XYLANASE FROM RAGI MALT AND IT'S USE IN THE PREPARATION OF XYLO-OLIGOSACCHARIDES FROM CEREAL BRANS

A thesis submitted to the Department of

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DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

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DECLARATION

I declare that the thesis entitled '**XYLANASE FROM RAGI MALT AND IT'S USE IN THE PREPARATION OF XYLO-OLIGOSACCHARIDES FROM CEREAL BRANS'** submitted to the **UNVERSITY OF MYSORE** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** is the result of the work carried out by me under the guidance of **Dr. G. MURALIKRISHNA**, Scientist E-II, Department of Biochemistry and Nutrition, Central Food Technological Research Institute (CFTRI), Mysore, during the period of February 2003- November 2007. I further declare that the results presented in the thesis have not been submitted for the award of any other degree or fellowship.

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Place: Mysore

Date: 19.12.2007

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CERTIFICATE

This is to certify that the thesis entitled **''XYLANASE FROM RAGI MALT AND IT'S USE IN THE PREPARATION OF XYLO-OLIGOSACCHARIDES FROM CEREAL BRANS'** submitted by **CHITHRA. M** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** to the **UNVERSITY OF MYSORE** is the result of the research work carried out by her in the department of Biochemistry and Nutrition under my guidance during the period of February 2003-November 2007.

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ABSTRACT

Xylanases (EC 3.2.1.8) are the major enzymes which bring about the depolymerization of heteroxylan, the major hemicellulosic component of plant cell walls. Xylanases from plant sources still remains unexplored and hence the present study. It focuses on the characterization of purified xylanase from finger millet (Ragi; *Eleusine coracana*, Indaf-15) malt and its utilization in obtaining xylo-oligosaccharides. Xylanase was purified to apparent homogeneity from 96 h ragi malt by a three step purification procedure *via* ammonium sulphate fractionation, DEAE-cellulose ion exchange and Sephadex G-75 gel filtration chromatographies with a recovery of 4.0 % and fold purification of 60. Xylanase, having a molecular weight of 29.5 kDa was found to be monomeric on SDS-PAGE. pH optimum of the enzyme was found to be in the range of pH 5.0-5.5. Xylanase showed maximum stability at 35°C in a pH range of 5.0-6.0. K_m and V_{max} of purified xylanase were found to be 0.2 % and 4.5 μmoles min^{-1} respectively. Metal ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Ag^{2+} and Ni^{2+} enhanced xylanase activity at 5 mM concentration. P-chloromercuribenzoate (PCMB), citric, oxalic and boric acids inhibited the enzyme in concentration dependent manner. Purified ragi xylanase hydrolyzed wheat and ragi bran soluble polysaccharides (SP) yielding xylo-oligosaccharides which were purified on Biogel P-2 column followed by HPLC and their composition was determined by GLC. The oligosaccharides consisted of arabinose and xylose. The purified oligosaccharides were characterized by ESI-MS and H^1NMR. Wheat bran SP yielded four oligosaccharides (WO-1, WO-2, WO-3 and WO-4). WO-1 and WO-2 were identified as arabinose containing xylotetraose and xylotriose respectively whereas, WO-3 and WO-4 were identified as unsubstituted xylotriose and xylobiose respectively. Ragi bran SP yielded only one oligosaccharide (RO-1), identified as xylobiose. The xylo-oligosaccharides liberated from both wheat and ragi brans were proved to be prebiotic by *in vitro* fermentation experiments using *Bifidobacteria, Lactobacilli* and *Pediococci* spp. in terms of growth characteristics such as absorbance (600 nm) and pH of the culture broth and dry cell mass. Acetic acid was the major short chain fatty acid (SCFA) liberated due to *in vitro* oligosaccharide fermentation. The thesis ends with summary and conclusions succeeded by references quoted.

To

My Beloved Parents

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(CHITHRA. M)

CONTENTS

ABBREVIATIONS

Abbreviations	Expansions
α	Alpha
β	Beta
µ	Micro
Ara	Arabinose
BOD	Biological oxygen demand
BSA	Bovine serum albumin
cm	Centimeter(s)
D_2O	Deuterium oxide
DEAE	Diethyl amino ethyl
DP	Degree of polymerization
^0C	Degree Celsius
EDTA	Ethylene diamine tetra acetic acid
ESI-MS	Electrospray ionization-mass spectrometry
EV	Electron volts
Fig.	Figure
FOS	Fructo-oligosaccharides
GA_3	Gibberellic acid
Gal	Galactose
GLC	Gas liquid chromatography
Glc	Glucose
GOS	Galacto-oligosaccharides
g	Gram
h	Hours
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
kDa	Kilo Dalton(s)

K_m	Michaelis constant
Kv	Kilo Volts
L	Litre (s)
Man	Mannose
ml	Millilitre (s)
mm	Millimetre (s)
min	Minute (s)
mM	Millimolar
mU	Milliunits
MS	Mass spectrometer
mg	Milligram (s)
M	Molar
µ g	Microgram
µ M	Micromolar
nm	Nanometre
N	Normal
NMR	Nuclear magnetic resonance
NSP	Non-starch polysaccharides
PAGE	Poly acrylamide gel electrophoresis
PCMB	Parachloro mercuric benzoate
pI	Isoelectric point
PVPP	Polyvinyl poly pyrrolidone
rpm	revolutions per minute
SCFA	Short chain fatty acid
SDS	Sodium dodecyl sulphate
sec	Second (s)
SP	Soluble polysaccharides
TEMED	N, N, N', N'- Tetra methyl ethylene diamine
TFA	Trifluoro acetic acid
U	Units

V	Volume
V_{max}	Maximum velocity
w/v	Weight/Volume
v/v	Volume/Volume
M_r	Molecular weight
UV	Ultra violet
XOS	Xylo-oligosaccharides
Xyl	Xylose
Xylp	Xylopyranose

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SYNOPSIS

Synopsis of the thesis submitted for the award of Ph.D degree (Biotechnology) of the University of Mysore, India.

Title of the thesis: 'Xylanase from ragi malt and it's use in the preparation of xylo-oligosaccharides from cereal brans'.

Candidate : Chithra. M.

Introduction

Xylanases (EC 3.2.1.8, 1, 4-β-D xylohydrolases) are hydrolases which depolymerize the xylan backbone of heteroxylans producing xylo-oligosaccharides varying in their degree of polymerization (D.P 2-10). Xylanases are wide spread in nature and have been reported to be present in marine and terrestrial bacteria, rumen and ruminant bacteria, fungi, marine algae, protozoa, snails, crustaceans, insects and seeds of terrestrial plants. Plant xylanases are less exploited compared to that of microbial origin especially from bacteria and fungi and the studies are mostly restricted to wheat and barley. No information is available on finger millet xylanase and its kinetic properties. In the present study malting has been employed to obtain maximum recovery of xylanase from finger millet. Malting is a biotechnological process in which the seeds are germinated under controlled environmental conditions. It brings about changes in protein quality and induction of hydrolytic enzymes such as endoxylanases, arabinofuranosidases, xylosidases and esterases which cause the depolymerization of the plant cell walls and mobilization of endosperm storage material.

Arabinoxylan constitute the major portion of the cereal cell wall polysaccharides after cellulose. The major difference in the chemical structure of the arabinoxylan isolated from different cereals or plant tissues occur in the ratio of the arabinose to xylose in the relative proportions, sequence of their linkage and the substuituents linked to it. Cereal brans which are the major byproduct of cereal industry serve as a good biosource for arabinoxylan and are widely used considering the increased environmental concern.

Isolation and structural elucidation of the arabinoxylan and their possible use in obtaining bioactive oligosaccharides by chemical/enzymatic methods are one of the hottest areas of carbohydrate research.

Functional food/functional food ingredients are attaining increased attention due to their possible health benefits. Of the currently known functional foods, non-digestible oligosaccharides have an important role with respect to their bifidogenic activity. The research on prebiotics in improving human health is mostly focused on the strains belonging to the genera *Bifidobacterium* and *Lactobacillus* spp inhabiting the human gastrointestinal tract. Even though there are many reports available on the prebiotic activity of fructo-oligosaccharides and galacto-oligosaccharides, xylo-oligosaccharides still remains as an unexploited area. Taking all the above aspects to consideration a study has been carried out which mainly deals with the isolation, purification and characterization of xylanase from finger millet malt and its use in obtaining bioactive xylo-oligosaccharides.

Objectives and plan of work

The major objectives of the present investigation are to characterize the xylan degrading enzyme/s from ragi malt and to utilize xylanase rich enzyme fraction to produce xylo-oligosaccharides from cereal brans as well as to determine their prebiotic activity and accordingly the following work plan has been envisaged.

1. Standardization of the experimental conditions with respect to the isolation of xylanase from finger millet malt, *i.e,* ionic strength, pH, number of extractions and temperature.
2. Purification of xylanase from finger millet malt using ammonium sulphate precipitation, ion exchange and gel filtration chromatography and ascertaining their homogeneity with respect to protein / activity staining and to determine its molecular weight. Determine the kinetic parameters. *i.e.,* pH, temperature

optima's, their stabilities and the effect of inhibitors on the activity of xylanase from finger millet malt.

3. Isolation and characterization of xylo-oligosaccharide(s) obtained from cereal brans using xylanase from finger millet malt with respect to their composition, reducing end, molecular weight and linkage.
4. Determining the prebiotic activity of major oligosaccharide(s) isolated from cereal brans and correlating with their composition.

Chapter I:
Introduction

This chapter deals with the general account on xylanases *i.e*. description on xylanolytic enzymes, plant cell walls and structure of heteroxylan, xylanase distribution, induction during malting and effect of gibberellic acid during germination, xylanase classification, three dimensional structure, catalytic properties, mode of action, isolation, purification procedures and kinetic properties. This chapter also describes on the soluble and insoluble polysaccharides from cereal bran, determination of their composition and use in obtaining xylo-oligosaccharides by xylanase treatment, purification and structural characterization of oligosaccharides. A description on human gastrointestinal tract, its microflora and prebiotic activity of non-digestible oligosaccharides are given in the chapter. At the end of the chapter a brief description of *Eleusine coracana*, Indaf-15 has been given and the scope of the present study is highlighted.

Chapter II:
Materials and Methods

This chapter encompasses the general procedures, various chemicals and instruments adopted in the present investigation. Described here are the malting of ragi, different analytical methods for the quantification of total / reducing sugar, protein uronic acid and enzyme assays such as xylanase, xylosidase, arabinofuranosidase, α/ β-

galactopyranosidase and O-acetyl esterase. Procedures pertaining to the isolation of xylanase, effect of different parameters on xylanase extraction, purification including ammonium sulphate fractionation, chromatographic methods such as ion exchange and gel filtration, purity criteria by poly acrylamide gel electrophoresis (PAGE), protein/ activity staining, characterization of xylanase *i.e.* molecular weight, isoelectric focusing, pH optima and stability, temperature optima and stability, activation energy, effect of substrate concentration, determination of K_m and V_{max} of purified xylanase, effect of metal ions and inhibitors are given in detail. Isolation procedure of the soluble and insoluble polysaccharides (SP & IP) from cereal brans, determination of their composition by gas liquid chromatography (GLC), liberation of oligosaccharides from soluble polysaccharides of wheat and ragi bran by purified ragi xylanase hydrolysis are also given. Identification and structural characterization of the oligosaccharides are carried out using state of art methods such as electrospray ionization mass spectrometry (ESI-MS) and proton nuclear magnetic resonance (H^1 NMR). Various microorganisms used for the i*n vitro* fermentation experiments, preparation of the inoculum, culture media composition are given under microbiological studies. Growth characteristics (increase in absorbance at 600 nm and cell mass, decrease in pH of the culture medium) and short chain fatty acid analysis are carried out to prove the bifidogenic nature of the oligosaccharides using *Bifidobacterium and Lactobacillus* spp. Enzyme activities in the 24 h culture broth of the microbial spp tested are also determined.

Chapter III:

Isolation and purification of xylanase from finger millet malt

This chapter deals with the isolation and purification of xylanase from finger millet (Ragi, *Eleusine coracana*, Indaf-15) malt. The method for obtaining maximum recovery of xylanase, xylosidase and arabinofuranosidase from finger millet malt is also described in this chapter. The enzyme activities were determined during different malting periods such as 24, 48, 72 and 96 h. xylanase activity is maximum at 96 h and no further increase in activity was observed at 120 h. Similar results are obtained for both

xylosidase and arabinofuranosidase. Xylosidase activity is found to be slightly higher than arabinofuranosidase. The effect of various parameters such as pH and ionic strength of the extraction medium, number of extractions, polyvinyl polypyrrolidine (PVPP), Triton X-100, reduced glutathione and metal ions are studied for isolating maximum activity of xylanolytic enzymes from ragi malt. Xylanase activity is maximum in acetate buffer at 0.1 M, pH 6.0 whereas, arabinofuranosidase and xylosidase activities are found to be maximum 0.2 M acetate buffer at pHs 5.0 and 5.5 respectively. The first two consecutive extractions yielded maximum enzyme activities from ragi malt. Addition of polyvinyl polypyrrolidine (PVPP), Triton X-100 reduced glutathione and metal ions such as calcium chloride and magnesium chloride to the extraction medium enhanced the enzyme activities and accordingly the best condition for the isolation of xylanase from ragi malt is determined.

The purification is achieved in three steps. In the first step the crude enzyme extract is fractionated using ammonium sulphate. The fraction with enriched xylanase activity (ASF 20-60 %) is separated on DEAE-cellulose ion exchange column into two activity peaks, P-1 and P-II using sodium chloride gradient (0-0.5 M). Since the yield of P-I is insignificant, it is not taken for further studies. The major peak (P-I) is further purified by gel filtration chromatography on Sephadex G-75 column which resulted in apparently homogenous xylanase. The purity of the xylanase is ascertained by native and SDS-PAGE, activity staining and ESI-MS.

Chapter IV:
Characterization of xylanase

This chapter describes the characterization of the kinetic properties of purified ragi xylanase. The pH and temperature optima of the purified xylanase are found to be of 5.0 and 50^0C respectively. The enzyme is inactivated at extreme pHs (pH 2.0 and 9.0). Xylanase showed maximum stability at 35^0C and above which the activity decreased. The activation energy of the enzyme is calculated from Arrhenius plot (25 $kJmol^{-1}$). The

activation/ inhibition effects of various metal ions on xylanases activity are determined. Ethylene diamine tetra acetic acid (EDTA) and mercuric chloride ($HgCl_2$) inhibited the enzyme activity. The low K_m value indicated the high specificity of xylanase towards larchwood xylan. Citric, oxalic and boric acids, P-Chloromercuribenzoate (PCMB) and iodoacetamide inhibited the xylanase activity in concentration dependent manner. The mode of action of purified xylanase is determined by characterizing its hydrolytic products liberated from larchwood xylan by ESI-MS and H^1 NMR.

Chapter V:
Structural characterization of oligosaccharides liberated from soluble polysaccharides of wheat and ragi bran

This chapter deals with the structural characterization of the oligosaccharides liberated from soluble polysaccharides (SP) of wheat and ragi brans. The bran isolated from the cereal grains are often contaminated with the endosperm starch. Destarching of the polysaccharides is carried out sequentially using termamyl and glucoamylase treatment. The non-starch polysaccharides are isolated in acetate buffer (pH 5.0, 0.05 M). Ragi bran SP (14.4 %) yield is less compared to wheat bran (35 %) and their monosaccharide composition is determined by gas liquid chromatography. The non-starch polysaccharides of cereal brans consisted preponderantly of arabinoxylans. Cereal bran SP are used to obtain bioactive oligosaccharides by enzymatic hydrolysis using purified ragi xylanase. The oligosaccharides liberated subsequently are purified by gel filtration chromatography followed by HPLC. The structural characterization of the oligosaccharides liberated from soluble polysaccharides of both wheat and ragi bran is carried out by ESI-MS and H^1 NMR and the probable structures of the above oligosaccharides are assigned.

Chapter VI:

Prebiotic activity of the oligosaccharides liberated from soluble polysaccharides of wheat and ragi bran

In vitro experiments using *Bifidobacteria, Lactobacilli* and *Pediococci* spp are carried out to determine the prebiotic activity of the oligosaccharides liberated from soluble polysaccharides of wheat and ragi bran. *In vitro* experiments are carried out since they are easy to perform and provide the result in short span of time and are consistent in their reproducibility compared to the *in vivo* methods. The oligosaccharides were incorporated into the culture medium at 0.25 % level and the growth of the microorganisms is monitored and compared with the control which is devoid of the carbohydrate source. The prebiotic activity of the oligosaccharides is determined in terms of growth characteristics such as absorbance at 600 nm, reduction in pH and increase in cell mass. The enzymes such as xylanase, xylosidase, arabinofuranosidase, α/β-galactopyranosidase and acetyl esterase produed by the microbes for the hydrolysis of the oligosaccharides are detected in the 24 h culture broth. The short chain fatty acids (SCFA) liberated from oligosaccharide fermentation are identified by GLC. Acetate is found to be the major SCFA liberated in the case of both wheat and ragi bran oligosaccharides fermentation by all the tested species of *Bifidobacteria, Lactobacilli* and *Pediococci*.

A summary and conclusions are given at the end of results and discussion section.

The thesis ends with a list of references arranged in alphabetical order.

CHAPTER I

Introduction

1.1. Introduction

Xylanases (EC 3.2.1.8; 1, 4-β-D-xylan xylohydrolases) are key enzymes in the metabolism of heteroxylans, which are enriched in the cell wall matrix of cereals. More than 200 1, 4-β-xylan endohydrolases have been identified till date among which the majority originate from saprophytic bacteria and fungi (Simpson *et al*., 2003; Krengel & Dijkstra, 1996). Over the last decade xylanases have attracted considerable research interest and impetus because of their potential applications in various biotechnological processes. Xylanases are well characterized from microbes with respect to their mode of action and genetic engineering (Lin *et al*., 2007; Hyeong *et al*., 2007; Wong *et al*., 1988; Coughaln & Hazlewood, 1993; Subramaniyan & Prema, 2002, Liu *et al*., 1998; Tuncer & Ball, 2003). Increased attention is laid on plant sources as they are under exploited compared to microbial sources. The information pertaining to millet xylanases is found to be completely lacking.

I.2. Xylanolytic enzymes

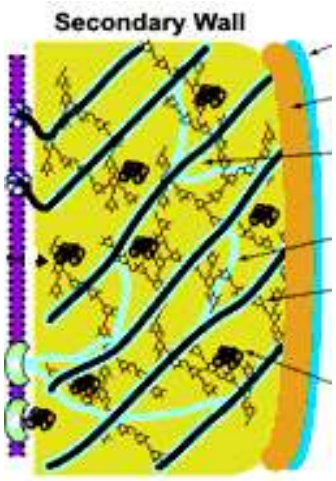
Xylanases are the major cell wall degrading enzymes after cellulases, induced during germination of cereals. Apart from xylanases (EC 3.2.1.8), xylosidases (EC 3.2.1. 37), arabinofuranosidases (EC 3.2.1.55), acetyl xylan esterases (EC 3.1.1.6) and ferulic acid esterases (EC 3.1.1.2) are also important in the degradation of cell wall and are collectively known as xylanolytic enzymes (Subramaniyan & Prema, 2002). The structure, composition and organization of plant cell walls are important for understanding the molecular mechanism of the cell wall degrading enzymes.

I.3. Components of the cell wall

The major components of plant cell walls are cellulose, lignin, hemicellulose and pectin. In addition, there are small amounts of extensins as well as structural proteins. The primary cell wall, laid down while the cells are dividing and expanding, constitutes the outermost part of the cell wall (Fig. 1). It is composed of cellulose microfibrils and

matrix polysaccharides. Contiguous cells are separated by a middle lamella (about 0.1-0.2 μm) of amorphous pectins and associated calcium ions. The secondary cell wall (1-3 μm) is laid down inside primary cell wall after cell expansion**.** It is comprised of several layers that are differentiated ultrastructurally by their different orientation of cellulose microfibrils, which are stabilized by hydrogen bonding within and between the glucan chains. Lignification is initiated in the middle lamella and primary wall and proceeds throughout the secondary wall as the cells age.

The tertiary wall is a thin membranous layer on the lumen side of the secondary cell wall and it appears to arise from the condensation of the protoplast together with lignin precursors. The occurrence of the different components differs between the primary and the secondary cell wall and also depends on the species and the type of cell (Timell, 1967). Pectins and hemicelluloses are synthesized in the golgi apparatus (Gibeaut, 2000) whereas, cellulose microfibrils are synthesized by rosette-like terminal complexes in the plasma membrane (Doblin *et al*., 2002). The cellulose microfibrils are embedded into the matrix polymers simultaneously with their synthesis (Darley *et al*., 2001).



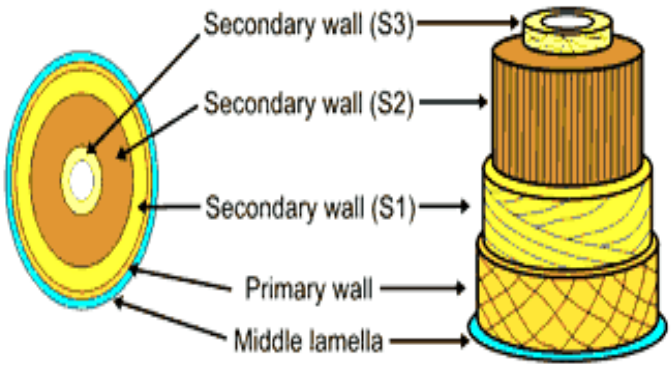


Fig. 1. Structure of plant cell wall

*(Courtesy to http://www.ccrc.uga.edu/~mao/intro/Secwall.gif)

I.4. Structure of heteroxylan and its depolymerization by xylanolytic enzymes

Hemicelluloses are the second most abundant polysaccharide constituents of plant cell walls after cellulose (Harris & Ferguson 1999). Heteroxylans constitute the major hemicellulose in the plant cell walls. Xylans are composed of a backbone of β-linked xylopyranose residues and may be classified on the basis of the nature of the linkage joining these residues. The β-1, 3-linked materials are found only in marine algae and those containing miuxture of β-1, 3 and -1,4 linkages are found in some of the sea weeds *(Rhodymenia*) while β-1, 4 linked xylans are characteristic of hard/soft woods and grasses (Barry & Dillon, 1940; Timell, 1967). Cereal hemicelluloses which are bracketed under soft wood xylans consist of arabinoxylans and 1, 3/ 1, 4 β-D glucans (Guillon *et al*., 2004; Dervilly *et al*., 2002). Xylan constitutes 15-30 % of the cell wall components of the hardwood, 7-10 % of the soft wood and up to 30 % of the annual plants. Arabinoxylans, the major non-cellulosic polysaccharides consist of β- (1, 4) linked D-xylopyranosyl residues in the backbone and occasionally substituted either at O-2 or O-3 with L-arabinofuranose residues in the side chain. Ferulic and p-coumaric acids are the minor constituents which are esterified to 5'-OH group of arabinose residues in the side chains (Barron *et al*., 2006; Grootaerta *et al.,* 2007; Sorensen *et al*., 2003). β -1, 4- xylans are mainly found in the secondary cell walls, the major component of mature cell walls in woody tissues. But in monocots they represent the major component of the primary cell wall (Timell, 1967).

The complete degradation of the heteroxylan is caused by the collective action of the xylanolytic enzymes (Fig. 2). Xylanases are endohydrolases, which depolymerize the xylan backbone liberating xylo-oligosaccharides, which vary in their degree of polymerization (D.P. 2-10). β-D-xylosidase catalyzes the hydrolysis of xylo-oligosaccharides by removing successive xylose residues from the non-reducing end (Sunna *et al*., 1997). α-L-arabinofuranosidase cleaves off the arabinose residues linked to the xylan backbone, resulting in the overall degradation of arabinoxylans. Acetyl xylan

esterase activity removes the acetate groups from the xylan backbone. Since the acetyl side groups might constitute a steric hindrance for the xylan backbone degradation, it has generally been considered that the side groups need to be removed before the complete degradation of the xylan backbone can take place (Gilbert *et al*., 1993). However, xylanase from *Thermoascus aurantiacus* was recently shown that the side groups are important determinants of the efficiency of the backbone degrading enzymes (Vardakou *et al*., 2005). Acetyl xylan esterases are having more specificity towards shorter xylo-oligosaccharides compared to linear xylans having high molecular weight (Thomson, 1993). Hence the xylan-degrading enzymes are collectively expressed for the individual xylan degradation *in vivo* during germination.

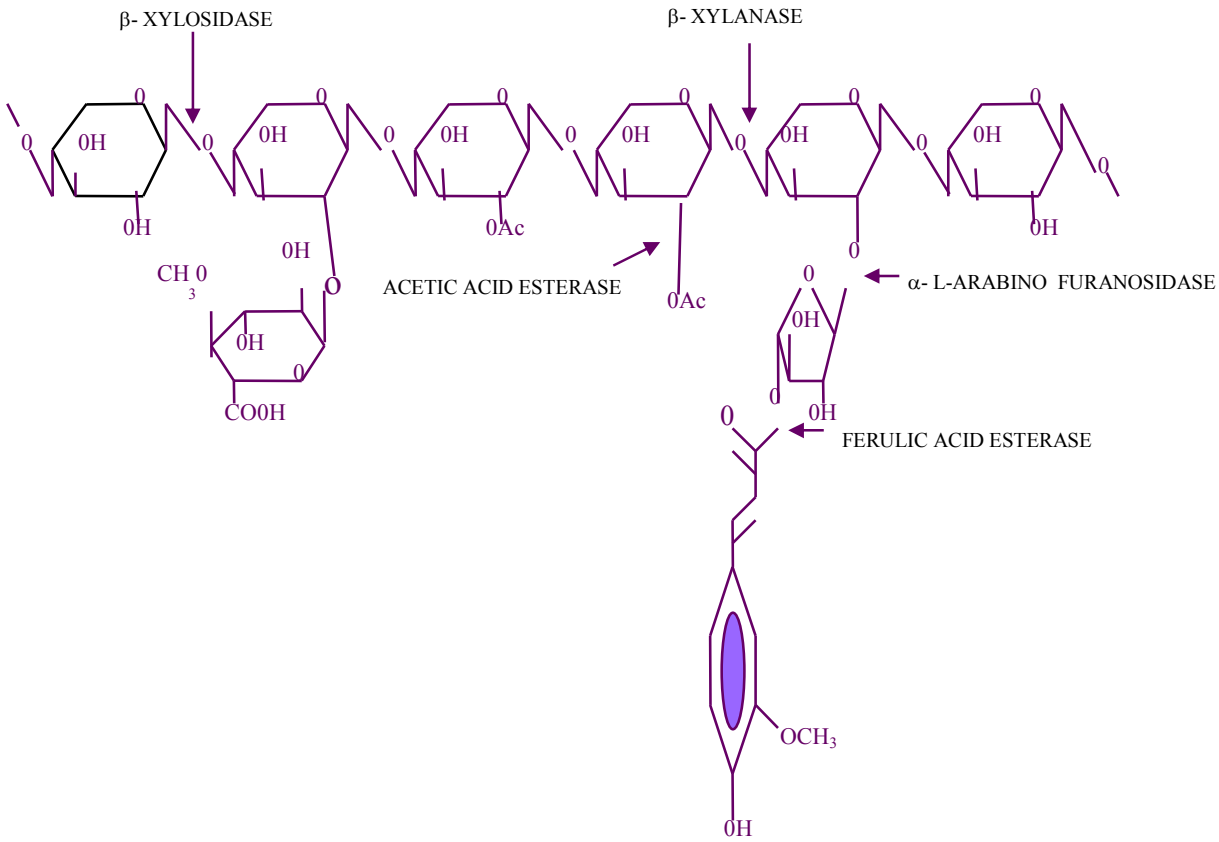


Fig. 2. Hydrolysis of heteroxylan by xylanolytic enzymes

I.5. Xylanase distribution

As mentioned earlier endoxylanases are reported to be produced mainly by microorganisms. Some members of higher animals are also able to produce xylanases (Yamura *et al.*, 1997). There are many reports on the purification and characterization of xylanases from microbial sources (Nakamura *et al*., 1993; Fernadez-Espinar *et al.,* 1994; Chen *et al*., 1997) (Table. 1).

I.5.1. Bacterial xylanases

Bacteria are the major producers of xylanases. They have attained potential applications in research due to their alkaline and thermostable nature. Members of *Bacillus* spp. such as *Bacillus* SSP-34, *Bacillus circulans* are reported to produce high xylanase activity at extreme conditions of pH and temperature (Subramaniyan & Prema 2000; Ratto *et al.*, 1992). A cellulase free xylanase was reported from *Bacillus stearothermophilus* strain T6 (Khasin *et al*., 1993). Extensive studies have been done on molecular biological aspects of bacterial xylanases with respect to *Pseudomonas fluorescens* done subsp. *Cellulosa, Butyrivibrio* and *Cladocellum saccharolyticum*. Four genes encoding two xylanases, one arabinofuranosidase and one acetyl xylan esterase have been isolated and sequenced from *Ps. fluorescens* subsp (Hazlewood & Gilbert, 1992; Luthi *et al.,* 1990). *Cellulosa* may be considered as the best characterized bacterial xylan degrading systems (Hazlewood & Gilbert, 1992).

I.5.2. Fungal xylanases

Compared to bacterial xylanases, fungi produce xylanases which tolerate temperatures below 50^0C. Fungal xylanases are also characterized with a lower pH optima compared to bacterial xylanases. *Trichoderma viride* and *Trichoderma reesei* have been reported to produce high xylanase activity. (Bailey *et al.,* 1993). Apart from *Trichoderma* spp., *Schizophyllum* are also known to produce high xylanase activity

(Steiner *et al*., 1987). Xylanases were purified and characterized from *Penicillium purpurogenum* (Belancic *et al*., 1995). Most of the fungal xylanases show considerable cellulase activity. However xylanase from *Thermomyces lanuginosus* was reported to be having negligible cellulase activity (Gomes *et al.,* 1993a, Gomes *et al.,* 1993b). Considerable analogy has been reported for bacterial and fungal xylanases. The sequence studies on xylanase from *Cryptococcus albidus* provide the first evidence that bacterial and fungal xylanases share a common origin (Boucher *et al.,* 1988).

Table. 1. Xylanases purified and characterized from bacteria and fungi.

Microorganism	Purification fold	Yield (%)	Reference
Bacteria			
Bacillus stearothermophilus T-6	38.9	46	Khasin *et al*., 1993
Bacillus amyloliquefaciens	7.3	53.9	Breccia *et al*., 1998
Bacillus sp. Strain 41-1(36)	3.6	15.3	Nakamura *et al*., 1993
Streptomyces T-7	41.3	6.7	Keskar *et al*., 1989
Fungi			
Aspergillus nidulans	24	7.5	Fernadez-Espinar *et al.,* 1994
Aureobasidium pullulans ATCC 42023	38	6.3	Vadi *et al*., 1996
Trichoderma viride	16	12.5	Ujiie *et al.*, 1991
Trichoderma longibrachiatum	55.8	5.1	Chen *et al*., 1997
Erwinia chrysanthemi	19.9	3.12	Braun and Rodrigues, 1993

I.5.3. Plant xylanases

The knowledge of plant 1, 4-β-xylan endohydrolases is restricted to wheat (Cleemput *et al*., 1997a; Cleemput *et al*., 1997b), barley (Slade *et al*., 1989; Benjavongkulchai & Spencer, 1986), maize (Bih *et al*., 1999) and *Acacia* (Lienart *et al*., 1985). Xylanase is induced during the germination of cereals and it helps in the digestion/ softening of the cell walls to facilitate the entry of α- amylase to its site of action *i.e.* endosperm wherein the storage polysaccharide, starch is degraded (Slade *et al.,* 1989; Gys *et al*., 2004). Three endo-β-1, 4-xylanase isoenzymes with an apparent M_r of 41 kDa have been purified from germinating barley (Slade *et al*., 1989) and its cDNA and genomic clone were characterized. The enzyme was expressed with a 32-amino-acid presequence having characteristics of a signal peptide and its N-terminal sequence was determined (Banik *et al*., 1996, Banik *et al*., 1997).

Two 1, 4-β -xylan endohydrolases were purified and partially characterized from wheat flour and their pH optimum was found to be pH 5.0 (Cleemput *et al*., 1997a & 1997b). The M_r of the purified wheat xylanase was 55 kDa and the enzyme was able to hydrolyze only unsubstituted xylans from oat spelts, whereas a 30 kDa xylanase showed preferential hydrolysis of wheat and rye xylan (Cleemput *et al*., 1997b). An endoxylanase of *Mr* 34 kDa was extracted from the surface of maize pollen with diethyl ether by Bih *et al*., 1999. But, antibodies raised against the 34 kDa proteins recognized a much larger (68 kDa) protein in the interior region of the pollen grains. Endoxylanase activity was enhanced in isolated barley aleurone layers treated with the phytohormone gibberellic acid (GA_3). A 29 kDa protein was detected in the extracellular medium of the aleurone layer culture (Dashek & Chrispeels, 1977). A 34 kDa endoxylanase from barley aleurone layers was purified by preparative isoelectric focusing followed by gel filtration chromatography on Sephadex G-200 (Benjavongkulchai & Spencer, 1986). The isoelectric point of the enzyme was found to be 4.6 and it showed maximum activity on xylan at pH 5.5 and at 35^0C. The enzyme showed maximum stability between pH 5 and pH 6.

