replaced. The mutation allows irreversible glycosylation of sugar acceptors using glycosyl fluoride donors to afford oligosaccharides without any enzymatic hydrolysis. Glycosynthase technology has proven fruitful for the facile synthesis of useful oligosaccharides; therefore the expansion of the glycosynthase repertoire is of the utmost importance. Kim *et al*. (2006) described for the first time a glycosynthase derived from a xylanase that synthesizes a range of XOS. The catalytic domain of the retaining endo-1,4-β-xylanase from *Cellulomonas fimi* (CFXcd) was successfully converted to the corresponding glycosynthase by mutation of the catalytic nucleophile to a glycine residue. The mutant enzyme (CFXcd-E235G) was found to catalyze the transfer of a xylobiosyl moiety from α-xylobiosyl fluoride to either *p*-nitrophenyl-β-xylobioside or benzylthio-β-xylobioside to afford oligosaccharides ranging in length from tetra- to dodecasaccharides. These products were purified by high performance liquid chromatography (HPLC) in greater than 60% combined yield. $^{1}$H-NMR and $^{13}$C-NMR spectroscopic analysis of the isolated *p*-nitrophenyl xylotetraoside and *p*-nitrophenyl xylohexaoside revealed that CFXcd-E235G catalyzes both the regio- and stereo-selective synthesis of XOS containing exclusively β-(1→4) linkages.

In one example, the donor repertoire of the *Agrobacterium sp*. β-glucosidase was expanded through directed evolution experiments, allowing it to transfer the donor α-xylosyl fluoride to various aryl glycosides *(Kim et al., 2006, Kim et al., 2004).* In another case, Honda and Kitaoka (2006) were able to produce xylotriose with α-xylobiosyl fluoride as a donor and xylose as an acceptor, by using a mutant of an inverting glycosyl hydrolase family 8 (GH8) xylanase from *Bacillus halodurans.* It was also demonstrated that GH10 xylanases can serve as glycosynthase and produce XOS when α-xylobiosyl fluoride is used as a donor *(Kim et al., 2006, Sugimura et al., 2006).*

In another study, Ben-David *et al.* (2007) demonstrated that *Geobacillus stearothermophilus* GH52 β-xylosidase can function as an efficient glycosynthase, using α-D-xylopyranosyl fluoride as a donor and various aryl sugars as acceptors. The mutant enzyme can also catalyze the self-condensation reaction of α-D-xylopyranosyl fluoride, providing mainly α-D-xylobiosyl fluoride. The self-condensation kinetics exhibited apparent classical Michaelis-Menten behavior, with kinetic constants of 1.3/s and 2.2 mm for $k_{cat}$ and $K_{M(acceptor)}$, respectively and a $k_{cat}/K_{M(acceptor)}$ value of 0.59/s/mm. When the β-xylosidase E335G mutant was combined with a GH10 glycosynthase, high-molecular weight XOS were readily obtained from the affordable α-D-xylopyranosyl fluoride as the sole substrate. To date, there has been no other report of a β-xylosidase that can function as a glycosynthase.



**2.6. Purification of XOS**

When XOS are produced by water or steam treatments, a variety of other compounds such as monosaccharides, acetic acid, products derived from the extractive and acid-soluble lignin fractions of the feedstock, furfural from pentose dehydration, soluble inorganic components of the feedstock and protein-derived products appear in the reaction media. In order to produce food-grade XOS, the autohydrolysis liquors have to be refined by removing both monosaccharides and non-saccharide compounds to obtain a concentrate with an XOS content as high as possible. The usual purity of commercial XOS lies in the range 75-95%. Purification of XOS obtained by enzymatic processing of

substrates containing susceptible xylan is facilitated by the previous chemical processing of the LCMs as well as by the specific action of xylanases.

### 2.6.1. Solvent extraction and precipitation for purification of XOS

Solvent extraction is useful for removing non-saccharide components of autohydrolysis liquors *(Vazquez et al., 2005, Vegas et al., 2005, Vegas et al., 2004)* yielding both a selectively refined aqueous phase and a solvent-soluble fraction mainly made up of phenolics and extractive-derived compounds. Solvent precipitation of liquors has been employed for refining XOS using ethanol, acetone and 2-propanol *(Swennen et al., 2005, Vazquez et al., 2005, Vegas et al., 2005, Vegas et al., 2004).* The degree of purification and the recovery yields depend on the solvent employed and on the LCMs, which control the XOS substitution pattern and the possible presence of stabilizing, non-saccharide components *(Vazquez et al., 2005).* As the presence of even minimal amounts of water limits the precipitation of hemicellulose-derived products, solvent extraction of freeze-dried autohydrolysis liquors has been carried out using the same solvents employed for precipitation *(Vazquez et al., 2005, Vegas et al., 2005, Vegas et al., 2004).* The best purification effects were achieved with ethanol but the process showed limited recovery yields *(Vazquez et al., 2005).*

### 2.6.2. Adsorption for purification of XOS

Adsorption has been used in combination with other treatments for the refining of XOS intending either the separation of oligosaccharides from monosaccharides *(Sanz et al., 2005, Ohsaki et al., 2003, Vazquez et al., 2000)* or the removal of undesired compounds *(Izumi et al., 2005, Izumi et al., 2004, Kokubo et al., 2004, Yuan et al., 2004).* Activated carbons were used for the purification of XOS produced by the autohydrolysis of almond shells *(Montane et al., 2006).* Adsorption equilibrium was measured in a batch system for three commercial activated carbons using a constant concentration of 20 g/l of crude XOS and loads of activated carbon from 1.5 to 50.0 mg/ml. Adsorption for lignin-related products was higher than those for XOS. The selectivity towards lignin adsorption was better when the carbon was highly microporous and had small mesopore diameters, a low volume of mesopores, a low concentration of basic surface groups to limit XOS

adsorption and acidic surface groups to favor the adsorption of the lignin-related products. Column tests were performed at a feed rate of crude XOS solution of 6.0 ml/min (35 g/l) in columns packed with 22 g of granular activated carbon and operated in up-flow mode. Average retention was around 64% for lignin products and 21% for carbohydrates for the fraction of treated solution collected during the first 2 h of operation. Retention for lignin-derived products was limited because part of them is linked to the XOS. On the basis of the analysis of the Freundlich isotherms, the purification of XOS required activated carbons with unit-capacity and site-energy parameters that are low for XOS and high for lignin-derived products.

### 2.6.3. Chromatographic separation for purification of XOS

Chromatographic separation has been carried out for XOS purification at an analytical level yielding high purity fractions. For example, samples from hydrothermally treated LCMs have been fractionated by anion-exchange chromatography and size-exclusion chromatography (SEC) *(Kabel et al., 2002a, Kabel et al., 2002b),* whereas chromatographic techniques have been employed for refining samples before structural characterization of XOS, for example by $^{13}$C-NMR *(Christakopoulos et al., 2003)* or matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) or nanospray mass spectrometry *(Kabel et al., 2002a).* Simulated moving bed, chromatographic separation has also been proposed for purification of oligosaccharides made up of xylose and arabinose units *(Ohsaki et al., 2003),* whereas SEC has been employed in combination with other techniques for purification of feruloylated oligosaccharides *(Katapodis et al., 2003).* Jacobs *et al.* (2003) purified hemicellulose-derived products from hydrothermal microwave treatments of flax shive employing ion exchange chromatography and/or SEC in combination with enzymatic processing. Ion exchange has been employed for purification of XOS *(Vegas et al., 2005, Yuan et al., 2005, Izumi et al., 2004a, Izumi et al., 2004b, Kokubo et al., 2004, Van Thorre 2004, Vegas et al., 2004, Endo and Kuroda, 2000)* alone or in multi-step processing, looking mainly for desalination and removal of other undesired compounds.

### 2.6.4. Membrane technology for purification of XOS

Ultrafiltration and nanofiltration, well known membrane separation processes, are the most promising methods for refining and concentrating oligosaccharides. The size-dependent selection mechanism of the membrane process results in the various concentrations of molecules with different molecular weights. Membrane separations have been used for the preparation of several oligosaccharides such as fructooligosaccharides *(Li et al., 2004),* maltooligosaccharides *(Stominska and Grzeskowiak-Przywecka, 2004),* soybean oligosaccharides *(Kim et al., 2003),* pectic oligosaccharides *(Iwasaki and Matsubara, 2000)* and chitooligosaccharides *(Jeon and Kim, 1998).* Little information exists on the utilization of membrane technologies for processing XOS containing solutions and some of them deal with the processing of media obtained by a hydrolytic treatment followed by enzymatic reaction. Recently, membrane technologies have been used successfully for processing XOS produced by enzymatic hydrolysis and autohydrolysis *(Nabarlatz et al., 2006, Vegas et al., 2006, Swennen et al., 2005, Yuan et al., 2004).* In this field, Yuan *et al.* (2004) reported the manufacture of XOS at the pilot plant scale by chemical-enzymatic processing of corncobs (steaming followed by xylanase treatment) and further purification by flocculation, ion exchange, nanofiltration, charcoal adsorption and vacuum evaporation. Izumi *et al.* (2005b & 2004a) employed both reverse osmosis and ultrafiltration in the processing of XOS containing solutions coming from the xylanase treatment of pulp slurry.

Swennen *et al.* (2005) compared ultrafiltration and ethanol precipitation for fractionating arabinose-substituted XOS obtained by enzymatic processing of wheat. They found that the ultrafiltered fractions were more heterogeneous and poly-disperse and less strictly separated than the fraction obtained with the 1 and 3 kDa ultrafiltration membranes used in their study. The ultrafiltration membrane with a narrow size distribution results in more selective separation. Membrane reactors have been employed for the one-step DP reduction and fractionation of XOS *(Yang et al., 2003, Freixo et al., 2002).*

Liquors from rice husk autohydrolysis containing XOS, other saccharides and non-saccharide compounds were subjected to two selected processing schemes to increase the

proportion of substituted XOS in refined liquors *(Vegas et al., 2006).* Nanofiltration through a ceramic membrane with a molecular mass cutoff of 1000 Da allowed simultaneous concentration and purification. When liquors were nanofiltered to achieve a volume reduction factor of 5 operating at a transmembrane pressure of 14 bar, 58.6% of the non-saccharide components and 20.9-46.9% of monosaccharides were kept in retentate in comparison with 92% of XOS and glucooligosaccharides**.** When nanofiltered liquors were subjected to double ion-exchange processing, a final product with non-saccharide content of nearly 9 kg/100 kg of nonvolatile components was obtained at a yield of 10.90 kg/100 kg of oven dried rice husks. Alternatively, when nanofiltered liquors were subjected to ethyl acetate extraction and further double ion-exchange processing, a purified product with a non-saccharide content of 5.66 kg/100 kg of nonvolatile components was obtained at a yield of 9.94 kg/100 kg of oven dry rice husks. The non-saccharide components remaining in the final concentrate were mainly made up of phenolic and nitrogen-containing compounds.

A two-step ultrafiltration process has been suggested by using membranes of 1, 3 and 10 kDa cutoff for the purification of XOS obtained by the hydrolysis of cotton stalk and the process fractionated XOS syrup without much loss *(Akpinar et al., 2007).* Liquors from rice husk autohydrolysis containing XOS, other saccharides and non-saccharide compounds were refined by membrane processing to increase the proportion of substituted XOS in refined liquors. XOS were assayed for composition and DP distribution and hydrolyzed with commercial enzymes for obtaining XOS with DP in the range of 2-6 *(Gullon et al., 2008).* Nanofiltered, hydrolyzed liquors were subjected to ion exchange processing to yield a final product containing monosaccharides, XOS (accounting for 55.6% of the non-volatile solutes) and other nonvolatile compounds.

### 2.7. Degradation and utilization of XOS by microorganisms

#### 2.7.1. Degradation and utilization of XOS by probiotics microorganisms

Okazaki *et al.* (1990) employed a mixture of xylose (22%), xylobiose (58%), xylotriose (13%) and other saccharides (7%) as a carbon source for *in vitro* fermentations with *Bifidobacterium adolescentis*, *Bifidobacterium longum* and *Bifidobacterium infantis* and

assessed the degree of utilization of the various saccharides by these microorganisms. In this study, *Bifidobacterium adolescentis* showed a remarkable ability to use both xylobiose and xylotriose. Hopkins *et al.* (1998) carried out fermentations with commercial XOS (from Suntory, Japan) with 70% purity and DP 2-4 and found that the ability of bifidobacteria for growing on XOS depended on the considered strain.

The influence of oat bran oligosaccharides on carbohydrate utilization and fermentation end-products was studied with reference to three different lactic acid bacteria (LAB) such as *Lactobacillus rhamnosus, Lactobacillus plantarum* and *Lactobacillus lactis (Kontula et al., 1998).* The result showed that all three LAB utilized oat β-glucooligosaccharides, while only *Lactobacillus plantarum* utilized XOS. The main products of LAB metabolism were lactic acid, acetic acid, formic acid and ethanol. The results indicated that oat β-glucooligosaccharides and XOS induce LAB to form the end-products of typical mixed-acid fermentation. The formation of mixed-acid production from XOS was mainly due to the starvation of cells. Some strains used XOS with DP 2, 3, and 4 preferentially, whereas other strains preferred xylose. Van Laere *et al.* (2000) studied the fermentation of arabino-XOS from wheat meal and reported that branched structures show increased selectivity for bifidobacteria with respect to linear structures.

The preference of bifidobacteria to ferment low-substituted XOS, both *in vitro* and *in vivo*, has been described previously *(Campbell et al., 1997, Okazaki et al., 1990).* On the other hand, oat XOS were not selective for bifidobacteria exclusively, because *Bacteroides* spp., *Clostridium* spp., *Lactobacillus acidophilus* and *Klebsiella pneumoniae* were also showed moderate growth on these substrates *(Van Laere et al., 2000, Jaskari et al., 1998).* Also, the more branched wheat arabinoxylan hydrolysates (singly and doubly substituted arabino-XOS) could only be (partly) fermented by the *Bifidobacterium* spp. and *Bacteroides* spp. tested *(Van Laere et al., 2000).*

Kabel *et al.* (2002) studied the *in vitro* fermentability of differently substituted XOS. Their study concluded that the neutral-XOS, arabino-XOS, acetylated-XOS and acidic-XOS obtained from hydrothermally treated xylan-rich byproducts were fermented by

human fecal inoculum. For all fermentations described, a distinction could be made between the first stage of the fermentation (0-40 h) and a second stage (>40 h). In the first stage of the fermentations the pH decreased, whereas in the second stage the pH remained constant or even increased slightly. In the first stage of fermentation of neutral-XOS and arabino-XOS, mainly acetate and lactate were formed. LAB may play an important role in this part of the fermentation, as they do not produce butyrate or propionate but they do produce acetate and lactate *(Schlegel, 1993).* A high concentration of acids formed might be desirable because, by a decrease in pH, the growth of potentially pathogenic microorganisms and the growth of putrefactive bacteria will be inhibited *(Gibson and Roberfroid, 1995, Degnan and MacFarlane, 1994, Schlegel, 1993).*

The ability of bifidobacteria to metabolize XOS depends on the efficiency of their xylanolytic enzyme systems. One xylosidase and a few arabinosidases have been purified and characterized from bifidobacteria. They are β-D-xylosidase from *Bifidobacterium breve* K- 110 and arabinosidases from *Bifidobacterium adolescentis* DSM20083 *(van Laere et al., 1999, van Laere et al., 1997)* and arabinosidase from *Bifidobacterium breve (Shin et al., 2003).* The arabinosidase genes from *Bifidobacterium longum* B667 *(Margolles and De Los Reyes-Gavilan, 2003)* and *Bifidobacterium adolescentis (Van den Broek et al., 2005)* have been cloned and sequenced. Zeng *et al*. examined the xylanolytic enzyme system in *Bifidobacterium adolescentis*, *Bifidobacterium infantis* and *Bifidobacterium bifidum* to determine their ability to utilize XOS *(Zeng et al., 2007).* All these species produced xylosidase and arabinosidase; however production of xylanase, α-glucuronidase and acetyl xylan esterase were not reported. The optimal activity of β-D-xylosidase from *Bifidobacterium adolescentis* was at pH 5.6 and 45 °C and α-L-arabinosidase was at pH 5.0 and 40 °C. The degradation products of cell-free extracts and the growth rate of *Bifidobacterium adolescentis* were tested over XOS and XOS de-branched by a recombinant α-glucuronidase. The results showed that debranching α-glucuronidase increased the production of xylose and the total cell density by 10% and accelerated the growth of *Bifidobacterium adolescentis* by 20%. Thus, efficient and

complete degradation of XOS requires the cooperation of different enzymes including β-xylosidase, α-glucuronidase, α-L-arabinosidase or acetyl xylan esterase.

Strains of *Leuconostoc lactis* SHO-47 and *Leuconostoc lactis* SHO-54 were cultivated with a hydrolyzed birch wood xylan as the unique carbon source to produce D-lactic acid *(Ohara et al., 2006).* In addition to the strains SHO-47 and SHO-54, *Lactococcus lactis* IO-1, well known as a good xylose-utilizing LAB, was used as a control to confirm the extent of hemicellulose hydrolysis. The fermentation time for the production of lactic acid by strains SHO-47 and SHO-54 was 12 h and produced 2.3 and 2.2 g/l lactic acid respectively from 8.5 g/l hydrolyzed xylan, whereas the fermentation time of strain IO-1 was 21 h, and produced 1.3 g/l lactic acid. XOS from xylobiose to xylohexose were utilized more rapidly than xylose in the cultures of strains SHO-47 and SHO-54. However, xylose concentration increased temporarily and then decreased in the culture of strain IO-1. On the other hand, XOS larger than xyloheptaose were not utilized by these three strains *(Ohara et al., 2006).* The xylosidase activities of SHO-47, SHO-54 and IO-1 were induced by xylose or a mixture of xylobiose and xylotriose. The study suggested that the xylosidase enzyme of these three strains were localized in their cytoplasm.

The effects of XOS and the combined administration of XOS and a probiotic culture of *Saccharomyces cerevisiae* on the piglet intestinal microbiota were investigated *(Moura et al., 2007).* Previously, Smiricky-Tjardes *et al.* (2003) investigated the fermentation characteristics of various oligosaccharides including XOS by fecal microbes of healthy pigs. Substrates studied included short-chain fructooligosaccharides, medium-chain fructooligosaccharides, long-chain fructooligosaccharides, raffinose, stachyose, soy solubles, granular and liquid forms of transgalactooligosaccharides, glucooligosaccharides, mannanoligosaccharides and XOS. All oligosaccharides studied were readily fermentable but varied in amount and type of SCFA produced. The results indicated that total SCFA production was greatest for XOS, stachyose and raffinose + stachyose and least for mannanoligosaccharides and raffinose.

The effect of water-unextractable arabinoxylan (WU-AX) derived from wheat on the modulation of gut bacterial composition was investigated using a mixed culture fermentation system *(Vardakou et al., 2008).* The study proved that the extracellular hydrolytic enzymes such as xylanase and ferulic acid esterase are required for bacterial metabolism of WU-AX and both activities were present in supernatants derived from the mixed batch cultures. Addition of the WU-AX substrates to the batch cultures produced several fold increases of bacterial synthesis of both enzymes and these increases were greater when the WU-AX substrate was pretreated with xylanase.

Gullon *et al.* (2008) evaluated the fermentability of XOS from rice husks by probiotic bacteria. Nanofiltered, hydrolyzed liquors (in which 82.8% of XOS were in the DP range of 2-6) were examined as a medium for promoting the growth of *Bifidobacterium adolescentis* CECT 5781, *Bifidobacterium longum* CECT 4503, *Bifidobacterium infantis* CECT 4551 and *Bifidobacterium breve* CECT 4839. The growth rate of *Bifidobacterium adolescentis* (0.58/h) was higher than the ones determined for *Bifidobacterium longum*, *Bifidobacterium infantis* and *Bifidobacterium breve* (0.37/h, 0.30/h and 0.40/h, respectively). The total XOS consumption by *Bifidobacterium adolescentis* was 77% after 24 h, the highest amount of utilization corresponding to xylotriose (90%), followed by xylobiose (84%), xylotetraose (83%) and xylopentaose (71%).

### 2.7.2. Degradation and utilization of XOS by other microorganisms

The endo-1,4-β-D-xylanase of the basidiomycete *Schizophyllum commune***,** designated as xylanase A**,** was studied to determine its action pattern, rates of reaction and bond-cleavage frequencies on XOS and xylo-alditol substrates ranging in DP from xylotriose to xyloheptaose *(Bray and Clarke, 1992).* Reduced XOS were used in enzymatic reactions to determine unambiguously the exact xylosidic linkage being cleaved as well as relative rates of cleavage for each linkage under conditions of unimolecular hydrolysis (*i.e.* the bond cleavage frequency). Based on the amount of xylanase A and time required to achieve comparable degrees of reaction of the various oligomers, it appears that $X_5$ is the smallest chain length broken rapidly by the xylanase. Therefore, the nature of the binding

site is such that the binding of oligomers of DP<*5* (*i.e.* $X_4$, $X_3$ & $X_2$) in productive complexes is energetically less favorable.

Co-culture experiments with *Selenomonas ruminantium* and xylanolytic ruminal microorganisms have demonstrated the ability of *Selenomonas ruminantium* to reduce the accumulation of XOS, in some cases leading to enhancement of xylan degradation *(Williams et al., 1991).* Fermentation of XOS prepared from the partial hydrolysis of oat spelt xylan by a few strains of *Selenomonas ruminantium* was examined *(Cotta et al., 1998).* Strains of *Selenomonas ruminantium* varied considerably in their capacity to ferment XOS. Strains GA192, GA31, H18 and D used arabinose, xylose and the oligosaccharides xylobiose through xylopentaose, as well as considerable quantities of larger, unidentified oligosaccharides. Other strains of *Selenomonas ruminantium* (HD4, HD1, 20-21a, H6a, W-21 & S23) were able to use only the simple sugars present in the substrate mixture. The ability of *Selenomonas ruminantium* strains to utilize XOS was correlated with the presence of xylosidase and arabinosidase activities. Both enzyme activities were induced by growth on XOS, but no activity was detected in glucose- or arabinose-grown cultures.

It was also reported later that strains of *Selenomonas ruminantium* vary considerably in their capacity to ferment XOS *(Terence et al., 2001).* A genetic locus from *Selenomonas ruminantium* GA192 was cloned into *Escherichia coli* JM83 that produced both xylosidase and arabinosidase. Analyses of crude extracts from the *Escherichia coli* clone and *Selenomonas ruminantium* GA192 by using native polyacrylamide gel electrophoresis and methylumbelliferyl substrates indicated that a single protein was responsible for both activities. The study showed that the enzyme expressed in *Escherichia coli* was capable of degrading XOS derived from xylan.

**2.8. Transport of XOS in microbial systems**

Plant cell wall-degrading microorganisms utilize an impressive variety of modular enzyme structures and different physiological strategies for the degradation of the plant cell wall. Anaerobic bacteria such as *Clostridium* spp. have evolved unique multienzyme

complexes called cellulosomes, that integrate many cellulolytic and hemicellulolytic enzymes that mediate the attachment of the cell to the crystalline polymer and its controlled hydrolysis *(Adelsberger et al., 2004, Han et al., 2004, Shallom et al., 2004, Shoham et al., 1999).* Aerobic fungi such as species of *Trichoderma* and *Aspergillus* secrete numerous cellulases and hemicellulases that work synergistically to completely degrade the polymers into mono- or disaccharides which may be utilized by the surrounding microorganisms *(de Vries et al., 2001).* Lastly, aerobic bacteria such as *Bacillus* and *Cellvibrio* (formerly *Pseudomonas fluorescens* subsp. *cellulosa*) secrete only a limited number of extracellular polysaccharide backbone-degrading enzymes that yield relatively large oligosaccharides. These oligosaccharides enter the cell *via* specific transporters and their final breakdown is carried out by cell-associated or intracellular enzymes *(Nagy et al., 2002, Beylot et al., 2001, Shulami et al., 1999).* This latter strategy has the advantage that the extracellular soluble products are not easily available to competing, non-hemicellulolytic microorganisms.

*Streptomyces thermoviolaceus* OPC-520, a thermophilic actinomycete isolated from decayed wood, grows actively on xylan as a sole carbon source and does not have cellulase activity *(Tsujibo et al., 1992).* The biosynthesis of xylanolytic enzymes in this organism was induced by xylan or xylobiose and repressed by readily metabolized sugars such as glucose *(Tsujibo et al., 2001).* The bacterium produces four extracellular enzymes (designated StxI through StxIV) in the presence of xylan *(Tsujibo et al., 2002, Tsujibo et al., 2001, Tsujibo et al., 1997, Tsujibo et al., 1992).* StxI and StxII are endo-β-1,4-xylanases, StxIII is an acetylxylan esterase, and StxIV is a α-L-arabinofuranosidase. These enzymes effectively convert xylan into XOS. The generated xylobiose and small amounts of other XOS enter the cells and are further hydrolyzed to xylose by an intracellular β-D-xylosidase (BxlA) *(Tsujibo et al., 2002).* The genes involved in the xylan degradation of the strain have been cloned and sequenced *(Tsujibo et al., 2002, Tsujibo et al., 2001, Tsujibo et al., 1997).* Later studies suggested that XOS are specifically transported to the cytoplasm through an ATP-binding cassette (ABC) transporter system and XOS are then degraded to xylose by an intracellular β-xylosidase *(Tsujibo et al., 2004).*

Although many hemicellulolytic enzymes have been studied extensively *(Shallom and Shoham, 2003)*, very little is known about the sensing systems for the hemicellulolytic products and their uptake systems. Several microbial transport systems, including UhpT *(Verhamme et al., 2002, Verhamme et al., 2001)*, DcuB *(Yurgel et al., 2004)*, UpgP *(Niu et al., 1995; Jiang et al., 1988)* and YxdLM have been shown to be regulated by two-component systems (TCSs). This type of regulation enables the cell to respond to environmental or intracellular signals and to alter gene expression. TCSs comprise a sensor histidine protein kinase and a response regulator, usually found in separate proteins. The kinase, typically a membrane protein, becomes autophosphorylated at a conserved histidine residue using ATP as a substrate, in response to a specific signal. The phosphoryl group is then transferred to a conserved aspartate residue on the response regulator, altering its ability to bind target DNA sequences *(Stock et al., 2000)*.

*Geobacillus stearothermophilus* T-6 is a soil bacterium that possesses a highly efficient and complete hemicellulolytic system. The 30 genes comprising the system appear to be organized in at least nine transcriptional units within a 39.7 kb chromosomal segment. When grown in the presence of xylan, strain T-6 secretes a single extracellular endo 1,4-β-xylanase that hydrolyzes the polymer's main backbone producing short modified XOS units of two or more sugars in length *(Shulami et al., 2007)*. These modified XOS enter the cell by specialized ABC sugar transporters *(Shulami et al., 1999)* and they are further degraded to monomers by intracellular hydrolases, including a GH10 xylanase *(Teplitsky et al., 2000)*, two α-L-arabinofuranosidases *(Hovel et al., 2003)*, an α-glucuronidase *(Shallom et al., 2004, Zaide et al., 2001)*, three β-xylosidases *(Brux et al., 2006; Czjzek et al., 2005; Bravman et al., 2003a, Bravman et al., 2003b, Bravman et al., 2001)* and two xylan acetyl esterases.

### 2.9. Biological properties of XOS

#### 2.9.1. Immuno-modulatory activity of XOS

Immunostimulating effects have been reported for arabino-(glucurono) xylans isolated from *Echinacea purpurea, Eupatorium perfoliatum* and *Sabal serrulata (Proksch and*

*Wagner, 1987, Wagner et al., 1985)* and anti-inflammatory activity for the 4-*O*-methylglucuronoxylan from *Chamommilla recutita (Whistler et al., 1976)* and the acidic, highly branched heteroxylan from *Plantago species (Samuelsen et al., 1995, Yamada et al., 1985).* Partially *O*-acetylated XOS and de-acetylated form showed direct mitogenic activity and enhancement of the T-mitogen-induced proliferation of rat thymocytes, indicating the immunostimulatory potential of the almond shell XOS *(Nabarlatz et al., 2007).* The immunostimulatory activity was assessed using the comitogenic rat thymocyte test which was proved to be applicable for polysaccharides of known immunomodulatory activities *(Ebringerova et al., 2002, Ebringerova et al., 1999, Rovensky et al., 1990).* The test is based on the capacity of adjuvant immunomodulators to augment the proliferate response of rat thymocytes to T-mitogens *in vitro*. The almond shell acetylated-XOS showed dose-dependent direct mitogenic as well as comitogenic activities, as was the case with the immunogenic water-soluble arabinoglucuronoxylan from corncobs (CCX) *(Ebringerova et al., 1999)* which was used as a positive control in this study. The activities in the whole doses range were about 30% lower in comparison to CCX.

### 2.9.2. Anti-cancerous activity of XOS

Glucuronic acid-containing (acidic) xylans have been reported to inhibit significantly the growth of sarcoma-180 and other tumors *(Hashi and Takeshita, 1979).* However, no systematic attempts have been carried out to determine the active principle of xylan-type polysaccharides which differ greatly in the type, proportions and distribution of glycosyl side-chains decorating the β-1,4-D-xylan backbone *(Ebringerova et al., 2002).* Comparison of the biological responses of different acidic xylans has not revealed any unequivocal relation either to the 4-*O*-methyl-D-glucuronic acid (MeGlcA) content or to the distribution pattern of MeGlcA *(Ebringerova et al., 2002).*

Ando *et al.* (2004) examined the effect of hot-compressed-water (HCW) extracted and fractionated bamboo products (named as fractions A and B) on the viability of human cultured cell lines derived from leukemia patients and human peripheral blood lymphocytes obtained from normal adults. Fraction A was composed of xylose, XOS

and water-soluble lignin and fraction B was composed of glucose and cellooligosaccharides. It was found that fraction B expressed a negligible cytotoxic effect against leukemia cells, while fraction A reduced markedly (in a dose-dependent manner) the viability of leukemia cell lines derived from acute lymphoblastic leukemia (ALL)-Jurkat and MOLT-4. Fraction A did not influence the viability of leukemia cells derived from myelogenous leukemia (ML-2) or lymphoma (SupT-1), as well as the viability of normal lymphocytes. Furthermore, microscopic examination of ALL-derived cells treated with fraction A showed typical apoptotic morphological changes such as a condensation of nucleus and membrane blebing, as well as phosphatidylserine exposure on the cell surface. The effect of decomposed products of commercially available xylan against ALL-derived Jurkat cells was significantly lower than that of fraction A. These results suggested that the cytotoxic effect of fraction A might be attributed to apoptosis, induced by XOS and it is specific for ALL-derived cells. Ando *et al*. speculated that the water-soluble lignin is an important factor, potentiating the cytotoxic effect of xylan in HCW-extracts from bamboo. Though many other biological activities of arabinoxylan have also been reported *(Migne et al., 1996, Ishii and Nishijima, 1995, Stevens and Selvendran, 1988),* there are no data about its effect on the homeostasis of cancer cells. This is the first report demonstrating the cytotoxic effect of XOS, obtained from natural products on cancer cells.

Certain nondigestible oligosaccharides can be selectively utilized by probiotics in the colon and reduce the risk of colon cancer. For XOS and arabinoxylan, only one experiment each exists investigating their anti-tumorigenic properties. Therefore, it is difficult to conclude their potential to reduce colon cancer incidence. Synbiotic intervention of 2% wheat bran oligosaccharides and $10^8$ cfu bifidobacteria significantly reduced aberrant crypts/$cm^2$ in colon of 1,2-dimethyl hydrazine (DMH) treated Wistar rats *(Gallaher and Khil, 1999).* But the effect of the wheat bran oligosaccharides remain uncertain, even when it was shown that bifidobacteria alone had no effect because no group was fed oligosaccharides alone.

Hsu *et al*. (2004) evaluated the effects of XOS and fructooligosaccharides on the alteration of cecal microbiota, cecal pH, cecal weight, serum lipid levels and also their inhibitory effect on precancerous colon lesions in male Sprague-Dawley rats. The rats were randomly assigned to 4 groups: control, treatment with DMH [15 mg/(kg body wt/wk) for 2 wk], treatment with DMH+60 g XOS/kg diet, and treatment with DMH+60 g fructooligosaccharides/kg diet. Rats were fed the experimental diets for 35 days, beginning 1wk after the second dose of DMH. Both XOS and fructooligosaccharides markedly decreased the cecal pH and serum triglyceride concentration and increased the total cecal weight and bifidobacteria population. XOS had a greater effect on the bacterial population than did fructooligosaccharides. Moreover, both XOS and fructooligosaccharides markedly reduced the number of aberrant crypt foci in the colon of DMH-treated rats. These results suggest that XOS and fructooligosaccharides dietary supplementation may be beneficial to gastrointestinal health and indicate that XOS is more effective than fructooligosaccharides. Further experiments are necessary to confirm an anti-tumorigenic effect of XOS and arabinoxylan.

### 2.9.3. Anti-microbial activity of XOS

Fooks and Gibson (2002) reported that *Lactobacillus plantarum* 0407 and *Lactobacillus pentosus* 905 combined with fructooligosaccharides, inulin, XOS and mixtures of inulin: fructooligosaccharides and fructooligosaccharides: XOS were effective in inhibiting growth of *Escherichia coli* and *Salmonella enteritidis.* The antimicrobial potential exhibited by each of the probiotics used here appeared to depend on the carbohydrate source used. Fructooligosaccharides, inulin, XOS and their mixtures, all caused greater inhibition than lactulose, lactitol, starch and dextran, perhaps suggesting a structure-to-function relationship in terms of the prebiotic used. The type of bond links the component monomers, in view of specific cleavage enzymes being required for fermentation of the carbohydrate, may effect fermentation rate and thereby determine the speed at which potential inhibitory metabolic end products are released.

Acidic XOS were produced from birch wood xylan by treatment with a *Thermoascus aurantiacus* family 10 and a *Sporotrichum thermophile* family 11 endoxylanases

*(Christakopoulos et al., 2003)* and these oligosaccharides were tested against three Gram-positive and three Gram-negative aerobic bacteria as well as against *Helicobacter pylori*. Aldopentauronic acid proved more active against the Gram-positive bacteria and *Helicobacter pylori*. Both acidic XOS compounds showed a moderate activity against the Gram-positive bacteria *Bacillus cereus* while aldopentauronic acid had a similar activity against *Staphylococcus aureus*. On the other hand, *Pseudomonas aeruginosa* and *Proteus mirabilis* were the most resistant strains as both compounds have been proven inactive against them *(Christakopoulos et al., 2003).* Compared to ampicillin, both alduronic acids showed inhibitory effects on growth of *Helicobacter pylori* at much higher concentrations but the results suggested that these constituents have an anti-*Helicobacter pylori* activity with aldopentauronic acid being more active.

**2.9.4. Plant growth regulatory activity of XOS**

Morphogenetic effects of oligosaccharides on tobacco explants were first reported by Van *et al*. (1985). Studies with *Pinus radiata* cell cultures indicated that medium conditioned by short term culture of rapidly dividing cells could assist the culture of cells at low inoculum densities. The low inoculum growth factor in such conditioned medium was considered to be a cell wall oligosaccharide of low molecular weight *(Teasdale and Richards, 1991).* The presence of XOS in the culture medium in the range 5-25 mg/l doubled the growth rate of *Leucaena leucocephara* seedlings, while increasing the concentration of XOS to 100 mg/l provided some growth inhibition *(Ishihara et al., 1991).* Ishihara *et al.* (1991) also showed that acidic XOS at a concentration of 10-50 mg/l were effective in promoting rooting of *Cryptomeria japonica* cuttings. These results indicated a physiological role for XOS in plant growth and development. However, little is known about their effects at the cellular level.

In *Pinus thunbergii,* XOS promoted the rooting of *in vitro* grown shoots *(Ishii et al., 1992).* Ishi *et al*. (1993) also showed that acidic XOS at a concentration of 2-5 mg/l were effective in promoting growth of tissue cultures of aspen (*Populus tremuloides*) and pine (*Pinus thunbergii*). Furthermore, small quantities of acidic xylan hydrolysate from *Betula*

*platyphylla* wood stimulate rooting of conifer cuttings in hydroponic culture solution *(Ishihara et al., 1995).*

Later, the effects of XOS isolated from the cell walls of *Betula platyphylla* var. japonica on cells and protoplasts of *Pinus radiata* were examined *(Ishii and Teasdale, 1997).* The addition of a semi-purified mixture of XOS at a concentration of 5 mg/ml promoted elongation of cultured cells, whereas the neutral fraction of this mixture had no effect. A similar effect was seen in the presence of conditioned medium. The unfractionated mixture of XOS was also found to enhance the viability of protoplasts prepared from cell cultures of *Pinus radiata* in a concentration dependent manner, highly similar to the effect provided by addition of medium conditioned by pine cells. Such effects are considered to be due to the addition of components that play a structural role in the cell wall of pines. It is inferred that the acidic components of the XOS mixture derived from *Betula platyphylla* are responsible for this effect in the distant pine species. Ishii and Teasdale speculated that acidic XOS operate either by replacing or mimicking the natural cell wall components required for growth and development of pine cultured cells.

Plant growth-promoting oligosaccharides produced from tomato wastes after acid hydrolysis were significantly promoted the growth of cock-scomp (*Celosia argenta* L.) and tomato (*Lycopersicon enculentum* L.) *(Suzuki et al., 2002).* Another study reported that the addition of aldotetrauronic acid remarkably improved callus induction from both mallow and cotton cultures. The best results were observed at 1.6 mg/l with tissue dedifferentiation declining considerably at higher concentrations (especially in cotton) *(Katapodis et al., 2002).* In conclusion, the preliminary results indicated the dose-dependent, species-specific growth regulatory activity of the isolated pure acidic oligosaccharide.

**2.9.5. Growth regulatory activity of XOS in aquaculture and poultry**

In a study, day-old chick were fed diets containing 0, 0.4, 4.0 or 40 g/kg XOS to 21 days of age *(Graham et al., 2004).* XOS did not influence chick growth, gut length or digesta dry matter. However, XOS decreased ileal lactic acid concentration, increased cecal

butyric acid and increased total volatile fatty acid concentrations. XOS were rapidly fermented in the caeca. The results showed that XOS had little influence on the overall bacterial community profile.

Xu *et al.* (2008) evaluated the application of XOS as a feed additive in the diet of allogynogenetic crucian carp, *Carassius auratus gibelio.* XOS were added to fish basal semi-purified diets at three concentrations by dry feed weight: diet 1:50 mg/kg; diet 2:100 mg/kg; diet 3: 200 mg/kg, respectively. Weights of all collected carp from each aquarium were determined at the initial phase and at the end of the experiment and the carp survival was also determined by counting the individuals in each aquarium. After 45 days, there were significant differences in the relative weight gain rate and daily weight gain rate of diets 1-3 as compared with the control. However, the survival rate was not affected by the dietary treatments. The protease activity in the intestine and hepatopancreas content of fish in diet 2 were significantly different from that in the control and diet 3 groups. Amylase activity in the intestine was significantly higher for diet 2 group compared to diet 1 group and the control group. As for amylase in the hepatopancreas, assays showed higher activity in diet 2 group compared to the rest. The study suggested the use of XOS as a feed additive in the diets of fishes.

### 2.9.6. Other biological effects of XOS

XOS (alone or as active components of pharmaceutical preparations) exhibit a range of biological activities different from the prebiotic effects related to gut modulation. The other applications claimed for XOS in the last few years, include antioxidant activity (conferred by phenolic substituents), blood- and skin-related effects, antiallergy, anti-infection and anti-inflammatory properties, immunomodulatory action, cosmetic and a variety of other properties. It can be noted that a significant part of the recent developments has been proposed for acidic oligosaccharides containing uronic substituents, which can be produced from hardwoods by a combination of enzymatic and/or chemical treatments. Besides biological effects concerning human health, XOS have been employed for phyto-pharmaceutical and feed applications.

**2.10. Human experimental data on XOS**

A large number of references are available on the effect of XOS in animal models. However, the human experimental data on XOS are limited and evidence of the prebiotic efficacy of XOS is sparse. Previous animal studies showed that oral administration of XOS could significantly increase the moisture content of feces, the total cecum weight, and the population of bifidobacteria and decrease the pH level of feces in Institute of Cancer Research (ICR) mice *(Chung et al., 2002).* Later, similar prebiotic effects were also found in Sprague-Dawley rats *(Chan et al., 2005).* Hsu *et al.* reported that XOS significantly inhibited the colon precancerous lesion induced by DMH in Sprague-Dawley rats and the supplementation of XOS was more effective than fructooligosaccharides on increasing the population of bifidobacteria *(Hsu et al., 2004).*

In an *in vivo* study of five healthy human volunteers, *Okazaki et al.* (1990) reported a 10-30% increase in the relative ratio of bifidobacteria to total intestinal microflora following consumption of XOS at 1-2 g/day, which dropped after administration of XOS ceased. Howard *et al.* (1995) studied the effect of XOS at 4.2 g/day on the colonic microflora of mice but did not observe any increase in bifidobacterial number. The same group reported a significant increase in bifidobacterial levels upon administration of XOS at 5 g/day to human volunteers. They suggested that lack of a prebiotic effect in the mice might be due to different bifidobacterial species inhabiting the gastrointestinal tract of human and mice.

Another study on the effects of XOS in the fecal content of men showed that fecal content was maintained within the normal range by daily intake of XOS *(Kobayashi et al., 1991).* SCFA (especially acetic acids) in the fecal matter of subjects were increased and the increased putrefactive products such as p-cresol, indole and skatole were decreased by the continuous intake of 0.7 g/day XOS. In a clinical study intended for adult woman, the relative percentage of bifidobacterium to the total intestinal microflora was significantly increased by the daily intake of 0.4 g of XOS and the stool frequency and abdominal conditions improved simultaneously *(Iino et al., 1997).*

Kajihara *et al.* (2000) evaluated the effects of XOS on blood ammonia levels and intestinal flora in patients with liver cirrhosis. Fourteen cirrhotic patients with clinically stable and mild hyperammonaemia but without apparent hepatic encephalopathy were given 3.0 g of XOS per day for two weeks. The levels of blood ammonia, along with other laboratory values such as serum aspartate aminotransferase (AST), alanine transaminase (ALT), bilirubin and albumin were assessed at the beginning and the end of trial. After faecal samples were collected at the beginning and the end of study, the diluted suspensions were anaerobically incubated and changes in the proportion of faecal bacteria were assessed. No modification on food intake or medication considered to be influential on intestinal microflora was made during the study. Results indicated that oral administration of XOS for two week resulted in reduced blood ammonia levels in patients with liver cirrhosis while other parameters such as AST, ALT, bilirubin and albumin were unaffected. Furthermore, XOS promoted the growth of intestinal bifidobacteria, while suppressing that of bactericides. No diarrhoea, flatulence or other complications were complained. Kajihara *et al.* concluded that XOS effectively improved mild hyperammonaemia in cirrhotic patients. Though the exact mechanism is not yet clear, the results implicated that XOS can exert its effect by inhibiting enteric colonization of ammonia-producing anaerobes such as bacteroides. Further clinical randomized-controlled studies examining a large number of cirrhotic patients should be necessary.

Tateyama *et al*. (2005) investigated the effect of XOS in preventing constipation in pregnant women. The study indicated that XOS intake was highly effective for the reduction of severe constipation in pregnant women, where occurrence of a very loose or hard stool decreased and the stool consistency normalized.

Recently, Chunga *et al.* (2007) studied the effect of XOS on the intestinal microbiota, gastrointestinal function and nutritional parameters of the elderly. Subjects (65 years and older) who did not have recent history of gastrointestinal disease were included and randomly divided as either a control or XOS group. The treatment group was supplemented with 4 g of XOS per day for 3 weeks, whereas the control group was given a placebo. The anthropometric and nutrient parameters, fecal moisture content, pH,

bifidobacterium count and *Clostridium perfringens* count of the subjects were determined. The results showed that XOS supplementation significantly increased the population of bifidobacteria and the fecal moisture content and decreased the fecal pH value. The nutrient intakes, gastrointestinal function and blood parameters were not significantly different between the XOS and control groups after 3 weeks of administration. In conclusion, XOS supplementation was effective in promoting the intestinal health and did not show adverse effects on nutritional status in the elderly.

**2.11. Chemical degradation of XOS**

The hypothesis that oligomers directly degrade at low acid concentration was also tested for a range of oligomers from xylobiose to xylopentose *(Lloyd, 2005, Li, 2002)*. Observations with xylobiose and xylotriose strongly suggest that direct degradation of oligomers occurs along with depolymerization to lower oligomers and monomer. However, xylotetraose and xylopentose modeling contradicts the hypothesis and direct degradation of these oligomers at low and high acid concentrations was found to be negligible. Thus, it may be interpreted that the direct degradation may only occur with lower oligomers such as xylobiose and xylotriose. Consistent with the finding in this research, Garrote *et al.* (2001) assumed for kinetic modeling of corncob autohydrolysis that xylan breaks down to higher DP oligomers and that it then forms lower DP oligomers, which can degrade directly to furfural and form xylose. However, this was an empirical fit in that the length of XOS that directly degrade was not defined. Therefore, it can be assumed that oligomers of DP 2-3 are decomposed directly to degradation products at high pH while DP 4 and 5 do not. However, further validation is needed with higher oligomers to determine if this trend is followed by higher DP oligomers.

Previously, a study on xylobiose degradation revealed that sulfuric acid dramatically increased the ratio of the depolymerization rate constant to the overall disappearance rate constant to close to one, significantly enhancing the selectivity of xylose formation *(Lloyd, 2005)*. In addition, the optimum acid concentration to maximize xylose monomer recovery during acid hydrolysis of xylan rich hemicellulose was determined to be between pH 2 and 3. The results for XOS reactions reported to date suggest that acid

enhances the selectivity of XOS breakdown to monomers through either dramatically reducing the role of side reactions that directly degrade oligomers or by significantly speeding the reactions to monomers relative to these direct degradation reactions.

Recently, Kumar and Wyman (2008) monitored the disappearance of xylose and XOS with DP ranging from 2 to 5 at 160 °C with sulfuric acid added to adjust the pH from near neutral to 1.45 and studied its impact on the yields of lower DP XOS and xylose monomer. In addition, the experimental data for the disappearance of XOS was kinetically modeled assuming first-order reaction kinetics for xylose degradation and XOS hydrolysis to evaluate how the pH affected the selectivity of monomer formation from XOS and direct oligomer degradation to unknown products. The yield of xylose from XOS increased appreciably with increasing acid concentration but decreased with increasing DP at a given acid concentration, resulting in more acid being required to release the same xylose yields for higher DP XOS. However, because published results are limited to a few temperatures, acid concentrations and oligomer chain lengths, it is difficult to draw broad conclusions.

**2.12. Structural characterization of XOS**

The analysis of oligosaccharides released from xylans has been done mainly by NMR, and also by mass spectrometry using MALDI-MS *(Reis et al., 2003a, Kabel et al., 2002, Deery et al., 2001, Jacobs et al., 2001, Vierhuis et al., 2001a, Huisman et al., 2000 )* and electrospray tandem mass spectrometry (ESI-MS) *(Reis et al., 2003b, Reis et al., 2002, Samuelsen et al., 2001).* Recently, positive tandem mass spectrometry using ESI was proposed for the characterization of underivatised neutral and acidic XOS formed by partial acid hydrolysis *(Reis et al., 2003b, Reis et al., 2002).*

Acidic oligosaccharides obtained from birch wood xylan by treatment with a *Thermoascus aurantiacus* family 10 (XYL I) and a *Sporotrichum thermophile* family 11 (XYL A) endoxylanases were characterized using $^{13}$C-NMR (*Christakopoulos et al., 2003).* The $^{13}$C-NMR spectrum of the acidic XOS liberated by XYL I was consistent with the structure of 2''-*O*-α-(4-*O*-methyl-α-D-glucuronosyl)-xylotriose (MeGlcAα1-2-Xyl1-

4Xyl-β1-4Xyl, MeGlcAXyl3). The structure of the shortest acidic fragment liberated by XYL A was established as aldopentauronic acid 2"- *O*-α-(4-*O*-methyl-α-D-glucuronosyl)-xylotetraose (Xyl–1,4-[MeGlcA-α-1,2-]Xyl–1,4-Xyl–1,4Xyl,MeGlcAXyl$_4$). The assignment of the most important signals was carried out on the basis of published data for analogue compounds *(Biely et al., 1997).*

Oligosaccharides have been shown to be suitable models for understanding the conformation in the solid state and in aqueous solution as well as intermolecular interactions. β-D-xylobiosides and XOS which consist of a (1→4)-linked-β-D-xylopyranoside substituted at O-2 or O-3 with a terminal β-D-xylopyranosyl or α-L-arabinofuranosyl residue were used in a previous study *(Kacurakova et al., 1994).* It was shown that the presence of a (1→3)-linkage in xylan molecules and in the corresponding oligosaccharide models had an effect on the Infrared (IR) spectral pattern of compounds in the freeze-dried state. These changes, occurring mainly in the 1200-900 cm$^{-1}$ region, have been shown to be useful for xylan type recognition and even for compositional analysis. However, the origin of the effect has not been elucidated. Kacurakova *et al.* (1998) hypothesized that the glycosidic linkage type and position also could play an important role in the swelling and gelling mechanism of xylans. The studied XOS models can be divided into two categories: those which readily crystallize into highly ordered hydrated structures (having prevalence of 1→4 and/or 1→2, β glycosidic links) and those which do not crystallize (1→3 and α-link contributions). The water-vapor treatment on the XOS models results in IR spectral pattern changes which are strongly affected by the type and position of the inter-glycosidic linkages and reflects the hydration properties of the models at the discrete relative humidity steps.

Oligo- and polysaccharides isolated from water-soluble hemicelluloses extracted from milled aspen wood (*Populus tremula*) were characterized using NMR techniques *(Teleman et al., 2000).* The polysaccharides present in the first two fractions eluted were *O*-acetyl-(4-*O*-methylglucurono) xylans. The structural element [4 -*O*-Me-α-D-Glc*p*A-(1.2)][3-*O*-Ac]-β-D-Xyl*p*-(1→ could also be identified. The third fraction was an oligosaccharide fraction contained acetylated XOS that might be a hydrolysis product of

acetylated 4-*O*-methylglucuronoxylan and was structurally characterized as *O*-acetyl-β-D-xylooligosaccharides.

The fragmentation of XOS has been studied significantly less. Reis *et al.* prepared a mixture of neutral and acidic XOS by partial acid hydrolysis from olive pulp and measured collision induced dissociation (CID) spectra in both positive and negative ionization modes for several ionic species *(Reis et al., 2005, Reis et al., 2004a, Reis et al., 2004b, Reis et al., 2003, Reis et al., 2002).* Olive pulp XOS and the correspondent alditol derivatives were analyzed by ESI-MS and ESI-MS/MS *(Reis et al., 2004).* ESI-MS spectrum of XOS and their alditols showed $[M+ Na]^+$ neutral ($Xyl_{3-6}$) and acidic ($Xyl_{2-3}$MeGlcA and $Xyl_{2-3}$GlcA) XOS. The ESI-MS/MS spectra of underivatised XOS presented fragments of glycosidic cleavages attributed to B/Z and C/Y ions. On the other hand, MS/MS spectra of the correspondent alditols showed glycosidic cleavages unambiguously identified as B-type and Y-type ions. Y-type fragment ions showed higher abundance in the MS/MS spectra of the alditol derivatives when compared to the non-reduced samples. The study of the oligoxylosyl alditols fragmentation permits to distinguish fragmentation pathways that occur both from the reducing end and from the non-reducing end of the xylan chain allowing to obtain more information about the localization of the acidic substituent along the glucuronoxylan backbone *(Reis et al., 2004).*

In addition, Quemener *et al.* (2006) and Matamoros Fernandez *et al.* (2004, 2003) studied arabinoxylans and also presented CID spectra for some precursor ions of neutral XOS. In order to study the effects of the precursor ion type and the carbohydrate structure on the fragmentation of neutral unsubstituted oligosaccharides in CID, a systematic study of deprotonated, protonated, ammoniated and alkali metal cationized cellooligosaccharides, maltooligosaccharides and XOS was carried out using a quadrupole ion trap (QIT) and Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry *(Pasanen et al., 2007).* The fragmentation pathway was highly dependent on the choice of the precursor ion type. Deprotonated precursors gave rise to both glycosidic and cross-ring fragmentation, with clear differences among the three oligosaccharides, therefore, being

the most prominent for structural analysis. The fragmentation behavior of the XOS differed from that of the cello- and maltooligosaccharides for all the precursor ions studied, most remarkably with the deprotonated and ammoniated precursors. Stereochemical differentiation of cello- and maltooligosaccharides was possible with the use of deprotonated, lithiated and sodiated precursors. In general, as the size of the alkali metal cation increased the amount of structurally informative cross-ring fragmentation increased, but the probability for metal ion loss from the precursor ion also increased. The CID spectra of XOS measured with the QIT and FT-ICR were surprisingly similar *(Pasanen et al., 2007).*

Partially *O*-acetylated XOS (DXO) isolated from almond shells by autohydrolysis as well as their de-acetylated form (DeXO) were subjected to chemical, molecular and structural analyses *(Nabarlatz et al., 2007).* The structural features of the almond shell oligosaccharides were examined by NMR spectroscopy of DXO and DeXO. The chemical shifts of the signals observed in the $^{13}$C-NMR spectra were in excellent agreement with values reported for various 4-*O*-methylglucuronoxylan oligomers and polymers and their acetylated forms *(Teleman, 2002, Teleman et al., 2000, Kardosova et al., 1998).* The relatively weak signals of the α- and β-anomers of reducing *Xylp* end groups were in accordance with the average molecular mass of DXO *(Nabarlatz et al., 2006)* confirming the presence of oligomeric and polymeric fragments. Further analysis of the almond shell XOS was performed by MALDI-TOF mass spectrometry of DXO and DeXO, which both contained more than 90% XOS. The results of both the NMR spectroscopy and MALDI-TOF mass spectrometry analyses revealed that the almond shell XOS comprise a mixture of partially *O*-acetylated neutral and acidic oligomers derived from the 4-*O*-methylglucuronoxylan-type polymers, known to be the dominant hemicelluloses of dicotyl plants *(Ebringerova and Heinze, 2000)* to which group the almond tree belongs. The use of MALDI-TOF-MS for the characterization of XOS was also reported by others *(Cano and Palet, 2007).*

### 2.13. Use of XOS in food

The most important applications of XOS in terms of current and potential market demand correspond to ingredients for functional foods, for example, in combination with soya milk, soft drinks, tea or cocoa drinks, nutritive preparations, dairy products with milk, milk powder and yoghurts, candies, cakes, biscuits, pastries, puddings, jellies, jam and honey products and special preparations for health food for elder people and children or as active components of synbiotic preparations. Enrichment of yoghurt with XOS at different levels was studied with physicochemical and sensory analysis *(Mumtaz et al., 2008).* Yoghurt prepared by incorporation of XOS was compared for these characteristics against the yoghurt containing stabilizer (gelatin, 0.4% w/w) in addition to XOS. Moisture contents, pH, acidity and total solids were studied and these attributes were significantly affected by the use of stabilizer and rate of XOS incorporation. The results also showed that addition of XOS up to 3.5% did not influence taste and overall acceptability but higher levels contributed aftertaste.

### 2.14. Global Market of XOS

The functional food market is growing rapidly, based on the consumers' awareness of the link between health, nutrition and diet as well as on the interest of food manufacturers due to the increased value that the added ingredients give to food. This is particularly applicable to XOS, for which a selling price of 2500 yen/kg has been reported (the highest one among 13 different types of oligosaccharides) *(Taniguchi et al., 2004).* The same reference reports a total production of 650 ton of XOS per year in Japan, which accounts for about one-half of the world market. However, the fastest growth rate is expected for the United States market.

XOS are available in market in different forms and purity and they mainly include XOS capsules, XOS powder (>95% XOS, >35% XOS, >20% XOS), XOS syrup (>70% XOS), XOS vinegar, XOS synbiotic products etc. Among the companies who manufactures XOS, Suntory Ltd. (Japan), Qindao Free Trade Zone United International Inc. (China), SF Chemical (China), CarboMer (USA) , Wako Pure Chemical Industries Ltd. (Japan),

Shandong Longlive Biotechnology Co Ltd. (China), HN Core Corporation (China), Zibo Younger Chemicals Co Ltd. (China), Naga Global Co Ltd. (Taiwan), China Jiangsu International Economic Technical Cooperation (China), Shandong Longli Biology Co Ltd. (China), Shandong Baolingbao Biology Co Ltd. (China), Yongchuntang Biology Co Ltd. (China), Anhui Xingwan Herding Technology (Group) Development Co Ltd. (China), Zibo Younger Chemicals Co Ltd. (China), Qingdao Iro Taihe International Trade Co Ltd. (China), Honest Joy Chemicals (China) are in the top list. In India there is no company who either manufactures or imports XOS.

# CHAPTER: 3

# EVALUATION OF MICROBIAL XYLANASES FOR THE ENZYMATIC PRODUCTION OF XYLOOLIGOSACCHARIDES

**Overview**

Endo-1,4-β-xylanases generally known as endoxylanases are the key enzyme components of microbial xylanolytic systems which hydrolyze β-(1,4)-xylosidic linkages of the xylan backbone to xylooligosaccharides (XOS) of various chain lengths. The first section of this chapter details the evaluation of eight different fungi to produce the endo-1,4-β-xylanases for XOS production. All the fungal species evaluated produced xylanases, among which *Aspergillus oryzae* MTCC 5154 showed significant titers of endoxylanases, which was further used for the production of XOS from birch wood xylan. This chapter also focuses on the utilization of corncob powder as an efficient inducer for xylanases in comparison with that of standard xylan. The disaccharide thus obtained, by the hydrolysis of xylan by corncob induced xylanase of *Aspergillus oryzae*, was characterized using ESI-MS and $^{13}$C-NMR techniques. The second section of the chapter details the molecular cloning and sequencing of a xylanase gene of *Aspergillus oryzae.*

## SECTION: 3.1

# PRODUCTION OF XYLOOLIGOSACCHARIDES FROM GLUCURONO-XYLAN USING CORNCOB-INDUCED ENDO-1,4-β-D-XYLANASE

### 3.1.1. Introduction

Xylan, a β-1,4-linked polymer of xylose, is the most abundant hemicellulose in the majority of plants. The β-1,4-linked D-xylosyl backbone of xylan is decorated through an α-1,2-linkage with glucuronic acid and 4-*O*-methyl-α-D-glucuronic acid, whereas α-L-arabinofuranosyl residues are linked through α-1,3 to the polymer *(Zui et al., 2004, Ishihara et al., 1997).* 1,4-β-D-xylan xylanohydrolase (EC 3.2.1.8) generally known as endoxylanase hydrolyzes β-1,4-xylosisdic bonds within these polysaccharide backbone, producing β-anomeric xylooligosaccharides (XOS) consisting of 4-*O*-methyl-α-D-glucuronic acid residues. These oligosaccharides are a class of functional food ingredients generally regarded as prebiotics *(Okazaki et al., 1990).* In addition, these oligosaccharides often possess more unique structural features or unusual properties that are of special value to the biological entity *(Pazur, 1970).*

XOS find potential applications in a variety of fields such as pharmaceuticals, feed formulations and food industries. In food industry XOS can be used as soluble dietary fiber since they are not metabolized by the human digestive system. They have diverse beneficial health effects such as revitalizing the growth of intestinal bifidobacteria, immunity activation and non-cariogenicity. They exhibit water retention capacity, antifreezing property and a variety of biological properties *(Moure et al., 2006).* XOS have acceptable organoleptic properties and do not exhibit toxicity or negative effects on human health *(Nabarlatz et al., 2005).* The preferred degree of polymerization (DP) of XOS is 2-4 for food related applications *(Loo et al., 1999).* The sweetness of xylobiose is equivalent to 30% that of sucrose and the sweetness of higher XOS is moderate and possesses no off-taste. XOS, being low calorific, find use in the preparation of anti-obesity diet *(Toshio et al., 1990).*

The production of XOS on an industrial scale is carried out from lignocellulosic materials (LCMs) rich in xylan. The strategies for the production of XOS from LCMs are grouped into three categories: enzyme treatment of native xylan containing LCMs, chemical fractionization of suitable LCMs followed by enzymatic hydrolysis for the production of

XOS and hydrolytic degradation of xylan to XOS by steam, water or dilute mineral acids *(Vazquez et al., 2000).* However, alkaline extraction and acidic pretreatment are not suitable for the production of XOS because the former results in the corrosion of equipments used and pollution and the latter produces an excess of xylose in the hydrolysate *(Yang et al., 2005).* Enzymatic production of XOS using endoxylanase is preferred in food industry because of the lack of undesirable side reactions and products.

