MICROBIAL PRODUCTION OF FRUCTOOLIGOSACCHARIDES

A Thesis submitted to the

UNIVERSITY OF MYSORE

For the award of the Degree of

DOCTOR OF PHILOSOPHY

In

BIOTECHNOLOGY

By

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November, 2003

Dedicated to my dear parents.....

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CERTIFICATE

This is to certify that the thesis titled **"Microbial production of Fructooligosaccharides"** submitted to the University of Mysore, Mysore by **Ms. Sangeetha. P. T,** for the award of the degree of **Doctor of Philosophy** in **Biotechnology**, is the result of research work carried out by her in the Department of Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore under my guidance during the period 1999 – 2003.

Mysore
Date: **(S. G. Prapulla)**

DECLARATION

I hereby declare that the thesis titled **"Microbial production of Fructooligosaccharides"** submitted to the University of Mysore, Mysore for the award of the degree of **Doctor of Philosophy** in **Biotechnology**, is the result of research work carried out by me in the Department of Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore under the guidance of **Dr. (Mrs.) S. G. Prapulla** during the period 1999 – 2003. I further declare that the results of the work have not been previously submitted for any other degree or fellowship.

Mysore
Date: **(Sangeetha. P. T)**

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection
a_W	Water Activity
BSA	Bovine Serum Albumin
CWS	Cold Water Solubility
2D-HMQCT	Two Dimensional Heteronuclear Multiple Quantum Coherence Transfer
DCW	Dry Cell Weight
DEAE	Diethyl Aminoethyl
DNA	Deoxyribonuleic acid
DNS	Dinitro Salicylic Acid
DP	Degree of Polymerization
E	Average Percent Error
F	Fructose
FFT	Fructan: fructan 1-Fructosyl Transferase
FOS	Fructooligosaccharides
FTase	Fructosyl Transferase
G	Glucose
GC	Gas Chromatography
GF	Sucrose
GF_2	Kestose
GF_3	Nystose
GF_4	Fructofuranosyl Nystose
GIT	Gastrointestinal Tract
GOD/ POD	Glucose Oxidase / Peroxidase
GRAS	Generally Regarded As Safe
HDL	High Density Lipoprotein
HMG	β-hydroxyl-β-methyl glutarate
HPAEC	High Performance Anion Exchange Chromatography
HPLC	High Performance Liquid Chromatography
LAB	Lactic Acid Bacteria
LC-MS	Liquid Chromatography – Mass Spectrometry

LDL	Low Density Lipoprotein
mRNA	Messenger Ribonucelic Acid
MRS	deMann, Rogosa and Sharpe
MSE	Moldy Substrate Extract
NDM	Nonfat Dry Milk
NDO	Non- digestible Oligosaccharides
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PAD	Pulsed Amperometric Detector
PAGE	Polyacrylamide Gel Electrophoresis
PDA	Potato Dextrose Agar
QDA	Quantitative Descriptive Analysis
RDA	Recommended Dietary Allowance
RDI	Recommended Daily Intake
RID	Refractive Index Detector
RMSD	Root Mean Square Deviation
RSM	Response Surface Methodology
SCFA	Short Chain Fatty Acids
S_D	Standard Deviation of Differences
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscope
SmF	Submerged Fermentation
SSF	Solid State Fermentation
SST	Sucrose:sucrose 1-Fructosyl Transferase
TAG	Triacyl Glycerol
TDF	Total Dietary Fibre
TEMED	Tetra Methyl Ethylene Diamine
TFA	Triflouro Acetic Acid
TLC	Thin Layer Chromatography
TMS	Tri Methyl Silane
U_h	Hydrolytic Activity
U_t	Transfructosylating Activity
UV	Ultraviolet
VLDL	Very Low Density Lipoprotein

CHAPTER 1
INTRODUCTION

1. INTRODUCTION

Food products are designed for taste, appearance, cost and convenience of the consumer. The design of food products that confer a health benefit is a relatively new trend, and recognizes the growing acceptance of the role of diet in disease prevention, treatment and well being. This change in attitude for product design and development has forced organizations and industries involved in formulating foods for health benefit into new areas of understanding. The concept of formulating foods for health benefits is a trend that is quickly becoming popular. A significant driving force in the 'functional food' market place is consumer demand- the quest by consumers to optimize their health through food.

It is becoming increasingly clear that there is a strong relationship between the food we eat and our health. Scientific knowledge of the beneficial role of various food ingredients (nutrients) for the prevention of specific diseases is rapidly accumulating. Functional foods, designer foods, pharma foods and nutraceuticals are synonyms for foods that can prevent and treat diseases. Generally, a functional food can be defined as 'any food that has a positive impact on an individual's health, physical performance or state of mind in addition to its nutritional content'. Functional foods in addition to their basic nutritional content and natural being, will contain the proper balance of ingredients which will help to improve many aspects of human lives, including the prevention and treatment of illness and disease (Goldberg, 1994).

So far, a large number of functional foods in various forms have already been introduced into the market. Many of them contain a number of characteristic functional ingredients. They include dietary fiber, oligosaccharides, sugar alcohols, peptides and proteins, prebiotics and probiotics, phytochemicals and antioxidants and polyunsaturated fatty acids (Stark and Madar, 1994). Substances, which can lead to the production of functional foods, can be classified as

(i) an essential micronutrient having specific physiological effects, such as resistant starch or omega – 3 – fatty acids

(ii) an essential micronutrient, if it confers a special benefit through the intake over and above recommended daily intake (RDI)

(iii) a non (essential) – nutrient giving specific physiological effects, such as some oligosaccharides and phytochemicals (Kwak and Jukes, 2001).

Oligosaccharides are very well recognized as 'functional food ingredients' because of their positive effects on human health. This research work focuses on the microbial production of Fructosyl Transferase (FTase) and the production of Fructooligosaccharides (FOS) by transfructosylation using this enzyme.

1.1. Oligosaccharides

Oligosaccharides are relatively new functional food ingredients that have great potential to improve the quality of many foods. In addition to providing useful modifications to food flavour and physicochemical characteristics, they possess properties that are beneficial to the health of the consumers. The production and application of food grade oligosaccharides are increasing and their major uses are in beverages, infant milk powders, confectionery, bakery products, yoghurts and dairy desserts.

Oligosaccharides of various types are found as natural components in many common foods including fruits, vegetables, milk and honey. The main claim made for these products is that they are 'foods designed to help maintain a good gastrointestinal environment, and increase intestinal bifidobacteria'. Along with dietary fibre, oligosaccharides are the most popular components to use in emerging functional foods (Crittenden and Playne, 1996).

Oligosaccharides are usually defined as carbohydrates with a low degree of polymerization (DP) and consequently low molecular weight. The concept of non-digestible oligosaccharides originates from the observation that the anomeric carbon atom (C_1 or C_2) of the monosaccharide units of some dietary oligosaccharides has a configuration that makes their osidic bonds nondigestible to the hydrolytic activity of the human digestive enzymes. The main categories of nondigestible oligosaccharides include carbohydrates in which the monosaccharide unit is fructose, galactose, glucose and xylose. Chemical differences among commercially available oligosaccharides include chain length, monosaccharide composition, degree of branching and purity. In general, food grade

oligosaccharides are not pure products, but are mixtures containing oligosaccharides of different degrees of polymerization, the parent polysaccharide or disaccharide and the monomer sugars (Roberfroid and Slavin, 2000).

1. 2. Fructooligosaccharides (FOS)

Of all the oligosaccharides known so far, FOS has attracted special attention. FOS is a common name for fructose oligomers that are mainly composed of 1- Kestose (GF_2), 1 - Nystose (GF_3) and 1^F – Fructofuranosyl nystose (GF_4) in which fructosyl units (F) are bound at the β - 2, 1 position of sucrose (GF) (Figure 1.1). However, recently many terms like fructans, glucofructosans, oligofructosides and inulin type oligosaccharides have emerged.

1. 2. 1. Occurrence

FOS derived from sucrose occur in many higher plants as reserve carbohydrates. They are found in a variety of edible plants, including banana, barley, garlic, honey, onion, rye, brown sugar, tomato, asparagus root, Jerusalem artichoke, wheat and triticale (Fishbein *et al*, 1988). The concentration of FOS in these foods is diagrammatically represented in Figure 1.2 (derived using data from the Environmental Protection Agency's Dietary Risk Evaluation system - EPA, 1984). FOS like 1- kestose, neokestose, 6- kestose and their derivatives have also been isolated from the plant *Agave vera cruz* (Satyanarayana, 1976).

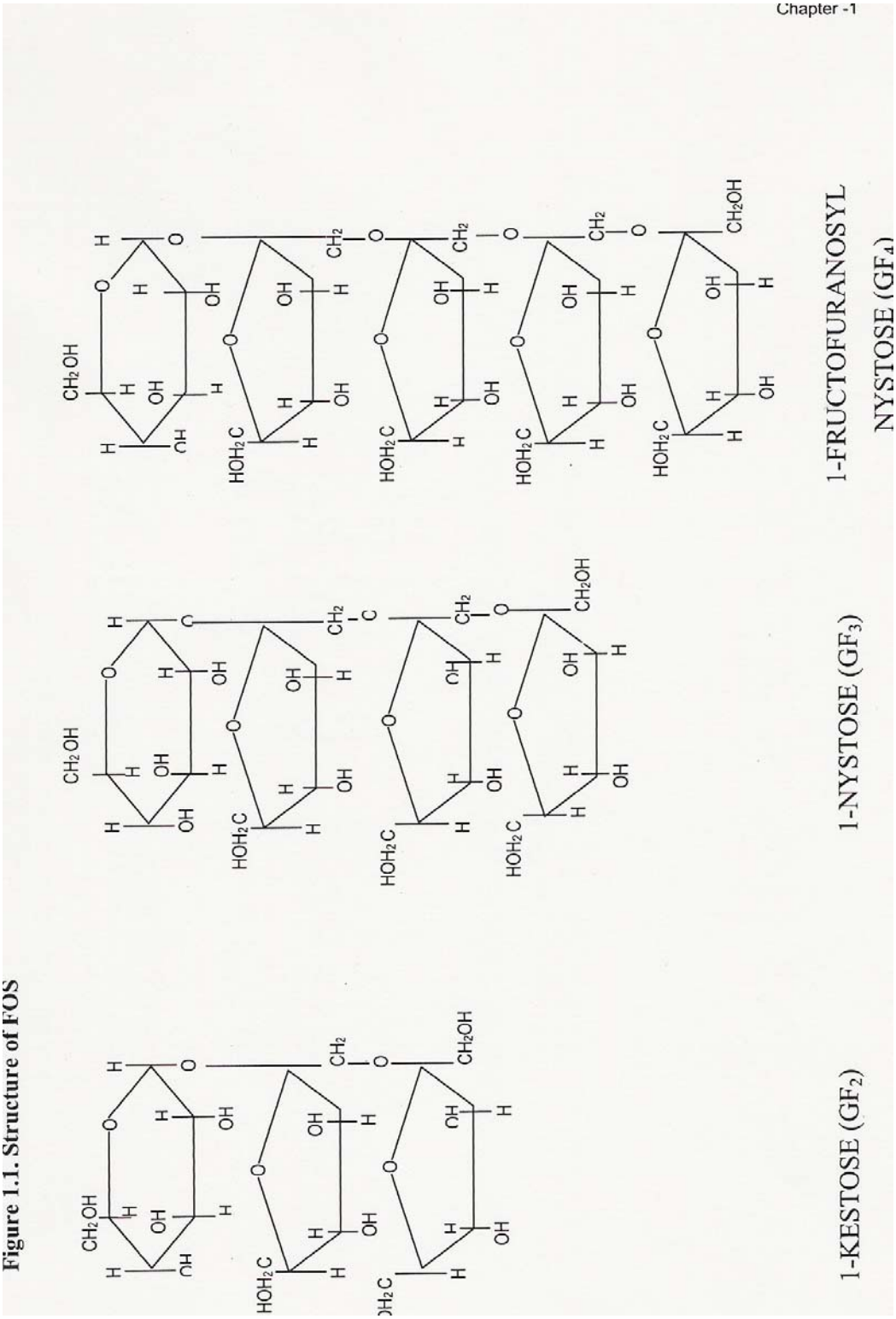
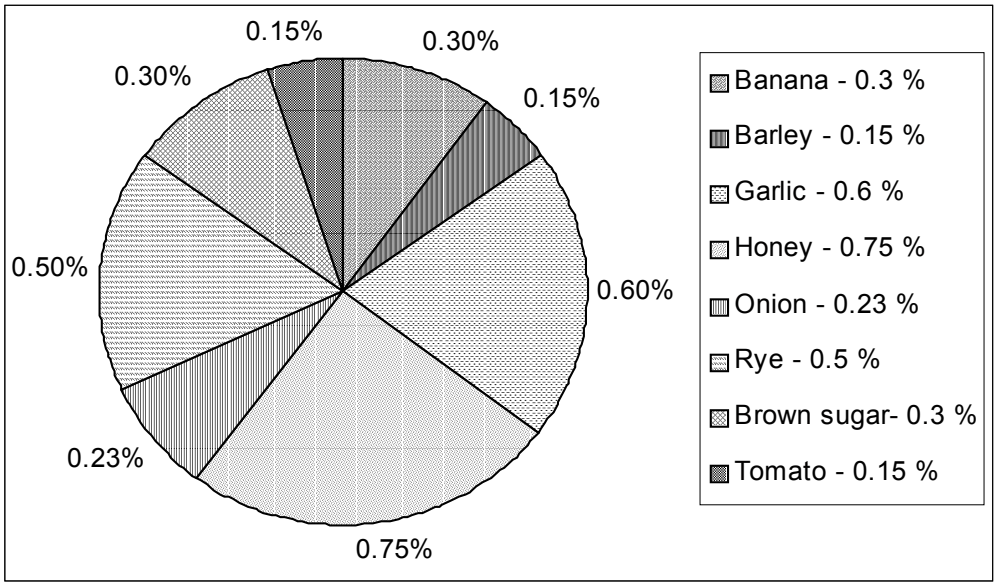


Figure 1.1. Structure of FOS

Figure 1. 2. Concentration of FOS in natural foods



1. 2. 2. Preparation

FOS can be produced by the transfructosylation activity of enzymes designated as Fructosyl Transferases (FTase) (EC. 2. 4. 1. 9). The enzyme source of FOS synthesis can be divided into two classes; one is plants such as asparagus, sugar beet, onion, Jerusalem artichoke etc; the other consists of bacteria and fungi such as *Arthrobacter* sp., *Fusarium* sp., *Aspergillus* sp., *Aureobasidium* sp. etc (Table 1.1). Depending on the enzyme sources, they have different linkages resulting in the production of 1^F – type [1^F (1-β-D-fructofuranosyl)$_{n-1}$ sucrose], 6^F – type [6^F (1-β-D-fructofuranosyl)$_{n-1}$ sucrose] and 6^G – type [6^G (1-β-D-fructofuranosyl)$_{n-1}$ sucrose] FOS (Figure 1.3). The yield of FOS produced using enzymes originated from plants is low and mass production of enzyme is limited by seasonal conditions. Therefore, industrial production depends chiefly on microbial enzymes (Yun, 1996).

Chapter -1

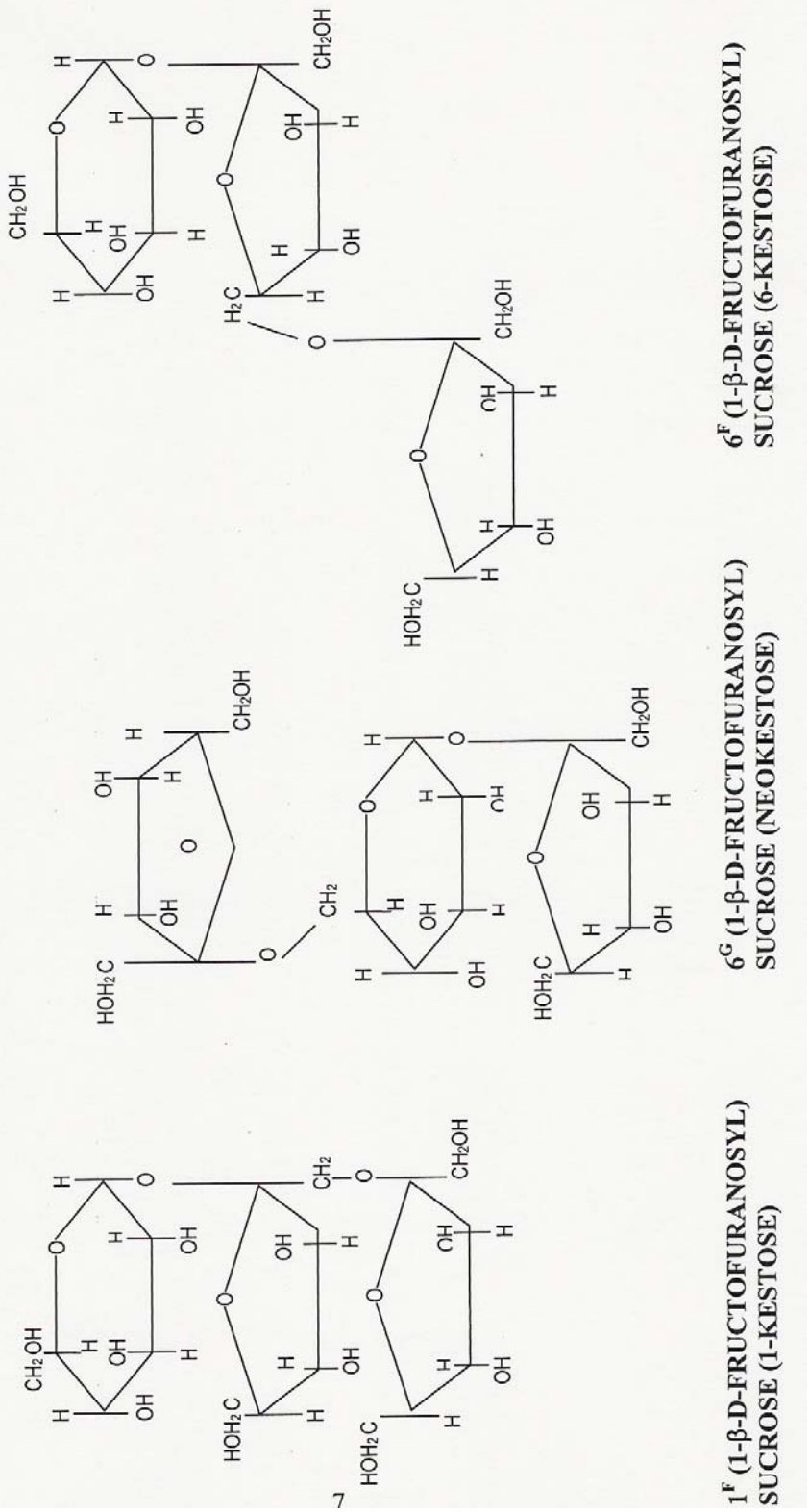


Figure 1.3. FOS with different types of linkages

1^F (1-β-D-FRUCTOFURANOSYL) SUCROSE (1-KESTOSE)

6^G (1-β-D-FRUCTOFURANOSYL) SUCROSE (NEOKESTOSE)

6^F (1-β-D-FRUCTOFURANOSYL) SUCROSE (6-KESTOSE)

Table 1.1. Microbial and Plant sources of FOS synthesizing enzymes

Fungal source	Plant source	Bacterial source
1. *Aureobasidium pullulans*	1. *Agave americana*	1. *Arthrobacter sp.*
2. *Aureobasidium sp.*	2. *Agave vera cruz*	2. *Bacillus macerans*
3. *Aspergillus japonicus*	3. *Asparagus officinalis*	
4. *Aspergillus niger*	4. *Allium cepa*	
5. *Aspergillus phoenicis*	5. *Cichorium intybus*	
6. *Aspergillus sydowi*	6. *Crinum longifolium*	
7. *Calviceps purpurea*	7. *Sugar beet leaves*	
8. *Fusarium oxysporum*	8. *Helianthus tuberosus*	
9. *Penicillium frequentans*	9. *Lactuca sativa*	
10. *Penicillium spinulosum*	10. *Lycoris radiata*	
11. *Phytophthora parasitica*	11. *Taraxacum officinale*	
12. *Scopulariopsis brevicaulis*		
13. *Saccharomyces cerevisiae*		
14. *Aspergillus foetidus*		
15. *Penicillium citrinum*		
1-13 - Yun, 1996	1 – 11 – Yun, 1996	1. Yun, 1996
14. Wang and Rakshit, 2000		2. Park *et al*, 2001
15. Hayashi *et al*, 2000		

1.2.3. Mechanism of enzyme action

The reaction mechanism of FTase depends on the source of the enzyme. In plants and some microorganisms, a series of enzymes act together whereas a single enzyme works in most other microorganisms. In Jerusalem artichoke, two enzymes – sucrose: sucrose 1- FTase (SST) and $\beta(2\rightarrow1)$ fructan: $\beta(2\rightarrow1)$ fructan 1- FTase (FFT) - were found to be involved in FOS production. The overall reaction mechanism can be expressed as follows:

$GF + GF \rightarrow GF\text{-}F + G$ ⟶ by SST

$GF\text{-}F_n + GF\text{-}F_m \rightarrow GF\text{-}F_{n-1} + GF\text{-}F_{m+1}$ ⟶ by FFT

Where G is glucose, F is fructose, GF is sucrose and n and m are the number of extrasucrosyl fructose residues (Edelman and Jefford, 1968).