An endoxylanase with a molecular weight and pI of 41 kDa and 5.2 respectively was purified from germinated barley. It showed maximum activity at pH 6.7 (Slade *et al*., 1989). The chromosomal location of the genes encoding barley endoxylanase and the appearance of mRNA encoding the enzyme was monitored in the aleurone layer during germination (Banik *et al*., 1996 & 1997). Thus the endoxylanases isolated from higher plants eventhough varied in their molecular weight (34 kDa to 60 kDa) exhibited essentially the same substrate specificity. However, recent studies have revealed that the different forms of endoxylanases found in germinated barley grain probably arise from the same gene (HvXYN-1) (Caspers *et al*., 2001). It is expressed as a precursor of M_r 61 kDa with both N- and C-terminal properties. XYN-1 is synthesized as an inactive enzyme in the cytoplasm, and only becomes active at later stages of germination when the aleurone ceases to secrete hydrolases. Upon subsequent proteolysis it generates a 41 kDa intermediate form of the enzyme having low activity and presumably corresponds to the enzyme described by Slade *et al*., 1989. A series of processing steps, mediated in part by aleurone cysteine endoproteases, yield a mature active enzyme with a M_r of 34 kDa. Processing and extracellular release of the mature enzyme coincide with the programmed cell death (PCD)-regulated disintegration of aleurone cells. Barley xylanases of apparent Mr 29 and 34 kDa were detected in the culture medium of gibberellic acid induced aleurone layers after 28-32 h incubation (Benjavongkulchai & Spencer, 1986; Dashek & Chrispeels, 1977).

In addition to these cereal enzymes, four putative endoxylanases (A_1, A_2, B and C) have been purified from *in vitro* cultured *Acacia* cells by LiCl extraction, anion-exchange chromatography, gel filtration chromatography and flat-bed electrofocusing procedures. A_2 was further purified and its molecular weight was determined as 55 kDa (Lienart *et al*., 1985).

I.6. Induction and regulation of xylanases during germination

In germinating cereal grains, the aleurone tissue secretes hydrolytic enzymes into the endosperm storage tissue resulting in the degradation of stored starch, protein and residual nucleic acids, providing supply of nutrients for the growing embryo (Caspers *et al.,* 2001; Jensen, 1994). Thus the depolymerization of endosperm cell walls is essential for the mobilization of endosperm reserves. Cereal endosperm cell walls and aleurone layers account for 95 % of arabinoxylans and (1, 3 & 1, 4)-β-glucans with approximately 2 % each of cellulose and glucomannans (Fincher, 1992).

Gibberellic acid (GA_3) is a natural plant hormone which induces the release of hydrolytic enzymes during germination of seeds (Taiz & Honigman, 1976). GA_3 may account for the induction of xylanase during germination. Caspers *et al*., 2001 has reported the synthesis, processing and export of cytoplasmic endo-1, 4- β- xylanase from barley aleurone during germination. In the initial stages of germination the aleurone layers are significantly thicker, comprising a thin inner wall and a thick outer wall. In the later stages of germination they showed strong autofluorescence due to the abundance of ferulic acid esterified with arabinoxylan polymers in the cell wall (Bacic & Stone, 1981). Induction of endoxylanase is reported to occur at a later stage in comparison to glucanase, xylosidase and arabinofuranosidase during germination of barley (Caspers *et al.*, 2001).

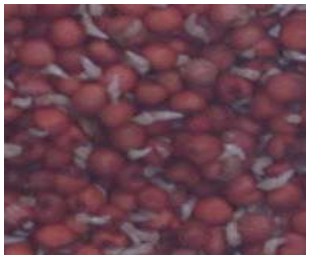
I.7. Malting

Malting is a biotechnological process applied to cereal grains, in which the grains are allowed to germinate under controlled conditions in a B.O.D incubator. Malting involves three major steps such as steeping, germination and kilning. During steeping the grain is hydrated to a level where it germinates. The extent of steeping is important for uniform germination. During the germination steps the induction of various hydrolases like amylases, proteases, esterases, pentosanases (xylanases, xylosidases and

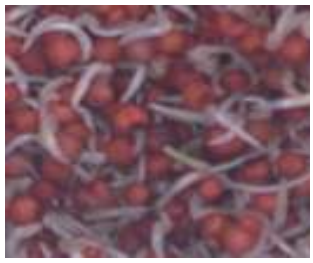
arabinofuranosidases), glucanases etc. take place. Kilning, controlled drying at 40-50^{0}C is carried out to arrest the growth of seedling. In the present study ragi seeds were malted for different time periods such as 24, 48, 72 and 96 h (Fig.3). Changes in hydrolytic pattern and protein quality during malting have been previously documented with respect to barley and sorghum (Woonton *et al.,* 2005, Ogbonna *et al*., 2003). Increased amylase activity has been reported during malting of finger millet which was maximum at 72 h (Nirmala & Muralikrishna, 2000; Nirmala & Muralikrishna, 2003 a).

Panicle

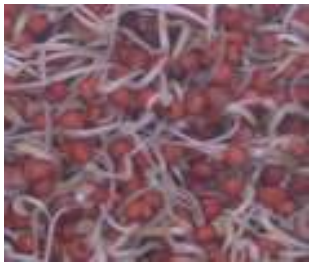
Ragi seeds (N)



24 h (M)



48 h (M)



72 h (M)

96 h (M)

Fig. 3. Malting of ragi

* N (Native), M (Malted)

I.8. Classification of xylanases

Endo (1, 4)- β- xylanases are found in the glycoside hydrolase (GH) families 5, 8, 10, 11 and 43 (Parkkinen *et al.,* 2002; Collins *et al*., 2002; Van Petegem *et al.*, 2003; Henrissat, 1991; Henrissat & Bairoch, 1993) whereas (1, 4)- β- xylosidases are found in GH families 3, 30, 39, 43, 52 and 54. Eventhough xylanases from microorganisms have been characterized in detail only a few plant xylanases have received attention (Lienart *et al.,* 1985; Benjavongkulchai *et al.,* 1986; Slade *et al.,* 1989; Cleemput *et al.,* 1997a, Cleemput *et al.,* 1997b; Bih *et al*., 1999; Suzuki *et al*., 2002). Initially xylanases were classified only into two GH families 10 (F) and 11 (G) based on amino acid sequence similarities (http://afmb.cnrs-mrs.fr/CAZY/) (Henrissat, 1991, Henrissat & Bairoch, 1993). Family 10 consist of proteins with molecular masses exceeding 35 kDa whereas family 11 consist of lower molecular weight proteins (20- 30 kDa) (Tanaka *et al*., 2004). Significant differences in three-dimensional structure and catalytic properties between the two families were also reported (Kulkarni *et al*., 1999; Biely *et al*., 1997). All plant endo-(1, 4)- β- xylanases identified so far belong to GH family 10, whereas all putative plant (1, 4)- β- xylosidases are classified into GH family 3 (Simpson *et al*., 1993).

I.9. Structure of xylanases

The three dimensional structure of GH family 10 and 11 endoxylanases were determined from both bacteria and fungi (Jeffries, 1996). The difference in their catalytic activities can be due to the difference in their tertiary structure. The family 11 endoxylanases are having a low molecular weight with molecular organization mainly of β- pleated sheets accommodating five to seven xylopyranosyl residues in the catalytic site. The overall structure of the catalytic domain of family 10 xylanase is an eight-folded barrel. The substrate binds to shallow groove on the bottom of the bowl. The substrate binding sites of family 10 endoxylanases are not as deep cleft as that of family 11 and may account for the lower substrate specificity of family 10. The endoxylanase IBCX

from *Bacillus circulans* belongs to the family 11 (Jeffries, 1996; Davoodi *et al*., 1995) (Fig. 4) wherein the catalytic domain folds into two β sheets (A & B) constituted mostly by antiparallel β strands and one short α helix (Sapag *et al*., 2002).

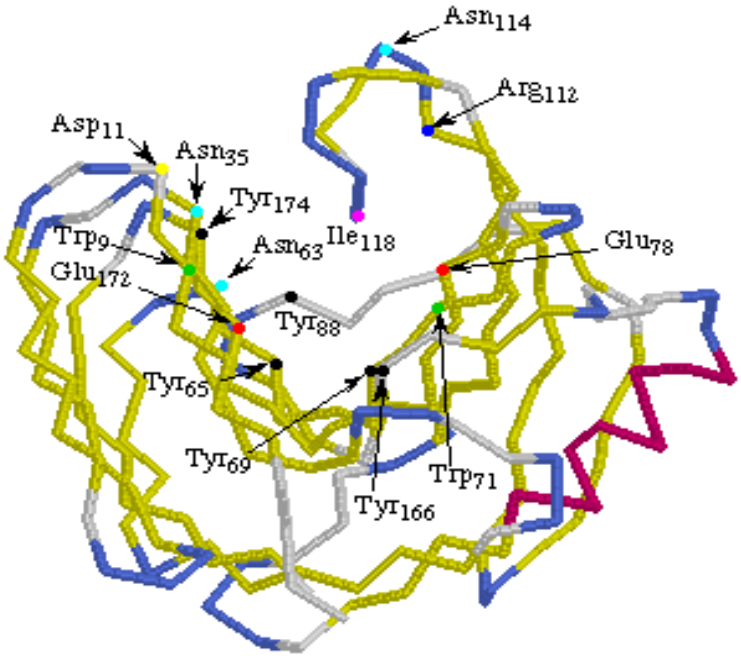


Fig. 4. The three dimensional structure of Family 11 xylanase from *Bacillus circulans*. (β- pleated structure is present more than 50 %, while extreme right side structure denotes α- helical structure. Glu 172 and Glu 78 are at catalytic site).
*(Courtesy to Protein Data Bank- PDB- http://www.rcsb.org.pdb/index.html.; http://www2.biotech.wisc.edu/jeffries/xylanase)

Catalytic properties

The catalytic mechanisms and substrate binding properties are mainly deduced from microbial xylanases since relatively few plant xylanases have been studied.

I.10. Catalytic sites

The catalytic domain of family 10 xylanases is a cylindrical [(α)/ (β)] barrel resembling a salad bowl, with the catalytic site at the narrower end, near the carboxyl terminus of the barrel (Kuno *et al*., 2000). Family 10 xylanases have relatively high molecular weights, and they tend to form oligosaccharides having low D.P. The overall structure of xylanase from *Cellulomonas fimi* resembles a tadpole with a catalytic (amino-terminal) 'head' and a cellulose-binding domain (carboxyl-terminal) 'tail' (White *et al*., 1994). Family 11 catalytic domains consist principally of β- pleated sheets formed into a two- layered trough that surrounds the catalytic site. Isoleucine is the terminating amino acid residue of the long loop protruding down into the trough at one side of the protein. The trough has been likened to the palm and fingers, and the loop to the thumb of a right hand (Jeffries, 1996). The positions of many amino acids are essentially identical in Family 11 xylanases from bacterial (e.g. *Bacillus circulans*) or fungal (e.g. *Trichoderma reesei*) origins (Davoodi *et al*., 1995; Torronen *et al*., 1994).

I.11. Mode of action of xylanase

In the case of Family 10 endoxylanases, hydrolysis occurs with retention of anomeric configuration by a double displacement mechanism (Fig. 5). The glycosidic O-atom is protonated by a catalytic acid/base which is usually an aspartic acid or glutamic acid residue. This cause cleavage of bond and the aglycone portion of the substrate diffuses away. A positively charged oxocarbonium ion-like transition state is formed simultaneously and reacts with a second catalytic amino acid residue, the catalytic nucleophile, to generate a covalent glycosyl-enzyme intermediate. The glycosyl-enzyme

intermediate is subsequently hydrolyzed to release the product and the catalytic acid/base is re-protonated for the subsequent catalytic action. Thus among the two amino acids one functions as a catalytic nucleophile, while the other functions as an acid/base. Other amino acid residues conserved in the active site region may also be important in the catalytic mechanism (Simpson *et al.,* 2003).

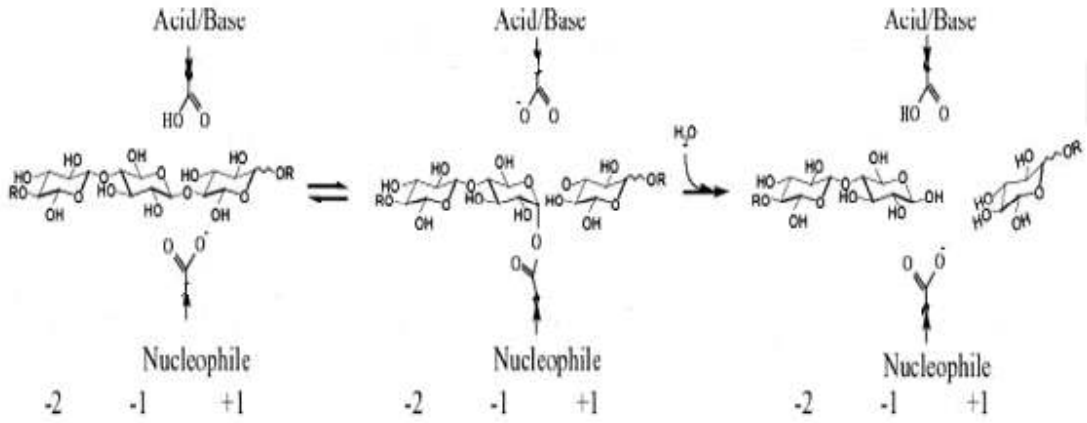


Fig. 5. Schematic representation of the proposed catalytic mechanism for the hydrolysis of glycosidic linkages by family 10 β- xylan endohydrolases.

*(Source- Simpson *et al*., (2003). J. Cereal. Sci. 37,111-127; Elsevier Science Ltd.)

I.12. Isolation of xylanase

Isolation of xylanases from cereal malts are generally carried out using buffers such as sodium phosphate, sodium acetate etc. The pH of the buffers is maintained in the range of 4.5-7.0 for maximum xylanase activity thereby preventing the inactivation of the enzyme at extremes of pHs. The ionic strength of the solution is an important parameter affecting the enzyme activity. Detergents have been used to disrupt membranes and

extract lipoprotein fragments into aqueous media as components of micelles. Detergents such as natural bile salts (cholate), non-ionic synthetic detergents (Triton X-100) and ionic synthetic detergents which may be zwitterionic as in the case of CHAPS, acidic (SDS) or basic (CTAB) are generally used in the isolation of the enzyme. The choice of the detergent is dependent whether the enzyme is needed in the active form or not. So non-ionic detergents are preferred for the extraction of an active enzyme. Phenolic adsorbents are also added to the extraction medium to prevent the co-extraction of phenolic acids (Hsu & Heatherbell, 1987).

I.13. Xylanase assay

Dinitrosalicylic acid (DNS) and arsenomolybdate (ARS) methods are the commonly used methods for xylanase assay. Xylanase activity was determined by measuring the release of reducing sugars from water soluble birchwood/larchwood xylans using ARS or DNS. DNS method is more specific and sensitive compared to that of ARS based on the released products' reactivity towards the respective substrates (Jeffries *et al*., 1998).

I.14. Purification of xylanase

I.14.1. Fractional precipitation

Fractional precipitation comes under the preliminary purification procedures of the enzymes. Large volumes of enzyme extracts can be concentrated by this method using salts such as ammonium sulphate or organic solvents such as ethanol or acetone. Ammonium sulphate precipitation has been used in the purification of cereal xylanases (Cleemput *et al.,* 1997; Slade *et al*., 1989). Ammonium sulphate fraction (ASF) 0-30 % was used in the purification of an endoxylanase from wheat flour (Cleemput *et al*., 1997). 20-40 % ASF was used in the purification of xylanase from germinated barley (Slade *et al.,* 1989). Ammonium sulphate fractionation was also employed in the purification of microbial xylanases (Chivero *et al*., 2001; Latif *et al.*, 2006; Jiang *et al.,* 2005). It is

carried out at lower temperatures to reduce the inactivation of the enzyme at high salt concentrations. The salt concentration is increased gradually and at a particular concentration the relevant enzyme will get precipitated. The principle behind this method is that the presence of salts decreases the solvating power of water by tying up many of the water molecules by interaction with the salt ions. Since water molecules are removed from the surface of proteins, the hydrophobic patches due to surface located residues such as leucine, valine, phenylalanine etc. are exposed which interact with each other resulting in the aggregation of the proteins present. Proteins which contain more hydrophobic residues on their surface precipitate at a relatively low salt concentration (Price, 1996).

I.14.2. Ion exchange chromatography

Ion exchange chromatography is one of the most widely used methods for the purification of xylanase. In ion exchange chromatography the protein separation is based on the charge. DEAE-cellulose is the most widely used anion exchange matrix for the purification of xylanases from the microbial sources. DEAE-cellulose was used in the purification of endo-1, 4-β-xylanase from *Trichosporon cutaneum* (Stüttgen & Sahm, 1982) and *Bacillus* sp. (Sapre *et al*., 2005). DEAE-sepharose was used in the purification of xylanase from *Enterobacter* sp (Khandeparker & Bhosle, 2006a). Anion exchange column (Mono-Q HR) was used in the purification of an endoxylanase from wheat flour (Cleemput *et al.,* 1997). Q-sepharose high performance column was used in the purification of wheat xylanase. Q-sepharose CL-RB and CM-sepharose CL-RB were used in the purification of xylanase from *Fibrobacter succinogenes* S85 (Matte & Forsberg, 1992)

I.14.3. Hydrophobic interaction chromatography

Phenyl sepharose column was used in the purification of wheat xylanase (Cleemput *et al.*, 1997). Phenyl sepharose 6 fast flow was used for the purification of xylanase from *Melanocarpus albomyces* which resulted in fold purification of 7 and

recovery of 60 % (Gupta *et al*., 2002). Affinity chromatography using eudragit S-100, a copolymer of methylacrylic acid and methylmethacrylate was employed in the purification of xylanase from *Aspergillus niger* (Sharma & Gupta, 2002).

I.14.4. Gel filtration chromatography

In gel filtration chromatography the proteins get separated according to their molecular weight. Sephadex G-75 is one of the most widely used matrixes for xylanase purification. Sephadex G-75 was used in the purification of two xylanases from *Bacillus licheniformis* 77-2 (Damiano *et al.,* 2006). Sephadex G-200 column was used in the purification of barley aleurone layer xylanase (Benjavongkulchai & Spencer, 1986). Sephacryl-200 was used in the purification of xylanase from *Bacillus stearothermophilus* strain (Nanmori *et al*., 1990) and *Bacillus thermantarcticus* (Lama *et al*., 2004)

I.14.5. Criteria of purity

There are several methods to ascertain the purity of the enzymes which mainly dependent on the charge, molecular mass, biological activity and other biophysical properties of proteins. Among them the most widely used methods are polyacrylamide gel electrophoresis (PAGE), activity straining, electrospray ionization mass spectrometry (ESI-MS) and gel filtration chromatography (Royer & Nakas, 1990; Winterhalter & Liebl, 1995).

I.15. Kinetic properties of xylanases

I.15.1. PH optima and pH stability

Enzymes are amphoteric molecules containing a large number of acidic and basic groups, mainly situated on their surface. The charges on these groups will vary according to their acid dissociation constants and pH of the surrounding environment. This will affect the total net charge of the enzymes and the distribution of charge on their exterior surfaces, in addition to the reactivity of the catalytically active groups. These effects are

especially important in the neighborhood of the active sites. Taken together, the changes in charges with pH also affect the activity, structural stability and solubility of the enzyme. The most favorable pH value where the enzyme is most active is known as the optimum pH. Extremely high or low pH values generally result in complete loss of activity for most of the enzymes. For each enzyme there is also a region of pH optimal stability. The optimum pH and stability values will vary greatly from one enzyme to another**.** Cereal xylanases are reported to have pH optima in the range of pH 5.0-7.0 (Slade *et al*., 1989; Benjavongkulchai & Spencer, 1986) whereas thermostable xylanases purified from microbial sources showed maximum activity at a higher pH range (Gessesse, 1998).

I.15.2. Temperature optima and thermal stability

Enzyme will have an optimum temperature at which it functions most rapidly. At low temperatures an enzyme is inactive and as the temperature increases towards the optimum the kinetic energy of the enzyme and substrate molecules will increase. Thus there will be more collisions between them and the rate of the reaction gets increased. At temperatures above the optimum the tertiary structure of the protein is altered causing alteration in the shape of the active site, finally resulting in the denaturation of the enzyme. Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases as the temperature is raised. A 10^0C rise in temperature will increase the activity of most enzymes by 50 to 100 %. Variations in reaction temperature as small as 1 or 2 degrees may introduce changes of 10 to 20 % in the results. In the case of enzymatic reactions, this is complicated by the fact that many enzymes are adversely affected by high temperatures. Cereal xylanases showed a temperature optimum below 50^0C (Cleemput et al., 1997; Benjavongkulchai & Spencer, 1986). Microbial xylanase are reported to have a high temperature optimum compared to plant xylanase (Nakamura *et al*., 1993; Gessesse, 1998).

Activation energy is the minimum energy required for the specific reaction. From the Arrhenius equation, the activation energy can be expressed as $K= Ae^{-Ea}/RT$,
where, A is the frequency factor for the reaction, R is the universal gas constant and T is the temperature (in Kelvin). The higher the temperature, the more likely the reaction will be able to overcome the energy of activation. A is a steric factor, which expresses the probability that the molecules contain a favorable orientation and will be able to proceed in a collision. In order to precede the reaction and overcome the activation energy, the temperature, orientation and energy of the molecules must be substantial and this equation manages to sum up all of these things. Arrhenius plot showed an increased value of activation energy for immobilized xylanase (227 kcal/mol) compared to free xylanase (210 kcal/mol) isolated from *Scytalidium thermophilum* which indicated the higher thermal stability of the free enzyme (Ruchi *et al*., 2005).

I.15.3. **Isoelectric focusing**

Isoelectric focusing is a method in which the proteins are separated in a pH gradient according to their isoelectric point. Isoelectric point (pI) is the pH at which the net charge on the protein molecule is zero. At this pH the protein molecules have no electrophoretic mobility and get focused into narrow zones. Proteins are introduced into polyacrylamide gels containing ampholytes. Ampholytes are synthetic, aliphatic polyaminopolycarboxylic acids available commercially whose individual pI values cover a preselected pH range. Commercial ampholytes include Ampholine, BioLyte and Pharmalyte. When an electric current is applied, the ampholyte molecules migrate to cathode or anode depending on their net charge. At a pH below its pI, proteins carry positive charge and above negative. The extraction of an enzyme is carried out at a pH value far from its isoelectric point since proteins are least soluble at their isoelectric point. Preparative isoelectric focusing was used in the purification of barley aleurone layer xylanase by Benjavongkulchai & Spencer, 1986. The pI of xylanase purified from wheat flour was reported to be 5.5 (Cleemput *et al.,* 1997).

I.15.4. Effect of activators/ stabilizers

Increase in xylanase activity was observed in the presence of metal ions such as Co^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Mg^{2+} and Ca^{2+}, whereas strong inhibition of the enzyme activity was noticed in the presence of Hg^{2+} and EDTA **(**Khandeparker & Bhosle, 2006a). Xylanases produced by *Bacillus circulans* BL53 and *Arthrobacter* sp. MTCC 5214 were found to be strongly inhibited by Hg^{2+} (Hecka *et al*., 2006; Khandeparker & Bhosle, 2006b; Bataillon *et al*., 2000; Nakamura *et al*., 1993). Chelating agent, EDTA was also found to be inhibiting the xylanase activity (Latif *et al.*, 2006).

I.16. Cereal bran polysaccharides

Destarched cereal bran soluble and insoluble polysaccharides (SP & IP) mainly consist of arabinoxylans. Arabinoxylans (AX) structurally constitute β- (1-4) linked D-xylopyranosyl residues in the backbone and occasionally substituted either at O-2 or O-3 with L-arabinofuranose residues in the side chain. Ferulic and p-coumaric acids are the minor constituents which are esterified to 5^1-OH group of arabinose residues in the side chains (Sorensen *et al*., 2003; Goesaert *et al*., 2001). Unsubstituted linear xylans have been isolated from esparto grass and seaweeds. Extensive studies have been carried out on barley and rye arabinoxylans (Debyser *et al.,* 1997; Rasmussen *et al.,* 2001). AX from wheat, rye, and barley on average have a relatively low degree of substitution (Dervilly *et al*., 2002; lzydorczyk *et al*., 1991). They contain higher proportions of unsubstituted xylose residues and lower levels of monosubstituted xylose residues than the more highly branched AX from rice (Shibuya & Iwasaki, 1985) and sorghum (Vietor *et al*., 1994). The highest levels of doubly substituted xylose have been reported from wheat pericarp AX (Maes & Delcour, 2002).

I.17. Composition of the polysaccharides

Determination of the composition of polysaccharide involves the identification of their sugar constituents. The first step is the depolymerization of the polysaccharide for which the most widely used method is acid hydrolysis. Aldose containing polysaccharides can be completely hydrolyzed with 0.5 or 1.0 M sulphuric acid at 100^0C for about 6 h (Selvendran *et al*., 1979) or by 1.0 M trifluoro acetic acid at 120^0C for 1 h. (Albersheim *et al*., 1967). Mild acid hydrolysis using 0.1 M oxalic acid at 70^0C for 1 h (Aspinall *et al*., 1953) is carried out for ketose containing sugars since they are unstable at conditions described for aldoses. Amino sugar containing polysaccharides are hydrolyzed completely by HCl (4 M) at 100^0C for about 6 h. The monosaccharides liberated after acid hydrolysis are derivatized and analyzed by gas liquid chromatography (GLC). GLC is more sensitive compared to high performance liquid chromatography (HPLC) and detects sample in nanograms. Alditol acetates and trimethyl silyl ethers and trifluoroacetyl esters are the most commonly prepared derivatives (Sawardekar *et al*., 1995).

I.18. Xylo-oligosaccharides- Isolation, purification and characterization

Both chemical and enzymatic methods are used for the isolation of xylo-oligosaccharides from heteroxylans (Reis *et al*., 2003; Reis *et al.,* 2005; Kabel *et al*., 2002). But enzymatic method has several advantages over chemical method such as their specificity, both to linkage type and substitution pattern, high reaction rates, control over the reaction etc. Gel filtration chromatography is employed for the separation of the oligosaccharides liberated by enzymatic hydrolysis. Biogel gel filtration matrixes (Biogel P-2, P-4 and P-6) are widely used for xylo-oligosaccharide purification (Kormelink *et al.,* 1993). The purity of the oligosaccharide recovered from Biogel column needs to be

ascertained on HPLC for further structural analyses such as ESI-MS (Reis *et al*., 2003 & 2004) and NMR (Vietor *et al*., 1994; Gruppen *et al*., 1992). HPLC method is non-destructive, requires no derivatization and the sample can be recovered after analysis. Here the separation of individual sugars is based on either on cation/anion exchange (water) or on partition (acetonitrile:water) chromatographies. Detection of oligosaccharides is done by using refractive index (RI) detector. The sample concentration should be in micrograms due to the low sensitivity of the RI detector.

I.19. Electrospray ionization mass spectrometry (ESI-MS)

ESI-MS is a powerful technique used to determine the mass of the biomolecules. Mass spectrometry is based on the principle that ions of different mass: charge ratio (m/Z) are separated because they are differently deflected by magnetic and electrostatic fields. It is especially useful in producing ions from macromolecules because it overcomes the propensity of these molecules to fragment when ionized. The method has also been employed to identify poly/oligosaccharides. But the method does not differentiate between the isomers (Fernandez *et al.,* 2004; Reis *et al.,* 2005).

I.20. NMR spectroscopy

It is a powerful technique that can provide detailed information on the topology, dynamics and three-dimensional structure of molecules in solution and solid state. It is a physical phenomenon involving the interaction of atomic nuclei placed in an external magnetic field with an applied electromagnetic field oscillating at a particular frequency. Magnetic field within the material is measured by monitoring radiation absorbed and emitted by atomic nuclei. H^1 NMR and C^{13} NMR are the most commonly used techniques to elucidate structure of poly as well as oligosaccharides (Gruppen *et al*., 1992; Vietor *et al*., 1994).

I.21. Biotechnological applications of xylanase

Xylanases are widely used for various biotechnological applications (Polizeli *et al*., 2005). The most common use of xylanase is as a bleaching agent in the pulp and paper Industry. The use of xylanase leads to a reduction in organo-chlorine pollutants such as dioxin from the paper making process. In addition, chlorine-free bleaching (such as peroxide or ozone bleaching) can achieve brighter results with the addition of xylanase. Because xylanase does not harm cellulose, the strength of the paper product is not adversely affected. Endoxylanases can be used to obtain xylo-oligosaccharides having great significance in food industry as prebiotics. There are also a number of other commercial applications (Kulkarni *et al*., 1999, Collins *et al.,* 2005, Polizeli *et al*., 2005). In juice-, wine- and beer-making, xylanases are used for clarification and in the feed industry both cellulases and xylanases have been shown to reduce the viscosity of the seed extracts, thereby ameliorating the uptake of nutrients. Also, xylanases can be used for the conversion of xylan into xylitol, a sweetener used for low-calorie products in the food industry.

I.22. The human gastrointestinal tract and its microflora

The human gut microflora constitutes a dynamic and ecologically diverse environment. It is estimated that there are about 10-100 trillion cells of bacteria in the intestinal tract. Thus, 10- 50 % of our total cells are due to bacteria in a normal individual who is not on antibiotics. The microflora of an adult human gut predominantly consists of facultative anaerobes and obligate anaerobes such as *Bacteroides, Bifidobacterium, Eubacterium, Clostridium, Peptococcus, Peptostreptococcus, Ruminococcus, Escherichia, Enterobacter, Enterococcus, Klebsiella, Lactobacillus and Proteus.* The intestinal flora of an adult is composed of approximately 100 species of bacteria, which are present at a level of 10^{10} to 10^{11} per gram of colonic content (Macfarlane & Macfarlane, 1997; O'Sullivan, 1996).

These intestinal bacteria can be classified into 3 groups depending on their effect on the intestinal environment; *i.e*. beneficial bacteria, harmful bacteria and bacteria exhibiting an intermediate property. Harmful bacteria are those that possess pathogenicity or transform food components into harmful substances, and they include *Clostridium, Veillonella, Proteus* and the *Enterobacteriaceae* family. Beneficial bacteria represented by *Bifidobacterium* and *Lactobacillus* suppress the harmful bacteria and exert many beneficial physiological effects. They have no harmful effect on the host. In the large intestine, the number of *Lactobacillus* is approximately 1/100 that of *Bifidobacterium,* and the influence on the intestinal environment is less than that of *Bifidobacterium.* Finally, *Bacteroides, Eubacterium* and anaerobic *streptococci* belong to the intermediate group. These bacteria do not show any virulence under normal conditions, but they may cause opportunistic infections when the host immunity or resistance is lowered. (Macfarlane & Macfarlane, 1997). The human gastrointestinal tract consists of mouth, oral cavity, esophagus, stomach, small intestine and colon (Fig. 6).