Endo-1,4-β-xylanases generally known as endoxylanases are the key enzyme components of microbial xylanolytic systems which hydrolyze β-1,4-xylosidic linkages of the xylan backbone to XOS of various chain lengths. A number of microorganisms, including bacteria, yeasts and filamentous fungi have been reported to produce xylanases *(Haltrich et al., 1996).* They are involved in the production of xylose, a primary carbon source for cell metabolism and in plant cell infection by plant pathogens. The microbial xylanases have been used commercially in the pulp and paper industry for the past several years. Apart from this, they are also used as feed additives in poultry, in wheat flour for improving dough handling and quality of baked products, for the extraction of coffee, plant oils and starch and in combination with cellulases and pectinases for clarification of fruit juices and degumming of plant fibers such as flax, hemp and jute. Xylanases from fungi have been well documented and studied *(Pham et al., 1998)* and there are a few reports on its use for the production of XOS *(Akpinar et al., 2007, Yang et al., 2007).*

For commercial applications, production of xylanase from simple and inexpensive substrates is economical. Abundantly available agro-residues such as corncob, wheat bran, wheat straw and rice straw as substrate are of obvious choice. Corncob is an important by-product of the corn industry which is used either as animal feed or returned to the harvested fields. In recent years, interest in the microbial utilization of food processing waste into value-added products has increased. The xylan content of corncob is about 35% *(Yang et al., 2005)* and can be used as a carbon source for various microorganisms for their growth and production of food grade enzymes.

The present section deals with the evaluation of eight different fungal strains for endoxylanases production, which are further used for the production of XOS by hydrolysis of birch wood xylan, 4-*O*-methyl-α-D-glucurono xylans. The section also focuses on the production of endoxylanase by a selected fungus *Aspergillus oryzae* MTCC 5154 using three different xylan sources including corncob under submerged cultivation conditions and evaluation of these endoxylanases for XOS production. The study also discusses the product characterization using HPLC, ESI-MS and $^{13}$C-NMR techniques.

### 3.1.2. Materials and methods

#### 3.1.2.1. Chemicals

All chemicals were of analytical grade. Standards of xylose, xylobiose, arabinose, glucose, oat spelt xylan and birch wood xylan were from Sigma-Aldrich (USA).Corncobs were obtained from a local maize field in Mysore, Karnataka, India and were sorted, dried at 40 °C for 24 h, milled (60-80 mesh) and stored in polycarbonate containers.

#### 3.1.2.2. Milling of corncob

A two stage grinding of corncob has been carried out to get a powder of 60-80 mesh size. Firstly the corncob chips were ground in a Multimill (Gansons Ltd, Bombay-55, India) using a 10 mm sieve followed by 6 mm sieve. Secondly the powder obtained was ground again in a Plate Mill (Madras Standard Engineering Works, Madras, India) to 60-80 mesh size. This powder was used as the inducer for xylanase production.

#### 3.1.2.3. Microorganisms and culture conditions

*Aspergillus oryzae* MTCC 5154 is a soil isolate from CFTRI and deposited in MTCC, IMTECH, Chandigarh, India; *Aspergillus ochraceus* MTCC 1877 and *Penicillium citrinum* MTCC 2553 were from MTCC, IMTECH, Chandigarh, India; *Trichoderma harzianum* ATCC 42459 was from ATCC, USA and *Aspergillus flavus*, *Aspergillus niger, Aureobasidium pullulans* CFR 77 *and Trichoderma viridae* were from the culture collection of CFTRI, Mysore, India. All the strains were maintained on potato dextrose agar (PDA, from Himedia Laboratories Ltd, India) slants. *Aspergillus ochraceus* and

*Penicillium citrinum* were maintained on malt extract agar and Czepadox yeast extract agar (CYEA from Himedia Laboratories Ltd, India) slants respectively at 4±1 °C.

### 3.1.2.4. Congo red staining method for detection of endoxylanase activity

The endoxylanase activity was detected by the Congo red staining method *(Theater and Wood, 1993)*. A loopful spore of each of the eight strains of fungi were inoculated separately onto PDA plates containing 1.5% agar (w/v) and 0.5% oat spelt xylan (w/v) and incubated at 30 °C for five days. The agar plates were flooded with 0.1% Congo red (w/v) in water for 15 min. The Congo red solution was then poured off and the plates were further washed with 1 M HCl, to arrest the enzyme activity.

### 3.1.2.5. Evaluation of different fungi for production of endoxylanase

A few fungal strains were evaluated for their endoxylanase activity under submerged fermentation conditions. A loopful of spores from five day old slants of the fungi were inoculated into 50 ml fermentation medium *(Kimura et al., 2002)* containing 1% oat spelt xylan, 0.5% yeast extract, 0.1% $NaNO_3$, 0.1% $KH_2PO_4$, 0.1% peptone and 0.03% $MgSO_4.7H_2O$ with an initial pH of 5.5. The flasks were incubated at 30±1 °C on a rotary shaker (Emenvee Rotary Shaker, 48N3, Pune, India) up to 144 h. Sampling was done at regular intervals of 24 h. Experiments were carried out in duplicates. At the end of respective periods of cultivation, the cultures were centrifuged at 4 °C using a refrigerated centrifuge at 5000 x g (Remi cooling centrifuge, C-30, Mumbai, India) and the supernatant was used as the source of endoxylanase and carboxy methyl cellulase (CMCase) without further purification. The pH of the culture fluids (CF) was recorded.

### 3.1.2.6. Production of endoxylanase by *Aspergillus oryzae*

The effect of different sources of xylan such as birch wood xylan (1%), oat spelt xylan (1%) and raw corncob powder (3%) on the production of endoxylanase, using the selected organism *Aspergillus oryzae* MTCC 5154 under submerged conditions was studied. The other media components were similar to those mentioned under section 3.1.2.5. The media was inoculated with 0.5 ml of spore suspension containing $6x10^6$ spores/ml. Growth parameters were maintained as those mentioned above and

fermentation was carried out up to 144 h. The enzyme activities were monitored at 24 h intervals. Experiments were carried out in duplicates. The CF was centrifuged as described previously. The endoxylanase activity, CMCase activity, total cellulase activity and protein *(Lowry et al., 1951)* content of the CF were estimated.

**3.1.2.7. Production of XOS from birch wood xylan using selected strains of fungi**

Out of the eight organisms evaluated for xylanase production under submerged fermentation conditions, *Aspergillus oryzae* MTCC 5154, *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus ochraceus* were showed significant xylanase activity and were further used for the production of XOS from standard birch wood xylan. The reaction was carried out in 250 ml conical flask containing 50 ml reaction mixture, consisting of 0.5 g birch wood xylan and CF corresponding to 100 U of endoxylanase, made up to a total volume of 50 ml using Mc Ilvain buffer (0.05 M, pH 5.4). The enzymatic reaction was carried out in a shaking water bath (Haake SWB 20, Haake, Germany) maintained at 50 °C for 24-36 h. At the end of specified incubation time, the reaction was stopped by keeping the reaction mixture in a boiling water bath for 10 min.

**3.1.2.8. Production of XOS by *Aspergillus oryzae***

XOS production was carried out from standard birch wood xylan using CF obtained after the submerged cultivation of *Aspergillus oryzae* MTCC 5154 using birch wood xylan, oat spelt xylan and corncob. The CF exhibiting maximum endoxylanase activity was selected for the reaction. The reaction parameters were as mentioned in the section 3.1.2.7. The reaction was carried out for 36 h with a regular sampling of 6 h interval followed by analysis of the products.

**3.1.2.9. Enzyme assays**

**3.1.2.9.1. Endoxylanase activity**

Endoxylanase activity of CF was assayed using standard birch wood xylan as the substrate *(Jiang et al., 2005).* Exactly 0.9 ml of 1% (w/v) birch wood xylan in 0.05 M, pH 5.4 Mc Ilvain buffer and 0.1 ml of suitably diluted CF was incubated at 50 °C in a water bath at 100 rpm for 10 min. Amount of reducing sugars liberated was measured by

3,5-dinitrosalicylic acid (DNS) method *(Miller, 1959).* One unit of endoxylanase activity is defined as the amount of enzyme required to produce 1 µmol of xylose per min under the above mentioned conditions.

**3.1.2.9.2. Cellulase activity**

CMCase activity of CF was assayed using standard carboxy methyl cellulose (CMC) as the substrate *(Wood and Bhat, 1988).* 1ml of 1% (w/v) CMC in 0.05 M, pH 4.8 citrate buffer and 0.5 ml of suitably diluted CF was incubated at 50 °C in a water bath at 100 rpm for 30 min. Amount of reducing sugars liberated was measured by DNS method *(Miller, 1959).* One unit of CMCase activity is defined as the amount of enzyme required to produce 1 µmol of glucose per minute under the above mentioned conditions. Total cellulase as filter paper activity (FPA) was also measured *(Wood and Bhat, 1988).* One unit of total cellulase activity is defined as the amount of enzyme required to release 1 µmol of glucose equivalents.

**3.1.2.10. Analysis of XOS using HPLC**

The reaction mixture was evaluated for XOS according to a method reported previously *(Jeong et al., 1998).* The reaction mixture was filtered through a cellulose nitrate membrane (0.45 µm) to remove the residual xylan. The products of hydrolysis were analyzed by HPLC LC-6A (Shimadzu, Japan) equipped with a refractive index detector RID 6A (Shimadzu, Japan) using an Aminopropyl column 250×4.6 mm SS Excil amino 5 µm (SGE, Australia). 20 µl of the hydrolysate was injected using HPLC injector syringe (Hamilton, Nevada, USA) and the analysis was carried out at room temperature (26±2 °C) using acetonitrile/water (75:25 v/v) as the mobile phase at a flow rate of 1.0 ml/min. Data acquisition was carried out by AIMIL Chromatography Data Station (AIMIL, New Delhi, India) and processed on computer using WINACDS software (AIMIL, New Delhi, India). In the preliminary experiments on the evaluation of eight different fungal strains, the final XOS yield was expressed as the percentage of the total sugars in the hydrolysate. In the further experiments, the XOS formed was quantified by comparing the peak area of XOS with standard peak area of xylose and is expressed as mg/ml of hydrolysate.

### 3.1.2.11. Purification of XOS using gel permeation chromatography (GPC)

100 ml of XOS mixture was prepared as described earlier and was concentrated to 5 ml by lyophilization and then filtered through a cellulose nitrate membrane (0.45 µm) and centrifuged. About 0.5 ml of this sample was loaded to a gel filtration column (100x1 cm) packed with BioGel P-2 (BioRad Laboratories, California, USA). Elution was carried out at room temperature (26±2 °C) using triple distilled water at a flow rate of 6 ml/h and 1 ml fractions were collected. The fractions were analyzed by HPLC as mentioned above. The major fractions were pooled up, lyophilized and used for ESI-MS and NMR studies.

### 3.1.2.12. Electrospray tandem mass spectrometry (ESI-MS) of XOS

ESI-MS analysis was carried out on a Waters platform ZMD 4000 system composed of a Micromass ZMD spectrophotometer, a Waters 2690 HPLC and a Waters 996 photodiode array detector (Waters Corporation, USA). Data were collected and processed *via* a personal computer running Mass Lynx software version 3.1 (Micromass, a diversion of Waters corporation, USA). The ESI-MS analysis of XOS has been performed using underivatised samples *(Reis et al., 2004)*. Positive ion mode ESI-MS was acquired, setting the needle voltage at 3000 V with ion source at 80 °C and desolvation temperature at 150 °C maintaining the cone voltage at 30 °C. ESI-MS was performed using argon as cone gas (50 l/h) and hydrogen as desolvation gas (50 l/h).The Tandem Mass Spectra (MS/MS) of molecular ions were obtained using Collision Induced Dissociation (CID). The electron spray probe flow was adjusted to 70 ml/min. Each spectrum was produced by accumulating data during 1-2 min. The hydrolysate was filtered through 0.45 µm cellulose nitrate membranes to remove the unreacted xylan and was further used for the analysis. Mass spectra of pure xylose and xylobiose were also recorded.

### 3.1.2.13. $^{13}$C-NMR spectrometry of xylobiose

The NMR spectrum was recorded at 27 °C with Bruker, AQS 500 MHz. About 10 mg of dried sample of xylobiose (major fraction) was dissolved in $D_2O$ (99.996%) and $^{13}C$ chemical shifts were measured relative to internal Deuterium nucleus. The spectra were

obtained at 125 MHz. The typical acquisition parameters for 1D $^{13}$C-NMR are a 90° pulse of 12 µs, a typical spectral width of 26455 Hz and a repetition time of 4 s. The repetition time was long enough to obtain quantitative data.

### 3.1.2.14. Statistical analysis

All experiments were conducted in duplicates (n=2) and repeated at least three times. The statistical significances and standard deviation were calculated using Microsoft Excel (Version 5.0, Microsoft, Corporation, Redmond, WA)

## 3.1.3. Results

### 3.1.3.1. Evaluation of xylanase production by different fungi

All the eight organisms studied were tested positive for endoxylanase by Congo red staining method (figure 3.1.1). However, the zone of hydrolysis was very less in the case of *Aureobasidium pullulans, Trichoderma viridae* and *Trichoderma harzianum.*

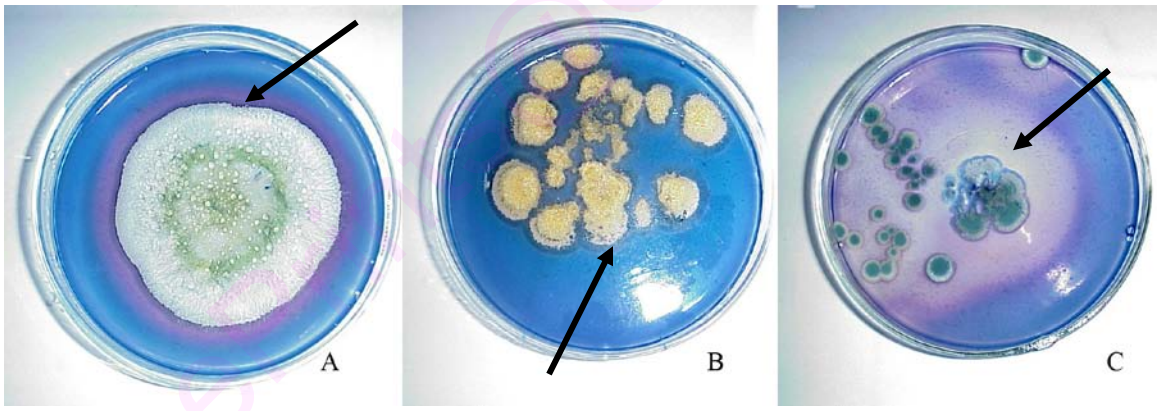



**Figure. 3.1.1:** Congo red staining for detection of endoxylanase activity. Clear zone around the colony shows the zone of hydrolysis. (A) *Aspergillus oryzae,* (B) *Aspergillus ochraceus* and (C) *Penicillium citrinum*

The endoxylanase activity and CMCase activity of the CF obtained by these strains under submerged fermentation were measured. The xylanase activities were estimated and pH was recorded during the course of 144 h of fermentation. Among the organisms evaluated for xylanase production, *Aspergillus oryzae*, *Aspergillus flavus*, *Aspergillus niger* and

*Aspergillus ochraceus* were found to produce high titers of extracellular endoxylanase (figure 3.1.2.)

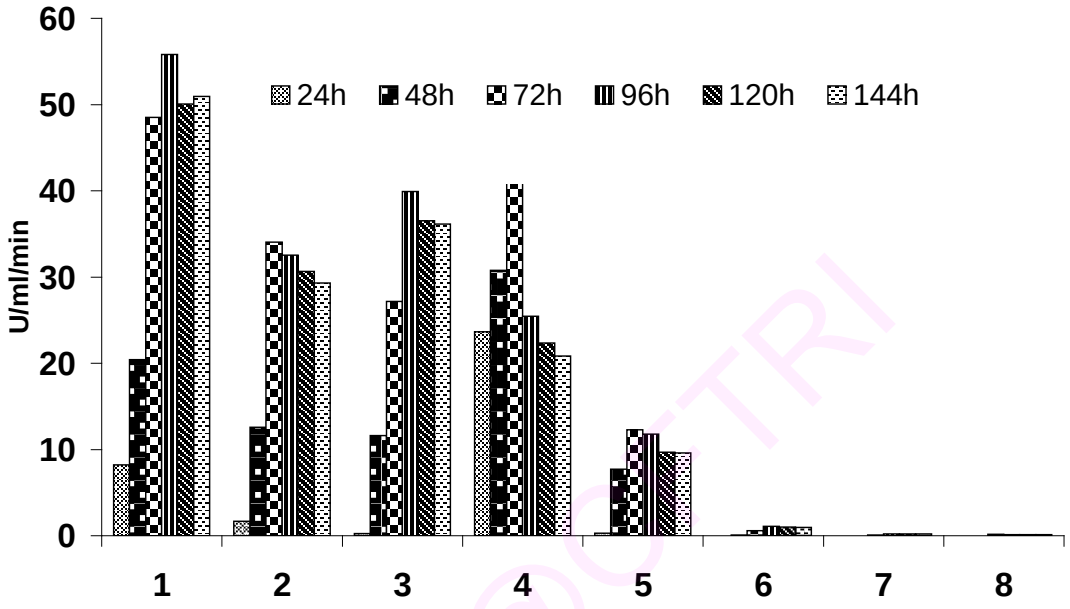



**Figure 3.1.2:** Endoxylanase activity in various fungi during 144 h of fermentation. 1) *Aspergillus oryzae* MTCC 5154, *2) Aspergillus flavus,* 3) *Aspergillus ochraceus,* 4) *Aspergillus niger,* 5) *Penicillium citrinum,* 6) *Aureobasidium pullulans, 7*) *Trichoderma viridae* and 8) *Trichoderma harzianum.*

### 3.1. 3.1.1. Evaluation of xylanase and XOS production by *Aspergillus oryzae*

Among the eight fungal strains studied, the endoxylanase activity was higher in *Aspergillus oryzae* MTCC 5154 as shown in table 3.1.1. The pH of CF increased from an initial value of 5.5 to 7.67 by 24 h and reached a maximum of 8.08 at 96 h of fermentation and thereafter, it decreased (data not shown). The maximum endoxylanase activity was observed at 96 h of fermentation, beyond which it decreased with increase in fermentation time. The extracellular CMCase activity at 96 h of fermentation was negligible (0.103±0.03 U/ml/min)

**Table 3.1.1:** Evaluation of fungi for the production of endoxylanase activity

| Fungus | Fermentation time (h) | Xylanase activity [a] (U/ml/min) | CMCase activity [b] (U/ml/min) |
|---|---|---|---|
| *Aspergillus oryzae* | 96 | 55.8±0.53 | 0.103±0.03 |
| *Aspergillus flavus* | 72 | 34.05±0.56 | 0.094±0.03 |
| *Aspergillus ochraceus* | 96 | 39.93±2.27 | 0.090±0.01 |
| *Aspergillus niger* | 72 | 40.97±0.55 | 0.087±0.04 |
| *Aureobasidium pullulans* | 72 | 1.09±0.01 | 0.066±0.02 |
| *Penicillium citrinum* | 72 | 12.30±0.13 | 0.069±0.02 |
| *Trichoderma viridae* | 96 | 0.20±0.02 | 0.059±0.03 |
| *Trichoderma harzianum* | 72 | 0.16±0.01 | 0.068±0.01 |

a, b: Mean and standard deviations for n =2.

The extracellular xylanase activity was 55.8±0.53 U/ml/min at 96 h of cultivation and the resultant XOS produced using this xylanase was 89.5% and 71.7% at 24 h and 36 h of reaction respectively at 50 °C (table 3.1.2).

**Table 3.1.2:** Evaluation of selected strains of fungi for XOS production

| Fungus | Xylooligosaccharides [a] | | | |
|---|---|---|---|---|
| | 24 h reaction | | 36 h reaction | |
| | % xylobiose | Total XOS (%) | %xylobiose | Total XOS (%) |
| *Aspergillus oryzae* | 62.57±1.83 | 89.5±1.13 | 67.10±0.84 | 71.7±1.15 |
| *Aspergillus flavus* | 53.01±1.56 | 79.0±1.12 | 49.4±1.13 | 79.3±1.13 |
| *Aspergillus ochraceus* | 19.95±1.19 | 83.92±2.24 | 24.82±2.00 | 81.04±0.59 |
| *Aspergillus niger* | 32.45±1.41 | 71.81±1.56 | 36.82±1.30 | 68.40±0.70 |

a: The values are mean and standard deviations for n =2.

Among the eight fungi studied, maximum percentage of XOS in the hydrolysate was obtained from *Aspergillus oryzae* endoxylanase. The xylobiose in the hydrolysate was 62.57±1.83% after 24 h of reaction and it increased to 67.10±0.84% by the end of 36 h of reaction indicating the hydrolysis of XOS of higher DP to xylose and xylobiose with an increase in the reaction time. Figure 3.1.3 represents a typical HPLC chromatogram of XOS mixture obtained.

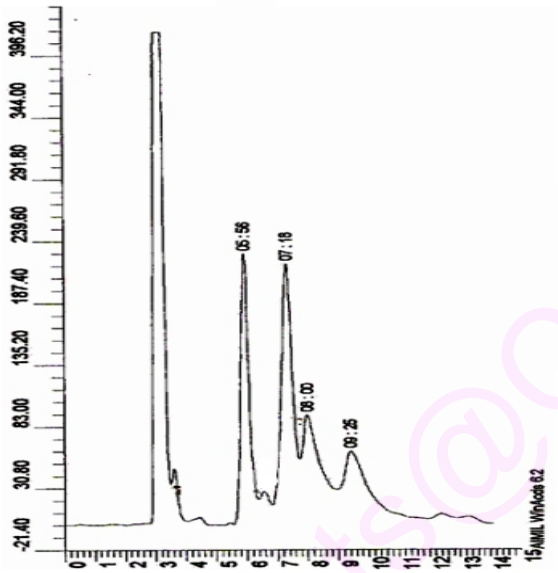



**Figure 3.1.3:** A typical HPLC chromatogram of XOS from birch wood xylan.

(X-axis: Retention time (RT) in minutes and Y-axis: milli-Volt area of peaks. The sugars corresponding to the RT 05:56, 07:18, 08:00 and 09:25 are xylose, xylobiose, xylotriose and xylotetraose respectively).

### 3.1.3.1.2. Evaluation of xylanase and XOS production by *Aspergillus flavus*, *Aspergillus ochraceus* and *Aspergillus niger*

In the case of *Aspergillus flavus* the endoxylanase activity was maximum (34.05±0.56 U/ml/min) at 72 h of cultivation (table.3.1.1). The percentage of XOS produced in the hydrolysate was 79.0±1.12% and 79.3±1.13% at 24 h and 36 h of incubation respectively at 50 °C. This indicates that there is no significant increase in the percentage of XOS beyond 24 h of reaction. *Aspergillus ochraceus* showed maximum endoxylanase activity

at 96 h of fermentation as in the case of *Aspergillus oryzae*; however, was less than that of *Aspergillus oryzae.* The endoxylanase activity at 96 h of cultivation was 39.93±2.27 U/ml/min. The percentage of XOS in the hydrolyzate was 83.92±2.24% and 81.04±0.59% at 24 h and 36 h of reaction respectively at 50 °C. The xylobiose in the hydrolysate was 19.95±1.19% by 24 h of reaction and increased to 24.82±2.0% by 36 h of incubation indicating the hydrolysis of XOS of higher DP to xylose and xylobiose with the increases in the reaction time.

Unlike other *Aspergillus* species evaluated, *Aspergillus niger* showed significant endoxylanase activity of 23.64±0.67 U/ml/min at 24 h of cultivation and a maximum activity of 40.97±0.55 U/ml/min at 72 h of fermentation. The percentage of XOS was 71.81±1.56 and 68.40±0.70 at 24 h and 36 h of incubation respectively at 50 °C. The percentage xylobiose was 32.45±1.41 by 24 h of reaction and increased to 36.82±1.30% by 36 h of reaction indicating the hydrolysis of XOS of higher DP to xylose and xylobiose with an increase in the reaction time.

The endoxylanase activities of the other four species of fungal strains- *Aureobasidium pullulans, Penicillium citrinum, Trichoderma viridae* and *Trichoderma harzianum*-were very insignificant and hence these were not evaluated for XOS production. *Aspergillus oryzae, Aspergillus flavus, Aspergillus niger* and *Aspergillus ochraceus* were found to be efficient for the production of XOS. Among these four *Aspergillus* species, *Aspergillus oryzae* has been selected for further studies as it exhibited maximum endoxylanase activity and high percentage of XOS in the hydrolysate by 24 h of incubation.

### 3.1.3.2. Evaluation of different xylan sources for the production of endoxylanase by *Aspergillus oryzae*

Based on literature *(Kimura et al., 2002),* oat spelt xylan was selected as the carbon source as well as the inducer in the cultivation medium for evaluation experiments and was replaced with 1% birch wood xylan or 3% corncob powder (to equalize the amount of xylan in the medium, as corncob contains only 30-35% xylan). The efficiency of these xylan sources for the xylanase production was evaluated. The cultivation media (50 ml)

was inoculated with 0.5 ml of spore suspension containing $6x10^6$ spores/ml from a five day old slant of *A. oryzae*.

Among the three xylan sources evaluated, the production of endoxylanase was more in media containing 3% corncob (table 3.1.3). The maximum endoxylanase activity was observed at 96 h of growth, beyond which it decreased with an increase in fermentation time. The CMCase activity (table 3.1.3) and total cellulase activity based on filter paper assay was negligible (data not shown).

**Table 3.1.3:** Xylanase and CMCase activity of the CF obtained from three different media by *Aspergillus oryzae* under submerged cultivation conditions.

| F T (h) | Activity in U/ml/min [a] | | | | | |
|---|---|---|---|---|---|---|
| | Media containing 3% raw corncob | | Media containing 1% BWX | | Media containing 1% OSX | |
| | Xylanase | CMCase | Xylanase | CMCase | Xylanase | CMCase |
| 24 | 32.3±4.24 | 0.207±0.07 | 3.12±0.28 | ND [b] | 3.56±0.28 | ND [b] |
| 48 | 60.2±3.46 | 0.201±0.12 | 35.72±1.48 | 0.111±0.08 | 16.56±1.10 | 0.036±0.01 |
| 72 | 71.3±3.39 | 0.179±0.05 | 54.98±0.91 | 0.173±0.06 | 24.01±1.41 | 0.063±0.03 |
| 96 | 81.1±3.74 | 0.120±0.10 | 62.78±2.46 | 0.151±0.06 | 76.73±0.28 | 0.178±0.05 |
| 120 | 70.7±3.74 | 0.189±0.08 | 51.23±2.82 | 0.094±0.02 | 65.17±0.98 | 0.158±0.06 |
| 144 | 69.9±1.83 | 0.203±0.05 | 40.95±5.13 | 0.106±0.05 | 47.33±0.94 | 0.175±0.04 |

a: The values are mean and standard deviations for n =2; b: ND, not detectable; FT: fermentation time; BWX: birch wood xylan; OSX: oat spelt xylan

As can be seen from the results, *Aspergillus oryzae* produced higher titers of xylanases when powdered corncob was used as the substrate in comparison to that of birch wood or oat spelt xylan. The endoxylanase activity at 96 h was 81.1±3.74 U/ml/min and the total maximum protein content was 1.79 mg/ml. Similarly, when birch wood xylan was used

as the xylan source, the maximum endoxylanase activity (62.78±2.46 U/ml/min) was at 96 h of cultivation (table 3.1.3). The CMCase and FPA activity of the CF were also recorded, however, was not significant. A maximum xylanase activity of 76.73±0.28 U/ml/min was observed at 96 h of cultivation when oat spelt xylan was the inducer in the media. The CMCase and FPA activity of the CF were found insignificant (table. 3.1.3).

**3.1.3.3. Production of XOS by *Aspergillus oryzae***

XOS production is maximum with xylanase obtained at 96 h of fermentation in medium containing corncob as the inducer (figure 3.1.4). Maximum XOS produced was 5.87±0.53 mg/ml from birch wood xylan (an initial substrate concentration of 10 mg/ml). The resultant hydrolysate consisted of 12±2.0% xylose, 48±2.43% xylobiose and 40±3.6% other XOS. The xylobiose content by 6 h of reaction was 1.33±0.16 mg/ml which has increased to a value of 5.8±0.33 mg/ml by 30 h. There was a slight increase in the concentration of XOS from 5.70±0.15 mg/ml at 24 h of reaction to 5.87±0.53 mg/ml at the end of 36 h. The amount of XOS produced using the enzyme obtained at 96 h of cultivation in the medium using birch wood xylan as the inducer was comparatively less and was 4.68±0.22 mg/ml at 18 h of reaction, thereafter it reduced slightly. The xylobiose was maximum at 36 h of reaction (3.12±0.21 mg/ml). The amount of XOS produced using oat spelt xylan as the inducer was much lesser when compared to the above two and was 4.03±0.15 mg/ml at 36 h of reaction. A maximum xylobiose of 3.12±0.22 mg/ml was obtained at 36 h of reaction.

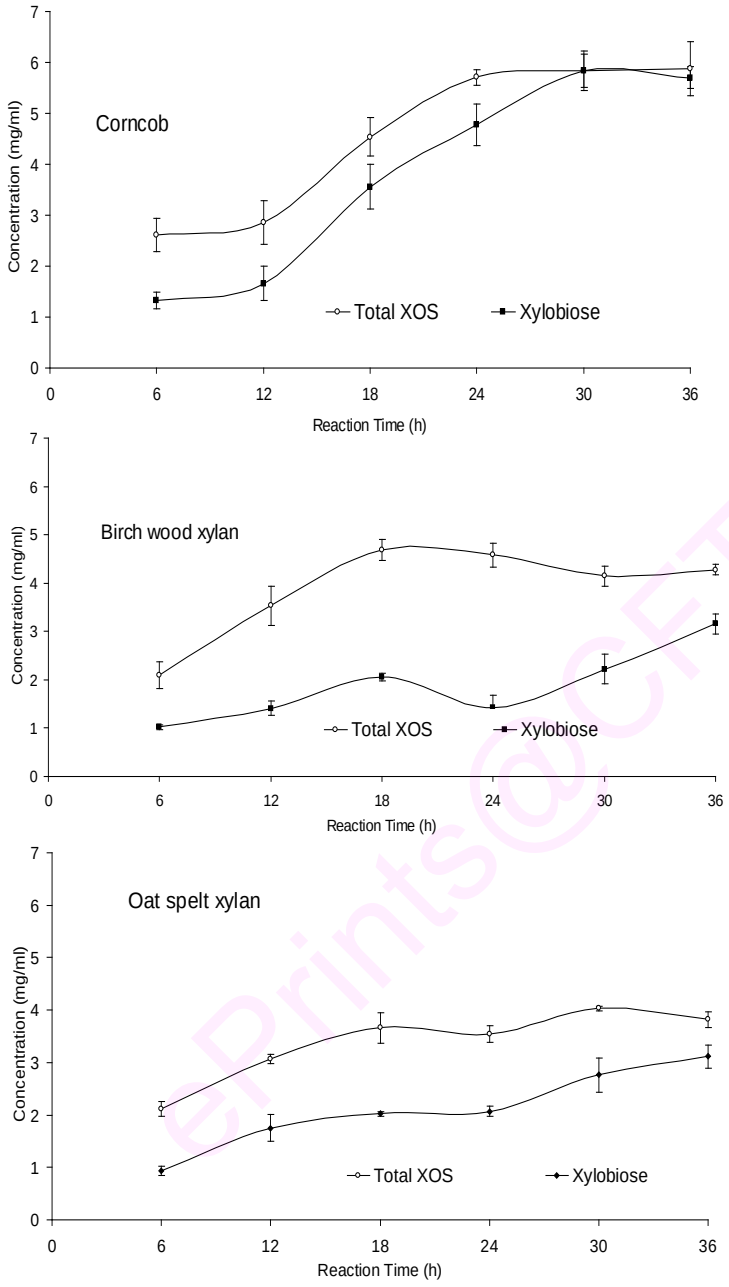



**Figure 3.1.4:** Production of XOS from birch wood xylan (10 mg/ml) using endoxylanases produced by *Aspergillus oryzae* in medium containing different xylan inducers at 96 h of fermentation.

### 3.1. 3.4. ESI-MS of XOS

All measurements were performed in water. In these conditions all the oligosaccharides appeared as $[M+Na]^+$ ions except xylotriose ($X_3$) which formed both $[M+Na]^+$ and $[M+H]^+$ ions. The sodium adducts are found to be more stable in the case of oligosaccharides with higher DP. On the basis of measured mass spectra six different oligosaccharides were identified. In addition, no in-source fragmentation of oligosaccharide ions appeared under the experimental condition used. The mass spectra (figure 3.1.5) showed the presence of various oligosaccharides at an m/z value of 305.19, 365.18, 413.34, 437.19, 569.25, 627.25 and 759.33. The mass spectra of the major fraction obtained by GPC showed the presence of two compounds at an m/z of 301.19 and 365.18.

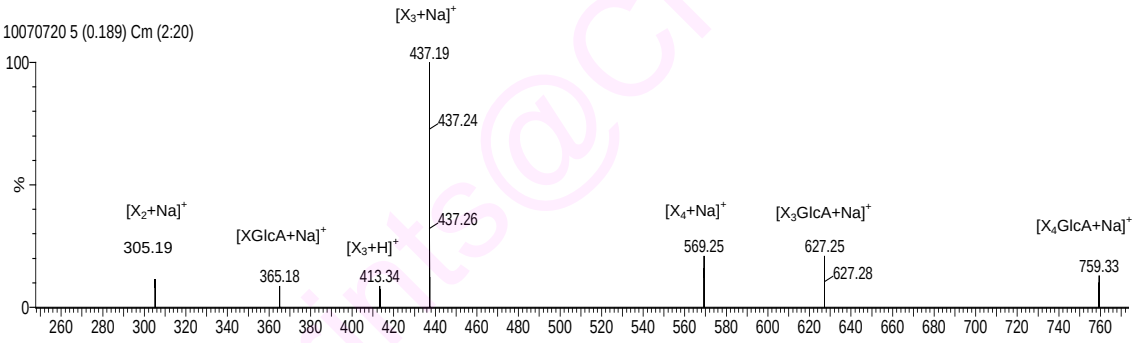



**Figure 3.1.5:** The ESI-MS spectrum of XOS obtained by hydrolyzing birch wood xylan using endoxylanase produced by *Aspergillus oryzae* in a medium containing corncob as inducer.

### 3.1.3.5. $^{13}$C-NMR spectrometry of xylobiose

Analysis of purified disaccharide by $^{13}$C-NMR spectrometry was carried out to specify the structure of the same (figure 3.1.6). Assignments of signals were carried out using $^{13}$C-NMR and were based on published values for some related compounds *(Takeshi et al., 1998)*. Table 3.1.4 gives the complete assignments of the signals which were made by $^{13}$C-NMR. The anomeric carbons of the probable disaccharide appeared at $\delta_{ppm}$ 91.97,

96.3 and 101.57, which were assigned as reducing α-D-xylopyranoside, reducing β-D-xylopyranoside and non reducing β-D-xylopyranoside respectively (figure 3.1.7).

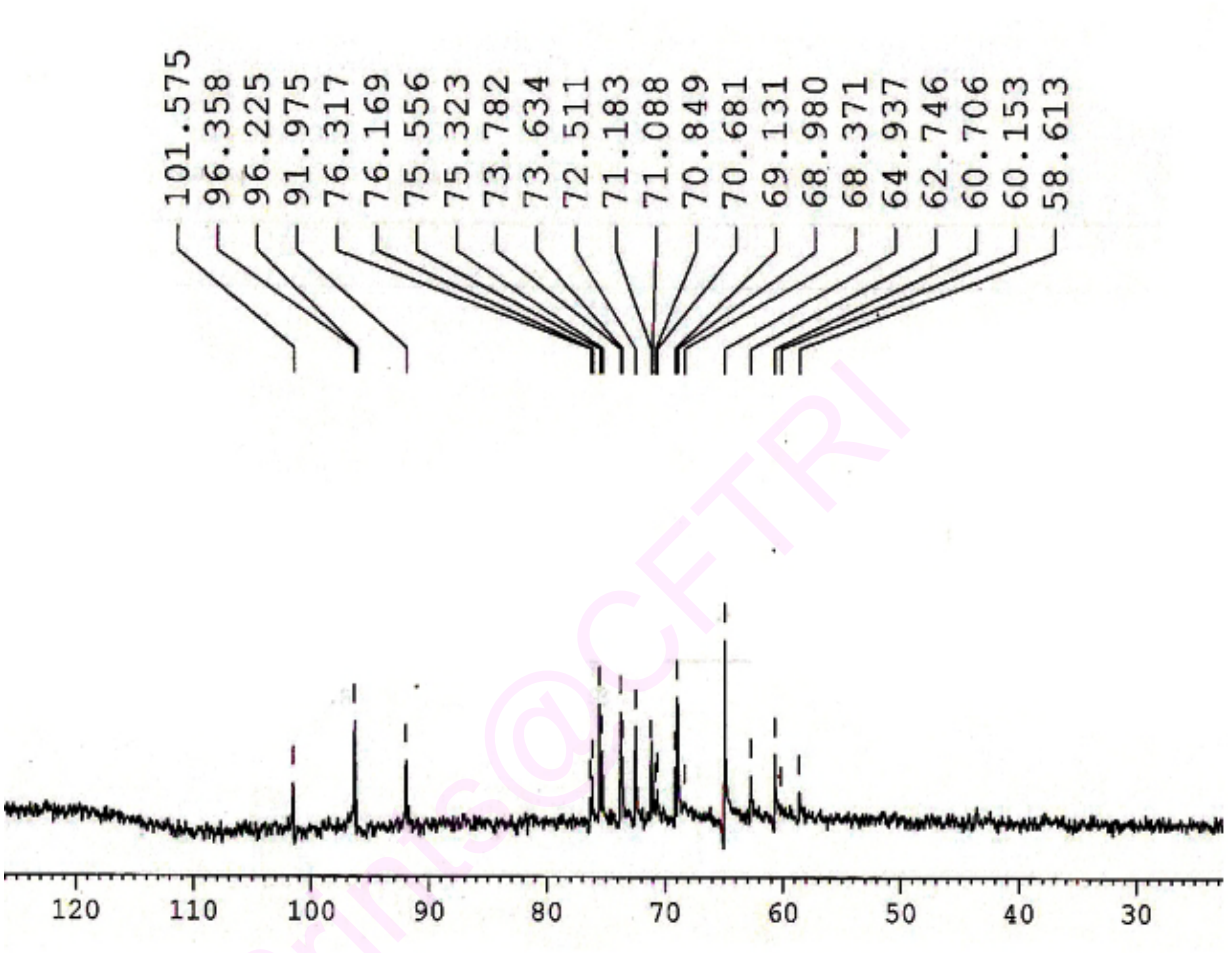



**Figure 3.1.6:** The $^{13}$C-NMR spectrum of xylobiose obtained from birch wood xylan

**Table 3.1.4:** Assignments of signals in the $^{13}$C-NMR [a] spectra of xylobiose obtained from birch wood xylan

| Xylobiose | Chemical shifts ($\delta_{ppm}$) [b] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | C5 | C1' | C2' | C3' | C4' | C5' |
| α- form [c] | 91.97 | 71.18 | 70.08 | 76.3 | 58.6 | 101.57 | 72.5 | 75.32 | 75.55 | 64.9 |
| β- form [d] | 96.3 | 73.6 | 73.7 | 76.1 | 62.7 | 101.57 | 72.5 | 75.32 | 75.55 | 64.9 |

a: In $D_2O$

b: Values are chemical shifts relative to acetone nucleus

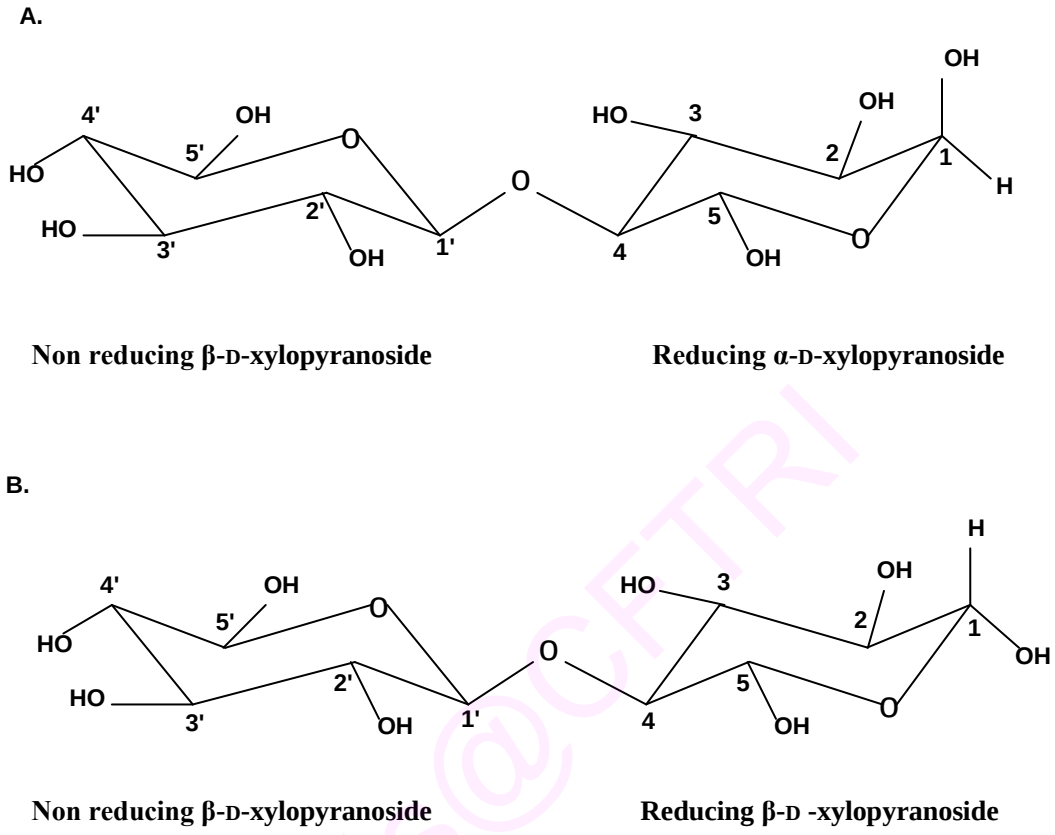



**Figure 3.1.7:** Structure of xylobiose

A. β-D-xylopyranosyl-(1→4)-α-D-xylanopyranose

B. β-D-xylopyranosyl-(1→4)-β- D-xylanopyranose

### 3.1.2. Discussion

Several species of *Aspergillus* have been reported to produce xylanase including *Aspergillus niger, Aspergillus ochraceus, Aspergillus oryzae, Aspergillus awamori, Aspergillus tamarii* and *Aspergillus fumigatus (Kadowaki et al., 1997, Haltrich et al., 1996, Bailey and Poutanen, 1989).* In the present study, all the strains evaluated were found to be positive for extracellular endoxylanase production as evident from the clear zone of xylan hydrolysis observed in Congo red plate assay. *Aspergillus oryzae* is well known for the production of a variety of industrially important enzymes. The xylanases

activity was maximum (55.8±0.53 U/ml/min) at 96 h of cultivation and the pH was 8.08. Alkaline pH is found to be suitable for the production of high titers of endoxylanase. The results of preliminary screening experiments with the other organisms indicated that a pH range of 6.5-8.5 is good for xylanase production, with an exception of *Aspergillus niger*, wherein the pH has drastically reduced during the initial hours of fermentation.

The extracellular CMCase activity of *Aspergillus oryzae* at 96 h of fermentation was negligible (0.103±0.03 U/ml/min) (table 3.1.1) which has an important bearing when LCMs are used as substrates for XOS production. In general, LCMs rich in cellulose and xylan are the preferred substrates for the production of XOS. Since most of the fungal cellulases and xylanases exhibit maximum activity at 50 °C, a cellulase-free xylanase or xylanase with less cellulase activity is advantageous because the glucose released by the action of cellulases on cellulose matrix is an undesirable calorific sugar in the final product, XOS. *Aspergillus oryzae* and *Aspergillus foetidus* have been reported to be non-cellulolytic but *Aspergillus fumigatus* and *Aspergillus terreus* are cellulolytic (*Bailey and Poutanen, 1989*). Similarly, *Aspergillus ochraceus* has been reported to produce cellulase free xylanolytic enzymes when grown on pulverized grass *(Das and Nanda, 1995).* In the present study, all the four species of *Aspergillus* showed insignificant cellulase activity.

The endoxylanase activity of the other four species of fungal strains- *Aureobasidium pullulans, Penicillium citrinum, Trichoderma viridae* and *Trichoderma harzianum*-were very insignificant, though some of them are already reported as good xylanases producers. The results clearly indicate that there are significant differences in the endoxylanase activity produced by the different strains of organisms. *Aspergillus oryzae*, *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus ochraceus* were found to be efficient for the production of XOS. *Aspergillus oryzae* and *Aspergillus flavus* produced more than 50% of xylobiose in the hydrolysate by 24 h of reaction, but *Aspergillus niger* and *Aspergillus ochraceus* produced comparatively less xylobiose even though their endoxylanase has resulted in 71.81±1.56% and 83.9±2.24% of XOS by 24 h.

The vast majority of xylanases are excreted into the extra cellular environment as the large size of the substrate (generally LCMs) prevents its penetration into the cell. Xylanases are usually inducible enzymes secreted into the media containing pure xylan or xylan rich residues *(Balakrishnan et al., 1997).* In fact, the current belief is that the xylanase production is induced by means of the products of their own action *(Singh et al., 2003, Defaye et al., 1992, Bailey, 1989).* It is believed that small amounts of constitutively produced enzymes liberate xylooligomers which may be transported into the cell where they are further degraded by β-xylosidases or by intracellular xylanases *(Shulami et al., 1999)* and where they induce further xylanase synthesis. Induction is mostly by xylan in *Trametes trogii (Levin and Forschiassin, 1998)* and *Aspergillus awamori*. Induction of xylanases by several other compounds such as L-sorbose, various XOS and xylose has been reported *(Beg et al., 2001).* In the evaluation experiment using eight different fungi, the xylan source in the cultivation medium was oat spelt xylan. The use of purified xylan as an inducer increases the cost of enzyme production. For this reason, different LCMs, including wheat bran, wheat straw, corncob and sugar cane bagasse have been used as substrates for the growth and production of xylanases *(Haltrich, 1996).* There are many reports on the different degree of xylanase induction by xylan from various sources. Induction is affected by the type of xylan particularly the extent of branching, the type and frequency of side group on the xylan backbone and the DP of the xylan molecule *(Anita, 1995).* In the present study, the efficiency of birch wood xylan and raw corncob to induce xylanase production was also studied.

The efficiency of corncob in the maximum induction of xylanase production over the other complex carbon sources such as sawdust, wheat bran, rice bran and bagasse has also been reported (*Rani and Nand,* 1996). As can be seen from the results, *Aspergillus oryzae* produced higher titers of xylanases when powdered corncob was used as the substrate in comparison to that of birch wood or oat spelt xylan. In the case of media containing corncob as inducer, a high xylanase activity of 32.2±4.24 U/ml/min was observed by 24 h of cultivation. This could be attributed to the presence of soluble fragments of xylooligomers in the corncob itself. The possibility of methyl xyloside, xylobiose and other xylooligomers inducing xylanase is reported in *Aspergillus oryzae,*

*Aspergillus foetidus, Aspergillus fumigatus* and *Aspergillus terreus* (*Bailey and Poutanen, 1989*). In all the cases, the maximum xylanase production was observed at 96 h of cultivation, with a considerable reduction in the activity as the fermentation proceeds. The CMCase and total cellulase activity of the culture fluids were found to be very insignificant. These results are in agreement with the literature reports *(Li et al., 2006, Samain et al., 1997).* Yet, the reason and regulation of xylanase secretion by microorganisms are still not completely understood. Since xylan is unable to enter the microbial cell, the induction of xylanase is stimulated by low molecular weight xylan fragments, which are produced in the medium by a small amount of constitutively produced xylanase *(Kulkarni et al., 1999, Bastawde, 1992).*

The results of XOS production using endoxylanase from *Aspergillus oryzae* indicated that the maximum production occurred using the enzyme produced in the medium containing corncob as inducer. In all the cases, the amount of XOS produced by 6 h of reaction was around 2 mg/ml and it reached a maximum of 5.84±0.39 mg/ml and 4.03 mg/ml by 30 h of reaction when enzymes obtained in medium containing corncob powder and oat spelt xylan respectively were used for hydrolysis. A maximum of 4.68±0.22 mg/ml of XOS was produced by 18 h of reaction when birch wood induced xylanase was used. In the initial stages of the enzymatic reaction the amount of XOS produced was almost the same; however, the maximum amount of XOS produced varied significantly. These results suggest the diversity in the mode of action of xylanases obtained from different sources.

Xylobiose was the major oligomer of the XOS obtained in all the cases. This is in agreement with the reports on XOS production by xylanase obtained from *Aspergillus kawachii (Ito et al., 1992).* This indicates the true endoxylanase activity of these enzymes, since for an exoenzyme the proportion of xylose should have increased soon after the start of hydrolysis and should have been the major product. The action of purified acidic xylanase from *Aspergillus nidulans* on oat spelt xylan has resulted in xylopentose with a lesser amount of xylotriose and xylobiose by 7 h where as xylobiose was the main product after prolonged incubation of 24 h and 72 h *(Fernandez- Espinar et*

*al., 1994).* The results indicate the potential use of *Aspergillus oryzae* MTCC 5154 for the production of xylanases (with low cellulase activity) by submerged cultivation using raw corncob powder as inducer which can be further used for the production of XOS with higher concentration of xylobiose for food applications.

The ESI-MS indicated the presence of two disaccharides in the XOS mixture mainly xylobiose with a m/z of 305.19 for its sodium adduct $[X_2+Na]^+$. Another disaccharide detected has an m/z value of 365.18 $[XGlcA+Na]^+$which is probably a disaccharide of xylose and 4-*O*-methyl-α-D-glucuronic acid. Both sodium and hydrogen adducts of xylotriose are detected with an m/z value of 437.19 $[X_3+Na]^+$ and 413.34 $[X_3+H]^+$ respectively. The mass spectrum shows the absence of a trisaccharide containing 4-*O*-methyl-α-D-glucuronic acid residue. Two types of tetrasaccharides were observed, xylotetraose and the other having one 4-*O*-methyl-α-D-glucuronic acid residue with an m/z value of 569.25 $[X_4+Na]^+$and 627.25 $[X_3GlcA+Na]^+$respectively. An m/z value of 759.33 $[X_4GlcA+Na]^+$ indicates the presence of a pentasaccharide with 4-*O*-methyl-α-D-glucuronic acid residue. The mass spectra of the major fraction obtained by GPC showed the presence of a disaccharide of xylose and 4-*O*-methyl-α-D-glucuronic acid in addition to the xylobiose. The $^{13}$C-NMR spectrometry of purified disaccharide, revealed the structural details of the same. The signal at $\delta_{ppm}$ 101.57 confirmed the β-(1→4) xylosidic linkage between the two xylose residues. In this way, the major disaccharide was determined as β-D-xylopyranosyl-(1→4)-D-xylanopyranose (xylobiose). The structure of xylobiose based on these data is given in the figure 3.1.6. The formation of xylobiose during the hydrolysis of birch wood xylan explains the structural features of the backbone of xylan and it will also be useful as a basic data for identification of analogous XOS *(Ishihara et al., 1997).*

SECTION: 3.2

# MOLECULAR CLONING AND SEQUENCING OF ENDOXYLANASE GENE OF *Aspergillus oryzae* MTCC 5154

### 3.2.1. Introduction

*Aspergillus oryzae* is a fungus widely used in traditional Japanese fermentation industries, including soy sauce, sake, bean curd seasoning and vinegar production. Filamentous fungi generally have the ability to produce various types and large amounts of enzymes in a secretory manner. Among filamentous fungi, *Aspergillus oryzae* is known to have prominent potential for the secretory production of various enzymes. In addition, developments in genetic engineering technology have led to the application of *Aspergillus oryzae* in the production of industrial enzymes in modern biotechnology. *Aspergillus oryzae* was used for the first example of commercial production of heterogonous enzyme, the lipase for laundry detergent in 1988 *(Christensen et al., 1988).*

The long history of extensive use of *Aspergillus oryzae* in food fermentation industries prompted industrial applications of *Aspergillus oryzae* to be listed as Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) in the USA. The safety of this organism is also supported by World Health Organization *(FAO-WHO, 1987).* Although *Aspergillus oryzae* is genetically very close to *Aspergillus flavus,* which is known to produce aflatoxin, *Aspergillus oryzae* has no record of producing aflatoxin or any other carcinogenic metabolites *(Barbesgaard et al., 1992).* Fermented foods produced by *Aspergillus oryzae* have been shown to be aflatoxin free *(Matsushima et al., 2001a, Matsushima et al., 2001b).* The two species, *Aspergillus oryzae* and *Aspergillus flavus* were traditionally distinguished based on morphological, physiological and culture-based characteristics *(Murakami, 1971).* Recent DNA based techniques have enhanced the potential for distinction *(Lee et al., 2006, Chang et al., 1995, Gomi et al., 1989).* Zhang *et al.* (2005) reported that homologs of aflatoxin biosynthesis gene cluster of *Aspergillus oryzae* were not expressed even under the conditions that are favorable to aflatoxin expression in *Aspergillus flavus* and *Aspergillus parasiticus.*.

Sequencing the genome of *Aspergillus oryzae* RIB40 (ATCC- 42149) was completed in 2005 *(Machida et al., 2005).* The sequenced strain is a wild-type strain, most similar to those used for sake brewing but still has the strong ability to produce proteases, which is

one of the most important characteristics for soy-sauce fermentation. *Aspergillus oryzae* was first isolated from *koji* by H. Ahlburg in 1876. Its original name, *Eurotium oryzae*, was later renamed *Aspergillus oryzae* by F. Cohn because he found that it lacked the ability of sexual reproduction. It is said that *Aspergillus oryzae* is a domesticated species and therefore, that *Aspergillus oryzae* may be found only in a domesticated form but not in nature. Some reports about the isolation of *Aspergillus oryzae* from soil, plants and food support the natural isolation of *Aspergillus oryzae* for fermentation *(Klich, 2002)*. However, these species might now be distributed in nature following domestication many years ago.

The *Aspergillus oryzae* genome consists of eight chromosomes with an entire genome size of 37.6 Mb. Comparison of the *Aspergillus oryzae* genome with two other *Aspergilli* species such as *Aspergillus nidulans* and *Aspergillus fumigatus*, sequenced in the similar timelines revealed that *Aspergillus oryzae* had a 25-30% bigger genome size than the other two species *(Machida et al., 2005)*. Accordingly, *Aspergillus oryzae* had 2000-3000 more genes than the other two species, indicating similar gene densities of the three species. The increase of gene number, in accordance with the increase of genome size, mainly derived from the gene expansion of metabolic genes. This includes secretory hydrolases, transporters and primary and secondary metabolism genes, among which the expansion of secondary metabolism genes is most prominent *(Machida et al., 2005). A. oryzae* is known to have a wide variety of hydrolytic enzymes and a strong capacity for degrading various materials. The gene expansion of secretory hydrolases explains these abilities well. The multiple transporters may be used for efficient degradation or further conversion of degraded materials.

Several genes encoding endoxylanases from *Aspergilli* have been cloned. The encoded enzymes have been assigned to glycosidase families 10 and 11 (table 3.2.1.) and they all work *via* a retaining mechanism. Based on the data of the *Aspergillus kawachii* endoxylanases, it would appear that the acidic endoxylanases belong to family 11 whereas the neutral endoxylanases belong to family 10. However, more data on other neutral and acidic endoxylanases are needed to verify this. Recently, a method was

developed to experimentally determine whether an endoxylanase belongs to family 10 or 11 *(Ntarima et al., 2000).* This method is based on the irreversible inhibition of family 11 endoxylanases by epoxyl glycosides of D-xylose and XOS, whereas family 10 endoxylanases are unaffected *(Ntarima et al., 2000).*

**Table 3.2.1:** Genes encoding *Aspergillus* endoxylanases and their assignment to the glycosidase families

| **Species** | **Activity/ Glycosidase family** | **Gene** | **Database accession no.** |
|---|---|---|---|
| *Aspergillus awamori* | EXL*/11 | *exlA* | X78115 |
| *Aspergillus kawachii* | EXL/10 | *xynA* | D14847 |
| *Aspergillus kawachii* | EXL/11 | *xynB, xynC* | D38070, S45138 |
| *Aspergillus nidulans* | EXL/11 | *xlnA, xlnB* | Z49892, Z49893 |
| *Aspergillus nidulans* | EXL/10 | *xlnC* | Z49894 |
| *Aspergillus niger* | EXL/11 | *xynB* | D38071 |
| *Aspergillus oryzae* | EXL/11 | *xynG1* | AB003085 |
| *Aspergillus oryzae* | EXL/10 | *F1* | AB011212 |
| *Aspergillus tubingensis* | EXL/11 | *xlnA* | L26988 |

*EXL: endoxylanase activity

Xylanolytic enzymes from *Aspergilli* have all been found to be produced on xylose-, xylan-, and crude-xylan-containing substrates but not on other monomers (e.g. glucose, galactose) or polymers (e.g., cellulose and pectin). However, several cellulolytic enzymes are also produced in the presence of xylan and xylose. This suggests a general system of regulation of the genes encoding these enzymes. Several genes encoding xylanolytic enzymes have been studied with respect to regulation of expression and all demonstrated expression in the presence of D-xylose, xylobiose or xylan *(Perez-Gonzalez et al., 1998, Gielkens et al., 1997, van Peij et al., 1997, Kumar and Ramon, 1996, Fernandez-Espinar et al, 1994)* but repression of expression in the presence of glucose. Expression of some

xylanolytic genes was also observed on L-arabinose *(de Vries and Visser, 1999, de Vries et al, 1998).* This might be due to the presence of small amounts of D-xylose in the L-arabinose preparations used, as detected in the L-arabinose preparation obtained from Sigma. Additionally, induction of xylanolytic enzymes was observed on cellobiose and cellulose and on a heterodisaccharide consisting of glucose and xylose (Glcβ1-2Xyl) in *Aspergillus terreus*, which are compounds that also induce the cellulolytic system from this fungus.

Sophorose and 2-*O*-β-D-xylopyranosyl-D-xylose specifically induced the synthesis of cellulolytic and xylanolytic enzymes, respectively, in this fungus. A xylose-induced, glucose-repressed, endoxylanase-encoding gene (*xynG1*) from *Aspergillus oryzae* was expressed in *Aspergillus nidulans*, resulting in expression of the gene on xylose as well as glucose *(Kimura et al., 1998).* This indicates that regulation of the expression of xylanolytic genes is not identical in *Aspergillus oryzae* and *Aspergillus nidulans*, although identical regulation of xylanolytic genes has been reported for *Aspergillus niger* and *Aspergillus tubingensis (de Graaff et al., 1994).*

From *Aspergillus niger* a gene encoding a transcriptional activator has been isolated by complementation of a mutant unable to degrade xylan *(van Peij et al., 1998a).* Sequence analysis of this factor, XlnR, demonstrated that it is a member of the GAL4-like family of transcriptional activators. Characterization of XlnR showed that it was responsible for the expression of genes encoding endoxylanase and β-xylosidase. Analysis of the promoter region of these genes identified a putative XlnR binding site, GGCTAAA, of which the second G was determined to be essential for XlnR binding by band mobility shift assays and *in vivo (van Peij et al., 1998a).* A more detailed analysis of the role of XlnR in the regulation of genes involved in xylan, arabinan and cellulose degradation indicated that this protein does not activate only the expression of xylanolytic genes *(van Peij 1998b).* Genes encoding two endoxylanases (*xlnB* and *xlnC*), a β-xylosidase (*xlnD*), an arabinoxylan arabinofuranohydrolase (*axhA*), an acetylxylan esterase (*axeA*), an α-glucuronidase (*aguA*), a feruloyl esterase (*faeA*) and two endoglucanase (*eglA* and *eglB*) were found to be regulated by XlnR *(van Peij et al., 1998a).* However, genes encoding α-

arabinofuranosidase (*abfB*) and β-glucosidase (*bglA*) were not regulated by this protein. This indicates that in addition to its role as a xylanolytic activator, XlnR also regulates the expression of some, but not all, genes encoding cellulolytic enzymes. A subsequent study demonstrated that XlnR is also involved in the regulation of α- and β-galactosidase genes (*aglB* and *lacA* respectively) *(de Vries et al., 1999),* two cellobiohydrolase-encoding genes (*cbhA* and *cbhB*) *(Gielkens et al., 1999)* and a gene encoding xylose reductas.