Agave enzyme catalyzed a stepwise reaction as follows for synthesis of FOS:

GF + fructosyl transferase ⟶ F-fructosyl transferase + G

F-fructosyl transferase + GF ⟶ GF_2 + fructosyl transferase.

Here instead of fructose, glucose acts as the acceptor of the fructose molecule from sucrose. GF_2, GF_3 and GF_4 act as acceptors of fructose from sucrose for the synthesis of higher oligosaccharides, but cannot act as donors of the fructosyl moiety (Arnold, 1965).

The reaction mechanism of *Claviceps purpurea* enzyme produces mainly neokestose-based oligosaccharides, which can be summarized as follows:

$(F2 \rightarrow 1G) + (F2 \rightarrow 1G)$ ⟶ $(F2 \rightarrow 6G1 \leftarrow 2F) + G$

$(F2 \rightarrow 1G) + (F2 \rightarrow 6G1 \leftarrow 2F)$ ⟶ $(F2 \rightarrow 1F2 \rightarrow 6G1 \leftarrow 2F) + G$

where number indicates the position of the carbonyl carbon atoms and arrows represent the direction of glycosidic linkage (Dickerson, 1972).

In the case of FTase derived from *A. pullulans*, the enzyme acts on sucrose in a disproportionate type reaction where one molecule of sucrose serves as a donor and another acts as an acceptor, which can be expressed as follows:

$GF_n + GF_n \rightarrow GF_{n-1} + GF_{n+1}$ where n = 1-3 (Jung *et al*, 1989).

1. 2. 4. Properties

FOS possesses several properties, which make their use as food ingredients particularly attractive. They are water-soluble and mildly sweet, typically 30 % as sweet as sucrose. Their relatively low sweetness is useful in food production when a bulking agent with reduced sweetness is desirable to enhance other food flavours. The viscosity and thermal stability of FOS solution is higher than that of sucrose at same concentration. Compared with mono- and disaccharides, the higher molecular weight of oligosaccharides provides increased viscosity, leading to improved body and mouthfeel. FOSs are highly stable in the normal pH range for food (4.0 – 7.0) and at refrigerated temperatures over one year. They can also be used to alter the freezing temperature of frozen foods, and to control the amount of browning due to Maillard reactions in heat - processed foods. They provide high moisture retaining capacity, preventing excessive drying, and a low water activity, which is convenient in controlling microbial contamination (Crittenden and Playne, 1996).

1. 2. 5. Functional properties

1. 2. 5. 1. Low calorific value

The β- configuration of the anomeric carbon, C_2 in their fructose monomers make FOS nondigestible by human digestive enzymes which are mostly specific for α-osidic linkages and hence they are not utilized as an energy source in the body. However, due to colonic fermentation, they have an energy contribution to food of about 1.5 kcal/ g. This property makes them suitable for use in sweet, low-calorie diet foods and safe for consumption by individuals with diabetes. In the case of very sweet foods, they may be used as bulking agents in conjunction with intense artificial sweeteners such as aspartame, phenylalanine or sucralose, thereby masking the aftertaste produced by some of these intense sweeteners (Crittenden and Playne, 1996).

1. 2. 5. 2. Non - cariogenicity

Unlike starch and simple sugars, FOSs are not utilized by oral microflora like *Streptococcus mutans* to form acids and insoluble β-glucans, that serve as a matrix for plaque formation and are the main culprits in causing dental caries

(Oku, 1994). Hence, FOSs are presently used as non – cariogenic sugar substitutes in confectionery, chewing gums, yoghurts and drinks.

1. 2. 5. 3. Dietary fibre effect

'Dietary fibre is the edible part of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine' (AACC Report, 2001). The nondigestible quality of FOS makes them act similar to dietary fibre, and thus prevent constipation. However, excessive consumption of FOS may cause flatulence or diarrhea, the minimum dose being 40-50 g/day. The advantages FOS has over dietary fibre are that they have a smaller daily requirement, do not cause diarrhea in recommended doses, are slightly sweet, have neither bad texture nor bad taste, are completely water soluble, do not build viscosity, do not bind minerals, are physically stable, and are easier to incorporate into processed foods and drinks (Tomomatsu H, 1994).

1. 2. 5. 4. Prebiotic effect

In recent years, the ability of FOS to promote the proliferation of bifidobacteria in the colon has been recognized. These intestinal bacteria metabolize FOS readily and produce large amounts of short - chain fatty acids resulting in an acidic pH in the lumen of the large intestine. The beneficial bacteria such as *Bifidobacterium* sp. and *Lactobacillus* sp. are resistant to the acidic pH, whereas the harmful bacteria such as *Clostridium* sp. are sensitive to the acidic conditions. Therefore the proliferation of useful bacteria is stimulated and that of harmful bacteria is suppressed. Subsequently, FOS has been described as one of several prebiotics, which can be defined as 'a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and / or the activity of one or a limited number of bacteria in the colon and thus improves host health' (Gibson and Roberfroid, 1995). FOS have been demonstrated to increase bifidobacteria numbers in the colon at doses of <15 g/day (Crittenden and Playne, 1996).

1. 2. 5. 5. Lipid lowering effect

FOS in diet has been found to decrease the levels of triglycerides, serum cholesterol and lipids. Hypotriglyceridemia is due to decrease in the hepatic synthesis of triglycerides and hypocholesterolemia is likely to result from the antagonistic effect of short chain fatty acids, especially propionate on cholesterol metabolism. Propionate has been found to be an inhibitor of HMG CoA reductase, an important enzyme in cholesterol synthesis (Roberfroid and Slavin, 2000).

1. 2. 5. 6. Effect on mineral absorption

FOS have been found to enhance the absorption of Ca^{2+} and Mg^{2+} ions and maintain balance of iron and Zn^{2+} ions. This has been found to be due to the osmotic effect, acidification of the colonic content due to fermentation and production of short chain carboxylic acids, formation of calcium and magnesium salts of these acids and hypertrophy of the colon wall (Roberfroid and Slavin, 2000).

1. 2. 5. 7. Anticancer effect

FOS has an indirect effect on prevention of cancer in human beings due to its prebiotic properties. This is due to immunity enhancements by the cells, cell wall components and extracellular components of bifidobacteria (Tomomatsu H, 1994).

1. 2. 5. 8. Production of nutrients

The presence of a good colonic environment with high bifidobacterial count leads to the production of Vitamins B-1, B-2, B-6, B-12, nicotinic acid and folic acid. Bifidobacteria – fermented dairy products also improve lactose tolerance, calcium absorbability and digestibility (Tomomatsu H, 1994).

1. 2. 5. 9. Antidiabetic effect

FOS have been claimed to have no effect on blood glucose levels in patients with type 2 diabetes, possibly due to effects of short chain fatty acids produced during fermentation. (Luo *et al*, 2000).

1. 2. 6. Dosage and side effects

The maximum effective daily doses of FOS were determined to be 0.30 and 0.40 g/kg body weight for males and females, respectively. Excessive flatus was caused by the intake of >30 g FOS/ day and borborygmus (Gaseous intestinal rumbling) and bloating occurred at higher FOS intakes (>40 g/ day). Abdominal cramps and diarrhea did not occur until ingestion exceeded 50 g FOS /day. *In vitro* and *in vivo* studies conducted with FOS have shown that there is no evidence of genotoxicity and the only effect noted was the occurrence of soft stools or diarrhea after ingestion of large quantities of FOS (Tomomatsu, 1994).

1. 2. 7. Applications and market trend

The major use of FOS is in beverages. Increasingly they are being included in probiotic yoghurts and yoghurt drinks to produce synbiotic products. 'Symbalance' (Toni Milch, Zurich, Switzerland), 'Fyos' (Nutricia, Bornem, Belgium) and 'Fysq' (Mona, Weerden, The Netherlands) are all products containing FOS. They are also widely used in confectionery. Other applications include desserts such as jellies, and ice creams; bakery products including biscuits, breads and pastries; spreads such as jams and marmalades; and infant milk formulae. The use of oligosaccharides in livestock industry is also increasing (Crittenden and Playne, 1996).

The US nutraceutical market has grown more than $ 30 billion by the year 2003 and the market of FOS is already substantial and continues to expand rapidly. At present Japanese companies still dominate worldwide FOS production, as well as research and development activity. However, European interest in FOS is also increasing with several companies currently producing, or planning to produce FOS products. In contrast, FOS production in US at present remains negligible. Meiji Seika Kaisha Co. (Tokyo, Japan) first introduced FOS into the market as foodstuff during 1984 and they are the major producers of transfructosylation fructooligosaccharides. These are marketed in Japan as 'Meioligo'. Meiji has also established joint ventures with Beghin Say in France (Beghin-Meiji Industries, Paris) producing FOS that is marketed as 'Actilight' and with Golden Technologies (Westminster Co.) in the USA, which

distributes Meiji Seika's FOS as 'Nutraflora'. Cheil Foods and Chemicals (Seol, Korea) also manufactures FOS. The Belgian Company ORAFTI (previously called Raffinerie Tirlemontoise SA, Brussels) produces inulin oligofructose from inulin extracted from chicory. It is marketed as 'Raftilose', either as powder or syrup. The FOS in Raftilose range from two to nine monosaccharide units in length, with an average length of four sugar moieties (Crittenden and Playne, 1996). In the Indian scenario, the only product marketed is 'Raftilose' imported from ORAFTI by SA Chemicals, Mumbai.

New physiological effects of FOS consumption continue to be elucidated, including possible protection against the development of colon cancer. The current high interest in the application of bifidobacteria to improve colonic health has made the bifidogenic property of FOS one of their strongest marketing points. The advantages provided to food manufacturers by the physicochemical properties of FOS, combined with the health benefits they impart to consumers, should ensure that FOS production and use would continue to expand.

1. 3. Scope of the work

The concepts in nutrition have changed in the recent years. In the past, emphasis was to avoid food ingredients that may cause adverse effects. Presently, the focus is on the use of foods that promote a state of well being, better health and reduction of the risk of diseases. These concepts have recently become popular as the consumer is becoming more and more health conscious. There is a growing awareness of the additional benefits and market potential for functional foods. The development of functional foods is a unique opportunity to contribute to the improvement of the quality of the consumer health and well being. Recently there has been a lot of attention paid to oligosaccharides and in particular FOS present in diet.

FOS is naturally present only in traces in fruits and vegetables, hence its preparation is more feasible by enzymes (FTases) produced by microbial fermentation. Many research groups have reported the production of FTase using microbial strains like *Aureobasidium* sp., *Arthrobacter* sp., *Penicillium* sp., *Fusarium* sp. etc. In view of the great demand for FOS as food ingredients,

scope exists for further screening and identification of newer strains capable of producing FTase. Hence, this research work focuses on the use of *Aspergillus oryzae* - which has not been reported so far for FTase production - as a novel source for FTase production and thereby FOS production using this enzyme.

Though there are a few reports on the production of FTase by a few species of Aspergillus like *A. niger*, *A. sydowi* and *A. japonicus*, there are no reports on the use of *Aspergillus oryzae* for FTase production. Hence, the potential of this organism to produce FTase and thereby FOS is investigated in this research work. *A. oryzae* is a well known koji mould, which has played a pivotal role in food manufacturing. For many years, it is being used for making fermented foods and beverages and production of industrial enzymes for food processing. *A. oryzae* is accepted as a microorganism having GRAS (generally regarded as safe) status (Gomi, 1999). Various parameters involved in the production of FTase and FOS have been selected and optimized using methods like Plackett Burman Model and Response Surface Methodology.

There are two methods of enzyme production by fermentation – Submerged Fermentation (SmF) and Solid State Fermentation (SSF). Production of enzymes by SSF has potential advantages over SmF with respect to simplicity in operation, high productivity fermentation, less favourable for growth of contaminants and concentrated product formation. SSF requires less space capital and operating costs, simpler equipment and the downstream processing is easier compared to SMF (Pandey *et al*, 2000). In addition, it permits the use of agro-industrial residues as substrates, which are converted in to bulk chemicals and fine products with high commercial value. There has been no reports on FTase production by SSF except for the use of apple pomace as substrate for FTase production (Hang *et al*, 1995). Different substrates have been tried in the present work for the production of FTase by *A. oryzae* under SSF conditions. In addition, various sources of sucrose have been used for the production of FOS using FTase.

Purification and characterization of the enzyme (FTase) are necessary steps to obtain the product (FOS) rapidly, with high purity, to improve our understanding of its mode of action and the nature of activity. Efforts have been

made to purify the FTase enzyme produced by *A. oryzae* CFR 202 to get high specific activity and fold of purification. Theoretically, purified enzyme should result in higher yields of the product. However, the peculiar reaction mechanism involved in FOS production does not lead to higher yields than the theoretical maxima of 56 – 58 % due to inhibition caused by accumulation of glucose. Nevertheless, the time involved in obtaining maximum FOS yield has been considerably reduced by using the purified FTase in comparison to the crude FTase. The product FOS formed has also been characterized for its physicochemical properties and structure using HPLC, NMR and LC-MS. Studies on the prebiotic effects of FOS are required to establish its functional properties and to demonstrate its use in product development. The present research work has also focussed on the scaling up of both the production of FTase as well as FOS.

The research work carried out is presented in nine chapters

Chapter 1 gives an introduction on fructooligosaccharides – its occurrence, structure, properties, mode of preparation, functional properties, application and market demand.

Chapter 2 deals with the literature survey relating to recent developments in the area of microbial production of fructooligosaccharides.

Chapter 3 comprises of the screening and selection of microorganisms capable of FTase production by Submerged Fermentation and FOS production using FTase from *A. oryzae* CFR 202 and *A. pullulans* CFR 77, selection and optimization of significant nutritional and cultural parameters involved in FTase and FOS production, recycling cell culture for FTase production by the selected microorganism (*A. oryzae* CFR 202) under the optimized conditions and the use of alternate substrates for FTase and FOS production.

Chapter 4 gives detailed account of the purification and characterization of FTase produced by *A. oryzae* CFR 202 by Submerged Fermentation.

Chapter 5 presents the results on the use of different agro - industrial byproducts for FTase production using *A. oryzae* CFR 202 by Solid State Fermentation and characterization of the crude enzyme produced thereby.

Chapter 6 deals with *in vitro* studies on prebiotic effects of FOS.

Chapter 7 deals with scale up studies for FTase production in 3 L and 15 L fermentors for FTase production by *A. oryzae* CFR 202 and 10 L level for FOS production. The chapter also discusses the preparation of FTase and FOS powder by spray drying.

Chapter 8 focuses on the applicability of FOS in formulating various food products like spread, honey and beverages, studies on the physicochemical properties of FOS, its purification by column chromatography and analysis by various methods like HPLC, LC - MS and NMR.

Chapter 9 gives the summary of the present research work.

CHAPTER 2
LITERATURE REVIEW

2. LITERATURE REVIEW

A lot of attention is being paid to dietary carbohydrates, especially oligosaccharides, in particular, FOS. The possible health benefits associated with the consumption of these compounds have led to their increased popularity as food ingredients and they are also being promoted as alternative sweeteners for diabetic formulations. Average daily consumption of FOS has been estimated to be 1 to 4 grams in US and 3 to 11 grams in Europe. The most common sources of FOS are wheat, onion, garlic and banana (Flamm *et al*, 2001). Research work is being carried out all over the world for the past two decades on the production, properties, analytical aspects and nutritional benefits of FOS. Many review articles describing the occurrence, preparation, properties and applications of FOS (Crittenden and Playne, 1996; Yun, 1996; Slavin, 1999) have been published. Flamm *et al* (2001) have critically reviewed the composition and source of FOS, its physiological effects upon consumption, and its relation to the dietary fiber concept. The following section focuses on the recent developments in the area of FOS research - its microbial production, functional properties and applications.