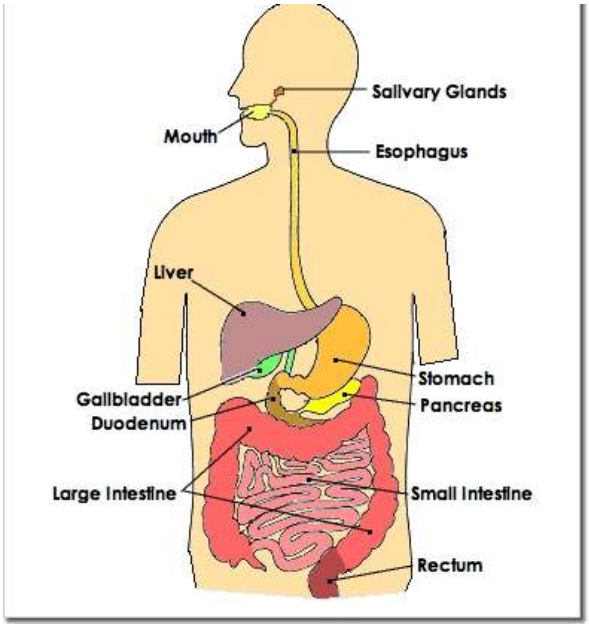


Fig. 6. Human digestive system

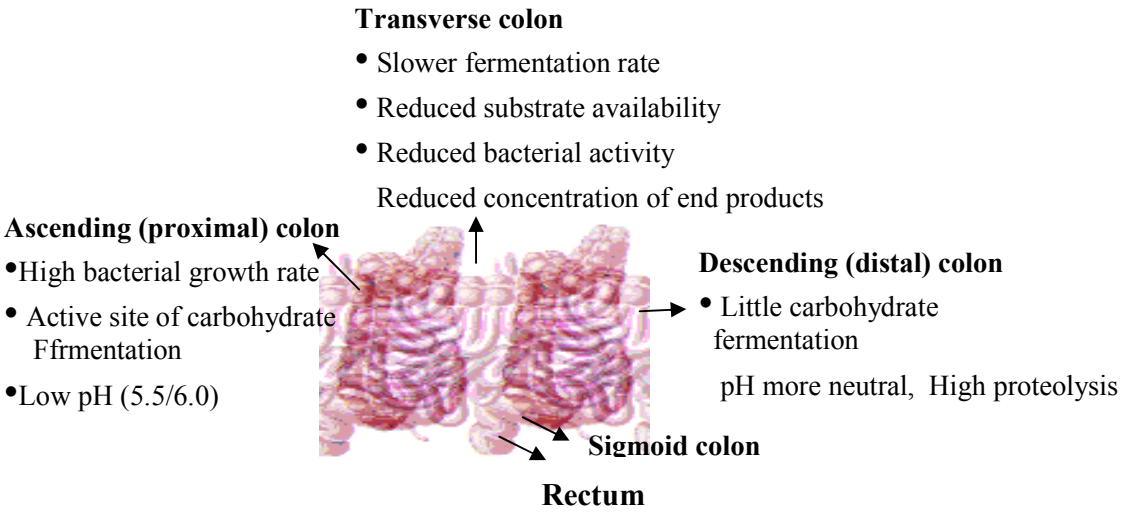


Fig. 7. Large intestine

Table. 2. The functions of human digestive system and factors that determine population levels of microbiota in gastro intestinal tract

	Mouth	**Stomach**	**Duodenum**	**Ileum**	**Colon**
pH	Alkaline	1.0-3.0	Acidic-Neutral	Neutral-Alkaline	5.5-7.2
Function	Mastication/ Partial digestion	Digestion	Digestion/ absorption	Digestion/ absorption	Digestion/ fluid and salts
Approximate no. of cells per ml or g content	10^8	10-100	10-1000	10^4-10^6	10^{11}-10^{12}

A balanced gut flora is important for our health and well-being. This microflora plays an important role in the digestive process and is governed by the various physiological conditions of the intestinal tract (Table. 2). The typical 'function' of the large intestine (Fig. 7) is often thought to be water absorption, storage and then excretion of waste material. However, because of the metabolic capacity of the gut flora (which ferments about 100 g of food each day), the hindgut is probably the most active organ in the body. However, some species are beneficial because they can repress the activities of the harmful types. This has led to the development of foods that serve to increase numbers of the latter.

Recently, the functional food research has moved progressively towards the development of dietary supplementation, introducing the concept of probiotics and prebiotics. The aim of these products is to affect the gut microbial composition and activities. International Scientific Association for Probiotics and Prebiotics (ISAPP, California, 2002) involved in research on fundamental and applied aspects of probiotics and prebiotics. Probiotics include live microorganisms in the food, while prebiotics are carbohydrates which have selective effects that enhance the growth of the 'beneficial'

flora already in the gut. It is estimated that at any one time 1 in 5 people are experiencing a digestive disorder. This may manifest itself in acute forms such as gastroenteritis. One key to this interaction is the targeting of beneficial microorganisms in the gut. In the present study we have given emphasis on prebiotics and *in vitro* experiments that have been conducted to prove the prebiotic activity of the xylo-oligosaccharides.

I.23. Prebiotics

The term prebiotics was coined by Gibson and Roberfroid. Prebiotics can be 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). '*Lactobacilli (L. acidophilus, L. casei, L. delbruekii)* and *Bifidobacteria (B. adolescentis, B. bifidum, B. longum, B. infantis)* are the two important beneficial bacteria inhabiting the human colon. The prebiotic effect of *Bifidobacteria, Lactobacilli and Pediococci* has been monitored in the present study. Some of the candidate prebiotics have been listed in Table. 2.

I.23.1. *Bifidobacteria*

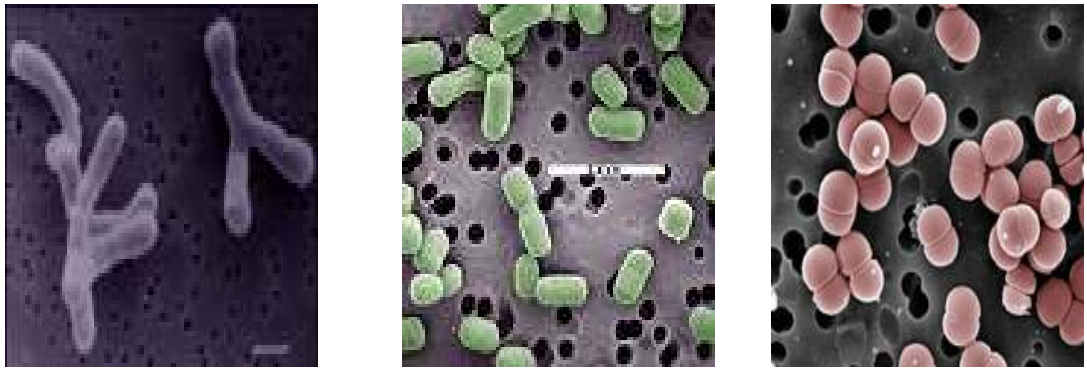
Bifidobacteria are the normal inhabitants of human and animal colon. The population of these bacteria remains almost stable until advanced when it appears to decline. They are gram positive anaerobes, non-motile and non-spore forming. They have various shapes, including short, curved rods, club-shaped rods and bifurcated Y-shaped rods (Fig. 8a). The name is derived from the observation that often they exist in bifid (Y) form. To date around 30 spp of *Bifidobacteria* have been isolated and some of them are *Bifidobacterium adolescentis*, *Bifidobacterium bifidum, Bifidobacterium animalis, Bifidobacterium breve* and *Bifidobacterium longum* (Roy, 2001; http://microbewiki.kenyon.edu/index.php/Bifidobacterium).

I.23.2. *Lactobacilli*

Lactobacilli are the inhabitants of human intestine. They are gram positive facultative anaerobes, non-spore forming and non-flagellated rod or *coccobacilli* (Fig. 8b) To date almost 56 species have been identified. Some of them *are Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus plantarum*, *Lactobacillus salivarus* etc **(**Axelsson, L. 1998; Moura *et al.*, 2007).

I.23.3. *Pediococci*

The genus *Pediococcus*, a member of *Streptococcaceae* is a gram positive *lactic acid bacteria* (Fig. 8c). Pediococcus pentosaceus show good growth in both aerobic micro aerophilic conditions and they have diverse and unique morphological, physiological, nutritional and genetic characteristics. They uniquely divide to form tetrads. Cultures usually show cocci in pairs. *Pediococcus pentosaceus, Pediococcus damnosus, Pediococcus parvulus, Pediococcus inopinatus, Pediococcus dextrinicus, Pediococcus halophilus, Pediococcus urinaeequi (*Osmanagaoglu *et al*., 2001)



a **b** **c**

Bifidobacteria ***Lactobacilli*** ***Pediococci***

Fig. 8. Beneficial bacteria

Table. 3. A few examples of the prebiotic oligosaccharides

Prebiotic	Isolation method	Trade name
Inulin Fructo-oligosaccharide, FOS)	Hot water extraction from chicory root (followed by enzymatic hydrolysis) or polymerization of fructose monomers (Bornet *et al.,* 2002	Meioligo NutraFlora Raftilose & Raftiline
Galacto-oligosaccharide GOS)	Enzymatic-lactose transglycosyolation (Teuri & Korpel, 1998)	Oligomate TOS-Syrup
Xylo-oligosaccharide (XOS)	Enzymatic hydrolysis of plant xylans (Imaizumi *et al*., 1991)	Xylo-oligo
Isomalto-oligosaccharide (IMO)	Transglycosylation of liquefied starch (Morgan *et al*., 1992)	Isomalto-900 Biotose and Panorich
Lactulose	Isomerization of lactose (Salminen & Salminen, 1997)	MLP/P/C

I.24. Non-digestible oligosaccharides (NDOs)

Functional food/functional food ingredients are having good market in today's global food industry. Of the currently known functional foods, non-digestible oligosaccharides hold an important position with respect to their prebiotic activity (Kolida & Gibson, 2007; Swennen *et al.,* 2006). Effective bifidogenic doses appear to vary among the different oligosaccharide types. However, most oligosaccharides have been demonstrated to increase beneficial bacteria numbers in the colon at doses of >15 g/d. In the large intestine prebiotics in addition to their selective growth enhancing effects on *Bifidobacteria* and *Lactobacilli*, influence many other important aspects of bowel function through *in vitro* fermentation (Moura *et al*., 2007). Human studies are time consuming and it would be unreasonable to expect that every new prebiotic undergo the same process of testing. *In vitro* methods hold the advantage that they relate the properties of the new prebiotics reasonably well to their physiological function and analytical results. So this stands as a better method to screen potential prebiotics.

Carbohydrates are found to be the first limiting nutrient for many bacterial species in the intestinal tract and thus the type of carbohydrates available influence the growth of the gut microflora. NDOs are oligomeric carbohydrates, whose glycosidic bond allows resistance to intestinal digestive enzymes in the upper gastrointestinal tract to become 'colonic nutrients' (Delzenne, 2003) for the selective growth of the beneficial microflora such as *Bifidobacteria* and *Lactobacilli*. They produce specific hydrolases which convert these oligosaccharides into short-chain fatty acids (SCFA) by colonic fermentation. Acetate, propionate and butyrate are the major SCFA liberated due to carbohydrate fermentation. *Bifidobacterium* strains are largely described as capable of efficiently fermenting xylose-based oligo and polysaccharides (Crittenden *et al.,* 2002). Growth characteristics of the beneficial bacteria such as decrease in the culture broth pH, increased bacterial cell mass and absorbance at 600 nm are monitored in the *in vitro* fermentation experiments. Butyrate serves as the preferred energy substrate and prevents colon cancer in humans (Rycroft *et al.,* 2001). Acetate is mainly metabolized in human

muscle, kidney, heart and brain. Propionate has been suggested to spare amino acids that would be used in gluconeogenesis in the postabsorptive state (Demigne & Remesy, 1991).

I.25. Health benefits of prebiotics

In addition to their bifidogenic nature the oligosaccharides have many health benefits at physiological level. The first is the improvement of gastrointestinal conditions, including a normal stool frequency, less constipation, and healthy intestinal microflora (Kurasawa *et al.,* 2000; Crittenden & Playne, 1996). The second is the promotion of mineral absorption, including an increase of bone density and relief of anemia. Short chain fatty acids (SCFA) resulting from colon fermentation lowers the colon pH and thus enhances the solubility/bioavailability of minerals, particularly calcium and magnesium (Chonan *et al.,* 2001). The third is an immunomodulation effect, such as allergy and cancer prevention (Rycroft *et al.,* 2001). They also help in lipid regulation (Costabile *et al*., 2007). Although the mechanism is currently unknown, studies have shown positive results and mechanistic hypothesis have been developed. Studies have been conducted using XOS and FOS. A study on diabetic rats found that when XOS replaced with simple sugars, serum cholesterol and triglycerides were found to be decreased. This was due to the inhibition of lipogenic enzymes in liver (Imaizumi *et al*., 1991).

I.26. Cereals/Millets

Cereals contribute over 60 % of the world food production and serve as biosource for many important enzymes in biotechnology. Finger millet *(Eleusine coracana*- Indaf-15) is used as the biosource to obtain xylanases in the present study.

Fig. 9. *Eleusine coracana*

Binomial name: *Eleusine coracana L.*

Division : Magnoliophyta
Class : Liliopsida
Order : Poales
Family : Poaceae
Subfamily: Chloridoideae
Genus : Eleusine
Species : E. coracana
Kingdom : Plantae

Synonyms: ragi, ragee, African millet, coracan, corakan, kurakkan, *Eleusine coracana*

Finger millet is an annual plant widely grown as a cereal in the arid areas of Africa and Asia. Finger millet is originally native to the Ethiopian Highlands and was introduced into India approximately 4000 years ago. It is very adaptable to higher elevations and is grown in the Himalaya up to 2300 meters altitude. Finger millet is especially valuable as it contains the amino acid methionine, which is lacking in the diets of hundreds of millions of the poor who live on staple foods such as cassava, plantain, polished rice, or maize meal. It is used in geriatric, infant and health foods both in native and malted conditions due to its high amount of calcium, dietary fiber and hydrolytic enzymes (Nirmala *et al.,* 2000, Nirmala & Muralikrishna, 2003a). Nutritive value of ragi per 100 gms: Protein 7.3(gm) Fat 1.3(gm) CHO 72(gm) Minerals 2.7(gm) Calcium 344(mg) Fibre 3.6(gm) Energy 328(K Cal).

I.27. Scope of the present study

Cereals are grown over 73 % of the total world harvested area providing dietary fibre, energy, proteins etc. required for human health (Charalampopoulos *et al*., 2002). Endogenous plant enzymes have been utilized in traditional food processes for a long time and high specificity and possible isolation of these enzymes from their natural sources offer several applications in food industry (Teichgraber *et al.,* 1993). The recovery of enzymes from plant sources has attained increased attention in recent research due to environmental concern. But the information pertaining to plant xylanases is limited compared to microbial sources. An authenticated variety of *Eleusine coracana*, *i.e.*,'Indaf-15', known to be fast germinating according to the breeders was chosen as the biosource for xylanase in the present study.

Cereal brans, an important by-product of cereal industry are rich in non-cellulosic polysaccharides such as arabinoxylans, 1, 3/1, 4-β-D-glucans and lignocellulose complexes which represent a vast renewable energy resource which can be enzymatically converted into bioactive compounds such as oligosaccharides and phenolic acids. Recently the functional food research has moved progressively towards the development of dietary supplementation introducing the concept of prebiotics which considerably affect the gut microbial composition and activities. Xylo-oligosaccharides have not yet been explored to the extent as candidate prebiotics in global food market compared to fructo-oligosaccharides and galacto-oligosaccharides. Apart from their prebiotic effect non-digestible oligosaccharides also possess various important physiological functions. They are believed to alleviate disease symptoms such as diabetes, arteriosclerosis and colon cancer.

The present study focus on the isolation, purification and characterization of an endoxylanase from finger millet (Ragi; *Eleusine coracana,* Indaf-15) malt. The kinetic properties of the enzyme are well studied for its best exploitation in obtaining prebiotic xylo-oligosaccharides from cereal brans which will surely contribute to objectives of the

today's progressing functional food industry.

The following constitutes the main objectives of the present study:

5. Standardization of the experimental conditions with respect to the isolation of xylanase from finger millet malt, *i.e,* ionic strength, pH, number of extractions and temperature.
6. Purification of xylanase from finger millet malt using ammonium sulphate precipitation, ion exchange and gel filtration chromatography and ascertaining their homogeneity with respect to protein / activity staining and to determine its molecular weight. Determine the kinetic parameters. *i.e.,* pH, temperature optima's, their stabilities and the effect of inhibitors on the activity of xylanase from finger millet malt.
7. Isolation and characterization of xylo-oligosaccharide(s) obtained from cereal brans using xylanase from finger millet malt with respect to their composition, reducing end, molecular weight and linkage.
8. Determining the prebiotic activity of major oligosaccharide(s) isolated from cereal brans and correlating with their composition.

CHAPTER II

Materials and methods

II.1. General

- All the results are average values of minimum of three experiments.
- Reagent preparations and extractions were carried out using double glass-distilled water.
- Room temperature was ~ 25^0C.
- Boiling water bath temperature was ~ 95^0C, unless otherwise mentioned.
- Dialysis against double distilled water or buffer was carried out at 4^0C by using dialysis bags with a cut off range of ~ 10 kDa.
- Concentration/ evaporation of samples was carried out using Buchi Rotavapor (RE 111) with a water bath temperature ranging from 30 to 40^0C.
- Spectrophotometric readings of test solutions with appropriate blanks were taken using shimadzu double beam spectrophotometer (UV-160 A), Japan.
- Lyophilization was carried out using Virtis Freeze Mobile (12 SL), Germany.
- Centrifugation was carried out either in Sigma (202 C), Hermle (Z 320 K) or Remi (RC 8) centrifuges.
- Ion exchange and gel filtration column chromatography fractions were collected by using Gilson FC 203 B fraction collector.
- Autoclaving was done at ~ 121^0C, ~ 15 lbs for ~ 20 min.

II.2. Materials

Finger millet (Ragi; *Eleusine coracana*, Gaertn. Indaf-15), rice (*Oryza sativa*, L.) and maize (*Zea mays*, L.) seeds were procured from V.C. Farm, University of Agricultural Sciences, Bangalore, located at Mandya, Karnataka.

II.3. Chemicals

Chemicals used in the present investigation were obtained from various agencies/companies as discussed below:

II.3.1. Sigma Chemical Company, St. Louis, USA:

Enzymes

Glucoamylase (EC 3.2.1.3) from *Aspergillus niger*, Termamyl (EC 3.2.1.1) from *Bacillus licheniformis*.

Substrates

Larchwood xylan, p- nitrophenyl β- D- xylopyranoside, p- nitrophenyl α- L- arabinofuranoside, p- nitrophenyl α/ β -D- galactopyranoside, and p- nitrophenyl acetate.

Gel matrices and ion exchange resins

DEAE- cellulose (0.99 meq/g) and Sephadex G-75 (super fine, 12-15 ml/g).

Other chemicals

Ethylene diamine tetra acetic acid (EDTA), Iodoacetamide, Bovine serum albumin (BSA) and Dinitro salicylic acid (DNS), Coomassie brilliant blue G-250/ R-250, Carbazole, sodium azide and sodium borohydride.

II.3.2. Other Sources

Protein molecular weight markers were obtained from Genei, Bangalore, India.
Sugar standards (xylose, glucose, maltose, arabinose, mannose, galactose, fucose) were from ICN Pharmaceuticals Inc., Life Sciences group, Cleveland, USA.
Biogel P-2 [fine, 40-90 μm (wet)] was obtained from Bio-Rad laboratories, Bangalore, India.
Microbiological culture media and media ingredients were obtained from HiMedia, Mumbai, India.
Microbiological filters (0.2 micron) were from Millipore, Bangalore, India.

Column for HPLC analysis (μ-Bondapak amino propyl column) was obtained from Waters Associates, Milford, USA.

HPLC grade solvents were obtained from Qualigens Fine Chemicals, Ranbaxy Fine chemicals Ltd. India.

All other chemicals used were of analytical grade and were obtained from E- Merck, BDH and SRL (India).

II.4. Instruments

Following instruments were used for various experiments:

B.O.D. incubator from Industrial and Laboratory Tools Corporation, Chennai, India.

GC-15 A, equipped with FID, from Shimadzu, Japan

HPLC- LC 10 A, equipped with RI and UV-photo diode array detectors, Shimadzu, Japan

Broviga mini slab gel electrophoresis unit, Balaji Scientific Services, Chennai, India.

UV-visible Spectrophotometer (UVH 60A) from Shimadzu, Japan.

ESI-MS by Alliance, Waters 2695 mass spectrometer.

NMR - Bruker AMX 500 MHz Spectrometer from Bruker Biospin, Germany.

II.5. Columns

II.5.1. GLC analysis

OV-225 (ϕ 1/8" x 8 ft), 3 % on Chromosorb W (80-100 mesh) and PEG- 20 M (ϕ 1/8" x 8 ft) were obtained from Pierce Chemical Company, Rockford, Illinois, USA.

II.5.2. HPLC analysis

μ-Bondapak aminopropyl column (4.1 mm x 30 cm) was obtained from Waters Associates, Milford, USA.

II.6. Methods

II.6.1. Distillation/ purification of solvents

Phenol, acetic anhydride and pyridine were purified by distilling in all glass distillation apparatus following standard distillation protocols.

II.6.2. Malting of ragi

Ragi seeds (100 g) were cleaned, surface sterilized with sodium hypochlorite (0.02 %) for 1-5 min and steeped for 16 h and germinated under controlled conditions on moist cloth at 25^0C in a B.O.D incubator. Germinated seeds were taken from the incubator at different time intervals such as 24, 48, 72, 96 and 120 h and dried in an air oven at 50^0C for 12 h and vegetative growth portions were removed by hand brushing. Devegetated seeds were weighed, powdered and used for the extraction of the enzyme (Nirmala *et al*., 2000).

II.6.3. Spectrophotometric methods

II.6.3.1. Total carbohydrate estimation

To a sample (0.5 ml) containing carbohydrate, phenol (0.3 ml, 5%) followed by concentrated sulphuric acid (H_2SO_4, 1.8 ml) were added. The absorbance was read at 480 nm after 20 min. incubation at room temperature. Xylose (5-25 µg) was taken as reference sugar for preparing the standard curve (Mckelvy & Lee, 1969).

II.6.3.2. Reducing sugar by dinitrosalicylic acid method

Preparation of DNS reagent:

Dinitrosalicylic acid (1 g) was dissolved in a solution containing sodium potassium tartrate (30 g) and 0.4 N NaOH (20 ml) in a 500 ml beaker and the content was made up to 100 ml with double distilled water. The reagent was filtered through a whatman No.1 filter paper and stored in a brown bottle at 4^0C.

To the sample (1 ml) in a test tube, DNS reagent (1 ml) was added, mixed well and incubated in a boiling water bath for 10 min. The contents were then cooled and diluted with double distilled water (2 ml) and the absorbance was read at 550 nm (Miller, 1959). Reducing sugar content was determined by referring to the standard curve prepared by using D- xylose (0.1-1.0 mg/ ml).

II.6.3.3. Uronic acid estimation by carbazole method

To a test tube containing sample solution (0.5 ml), kept in ice-cold temperature was added conc. sulphuric acid (3 ml) and the mixture was kept in a boiling water bath for 20 min., cooled and then carbazole solution (0.1 ml, 0.1 %- prepared by dissolving recrystrallized carbazole in alcohol) was added. The tubes were kept in dark for 2 h and the O.D was taken at 530 nm (Knutson & Jeanes, 1968). Uronic acid content was determined by referring to the standard graph prepared by using D-galacturonic acid.

II.6.3.4. Protein estimation

II.6.3.4.1. Bradford method

Bradford reagent preparation:

Coomassie brilliant blue G-250 (10 mg) was dissolved in ethanol (5 ml, 95%) and phosphoric acid (10 ml, 85%) was added to it. The solution was made up to 100 ml with double distilled water, filtered through whatman No.1 filter paper and stored in a brown bottle at 4^0C.

To the sample (0.2 ml) Bradford reagent (0.8 ml) was added and mixed well. The absorbance was read at 590 nm. BSA (2-10 µg/0.2 ml) was used for preparing standard curve (Bradford, 1976).

II.6.3.4.2. Spectrophotometric method

The presence of protein/ peptide bonds in column fractions was monitored by taking absorbance at 280/ 220 nm, respectively (Peterson, 1983).

II.6.4. Enzyme assays

II.6.4.1. Xylanase

Xylanase activity was determined by measuring the release of reducing sugar from Larchwood xylan by DNS (Dinitro salicylic acid) method. Larchwood xylan (0.5 % in acetate buffer) was incubated with the enzyme (100 µl) at 50^0C for 1 h. The reaction was stopped by the addition of DNS (1.0 ml) followed by boiling for 10 min. and the colour developed was quantified colorimetrically at 550 nm. Activity is expressed as µmoles of xylose released under assay conditions. One unit of enzyme activity is defined as the amount of enzyme required to release 1 µmole of xylose/min under the experimental conditions (Cleemput *et al*., 1997).

II.6.4.2. β - D- xylopyranosidase

The substrate, p-nitrophenyl β-D-xylopyranoside (0.5 ml, 2 mmol in sodium phosphate buffer) was incubated with sample (100 µl) for 1 h at 37^0C. The reaction was stopped by adding saturated solution of sodium tetraborate (0.5 ml). Absorbance was read at 400 nm. One unit of activity is defined as the amount of enzyme required to liberate 1 µmol of p-nitrophenol/min under assay conditions (Beldman *et al*., 1996).

p- nitrophenol (2-10 µg/ 0.5 ml) was mixed with equal volumes of saturated solution of sodium tetraborate (0.5 ml). Absorbance was read at 400 nm and the standard curve was prepared.

II.6.4.3. α-L- arabinofuranosidase

p- nitrophenyl α-L- arabinofuranoside was taken as the substrate (Beldman et al., 1996) and the assay was performed as mentioned above (Section II.6.4.2.).

II.6.4.4. α-D-galactopyranosidase

p- nitrophenyl α-D-galactopyranoside was taken as the substrate and assay was performed as mentioned above (Section II.6.4.2.).

II.6.4.5. β - D- galactopyranosidase

p- nitrophenyl β -D-galactopyranoside was taken as the substrate and assay was performed as mentioned above (Section II.6.4.2.).

II.6.4.6. Acetyl esterase

The substrate, p-nitrophenyl acetate (0.5 ml, 2 mmol in sodium phosphate buffer) was incubated with sample (20 µl) for 30 min at 37^0C. The reaction was stopped by the addition of saturated solution of sodium tetraborate (0.5 ml). Absorbance was read at 400 nm. One unit of activity is defined as the amount of enzyme required to liberate 1 µmol of p-nitrophenol/min under assay conditions (Biely & Schneider, 1985).

II.6.4.7. Amylase

Gelatinized soluble starch (1 %, 1 ml) in sodium acetate buffer (50 mM, pH 5.0) was incubated with appropriately diluted enzyme (50 µl) at 45^0C for 30 min. The reaction was stopped by adding DNS reagent (1 ml). The reducing sugar was estimated by DNS method (Miller, 1959). One unit of enzyme activity was defined as µmoles of maltose equivalent released/ min under the assay conditions.

II.6.5. Isolation of xylanase from malted ragi

II.6.5.1. Determination of xylanase activity in different malts

Ragi malts *viz*, 24, 48, 72, 96 and 120 h were extracted with acetate buffer (pH 5.0, 0.1 M) for 1 h at 4^0C separately, followed by centrifugation (10000 g) at 4^0C for 10 min. Supernatants were collected and dialyzed overnight against the extraction buffer at 4^0C. Xylanase activity in the supernatants was determined by DNS (Dinitro salicylic acid) method using larchwood xylan as the substrate.

II.6.5.2. Effect of different parameters on xylanase, xylosidase and arabinofuranosidase extraction

II.6.5.2.1. Effect of pH and ionic strength

Ragi malt (1 g, 96 h) was extracted with acetate buffer (pH 4.5, 5.0, 5.5 & 6.0) separately for 1 h at 4^0C, followed by centrifugation (10000 g) at 4^0C for 10 min. Supernatants were collected and dialyzed overnight against the respective extraction buffer at 4^0C. Xylanase activities in the supernatants were determined by DNS (Dinitro salicylic acid) method using larchwood xylan as the substrate.

To determine the effect of ionic strength of the extractant, ragi malt (1 g, 96 h) was dispersed in acetate buffer (pH 6.0) varying in ionic strength (0.05, 0.1, 0.15 & 0.2 M) and the extractions were carried out as mentioned above. Xylanase activity was determined by DNS method.

II.6.5.2.2. Effect of PVPP

Ragi malt (1 g, 96 h) was extracted with acetate buffer (pH 6.0, 0.1 M) consisting of polyvinyl polypyrrolidine (PVPP, 0.25, 0.5, 0.75 & 1.0 %) for 1 h at 4^0C separately, followed by centrifugation (10000 g) at 4^0C for 10 min. (Machiah & Vakil, 1984).

Supernatants were collected and dialyzed overnight against 0.1 M acetate buffer (pH 6.0) at 4^0C. Xylanase activity was determined by DNS method using Larchwood xylan as the substrate.

II.6.5.2.3. Effect of reduced glutathione and Triton X-100

Ragi malt (1 g, 96 h) was extracted with acetate buffer (pH 6.0, 0.1 M) consisting of different concentrations of reduced glutathione (2.5, 5.0, 7.5 & 10 mM) for 1 h at 4^0C, followed by centrifugation (10000 g) at 4^0C for 10 min. Supernatants were collected and dialyzed overnight against 0.1 M acetate buffer (pH 6.0) at 4^0C. Xylanase activity was determined by DNS method using Larchwood xylan as the substrate.

Ragi malt (1 g, 96 h), dispersed in separate beakers containing acetate buffer (pH 6.0, 0.1 M) and Triton-X 100 (0.25- 1.0 %) was extracted as mentioned above. Xylanase activity was determined by DNS method.

II.6.5.2.4. Effect of metal ions

Ragi malt (1 g, 96 h) was dispersed in different beakers containing 3 ml of acetate buffer (pH 6.0, 0.1 M) to which metal ions such as calcium chloride ($CaCl_2$, 5, 10, 15, 20 & 25 mM) and magnesium chloride ($MgCl_2$, 5, 10, 15, 20 & 25 mM) were added individually and extractions were carried out for 1 h at 4^0C, followed by centrifugation (10000 g at 4^0C for 10 min.) (Machiah & Vakil, 1984). Supernatants were collected and dialyzed overnight against acetate buffer (0.1 M, pH 6.0) at 4^0C. Xylanase activity was determined by DNS method using Larchwood xylan as the substrate.

II.6.6. Purification and characterization of xylanase

II.6.6.1. Preparation of the crude enzyme extract

96 h ragi malt (50 g) was suspended in 0.1 M sodium acetate buffer (150 ml, pH

6.0, 0.1 M) containing 1 % PVPP and extracted for 2 h at 4^0C. The twice extracted 96 h ragi malt was centrifuged and the supernatants obtained after centrifugation were pooled and dialyzed overnight against the extraction buffer and the resultant enzyme solution was hereafter referred to as crude enzyme extract.

II.6.6.2. Fractionation by ammonium sulphate (AS)

To the crude enzyme extract solid ammonium sulphate was added (20 %) and the precipitate was removed by centrifugation (10,000 g) in a refrigerated centrifuge and further subjected to fractional precipitation resulting in fractions, 0-20 %, 20-40 %, 40-60 % and 60-80 %. Ammonium sulphate fraction (ASF 20-60 %) having approximately 80 % of the total enzyme activity was dialyzed against 20 mM phosphate buffer (pH 7.0) and xylanase activity was estimated.

II.6.6.3. Chromatographic methods

II.6.6.3.1. Ion exchange chromatography

Ion exchange chromatography was carried out on DEAE-cellulose column.

Regeneration of DEAE-cellulose

Anion exchanger (30 g) was kept in water at room temperature for 24 h for swelling, followed by washing with distilled water to remove the fines. DEAE-cellulose was treated with HCl (0.1 N) for 30 min. at room temperature with occasional stirring using a glass rod. The excess acid was washed with water till it comes to neutral pH and subsequently treated with NaOH (0.1 M) as mentioned above. DEAE-cellulose was then thoroughly washed with water and preserved in sodium phosphate buffer (20 mM, pH 7.0) at 4^0 C.

Purification of xylanase (ASF 20-60 %) on DEAE-cellulose

Regenerated and degassed DEAE-cellulose (60 ml) was packed in a glass column (2.5 X 28 cm) equilibrated with sodium phosphate buffer (20 mM, pH 7.0, 500 ml) at a flow rate of 19.2 ml/h. ASF (20-60 %) having enriched xylanase activity was dialyzed against 20 mM phosphate buffer (pH 7.0) and subsequently loaded on DEAE-cellulose column. The column was washed with the equilibrating buffer to remove the unbound proteins. The bound proteins were eluted using a linear gradient of NaCl (0-0.5 M) in equilibrating buffer and monitored for protein as well as xylanase activity. Fractions were collected (18 ml/h) and monitored for protein as well as xylanase.

II.6.6.3.2. Gel filtration chromatography

Purification on Sephadex G-75

Sephadex G-75 superfine (10 g) was kept in water for 24 h at room temperature for swelling. The swollen gel was washed with water to remove the fine particles, degassed and packed in a glass column (0.9X100 cm) and equilibrated with sodium acetate buffer (50 mM, pH 5.0, 500 ml) at a flow rate of 7.5 ml/h. The active fraction (P-1) of DEAE-cellulose was concentrated and loaded (1 ml) on Sephadex G-75 column. Fractions were collected (7.2 ml/h) and monitored for protein and xylanase activity.

II.6.7. Purity criteria

II.6.7.1. SDS-Poly Acrylamide Gel Electrophoresis (PAGE)

Vertical slab gel electrophoresis was carried out at room temperature (Laemmli, 1970) in a Broviga mini model electrophoresis unit at room temperature.

Reagents

a) *Monomer*: -Acrylamide (29.2 g) and Bis acrylamide (0.8 g) were dissolved in water (50 ml) and final volume was made to 100 ml, filtered and stored in a brown bottle at 4^0C.

b) *Separating gel buffer*: Tris (18.15 g) was dissolved in water, pH was adjusted to 8.8 with 6 N HCl and made upto 100 ml and stored at 4^0C.

c) *Stacking gel buffer*: Tris (3 g) was dissolved in water, pH was adjusted to 6.8 with 6 N HCl and made upto 50 ml and stored at 4^0C.

d) *Sodium dodecyl sulphate (SDS):* 10 g was dissolved in 100 ml water.

e) *Ammonium persulphate (APS)*: 100 mg was dissolved in 1 ml of water.

f) *Tank buffer*: Tris (0.3 g) and glycine (1.44) and SDS (0.1 g) were dissolved in water and made upto 100 ml.

g) *Cocktail buffer:* Mixed 2.5 ml of solution (c), 2 ml solution (d), glycerol (2 ml), β-mercaptoethanol (1 ml) and bromophenol blue (0.01 %, 0.5 ml) and stored at 4^0C.