Analysis of the promoter regions of the genes that are regulated by XlnR demonstrated that the third A in the consensus for the binding site is variable and the consensus sequence was therefore shortened to GGCTAA *(van Peij et al., 1998a).* However, the presence of a putative XlnR binding site does not automatically imply regulation by XlnR. A putative XlnR binding site was detected in the promoter of an endoglucanase from *Aspergillus nidulans*, but no expression of this gene was detected on xylose *(Chikamatsu et al., 1999).* Introduction of a large number of copies of a xylanolytic gene or the promoter of a xylanolytic gene results in a decrease in the expression of other XlnR-regulated genes *(van Peij, 1999).* This indicates that the production of XlnR is tightly balanced with the number of genes this protein regulates. Indications for a transcription activator from *Aspergillus oryzae* binding to a similar DNA region have been obtained *(Kato et al., 1999).*

Recently a model was suggested for the role of XlnR in the regulation of (hemi) cellulose degradation by *Aspergillus niger***.** XlnR is activated during growth of *Aspergillus niger* in the presence of arabinoxylan by monomeric xylose which is already present in the substrate or released by endoxylanase B and β-xylosidase that are present at low constitutive levels. XlnR then activates the expression of (hemi) cellulolytic genes *(de Vries et al., 1999, Gielkens et al., 1999, van Peij et al., 1998b).* However, if the concentration of xylose is high, this causes CreA-mediated repression, resulting in reduced expression of these genes. This effect is stronger in the presence of glucose, which prevents the expression of (hemi) cellulolytic genes to a large extent *(de Vries et al., 1998, de Graaff 1994).* XlnR-mediated expression of (hemi) cellulolytic genes results

in the release of arabinose, cellobiose, ferulic acid, and release galactose by the enzymes encoded by these genes. These compounds induce the expression of other genes.

The present investigation focuses on the molecular cloning and sequencing of exons of xylanase gene of *Aspergillus oryzae* MTCC 5154 and the homology analysis with the available *Aspergillus* xylanase sequences.

### 3.2.2. Materials and methods

#### 3.2.2.1. Chemicals

All chemicals were of analytical grade. Standards of xylose, glucose and oat spelt xylan were from Sigma-Aldrich (USA).

#### 3.2.2.3. Microorganisms and culture conditions

*Aspergillus oryzae* MTCC 5154, a soil isolate was maintained on potato dextrose agar (Himedia Laboratories Ltd, India) slants at 4 °C as mentioned in the section 3.1.2.5.

#### 3.2.2.4. Isolation of chromosomal DNA from *Aspergillus oryzae* MTCC 5154

Mycelia were obtained after inoculating 20 ml PDB (HiMedia, Mumbai, India) in 100 ml flasks by transferring a loopful of mycelia from a five day old slant and incubated at 28 °C for 3-4 days. Mycelia were collected by filtration and washed with sterile deionized water. DNA isolation was performed following the modified method of Lee *et al.* (2001). Approximately 100 mg of mycelia was ground in 500 µl lysis buffer that has been preheated at 65 °C for 90 min. The contents were transferred to sterile micro-centrifuge tube and vortexed. The tubes were incubated for 90 min in a water bath set at 65 °C with intermittent vortexing of the tubes at intervals of 30 min. Equal volume of phenol:chloroform:isoamyl alcohol was added and the tubes were centrifuged at 10,000 rpm for 15 min. The supernatant was transferred to a fresh micro-centrifuge tube. Phenol:chloroform:isoamyl alcohol extraction was repeated 3-4 times which helped in minimizing protein contamination to a greater extent. 200 µl of 1M NaCl and 800 µl of ice cold ethanol were added and incubated overnight at -20 °C for precipitation of DNA. The tubes were centrifuged at 10000 rpm for 15 min. The pellet was washed in 70 % ice

cold ethanol, finally suspended in suitable volume of TE buffer and stored at -20 °C thereafter. The quality of DNA preparation was checked by 0.8% agarose gel electrophoresis

**3.2.2.5. Designing of oligonucleotides**

The xylanase gene is 725 bp in length with two exons of length 263 and 400 bp respectively and one intron of length 62 bp. The complete xylanase gene sequence of different xylanase producers available in the NCBI databank were compared using Dialign 2 *(Morgenstern, 1999),* a program which gives base to base alignment of the given sequences.

The xylanase gene sequences of various xylanolytic *Aspergilli* were highly conserved (97-99 %) at the exons with some variations at the intron. Two sets of primers were synthesized based on the cDNA sequences available in the GenBank (accession nos. AB003085). Primers XYLF1, XYLR1, XYLF2 and XYLR2 for amplification of exons were picked up from the highly conserved regions of xylanase gene. The nucleotide sequence of the primers designed is presented in table 3.2.2.

**Table 3.2.2:** The list of primers designed for amplification of exons of XYL gene

| Sl. No | Oligo name | 5′-sequence-3′ | Length |
|---|---|---|---|
| 1 | XYLF1 | 5′-ATGGTTAGCTTCTCTTCTCT -3′ | 20 |
| 2 | XYLR1 | 5′-TTCGCACTGCCGGGGTTCCA -3′ | 20 |
| 3 | XYLF2 | 5′-GACTGTGACCTACAGCGGTG -3′ | 20 |
| 4 | XYLR2 | 5′-TTACTCAACAGTGATAGTGG -3′ | 20 |

**3.2.2.6. PCR amplification of exons of XYL gene of *Aspergillus oryzae* MTCC 5154**

Various combinations of primers were tried to get the possible amplicons for XYL gene. The PCR reaction was carried out by combining the following reaction components in 50µl reaction volume:

| Components | Volume (μl) | Final concentration |
|---|---|---|
| Nuclease-free water | 37.5 | |
| 10 X Reaction Buffer | 5.0 | 1 X |
| dNTP mix (10 mM) | 1.0 | 0.2 mM |
| DNA-Taq polymerase (3U/ μl) | 0.5 | 0.03 U/μl |
| Primer XYLF1 or XYLF2 (Forward) | 2.0 | 0.2 μM |
| Primer XYLR1 or XYLR2 (Reverse) | 2.0 | 0.2 μM |
| Template (~100 ng) | 2.0 | |

The contents of the tube were mixed by a brief spin in a micro centrifuge. The reaction was carried out in a thermocycler GeneAmp PCR System 9700 (Perkin-Elmer, USA) and the reaction parameters were as follows:

a. Initial Denaturation: 94 °C for 4 min
b. Denaturation: 94 °C for 1 min
c. Annealing: 50 °C for 1 min
d. Extension: 72 °C for 1 min

(b–d: 30 cycles)

e. Final extension: 72 °C for 10 min

**3.2.2.7. Analysis of PCR products by agarose gel electrophoresis**

A 10 μl aliquot of the PCR product was analyzed by agarose gel (1.2%) electrophoresis as described below. The size of the exons of XYL gene amplicon was checked by comparing with a 3kb DNA Marker (MBI Fermentas, Lithuania). The boat was sealed with an adhesive tape and the comb was placed for the wells. 1.2 g of agarose was added to 100 ml of 1 X TAE buffer. The mixture was heated on a hot plate to allow the agarose to dissolve. The solution was cooled to 50 °C and poured into the sealed boat. The gel was allowed to polymerize. The comb and the adhesive tape were removed and the gel was placed in the electrophoresis tank with sufficient volume of 1 X TAE buffer to cover the surface of the gel. The PCR reaction sample and the standard DNA size marker were loaded in the wells.

Electrophoresis was carried out at 50 volts till the dye reached 3/4$^{th}$ of the gel. The gel was removed from the tank and stained by soaking in a solution of 0.5 μg/ml ethidium bromide for 30 min at room temperature. The gel was de-stained in distilled water for 10 min, examined on a UV transilluminator and documented using Gel Documentation system (Herolab, Germany).

### 3.2.2.8. Purification of amplicons of exons of XYL gene of *Aspergillus oryzae*

The amplicons for exons of XYL gene were purified using GenElute PCR Clean - Up Kit (Sigma, USA) following manufacturers instructions.

### 3.2.2.9. Cloning of amplicons of exons of XYL gene from *Aspergillus oryzae*

#### 3.2.2.9.1. Ligation of PCR product to vector

The purified PCR products for exons of XYL gene were cloned by ligating to pTZ57R/T vector using InsT/A Clone PCR Product Cloning Kit (MBI Fermentas, Lithuania) and transforming competent cells of *Escherichia coli* strain DH5α. The following components were added in a thin-walled 0.2 ml PCR reaction tube:

| | | |
|---|---|---|
| Plasmid vector pTZ57R/T DNA | : | 2.0 μl |
| Purified PCR fragment | : | 10.0 μl |
| 10X Ligase Buffer | : | 3.0 μl |
| PEG 4000 solution | : | 3.0 μl |
| T4 DNA Ligase, 5U/μl | : | 1.0 μl |
| Deionized water (to make upto 30.0 μl) | : | 11.0 μl |

The reaction components were mixed by pipetting or brief spin. The reaction was incubated at 22 °C for 4 h. The enzyme was inactivated by heating the reaction to 65 °C for 10 min.

**3.2.2.9.2. Transformation of *Escherichia coli* using the ligation reaction mix**

**3.2.2.9.2.1. Preparation of competent cells using $CaCl_2$**

Competent cell preparation was carried out following the procedure of Sambrook and Russell (2001). A single colony of *Escherichia coli* (DH5α strain) from a plate, freshly grown for 16-20 h at 37 °C was picked and transferred into 50 ml of LB broth in a 250 ml conical flask. The culture was incubated at 37 °C with rigorous shaking. The $OD_{600}$ of the culture was determined periodically to monitor cell growth. When the $OD_{600}$ reached 0.40-0.50, the cells were transferred aseptically to 50 ml sterile polypropylene tube. The culture was cooled by storing the tube on ice for 10 min. The cells were recovered by centrifugation at 4000 rpm for 10 min at 4 °C. The media was decanted from the cell pellet. The cell pellet was re-suspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice. The cells were recovered by centrifugation at 4000 rpm for 10 min at 4 °C. The fluid from the cell pellet was decanted and the tubes were kept in an inverted position for 1 minute to allow the last traces of fluid to drain away. The cell pellet was re-suspended in 2.0 ml of ice-cold 0.1 M $CaCl_2$ and cells were stored at 4 °C overnight.

**3.2.2.9.2.2. Transformation of competent cells**

Transformation of *Escherichia coli* was carried out by $CaCl_2$ method *(Sambrook and Russel, 2001).* About 200 µl suspensions of competent cells were added to sterile micro-centrifuge tubes. Plasmid DNA (~50 ng) or 2 to 5 µl of ligation mixture was added to each tube. The contents of the tubes were mixed by swirling gently and the tubes were stored on ice for 30 min. Control samples were included as following: (a) competent cells that received standard supercoiled plasmid DNA and (b) competent cells that received no plasmid DNA. The tubes were transferred to water bath set at 42 °C for 90 seconds to subject the cells to heat shock. The tubes were rapidly transferred to ice and the cells were allowed to chill for 1-2 min. 800 µl of SOC medium was added to each tube and the cultures were incubated for 45 min at 37 °C in a shaker incubator set at 150 rpm.

**3.2.2.10. Selection of transformants/recombinants**

The transformants were selected on ampicillin containing LB agar plates. 30 µl each of X-Gal and isopropyl-beta-thiogalactopyranoside (IPTG) were spread on the agar surface

(before plating bacteria) for colour selection. 75-100 µl aliquots of the transformed bacterial cells were plated on to the agar surface. Control *Escherichia coli* competent cells were plated on to LB plates containing ampicillin, X-Gal-IPTG and those devoid of them. The plates were incubated at 37 °C for 15-17 h.

**3.2.2.11. Analysis of transformants/recombinants**

The transformants were checked for the presence of plasmid by isolating plasmid DNA and performing 0.8% agarose gel electrophoresis. Restriction digestion experiments were set up to check for insert release from recombinants.

**3.2.2.11.1. Isolation of plasmid DNA from the transformed colonies**

The plasmids were isolated from the transformed *Escherichia coli* cells by alkali lysis method *(Birnboim and Doly, 1979).* Single colonies of appropriate strain were inoculated in 2 ml of LB broth containing required antibiotic and grown overnight in a shaker incubator at 37 °C and 180 rpm. 1.5 ml of the overnight culture was transferred to a 1.5 ml micro centrifuge tube and the cells were harvested by centrifugation at 10000 rpm for 2 min. After discarding the supernatant, 100 µl of solution I was added and vortexed vigorously until no visible clumps of cells were observed. The samples were kept on ice for 5 min. About 200 µl of freshly prepared alkaline solution (solution II) was added to the tube and mixed gently by inverting the tubes several times until the lysed cell suspension became clear. The samples were kept on ice for 5 min, 150 µl of ice-cold potassium acetate solution (solution III) was added, and tubes were inverted gently. The tubes were centrifuged at 10000 rpm for 10 min. The supernatant was transferred to a fresh tube and equal volume of phenol- chloroform was added and vortexed thoroughly. Centrifugation at 10000 rpm for 10 min separated the two phases. The upper aqueous phase was transferred to a fresh tube and equal volume of chloroform was added. The tubes were centrifuged at 10000 rpm for 10 min. The upper aqueous phase was transferred to a fresh tube and 2 volumes of absolute ethanol were added. The tubes were kept at -20 °C for 1 h to overnight for precipitation. The tubes were centrifuged at 10000 rpm for 10 min and the supernatant was discarded. The pellet was washed with 300 µl of

70% ethanol and the air-dried pellet was dissolved in 20 µl of TE buffer. Samples were tested by carrying out agarose gel (0.8%) electrophoresis along with control plasmid.

**3.2.2.11.2. Double restriction digestion of recombinant plasmids for insert release**

Restriction enzymes such as *Eco RI and Pst I* (MBI Fermentas, Lithuania) were used for double digestion experiment. The following constituents were added in a micro centrifuge tube in the order stated:

| Constituents | Volume (µl) |
|---|---|
| Nuclease-free water | 13.0 |
| Restriction enzyme 10 x buffer | 2.0 |
| Plasmid DNA | 3.0 |
| Restriction enzyme 1 (*Eco RI)* | 1.0 |
| Restriction enzyme 2 *(Pst I)* | 1.0 |
| Final volume | 20.0 |

The contents of the tube were mixed gently by pipetting and the tube was centrifuged briefly at 10000 x g to collect the contents at the bottom of the tube. The reactions were incubated at 37 °C for 4-8 h. The samples were analyzed by agarose gel (1.5%) electrophoresis.

**3.2.2.12. Sequencing of the clones**

DNA sequencing was carried out using M13F and M13R primers by dideoxy chain termination method *(Sanger et al., 1977)*. The reaction was carried out in an automatic DNA sequencer (ABI prism, Applied Biosystems, USA) with fluorescent dideoxy chain terminators at Bangalore Genie, Bangalore, India.

### 3.2.3. Results

#### 3.2.3.1. PCR amplification of exons of XYL gene of *Aspergillus oryzae* MTCC 5154

Two sets of primers were tried to get amplicons for XYL gene. Primer combination of XYLF1 and XYLR1 resulted in amplification of a product approximately 250 kb in size and primer combination of XYLF2 and XYLR2 resulted in amplification of a product approximately 400 kb in size. Approximate size of the amplicons were obtained from agarose gel electrophoresis wherein the size of the amplicons were checked against a 3 kb DNA Marker (figure 3.2.1).

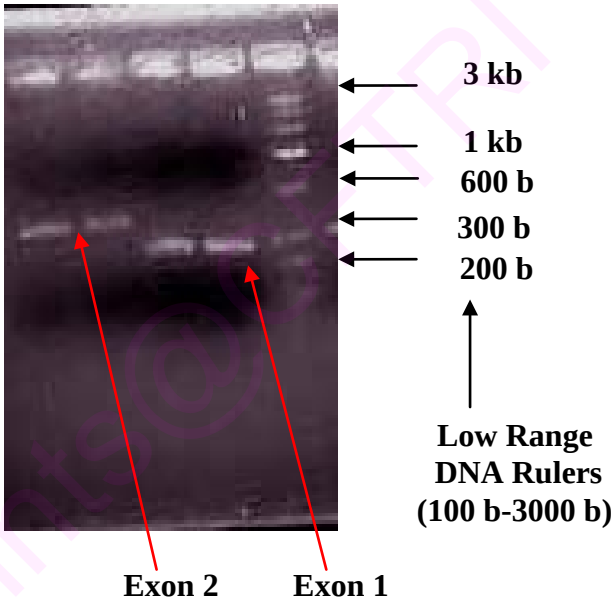



**Figure 3.2.1:** Amplification of exon 1 and exon 2 from xylanase gene of *Aspergillus oryzae* MTCC 5154

#### 3.2.3.2: Cloning of amplicons of XYL gene from *Aspergillus oryzae* MTCC 5154

The transformants were selected on ampicillin containing LB agar plates. In this study, PCR fragments which are expected to be the exons of the XYL gene were amplified from the genomic DNA of *Aspergillus oryzae* MTCC 5154 and their size were found to be approximately 250 and 400 bp, respectively, by using agarose gel electrophoresis. The PAX1 and PAX2 were used for the amplification of the inserted fragments and this resulted in fragments of approximate sizes of 250 and 400 bp respectively. These

fragments were cloned into pTZ57R/T to obtain PAX1 and PAX2 (figure 3.2.2). Amplification of the 260 and 400 bp PCR fragments were obtained from PAX1 and PAX2 respectively. Restriction digestion of PAX1 and PAX2 using *Eco RI and Pst I* enzymes also resulted in the release of 250 and 400 bp fragments respectively (figure 3.2.3).

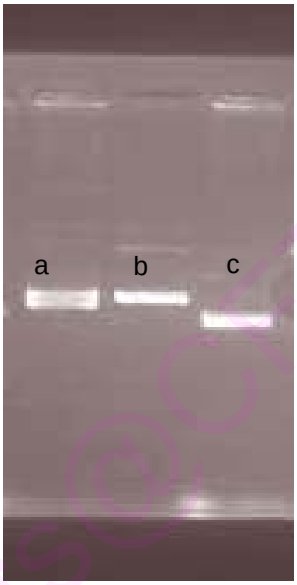



**Figure 3.2.2:** Ligation of exon1 and exon 2 in pTZ57R/T (a: PAX1, b: PAX2, c: pTZ 57R/T control vector)

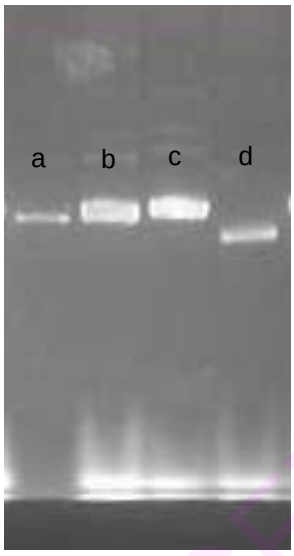



**Figure 3.2.3:** Release of insert from PAX1 and PAX2 (a: PAX1 after restriction digestion, b: PAX1, c: PAX2 after restriction digestion, d: PAX2)



### 3.2.3.3. Sequencing of amplicons of XYL gene from *Aspergillus oryzae* MTCC 5154

The sequences of 203 and 397 bp fragments of XYL gene obtained after sequencing using M13 forward primer is provided in figure 3.2.4. The sequences were homologous (97-99%) to the XYL gene sequence of various *Aspergillus oryzae* strains (table 3.2.3 and 3.2.4). Multiple alignment of the sequences with that of other *Aspergillus* species also indicated greater homology of the XYL gene fragments with that of *Aspergillus oryzae* (figure 3.2.5 and figure 3.2.6).

ATGGTTAGCTTCTCTTCTCTCCTCCTTGCGGTTTCCGCTGTCTCCGGCGCTTTGGCTGCCCCTGGCGATTCCACTCTTG
TTGAGCTCGCCAAGCGCGCTATCACCAGCTCCGAGACAGGCACCAACAACGGCTACTACTACTCCTTCTGGACCAACGG
CGGTGGTGATGTCAAATACACCAATGGCAACGGCGGCCAGTACAG

a) Sequence of exon 1 of XYL gene

TACTCAACAGTGATAGTGGCAGACCCGTCTGCTCTGGTAGCCCTCGGTGGCAAGGATCATGTAGTTATGGGTACCCAGC
TGCAAACCAACGTTGGCCCAGCATCGAAGTGGTTCTGAGCAGTGATGGTGCCACCAACAGGCCGCTCTGGCGGACCGAC
CAGTACTGGTTGAAGGTCGAGGTGCCCTCAATGGAGGGACGTTTTCACGAGTAGTCTGGTAGATCTTGTAGGTGCCGCC
ATCGCTCTCGACGGTGCCGAGCTCGGTAGCGCCGGTGGAGGGATCGTAGTCGCCGTACTTGTCAACGATGTAGTATTCG
ACGAGGGGGTTCTGGGTCCAGCCATAGAGAGAGACGTAGCTGTTGCTGTTGCTCTCCCACTCACCGCTGTAGGTCACAG
TC

b) Sequence of exon 2 of XYL gene

**Figure 3.2.4:** Sequences of exon 1 and exon 2 of XYL gene of *Aspergillus oryzae* MTCC 5154

**Table 3.2.3:** Sequences producing significant alignments with exon1 of XYL gene of *Aspergillus oryzae* MTCC 5154

| Accession No: | Details | Homology |
|---|---|---|
| | **Alignment with exon 1** | |
| **EU586113.1** | *Aspergillus oryzae* strain AS 3.4382 xylanase (xynG) mRNA, complete cds | 99% |
| **XM_001818614.1** | *Aspergillus oryzae* RIB40 hypothetical protein partial mRNA | 99% |
| **AB228619.1** | *Aspergillus oryzae* cDNA, contig sequence: AoEST5492 | 99% |
| **AB003085.1** | *Aspergillus oryzae* DNA for XynG1, complete cds | 99% |
| **EF375873.1** | *Aspergillus fumigatus* strain MKU1 endoxylanase (xynf11a) gene, complete cds | 85% |
| **DQ156553.1** | *Aspergillus fumigatus* XynB mRNA, complete cds | 85% |

**Table 3.2.4:** Sequences producing significant alignments with exon 2 of XYL gene of *Aspergillus oryzae* MTCC 5154

| Accession No: | Details | Homology |
|---|---|---|
| | **Alignment with exon 2** | |
| **EU586113.1** | *Aspergillus oryzae* strain AS 3.4382 xylanase (xynG) mRNA, complete cds | 97% |
| **XM_001818614.1** | *Aspergillus oryzae* RIB40 hypothetical protein partial mRNA | 97% |
| **AB003085.1** | *Aspergillus oryzae* DNA for XynG1, complete cds | 97% |
| **AB228619.1** | *Aspergillus oryzae* cDNA, coding sequence: AoEST5492 | 98% |
| **XM_001214121.1** | *Aspergillus terreus* NIH2624 endo-1,4- beta-xylanase B precursor (ATEG_04943) partial mRNA | 77% |

```
EXON1     ---------------------------------ATGGTTAGCTTCTCTTCTCTCCTCCTT
1         ---------------------------------------------------------
2         ---------------------------------ATGGTTAGCTTCTCTTCTCTCCTCCTT
3         CAGCTTACACACCACAAGACCAACACCGTCGCCATGGTTAGCTTCTCTTCTCTCCTCCTT
4         --------------------------CGTCGCCATGGTTAGCTTCTCTTCTCTCCTCCTT
5         ---------------------------------------------------------
6         ---------------------------------ATGGTTTCTTTCTCCTACCTGCTGCTG

EXON1     GCGGTTTCCGCTGTCTCCGGCGCTTTGGCTGCCCCTG----------------------
1         ---------------------------------------------------------
2         GCCGTTTCCGCTGTCTCCGGCGCTTTGGCTGCCCCTG----------------------
3         GCCGTTTCCGCTGTCTCCGGCGCTTTGGCTGCCCCTG----------------------
4         GCCGTTTCCGCTGTCTCCGGCGCTTTGGCTGCCCCTG----------------------
5         ---------------------------------------------AGACCACCTCGTTC
6         GCGTGCTCCGCCATT---GGAGCTCTGGCTGCCCCCGTCGAACCCGAGACCACCTCGTTC

EXON1     -GCGATTCCACTCTTGTTGAGCTCGCCAAGCGCGCTATCACCAGCTCCGAGACAGGCACC
1         -------CCACTCTTGTTGAGCTCGCCAAGCGCGCTATCACCAGCTCCGAGACAGGCACC
2         -GCGATTCCACTCTTGTTGAGCTCGCCAAGCGCGCTATCACCAGCTCCGAGACAGGCACC
3         -GCGATTCCACTCTTGTTGAGCTCGCCAAGCGCGCTATCACCAGCTCCGAGACAGGCACC
4         -GCGATTCCACTCTTGTTGAGCTCGCCAAGCGCGCTATCACCAGCTCCGAGACAGGCACC
5         AATGAGACTGCTCTTCATGAGTTCGCTGAGCGCGCCGGCACCCCAAGCTCCACCGGCTGG
6         AATGAGACTGCTCTTCATGAGTTCGCTGAGCGCGCCGGCACCCCAAGCTCCACCGGCTGG

EXON1     AACAACGGCTACTACTACTCCTTCTGGACCAACGGCGGTGGTGATGTCAAATACACCAAT
1         AACAACGGCTACTACTACTCCTTCTGGACCAACGGCGGTGGTGATGTCGAATACACCAAT
2         AACAACGGCTACTACTACTCCTTCTGGACCAACGGCGGTGGTGATGTCGAATACACCAAT
3         AACAACGGCTACTACTACTCCTTCTGGACCAACGGCGGTGGTGATGTCGAATACACCAAT
4         AACAACGGCTACTACTACTCCTTCTGGACCAACGGCGGTGGTGATGTCGAATACACCAAT
5         AACAACGGCTACTACTACTCCTTCTGGACTGATGGCGGCGGCGACGTGACCTACACCAAT
6         AACAACGGCTACTACTACTCCTTCTGGACTGATGGCGGCGGCGACGTGACCTACACCAAT

EXON1     GGCAACGGCGGCCAGTACAG---------------------------------
1         GGCAACGGCGGCCAGTACAGCGTCAAA--------------------------
2         GGCAACGGCGGCCAGTACAGCGTCAAATGGACCA-------------------
3         GGCAACGGCGGCCAGTACAGCGTCAAATGGACCAACTGCGACAA---------
4         GGCAACGGCGGCCAGTACAGCGTCAAATGGACCAACTGCGACAACTTTGTCGCC
5         GGCGCCGGTGGCTCGTACTCCGTCAACTGGA----------------------
6         GGCGCCGGTGGCTCGTACTCCGTCAACTGGA----------------------
```

**Figure 3.2.5:** Alignment of the sequence of exon 1 of XYL gene of *Aspergillus oryzae* MTCC 5154 with that of other *Aspergilli.*

[Exon 1: from *Aspergillus oryzae* MTCC 5154 XYL gene, 1) Accession No: U586113.1, *Aspergillus oryzae* xylanase (xynG), 2) Accession No: XM_001818614.1, *Aspergillus oryzae* RIB40, 3) Accession No: AB228619.1, *Aspergillus oryzae* cDNA, 4) Accession No: AB003085.1, *Aspergillus oryzae* DNA for XynG1, 5) Accession No: EF375873.1, *Aspergillus fumigatus* strain MKU1, 6) Accession No: DQ156553.1, *Aspergillus fumigatus* XynB].

```
Exon    ------------------------TACTCAACAGTGATAGTGGCAGACCCGTCTGCTCT
1       ------------------------------------------------------------
2       -------------GCGGTGAGTGGGAGAGCAACAGCAACAGCTACGTCTCTCTCTACGGC
3       ACTGTGACCTACAGCGGTGAGTGGGAGAGCAACAGCAACAGCTACGTCTCTCTCTACGGC
4       ACCATCACCTACAGCGGCGAGTGGAACCCCAACGGAAACAGCTACCTGTCAGTCTACGGC
5       ACTGTGACCTACAGCGGTGAGTGGGAGAGCAACAGCAACAGCTACGTCTCTCTCTACGGC

Exon    GGTAGCC-----------------------------------CTCGGTGGCAAGGATCAT
1       ----------------------ATACTACATCGTTGACAAGTACGGCGACTACGATCCC
2       TGGACCCAGAACCCCCTCGTCGAATACTACATCGTTGACAAGTACGGCGACTACGATCCC
3       TGGACCCAGAACCCCCTCGTCGAATACTACATCGTTGACAAGTACGGCGACTACGATCCC
4       TGGACCCAGAACCCGCTGGTCGAGTATTACATTGTTGAATCGTTCAGCACCTACGATCCT
5       TGGACCCAGAACCCCCTCGTCGAATACTACATCGTTGACAAGTACGGCGACTACGATCCC

Exon    GTAGTTATGGGTACCCAGCTGCAAACCAACGTTGGCCCAGCATCGAAGTGGTTCTGAGCA
1       TCCACCGGCGCTACCGAGCTTGGCACCGTCG---------------------------AGA
2       TCCACCGGCGCTACCGAGCTTGGCACCGTCG---------------------------AGA
3       TCCACCGGCGCTACCGAGCTTGGCACCGTCG---------------------------AGA
4       TCCACCGGCGCCCAGGAGCTCGGCACCGTGG---------------------------AGA
5       TCCACCGGCGCTACCGAGCTTGGCACCGTCG---------------------------AGA

Exon    GTGATGGTGCCACCAACAGGCCGCTCTGGCGGACCGACCAGTACTGGTTGAAGGTCGAGG
1       GCGATGGCGGCACCTACAAGATCTACAAGACTACTCGTGAAAACGCTCCCTCCATTGAGG
2       GCGATGGCGGCACCTACAAGATCTACAAGACTACTCGTGAAAACGCTCCCTCCATTGAGG
3       GCGATGGCGGCACCTACAAGATCTACAAGACTACTCGTGAAAACGCTCCCTCCATTGAGG
4       CCGACGACGGCACCTACAAGATCTACAAGACCACCCGCGAGAATGCGCCGTCTATCGAAG
5       GCGATGGCGGCACCTACAAGATCTACAAGACTACTCGTGAAAACGCTCCCTCCATTGAGG

Exon    TGCCCTCAATGGAGGGACGTTTTCA--CGAGTAGTCTGGTAGATCTTGTAGGTGCCGCCA
1       GCACCTCGACCTTCAACCAGTACTGGTCGGTCCGCCAGAGCGGCCGTGTTGGTGGCACCA
2       GCACCTCGACCTTCAACCAGTACTGGTCGGTCCGCCAGAGCGGCCGTGTTGGTGGCACCA
3       GCACCTCGACCTTCAACCAGTACTGGTCGGTCCGCCAGAGCGGCCGTGTTGGTGGCACCA
4       GCACTGCGACCTTCAACCAGTACTGGTCGGTCCGCACCGACCACCGCGTGGGCGGTACCG
5       GCACCTCGACCTT-----------------------------------------------

Exon    TCGCTCT--------------------------CGACGGTGCCGAGCTCGGTAGCGCCG
1       TCACTGCTCAGAACCACTTCGATGCCTGGGCCAACGTTGGTTTGCAGTTGGGTACCCATA
2       TCACTGCTCAGAACCACTTCGATGCCTGGGCCAACGTTGGTTTGCAGTTGGGTACCCATA
3       TCACTGCTCAGAACCACTTCGATGCCTGGGCCAACGTTGGTTTGCAGTTGGGTACCCATA
4       TCACCACGCAGAACCATTTCGATGCTTGGAAAAATGCTGGCCTCGAGATGGGAACCGCCA
5       ------------------------------------------------------------

Exon    GTGGAGGGATCGTAGTCGCCGTACTTGTCAACGATGTAGTATTCGACGAGGGGGTTCTGG
1       ACTACATGATCCTTGCCACCGAGGGCTACAAGAGCAGCGGGTCTGCCACTATCACTGTTG
2       ACTACATGATCCTTGCCACCGAGGGCTACAAGAGCAGCGGGTCTGCCACTATCACTGTTG
3       ACTACATGATCCTTGCCACCGAGGGCTACAAGAGCAGCGGGTCTGCCACTATCACTGTTG
4       ACTACATGATTGTCGCGACCGAGGGCTACCAGTCGAGCGGATCGGCCTCCATCACTGTGC
5       ------------------------------------------------------------

Exon    GTCCA-------------------------------------------------------
1       AGTAAATGGGGTTGTCTTTAGTTGCGGGATACCGTGGTCAACTTGCTTTGTAGGGTGATC
2       AGTAA-------------------------------------------------------
3       AGTAAATGGGGTTGTCTTTAGTTGCGGGATACCGTGGTCAACTTGCTTTGTAGGGTGATC
4       AGTAA-------------------------------------------------------
5       ------------------------------------------------------------
```

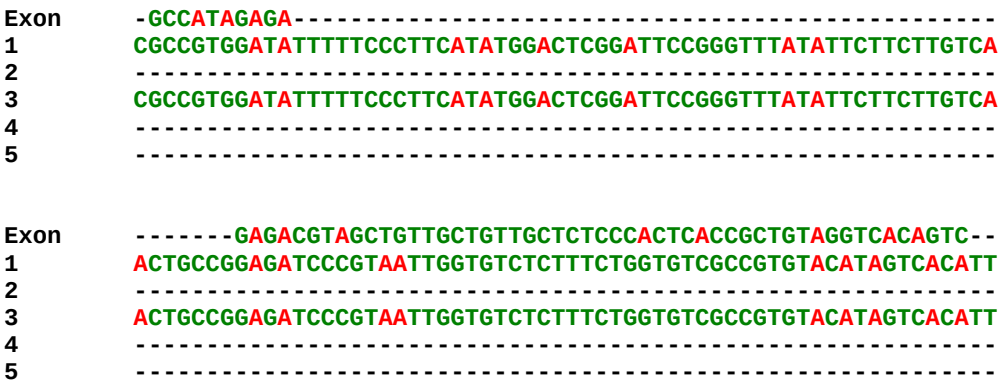



**Figure 3.2.6:** Alignment of the sequence of exon 2 of XYL gene of *Aspergillus oryzae* MTCC 5154 with that of other *Aspergilli.*

[Exon 1: from *Aspergillus oryzae* MTCC 5154 XYL gene, 1) Accession No: EU586113.1, *Aspergillus oryzae* strain, 2) Accession No: XM_001818614.1, *Aspergillus oryzae* RIB40, 3) Accession No: AB003085.1, *Aspergillus oryzae* DNA for XynG1, 4) Accession No: XM_001214121.1, *Aspergillus terreus* NIH2624 partial mRNA, 5) Accession No: AB228619.1, *Aspergillus oryzae* cDNA].

### 3.2.4. Discussion

Xylan- and cellulose-dependent inductive expression of the genes encoding xylanolytic and cellulolytic enzymes in the industrial fungus *Aspergillus oryzae* are mediated by a transcriptional activator, AoXlnR *(Marui et al., 2002a, Marui et al., 2002b).* In addition, XlnR is suggested to be involved in the regulation of the *cgxA* gene encoding a xylanase in the phytopathogenic fungus, *Chaetomium gracile (Rao et al., 2002).* The *xynF1* gene encoding the major xylanase in *Aspergillus oryzae* contains two different affinity binding sequences for AoXlnR and one analogous sequence in a short promoter region *(Kitamoto et al., 1998, Marui et al., 2002a, b).*

The structural part of XynG1 of *Aspergillus oryzae* KBN 616 has been reported earlier and it appeared to be 725 bp long and was interrupted by a single intron of 62 bp *(Kimura et al., 1998).* In the present study the PCR reactions with genomic DNA of *Aspergillus oryzae* MTCC 5154 and the designed primers resulted in two amplicons and later their

sizes have been confirmed with agarose gel electrophoresis as approximately 260 and 400bp. Later the sequence analysis of the amplicons showed that exon 1 contains 203 bp and exon 2 contains 397 bp. A comparison was made between the nucleotide sequences of the exon 1 and 2 of XYL gene of *Aspergillus oryzae* MTCC 5154 and the published nucleotide sequences for xylanases from the *Aspergillus* species (figure. 3.2.5 and figure 3.2.6). The results showed that exon 1 of *Aspergillus oryzae* MTCC 5154 is 99% homologous to other *Aspergillus oryzae* strains reported. At the same time, it showed only 85% similarity with *Aspergillus fumigatus*. Similarly, exon 2 showed 97% homology with that of other *Aspergillus oryzae* strains reported. It showed only 77% similarity with *Aspergillus terreus*. The results clearly indicate that the xylanase gene in *Aspergillus* species, especially in *Aspergillus oryzae* is conserved to a greater extent.

# CHAPTER: 4

# ENZYMATIC PRODUCTION OF XYLOOLIGOSACCHARIDES FROM CORNCOB

**Overview**

In the current trend of a complex and more effective utilization of biomass and developments of environmentally friendly industrial processes, increasing research activities have been directed towards the exploitation of the xylan component of economically important plants and plant residues as biopolymer resources. Corncob is rich in xylan and is an important by-product of the corn industry that is used either as animal feed or returned to the harvested fields. The present chapter details the value addition of corncob to xylooligosaccharides (XOS) through biotechnological routes. The first section of the chapter details the evaluation of different methods of pretreatment of corncobs for the efficient production of XOS. It also focuses on the changes in the super molecular structure of lignin-saccharide complex during pretreatment. The second section of the chapter focuses on the production of XOS from alkali pretreated corncob powder using endoxylanase produced by *Aspergillus oryzae* MTCC 5154 under submerged fermentation conditions. The study also details optimization, purification and characterization of XOS.

**SECTION: 4:1**

# EVALUATION OF PRETREATMENT OF CORNCOB FOR ENHANCED AVAILABILITY OF XYLAN FOR XYLOOLIGOSACCHARIDE PRODUCTION

### 4.1.1. Introduction

The plant cell wall is a complex, multifunctional extracellular organelle which plays an essential role in nearly every aspect of plant physiology, from cell division and support through to ripening and senescence *(Waldron and Faulds, 2007).* The wall also provides the basis for exploitation, from structuring agents and fuels through to nutritional and bioactive functionality. In the current trend of a complex and more effective utilization of biomass and developments of environmentally friendly industrial processes, increasing research activities have been directed towards the exploitation of the xylan component of economically important plants and plant residues as biopolymer resources.

Xylans are plant polysaccharides that represent an immense resource of biopolymers for practical applications, accounting for 25-35% of the dry biomass of woody tissues of dicots and lignified tissues of monocots and occur up to 50% in some tissues of cereal grains *(Ebringerova and Heinze, 2000).* The occurrence of xylans can be traced up to the botanically oldest plant families. The structures of xylans depend on the source considered. The most common xylans are made up of a main backbone of xylose linked by β-(1,4)-linkages, where the structural units are often substituted at positions C2 or C3 with arabinofuranosyl, 4-*O*-methylglucuronic acid, acetyl or phenolic substituents *(Stephen, 1983).*

Corncob is the central core of a maize (*Zea mays* ssp mays L.) ear. As the plant matures, the cob becomes tougher until only kernels are edible and when harvesting corn, the cob is collected as a part of ear. Corncob is an important by-product of the corn industry that is used either as animal feed or returned to the harvested fields. Corncobs are reported as an excellent substrate for the growth of various industrially important bacteria and fungi for the production of pharmaceutically and nutraceutically important enzymes. Alkali pretreated corncobs are used as a carbohydrate source for bacterial protein production *(Pece et al., 1994).* Corncobs are relatively new candidate for water decontamination and studies have been carried out to study efficiency of succinic anhydride and maleic anhydride derivatives of corncobs for efficient removal of copper ions from water of

boilers *(Billon et al., 2006).* Enough scope exists for value addition to corncob and its utilization for food applications such as production of xylooligosaccharides (XOS), xylose, xylitol and xylanase.

XOS are produced from xylan rich agro-residues either by chemical methods, autohydrolysis, direct enzymatic hydrolysis of a susceptible substrate or a combination of chemical and enzymatic treatments *(Vazquez et al., 2000).* Enzymatic production of XOS is preferred in food industry because of the problems associated with other strategies of XOS production. 1,4-β-D-xylan xylanohydrolase hydrolyze β-1,4-xylosidic bonds within the β-(1,4)-linked D-xylosyl backbone of xylan producing β-anomeric XOS. For the efficient production of XOS from agro-residues, the xylan should be exposed to the endoxylanase action. Since the complete extraction of xylan is time consuming and is often related with environmental issues, a mild preatment method is suggested by various authors for making the xylan available for enzymatic reaction.

Most pretreatment methods for ligno-cellulose conversions are aimed at the removal of hemicelluloses or lignin. Several physical/chemical pretreatment methods (or their combination) are possible including ball milling, dilute acid pretreatment, steam pretreatment, ammonia fiber explosion, alkali pretreatment and organosolvent process. There are several desirable goals for pretreatment process, however, in practice not all of them are achieved with any current treatment. Generally, the pretreatment should promote high product yields in a subsequent enzymatic hydrolysis. The formation of degradation products from lignin and sugars, such as furfural and hydroxyl methyl furfural (HMF) should be minimized. Down stream processing of pretreated raw material, including filtering should be simple.

Steaming or hydrothermal treatments (150-240 °C) are good methods for the fractionation of the xylans from ligno-cellulosics. Xylan hydrolysis can be improved by pretreatment of the substrates with diluted acids like sulfuric acid, whereas both cellulose and lignin remain in solid phase *(Parajo et al., 1995, Saska et al., 1995, Prajo et al., 1994, Parajo et al., 1993).* However, the use of strong acids as pretreatment is not very

environmentally friendly and it results in higher processing costs due to the requirement of corrosion-proof equipment. Therefore, a milder hydrothermal treatment may be advantageous.

In the presence of water at elevated temperatures the xylans in the treated products will be hydrolyzed and dissolved. Additionally, *O*-acetyl substituents present are (partly) hydrolyzed from the xylan, which result in the generation of acetic acid. The acetic acid formed acts as a catalyst in the further hydrolysis of the glycosidic linkages in the xylans present. Depending on the operational conditions used, the hydrolysates obtained consist mainly of soluble xylan, XOS and/or xylose *(Aoyama et al., 1995, Korte et al., 1991, Puls et al., 1985).* To a lesser degree, oligomeric fragments of degraded cellulose occur in the hydrolysates. Products of side reactions of pentoses and hexoses, such as furfural and HMF respectively, can be observed as well *(Ramose et al., 1992, Bonn et al., 1984).*

The present investigation deals with the evaluation of different methods of pretreatment of corncobs such as dilute alkali treatment, dilute acid treatment and cooking for the efficient production of XOS. The study also focuses on the chemical and physicochemical characterization of native and pretreated corncob.

### 4.1.2. Materials and Methods

#### 4.1.2.1. Chemicals

All chemicals were of analytical grade. Standards of xylose, xylobiose, arabinose, glucose and birch wood xylan were from Sigma-Aldrich (USA). A commercial food grade xylanase, Bioxyl P 40 was a gift from Biocon India Pvt Ltd, India. Corncobs were obtained from a local maize field in Mysore, Karnataka, India and were sorted, dried at 40 °C for 24 h, milled (60-80 mesh) and stored in polycarbonate containers.

#### 4.1.2.2. Milling of corncob

A two stage powdering of corncob has been carried out to get a powder of 60-80 mesh size as explained under section 3.1.2.2. This powder was used as the inducer for xylanase production and also for various pretreatment studies and XOS production.

#### 4.1.2.3. Pretreatments of corncob

##### 4.1.2.3.1. Alkali treatment

Corncob powder was treated with 2% NaOH solution at a solid to liquid ratio of 1:6 (w/w) for 12-15 h at room temperature with occasional stirring *(Ai et al., 2005).* Excess alkali was washed with deionized water till the pH of the washings was 6.0 and the pretreated corncob powder was dried at 40 °C and stored till further use.

##### 4.1.2.3.2. Acid treatment

Corncob powder was soaked in 0.1% $H_2SO_4$ solution at a solid to liquid ratio of 1:10 (w/w) for 12 h at 60 °C *(Yang et al., 2005).* The excess acid was washed with deionized water till the pH of the washings was 6.0 and pretreated corncob powder was dried at 40 °C and stored till further use.

##### 4.1.2.3.3. Acid treatment followed by pressure cooking

The acid treated corncob obtained as mentioned in section 4.1.2.3.2 was further cooked under pressure at 121 °C for 30 min and was dried at 40 °C and stored until use.

#### 4.1.2.4. Scanning Electron Microscopy (SEM) of lignin-saccharide complex of untreated and pretreated corncob powder

The physical structure of lignin-saccharidic complex of untreated and pretreated lignocellulosic materials (LCMs) was observed by SEM (magnifications 8000x) using a standard procedure *(Kosikoba et al., 1978).* The samples were soaked in 1N NaOH solution at 30 °C for 12 h. After filtration, the filtrate was neutralized with acetic acid to pH 7.5. The lignin-saccharide complex was precipitated by an addition of equal volume of methanol. After filtration, the lignin-saccharide complex was washed with methanol twice and suspended in methanol. The suspension was homogenized and a small portion of it was placed on the cover slips. Before observation by SEM, the samples were sputted with a thick layer of gold, spread uniformly from all sides under two different angles. The interpretation of result is based on the model of lignin-saccharide complex proposed earlier *(Fengel, 1976).*

### 4.1.2.5. Endoxylanase Assay

Endoxylanase activity of the suitably diluted commercial xylanase Bioxyl P40 was assayed using the protocol explained in the section 3.1.2.9.1.

### 4.1.2.6. Production of XOS from untreated and pretreated corncob

For evaluating the efficiency of various pretreatment methods, XOS production was carried out from untreated/pretreated corncob powder using Bioxyl P40, a commercial food grade xylanase. The reaction was carried out in 250 ml conical flask containing 50 ml reaction mixture, consisting of 1.5 g untreated/pretreated corncob powder and 300 U of endoxylanase and the volume was made up to 50 ml using Mc Ilvain buffer (0.05 M, pH 5.4). The enzymatic reaction was carried out in a shaking water bath maintained at 50 °C for 24 h. At the end of specified incubation time, the reaction was stopped by keeping the reaction mixture in a boiling water bath for 10-15 min.

### 4.1.2.7. Chemical analysis of untreated and pretreated corncob

Native and pretreated corncobs were subjected to moisture determination by drying at 105 °C to a constant weight. Carbohydrate and lignin analysis of the samples were carried out using a slightly modified version of the summative analysis procedure *(Karr et al., 1991a, Karr et al., 1991b).* Solid samples were ground to 40-mesh size particles and dried at 105 °C for 18 h. Powdered corncob (0.1 g) was taken in 50 ml autoclavable bombs to which 1 ml of 14.6 N $H_2SO_4$ was added and incubated in a 30 °C in a water bath for 1 h. After incubation, 30 ml of distilled water was added, sealed and autoclaved at 121 °C for 1 h. The filtrate was neutralized, diluted and analyzed for monosaccharides by HPLC. Xylose was used as the standard. The weight of the xylan in the sample was calculated using the following formula, assuming that the hydrolysis is total, where 0.88 accounts for the loss of water during xylosidic bond formation *(Yang et al., 2005).*

Weight of xylan = 0.88 x weight of xylose produced

#### 4.1.2.8. Analysis of XOS

XOS was estimated by HPLC method *(Jeong et al., 1998)* and was detailed in section 3.1.2.10. The XOS produced are expressed in two ways. The chromatographic percentage of the monosaccharides and XOS indicates the percentage distribution sugars in the hydrolyzate. The XOS formed are quantified by comparing the peak area of XOS with that of standard xylose and is expressed as mg/ml of hydrolyzate.

#### 4.1.2.9. Statistical analysis

All experiments were conducted in triplicates (n=3) and repeated at least three times. The statistical significances and standard deviations were done using Microsoft Excel (Version 5.0, Microsoft, Corporation, Redmond, WA)

### 4.1.3. Results

#### 4.1.3.1. Evaluation of pretreatment methods of corncob

The xylan content of native corncob was found to be 31.9±2.3%. The alkali pretreated, acid pretreated and pressure cooked corncob powder have a xylan content of 40.8±1.2, 39.2±1.1 and 40.0±0.8% respectively. XOS were produced by enzymatic conversion of untreated/pretreated corncob powder using a commercial food grade xylanase (Bioxyl P40). The effects of various pretreatment methods on XOS production are presented in table 4.1.1.

The percentage of XOS obtained by the enzymatic hydrolysis of raw corncob was 89±1.1% where as 81±1.5% of XOS was obtained by the enzymatic hydrolysis of alkali pretreated corncob powder. The choice of pretreatment method for further studies was based on the amount of XOS produced on weight basis. The results indicated that a maximum of 5.8±0.14 mg/ml of XOS was obtained when alkali pretreated corncob was used in comparison to 3.7±0.17 mg/ml XOS obtained from steam cooked corncob. Based on these results alkali pretreatment was found to be more efficient for XOS production and hence was selected as the pretreatment method for further studies.

**Table 4.1.1:** The xylan content and the amount of XOS[a] produced from untreated and pretreated corncob

| Substrate | %xylan [b] | %XOS [c] | XOS (mg/ml) [d] |
|---|---|---|---|
| Native corncob | 31.9±2.3 | 89±1.1 | 2.06±0.13 |
| Alkali pretreated corncob | 40.8±1.2 | 81±1.5 | 5.80±0.14 |
| Acid pretreated corncob | 39.2±1.1 | 52±3.2 | 2.70±0.14 |
| Steam cooked corncob | 40.0±0.8 | 77±1.8 | 3.70±0.17 |

a: By using Bioxyl P40, a commercial food grade endoxylanase

b, c, d: Mean and standard deviations for n=3

#### 4.1.3.2. Chemical characterization of untreated and pretreated corncob powder

The chemical composition of native and pretreated corncob is briefed in the table 4.1.2. From the results, it can be found that the total percentage of xylan was found to increase from 31.9±1.1 to 40.8±0.9% during the alkali pretreatment, while that of cellulose and lignin was found to decrease from 34.6±0.7 to 30.7±0.6% and 16.4±0.4 to 10.4±0.7% respectively. The ash and moisture content were also found to reduce during the pretreatment.

**Table 4.1.2:** The chemical composition of native and alkali pretreated corncob powder

| Component % (w/w)[a] | Native corncob | Alkali pretreated corncob |
|---|---|---|
| Xylan | 31.9±1.1 | 40.8±0.9 |
| Cellulose | 34.6±0.7 | 30.7±0.6 |
| Klason Lignin | 16.4±0.4 | 10.4±0.7 |
| Ash | 1.9±0.1 | 1.4±0.1 |
| Moisture | 8.0±0.2 | 7.7±0.3 |
| Others by difference | 7.2±0.2 | 9±0.4 |

a: Mean and standard deviations for n=3

### 4.1.3.3. SEM structure of lignin-saccharide complex of native and pretreated corncob powder

The supermolecular structure of lignin-saccharide complex of the raw corncob, pretreated corncob and the residue after enzymatic hydrolysis was investigated by SEM using a slightly modified procedure as reported by Kosikoba *et al*. (1978). Based on the model of lignin-saccharide complex proposed by Fengel (1976) and based on the SEM micrographs obtained in the present study, it is postulated that the spherical particles represents the lignin portion and the fibril represents the saccharidic portion of lignin-saccharide complex. Figure 4.1.1 shows the lignin-saccharide complex of the native corncob where the complex is intact and the lignin is not visible. Figure 4.1.2 represents the scanning electron micrographs of alkali pretreated corncob where the lignin is visible. Figure 4.1.3 represents scanning electron micrographs of the residue obtained after the enzymatic hydrolysis of pretreated corncob for XOS production. It can be clearly seen that the treatment has resulted in the separation of xylan from lignin making it more accessible for enzymatic hydrolysis.

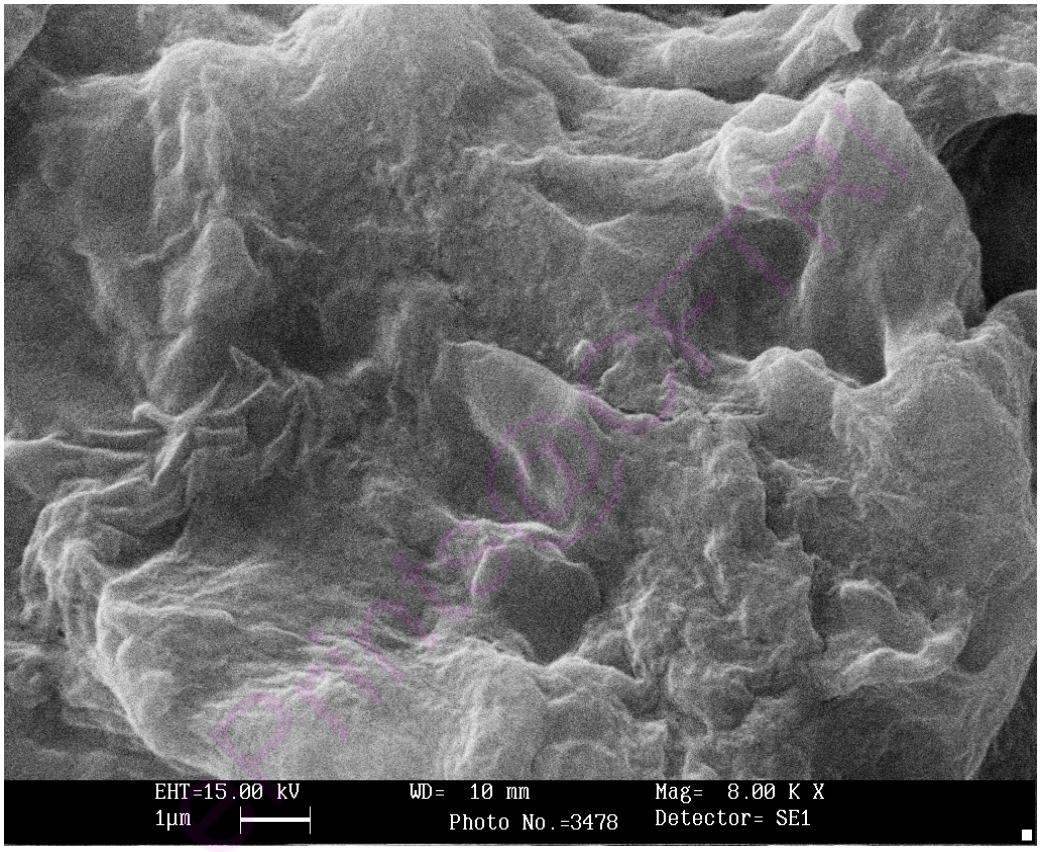



**Figure 4.1.1:** Scanning electron micrographs of lignin-saccharide complex of native corncob powder (Magnification 8000x)

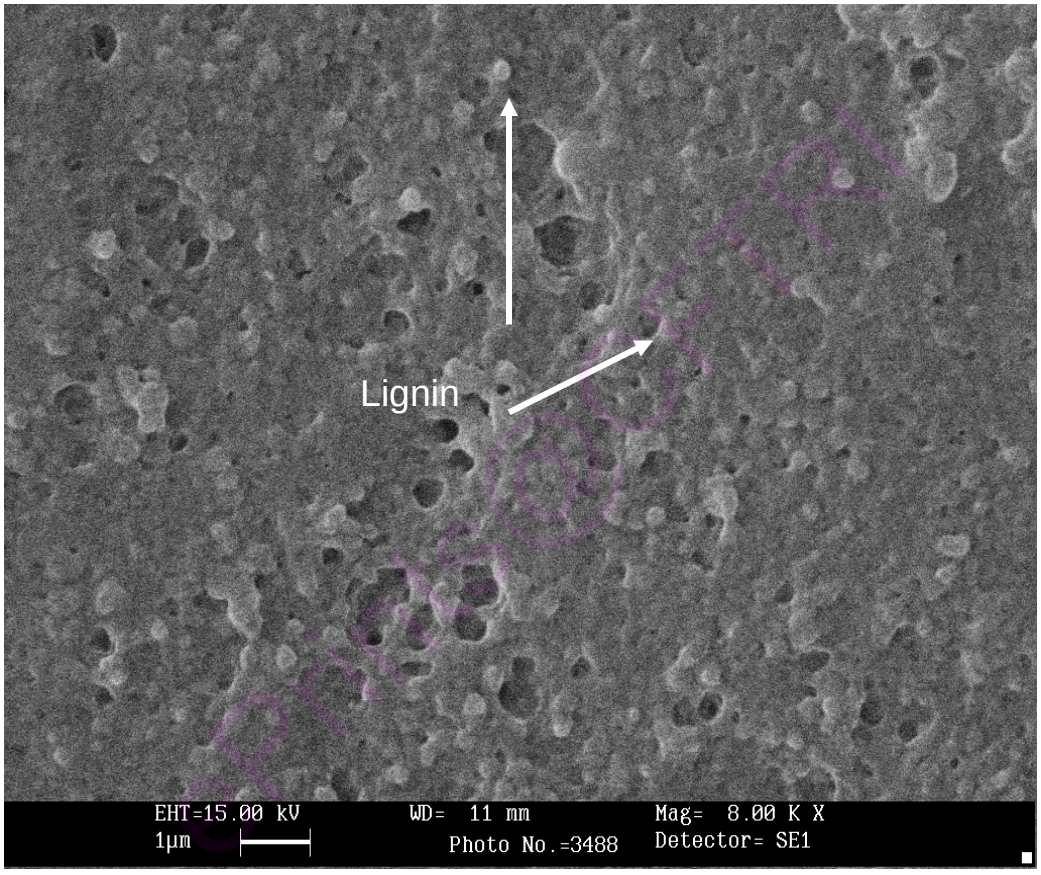



**Figure 4.1.2:** Scanning electron micrographs of lignin-saccharide complex of alkali pretreated corncob powder (Magnification 8000x)

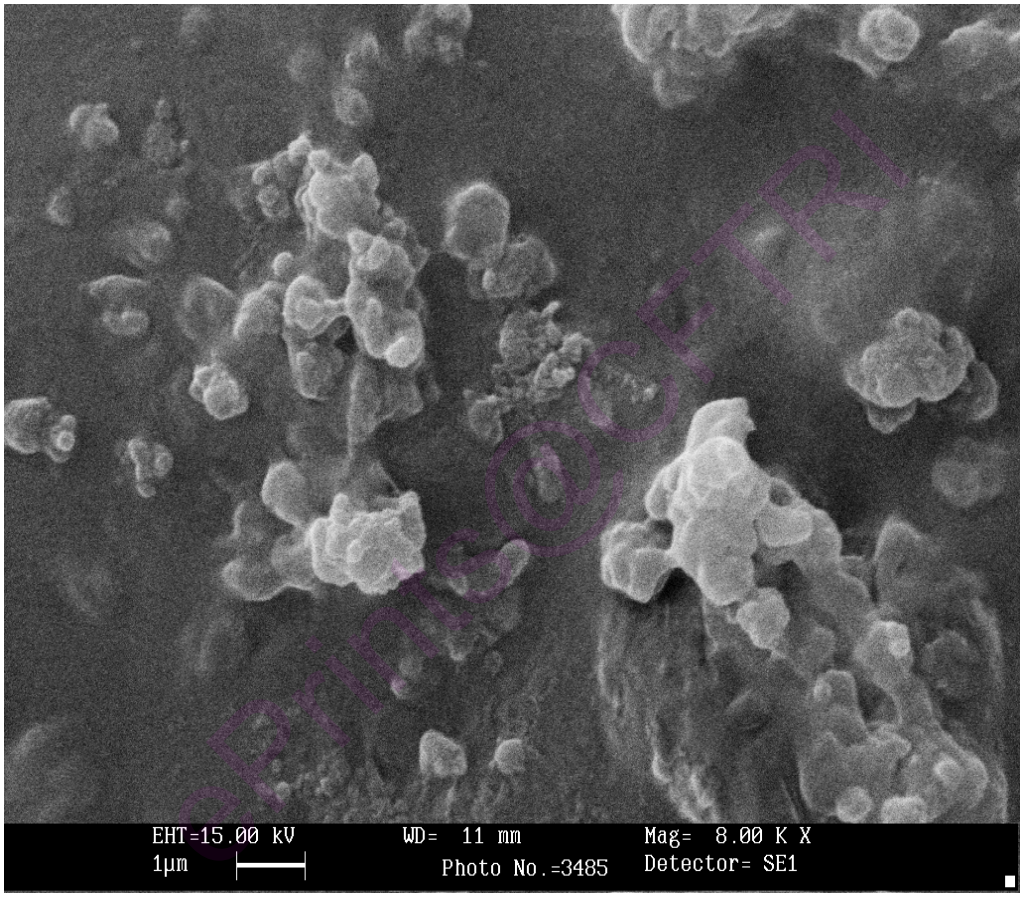



**Figure 4.1.3:** Scanning electron micrographs of lignin-saccharide complex of the residue obtained after enzymatic hydrolysis of alkali pretreated corncob powder (Magnification 8000x)

### 4.1.4. Discussion

Generally, production of XOS involves the extraction of xylan from corncob or any other suitable LCMs with alkali such as KOH or NaOH and further conversion of extracted xylan to XOS using xylanase having low exo-xylanase and/or β-xylosidase activity *(Ozlem et al., 2007).* In contrast to autohydrolysis, this method is desirable, as it does not produce undesirable by-products or high amount of monosaccharides. Complete extraction of xylan is cost prohibitive and is not environmentally friendly. Hence different pretreatment methods have been evaluated for the purpose of making corncob xylan more accessible for the enzymatic reaction instead of complete extraction of xylan.