2.1. Microbial production of FOS

FTases are the enzymes responsible for the microbial production of FOS. FTase produces FOS (GF_n) from sucrose (GF) in a disproportionate mode, thereby forming 1 - kestose (GF_2) initially, then 1 - nystose (GF_3), followed by 1 - fructofuranosyl nystose (GF_4). This mechanism has already been explained (Yun, 1996). Microbial production of oligosaccharides has been extensively reviewed by Prapulla *et al* (2000). Microbial FTases are derived from fungal and bacterial sources. Enzymes derived from microorganisms like *Aspergillus phoenicis, Aspergillus japonicus, Aspergillus niger, Fusarium oxysporum, Scopulariopsis brevicaulis, Penicillium frequentens, Penicillium rugulosum, Aureobasidium pullulans* and *Arthrobacter* sp. have been reported to produce FOS from sucrose. *S. brevicaulis* has been found to produce only 1-kestose (Prapulla *et al*, 2000).

2.1.1. Fungal FTases

Several fungal strains, especially of *Aspergillus* sp. are known to produce extracellular or intracellular FTase. *Aspergillus niger* AS 0023 has

been reported to produce an intracellular FTase which yielded 54 % FOS using 50 % sucrose as substrate (L'Hocine *et al*, 2000). The cells of *Penicillium citrinum* have been reported to produce a syrup containing neofructooligosaccharides wherein the efficiency of FOS production was more than 55 % using 70 % sucrose as substrate. The product mixture comprised of 1-kestose (22 %), nystose (14 %) and neokestose (11 %) (Hayashi *et al*, 2000).

Production of FOS from sucrose catalyzed by β-D- Fructofuranosidase (Ffase) was achieved by Chien *et al* (2001) with the use of mycelia of *Aspergillus japonicus* immobilized in gluten. One gram of mycelia – immobilized particles having a cell content of 20 % (w/w) were incubated with 100 ml sucrose solution with an initial concentration of 400 g/ L. After a reaction period of 5 h, the FOS yield was 61 % of the total sugars. The reaction velocity increased with the cell content in the gluten matrix and a maximum value was obtained when the cell content was as high as 20 % (w/w).

2. 1. 2. Bacterial FTases

Bacterial strains have been reported to produce inulinases but, FOS producing enzymes are very rare in bacterial strains. A transfructosylating enzyme, which produces FOS from sucrose, has been isolated from *Bacillus macerans* EG-6 which, unlike other FTases, produced selectively GF_5 and GF_6 fructooligosaccharide. The final yield of FOS was reported to be 33 % when 50 % sucrose was used as substrate (Park *et al*, 2001).

The ethanol producing bacteria *Zymomonas mobilis* has been reported to produce a levansucrase capable of producing FOS and levan. The extracellular levansucrase that precipitated along with levan after ethanol treatment of culture fluid has been used as a biocatalyst for FOS production in sugar syrup. The yield of FOS was found to be 24 – 32 %, which constituted a mixture of 1-kestose, 6-kestose, neokestose and nystose. Glucose content was found to increase during all 24 h of reaction. The presence of ethanol (7.0 %) in sucrose syrup limited the enzyme's FOS forming activity to 24 % during the first 24 h of incubation. Fructan syrup produced from sucrose by using levan-levansucrase sediment as biocatalyst was reported to have satisfactory taste,

reduced energetic value and therefore, may be used as source of prebiotics (Beker *et al*, 2002).

Lactobacillus reutri strain 121 has been reported to produce 10 gL^{-1}FOS (95 % kestose and 5 % nystose) in the supernatants when grown on sucrose containing medium. FTase isolated from the strain when incubated with sucrose, produced FOS as well as inulin. After 17 h of incubation with sucrose, 5.1 gL^{-1} FOS and 0.8 gL^{-1} inulin were synthesized (Van Hijum *et al*, 2002). With the increasing demand for FOS in the functional foods market, there is always scope for discovery of novel sources of FTase.

2. 2. Continuous production of FOS

The use of immobilized enzymes and cells has led to the development of effective and economic methods for large - scale production of FOS. This technique has imparted operational stability to the enzyme thereby resulting in continuous production of FOS. A method for the continuous production of FOS was studied by Chien *et al* (2001) immobilizing the mycelia on gluten particles and packing it into a column reactor. FOS yield of 173 $gh^{-1}L^{-1}$ of reaction volume was achieved at a flow rate of 0.8 ml min^{-1}. The mass fraction of FOS increased from 0.2 to 0.54 w/w as the flow rate decreased from 1 to 0.1 ml min^{-1}, corresponding to an increase in the residence time from 0.35 to 3.5 h. The immobilized preparation was reported to be stable in long term operation since gluten was found to be adequate as the base material to immobilize mycelia - associated enzymes. However, the half - life of the enzyme was found to be 34 days.

A forced flow membrane reactor system for transfructosylation was investigated by Nishizawa *et al* (2000) using several ceramic membranes having different pore sizes. β-Fructofuranosidase from *A. niger* ATCC 20611 was immobilized chemically to the inner surface of a ceramic membrane activated by a silane coupling reagent. Transfructosylation took place while sucrose solution was forced through the ceramic membrane by cross flow filtration and the yield of FOS was reported to be 560 times higher than that is

reported in a batch system. The half-life of the immobilized enzyme on the membrane was estimated to be 35 days by a long-term operation.

Sheu *et al* (2002) have reported a complex biocatalyst system with a bioreactor equipped with a microfiltration (MF) module to produce high-content FOS in a continuous process initiated by a batch process. The system used mycelia of *Aspergillus japonicus* CCRC 93007 or *Aureobasidium pullulans* ATCC 9348 with β-fructofuranosidase activity and *Gluconobacter oxydans* ATCC 23771 with glucose dehydrogenase activity. Calcium carbonate slurry was used to maintain pH at 5.5 and gluconic acid in the reaction mixture was precipitated as calcium gluconate. Sucrose solution with an optimum concentration of 30 % (w/v) was employed as feed for the complex cell system and high content FOS was discharged continuously from a MF module. The complex cell system was run at 30 °C with an aeration rate of 5 vvm and produced more than 80 % FOS with the remainder being 5-7 % glucose and 8-10 % sucrose on a dry weight basis, plus a small amount of calcium gluconate. The system was operated for a 7-day continuous production process with a volumetric productivity of more than 160 g L^{-1} h^{-1} FOS. The complex cell system with both β-fructofuranosidase and glucose dehydrogenase activities was proved to be as effective as a two enzyme system. Since the enzyme activities were retained upto 6 days, the complex cell system might be more economical than two-enzyme system (Sheu *et al*, 2002).

2. 3. Production of High Content FOS

Industrial production of FOS carried out with microbial FTases has been found to give a maximum theoretical yield of 55 – 60 % based on the initial sucrose concentration. The FOS yield does not increase beyond this value because glucose liberated during the enzymatic reaction acts as a competitive inhibitor (Yun, 1996). To enhance the FOS conversion by removing the liberated glucose, the use of mixed enzyme systems has been recommended by many authors.

Studies were carried out on mixed enzyme systems using a commercial enzyme, with glucose oxidase and catalase, and mycelia of *Aspergillus*

japonicus CCRC 93007 and *Aspergillus niger* ATCC 20611 with β-fructofuranosidase activity to produce high yields of FOS. The reaction was performed in an aerated stirred tank reactor maintained at pH 5.5 by a slurry of $CaCO_3$. Glucose, an inhibitor of β-fructofuranosidase, produced was converted by glucose oxidase to gluconic acid, which was then precipitated by slurry of $CaCO_3$ to calcium gluconate in solution. The system produced more than 90 % (w/w) FOS on a dry weight basis, the remainder was glucose, sucrose and a small amount of calcium gluconate (Sheu *et al*, 2001).

Nishizawa *et al* (2001) have achieved higher yields of FOS with a simultaneous removal of glucose using a membrane reactor system with a nano-filtration membrane, through which glucose permeated but, not sucrose and FOS. FOS percentage of the reaction product was increased to above 90 %, which was much higher than that of the batch reaction product (55 – 60 %).

Studies have been carried out by Crittenden and Playne (2002) to remove glucose, fructose and sucrose present in food grade oligosaccharide mixtures using immobilized cells of the bacterium *Zymomonas mobilis*. Unpurified fructo, malto, isomalto, gentio and inulin oligosaccharides containing total carbohydrate concentrations of 300 g L^{-1} were added to immobilized cells, in 100 ml batch reactors. Glucose, fructose, and sucrose present in the mixtures were completely fermented within 12 h without any pH control or nutrient addition. The fermentation end products were ethanol and carbon dioxide without any degradation of the oligosaccharides in the mixtures. A minor amount of sorbitol was also produced as a fermentation by- product. The methods using mixed enzyme systems and mixed cultures have facilitated the removal of the residual sucrose as well as the inhibitory byproduct glucose, thereby improving the final FOS yields.

Mineral salts in the fermentation media have been found to improve FOS production by microbes. The effect of salt concentrations on the synthesis of FOS has been studied by Vigants *et al* (2000). It has been reported that 0.6 M NaCl concentration led to an increase of FOS production by 3.5-fold by *Zymomonas mobilis* 113S during fermentation in a medium containing 10 %

sucrose. Sorbitol was also produced as a fermentation by-product in the presence of mineral salts. In a medium with high (65 %, w/w) sucrose content, the salts had an inhibitory effect on FOS production by lyophilized *Z. mobilis* cells. The enhanced production of FOS and formation of sorbitol has been concluded to play an osmoprotective role to *Z. mobilis*.

2. 4. Purification and properties of FTase

Purification and characterization of an enzyme is a necessary step to improve one's understanding of its mode of action. Many authors have reported the purification and characterization of FTases from various sources. FTases have been found to differ in their molecular weight and properties from one source to another. Park *et al* (2001) have purified FTase from *B. macerans* EG – 6, 63.5 fold by ammonium sulfate precipitation (20-60 %), CM Sepharose CL 6B and fast protein liquid chromatographies on Resource Q, Phenyl Superose HR 5/5 and Mono S. The purified enzyme had a molecular mass of 66 kDa by SDS PAGE. The enzyme was stable at the pH range of 5-7 and had an optimum pH at 5. The optimum temperature for enzyme activity was at 50 °C. An important feature of this purified FTase is that the oligosaccharide composition in reaction products was significantly different on using the enzyme obtained from each purification step. The purified enzyme was found to produce kestose and nystose unlike the crude enzyme which produced GF_5 and GF_6 oligosaccharides (Park *et al*, 2001).

Purification of FTase from the crude extract of *A. niger* AS 0023 has been detailed by L'Hocine *et al* (2000) by successive chromatographies on DEAE Sephadex A – 25, Sepharose 6B, Sephacryl S-200, and concanavalin A-Sepharose 4B columns. FOS yield was increased by 8 % when purified enzyme was used. On native and SDS PAGE, the enzyme migrated as polydisperse aggregates yielding broad and diffused bands, which showed that the enzyme is a typical glycoprotein. FTase on native PAGE migrated as two enzymatically active bands with different electrophoretic mobility, one around 600 kDa and other from 193 to 425 kDa. On SDS PAGE these two fractions yielded one band corresponding to a molecular weight range from 81 to 168 kDa. The optimum pH and temperature for FTase were found to be 5.8 and 50

°C, respectively. Studies on the effect of metal ions showed that FTase was completely inhibited with 1 mM Hg^{2+} and Ag^{2+} (L'Hocine *et al*, 2000).

Wang and Rakshit (2000) have partially purified four fractions of an enzyme with transferase activity from *A. foetidus* NRRL 337. After ammonium sulphate precipitation and DEAE cellulose column chromatography, the purification fold of the fractions were 64, 25, 29 and 43. The optimum temperature was 60 °C and pH stability was in the range of 4-6. The pH optima, heat sensitivity and kinetic parameters for the four fractions were however, not the same. The substrate and product specificity and kinetic parameters of the multiple forms of the transferase enzymes produced by this organism has also been described.

β - fructofuranosidase was purified from *A. niger* ATCC 20611 with 76 % recovery by Nishizawa *et al* (2001) after calcium acetate precipitation, anion exchange chromatography and gel filtration chromatography. Kinetic parameters (V_m, K_m, and K_i) of the enzyme were determined from experimental data on the transfructosylation rate at various substrate concentrations with and without addition of glucose. Transfructosylation reaction was found to be inhibited non- competitively (K_I = 0.12 mol L^{-1}) by glucose.

Gorrec *et al* (2002) have reported about an extracellular FTase from *Bacillus subtilis* NCIMB 11871 and 11872 with sucrase and polymerase activities synthesizing fructose polymers. The production and degradation of the three enzymatic activities was tightly coupled during the growth of the microorganism. Since ammonium sulphate precipitation, ultrafiltration and other separation techniques could not achieve separation of the activities, it is confirmed that the same enzyme supports these three activities, and that the enzyme is a levansucrase.

2. 5. FOS production using recombinant enzymes

Advances in recombinant DNA research have made its impact in every field of biological research and microbial enzymes are no exception. Modern biotechnology has paved the way to studies on recombinant FTases. A

recombinant yeast - *Pichia pastoris* - with a levan sucrase activity was reported to produce FOS in a 50 % sucrose solution as substrate resulting in 43 % 1-kestose. The levan sucrase activity was detected in the periplasmic fraction (81 %) and in the supernatant (18 %) of the yeast. Intact recombinant yeast cells also converted sucrose to FOS with 30 % efficiency (Trujillo *et al*, 2001).

Van Hijum *et al* (2002) have reported the cloning and expression of FTase gene (ftf) from *Lactobacillus reutri* in *Eschericia coli*. When incubated with sucrose, the purified recombinant FTF enzyme produced large amounts of FOS and a high molecular weight fructan polymer, an inulin. Nucleotide and amino acid sequence analysis of the recombinant enzyme has been reported.

2. 6. Production of Isooligosaccharides

Although FTases are known to produce only FOS from sucrose, certain microorganisms are characterized by the presence of transferases with multiple substrate specificity. Wang and Rakshit (2000) have reported the production of a transferase enzyme by *A. foetidus* NRRL 337 with four fractions capable of producing isooligosaccharides using different substrates. HPLC analysis of the product of the transferase enzymes revealed that the four forms of enzymes are distinct, producing oligosaccharides like panose, kestose and nystose or acting as hydrolytic enzymes depending on the conditions.

There are reports on the production of different oligosaccharides by transferring the fructose residue of sucrose to maltose, cellobiose, lactose and sucrose where, the yields of fructosylated acceptor products accounted for 26 – 30 % (w/w). The maximum yield (30 %) was obtained in fructosyl celllobioside formation with 500 g sucrose L^{-1} (substrate) and 200 g cellobiose L^{-1} (acceptor). These four acceptors gave various products having DP (degree of polymerization) 2-7 by successive transfer reactions (Kim *et al*, 2001).

2. 7. FOS Hydrolysis

The β - 2,1 linkage in FOS renders them resistant to hydrolysis by digestive enzymes and thereby imparts a dietary fibre effect. However, recently studies have been carried out on the effect of pH and temperature on

hydrolysis of FOS. A kinetic study was carried out at 80, 90, 100, 110 and 120 °C in aqueous solutions buffered at pH values of 4.0, 7.0 and 9.0 by L'Homme *et al* (2003). Under each experimental condition, the determination of the respective amounts of reactants and hydrolysis products showed that FOS hydrolysis obeyed pseudo-first –order kinetics as the extent of hydrolysis, which decreased at increasing pH values, increased with temperature. The three oligomers were found to be degraded mainly under acidic conditions, and at the highest temperature value (120 °C), a quick and complete acid degradation of each FOS was observed. Using Arrhenius equation, rate constants, half-life values, and activation energies were calculated and compared with those obtained from sucrose under the same experimental conditions. It appeared that the hydrolysis of FOS took place much more easily at acidic pH than at neutral or basic pH values. This study was aimed at putting forward the mechanism whereby this reaction takes place in order to evaluate the consequence of heating food products on their FOS content. High performance anion exchange chromatography coupled with pulsed amperometric detection system (HPAEC-PAD) was used to evaluate the extent of chemical hydrolysis of three FOS including 1- kestose, nystose, and fructofuranosyl nystose (L'Homme *et al*, 2003).

The kinetics of acid hydrolysis of five commercially available oligofructose samples used as food ingredients has been investigated by Blecker *et al* (2002) as a function of dry matter concentration, reaction pH and temperature. The initial fructose release rate is roughly proportional to the inverse of the average polymerization degree in number. A pseudo first order kinetic is found with respect to the fructosyl chain concentration and to the proton concentration. The data in a relatively wide temperature range (7 – 130 °C) is found to reasonably fit in an Arrhenius plot (Blecker *et al*, 2002). Studies on the hydrolysis of FOS under different pH and temperature conditions helps to understand the fate of them in food products subjected to processing conditions.

2. 8. Analysis of FOS

Advances in analytical techniques like chromatography, with a particular reference to High Performance Liquid Chromatography (HPLC) has led to rapid and accurate analysis of FOS.

2.8.1. High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) has been the most popular technique for analysis of FOS. Both polar-bonded phase and resin – based HPLC columns are commonly used with Refractive Index Detector (RID) for separation of FOS of different Degree of Polymerization (DP) (Prapulla *et al*, 2000). The polar bonded phases are efficient, and carbohydrates elute in order of increasing monosaccharide chain length. On the other hand, components elute in order of decreasing molecular size from resin based columns.

There have been many reports on the use of polar bonded phase columns like NH_2 column at 30 °C with acetonitrile: water (75:25) as mobile phase at a flow rate of 1 or 1.5 ml/min (Vigants *et al*, 2000; Nishizawa *et al*, 2000, L'hocine *et al*, 2000, Sheu *et al*, 2001; Chien *et al*, 2001). Gorrec *et al* (2002) have used the resin based ion exchange K^+ column Aminex HPX-87 K column at 65 °C with water as mobile phase at a flow rate of 0.6 ml min^{-1} whereas Kim *et al* (2001) and Park *et al* (2001) have used Aminex HPX 42 C column at 85 °C using the same mobile phase and flow rate. Trujillo *et al* (2001) have reported the use of Aminex HPX 87 N column at the same conditions with 10 mM Na_2SO_4 as mobile phase at 0.5 ml min^{-1} flow rate. Another resin based column, Aminex HPX 87C has also been widely used for FOS analysis using water as mobile phase (Crittenden and Playne, 2002).