Staining reagents

a) Coomassie staining

a.1) *Staining reagent*: Coomassie brilliant blue R-250 (100 mg) was dissolved in methanol: acetic acid: water (100 ml, 12.5:7.5:30, v/v). The reagent was filtered and stored at room temperature.

a. 2) *Destaining solution*: Methanol: acetic acid: water (12.5: 7.5:30 v/v).

b) Silver staining

b.1) *Fixing*: Acetic acid (10 ml), methanol (30 ml) and formaldehyde (75 µl) were mixed and made upto 100 ml with water.

b. 2) *Ethanol* (50 %)

b. 3*) Pretreatment solution*: Sodium thiosulphate (200 mg) in 100 ml water.

b. 4) *Impregnating solution*: Silver nitrate (200 mg) dissolved in water, added formaldehyde (25 µl) and made upto 100 ml.

b. 5) *Developing solution*: Sodium carbonate (6 g) was dissolved in water containing sodium thiosulphate (1 mg), formaldehyde (25 µl) and made upto 100 ml.

b. 6*) Stopping solution*: Acetic acid (7 ml) and methanol (10 ml) were mixed and made upto 100 ml.

Running gel (12 %, 0.7 mm) was prepared by mixing solution 'a' (2.4 ml), solution 'b' (1.51 ml) and water (1.96 ml) and degassed. Solution 'd' (60 µl), TEMED (10 µl) and solution 'e' (60 µl) were added to this, mixed well and poured between the assembled glass plates with the edges sealed with 2 % agar. Gels were allowed to polymerize at room temperature for 2 h.

Stacking gel (5 %) was prepared by mixing solution 'a' (0.347 ml) and 'c' (0.5 ml) with water (1.107 ml) and degassed. To this mixture was added solution 'd' (20 µl), TEMED (10 µl) and solution 'e' (20 µl) and poured above the polymerized running gel. The polymerization was facilitated at room temperature for 2 h.

Protein samples (15 µg) were prepared by dissolving in solution 'g' (10 µl). This was heated in a boiling water bath for 3 min. Cooled samples were loaded (25 µl) on to the wells immersed in solution 'f' and were run at a constant voltage (50 V) for 3 h or until the tracking dye was just above the lower end of the gel.

II.6.7.2. Native PAGE

PAGE (12 %) under native conditions was carried out to evaluate the purity of xylanase. The electrophoresis was carried out as described above without SDS. The protein sample (15 µg) was dissolved in sample buffer devoid of SDS and β-mercaptoethanol. Duplicate samples (25 µl) were loaded on the wells and run for simultaneous protein and

enzyme stainings at a constant voltage (50 V) for 3 h. The gels were stained for both protein (Wray *et al*., 1981) as well as activity stainings (Royer & Nakas, 1990).

Gel staining

After electrophoresis the gels were stained for protein by any one of the following methods:

a) *Coomassie blue staining*: After electrophoresis gel was kept in solution (a.1) for 8 h and sustained by solution (a. 2).
b) *Silver staining*: Gel after electrophoresis was mixed in solution (b.1) for 45 min. followed with washing by solution (b. 2) twice for 30 min. each. Subsequently the gel was placed in solution (b. 3) for 1 min. with continuous shaking. After washing thoroughly with water, the gel was kept in solution (b. 4) for 20 min. with shaking. The protein bands were visualized by treating with solution (b. 5) for 10-15 min. or till the bands appear. Staining was arrested by placing the gel in solution (b. 6) and stored in 6 % acetic acid (Wray *et al*., 1981).

II.6.7.3. Activity staining

Zymogram analysis was done using Remazole Brilliant Blue (RBB) - dyed xylan (sigma) as the substrate. To prepare the substrate gel of RBB-xylan (45 mg) was dissolved in deionized water (3 ml), heated to 50^0C and mixed with 3 % agarose (sigma) in sodium acetate buffer (0.1 M, pH 5.0). After hardening, the substrate gel was carefully overlaid with a protein gel and incubated at 50^0C for 1 ½ h. After the incubation period the substrate and running gels were separated and the former was immersed in ethanol- sodium acetate buffer (2:1) for 10- 20 min. Activity bands were observed as clear colorless areas in the gel against the bluish background (Royer & Nakas, 1990).

II.6.8. Determination of Molecular weight

II.6.8.1. Molecular weight determination by SDS-PAGE

SDS-PAGE was performed on 15% gel according to the method of Laemmli (Laemmli, 1970). Phosphorylase (97 kDa), BSA (63 kDa), ovalbumin (43 kDa), Soybean trypsin inhibitor (20 kDa) and Lysozyme (14 kDa) were used as SDS-PAGE standards. After the run, gels were stained for protein with coomassie staining.

II.6.8.2. ESI-MS of xylanase

Mass spectra of the purified xylanase was measured by Alliance, Waters 2695 mass spectrometer using positive mode electro spray ionization. Capillary voltage was 3.5 kV, core voltage 100 V, source temperature 120^0C, disolvation temperature 300^0C, core gas (Argon) 50 lt h^{-1} and disolvation gas (Nitrogen) 500 lt h^{-1} (Stapels *et al.,* 2004).

II.6.9. Isoelectric focusing

The pI of the enzyme was determined using Multiphor II (LKB) system using polyacrylamide gels (7.5 %) containing ampholines (pH 3 - 9, Sigma). pI markers (Sigma) ranging from 3.6-9.3 were used (Cleemput *et al*., 1997). The polyacrylamide gel was run at different voltages starting from 200 V in the pre-run (30 min.) and after sample loading at 400 (I h), 600 and 800 V (30 min. each) followed by 1000 V (1 h). The gel was washed with 10 % trichloroacetic acid (TCA) to remove the ampholines followed by water wash and stained by coomassie staining.

II.6.10. Effect of temperature

II.6.10.1. Temperature optima

The activity of xylanase was determined at a temperature range of 30- 60^0C with an interval of 5^0C. Larchwood xylan (0.5 %) was used as the substrate. The maximum activity was taken as 100 % and relative activities were plotted against different temperatures.

II.6.10.2. Thermal stability

Thermal stability of xylanase was determined by pre-incubating the enzyme in acetate buffer (0.1 M, pH 5.0) at different temperatures (ranging from 30-60^{0}C) for 15 min. followed by measuring the residual activity. The original activity was taken as 100 % and relative activity was plotted against different temperatures.

II.6.10.3. Activation energy

The activation energy for xylanase was calculated from Arrhenius plot at a temperature range of 30- 60^{0}C with an interval of 5^{0}C. A graph is plotted taking *lnk* against 1/T. The slope of the graph is –Ea/RT from which activation energy (Ea) was calculated. (R-gas constant, 8.31432 J/mol; T-Temperature in Kelvin) (Baral *et al*., 1995).

II.6.11. Effect of pH

II.6.11.1. pH optima

The activity of xylanase at various pH values were determined using different buffers such as sodium acetate and sodium succinate (pH 4.0-6.0), sodium phosphate (pH 6.0-7.5) and Tris-HCl (pH 7.0-9.0) at 0.1 M concentration. The maximum activity was taken as 100 % and relative activity plotted against different pH values.

II.6.11.2. pH stability

The stability of xylanase was determined using different buffers such as glycine-HCl (pH 2.0-3.0), sodium acetate (pH 4.0-5.0). Sodium phosphate (pH 6.0-7.0) and Tris-HCl (pH 7.0-9.0) at 0.1 M concentrations. Xylanase was incubated in different buffers for varying time intervals (15, 30, 60, 120 & 240 min) and the residual activity was determined at 50^{0}C. The original activity was taken as 100 % and the relative activity was plotted against different time intervals.

II.6.12. Effect of metal ions

Purified xylanase was incubated with 5 mM solution of metal ions such as $CaCl_2$, $MgCl_2$, $CuSO_4$, $MnCl_2$, $NiCl_2$, KCl and $HgCl_2$ followed by determining the enzyme activity. The enzyme activity without metal ions was taken as 100 % and the relative activities determined in the presence of metal ions were calculated (Chaudhary & Deobagkar, 1997).

II.6.13. Effect of inhibitors

The inhibitors such as citric acid (2.5-12.5 mM), oxalic acid (2.5-12.5 mM), boric acid (2.5-12.5 mM), P-chloromercuribenzoate (PCMB, 50 mM) and iodoacetamide (50 mM) were pre-incubated with the purified xylanase at 50^0C for 15 min. and the mixture was added to the substrate and residual activity was determined at 50^0C . The enzyme activity without inhibitor was taken as 100 % and relative activity was plotted against different inhibitor concentrations (Nirmala & Muralikrishna, 2003c).

II.6.14. Effect of substrate concentration

For kinetic experiments different concentrations (0.05, 0.1, 0.15 and 0.2 %) of soluble larchwood xylan were prepared. The purified enzyme (25 µg) was incubated with the substrate at 50^0C for 60 min. V_0 was determined for each substrate concentration. Kinetic constant K_m and V_{max} were found out from the Lineweaver-Burk plot (Lineweaver & Burk, 1934). One unit of xylanase activity is defined as the amount of protein that released 1µmol of xylose / min under the experimental conditions.

II.6.15. Isolation of soluble and insoluble polysaccharides (SP & IP) from wheat and ragi brans

Bran (100 g) were extracted with acetate buffer (400 ml, 0.05 M, pH 5.0) for 2 h

each and digested with termamyl (500 µl) at 95^0C for 2 h followed by glucoamylase at 55^0C for 48 h. The supernatant obtained after centrifugation was concentrated by flash evaporation to 50 ml and precipitated with 3 volumes of ethanol. Precipitate was separated out by centrifugation (10000 g), dialyzed and lyophilized to obtain soluble polysaccharides (SP). The residue obtained after centrifugation was dried by solvent exchange and designated as insoluble polysaccharides (IP) (Rao & Muralikrishna, 2004).

II.6.16. Determination of neutral sugar composition of SP & IP by GLC:

II.6.16.1. ***Hydrolysis of polysaccharides by sulphuric acid:*** **(Selvendran & O'Neil, 1998)**

The polysaccharide (10 mg) suspended in water was completely hydrolyzed by prior solubilization with 72 % sulphuric acid at ice cold temperature followed by diluting to 8% acid and heating at 100^0 C for 10-12 h. The above mixture was neutralized with barium carbonate (solid), filtered, deionized with Amberlite IR 120 H^+ resin and concentrated.

II.6.16.2. ***Preparation of alditol acetate derivatives*** **(Sawardekar *et al*., 1965)**

To the polysaccharide hydrolyzate was added inositol (10 mg) as an internal standard, followed by the addition of sodium carbonate solution (0.02 M) to saponify any glucuronolactones. After 15 min. sodium borohydride (20 mg) or sodium borodeuteride in D_2O (in case of methylated samples) was added to reduce the monosaccharides and the tubes were kept at room temperature for 4-6 h (or overnight). The excess borohydride was decomposed by adding acetic acid (2 N) drop wise till the effervescence of hydrogen stops. The boric acid formed was removed by co-distillation with methanol (1 ml x 4). To the dry glycitols were added acetic anhydride and pyridine (1: 1 ratio) and the mixture was kept at 100^0C for 2 h. After acetylation excess reagents were removed by co-distillation with water and toluene (4 x 1 ml each). The alditol acetates were extracted with chloroform, filtered through glass wool and dried by flushing nitrogen. The

derivatives were taken in known amount of chloroform and analyzed by GLC for qualitative and quantitative analysis.

II.6.16.3. *GLC*

Analysis of alditol acetates were carried out by using Shimadzu GLC system (GC-15 A) fitted with flame ionization detector and CR4-A monitor. OV-225 (3 %) stainless steel column (1/8 x 8 ft).

II.6.17. Hydrolysis of Larchwood xylan, ragi and wheat bran soluble polysaccharides (SP) by purified xylanase

Soluble larchwood xylan (0.35 %, 10 ml) and polysaccharides (20 mg) isolated from wheat and ragi brans were subjected to enzymatic hydrolysis using purified ragi xylanase (240 μg) at 50^0 C for 150 min. The reaction was stopped by the addition of three volumes of ethanol in order to precipitate the undegraded polysaccharides which were subsequently separated from the hydrolytic products, *i.e.,* oligosaccharides in the supernatant by centrifugation (10000 g for 10 min) (Minic *et al*., 2004).

II.6.18. Purification of oligosaccharides on Biogel P-2

The supernatant as obtained above (Section II.6.18.) containing the oligosaccharides was concentrated to 1 ml by flash evaporation at 30^0C and loaded on Biogel P-2 column (0.9X 105 cm) using water as the eluent at a flow rate of 6 ml h^{-1} (Guillon *et al*., 2004).

II.6.19. Purification of oligosaccharides by HPLC

The purified and concentrated oligosaccharides as obtained above (Section II.6.19) were passed through a Millipore filter (0.2 micron), resolved and identified on

μ-Bondapak-NH_2 column (4.1 mm x 30 cm) using acetonitrile-water (75:25) solvent system at a flow rate of 0.7 ml/min using Shimadzu HPLC system equipped with refractive index detector. Xylose, glucose and maltose were the reference sugars (Nirmala & Muralikrishna, 2003b).

II.6.20. Structural characterization of oligosaccharides

II.6.20.1. Electro spray ionization mass spectrometry (ESI-MS)

Mass spectrum of the purified oligosaccharide was measured by Alliance, Waters 2695 mass spectrometer using positive mode electro spray ionization with the following operational conditions i.e. capillary voltage 3.5 kV, core voltage 100 V, source temperature 80^0C, disolvation temperature 150^0C, core gas (Argon) 35 lt h^{-1} and disolvation gas (Nitrogen) 500 lt h^{-1} (Fernandez *et al*., 2004).

II.6.20.2. H^1 NMR spectrum

The H^1 NMR spectrum of purified oligosaccharide (1.5 mg in D_2O) was recorded using Bruker 500 spectrometer operating at 500 MHz at 27^0C with tetra methyl silane (TMS) as the internal standard. 16 pulses were collected with pulse retention time of 5 s and pulse angle 30^0 (Dervilly *et al*., 2002).

II.6.21. Microbiological methods

II.6.21.1. Microorganisms

Bifidobacterium adolescentis NDRI 236, *Bifidobacterium bifidum* 229 ATCC 29521, *Bifidobacterium bifidum* NCDO 2715, *Lactobacillus brevis* 01 NDRI strain RTS, *Lactobacillus plantarum* 020 NDRI strain 184, *Pediococcus pentosaceus* 035 NCDO 813, *Pediococcus pentosaceus* ATCC 8081 were the microorganisms used for the present study.

II.6.21.2. Inoculum

Individual cultures were grown in MRS broth and subjected to centrifugation after 24 h of incubation at 37^0C at 3000 g for 15 min. at 15^0C. The resultant cells were suspended in 0.85 % saline. Serial dilutions (10^{-6}) were prepared to get the requisite cell population.

II.6.21.3. Media composition and preparation

Lactobacillus MRS Broth

Ingredients (g/L)	
Protease peptone	10.00
Beef extract	10.00
Yeast extract	5.00
Dextrose	20.00
Polysorbate 80	1.0
Ammonium sulphate	2.0
Sodium acetate	5.0
Magnesium sulphate	0.10
Manganese sulphate	0.05
Dipotassium phosphate	2.0

Final pH (at 25^0 C) 7.0 ±0.2

The broth was prepared (55.15 g/L) and sterilized by autoclaving at 15 lbs pressure (121^0C) for 15 min. 10 ml broth was inoculated with inoculum (0.1 % v/v- 100 µl) and incubated at 37^0C for 48 h. The MRS broth for fermentation experiments was formulated by excluding beef extract, yeast extract and dextrose and replacing protease peptone with tryptone.

II.6.21.4. *In vitro* fermentation experiments

In vitro experiments were carried out using crude xylo-oligosaccharides as well as purified xylo-oligosaccharide liberated from ragi bran SP. The xylo-oligosaccharides liberated from ragi bran by xylanase treatment were filtered through membrane filter (0.22 mμ, Millipore) and added at 0.25 % level to MRS broth (without dextrose, 2 ml) and inoculated with 100 μl of culture suspension giving 200 CFU (Colony Forming Unit) and incubated at 37^0C for 48 h. With respect to *Bifidobacterium* cultures Cystein-HCl (20 μl) was added to the culture broth and incubation was carried out in an anaerobic chamber. Growth characteristics were monitored by measuring pH and absorbance (600 nm) of culture broth after 48 h of incubation. Microbial cultures were taken out after 48 h of incubation, centrifuged (3000 x g for 20 min at 15^0C) and oven dried to determine the dry cell mass. The resultant supernatants were analyzed for short chain fatty acids (SCFA) (Van der Meulen *et al.,* 2004).

II.6.21.5. Enzyme assays

Aliquots (20 μl) were taken out from culture broth (24 h old) and assayed for xylanase, β- D- xylopyranosidase, α- L- arabinofuranosidase**,** α- D- galactopyranosidase, β- D-galactopyranosidase and acetyl esterase as described earlier in section II.6.8.

II.6.21.6. SCFA analysis

The culture supernatant obtained by centrifuging (3000 x g, for 20 min at 15^0C) the culture broth after 48 h of incubation was acidified with 50 % sulphuric acid and extracted with diethyl ether (Karppinen *et al*., 2000) and analyzed for SCFA by GLC on PEG-20 M with column, injector and detector temperatures of 120, 220 and 230^0C respectively (Silvi *et al.,* 1999) Nitrogen (40 ml/ min) was used as the carrier gas. Acetate, propionate and butyrate were used as the standards to identify the SCFA in the microbial culture broth.

CHAPTER III

Isolation and purification of xylanase from finger millet malt

III. 1. Introduction

Endogenous plant enzymes have been utilized in traditional food processes for a long time and the high specificity and possible isolation of these enzymes from their natural sources offer potential applications in food industry (Teichgraber *et al.,* 1993). But the effective isolation of these enzymes from their natural sources is affected by various parameters and experimental conditions. Column chromatographic techniques such as ion exchange and gel filtration are the generally applied methods for the purification of xylanases. Hydrophobic interaction column chromatography was employed in the purification of *Trichoderma* xylanases (Wong *et al.,* 1992). A low molecular weight xylanase was purified from *Bacillus* sp. strain TAR-1 by using CM Toyopearl 650 M column (Takahashi *et al*., 2000). Affinity chromatography was employed in the purification of xylanase from *Bacillus* sp. strain SPS-0 (Bataillon *et al*., 2000). But this technique is not widely used for the purification of xylanases due to the expensive affinity matrixes. However the yield and purification fold vary depending on the efficiency of the method used for enzyme purification.

The present study deals with the isolation and purification of xylanase from finger millet (Ragi, *Eleusine coracana*, Indaf-15) malt. The method for obtaining maximum recovery of xylanase, xylosidase and arabinofuranosidase from finger millet malt is also described in this chapter.

III. 2. Xylanase, xylosidase and arabinofuranosidase activities during malting of finger millet

Xylanase, xylosidase and arabinofuranosidase activities were increased during the malting of ragi. Xylanase activity was maximum in 96 h ragi malt compared to 24, 48 and 72 h malts (Fig. 10). Steeping hydrates the ragi seeds to a moisture level that will meet the water requirement of the aleurone tissue for both enzyme production and migration through the multicellular endosperm complex (Palmer & Bathgate, 1976).

Negligible activity was observed after 16 h of steeping for xylanase (1 %), xylosidase (0.5 %) and arabinofuranosidase (0.3%). There was no significant increase in xylanase activity at 120 h compared to 96 h of malting. Moreover the vegetative loss was found to be high at 120 h.

Xylosidase and arabinofuranosidase activities were increased during the malting period and maximum activity was observed in 96 h malt. But xylosidase activity was found to be slightly higher than the arabinofuranosidase. Similar results were reported for rye (*Secale cerale*) kernels wherein 96 h malt showed maximum activity of xylanolytic enzymes wherein xylanase activity was the highest followed by xylosidase and arabinofuranosidase activities (Kołodziejczyk & Michniewicz, 2004).

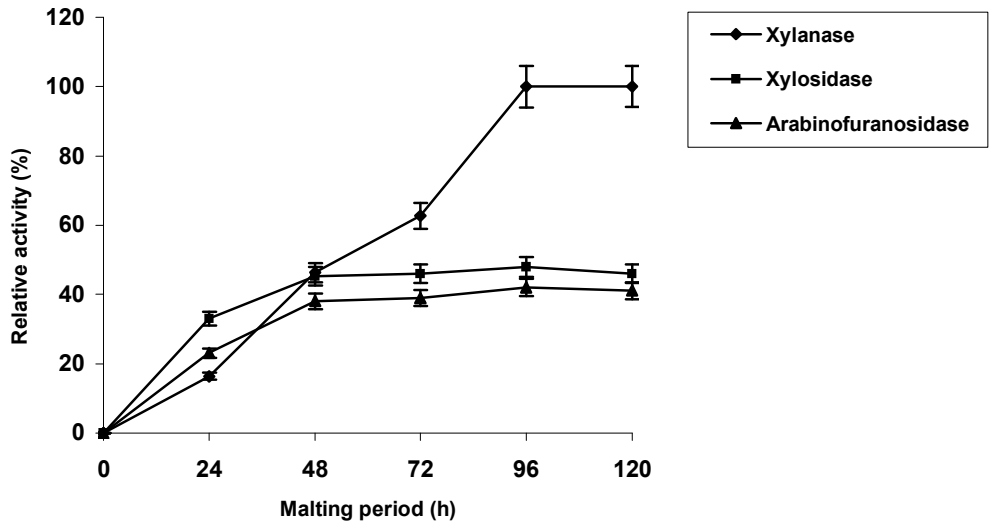


Fig. 10. Changes in Xylanase, xylosidase and arabinofuranosidase activities during malting of finger millet

During the first two days of germination endoxylanase activity was low, but thereafter it increased sharply till the 6 th day of malting with respect to barley. However, xylosidase and arabinofuranosidase activities increased steeply from day 1 to 7 (Sungurtas *et al*., 2004). Protein modifications occurring in sorghum during malting have been reported by Ogbonna et al. (Ogbonna *et al.*, 2003).

III.3. Factors affecting the isolation of xylanase, xylosidase and arabinofuranosidase from finger millet

The effects of various parameters such as pH and ionic strength of the extraction medium, number of extractions, exogenous addition of polyvinyl polypyrrolidine (PVPP), Triton X-100, reduced glutathione and metal ions were studied for isolating the maximum activity of xylanolytic enzymes from ragi malt.

III. 3. 1. pH & ionic strength of the extraction medium

Extraction medium plays an important role in the maximum recovery of the enzymes from their biosource. For the isolation of xylanolytic enzymes such as xylanase, xylosidase and arabinofuranosidase acetate buffer in a pH range of 4.5-6.0 was used. (Fig. 11) Xylanase activity was maximum in acetate buffer pH 6.0. Reduction in xylanase activity was observed at pHs beyond 6.0. Xylosidase and arabinofuranosidase activities were maximum at pH 5.5 and 5.0 respectively. The pH of a solution can influence the structure and activity of enzymes. pH affects the state of ionization of acidic and basic amino acids. If the state of ionization of amino acids in a protein is changed, the ionic bonds that help to stabilize the 3-D shape of the protein will be altered. This can result in the inactivation/ denaturation of the enzyme and may eventually lead to poor extraction, thereby low yield (Palmer, 2001a).

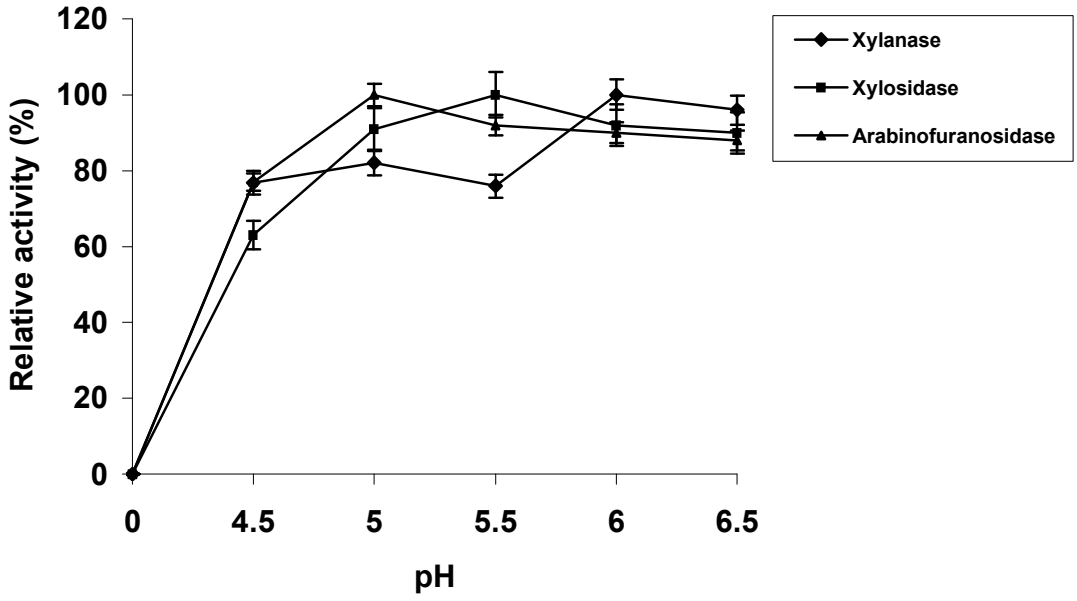


Fig. 11. Effect of pH of the extraction medium on enzyme activity

III. 3. 2. Ionic strength

Ionic strength of the extraction medium also plays an important role in protein extraction. 0.1M was the optimum concentration for obtaining maximum xylanase activity compared to 0.5, 0.15 and 0.2 M concentrations. Xylosidase and arabinofuranosidase recovery was maximum in 0.2 M acetate buffer eventhough the difference in activity was very less compared to 0.15 M (Fig. 12).

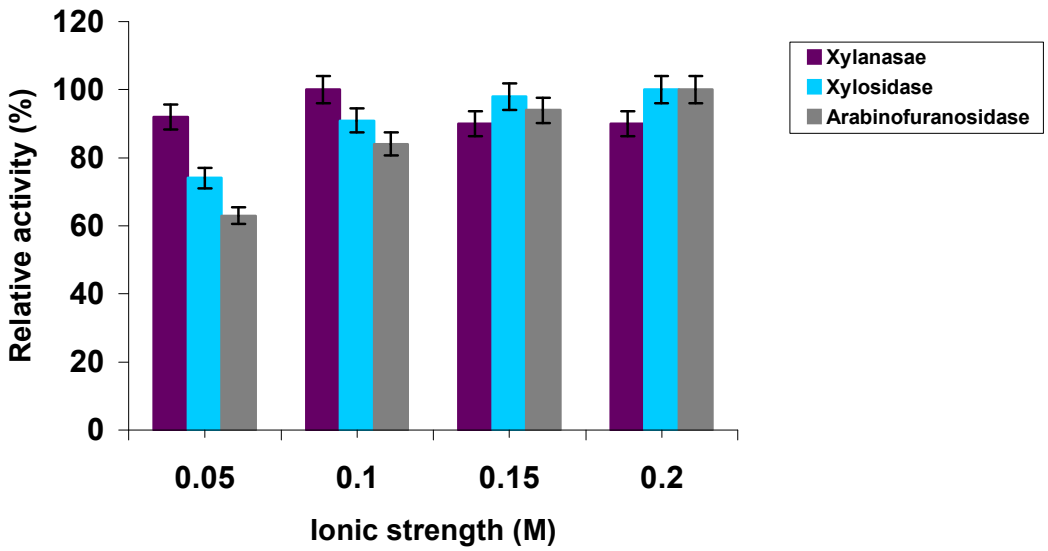


Fig. 12. Effect of ionic strength of the extraction medium on enzyme activity

III. 3. 3. Number of extractions

The complete recovery of xylanolytic enzymes was not achieved in the first extraction. The relative activity of xylanase in the first extraction was taken as 100 % and accordingly the second extraction yielded ~ 32 % of activity. Similarly xylosidase and arabinofuranosidase yielded 41 and 47 % of activities respectively compared to their respective first extractions. The third extraction did not yield any significant enzyme activity (Fig. 13). Hence the first two consecutive extractions yielding more than 90 % of enzyme activities were chosen for the effective isolation of the enzymes from ragi malt.

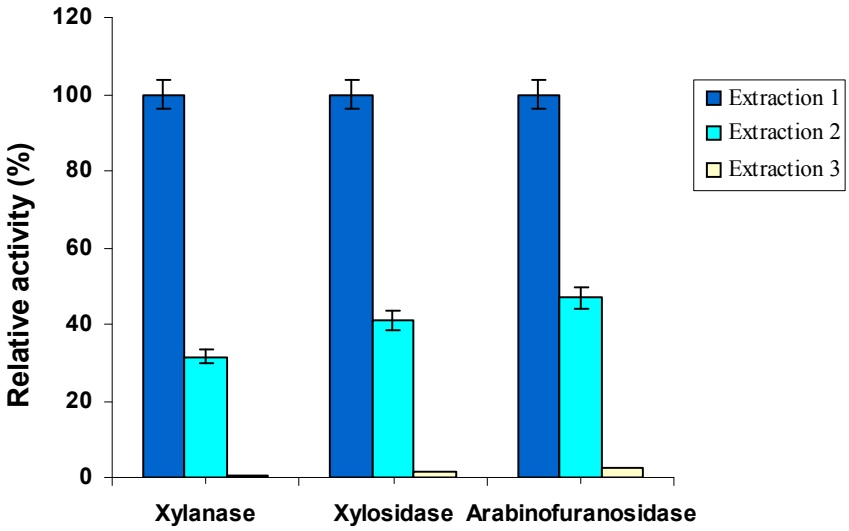


Fig. 13. Effect of number of extractions on enzyme activity

III. 3. 4. Effect of polyvinyl polypyrrolidine (PVPP)

Polyvinyl polypyrrolidine (PVPP) is used as a phenolic scavenger in protein extraction to prevent the co-extraction of the phenolic compounds which may inhibit the enzyme action. Addition of PVPP enhanced the xylanase activity in the 96 h ragi malt extracts. Even though there was no significant difference in the enzyme activities at different concentrations of PVPP (0.25, 0.5, 0.75 and 1 %) added to the extractant, PVPP (0.5 %) yielded maximum xylanase activity (Table. 4). Xylosidase activity was maximum at 0.75 % concentration of PVPP and further increase in the concentration did not show any effect on the enzyme activity. Similar result was observed with respect to arabinofuranosidase wherein the activity was maximum at 0.75 % concentration of PVPP. This was employed in the case of wheat α-amylases (Hsu & Heatherbell, 1987). Addition of PVPP to the extraction medium yielded enhanced xylanase activity from

barley malts (Li *et al.,* 2005). Sungurtas *et al*., 2004, has also reported the enhanced recovery of xylanase, arabinofuranosidase and xylosidase in barley malt samples by the addition of PVPP (0.1 %) to the extraction medium.

Table.4. Effect of PVPP on enzyme activity (μmolmin^{-1})

Conc. (%)	Xylanase	xylosidase	Arabinofuranosidase
0.25	1.12± 0.02	0.40±0.01	0.30± 0.01
0.5	1.38± 0.04	0.42±0.01	0.30±0.02
0.75	1.17± 0.02	0.48±0.04	0.38±0.01
1.0	1.14± 0.01	0.48±0.02	0.32±0.03

Mean±SD; n=3

III. 3. 5. Triton X-100

Triton X-100 is a non-ionic detergent. It helps in the solubilization of membrane proteins and thus increases the protein extractability. Triton X-100 (1 %) added to the extraction medium yielded maximum xylanase activity (1.33 μmol min-1) (Table. 5). There was no difference in the enzyme activity by the addition of 0.25, 0.5 and 0.75 % of Triton X-100 to the extraction medium. Xylosidase activity was maximum at 0.75% concentration of Triton X-100. Arabinofuranosidase activity remained the same at 0.25 & 0.5 % concentrations and was maximum at 1 % concentration of triton X-100. 0.75 % triton X-100 was used for the isolation of membrane bound xylanases from *Thermotoga maritima* MSB8 (Winterhalter & Liebl, 1995). Addition of xylanase was also found to enhance the xylanase activity in *Bacillus* sp. (Hattori *et al*., 2002).

Table. 5. **Effect of Triton X-100 on enzyme activity (μmol min^{-1})**

Conc. (%)	Xylanase	Xylosidase	Arabinofuranosidase
0.25	1.23±0.01	0.38±0.01	0.3±0.02
0.5	1.23±0.02	0.40±0.01	0.3±0.01
0.75	1.23± 0.01	0.48±0.01	0.38±0.01
1.0	1.33± 0.03	0.48±0.02	0.41±0.03

Mean±SD; n=3

III. 3. 6. Effect of metal ions

Xylanase activity was increased by the addition of $CaCl_2$ and $MgCl_2$ to the extraction medium. Maximum enzyme activity was obtained at $CaCl_2$ (20 mM) and $MgCl_2$ (10 mM) (Table. 6). Metal ions play a significant role in enhancing the enzyme activity by bringing together enzyme and substrate molecules by means of co-ordinate bonds, possibly causing strain to the substrate in the process and holding the reacting groups in the required three dimensional orientation. Xylosidase and arabinofuranosidase activities were enhanced by the addition of $CaCl_2$ to the extraction medium and were maximum at 15 mM concentration (Table. 7). $CaCl_2$ was employed for the extraction of α-amylases from immature barley and wheat (Hsu & Heatherbell, 1987). The divalent cations Ca^{2+} and Mg^{2+} can form six co-ordinate bonds to produce octahedral complexes (Palmer, 2001b).