The evaluation of pretreatment method was based on two criteria: the xylan content after pretreatment and the amount of XOS produced from it under certain standard conditions. The percent xylan in the untreated and pretreated corncob powder was compared. Native corncob was found to have 31.9±2.3% xylan and there is an apparent increase in the xylan content of alkali pretreated, acid pretreated and steam cooked corncob probably due to the removal of some cellulose and lignin components during pretreatment. This indicated the efficiency of pretreatment methods. The results of XOS production from pretreated corncob indicated that a maximum of 5.8±0.14 mg/ml of XOS was obtained when alkali pretreated corncob was used in comparison to 3.7±0.17 mg/ml XOS obtained from steam cooked corncob. The percentage of XOS obtained from acid pretreated corncob powder was considerably less and this is probably because of the degradation of xylan to xylose under acidic conditions of pretreatment.

The availability of xylan component of the cell wall matrix for the enzymatic reaction is restricted by the surrounding lignin network as well as ester and ether lignin-carbohydrate linkages. In the latter case, ferulic acid is known to be a bridging unit between polysaccharide and lignin. Also extensive hydrogen bonding between the individual polysaccharide cell wall components may impede isolation as well as the availability of the xylan component. Thus, before enzyme hydrolysis the xylan-lignin bonds needs to be broken. Most of the hemicellulose preparations are soluble in water after alkaline

extraction or treatment. Their isolation actually involves alkaline hydrolysis of ester linkages to liberate them from ligno-cellulosic matrix followed by extraction in aqueous media *(Sun et al., 2000).*

In native corncob, xylans tend to lie perpendicular to the longitudinal axis of the cell, that is perpendicular to the orientation of cellulose in the epidermal wall and all xylans are thought to cross-link the cellulose microfibrils and contribute to the mechanical properties of the cell wall. During pretreatments with alkali, these alignments will be disturbed. The pretreatment removes some saccharides surrounding the lignin and the lignin become more visible indicating that saccharides have been removed to a certain degree. Eventhough arabinoxylans are closely associated through covalent and other linkages with other cell wall constituents, such as lignin, protein, cellulose and pectin, the removal of some saccharides during the pretreatment indicates the presence of some loosely bound saccharides which are easily hydrolyzed by dilute alkali. This indicates that the xylan-lignin structure is greatly altered during the pretreatment. Breaking of α-benzyl ether linkages between lignin and hemicelluloses from the cell walls of rice straw by alkali is also reported *(Sun et al., 2000).* It can be clearly seen from the electron micrographs of lignin-saccharide complex of pretreated corncob that the treatment has resulted in the separation of xylan from lignin making it more accessible for enzymatic hydrolysis.

SECTION: 4.2

# BIOCONVERSION OF PRETREATED CORNCOB TO XYLOOLIGOSACCHARIDES USING ENDOXYLANASE FROM *Aspergillus oryzae* MTCC 5154: OPTIMIZATION, PURIFICATION AND CHARACTERIZATION

### 4.2.1. Introduction

There is an increasing interest in the application potential of xylan polymers isolated from industrial and field grown crops both in the food and non-food area *(Ebringerova and Heinze, 2000).* The variability in sugar constituents, glycosidic linkages and structure of glycosyl side chains as well as two reactive hydroxyl groups at the xylose repeating unit of the main chain offer various possibilities for regioselective, chemical and enzymatic modifications. Functionalization creates novel opportunities to exploit the various valuable properties of xylans for previously unconceived applications. Sulfated xylans are used as a biologically active component in drugs *(Ebringerova and Hromadkova, 1999).* Indigestible polysaccharides, including xylans were shown to exhibit inhibitory activity on mutagenicity and heating seems to increase the detoxification ability of dietary fibers.

The richest sources of xylans are represented by the woody tissues of dicots and non-graminaceous monocots where heteroxylans comprise 25-35% of the biomass *(Thomas, 1977),* as well as seeds and cereal grains which contain 30-50% of the arabinoxylan, glucuronoarabinoxylan and heteroxylan types *(Wilkie, 1979).* Several fiber crops of agricultural plants available in huge amounts have been reported as xylan sources such as bagasse, wheat straw, sunflower hulls, corncobs, husks of guar seeds, sweet sorghum stalks and husks of red gram.

Xylooligosaccharides (XOS) are a class of nondigestible food ingredient that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health *(Moure, 2006).* In the large bowel, these prebiotic oligosaccharides can be fermented by the intestinal flora into mainly short-chain fatty acids (SCFA; acetate, propionate and butyrate), lactate, $CO_2$ and $H_2$. The production of SCFA is related with a number of health effects, for example, bowel function, calcium absorption, lipid metabolism and reduction of the risk of colon cancer. As a food ingredient, XOS show favorable technological features, including stability in acidic pH, resistance to heat and ability for offering lower available energy

and for achieving significant biological effects at low daily intakes *(Vazquez et al., 2000).* XOS are non cariogenic, stimulate intestinal mineral absorption and does not invoke insulin secretion from the pancreas. XOS exhibit a range of biological activities different from the prebiotic effects related to gut modulation including antioxidant activity, blood and skin related effects, antiallergy, antimicrobial, antiinfection and antiinflamatory properties, selective cytotoxic activity, immunomodulatory action, cosmetic and a variety of other properties. XOS improve hyperammonaemia produced by high-protein diet in rats, while it lowers blood nitrogen levels and increased faecal nitrogen excretion.

XOS are produced from xylan rich agro-residues either by chemical methods, autohydrolysis, direct enzymatic hydrolysis of a susceptible substrate or a combination of chemical and enzymatic treatments *(Vazquez et al., 2000).* Enzymatic production of XOS is preferred in food industry because of the problems associated with other strategies of XOS production. 1,4-β-D-xylan xylanohydrolase (EC 3.2.1.8) generally known as endoxylanases are the enzymes which hydrolyze β-1,4-xylosidic bonds within the β-1,4-linked D-xylosyl backbone of xylan producing β-anomeric XOS. For the efficient production of XOS from agro-residues, the xylan should be exposed to the endoxylanase action. Since the complete extraction of xylan is time consuming and is often related with environmental issues, a mild pretreatment method is suggested by various authors for making the xylan available for enzymatic reaction.

The present investigation focuses on production of XOS from alkali pretreated corncob powder using endoxylanase produced by *Aspergillus oryzae* MTCC 5154 under submerged fermentation conditions. The study also details optimization, purification and characterization of XOS.

### 4.2.2. Materials and Methods

#### 4.2.2.1. Chemicals

All chemicals were of analytical grade. Standards of xylose, xylobiose, arabinose, glucose and birch wood xylan were from Sigma-Aldrich (USA). Corncobs were obtained

from a local maize field in Mysore, Karnataka, India and were sorted, dried at 40 °C for 24 h, milled (60-80 mesh) and stored in polycarbonate containers.

#### 4.2.2.2. Milling and alkali pretreatment of corncob

A two stage powdering of corncob has been carried out to get a powder of 60-80 mesh size as explained in the section 3.1.2.2. This powder was used as the inducer for xylanase production and substrate for XOS production. Alkali pretreatment of the corncob powder has been carried out using the method described in the section 4.1.2.3.1.

#### 4.2.2.3. Microorganism and endoxylanase production

*Aspergillus oryzae* MTCC 5154, a soil isolate was maintained on potato dextrose agar slants at 4 °C. Endoxylanase production has been carried out as explained under the section 3.1.2.5.

#### 4.2.2.4. Endoxylanase Assay

Endoxylanase activity of culture fluid was assayed using standard birch wood xylan as the substrate *(Jiang et al., 2005)* as explained in the section 3.1.2.9.1.

#### 4.2.2.5. Production of XOS from untreated and pretreated corncob

The effects of various reaction parameters on the XOS production from alkali pretreated corncob powder was carried out using extracellular endoxylanase obtained by submerged fermentation of *Aspergillus oryzae* MTCC 5154. The reaction was carried out in 250 ml conical flask containing 50 ml reaction mixture, consisting of 1.5 g untreated/pretreated corncob powder and 300 U of endoxylanase and the volume was made up to 50 ml using Mc Ilvain buffer (0.05 M, pH 5.4). The enzymatic reaction was carried out in a shaking water bath maintained at 50 °C for 24-36 h. At the end of specified incubation time, the reaction was stopped by keeping the reaction mixture in a boiling water bath for 10-15 min. The effects of substrate concentration (2-10%, w/v), enzyme concentration (4-20 U/ml), pH (4.8-6.0), temperature (40-60 °C) and reaction time (6-42 h) on the yield of XOS have been investigated.

**4.2.2.6. Analysis of XOS**

The composition of XOS was estimated by HPLC method *(Jeong et al., 1998)* as detailed in section 3.1.2.10. The XOS produced are expressed in two ways. The chromatographic percentage of the monosaccharides and XOS indicates the percentage distribution of sugars in the hydrolyzate. The quantity of XOS formed is estimated by comparing the peak area of XOS with that of standard xylose and is expressed as mg/ml of hydrolyzate.

**4.2.2.7. Purification of XOS by gel permeation chromatography (GPC)**

100 ml of XOS mixture was purified by following the method explained in the section 3.1.2.11. The fractions were analyzed by HPLC as mentioned in section 3.1.2.10. The major fractions were pooled for ESI-MS and NMR analysis.

**4.2.2.8. Electrospray tandem mass spectrometry (ESI-MS) of XOS**

ESI-MS analysis was carried out using a standard protocol as explained in the section 3.1.2.12. Each spectrum was produced by accumulating data during 1-2 min. The hydrolyzate was filtered through 0.45 µm cellulose nitrate membranes to remove the unreacted xylan prior to further analysis. The major fraction obtained by GPC was also analyzed. Mass spectra of pure xylose and xylobiose were also recorded.

**4.2.2.9. $^{13}$C and 2D-HSQC NMR spectrometry of xylobiose**

The NMR spectra were recorded at 27 °C with Bruker, AQS 500 MHz. About 10 mg of dried sample of xylobiose (major fraction) was dissolved in $D_2O$ (99.996%) and $^{13}$C chemical shifts were measured relative to internal Deuterium nucleus. The spectra were obtained at 125 MHz. The typical acquisition parameters for $^{13}$C-NMR and 2D-HSQC NMR are a 90° pulse of 12 µs, a typical spectral width of 26455 Hz and a repetition time of 4s. The repetition time was long enough to obtain quantitative data.

**4.2.2.10. Statistical analysis**

All experiments were conducted in triplicates (n=3) and repeated at least three times. The statistical significances and standard deviations were done using Microsoft Excel (Version 5.0, Microsoft, Corporation, Redmond, WA)

### 4.2.3. Results

#### 4.2.3.1. Production of XOS from alkali pretreated corncob

The effects of various parameters such as substrate concentration (2-10%, w/v), enzyme concentration (2-20 U/ml reaction mixture), pH (4.8-6.0), temperature (40-60 °C) and time (6-42 h) on the production of XOS have been investigated.

##### 4.2.3.1.1. The effect of substrate concentration on XOS production

The effect of varying concentration of substrate (2-10%, w/v) has been evaluated for XOS production using the endoxylanase (6 U/ml of reaction mixture) of *Aspergillus oryzae* produced under submerged fermentation conditions using corncob as a xylanase inducer (figure 4.2.1).

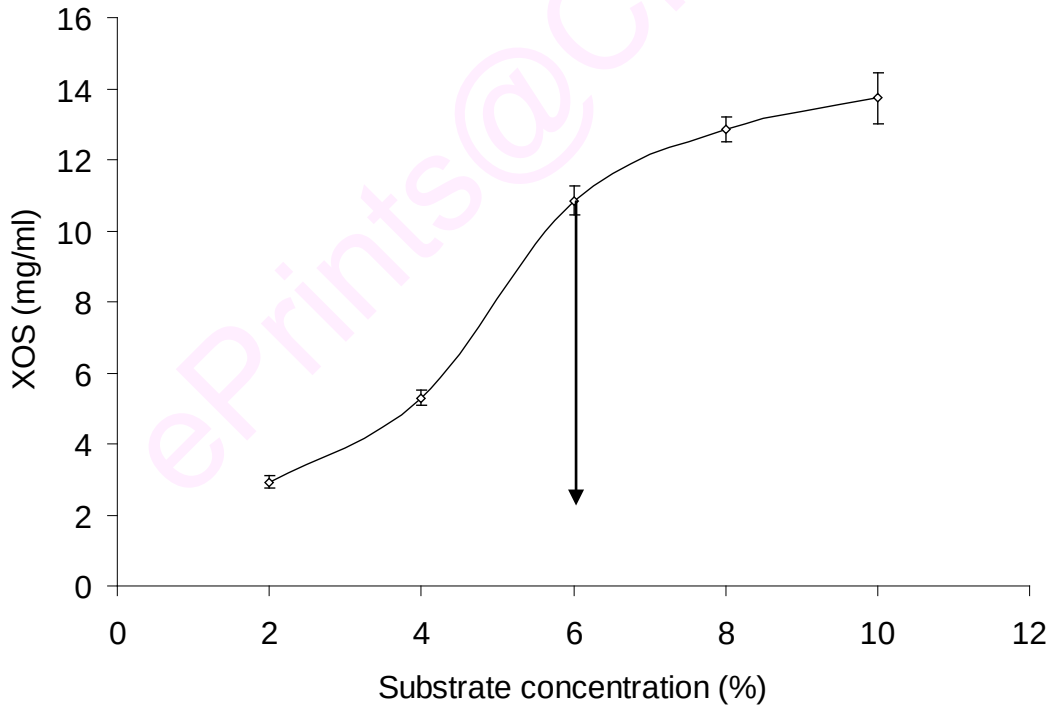



**Figure 4.2.1:** Effect of substrate concentration on the production of XOS from alkali pretreated corncob powder using endoxylanase (6 U/ml of reaction mixture) from *Aspergillus oryzae* MTCC 5154. The results were expressed as average of triplicate determinations with ±SD.

At 2% substrate concentration, 2.9±0.18 mg/ml of XOS were produced which gradually increased as the concentration of corncob powder increased. At 6% substrate concentration, the amount of XOS formed was 10.85±0.42 mg/ml and at 8% and 10% concentration of substrates their quantities were around 13 mg/ml.

**4.2.3.1.2. The effect of enzyme concentration on XOS production**

The effect of enzyme concentration on XOS production was studied in the range of 4-20 U/ml of reaction mixture (figure 4.2.2).

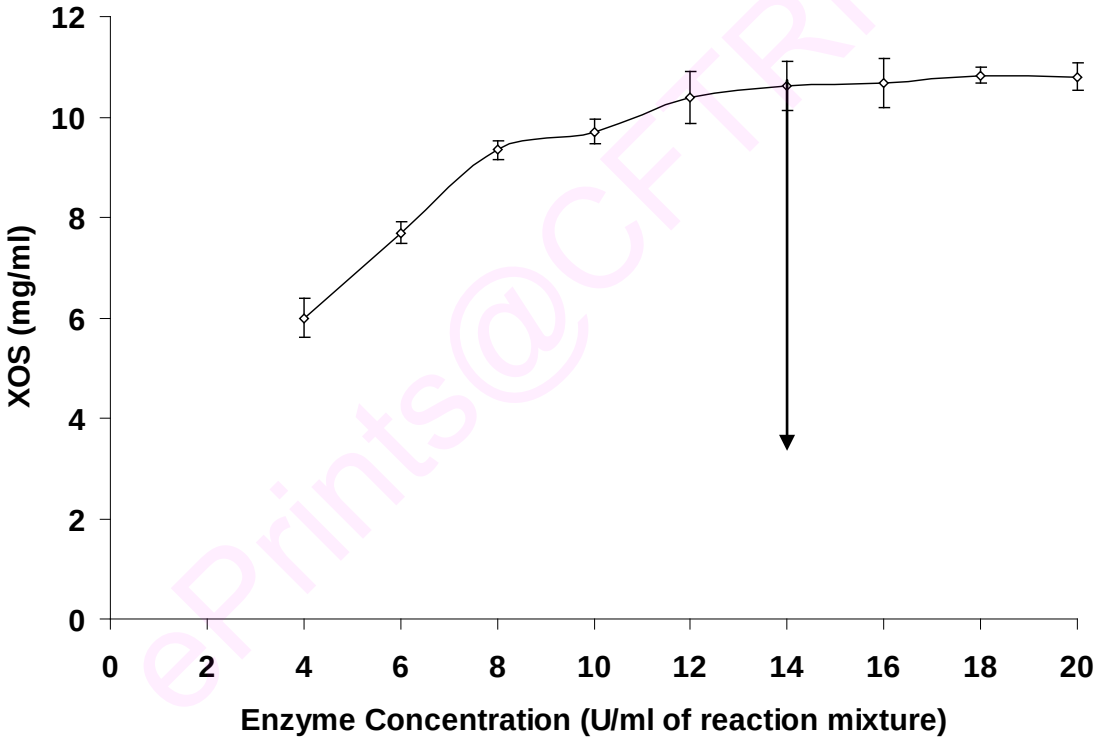



**Figure 4.2.2:** Effect of enzyme concentration on the production of XOS from alkali pretreated corncob powder (6%, w/v) using endoxylanase from *Aspergillus oryzae* MTCC 5154. The results were expressed as average of triplicate determinations with ±SD.

At 4 U/ml enzyme concentration, the amount of XOS produced was around 5.9±0.39 mg/ml which increased to a value of 10.62±0.48 mg/ml at an enzyme concentration of 14

U/ml and thereafter, the increase was very insignificant with respect to increase in enzyme concentration. At this concentration of endoxylanase activity, the percentage XOS formed was around 80.9±1.4% of the total sugars in the hydrolyzate.

**4.2.3.1.3. The effect of pH and temperature on XOS production**

The effect of pH in the range of 4.8-6.0 was studied (figure 4.2.3). In the present investigation, the maximum production of XOS was observed at pH 5.4. At this pH the percentage of XOS formed was around 82.6±1.8% of the total sugars in the hydrolyzate which corresponded to 10.31±0.42 mg/ml. The effect of temperature in the range of 40-60 °C has been investigated (figure 4.2.4). At 40 °C, the amount of XOS formed was 9.43±0.31 mg/ml and increased to a maximum concentration of 10.38±0.15 mg/ml at 50 °C and there after it is found to decrease.

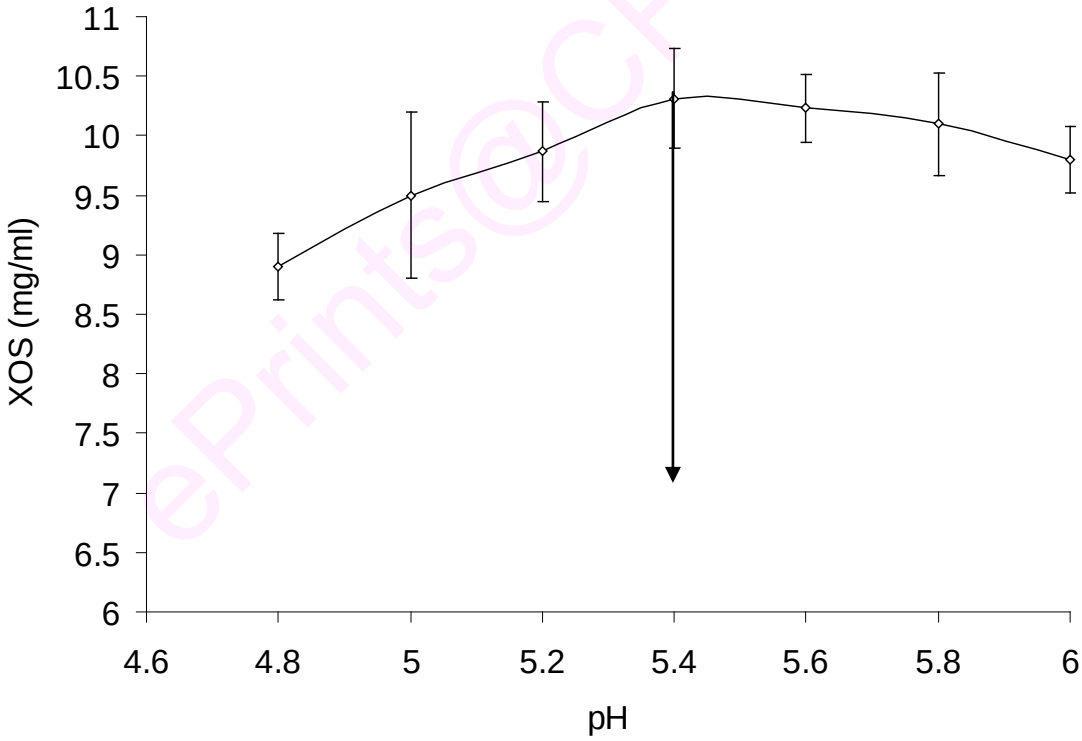



**Figure 4.2.3:** Effect pH of reaction medium (Mc Ilvain Buffer, 0.05 M) on the production of XOS from alkali pretreated corncob powder (6%, w/v) using endoxylanase (14 U/ml of reaction mixture) from *Aspergillus oryzae* MTCC 5154. The results were expressed as average of triplicate determinations with ±SD.

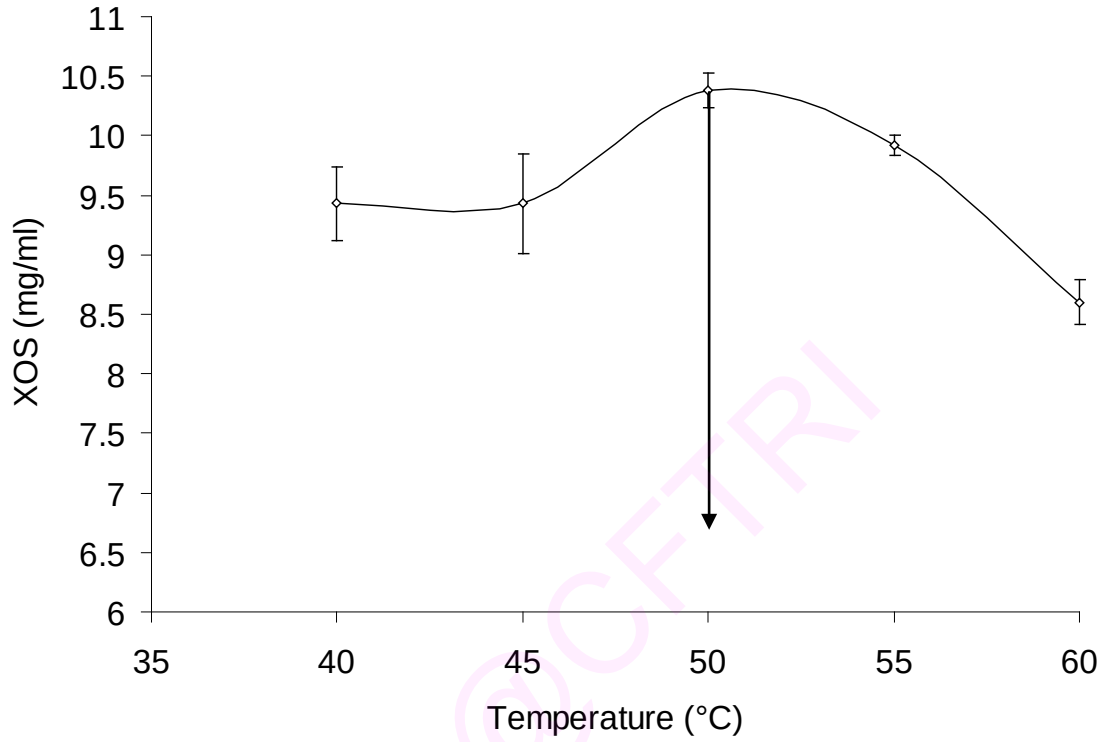



**Figure 4.2.4:** Effect of reaction temperature on the production of XOS from alkali pretreated corncob powder (6%, w/v) using endoxylanase (14 U/ml of reaction mixture) from *Aspergillus oryzae* MTCC 5154 in Mc Ilvain Buffer, pH 5.4, 0.05 M. The results were expressed as average of triplicate determinations with ±SD.



**4.2.3.1.4. The effect of reaction time on XOS production**

A time course study of XOS production has been carried out for 42 h with a sampling interval of 6 h. The effect of reaction time is presented in figure 4.2.5, wherein the maximum XOS production of 10.2±0.14 mg/ml was observed after 14 h of reaction. A detailed data on the varying concentration of different oligosaccharides as a function of time is depicted in table 4.2.1.

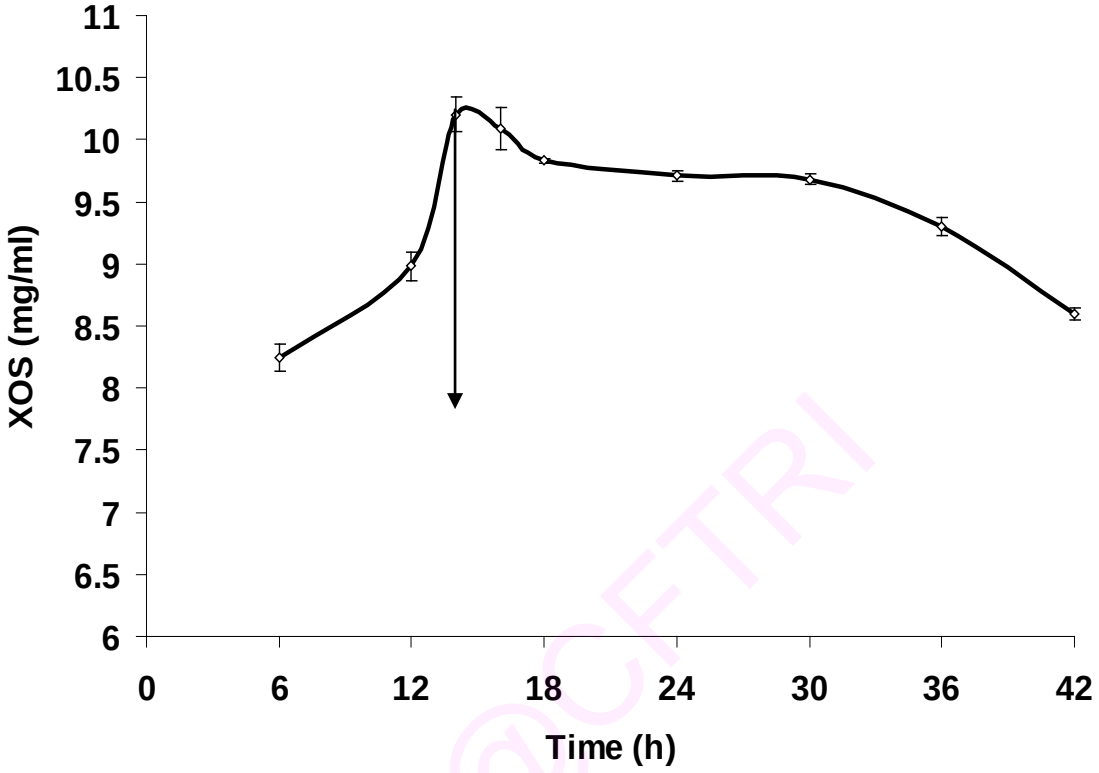



**Figure 4.2.5:** Effect of reaction time on the production of XOS at 50 °C from alkali pretreated corncob powder (6%, w/v) using endoxylanase (14 U/ml of reaction mixture) from *Aspergillus oryzae* MTCC 5154 in Mc Ilvain Buffer, pH 5.4, 0.05 M. The results were expressed as average of triplicate determinations with ±SD

At the end of 6 h of reaction, the amount of XOS formed was 8.24±0.11 mg/ml which is equivalent to about 86.7±3.5% of the total sugars in the hydrolyzate. Monosaccharides, mainly xylose constituted the remaining 13.3%. The percentage of XOS in hydrolyzate was found to decrease as the reaction time increased. At the end of 42 h of reaction, a considerable reduction in the percentage amount of XOS from a maximum value of 86.7±3.5% to 69.6±3.3% was observed. The actual concentration of XOS in the hydrolyzate was found to increase from an initial value of 8.24±0.11 mg/ml at 6 h to a maximum of 10.2±0.14 mg/ml at 14 h of reaction. There after it was found to decrease gradually to a value of 8.6±0.05 mg/ml at 42 h of reaction. Figure 4.2.6 represents a typical HPLC chromatogram of purified xylobiose obtained by GPC.

**Table 4.2.1:** Time course study of XOS production from alkali pretreated corncob powder using endoxylanase from *Aspergillus oryzae* MTCC 5154

| Reaction Time (h) | Carbohydrate profile (%) of the hydrolyzate | | | | Concentration of XOS (mg/ml) [b] |
|---|---|---|---|---|---|
| | Xylose | Xylobiose | Other XOS | Total XOS [a] | |
| 6 | 13.3 | 69.5 | 17.2 | 86.7±3.5 | 8.24±0.11 |
| 12 | 19.6 | 73.2 | 7.2 | 80.4±2.9 | 8.98±0.11 |
| 14 | 19.0 | 73.5 | 7.5 | 81.0±3.9 | 10.20±0.14 |
| 16 | 20.3 | 70.5 | 9.2 | 79.7±4.9 | 10.09±0.17 |
| 18 | 20.0 | 70.3 | 9.7 | 80.0±1.2 | 9.83±0.02 |
| 24 | 20.8 | 68.6 | 10.6 | 79.2±1.6 | 9.71±0.04 |
| 30 | 23.9 | 67.2 | 8.9 | 76.1±1.8 | 9.68±0.04 |
| 36 | 26.7 | 67.9 | 5.4 | 73.3±1.8 | 9.30±0.07 |
| 42 | 30.3 | 67.6 | 2.0 | 69.6±3.3 | 8.60±0.05 |

a, b: Mean and standard deviations for n=3

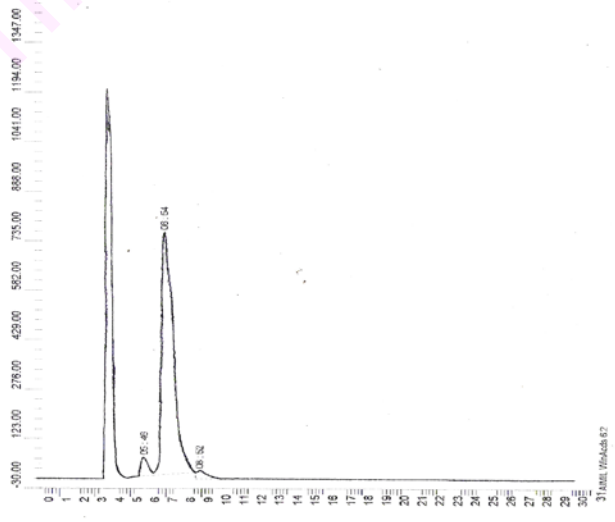


**Figure 4.2.6:** The HPLC profile of purified xylobiose obtained in GPC using BioGel P-2.

### 4.2.3.2. ESI-MS of XOS

The ESI-MS spectra of XOS mixture were acquired in order to identify their m/z (mass/charge) peaks. All measurements were performed in water. It was observed that the m/z peaks found in the XOS mixture obtained from corncob corresponded to adduct of XOS with sodium atom ($[M+Na]^+$). The sodium adducts are found to be more stable in the case of oligosaccharides with higher degree of polymerization. On the basis of measured mass spectra, six different oligosaccharides were identified. In addition, no in-source fragmentation of oligosaccharide ions appeared under the experimental condition used. The mass spectra (figure 4.2.7) showed the presence of various oligosaccharides at an m/z value of 305.19, 437.25, 569.32, 701.38, 833.46 and 965.51.

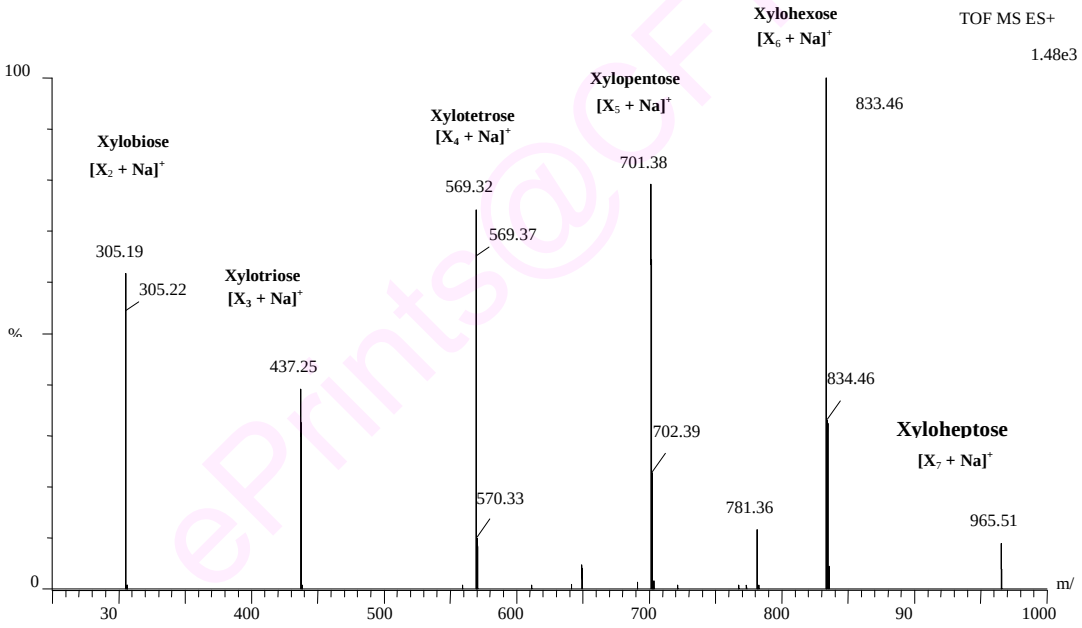



**Figure 4.2.7:** ESI-MS spectra of XOS mixture obtained from alkali pretreated corncob powder (6%, w/v) using endoxylanase (14 U/ml of reaction mixture) from *Aspergillus oryzae* MTCC 5154 in Mc Ilvain Buffer, pH 5.4, 0.05 M

### 4.2.3.3. $^{13}$C and 2D-HSQC NMR spectrometry of xylobiose

The $^{13}$C-NMR and 2D-HSQC NMR spectra of the purified disaccharide are shown in the figure 4.2.8 and 4.2.9 respectively. The spectrum was interpreted and the signals were

assigned (table 4.2.2) on the basis of reported data for structurally defined arabinoxylan and glucuronoxylan as well as those of xylobiose, xylotriose and xylotetrose and xylopentose *(Nabarlatz et al., 2007, Sun et al., 2000, Teleman et al., 2000, Ebringerova et al., 1998, Sun, 1996, Immamura et al., 1994, Ebringerova et al., 1992).*

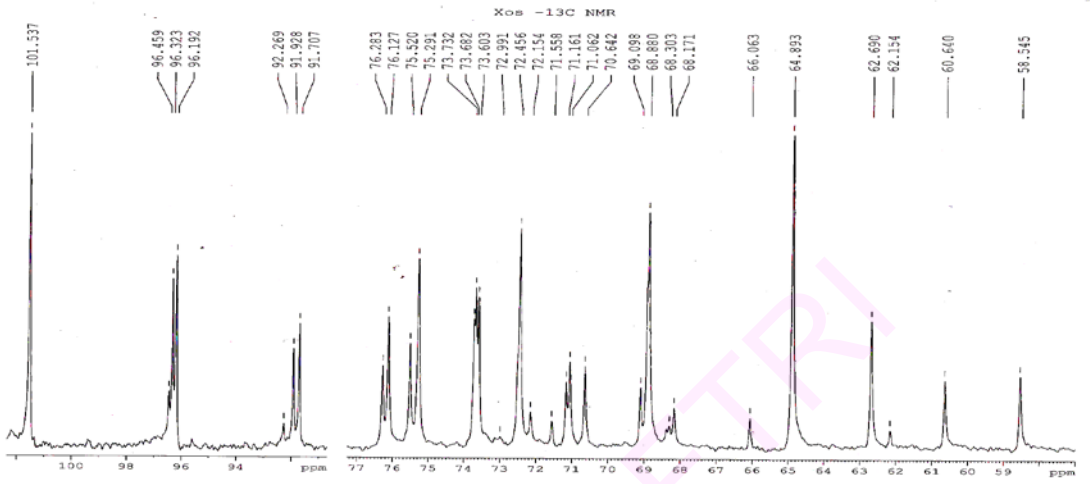



**Figure 4.2.8:** $^{13}$C-NMR spectra of purified xylobiose obtained from corncob

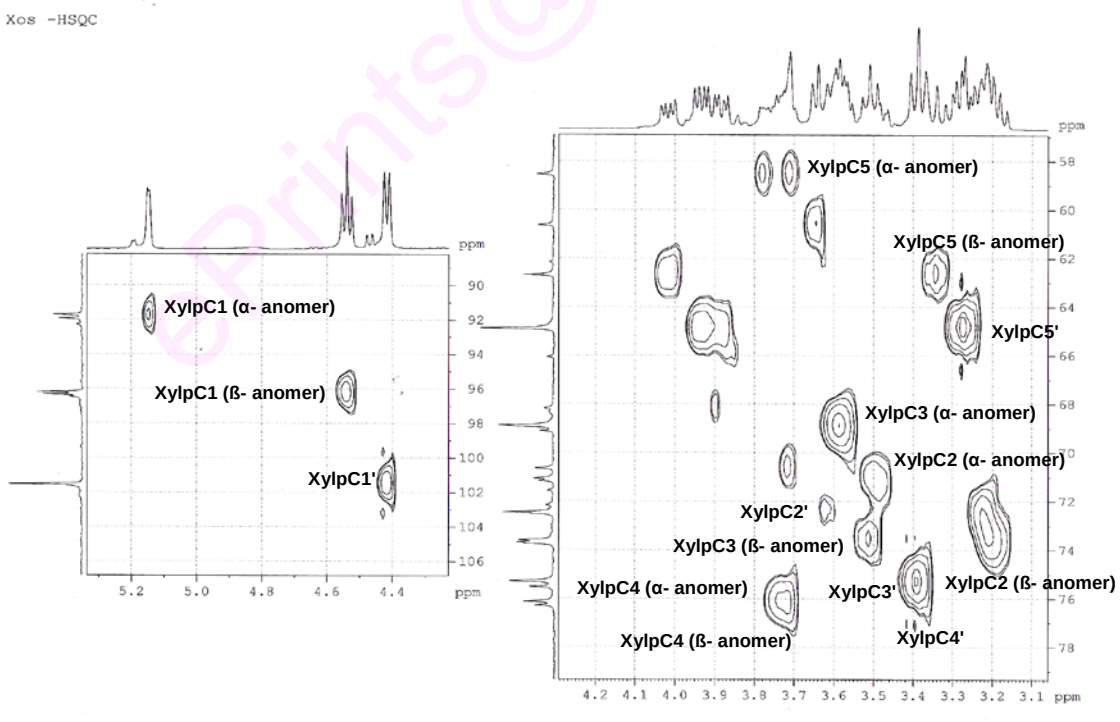



**Figure 4.2.9:** 2D-HSQC spectra of xylobiose purified using GPC

**Table: 4.2.2.** Assignments of signals in the $^{13}$C-NMR [a] spectrum of the purified xylobiose obtained from corncob.

| Xylobiose | Chemical shifts ($\delta_{ppm}$) [b] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | C5 | C1' | C2' | C3' | C4' | C5' |
| α- form [c] | 91.92 | 71.16 | 70.64 | 76.28 | 58.54 | 101.53 | 72.45 | 75.29 | 75.52 | 64.89 |
| β- form [d] | 96.32 | 73.68 | 73.73 | 76.12 | 62.69 | 101.53 | 72.45 | 75.52 | 75.52 | 64.89 |

a: In $D_2O$

b: Values are chemical shifts relative to acetone nucleus

c: β-D-xylopyranosyl-(1→4)-α-D-xylanopyranose

d: β-D-xylopyranosyl-(1→4)-β-D-xylanopyranose

### 4.2.4. Discussion

Kusakabe *et al.* (1977) reported the pretreatment of xylan containing natural materials with dilute alkali solution to remove lignin so that xylan in the pretreated materials could be easily hydrolyzed by xylanase. Therefore, attempts were made to produce XOS from alkali pretreated corncob powder using endoxylanase from *Aspergillus oryzae* MTCC 5154. The corncob is a heterogonous substrate which is sparingly soluble in water and hence encountered practical difficulties when higher concentrations of substrate were used. The mixing of substrate and the enzyme was not uniform at higher concentrations of substrate, which may lead to the poor conversion, resulting in lower yields and hence, further optimization studies were carried out using 6% pretreated corncob powder, though higher concentration of XOS was obtained at 8% and 10% substrate level. At 6% substrate concentration, the XOS formed were around 83.8±2.1% of the total sugars in the hydrolyzate which corresponded to 10.85±0.42 mg/ml XOS. In the present investigation, the maximum production of XOS was observed at pH 5.4 in accordance with the literature reports wherein the optimum pH of most of the fungal xylanases is reported to range from 5.0-6.0.

Jeong *et al.* (1998) studied the effects of temperature and reaction time on the production of xylobiose from xylan by the purified endoxylanase of *Bacillus subtilis* and a temperature less than 60 °C was found to be the optimum for xylobiose production. In the present study also, the results indicate a distinct effect of temperature on XOS production. As can be seen from the results, a maximum of 10.38±0.15 mg/ml XOS production was observed at 50 °C, which is the optimum temperature for endoxylanases of most of the fungi. Beyond 50 °C, the XOS production was found to decrease probably due to the inactivation of xylanases at higher temperatures. This observation is in agreement with the studies on the enzymatic hydrolysis of corncob xylan for the production of XOS where a maximum XOS production was recorded at 50 °C *(Yang et al., 2005).*

After 6 h of reaction, the percentage of XOS in the hydrolyzate was found to decrease as the reaction time increased. This clearly indicates that as the reaction time increases, XOS formed gets hydrolyzed into xylose and oligosaccharides of lower degree of polymerization. Thus at the end of 42 h of reaction, a considerable reduction in the percentage of XOS from a maximum value of 86.7±3.5% to 69.6±3.3% was observed.

The percentage xylobiose followed the same trend as that of total XOS. Xylobiose has been found to have a stimulatory effect on the selective growth of human intestinal bifidobacteria which are important for the maintenance of a healthy intestinal microflora *(Jiang et al., 2004).* At 14 h of reaction, the percentage xylobiose was about 73.5 and is considerably high in comparison with the literature reports *(Jiang et al., 2004).* Since the production of high content xylobiose is a time consuming and expensive process *(Jeong et al., 1998),* the leads obtained from the present investigation can further be explored for large scale production of XOS with a high content of xylobiose. On the other hand the percentage xylose was found to increase with time from an initial value of 13.3% at 6 h to 19.6% at 12 h and 30.3% at 42 h. The percentage of other XOS was higher at the 6 h indicating the random hydrolysis of xylan during the initial stages of reaction. This

primarily results in long chain oligosaccharides which on further hydrolysis results in the formation of smaller oligosaccharides.

Ai *et al*. (2005) have reported that the native and immobilized xylanase of *Streptomyces olivaceoviridis* produced 3.9 mg/ml and 15.5 mg/ml of XOS respectively from pretreated corncob by 24 h of reaction time. A reaction time of 24 h can be a bottle neck in the development of an economically viable process. The present study has a distinct advantage in terms of the lesser period of reaction for obtaining 81±3.9% of XOS corresponding to 10.2±0.14 mg/ml in the hydrolyzate at 14 h of reaction.

Pellerin *et al.* (1991) have studied the hydrolysis of xylan obtained by alkali extraction of corncob using a partially purified endoxylanase (*Clostridium thermolacticum*) and the maximum amount of reducing sugar and total sugars were reported at 48 h. The reason for long hours of reaction time to obtain maximum XOS could probably be due to the inhibition of enzymatic activity in high salt concentration in the pretreated corncob meal which has resulted from the neutralization with acetic acid and concomitant formation of potassium acetate. This aspect has been addressed in the present investigation by washing the alkali pretreated corncob residue with deionized water to pH 6-7.

The ESI-MS spectra of XOS mixture showed presence of six oligosaccharides. The molecular weights of xylooligomer adducts were calculated considering that, during the glycosidic bond formation between two xylose residues one water molecule is released and that the xylooligomeric compounds form adduct with a sodium atom *(Cano and Palet, 2007).* These peaks were identified as xylobiose (305.19), xylotriose (437.25), xylotetrose, (569.32), xylopentose (701.38), xylohexose (833.46) and xyloheptose (965.51). The mass spectra of the major fraction obtained by GPC corresponded to xylobiose.

The $^{13}$C-NMR spectrum obtained was rather simpler when compared to the complicated spectra obtained by hemicelluloses probably because of the relatively large molecular weight hemicelluloses and higher oligosaccharides. The main 1,4-linked β-xylopyranoses

are obviously characterized by the signals at 101.53, 72.45, 75.29, 75.52 and 64.89 ppm which contribute to C1', C2', C3', C4' and C5' of the xylobiose respectively. The signal at $\delta_{ppm}$ 101.53 confirmed the β-(1→4) xylosidic linkage between the two xylose residues. The anomeric carbons of the probable disaccharide appeared at $\delta_{ppm}$ 91.92, 96.32 and 101.53, which were assigned as reducing α-D-xylopyranoside, reducing β-D-xylopyranoside and non reducing β-D-xylopyranoside, respectively.

A detailed assignment of signals is obtained in 2D-HSQC spectrum (figure 4.2.9). There is no $^{1}H/^{13}C$ cross peaks in the 2D-HSQC spectrum at $\delta_{ppm}$ 5.34/98.4 and this clearly indicates the absence of xylose residues with methyl glucuronic acid *(Nabarlatz et al., 2007, Teleman et al., 2000).* The presence of a comparatively weak signal at δ 62.15 indicates the C5 of *Araf* and shows the presence of some disaccharides with xylose and arabinose, but not sufficient to produce a cross peak in 2D-HSQC. This confirms that the purified disaccharide from the XOS mixture is primarily a disaccharide of xylose and is identified as β-D-xylopyranosyl-(1→4)-D-xylanopyranose (xylobiose).

# CHAPTER: 5

## *IN -VIVO* INVESTIGATIONS ON THE EFFECT OF XYLOOLIGOSACCHARIDES ON 1,2-DIMETHYLHYDRAZINE INDUCED COLON CANCER IN RATS

**Overview**

Diet interventions and natural bioactive supplements have now been extensively evaluated for reducing the risks of colon cancer, especially for possible prevention. Prebiotics, being indigestible, have been associated with improved bowel functions and metabolisms of the distal colon, including a reduced risk of colon cancer. Most of the protective effects of prebiotics on colon cancer have emphasized on the oligofructose based prebiotics such as fructooligosaccharides and inulin. The present study describes alterations in endogenous antioxidant components in colonic mucosa of rats treated with the carcinogen DMH and maintained on diet supplemented with or without XOS. The present chapter also discusses the changes in the profile of colonic microbiota on DMH treatment followed by XOS supplementation. The study also focuses on the reduction in the number of DMH induced aberrant crypt foci in the colon of Wistar rats by dietary XOS supplementation.

## 5.1. Introduction

Colorectal cancer represents a major public health problem accounting for over 1 million cases and about half a million deaths worldwide *(Chau and Cunningham, 2006).* Survival from colon cancer at 5 years has been found to vary demographically and estimated to be 65% in North America, 54% in Western Europe, 34% in Eastern Europe and 30% in India *(Parkin et al., 2005).* Although chemotherapy and radiotherapy are generally recommended as surgical adjuvant treatments for colon cancer, they vary in success rates for local recurrence, disease-free survival and overall survival *(Martenson et al., 2004).* Diet interventions and natural bioactive supplements have been extensively evaluated for reducing the risks of colon cancer, especially for possible prevention. Prebiotics, being indigestible, have been associated with improved bowel functions and metabolisms of the distal colon, including a reduced risk of colon cancer.

The prebiotics have a strong chemopreventive effect on chemically induced colorectal cancer in rodent models. The mechanisms of chemoprevention by prebiotics have been elucidated *in vivo* in animal models of colorectal carcinogenesis and by *in vitro* experiments. These experiments may involve the protection from DNA damage by reducing the carcinogen burden of the gut through a decrease of the activity of certain microbial enzymes associated with the conversion of procarcinogens to carcinogens as well as the induction of detoxicifying enzymes in the mucosal cells. Additional mechanisms of chemoprevention by prebiotics include an increase in apoptosis to remove DNA damaged cells, inhibition of proliferation of dysplastic cells, improvement of the barrier function, an activation of the immune system and modulation of the genes involved in oxidative and metabolic stress. A couple of in-depth studies have indicated that prebiotics possess anticarcinogenic potential in humans *(Roberfroid, 2008).*

Most of the protective effects of prebiotics on colon cancer have emphasized on the oligofructose based prebiotics such as fructooligosaccharides and inulin. In one of the animal trials conducted (trial of 31 weeks), it was reported that the protective effect of probiotics on azoxymethane-induced carcinogenesis was less compared to the effects of

prebiotics *(Femia et al., 2002).* The authors subsequently conducted further studies on the expression of genes encoding enzymes involved in colon carcinogenesis processes. Glutathione *S*-transferase (GST) and placental GST pi type were found to be expressed to a lesser extent when rats were fed with prebiotic alone or in combination with *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12. In addition, the inducible nitric-oxide synthase which is reported to have an important role in colon tumor growth and progression *(Ahn and Ohshsima, 2001),* was also reduced in the tumors from rats in the prebiotic group. The authors also evaluated the levels of cyclooxygenase-2, an enzyme found to be up-regulated in cancers *(DuBois et al., 1996)* and cyclooxygenase-2 inhibitors which are often associated with chemopreventive activities. Cycloxygenase-2 expression was found to be increased in the tumors of the control rats but not in those fed with prebiotics. Although the exact mechanisms remain unknown, Femia *et al.* (2002) postulated that prebiotic reduced carcinogenesis occur *via* modification of gene-expressions.

The fermentation of prebiotics in the colon often generates short chain fatty acids (SCFA). Considering that butyrate is not a common end-product from the fermentation by lactobacilli and bifidobacteria, its production would originate from the fermentation by other intestinal flora. It has been found that butyrate is produced in the colon at varying concentrations depending on the type of prebiotics *(Liong et al., 2007).* Although the production of butyrate is approximately 5% of total SCFA, it is of particular interest because butyrate has been found to induce differentiation of colorectal tumor cells *(Reddy, 1999).* The reduction of colonic cell proliferation and induction of differentiation in colonic epithelial cells have now led to increased clinical trials using butyrate in the treatment of ulcerative colitis *(Reddy et al., 1997).* In addition, sodium butyrate has been reported as a powerful inhibitor of growth and inducer of phenotype differentiation and apoptosis. It is also considered to exert beneficial effects in reducing risk factors involved in the etiology of colon cancer and adenoma development *(Kotunia et al., 2004).* Treptow-van Lishault *et al.* (1999) found that the fermentation of gut bacteria on a retrograded, high amylose starch had produced butyrate that may increase the detoxification of both electrophilic products and compounds associated with oxidative

stress. The specific enzyme induction by butyrate, or by the microflora and increased activity by prebiotics may be an important mechanism of protection against carcinogen-induced colon cancer *(Wollowski et al., 2001).*

A combination of suitable prebiotics with probiotics ( known as synbiotics) has been found to enhance the survival and activity of the organism, both in *in vitro* and *in vivo* experiments, for example fructooligosaccharides in conjunction with a bifidobacterium strain or lactitol in conjunction with a lactobacillus *(Gibson and Roberfroid, 1995).* The synbiotics has synergistic effects because, in addition to promoting growth of existing strains of beneficial bacteria in the colon, synbiotics also act to improve the survival, implantation and growth of newly added probiotic strains. The combination of bifidobacterium and oligofructose synergistically retarded colon carcinogenesis in rats compared to when both were given individually *(Gallaher and Khil, 1999).*

In a randomized, double-blind and placebo-controlled trial, Rafter *et al.* (2007) evaluated the effect of synbiotics in reducing cancer risk factors in 37 colon cancer patients and 43 polypectomized patients. The synbiotic used for the study was a mixture of *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 as the probiotic and oligofructose-enriched inulin as the prebiotic. The authors found that certain colorectal cancer intermediate biomarkers can be altered *via* synbiotic intervention, where colorectal proliferation and the capacity of fecal water to induce necrosis in colonic cells were reduced. In addition, polypectomized patients showed improved epithelial barrier functions. The authors conducted genotoxicity assays using colonic biopsy samples and found that exposure to genotoxins in polypectomized patients decreased at the end of the intervention period. Although the exact mechanisms of these remain unknown, the authors postulated that the synbiotic intervention had contributed to the alterations in composition of the colonic bacterial ecosystem and subsequently the metabolic activity of the colon.

Studies on the anticancerous effect of xylan and xylooligosaccharides (XOS) have been very much limited. Glucuronic acid-containing xylans have been reported to markedly

inhibit the growth of sarcoma-180 and other tumors *(Hashi and Takeshita, 1979).* However, no systematic attempts have been carried out to determine the active principle of xylan-type polysaccharides which differ greatly in the type, proportions and distribution of glycosyl side-chains decorating the *β*-1,4-D-xylan backbone *(Ebringerova et al., 2002).* Comparison of the biological responses of different acidic xylans has not revealed any unequivocal relation either to the 4-O-methyl-D-glucuronic acid (MeGlcA) content or to the distribution pattern of MeGlcA *(Ebringerova et al., 2002).*

There exist only few studies for investigating the anti-tumorigenic properties of XOS and arabinoxylan. Therefore it is difficult to evaluate their potential in reducing the colon cancer incidence. Synbiotic intervention of 2% wheat bran oligosaccharides (arabinoxylan) and $10^8$ cfu bifidobacteria significantly reduced aberrant crypts/$cm^2$ in colon of DMH treated Wistar rats *(Gallaher and Khil, 1999).* But the effect of wheat bran oligosaccharides remain uncertain, even when it was shown that bifidobacteria alone had no effect because no group was fed with oligosaccharides alone. Further experiments are necessary to confirm an anti-tumorigenic effect of XOS and arabinoxylan.

Hsu *et al.* (2004) evaluated the effects of XOS and fructooligosaccharides on the alteration of cecal microbiota, pH and weight, serum lipid levels in addition to their inhibitory effect on precancerous colon lesions in male Sprague-Dawley rats. The rats were randomly assigned to 4 groups: control, treatment with DMH [15 mg/kg body weight/wk for 2 wk], treatment with DMH+60 g XOS/kg diet, and treatment with DMH+60 g FOS/kg diet. Rats were fed with the experimental diets for 35 days, beginning, 1week after the second dose of DMH. Both XOS and FOS markedly decreased the cecal pH and serum triglyceride concentration and increased the total cecal weight and bifidobacterial population. XOS had a greater effect on the bacterial population than fructooligosaccharides. Moreover, both XOS and fructooligosaccharides markedly reduced the number of aberrant crypt foci (ACF) in the colon of DMH-treated rats. These results suggest that dietary supplementation of XOS and fructooligosaccharides may be beneficial to gastrointestinal health and indicate that XOS is more effective than fructooligosaccharides.

Genetic changes in the malignant transformation process of colorectal mucosa include deletions, rearrangements and mutations leading to either inactivation or activation of specific target genes *(Kinzler and Vogelstein, 1996, Boland, 1993, Fearon et al., 1990).* A number of biomarkers associated with genetic changes have been identified for early detection of colorectal cancer. Identification of ACF as an early preinvasive lesion and its relationship to the development of cancer have aroused an increasing interest in recent years. ACF was first reported by Bird (1987) in the colons of carcinogen-treated C57BL/6J or CF1 female mice and the assumption of ACF as potential preneoplastic lesions in murine colon was put forward one year later, coming up with the methodological approach to detect ACF *(McLellan and Bird, 1988).* Under microscope, aberrant crypts appeared larger and had a thicker epithelial lining compared to normal crypts and usually gathered into a focus, consisting of aberrant crypts from one to hundreds *(Otori et al., 1995, Roncucci et al., 1991).* These aberrant crypts sometimes were slightly elevated from the surrounding normal mucosa and often had oval or slit-like lumens *(Pretlow et al., 1992, Pretlow et al., 1991, Roncucci et al., 1991).* It has been described as single or clusters of abnormally large crypts of the colon mucosal surface after staining with methylene blue. On colonoscopy, ACF appeared darker with methylene blue staining than normal surrounding mucosa and as a cluster of two or more crypts with dilated or slit-like openings rising above the surrounding mucosa *(Adler et al., 2002, Yokota et al., 1997).*

ACF with a single crypt met the following criteria by McLellan *et al.* (1991). The size of the crypt was at least twice that of the normal surrounding ones, the luminal opening was more elliptical than circular and the epithelial lining was thicker than that of the normal surrounding crypts. ACF consisting of more than one crypt were defined as crypts to form a distinct focus. Individual crypts within the focus had a thicker epithelial lining and an elliptical luminal opening and the total area occupied by the crypts of ACF was greater than that occupied by an equivalent number of the surrounding morphologically normal crypts. As ACF are characterized by a larger size and thicker lining of epithelial cells than normal crypts, they could be used as a proliferation marker to study colon

carcinogenesis *(Bird, 1995, McLellan et al., 1991, Bird, 1987).* Several studies indicate that the administration of bifidobacteria or lactobacilli alone or with fermentable oligosaccharide could modify colonic microflora populations and decrease the development of aberrant crypts and tumors in the colon *(Buddington et al., 2002, Kleessen et al., 2001, Gallaher and Khil, 1999, Reddy, 1999, Goldin and Gorbach, 1980).*

The present chapter focuses on the inhibitory effect of XOS supplementation on the neoplastic transformation at the early stages in a defined DMH-induced Wistar rat colon carcinogenesis model. The effects of XOS have been studied by morphometric evaluation of the incidence and multiplicity of ACF in colonic mucosa. The study also focuses on the effect of DMH treatment on the colonic microbiota and further associated changes. Furthermore, the chemopreventive efficacies of XOS are also investigated on certain drug metabolizing and phase II detoxifying enzymes of liver and colonic mucosa.

## 5. 2. Materials and methods

### 5. 2.1. Materials

All chemicals were of analytical grade. 1,2-dimethyl hydrazine (DMH) was obtained from Sigma-Aldrich (Germany). Corncobs were obtained from a local maize field in Mysore, Karnataka, India and were sorted, dried at 40 °C for 24 h, milled (60-80 mesh) and stored in polycarbonate containers.

### 5. 2.2. Production of xylooligosaccharides

XOS were produced from alkali pretreated corncob by the action of endoxylanase produced by *Aspergillus oryzae* MTCC 5154 as reported earlier *(Aachary and Prapulla, 2009).* The products of hydrolysis were analyzed by HPLC equipped with a refractive index detector using an Aminopropyl column *(Jeong et al., 1998)* and the sample preparations and conditions were based on previous reports *(Aachary and Prapulla, 2008).* The XOS formed were quantified by comparing the peak area of XOS with that of standard xylose and is expressed as mg/ml of hydrolyzate. The XOS obtained were having 73.5% of xylobiose, 13.2 % other oligosaccharides and 13.3% xylose residues.