High Performance Anion Exchange Chromatography with Pulsed Amperometric Detector (HPAEC – PAD) is another widely used technique for analysis of FOS. L'homme *et al* (2003) have used a Carbopac PA 100 analytical anion exchange column using a 20 min linear gradient from 0 % to 40 % of a 80 mM NaOH – 500 mM sodium acetate in 80 mM NaOH – 5 mM sodium acetate whereas Finke *et al* (2002) have used a Dionex DX-300 chromatograph equipped with a pulsed electrochemical detector with gold electrode operating in the integrated amperometry mode. Fructans originally

extracted from chicory roots were separated by continuous annular and fixed bed conventional gel chromatography. Both columns were packed with Toyopearl HW 40 (S) and eluted with deionized water. A multicomponent fractionation was established to obtain single oligosaccharides in a low molecular weight range up to a chain length of 90 monosaccharide units. The productivity and resolution of the continuous annular size exclusion chromatograph (40 cm bed height) were investigated and compared with those of the fixed bed counterpart (2 x 100 cm bed height). The eluting fractions were analyzed by high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The productivity of the annular system was found 25 fold higher than the conventional system. Thus, annular chromatography exemplified for the fractionation of fructans is suggested to be a powerful method for the large scale and continuous fractionation of oligomeric and polymeric carbohydrates (Finke *et al*, 2002).

An ion chromatographic method has been reported in which sugar concentrations were measured using integrated amperometry. The samples were simply prepared by blending with water and filtering the suspensions through a 10,000 Da cut off centrifugal filter. These samples were then injected into the chromatograph, which had been programmed for gradient elution, and the areas of the sugar peaks obtained compared to those of the standard sugars on a calibration curve. Selected samples were prepared both with and without standard spikes in order to assess the efficiency of the determination. Of the vegetables investigated, artichokes contained the most of FOS followed by onions, bananas contained more FOS than other fruits. Method is simple, economical and relatively fast for the quantification of FOS (Hogarth *et al*, 2000).

Several other methods like paper chromatography, thin layer chromatography, gas liquid chromatography, NMR analysis and Mass Spectrometry have been discussed in the literature (Prapulla *et al*, 2000).

2. 8. 2. Thin Layer Chromatography (TLC)

Park *et al* (2001) have reported the quantitative analysis of FOS by TLC using the solvent systems; isopropyl alcohol: ethyl acetate: water (2:2:1). The products were visualized by heating the plates after spraying phenol sulfuric acid. A routine method has been proposed by Vaccari *et al* (2000) for the analysis of FOS utilizing modern instrumental thin layer chromatography, which meets most of the criteria and gives a rapid method for the detection and quantitative determination of the oligosaccharides in beet molasses and other products. Diol HPTLC plates were used and development was done using solvents like acetonitrile and acetone. A nine-step gradient was performed by mixing the two solvents using a Camag Automated Multiple Development apparatus. Derivatization was performed with 4- aminobenzoic acid reagent, glacial acetic acid, water, 85 % phosphoric acid and acetone added to 4-aminobenzoic acid. The developed plates on heating at 115 °C for 15 min showed yellowish to brown spots corresponding to FOS (Vaccari *et al*, 2000).

2.8.3. Gas Chromatography – Mass Spectrometry (GC – MS)

Hayashi *et al* (2000) have reported the analysis of FOS using GC-MS after methylation of the samples by methyl iodide and hydrolysis with 1 M H_2SO_4 for 1 h. The samples were then reduced by the addition of $NaBD_4$ and then alditol acetylated with acetic anhydride at 110 °C for 3 h. GC-MS was performed on a Hitachi M-2000 AM instrument fitted with an OB 225 fused silicone column at 170 – 200 °C using Helium as carrier gas with a temperature program of 1 °C min^{-1}. ^{13}C NMR analysis of FOS has also been reported by the same authors.

2.8.4. AOAC Method for fructan analysis

Although HPLC has been the most widely used technique for the analysis of FOS, the lack of availability of pure standards has been a major setback in determination of FOS. In this context, an enzymatic method has been reported by AOAC for FOS analysis. The official AOAC method for determination of fructans in foods and food products relies on the enzymatic treatment of the sample with an inulinase enzyme. Inulin and oligofructose are extracted from the sample with boiling water. One aliquot is kept untreated as the initial sample. A second aliquot of the extract is hydrolyzed using an

amyloglucosidase enzyme. A sample of the hydrolysate is kept as the second sample and the rest is further hydrolyzed using an inulinase (Fructozyme SP 230). Glucose, fructose and sucrose are quantified in the three samples by capillary gas chromatography, high performance liquid chromatography or preferably high performance anion exchange chromatography-pulse amperometric detection. Inulin is then calculated by subtracting the sugars in the first and second aliquots from the third. Because the oligofructose is not recovered by the AOAC-TDF (Total Dietary Fibre) methods and only a small fraction of inulin is recovered by the AOAC - TDF methods, a correction can be made for inulin that would be double counted. On the one hand, inulinase can be added to the enzyme complex in the TDF method. This removes all fructans from the sample. Another approach is to determine the fructan content in precipitates of the TDF methods, and then to subtract this amount from the TDF amount (Flamm *et al*, 2001).

2.9. Functional properties of FOS

FOS has a number of interesting functional properties that make them important food ingredients. The nutritional and health benefits of FOS have been the subject to many reviews in the recent years (Flamm *et al*, 2001). Flickinger *et al* (2003) have extensively reviewed the nutritional responses to the presence of inulin and oligofructose in the diets of domesticated animals.

2.9.1. FOS as prebiotic

Prebiotics are non- digestible food ingredients that selectively stimulate the growth and / or activity of potentially health – enhancing intestinal bacteria. Since FOS is not hydrolyzed by the human digestive enzymes, it undergoes fermentation in the colon and encourages the growth of beneficial bacteria in the colon. This in turn discourages the growth of potentially putrefactive microorganisms in the colon resulting in a healthy gut environment. FOS has been demonstrated as an effective prebiotic through both *in vivo* and *in vitro* assessments. Durieux *et al* (2001) have investigated the prebiotic effect of FOS by studying the metabolism of two types of chicory FOS (Fibruline Instant and Fibrulose F 97) by *Bifodobaterium longum, B. infantis* and *B. angulatum*. Chromatographic analysis of the medium after 120 h revealed consumption of

all the fructose oligomers present in the commercial chicory FOS by all the strains. Maximum measurable degree of polymerisation of the substrates before fermentation was 41. Biomass production was highest with *B. infantis* (1.4 and 1.7 g dry wt L^{-1}) for its cultivation in a medium supplemented with Fibruline instant and Fibrulose F 97 respectively as substrate. These results indicate that Chicory FOS can be used as prebiotic (Durieux *et al*, 2001).

A comparative evaluation of the fermentation properties of prebiotic oligosaccharides has been carried out by Rycroft *et al* (2001). Populations of predominant gut bacterial groups were monitored over 24 h batch culture through fluorescent in-situ hybridization. Short chain fatty acid (SCFA) and gas production was also measured. All prebiotics increased the numbers of bifidobacteria and most decreased clostridia. The oligosaccharides were found to differ in their fermentation characteristics. FOS produced the highest populations of lactobacilli with least flatulence.

Studies were carried out by Perrin *et al* (2001) to compare the physiological behaviour *of Bifidobacterium infantis* ATCC 15697 growing on synthetic oligofructose. The studies were carried out in regulated and non-regulated batch cultures on semi-synthetic media. Differences between the carbohydrate utilization patterns were determined. Glucose was the preferred substrate for growth and biomass production, whereas fructose was the best for lactate and acetate production. With sucrose, biomass production reached the level obtained with glucose, where as with FOS and fructose more metabolites were produced. In a mixture of FOS, the shorter saccharides were used first and fructose was released in the medium (Perrin *et al*, 2001).

Studies were carried out on two commercial strains of *Bifidobacterium* spp (Bf-1 and Bf-6) cultured anaerobically at 37 °C for 48 h in 12 % (w/w) reconstituted Nonfat Dry Milk (NDM) containing 0, 0.5, 1.0, 3.0 or 5.0 % (w/v) FOS. Growth and activity of the cultures in the presence of FOS was determined. Viability of each strain was assessed after 4 weeks of refrigerated storage at 4 ^{o}C. Growth promotion, enhancement of activity and retention of viability was more when *Biifidobacterium* Bf-1 (67 %) and Bf-6 (45 %) were

grown in the presence of FOS. The effects of FOS increased with its increasing concentration and was maximal at 5 % (w/v) (Shin *et al*, 2000).

The effect of ingesting a low dose of FOS (5 g/ day) by healthy human subjects on the faecal microflora especially bifidobacteria was investigated and compared with the ingestion of a placebo – sucrose (Rao, 2001). In a placebo controlled study design, faecal samples were collected from healthy human subjects, who were not on any medication, and immediately enumerated for bifidobacteria, bacteriodes, coliforms, total anaerobes and total aerobes. Faecal samples were collected from the subjects after administering FOS (5 g daily for 3 weeks). Samples subjected to microbial enumeration showed that ingestion of sucrose (5 g per day) was without effect on all faecal bacteria enumerated, whereas consumption of FOS (5 g per day) for 11 days resulted in close to one log cycle increase in bifidobacteria numbers. No further increase was observed after the next 10 days. At 2 weeks after termination of FOS ingestion, bifidobacteria numbers had decreased to almost that of the period before treatment. Increase in number of bacteriodes and total anaerobic bacteria also occurred but not aerobic bacteria.

Kaplan and Hutkins (2000) screened twenty eight strains of Lactic Acid Bacteria (LAB) and bifidobacteria for their ability to ferment FOS on MRS agar. Twelve of 16 *Lactobacillus* strains and 7 of 8 bifidobacterial strains tested were able to ferment the substrate. It was found that like glucose, FOS was equally a good substrate in supporting growth. When individual oligosaccharides like GF_2, GF_3 and GF_4 were used, their utilization was found to be minimal and the pH decreased to only 6.0 and none of the strains were able to use GF_4. The tolerance and threshold dose of FOS that significantly increased fecal bifidobacteria were assessed and the optimal dose for increased bifidobacterial counts without significant side effects such as flatulence was reported to be 10 g/ day. Supplementation with FOS led to an increase in LAB after 2 weeks with out changing anaerobic bacterial levels. LAB is considered to be immunomodulatory and directly or indirectly influence the Gastrointestinal Tract (GIT) and systemic defense functions. Corresponding with this, supplementing the diet with FOS that increase the densities and metabolic capacities of the

LAB enhances the defense mechanisms of the host, increases resistance to various health challenges and accelerates recovery of the gastrointestinal tract after disturbances (Kolida *et al* 2002).

2.9.2. FOS as dietary fiber

Dietary fibre consists of remnants of edible plant cell polysaccharides and associated substances resistant to hydrolysis by human alimentary enzymes, which may benefit health through a wide range of physiological effects. FOSs are storage carbohydrates found in a number of vegetables, fruits and whole grains. They resist digestion and absorption in the stomach and small intestine of humans, as shown by their full recovery at the end of the ileum of healthy or ileostomised volunteers. Studies in patients with a conventional ileostomy by Cherbut (2002) have shown that mean excretion of FOS at the end of ileum was about 90 % of the ingested dose. Thus, they enter the large intestine where they will be available for fermentation, as demonstrated by increased breath hydrogen. Increased lactate concentration has been found in colonic and fecal contents of rats, fed with FOS. Fermentation is complete and no residue has been found in human stools. They also improve laxation. Their bulking capacity comprises between 1.2 to 2.1 g of stool per gram of ingested substrate, resulting mainly from increase in microbial biomass in the colon. In addition, due to their fermentation properties, they also affect the intestinal epithelium that may strengthen mucosal protection and reduce the risk of gastrointestinal diseases. Therefore, FOS has been found to fit well within the concept of dietary fibre (Cherbut, 2002).

2.9.3. FOS and mineral absorption

Colonic fermentation of FOS leads to decrease in pH in the colon and this facilitates the absorption of mineral ions from the intestine, mainly calcium and magnesium. This has been indicated by long term beneficial effects on bone health such as accumulation of bone mineral content in growing rats or prevention of bone loss in ovariectomised rats. The addition of 5 % FOS prevented bone loss significantly in the femur and lumbar vertebra in the presence of dietary calcium (1 %). At 0.5 % calcium, 10 % oligofructose was needed to significantly increase bone mineralization. The effect may be due to

enhancement of passive and active mineral transport across the intestinal epithelium, mediated by an increase in certain metabolites of the intestinal flora and a reduction in pH (Scholez Ahrens and Schrezenmeir, 2002).

The effect of FOS on protein digestibility and mineral absorption was studied by Gudieal – Urabano and Goni (2002) in rats fed with diets containing 5 gkg^{-1} FOS, 5 gkg^{-1} cellulose/ FOS (1:1) or 5 gkg^{-1} cellulose as a source of dietary fibre. Addition of cellulose/ FOS or FOS to the diet did not significantly modify the daily food intake and food efficiency. However, FOS fed group showed significant decrease in body weight gain compared with cellulose fed groups. Faecal excretion was significantly lower when there was FOS intake, despite there being no significant difference in cellulose fed groups. Intake of FOS produced an increase in caecal content and an enlargement of the ceacal wall. This trophic effect could be attributed to the short chain fatty acids produced from the anaerobic fermentation of FOS by intestinal bacteria. Cellulose/ FOS enhanced apparent absorption and apparent retention of Ca, Mg, Zn and Fe. FOS fed rats experienced an increase in apparent absorption and apparent retention of Mg compared with cellulose fed rats. FOS intake at the lowest dose was enough to provide a desirable effect on mineral bioavailability in rats without any modification of nutritional parameters (Gudieal – Urabano and Goni, 2002).

2.9.4. Role of FOS in defense functions

FOS is known to prevent the colonization of human gut by pathogenic microorganisms because it encourages the growth of beneficial bacteria. This effect is attributed to the low pH environment created during fermentation of FOS in the colon and due to the secretion of antibiotic like substances by the beneficial bacteria. Studies have shown that supplementing the diets of chicken, pigs and rats with oligofructose and other Non – Digestible Oligosaccharides (NDOs) reduces fecal densities of *Salmonella* (Letllier *et al*, 2000). Supplementing the diet of mice with inulin and oligofructose reduces the densities of *Candida* in the small intestine of mice 7 days after infection. Mice infected systemically with virulent strains of *Listeria monocytogenes* and *Salmonella typhimurium* after being fed a diet with inulin and oligfructose (at

100g/kg) had lower mortality than mice fed a diet with cellulose as the source of fiber (Buddington *et al*, 2002). FOS stimulates higher rates of colonocyte proliferation than cellulose and other NDOs without increasing the total amount of mucosa. Feeding mice with diets supplemented with inulin and oligofructose increased activities of natural killer cells and phagocytes and enhanced T-lymphocyte functions compared to mice fed diets with cellulose or lacking fiber. These results are consistent with the observations of heightened resistance to systemic infections with *Listeria* and *Salmonella,* the lower incidence and growth of tumors after exposure to carcinogens and transplanted tumor cells and are in agreement with enhanced innate and acquired immune functions provided by *Lactobacillus* and other LAB. Supplementing diets with FOS should increase production of SCFA, particularly butyrate, and can be predicted to strengthen mucosal defenses and enhance response to health challenges.

2.9.5. FOS and lipid metabolism

FOS, besides its effect on the GIT, is also able to exert systemic effect, namely by modifying the hepatic metabolism of lipids in several animal models (Delzenne *et al*, 2002). Colonic fermentation of FOS results in the synthesis of short chain fatty acids, which influences the lipid metabolism in human beings. Feeding male Wistar rats on a carbohydrate rich diet containing 10 % FOS significantly lowers serum triacylglycerol (TAG) and phospholipid concentration. A lower hepatic lipogenesis, through a coordinate reduction of the activity and mRNA of lipogenic enzymes is an important event in the reduction of very low density lipoprotein TAG secretion by FOS. FOS is able to counteract triglyceride metabolism disorder occurring through dietary manipulation in animals and sometimes independently on lipogenesis modulation. FOS reduces post-prandial triglyceridemia by 50 % and avoids the increase in serum free cholesterol level occurring in rats fed with a Western type high fat diet. FOS protects rats against steatosis (liver TAG accumulation) induced by fructose, or occurring in obese Zucker fa/fa rats. FOS given at the dose of 10 % in the diet of male Wistar rats for 30 days reduces postprandial insulinemia by 26 %. However, the glycemic response during a glucose tolerance test after overnight fasting is identical in control and FOS fed rats (Daubiol, 2000). In streptozotocin-treated diabetic rats, feeding a diet containing 20 % FOS for 2

months decreases postprandial glycemia despite lack of modification of the glycemic /insulinemic response to a saccharose or maltose load.

Out of nine studies reported on the response of blood lipids to inulin and FOS, three have shown no effects on blood levels of cholesterol or triacyl glycerol, three have shown significant reductions in TAG, whilst four have shown modest reductions in total and LDL cholesterol (Williams and Jackson, 2002). Animal studies provide strong evidence that FOS inhibit secretion of TAG rich Very Low Density Lipoprotein (VLDL) particles via inhibition of de novo fatty acid synthesis. High levels of fat present in most human diets mean that rates of hepatic de novo fatty acid synthesis are extremely low, since exogenous dietary fatty acids provide all the required substrate for hepatic triacylglycerol synthesis (Parks, 2002). Although there is some evidence to suggest that FOS may also inhibit esterification step of fatty acids into TAG, this is relatively modest in comparison with the marked inhibition of fatty acid synthetase which characterizes the response to inulin and oligofructose in animals. Although convincing lipid lowering effects have been observed in animals, the studies have used relatively high levels, equivalent doses of which could not be used in man because of known adverse gastrointestinal side effects at intake levels greater than 15 g/day. Feeding rats with 10 % FOS significantly lowers serum triglycerides and phospholipids concentrations but does not modify free fatty acid concentration in the serum. Shot chain fructans have been shown to lower serum total and LDL cholesterol in non-insulin dependent diabetic patients, but not in healthy subjects (Roberfroid, 2000).

2.9.6. Anticancerous effect of FOS

Studies with inulin and FOS have shown reduction of chemically induced aberrant crypts and prevention of colon cancer. According to Pool-Zobel *et al* (2002), in rats, a prebiotic effect resulting in the proliferation of bifidobacteria (with the major metabolites lactate or acetate) as well as of other bacteria could be responsible for the observed anticancer effects.

Dietary treatment with inulin/ oligofructose (15 %) incorporated in the basal diets for experimental animals resulted in (a) reduction of the incidence of mammary tumors induced in Sprague Dawley rats by methylnitrosourea (b)

inhibited the growth of transplantable malignant tumors in mice and (c) decreased the incidence of lung metastases of a malignant tumor implanted intramuscularly in mice. It is reported that the dietary treatment with FOS/ inulin significantly potentiated the effects of subtherapeutic doses of six different cytotoxic drugs commonly utilized in human cancer treatment (Taper and Roberfroid, 2002).

2.9.7. Additional health effects of FOS

It has been reported by Luo *et al* (2000) that the daily consumption of 20 g FOS decreased basal hepatic glucose production in healthy subjects without any effect on insulin stimulated glucose metabolism. When the effect of chronic ingestion of FOS on plasma lipid and glucose concentrations, hepatic glucose production and insulin resistance in type 2 diabetics was evaluated, it was found that FOS did not modify fasting plasma blood glucose and insulin concentrations or basal hepatic glucose production. Also, serum triacylglycerol, total and HDL cholesterol, free fatty acid, apolipoproteins A1 and B concentrations were not modified by the chronic ingestion of FOS.