Table. 6. **Effect of metal ions on xylanase activity (μmol min^{-1})**

Conc. (mM)	$CaCl_2$	$MgCl_2$
5	1.14±0.02	1.04±0.01
10	1.14±0.02	1.22±0.02
15	1.17±0.01	1.14±0.01
20	1.29±0.04	1.14±0.02
25	1.28±0.02	1.1±0.02

Mean±SD; n=3

Table. 7. Effect of $CaCl_2$ on xylosidase and arabinofuranosidase activity (μmol min^{-1})

Conc. (mM)	Xylosidase	Arabinofuranosidase
5	0.4±0.02	0.32±0.01
10	0.46±0.01	0.34±0.01
15	0.48±0.02	0.41±0.02
20	0.48±0.02	0.4±0.01

Mean±SD; n=3

III. 3. 7. Effect of reduced glutathione

The effect of reduced glutathione on enzyme activity is represented in Table. 8. Xylanase activity increased in the presence of reduced glutathione and maximum activity was obtained at 5 mM concentration. At higher concentration of reduced glutathione xylanase activity was found to be decreased. Reduced glutathione breaks down the disulphide bonds and helps in the solubilization of proteins. It also possesses a protective role during protein extraction. Xylosidase and arabinofuranosidase activities were maximum at 7.5 mM concentration of reduced glutathione. There was no further increase in xylosidase activity whereas arabinofuranosidase activity slightly reduced at higher concentration of reduced glutathione (10 mM). Increase in xylanase activity of *Bacillus pumilus* was observed in the presence of reduced glutathione (Evans *et al*., 1996).

Table. 8. Effect of reduced glutathione on enzyme activity (μmol min^{-1})

Conc. (mM)	Xylanase	Xylosidase	Arabinofuranosidase
2.5	1.26±0.01	0.40±0.01	0.32±0.04
5.0	1.41±0.04	0.46±0.01	0.34±0.01
7.5	1.18±0.02	0.48±0.02	0.41±0.02
10.0	1.27±0.02	0.48±0.02	0.40±0.01

Mean±SD; n=3

Optimum experimental condition for obtaining maximum enzyme activity

The extractant (acetate buffer, 0.1 M, pH 6.0) consisting of polyvinyl polypyrrolidine (PVPP, 0.5 %), Triton X-100 (1.0 %), calcium chloride (20 mM) and reduced glutathione (5.0 mM) was selected as the optimum condition to isolate maximum

xylanase activity (1.55 μmolmin^{-1}) from 96 h ragi malt. Xylosidase activity was maximum (0.55 μmolmin^{-1}) in acetate buffer (0.2 M, pH 5.5) consisting of polyvinyl polypyrrolidine (PVPP, 0.75 %), Triton X-100 (0.75 %), calcium chloride (15 mM) and reduced glutathione (7.5mM).

Acetate buffer (0.2 M, pH 5.0) containing reduced glutathione (7.5mM), polyvinyl polypyrrolidine (PVPP, 0.75 %), Triton X-100 (1 %) and calcium chloride (15 mM) was the optimum condition to obtain maximum arabinofuranosidase activity (0.47 μmolmin^{-1}) from 96 h ragi malt.

Further studies with respect to xylosidase and arabinofuranosidase were not carried out due to their low yields and the studies were restricted to only on xylanase with respect to its purification and characterization.

III. 5. Purification of xylanase

96 h ragi malt (50 g) was extracted with 0.1 M sodium acetate buffer (pH 6.0) containing PVPP (1 %), Triton X-100 (1.0 %), reduced glutathione (5.0 mM) and $CaCl_2$ (20 mM) for 2 h at 4^0C. The extraction was carried out consecutively twice and the supernatants obtained after centrifugation were pooled and dialyzed overnight against the extraction buffer and the resultant enzyme solution was hereafter referred to as crude enzyme extract.

The crude xylanase extract from 96 h ragi malt was purified by employing a three step purification procedure *viz* ammonium sulphate fractionation followed by DEAE-cellulose and Sephadex G-75 column chromatographies. Detailed studies on the purification and characterization of xylanases from cereal malts are limited compared to microbial sources. Three endoxylanases were purified and characterized from barley malt (Slade *et al.,* 1989) and an endoxylanase was purified from wheat flour (Cleemput *et al*., 1997).

The purification scheme employed for ragi xylanase in the present investigation can be summarized as follows:

Crude enzyme extract from 96 h ragi malt

↓

Ammonium sulphate fractional precipitation

0-20%, 20-40%, 40-60%, 60-80%

↓

Anion exchange chromatography (DEAE-cellulose)

↓

Gel filtration chromatography (Sephadex G-75)

III. 5. 1. Step 1. Ammonium sulphate fractional precipitation

Fractional precipitation of proteins by ammonium sulphate is one of the most widely used preliminary enzyme purification procedures. The crude enzyme extract from 96 h ragi malt was fractionated by ammonium sulphate into four fractions namely, ASF 0-20 %, 20-40 %, 40-60 % and 60-80 %. Maximum activity was obtained for 40-60 % fraction and was represented as 100 %. Relative activity obtained for ASF 20-40 % was around 30 %. ASF 0-20 % and 60-80 % yielded very less activity *i.e.* 0.8 % and 8 % respectively. Hence 20-40% and 40-60 % fractions were pooled since they represented 80% of xylanase activity. Ammonium sulphate fractionation was employed for the purification of endoxylanase from wheat flour (Cleemput *et al.,* 1997) wherein, ASF 30-70 % yielded maximum xylanase activity. ASF 30-40 % was utilized in the purification of endo xylanases from germinated barley (Slade *et al*., 1989).

III. 5. 2. Step 2. Ion Exchange Chromatography: DEAE-cellulose

ASF 20-60 % was dialyzed against phosphate buffer (0.1 M, pH 7.0) and loaded on DEAE– cellulose column. The ion exchange chromatography efficiently removed the contaminating unbound proteins and the bound proteins were eluted with a linear NaCl gradient (0-1.0 M). Xylanase was separated into two activity peaks designated as P-1 and P-II at 0.25 M and 0.35 M respectively (Fig. 14). P-II was not taken for further purification studies due to its low yield (<0.03 %). The specific activity of P-1 was found to be to 34.7 with a percent recovery of 4.8 and fold purification 60 (Table. 9).

Ion exchange chromatography was used effectively in the purification of xylanases. In the case of barley malt xylanase both anion (DEAE-Trisacryl) and cation (CM-cellulose) exchange chromatography methods were employed, but the percent recovery was found to be less compared to the present study (Slade *et al*., 1989). Wheat xylanase was purified using Mono Q-HR (5/5, 5x 50 mm, Pharmacia) column with a fold purification of 15 and percentage yield 0.2 % (Cleemput *et al.,* 1997). DEAE-sephadexA-25 was used in the purification of an endoxylanase from *Aspergillus niger* (Shei *et al*., 2004). DEAE- cellulose was employed in the purification of xylanases from *Penicillium purpurogenum* (Belancic *et al.,* 1995). DEAE Bio-Gel A and CM Bio-Gel A were used for the purification of xylanase from *Bacillus* sp. (Hattori *et al*., 2002).

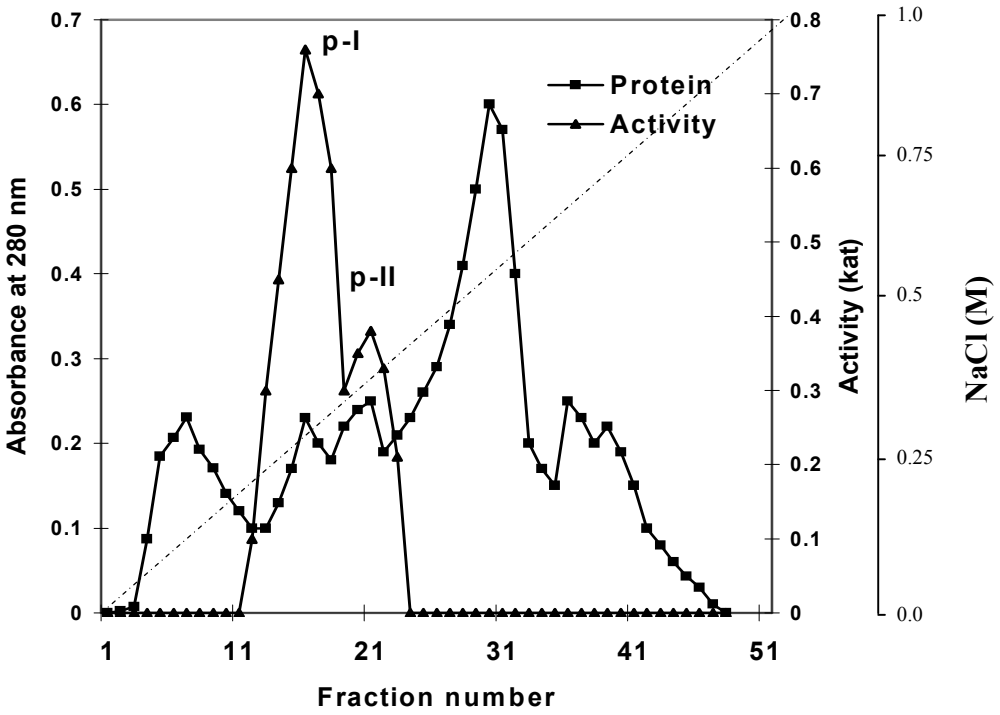


Fig. 14. Elution profile of Ammonium Sulphate Fraction (20-60%) of ragi xylanase on DEAE-cellulose column

III. 5. 3. Step 3. Gel-filtration Chromatography

Gel filtration chromatography has been effectively used in the purification of ragi xylanase. P-1 of DEAE-cellulose column was further purified by gel filtration chromatography on Sephadex G-75 column, resulting in a single activity peak (Fig. 15). Two major contaminating proteins were separated out in the void volume. The specific activity of xylanase increased 8 times than that of P-1 (DEAE- cellulose) with a fold purification of 60 and recovery of 4 % (Table. 9).

The three step purification procedure adopted in this investigation yielded high amount of xylanase activity compared to the published literature on endoxylanase from wheat flour where the yield is only 0.2 %. Moreover the fold purification of ragi xylanase (60) is four times more than that of wheat xylanase (15) (Cleemput *et al.,* 1997). Gel filtration chromatography was employed in the purification of barley malt xylanase wherein ultrogel AcA44 was used as the matrix (Slade *et al*., 1989). Sephadex G-200 was employed in the purification of xylanase isolated from barley aleurone layers (Benjavongkulchai & Spencer, 1986). Sephadex G-50 was used in the purification of xylanase from *Aspergillus niger* (Shei *et al.*, 2004). Two Thermostable Alkaline xylanases were purified from Alkaliphilic *Bacillus* sp using Sephadex G-75 (Gessesse, 1998).

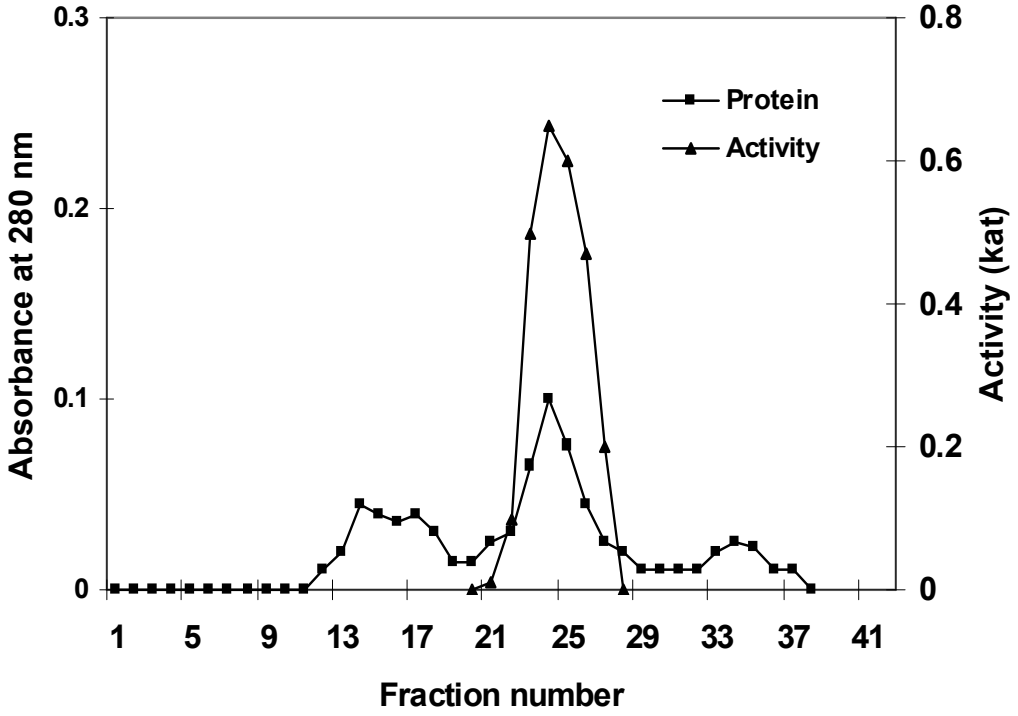


Fig. 15. Elution profile of P-1 obtained from DEAE-cellulose column on Sephadex G-75

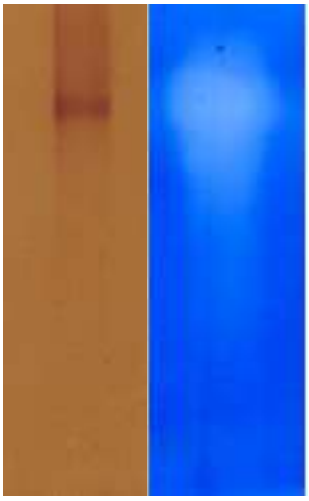
Table. 9. Summary of the purification of xylanase from ragi malt

Step	Total activity (kat)	Total protein (mg)	Specific activity (kat/mg)	Fold purification	Percent recovery
Crude	430	89	4.8	1	100
$(NH_4)_2SO_4$	100	15	6.7	1.4	23.3
DEAE-cellulose (P-1)	20.8	0.6	34.7	7.2	4.8
Sephadex G-75	17.5	0.06	291.7	60.0	4.0

III. 5. 4. Native PAGE and Activity staining

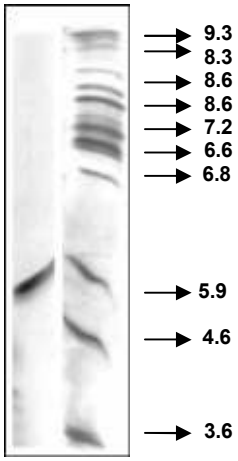
Purified ragi xylanase was found to be homogenous by both protein (silver) as well as activity stainings (Fig. 16a). The isoelectric point (pI) of purified ragi xylanase was found to be 5.9 (Fig. 16b). A pI of 5.5 was reported for wheat xylanase (Cleemput *et al.,* 1997). The pI of endoxylanases purified from germinated barley was found to be 5.2 (Slade *et al*., 1989). A 34 kDa xylanase was purified from barley aleurone layers and the pI of the enzyme was found to be 4.6 (Benjavongkulchai & Spencer, 1986). Xylanase purified from *Cellulomonas flavigena* showed a pI of 6.0 (Horcasitas *et al*., 1998). Endoxylanases having pI values 7.1 and 8.1 were reported from *Trichoderma reesei* (Lappalainen *et al*., 2000).

The M_r of the enzyme was estimated to be 29 kDa based on its mobility on SDS-PAGE, which showed its monomeric nature (Fig. 17a). The molecular weight of the purified ragi xylanases was detected to be 29,500 by ESI-MS (Fig. 17b). But, comparatively high molecular weight was reported for xylanases from barley (41 kDa) and wheat (55 kDa) (Slade et al., 1989; Cleemput *et al.,* 1997).



a b

Fig. 16a. PAGE of purified ragi xylanase (a) protein and (b) Activity staining



a b

Fig. 16b. Isoelectric focusing of purified ragi xylanase

a) Purified ragi xylanase; **b**) pI markers (3.6-9.3, Sigma)

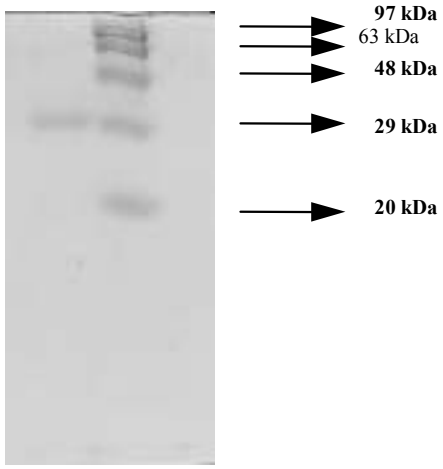


Fig. 17a. SDS-PAGE of the purified ragi xylanase

(**a**: purified ragi xylanase, **b**: standard molecular weight markers - 97 KDa- Phosphorylase, 68 KDa- Bovine Serum Albumin, 43- Ovalbumin, 29- Carbonic anhydrase, 20- Soybean trypsin inhibitor)

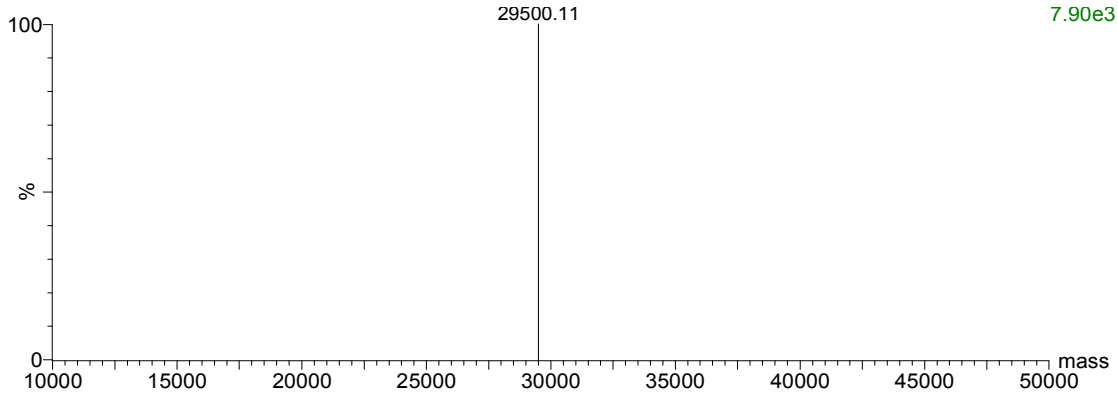


Fig. 17b. ESI-MS of purified ragi xylanase

CHAPTER IV

Characterization of xylanase

IV.1. Introduction

The kinetic characteristics of cereal xylanases have not yet been thoroughly investigated and the information available is restricted to barley and wheat (Slade *et al*., 1989; Cleemput *et al*., 1997). Xylanases have potential applications in food, feed, pulp and paper industries (Liu *et al.,* 1998; Tuncer & Ball, 2003). The information on the kinetic parameters such as pH and temperature optima, their stability, substrate specificity, various activators/stabilizers of xylanase is essential for their better utilization in various biotechnological processes. Increased attention is laid on plant sources, as they are under-exploited compared to microbial sources. One of the important plant sources is cereal malt, which is widely used with respect to α- amylase in baking and brewing industries (Nirmala & Muralikrishna, 2003a). During our initial studies on rice, maize and ragi malts, relatively high activity of xylanase was obtained in ragi compared to rice and maize malts. But in depth studies revealing their kinetic properties are found to be lacking. The present chapter deals with the characterization of the kinetic properties of purified ragi xylanase.

IV.2. Effect of pH on xylanase activity

a) pH optima

Purified ragi xylanase showed a pH optimum of 5.0 .The activity of the enzyme decreased both in phosphate and Tris–HCl buffers at a higher pH compared to sodium acetate and sodium succinate buffers (Fig. 18 a) indicating its labile nature in alkaline pH. However the drop in activity was maximum at pH 8.0 and 8.5 in phosphate and Tris-HCl buffers respectively. The change in enzymatic activity at different pHs is due to the changes in the ionization of the enzyme, substrate or the enzyme–substrate complex. The effect of pH on an enzyme reaction is crucial for most enzyme activities, since substrate binding and catalysis are dependent on the charge distribution of substrate and enzyme molecules. Some enzymes are active over a broad range of pH values, while most are active only over a relatively narrow range. A slightly higher pH optimum (pH 6.7) was

reported for xylanase purified from germinated barley (Slade *et al.,* 1989). An endoxylanase purified from aleurone layers of barley showed a pH optimum of 5.5 (Benjavongkulchai & Spencer, 1986). A pH optimum of 6.5 was reported for xylanase from *Bacillus* sp (Hattori *et al*., 2002). Xylanase purified from a thermostable alkaliphilic *Bacillus* sp showed maximum activity at a slightly higher pH, 9.0 (Gessesse, 1998).

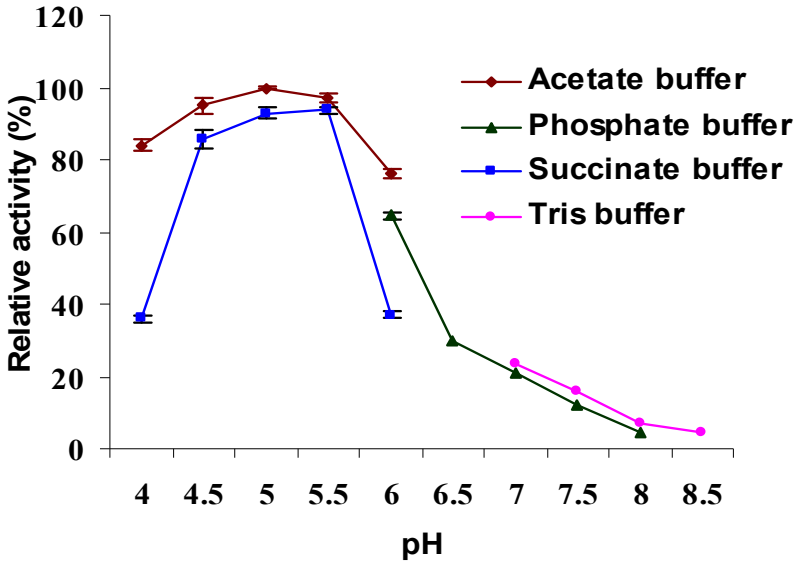


Fig. 18 a. pH optima of purified ragi xylanase

In addition, enzyme stability is affected by pH and typically the stability range tends to be much greater than the activity range. Since enzymes are not stable at all pH levels, it is important to determine the pH range of enzyme activity. Xylanase showed maximum stability in a pH range of 5.0-6.0 (Fig. 18 b). Xylanase activity was completely lost at pH 2.0 and only 15 % of the activity was retained at pH 9.0 after 15 min. of incubation.

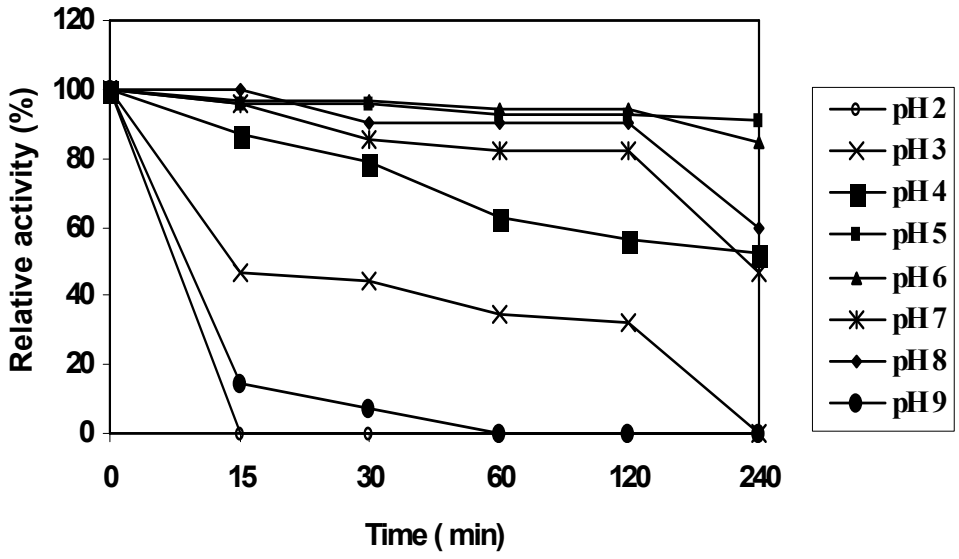


Fig. 18 b. pH stability of purified ragi xylanase**.**

At extreme pHs, the pattern of charges carried out by the ionizable side chains will be very different from normal conditions, so the compact tertiary structure will be disrupted and a more random structure will be formed. This accounts for the denaturation of the protein as well as inactivation of the enzyme (Palmer, 2001a). Around 50-60 % of the maximal activity was retained in a pH range of 4.0-5.0 and 7.0-8.0 after 4 h of incubation. Xylanase purified from barley aleurone layers showed maximum stability in a pH range of 5.0-6.0 (Benjavongkulchai & Spencer, 1986).

IV.3. Effect of temperature on xylanase activity

a) Temperature optimum and activation energy

The effect of temperature on xylanase activity was studied in the range of 35-60^{0}C. Temperature optimum of purified xylanase was 50^{0}C, above which the activity was decreased. However almost 70 % of the activity was retained at 60^{0}C (Fig. 19 a). The Arrhenius plot was linear over the temperature range of 30-50^{0}C. A temperature optimum of 35^{0}C was reported for barley aleurone xylanase (Benjavongkulchai & Spencer, 1986). Xylanase purified from Bacillus sp showed maximum activity at a high temperature of 70^{0}C (Gessesse, 1998). The activation energy (E_a), which is the minimum energy required by the reactants to pass into transition state finally resulting in the formation of products, was calculated from the Arrhenius plot and was found to be 25 kJ mol^{-1} (Fig. 19b).

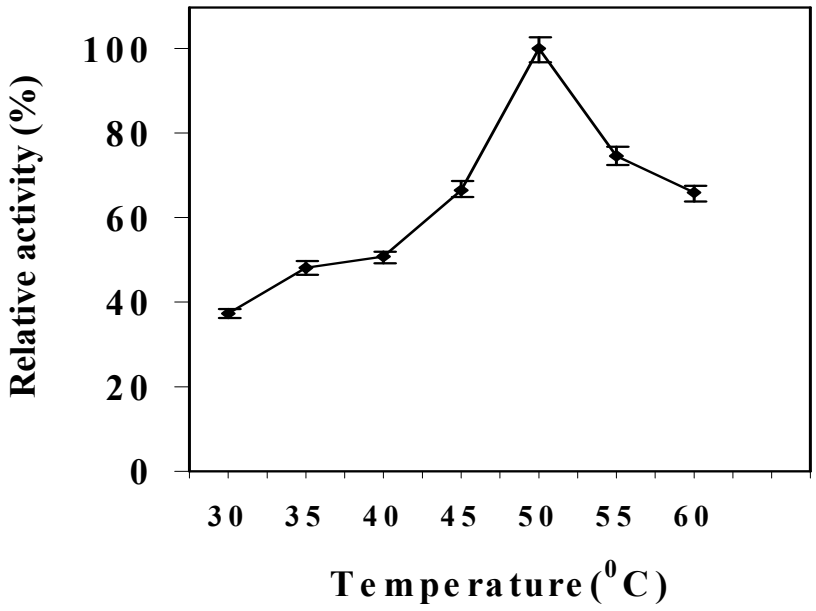


Fig. 19 a. Temperature optima of purified ragi xylanase

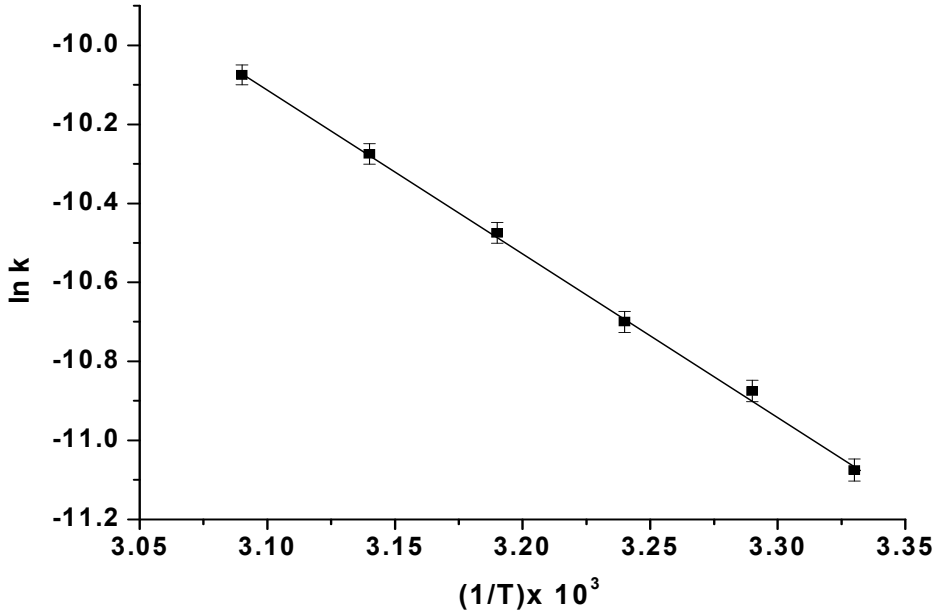


Fig. 19 b. Arrhenius plot. Each point shows the mean ± SD of at least three independent experiments carried out in triplicates.

b) Thermal stability

Xylanase was found to be more stable at 35^0 C and only 25 % of the activity was retained at 60^0C (Fig. 19 c). As the temperature increases, the three dimensional structure of the protein which is maintained by a number of forces, mainly hydrophobic interactions, hydrogen bonds and sometimes disulphide bonds will be disrupted which result in the denaturation of the protein and lead to the inactivation of the enzyme (Heinemann, 1992). Microbial xylanases were found to be stable at higher temperatures compared to ragi xylanase (Khasin *et al*., 1993).

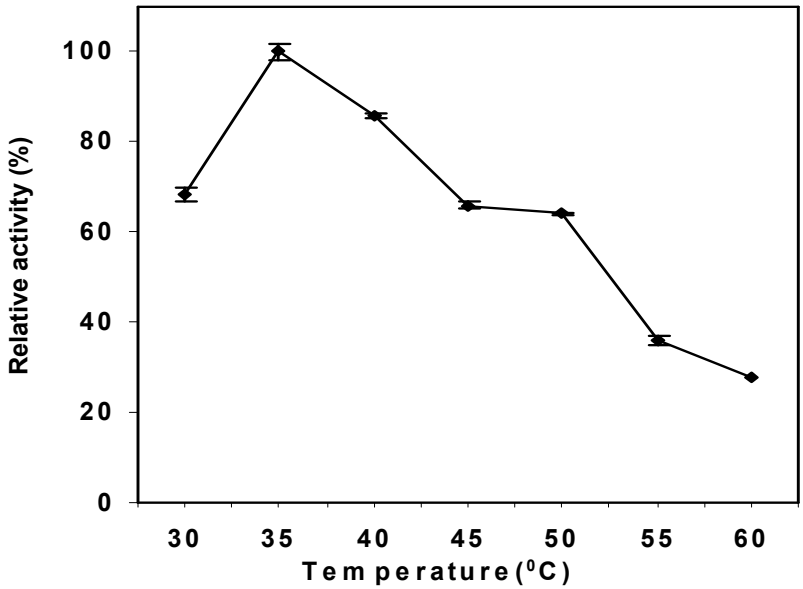


Fig. 19 c. Thermal stability of purified ragi xylanase

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IV.4. Effect of metal ions

Various metal ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Ag^{2+}, Ni^{2+} and Hg^{2+} at 5mM concentration were tested to find out their activation/ inhibition effect on xylanase activity (Table. 10). Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Ag^{2+} and Ni^{2+} were found to have both activating and stabilizing effects, whereas Hg^{2+} was found to be completely inactivating the enzyme. Enhancement of xylanase activity by the cations such as Fe^{2+}, Ca^{2+} and Mg^{2+} has been previously documented (Ratanakhanokchai *et al*., 1999). These metal ions can accept or donate electrons to activate electrophiles or nucleophiles and they may bring together enzyme and substrate molecules by means of co-ordinate bonds, possibly causing strain to the substrate in the process and holding the reacting groups in the required three dimensional orientation and thus catalyze the enzymatic reactions. The

divalent cations Ca^{2+} and Mg^{2+} can form six co-ordinate bonds to produce octahedral complexes (Palmer, 2001b).