**5. 2.3. Animals, treatments and diets**

Male Albino Wistar rats weighing 230-250 g were used for the study. The animal study was conducted with due approval from the Institutional Animal Ethics Committee and by following all the procedures with regard to care and use of animals. The animals (n=40) were housed individually in stainless-steel wire-bottom cages in an environmentally controlled room (25±2 °C) with a 12-h light-dark cycle. The rats were fed with a standard semi-synthetic diet for 1 week and the basal diet consisted of (%): casein, 21; cane sugar, 10; corn starch, 54; refined peanut oil, 10; NRC vitamin mixture, 1; and Bernhardt-Tommarelli salt mixture, 4. After one week, the rats were randomly assigned to 5 treatment groups (8 rats/group): control, DMH (positive control), XOS (10%), DMH+XOS (5%) and DMH+XOS (10%). XOS containing diets were prepared by replacing the starch in the basal diet by equivalent amount of XOS syrup (50 g/kg diet and 100g/kg diet). The treatment protocol of this animal experiment is schematically represented in figure 5.1.

A preliminary study confirmed that 3 injections of DMH (20 mg/kg body wt) at weekly intervals induced aberrant crypt formation in rats. The rats in the DMH, DMH+XOS (5%) and DMH+XOS (10%) groups received 3 weekly doses of DMH dissolved in saline by subcutaneous injection in the groin region. Dietary treatment began 1 week after the third application of DMH. The XOS groups were fed with XOS for 45 days and the normal control and DMH control groups were fed with basal diet. The rats were allowed to water and food *ad libitum*. The rats were weighed at weekly intervals and the feed intake was recorded daily. At the end of the experimental period, the rats were anesthetized with ether and sacrificed by decapitation.

Immediately after sacrifice, liver and large intestines were quickly excised and washed with ice cold saline. The tissues were homogenized with three volumes (w/v) of the appropriate buffer using a Potter–Elvehjam homogenizer with a Teflon pestle and centrifuged at 12,000×g for 20 min at 4 °C. The supernatant was used for the biochemical analyses.

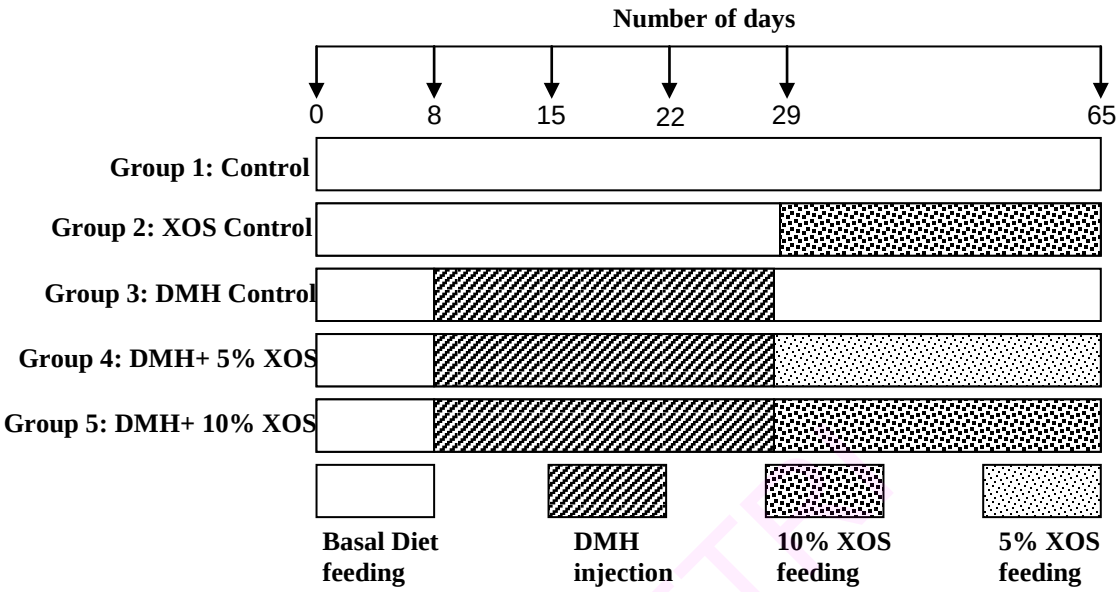



**Figure 5.1:** DMH induced colorectal cancer in rats: Experimental protocol

### 5. 2.4. Biochemical assays

Lipid peroxidation was estimated by measuring the level of tissue lipid peroxidation byproduct, thiobarbituricacid reactive substances (TBARS) by using the method of Jiang *et al.* (1992). The values are expressed as nmole per mg protein. The activity of catalase (EC.1.11.1.6) was determined by the method of Sinha (1972). The values of catalase activity are expressed as µmol of $H_2O_2$ utilized per min per mg protein. The activity of glutathione-*S*-transferase (EC. 2.5.1.18) was estimated by the method of Habig *et al.* (1974) and the values are expressed as µmol of 1-chloro-2,4-dinitrobenzene (CDNB)-GSH conjugate formed per min per mg protein. Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

### 5. 2.5. Bacteriological analysis of caecal material

Bifidobacterium iodoacetate medium 25 (BIM-25) *(Reeves, 1997)* was used to culture *Bifidobacterium* species, comprising of 51 g/l reinforced clostridial agar, 2 g/l nalidixic acid, 0.85 g/l polymyxin B sulfate, 5 g/l kanamycin sulfate, 0.25 g/l iodoacetic acid, and

2.5 g/l 2,3,5-triphenyltetrazolium chloride. The medium used for *Clostridium perfringens* was Tryptose-sulfite-cycloserine (TSC), containing 15 g/l tryptose, 5 g/l yeast extract, 5 g/l soytone, 1 g/l ferric ammonium citrate, 1 g/l sodium metabisulfite, 0.1 g/l D-cycloserine, 20 g/l agar, and 1 g/l egg yolk. The anaerobic dilution buffer contained 0.2 g/l gelatin, 20 g/l $MgSO_4.7H_2O$, 0.25 g/l $FeSO_4.7H_2O$, 0.4 g/l $MnSO_4.2H_2O$, 0.5 g/l NaCl, and 1 g/l resazurin. The anaerobic culture media and anaerobic dilution buffer were prepared by a prereduced anaerobically sterilized method.

To determine the microbial counts of *Bifidobacterium* species, *Clostridium perfringens,* and *Escherichia coli*, a serial dilution was made by mixing the cecal contents with dilution buffer and 0.1 ml of the mixture was cultured using the spread-plate method. The *Bifidobacterium* species and *Clostridium perfringens* were cultured anaerobically in an oxygen-free mixed-gas atmosphere at 37 °C for 48 h. *Escherichia coli* was cultivated in Desoxycholate agar at 37 °C for 24 h in an aerobic incubator. Cecal contents were cultured with triplicate plates for the microbial colony count. The microbial count data were expressed as colony forming units/g wet sample (cfu/g).

**5. 2.6. Proliferation markers: Analysis of ACF**

The development of colonic preneoplastic lesions (aberrant crypts) in the distal colon was used as a biomarker of colon cancer. The efficacy endpoints used in this study were reductions in total ACF/colon and multicrypt clusters of aberrant crypts ($\geq$2 crypts/focus), as described by Reddy (1999). The colon was removed, slit open from the anus to the cecum along the longitudinal axis, spread flat between sheets of filter paper and fixed in buffered 10% formalin. It was stained with 0.2% methylene blue in saline after fixation as per the procedure of Bird (1987) to observe aberrant crypts. The number of ACF per colon, the number of aberrant crypts in each focus and the location of each focus were determined by microscopy at a magnification of x40. To determine the distribution of ACF, the rectum is defined as the segment 2 cm proximal to the anus and the remaining colon was divided into three segments of about 6 cm lengths, the distal colon the middle colon, and the proximal colon.

### 5. 2.7. Data and statistical analysis

The averages and standard deviations were done using Microsoft Excel (Version 5.0, Microsoft, Corporation, Redmond, WA). The significance of the differences between values for different groups was analyzed by One way ANOVA, and a P value of $\leq 0.05$ was regarded as significant.

## 5. 3. Results

### 5. 3.1. Effect of XOS supplementation on body weight

The influences of XOS supplementation on the weight of DMH-treated and normal rats were monitored. The changes in body weight are expressed as a ratio of average initial and final body weight and are presented in table 5.1. Control rats showed an average weight increase index of 1.17 ± 0.09 while that of the DMH control group was found to be significantly decreased ($P \leq 0.05$). Significant increases ($P \leq 0.05$) in the weight increase index were observed when the animals (control and DMH-treated) were fed with XOS. Interestingly, it is observed that the weight increase index was more in groups fed with 5% XOS than 10% XOS.

**Table 5.1:** Effect of dietary XOS supplementation on weight* of normal and DMH-treated Wistar rats

| Group | Final weight (Wt) | Final weight ($W_0$) | Increase in body weight ($Wt/W_0$) |
|---|---|---|---|
| Control | 347.2 ± 2.1 | 296.0 ± 3.3 | 1.17 ± 0.09 |
| XOS Control | 345.5 ± 3.8 | 256.6 ± 3.1 | 1.35 ± 0.10 |
| DMH Control | 329.3 ± 2.4 | 292.7 ± 1.6 | 1.12 ± 0.08 |
| DMH+ 5% XOS | 357.3 ± 4.2 | 233.3 ± 3.9 | 1.53 ± 0.12 ** |
| DMH+ 10% XOS | 364.2 ± 2.1 | 285.9 ± 2.2 | 1.27 ± 0.08 ** |

* Mean and standard deviations for n=8

* *The values are significantly ($P \leq 0.05$) different from the DMH-control group.

### 5. 3.2. Effect of XOS supplementation on cecum and liver weight

The total cecum weights of the XOS groups were markedly greater than those of the control and DMH (positive control) groups as indicated in table 5.2. The values are expressed as g/kg body weight. The cecum weights of the DMH+XOS (5 and 10%) groups were significantly greater ($P \leq 0.05$) than those of the DMH control group. It is observed that DMH treatment significantly reduced the caecum weight, however, it was found to increase markedly on XOS supplementation. DMH treatment reduced the liver weight in comparison with normal control and this effect has been reversed by the XOS supplementation as shown in table 5.2.

**Table 5.2:** Effect of XOS supplementation on liver and caecum weight* of normal and DMH- treated Wistar rats.

| Group | Weight of liver (g/ kg body weight) | Weight of caecum (g/ kg body weight) |
|---|---|---|
| Control | 29.2 ± 0.9 | 15.3 ± 0.9 |
| XOS Control | 29.1 ± 0.8 | 17.3 ± 0.1 |
| DMH Control | 28.7 ± 0.5 | 14.1 ± 0.7 |
| DMH+ 5% XOS | 29.8 ± 0.8** | 16.7 ± 0.7** |
| DMH+ 10% XOS | 29.7 ± 0.4** | 16.9 ± 0.4** |

* Mean and standard deviations for n=8

** The values in each column are significantly ($P \leq 0.05$) different from the DMH-control group in the same column.

### 5. 3.3. Microbiota of the colon contents

Table 5.3 indicates the microbial profile of caecal contents of various groups of rats. The DMH treatment markedly reduced the population of bifidobacteria and increased the number of *Clostridium perfringens* and *Escherichia coli.* XOS supplementation resulted in an increased population of bifidobacteria which is significant ($P \leq 0.05$) when compared with the control and DMH treated groups. Though DMH treatment increased the number of *Clostridium perfringens,* XOS supplementation effectively reduced their number to

normal and beyond. The XOS group had a smaller population of *Escherichia coli* than the DMH group. The increased weights of cecal content (wet basis) of XOS fed rats can be correlated to the increased cecal bifidobacterial population. These results indicate that XOS supplementation markedly lowered cecal pH (data not shown) and increased both cecal weight and the bifidobacterial population, effects which in turn may promote the health of intestinal tract.

**Table: 5.3:** Effect of XOS supplementation on cecal microbial profile* of normal and DMH- treated Wistar rats

| Group | Microbial profile of cecal content (log cfu/g wet weight) | | |
|---|---|---|---|
| | *Bifidobacterium* | *Clostridium* | *E.coli* |
| Control | 10.1 ± 0.24 | 8.48 ± 0.23 | 8.52 ± 0.22 |
| XOS Control | 11.79 ± 0.15 | 8.61 ± 0.14 | 8.09 ± 0.10 |
| DMH Control | 8.38 ± 0.15 | 9.01 ± 0.25 | 9.05 ± 0.22 |
| DMH+ 5% XOS | 10.31 ± 0.09** | 8.71 ± 0.21** | 8.62 ± 0.16** |
| DMH+ 10% XOS | 11.01 ± 0.16** | 8.27 ± 0.17** | 8.09 ± 0.20** |

* Mean and standard deviations for n=8

** The values in each column are significantly ($P \leq 0.05$) different from the DMH-control group in the same column.

### 5. 3.4. Proliferation markers: Analysis of ACF

Table 5.4 represents the average number of ACF observed in various groups. Both the normal and XOS control groups had no ACF. ACF were found only in the colons of all animals treated with DMH. ACF were located mainly in the distal colon, with some in the middle colon, a few in the rectum and very few in the proximal colon. The mean size of ACF, measured as the number of crypts per focus, was also significantly smaller in the group fed with XOS than in the DMH-control group at the end of experimental period. A 50% reduction in the incidence of crypt multiplicity for 1, 2, 3, and 4 crypts/focus was observed on supplementation with XOS. It was also observed that 10% XOS drastically

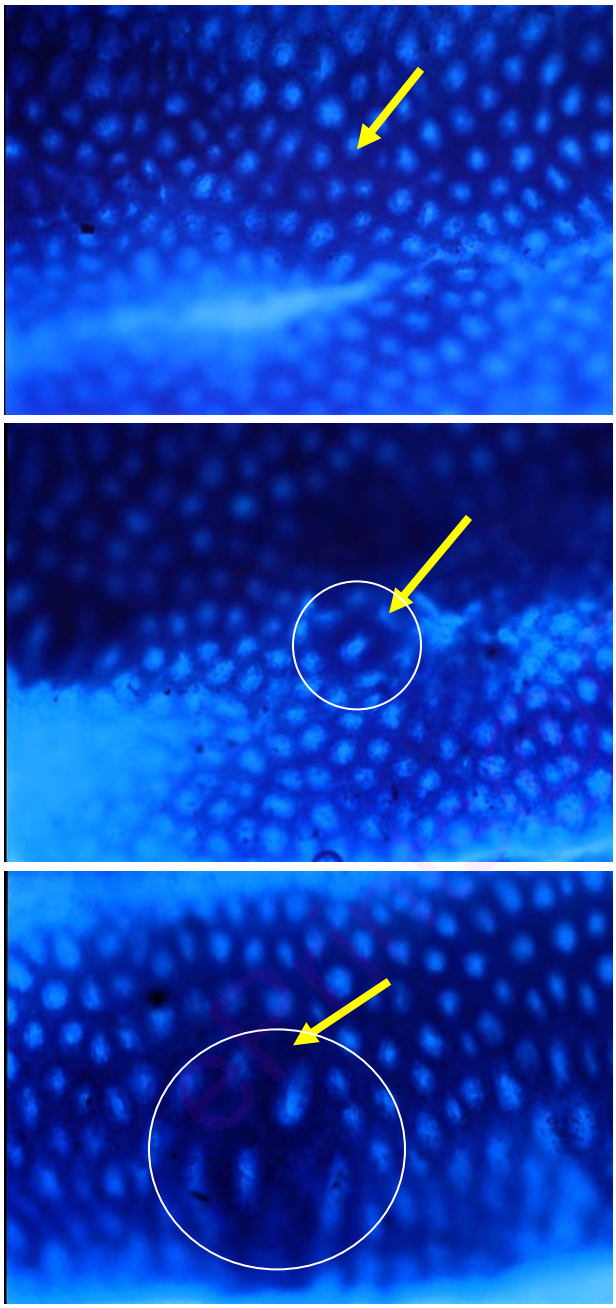
reduced the ACF and there were no crypt multiplicity for 3, and 4 crypts/focus. The microscopic pictures of normal crypt foci and ACF are given in figure 5.2.

**Table 5.4:** Effect of XOS supplementation on the number and multiplicity of ACF* in the distal colon in DMH treated Wistar rats.

| Group | Number of ACF/foci* in colon | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Control | ND | ND | ND | ND |
| XOS control | ND | ND | ND | ND |
| DMH control | 8.3 ± 1.0 | 6.2 ± 1.0 | 3.1 ± 0.9 | 2.0 ± 0.4 |
| DMH+ 5% XOS | 4.3 ± 0.9 | 3.2 ± 0.7 | 1.2 ± 0.2 | 0.4 ± 0.1 |
| DMH+ 10% XOS | 2.7 ± 0.1 | 2.0 ± 0.1 | ND | ND |

* Mean and standard deviations for n=8

ND: Not detected



**a. Normal Crypt Foci**

**b. Single aberrant Crypt Foci (ACF)**

**c. Multiple aberrant Crypt Foci (ACF)**

**Figure 5.2:** Photomicrographs of transilluminated, methylene blue-stained, whole mount of distal colon from a rat treated with DMH showing (a) normal crypt foci, (b) single ACF and (c) multiple ACF***.**

* Increased density of staining around the crypt mouth and the altered morphology of the mouths of the crypts in the ACF.

### 5. 3.5. Changes in the levels of lipid peroxidation in the colonic mucosa and liver

Table 5.5 shows the tissue levels of TBARS, in the control and experimental rats. The levels of TBARS decreased in the colon of DMH-treated control rats relative to the levels of that of the normal control rats. XOS supplementation, after DMH treatment significantly restored the levels of colonic TBARS to near control levels. The results also show the effect of DMH treatment and XOS supplementation on liver lipid peroxidation values. It is observed that the DMH treatment markedly increased the liver lipid peroxidation value and was significantly ($P \leq 0.05$) reversed on XOS supplementation.

**Table 5.5:** Effect of XOS supplementation on the lipid peroxidation values* of colonic mucosa and liver of normal and DMH treated Wistar rats.

| Group | TBARS (n mol of MDA/mg of protein) | |
|---|---|---|
| | Liver | Colonic mucosa |
| Control | 32.23 ± 0.01 | 21.45 ± 0.13 |
| XOS Control | 24.63± 0.90 | 20.20 ± 0.08 |
| DMH Control | 55.87± 0.12 | 16.07 ± 0.01 |
| DMH+ 5% XOS | 48.40± 0.14** | 19.87 ± 0.12** |
| DMH+ 10% XOS | 41.06 ± 0.14** | 18.34 ± 0.09** |

* Mean and standard deviations for n=8

** The values in each column are significantly ($P \leq 0.05$) different from the DMH-control group in the same column.

### 5. 3.6. Changes in the level of Glutathione-*S*-transferase (GST) activity in the colonic mucosa and liver

Table 5.6 shows the activity of GST in colonic mucosa and liver of control and experimental rats. In the colonic mucosa, the activity of GST was significantly ($P \leq 0.05$) decreased on DMH treatment. Activity of GST was significantly decreased in the colonic mucosa of rats fed with XOS in comparison to that of DMH control group. Similar effects were observed with liver tissue.

**Table: 5.6:** Effect of XOS supplementation on the GST activity* of colonic mucosa and liver of normal and DMH treated Wistar rats.

| Group | GST (µmol CDNB-GSH conjugate formed /minute/ mg protein) | |
|---|---|---|
| | Colonic mucosa | Liver |
| Control | 2.50± 0.30 | 7.33 ± 0.92 |
| XOS Control | 2.62± 0.20 | 6.94 ± 0.01 |
| DMH Control | 1.93 ± 0.60 | 6.04 ± 0.84 |
| DMH+ 5% XOS | 2.35± 0.90** | 6.14 ± 0.12** |
| DMH+ 10% XOS | 2.34± 0.40** | 6.68 ± 0.08** |

* Mean and standard deviations for n=8

** The values in each column are significantly ($P \leq 0.05$) different from the DMH-control group in the same column.

**5. 3.7. Changes in the level of catalase activity in the colonic mucosa and liver**

Table 7 represents the activity of catalase in the colonic mucosa and liver of control and experimental rats. The activity of catalase in the colon was markedly decreased on DMH treatment. The catalase activity was significantly ($P \leq 0.05$) elevated in the colonic mucosa of rats fed with XOS in comparison to that of DMH treated control rats. In liver tissues also, the catalase activity decreased on DMH treatment but the values were restored to normal level on XOS supplementation to significant ($P \leq 0.05$) extent.

**Table 5.7:** Effect of XOS supplementation on the catalase activity* of colonic mucosa and liver of normal and DMH administered Wistar rats.

| Group | Catalase (μmol of $H_2O_2$ utilized/ minute/ mg protein) | |
|---|---|---|
| | Colonic mucosa | Liver |
| Control | 46.85± 0.30 | 54.56± 0.92 |
| XOS Control | 48.79± 0.21 | 56.02 ± 0.01 |
| DMH Control | 35.20 ± 0.60 | 36.41 ± 0.84 |
| DMH+ 5% XOS | 38.39± 0.91** | 44.54 ± 0.12** |
| DMH+ 10% XOS | 45.44± 0.42** | 54.15± 0.08** |

* Mean and standard deviations for n=8

** The values in each column are significantly ($P \leq 0.05$) different from the DMH-control group in the same column.



**5. 4. Discussion**

The organotropic colon carcinogen DMH is an alkylating agent widely used to induce benign and malignant neoplasms in the colon of rodents *(Cooper et al., 1978).* DMH is a procarcinogen, requires metabolic activation within the host to become an active carcinogen. The cells at the subcutaneous site do not possess the enzymes capable of reacting with DMH. Hence, subcutaneously administered DMH is released slowly into the circulation, reaches the liver and gets metabolized into various intermediates which can potentially carcinogenic *(Fiala, 1977).*

The induction of colon tumours by DMH is a widely studied experimental model because of a number of factors including the pronounced organotropic properties of the carcinogen *(Weisburger and Fiala, 1983),* the wide variation in tumour response in different rodent strains *(Van Wezel et al., 1999, Moen et al., 1996, Van Wezel et al., 1996, Jacoby et al., 1994),* the dietary induced modulation in tumour incidence *(Kim et al., 1998),* similar distribution of tumour within the human colon and the colon of treated

animals *(Park et al., 1997, Carter et al., 1994)* and the similarity between animal and human pathology *(Chang, 1984)*. Implicit in such work is the assumption that DNA alkylation may also be important in human colon cancer formation *(Esteller et al., 2000, Povey et al., 2000, Jackson et al., 1997)* so that by understanding the mechanisms through which DMH induces colon tumours, new approaches for identifying high risk populations or preventing human colon cancer may be developed. DMH produces predominantly distal colon tumours but at widely varying rates depending upon the strain of the animal *(Moen et al., 1996, Van Wezel et al., 1996, Jacoby et al., 1994, Turusov et al., 1982, Diwan et al., 1977, Evans et al., 1974).* This differential susceptibility has been ascribed to variations in the ability to metabolize DMH *(Delker et al., 1996, Sohn et al., 1985)* or in proliferative responses to the carcinogen *(Deschner et al., 1984).*

The significant reduction in body weights of rats on exposure to DMH was observed in comparison with that of control throughout the experimental period. The reduction in body weight observed in carcinogen treated rats may be an integral part of the carcinogenic process. XOS supplementation increased the body weight in DMH-administered rats. No significant pathological alterations in the major organs (liver, kidney, lung, heart, etc.) were noticed in rats treated with XOS alone, ascertaining absence of any toxicity of XOS. The beneficial effect of XOS supplementation is also clear from the observation that XOS significantly increased the body weight in DMH-administered rats to the normal values.

In the present study, the total cecal weights relative to body weight of both 5% and 10% XOS groups was markedly higher than that of the control and DMH groups. Campbell *et al.* (1997) also found that supplementation with 60 g/kg diet of fructooligosaccharides, oligofructose or XOS for 14 days markedly increased the cecal total and wall weights. The authors suggested that this increase might be caused *via* the normalization of epithelial cell proliferation by SCFA, the metabolites formed from these non-digestible oligosaccharides by colonic microbes. In an *in vivo* study, Frankel *et al.* (1994) documented the trophic effect of SCFA on epithelial cell proliferation in rats. Howard *et al.* (1995) also reported that XOS increase cecal cell density *via* a modest enhancement of

cecal epithelial cell proliferation. Therefore, the production of SCFAs from XOS fermentation may normalize epithelial cell proliferation, which would decrease mucosal atrophy and account for the observed increases in cecal weights.

Several *in vivo* studies demonstrated that diets that supply prebiotic oligosaccharides selectively increase the intestinal tract population of bifidobacteria in animals and humans *(Delzenne and Roberfroid, 1994, Okazaki et al., 1990).* In the present study, XOS fed groups had greater intestinal bifidobacterial populations than the DMH-control group, which are in consistence with the published studies. The higher intestinal bifidobacterial counts in the oligosaccharide groups might be explained by the fact that the oligosaccharides cannot be digested by enzymes in the small intestine and cannot be utilized by most intestinal microflora other than probiotic species, such as bifidobacteria and propionibacteria *(Okazaki et al., 1990, Mitsuoka et al., 1987, Hidaka et al., 1986).* The XOS groups also had lower cecal pH levels than the control and DMH groups, due to an increase in the bifidobacterial population. Bifidobacteria can digest XOS to produce lactate and SCFA such as acetate, butyrate and propionate *(Okazaki et al., 1991, Modler et al., 1990).* The production of SCFA lowers the pH in the intestinal tract and this decrease in colon pH is one of the probable ways for bifidobacterial effect on other colonic bacteria *(Gibson and Roberfroid, 1995, Gibson and Wang, 1994).* Therefore, XOS might act as a source of SCFA to the large bowel, resulting in a lower pH in the intestine. The aforementioned production of SCFA could lower the pH and also normalize epithelial cell proliferation.

Tomomatsu (1994) reported that the effective daily doses of oligosaccharides (pure form) in humans are 3.0 g for fructooligosaccharides and 0.7 g for XOS, indicating that XOS may be more effective than fructooligosaccharides. In a study, comparing the effectiveness of XOS and fructooligosaccharides, results revealed that the XOS fed group had markedly greater colonic wall and cecal wall relative weights and also a greater bifidobacterial population *(Hsu et al., 2004).* The authors concluded that the greater relative weights of colonic wall and cecal wall found in the XOS group may be an indirect result of the higher bifidobacterial population in this group.

Colon carcinogenesis models using DMH or the related azoxymethane, with putative preneoplastic ACF as end-point marker lesions have been used to assess the influence of modulatory factors *(Bird, 1995, McLellan and Bird, 1988).* ACF are readily discernible 'preadenomatous' morphological putative lesions within the colonic mucosa of rodents and even in cancer patients that may contribute to the stepwise progression to colon cancer *(McLellan et al., 1991a, McLellan et al., 1991b).* The formation and growth of ACF are associated with the induction of colon tumors in rats and are influenced by exposure to chemopreventive agents *(Pereira et al., 1994, Pretlow et al., 1992).* Natural compounds that inhibit ACF induced colon carcinogenesis have proved to be protective against colon cancer in rodents *(Tanaka and Mori, 1996).*

Treatment with DMH causes a continuum of morphological changes from normal colonic epithelium to carcinoma. One injection of DMH causes the development of aberrant crypts within 2 weeks *(McLellan and Bird, 1988),* while 6 injections of DMH, 1 per week for 6 weeks typically causes grossly visible adenomas and carcinomas of the colon within 4-6months *(Deschner et al., 1979, Thurnherr et al., 1973).* In the present study, three weekly doses of DMH at 20mg/kg body weight induced the ACF formation. As there is a strong correlation between ACF formation and colon carcinogenesis, the observation amply implies that supplementation by XOS under the conditions of the experiment, can greatly affect the post-initiation stages of colon carcinogenesis by altering the efficacy at which DMH can initiate foci appearance. Supplementation with XOS greatly restored normalcy in the colonic epithelial cells. The ability of XOS to reduce the number of ACF per colon also indicates that the anti-carcinogenic potential of XOS could be mediated through an enhanced repair or remodeling of preneoplastic lesions (*Farber et al., 1976).*

Numerous studies report a high correlation between the number of aberrant crypts and the number of tumors that subsequently develop *(Alabaster et al., 1995, Shivapurkar et al., 1992).* Hsu *et al.* (2004) reported that a supplementation with 60 g/kg diet of XOS and fructooligosaccharides to 6-wk-old male Sprague-Dawley rats for 5 wk decreased the mean number of multicrypt clusters of aberrant crypts (>2 crypts/focus) by 81 and 56%,

respectively. In a different study on the effects of dietary oligofructose and inulin on azoxymethane-induced aberrant crypts in 7-wk-old male F344 rats, Reddy *et al.* (1999) found that ingestion of 100 g/ kg diet of oligofructose and inulin for 7 week decreased the mean number of multicrypt clusters (>2 aberrant crypts/focus) by 23 and 36%, respectively. The present study involved the use of two different levels of XOS to assess the quantitative effects of these prebiotics on the development of ACF as compared to a study by Hsu *et al.* (2004) wherein only one level of XOS is studied. The data suggest that the inhibition of the ACF development in the colon was more effective at 10% XOS than at 5% XOS. It is reported that, the administration of bifidobacteria and *Lactobacillus acidophilus* also decreases tumor incidence and aberrant crypt and ACF counts in the colon *(Koo and Rao, 1991, Goldin and Gorbach, 1980).* It is also reported that the combination of bifidobacteria and oligofructose reduces the incidence of colon cancer in rats treated with a carcinogen *(Gallaher and Khil, 1999).* The current data and the results of earlier studies suggest that either dietary oligosaccharides or consumption of bifidobacteria could decrease the incidence of colon cancer *(Buddington et al., 2002, Kleessen et al., 2001, Gallaher and Khil, 1999).* Bifidobacteria also decrease the formation of toxic fermentation products in the gastrointestinal tract *(Williams et al., 1994, Hidaka et al., 1991, Okazaki et al., 1990),* correlating with the lower ACF counts in the XOS fed-DMH treated rats.

The present study clearly indicates that administration of the procarcinogen DMH and subsequent supplementation of XOS brings about profound alterations in the tissue lipid peroxidation and antioxidant status. DMH, a potent colon-specific carcinogen used in this study, is metabolized in the liver to azoxymethane (a known colon carcinogen), ultimately leading to the generation of methyldiazonium ions and carbonium ions, which are active carcinogenic electrophiles *(Fiala et al., 1987)* whose action gets manifested in the colon. Earlier reports suggested an inverse relationship between the concentrations of lipid peroxides and the rate of cell proliferation, *i.e.* the higher rate of lipid peroxidation in the cells with lower the rate of cell division *(Das, 2002).* Enhanced levels of lipid peroxides along with the lowering of catalase activities indicated an increased oxidative stress during DMH induced malignant alterations *(Oshima et al., 1996).* The present

study also demonstrates enhanced lipid peroxidation in the liver of DMH treated rats. Liver catalase was also significantly decreased in DMH treated rats as compared to the control rats, which in turn, might have contributed to the increased liver lipid peroxidation in DMH treated rats. The results indicate that XOS supplementation to DMH-treated rats markedly restored the liver lipid peroxidation levels to the normal.

On the other hand, the decreased lipid peroxidation in the colonic mucosa reported here is based on the assessment of the levels of TBARS formed during the chain reaction of lipid peroxidation. Earlier studies have shown reduced rates of lipid peroxidation in the tumor tissue of various types of cancer *(Manoj et al., 1999, Tanaka et al., 1998, Tanaka 1997, Cheesman et al., 1986).* Increased cell proliferation is thought to be involved in the pathogenesis of colon cancer. Cancer cells acquire particular characteristics that benefit their proliferation *(Schmelz et al., 2000)* and they tend to proliferate faster when the lipid peroxidation level is low. Therefore, the decreased colon lipid peroxidation observed in DMH-treated rats could be due to increased cell proliferation. Thus, malignant tissues are less susceptible and more resistant to free radical attack and hence lipid peroxidation is less intense *(Nakagami et al., 1990).* The results from the present study correlate with previous findings on malignant cells being better protected than their normal counterparts against free radicals *(Masotti et al., 1998).* In addition to this, the decreased levels of lipid peroxidation in DMH-treated rats may also be due to increased resistance and/or decreased susceptibility of the target organs to free radical attack. XOS supplementation to DMH-treated rats restored the lipid peroxidation levels near to that of the control. Kamaleeswari and Nalini (2006) have previously proved that rats on DMH treatment for a long period showed decreased intestinal, colonic and caecum lipid peroxidation as compared to the control rats. Cancer cells are known to have highly evolved protective mechanisms to prevent lipid peroxidation so that rapid cell proliferation can occur *(Kenneth, 2000).* Similar to this line, several studies have demonstrated that lipid peroxidation decreases significantly in tumor cells and tissues when compared to that in corresponding normal cells and tissues *(Bartoli and Galeotti, 1979).*

Phase II enzymes help to inhibit the formation of electrophiles and catalyze their conversion to inactivate conjugates making them more water soluble and readily excretable from the cell. It is the cellular balance between the Phase I activating enzymes and Phase II detoxifying enzymes that contribute to one's risk of developing cancer *(Wilkinson and Clapper, 1997).* GSTs are a multigene family of proteins functioning as detoxification enzymes by catalyzing the conjugation of potentially mutagenic electrophilic compounds with reduced glutathione. GSTs catalyze the reaction of the compounds with thiol group of GSH, thus neutralize their electrophilic sites and render the product more water soluble *(Habig et al., 1974).* DMH is metabolized to the active carcinogen in the liver through a sequential radical generating mechanism *(Albano et al., 1989)* implying a need for detoxification through antioxidant as well as biotransformation mechanism. Cellular GSH by itself or together with GST can function as a non-critical nucleophile for conjugation reactions and play an important role in the inactivation of electrophilic compounds *(Uhlig and Wendel, 1992).* Therefore, an elevation of GSH level indicates an increase in the systemic ability to detoxify electrophilic compounds including carcinogens.

In the intestinal tract, the induction of the GST isoenzyme has recently been discussed as a possible anticancer mechanism of dietary compounds. In addition, GST has been shown to protect against the damaging of DNA by metabolites of poly-cyclic hydrocarbons or aflatoxin B, both by catalyzing the conjugation of reactive metabolites with glutathione and by covalent binding of reactive species. It is also believed that GST as a membrane–associated phosphoglycoprotein has an important role in the detoxification in the tissues of colon, kidney and liver. The present study also revealed decreased colonic GST activities in the DMH treated rats as compared to control rats. Similar effects have already been reported *(Kamaleeswari and Nalini, 2006).* Tanaka *et al.* (1999) have also shown that azoxymethane (an intermediate formed during DMH metabolism) treatment significantly reduces GST activity. XOS supplementation to DMH treated rats increased the GST enzyme activities in the colonic mucosa as compared to control rats. This may be due to the ability of XOS to contribute to the detoxification of the carcinogen directly or indirectly by the metabolites produced from the same by bacterial fermentation. Thus

the decreased GST levels in colonic mucosa observed in the present study may be used as marker of cell proliferation.

Catalase prevents oxidative hazard by catalyzing the formation of water and oxygen from hydrogen peroxide *(Rajeshkumar and Kuttan, 2003).* The results show decreased levels of liver and colonic mucosal catalase activities in DMH treated rats when compared with control rats. A low level of catalase activity in the cancerous tissue promotes the growth of cancer and its infiltration into the surrounding tissues, which is important for invasion and metastasis *(Janssen et al., 1999).* In the present study, XOS significantly ($P<0.05$) elevated the catalase activities of the colonic mucosa which could be important in inhibiting the DMH induced carcinogenesis.

# CHAPTER: 6

# EFFECTS OF XYLOOLIGOSACCHARIDES ON QUALITY ATTRIBUTES OF *IDLI*, A CEREAL-LEGUME BASED INDIAN TRADITIONAL FOOD

**Overview**

The food industry is constantly looking for economic ways of formulating products with even more desirable textural and organoleptic properties than are currently provided by available functional food ingredients. Prebiotics show both important technological characteristics and interesting nutritional properties. In food formulations, they can significantly improve organoleptic characteristics, upgrading both taste and mouth-feel. Among several of the Indian traditional foods, *idli*, a fermented steamed product with a soft and spongy texture is highly popular and very widely consumed throughout India and is also becoming popular in other countries. From the nutritional and health point, *idli* appears to be an ideal human food for all ages and at all times. Being a cereal-legume based fermented product, it has improved nutritive value as evident from the higher protein efficiency ratio and increased essential amino acid and vitamin contents. The present chapter deals with the utility of XOS obtained from corncob xylan to improve quality attributes of *idli*.

## 6.1. Introduction

The food industry is constantly looking for economic ways of formulating products with more desirable textural and organoleptic properties than are currently provided by available hydrocolloids. Thus, despite the diversity of hydrocolloid functionalities at hand, there is an onus on researchers to investigate new sources of hydrocolloids with more specific functionalities, or to use existing hydrocolloids in innovative ways to improve and optimize the textural and organoleptic properties of food products *(Redgwell and Fischer, 2005).* There is an increasing interest in the application potential of xylan polymers isolated from industrial and field grown crops both in the food and non-food area *(Ebringerova and Heinze, 2000).* The variability in sugar constituents, glycosidic linkages and structure of glycosyl side chains, as well as two reactive hydroxyl groups at the xylose repeating unit of the main chain offer various possibilities for regioselective, chemical and enzymatic modifications and applications. Functionalization creates novel opportunities to exploit the various valuable properties of xylans for previously unconceived applications.

The β-(1,4)-linked D-xylosyl backbone of xylan is hydrolyzed by 1,4-β-D-xylan xylanohydrolase (EC 3.2.1.8) generally known as endoxylanases, producing β-anomeric xylooligosaccharides (XOS). These oligomers are a class of non-digestible food ingredient generally regarded as prebiotics. They beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve host health *(Moure, 2006).* XOS are non cariogenic, stimulate intestinal mineral absorption and does not invoke insulin secretion from the pancreas. XOS exhibit a range of biological activities different from prebiotic effects related to gut modulation including antioxidant activity, blood and skin related effects, antiallergy, antimicrobial, antiinfection and antiinflamatory properties, selective cytotoxic activity, immunomodulatory action, cosmetic and a variety of other properties. As a food ingredient, XOS show favorable technological features, including stability in acidic pH, resistance to heat and ability for offering lower available energy and for achieving significant biological effects at low daily intakes *(Vazquez et al., 2000).*

Polysaccharides and oligosaccharides usually have a strong affinity for water and in the solid state these molecules may have disordered structures which can easily be hydrated. The interaction of water molecules with amorphous zones increases the mobility of molecular chains and hence modifies the swelling and glass transition properties *(Fringant et al., 1995).* Kacurakova *et al.* (1998) hypothesized that glycosidic linkage type and position could play an important role also in the swelling and gelling mechanism of xylans and XOS. Protein-polysaccharide mixtures often show better and more versatile functional properties than the individual components alone *(Schmitt et al., 1998).* The interaction and spatial arrangement of proteins and polysaccharides confer the specific structure and texture of food material. As a consequence, novel hydration, structure forming and surface properties of the mixtures lend themselves to applications in food formulations as fat replacers, texturizers and stabilizers of dispersed systems *(Redgwell and Fischer, 2005).*

From a nutritional point of view, it is advantageous to consume mixtures of cereals and legumes because this results in an improved balance of carbohydrates and proteins as well as a better balance of dietary essential amino acids *(Nout, 1993).* Acidified and leavened foods constitute an interesting group of products made from cereal-legume mixtures. The diversity of the population of India has given rise to a large number of traditional fermented foods which have been extensively reviewed *(Soni and Sandhu 1990).* Among several of the Indian traditional foods, *idli*, a fermented steamed product with a soft and spongy texture is highly popular and very widely consumed throughout India and is also becoming popular in other countries. An early history on the use of this traditional food dates back to 1100 A.D. *(Ramakrishnan, 1979).* From the nutritional and health point, *idli* appears to be an ideal human food for all ages and at all times. Being a cereal-legume based fermented product, it has an improved nutritive value as evident from the higher protein efficiency ratio (PER) and increased essential amino acid and vitamin contents *(Desikachar et al., 1960). Idli* preparation in the conventional manner takes at least 18 h. Although instant *idli* pre-mixes are available, these do not possess the characteristic texture and also lack the typical fermented aroma.

*Idli* fermentation is mixed auto fermentation as the organisms present in the ingredients as well as the environment determine the nature of microflora involved *(Desikachar et al., 1960).* Black gram is the main ingredient responsible for the characteristic texture of *idli*. The surface-active proteins and polysaccharides of black gram are well conditioned to retain a large volume of gases to give soft and fluffy texture *(Susheelamma and Rao, 1978).* It is observed that, after a certain period of fermentation the batter starts collapsing and with further days of storage, there is whey separation, resulting in *idlis* with a very hard texture. On storage at refrigerated conditions, the batter without stabilizers or preservatives has a limited shelf life of few days after which there is collapse in batter volume causing whey separation and consequently very hard *idlis*.

The stabilization of foam is very important with respect to *idli* texture. The polysaccharide in black gram is characterized as arabinogalactan, which stabilizes the soft porous texture of *idli*. The arabinogalactan and guar gum are speculated to have similarity in their primary structure *(Susheelamma and Rao, 1978).* An important way to stabilize the foam is through the formation of a rigid system by means of crystallization, denaturation or gelatinization of the continuous phase. Hydrocolloids are very effective functional agents in making stable foam products. They act as whipping agents to permit aeration, formation of foam and then stabilize the interfacial film and thus prevent the leakage of air and collapse of the structure *(Glicksman, 1986).* Addition of 0.1% pre-swollen xanthan to instant *idli* batter is known to give desirable *idli* texture *(Thakur et al., 1995).*

In the present investigation, the utility of XOS obtained from corncob xylan to improve the quality attributes of *idli* has been studied.

## 6. 2. Materials and methods

### 6.2.1. Materials

All chemicals were of analytical grade. Standards of xylose, xylobiose, arabinose, glucose and birch wood xylan were from Sigma-Aldrich (USA). Corncobs were obtained

from a local maize field in Mysore, Karnataka, India and were sorted, dried at 40 °C for 24 h, milled (60-80 mesh) and stored in polycarbonate containers. Parboiled rice and black gram dhal were procured from local market.

### 6.2.2. Milling and alkali pretreatment of corncob

A two stage powdering of corncob has been carried out to get a powder of 60-80 mesh size as explained under section 3.1.2.2. This powder was used as the inducer for xylanase production and also for various pretreatment studies and XOS production.

### 6.2.3. Microorganism and endoxylanase production

*Aspergillus oryzae* MTCC 5154, an isolate from our lab was maintained on potato dextrose agar slants at 4 °C. Exactly 0.5 ml of spore suspension containing $6x10^6$ spores/ml from a 5 day old slant was inoculated into 50 ml fermentation medium containing 3% native corncob powder, 0.5% yeast extract, 0.1% $NaNO_3$, 0.1% $KH_2PO_4$, 0.1% peptone and 0.03% $MgSO_4.7H_2O$ with an initial pH of 5.5. The fermentation parameters were maintained as explained under the section 3.1.2.5.

### 6.2.4. Endoxylanase Assay

Endoxylanase activity of culture fluid was assayed using standard birch wood xylan as the substrate *(Jiang et al., 2005)* as explained in the section 3.1.2.9.1.

### 6.2.5. XOS production

XOS were produced from alkali pretreated corncob by the action of *A. oryzae* endoxylanase as explained in the section 4.2.2.5. XOS were estimated by HPLC method *(Jeong et al., 1998).* The products of hydrolysis were analyzed by HPLC equipped with a refractive index detector using an Aminopropyl column. The XOS formed were quantified by comparing the peak area of XOS with that of standard xylose and is expressed as mg/ ml of hydrolyzate. The XOS obtained were a mixture of 73.5% of xylobiose, 13.2 % other oligosaccharides and 13.3% xylose residues.

### 6.2.6. Preparation of *idli* batter and addition of XOS

*Idli* batter was prepared with parboiled rice and black gram dhal in 3:1 proportions (w/w). The ingredients, black gram dhal and rice, were washed three times with water to remove adhering dirt and dust particles from the surface, soaked for 3 h and ground in a kitchen mixer grinder separately. Rice was ground coarsely and black gram finely to a smooth batter and both were mixed together with NaCl (1.0% w/v). The batter was allowed to ferment for 4, 6, 12 and 18 h at 29±1°C with or without XOS.

XOS were added at three levels (0.2, 0.4 and 0.6% w/v). The levels of addition of XOS were decided on the basis of their level of usage in different foods and also considering their Recommended Daily Allowance. Known concentrations of XOS were mixed well with the freshly prepared batter and homogenized with a hand mixer to facilitate uniform mixing of XOS.

### 6.2.7. Physicochemical parameters of *idli* batter

The fermented *idli* batter was observed for certain physicochemical parameters like specific gravity, pH and percent total acidity. The specific gravity of idli batter was determined by dividing the weight of a glass beaker (200 ml) filled with batter to the weight of the same beaker filled with water *(Kaur et al., 2000).* The pH was measured with a precalibrated pH meter. For acidity, total percentage of lactic acid was determined by titration against NaOH of known normality *(Ranganna, 1977).*

### 6.2.8. Microbiological analysis of idli batter

Fermented *idli* batters with or without XOS were used for plating to determine lactic acid bacteria, total plate count and yeast and molds. The microbial load at 4, 6, 12 and 18 h fermentation was measured by suspending the respective batter in 0.5% sterile saline and plating it out at appropriate dilutions. de Man, Rogos and Sharpe (MRS) media, potato dextrose agar (PDA) and nutrient agar (NA) media were used for enumerating the number of lactic acid bacteria, yeast and molds, and mesophilic aerobes respectively employing spread plate and pour plate method. Colonies were counted after 24 h

incubation at 30 °C (PDA) and 37 °C (NA and MRS) and the results were expressed as cfu/g of batter.

### 6.2.9. Preparation and evaluation of *idli*

The *idli* cooking stand was placed in a pressure cooker containing a small quantity of water. The batter was poured into molds and steamed at 100 °C for 10 min *(Singh et al. 1995).*

#### 6.2.9.1. Texture

*Idli* has a circular shape of approximately 7-10 cm diameter (depending on the mold size), flat with lower and upper surface bulging, so that the product is thick at the center (2-3 cm) and tapering towards periphery. Texture of the *idlis* was analyzed on Universal Texture Measuring System (LLOYD Instruments, LR 5K, England). The shear test was conducted in the centre were the average thickness is 2-3 cm using knife probe in the normal mode at 100 mm/min at a load of 50 N. Texture was expressed as the load in grams required to cut the product *(Nisha et al., 2005).*

#### 6.2.9.2. Colour

Colour of the *idlis* was measured using Hunter lab colour measuring system (Labscan XE, Hunter Ass. Lab., Virginia, USA), in terms of Hunter L (lightness, ranging 0-100 indicating black to white), a (+a; redness and -a; greenness) and b (+b; yellowness and -b; blueness) *(Krishnamurthy and Kantha, 2005).*

#### 6.2.9.3. Moisture content

The average initial moisture content of *idli* was determined using AOAC method *(AOAC, 1990).*

#### 6.2.9.4. Sensory evaluation of *idli*

Acceptability of *idlis* made from the batter with or without XOS and with varying fermentation time was determined by sensory evaluation. *Idli* samples were coded and presented to 20 panel members for sensory scoring. The panel members, who were

familiar with sensory analysis techniques, were research scholars of Central Food Technological Research Institute, Mysore, India. There were five samples: conventional *idli* (18 h fermentation) and *idli* made from batter with different concentrations of XOS (0, 0.2, 0.4 and 0.6% w/v) after 6 h fermentation. Samples were scored for appearance, color, texture, flavor, taste and overall quality according to numerical scoring system. A score card was developed based on quality scaling with a maximum score of 10 *(ISI, 1972).* The quality grade description indicative of scores given were excellent (9-10), very good (7-8), good (5-6), fair (3-4) and poor (1-2). The sensory analysis data were subjected to statistical analysis. Mean and standard deviation were individually calculated for scores obtained for all quality attributes of each product.

## 6. 3. Results

### 6.3.1. Effects of addition of XOS on the specific gravity of batter during fermentation

The coefficients obtained for specific gravity of *idli* batter are summarized in table 6.1. The specific gravity of *idli* batter decreased with an increase in fermentation time. In the conventional way of *idli* batter fermentation for 18 h, the specific gravity was found to be 0.632±0.015 and almost the same range of specific gravity was reached at 12 h and 6 h of fermentation in the presence of 0.2 and 0.4% XOS respectively.

**Table: 6.1.** Specific gravity* of *idli* batter with or without XOS (0, 0.2, 0.4 and 0.6% w/v) subjected at different fermentation times (4, 6, 12 and 18 h)

| Concentration of XOS | Specific gravity of *idli* batter at various fermentation time | | | |
|---|---|---|---|---|
| (% w/v) | 4 h | 6 h | 12 h | 18 h |
| 0 | 0.832±0.001 | 0.751±0.003 | 0.717±0.007 | 0.632±0.015 |
| 0.2 | 0.763±1.004 | 0.710±0.006 | 0.639±0.006 | 0.578±0.013 |
| 0.4 | 0.723±0.003 | 0.693±0.015 | 0.608±0.008 | 0.546±0.007 |
| 0.6 | 0.718±0.003 | 0.79±0.006 | 0.598±0.007 | 0.542±0.008 |

*Values are mean and standard deviations for n =3

### 6.3.2. Effects of addition of XOS on the pH and total acidity of batter during fermentation

Table 6.2 summarizes the pH and percent total acidity of *idli* batter with various concentrations of XOS at different fermentation time. A drop in pH of batter from 6.0±0.16 to 4.18±0.02 and 4.57±0.08 was observed at 18 h fermentation with or without XOS respectively. The percent total acidity of *idli* batter at different fermentation times was in the range of 0.468±0.016 and 1.116±0.016 with or without XOS, respectively.

### 6.3.3. Effects of addition of XOS on the microbial profile of fermented batter

The microbial loads of batter with or without XOS at various fermentation times were determined by counting the colonies developed in specific media after 24 h of incubation and the results are presented in table 6.3. The number of mesophilic aerobes increased in all cases; however, no significant difference was observed between *idli* made from batter containing XOS and *idli* made by the conventional method. The number of lactic acid bacteria in batter without XOS at 4, 6, 12 and 18 h of fermentation was 8.99±0.115, 9.41±0.017, 9.44±0.036 and 9.46±0.060 cfu/g of batter respectively. In the case of conventional *idli* batter without XOS, the number of yeasts and mould were found to be increased with an increase in time. However, their number is found to be decreased when XOS were used in the batter.

**Table: 6.2.** pH and percent total acidity* of *idli* batter with or without XOS (0, 0.2, 0.4 and 0.6% w/v) subjected at different fermentation times (4, 6, 12 and 18 h)

| Concentration of XOS (% w/v) | pH and percent total acidity of *idli* batter at various fermentation time | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | pH | | | | Total acidity | | | |
| | 4 h | 6 h | 12 h | 18 h | 4 h | 6 h | 12 h | 18 h |
| 0.0 | 4.91±0.02 | 4.83±0.01 | 4.68±0.05 | 4.57±0.08 | 0.456±0.021 | 0.516±0.007 | 0.648±0.006 | 0.882±0.021 |
| 0.2 | 4.86±0.06 | 4.74±0.02 | 4.56±0.04 | 4.34±0.04 | 0.492±0.021 | 0.732±0.008 | 0.984±0.005 | 1.020±0.008 |
| 0.4 | 4.84±0.04 | 4.61±0.03 | 4.49±0.02 | 4.21±0.02 | 0.564±0.011 | 0.846±0.033 | 1.056±0.019 | 1.080±0.004 |
| 0.6 | 4.83±0.05 | 4.70±0.20 | 4.54±0.03 | 4.18±0.02 | 0.612± 0.029 | 0.960±0.004 | 1.080±0.046 | 1.104±0.006 |

*Values are mean and standard deviations for n =3

**Table: 6.3.** Microbial loads* of *idli* batter with or without XOS (0, 0.2, 0.4 and 0.6% w/v) subjected at different fermentation times (4, 6, 12 and 18 h)

| Fermentation time (h) | XOS (% w/v) | cfu/g of batter | | |
|---|---|---|---|---|
| | | Lactic acid bacteria | Yeast and moulds | Mesophiles |
| 4 | 0.0 | 8.99±0.115 | 8.20±0.017 | 8.25±0.010 |
| | 0.2 | 9.34±0.043 | 8.14±0.026 | 8.20±0.036 |
| | 0.4 | 9.36±0.036 | 8.08±0.026 | 8.17±0.026 |
| | 0.6 | 9.40±0.010 | 7.90± 0.400 | 8.20±0.020 |
| 6 | 0.0 | 9.41±0.017 | 8.23±0.043 | 8.34±0.040 |
| | 0.2 | 9.68±0.026 | 7.97±0.105 | 8.33±0.034 |
| | 0.4 | 9.88±0.080 | 7.84±0.52 | 8.36±0.026 |
| | 0.6 | 9.94±0.034 | 7.79±0.036 | 8.38±0.030 |
| 12 | 0.0 | 9.44±0.036 | 8.23±0.026 | 8.55±0.045 |
| | 0.2 | 9.69±0.060 | 7.96±0.026 | 8.52±0.026 |
| | 0.4 | 9.89±0.030 | 7.83±0.030 | 8.50±0.046 |
| | 0.6 | 9.95±0.026 | 7.78±0.026 | 8.52±0.020 |
| 18 | 0.0 | 9.46±0.060 | 8.26±0.043 | 8.49±0.026 |
| | 0.2 | 9.70±0.020 | 7.96±0.026 | 8.53±0.026 |
| | 0.4 | 9.90±0.040 | 7.85±0.036 | 8.49±0.043 |
| | 0.6 | 9.95±0.034 | 7.80±0.095 | 8.49±0.010 |

*Values are mean and standard deviations for n =3

### 6.3.4. Effects of addition of XOS on the textural quality of *idli*

The shear values as given in table 6.4 indicated a lower range of values for *idlis* prepared using batter supplemented with XOS. It is observed that *idlis* prepared conventionally exhibited a normal texture (4.67±0.05 N). When 0.2, 0.4 and 0.6% XOS were used in the batter, *idlis* with a more spongy texture (4.27±0.01, 4.18±0.03 and 4.06±0.03 N,

respectively) were obtained within a short span of 6 h fermentation and accounted for a softer texture with the increase in fermentation time.

**Table: 6.4.** Textural properties (shear*) of *idli* prepared from batter with or without XOS (0, 0.2, 0.4 and 0.6% w/v) subjected at different fermentation times (4, 6, 12 and 18 h)

| Concentration of XOS (% w/v) | Shear values (N) of *idli* prepared from batter at various fermentation time | | | |
|---|---|---|---|---|
| | 4 h | 6 h | 12 h | 18 h |
| 0.0 | 5.58±0.03 | 5.26±0.05 | 5.15±0.05 | 4.67±0.05 |
| 0.2 | 4.73±0.03 | 4.27±0.01 | 4.27±0.02 | 4.28±0.03 |
| 0.4 | 4.34±0.02 | 4.18±0.03 | 3.96±0.01 | 3.87±0.04 |
| 0.6 | 4.22±0.03 | 4.06±0.03 | 3.95±0.04 | 3.73±0.03 |

*Values are mean and standard deviations for n =3

### 6.3.5. Effects of addition of XOS on the colour characteristics of *idli*

The colour values of *idlis* (Hunter L, a and b) are given in table 6.5. The colour was measured in terms of Hunter L (lightness ranging from 0 to 100 indicating black to white), a (+a: redness and –a: greenness) and b (+b: yellowness and –b: blueness). The L, a and b values for *idli* prepared in the conventional way (18 h fermentation) were 66.56±0.30and -1.17±0.19 and 12.11±0.14, respectively and were taken as the control readings. In the conventional *idli* batter fermentation, the L value was reduced from 69.94±0.26 to 66.56±0.30 from 4 h to 18 h fermentation. A similar reduction in the L values was also observed in the case of batter with XOS which indicated a reduction in the whiteness of *idli*. Similarly, the Hunter b value, which indicates the yellowness increased during fermentation irrespective of the presence or absence of XOS in the batter. It was observed that b value of idlis made from batter supplemented with 0.2, 0.4 and 0.6% XOS showed less value (10.59±0.68, 11.67±1.29 and 11.80±0.91) compared to that of *idli* prepared from batter without XOS (12.11±0.14) indicated a slight yellowness of the finished product.

**Table: 6.5.** Colour properties* (measured as Hunter L, a and b) of *idli* prepared from batter with or without XOS (0, 0.2, 0.4 and 0.6% w/v) subjected at different fermentation times (4, 6, 12 and 18 h)

| Fermentation time (h) | Concentration of XOS (% w/v) | Visual colour Parameters of *idli* | | |
|---|---|---|---|---|
| | | L | a | b |
| 4 | 0.0 | 69.94±0.26 | -1.04±0.18 | 10.88±0.10 |
| | 0.2 | 70.92±0.24 | -1.09±0.21 | 11.29±0.48 |
| | 0.4 | 69.39±0.71 | -1.00±0.14 | 12.00±0.47 |
| | 0.6 | 68.21±2.08 | -0.82±0.40 | 11.96±0.37 |
| 6 | 0.0 | 71.06±1.15 | -1.74±0.07 | 9.96±0.67 |
| | 0.2 | 69.90±1.43 | -1.62±0.17 | 10.59±0.68 |
| | 0.4 | 69.25±1.17 | -1.63±0.17 | 11.67±1.29 |
| | 0.6 | 68.24±0.79 | -1.44±0.21 | 11.80±0.91 |
| 12 | 0.0 | 67.63±1.78 | -0.96±0.17 | 10.04±0.27 |
| | 0.2 | 66.51±2.02 | -0.93±0.11 | 10.97±0.54 |
| | 0.4 | 66.31±0.44 | -0.95±0.13 | 11.72±0.94 |
| | 0.6 | 64.71±0.87 | -0.80±0.19 | 11.96±0.76 |
| 18 | 0.0 | 66.56±0.30 | -1.17±0.19 | 12.11±0.14 |
| | 0.2 | 63.20±0.90 | -0.90±0.39 | 13.01±0.14 |
| | 0.4 | 62.03±0.75 | -0.49±0.41 | 14.69±1.34 |
| | 0.6 | 62.68±0.52 | -0.62±0.35 | 14.14±0.74 |

*Values are mean and standard deviations for n =3

### 6.3.6. Effects of addition of XOS on the moisture content of *idli*

The *idli* made in the conventional way (18 h fermentation) exhibited a moisture content of 66.38±0.11%. It is clear from the table 6.6 that the moisture content slightly reduced with an increase in fermentation time.

**Table: 6.6.** Moisture content* of *idli* prepared from batter with or without XOS (0, 0.2, 0.4 and 0.6% w/v) subjected at different fermentation times (4, 6, 12 and 18 h)

| Concentration of XOS (% w/v) | Moisture content (%) of *idli* prepared from batter at various fermentation time | | | |
|---|---|---|---|---|
| | 4 h | 6 h | 12 h | 18 h |
| 0.0 | 67.66±0.08 | 67.34±0.12 | 66.30±0.07 | 66.38±0.11 |
| 0.2 | 67.67±0.11 | 68.57±0.15 | 68.24±0.07 | 66.76±0.05 |
| 0.4 | 69.83±0.16 | 68.98±0.22 | 67.91±0.04 | 67.79±0.80 |
| 0.6 | 69.87±0.09 | 68.97±0.09 | 68.17±0.07 | 67.98±0.04 |

*Values are mean and standard deviations for n =3

### 6.3.7. Effects of addition of XOS on the sensory attributes of *idli*

The sensory analysis of control *idlis* and *idlis* made from batter with or without XOS have been carried out on a 10-point hedonic scale and graded as excellent (8-10), very good (points 6-7.9), good (points 4-5.9), fair (points 2-3.9) and poor (points 1-1.9) and mean sensory scores and standard deviation obtained for various quality parameters of *idlis* are shown in the table 6.7. *Idlis* made with batter supplemented with XOS after 6 h of fermentation was selected for sensory studies, because the addition of XOS reduced a normal fermentation time of 18 h to 6 h to get *idlis* with desired quality attributes.