Roberfroid (2000) has reported that feeding rats with FOS (10 %) for a few weeks decreased uremia in both normal and nephrectomized rats. Dietary FOS enhanced fecal nitrogen excretion and reduced renal excretion of nitrogen in rats. This occurs because these fermentable carbohydrates serve as energy source for the intestinal bacteria, which during growth also require a source of nitrogen for protein synthesis.

2.10. Applications of FOS in food formulations

Inulin and oligofructose are ingredients that deliver a number of important nutritional benefits as well as contribute functional properties that enhance shelf life and taste profile of various food products like nutrition bars (Izzo and Niness, 2001).

Examples of the use of FOS in food products include the following

1. Light jam products: FOS can be used as the sole sweetening agent and gives 34 % calorie reduction compared with sucrose standard. Organoleptic

characteristics of the products are claimed to be very similar, with the test sample having a lower sweetness and a softer texture.

2. Ice cream: FOS can be used with inulin to replace all the sugar and reduce the fat content and give excellent mouthfeel characteristics. Since the freezing point depression is less with oligofructose than with sugar, the texture can be harder.
3. Confectionery applications: Hard candies, gums, and marshmallows can be made while achieving significantly reduced energy values (Murphy, 2001).

In the present investigation, efforts have been made to screen fungal cultures for FTase activity by SmF. *Aspergillus oryzae* CFR 202 has been found to produce high titres of FTase and it was selected as a novel source of FTase. An equally efficient strain *Aureobasidium pullulans* CFR 77 has also been used for production of FTase – the enzyme source being the culture broth homogenate. Attempts have been made to select the significant parameters influencing the production of FTase as well as FOS by statistical methods using Plackett Burman design. Further, optimization of the significant parameters was carried out using Response Surface Methodology. Although the effect of various parameters on FTase and FOS production has been studied individually, the use of a statistical approach is novel in the optimization of FTase and FOS production. Another novel approach has been the development of recycling culture of *A. oryzae* CFR 202 pellets for the repeated production of FTase and FOS. This system has been found to be highly efficient for industrial production of FOS since it facilitates the reuse of the pellets six times without development of fresh inoculum.

Although this literature survey has covered most of the recent reports on various aspects of microbial production of FOS, it is obvious that all of them are based on Submerged Fermentation (SmF). There have been no reports on efforts to produce FTase by Solid State Fermentation (SSF). Attempts have been made to carry out production of FTase by SSF. Various agroindustrial by – products such as cereal bran, corn products, coffee and tea processing by - products, sugarcane bagasse and cassava fibrous residue were used as solid substrates to grow *A. oryzae* CFR 202 for FTase production. Cereal bran like

wheat bran, rice bran and oat bran, which otherwise find use as animal feed have been used for the production of the enzyme, thereby giving value addition to the same. The different components obtained after milling of corn like corn bran, corn meal, corn grits, corn cob and corn germ have also been used for FTase production. This ensures the use of all the components obtained after corn milling. The by – products of coffee processing industries like coffee pulp, coffee parchment (husk) etc pose serious threat to the environment due to disposal problems. Development of a process for the production of FTase using these gives value addition to these by – products. Spent coffee and spent tea also has served as substrates for FTase production up on supplementation with nitrogen source. Various alternate sources of sucrose like jaggery have also been tried for FOS production. Jaggery served as a carbon source in the medium for FTase production as well as substrate for FOS production. In addition, studies have been carried out on the physicochemical characteristics of FOS and development of various products like spread, honey – like product and beverages based on FOS. Studies are also being carried out on the preparation of a powder form of FOS. Due to the increasing demand for FOS in the functional food market, the field of FOS research is also expected to come up with new developments.

CHAPTER 3

FTase PRODUCTION BY SUBMERGED FERMENTATION

a. Screening and selection of microorganisms for FTase activity.

b. FOS production by *Aspergillus oryzae* CFR 202 and *Aureobasidium pullulans* CFR 77.

c. Optimization studies on FTase and FOS production by *A. oryzae* CFR 202.

   1. Selection of significant cultural and nutritional parameters influencing FTase and FOS production by Plackett – Burman design.

   2. Optimization of FOS production by *A. oryzae* CFR 202 using Response Surface Methodology

d. FTase production by *A. oryzae* CFR 202 during recycling cell culture.

e. Alternate substrates for the production of FTase and FOS.

3. a. SCREENING AND SELECTION OF MICROORGANISMS FOR FTase ACTIVITY

3. a. 1. INTRODUCTION

FOS is commercially produced from sucrose by enzymatic transfructosylation. The enzyme, fructosyl transferase, responsible for the production of FOS from sucrose can be derived from plants or microorganisms. Production of FOS was observed during the growth of several fungi in sucrose medium. Microbial production of oligosaccharides has been extensively reviewed by many authors (Crittenden and Playne, 1996; Yun, 1996 and Prapulla *et al*, 2000).

This chapter deals with the screening of a few fungal strains for FTase activity that can produce FOS yields of 50 % and above for industrial application. Several fungal strains have been found to produce FTase (Table 1.1, Chapter 1). They include *Aureobasidium* sp., *Aspergillus* sp., and *Penicillium* sp. However, scope exists for further screening for FTase production. Attempts have been made to screen a few fungal strains for extracellular as well as intracellular FTase activity and the study has revealed that *Aspergillus oryzae*, *Aspergillus flavus*, and *Mucor miehei*, which are hitherto unreported could be used as novel sources of FTase.

3. a. 2. MATERIALS AND METHODS

3. a. 2.1 Chemicals

Sucrose, sodium chloride, magnesium sulphate, di potassium hydrogen phosphate, potassium di hydrogen phosphate, ammonium chloride, sodium nitrate, citric acid, tri sodium citrate were all from Qualigens Fine Chemicals (Mumbai, India). Yeast extract, glucose and agar were from Himedia Laboratories (Mumbai, India). Fructooligosaccharide standards- 1-kestose (GF_2), 1- nystose (GF_3) and 1-fructofuranosyl nystose (GF_4) were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Membrane filters (0.45 μm) were from Millipore (India) Private Ltd. (Bangalore, India). Acetonitrile (HPLC grade) was from E. Merck (India) Ltd. (Mumbai, India).

3. a. 2.2 Microorganisms and Culture conditions

The six fungal strains – *Aspergillus niger, Aspergillus flavus* CFR 203*, Aspergillus oryzae* CFR 202*, Penicillium* sp.*, Aureobasidium pullulans* CFR 77 and *Mucor miehei* – were from the type culture collection of CFTRI, Mysore. The strains were maintained on potato dextrose agar slants at 4 °C.

3. a. 2.3 Inoculum Development

The inoculum was prepared by transferring a loopful of spores/mycelia from a five day old slant to 100 ml medium containing 1 % sucrose and 0.2 % yeast extract (pH 5.5) in 500 ml flasks. The flasks were incubated at 30 ± 1 °C on a rotary shaker (Emenvee Rotary Shaker, 48N3, Pune, India) at 250-rpm for 24 hours.

3. a. 2.4 Production of Fructosyl transferase enzyme

A 24 h old inoculum (10 %v/v) was transferred to 100 ml of fermentation medium containing 20 % sucrose, 0.5 % yeast extract, 1 % $NaNO_3$, 0.05 % $MgSO_4.7H_2O$, 0.25 % K_2HPO_4, 0.25 % KH_2PO_4, 0.5 % NH_4Cl and 0.25 % NaCl with an initial pH of 5.5 in 500 ml flasks. The flasks were incubated at 30 ± 1 °C on a rotary shaker at 250 rpm for 48, 72, 96 and 120 hours. Experiments were carried out in triplicates. At the end of respective periods of incubation, the cultures were centrifuged (4 °C, 6000 rpm) using a refrigerated centrifuge (Remi cooling centrifuge C-30, Mumbai, India), the supernatant and the cells were separated for enzyme assay. In the case of strains that formed pellets, the pellets were separated from the culture broth by filtration using filter paper (Whatman No-2, 110 mm diameter). The pH of the culture fluid was recorded. The cells / mycelial mats were washed with distilled water and dried at 110 °C to constant weight in a hot air oven (The Andhra Scientific company Ltd., Andhra Pradesh, India) for dry cell weight (DCW) determination. The results were expressed as g/L. The culture fluids as well as the cells were used as enzyme source with out any pretreatment (Hidaka *et al*, 1987).

3. a. 2. 5 Enzyme Assay

3. a. 2.5.1 Fructosyl transferase activity

The reaction mixture for the assay of FTase activity consisted of 1.5 ml of 55 % (w/v) sucrose in 0.1 M citrate buffer (prepared by mixing 0.1 M citric

acid and 0.1 M tri sodium citrate to get pH 5.5) and 0.5 ml crude enzyme - either culture fluid or 1 g (wet weight) of cells suspended in 0.5 ml of 0.1 M citrate buffer, pH 5.5. The reaction was carried out at 55 ± 1 °C for 1 h using a water bath (Inlab Equipments (M) Private Limited, Chennai, India). The reaction was terminated by keeping the reaction mixture in boiling water bath for 15 minutes. Quantitative analysis of the products was done using HPLC (LC –10 A, Shimadzu, Japan) with a refractive index detector using the polar bonded phase column (Supelcosil LC-NH_2, 4.6 mm x 25 cm, 5 μ) and the retention times of the individual FOS were compared with that of standards for identification. The samples were diluted appropriately and filtered through a membrane filter with a pore size of 0.45 μm (Millipore) before injection. One unit of fructosyl transferase activity is defined as the amount of enzyme required to produce 1 μmol of glucose per minute at 55 °C with 55 % sucrose at pH 5.5.

3. a. 2. 5. 2 Hydrolytic activity

The hydrolytic activity was determined following the same procedure used for transfructosylating activity except that the substrate used was 0.5 % sucrose. At the end of the reaction, the amount of glucose released was determined using HPLC. One unit of hydrolytic activity is defined as the amount of enzyme required to release 1μmol of glucose per minute at 55 °C with 0.5 % sucrose at pH 5.5 (Hidaka *et al*, 1987).

3. a. 2. 6 Analytical method

The FOS was analyzed by HPLC at room temperature with acetonitrile:water (75:25) as mobile phase at a flow rate of 1.0 ml min^{-1}. The final FOS was expressed as yield (% w/w) based on the initial sucrose concentration. Data were reported as mean values of triplicate analysis along with standard deviation values.

3. a. 3 RESULTS AND DISCUSSION

3. a. 3.1 Distribution of transfructosylating activity (U_t) and hydrolyzing activity (U_h) in microorganisms

The transfructosylating and hydrolyzing activities in the intracellular (cells) and the extracellular (culture fluid) fractions of all the fungal strains were quantified. The changes in the two activities, pH and cell growth were followed

during 120 hours of fermentation. The distribution of the two activities in the six microorganisms was examined at the end of 48, 72, 96 and 120 hours of incubation.

3.a. 3. 1. a. Aureobasidium pullulans CFR 77

Among the six fungal strains studied, the transfructosylating activity was more in *A. pullulans,* in both extracellular and intracellular fractions as shown in Figure 3. a. 1. a & 3. a. 1. b respectively. The maximum enzyme activity in both extracellular and intracellular fractions was observed at the end of 48 h of growth beyond which it decreased with an increase in fermentation time. Using extracellular FTase, the trisaccharide (GF_2) produced was highest followed by the tetrasaccharide (GF_3) and pentasaccharide (GF_4). However, when intracellular FTase was used, the GF_3 concentration was higher than other constituents. The GF_4 concentration was higher when intracellular FTase was used in comparison with the use of extracellular FTase for the production of FOS. The hydrolytic activity was less compared to transfructosylating activity and varied between 1 & 5 U g cells^{-1} min^{-1} in both extracellular as well as intracellular fraction during the fermentation period. The pH remained almost constant throughout fermentation (3.0 $\pm$ 0.5). The maximum biomass of 21.64 g/L was attained at the end of 72 h of fermentation. The results are presented in Figure 3. a. 2.

3. a. 3. 1. b. Aspergillus oryzae CFR 202

The maximum FTase activity exhibited was almost the same in both extracellular and intracellular fractions in both *A. oryzae* CFR 202 and *A. pullulans* CFR 77 as illustrated in Figures 3. a. 1. a & 3. a. 1. b respectively. The extracellular enzyme activity in *A. oryzae* CFR 202 was maximum at the end of 120 h of growth whereas the intracellular enzyme activity was maximum at the end of 96 h of growth beyond which it decreased. As in the case of *A. pullulans* CFR 77, the concentration of GF_3 and GF_4 were more in the reaction mixture when intracellular enzyme was used. The extracellular hydrolytic activity remained almost the same through out the growth but intracellular activity increased marginally at the end of 120 h. The pH of the culture broth remained almost the same throughout the growth (4.5 $\pm$ 0.5). The biomass formation was maximum (16 g/L) at the end of 72 h of fermentation. The above results are illustrated in Figure 3. a. 3.

Figure 3. a. 1. Fructosyl transferase activity in various fungi by submerge fermentation. (a) Extracellular, (b) Intracellular. AP-*A. pullulans* CFR 77, AO-*A oryzae* CFR 202, AF-*A. flavus* CFR 203, AN-*A. niger*, P-*Penicillium* sp., MM-*M miehei*.

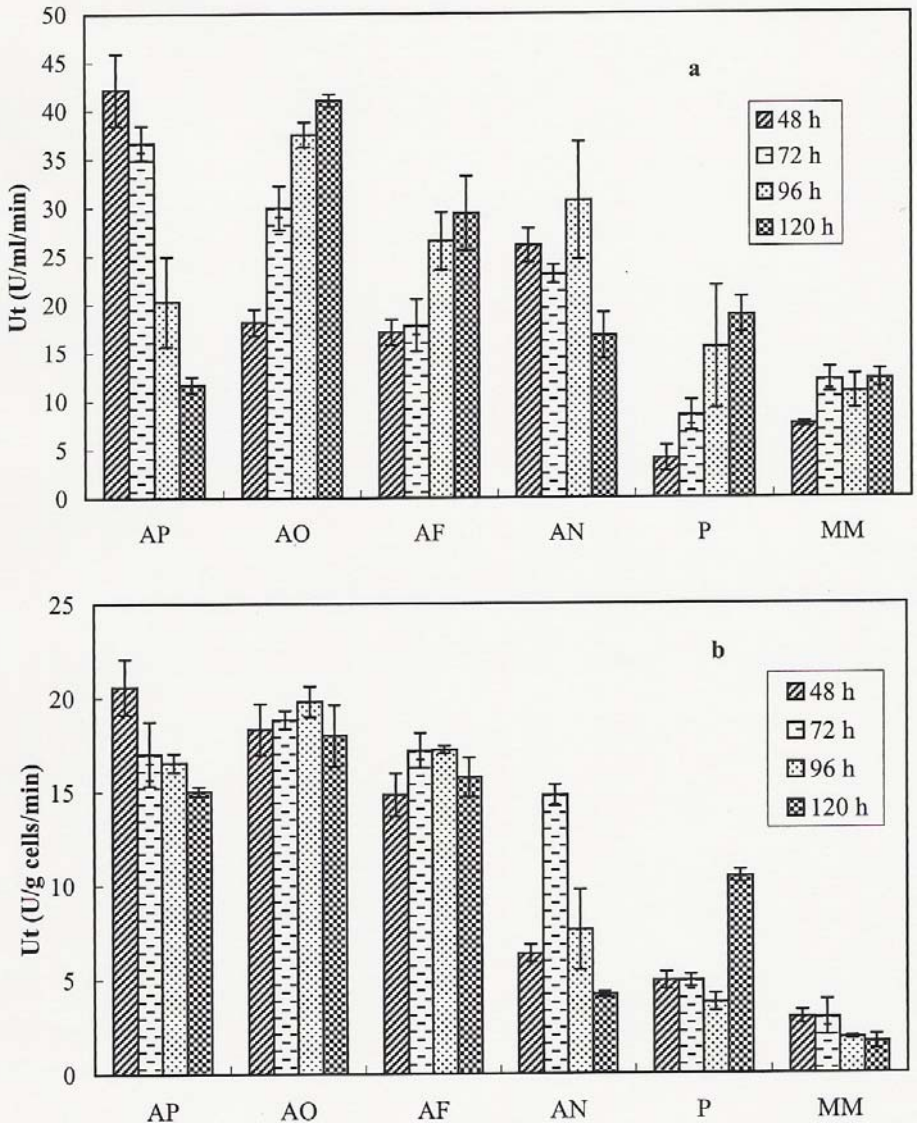


Figure 3. a. 2. Biomass, pH profile and comparison of FOS production using extracellular and intracellular FTase during the growth of *A. pullulans* CFR 77.