Table. 10. Effect of metal ions on purified ragi xylanase

Metal ions (5mM)	Relative activity (%)
Control	*100*
$CaCl_2$	135
$MgCl_2$	111
$MnCl_2$	118
$NiCl_2$	125
$FeSO_4$	125
$CuSO_4$	107

IV.5. Effect of substrate concentration, EDTA and $HgCl_2$ on enzyme kinetics

The effect of different concentrations of the substrate (larchwood xylan) on the initial velocity was calculated. K_m and V_{max} were found out from the double reciprocal plots (Lineweaver & Burk, 1934). K_m and V_{max} of the purified ragi xylanase were found to be 0.2 % and 4.5 micromoles min^{-1} respectively (Fig. 20). The low K_m value indicates its high substrate specificity. Detailed information has not been reported on the kinetic properties of wheat xylanase. The K_m value reported for barley aleurone xylanase was 0.86 mg xylan/ml (Benjavongkulchai & Spencer, 1986). A V_{max} value of 88 µmol xylose equivalents/min/µmol enzyme and a K_m of 4.5 mg/ml were reported for an endoxylanase

XH2 purified from germinated barley (Slade *et al.,* 1989). However, it should be emphasized that it is not generally possible to assign single K_m and V_{max} values to polysaccharide hydrolases because of the heterogeneity of their respective polysaccharide substrates (Slade *et al.,* 1989).

Purified ragi xylanase exhibited classical, linear Michaelis-Menten kinetics in the substrate concentration range of 0.05-0.35 % larchwood xylan. The effect of EDTA and $HgCl_2$ on xylanase activity was determined at various concentrations (1.0-6.0 mM). Xylanase activity was completely inhibited by 3 mM concentration of EDTA and 4 mM $HgCl_2$. EDTA was found to be a competitive inhibitor whereas $HgCl_2$ showed non-competitive inhibition. The inhibitory effect of EDTA was previously reported in the case of xylanases from *Bacillus subtilis* (Yuan, *et al.,* 2005). The inhibitory effect of Hg^{2+} was reported in the case of xylanase from *Cellulomonas* sp. NCIM 2353 (Chaudhary & Deobagkar, 1997) and *Trichosporon cutaneum* SL409 (Liu *et al.,* 1998). The inactivation of the enzyme by Hg^{2+} indicates that thiol-containing amino acid may be involved in the active site region of the enzyme (Bell & Bell, 1998).

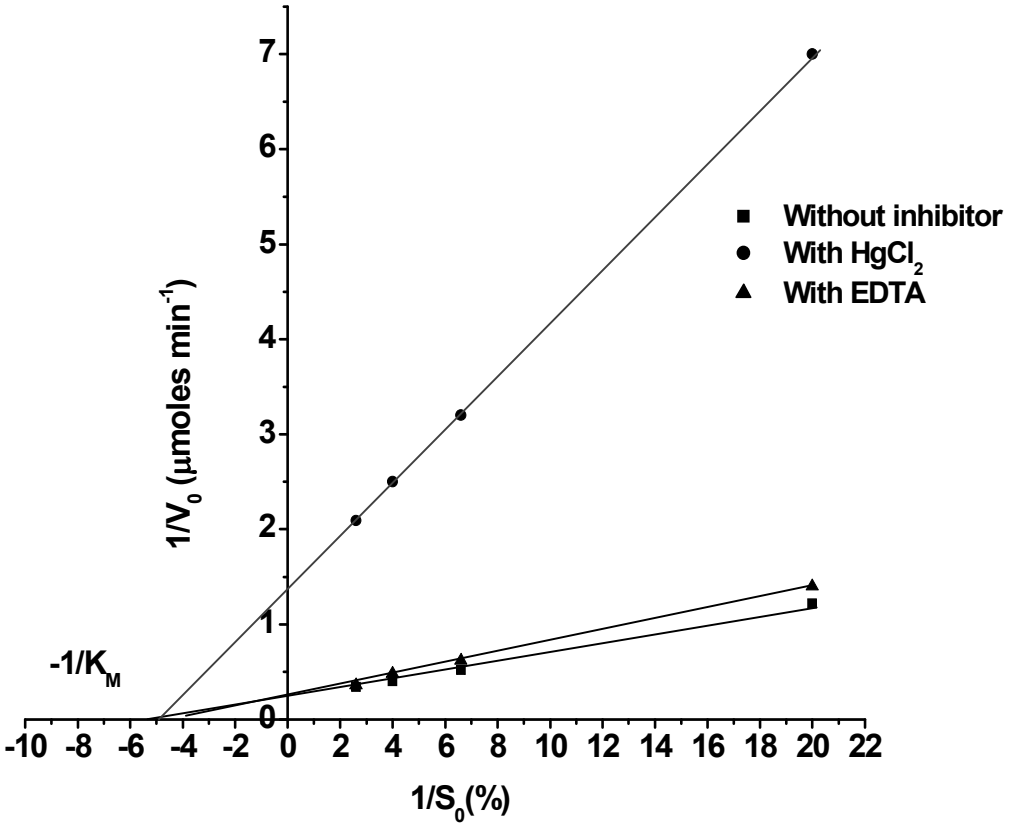


Fig. 20. Determination of K_m and V_{max} of ragi xylanase by Lineweaver-Burk plot with and without inhibitors (substrate used- Larchwood xylan)

IV.6. Effect of inhibitors

IV.6.1. Effect of citric, oxalic and boric acids

The effect of citric, oxalic and boric acids on xylanase activity was determined in a concentration range of 0-12.5 mM (Fig. 21). It was found that all the above mentioned acids inhibited the enzyme activity completely at 12.5 mM concentration. Boric acid proved to be an effective inhibitor compared to citric and oxalic acids wherein only 18% of the activity was retained at 2.5 mM concentration. Boric acid inhibits enzyme activity by binding covalently to the active-site serine residue**.** Carboxylic acids such as citric and oxalic acids inhibit the enzyme by selective removal of Ca^{2+} which form co-ordinate bonds with the active site of the enzyme and stabilize the three dimensional structure of the enzyme (Nirmala & Muralikrishna, 2003b).

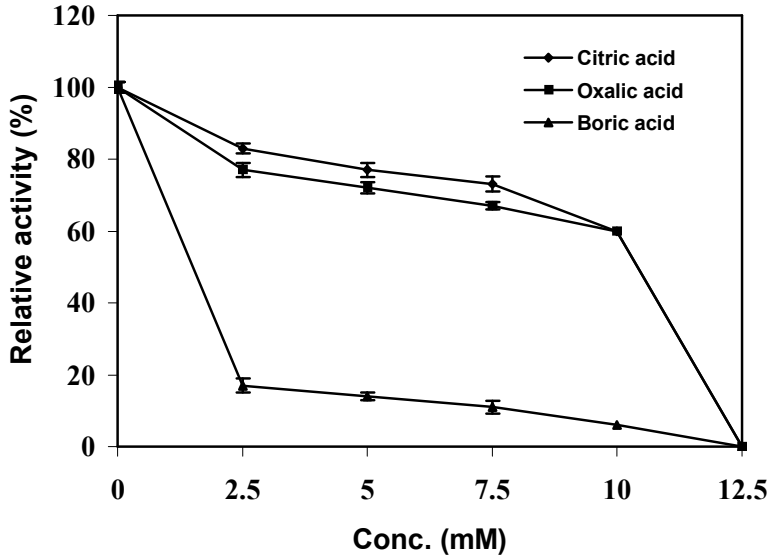


Fig. 21 Effect of Citric, Oxalic and Boric acids on xylanase activity. Each point shows the mean ± SD of at least three independent experiments carried out in triplicates.

IV.6.2. Effect of P-Chloromercuribenzoate (PCMB) and Iodoacetamide

Xylanase activity was determined in the presence of chemicals such as P-Chloromercuribenzoate (PCMB) and Iodoacetamide. PCMB at 50 mM concentration completely inhibited the enzyme whereas, 71 % of inhibition was observed with iodoacetamide (50 mM) (Fig. 22). Haga *et al.,* 1991 have reported that xylanase activity was completely inhibited by PCMB. Both PCMB and Iodoacetamide are specific cysteine residue modifiers and result in inactivation of the enzyme by forming complexes with cysteine present in the active site region of the enzyme (Bell & Bell, 1998).

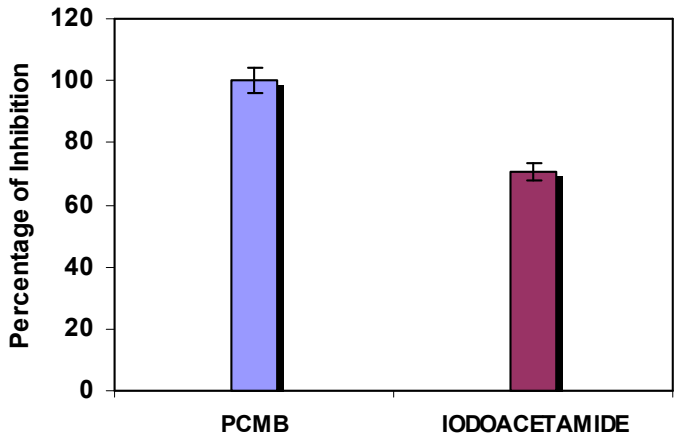


Fig. 22. Effect of P-Chloromercuribenzoate (PCMB) and Iodoacetamide on purified ragi xylanase

IV.7. Determination of the mode of action of purified ragi xylanase

A study on the hydrolytic pattern of purified ragi xylanase on larchwood xylan, universally used substrate for xylanase, has been carried out to determine its mode of

action. The identification and structural characterization of the resultant hydrolytic products were carried out by ESI-MS and H^1 NMR to substantiate the enzyme activity. Several models have been proposed to explain the mode of action of xylanases (Subrahmaniyan & Prema, 2002). Endoxylanases may differ from one another depending on the substrates upon which they act and their hydrolytic products (Coughlan & Hazlewood, 1993). Xylanolytic enzymes have been purified from many microorganisms and their hydrolytic properties have been thoroughly investigated (Tuncer & Ball, 2003). Xylanase purified from wheat flour showed endo-mode of action towards oat spelt xylan (OSX) and rye arabinoxylan (RAX). Similarly xylanases from germinated barley also showed endoactivity towards larchwood xylan and wheat endosperm arabinoxylan. Xylanase isolated from the culture medium of *Aeromonas caviae* ME-1 produced only xylotetraose as its hydrolytic product from oat spelt xylan has been reported by Kubata *et al*., 1995.

IV.8. Hydrolysis of larchwood xylan by purified ragi xylanase and purification of the products of hydrolysis

The hydrolytic products (57 %) liberated from larchwood xylan by the action of purified ragi xylanase were separated on Biogel P-2 column (Kormelink *et al.,* 1993) into four major peaks designated as P-1, P-2, P-3 & P-4 (Fig. 23). Since the yield of p-1 was less, it was not taken for further characterization. P-2, P-3 & P-4 were characterized by ESI-MS and H^1 NMR studies.

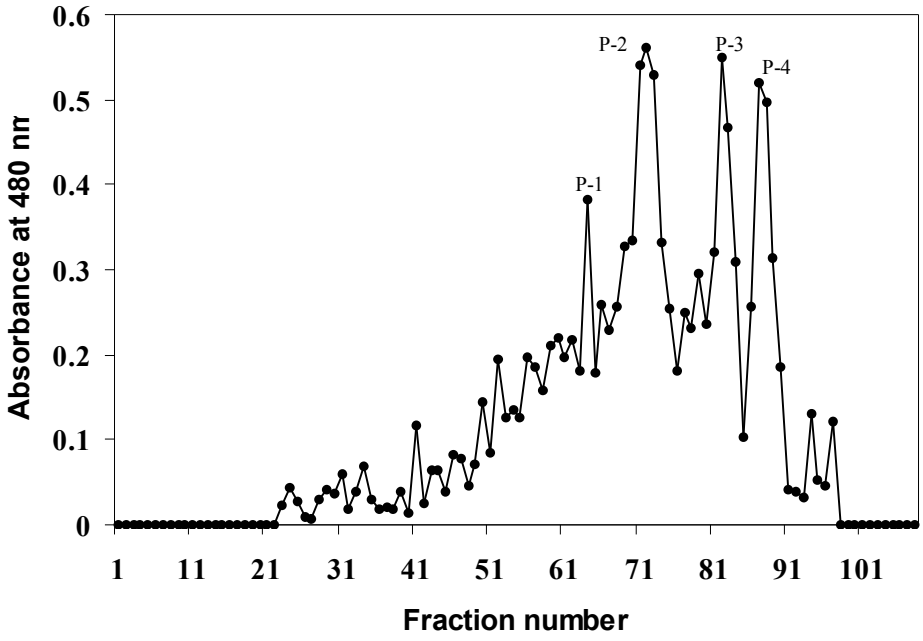
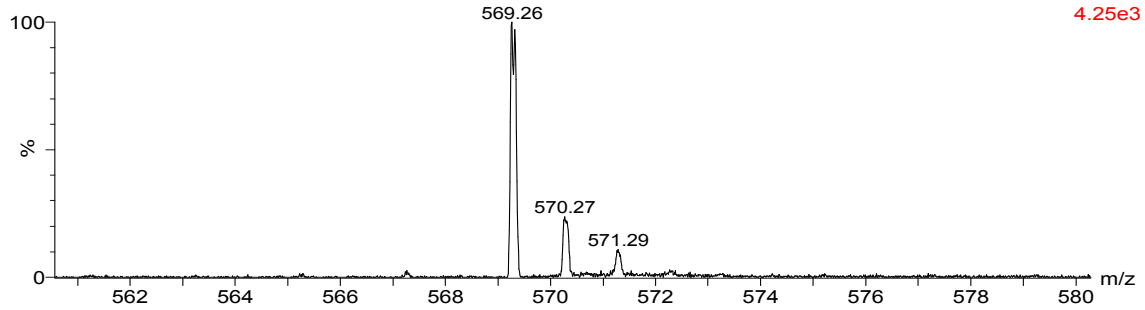


Fig. 23. Elution profile of xylo-oligosaccharides liberated from larchwood xylan by the action of purified ragi xylanase on Biogel P-2 column

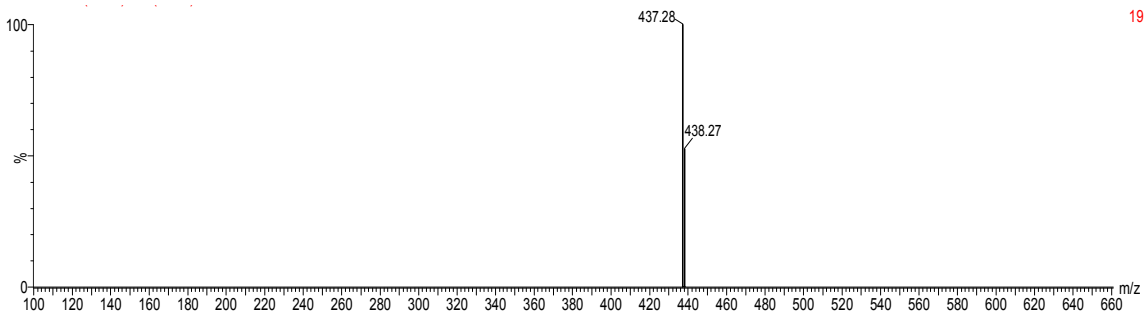
IV.9. Electro Spray Ion – Mass Spectrometry (ESI-MS)

The cleavage pattern of larchwood xylan oligosaccharides done by the method (Fernandez *et al.,* 2004) confirmed the endo mode of action of xylanase as analyzed by ESI-MS (Fig. 24 a, b & c). P-2 showed the MS spectrum of the ion at *m/z* 569.28 which was assumed to be a tetrasaccharide (150X4→ 600-4→546+23→569). The analysis of the signals at *m/z* for P3 (437.17) and P4 (305.14) were corresponding to xylotriose (X_3, 150X3→ 450-36→ 414+23→ 437) and xylobiose (X_2, 150X2→ 300-18→ 282+23→ 305) respectively (Levigne *et al*., 2004). Trace amounts of higher oligosaccharides (X_5 & X_6)

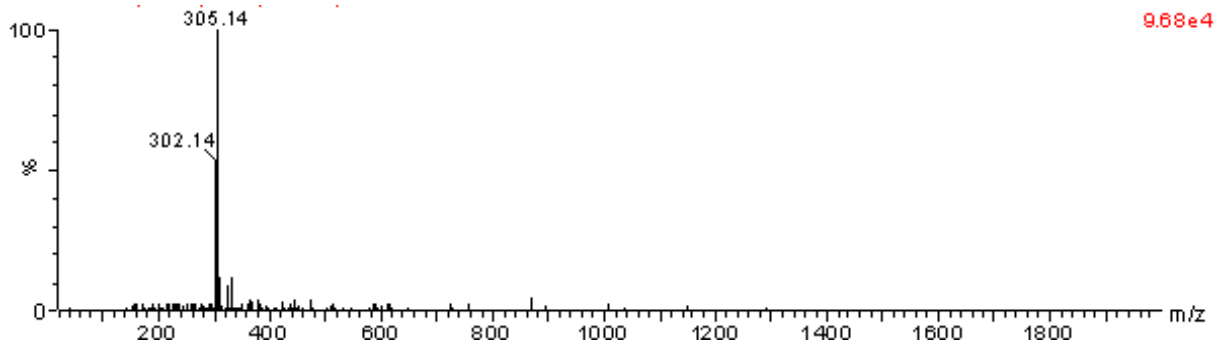
were also detected which indicated that they might be liberated in the early stages of hydrolysis and slowly hydrolyzed to oligomers of low D.P in the extended hydrolysis time (Ishihara *et al*., 1997). However from the spectra, it was not possible to obtain information on the arabinosyl residue linked to the xylopyranosyl backbone since both component monosaccharides (xylose and arabinose) are isomeric and fragmentation cannot differentiate between xylose or arabinose. To resolve this problem, H^1 NMR was carried out in order to obtain complete information on the structural characteristics of the oligosaccharides.



(a)



(b)



(c)

Fig. 24. ESI – MS of purified oligosaccharides a) P-2; b) P-3; c) P-4

IV.10. H^1 NMR spectra

The structures of P-2, P-3 and P-4 were elucidated by H^1 NMR and the signals were falling in the range of 3.0-5.0 ppm [Fig. 25 a (P-2), 25 b (P-3) & 25 c (P-4)]. The H^1 NMR spectra of oligosaccharide P-2 showed prominent chemical shifts for anomeric protons at 4.5097, 3.437, 3.74, 3.826 and 3.40 ppm corresponding to H-1, H-2, H-3, H-4 and H-5 of β-D-xylopyranose residue respectively (Hoffmann *et al.,* 1991; Gruppen *et al*., 1992; Vietor *et al.,* 1994). The chemical shift observed at 5.18 ppm can be attributed to O-3 linked α- arabinoside residue linked to the xylan backbone (Subba Rao & Muralikrishna, 2004) and chemical shifts at 4.1, 3.904, 4.25 and 3.78 correspond to H-2, H-3, H-4 and H-5 of arabinofuranose linked to xylopyranose residue. Accordingly following tentative structure can be assigned to **P-2**;

β-Xyl*p*- (1→4) β-Xyl*p*- (1→4) β-Xyl*p*
α- Ara*f*- (1→3)

Oligosaccharide, P-3 was identified as xylotriose with characteristic chemical shifts of 3.2, 3.54 and 3.77 ppm which can be attributed to H-2, H-3 and H-4 respectively of β-Xyl*p*-1.

P-3:

β-Xyl*p*- (1→4) β-Xyl*p*- (1→4) β-Xyl*p*).

P-4 was identified as xylobiose with chemical shifts of 3.22, 3.547 and 3.771 ppm which can be assigned to H-2, H-3 & H-4 respectively of un-substituted internal (1-4)-linked β-D-xyl*p*-1 residue .

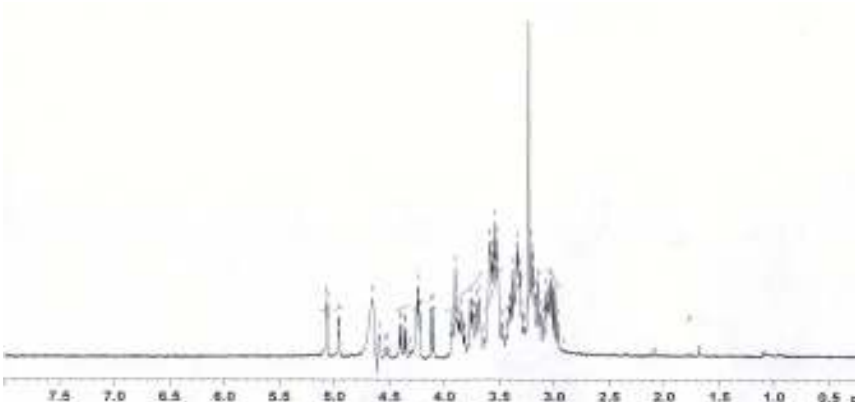
P-4:

β-Xyl*p*- (1→4) β-Xyl*p*).

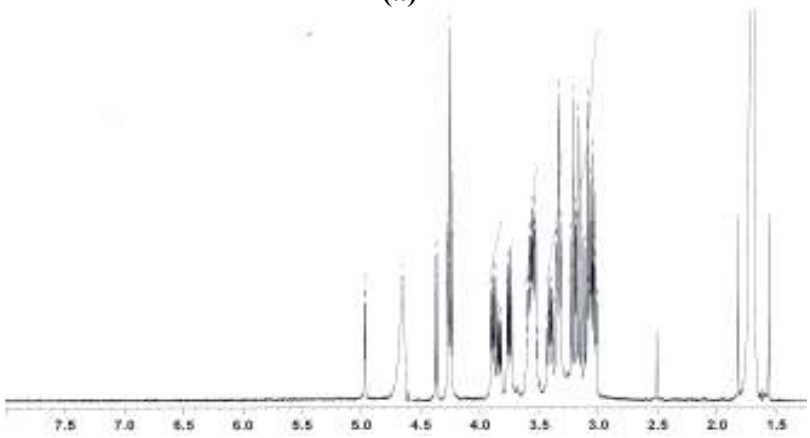
The proton resonance corresponding to acetyl residue (2.22 ppm) and 4-O-methyl α-D-glucuronic acid (H1- 5.28 ppm) was not observed as reported for the arabinoxylan of *Eucalyptus globulus* Labill (Shatalov *et al.,* 1999).

Table 11. H^1 NMR data of purified oligosaccharides liberated from larchwood xylan

Oligosaccharide	Residue	Chemical shift (δ)
P-2	β-xylp-2	H-1 (4.5097), H-2 (3.437),
		H-3 (3.74), H-4 (3.826) and H-5 (3.40)
	α-arabinofuranose linked to o-3 of xylopyranose	H-2 (4.1), H-3 (3.904), H-4 (4.25), H-5 (3.78)
P-3	β-xylp-1	H-2 (3.2), H-3 (3.54), H-4 (3.77)
P-4	β-xylp-1	H-2 (3.22), H-3 (3.547), H-4 (3.771)



(a)



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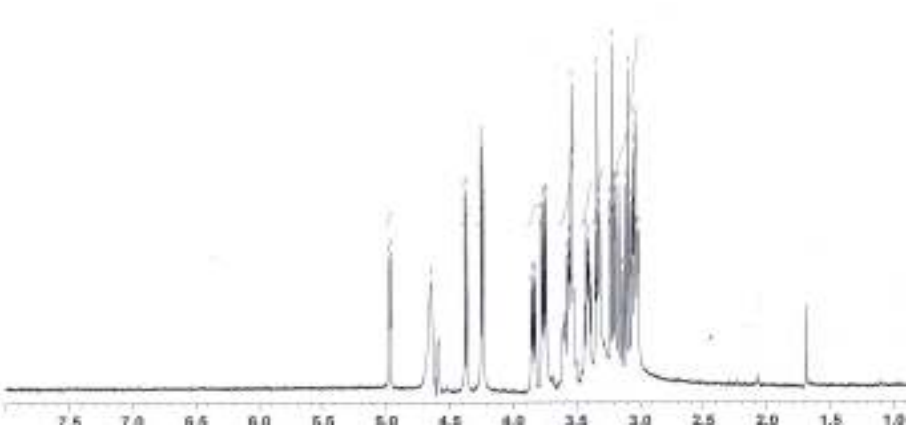


(c)

Fig. 25. H^1 NMR spectra of Purified oligosaccharides a) P-2, b) P-3, c) P-4

CHAPTER V

Structural characterization of oligosaccharides liberated from soluble polysaccharides of wheat and ragi brans

V.1. Introduction

Cereal brans, an important by-product of cereal industry, in addition to cellulose are rich in non-cellulosic polysaccharides such as arabinoxylans, 1, 3/1, 4-β-D-glucans and lignocellulose complexes which represent a vast renewable energy resource that can be enzymatically converted into bioactive compounds such as oligosaccharides and phenolic acids. Arabinoxylans are designated as water extractable or water un-extractable based on their extractability. (Trogh *et al.*, 2004; Brijs *et al.,* 2004). The difference in their extractability accounts for various physicochemical properties. Water unextractable nature is due to the involvement of non-covalent interactions of arabinoxylans with other cell wall components such as proteins, lignins etc. (Courtin & Delcour, 2002). Studies have been carried out on barley and rye water soluble/insoluble arabinoxylans (Debyser *et al*., 1997; Rasmussen *et al.*, 2001). A histological study on the effect of endoxylanase on wheat bran was carried out by Benamrouche *et al* (Benamrouche *et al.,* 2002). They have reported that the endoxylanase treatment liberated 80 % and 50 % of the carbohydrate from aleurone layer and inner bran respectively. Many studies were carried out on the characterization of arabinoxylans from cereal brans and their degradation products i.e. xylo-oligosaccharides with respect to their structure. However no study has been carried out with respect to the oligosaccharides from ragi bran. In the present study both wheat and ragi bran soluble polysaccharides were isolated and oligosaccharides liberated from them by purified ragi xylanase (Chapter IV) were characterized using ESI-MS and H^1NMR analyses.

V.2. Isolation of soluble polysaccharides (SP) from wheat and ragi bran

The major problem associated with the isolation of soluble arabinoxylans from wheat and ragi brans is associated starch contamination. Termamyl followed by glucoamylase treatments efficiently removed starch and yielded wheat bran SP (35 %, w/w) and ragi bran SP (14.4 % w/w) devoid of starch. The neutral sugar composition of

the SP isolated from wheat consisted of arabinose and xylose in the ratio of 1:0.86 (Table. 12). Arabinose to xylose ratio of the arabinoxylans from barley and malt were close to 1: 0.65 (Dervilly *et al.,* 2002). In addition to arabinose (41.8 %) and xylose (48.6 %), rhamnose (0.72 %), mannose (0.44 %), galactose (5.6 %) and glucose (2.9 %) were found in minor amounts. Studies were carried out on the water extractable non-starch polysaccharides from native and malted ragi and rice (Rao & Muralikrishna, 2006).

Table. 12. Neutral sugar composition of non-starch polysaccharides isolated from wheat bran

Sample		Rha	Ara	Xyl	Man	Gal	Glu
Wheat	**SP**	0.72	41.8	48.6	0.44	5.6	2.9
	IP	0.44	34.6	44.6	**-**	0.84	19.6

Rha- Rhamnose; Ara- Arabinose; Xyl- xylose; Man- Mannose; Gal- Galactose; Glu- Glucose; SP- Soluble Polysaccharides; IP- Insoluble Polysaccharides

A large portion of cereal arabinoxylans is water unextractable (Maes & Delcour, 2002). Water insoluble polysaccharides of wheat bran consisted mainly of arabinose and xylose in a ratio of 1: 0.78 with trace amounts of rhamnose (0.44 %), galactose (0.84 %) and glucose (19.6 %). The uronic acid content of wheat bran SP and IP were found to be 1.0 and 3.5 % respectively. Chemical /enzymatic methods are also used for the extraction of arabinoxylans (Saulnier *et al*. 1995)**.**

The yield of soluble polysaccharide from ragi bran was found to be less compared to that of wheat bran. The neutral sugar composition of SP and IP isolated from ragi bran were shown in Table. 13. Arabinose and xylose were found to be the major

monosaccharides in the SP of ragi bran with trace amounts of galactose, glucose, rhamnose and mannose. Ara/xyl ratio in SP was found to be 1:1.8, while in the case of IP it is 1:1.2. The ratio of Ara/xyl obtained for wheat bran arabinoxylans was in accordance with the earlier report (Benamrouche *et al.* 2002). Studies were carried out on maize bran polysaccharides (Saulnier *et al*. 1995).

Table. 13. Neutral sugar composition of non-starch polysaccharides obtained from ragi bran

Sample		Rha	Ara	Xyl	Man	Gal	Glu
Ragi	**SP**	5.4	41.8	22.9	4.71	14.9	10.4
	IP	0.92	10.4	9.1	4.3	2.9	72.4

Rha- Rhamnose; Ara- Arabinose; Xyl- xylose; Man- Mannose; Gal- Galactose; Glu- Glucose; SP- Soluble Polysaccharides; IP- Insoluble Polysaccharides

V.3. Liberation of oligosaccharides from wheat bran SP, their purification and characterization

Soluble polysaccharides (SP) isolated from wheat bran were subjected to purified ragi xylanase treatment in order to obtain xylo oligosaccharides (30 %). The crude oligosaccharides were fractionated on Biogel P-2 column (Fig. 26). Undegraded or partially degraded polysaccharides were eluted out in the void volume whereas the oligosaccharides eluted in the bed volume were separated out into four major peaks designated as WO-1, WO-2, WO-3 and WO-4.

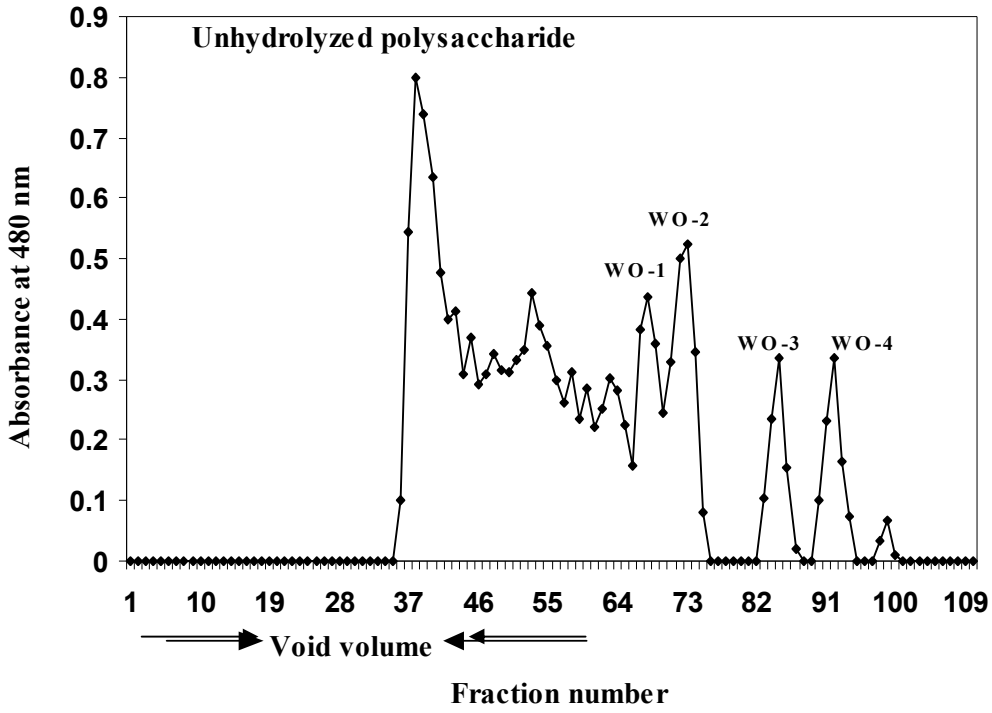
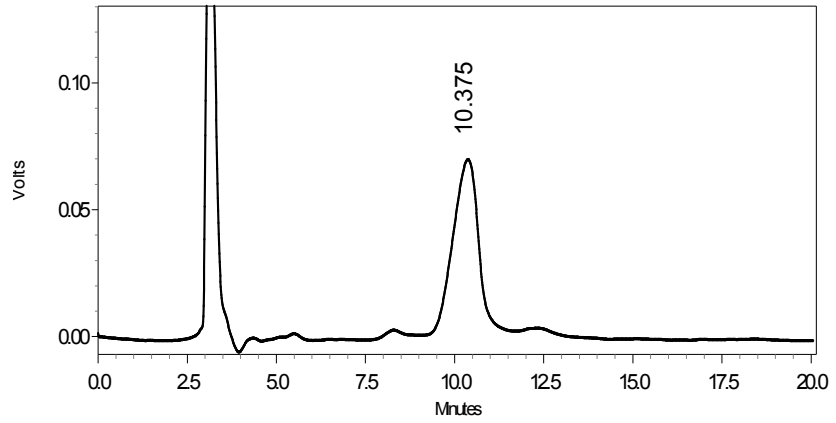


Fig. 26. Elution profile of oligosaccharides liberated from wheat bran SP on Biogel P-2

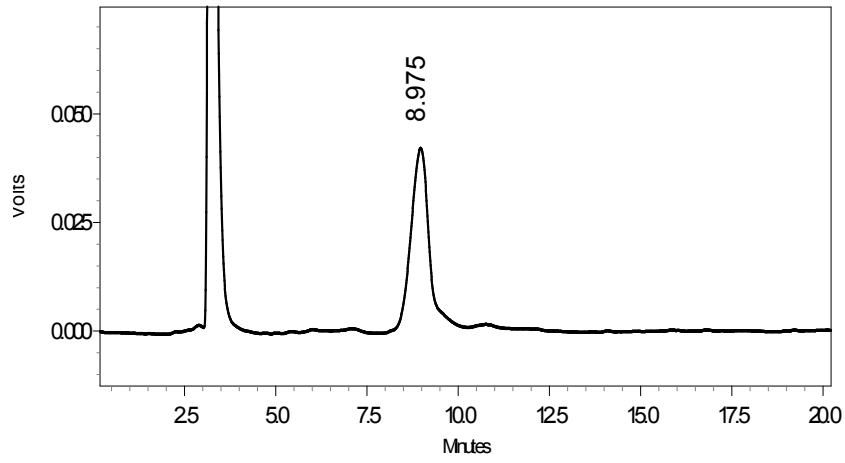
Biogel P-2 column chromatography was employed in the purification of xylo-oligosaccharides from soy arabinogalactan (Van Laere *et al*., 2000). Heavily substituted arabinoxylans from barley malt were less prone to the xylanase degradation (Dervilly *et al*., 2002). Biogel P-6 was used in the purification of oligosaccharides liberated from arabinoxylans of white endosperm of wheat (Hoffmann *et al.,* 1991).