**Table: 6.7.** Sensory attributes* of *idli* prepared from batter with or without XOS (0, 0.2, 0.4 and 0.6% w/v) subjected at different fermentation times (4, 6, 12 and 18 h)

| *Idli* sample | Fermentation time (h) | Concentration of XOS (% w/v) | Sensory parameters of *idli* | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Appearance | Colour | Texture | Flavor | Taste | Overall quality |
| Conventional | 18 | 0 | 5.45±1.79 | 5.55±2.18 | 5.15±1.78 | 4.75±2.06 | 5.35±1.78 | 5.50±1.57 |
| Present study | 6 | 0 | 5.9±1.86 | 6.10±1.89 | 5.50±2.18 | 4.75±2.40 | 4.95±1.19 | 5.25± 0.65 |
| Present study | 6 | 0.2 | 6.25±1.51 | 5.70±1.75 | 6.05±1.57 | 5.70±2.25 | 5.80±1.64 | 5.90±1.87 |
| Present study | 6 | 0.4 | 6.35±2.17 | 6.55±1.66 | 7.25±1.68 | 5.65±2.30 | 5.65±2.45 | 7.10±1.68 |
| Present study | 6 | 0.6 | 5.4±2.21 | 4.55±2.45 | 5.95±2.08 | 5.30±2.02 | 5.20±2.19 | 5.30±1.94 |

*Values are mean and standard deviations of response of 20 panelists and are indicative of following grades- very good (7-7.9), good (5-6.9) and fair (3-4.9).

**6.4. Discussion**

Specific gravity is used as an index to evaluate foam formation of *idli* batter that occurs during fermentation due to microbial activity. Formation of foam during batter fermentation is essential in the preparation of *idli*. The foam consists of gas (air) droplets encapsulated in a liquid film containing soluble proteins *(Kaur et al., 2000).* The specific gravity of *idli* batter decreased with an increase in fermentation time. Similar effects of fermentation time on specific gravity have been reported earlier *(Padhye and Salunkhe 1978, Steinkraus et al., 1967).* The addition of XOS to *idli* batter considerably reduced the specific gravity during fermentation and the effect was more significant as the concentration of XOS increased from 0.2% to 0.4%.

The decrease in specific gravity with the increase in fermentation time corroborated well the decrease in pH. This indicated an enhanced microbial lactic acid metabolism triggered by XOS during *idli* batter fermentation which resulted in the formation of more gas and acids. With the increase in concentration of XOS and fermentation time, the foam stability improved as evident from a decreased specific gravity of *idli* batter. This effect on foam stability might be attributed to the effect of pH on net charge and conformation of proteins *(Kinsella, 1981).* The results also showed that a further increase in the concentration of XOS at 0.6% did not result in any significant change in specific gravity and the value was similar to specific gravity obtained at 0.4% XOS level.

The change in pH and acidity reflects the activity of lactic acid bacteria in the *idli* batter during fermentation. The results indicated that fermentation time and concentration of XOS had the most pronounced effect on pH of *idli* batter. A drop in pH from 6.0 to 4.3-5.5 during 20-24 h *idli* batter fermentation has been observed earlier *(Reddy and Salunkhe, 1980, Steinkraus et al., 1967).* Similar results were obtained in the present study where the pH dropped to 4.18±0.02 and 4.57±0.08 at 18 h fermentation with or without XOS respectively.

It was observed that in *idli* batter supplemented with XOS, the fermentation was rapid as evident from a significant reduction in pH in comparison to conventional way of batter fermentation. It was also evident that the reduction in pH was more prominent with the increase in XOS concentration. This could be related to the prebiotic activity of XOS, which might selectively increase the growth of lactic acid bacteria and hence a resultant increase in acid production.

The increase in acid level would enhance the proliferation of *Saccharomyces* and thereby an increased production of carbon dioxide, which leaven the batter. Black gram soaked in water has a higher concentration of soluble nutrients to support the growth of lactic acid bacteria. The role of lactic acid bacteria is to reduce the pH of the batter to an optimum level (4.4-4.7) for yeast activity *(Soni and Sandhu, 1990).* The present study clearly indicated that the use of XOS in addition to the soluble nutrients from black gram could considerably reduce the fermentation time. The result also indicated that an optimum value of acidity of batter for facilitating the growth of yeast was achieved within a short span of 6 h fermentation when XOS either at 0.4 or 0.6% were added to the batter. However, with the increase in fermentation time, the pH was reduced to 4.3 and total acidity increased to 1.0, at higher concentration of XOS. This adversely affected the growth of yeast as discussed further.

*Idli* is an auto-fermented food and no inoculum is added generally for fermentation. This is because the essential microorganisms have been found to be naturally present in the ingredients. When the product is made daily, it is often the practice of adding a bit of freshly fermented batter (‘backslop’) to the newly ground one. It has been reported that *Leuconostoc mesenteroides* is the microorganism essential for leavening of the batter and also responsible, along with *Enterococus faecalis,* for acid production in *idli (Mukherjee et al., 1965).* The major functions of the fermentation include the leavening of the batter and the improvement of taste and nutritional value of *idli*. *L. mesenteroides* is the main strain responsible for the production of $CO_2$ which results in about 2-3 times increase in the original volume of batter *(Soni and Sandhu, 1989).* The number of mesophilic

aerobes increased in all cases, however no significant differences were observed between *idli* made from batter with or without XOS.

Soni and Sandhu (1989) reported the role of *Lactobacillus fermentum* and *Pediococcus cerevisiae* in addition to the aforementioned bacteria in *idli* batter fermentation. Certain oligosaccharides such as raffinose, stachyose and verbascose are prevalent in legumes and are associated with abdominal distention and flatulence. Lactic acid fermentation reduces these flatulence factors. Cereals and legumes have restricted bio-availability of nutrients as a result of the presence of anti-nutritional factors such as phytates and polyphenols (tannins). Lactic acid bacteria are able to reduce their levels and it has been demonstrated that, this results in improved bio-availability of minerals and starch, as well as increased PER *(Nout and Sarkar, 1999).* The number of lactic acid bacteria was found to increase considerably in the case of batter supplemented with XOS. This could be due to the bifidogenic effects of XOS. This corroborated well with the reduction in the pH, increase in the total acidity of batter and an increase in the number of lactic acid bacteria with XOS as a function of fermentation time. It was also observed that the total lactic acid bacterial count (cfu/g) was markedly higher at 6 h of fermentation.

The slightly acidic environment favors the growth and activity of yeasts *(Venkatasubbaiah et al, 1985).* The principal yeasts in the fermenting *idli* batter are *S. cerevisiae*, *Debaromyces hansenii*, *Hansenula anomala*, *Torulopsis candida* and *Trichosporon beigelii*. In the case of conventional *idli* batter without XOS, the number of yeasts and mould were found to be increased with an increase in time. However, their number was decreased when XOS were used in the batter, indicating that lowering of pH below 4.4 inhibited the growth of moulds and yeasts.

Soft texture of *idli* is a desirable quality. The shear values indicated a lower range of values for *idlis* prepared using batter supplemented with XOS. This also confirmed the observation that the presence of these oligosaccharides increased the lactic acid bacteria, thereby an enhanced fermentation and a softer product. The results obtained are correlated with the changes in specific gravity of batter. Similar results on improvement

in the textural quality by the addition of various carbohydrate hydrocolloids to the batter have been reported *(Nisha et al., 2005).* The soft texture of *idli* is also attributed to the higher retention of its moisture content as explained further.

The colour of idli was measured in terms of Hunter L (lightness ranging from 0 to 100 indicating black to white), a (+a: redness and –a: greenness) and b (+b: yellowness and –b: blueness). A decrease in L value indicated a decrease in whiteness of the *idli* prepared from batter with or without XOS. XOS containing hydrolyzate which was obtained from the enzymatic hydrolysis of corncob xylan was golden brown in colour and hence it imparted an off-white colour to the *idli* and the intensity increased with an increase in concentration. In the conventional *idli* batter fermentation for 18 h, the L value was reduced as fermentation time increased. A similar reduction in the L values was also observed in the case of batter with XOS which indicated a reduction in the whiteness of *idli*. However, sensory analysis showed that because of the lightness values of *idli* prepared from batter with XOS the panelist rated it as acceptable. Similarly, the Hunter b value, which indicates the yellowness increased during fermentation irrespective of the presence or absence of XOS in the batter. However, the increase in the yellowness was more prominent with an increase in XOS concentration.

The water absorption property of hydrocolloids has been attributed to the presence of hydroxyl groups in the hydrocolloid structure, which allows more water interactions through hydrogen bonding *(Guarda et al., 2004, Friend et al., 1993).* The moisture content of *idli* had a positive impact on its texture and it mostly depends on the carbohydrate constituents of the batter. It was clear that the moisture content slightly reduced with an increase in fermentation time. It was evident that XOS could result in an increase the moisture content of the *idlis* which could be attributed to its hydration property. It was also found that with an increase in XOS concentration from 0.2 to 0.4% the water holding capacity also increased. On the other hand, the increase in moisture content of *idli* was less significant at higher concentrations of XOS (0.6%). Similarly, the softening effect of hydrocolloids on the parottas, due to an increase in the moisture has been reported *(Smitha et al., 2008).*

The *idli* made from batter without XOS after 6 h and 18 h (conventional) fermentation scored 5.90±1.86 and 5.45±1.79 respectively for the quality of appearance representing a quality grade of 'good'. The corresponding ranges of scores 6.25±1.51 and 6.35±2.17 for *idlis* made from batter with 0.2 and 0.4% XOS represented a quality grade of 'very good'. On the contrary, a higher concentration of XOS (0.6%) reduced the acceptability to a quality grade of 'good'. The scores for color were 6.10±1.89 and 5.5±2.18 for control *idlis* (6h and 18 h fermentation without XOS) and this corresponded to 'very good' and 'good' grade respectively. On the other hand, *idli* made from batter with 0.2 and 0.4% XOS got a score of 5.70± 1.75 and 6.55±1.66 respectively and indicated that the former comes under the quality grade of 'good' and the latter in the grade of 'very good'. In line with appearance, *idlis* made from batter with 0.6% XOS scored less (4.55±2.45) for colour in comparison with that of supplemented with 0.2 and 0.4% XOS. Because the *idli* is generally associated with white or off-white color, products with darker color scored less. This was also evident from Hunter b values of the *idli* made from batter with 0.6% XOS wherein a higher yellowness value was obtained.

The texture quality increased considerably with an increase in XOS concentration from 0 to 0.4%. The scores for texture were 5.50±2.18 and 5.15±1.78 for control *idlis* (6h and 18 h fermentation without XOS) and this corresponded to 'good' quality grade. On the other hand *idli* made from batter with 0.2 and 0.4% XOS scored 6.05±1.57 and 7.25±1.68 respectively which corresponded to the quality grade of 'very good'. Similar observations were made with instrument analysis where it was found that with an increase in XOS concentration from 0-0.4%, the shear value decreased considerably indicating a more spongy texture.

The sensory scores for flavor of *idlis* made from batter containing 0.2 and 0.4% XOS were 5.7±2.25 and 5.65±2.30 and graded as good, whereas those made from batter without XOS (6 h and 18 h fermentation) scored slightly less compared to others. However, XOS at 0.6% level imparted a woody flavor to the *idli* and hence, scored less (5.30±2.02). For the taste, all the *idlis* were graded as 'good'. The overall quality of the

conventional *idli* was 5.50±1.57 and was graded as good. *Idli* made from batter containing 0.6% XOS scored a slightly less value (5.30±1.94) compared with control. On the other hand, 0.2 and 0.4% resulted in an increased overall quality score of 5.90±1.87 and 7.10±1.68 respectively and were graded as 'good' and 'very good' category. The instrumental and sensory analysis of various *idli*, showed that *idli* made from batter containing 0.4% XOS has supreme quality attributes even at 6 h of batter fermentation.

# CHAPTER: 7

# SUMMARY AND CONCLUSION

**Summary and conclusion**

The science of prebiotics has come a long way since initiation of the concept in 1995. Concomitantly, new product developments have also moved at a rapid pace. This thesis titled 'Bioactive xylooligosaccharides from corncob: Enzymatic production and applications' has attempted to bridge certain gaps in the field of XOS as a promising prebiotic. The important observations made in this study are summarized as follows.

- All the fungal species evaluated produced xylanases, among which *Aspergillus oryzae* MTCC 5154 showed significant titers of endoxylanases. The activity was maximum at 96 h of cultivation. Alkaline pH was found to be suitable for the production of high titers of endoxylanase.
- The extracellular CMCase activity of *Aspergillus oryzae* at 96 h of fermentation was negligible which has an important bearing for use of LCMs as substrates for XOS production.
- *Aspergillus oryzae* produced higher titers of xylanases when powdered corncob was used as the substrate in comparison to that of birch wood or oat spelt xylan. This could be attributed to the presence of soluble fragments of xylooligomers in the corncob, which could have induced the enhanced xylanase production.
- The results of XOS production from birch wood xylan using corncob-induced endoxylanase from *Aspergillus oryzae* indicated that the major product formed was xylobiose which can be further used for food applications.
- The mass spectra of the major fraction obtained from XOS produced by corncob-induced endoxylanase using birch wood xylan as substrate showed the presence of a disaccharide of xylose and 4-*O*-methyl-α-D-glucuronic acid, in addition to the xylobiose. The $^{13}$C-NMR spectrometry of purified disaccharide confirmed this result.
- A comparison was made between the nucleotide sequences of the exon 1 and 2 of XYL gene of *Aspergillus oryzae* MTCC 5154 and the reported nucleotide sequences for xylanases. The results showed that exon 1 of *Aspergillus oryzae* MTCC 5154 is 99% homologous to other *Aspergillus oryzae* strains reported. At

the same time, it showed only 85% similarity with *Aspergillus fumigatus*. Similarly, exon 2 showed 97% homology with that of other *Aspergillus oryzae* strains reported. It showed only 77% similarity with *Aspergillus terreus*. The results clearly indicate that the xylanase gene in *Aspergillus* species, especially in *Aspergillus oryzae* is conserved to a greater extent.

- Evaluation of different pretreatment methods showed that maximum XOS was obtained when alkali pretreated corncob was used as the substrate.
- The electron micrographs of lignin-saccharide complex of pretreated corncob indicated that the alkali pretreatment resulted in the separation of xylan from lignin making it more accessible for enzymatic hydrolysis.
- The maximum production of XOS from pretreated corncob at a substrate concentration of 6% (w/v) using corncob-induced xylanase was observed at pH 5.4 which is in accordance with the literature reports wherein the optimum pH of most of the fungal xylanases is reported to range from 5.0-6.0.
- The results also indicate a distinct effect of temperature on XOS production. As indicated by the results, a maximum XOS production from pretreated corncob was observed at 50 °C, which is the optimum temperature for endoxylanases of most of the fungi.
- The present study has a distinct advantage in terms of the shorter reaction period of reaction for obtaining 81±3.9% of XOS from pretreated corncob corresponding to 10.2±0.14 mg/ml in the hydrolyzate at 14 h of reaction, in comparison to 24-36 h of reaction period reported in literature.
- At 14 h of reaction, the percentage xylobiose was about 73.5 and is considerably high in comparison with the literature reports. Since the production of high content xylobiose is a time consuming and expensive process, the leads obtained from the present investigation can further be explored for large scale production of XOS with a high content of xylobiose.
- The ESI-MS spectra of XOS mixture showed presence of six oligosaccharides and were identified as xylobiose, xylotriose, xylotetraose, xylopentose, xylohexose and xyloheptaose. The mass spectra of the major fraction obtained by GPC corresponded to xylobiose.

- There is no $^{1}H/^{13}C$ cross peaks in the 2D-HSQC spectrum of purified disaccharide obtained from pretreated corncob at $\delta_{ppm}$ 5.34/98.4 and this clearly indicates the absence of xylose residues with methyl glucuronic acid. The presence of a comparatively weak signal at δ 62.15 indicates the C5 of *Araf* and shows the presence of some disaccharides with xylose and arabinose, but not sufficient to produce a cross peak in 2D-HSQC. This confirms that the purified disaccharide from the XOS mixture is primarily a disaccharide of xylose and is identified as β-D-xylopyranosyl-(1→4)-D-xylanopyranose (xylobiose).
- *In vivo* evaluation of the effect of XOS on DMH induced colon cancer in rats indicated no significant pathological alterations in the major organs (liver, kidney, lung, heart, etc.) when rats were fed with XOS, ascertaining absence of any toxicity of XOS. The beneficial effect of XOS supplementation is also clear from the observation that XOS significantly increased the body weight in DMH-treated rats to the normal values.
- The study also indicates that XOS fed groups have greater intestinal bifidobacterial populations than the DMH-control group. XOS might act as a source of SCFA, resulting in a lower pH in the intestine and also normalize epithelial cell proliferation.
- The observation amply implies that XOS supplementation greatly restored normalcy in the colonic epithelial cells. The ability of XOS to reduce the number of ACF per colon could be mediated through an enhanced repair or remodeling of preneoplastic lesions.
- The *in vivo* study involved the use of two different levels of XOS to assess the quantitative effects of these prebiotics on the development of ACF. The data suggest that the inhibition of the ACF development in the colon was more effective at 10% XOS than at 5% XOS.
- The results also indicate that XOS supplementation to DMH-treated rats markedly restored the liver and colonic lipid peroxidation levels to normal levels.
- XOS supplementation to DMH treated rats increased the GST enzyme activities in the colonic mucosa as compared to control rats. This may be due to the ability of XOS to contribute to the detoxification of the carcinogen directly or indirectly

by the metabolites produced from the same by bacterial fermentation. Thus the decreased GST levels in colonic mucosa observed in the present study may be used as marker of cell proliferation.

- The results of animal study also showed that XOS significantly elevated the catalase activities of the colonic mucosa which could be important in inhibiting the DMH induced carcinogenesis.
- The observations of the study intended to evaluate the effects of XOS on the quality attributes of *idli*, indicated that with the increase in concentration of XOS and fermentation time, the foam stability improved as evident from a decreased specific gravity of *idli* batter.
- The change in pH and acidity reflects the activity of lactic acid bacteria in the *idli* batter during fermentation. The results indicated that fermentation time and concentration of XOS had the most pronounced effect on pH of *idli* batter. It was observed that in *idli* batter supplemented with XOS, the fermentation was rapid as evident from a significant reduction in pH in comparison to conventional way of batter fermentation.
- The number of lactic acid bacteria was found to increase considerably in the case of batter supplemented with XOS. This could be due to the bifidogenic effects of XOS. This corroborated well with the reduction in the pH, increase in the total acidity of batter and an increase in the number of lactic acid bacteria with XOS as a function of fermentation time.
- Soft texture of *idli* is a desirable quality. The shear values indicated a lower range of values for *idlis* prepared using batter supplemented with XOS.
- A similar reduction in the L values was also observed in the case of batter with XOS which indicated a reduction in the whiteness of *idli*. However, sensory analysis showed that the *idlis* prepared from batter with XOS was rated as acceptable by the panelists.
- The moisture content of *idli* had a positive impact on its texture and it mostly depends on the carbohydrate constituents of the batter. It was evident that XOS could result in an increase in moisture content of the *idlis* which could be attributed to its hydration property.

- The overall quality of the conventional *idli* was graded as 'good'. *Idli* made from batter containing 0.6% XOS scored slightly less when compared with control. On the other hand, 0.2 and 0.4% XOS addition resulted in an increased overall quality score and were graded as 'good' and 'very good' respectively.
- The instrumental and sensory analysis of various *idli*, showed that *idli* made from batter containing 0.4% XOS has supreme quality attributes within a very short period of 6 h of batter fermentation.

The research work carried out encompasses on the evaluation of microbial endoxylanases for XOS production, followed by the selection of *Aspergillus oryzae* MTCC 5154 as a potent endoxylanase source, production and characterization of XOS from pretreated corncob, evaluation of efficacy of XOS in the reduction of DMH induced colon cancer in rodent model and the use of XOS to improve the fermentation characteristics of *idli* batter and the quality attributes of *idli.*

**Future perspectives:**

XOS have great potential as agents to improve or maintain a balanced intestinal microflora for enhanced health and well-being. They can be incorporated into many food products. There are, however, several questions that still need to be answered. The observations made in the present investigation and published information on nutritional, physiological and microbial benefits of XOS gives a distinct direction to future research. Furthermore, the challenge of the future exploitation of these benefits into authentic health issues remains. Some of these are listed below.

- XOS seem to exert their nutritional benefits in various animal species, which by definition have an intestinal tract populated by a complex, bacterial intestinal ecosystem. The realization that the increase in the beneficial members of fecal microbiota can actually mediate health effects to improve disease states or susceptibility to disease led to the refinement of the definition of prebiotics by *Gibson et al.* (2004), to shift the focus of prebiotic efficacy from the increase of beneficial bacteria in the human gut microflora to the human well-being in general. The information currently available does not provide an exact explanation about effects of XOS. More studies are needed in this direction.

- The concept of XOS as a prebiotic currently targets microbial changes at the genus level. Future developments may elucidate molecules that induce species level effects. This is because certain species of bifidobacteria/lactobacilli may be more desirable than others. With the new generation of molecular microbiological techniques now becoming available, it will be possible to gain definitive information on species rather than genera that are influenced by XOS.

- Available experimental evidence supports the hypothesis that XOS and other prebiotics can offer an opportunity to prevent or mitigate gastrointestinal disorders. Even though encouraging results have been obtained for other prebiotics in preliminary clinical trials, the data on XOS is very much limited.

More advanced investigations are necessary to further elucidate the mechanisms involved in the reduction of cancer risk and in the cancer chemo- and/or radiotherapy-potentiating effects of XOS. However, these effects need to be confirmed in other experimental models as well as in clinical trials before XOS can be recommended as non-toxic and easily applicable adjuvant for cancer therapy without any additional risk for the patients.

- The properties of XOS offer a new dimension for the development of functional foods. One approach that may be encouraged for future research is the combination of prebiotics and probiotics (as synbiotics). Opportunities exist in exploring the improved knowledge of the synbiotic relationships between colonic microbiota, XOS and whole body physiopathology.

***What next?*** The research on XOS is moving well and has caught the attention of scientists all around the globe. However, there could be a new research development that has the potential to outstrip even the molecular approaches to gut microbiology and has prebiotics like XOS in the forefront. This is the science of "metabolomics". Nevertheless, the capabilities of XOS (and other prebiotics)-induced gut microbiota changes are now known, as is the potential to determine metabolome impact. Coupling the two approaches may mean that the prebiotic story for improving human health/well-being standards is only just a beginning.

# BIBLIOGRAPHY

## REVIEWS & RESEARCH ARTICLES

**Aachary, 2009**

Aachary AA & Prapulla SG. Value addition to corncob: production and characterization of xylooligosaccharides from alkali pretreated lignin-saccharide complex using *Aspergillus oryzae* MTCC 5154. *Bioresource Technology*, 2009, 100 (2): 991-995.

**Aachary, 2008**

Aachary AA & Prapulla SG. Corncob-induced endo-1,4-β-D-xylanase of *Aspergillus oryzae* MTCC 5154: production and characterization of xylobiose from glucuronoxylan. *Journal of Agricultural and Food Chemistry*, 2008, 56(11): 3981-3988.

**Abrams, 2005**

Abrams SA, Griffin IJ, Hawthorne KM, Liang L, Gunn SK, Darlington G & Ellis KJ. A combination of prebiotic short and long-chain inulin-type fructans enhances calcium absorption and bone mineralization in young adolescents. *American Journal of Clinical Nutrition*, 2005, 82: 471-476.

**Adelsberger, 2005**

Adelsberger H, Hertel C, Glawischnig E, Zverlov VV & Schwarz WH. Enzyme system of *Clostridium stercorarium* for hydrolysis of arabinoxylan: reconstitution of the *in vivo* system from recombinant enzymes. *Microbiology*, 2004, 150: 2257-2266.

**Adler, 2002**

Adler DG, Gostout CJ, Sorbi D, Burgart LJ, Wang L & Harmsen WS. Endoscopic identification and quantification of aberrant crypt foci in the human colon. *Gastrointestinal Endoscopy*, 2002, 56: 657-662.

**Ahn, 2001**

Ahn B & Ohshsima H. Suppression of intestinal polyposis in APCMin/+ mice by inhibiting nitric oxide production. *Cancer Research*, 2001, 61: 8357-8360.

**Ai, 2005**

Ai Z, Jiang Z, Li L, Deng W, Kusakabe I & Li H. Immobilization of *Streptomyces olivaceoviridis* E-86 xylanase on Eudragit S-100 for xylooligosaccharide production. *Process Biochemistry*, 2005, 40: 2707-2714.

**Akpinar, 2007**

Akpinar O, Ak O, Kavas A, Bakir U & Yilmaz L. Enzymatic production of xylooligosaccharides from cotton stalks. *Journal of Agricultural and Food Chemistry*, 2007, 55: 5544-5551.

**Al Jassim, 2005**

Al Jassim RA, Scott PT, Trebbin AL, Trott DJ & Pollitt CC. The genetic diversity of lactic acid producing bacteria in the equine gastrointestinal tract. *FEMS Microbiology Letters*, 2005, 248: 75-81.

**Alabaster, 1995**

Alabaster O, Tang Z, Frost A & Shivapurkar N. Effect of β-carotene and wheat bran fiber on colonic aberrant crypt and tumor formation in rats exposed to azoxymethane and high dietary fat. *Carcinogenesis*, 1995, 16: 127-132.

**Alander, 2001**

Alander M, Matto J, Kneifel W, Johansson M, Kogler B, Crittenden R, Mattila-Sandholm T & Saarela M. Effect of galactooligosaccharide supplementation on human faecal microflora and on survival and persistence of *Bifidobacterium lactis* Bb-12 in the gastrointestinal tract. *International Dairy Journal*, 2001, 11: 817-825.

**Albano, 1989**

Albano E, Tomasi A, Goria-Gatti L & Iannone A. Free radicals activation of monomethyl and dimethylhydrazines in isolated hepatocytes and liver micrososmes. *Free Radical Biology and Medicine*, 1989, 6: 3-8.

**Alles, 1999**

Alles MS, de Roos NM, Bakx JC, van de Lisdonk E, Zock PL & Hautvast JGAJ. Consumption of fructooligosaccharides does not favorably affect blood glucose and serum lipid concentrations in patients with type 2 diabetes. *American Journal of Clinical Nutrition*, 1999, 69(1): 64-69.

**Alonso, 2003**

Alonso JL, Dominguez H, Garrote G, Parajo JC & Vazquez MJ. Xylooligosaccharides: properties and production technologies. *Electronic Journal of Environmental, Agricultural and Food Chemistry*, 2003, 2(1): 230-232.

**Ando, 2004**

Ando H , Ohba H, Sakaki T, Takamine K, Kamino Y, Moriwaki S, Bakalova R, Uemura Y & Hatate Y. Hot-compressed-water decomposed products from bamboo

manifest a selective cytotoxicity against acute lymphoblastic leukemia cells. *Toxicology in Vitro*, 2004, 18: 765-771.

**Ando, 2003**

Ando H, Morita S, Furukawa I, Kamino Y, Sasaki T & Hirose H. Generation of xylooligosaccharides from moso bamboo (*Phyllostachys pubescens*) using hot-compressed-water. *Mokuzai Gakkaishi,* 2003, 49: 293-300.

**Anita, 1995**

Anita J. Production of xylanase by Thermophilic *Melanocarpus albomyces* IIS-68. *Process Biochemistry*, 1995, 30(8): 705-709.

**Aoyama, 1995**

Aoyama M, Seki K & Saito N. Solubilization of bamboo grass xylan by steaming treatment. *Holzforschung,* 1995, 49: 193-196.

**Arai, 2001**

Arai, Osawa T, Ohigashi H, Yoshikawa M, Kaminogawa S, Watanabe M, Ogawa T, Okubo K, Watanabe S, Nishino H, Shinohara K, Esaki T & Hirahara T. A mainstay of functional food science in Japan- history, present status, and future outlook. *Bioscience, Biotechnology and Biochemistry*, 2001, 65: 1-13.

**Asahara, 2001**

Asahara T, Nomoto K, Shimizu K, Watanuki M & Tanaka R. Increased resistance of mice to *Salmonella enterica serovar* Typhimurium infection by synbiotic administration of bifidobacteria and transgalactosylated oligosaccharides. *Journal of Applied Microbiology,* 2001, 91: 985-996.

**Bailey, 1989**

Bailey MJ & Poutanen K. Production of xylanases by strains of *Aspergillus*. *Applied Microbiology and Biotechnology,* 1989, 30: 5-10.

**Balakrishnan, 1997**

Balakrishnan H, Srinivasan MC & Rele MV. Extracellular protease activities in relation to xylanase secretion in an alkilophilic *Bacillus* sp. *Biotechnology Letters*, 1997, 18: 599-601.

**Barbesgaard, 1992**

Barbesgaard P, Heldt-Hansen HP & Diderichsen B. On the safety of *Aspergillus oryzae*: a review. *Applied Microbiology and Biotechnology,* 1992, 36: 569-572.

**Bartoli, 1979**

Bartoli GM & Galeotti T. Growth related lipid peroxidation in tumor microsomal membranes and mitochondria. *Biochimica et Biophysica Acta*, 1979, 574: 537-541.

**Bastawde, 1992**

Bastawde KB. Xylan structure, microbial xylanases and their mode of action. *World Journal of Microbiology and Biotechnology*, 1992, 8: 353-368.

**Bazus, 1993**

Bazus A, Rigal L, Gaset A, Fontaine T, Wieruszeski JM & Fournet B. Isolation and characterisation of hemicelluloses from sunflower hulls. *Carbohydrate Research*, 1993, 243: 323-332.

**Beg, 2001**

Beg QK, Kapoor M, Mahajan L & Hoondal GS. Microbial xylanases and their industrial applications: a review. *Applied Microbiology and Biotechnology*, 2001, 56: 326-338.

**Ben-David, 2007**

Ben-David A, Bravman T, Balazs YS, Czjzek M, Schomburg D, Shoham G & Shoham Y. Glycosynthase activity of *Geobacillus stearothermophilus* GH52 β-xylosidase: Efficient synthesis of xylooligosaccharides fro m α-D-xylopyranosyl fluoride through a conjugated reaction. *ChemBioChem*, 2007, 8: 2145-2151.

**Benno, 1984**

Benno Y, Sawada K & Mitsuoka T. The intestinal microflora of infants: composition of fecal flora in breastfed and bottle-fed infants. *Microbiology and Immunology*, 1984, 28: 975-986.

**Beylot, 2005**

Beylot M. Effects of inulin-type fructans on lipid metabolism in man and in animal models. *British Journal of Nutrition*, 2005, 93: S163-S168.

**Beylot, 2001**

Beylot MH, Emami K, Mc Kie VA, Gilbert HJ & Pell G. *Pseudomonas cellulosa* expresses a single membrane-bound glycoside hydrolase family51 arabinofuranosidase. *Biochemistry Journal*, 2001, 358: 599-605.

**Beynen, 2002**

Beynen AC, Baas JC, Hoekemeijer PE, Kappert HJ, Bakker MH, Koopman JP & Lemmens AG. Faecal bacterial profile, nitrogen excretion and mineral absorption in

health dogs fed supplemental oligofructose. *Journal of Animal Physiology and Animal Nutrition*, 2002, 86: 298-305.

**Beynen, 2001**

Beynen AC, Kappert HJ & Yu S. Dietary lactulose decreases apparent nitrogen absorption and increases apparent calcium and magnesium absorption in healthy dogs. *Journal of Animal Physiology and Animal Nutrition*, 2001, 85: 67-72.

**Bhaduri, 1995**

Bhaduri SK, Ghosh IN & Deb Sarkar NL. Ramie hemicellulose as beater additive in paper making from jute-stick kraft pulp. *Industrial Crops and Products*, 1995, 4: 79-84.

**Bielecka, 2002**

Bielecka M, Biedrzycka E & Majkowska A. Selection of probiotics and prebiotics for synbiotics and confirmation of their *in vivo* effectiveness. *Food Research International*, 2002, 35: 125-131.

**Biely, 1997**

Biely P, Vrsanska M, Tenkanen M & Kluepfel D. Endo-β-1,4-xylanase families: differences in catalytic properties. *Journal of Biotechnology*, 1997, 57: 151-166.

**Biely, 1985**

Biely P. Microbial xylanolytic systems. *Trends in Biotechnology*, 1985, 9: 83-88.

**Biely, 1981**

Biely P, Kratky Z & Vrasanska M. Substrate-binding site of endo-1,4-β-xylanase of the yeast *Cryptococcus albidus*. *European Journal of Biochemistry*, 1981, 119: 559-564.

**Billa, 1997**

Billa E, Koullas DP, Monties B & Koukios EG. Structure and composition of sweet sorghum stalk components. *Industrial Crops and Products*, 1997, 6: 297-302.

**Billon, 2006**

Billon L, Meric V, Castetbon A, Francois J & Desbrieres J. Removal of copper ions from water of broilers by a modified natural based corncobs. *Journal of Applied Polymer Science*, 2006, 102: 4637-4645.

**Bird, 1995a**

Bird RP. Further investigation of the effect of cholic acid on the induction, growth characteristics and stability of aberrant crypt foci in rat colon. *Cancer Letters*, 1995, 88: 201-209.

**Bird, 1995b**

Bird RP. Role of ACF in understanding the pathogenesis of colon cancer. *Cancer Letters*, 1995, 93: 55-71.

**Bird, 1987**

Bird RP. Observation and quantification of aberrant crypts in the murine colon treated with a carcinogen: preliminary finding. *Cancer Letters*, 1987, 37: 147-151.

**Birnboim, 1979**

Birnboim HC & Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research*, 1979, 7: 1513-1523.

**Boland, 1993**

Boland CR. The biology of colorectal cancer. *Cancer Supplement*, 1993, 71: 4180-4186.

**Bonn, 1984**

Bonn G, Pecina R, Burtscher E & Bobleter O. Separation of wood degradation products by high performance liquid chromatography. *Journal of Chromatography*, 1984, 287: 215-221.

**Bouhnik, 1999**

Bouhnik Y, Vahedi K, Achour L, Attar A, Salfati J, Pochart P, Marteau P, Flourie B, Bornet F & Rambaud JC. Short-chain fructooligosaccharide administration dose-dependently increases fecal bifidobacteria in healthy humans. *Journal of Nutrition*, 1999, 129: 113-116.

**Bouhnik, 1996**

Bouhnik Y, Flourie B, Riottot M, Bisetti N, Gailing MF, Guibert A, Bornet F & Rambaud JC. Effects of fructooligosaccharides ingestion on fecal bifidobacteria and selected metabolic indexes of colon carcinogenesis in healthy humans. *Nutrition and Cancer*, 1996, 26: 21-29.

**Bravman, 2003a**

Bravman T, Belakhov V, Solomon D, Shoham G, Henrissat B, Baasov T & Shoham Y. Identification of the catalytic residues in family 52 glycoside hydrolase, a β-xylosidase from *Geobacillus stearothermophilus* T-6. *Journal of Biological Chemistry,* 2003a, 278: 26742-26749.

**Bravman, 2003b**

Bravman T, Zolotnitsky G, Belakhov V, Shoham G, Henrissat B, Baasov T & Shoham Y. Detailed kinetic analysis of a family 52 glycoside hydrolase: a β-xylosidase from *Geobacillus stearothermophilus*. *Biochemistry,* 2003b, 42: 10528-10536.

**Bravman, 2001**

Bravman T, Zolotnitsky G, Shulami S, Belakhov V, Solomon D, Baasov T, Shoham G & Shoham Y. Stereochemistry of family 52 glycosyl hydrolases: a β-xylosidase from *Bacillus stearothermophilus* T-6 is a retaining enzyme. *FEBS Letters,* 2001, 495: 39-43.

**Bray, 1992**

Bray MR & Clarke AJ. Action pattern of xylooligosaccharide hydrolysis by *Schizophyllum commune* xylanase A. *European Journal of Biochemistry,* 1992, 204: 191-196.

**Brux, 2006**

Brux C, Ben-David A, Shallom-Shezifi D, Leon M, Niefind K, Shoham G, Shoham Y & Schomburg D. The structure of an inverting GH43 β-xylosidase from *Geobacillus stearothermophilus* with its substrate reveals the role of the three catalytic residues. *Journal Molecular of Biology,* 2006, 359: 97-109.

**Buddington, 2002**

Buddington KK, Danohoo JB & Buddington RK. Dietary oligofructose and inulin protect mice from enteric and systemic pathogens and tumor inducers. *Journal of Nutrition,* 2002, 132: 472-477.

**Busserolles, 2003**

Busserolles J, Gueux E, Rock E, Demigne C, Mazur A & Rayssiguier Y**.** Oligofructose protect against the hypertriglyceridemic and pro-oxidative effects of a high fructose diet in rats. *Journal of Nutrition*, 2003, 133: 1903-1908.

**Campbell, 1997**

Campbell JM, Fahey GC Jr. & Wolf BW. Selected indigestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acids, pH and microflora in rats. *Journal of Nutrition*, 1997, 27: 130-136.

**Cano, 2007**

Cano A & Palet C. Xylooligosaccharide recovery from agricultural biomass waste treatment with enzymatic polymeric membranes and characterization of products with MALDI-TOF-MS. *Journal of Membrane Science*, 2007, 291: 96-105.

**Caparros, 2007**

Caparros S, Garrote G, Ariza J, Diaaz MJS & Loapez F. Xylooligosaccharides production from *Arundo donax*. *Journal of Agricultural and Food Chemistry*, 2007, 55: 5536-5543.

**Carter, 1994**

Carter JW, Lancaster HK, Hardman WE & Cameron IL. Distribution of intestine-associated lymphoid tissue, aberrant crypt foci and tumors in the large bowel of 1,2-dimethylhydrazine-treated mice. *Cancer Research*, 1994, 54: 4304-4307.

**Carvalheiro, 2005**

Carvalheiro F, Garrote G, Parajo JC, Pereira H & Girio FM. Kinetic modeling of brewery's spent grain autohydrolysis. *Biotechnol*ogy *Progress*, 2005, 21: 233-243.

**Carvalheiro, 2004**

Carvalheiro F, Esteves MP, Parajo JC, Pereira H & Girio FM. Production of oligosaccharides by autohydrolysis of brewery's spent grain. *Bioresource Technology*, 2004, 91: 93-100.

**Cavaglieri, 2003**

Cavaglieri CR, Nishiyama A, Fernandes LC, Curi R, Miles EA & Calder PC. Differential effects of short-chain fatty acids on proliferation and production of pro- and anti-inflammatory cytokines by cultured lymphocytes. *Life Sciences*, 2003, 73: 1683-1690.

**Chan, 2005**

Chan SH, Chung YC, Chang CT & Chan KC. Effect of oligosaccharides on fecal content and intestinal microflora in adult Sprague-Dawley rats. *Taiwanese Journal of Agriculture Chemistry and Food Science*, 2005, 42: 440-447.

**Chang, 1995**

Chang PK, Bhatnagar D, Cleveland TE & Bennett JW. Sequence variability in homologs of the aflatoxin pathway gene aflR distinguishes species in *Aspergillus* section Flavi. *Applied and Environmental Microbiology*, 1995, 61: 40-43.

**Chang, 1984**

Chang WWL. Histogenesis of colon cancer in experimental animals. *Scandinavian Journal of Gastroenterology*, 1984, 19: 27-43.

**Chaturvedi, 2001**

Chaturvedi P, Warren CD, Buescher CR, Pickering LK & Newburg DS**.** Survival of human milk oligosaccharides in the intestine of infants. *Advances in Experimental Medicine and Biology*, 2001, 501: 315-323.

**Chau, 2006**

Chau I & Cunningham D. Adjuvant therapy in colon cancer-what, when and how? *Annals of Oncology*, 2006, 17: 1347-1359.

**Cheesman, 1986**

Cheesman H, Collins H, Proudfoot K, Slater TF, Burton W, Webb AC & Ingold KU. Studies on lipid peroxidation in normal and tumor tissues. *Journal of Biological Chemistry*, 1986, 235: 507-514.

**Chen, 2005**

Chen YC, Nakthong C & Chen TC. Improvement of laying hen performance by dietary prebiotic chicory oligofructose and inulin. *International Journal of Poultry Science*, 2005, 4: 103-108.

**Chen, 2004**

Chen YC & Chen TC. Mineral utilization in layers as influenced by dietary oligofructose and inulin. *International Journal of Poultry Science*, 2004, 3: 442-445.

**Chen, 2001**

Chen HL, Lu YH, Lin JJ & Ko LY. Effects of isomalto-oligosaccharides on bowel functions and indicators of nutritional status in constipated elderly men. *Journal of American College of Nutrition*, 2001, 20: 44-49.

**Chen, 1997**

Chen C, Chen J & Lin T. Purification and characterization of a xylanase from *Trichoderma longibrachiatum* for xylooligosaccharide production. *Enzyme and Microbial Technology*, 1997, 21: 91- 96.

**Chiang, 2000**

Chiang BL, Sheih YH, Wang LH, Liao CK & Gill HS. Enhancing immunity by dietary consumption of probiotic lactic acid bacterium (*Bifidobacterium lactis* HN019): optimization and definition of cellular immune responses. *European Journal of Clinical Nutrition*, 2000, 54: 849-855.

**Chikamatsu, 1999**

Chikamatsu G, Shirai K, Kato M, Kobayashi T & Tsukagoshi N. Structure and expression properties of the endo-β-1,4-glucanase A gene from the filamentous fungus *Aspergillus nidulans*. *FEMS Microbiology Letters*, 1999, 175: 239-245.

**Christakopoulos, 2003**

Christakopoulos P, Katapodis P, Kalogeris E, Kekos D, Macris BJ, Stamatis H & Skaltsa H. Antimicrobial activity of acidic xylooligosaccharides produced by family 10 and 11 endoxylanases. *International Journal of Biological Macromolecules*, 2003, 31: 171-175.

**Christensen, 1988**

Christensen T, Woeldike H, Boel E, Mortensen SB, Hjortshoej K, Thim L & Hansen MT. High level expression of recombinant genes in *Aspergillus oryzae*. *Bio/Technology*, 1988, 6: 1419-1422.

**Chung, 2002**

Chung YC, Hsieh CP & Chan YC. Effect of xylooligosaccharides on the intestinal properties of ICR mice. *Taiwanese Journal of Agriculture Chemistry and Food Science*, 2002, 40: 377-384.

**Chunga, 2007**

Chunga CY, Hsub CK, Koa CY & Chan YC. Dietary intake of xylooligosaccharides improves the intestinal microbiota, fecal moisture and pH value in the elderly. *Nutrition Research*, 2007, 27: 756-761.

**Coimbra, 1994**

Coimbra MA, Waldron KW & Selvendran RR. Isolation and characterisation of cell wall polymers from olive pulp (*Olea europaea* L). *Carbohydrate Research*, 1994, 252: 245-262.

**Cooper, 1978**

Cooper HK, Buecheler J & Kleihues P. DNA alkylation in mice with genetically difference susceptibility to 1,2-dimethylhydrazine-induced colon carcinogenesis. *Cancer Research*, 1978, 38: 3063-3065.

**Coppa, 2006**

Coppa GV, Zampini L, Galeazzi T & Gabrielli O. Prebiotics in human milk: a review. *Digestive and Liver Disease*, 2006, 38(2): S291-S294

**Coppa, 2004**

Coppa GV, Bruni S, Morelli L, Soldi S & Gabrielli O. The first prebiotics in humans: human milk oligosaccharides. *Journal of Clinical Gastroenterology*, 2004, 38: S80-S83.

**Coppa, 2001**

Coppa GV, Pierani P, Zampini L, Bruni S, Carloni I & Gabrielli O. Characterization of oligosaccharides in milk and faeces of breast-fed infants by high-performance anion-exchange chromatography. *Advances in Experimental Medicine and Biology*, 2001, 501: 307-314.

**Coppa, 1999**

Coppa GV, Pierani P, Zampini L, Carloni I, Carlucci A & Gabrielli O. Oligosaccharides in human milk during different phases of lactation. *Acta Paediatrica*, 1999, 430: 89-94.

**Coppa, 1993**

Coppa GV, Gabrielli O, Pierani P, Catassi C, Carlucci A & Giorgi PL. Changes in carbohydrates composition in human milk over 4 months of lactation. *Pediatrica*, 1993, 91: 637-641.

**Cotta, 1998**

Cotta MA & Whitehead TR. Xylooligosaccharide utilization by the ruminal anaerobic bacterium *Selenomonas ruminantium. Current Microbiology*, 1998, 36: 183-189.

**Coudray, 2006**

Coudray C, Feillet-Coudray C, Gueux E, Mazur A & Rayssiguier Y. Dietary inulin intake and age can affect intestinal absorption of zinc and copper in rats. *Journal of Nutrition*, 2006, 136: 117-122.

**Coudray, 2005a**

Coudray C, Feillet-Coudray C, Tressol JC, Gueux E, Thien S, Jaffrelo L, Mazur A & Rayssiguier Y. Stimulatory effect of inulin on intestinal absorption of calcium and magnesium in rats is modulated by dietary calcium intakes short- and long-term balance studies. *European Journal of Nutrition*, 2005a, 44: 293-302.

**Coudray, 2005b**

Coudray C, Rambeau M, Feillet-Coudray C, Tressol JC, Demigne C, Gueux E, Mazur A & Rayssiguier Y. Dietary inulin intake and age can significantly affect intestinal absorption of calcium and magnesium in rats: A stable isotope approach. *Nutrition Journal*, 2005b, 4: 29-37.

**Coudray, 2003**

Coudray C, Demigne C & Rayssiguier Y. Effects of dietary fibres on magnesium absorption in animals and humans. *Journal of Nutrition*, 2003, 133: 1-4.

**Coudray, 1997**

Coudray C, Bellanger J, Castglia-Delavaud C, Remesy C, Vermorel M & Rayssiguier Y. Effect of soluble or partly soluble dietary fibers supplementation on absorption and balance of calcium, magnesium, iron and zinc in healthy young men. *European Journal of Clinical Nutrition*, 1997, 51: 375-380.

**Crittenden, 2001a**

Crittenden RG, Morris LF, Harvey ML, Tran LT, Mitchell HL & Playne MJ. Selection of a *Bifidobacterium* strain to complement resistant starch in synbiotic yoghurt. *Journal of Applied Microbiology*, 2001a, 90: 268-278.

**Crittenden, 2001b**

Crittenden RG, Laitila A, Forssell P, Matto J, Saarela M, Mattila-Sandholm T & Myllarinen P. Adhesion of bifidobacteria to granular starch and its implications in probiotic technologies. *Applied Environmental Microbiology*, 2001b, 67: 3469-3475

**Crittenden, 1996**

Crittenden RG & Playne MJ. Production, properties and applications of food-grade oligosaccharides. *Trends in Food Science and Technology*, 1996, 7: 353-361.

**Czjzek, 2005**

Czjzek M, Ben David A, Bravman T, Shoham G, Henrissat B & Shoham Y. Enzyme-substrate complex structures of a GH39 β-xylosidase from *Geobacillus stearothermophilus*. *Journal of Molecular Biology*, 2005, 353: 838-846.

**Das, 2002**

Das U. A radical approach to cancer. *Medical science monitor: international medical journal of experimental and clinical research*, 2002, 8(4): RA79-RA92.

**Das, 1995**

Das A & Nanda G. Production of xylanolytic enzymes during growth on pulverized grass by *Aspergillus ochraceus*-42. *Letters in Applied Microbiology*, 1995, 20: 141-144.

**Daubioul, 2005**

Daubioul CA, Horsmans Y, Lambert P, Danse E & Delzenne NM. Effects of oligofructose on glucose and lipid metabolism in patients with nonalcoholic steatohepatitis: result of a pilot study. *European Journal of Clinical Nutrition*, 2005, 59: 723-726.

**Daubioul, 2002**

Daubioul CA, Rousseau N, Demeure R, Gallez B , Taper H, Declerck B & Delzenne N. Dietary fructans, but not cellulose, decrease triglyceride accumulation in the liver of obese Zucker *fa/fa* rats. *Journal of Nutrition*, 2002, 132: 967-973.

**Daubioul, 2000**

Daubioul CA, Taper HS, De Wispelaere LD & Delzenne NM. Dietary oligofructose lessens hepatic steatosis, but does not prevent hypertriglyceridemia in obese Zucker rats. *Journal of Nutrition*, 2000, 130: 1314-1319.

**de Boever, 2001**

de Boever P, Wouters R & Verstraete W. Combined use of *Lactobacillus reuteri* and soygerm powder as food supplement. *Letters in Applied Microbiology*, 2001, 33: 420-424.

**de Graaff, 1994**

de Graaff LH, van den Broeck HC, van Ooijen AJJ & Visser J. Regulation of the xylanase-encoding *xlnA* gene of *Aspergillus tubingensis*. *Molecular Microbiology*, 1994, 12: 479-490.

**de Vries, 2001**

de Vries RP & Visser J. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiology and Molecular Biology Reviews*, 2001, 65: 497-522.

**de Vries, 1999**

de Vries RP & Visser J. Regulation of the feruloyl esterase (*faeA*) gene from *Aspergillus niger*. *Applied and Environmental Microbiology,* 1999, 65: 5500-5503.

**Debyser, 1997**

Debyser W, Schooneveld-Bergmans MEF, Derdelinckx G, Grobet PJ, & Delcour JA. Nuclear magnetic resonance and methylation analysis-derived structural features of water-extractable arabinoxylans from barely (*Hordeum vulgare* L.) malts. *Journal of Agricultural and Food Chemistry*, 1997, 45: 2914-2918.

**Deery, 2001**

Deery MJ, Stimson E & Chapell CG. Size exclusion chromatography/mass spectrometry applied to the analysis of polysaccharides. *Rapid Communications in Mass Spectrometry*, 2001, 15: 2273-2283.

**Defaye, 1992**

Defaye J, Guillot JM, Biely P & Vrsanska M. Positional isomers of thioxylobiose, their synthesizing and inducing ability for D-xylan-degrading enzymes in the yeast *Cryptococcus albidus*. *Carbohydrate Research*, 1992, 228: 47-64.

**Degnan,1992**

Degnan BA & MacFarlane GT. Effect of dilution rate and carbon availability on *Bifidobacterium breve* fermentation. *Applied Microbiology and Biotechnology*, 1994, 40: 800-805.

**Delker, 1996**

Delker DA, Bammler TK & Rosenberg DW. A comparative study of hepatic and colonic metabolic enzymes in inbred mouse lines before and after treatment with the colon carcinogen, 1,2-dimethylhydrazine. *Drug Metabolism and Disposition*, 1996, 24: 408-413.

**Delzenne, 2002a**

Delzenne NM, Daubioul C, Neyrinck A, Lasa M & Taper HS. Inulin and oligofructose modulate lipid metabolism in animals: review of biochemical events and future prospects. *British Journal of Nutrition*, 2002, 87: S255-S259.

**Delzenne, 2002b**

Delzenne NM & Williams CM. Prebiotics and lipid metabolism. *Current Opinions in Lipidology*, 2002, 13: 61-67.

**Delzenne, 1995**

Delzenne NM, Aertssens J, Verplaetse H, Roccaro M & Roberfroid MR. Effect of fermentable fructooligosaccharides on mineral, nitrogen and energy digestive balance in the rat. *Life sciences*, 1995, 57: 1579-1587.

**Delzenne, 1994**

Delzenne NM & Roberfroid MR. Physiological effects of non-digestible oligosaccharides. *LWT-Food Science and Technology*, 1994, 27: 1-6.

**Deschner, 1984**

Deschner EE, Long FC, Hakissian M & Cupo SH. Differential susceptibility of inbred mouse strains forecast by acute colonic proliferative response to methylazoxymethanol. *Journal of National Cancer Institute*, 1984, 72: 195-198.

**Deschner, 1979**

Deschner EE, Long FC & Maskens AP. Relationship between dose, time, and tumor yield in mouse dimethyihydrazine-induced colon tumongenesis. *Cancer Letters*, 1979, 8: 23-28.

**Desikachar, 1960**

Desikachar HSR, Radhakrishnamurthy R, Rama Rao G, Kadkol SB, Srinivasan M & Subrahmanyan V. Studies on idli fermentation: Part 1. Some accompanying changes in the batter. *Journal of Scientific and Industrial Research*, 1960, 19C: 168-172.

**Diez, 1997**

Diez M, Hornick JL, Baldwin P & Istasse L. Influence of a blend of fructooligosaccharides and sugar beet fiber on nutrient digestibility and plasma metabolite concentrations in healthy beagles. *American Journal of Vetenary Research*, 1997, 58(11): 1238-1242.

**Diwan, 1977**

Diwan BA, Meier H & Blackman KE. Genetic differences in the induction of colorectal tumors by 1,2-dimethylhydrazine in inbred mice. *Journal of National Cancer Institute*, 1977, 59: 455-458.

**Domon, 1988**

Domon B & Costello CE. A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. *Glycoconjugate Journal*, 1988, 5: 397-409.

**Doner, 1997**

Doner LW & Hicks KB. Isolation of hemicellulose from corn fiber by alkaline hydrogen peroxide extraction. *Cereal Chemistry,* 1997, 74: 176-181.

**Druckrey, 1967**

Druckrey H, Preussmann R, Matzkles F, and Ivankovic S. Selektive Erzeugung von Darmkrebs bei Rattan durch 1,2-Dlmethylhydrazln. *Naturwissenschaften,* 1967, 54: 285-286.

**DuBois, 1996**

DuBois RN, Radhika A, Reddy BS & Entingh AJ. Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors. *Gastroenterology*, 1996, 110: 1259-1262.

**Ebringerova, 2002**

Ebringerova A, Kardosova A, Hromadkova Z, Malovıkova A & Hrıbalova V. Immunomodulatory activity of acidic xylans in relation to their structural and molecular properties. *International Journal of Biological Macromolecules*, 2002, 30, 1-6.

**Ebringerova, 2000**

Ebringerova A & Heinze T. Xylan and xylan derivatives- biopolymers with valuable properties, 1- Naturally occurring xylans structures, isolation procedures and properties. *Macromolecule Rapid Communications*, 2000, 21: 542-556.

**Ebringerova, 1998**

Ebringerova A, Hromadkova Z, Alfodi J & Hribalova V. The immunologically active xylan from ultrasound-treated corncobs: extractability, structure and properties. *Carbohydrate Polymers,* 1998, 37: 231-239.

**Ebringerova, 1995**

Ebringerova A, Hromadkova Z & Hrıbalova V. Structure and mitogenic activities of corncob heteroxylans. *International Journal of Biological Macromolecules*, 1995, 17: 327-332.

**Ebringerova, 1992**

Ebringerova A, Hromadkova Z, Alfodi J & Berth G. Structural and solution properties of corncob heteroxylans. *Carbohydrate Polymers,* 1992, 19: 99-105.

**Ellegard, 1997**

Ellegard L, Andersson H & Bosaeus I. Inulin and oligofructose do not influence the absorption of cholesterol, or the excretion of cholesterol, Ca, Mg, Zn, Fe or bile acids

but increases energy excretion in ileostomy subjects. *European Journal of Clinical Nutrition*, 1997, 51: 1-5.

**Ellman, 1959**

Ellman GL. Tissue sulphydryl groups. *Archives in Biochemistry and Biophysics*, 1959, 82: 72-77.

**Engfer, 2000**

Engfer MB, Stahl B, Finke B, Sawatzki G & Daniel H. Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *American Journal of Clinical Nutrition*, 2000, 71: 1589-1596.

**Erlandson, 2001**

Erlandson KA, Delamarre SC & Batt CA. Genetic evidence for a defective xylan degradation pathway in *Lactococcus lactis*. *Applied and Environmental Microbiology*, 2001, 67: 1445-1452.

**Esteller, 2000**

Esteller M, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Watkins DN, Issa JP, Sidransky D, Baylin SB & Herman JG. Inactivation of the DNA repair gene *O*-6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in *K*-ras in colorectal tumorigenesis. *Cancer Research*, 2000, 60: 2368-2371.

**Evans, 1974**

Evans JT, Hauschka TS & Mittelman A. Differential susceptibility of four mouse strains to induction of multiple large-bowel neoplasms by 1,2-dimethylhydrazine. *Journal of National Cancer Institute*, 1974, 52: 999-1000.

**Farber, 1976**

Farber E, Parker S & Gruenstein M. The resistance of putative premalignant liver cell populations, hyperplastic nodules to the acute cytotoxic effects of some hepatocarcinogenesis. *Cancer Research*, 1976, 36: 3879-3887.

**Fava, 2006**

Fava F, Lovegrove JA, Gitau R, Jackson KG & Tuohy KM. The gut microbiota and lipid metabolism: implications for human health and coronary heart disease. *Current Medicinal Chemistry*, 2006, 13: 3005-3021.

**Fearon, 1990**

Fearon ER & Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell,* 1990, 61: 759-767.

**Femia, 2002**

Femia AP, Luceri C, Dolara P, Giannini A, Biggeri A, Salvadori M, Clune Y, Collins KJ, Paglierani M & Caderni G. Antitumorigenic activity of the prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* on azoxymethane-induced colon carcinogenesis in rats. *Carcinogenesis,* 2002, 23: 1953-1960.

**Fengel, 1994**

Fengel D. Studies on the super molecular structure of cell wall components. Part 5. Ultrastructure of the fractions from ion exchange chromatography and of isolated lignin, *Svensk Papperstidn,* 1976, 79(1): 24-28.

**Fernandez- Espinar, 1994**

Fernandez- Espinar M, Pinaga F, de Graaff L & Valles S. Purification, characterization and regulation of the synthesis of an *Aspergillus nidulans* acidic xylanase. *Applied Microbiology and Biotechnology,* 1994, 42: 555-562.

**Fiala, 1987**

Fiala ES, Sohn OS & Hamilton SR. Effects of chronic dietary ethanol on the *in vivo* and *in vitro* metabolism of methylazoxymethonol and methylazoxymethanol induced DNA methylation in the rat colon and liver. *Cancer Research,* 1987, 47: 5939-5943.

**Fiala, 1977**

Fiala ES. Investigations into the metabolism and mode of action of the colon carcinogens 1,2-dimethylhydrazine and azoxymethane. *Cancer,* 1977, 40: 2436-2445.

**Fiordaliso, 1995**

Fiordaliso M, Kok N, Desager JP, Goethals F, Deboyser D, Roberfroid M & Delzenne N. Dietary oligofructose lowers triglycerides, phospholipids and cholesterol in serum and very low density lipoproteins of rats. *Lipids,* 1995, 30: 163-167.

**Flickinger, 2003a**

Flickinger EA, Van Loo J & Fahey GC. Nutritional response to the presence of inulin and oligofructose in the diets of domesticated animals: a review. *Critical Reviews in Food Science and Nutrition*, 2003a, 43: 19-60.

**Flickinger, 2003b**

Flickinger EA, Schreijen EM, Patil AR, Hussein HS, Grieshop CM, Merchen NR & Fahey GC Jr. Nutrient digestibilities, microbial populations and protein catabolites as affected by fructan supplementation of dog diets. *Journal of Animal Science*, 2003b, 81: 2008-2018.

**Frankel, 1994**

Frankel WL, Zhang W, Singh A, Klurfeld DM, Don S, Sakata T, Modlin I & Rombeau JL. Mediation of the trophic effects of short chain fatty acids on the rat jejunum and colon. *Gastroenterology*, 1994, 106: 375-380.

**Freixo, 2002**

Freixo MR & de Pinho M. Enzymatic hydrolysis of beech wood xylan in a membrane reactor. *Desalination*, 2002, 149: 237-242.

**Friend, 1993**

Friend CP, Waniska RD & Rooney LW. Effects of hydrocolloids on processing and qualities of wheat tortillas. *Cereal Chemistry*, 1993, 70: 252-256.

**Fringant, 1995**

Fringant CH, Tvaroska I, Mazeau K, Rinaudo M & Desbrieres J. Hydration of maltose and amylose: molecular modeling and thermodynamics study. *Carbohydrate Research*, 1995, 278: 27-41.

**Fukushima, 1999**

Fukushima Y, Kawata Y, Mizumachi K, Kurisaki J & Mitsuoka T. Effect of Bifidobacteria feeding on fecal flora and production of immunoglobulins in lactating mouse. *International Journal of Food Microbiology*, 1999, 46: 193-197.

**Furrie, 2006**

Furrie E, Macfarlane S, Kennedy A, Cummings JH, Walsh SV, O'neil DA & Macfarlane GT. Synbiotic therapy (*Bifidobacterium longum*/ Synergy1) initiates resolution of inflammation in patients with active ulcerative colitis: A randomized controlled pilot trial. *Gut*, 2006, 54: 242-249.

**Gallaher, 1999**

Gallaher DD & Khil J. Effects of synbiotics on colon carcinogenesis in rats. *Journal of Nutrition,* 1999, 129: S1483- S1487.