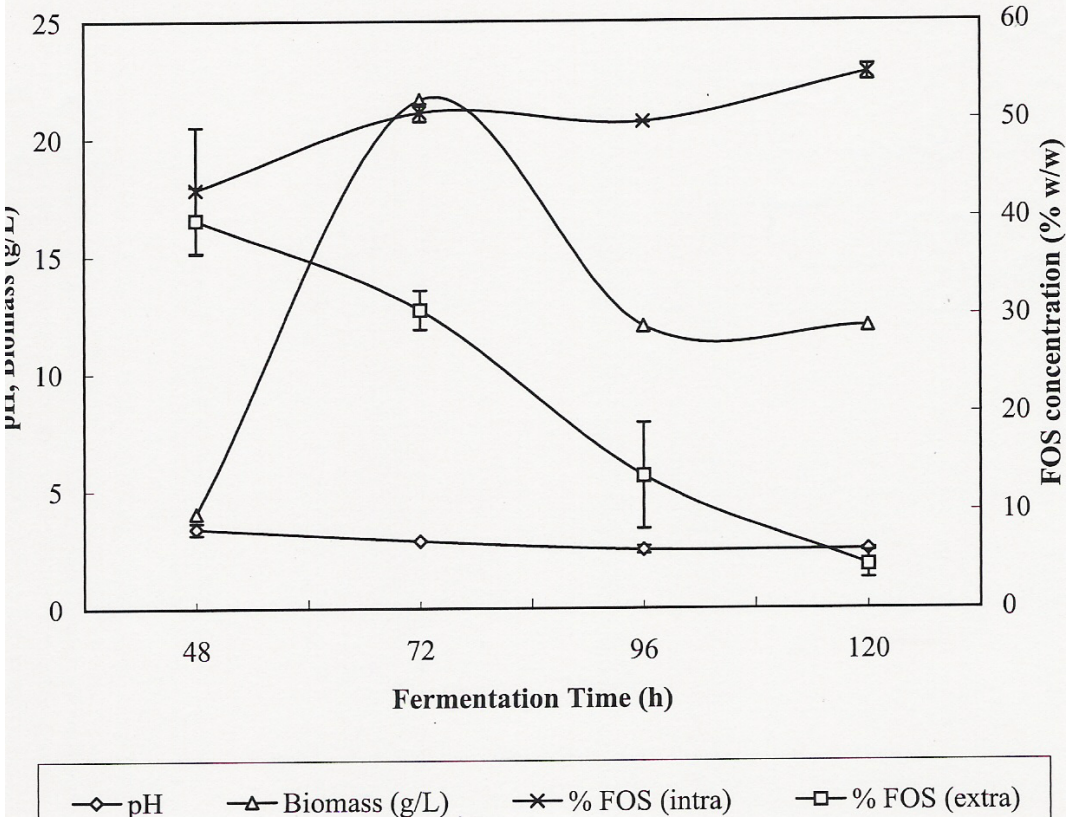
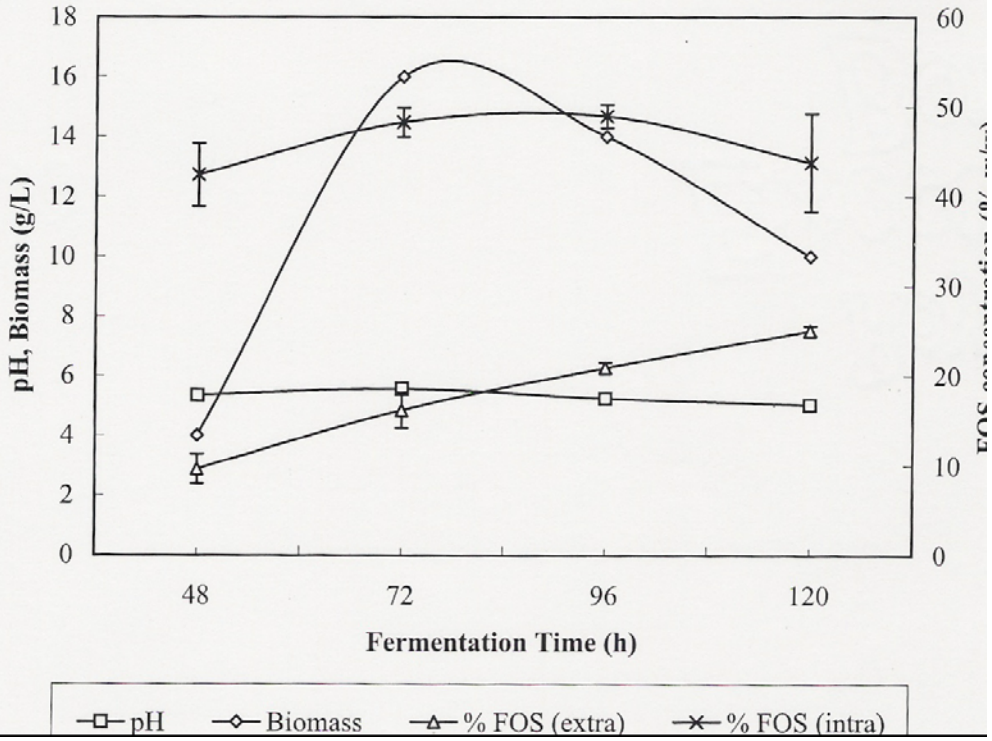


Figure 3. a. 3. Biomass, pH profile and comparison of FOS production using extracellular and intracellular FTase during the growth of *A. oryzae* CFR 202.



3. a. 3. 1. c. Aspergillus flavus CFR 203

In *A. flavus* CFR 203, the extracellular FTase activity was maximum at the end of 120 h of growth and the intracellular activity was maximum at the end of 96 h of growth as shown in Figure 3. a. 1. a & 3. a. 1. b respectively. The concentration of GF_3 and GF_4 were less than GF_2. The hydrolytic activity remained the same through out growth in both intracellular and extracellular fractions. The pH of the culture broth remained the same through out growth (5.57 $\pm$ 0.1), while biomass increased significantly at the end of 72 h and reached a maximum of 52 g/L at the end of 120 h of fermentation. The results are illustrated in Figure 3. a. 4.

The other three strains – *A. niger*, *Penicillium* sp. and *M. miehei* exhibited comparatively less FTase activity and were not suitable for FOS production The extracellular and intracellular FTase activities present in these strains are presented in Fig 3. a. 1. a & 3. a. 1. b). The FOS yield (% w/w) produced using the FTase from these strains is reported in Table 3. a. 1.

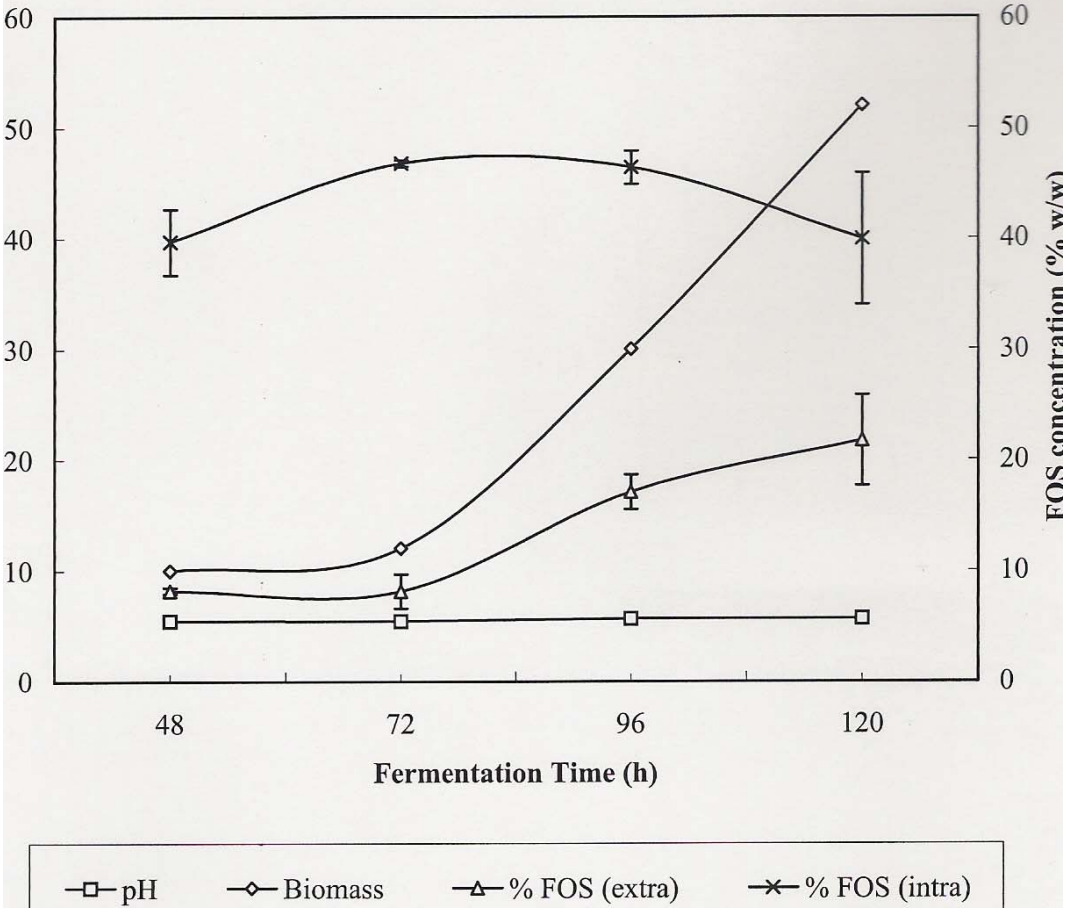
Table 3.a.1. FOS yields (% w/w) obtained using extracellular and intracellular FTase from *Penicillium* sp., *M. miehei* and *A. niger*.

Microorganism	Source of FTase	FOS (% w/w)			
		Fermentation time (h)			
		48	72	96	120
Penicillium sp.	E	2.54 $\pm$ 1.01	4.63 $\pm$ 0.33	4.39 $\pm$ 3.35	0.96 $\pm$ 0.53
	I	2.26 $\pm$0.24	2.54 $\pm$ 0.11	7.22 $\pm$ 0.99	1.45 $\pm$ 0.74
M. miehei	E	1.29 $\pm$ 0.8	6.37 $\pm$ 1.81	0.72 $\pm$ 0.41	3.17 $\pm$ 0.37
	I	9.3 $\pm$ 0.88	8.36 $\pm$ 4.37	4.19 $\pm$ 2.54	3.88 $\pm$ 0.29
A. niger	E	0.33 $\pm$ 0.26	1.52 $\pm$ 1.9	5.18 $\pm$ 1.95	4.88 $\pm$ 3.42
	I	1.61 $\pm$ 0.24	11.8 $\pm$ 3.51	2.35 $\pm$ 0.4	4.32 $\pm$ 1.29

E - extracellular, I - intracellular

The screening presented here demonstrates that transfructosylating activity, which is responsible for the production of FOS from sucrose exists in various microorganisms. However, there were significant differences among

Figure 3. a. 4. Biomass, pH profile and comparison of FOS production using extracellular and intracellular FTase during the growth of *A. flavus* CFR 203.



the activity, the enzyme of *A. pullulans* CFR 77*, A. oryzae* CFR 202 and *A. flavus* CFR 203 being the most efficient for the production of FOS.

Both the transfructosylating and hydrolyzing activities in extracellular and intracellular fractions are widely distributed in various strains of microorganisms. *A. pullulans* CFR 77*, A. oryzae* CFR 202 *and A. flavus* CFR 203 exhibited high FTase activity compared to *A. niger, Penicillium sp.* and *M. miehei.* In *A. pullulans* CFR 77 and *A. oryzae* CFR 202, the amount of tetrasaccharide (GF_3) produced in the reaction mixture was more when intracellular enzyme was used. This could be because the trisaccharide (GF_2) is produced in more quantities, which thereby act as the acceptor for further oligomerization to produce more of GF_3. This is in accordance with the observation made earlier (Jung *et al*, 1989) where the enzyme is said to follow disproportionation type reaction. In the case of *A. flavus* CFR 203, the concentration of GF_3 and GF_4 were less than GF_2. This is because the concentration of GF_2 formed was not enough to act as acceptor to promote the formation of more of the higher oligomers.

From the results presented here, it is clear that *A. oryzae* CFR 202 and *A. flavus* CFR 203 enroll as new sources of FTase. The safe commercial use of *A. oryzae* CFR 202 as a 'koji mold' is well established. These new sources can add up to the existing sources of FTase. Fructosyl transferases commonly possess both transfructosylating (U_t) and hydrolyzing activity (U_h) (Edelman, 1956). Transfructosylating activity is responsible for the production of FOS and hydrolytic activity results in hydrolysis of sucrose to glucose and fructose. Although transfructosylating activity exists in three of these fungal strains, it is desirable to have a high U_t / U_h ratio for the efficient production of FOS (Hidaka *et al*, 1987). Therefore, the strength of transfructosylating activity of a strain is indicated by the ratio of transfructosylating activity to hydrolytic activity, which can be represented as U_t/U_h. As can be seen from the data in Table 3. a. 2, the U_t/ U_h ratio in *A. oryzae* CFR 202 and *A. flavus* CFR 203 are higher than that of *A. pullulans* CFR 77. These data indicate that these strains are more suitable for the preparation of FOS.

Since the production and application of FOS have gained tremendous commercial importance because of their favorable functional properties, there is always scope to search for newer and potential sources of fructosyl transferase. From the present study, it is evident that FTase from *A. oryzae* CFR 202 and *A. flavus* CFR 203 can add to the existing sources of FTase for FOS production.

A high sucrose concentration is required for transfructosylation reaction to take place in order to deviate the enzyme from its hydrolytic mode of action to transfructosylating mode. There have been many reports specifying that sucrose concentrations ranging from 40 % (w/v) to 77 % (w/v) are used as substrate for both batch and continuous process of FOS production to obtain high yields of FOS. The present study has indicated that transfructosylation reaction proceeded even at 55 % (w/v) sucrose concentration leading to the formation of 50 % (w/w) of FOS.

Table 3. a. 2. U_t/U_h ratio in different microorganisms screened for FTase activity

Microorganism	Fermentation Time (h)			
	48	72	96	120
A. pullulans CFR 77	13.8	13.39	6.42	9.48
A. oryzae CFR 202	11.33	16.95	20.36	22
A. flavus CFR 203	12.74	11.01	17.87	16.27
Penicillium sp.	10.83	13.28	15.6	11.4
M. meihei	10.28	11.86	11.64	11.05
A. niger	20.73	9.54	11.6	8.67

U_t – Transfructosylating activity; U_h – Hydrolytic activity

3. a. 4. CONCLUSIONS

A few fungal strains were screened for fructosyl transferase activity and *Aureobasidium pullulans* CFR 77, *Aspergillus oryzae* CFR 202 and *Aspergillus*

flavus CFR 203 were found to be with maximum titres of FTase activity. Based on high U_t/U_h ratio, *A. oryzae* CFR 202 was selected as the best source for further studies. *A. niger*, *Penicillium* sp. and *M. miehei* exhibited comparatively less FTase activity and hence are not suitable for FOS production

3. b. FOS PRODUCTION BY *A. ORYZAE* CFR 202 AND *A. PULLULANS* CFR 77.

3. b. 1. INTRODUCTION

Several microorganisms have been reported to possess transfructosylating activity to produce FOS from sucrose (Prapulla *et al*, 2000). Earlier studies indicated that *A. oryzae* CFR 202, *A. pullulans* CFR 77 and *A. flavus* CFR 203 are potential sources of FTase based on high U_t/U_h ratio. This section deals with the production of FOS using culture fluid, cells as well as culture broth homogenate from *A. oryzae* CFR 202 and *A. pullulans* CFR 77 grown in media containing sucrose as the major carbon source. The effect of two different concentrations of sucrose used as substrate for the production of FOS is also described.

3. b. 2. MATERIALS AND METHODS

Chemicals, Microorganisms and culture conditions, Production of FTase enzyme and Enzyme assay were as described in Sections 3. a. 2.1 to 3. a. 2. 5.

3. b. 2. 1. Crude enzyme preparation

The culture fluid, cells and culture broth homogenate were used as enzyme source. The culture fluids and cells were used with out any pretreatment (Hidaka *et al*, 1987). The culture broth homogenate was prepared as follows. *A. oryzae* CFR 202 formed pellets during submerged fermentation. These pellets were separated by filtration, macerated and sonicated with the culture broth (B Braun Labsonic U Sonicator, Germany) at 20 kHz for 15 min at 4 °C. The homogenate obtained was then centrifuged at 6000 rpm for 20 min at 4 °C to get the cell free lysate which was used as the enzyme source. The culture broth homogenate of *A. pullulans* CFR 77 was prepared by subjecting the culture broth to sonication and centrifugation as described above to get the cell free lysate which was used as the enzyme source.

3. b. 2. 2. Production of FOS

FOS production was carried out using sucrose at two different concentrations (55 % and 80 % w/v). The extracellular and intracellular enzymes as well as the cell free lysate from *A. oryzae* CFR 202 (0.5 ml culture fluid, 0.5 ml cell free lysate and 1 g wet cells in 0.5 ml 0.1 M citrate buffer of pH

5.5 respectively) were mixed with 1.5 ml sucrose and incubated at 55 °C in a water bath. The reaction was carried out as a function of time from 1 to 36 h. Analysis of the reaction products was done as described in section 3. a. 2. 5. 1 and 3. a. 2. 6.

3. b. 3. RESULTS AND DISCUSSION

3. b. 3. 1 Production of FOS by A. oryzae CFR 202

Production of FOS catalyzed by extracellular FTase from *A. oryzae* CFR 202 at two different sucrose concentrations (55 % and 80 % w/v) was studied (Figure 3. b. 1). When extracellular enzyme was used, sucrose was rapidly converted into FOS as the reaction progressed beyond 1 h. The maximum FOS concentration was observed at the end of 12 h of reaction, which corresponded to 54 % of the initial sucrose. At the end of 12 h, the concentration of GF_2 decreased gradually while GF_3 and GF_4 increased. Transfructosylating reactions were substantially inhibited by glucose due to accumulation in the later stages of reaction (Chen and Liu, 1996). Hence, the maximum yield of FOS obtained was 54 % of the initial sucrose concentration. It has been demonstrated that the yield obtained using fructosyl transferase is limited to only 55- 60 %, as the glucose liberated during the enzymic reaction acts as a competitive inhibitor (Yun and Song, 1993). The forward reaction leading to the formation of FOS can be enhanced if glucose inhibition is removed. However, in the present study, the maximum yield of FOS was obtained at the end of 12 h of reaction, thereby reducing 50 % of the reaction time. When cells were used as the enzyme source, the FOS concentration decreased as the reaction proceeded beyond 1 h as shown in Figure 3. b. 2 using 55 % and 80 % sucrose as substrate respectively.

Figure 3. b. 1. Time course of the production of FOS using extracellular FTase from *A. oryzae* CFR 202 using different sucrose concentrations. (Line graph represents the FOS yields using 80 % sucrose and bar graphs indicate the FOS yields using 55 % sucrose).