The purity of the individual oligosaccharides recovered from Biogel P-2 column was confirmed by HPLC (Fig. 27 a, b, c & d)). The monosaccharide composition of the HPLC purified oligosaccharides was determined by GLC. WO-1 consisted exclusively of arabinose (31 %) and xylose (69 %). Similarly, Arabinose (42 %) and xylose (58 %) were

found as the constituents of WO-2 whereas, WO-3 and WO-4 consisted exclusively of xylose (100 %). HPLC and High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC PAD) have been widely used in the separation of oligosaccharides (Katapodis *et al*., 2002; Sorensen *et al*., 2003). Amberlite XAD-2 column was used in the purification of feruloyl oligosaccharides liberated from wheat bran insoluble dietary fibre by endoxylanase isolated from *Bacillus subtilis* (Yuan *et al*., 2005). Amberlite XAD-2 and SephadexLH-20 were employed in the purification of feruloyl oligosaccharides isolated from maize bran (Saulnier *et al*., 1995).

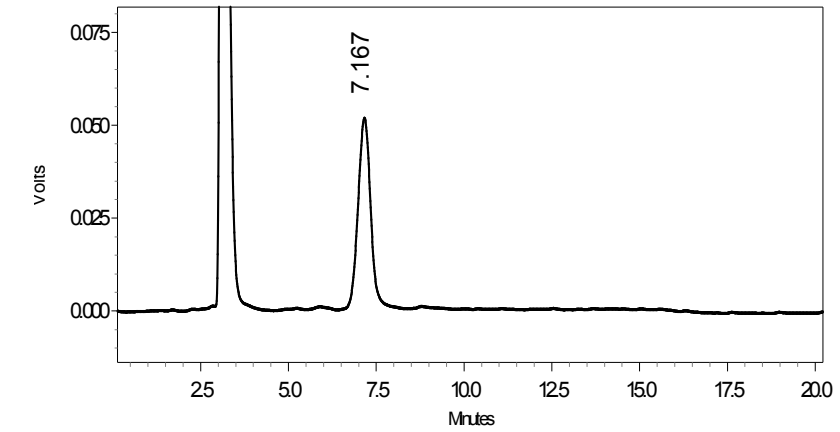


(27a)

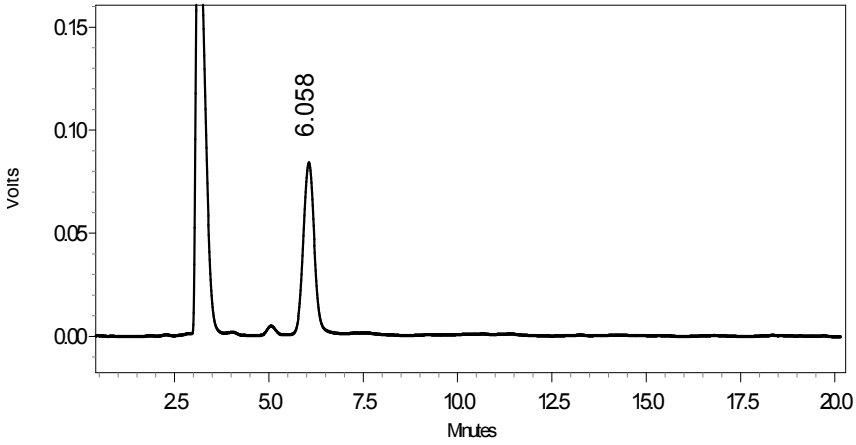


(27b)

Fig. 27. HPLC profile of purified oligosaccharides liberated from wheat bran SP: 27a) WO-1; 27b) WO-2



(27c)



(27d)

Fig. 27. HPLC profile of purified oligosaccharides liberated from wheat bran SP: 27c) WO-3; 27d) WO-4.

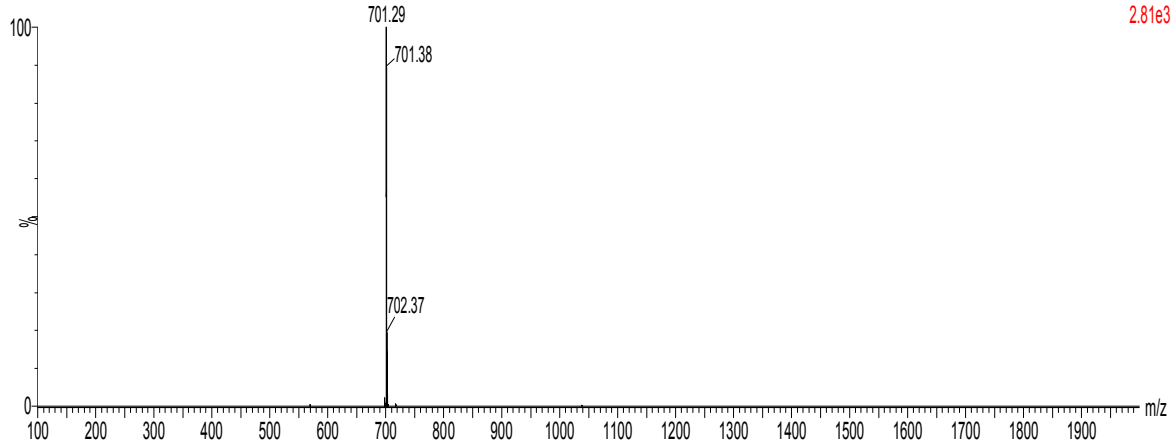
V.3.1. Structural characterization of the purified oligosaccharides from wheat bran soluble polysaccharides

The structural characterization of the purified oligosaccharides obtained from wheat bran SP was done by ESI-MS and H^1 NMR studies. These techniques are attaining paramount importance in the carbohydrate field and they are used in the structural elucidation of many of the biomolecules.

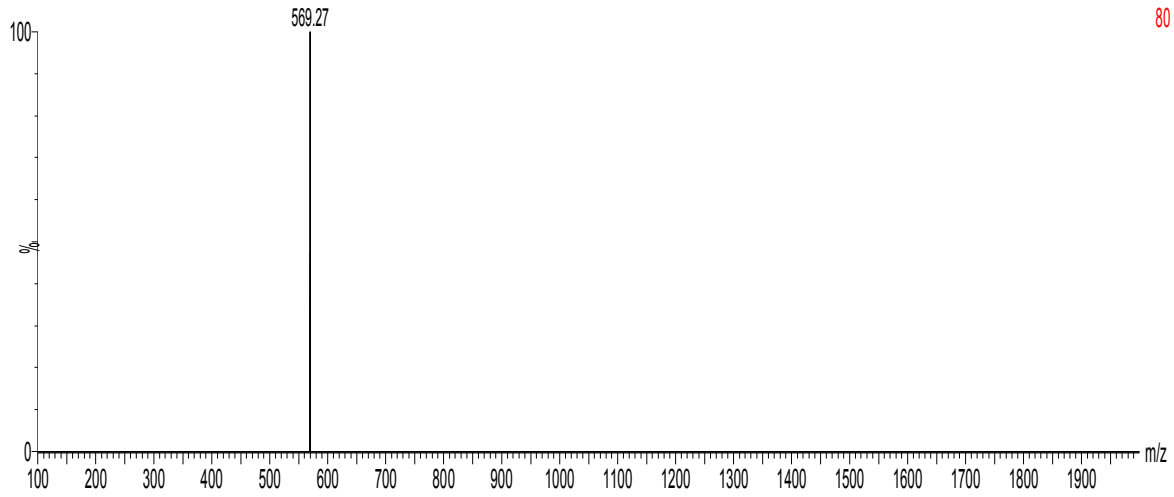
V.3.1.1. ESI-MS

The HPLC purified oligosaccharides were identified as cationised molecules $[M+Na]^+$ in the ESI mass spectrum (Fig. 28 a, b, c & d). WO-1 showed preponderantly the presence of ion at *m/z* 701.29 which was identified as a pentasaccharide[678+23 (sodium adduct)→701]. The ions observed at *m/z* 569.27 for WO-2 corresponds to a tetrasaccharide (546+23→569). Similarly the signal at *m/z* 437.28 (WO-3) and 305.14 (W)-4) were identified as trisaccharide (414+23→437) and disaccharide respectively (282+23→305). ESI-MS was employed in the analysis of a mixture of arabinoxylan oligosaccharides from wheat seedling and the spectra corresponding to xylopentaose, xylotetraose and xylotriose were identified (Fernandez *et al*., 2004). To our knowledge there is no information available on the identification of xylo-oligosaccharides derived from wheat and ragi brans by ESI-MS analysis. The fragmentation pattern of xylo-oligosaccharides liberated from olive pulp was identified by ESI-MS (Reis *et al*., 2004). Both positive and negative mode ESI-MS were employed in the identification of xylo-oligosaccharides with degrees of polymerization 5-13, formed by partial acid hydrolysis from olive pulp extract (Reis *et al*., 2003).

Mass spectra will not provide the unequivocal identification of the oligosaccharides since it fails to differentiate the mass losses due to the isobaric structures such as arabinose and xylose. (Fernandez *et al.,* 2004). Hence, H^1 NMR studies have been carried out for the complete structural elucidation of the purified oligosaccharides.

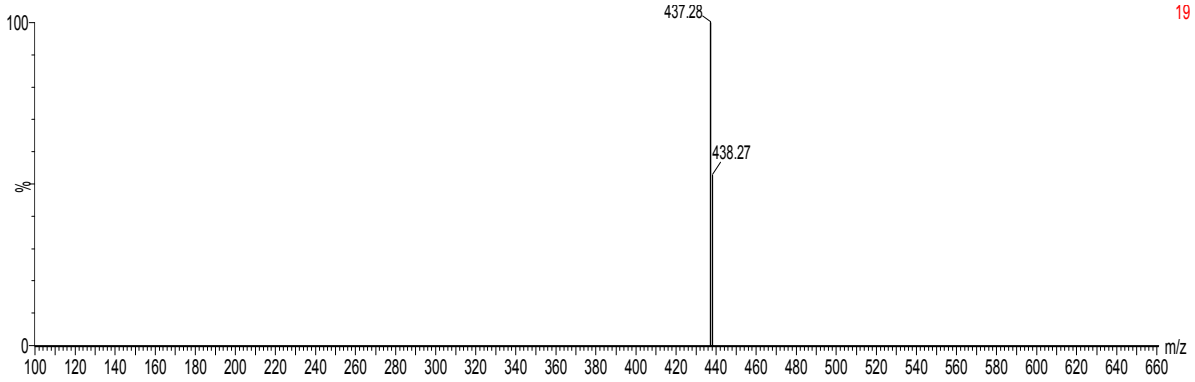


(28 a)

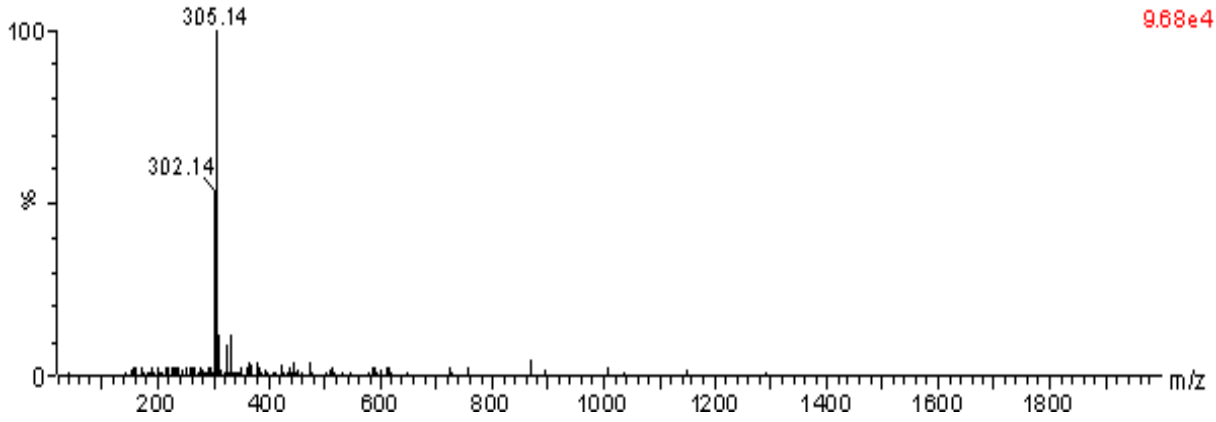


(28 b)

Fig.28. ESI – MS of purified oligosaccharides: 28a) WO-1; 28b) WO-2



(28 c)



(28 d)

Fig.28. ESI – MS of purified oligosaccharides: 28c) WO-3; 28d) WO-4

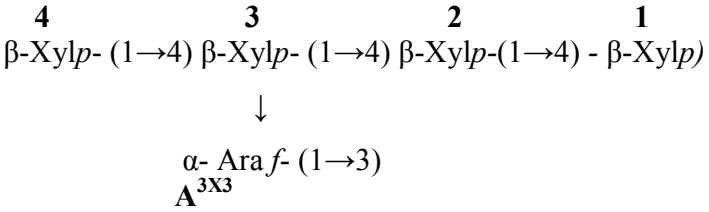
V.3.1.2. H^1 NMR

The details of the H^1 NMR spectra of the purified oligosaccharides liberated from wheat bran are given in Fig. 29. Chemical shift of anomeric proton at 3.41, 3.583, 3.67, 3.95 correspond to H-2, H-3, H-4 and H-5 of *β-xylp-3*^{II} (Table. 14). Chemical shifts at 3.957, 4.2, and 3.82 correspond to H-3, H-4 and H-5 of arabinofuranose linked to xylopyranose (*α-Araf-A*^{3X3}) (Gruppen *et al*., 1992; Vietor *et al.,* 1994). Oligosaccharide, WO-2 was identified as xylotetraose with characteristic chemical shifts of anomeric proton at 3.349, 3.748, 3.8226 and 4.1212 indicates that of H-2, H-3, H-4 and H-5 of *β- xyl-p-2*^{II}. Apart from these signals the spectra showed signals at 4.1497 (H-2), 3.907 (H-3), 4.2739 (H-4) and 3.7944 (H-5) indicated the presence of arabinofuranose unit linked to O-3 of xylopyranose residue.

WO-3 was identified as xylotriose with chemical shifts of 3.2, 3.5494 and 3.77 corresponding to H-2, H-3 and H-4 of *β- xyl-p-1*. The chemical shift observed for WO-4 at 3.2, 3.547 and 3.771 corresponds to that of H-2 H-3 and H-4 of *β- xyl-p-1*.

Probable structures of the purified xylo-oligosaccharides obtained from wheat bran SP are as follows:

WO-I:



WO-2:

3 **2** **1**
β-Xyl*p*- (1→4) β-Xyl*p*- (1→4) β-Xyl*p)*
↓
α - Ara *f*- (1→3)
A^{3X2}

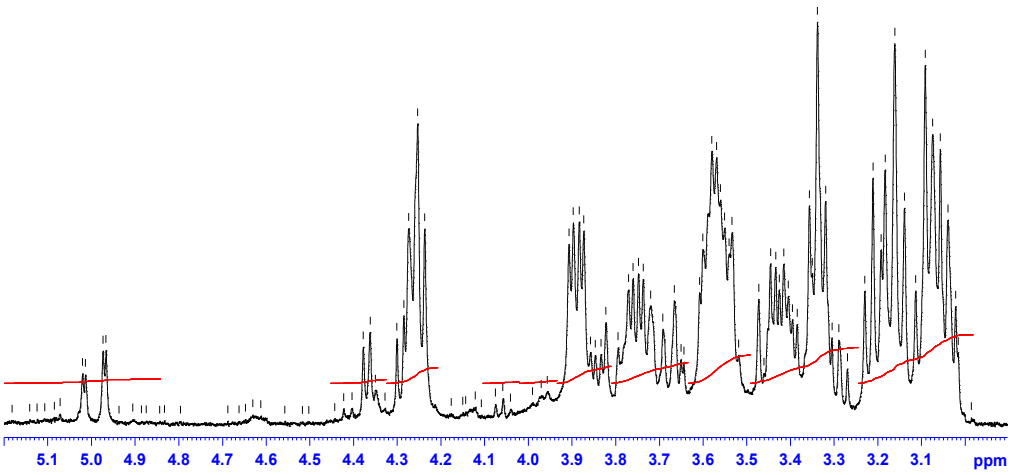
WO-3:

Xyl*p*- (1→4) β-Xyl*p*- (1→4) β-Xyl*p*

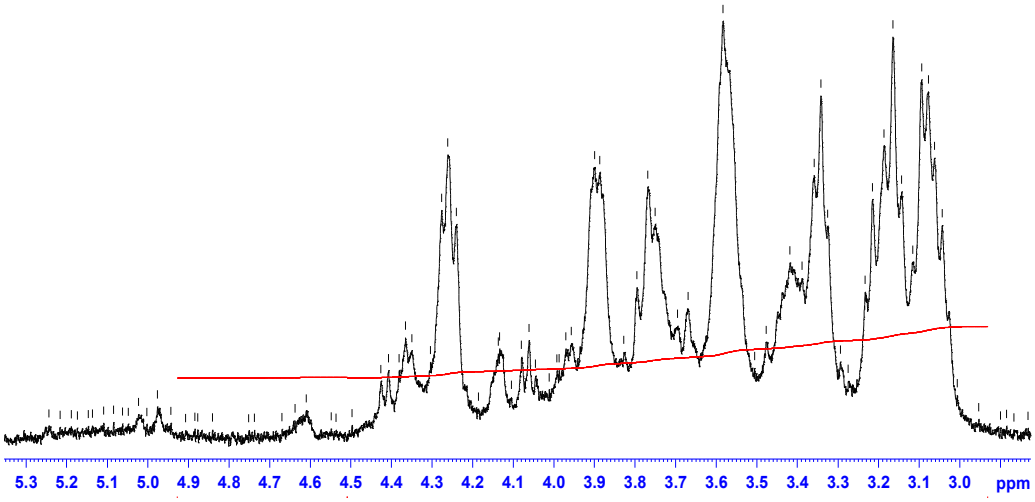
WO-4:

Xyl*p*- (1→4) β-Xyl*p*

The proton resonances corresponding to acetyl residue (2.22 ppm) and 4-O-methyl α-D-glucuronic acid (H1- 5.28 ppm) were not observed in the H^1 NMR spectra of any of the oligosaccharide liberated from wheat bran SP. H^1 NMR spectroscopy was employed in the identification of small oligomers liberated from barley arabinoxylans by endoxylanase from *Aspergillus awamori* (Vietor et al., 1994). The oligosaccharides identified consisted of 1, 4-linked β-D-xylopyranosyl residues, some of which were substituted with α-L-arabinofuranosyl residues at O-2/O-3 or at both at O-2 and O-3. H^1 NMR was also used in the structural characterization of oligosaccharides derived from arabinoxylans of white endosperm of wheat (Hoffmann *et al*., 1991). The primary structure of feruloyl arabinoxylopentasaccharide from wheat bran was determined using 2D NMR spectroscopy (Lequart *et al*., 1999). The oligosaccharides were identified as 1,4-linked β-D-xylopyranosyl residues substituted with α-L-arabinofuranose residues. C^{13} NMR spectroscopy was used in the structural elucidation of feruloylated oligosaccharides from maize bran (Saulnier *et al*., 1995).

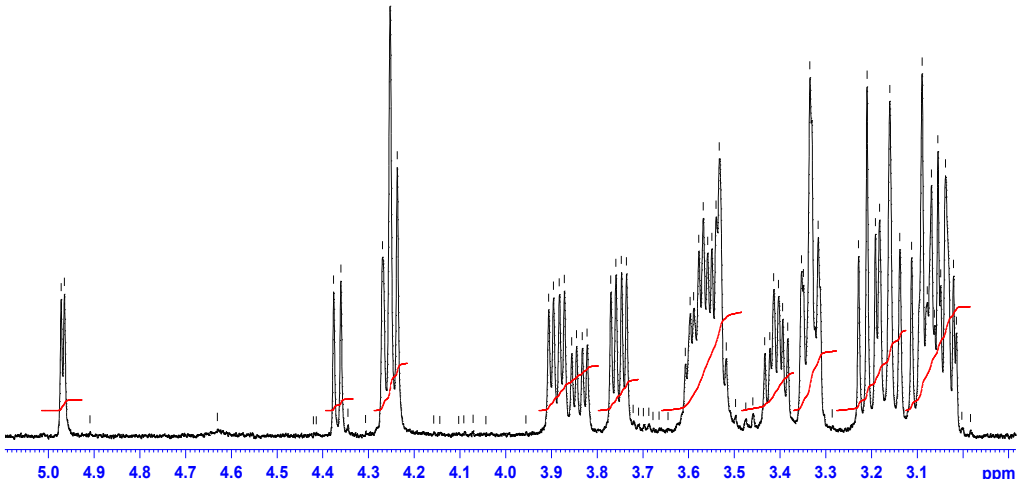


(29 a)

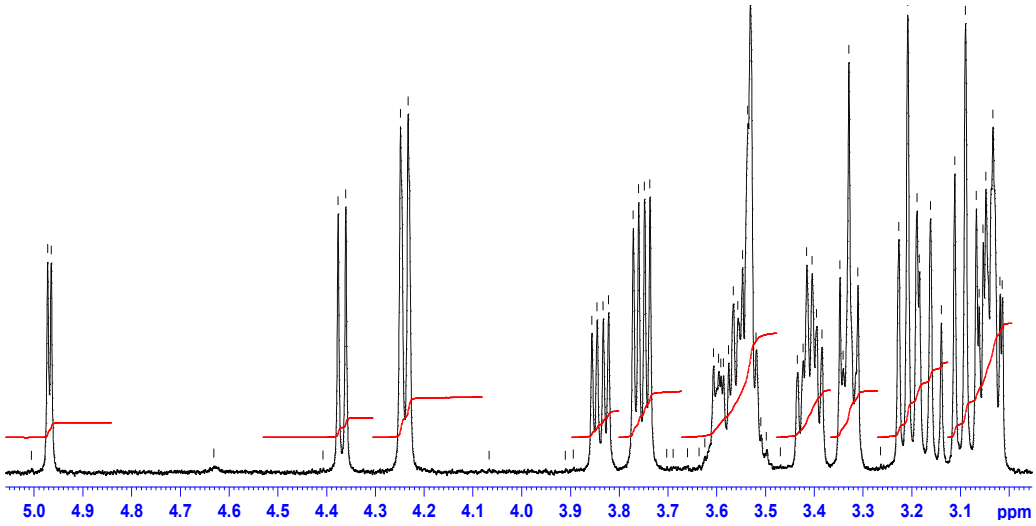


(29 b)

Fig. 29. H^1 NMR spectra of purified xylo-oligosaccharides: 29a) WO-1; 29b) WO-2



(29 c)



(29 d)

Fig. 29. H^1 NMR spectra of purified xylo-oligosaccharides: 29 c) WO-3; 29 d) WO-4

Table. 14. H^1 NMR data of purified oligosaccharides liberated from wheat bran SP

Fractions	Residue	Chemical Shift (δ)
WO-1	β-xylp-3	3.41 (H-2), 3.583 (H-3), 3.67 (H-4), 3.95 (H-5)
	α-arabinofuranose linked to O-3 of xylopyranose	3.957 (H-3), 4.2 (H-4), 3.82 (H-5)
WO-2	β-xylp-2	3.349 (H-2), 3.748 (H-3), 3.8226 (H-4), 4.1212 (H-5)
	α-arabinofuranose linked to-3 of xylopyranose	4.1497 (H-2), 3.907 (H-3), 4.2739 (H-4), 3.7944 (H-5)
WO-3	β-xylp-1	3.2 (H-2), 3.5494 (H-3), 3.77 (H-4)
WO-4	β-xylp-1	3.2 (H-2), 3.547 (H-3), 3.771 (H-4)

V.4. Liberation of oligosaccharide from ragi bran SP by xylanase treatment and their purification

Oligosaccharides (17.9 %) liberated from ragi bran by the action of purified ragi xylanase were separated on Biogel P-2 column chromatography (Kormelink *et al.* 1993) (Fig.30). The partially degraded polysaccharides (molecular weight>1.8 kDa) were separated out in the void volume and single oligosaccharide designated as RO-1 was collected and its purity was confirmed by HPLC (Fig. 31). RO-1 was found to be exclusively consisting of xylose as analyzed by GLC. Enzymatic hydrolysis of ragi bran SP confirmed the endo mode of action of purified ragi xylanase yielding xylobiose as the major product. Wheat bran subjected to endoxylanase treatment also showed the similar pattern of hydrolysis wherein xylotriose and xylobiose were found to be the major products (Benamrouche *et al*. 2002).

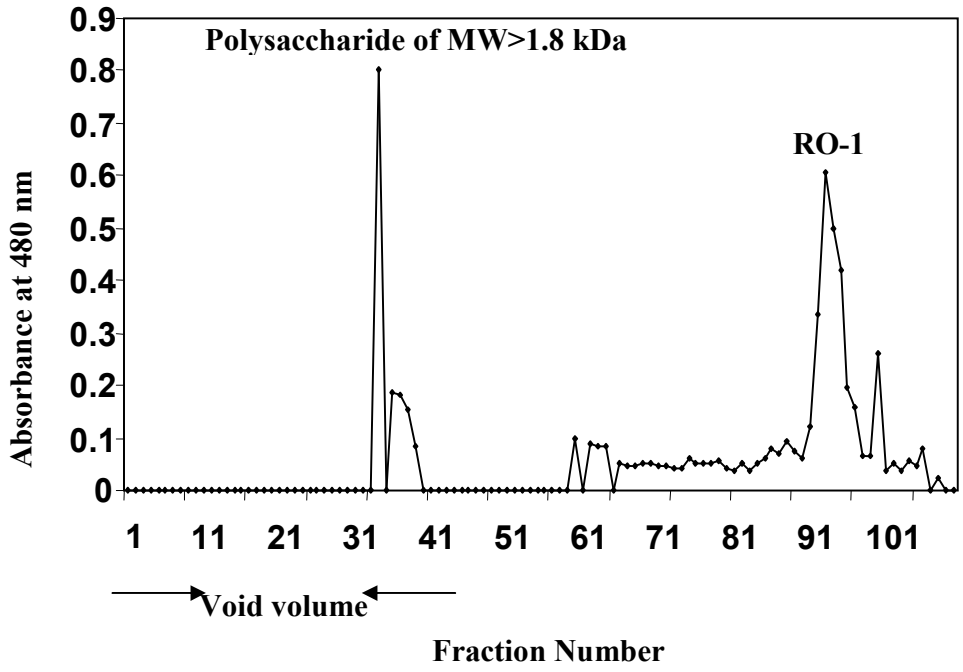


Fig. 30. Elution profile of oligosaccharides liberated from wheat bran SP on Biogel P-2

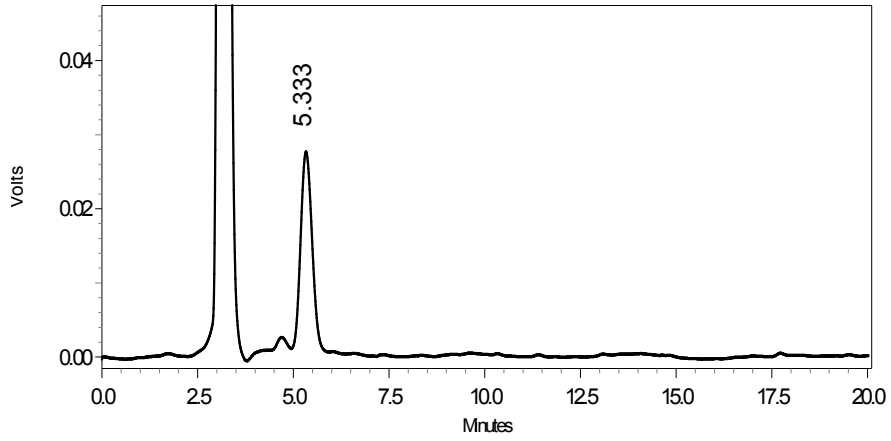


Fig. 31. HPLC profile of purified oligosaccharide (RO-1) liberated from ragi bran SP

V.4.1. Structural characterization of the purified oligosaccharide liberated from ragi bran SP

V.4.1. ESI-MS

RO-1 showed preponderantly the presence of ion at *m/z* 305.14 which was identified as a disaccharide (150X2→300-18 (elimination of water molecule)→282+23 (sodium adduct)→305) (Fig. 32). ESI-MS/MS was found to be a powerful tool for the characterisation of acetylated patterns in complex mixtures of oligosaccharides. ESI-MS was used in the identification of acetylated neutral and acidic xylo-oligosaccharides obtained by partial acid hydrolysis of *Eucalyptus globulus* wood glucuronoxylans (Reis *et al*., 2005). Electrospray tandem mass spectrometry was used in the differentiation of the structures of two oligomers of acidic xylo-oligosaccharides (XOS) of the same molecular weight (634 Da) extracted from *Eucalyptus globulus* wood (Reis *et al*., 2004). Negative ion electrospray mass spectrometry was also employed in the structural characterization of underivatized arabino-xylo-oligosaccharides (Reis *et al.,* 2005).

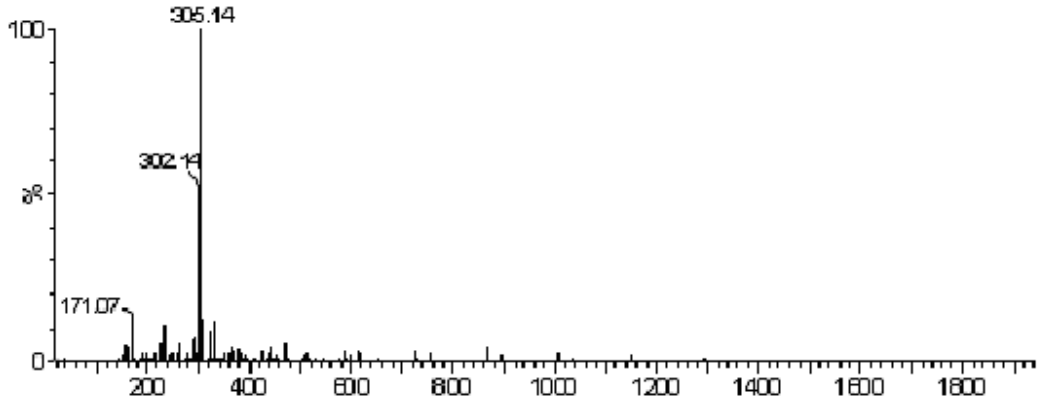


Fig. 32. ESI – MS of RO-1

V.4.2. H^1 NMR

The structure of the purified oligosaccharide (RO-1) was elucidated by H^1 NMR and the signals were falling in the range of 3.0-5.0 ppm (Fig. 33). The spectrum showed prominent chemical shifts for anomeric protons at 3.248, 3.542, and 3.7 ppm corresponding to H-2, H-3 and H-4 of β-D-xylopyranose residue respectively (Hoffmann *et al*. 1991; Gruppen *et al*. 1992; Vietor *et al*. 1994). The chemical shifts corresponding to arabinose residues and glucuronic acids were not detected in the spectrum. Hence the probable structure of the purified oligosaccharide is as follows, (1, 4-linked β-D-xylobiose).