**Garrote, 2004a**

Garrote G, Cruz JM, Dominguez H & Parajo JC. Production of xylooligosaccharides by autohydrolysis of lignocellulosic materials. *Trends in Food Sci*ence *and Technology*, 2004, 15: 115-120.

**Garrote, 2004b**

Garrote G, Domınguez H & Parajo JC. Production of substituted oligosaccharides by hydrolytic processing of barley husks. *Industrial and Engineering Chemistry Research,* 2004, 43: 1608-1614.

**Garrote, 2002a**

Garrote G & Parajo JC. Non-isothermal autohydrolysis of Eucalyptus wood. *Wood Science and Technology,* 2002, 36: 111-123.

**Garrote, 2002a**

Garrote G, Dominguez H & Parajo JC. Autohydrolysis of corncob: study of non-isothermal operation for xylooligosaccharides production. *Journal of Food Engineering,* 2002, 52: 211-218.

**Garrote, 2001**

Garrote G, Dominguez H & Parajo JC. Kinetic modelling of corncob autohydrolysis. *Process Biochemistry*, 2001, 36: 571-578.

**Garrote, 1999**

Garrote G, Dominguez H & Parajo JC. Mild autohydrolysis: an environmentally friendly technology from xylooligosaccharide production from wood. *Journal of Chemical Technology and Biotechnology,* 1999, 74: 1101-1109.

**Gibson, 1995**

Gibson GR & Roberfroid MB. Dietary modulation of the human colon microbiota: introducing the concept of prebiotics. *Journal of Nutrition,* 1995, 125: 1401-1412.

**Gibson, 1994**

Gibson GR & Wang X. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. *Journal of Applied Bacteriology,* 1994, 77: 412-420.

**Gielkens, 1999**

Gielkens MMC, Dekkers E, Visser J & de Graaff LH. Two cellobiohydrolase-encoding genes from *Aspergillus niger* require D-xylose and the xylanolytic transcriptional activator XlnR for their expression. *Applied and Environmental Microbiology,* 1999, 65: 4340-4345.

**Gielkens, 1997**

Gielkens MMC, Visser J & de Graaff LH. Arabinoxylan degradation by fungi: characterization of the arabinoxylan arabinofuranohydrolase encoding genes from *Aspergillus niger* and *Aspergillus tubingensis*. *Current Genetics,* 1997, 31: 22-29.

**Gionchetti, 2000**

Gionchetti P, Rizzello F, Venturi A & Campieri M. Probiotics in infective diarrhoea and inflammatory bowel diseases. *Journal of Gastroenterology and Hepatology,* 2000, 15: 489-493.

**Gmeiner, 2000**

Gmeiner M, Kneifel W, Kulbe KD, Wouters R, De Boever P, Nollet L & Verstraete W. Influence of a synbiotic mixture consisting of *Lactobacillus acidophilus* 74-2 and a fructooligosaccharides preparation on the microbial ecology sustained in a simulation of the human intestinal microbial ecosystem (SHIME reactor). *Applied Microbiology and Biotechnology,* 2000, 53: 219-223.

**Golan, 2004**

Golan G, Shallom D, Teplitsky A, Zaide G, Shulami S, Baasov T, Stojanoff V, Thompson A, Shoham Y & Shoham G. Crystal structures of *Geobacillus stearothermophilus* α-glucuronidase complexed with its substrate and products: mechanistic implications. *Journal of Biological Chemistry,* 2004, 279: 3014-3024.

**Goldin, 1980**

Goldin BR & Gorbach SL. Effect of *Lactobacillus acidophilus* dietary supplements on 1, 2-dimethylhydrazine dihydrochloride-induced intestinal cancer in rats. *Journal of National Cancer Institute,* 1980, 64: 263-265.

**Gomi, 1989**

Gomi K, Tanaka A, Iimura Y & Takahashi K. Rapid differentiation of four related species of koji molds by agarose gel electrophoresis of genomic DNA digested with SmaI restriction enzyme. *Journal of General and Applied Microbiology,* 1989, 35: 225-232.

**Griebl, 2006**

Griebl A, Lange T, Weber H, Milacher W & Herbert S. Xylooligosaccharide (XOS) formation through hydro-thermolysis of xylan derived from viscous process. *Macromolecular Symposia,* 2006, 232: 107-120.

**Grieshop, 2004**

Grieshop CM, Flickinger EA, Bruce KJ, Patil AR, Czarnecki-maulden GL & Fahey GC Jr. Gastrointestinal and immunological responses of senior dogs to chicory and mannan-oligosaccharides. *Archives of Animal Nutrition,* 2004, 58: 483-493.

**Griffin, 2003**

Griffin IJ, Hicks PMD, Heaney RP & Abrams SA. Enriched chicory inulin increases calcium absorption mainly in girls with lower calcium absorption. *Nutrition Research,* 2003, 23: 901-909.

**Griffin, 2002**

Griffin IJ, Davila PM & Abrams SA. Non-digestible oligosaccharides and calcium absorption in girls with adequate calcium intakes. *British Journal of Nutrition,* 2002, 87(2): S187-S191.

**Gruppen, 1992**

Gruppen H, Hoffman RA, Kormelink FJM, Voragen AGJ, Kamerling JP & Vliegenthart JFG. Characterisation of $^{1}$H NMR spectroscopy of enzymatically derived oligosaccharides from alkali-extractable wheat flour arabinoxylan. *Carbohydrate Research,* 1992, 233: 45-64.

**Guarda, 2004**

Guarda A, Rosell CM, Benedito C & Galotto MJ. Different hydrocolloids as bread improvers and antistaling agents. *Food Hydrocolloids*, 2004, 18: 241-247.

**Gullon, 2008**

Gullon P, Moura P, Esteves MP, Girio FM, Dominguez H & Parajo JC. Assessment on the fermentability of xylooligosaccharides from rice husks by probiotic bacteria. *Journal of Agricultural and Food Chemistry,* 2008, 56: 7482-7487.

**Gyorgy, 1954**

Gyorgy P, Norris RF & Rose CS. Bifidus factor I: a variant of *Lactobacillus bifidus* requiring a special growth factor. *Archives in Biochemistry and Biophysics*, 1954, 48: 193-201.

**Habig, 1974**

Habig WH, Pabst MJ & Jakoby WB. Glutathione-S-transferases, the first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, 1974, 249: 7130-7139.

**Haltrich, 1996**

Haltrich D, Nidezky B, Kulbe KD, Steiner W & Zupancic S. Production of fungal xylanases. *Bioresource Technology*, 1996, 58: 137-161.

**Han, 2004**

Han SO, Cho HY, Yukawa H, Inui M & Doi RH. Regulation of expression of cellulosomes and noncellulosomal (hemi) cellulolytic enzymes in *Clostridium cellulovorans* during growth on different carbon sources. *Journal of Bacteriology*, 2004, 186: 4218-4227.

**Harkonen, 1997**

Harkonen H, Pessa E, Suortti T & Poutanen K. Distribution and some properties of cell wall polysaccharides in Rye milling fractions. *Journal of Cereal Science*, 1997, 26: 95-104.

**Hashi, 1979**

Hashi M & Takeshita T. Studies on anti-tumor activity of wood hemicelluloses: II. The host mediated anti-tumor effect of 4-*O*-methylglucuronoxylan. *Agricultural and Biological Chemistry*, 1979, 43: 961-967.

**Hidaka, 1991**

Hidaka H, Tashiro Y & Eida T. Proliferation of bifidobacteria by oligosaccharides and their useful effect on human health. *Bifidobacteria Microflora*, 1991, 10: 65-79.

**Hidaka, 1986**

Hidaka H, Eida T, Takizawa T, Tokunage T & Tashiro Y. Effects of fructooligosaccharides on intestinal flora and human health. *Bifidobacteria Microflora*, 1986, 5: 37-50.

**Honda, 2006**

Honda Y & Kitaoka M. The First glycosynthase derived from an inverting glycoside hydrolase. *Journal of Biological Chemistry*, 2006, 281: 1426-1431.

**Hopkins, 1998**

Hopkins M, Cummings J & MacFarlane G. Inter-species differences in maximum specific growth rates and cell yields of bifidobacteria cultured on oligosaccharides

and other simple carbohydrate sources. *Journal of Applied Microbiology,* 1998, 85: 381-386.

**Hovel, 2003**

Hovel K, Shallom D, Niefind K, Belakhov V, Shoham G, Baasov T, Shoham Y & Schomburg D. Crystal structure and snapshots along the reaction pathway of a family 51 α-L-arabinofuranosidase. *EMBO Journal,* 2003, 22: 4922-4932.

**Howard, 2000**

Howard MD, Kerley MS, Sunvold GD & Reinhart GA. Sources of dietary fiber fed to dogs affects nitrogen and energy metabolism and intestinal microflora populations. *Nutrition Research*, 2000, 20: 1473-1484.

**Howard, 1995**

Howard MD, Gordon DT, Garleb KA & Kerley MS. Dietary fructooligosaccharide, xylooligosaccharide and gum arabic have variable effects on cecal and colonic microbiota and epithelial cell proliferation in mice and rats. *Journal of Nutrition*, 1995, 125: 2604-2609.

**Hromadkova, 1999**

Hromadkova Z, Kovacikova J & Ebringerova A. Study of the classical and ultrasound-assisted extraction of corn cob xylan. *Industrial Crops and Products*, 1999, 9: 101-109.

**Hsu, 2004**

Hsu CK, Liao JW, Chung YC, Hsieh C & Chan YC. Xylooligosaccharides and fructooligosaccharides affect the intestinal microbiota and precancerous colonic lesion development in rats. *Journal of Nutrition*, 2004, 134: 1523-1528.

**Huisman, 2000**

Huisman MMH, Schols HA & Voragen AGJ. Glucurono-arabinoxylans from maize kernel cell walls are more complex than those from sorghum kernel cell walls. *Carbohydrate Polymers*, 2000, 43: 269-279.

**Ichikawa, 2002**

Ichikawa H, Shineha R, Satomi S & Sakata T. Gastric or rectal instillation of short-chain fatty acids stimulates epithelial cell proliferation of small and large intestine in rats. *Digestive Diseases and Sciences*, 2002, 47: 1141-1146.

**Iino, 1997**

Iino T, Nishijima Y, Sawada S, Sasaki H, Harada H, Suwa Y & Kiso Y. Improvement of constipation by small amount of xylooligosaccharides ingestion in adult women. *Journal of Japanese Association of Dietary Fibre Research*, 1997, 1: 19-24.

**Immamura, 1994**

Immamura T, Watanabe T, Kuwahara M & Koshijima T. Ester linkages between lignin and glucuronic acid in lignin-carbohydrate complexes from *Fagus crenata. Phytochemistry*, 1994, 37: 1165-1173.

**Inan, 2000**

Inan MS, Rasoulpour RJ, Yin L, Hubbard AK, Rosenberg DW & Giardina C. The luminal short-chain fatty acid butyrate modulates *NF*-kappa*B* activity in a human colonic epithelial cell line. *Gastroenterology,* 2000, 118: 724-734.

**Iribe, 1984**

Iribe H & Koba T. Augmentation of the proliferative response of thymocytes to phytohemagglutinin by the muramyl dipeptide. *Cell Immunology,* 1984, 88: 9-15.

**Ishi, 1993**

Ishi K, Mohri T, Kinoshita I & Ishihara M. The effects of acidic oligosaccharides to tissue culture of aspen and Japanese black pine. *Transactions of the Japanese Forestry Society*, 1993, 104: 609-110.

**Ishihara, 1997**

Ishihara M, Nojiri M, Hayashi N, Nishimura T & Shimizu K. Screening of fungal β-xylanases for production of acidic xylooligosaccharides using *in situ* reduced 4-O-methylglucuronoxylan as substrate. *Enzyme and Microbial Technology,* 1997, 21: 170-175.

**Ishii, 1997**

Ishii K & Teasdale RD. Effects of xylooligosaccharides on suspension-cultured cells and protoplasts of *Pinus radiate. Plant Cell, Tissue and Organ Culture,* 1997, 49: 189-193.

**Ishii, 1995**

Ishii T & Nishijima T. Inhibition of gibberellin-induced elongation growth of rice by feruloyl oligosaccharides. *Plant Cell Physiology,* 1995, 36: 1447-1451.

**Ishii, 1992**

Ishii K, Kinoshita I, Shigenaga H, IshiharaM, Shimizu K, Nishihara H & Tanaka K. The effects of oligosaccharides on tissue culture of white birch and black pine. *Transactions of the Japanese Forestry Society*, 1992, 103: 485-486.

**Ishizaka, 1993**

Ishizaka S, Kikuchi E & Tsujii T. Effects of acetate on human immune system. *Immunopharmacology and Immunotoxicology*, 1993, 15: 151-162.

**Ito, 1992**

Ito T, Ogasawara H, Sugimoto T & Ishikawa T. Purification and properties of acid stable xylanase from *Aspergillus kawachii*. *Journal of Biotechnology and Biochemistry*, 1992, 56: 547-550.

**Ito, 1990**

Ito M, Deguchi Y, Miyamori A, Matsumoto K, Kikuchi K, Matsumoto K, Kobayashi Y, Yajima T & Kan T. Effects of administration of galactooligosaccharides on the human faecal microflora, stool weight and abdominal sensation. *Microbial Ecology in Health and Disease*, 1990, 3: 285-292.

**Iwasaki, 2000**

Iwasaki K & Matsubara Y. Purification of pectic oligosaccharides showing root-growth-promoting activity in lettuce using ultrafiltration and nanofiltration membranes. *Journal of Bioscience and Bioengineering*, 2000, 89: 495-497.

**Izumi, 2000**

Izumi T, Piskula MK, Osawa S, Obata A, Tobe K, Saito M, Katoaka S, Kubota Y & Kikuchi M. Soy isoflavone aglycones are absorbed faster and in higher amounts than their glycosides in humans. *Journal of Nutrition*, 2000, 130: 1695-1699.

**Izydorczyk, 1998**

Izydorzyk M, Macri L & MacGregor W. Structure and physicochemical properties of barely non-starch polysaccharides-I: Water-extractable β-glucans and arabinoxylans. *Carbohydrate Polymers*, 1998, 35: 249-258.

**Izydorczyk, 1995**

Izydorczyk MS & Biliarderis CG. Cereal arabinoxylan: advances in structure and physicochemical properties. *Carbohydrate Polymers*, 1995, 28: 33-48.

**Jackson, 1997**

Jackson PE, Hall CN, O'Connor PJ, Cooper DP, Margison GP & Povey AC. Low O6-alkylguanine DNA-alkyltransferase activity in normal colorectal tissue is associated with colorectal tumours containing a G→AT transition in the *K*-ras oncogene. *Carcinogenesis*, 1997, 18: 1299-1302.

**Jacobs, 2003**

Jacobs A, Palm M, Zacchi G & Dahlman O. Isolation and characterization of water-soluble hemicelluloses from flax shive. *Carbohydrate Research,* 2003, 338: 1869-1876.

**Jacobs, 2001**

Jacobs A, Larsson PT & Dahlman O. Distribution of uronic acids in xylans from various species of soft- and hardwood as determined by MALDI mass spectrometry. *Biomacromolecules,* 2001, 2: 979-990.

**Jacobsen, 2002**

Jacobsen SE & Wyman CE. Xylose monomer and oligomer yields for uncatalyzed hydrolysis of sugarcane bagasse hemicellulose at varying solids concentration. *Industrial and Engineering Chemistry Research,* 2002, 41: 1454-1461.

**Jacoby, 1994**

Jacoby RF, Hohman C, Marshall DJ, Frick TJ, Schlack S, Broda M, Smutko J & Elliott RW. Genetic analysis of colon cancer susceptibility in mice. *Genomics,* 1994, 22: 381-387.

**Janssen, 1999**

Janssen AM, Bosman CB, Kruidenier L, Griffioxn G, Lamers CB, Van krieken JH, Van de Velde CJ & Verspaget HW. Superoxide dismutases in the human colorectal cancer sequence. *Journal of Cancer Research and Clinical Oncology,* 1999, 125: 327-335.

**Jaskari, 1998**

Jaskari J, Kontula P, Siitonen A, Jousimies-Somer H, Mattila-Sandholm T & Poutanen K. Oat α-glucan and xylan hydrolysates as selective substrates for *Bifidobacterium* and *Lactobacillus* strains. *Applied Microbiology and Biotechnology,* 1998, 49: 175-181.

**Jeon, 1998**

Jeon Y & Kim S. Production of chitooligosaccharides using an ultrafiltration membrane reactor and their antibacterial activity. *Carbohydrate Polymers,* 1998, 41: 133-141.

**Jeong, 1998**

Jeong KJ, Park IY, Kim MS & Kim SC. High-level expression of an endoxylanase from gene *Bacillus sp.* in *Bacillus subtilis* DB104 for the production of xylobiose from xylan. *Applied Microbiology and Biotechnology*, 1998, 50: 113-118.

**Jeusette, 2004**

Jeusette I, Grauwels M, Cuvelier C, Tonglet C, Istasse L & Diez M. Hypercholesterolaemia in a family of rough collie dogs. *Journal of Small Animal Practice*, 2004, 45: 319-324.

**Jiang, 2005**

Jiang Z, Yang S, Yang Q, Li L & Tan S. Optimization of xylanase production by a newly isolated strain CAU 44 of the Thermophile *Thermomyces lanuginosus*. *World Journal Microbiology and Biotechnology,* 2005, 21(6,7): 863-867.

**Jiang, 2004**

Jiang ZQ, Denga W, Zhua YP, Li LT, Sheng YJ & Hayashi K. The recombinant xylanase B of *Thermotoga maritima* is highly xylan specific and produces exclusively xylobiose from xylans, a unique character for industrial applications. *Journal of Molecular Catalysis B: Enzymatic*, 2004, 27: 207-213.

**Jiang, 1992**

Jiang ZY, Hunt JV, & Wolff SP. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxides in low density lipoprotein. *Analytical Biochemistry*, 1992, 202: 384-389.

**Jiang, 1988**

Jiang SQ, Yu GQ, Li ZG & Hong JS. Genetic evidence for modulation of the activator by two regulatory proteins involved in the exogenous induction of phosphoglycerate transport in *Salmonella typhimurium*. *Journal of Bacteriology,* 1988, 170: 4304-4308.

**Johansen, 1996**

Johansen HN, Glitso V & Knudsen KEB. Influence of extraction solvent and temperature on the quantitative determination of oligosaccharides from plant

materials by high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry,* 1996, 44: 1470-1474.

**Kabel, 2002a**

Kabel MA, Carvalheiro F, Garrote G, Avgerinos E, Koukios E, Parajo JC, Gırio FM, Schols HA & Voragen AGJ. Hydrothermally treated xylan rich by-products yield different classes of xylooligosaccharides. *Carbohydrate Polymers,* 2002a, 50: 47-56.

**Kabel, 2002b**

Kabel MA, Kortenoeven L, Schols HA & Voragen AGJ. *In vitro* fermentability of differently substituted xylooligosaccharides. *Journal of Agricultural and Food Chemistry*, 2002b, 50: 6205-6210.

**Kacurakova, 1998**

Kacurakova M, Belton PS, Wilson RH, Hirsch J & Ebringerova A. Hydration properties of xylan-type structures: an FTIR study of xylooligosaccharides. *Journal of Science of Food and Agriculture*, 1998, 77: 38-44.

**Kadowaki, 1997**

Kadowaki MK, Souza CGM, Simao RCG & Peralta RM. Xylanase production by *Aspergillus tamari*. *Applied Biochemistry and Biotechnology*, 1997, 66: 97-106.

**Kacurakova, 1994**

Kacurakova M, Ebringerova A, Hirsch J & Hromadkova Z. Infrared study of arabinoxylans. *Journal of the Science of Food and Agriculture,* 1994, 66: 423-427.

**Kajihara, 2000**

Kajihara M, Kato S, Konishi M, Yamagishi Y, Horie Y & Ishii H, Xylooligosaccharide decreases blood ammonia levels in patients with liver cirrhosis. *American Journal of Gastroenterology,* 2000, 95(9): 2514-2514.

**Kamaleeswari, 2006**

Kamaleeswari M & Nalini N. Dose response efficacy of Caraway *(Carum carvi* L.) on tissue lipid peroxidation and antioxidant profile in rat colon carcinogenesis. *Journal of Pharmacy and Pharmacology,* 2006, 58: 1121-1130.

**Kaneko, 1994**

Kaneko T, Kohmoto T, Kikuchi H, Shiota M, Iino H & Mitsuoka T. Effects of isomaltooligosaccharides with different degrees of polymerization on human fecal bifidobacteria. *Bioscience, Biotechnology and Biochemistry*, 1994, 58: 2288-2290.

**Kardosova, 1998**

Kardosova A, Matulova M & Malovikova A. (4-*O*-Methyl-α-D-glucurono)-D-xylan from *Rudbeckia fulgida* var. sullivantii (Boynton et Beadle). *Carbohydrate Research*, 1998, 308: 99-105.

**Karr, 1991a**

Karr WE & Brink DL. Simplified analysis of acid soluble lignin. *Journal of Wood Chemistry and Technology*, 1991a, 11: 465-477.

**Karr, 1991b**

Karr WE, Cool LG, Merriman MM & Brink DL. The complete analysis of wood polysaccharides using HPLC. *Journal of Wood Chemistry and Technology*, 1991b, 11: 447-463.

**Karr-Lilienthal, 2005**

Karr-Lilienthal LK, Kadzere CT, Grieshop CM & Fahey Jr. GC. Chemical and nutritional properties of soybean carbohydrates as related to non-ruminants. *Livestock Production Science*, 2005, 97: 1-12.

**Katapodis, 2005**

Katapodis P & Christakopoulos P. Xylanases as a tool for the production of novel phytopharmaceuticals. *NutraCos*, 2005, 4: 17-21.

**Katapodis, 2003a**

Katapodis P, Kintzios S, Konstas J, Kekos D, Macris BJ & Christakopoulos P. Enzymic production of aldopentauronic acid and use as a bioregulator in plant airlift bioreactors. *Journal of Bioscience and Bioengineering*, 2003, 95: 630-632.

**Katapodis, 2003b**

Katapodis P, Vardakou M, Kalogeris E, Kekos D, Macris BJ & Christakopoulos P. Enzymic production of a feruloylated oligosaccharide with antioxidant activity from wheat flour arabinoxylan. *European Journal of Nutrition*, 2003, 42: 55-60.

**Katapodis, 2002**

Katapodis P, Kavarnou A, Kintzios S, Pistola E, Kekos D, Macris BJ & Christakopoulos P. Production of acidic xylooligosaccharides by a family 10 endoxylanase from *Thermoascus aurantiacus* and use as plant growth regulators. *Biotechnology Letters*, 2002, 24: 1413-1416.

**Kaur, 2000**

Kaur K, Singh N & Singh H. The effect of extruded flour and fermentation time on some quality parameters of idli. *Journal of Food Quality*, 2000, 23: 15-25.

**Kenneth, 2000**

Kenneth AC. Dietary antioxidants during cancer chemotherapy: impact on chemotherapeutic effectiveness and development of side effects. *Nutrition and Cancer*, 2000, 37: 1-18.

**Kim, 2006**

Kim YW, Fox DT, Hekmat O, Kantner T, McIntosh LP, Antony RJ & Withers SG. Glycosynthase-based synthesis of xylooligosaccharides using an engineered retaining xylanase from *Cellulomonas fimi*. *Organic and Biomolecular Chemistry*, 2006, 4: 2025-2032.

**Kim, 2005a**

Kim Y, Hendrickson R, Mosier N & Ladisch MR. Plug flow reactor for continuous hydrolysis of glucans and xylans from pretreated corn fiber. *Energy Fuels*, 2005, 19: 2189-2200.

**Kim, 2005b**

Kim YW, Chen H & Withers SG. Enzymatic transglycosylation of xylose using a glycosynthase. *Carbohydrate Research*, 2005, 340: 2735-2741.

**Kim, 2004**

Kim YW, Lee SS, Warren RA & Withers SG. Directed evolution of glycosynthase from *Agrobacterium* sp. increases its catalytic activity dramatically and expands its substrate repertoire. *The Journal of Biological Chemistry*, 2004, 279: 42787-42793.

**Kim, 2003**

Kim S, Kim W & Hwang IK. Optimization of the extraction and purification of oligosaccharides from defatted soybean meal. *Int*ernational *Journal of Food Science and Technology*, 2003, 38: 337-342.

**Kim, 1998**

Kim JM, Araki S, Kim DJ, Park CB, Takasuka N, Baba-Toriyama H, Ota T, Nir Z, Khachik F, Shimidzu N, Tanaka Y, Osawa T, Uraji T, Murakoshi M, Nishino H & Tsuda H. Chemopreventive effects of carotenoids and curcumin on mouse colon carcinogenesis after 1,2-dimethylhydrazine initiation. *Carcinogenesis*, 1998, 19: 81-85.

**Kimura, 2002**

Kimura T, Suzuki H, Furuhashi H, Aburatani T, Morimoto K, Sakka K & Ohmiya K. Molecular cloning, characterization and expression analysis of the xynF3 gene from *Aspergillus oryzae*. *Bioscience, Biotechnology and Biochemistry*, 2002, 66(2): 285-292.

**Kimura, 1998**

Kimura I & Tajima S. The modes of action of two endo-1,4-β-D-xylanases from *Aspergillus sojae* on various xylooligosaccharides. *Journal of Fermentation and Bioengineering*, 1998, 85: 283-288.

**Kinzler, 1996**

Kinzler KW & Vogelstein B. Lessons from hereditary colorectal cancer. *Cell*, 1996, 87: 159-170.

**Kitamoto, 1998**

Kitamoto N, Yoshino S, Ito M, Kimura T, Ohmiya K & Tsukagoshi N. Repression of the expression of genes encoding xylanolytic enzymes in *Aspergillus oryzae* by introduction of multiple copies of the *xynF1* promoter. *Applied Microbiology and Biotechnology*, 1998, 50: 558-563.

**Kizawa, 1991**

Kizawa H, Shinoyama H & Yasui T. The synthesis of new xylooligosaccharides by transxylosylation with *Aspergillus niger* β-xylosidase. *Agriculture and Biological Chemistry*, 1991, 55 (3): 671-678.

**Kleessen, 2001**

Kleessen B, Hartmann L & Blaut M. Oligofructose and long-chain inulin: influence on the gut microbial ecology of rats associated with a human faecal flora. *British Journal Nutrition*, 2001, 86: 291-300.

**Kleessen, 1997**

Kleessen B, Sykura B, Zunft HJ & Blaut M. Effects of inulin and lactose on fecal microflora, microbial activity and bowel habit in elderly constipated persons. *American Journal of Clinical Nutrition*, 1997, 65: 1397-1402.

**Klich, 2002**

Klich MA. Biogeography of *Aspergillus* species in soil and litter. *Mycologia*, 2002, 94: 21-27.

**Kobayashi, 1991**

Kobayashi T, Okazaki M, Fujikawa S & Koga K. Effect of xylooligosaccharides on feces of men. *Journal of Japanese Society of Bioscience, Biotechnology and Agrochemistry*, 1991, 65: 1651-1653 (in Japanese).

**Kontula, 1998**

Kontula P, Von Wright A & Mattila-Sandholm T. Oat bran β-gluco- and xylooligosaccharides as fermentative substrates for lactic acid bacteria. *International Journal of Food Microbiology*, 1998, 45: 163-169.

**Koo, 1991**

Koo M & Rao AV. Long term effect of bifidobacteria and neosugar on precursor lesions of colonic cancer in mice. *Nutrition and Cancer*, 1991, 16: 249-257.

**Korte, 1991**

Korte HE, Offermann W & Puls J. Characterization and preparation of substituted xylooligosaccharides from steamed birch wood. *Holzforschung*, 1991, 45: 419-425.

**Kosikoba, 1978**

Kosikoba B, Zakutna L & Joniak D. Investigation of the lignin-saccharidic complex by electron microscopy. *Holzforschung*, 1978, 32(1): 15-18.

**Kotunia, 2004**

Kotunia A, Wolinski J, Laubits D, Jurkowska M, Rome V, Guilloteau P & Zabielski R. Effect of sodium butyrate in the small intestine development in neonatal piglets fed by artificial sow. *Journal of Physiology and Pharmacology***,** 2004, 55: 59-68.

**Kripke, 1989**

Kripke SA, Fox AD, Berman JM, Settle RG & Rombeau JL. Stimulation of intestinal mucosal growth with intracolonic infusion of short-chain fatty acids. *Journal of Parenteral and Enteral Nutrition*, 1989, 13: 109-116.

**Krishnamurthy, 2005**

Krishnamurthy S and Kantha HJ. Food colour measurement: Instrumentation and techniques. *Journal of Instrument Society of India*, 2005, 35(2): 227-238.

**Kukkonen, 2007**

Kukkonen K, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T & Kuitunen M. Probiotics and prebiotic galactooligosaccharides in the prevention of allergic diseases: A randomized, double-blind placebo-controlled trial. *Journal of Allergy and Clinical Immunology*, 2007, 119: 192-198.

**Kulkarni, 1999**

Kulkarni N, Shendye A & Rao M. Molecular and biotechnological aspects of xylanases. *FEMS Microbiology Reviews,* 1999, 23: 411-456.

**Kumagai, 2004**

Kumagai S, Hayashi N, Sakai T, Nakada M & Shibata M. Fractionation and saccharification of cellulose and hemicellulose in rice hull by hot-compressed-water treatment with two-step heating. *Journal of the Japan Institute of Energy,* 2004, 83: 776-781.

**Kumar, 2008**

Kumar R & Wyman CE. The impact of dilute sulfuric acid on the selectivity of xylooligomer depolymerization to monomers. *Carbohydrate Research,* 2008, 343: 290-300.

**Kumar, 1996**

Kumar S & Ramón D. Purification and regulation of the synthesis of β -xylosidase from *Aspergillus nidulans*. *FEMS Microbiology Letters,* 1996, 135: 287-293.

**Kurakake, 2001**

Kurakake M, Kisaka W, Ouchi K & Komaki T. Pretreatment with ammonia water for enzymatic hydrolysis of corn husk, bagasse, and switch grass. *Applied Biochemistry and Biotechnology,* 2001, 90: 251-259.

**Kurita-Ochiai, 2003**

Kurita-Ochiai T, Amano S, Fukushima K, Ochiai K. Cellular events involved in butyric acid-induced T cell apoptosis. *Journal of Immunology,* 2003, 171: 3576-3584.

**Kusakabe, 1983**

Kusakabe I, Ohgushi S, Yasui T & Kobayashi T. Structures of the arabinoxylooligosaccharides from the hydrolytic products of corncob arabinoxylan by a xylanase from *Streptomyces*. *Agricultural and Biological Chemistry,* 1983, 47: 2713-2723.

**Kusakabe, 1977**

Kusakabe I, Kawaguchi M, Yasui T & Kobayashi T. Purification and some properties of extracellular xylanase from *Streptomyces* sp. E-86. *Nippon Nogeikagaku Kaishi,* 1977, 51: 429-437.

**Lee, 2006**

Lee CZ, Liou GY & Yuan GF. Comparison of the aflR gene sequences of strains in *Aspergillus* section Flavi. *Microbiology,* 2006, 152: 161-170.

**Lee, 2001**

Lee T, Oh DW, Kim HS, Lee J, Kim YH, Yun SH & Lee YW. Identification of deoxynivalenol- and nivalenol-producing chemotypes of *Giberella zeae* by using PCR. *Applied and Environmental Microbiology,* 2001, 67(7): 2966-2972

**Lemmel, 1986**

Lemmel SA, Datta R & Frankiewicz JR. Fermentation of xylan by *Clostridium acetobutylicum*. *Enzyme and Microbial Technology,* 1986, 8: 217-221.

**Lenz, 1984**

Lenz J, Schurz J & Bauer J. Die Dickungs- und Geliereigenschaften von Hemicellulosen aus dem Viscoseprozeß. *Das Papier*, 1984, 38: 45-54.

**Levin, 1998**

Levin L & Forschiassin F. Influence of growth conditions on the production of xylanolytic enzymes by *Trametes trogii*. *World Journal of Microbiology and Biotechnology,* 1998, 14: 443-446.

**Levrat, 1991**

Levrat MA, Remesy C & Demigne C. High propionic acid fermentations and mineral accumulation in the cecum of rats adapted to different levels of inulin. *Journal of Nutrition,* 1991, 121: 1730

**Li, 2006**

Li L, Tian H, Cheng Y, Jiang Y & Yang S. Purification and characterization of a thermostable cellulase free xylanase from newly isolated *Paecilomyces themophila*. *Enzyme and Microbial Technology,* 2006, 38: 780-787.

**Li, 2004**

Li W, Li J, Chen T & Chen C. Study on nanofiltration for purifying fructooligosaccharides. *Journal of Membrane Science*, 2004, 245: 123-129.

**Lina, 2002**

Lina BAR, Jonker D & Kozianowsky G. Isomaltulose (Palatinose): A review of biological and toxicological studies. *Food and Chemical Toxicology,* 2002, 40: 1375-1381.

**Lindsay, 2006**

Lindsay JO, Whelan K, Stagg AJ, Gobin P, Al-Hassi HO, Rayment N, Kamm MA, Knight SC & Forbes A. Clinical, microbiological, and immunological effects of fructooligosaccharides in patients with Crohn's disease. *Gut,* 2006, 55, 348-355.

**Liong, 2007**

Liong MT & Shah NP. Sorbitol, maltodextrin, inulin and *Bifidobacterium infantis* modify serum lipid profiles, intestinal microbial population and organic acids concentration in rats. *International Journal of Probiotics and Prebiotics,* 2007, 1: 121-130.

**Liu, 2008**

Liu MQ & Liu GF. Expression of recombinant *Bacillus licheniformis* xylanase A in, *Pichia pastoris* and xylooligosaccharides released from xylans by it. *Protein Expression and Purification,* 2008, 57 101-107.

**Loh, 2006**

Loh G, Eberhard M, Brunner RM, Hennig U, Kuhla S, Kleessen B & Metges CC. Inulin alters the intestinal microbiota and short-chain fatty acid concentrations in growing pigs regardless of their basal diet. *Journal of Nutrition,* 2006, 136: 1198-1202.

**Loo, 1999**

Loo JV, Cummings J, Delzenne N, Englyst H, Franck A, Hopkins M, Kok N, Macfarlane G, Newton D, Quigley M, Roberfroid M, van Vliet T & van den Heuvel E. Functional food properties of non digestible oligosaccharides: A Consensus Report from the ENDO Project (DGXII- AIRII-CT94-1095). *British Journal of Nutrition,* 1999, 81: 121-132.

**Lopez, 1999**

Lopez HW, Levrat MA, Guy C, Messager A, Demigné C & Rémésy C. Effects of soluble corn bran arabinoxylans on cecal digestion, lipid metabolism, and mineral balance (Ca, Mg) in rats. *The Journal of Nutritional Biochemistry,* 1999, 10: 500-509.

**Lowry, 1951**

Lowry OH, Rosebrough NJ, Farr AL & Randall RJ. Protein measurement with Folin's phenol reagent. *Journal of Biological Chemistry,* 1951, 193: 265-275.

**Luo, 2000**

Luo J, Van Yperselle M, Rizkalla SW, Rossi F, Bornet FRJ and Slama G**.** Chronic consumption of short-chain fructooligosaccharides does not affect basal hepatic glucose production or insulin resistance in type 2 diabetes. *Journal of Nutrition,* 2000, 130: 1572-1577.

**Machida, 2005**

Machida M, Asai K, Sano M, Tanaka T, Kumagai T, Terai G, Kusumoto K, Arima T, Akita O, Kashiwagi Y, Abe K, Gomi K, Horiuchi H, Kitamoto K, Kobayashi T, Takeuchi M, Denning DW, Galagan JE, Nierman WC, Yu J, Archer DB, Bennett JW, Bhatnagar D, Cleveland TE, Fedorova ND, Gotoh O, Horikawa H, Hosoyama A, Ichinomiya M, Igarashi R, Iwashita K, Juvvadi PR, Kato M, Kato Y, Kin T, Kokubun A, Maeda H, Maeyama N, Maruyama J, Nagasaki H, Nakajima T, Oda K, Okada K, Paulsen I, Sakamoto K, Sawano T, Takahashi M, Takase K, Terabayashi Y, Wortman JR, Yamada O, Yamagata Y, Anazawa H, Hata Y, Koide Y, Komori T, Koyama Y, Minetoki T, Suharnan S, Tanaka A, Isono K, Kuhara S, Ogasawara N & Kikuchi H. Genome sequencing and analysis of *Aspergillus oryzae*. *Nature*, 2005, 438: 1157-1161.

**Mackey, 1997**

Mackey BM & Gibson GR. *Escherichia coli* 0157 from farm to fork and beyond. *Society of General Microbiology Quarterly*, 1997, 24: 55-57.

**Maeda, 2004**

Maeda H, Ichihashi K, Fujii T, Omura K, Zhu X, Anazawa M & Tazawa K. Oral administration of hydrolyzed rice bran prevents the common cold syndrome in the elderly based on its immunomodulatory action. *Bio Factors*, 2004, 21: 185-187.

**Manning, 2004**

Manning TS & Gibson GR. Prebiotics. *Best Practice and Research Clinical Gastroenterology*, 2004, 18(2): 287-298.

**Manoj, 1999**

Manoj GR, Thampi BSH, Menon VP & Leelamma S. Influence of dietary fiber from coconut kernel (*Cocus nucifera*) on the 1,2-dimethylhydrazine-induced lipid peroxidation in rats. *Journal of Nutrition and Biochemistry,* 1999, 10: 555-560.

**Martenson, 2004**

Martenson JA, Willett CG, Sargent DJ, Mailliard JA, Donohue JH, Gunderson LL, Thomas CR, Fisher B, Benson AB, Myerson R & Goldberg RM. Phase III study of adjuvant chemotherapy and radiation therapy compared with chemotherapy alone in the surgical adjuvant treatment of colon cancer: Results of inter group protocol 0130. *Journal of Clinical Oncology*, 2004, 22: 3277-3283.

**Marui, 2002a**

Marui J, Tanaka A, Mimura S, de Graaff LH, Visser J, Kitamoto N, Kato M, Kobayashi T & Tsukagoshi N. A transcriptional activator, AoXlnR, controls the expression of genes encoding xylanolytic enzymes in *Aspergillus oryzae*. *Fungal Genetics and Biology*, 2002a, 35: 157-169.

**Marui, 2002b**

Marui J, Kitamoto N, Kato M, Kobayashi T & Tsukagoshi N. Transcriptional activator, AoXlnR, mediates cellulose-inductive expression of the xylanolytic and cellulolytic genes in *Aspergillus oryzae*. *FEBS Letters*, 2002b, 528: 279-282.

**Masotti, 1998**

Masotti L, Casali E & Galconi T. Lipid peroxidation in tumor cells. *Free Radical Biology and Medicine*, 1998, 4: 377-386.

**Matamoros Fernandez, 2004**

Matamoros Fernandez LE, Obel N, Scheller HV & Roepstorff P. Differentiation of isomeric oligosaccharide structures by ESI tandem MS and GC-MS. *Carbohydrate Research*, 2004, 339: 655-664.

**Matamoros Fernandez, 2003**

Matamoros Fernandez LE, Obel N, Scheller HV & Roepstorff P. Characterization of plant oligosaccharides by matrix-assisted laser desorption/ionization and electrospray mass spectrometry. *Journal of Mass Spectrometry*, 2003, 38: 427-437.

**Matsubara, 1996**

Matsubara Y, Iwasaki K, Nakajima M, Nabetani H & Nakao S. Recovery of oligosaccharides from steamed soybean waste water in tofu processing by reverse osmosis and nanofiltration membranes. *Biosci*ence, *Biotechnology and Biochemistry*, 1996, 60: 421-428.

**Matsushima, 2001a**

Matsushima K, Chang PK, Yu J, Abe K, Bhatnagar D & Cleveland TE. Pre-termination in aflR of *Aspergillus sojae* inhibits aflatoxin biosynthesis. *Applied Microbiology and Biotechnology*, 2001a, 55: 585-589.

**Matsushima, 2001b**

Matsushima K, Yashiro K, Hanya Y, Abe K, Yabe K & Hamasaki T. Absence of aflatoxin biosynthesis in koji mold *(Aspergillus sojae). Applied Microbiology and Biotechnology*, 2001b, 55: 771-776.

**Mc Lellan, 1991a**

Mc Lellan EA, Medline A & Bird RP. Dose response and proliferate characteristics of aberrant crypt foci: putative preneoplastic lesions in rat colon. *Carcinogenesis*, 1991a, 12: 2093-2098.

**Mc Lellan, 1991b**

Mc Lellan EA, Medline A & Bird RP. Sequential analyses of the growth and morphological characteristics of aberrant crypt foci: putative preneoplastic lesions. *Cancer Research*, 1991b, 51: 5270-5274.

**Mc Lellan, 1988a**

Mc Lellan EA & Bird RP. Aberrant crypts: Potential preneoplastic lesions in the murine colon. *Cancer Research*, 1988, 48: 6187-6192.

**Mc Lellan, 1988b**

Mc Lellan EA & Bird RP. Specificity study to evaluate induction of aberrant crypts in murine colons. *Cancer Research*, 1988, 48: 6183-6186.

**Meyer, 2004**

Meyer PD. Nondigestible oligosaccharides as dietary fiber. *Journal of AOAC International*, 2004, 87: 718-726.

**Migne, 1996**

Migne C, Prensier G, Cornu A & Grenet E. Immunocytochemical localization of arabinoxylans in the cell wall of maize apical inter node after microbial degradation in the rumen. *Biology of the Cell*, 1996, 88: 137-144.

**Mikkelson, 2003**

Mikkelson LL, Jakobsen M & Jensen BB. Effects of dietary oligosaccharides on microbial diversity and fructooligosaccharides degrading bacteria in feces of piglets post-weaning. *Animal Feed Science and Technology*, 2003, 109: 133-150.

**Millard, 2002**

Millard AL, Mertes PM, Ittelet D, Villard F, Jeannesson P & Bernard J. Butyrate affects differentiation, maturation and function of human monocyte-derived dendritic cells and macrophages. *Clinical and Experimental Immunology*, 2002, 130: 245-255.

**Miller, 1959**

Miller GL. Use of dinitrosalicylic acid reagent for the determination of reducing sugar. *Analytical Chemistry*, 1959, 31(3): 426-428.

**Mitsuoka, 1990**

Mitsuoka T. Bifidobacteria and their role in human health. *Journal of Industrial Microbiology*, 1990, 6: 263-268.

**Mitsuoka, 1987**

Mitsuoka T, Hidaka H & Eida T. Effect of fructooligosaccharides on intestinal microflora. *Nahrung*, 1987, 31: 427-436.

**Modler, 1990**

Modler HW, Mckellar RC & Yaguchi M. Bifidobacteria and bifidogenic factors-review. *Canadian Institute of Food Science and Technology Journal*, 1990, 23: 29-41.

**Moen, 1996**

Moen CJA, Groot PC, Hart AAM, Snoek M & Demant P. Fine mapping of colon tumor susceptibility (Scc) genes in the mouse, different from the genes known to be somatically mutated in colon cancer. *Proceedings of the National Academy of Sciences*, 1996, 93: 1082-1086.

**Montane, 2006**

Montane D, Nabarlatz D, Martorell A, Torne-Fernandez V & Fierro V. Removal of lignin and associated impurities from xylooligosaccharides by activated carbon adsorption. *Industrial and Engineering Chemical Research*, 2006, 45: 2294-2302.

**Moore, 2003**

Moore N, Chao C, Yang LP, Storm H, Oliva-Hemker M & Saavedra JM. Effects of fructooligosaccharides-supplemented infant cereal: double-blind, randomized trial. *British Journal of Nutrition*, 2003, 90: 581-587.

**Moro, 2002**

Moro G, Minoli I, Mosca M, Fanaro S, Jelinek J, Stahl B & Boehm G. Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. *Journal of Pediatric Gastroenterology and Nutrition*, 2002, 34: 291-295.

**Morosoli, 1989**

Morosoli R, Durand S & Boucher F. Stimulation of xylanase synthesis in *Cryptococcus albidus* by cyclic AMP. *FEMS Microbiology Letters*, 1989, 57: 57-60.

**Mortensen, 2002**

Mortensen A, Poulsen M & Frandsen H. Effect of a long-chained fructan Raftiline HP on blood lipids and spontaneous atherosclerosis in low density receptor knockout mice. *Nutrition Research*, 2002, 22: 473-480.

**Mosier, 2005**

Mosier NS, Hendrickson R, Brewer M, Ho N, Sedlak M, Dreshel R, Welch G, Dien BS, Aden A & Ladisch MR. Industrial scale-up of pH-controlled liquid hot water pretreatment of corn fiber for fuel ethanol production. *Applied Biochemistry and Biotechnology*, 2005, 125: 77-97.

**Mountzouris, 2006**

Mountzouris KC, Balaskas C, Fava F, Tuohy KM, Gibson GR & Fegeros K. Profiling of composition and metabolic activities of the colonic microflora of growing pigs fed diets supplemented with prebiotic oligosaccharides. *Anaerobe*, 2006, 12: 178-185.

**Moura, 2007**

Moura P, Marques S, Alves L, Freire JPB, Cunha LF & Esteves MP. Effect of xylooligosaccharides from corncobs autohydrolysis on the intestinal microbiota of piglets after weaning. *Livestock Science*, 2007, 108: 244-248.

**Moure, 2006**

Moure A, Gullon P, Dominiguez H & Parajo JC. Advances in the manufacture, purification and applications of xylooligosaccharides as food additives and nutraceuticals. *Process Biochemistry*, 2006, 41(9): 1913-1923.

**Mukherjee, 1965**

Mukherjee SK, Albury MN, Pederson CS, van Veen AG & Steinkraus KH. Role of *Leuconostoc mesenteroides* in leavening the batter of idli, a fermented food of India. *Applied Microbiology*, 1965, 13: 227-231.

**Mumtaz, 2008**

Mumtaz S, Rehman SU, Huma N, Jamil A and Nawaz H. Xylooligosaccharide enriched yoghurt: physicochemical and sensory evaluation. *Pakistan Journal of Nutrition*, 2008, 7(4): 566-569.

**Murakami, 1971**

Murakami H. Classification of the koji mold. *Journal of General and Applied Microbiology*, 1971, 17: 281-309.

**Murosaki, 1999**

Murosaki S, Muroyama K, Yamamoto Y, Kusaka H, Liu T, & Yyoshikai Y. Immunopotentiating activity of nigerooligosaccharides for the T helper 1-like immune response in mice. *Bioscience, Biotechnology and Biochemistry*, 1999, 63, 373-378.

**Mussatto, 2007**

Mussatto SI & Mancilha IM. Non-digestible oligosaccharides: a review. *Carbohydrate Polymers*, 2007, 68: 587-597.

**Nabarlatz, 2007a**

Nabarlatz D, Ebringerova A & Montane D. Autohydrolysis of agricultural by-products for the production of xylooligosaccharides. *Carbohydrate Polymers*, 2007, 69(1): 20-28.

**Nabarlatz, 2007b**

Nabarlatz D, Montane D, Kardosova A, Bekesova S, Hrıbalova V & Ebringerova A. Almond shell xylooligosaccharides exhibiting immunostimulatory activity. *Carbohydrate Research*, 2007, 342: 1122-1128.

**Nabarlatz, 2007c**

Nabarlatz D, Torras C, Garcia-Valls R & Montane D. Purification of xylooligosaccharides from almond shells by ultrafiltration. *Separation and Purification Technology*, 2007, 53(3): 235-243.

**Nabarlatz, 2005**

Nabarlatz D, Farriol X & Montane D. Autohydrolysis of almond shells for the production of xylooligosaccharides: product characteristics and reaction kinetics. *Industrial and Engineering Chemical Research*, 2005, 44: 7746-7755.

**Nabarlatz, 2004**

Nabarlatz D, Farriol X & Montane D. Kinetic modeling of the autohydrolysis of lignocellulosic biomass for the production of hemicellulose derived oligosaccharides. *Industrial and Engineering Chemical Research,* 2004, 43: 4124-4131.

**Nagy, 2002**

Nagy T, Emami K, Fontes CM, Ferreira LM, Humphry DR & Gilbert HJ. The membrane-bound α-glucuronidase from *Pseudomonas cellulosa* hydrolyzes 4-*O*-methyl-D-glucuronoxylooligosaccharides but not 4-*O*-methyl-D-glucuronoxylan. *Journal of Bacteriology*, 2002, 184: 4925-4929.

**Nakagami, 1990**

Nakagami K, Uchida T, Ohwada S, Koibuchi Y & Morishita Y. Increased choline kinase activity in 1,2-dimethylhydrazine-induced rat colon cancer. *Japanese Journal of Cancer Research,* 1990, 90: 1212-1217.

**Naughton, 2001**

Naughton PJ, Mikkelsen LL & Jensen BB. Effects of nondigestible oligosaccharides on *Salmonella enterica* Serovar Typhimurium and non pathogenic *Escherichia coli* in the pig small intestine *in vitro*. *Applied and Environmental Microbiology,* 2001, 67: 3391-3395.

**Neto, 1996**

Neto CP, Seca A, Fradinho D, Coimbra MA, Dominigues F, Evtuguin D, Silvestre A & Cavaleiro JAS. Chemical composition and structural features of the macromolecular components of *Hibiscus cannabinus* grown in Portugal. *Industrial Crops and Products,* 1996, 5: 189-196.

**Nilsson, 1996**

Nilsson M, Saulnier L, Andersson R & Aman PM. Water unextractable polysaccharides from three milling fractions of rye grain. *Carbohydrate Polymers*, 1996, 30: 229-237.

**Nisha, 2005**

Nisha P, Ananthanarayan L & Singhal RS. Effect of stabilizers on stabilization of idli (traditional south Indian food) batter during storage. *Food Hydrocolloids,* 2005, 19, 179-186.

**Nishimura, 1998**

Nishimura T, Ishihara N, Ishii T & Kato K. Structure of neutral branched xylooligosaccharides produced by xylanase from *in situ* reduced hardwood xylan. *Carbohydrate Polymers*, 1998, 308: 117-122.

**Niu, 1995**

Niu S, Jiang SQ & Hong J. *Salmonella typhimurium pgtB* mutants conferring constitutive expression of phosphoglycerate transporter *pgtP* independent of *pgtC*. *Journal of Bacteriology*, 1995, 177: 4297-4302.

**Nout, 1999**

Nout MJR & Sarkar PK. Lactic acid food fermentation in tropical climates. *Antonie van Leeuwenhoek*, 1999, 76, 395-401.

**Nout, 1993**

Nout MJR. Processed weaning foods for tropical climates. *International Journal of Food Science and Nutrition*, 1993, 43: 213-221.

**Ntarima, 2000**

Ntarima P, Nerinckx W, Klarslov K, Devreese B, Bhat MK, Van Beeumen J & Claeyssens M. Epoxy alkyl glycosides of D-xylose and xylooligosaccharides are active-site markers of xylanases from glycoside family 11, not from family 10. *Biochemistry Journal*, 2000, 347: 865-873.

**Ohara, 2006**

Ohara H, Owaki M & Sonomoto K. Xylooligosaccharide fermentation with *Leuconostoc lactis*. *Journal of Bioscience and Bioengineering*, 2006, 101(5): 415-420.

**Ohta, 1998a**

Ohta A, Ohtsuki M, Hosono A, Adachi T, Hara H & Sakata T. Dietary fructooligosaccharides prevent osteopenia after gastrectomy in rats. *Journal of Nutrition*, 1998, 128: 106-110.

**Ohta, 1998b**

Ohta A, Ohtsuki M, Uehara M, Hosono A, Hirayama M, Adachi T & Hara H. Dietary fructooligosaccharides prevent postgastrectomy anemia and osteopenia in rats. *Journal of Nutrition*, 1998, 128: 485-490.

**Ohta, 1995**

Ohta A, Ohtsuki M, Baba S, Adachi T, Sakata T & Sakaguchi E. Calcium and magnesium absorption from the colon and rectum are increased in rats fed fructooligosaccharides. *Journal of Nutrition*, 1995, 125: 2417-2424.

**Ohta, 1994**

Ohta A, Ohtsuki M, Takizawa T, Inaba H, Adachi T & Kimura S. Effects of fructooligosaccharides on the absorption of magnesium and calcium by cecectomized rats. *International Journal of Vitamin and Nutrition Research*, 1994, 64: 316-323.

**Okazaki, 1991**

Okazaki M, Koda H, Izumi R, Fujikawa S & Matsumoto N. *In vitro* digestibility and *in vivo* utilization of xylobiose. *Journal of the Japanese Society of Nutrition and Food Science*, 1991, 44: 41-44.

**Okazaki, 1990a**

Okazaki M, Fujikawa S & Mastumoto N. Effects of xylooligosaccharides on growth of *Bifidobacteria*. *Bifidobacteria Microflora*, 1990, 9: 77-86.

**Okazaki, 1990b**

Okazaki M, Fujikawa S & Matsumoto N. Effects of xylooligosaccharide on growth of bifidobacteria. *Journal of the Japanese Society of Nutrition and Food Science*, 1990, 43: 395-401.

**Olano-Martin, 2001**

Olano-Martin E, Mountzouris KC, Gibson GR & Rastall RA. Continuous production of oligosaccharides from pectin in an enzyme membrane reactor. *Journal of Food Science*, 2001, 66: 966-971.

**Olano-Martin, 2000**

Olano-Martin E, Mountzouris KC, Gibson GR & Rastall RA. *In vitro* fermentability of dextran, oligodextran and maltodextrin by human gut bacteria. *British Journal of Nutrition*, 2000, 83: 247-255.

**Oshima, 1996**

Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF & Taketo MM. Suppression of intestinal polyposis in Apc delta-716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell*, 1996, 29: 803-809.

**Otori, 1995**

Otori K, Sugiyama K, Hasebe T, Fukushima S & Esumi H. Emergence of adenomatous aberrant crypt foci (ACF) from hyperplastic ACF with concomitant increase in cell proliferation. *Cancer Research*, 1995, 55: 4743-4746.

**Ozlem, 2007**

Ozlem A, Ak O, Kavas A, Bakir U & Yilmaz L. Enzymatic production of xylooligosaccharides from cotton stalks. *Journal of Agricultural and Food Chemistry*, 2007, 55: 5544-5551.

**Padhye, 1978**

Padhye VW & Salunkhe DK. Biochemical studies on black gram *(Phaseolus mungo* L.): Fermentation of black gram and rice blend and its influence on the *in vitro* digestibility of the proteins. *Journal of Food Biochemistry*, 1978, 2: 327-347.

**Palm, 2003**

Palm M & Zacchi G. Extraction of hemicellulosic oligosaccharides from spruce using microwave oven or steam treatment. *Biomacromolecules*, 2003, 4: 617-623.

**Parajo, 2004**

Parajo JC, Garrote G, Cruz JM & Domínguez H. Production of xylooligosaccharides by autohydrolysis of lignocellulosic materials. *Trends in Food Science and Technology*, 2004, 15: 115-120.

**Parajo, 1995**

Parajo JC, Alonso JL & Santos V. Kinetics of *Eucalyptus* wood fractionation in acetic acid-HCl-water media. *Bioresource Technology*, 1995**,** 51: 153-162.

**Parajo, 1994**

Parajo JC, Vazquez D, Alonso JL, Santos V & Dominguez H. Prehydrolysis of *Eucalyptus* wood with dilute sulphuric acid: operation in autoclave. *Holz als Roh-und Werkstoff*, 1994, 52: 102-108.

**Parajo, 1993**

Parajo JC, Vazquez D, Alonso JL, Santos V & Dominguez, H Prehydrolysis of *Eucalyptus* wood with dilute sulphuric acid: operation at atmospheric pressure. *Holz als Roh-und Werkstoff*, 1993, 51: 357- 363.

**Park, 1997**

Park HS, Goodlad RA & Wright NA. The incidence of aberrant crypt foci and colonic carcinoma in dimethylhydrazine-treated rats varies in a site-specific manner and depends on tumor histology. *Cancer Research*, 1997, 57: 4507-4510.

**Park, 1991**

Park YK & Almeida MM. Production of fructooligosaccharides from sucrose by a transfructosylase from *Aspergillus niger*. *World Journal of Microbiology and Biotechnology*, 1991, 7: 331-334.

**Parkin, 2005**

Parkin DM, Bray F, Ferlay J & Pisani P. Global cancer statistics 2002. *CA Cancer Journal for Clinicians*, 2005, 55: 74-108.

**Paronen, 1985**

Paronen P, Juslin M & Kasnanen K. Comparison of xylan and some commercial materials as disintegrants in tablets. *Drug Development and Industrial Pharmacy*, 1985, 11: 405-429.

**Pasanen, 2007**

Pasanen S, Janis J & Vainiotalo P. Cello-, malto- and xylooligosaccharide fragmentation by collision-induced dissociation using QIT and FT-ICR mass spectrometry: A systematic study. *International Journal of Mass Spectrometry*, 2007, *263*: 22–29.

**Pece, 1994**

Pece N, Perotti N & Molina O. Bacterial protein production from corncob pretreated with NaOH at room temperature. *World Journal of Microbiology*, 1994, 10: 593-594.

**Peciarová, 1982**

Peciarová A & Biely P. β-xylosidases and a nonspecific wall-bound β-glucosidase of the yeast *Cryptococcus albidus*. *Biochimica et Biophysica Acta*, 1982, 716: 391-399.

**Pellerin, 1991**

Pellerin P, Gosselin M, Lepoutre JP, Samain E & Debeire P. Enzymatic production of oligosaccharides from corncob xylan. *Enzyme and Microbial Technology*, 1991, 13: 617-622.

**Pereira, 1994**

Pereira MA, Barnes LH, Rassman VL, Kolloff GV & Steele VE. Use of azoxymethane-induced foci of aberrant crypts in rat colon to identify potential cancer chemopreventive agents. *Carcinogenesis*, 1994, 15: 1049-1054.

**Perez-Gonzalez, 1998**

Perez-Gonzalez JA, van Peij NNME, Bezoen A, MacCabe AP, Ramon D & de Graaff LH. Molecular cloning and transcriptional regulation of the *Aspergillus nidulans xlnD* gene encoding β-xylosidase. *Applied and Environmental Microbiology*, 1998, 64: 1412-1419.

**Perrin, 2000**

Perrin S, Grill JP & Schneider F. Effects of fructooligosaccharides and their monomeric components on bile salt resistance in three species of bifidobacteria. *Journal of Applied Microbiology*, 2000, 88: 968-974.

**Pham, 1998**

Pham PL, Taillandier P, Delmas M & Strehaiano P. Production of xylanases by *Bacillus polymyxa* using lignocellulosic wastes. *Industrial Crops and Products*, 1998, 7: 195-203.

**Povey, 2000**

Povey AC, Hall CN, Badawi AF, Cooper DP & O'Connor PJ. Elevated levels of the pro-carcinogenic adduct, *O*-6-methylguanine, in normal DNA from the cancer prone regions of the large bowel. *Gut*, 2000, 47: 362-365.

**Pretlow, 1992a**

Pretlow TP, O' Riordan MA, Somich GA, Amini SB & Pretlow TG. Aberrant crypts correlate with tumor incidence in F344 rats treated with azoxymethane and phytate. *Carcinogenesis*, 1992, 13: 1509-1512.

**Pretlow, 1992b**

Pretlow TP, O'Riordan MA, Pretlow TG & Stellato TA. Aberrant crypts in human colonic mucosa: putative preneoplastic lesions. *Journal of Cellular Biochemistry-Supplement*, 1992, 16G: 55-62.

**Pretlow, 1991**

Pretlow TP, Barrow BJ, Ashton WS, O'Riordan MA, Pretlow TG, Jurcisek JA & Stellato TA. Aberrant crypt foci: Putative preneoplastic foci in human colonic mucosa. *Cancer Research*, 1991, 51: 1564-1567.

**Proksch, 1987**

Proksch A & Wagner H. Structural analysis of a 4-*O*-methy-glucuronoarabinoxylan with immunostimulating activity from *Echinacea purpurea*. *Phytochemistry*, 1987, 26: 1989-1993.

**Puls, 1985**

Puls J, Poutanen R, Korner HU & Viikari L. Biotechnical utilization of wood carbohydrates after steaming pretreatment. *Applied Microbiology and Biotechnology*, 1985, 22: 416-423.

**Qiao, 2002**

Qiao H, Duffy LC, Griffiths E, Dryja D, Leavens A, Rossman J, Rich G, Riepenhoff-Talty M & Locniskar M. Immune responses in rhesus rotavirus-challenged Balb/c mice treated with bifidobacteria and prebiotic supplements. *Pediatric Research*, 2002, 51: 750-755.