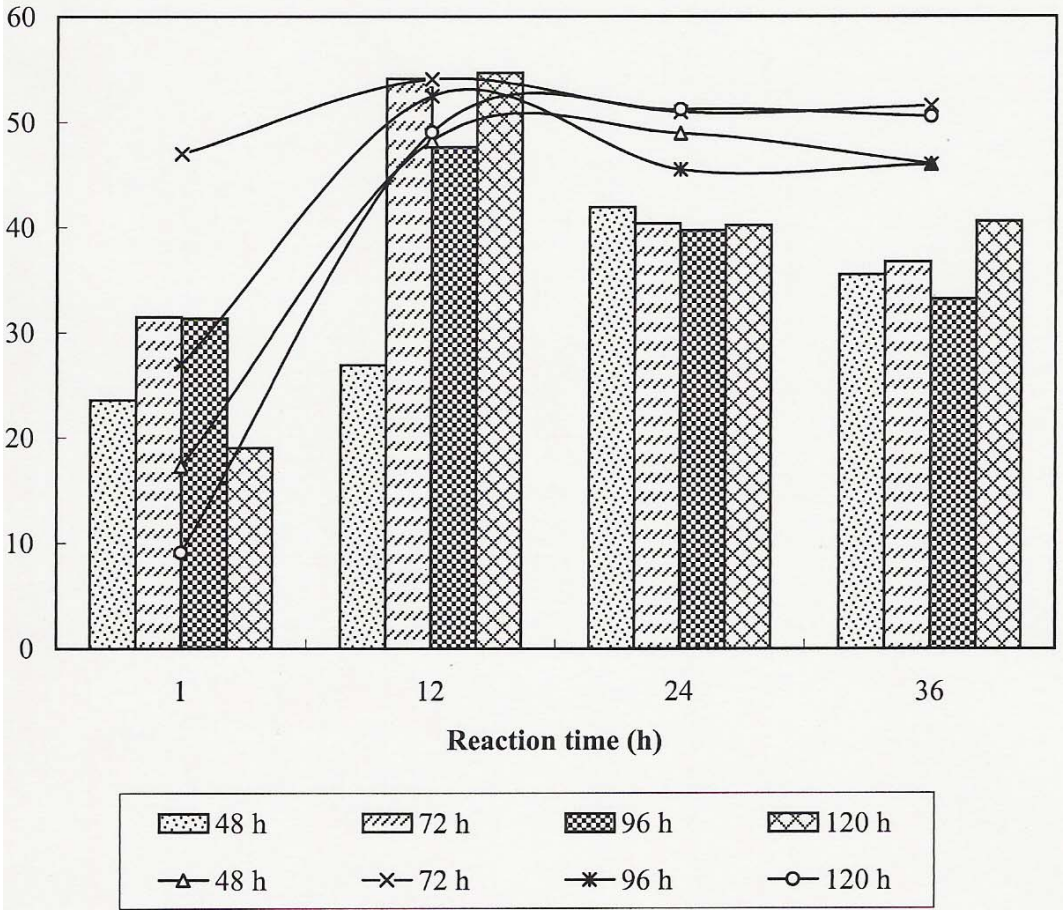
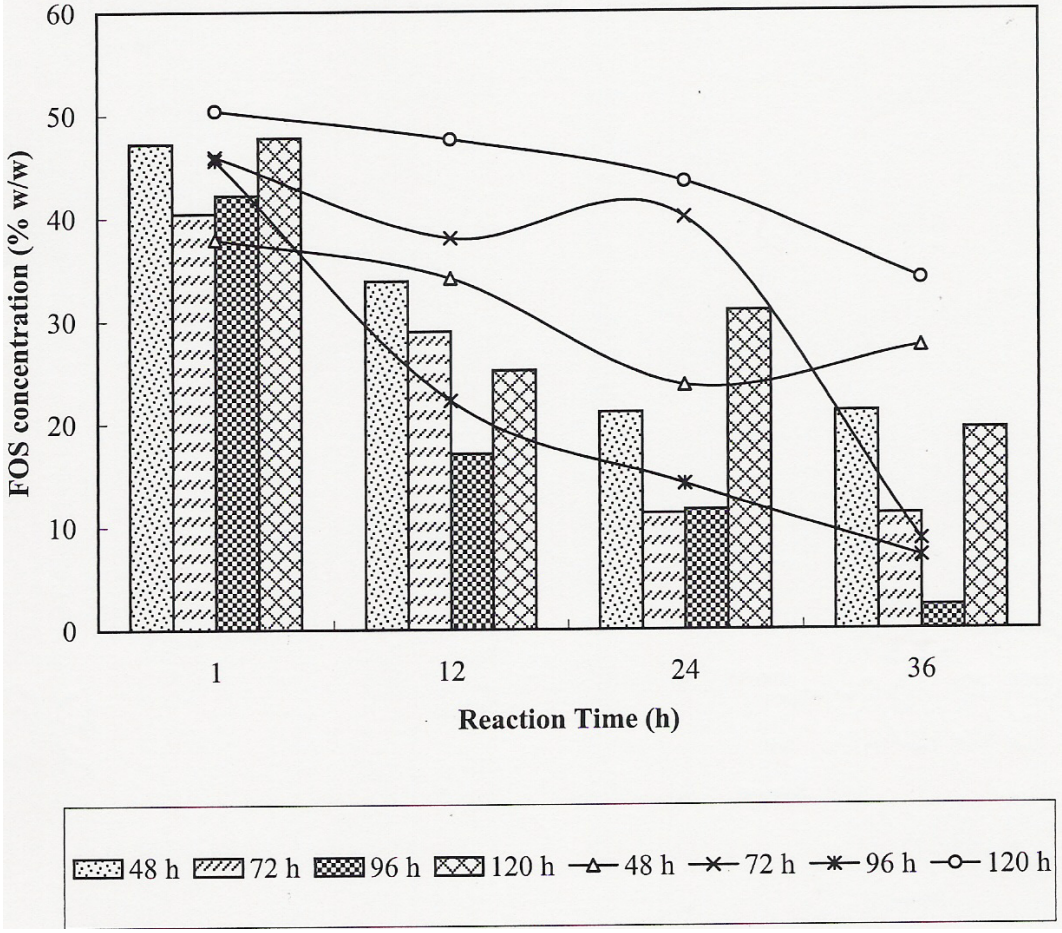


Figure 3. b. 2. Time course of the production of FOS using cells of *A. oryzae* CFR 202 using different sucrose concentrations. (Line graph represents the FOS yields using 80 % sucrose and bar graphs indicate the FOS yields using 55 % sucrose).



When culture broth homogenate of *A. oryzae* CFR 202 was used as enzyme source, the maximum yield of FOS was obtained at the end of 12 h of reaction with both 55 % and 80 % sucrose (Figure 3. b. 3). Production of FOS using homogenized cell suspension of *A. japonicus* has been demonstrated and the maximum FOS yield obtained was 60 % of the initial sucrose when 5 % and 50 % sucrose were used as substrate (Cheng *et al,* 1996). The present study is more elaborate giving the details of composition of FOS obtained, with an emphasis on the presence of GF_5 (Table 3. b. 1). Figure 3. b. 4 illustrates the HPLC profile of the reaction mixture obtained using extracellular enzyme at the end of 12 h of incubation with 55 % sucrose.

Table 3.b.1. Composition of FOS # (% w/w) obtained using culture fluid, cells and culture broth homogenate of *A. oryzae* CFR 202 as enzyme source at different sucrose concentrations.

Source of enzyme	F	G	GF	GF_2	GF_3	GF_4	GF_5	Total FOS
A. oryzae CF 55 % sucrose	---	31	14.6	31.67	17.57	4.88	--	54.12
A. oryzae CF 80 % sucrose	---	25	21	36.5	15.9	1.71	---	54.11
A. oryzae CBH 55 % sucrose	---	35	11.28	26.09	21.44	4.53	1.17	53.23
A. oryzae CBH 80 % sucrose	---	41.9	11.1	20.02	22.41	4.32	0.25	47

- FOS yield obtained after a reaction time of 12 h; CF-Culture Fluid; CBH-culture broth homogenate

3. b. 3. 2 Production of FOS by A. pullulans CFR 77

When extracellular enzyme was used, the maximum FOS concentration was observed at the end of 24 h of reaction (Figure 3. b. 5) which, corresponded to 56 % of the initial sucrose (55 % w/v). The composition of the reaction mixture was as follows. GF_2 –23.54 %, GF_3 – 25.76 %, GF_4 – 5.88 % and GF_5 – 0.99 % (Table 3. b. 2).

Figure 3. b. 3. Time course of the production of FOS using culture broth homogenate of *A. oryzae* CFR 202 using different sucrose concentrations (Line graph represents the FOS yields using 80 % sucrose and bar graphs indicate the FOS yields using 55 % sucrose).

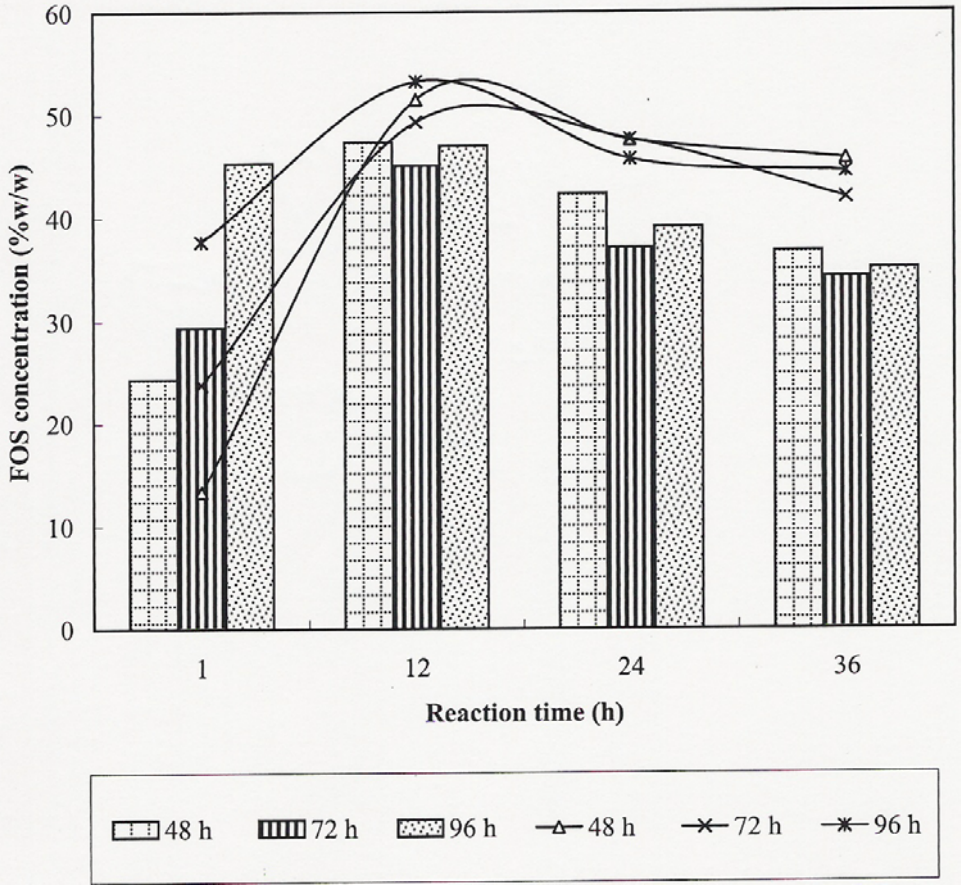


Figure 3. b. 4. Typical HPLC chromatogram of reaction mixture at the end of 12 h incubation with extracellular enzyme from *A. oryzae* CFR 202 incubated with 55 % sucrose under the conditions defined in Section 3. a. 2. 5. 1. G-Glucose, GF- Sucrose, GF_2-Kestose, GF_3-Nystose and GF_4-Fructofuranosyl nystose.

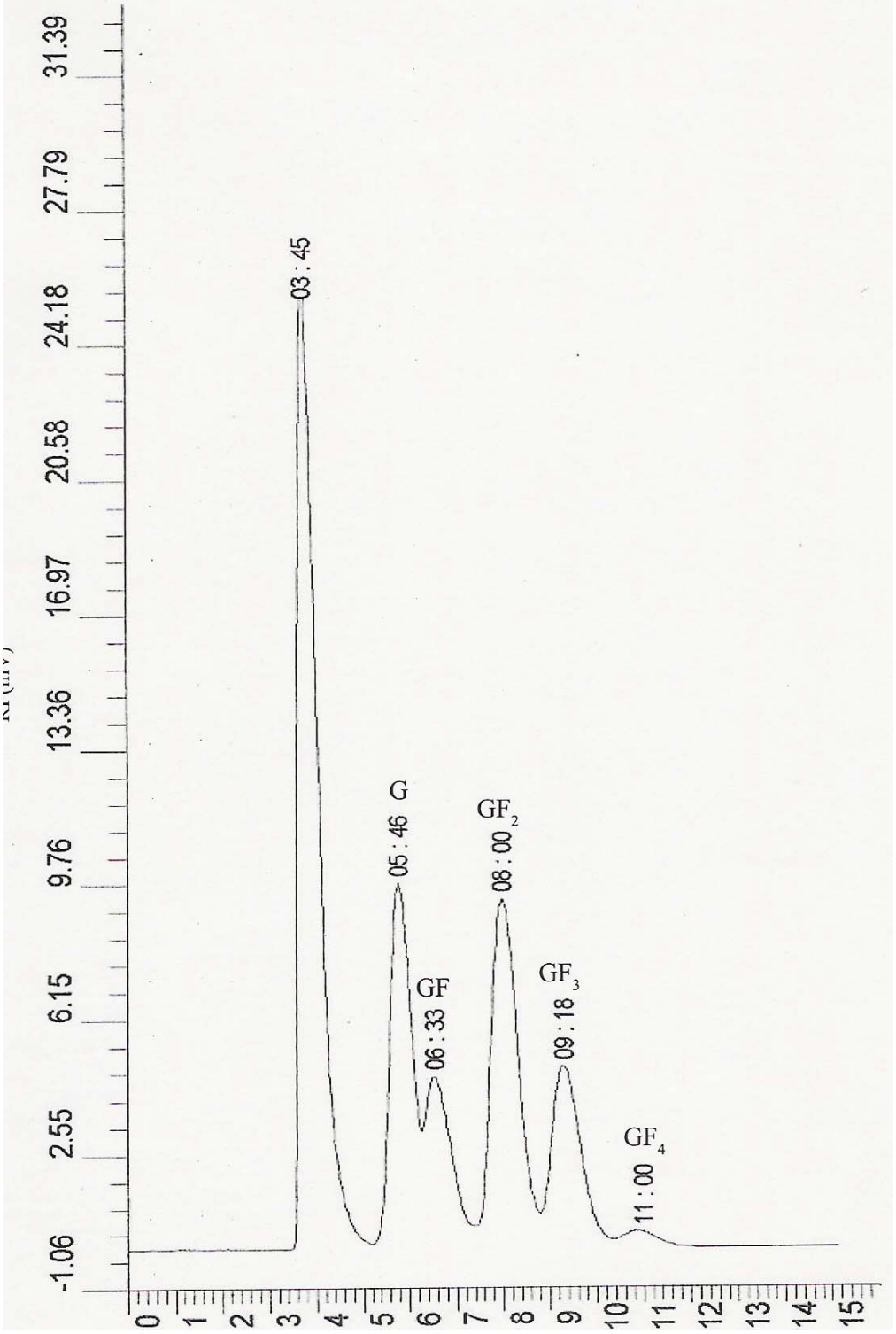


Figure 3. b. 5. Time course of the production of FOS using extracellular FTase from *A. pullulans* CFR 77 using different sucrose concentrations. (Line graph represents the FOS yields using 80 % sucrose and bar graphs indicate the FOS yields using 55 % sucrose).

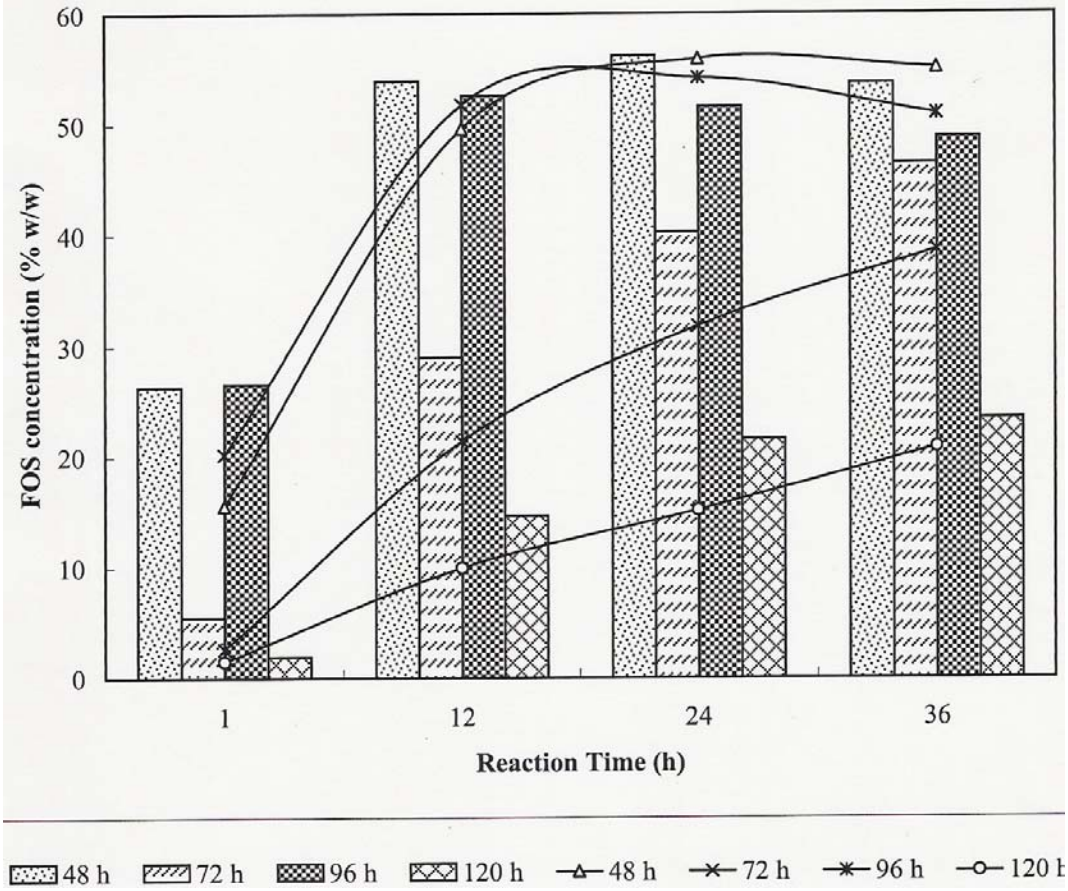


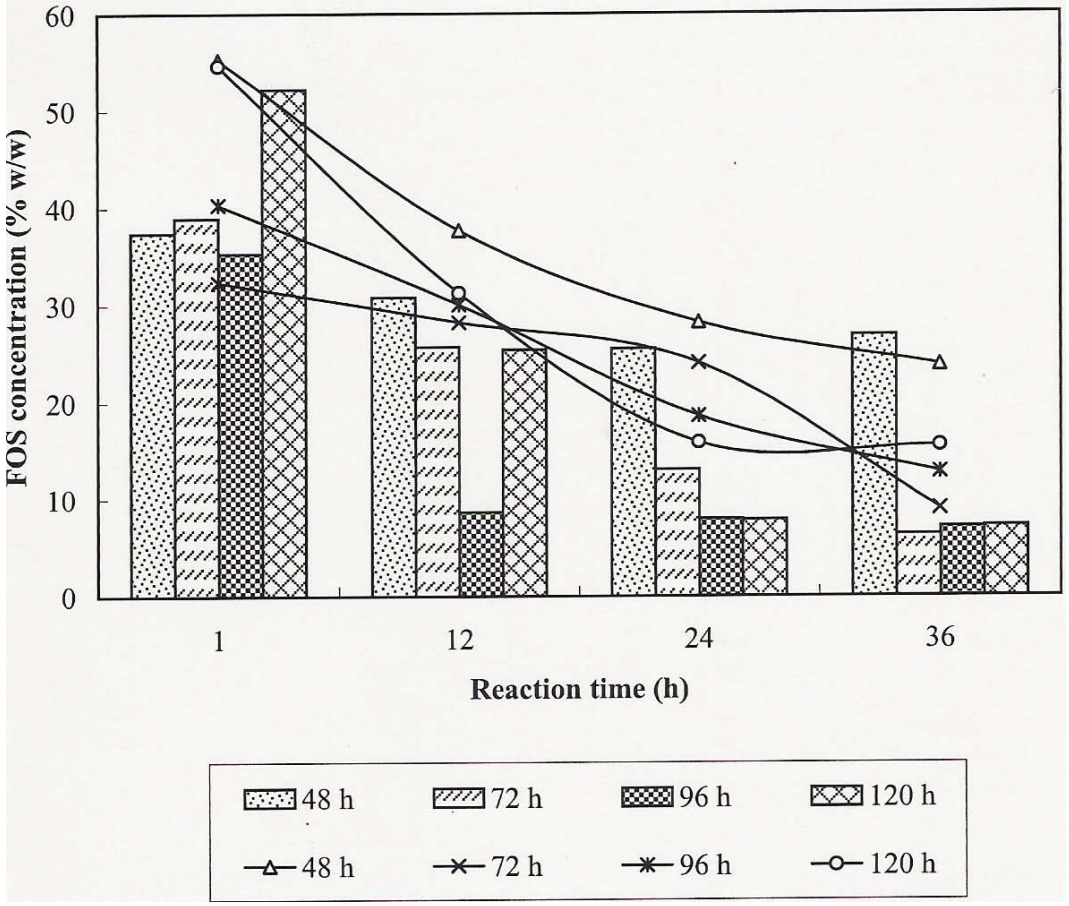
Table 3.b.2. Composition of FOS (% w/w) in the reaction mixture produced using culture fluid * and culture broth homogenate $ of *A. pullulans* CFR 77