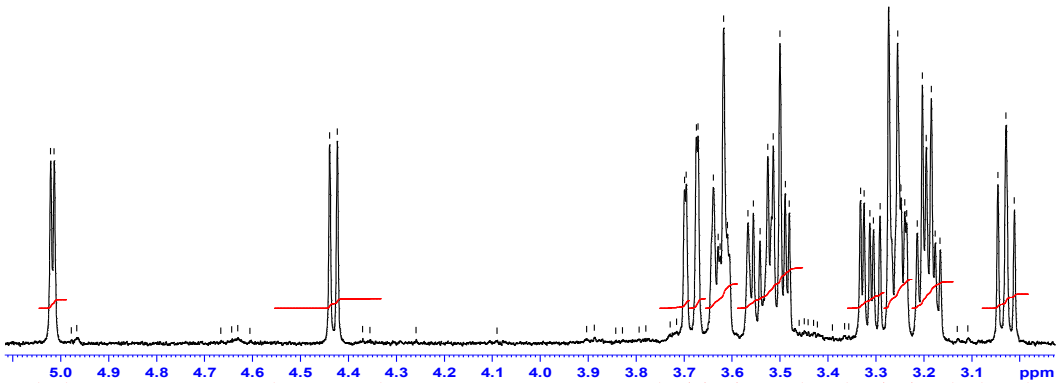


Fig. 33. H^1NMR spectra of RO-1

RO-1:

Xylp- (1→4) β-Xylp

C^{13} NMR spectroscopy was also employed in the characterization of oligosaccharides. The structural characterization of the acidic xylo-oligosaccharides liberated from birchwood xylan by treatment with an endoxylanase from *Thermoascus aurantiacus* was done by C^{13} NMR (Katapodis *et al*., 2002).

In conclusion, the purified oligosaccharides from wheat bran SP *viz,* WO-1 and WO-2 were characterized as xylotetraose and xylotriose respectively with monosubstituted α-D- arabinofuranose residue, whereas WO-3 and WO-4 were identified as unsubstituted xylotriose and xylobiose respectively by ESI-MS and H^1 NMR studies.

The purified oligosaccharide from ragi bran SP was identified as unsubstituted xylobiose. Thus the polysaccharides can be efficiently converted into oligosaccharides by enzymatic hydrolysis and the liberated oligosaccharides can be identified by modern analytical techniques such as ESI-MS and H^1 NMR.

CHAPTER VI

Prebiotic activity of xylo-oligosaccharides liberated from soluble polysaccharides of wheat and ragi brans

VI.1. Introduction

The role of prebiotics in improving human health has attracted global attention and the research is mostly focused on the strains belonging to the genera of *Bifidobacterium* and *Lactobacillus.* In the large intestine, prebiotics, in addition to their selective effects on *Bifidobacteria* and *Lactobacilli*, influence many aspects of bowel function through fermentation. Most of the interest in the development of new prebiotic ingredients is focused on oligosaccharides which can not be digested by small intestinal enzymes and are receiving increased attention all over the world due to their possible health benefits. Prebiotic effect of fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) were previously reported (Bouhnik *et al*. 1997; Delzenne & Kok, 2001). The prebiotic activity of xylo-oligosaccharides is less studied compared to FOS and GOS. A study based on the i*n vitro* fermentation experiments of the oligosaccharides obtained from wheat and ragi bran (chapter V) has been carried out in this chapter and the results are displayed.

VI. 2. *In vitro* fermentation studies

Xylo-oligosaccharide mixture as well as purified oligosaccharides (0.25 %) liberated from wheat and ragi bran soluble polysaccharides and larchwood xylan by purified ragi xylanase treatment were tested for their prebiotic activity *in vitro* using *Bifidobacteria* and *Lactobacilli* spp.

VI.2.1. Growth characteristics of Bifidogenic bacteria on xylo-oligosaccharides

VI.2.1.1. Wheat bran xylo-oligosaccharides

In the case of wheat bran xylo-oligosaccharides maximum absorbance was observed at 600 nm with respect to *Bifidobacterium adolescentis* NDRI 236 and *Lactobacillus plantarum* NDRI strain 184 (Table.15).

Purified individual oligosaccharides (WO-1, WO-2, WO-3, and WO-4) showed the same growth characteristics as crude oligosaccharides. The bifidogenic effect of the xylo-oligosaccharides was further confirmed by an increase in the dry cell mass of the tested microorganisms after 48 h of incubation period compared to the control. A reduction in pH of the culture broth was noticed for all the microorganisms grown on both crude as well as purified xylo-oligosaccharides. The reduction in pH of the culture broth is due to the production of short chain fatty acids (SCFA) as a result of the xylo-oligosaccharide fermentation by the tested microorganisms (Ruppin *et al*., 1980). Xylobiose was efficiently utilized by the beneficial bacterial spp compared to the crude oligosaccharides indicating a slightly high O.D. and dry cell mass. Similar results were reported for *in vitro* fermentation of arabinoxylan fractions from wheat by human faecal microflora, wherein low molecular weight AX fraction was effectively utilized by beneficial microbes compared to AX of high degree of polymerization (Hughes *et al*., 2007). It was reported that xylo-oligosaccharides are more effective for the gastrointestinal health compared to fructo-oligosaccharides (Hsu *et al*., 2004). The growth was found to be comparatively low in the case of *Lactobacillus brevis* and *Pediococcus pentosaceus* NCDO 8081 indicating their lower efficiency to utilize the xylo-oligosaccharides liberated from wheat bran SP. The growth of the microorganism on particular oligosaccharide may be strain specific (Holtl, *et al*., 2005). *Bifidobacterium bifidum* NCDO 2715 showed increased growth compared to *Bifidobacterium bifidum* ATCC 29521. Similarly *Pediococcus pentosaceus* ATCC 8081 was effectively grown on the oligosaccharides than *Pediococcus pentosaceus* NCDO 813.

Table. 15. Growth characteristics of microorganisms grown on crude as well as purified oligosaccharide(s) liberated from wheat bran SP

Microorganism	Xylo-oligosaccharide	O.D.	pH	Cell mass (mg)
		0.070[a]	7.2	1.3
	Crude	2.38	5.1	10.0
B. adolescentis	WO-1	2.32	5.3	10.0
NDRI 236	WO-2	2.41	5.1	11.0
	WO-3	2.48	5.0	11.0
	WO-4	2.48	5.0	11.0
		0.068 [a]	6.9	1.0
	Crude	0.773	5.8	6.0
B. bifidum				
ATCC 29521	WO-3	0.79	5.8	6.0
		0.06 [a]	7.5	1.1
B. bifidum,	Crude	1.892	5.5	8.0
NCDO 2715	WO-3	2.0	5.3	8.0
		0.039 [a]	6.9	1.0
	Crude	2.408	5.1	10.5
L. plantarum	WO-1	2.4	5.2	11.0
NDRI strain 184	WO-2	2.42	5.2	11.0
	WO-3	2.46	5.2	11.0
	WO-4	2.46	5.2	11.0
		0.04 [a]	7.1	1.4
L. brevis 01	Crude	0.420	6.4	4.0
	WO-3	0.44	6.4	4.0
		0.031 [a]	7.5	1.0
P. pentosaceus,	Crude	0.417	6.2	4.0
NCDO 813	WO-3	0.45	6.0	4.0
		0.35 [a]	6.9	1.2
P .pentosaceus,	Crude	0.924	5.8	7.4
ATCC 8081	WO-3	0.963	5.8	7.5

[a] Represents control

The bifidogenic nature of xylo-oligosaccharides was reported in human beings (Rycroft *et al*., 2001) and in rats (Campbell *et al*., 1997). Static batch culture fermentation experiments conducted with human faecal microflora shown that xylo-oligosaccharides alter the gut microflora increasing the number of *Bifidobacteria* and/ or *Lactobacilli* spp (Rycroft *et al*., 2001). Increase in the *Bifidobacteria* due to the dietary oligosaccharides induced a reduction in the pathogenic germs and it is reported that fewer number of clinically relevant pathogens have been detected in the fecal samples of infants supplemented with the GOS and FOS (Moura *et al.*, 2007).

VI.2.1.2. Ragi bran oligosaccharides

A decrease in the pH of the culture broth and increase in O.D were observed after 48 h of incubation for all the bacterial strains grown on crude xylo-oligosaccharides as well as RO-1 (Table. 16). Maximum O.D was observed with respect to *Pediococcus pentosaceus,* ATCC 8081. A concomitant increase in the dry cell mass was also observed compared to the control after 48 h of incubation proving that xylo-oligosaccharides were utilized by the beneficial microbes and has enhanced their growth. Purified xylobiose is more effective than crude oligosaccharides wherein the O.D of the culture broth and bacterial cell mass were found to be slightly high. *Pediococcus pentosaceus* NCDO 8081 showed increased growth compared to *Pediococcus pentosaceus* ATCC 813. But with respect to *Bifidobacterium bifidum* a slightly increased growth was noticed for *Bifidobacterium bifidum* ATCC 29521 compared to *Bifidobacterium bifidum* NCDO 2715. Apart from xylo-oligosaccharides, the bifidogenic nature of the arabinoxylan (AX) was also reported (Crittenden *et al*., 2002).

Table. 16. Growth characteristics of microorganisms grown on crude as well as purified oligosaccharide(s) liberated from ragi bran WEP

Microorganism	O.D.	pH	Cell mass (mg)
B. adolescentis NDRI 236	[a] 0.070	7.2	1.3
	[b] 0.483	5.8	6.0
	[c] (0.563)	(5.6)	(7.0)
B. bifidum ATCC 29521	[a] 0.068	6.9	1.0
	[b] 0.737	5.7	7.0
B. bifidum, NCDO 2715	[a] 0.06	7.5	1.1
	[b] 0.705	5.8	6.0
L. plantarum NDRI strain 184	[a] 0.039	6.9	1.0
	[b] 0.496	5.9	5.3
	[c] (0.634)	(5.8)	(7.0)
L. brevis 01	[a] 0.04	7.1	1.4
	[b] 0.517	6.0	5.0
P.pentosaceus, NCDO 813	[a] 0.031	7.5	1.0
	[b] 0.388	6.9	6.0
P.pentosaceus, ATCC 8081	[a] 0.035	6.9	1.2
	[b] 0.752	5.6	7.0

[a] Represents control; [b] Represents crude oligosaccharides
[c] Values in parenthesis are for purified xylobiose

VI.2.1.3. Larchwood xylan oligosaccharides

The bifidogenic nature of the xylo-oligosaccharides liberated from cereal brans was compared with the crude xylo-oligosaccharides from larchwood xylan, the commonly used commercial substrate of xylanase. After 48 h of incubation an increase in absorbance at 600 nm and decrease in pH of the culture broth were observed for all the bacterial strains grown on xylo-oligosaccharide mixture (Table. 17) and maximum absorbance at 600 nm was observed with respect to *Bifidobacterium bifidum ATCC 29521*. *Lactobacillus brevis* 01 was not able to effectively utilize the xylo-oligosaccharides liberated from larch wood xylan. The dry cell mass was also increased compared to the control after 48 h of incubation indicating the xylo-oligosaccharides utilization by the beneficial microbes.

A study on swine fecal microflora showed that the decrease in caecal pH was more with respect to fructo and xylo-oligosaccharides indicating their better utilization compared to trans-galacto, gluco and manno-oligosaccharides (Smiricky-Tjardes *et al.*, 2003). They also reported that the SCFA production was more for xylo-oligosaccharides compared to fructo and trans-galacto-oligosaccharides. Short chain fructo-oligosaccharides were reported to increase the faecal *Bifidobacteria* in healthy humans (Bouhnik *et al*., 1999). It was reported that the fecal *Bifidobacteria* concentration increased with 10 or 20 g FOS/d in healthy humans (Mitsuoka *et al*., 1987). *In vitro* studies as well as *in vivo* experiments in rats and humans have displayed the prebiotic effect of both inulin and FOS consistently (Ten Bruggencate *et al*., 2005; Ten Bruggencate *et al*., 2003; Bouhnik *et al*., 1999; Campbell *et al*., 1997). Galacto-oligosaccharides are reported to stimulate the growth of *Bifidobacteria* (Holma *et al*., 2002, Ito *et al*., 1990; Rowland *et al.,* 1993)**.** Inulin was found to increase the number of *Lactobacilli* and to a lesser extent *Bifidobacteria* (Mcbain & Macfarlane, 2001).

Table. 17. Growth characteristics of microorganisms grown on xylo-oligosaccharides liberated from larchwood xylan

Microorganism	O.D.at 600 nm	pH	Cell mass (mg)
B. adolescentis NDRI 236	0.070 [a]	7.2	1.3
	0.403 [b]	6.0	5.0
B. bifidum ATCC 29521	0.068 [a]	6.9	1.0
	0.643 [b]	5.7	6.3
L. plantarum NDRI strain 184	0.039 [a]	6.9	1.0
	0.412 [b]	5.9	5.1
L. brevis 01	0.031 [a]	7.5	1.0
	0.353 [b]	6.2	4.7

a Represents control
b Represents xylo-oligosaccharide mixture

Xylo-oligosaccharides, especially xylobiose and xylotriose, have been found to have a stimulatory and regulatory effect on the selective growth of human intestinal *Bifidobacteria* (Okazaki *et al.* 1990; Degnan & Macfarlane 1991). Xylo-oligosaccharides liberated from wheat bran SP were found to be more effective in enhancing the growth of *Bifidobacteria* compared to that of ragi bran and larchwood xylan wherein maximum growth was obtained for *Bifidobacterium adolescentis* NDRI 236.

VI.3. Enzyme activity

Bifidobacteria, Lactobacilli and *Pediococci* produce the hydrolytic enzymes which help in the fermentation of the xylo-oligosaccharides. Wheat and ragi bran xylo-oligosaccharides incorporated 24 h old cultures of bifidogenic bacteria showed xylanase, xylosidase, arabinofuranosidases, α and β-galactosidase and acetyl esterase activities. High activity of xylanase (440-694 μU ml^{-1}) was detected in the culture broth of all the tested microorganisms grown on crude as well as purified oligosaccharides (Table. 18).

Xylanase activity was maximum in the culture broth of microorganisms grown on purified oligosaccharides compared to the crude. High xylanase activity was detected in the culture broth of *Bifidobacterium adolescentis* NDRI 236 (830 μU ml^{-1}) grown on purified xylo-oligosaccharide (WO-4), which was identified as xylobiose by ESI-MS and H^1 NMR studies (Chapter V). Similarly, high activity of xylanase (1020-1690 μU ml^{-1}) was detected in the culture broth of all the tested microorganisms grown xylo-oligosaccharides liberated from ragi bran SP (Table. 19). Arabinofuranosidase activity was found to be higher than xylosidase whereas α/β- galactosidase activity was almost in the same range. High arabinofuranosidase activity was detected in the culture broth of *Lactobacillus* spp grown on wheat bran xylo-oligosaccharides. Maximum arabinofuranosidase activity was determined in the culture broth of *Bifidobacterium adolescentis* and *Lactobacillus plantarum* grown on purified xylobiose liberated from ragi bran. Arabinofuranosidase produced by the microorganisms cleave the arabinose residue from the xylo-oligosaccharides and help in their effective utilization for the bacterial growth. With respect to wheat bran oligosaccharides high xylosidase and α-galactosidase activities were detected culture broth of *Bifidobacterium bifidum*.

β- galactosidase activity was also found to be high in the culture broth of *Bifidobacterium* spp grown on wheat bran oligosaccharides. β- galactosidase was reported from *Bifidobacterium adolescentis* DSM 20083 which was active towards transgalacto-oligosaccharides (Van Laere *et al*., 2000). Acetyl esterase activity was found to be negligible (0.24-0.36 μU ml^{-1}) in the culture broth of all the tested microorganisms grown on both wheat as well as ragi bran oligosaccharides. *Bifidobacterium adolescentis* was shown to produce arabinofuranohydrolase which efficiently removed the arabinose residues from the doubly substituted xylose subunits (Van Laere *et al.,* 1997).

Table.18. Enzyme activities (μU/ml) in 24 h old culture broth of microorganisms grown on crude as well as purified xylo-oligosaccharides liberated from wheat bran SP

Microorganism	Xylo-oligosaccharide	Xylanase	Xylosidase	Arabino furanosidase	α-galactosidase	β-galactosidase	Acetyl esterase
B. dolescentis NDRI 236	Crude	560	5.4	9.6	5.1	5.1	0.24
	WO-1	570	5.5	9.0	5.0	5.2	0.24
	WO-2	570	5.7	9.0	5.1	5.2	0.24
	WO-3	780	6.6	11.0	5.1	5.2	3.5
	WO-4	830	7.2	11.0	5.1	5.2	3.5
B. bifidum ATCC 29521	Crude	500	8.9	9.6	5.4	7.7	0.24
	WO-3	510	8.9	11.5	5.4	7.7	0.46
B. bifidum NCDO 2715	Crude	666	7.0	10.0	5.4	6.6	0.24
	WO-3	680	8.4	11.0	5.1	7.7	0.32
L. plantarum NDRI strain 184	Crude	440	2.0	7.7	2.7	2.6	0.2
	WO-1	500	2.3	7.7	2.7	2.5	0.28
	WO-2	500	2.5	8.1	2.7	2.5	0.28
	WO-3	666	2.6	8.99	2.9	3.6	0.36
	WO-4	694	2.6	12	3.0	3.6	0.36
L. brevis 01	Crude	666	2.3	12.5	2.7	5.1	0.2
	WO-3	694	2.4	12.0	3.0	4.9	0.24
P.pentosaceus NCDO 813	Crude	690	3.3	8.99	2.7	3.9	0.35
	WO-3	694	3.3	11.0	2.7	3.9	0.36
P.pentosaceus ATCC 8081	Crude	560	2.5	7.7	2.7	3.6	0.38
	WO-3	680	2.5	7.7	2.9	3.9	0.4

Table. 19. Enzyme activities (μ U/ml) in 24 h old culture broth of microorganisms grown on xylo-oligosaccharide mixture as well as xylobiose liberated from ragi bran

Microorganism	Xylanase	Xylosidase	Arabinofuranosidase	α-galactosidase	β-galactosidase	Acetyl esterase
B. adolescentis NDRI 236	1020 [a](1090)	3.9 (3.8)	11.4 (12.6)	3.6 (3.1)	5.1 (3.6)	0.24 (0.38)
B. bifidum ATCC 29521	1160	3.3	5.4	2.7	4.5	0.36
B. bifidum NCDO 2715	1690	2.4	6.6	2.7	4.2	0.24
L. plantarum NDRI strain 184	1260 [a] (1690)	2.7 (2.7)	6.9 (12.6)	2.6 (3.1)	3.4 (3.6)	0.35 (0.38)
L. brevis 01	1200	2.7	8.1	3.0	3.3	0.24
P.pentosaceus, NCDO 813	1200	3.1	7.2	2.7	3.9	0.35
P.pentosaceus, ATCC 8081	1200	2.7	7.5	2.9	4.5	0.36

[a] Values in parenthesis are for purified xylobiose

VI.4. *In vitro* fermentation and short chain fatty acid (SCFA) analysis

Lactobacilli and *Bifidobacteria* ferment carbohydrates through a pathway mediated by the glycolytic enzymes they produce in which the main end products are SCFA (Grootaert *et al.,* 2007). Acetate was the chief SCFA released by the microorganisms due to fermentation of both crude as well as purified xylo-oligosaccharides liberated from wheat bran SP (Table. 20). This is in accordance with the earlier report **(**Smiricky-Tjardes *et al*., 2003). Butyrate was detected only in the culture broth of *Bifidobacterium bifidum* ATCC 29521 whereas, propionate was present in the culture broth of *Pediococcus pentosaceus* ATCC 8081 and *Pediococcus pentosaceus* NCDO 813. But, the amount of butyrate and propionate liberated as a result of *in vitro* fermentation was found to be very less. Fermentation of cross-Linked and non-cross-linked arabinoxylans by the intestinal microbiota in children resulted in more propionate production (Hopkins *et al*., 2003). SCFA liberated due to *in vitro* fermentation resulted in the reduction of pH of the culture broth. Such decrease in pH correlates with the population growth of the beneficial microbes (Berggren *et al*. 1993; Morisse *et al*. 1993; Choct *et al*. 1998). Moreover the shift in intestinal pH due to the acidic metabolites as a result of carbohydrate fermentation inhibits the growth of the undesirable pathogenic bacteria (Gibson & Wang, 1994). Short-chain fatty acids have been implicated in a number of important physiological events. An increased production of SCFA was reported when inulin was given in the diet of mice caecal bacteria causing a reduction in pH (Apajalahti *et al*., 2002). Oligosaccharide fermentation patterns obtained *in vitro* might be used to predict behavior *in vivo*. Acetate was found to be the major SCFA liberated due to the fermentation of xylo-oligosaccharides by swine faecal microflora (Smiricky-Tjardes *et al*., 2003).

Table. 20. Proportions of acetic, propionic and butyric acid as a percentage of total SCFA formed after 48 h of incubation in the culture broth of microbes incorporated with crude as well as purified oligosaccharides liberated from wheat bran SP

Microorganism		**Acetate**	**Propionate**	**Butyrate**
B. adolescentis NDRI 236	Crude	100	-	-
	WO-1	100	-	-
	WO-2	100	-	-
	WO-3	100	-	-
	WO-4	100	-	-
B. bifidum ATCC 29521	Crude	100	-	-
	WO-3	99.14	-	0.86
B.bifidum, NCDO 2715	Crude	100	-	-
	WO-3	100	-	-
L. plantarum NDRI strain 184	Crude	100	-	-
	WO-1	100	-	-
	WO-2	100	-	-
	WO-3	100	-	-
	WO-4	100	-	-
L. brevis 01	Crude	100	-	-
	WO-3	100	-	-
P.pentosaceus, NCDO 813	Crude	100	-	-
	WO-3	98.63	1.37	-
P.pentosaceus, ATCC 8081	Crude	99.1	0.89	-
	WO-3	93.78	6.22	-

Acetate was the chief SCFA released by the microorganisms due to fermentation of both crude xylo-oligosaccharides and RO-1 (Table. 21). Butyrate was analyzed in the culture broth of *Bifidobacterium bifidum,* ATCC 29521 (0.86 %) and *Lactobacillus plantarum* whereas, propionate was detected only in the culture broth of *Lactobacillus plantarum* NDRI strain 184 grown on crude xylooligosaccharides. *Bifidobacterium adolescentis* NDRI 236 and *Lactobacillus plantarum* were taken to study the prebiotic effect of the purified oligosaccharide due to its less quantity. Acetate (100 %) was detected in the culture broth of *Bifidobacterium adolescentis* NDRI 236, grown on RO-1, whereas acetate (81.4 %) and butyrate (3.0 %) were the end products of fermentation by *Lactobacillus plantarum.*

Table. 21. Proportions of acetic, propionic and butyric acid as a percentage of total SCFA formed after 48 h of incubation in the culture broth of microbes incorporated with crude as well as purified oligosaccharide(s) liberated from ragi bran SP

Microorganism	Acetate	Propionate	Butyrate
B. adolescentis NDRI 236	100 [a](100)	- -	- -
B. bifidum ATCC 29521	99.1	-	0.86
B.bifidum, NCDO 2715	100	-	-
L. plantarum NDRI strain 184	57.7 [a] (96.43)	24.4 -	18.0 (3.57)
L. brevis 01	100	-	-
P.pentosaceus, NCDO 813	100	-	-
P.pentosaceus, ATCC 8081	100	-	-

[a] Values in parenthesis are for purified xylobiose

In vitro fermentation pattern of the oligosaccharides liberated from larchwood xylan was also monitored in the present study. Acetate (100 %) was detected in the culture broth of *Bifidobacterium adolescentis* NDRI 236 and *Lactobacillus brevis*. The culture broth of *Bifidobacterium bifidum*, ATCC 29521 showed the presence of acetate (99.3 %) and butyrate (0.7 %). Propionate was detected only in the culture broth of *Lactobacillus plantarum* NDRI strain 184 and the percentage proportion of acetate: propionate: butyrate was 64.3: 25.2: 10.5.

A variety of model systems have been developed to study the colonic fermentation of prebiotics (Rumney & Rowland, 1992; Conway, 1995; Rycroft *et al*., 1999). *Bifidobacterium* strains are largely described as capable of efficiently fermenting xylose-based oligo and polysaccharides (Crittenden *et al*., 2002). Apart from their prebiotic effect GOS and FOS are reported to be effective in increasing the mineral absorption (Chonan *et al*. 2001; Chonan *et al*. 1995; Roberfroid, 2002).

Thus the bifidogenic nature of the xylo-oligosaccharides liberated from cereal brans was proved in the present study. The growth characteristic pattern showed that *Bifidobacterium adolescentis* NDRI 236 and *Lactobacillus plantarum* NDRI strain 184 were able to efficiently utilize the xylo-oligosaccharides liberated from wheat bran SP compared to other tested microorganisms. Eventhough no significant difference is observed between crude and purified oligosaccharides consumption by the microorganisms, a slight increase in bacterial growth was noticed for oligosaccharide having low degree of polymerization (xylobiose & xylotriose) and the utilization of the oligosaccharides by the bacterial spp was found to be strain specific.

SUMMARY AND CONCLUSIONS

The results of the present investigation on 'Xylanase from ragi malt and its use in the preparation of xylo-oligosaccharides from cereal brans' have been summarized and concluded as follows:

- Xylanase, xylosidase and arabinofuranosidase activities were increased during the malting of ragi and were maximum in 96 h ragi malt compared to 24, 48 and 72 h malts. The activity of xylanase was taken as 100 % and accordingly relative activities of xylosidase and arabinofuranosidase were found to be 48 and 42 % respectively **(Page 61-62, Chapter III).**

- The first two consecutive extractions yielding more than 90 % of xylanase, xylosidase and arabinofuranosidase activities were chosen for the effective isolation of the enzymes from ragi malt **(Page 65-66, Chapter III).**

- Xylanase activity was maximum (1.55 $\mu molmin^{-1}$) in acetate buffer (0.1 M, pH 6.0) consisting of polyvinyl polypyrrolidine (PVPP, 0.5 %), Triton X-100 (1.0 %), calcium chloride (20 mM) and reduced glutathione (5.0 mM) **(Page 70-71, Chapter III).**

- Xylosidase activity was maximum (0.55 $\mu molmin^{-1}$) in acetate buffer (0.2 M, pH 5.5) consisting of polyvinyl polypyrrolidine (PVPP, 0.75 %), Triton X-100 (0.75 %), calcium chloride (15 mM) and reduced glutathione (7.5mM) **(Page 71, Chapter III).**

- Arabinofuranosidase activity was maximum (0.47 $\mu molmin^{-1}$) in acetate buffer (0.2 M, pH 5.0) consisting of polyvinyl polypyrrolidine (PVPP, 0.75 %), Triton X-100 (1 %), calcium chloride (15 mM) and reduced glutathione (7.5 mM) **(Page 71, Chapter III).**

- Xylanase (E.C. 3.2.1.8) was purified to apparent homogeneity from 96 h finger millet (*Eleusine coracana*, Indaf-15) malt by a three step purification procedure *via* ammonium sulphate fractionation, DEAE-cellulose ion exchange and Sephadex G-75 gel permeation chromatographies with a recovery of 4.0 % and fold purification of 60. The first two consecutive extractions yielding more than 90 % of xylanase, xylosidase and arabinofuranosidase activities were chosen for the effective isolation of the enzymes from ragi malt **(Page 71-77, Chapter III).**

- The purified ragi xylanase with a molecular weight of 29.5 kDa was found to be monomeric on SDS-PAGE **(Page 78, 80, Chapter III).**

- pH optimum of the purified xylanase was found to be in the range of 5.0-5.5. The activation energy was 25 $kJmol^{-1}$. Temperature optimum of the purified xylanase was found to be 50°C. Xylanase showed maximum stability at 35°C in a pH range of 5.0-6.0 **(Page 81-86, Chapter IV).**

- Metal ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Ag^{2+} and Ni^{2+} enhanced xylanase activity at 5 mM concentration. P-chloromercuribenzoate (PCMB), citric, oxalic and boric acids inhibited the enzyme in concentration dependent manner **(Page 86-87 & 90- 91, Chapter IV).**

- K_m and V_{max} of purified xylanase were found to be 0.2 % and 4.5 µmoles min^{-1} respectively. EDTA and $HgCl_2$ were found to be competitive and non-competitive inhibitors respectively **(Page 87-89, Chapter IV).**

- The mode of action of xylanase was found to be "endo" as determined by the analysis of products liberated from larchwood xylan by ESI-MS and H^1NMR **(Page 92-97, Chapter IV).**

- Soluble polysaccharides isolated from wheat (SP, 35 %) and ragi (14.4 %) brans consisted mainly of arabinose and xylose with minor quantities of rhamnose, mannose, galactose and glucose **(Page 99- 101, Chapter V).**

- Soluble polysaccharides isolated from wheat and ragi brans were subjected to purified endoxylanase (from 96 h ragi malt) treatment to obtain xylo-oligosaccharides. The yield of ragi bran (17.9 %) oligosaccharides was less compared to that of wheat bran (30 %). The crude oligosaccharide mixture was purified on Biogel P-2 column followed by HPLC. Four major oligosaccharides designated as WO-1, WO-2, WO-3 and WO-4 were liberated from soluble polysaccharides of wheat bran whereas a singe oligosaccharide (RO-I) was obtained from that of ragi bran. The structural characterization of the purified oligosaccharides liberated from both wheat and ragi brans were done by ESI-MS and H1 NMR. WO-1 and WO-2 were identified as arabinose containing xylotetraose and xylotriose respectively whereas, WO-3 and WO-4 were identified as unsubstituted xylotriose and xylobiose respectively. RO-1 was identified as xylobiose **(Page 101-118 Chapter V).**

- The oligosaccharides incorporated into the culture broth at 0.25 % level showed prebiotic activity in terms of growth characteristics such as increase in absorbance at 600 nm, reduction in culture broth pH and increase in bacterial cell mass (**Page 119, Chapter VI).**

- *Bifidobacterium adolescentis* NDRI 236 and *Lactobacillus plantarum* NDRI strain 184 were able to efficiently utilize the xylo-oligosaccharides liberated from wheat bran SP and the utilization of the oligosaccharides by the bacterial spp was found to be strain specific. Xylo-oligosacharides liberated from ragi bran SP were efficiently utilized by *Pediococus pentosaceus*, ATCC 8081. A slight increase in the O.D of the culture broth and cell mass was noticed for microorganisms grown on xylobiose and xylotriose) **(Page 119- 123, Chapter VI).**

- Xylanase, xylosidase, arabinofuranosidase, α/β- galactopyranosidase and acetyl esterase were detected in the 24 h culture broth of the tested microorganisms wherein the xylanase activity was found to be maximum (**Page 126-129, Chapter VI).**

- Acetate was the chief short chain fatty acid released during fermentation of both crude as well as purified xylo-oligosaccharides by all the tested microorganisms **(Page 130-134, Chapter VI).**

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LIST OF PUBLICATIONS, PATENT AND POSTERS PRESENTED IN NATIONAL/INTERNATIONAL SYMPOSIA

Manuscripts from Ph.D Programme:

1. **Chithra Manisseri . Muralikrishna Gudipati**
   'Characterization of purified xylanase from finger millet (*Eleusine coracana*-Indaf 15) malt' – Published online on 11th October 2007 in European Food Research and Technology.
2. **Chithra Manisseri . Muralikrishna Gudipati**
   'An improved method for obtaining xylanase from Finger millet (*Eleusine coracana*, Var.'Indaf-15') malt'- Journal of Food Science and Technology (in press, March-April 2008).
3. **Chithra Manisseri . Muralikrishna Gudipati**
   'Bioactive xylo-oligosaccharides from wheat bran soluble polysaccharides'- Communicated to Food Chemistry.
4. **Chithra Manisseri . Muralikrishna Gudipati**
   'Prebiotic activity of xylo-oligosaccharides obtained from ragi (Eleusine coracana, Indaf-15) bran'- Communicated to Journal of Food Biochemistry.

PATENT:

M. Chithra and G. Muralikrishna

A patent entitled '**A process for the preparation of xylanase from ragi malt'** was filed. DEL No: 326/DEL/2006

Posters presented in National/ International Symposia

1. **M. Chithra**, G. Madhavilatha and G. Muralikrishna
   '**Cell wall degrading enzymes from cereal malts'**- Presented in *5th International Food Convention (*IFCON) held at Central Food Technological Research Institute (CFTRI), Mysore, Karnataka, India between 5-12-2003 to 8-12-2003.

2. **M. Chithra** and G Muralikrishna

'**Purification and partial characterization of xylanase from finger millet malt'** *Presented in colloquium on Novel proteins in Nutrition and Health* organized by the *Solae Company* and *Central Food Technological Research Institute*, Mysore, 22nd March 2005.

3. **M. Chithra** and G Muralikrishna

'Kinetic studies of a purified xylanase from ragi malt'- Presented in the 74th Annual Meeting, Society of Biological Chemists (India) held at Central Drug Research Institute, Lucknow, India between 7.11.2005 to 10.11.2005.

4. **M. Chithra and G. Muralikrishna**

'Purification and structural characterization of bioactive xylo-oligosaccharides from cereal brans'- Presented in CARBO-XXI**,** held at Delhi University, New Delhi, between 26 th to 29 November, 2006.

5 M. Chithra and G. Muralikrishna

'**Prebiotic activity of xylo-oligosaccharides liberated from soluble polysaccharides of wheat and ragi brans'-** Presented in 77th Annual Session and Symposium of the National Academy of Sciences India, held at CFTRI, Mysore, between December 6-8, 2007.