**Quemener, 2006**

Quemener B, Ordaz-Ortiz JJ & Saulnier L. Structural characterization of underivatized arabino-xylo-oligosaccharides by negative-ion electrospray mass spectrometry. *Carbohydrate Research*, 2006, 341(11): 1834-1847.

**Rafter, 2007**

Rafter J, Bennett M, Caderni G, Clune Y, Hughes R, Karlsson PC, Klinder A, O'Riordan M, O'Sullivan GC, Pool-Zobel B, Rechkemmer G, Roller M, Rowland I, Salvadori M, Thijs H, Loo JV, Watzl B & Collins JK. Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. *American Journal of Clinical Nutrition*, *2007*, 85: 488-496.

**Rajeshkumar, 2003**

Rajeshkumar NV & Kuttan R. Modulation of carcinogenic response and antioxidant enzymes of rats administered with 1,2- dimethylhydrazine by Picroliv. *Cancer Letters*, 2003, 191: 137-143.

**Ramos, 1992**

Ramos LP, Breuil C, Kushner DJ & Saddler JN. Steam pretreatment conditions for effective enzymatic hydrolysis and recovery of *Eucalyptus viminalis* wood chips. *Holzforschung*, 1992, 46: 149-154.

**Rani, 1996**

Rani S & Nand K. Development of cellulase-free xylanase producing anaerobic consortia for the use of lignocellulosic wastes. *Enzyme and Microbial Technology*, 1996, 18: 23-28.

**Rao, 2002**

Rao U, Marui J, Kato M, Kobayashi T & Tsukagoshi N. Regulation of the xylanase gene, *cgxA*, from *Chaetomium gracile* by transcriptional factors, XlnR and AnRP. *Biotechnology Letters*, 2002, 24: 1089-1096.

**Rault-Nania, 2006**

Rault-Nania MH, Gueux E, Demougeot C, Demigne C, Rock E & Mazur A. Inulin attenuates atherosclerosis in apolipoprotein E-deficient mice. *British Journal of Nutrition*, 2006, 96: 840-844.

**Reddy, 1999**

Reddy BS. Possible mechanisms by which pro- and prebiotics influence colon carcinogenesis and tumor growth. *Journal of Nutrition***,** 1999, 129: 1478S-1482S.

**Reddy, 1997**

Reddy BS, Hamid R & Rao CV. Effect of dietary oligofructose and inulin on colonic preneoplastic aberrant crypt foci inhibition. *Carcinogenesis*, 1997, 18: 1371-1374.

**Reddy, 1980**

Reddy NR & Salunkhe DK. Changes in oligosaccharides during germination and cooking of black gram and fermentation of black gram-rice blend. *Cereal Chemistry*, 1980, 57*:* 356-360.

**Redgwell, 2005**

Redgwell RJ & Fischer M. Dietary fiber as a versatile food component: An industrial perspective. *Molecular Nutrition and Food Research*, 2005, 49: 421-535.

**Reeves, 1997**

Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. *Journal of Nutrition*, 1997, 127: S838-S841.

**Reis, 2004a**

Reis A, Pinto P, Evtuguin DV, Neto CP, Domingues P, Ferrer- Correia AJ & Domingues MRM. Electrospray tandem mass spectrometry of underivatised acetylated xylooligosaccharides. *Rapid Communications in Mass Spectrometry*, 2005, 19: 3589-3599.

**Reis, 2004b**

Reis A, Pinto P, Coimbra MA, Evtuguin DV, Neto CP, Ferrer-Correia AJ & Domingues MRM. Structural differentiation of uronosyl sbstitution patters in acidic heteroxylans by electrospray tandem mass spectrometry. *Journal of the American Society for Mass Spectrometry,* 2004b, 15: 43-47.

**Reis, 2004c**

Reis A, Coimbra MA, Domingues P, Correia AJF, Rosario M & Domingues M. Fragmentation pattern of underivatised xylooligosaccharides and their alditol derivatives by electrospray tandem mass spectrometry. *Carbohydrate Polymers*, 2004, 55: 401-409.

**Reis, 2003b**

Reis A, Domingues MRM, Domingues P, Ferrer-Correia AJ & Coimbra MA. Positive and negative electrospray ionization tandem mass spectrometry as a tool for structural characterization of acid released oligosaccharides from olive pulp glucuronoxylans. *Carbohydrate Research,* 2003b, 338: 1497-1505.

**Reis, 2003a**

Reis A, Domingues MRM, Ferrer-Correia AJ & Coimbra MA. Structural characterization by MALDI-MS of olive xylooligosaccharides obtained by partial acid hydrolysis. *Carbohydrate Polymers*, 2003a, 53: 101-107.

**Reis, 2002**

Reis A, Coimbra MA, Domingues P, Ferrer-Correia AJ & Domingues MRM. Structural characterization of underivatised olive pulp xylooligosaccharides by mass spectrometry using matrix assisted laser desorption/ionisation and electrospray ionisation. *Rapid Communications in Mass Spectrometry*, 2002, 16: 2124-2132.

**Roberfroid, 2000**

Roberfroid MB & Slavin J. Nondigestible oligosaccharides. *Critical Reviews in Food Science and Nutrition,* 2000, 40: 461-480.

**Roberfroid, 1998a**

Roberfroid MB and Delzenne NM. Dietary fructans. *Annual Review of Nutrition*, 1998, 18 (1): 117-143.

**Roberfroid, 1998b**

Roberfroid MB, van Loo JAE & Gibson GR. The bifidogenic nature of chicory inulin and its hydrolysis products. *Journal of Nutrition*, 1998, 128: 11-19.

**Roe, 1943**

Roe JM & Kuether CA. Detection of ascorbic acid in whole blood, and urine through 2,4-DNPH derivative of dehydroascorbic acid. *Journal of Biological Chemistry,* 1943, 147: 399-407.

**Roncucci, 1991**

Roncucci L, Medline A & Bruce WR. Classification of aberrant crypt foci and microadenomas in human colon. *Cancer Epidemiology Biomarkers and Prevention,* 1991, 1: 57-60.

**Roncucci , 1991**

Roncucci L, Stamp D, Medline A, Cullen JB & Bruce WR. Identification and qualification of aberrant crypt foci and adenoma in the human colon. *Human Pathology,* 1991, 22: 287-294.

**Rycroft, 2001**

Rycroft CE, Jones MR, Gibson GR & Rastall RA. A comparative *in vitro* evaluation of the fermentation properties of prebiotic oligosaccharides. *Journal of Applied Microbiology,* 2001, 91: 878-887.

**Saemann, 2000**

Saemann MD, Bohmig GA, Sterreicher CH, Burtscher H, Parolini O, Diakos C, Stockl J, Horl WH & Zlabinger GJ. Anti-inflammatory effects of sodium butyrate on human monocytes: Potent inhibition of IL-12 and up-regulation of IL-10 production. *The Federation of American Societies for Experimental Biology Journal,* 2000, 14: 2380-2382.

**Sajjan, 1986**

Sajjan SU & Salimath PV. Isolation of a neutral linear xylan from guar seed husk. *Carbohydrate Research,* 1986, 145: 348-350.

**Sako, 1999**

Sako T, Matsumoto K & Tanaka R. Recent progress on research and applications of non-digestible galactooligosaccharides. *International Dairy Journal,* 1999, 9: 69-80.

**Samain, 1997**

Samain E, Debeire PH & Touzel JP. High level production of a cellulase-free xylanase in glucose-limited fed batch cultures of a thermophilic bacillus strain. *Journal of Biotechnology,* 1997, 58: 71-80.

**Samuelsen, 1999**

Samuelsen AB, Cohen EH, Paulsen BS, Brull LP & Thomas- Oates JE. Structural studies of a heteroxylan from *Plantago major* L. seeds by partial acid hydrolysis, HPAED-PAD, methylation and GC-MS, ES-MS and ESMS/MS. *Carbohydrate Research,* 1999, 315: 312-318.

**Samuelsen, 1995**

Samuelsen AB, Paulsen BS, Wold JK, Otsuka H, Yamada H & Espevik T. Isolation and partial characterization of biologically active polysaccharides from *Plantago major* L. *Phytotherapy Research,* 1995, 9: 211-218.

**Sanchez-Mata, 1998**

Sanchez-Mata MC, Penuela-Teruel MJ, Camara-Hurtado M, Daez- Marques C & Torija-Isasa ME. Determination of mono-, di-, and oligosaccharides in legumes by high-performance liquid chromatography using an amino-bonded silica column. *Journal of Agricultural and Food Chemistry,* 1998, 46: 3648-3652.

**Sangeetha, 2005**

Sangeetha PT, Ramesh MN & Prapulla SG. Recent trends in the microbial production, analysis and application of fructooligosaccharides. *Trends in Food Science and Technology,* 2005, 16: 442-457.

**Sanger, 1977**

Sanger F, Nicklen S & Coulson AR. DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences,* 1977, 74: 5463-5467.

**Sanz, 2005**

Sanz ML, Polemis N, Morales V, Corzo N, Drakoularakou A, Gibson GR & Rastall RA. *In vitro* investigation into the potential prebiotic activity of honey oligosaccharides. *Journal of Agricultural and Food Chemistry,* 2005, 53: 2914-2921.

**Saska, 1995**

Saska M & Ozer E. Aqueous extraction of sugarcane bagasse hemicellulose and production of xylose syrup. *Biotechnology and Bioengineering,* 1995, 45: 517-523.

**Schmelz, 2000**

Schmelz EM, Sullards MC & Dillachay DL. Colonic cell proliferation and aberrant crypt foci formation are inhibited by dairy glycerosphingolipid in 1,2-dimethylhydrazine-treated CFI mice. *Journal of Nutrition,* 2000, 130: 522-527.

**Schmitt, 1998**

Schmitt C, Sanchez C, Desobry-Banon S & Hardy J. Structure and techno-functional properties of protein-polysaccharide complexes: A review. *Critical Reviews in Food Science and Nutrition*, 1998, 38*:* 689-753.

**Schonfeld, 1926**

Schonfeld H. Uber die Beziehungen der einzelnen Bestandteile der Frauenmilch zur Bifidusflora. *Jahrb Kinderheilkd,* 1926, 113: 19-60.

**Schwarz, 2001**

Schwarz WH. The cellulosome and cellulose degradation by anaerobic bacteria. *Applied Microbiology and Biotechnology,* 2001, 56: 634-649.

**Shallom, 2004**

Shallom D, Golan G, Shoham G & Shoham Y. Effect of dimmer dissociation on activity and thermostability of the α-glucuronidase from *Geobacillus stearothermophilus*: dissecting the different oligomeric forms of family 67 glycoside hydrolases. *Journal of Bacteriology*, 2004, 186: 6928-6937.

**Shallom, 2003**

Shallom D & Shoham Y. Microbial hemicellulases. *Current Opinions in Microbiology*, 2003, 6: 219-228.

**Sheih, 2001**

Sheih YH, Chiang BL, Wang LH, Liao CK & Gill HS. Systemic immunity-enhancing effects in healthy subjects following dietary consumption of lactic acid bacterium *Lactobacillus rhamnosus* HN001. *Journal of American College of Nutrition*, 2001, 20: 149-156.

**Shim, 2005**

Shim SB, Verstegen MWA, Kim IH, Kwon OS & Verdonk JMAJ. Effects of feeding antibiotic-free creep feed supplemented with oligofructose, probiotics or synbiotics to suckling piglets increases the preweaning weight gain and composition of intestinal microbiota. *Archives of Animal Nutrition*, 2005, 59: 419-427.

**Shin, 2003**

Shin HY, Lee JH, Han YO, Han MJ & Kim DH. Purification and characterization of α-L-arabinopyranosidase and α-L-arabinofuranosidase from *Bifidobacterium breve* K-110, a human intestinal anaerobic bacterium metabolizing ginsenoside Rb2 and Rc. *Applied Environmental and Microbiology*, 2003, 69: 7116-7123.

**Shinoyama, 1988a**

Shinoyama H & Yasui T. Superiority of *Aspergillus niger* β-xylosidase for the enzymatic synthesis of alkyl β-xylosides in the presence of a variety of alcohols. *Agricultural and Biological Chemistry*, 1988a, 52: 2375-2377.

**Shinoyama, 1988b**

Shinoyama H, Kamiyama Y & Yasui T. Enzymatic synthesis of alkyl β-xylosides from xylobiose by application of the transxylosylation reaction of *Aspergillus niger* β-xylosidase. *Agricultural and Biological Chemistry*, 1988b, 52: 2197-2202.

**Shivapurkar, 1992**

Shivapurkar N, Tang ZC & Alabaster O. The effect of high-risk and low-risk diets on aberrant crypt and colonic tumor formation in Fischer-344 rats. *Carcinogenesis*, 1992, 13: 887-890.

**Shoham, 1999**

Shoham Y, Lamed R & Bayer EA. The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. *Trends in Microbiology*, 1999, 7: 275-281.

**Shulami, 2007**

Shulami S, Zaide G, Zolotnitsky G, Langut Y, Feld G, Sonenshein AL, & Shoham Y. A two-component system regulates the expression of an ABC transporter for xylooligosaccharides in *Geobacillus stearothermophilus*. *Applied and Environmental Microbiology*, 2007, 73: 874-884.

**Shulami, 1999**

Shulami S, Gat O, Sonenshein AL & Shoham Y. The glucuronic acid utilization gene cluster from *Bacillus stearothermophilus* T-6. *Journal of Bacteriology*, 1999, 181: 3695-3704.

**Silvi, 1999**

Silvi S, Rumney CJ, Cresci A & Rowland IR. Resistant starch modifies gut microflora and microbial metabolism in human flora-associated rats inoculated with faeces from Italian and UK donors. *Journal of Applied Microbiology*, 1999, 86: 521-530.

**Singh, 2003**

Singh S, Madlala AM & Prior BA. *Thermomyces lanuginosus*: properties of their strains and their hemicellulases. *FEMS Microbiology Reviews*, 2003, 27: 3-16.

**Singh, 2002**

Singh M, Sharma R & Banerjee VC. Biotechnological applications of cyclodextrins. *Biotechnology Advances,* 2002, 20: 341-359.

**Singh, 1995**

Singh N, Bawa AS & Sekhon KS. Quality improvement of idli using extruded rice flour. *Journal of Food Quality,* 1995, 18: 193-202.

**Sinha, 1972**

Sinha KA. Colorimetric assay of catalase. *Analytical Biochemistry*, 1972, 47: 389-394.

**Smiricky-Tjardes, 2003a**

Smiricky-Tjardes MR, Flickinger EA, Grieshop CM, Bauer LL, Murphy MR & Fahey GC Jr. *In vitro* fermentation characteristics of selected oligosaccharides by swine fecal microflora. *Journal of Animal Science,* 2003, 81: 2505-2514.

**Smiricky-Tjardes, 2003b**

Smiricky-Tjardes MR, Grieshop CM, Flickinger EA, Bauer LL & Fahey GC. Dietary galactooligosaccharides affect ileal and total-tract nutrient digestibility, ileal and fecal bacterial concentrations, and ileal fermentative characteristics of growing pigs. *Journal of Animal Science,* 2003, 81: 2535-2545.

**Smitha, 2008**

Smitha S, Rajiv J, Begum K & Indrani D. Effect of hydrocolloids on rheological, microstructural and quality characteristics of parotta- unleavened Indian flat bread. *Journal of Texture Studies,* 2008, 39: 267-283.

**Sohn, 1985**

Sohn OS, Puz C, Caswell N & Fiala ES. Differential susceptibility of rat and guinea pig colon mucosa DNA to methylation by methylazoxymethyl acetate *in vivo*. *Cancer Letters,* 1985, 29: 293-300.

**Soni, 1990**

Soni SK & Sandhu DK. Indian fermented foods: Microbiological and biochemical aspects. *Indian Journal of Microbiology,* 1990, 30(2): 135-157.

**Soni, 1989**

Soni SK & Sandhu DK. Fermentation of idli: Effects of changes in raw materials and physico-chemical conditions. *Journal of Cereal Science,* 1989, 10: 227-238.

**Sparkes, 1998**

Sparkes AH, Papasouliotis K, Sunvold G, Werrett G, Gruffyd-Jones EA, Egan K, Gruffyd-Jones TJ & Reinhart G. Effects of dietary supplementation with fructooligosaccharides on fecal flora of healthy cats. *American Journal of Veterinary Research*, 1998, 59: 436-440.

**Steinkraus, 1967**

Steinkraus KH, van **V**een AG & Thiebeau DB. Studies on idli-An Indian fermented black gram-rice food. *Food Technology*, 1967, 21: 110-113.

**Stevens, 1988**

Stevens BJ & Selvendran RR. Changes in composition and structure of wheat bran resulting from the action of human faecal bacteria *in vitro*. *Carbohydrate Research*, 1988, 183: 311-319.

**Stewart, 1997**

Stewart D, Azzini A, Hall AT & Morrison IM. Sisal fibres and their constituent non-cellulosic polymers. *Industrial Crops and Products*, 1997, 6: 17-26.

**Stock, 2000**

Stock AM, Robinson VL & Goudreau PN. Two-component signal transduction. *Annual Reviews in Biochemistry*, 2000, 69: 183-215.

**Stominska, 2004**

Stominska L & Grzeskowiak-Przywecka A. Study on the membrane filtration of starch hydrolysates. *Desalination*, 2004, 162: 255-261.

**Sugimura, 2006**

Sugimura M, Nishimoto M & Kitaoka M. Characterization of glycosynthase mutants derived from glycoside hydrolase family 10 xylanases. *Bioscience, Biotechnology and Biochemistry*, 2006, 70: 1210-1217.

**Sun, 2007**

Sun JY, Liu MQ, Weng XY, Qian LC & Gu SH. Expression of recombinant *Thermomonospora fusca* xylanase A in *Pichia pastoris* and xylooligosaccharides released from xylans by it. *Food Chemistry*, 2007, 104: 1055-1064.

**Sun, 2005**

Sun XF, Xu F, Sun RC, Geng ZC, Fowler P & Baird MS. Characteristics of degraded hemicellulosic polymers obtained from steam exploded wheat straw. *Carbohydrate Polymers*, 2005, 60: 15-26.

**Sun, 2000**

Sun RC, Tomkinson J, Ma PL & Liang SF. Comparative study of hemicelluloses from rice straw by alkali and hydrogen peroxide treatments. *Carbohydrate Polymers*, 2000, 42: 111-122.

**Sun, 1998**

Sun R, Lawther JM & Banks WB. Isolation and characterization of hemicellulose B and cellulose from pressure refined wheat straw. *Industrial Crops and Products*, 1998, 7: 121-128.

**Susheelamma, 1978**

Susheelamma NS & Rao MVL. Isolation and characterization of arabino-galactan from black gram (*Phaseolus mungo*). *Journal of Agricultural and Food Chemistry*, 1978, 26: 1434-1437.

**Suzuki, 2002**

Suzuki T, Tomita-Yokotami K, Tsubura H, Yoshida S, Kusakabe I, Yamada K, Miki Y & Hasegawa K. Plant growth-promoting oligosaccharides produced from tomato waste. *Bioresource Technology*, 2002, 91-96.

**Swamy, 1990**

Swamy NR & Salimath PV. Structural features of acidic xylans isolated from red gram (*Cajanus cajan*) husk. *Carbohydrate Research*, 1990, 190: 327-337.

**Swennen, 2005**

Swennen K, Courtin CM, Van der Bruggen B, Vandecasteele C & Delcour JA. Ultrafiltration and ethanol precipitation for isolation of arabino xylooligosaccharides with different structures. *Carbohydrate Polymers*, 2005, 62: 283-292.

**Taguchi, 1996**

Taguchi F, Hasegawa K, Saito-Taki T & Hara K. Simultaneous production of xylanase and hydrogen using xylan in batch culture of *Clostridium* sp. strain X53. *Journal of Fermentation and Bioengineering*, 1996, 81: 178-180.

**Tahiri, 2001**

Tahiri M, Tressol JC, Arnaud J, Bornet F, Bouteloup-Demange C, Feillet-Coudray C, Ducros V, Pepin D, Brouns F, Rayssiguier AM & Coudray C. Five-week intake of short-chain fructooligosaccharides increases intestinal absorption and status of magnesium in postmenopausal women. *Journal of Bone and Mineral Research*, 2001, 16: 2152-2160.

**Takahashi, 1998**

Takahashi T, Nakagawa E, Nara T, Yajima T & Kuwata T. Effects of orally ingested *Bifidobacterium longum* on the mucosal IgA response of mice to dietary antigens. *Bioscience, Biotechnology and Biochemistry,* 1998, 62: 10-15.

**Takeshi, 1998**

Takeshi N, Ishihara M, Ishii T & Kato A. Structure of neutral branched xylooligosaccharides produced by xylanase from *in situ* reduced hard wood xylan. *Carbohydrate Research*, 1998, 308: 117-122.

**Tan, 2008**

Tan SS, Li DY, Jiang ZQ, Zhu YP, Shi B & Li LT. Production of xylobiose from the autohydrolysis explosion liquor of corncob using *Thermotoga maritima* xylanase B (XynB) immobilized on nickel-chelated Eupergit C. *Bioresource Technology*, 2008, 99: 200-204.

**Tanaka, 1996**

Tanaka T & Mori H. Inhibition of colon carcinogenesis by nonnutritive constituents in foods. *Journal of Toxicology and Pathology*, 1996, 9: 139-149.

**Tanaka, 1999**

Tanaka T, Kawabata K, Kakumoto M, Makita H, Ushida J, Honjo S, Hara A, Tsuda H & Mori H. Modifying effects of a flavonoid morin on azoxymethane-induced large bowel tumorigenesis in rats. *Carcinogenesis*, 1999, 20: 1477-1484.

**Tanaka, 1998**

Tanaka T, Kawabata K & Kakumoto M. *Citrus auraptene* exerts dose dependent chemopreventive activity in rat large bowel tumorigenesis: The inhibition correlates with suppression of cell proliferation and lipid peroxidation and with induction of Phase II drug metabolizing enzymes. *Cancer Research*, 1998, 58: 2550-2556.

**Tanaka, 1997**

Tanaka T. Effect of diet on human carcinogenesis. *Critical Reviews in Oncology/ Hematology*, 1997, 25: 73-95.

**Taniguchi, 2004**

Taniguchi H. Carbohydrate research and industry in Japan and the Japanese Society of Applied Glycoscience. *Starch*, 2004, 56: 1-5.

**Tateyama, 2005**

Tateyama I, Hashii K, Johno I, Iino T, Hirai K, Suwa Y & Kiso Y. Effect of xylooligosaccharides intake on severe constipation in pregnant woman. *Journal of Nutrition Science and Vitaminology*, 2005, 51: 445-448.

**Teasdale, 1991**

Teasdale RD & Richards DK. Study of a factor produced by suspension cultured *Pinus radiata* cells which enhances cell growth at low inoculum densities. *Plant Cell, Tissue and Organ Culture*, 1991, 26: 53-59.

**Teleman, 2002**

Teleman A, Tenkanen M, Jacobs A & Dahlman O. Characterization of *O*-acetyl-(4-*O*-methylglucurono) xylan isolated from birch and beech. *Carbohydrate Research*, 2002, 337: 373-377.

**Teleman, 2000**

Teleman A, Lundqvist J, Tjerneld F, Stalbrand H & Dahlman O. Characterization of acetylated 4-*O*-methylglucuronoxylan isolated from aspen employing $^{1}H$ and $^{13}C$ NMR spectroscopy. *Carbohydrate Research*, 2000, 329, 807-815.

**Teplitsky, 2000**

Teplitsky A, Shulami S, Moryles S, Shoham Y & Shoham G. Crystallization and preliminary X-ray analysis of an intracellular xylanase from *Bacillus stearothermophilus* T-6. *Acta Crystallographica*, 2000, 56: 181-184.

**Thakur, 1995**

Thakur S, Prasad MS & Rastogi NK. Effect of xanthan on textural properties of idli (traditional south Indian foods). *Food Hydrocolloids*, 1995, 9: 141-145.

**Theater, 1993**

Theater RM & Wood PJ. Use of Congo Red- polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Applied Environmental Microbiology*, 1993, 43: 777-780.

**Thurnherr, 1973**

Thurnherr N, Deschner EE & Stonehill EH. Induction of adenocarcinomas of the colon in mice by weekly injections of 1,2-dimethylhydrazine. *Cancer Research*, 1973, 33: 940-945.

**Tomomatsu, 1994**

Tomomatsu H. Health effects of oligosaccharides. *Food Technology*, 1994, 48: 61-65.

**Topping, 2001**

Topping DL & Clifton PM. Short-chain fatty acids and human colonic function: roles of resistant starch and non-starch polysaccharides. *Physiological Reviews*, 2001, 81: 1031-1064.

**Trautwein, 1998**

Trautwein EA, Rieckhoff D & Erbersdobler HF. Dietary inulin lowers plasma cholesterol and triacylglycerol and alters biliary bile acid profile in hamsters. *Journal of Nutrition*, 1998, 128: 1937-1943.

**Treptow-van Lishaut, 1999**

Treptow-van Lishaut S, Rechkemmer G, Rowland I, Dolara P & Pool-Zobel BL. The Carbohydrate crystalean and colonic microflora modulate expression of glutathione S-transferase subunits in colon of rats. *European Journal of Nutrition*, 1999, 38: 76-83.

**Tsujibo, 2004**

Tsujibo H, Kosaka M, Ikenishi S, Sato T, Miyamoto K & Inamori Y. Molecular characterization of a high-affinity xylobiose transporter of *Streptomyces thermoviolaceus* OPC-520 and its transcriptional regulation. *Journal of Bacteriology*, 2004, 186(4): 1029-1037.

**Tsujibo, 2002**

Tsujibo H, Takada C, Wakamatsu Y, Kosaka M, Tsuji A, Miyamoto K & Inamori Y. Cloning and expression of an α-L-arabinofuranosidase gene (*stxIV*) from *Streptomyces thermoviolaceus* OPC-520, and characterization of the enzyme. *Bioscience, Biotechnology and Biochemistry*, 2002, 66: 434-438.

**Tsujibo, 2001**

Tsujibo H, Takada C, Tsuji A, Kosaka M, Miyamoto K & Inamori Y. Cloning, sequencing, and expression of the gene encoding an intracellular β-D-xylosidase from *Streptomyces thermoviolaceus* OPC-520. *Bioscience, Biotechnology and Biochemistry*, 2001, 65: 1824-1831.

**Tsujibo, 1997**

Tsujibo H, Ohtsuki T, Iio T, Yamazaki I, Miyamoto K, Sugiyama M & Inamori Y. Cloning and sequence analysis of genes encoding xylanases and acetyl xylan esterase from *Streptomyces thermoviolaceus* OPC-520. *Applied and Environmental Microbiology*, 1997, 63: 661-664.

**Tsujibo, 1992**

Tsujibo H, Miyamoto K, Kuda T, Minami K, Sakamoto T, Hasegawa T & Inamori Y. Purification, properties, and partial amino acid sequences of thermostable xylanases from *Streptomyces thermoviolaceus* OPC- 520. *Applied and Environmental Microbiology*, 1992, 58: 371-375.

**Tuohy, 2005**

Tuohy KM, Rouzaud GCM, Brueck WM & Gibson GR. Modulation of the human gut microflora towards improved health using prebiotics-assessment of efficacy. *Current Pharmaceutical Design*, 2005, 11: 75-90.

**Tuohy, 2002**

Tuohy KM, Ziemer CJ, Klinder A, Knobel Y, Pool-Zobel BL & Gibson GR. A human volunteer study to determine the prebiotic effects of lactulose powder on human colonic microbiota. *Microbial Ecology in Health and Disease*, 2002, 14: 165-173.

**Turusov, 1982**

Turusov VS, Lanko NS, Krutovskikh VA & Parfenov YD. Strain differences in susceptibility of female mice to 1,2-dimethylhydrazine. *Carcinogenesis*, 1982, 3: 603-608.

**Tzortzis, 2005**

Tzortzis G, Goulas AK, Gee JM & Gibson GR. A novel galactooligosaccharide mixture increases the bifidobacterial population numbers in a continuous *in vitro* fermentation system and in proximal colonic contents of pigs *in vivo*. *Journal of Nutrition*, 2005, 135: 1726-1731.

**Uhlig, 1992**

Uhlig S & Wendel A. The physiological consequences of glutathione variations. *Life Science*, 1992, 51: 1083-1094.

**van den Broek, 2005**

Van den Broek LAM, Lloyd RM, Beldman G, Verdoes JC, McCleary BV & Voragen AGJ. Cloning and characterization of arabinoxylan arabinofuranohydrolase-D3 (AXHd3) from *Bifidobacterium adolescentis* DSM20083. *Applied Microbiology and Biotechnology*, 2005, 67: 641-647.

**van den Heuvel, 1999**

Van den Heuvel EG, Muys T, van Dokkum W & Schaafsma G. Oligofructose stimulates calcium absorption in adolescents. *American Journal of Clinical Nutrition*, 1999, 69: 544-548.

**van Hazendonk, 1996**

Van Hazendonk JM, Reinerink EJM, de Waard P & van Dam JEG. Structural analysis of hemicellulose components of flax bast fibres (*Linum usitatissimum* L.). *Carbohydrate Research*, 1996, 291: 141-154.

**Van, 1985**

Van KTT, Toubart P, Ccousson A, Darvill A, Gollin DJ, Chelf P & Albersheim P. Manipulation of the morphogenetic pathways of tobacco explants by oligosaccharins. *Nature*, 1985, 314: 615-617.

**van Laere, 2000**

Van Laere KM, Hartemink R, Bosveld M, Schols H & Voragen A. Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria. *Journal of Agricultural and Food Chemistry*, 2000, 48: 1644-1652.

**van Laere, 1999**

Van Laere KM, Voragen CHL, Kroef T, Van den Broek LAM, Beldman G & Voragen AGJ. Purification and mode of action of two different arabinoxylan arabinofuranohydrolases from *Bifidobacterium adolescentis* DSM 20083. *Applied Microbiology*, 1999, 51: 606-613.

**van Laere, 1997**

Van Laere KM, Beldman G & Voragen AGJ. A new arabinofuranohydrolase from *Bifidobacterium adolescentis* able to remove arabinosyl residues from double-substituted xylose units in arabinoxylan. *Applied Microbiology*, 1997, 47: 231-235.

**van Loo, 1995**

Van Loo J, Coussement P, De Leenheer L, Hoebregs H & Smits G. On the presence of inulin and oligofructose as natural ingredients in the Western diet, *CRC Critical Reviews in Food Science and Nutrition*, 1995, 35: 525-352.

**van Peij, 1998a**

van Peij NNME, Gielkens MMC, de Vries RP, Visser J & de Graaff LH. The transcriptional activator *xlnR* regulates both xylanolytic and endoglucanase expression in *Aspergillus niger*. *Applied and Environmental Microbiology*, 1998a, 64: 3615-3619.

**van Peij, 1998b**

van Peij NNME, Visser J & de Graaff LH. Isolation and analysis of *xlnR*, encoding a transcriptional activator coordinating xylanolytic expression in *Aspergillus niger*. *Molecular Microbiology*, 1998b, 27: 131-142.

**van Peij, 1997**

van Peij NNME, Brinkmann J, Vrsanska M, Visser J & De Graaff LH. β-xylosidase activity, encoded by *xlnD*, is essential for complete hydrolysis of xylan by *Aspergillus niger* but not for induction of the xylanolytic enzyme spectrum. *European Journal of Biochemistry*, 1997, 245: 164-173.

**van Wezel, 1999**

Van Wezel T, Ruivenkamp CA, Stassen AP, Moen CJ & Demant P. Four new colon cancer susceptibility loci, Scc6 to Scc9 in the mouse. *Cancer Research*, 1999, 59: 4216-4218.

**van Wezel, 1996**

Van Wezel T, Stassen APM, Moen CJA, Hart AAM, Van der Valk MA & Demant P. Gene interaction and single gene effects in colon tumour susceptibility in mice. *Nature Genetics*, 1996, 14: 468-470.

**Vanhoutte, 2005**

Vanhoutte T, Huys G, De Brandt E, Fahey Jr. GC & Swings J. Molecular monitoring and characterization of the fecal microbiota of healthy dogs during fructan supplementation. *FEMS Microbiology Letters*, 2005, 249: 65-71.

**Vardakou, 2008**

Vardakou M, Palop CN, Christakopoulos P, Faulds CB, Gasson MA & Narbad A. Evaluation of the prebiotic properties of wheat arabinoxylan fractions and induction

of hydrolase activity in gut microflora. *International Journal of Food Microbiology*, 2008, 123: 166-170.

**Vardakou, 2004**

Vardakou M, Katapodis P, Topakas E, Kekos D, Macris BJ & Christakopoulos P. Synergy between enzymes involved in the degradation of insoluble wheat flour arabinoxylan. *Innovative Food Science and Emerging Technologies,* 2004, 5: 107-112.

**Vazquez, 2005**

Vazquez MJ, Garrote G, Alonso JL, Domınguez H & Parajo JC. Refining of autohydrolysis liquors for manufacturing xylooligosaccharides: Evaluation of operational strategies. *Bioresource Technology,* 2005, 96: 889-896.

**Vazquez, 2000**

Vazquez MJ, Alonso JL, Domınguez H & Parajo JC. Xylooligosaccharides: manufacture and applications. *Trends in Food Science and Technology*, 2000, 11: 387-393.

**Vegas, 2006**

Vegas R, Luque S, Alvarez RJ, Alonso JL, Domiänguez H & Parajoa JC. Membrane-assisted processing of xylooligosaccharide-containing liquors. *Journal of Agricultural and Food Chemistry,* 2006, 54: 5430-5436.

**Vegas, 2005**

Vegas R, Alonso JL, Domınguez H & Parajo JC. Manufacture and refining of oligosaccharides from industrial solid wastes. *Industrial and Engineering Chemical Research*, 2005, 44: 614-620.

**Vegas, 2004**

Vegas R, Alonso JL, Domınguez H & Parajo JC. Processing of rice husk autohydrolysis liquors for obtaining food ingredients. *Journal of Agricultural and Food Chemistry,* 2004, 52: 7311-7317.

**Venkatasubbaiah, 1985**

Venkatasubbaiah P, Dwarakanath CT & Sreenivasamurthy V. Involvement of yeast flora in idli batter fermentation. *Journal of Food Science and Technology,* 1985, 22: 88-90.

**Verhamme, 2001**

Verhamme DT, Arents JC, Postma PW, Crielaard W & Hellingwerf KJ. Glucose-6-phosphate-dependent phosphoryl flow through the Uhp two-component regulatory system. *Microbiology*, 2001, 147: 3345-3352.

**Verhamme, 2002**

Verhamme DT, Arents JC, Postma PW, Crielaard W & Hellingwerf KJ. Investigation of *in vivo* cross-talk between key two-component systems of *Escherichia coli*. *Microbiology*, 2002, 148: 69-78.

**Vila, 2002**

Vila C, Garrote G, Domınguez H & Parajo JC. Hydrolytic processing of rice husks in aqueous media: A kinetic assessment. *Collection of Czechoslovak Chemical Communications*, 2002**,** 67: 509-529.

**Vinkx, 1996**

Vinkx CJA & Delcour JA. Rye (*Secale cereale* L.) arabinoxylans: a critical review. *Journal of Cereal Science*, 1996, 24: 1-14.

**Virupakshi, 2005**

Virupakshi S, Babu KG, Gaikwad SR & Naik GR. Production of a xylanolytic enzyme by a thermoalkaliphilic *Bacillus* sp. JB-99 in solid state fermentation. *Process Biochemistry*, 2005, 40: 431-435.

**Voragen, 1998**

Voragen AGJ. Technological aspects of functional food-related carbohydrates. *Trends in Food Science and Technology*, 1998, 9: 328-335.

**Voughan, 2001**

Voughan T, Seo CW & Marshall WE. Removal of metal ions from aqueous solution using modified corncobs. *Bioresource Technology*, 2001, 78: 133-139.

**Wagner, 1985**

Wagner H, Proksch A, Riess-Maurer I, Vollmar A, Odenthal S, Stuppner S, Stuppner H. Immunostimulating polysaccharides (Heteroglycanes) of higher plants. *Arzneimittel Forschung/Drug Research*, 1985, 35:1069-1073.

**Wang, 1993**

Wang X & Gibson GR. Effects of the *in vitro* fermentation of oligofructose and inulin by bacteria growing in the human large intestine. *Journal of Applied Bacteriology*, 1993, 75: 373-380.

**Wende, 1997**

Wende G & Fry SC. *O*-feruloylated, *O*-acetylated oligosaccharides as side-chains of grass xylans. *Phytochemistry,* 1997, 44: 1011-1018.

**Westerlund, 1993**

Westerlund E, Andersson R & Aaman P. Isolation and chemical characterization of water-soluble mixed-linked β-glucans and arabinoxylans in oat milling fractions. *Carbohydrate Polymers,* 1993, 20: 115-123.

**Whistler, 1976**

Whistler RL, Bushway A, Singh PP, Nakahara W & Tokuzen R. Noncytotoxic, antitumor polysaccharides. *Advances in Carbohydrate Chemistry and Biochemistry,* 1976, 32: 235-275.

**Whitehead, 2001**

Whitehead TR & Cotta MA. Identification of a broad-specificity xylosidase/arabinosidase important for xylooligosaccharide fermentation by the ruminal anaerobe *Selenomonas ruminantium* GA192. *Current Microbiology*, 2001, 43, 293-298.

**Wilkie, 1979**

Wilkie KCB. The hemicelluloses of grasses and cereals**.** *Advance in Carbohydrate Chemistry and Biochemistry*, 1979, 36: 215-264.

**Wilkinson, 1997**

Wilkinson J & Clapper ML. Detoxification enzymes and chemoprevention. *Proceedings of the Society for Experimental Biology and Medicine*, 1997, 216: 192-200.

**Willard, 2000**

Willard MD, Simpson RB, Cohen ND & Clancy JS. Effects of dietary fructooligosaccharides on selected bacterial populations in feces of dogs. *American Journal of Veterinary Research,* 2000, 61: 820-825.

**Williams, 2002**

Williams CM & Jackson KG. Inulin and oligofructose: Effects on lipid metabolism from human studies. *British Journal of Nutrition,* 2002, 87: S261-S264

**Williams, 1994**

Williams CH, Witherly SA & Buddington RK. Influence of dietary neosugar on selected bacterial groups of the human fecal microbiota. *Microbial Ecology in Health and Disease*, 1994, 7: 91-97.

**Williams, 1991**

Williams AG, Withers SE & Joblin KN. Xylanolysis by cocultures of the rumen fungus *Neocallimastix frontalis* and ruminal bacteria. *Letters in Applied Microbiology*, 1991, 12: 232-235.

**Wollowski, 2001**

Wollowski I, Rechkemmer G & Pool-Zobel BL. Protective role of probiotics and prebiotics in colon cancer. *American Journal of Clinical Nutrition*, 2001, 73: S451-S455.

**Xu, 2008**

Xu B, WangY, Li J & Lin Q. Effect of prebiotic xylooligosaccharides on growth performances and digestive enzyme activities of allogynogenetic crucian carp (*Carassius auratus gibelio*). *Fish Physiology and Biochemistry*, 2008 Aug 13 [Epub ahead of print], DOI 10.1007/s10695-008-9248-8.

**Yamada, 1985**

Yamada H, Nagai T, Cyong JC & Otsuka Y. Relationship between chemical structure and anti-complementary activity of plant polysaccharides. *Carbohydrate Research*, 1985, 144: 101-111.

**Yang, 2007**

Yang, CH, Yang SF & Liu WH. Production of xylooligosaccharides from xylans by extracellular xylanase from *Thermobifida fusca. Journal of Agricultural and Food Chemistry*, 2007, 55: 3955-3959.

**Yang, 2005**

Yang R, Xu S, Wang Z & Yang W. Aqueous extraction of corncob xylan and production of xylooligosaccharides. *LWT-Food Science and Technology*, 2005, 38: 677-682.

**Yang, 2004**

Yang JK, Yoon HJ, Ahn HJ, Lee B, Pedelacq JD, Liong EC, Berendzen J, Laivenieks M, Vieille C, Zeikus GZ, Vocadlo DJ, Withers SG & Suh SW. Crystal structure of β-

D-xylosidase from *Thermoanaerobacterium saccharolyticum*, a family 39 glycoside hydrolase. *Journal of Molecular Biology*, 2004, 335: 155-165.

**Yang, 2003**

Yang F, Fang Z, Xu Y, Yao C, Yu S & Zhu Q. Separation of xylo-oligosaccharides from enzymatic hydrolytes using membrane reactor. *Journal of Central South University of Technology,* 2003, 10: 122- 125.

**Yasuda, 2006**

Yasuda K, Roneker KR, Miller DD, Welch RM & Lei XG. Supplemental dietary inulin affects the bioavailability of iron in corn and soybean meal to young pigs. *Journal of Nutrition,* 2006, 136: 3033-3308.

**Yasui, 1989**

Yasui T, Kizawa H, Masada Y & Shinoyama H. A novel non-reducing disaccharide, *O*-β-D-xylopyranosyl-(l>l')-β-D-xylopyranose, produced from xylobiose by transglycosylation with *Aspergillus niger* β-xylosidase. *Agricultural and Biological Chemistry,* 1989, 53: 3381-3382.

**Yokota, 1997**

Yokota T, Sugano K, Kondo H, Saito D, Sugihara K, Fukayama N, Ohkura H, Ochiai A & Yoshida S. Detection of aberrant crypt foci by magnifying colonoscopy. *Gastrointestinal Endoscopy,* 1997, 46: 61-65.

**Yoon, 2006**

Yoon KY, Woodams EE & Hang YD. Enzymatic production of pentoses from the hemicellulose fraction of corn residues. *LWT-Food Science and Technology*, 2006, 39: 388-392.

**Yuan, 2004a**

Yuan QP, Zhang H, Qian ZM & Yang XJ. Pilot-plant production of xylooligosaccharides from corncob by steaming, enzymatic hydrolysis and nanofiltration. *Journal of Chemical Technology and Biotechnology*, 2004, 79: 1073-1079.

**Yuan, 2004b**

Yuan X, Wang J & Yao H. Antioxidant activity of feruloylated oligosaccharides from wheat bran. *Food Chemistry,* 2004, 90: 759-764.

**Yuan, 1996**

Yun JW. Fructooligosaccharides- occurrence, preparation and application. *Enzyme and Microbial Technology,* 1996, 19: 107-117.

**Yurgel, 2004**

Yurgel SN & Kahn ML. Dicarboxylate transport by *Rhizobia*. *FEMS Microbiology Reviews,* 2004, 28: 489-501.

**Yusrizal, 2003**

Yusrizal & Chen TC. Effect of adding chicory fructans in feed on fecal and intestinal microflora and excreta volatile ammonia. *International Journal of Poultry Science,* 2003, 2: 188-194.

**Zaide, 2001**

Zaide G, Shallom D, Shulami S, Zolotnitsky G, Golan G, Baasov T, Shoham G & Shoham Y. Biochemical characterization and identification of catalytic residues in alpha-glucuronidase from *Bacillus stearothermophilus* T-6. *European Journal of Biochemistry*, 2001, 268: 3006-3016.

**Zeng, 2007**

Zeng H, Xue Y, Peng T & Shao W. Properties of xylanolytic enzyme system in bifidobacteria and their effects on the utilization of xylooligosaccharides. *Food Chemistry*, 2007, 101: 1172-1177.

**Ziemer, 1998**

Ziemer CJ & Gibson GR. An overview of probiotics, prebiotics and synbiotics in the functional food concept: Perspectives and future strategies. *International Dairy Journal*, 1998, 8: 473-479.

**Zilliox, 1998**

Zilliox C & Debeire P. Hydrolysis of wheat straw by a thermostable endoxylanase: adsorption and kinetic studies. *Enzyme and Microbial Technology,* 1998, 22: 58-63.

**Zui, 2004**

Zui F, Kaneko S, Kuno A, Kobayashi H, Kusakabe I & Mizuno H. Crystal structure of decorated xylooligosaccharides bound to a family 10 xylanase from *Streptomyces olivaceoviridis* E-86. *The Journal of Biological Chemistry,* 2004, 279 (10): 9606-9614.

## BOOKS, THESIS & WEB PAGES

**Carlberg, 1985**

Carlberg I & Mannervik B. Glutathione reductase. In: Meister A (editor), *Methods in Enzymology*. San Diego: Academic Press, 1985, vol: 113, pp 484-490.

**Cooperstock, 1983**

Cooperstock MS & Zedd AJ. Intestinal flora of infants. In: Hentges DJ (editor), *Human intestinal microflora in health and disease*. London: Academic Press, 1983, pp 79-99.

**Coppa, 2000**

Coppa GV, Bruni S, Zampini L, Varaldo PE & Gabrielli O. Oligosaccharides of human milk. In: Muzzarelli RAA (editor), *Chitosan per os: From dietary supplement to drug carrier*. Grottammare Atec, 2000, pp 313-30.

**Cygnus, 2008**

Cygnus. *Industry Insight: Indian Nutraceuticals.* India: Cygnus business consulting and research, 2008, www.cygnusindia.com/

**De Leenheer, 1994**

De Leenheer L. Production and use if inulin: industrial reality with a promising future. In: van Bekkum H, Roper H & Voragen AGJ (editors), *Carbohydrates as organic raw materials III*. Weinheim, Germany: Wiley-VCH, 1994, pp 67-92.

**Delzenne, 1999**

Delzenne NM & Williams CM. Actions of non-digestible carbohydrates on blood lipids in humans and animals. In: Gibson GR & Roberfroid MB (editors), *Colonic Microbiota, nutrition and health.* The Netherlands: Kluwer Academic Publisher, 1999, pp 213-232.

**Ebringerova, 1999**

Ebringerova A & Hromadkova Z. Xylans of industrial and biomedical importance. In: Harding SE (editor), *Biotechnology and genetic engineering reviews.* England: Intercept Ltd, 1999, vol: 16, 325-346.

**FAO, 2007**

FAO. *FAO Technical meetings on prebiotics: Food Quality and Standards Service (AGNS).* Rome: Food and Agricultural Organization, 2007, pp 1-11, www.fao.org/ag/agn/agns/index_en.stm

**FAO-WHO, 1987**

FAO-WHO. Committee on food additives 31. *World Health Organization Technical Report Series*: Geneva, 1987.

**Glicksman, 1982**

Glicksman M. Functional properties of food hydrocolloids. In: Glicksman M (editor), *Food hydrocolloids*. Boca Raton, Florida, USA: CRC Press, 1982, vol: II, pp 47-99.

**Gibson, 1997**

Gibson GR, Saavedra JM, MacFarlane S & Mac Farlane GT. Probiotics and intestinal applications. In: Fuller R (editor), *Probiotics 2: Application and practical aspects.* London: Chapman & Hall, 1997, pp 10-39.

**Graham, 2004**

Graham H, Apajalahti J & Peuranen S. Xylooligosaccharides alter metabolism of gut microbes and blood xylose levels in chicks. In: van der Kamp JW, Asp NG, Miller Jones J & Schaafsma G (editors), *Dietary fiber: Bioactive carbohydrates for food and feed*. The Netherlands: Wageningen Academic publishers, 2004, pp 329-332.

**Indian Standards, 1971**

Indian Standards. *Indian Standard Guide for Sensory Evaluation of Foods (Part II. Methods and Evaluation Cards).* New Delhi: Indian Standard Institution, 1971, p 36.

**Ishihara, 1995**

Ishihara M, Nagao Y & Shimizu K. Physiological activities of xylooligosaccharides. In: *Proceedings of the 8th International Symposium on Wood Pulping Chemistry*, 1995, 2, pp 17-22.

**Ishihara, 1991**

Ishihara M, Nagao Y & Shimizu K. Physiological activity of xylooligosaccharides. In: *Research Report of Biomass Conversion Program*, 1991, 27, pp 20-32.

**Kabel, 2002**

Kabel MA, Schols HA & Voragen AGJ. Structural features of acetylated xylooligosaccharides. In: *Proceedings of the 223rd American Chemical Society National Meeting*, Orlando, USA, 2002.

**Kato, 1999**

Kato M, Mimura S, Rao U, Tanaka A, Kitamoto N, Yoshino S & Tsukagoshi N. Regulation of the *xynF1* gene encoding the major family F xylanase of *Aspergillus oryzae*. In: Ohmiya K, Hayashi K, Sakka K, Koyabashi Y, Karita S & Kimura T (editors). *Genetics, biochemistry and ecology of cellulose degradation*. Tokyo, Japan: Uni Publishers Co. Ltd., 1999, pp 331-335.

**Klemm, 1998**

Klemm D, Philipp B, Heinze TH, Heinze U & Wagenknecht W. In: *Comprehensive Cellulose Chemistry: Fundamentals and Analytical Methods.* Germany: Wiley-VCH, 1998, vol: 1, pp 9-25.

**Kuhn, 1958**

Kuhn R. Les oligosaccharides du lait. *Bulletin de la Societe Chimie Biologique*, 1958, 40, pp 297-314.

**Lee, 1999**

Lee YK, Nomoto K, Salminen S & Gorbach SL. Role of probiotics in health and diseases. In: Lee YK, Nomoto K, Salminen S & Gorbach SL (editors), *Handbook of Probiotics.* New York: John Wiley & Sons Inc., 1999, pp 67-146.

**Painter, 1983**

Painter TJ. Algal Polysaccharides. In: Aspinall GO (editor), *The polysaccharides.* New York: Academic Press, 1983, pp 195-285.

**Pazur, 1970**

Pazur JH. Oligosaccharides. In: Pigman W and Horta D (editors), *The carbohydrates-chemistry and biochemistry.* New York: Academic Press, 1970, IIA, pp 69-73.

**Ramakrishnan, 1979**

Ramakrishnan CV. Terminal Report of American PL480- project. Study of Indian fermented foods from legumes and production of similar fermented foods from U.S. soybean. *PhD Thesis.* M.S. University of Baroda, Baroda, India, 1979.

**Rastall, 2002**

Rastall RA and Gibson GR. Prebiotic oligosaccharides: evaluation of biological activities and potential future developments. In: Tannock W (editor), *Probiotics and prebiotics: where are we going?* England: Caister Academic Press, 2002, p 107-148.

**Roberfroid, 2008**

Roberfroid MB. General introduction: Prebiotics in nutrition. In: Gibson BR & Roberfroid MB (editors), *Handbook of Prebiotics*. Boca Raton: CRC Press, Taylor and Francis Group, 2008, edition 1, pp 1-12.

**Roberfroid, 2008**

Roberfroid MB. Prebiotics: Concept, definition, criteria, methodologies and products. In: Gibson BR & Roberfroid MB (editors), *Handbook of Prebiotics*. Boca Raton: CRC Press, Taylor and Francis Group, 2008, 1st edition, pp 39-68.

**Rovensky, 1990**

Rovensky J, Pekarek J, Mlynarcık D, Kasafırek E, Lackovic V, Hrıbalova V & Buc M. In: Ram BP, Harris MC & Tyle P (editors). *Immunology: clinical, fundamental and therapeutic aspects.* New York: VCH, 1990, pp 344-354.

**RTS Resource Ltd., 2008**

RTS Resource Ltd. *Food and ingredients market research and analysis*. England: RTS Resource Ltd., 2008, www.rts.resource.com/

**Sambrook, 2001**

Sambrook J & Russell DW. *Molecular cloning: a laboratory manual.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 2001, 3rd edition.

**Schlegel, 1993**

Schlegel HG. Lactic acid fermentation and Lactobacteriaceae. In: Schlegel HG (editor). *General Microbiology*. Cambridge, England: Cambridge University Press, 1993, 7nth edition, pp 300-307.

**Stephen, 1983**

Stephen AM. Other plant polysaccharides. In: Aspinall GO (editor), *The Polysaccharides.* New York: Academic Press, 1983, vol: 2, pp 98-193.

**Sun, 1996**

Sun RC. Extraction, fractionization, isolation and structural characterization of polysaccharides and lignin from wheat straw. *PhD thesis*, University of Wales, Bangor, 1996, pp 5-10.

**Svensson, 1999**

Svensson U. Industrial perspectives. In: Tannock GW (editor), *Probiotics: a critical review*. Wymondham, England: Horizon Scientific Press, 1999, pp 57-64.

**Thomas, 1977**

Thomas RJ. Wood: structure and chemical composition. In: Goldstein IS (editor), *Wood technology: chemical aspects*. Washington, DC: ACS Symposium Series, 1977, Number 23, pp 1-23.

**Waldron, 2007**

Waldron KW & Faulds CB. Cell wall polysaccharides: composition and structure. In: Kamerling JP (editor), *Comprehensive glycoscience (from chemistry to systems biology)*, Oxford, England: Elsevier Ltd, The Boulevard Langford Lane Kidlington, 2007, vol: 1, pp182-189.

**Weisburger, 1983**

Weisburger JH & Fiala ES. Experimental colon carcinogens and their mode of action. In: Autrup H & Williams GM (editors), *Experimental colon carcinogenesis*. Florida: CRC Press, 1983, pp 27-50.

**Whistler, 1993**

Whistler RL. Hemicelluloses. In: BeMiller JN & Whistler RL (editors), *Industrial Gums*. New York: Academic Press, 1993, pp 295-303.

**Wood, 1988**

Wood TM & Bhat KM. Methods for measuring cellulase activities, In: Kellog ST (editor), *Methods in Enzymology*. San Diego: Academic Press, 1988, vol: 160, pp 94-102.

**Zhang, 2005**

Zhang YQ, Wilkinson H, Keller NP and Tsitsigiannis D. Secondary metabolite gene clusters. In: An Z (editor). *Handbook of industrial microbiology*. New York: Marcel Dekker, 2005, pp 355-386.

## PATENTS

**Azumi, 2004a**

Azumi N & Ikemizu S. Collagen production promoters containing acidic xylooligosaccharides. *Japan Patent*, JP 2004059478: 2004a.

**Azumi, 2004b**

Azumi N & Ikemizu S. Hyaluronic acid-formation promoters containing xylooligosaccharides. *Japan Patent*, JP 2004059480: 2004b.

**Azumi, 2004c**

Azumi N & Ikemizu S. Topical preparations for atopic dermatitis containing acidic xylooligosaccharides. *Japan Patent*, JP 2004210664: 2004c.

**Clayton, 2005**

Clayton P & Conn H. Carbohydrate substitute comprising a low glycemic index sugar and a flavonoid. *International Patent*, WO 2005006891: 2005.

**Endo, 2000**

Endo M & Kuroda Y. Production method of xylose and xylooligosaccharides from natural compounds containing xylan by hot water pretreatment. *Japan Patent*, JP 2000236899: 2000.

**Hayashi, 2005a**

Hayashi N, Sakaki T & Doi K. Water-soluble saccharides useful as health foods and their manufacture by hydrolysis of hemicellulose- containing plants with pressurized hot water. *Japan Patent*, JP 2005023041: 2005a.

**Hayashi, 2005b**

Hayashi N, Sakaki T & Doi K. Carbohydrate-based food having radical-scavenging activity and its manufacture. *Japan Patent*, JP 2005021111: 2005b.

**Ikemizu, 2002**

Ikemizu S & Azumi N. Melanin formation inhibitors containing acidic xylooligosaccharides. *Japan Patent*, JP 200217137: 2002.

**Ikemizu, 2003**

Ikemizu S & Kojo A. Hair growth stimulants containing acidic xylooligosaccharides. *Japan Patent*, JP 2003183133: 2003.

**Inafuku, 2002**

Inafuku M, Fujino T, Kashiwagi Y & Ohara S. Antioxidant dietary fiber, its manufacture, and processed food using it. *Japan Patent,* JP 2002204674: 2002.

**Izumi, 2001**

Izumi K & Azumi N. Xylooligosaccharide compositions useful as food and feed additives. *Japan Patent,* JP 2001226409: 2001.

**Izumi, 2002**

Izumi Y, Sugiura J, Kagawa H & Azumi N. Process for producing xylooligosaccharides from lignocellulose pulp. *U.S. Patent,* US 2002195213: 2002.

**Izumi, 2003a**

Izumi K, Nakagame S, Kido Y & Nakabo Y. Enteral nutrient containing xylooligosaccharides. *Japan Patent,* JP 2003055231: 2003.

**Izumi, 2003b**

Izumi Y & Kojo A. Long-chain xylooligosaccharides compositions with intestinal function-improving and hypolipemic activities and their manufacture. *Japan Patent,* JP 2003048901: 2003.

**Izumi, 2004**

Izumi K, Ikemizu S & Shizuka F. Anti-hyperlipidemics containing acidic xylooligosaccharides. *Japan Patent,* JP 2004182615: 2004.

**Izumi, 2004a**

Izumi Y, Azumi N, Kido Y & Nakabo Y. Oral preparations for atopic dermatitis containing acidic xylooligosaccharides. *Japan Patent,* JP 2004210666: 2004a.

**Izumi, 2004b**

Izumi Y, Ikemizu S & Shizuka F. Intestinal environment improving agents containing acidic xylooligosaccharides. *Japan Patent,* JP 2004182609: 2004b.

**Kokubo, 2003**

Kokubo I & Ikemizu S. Acidic xylooligosaccharides as topical anti-inflammatory agents. *Japan Patent,* JP 2003221339: 2003.

**Kokubo, 2004a**

Kokubo I & Ikemizu S. Histamine-release inhibitors containing xylooligosaccharides. *Japan Patent,* JP 2004059481: 2004.

**Kokubo, 2004b**

Kokubo I, Izumi K & Shizuka F. Preventive agents for osteoporosis containing acidic xylooligosaccharides. *Japan Patent*, JP 2004182621: 2004.

**Kokubo, 2004c**

Kokubo I & Ikemizu S. Therapeutic agents for osteoporosis containing acidic xylooligosaccharides. *Japan Patent*, JP 2004182618: 2004.

**Matsuoka, 2005**

Matsuoka M. Xylooligosaccharides for manufacture of dietary fiber health foods. *International Patent*, WO 2005092124: 2005.

**Obuchi, 2005**

Obuchi T & Takahashi T. Moisturizing agents comprising acidic xylooligosaccharides. *Japan Patent*, JP 2005187388: 2005.

**Ohsaki, 2003**

Ohsaki S, Tamura M & Yamaura T. Method for purification of saccharide. *U.S. Patent*, US 6548662: 2003.

**Stahl, 2005**

Stahl B, M'Rabet L, Vos AP, Garssen J & Boehm G. Immunomodulating oligosaccharides, and therapeutic use thereof. *International Patent*, WO 2005039597: 2005.

**Takahashi, 2005**

Takahashi T & Ikemizu S. Antiallergy agents containing acidic xylooligosaccharides for alleviation of allergic rhinitis. *Japan Patent*, JP 200112826: 2005.

**Toshio, 1990**

Toshio I, Noriyoshi I, Toshiaki K, Toshiyuki N & Kunimasa K. Production of xylobiose. *Japan Patent*, JP 2119790: 1990.

**Van Thorre, 2004**

Van Thorre D. Process for obtaining bio-functional fractions from biomass. U.S. *Patent*, US 2004138445: 2004.

**Yukawa, 2005**

Yukawa M, Okuda S & Ozeki K. Production of health food, drug, and cosmetic from brewing by-products. *Japan Patent*, JP 2005263722: 2005.

# PUBLICATIONS

**PAPERS: Under preparation**

1. *Impact of short chain xylan fragments of corncob: reduction of DMH induced aberrant crypt foci formation and colon tumor development in Wistar rats.* Ayyappan Appukuttan Aachary, D. Gopinath, K. Srinivasan and S. G. Prapulla.

2. *Prebiotic and hydrocolloidal properties of xylooligosaccharides affects the quality attributes of idli, an Indian traditional food.* Ayyappan Appukuttan Aachary and S.G.Prapulla.

3. *Hydrolytic-transxylosylation reactions mediated by microbial xylanolytic enzyme systems: production and utilization of xylooligosaccharides (Review).* Ayyappan Appukuttan Aachary, D. Gopinath and S. G. Prapulla.

**PAPERS PRESENTED IN SYMPOSIUM:**

1. Ayyappan Appukuttan Aachary and S.G. Prapulla. *Bioconversion of corncob to prebiotic xylooligosaccharides*, 48$^{th}$ AMI Annual Conference, IIT Madras, India, December, 2007

2. Ayyappan Appukuttan Aachary and S.G. Prapulla. *Production of xylooligosaccharides: a biotechnological approach*, ICFOST, ANGRAU, Hyderabad, India, November, 2006.