Source of enzyme	F	G	GF	GF_2	GF_3	GF_4	GF_5	Total FOS
A. pullulans CF 55 % sucrose	---	31.25	12.6	23.54	25.76	5.88	0.99	56.17
A. pullulans CF 80 % sucrose	---	30.19	13.9	28.11	23.36	4.00	0.42	55.89
A. pullulans CBH 55 % sucrose	0.11	35.42	10.72	26.30	22.55	4.35	0.56	53.76
A. pullulans CBH 80 % sucrose	0.09	33.16	10.12	21.84	25.46	7.71	1.62	56.63

* - FOS yield obtained after a reaction time of 18 h; $ - FOS yield obtained after a reaction time of 12 h; CF-Culture Fluid; CBH-culture broth homogenate

When 80 % sucrose was used as substrate, the maximum FOS yield was obtained at the end of 24 h of reaction using 48-h old culture fluid (Figure 3. b. 5). The total FOS yield was 55.9 % in which GF_2 concentration was more thereby resulting in lesser concentration of the higher saccharides (Table 3. b. 2). When cells of *A. pullulans* CFR 77 were used for FOS production, the FOS concentration was found to decrease at the end of 1 h of reaction (Figure 3. b. 6). The cells of *A. pullulans* CFR 77 did not give high FOS yields at the end of 1 h of reaction. This indicates that the enzyme is intracellular and some pretreatment of cells is required for it to be available for reaction. Hence, cell lysis is required for the reaction to proceed because cells per se are unable to carry out the reaction beyond 1 h. When intracellular enzyme from *A. pullulans* KFCC 10524 was used for FOS production, the maximum yield of FOS ranged from 55-60 % at the end of 25 h of reaction (Yun and Song, 1993).

Figure 3. b. 6. Time course of the production of FOS using cells from *A. pullulans* CFR 77 using different sucrose concentrations. (Line graph represents the FOS yields using 80 % sucrose and bar graphs indicate the FOS yields using 55 % sucrose).



When culture broth homogenate of *A. pullulans* CFR 77 was used for FOS production, the maximum FOS yields were obtained at the end of 12 h of reaction with both 55 % sucrose and 80 % sucrose (Figure 3. b. 7). The maximum FOS yield obtained was up to 54 % using 55 % sucrose and up to 57 % using 80 % sucrose. The composition of these FOS mixtures is given in Table 3. b. 2. In the present study using culture broth homogenate, up to 57 % yield has been obtained just at the end of 12 h of reaction. Also, so far there has been no report on the use of culture broth homogenate of *A. pullulans* for FOS production.

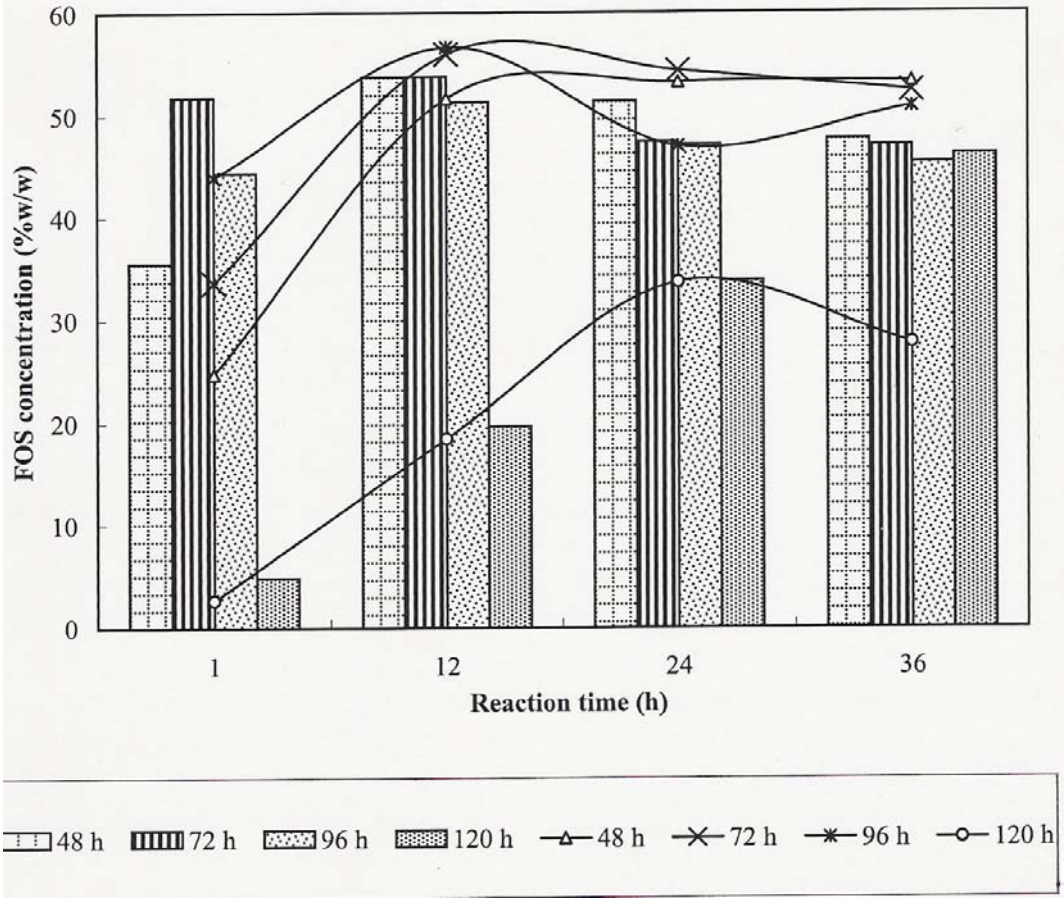
3. b. 3. 3 Effect of sucrose concentration on FOS production

The effect of sucrose concentration on the reaction products was studied by analyzing the saccharide compositions in the reaction mixture at two different sucrose concentrations. As shown in Table 3. b. 1, when extracellular FTase from *A. oryzae* CFR 202 was used, the concentration of individual FOS oligomers produced with 55 % sucrose as substrate were 31.67 $\pm$ 2 % GF_2, 17.57 $\pm$ 1 % GF_3 and 4.88 $\pm$ 0.5 % GF_4. Using 80 % sucrose as substrate, the composition of FOS were 36.46 $\pm$ 1.6 % GF_2, 15.89 $\pm$ 1.5 % GF_3 and 1.71 $\pm$ 0.6 % GF_4. This shows that transfructosylation reaction proceeded even in a 55 % sucrose solution. When culture broth homogenate from *A. oryzae* CFR 202 was used as FTase source, the concentrations of GF_2, GF_3, GF_4 and GF_5 were 26.1 $\pm$ 2 %, 21.44 $\pm$ 1.6 %, 4.48 $\pm$ 0.5 % and 1.17 $\pm$ 0.2 % respectively when 55 % sucrose was used as substrate (Table 3. b. 1). The presence of GF_5 in trace amounts is the highlight of this experiment. Studies on the effect of two different concentrations of sucrose showed that transfructosylation reaction proceeded even in a 55 % sucrose solution. At the end of 1 h reaction, the amount of FOS formed was higher when a substrate concentration of 55 % sucrose was used (illustrated as histogram), in comparison to that at 80 % sucrose concentration (Figure 3. b. 3 and Figure 3. b. 7).

When the cells, culture fluid and culture broth homogenate of *A. oryzae* CFR 202 were used as sources of FTase, it was found that extracellular enzyme gave the maximum FOS yield at the end of a reaction time of 12 h. The

cells were not found to be suitable for FOS production since the FOS yields

Figure 3. b. 7. Time course of the production of FOS using the culture broth homogenate of *A. pullulans* CFR 77 using different sucrose concentrations. (Line graph represents the FOS yields using 80 % sucrose and bar graphs indicate the FOS yields using 55 % sucrose).



decreased as the reaction time proceeded beyond 1 h. The culture broth homogenate also gave almost the same yield like the extracellular enzyme. The presence of higher oligomers like GF_5 was noted when culture broth homogenate was used. In the case of *A. pullulans* CFR 77, culture fluid and culture broth homogenate proved to be good for obtaining more than 55 % yield of FOS. There have been many reports on the production of FOS by FTases from *Aureobasidium* sp. (Yun, 1996). The source of enzyme had been either the culture fluid (extracellular) or the cells (intracellular). However, in the present study, since FTase activity was found in both extra and intracellular fractions, the whole culture broth was homogenized and the homogenate was used as the enzyme source for FOS production. This reduced the reaction time from 25 h to 12 h to obtain yield of FOS up to 57 %.

This study has thus demonstrated the use of different sources of FTase from *A. oryzae* CFR 202 and *A. pullulans* CFR 77 – in the form of culture fluid, cells and culture broth homogenate - for FOS production. Both the culture fluid and culture broth homogenate have resulted in the production of 54 – 57 % w/w of FOS based on the initial sucrose concentration.

3. c. OPTIMIZATION STUDIES ON FTase AND FOS PRODUCTION BY *A. ORYZAE* CFR 202.

3. c. 1. SELECTION OF SIGNIFICANT CULTURAL AND NUTRITIONAL PARAMETERS INFLUENCING FTase AND FOS PRODUCTION BY PLACKETT – BURMAN DESIGN.

3. c. 1. 1. INTRODUCTION

A. oryzae CFR 202 was selected as a potent strain for FOS production based on the high U_t / U_h ratio exhibited by the enzyme. The enzyme was further used for the production of FOS using different concentrations of substrate (sucrose). The extracellular FTase from *A. oryzae* CFR 202 was found to be suitable for carrying out efficient production of FOS in less than 24 h. Development of an efficient process for the production of FOS involves a study on the influence of nutritional parameters on high FTase titers as well as the reaction parameters for the maximization of FOS yields.

Studies on the influence of various nutrients by conventional single dimensional search involve changing one independent variable at a time while fixing the others at constant levels. This practice is time consuming, tedious and practically impossible when the number of variables is more. Application of statistical methods involving specific design of experiments can effectively tackle this problem. Plackett-Burman design (Plackett and Burman, 1944) is a well-established and widely used statistical technique for such an application (Rosiero *et al*, 1992). This design identifies the important variables affecting the production of fructosyl transferase as well as FOS on incubation with sucrose as substrate.

Plackett-Burman saturated orthogonal designs work at two levels, and can be constructed based on fractional replication of a full factorial design. This allows a reliable short listing of a small number of ingredients for further optimization. It also facilitates to obtain unbiased estimates of linear effects of all the factors with maximum accuracy for a given number of observations, the accuracy being the same for all effects (Akhnazarova and Kafarov, 1982). Plackett Burman design has been applied in several studies to optimize the media components in both submerged (Krishnan *et al*, 1998; Venugopal and Chandra, 2000) and solid state fermentation (Srinivas *et al*, 1994).

A number of reports are available on the production of FOS using fructosyl transferase and β-fructofuranosidase enzymes from different microbial sources (Patil and Patil, 1999; Yun, 1996). The effect of magnesium sulfate on the ratio of intra- to extracellular enzyme production in *Aureobasidium pullulans* has been reported (Jung *et al*, 1987). The effect of nutritional conditions on the production of β-fructofuranosidase by *Aureobasidium* sp. ATCC 20524 for the optimization of enzyme production has also been reported (Hayashi *et al*, 1993). However, not many reports are available on the effect of media components for the production of fructosyl transferase enzyme and reaction conditions for the production of FOS. The section deals with the study of the effect of media components on FTase production and reaction conditions on FOS production by *Aspergillus oryzae* CFR 202 using Plackett-Burman design.

3. c. 1. 2. MATERIALS AND METHODS

3. c. 1. 2. 1. Microorganism and characteristics

The microorganism used in this study is *A. oryzae* CFR 202. The organism was grown on PDA slants as well as plates to determine its growth characteristics.

3. c. 1. 2. 1. 1. Scanning electron microscopy

Scanning electron microscopy of pellets of *A. oryzae CFR 202* produced by submerged fermentation was carried out by the following procedure. The pellets obtained were first fixed using 2.5 % (v/v) glutaraldehyde for 4 – 24 h and washed with 0.1 M phosphate buffer (pH – 7.0). The pellets were then dehydrated by washing with a gradient of ethanol (20, 40, 60, 80 and 100 % v/v) for 15 min each and dried in a dessicator. The specimen coated with a thin layer of gold using Polaron SEM coating system was observed with a Scanning Electron Microscope (LEO 435, LEO Electron Microscopy Ltd., Cambridge, England).

3. c. 1. 2. 1. 2. Aflatoxin analysis

Aflatoxin analysis was carried out in the culture fluid of *A. oryzae* CFR 202 by Thin Layer Chromatography (TLC). To extract aflatoxin, equal volume of methanol was added to the culture fluid and kept until extraction. The sample was then transferred to a separating funnel and extracted three to four times with dichloromethane. The dichloromethane fraction containing aflatoxin was evaporated to dryness and dissolved in chloroform to do TLC. TLC was carried out using 95 % chloroform and 5 % methanol as mobile phase. The plates were viewed in UV chamber.

Chemicals and inoculum development were as mentioned in Section 3. a. 2. 1 and 3. a. 2. 3.

3. c. 1. 2. 2. Fermentation:

Fermentation medium (100 ml in 500 ml flasks) containing sucrose, yeast extract, magnesium sulfate, sodium nitrate, di-potassium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride and ammonium chloride (composition based on the design) was sterilized at 121 °C for 15 min and inoculated with the appropriate quantity of inoculum as per the design. These flasks were incubated at 30 ± 1 °C at 250 rpm for the specified time period. The cultures were harvested and filtered using Whatman filter paper No. 2 to separate the pellets from the culture fluid, which was later used as a source of fructosyl transferase. The pH of the culture fluid was recorded and the dry cell weight was obtained after washing the pellets with distilled water and drying to constant weight at 110 °C in a hot air oven (The Andhra Scientific Company, India).

Fructosyl transferase activity and hydrolytic activity were assayed as described in Section 3. a. 2. 5. 1 and 3. a. 2. 5. 2.

3. c. 1. 2. 3. FOS production:

FOS production was carried out by incubating the extracellular fructosyl transferase with sucrose in 0.1 M citrate buffer. The concentration of substrate

(sucrose) and enzyme, the temperature, pH and the duration of reaction were according to the design. The reaction was stopped by keeping the reaction mixture in boiling water bath for 15 min.

3. c. 1. 2. 4. Studies on the influence of various parameters:

Studies on the influence of media components on FTase production and reaction parameters on FOS production were carried out in two steps *i.e.* model development and model validation. The experiments and the responses under model development were utilized to develop the appropriate equation. Using this equation, values were predicted for the second set of experiments for model validation, for different values of the variables. The high and low values of the variables in the two experiments are shown in Table 3. c. 1. 1.

Plackett Burman design with sixteen variables comprising of eight media components and eight reaction parameters was used for model development. The sequences of level + and level – for the first experiment was in the following order

+ - + + - - - - + - + - + + + + - - + +

and the consecutive sequences were obtained by circular permutation by shifting the first row cyclically to the left one place and then finally adding a row of minuses. The total number of experiments to be carried out was K+1 where K is the number of variables. Each variable was represented at two levels, a high level denoted by (+) and a low level designated by (-). The design corresponding to 20 experiments was chosen including three dummy variables to estimate the variance and standard error. The dummy variables are unassigned factors, and are used to obtain an estimate of variance. The lower and upper levels of concentration of each parameter were selected based on literature information and the preliminary screening experiment. The first set of experiments are carried out based on the values taken for model development. From the responses obtained, the effects of each variable is calculated as follows

$$E_1 = \frac{\sum \text{Responses at (+)}}{\text{Number of values (+)}} - \frac{\sum \text{Responses at (-)}}{\text{Number of values (-)}}$$

Where E_1 is the effect of variable 1. From this the equation for predicting the responses is obtained as follows

$y_p = y_a + ½ (E_1X_1 + E_2X_2 + \ldots\ldots + E_nX_n)$

where y_p is the predicted value of response, y_a is the average value of response, E_1, E_2, ….E_n are the effects of variables X_1, X_2, ….X_n respectively. The equation is used to predict the responses in the model validation experiments also. The effect of each variable was determined and those with confidence levels greater than 80 % were considered to influence FOS production significantly.

Table 3. c. 1. 1. Ingredients/parameters chosen for the model development and model validation experiments with their maximum (+) and minimum (-) values.

	Variables	Model development		Model validation	
		+	-	+	-
X_1	Sucrose (%)	25.00	15.00	20.00	10.00
X_2	Yeast extract (%)	00.60	00.20	00.80	00.40
X_3	$NaNO_3$ (%)	03.00	01.00	05.00	02.00
X_4	Mg $SO_4.7H_2O$ (%)	00.08	00.02	00.05	00.03
X_5	K_2HPO_4 (%)	00.50	00.10	00.60	00.40
X_6	KH_2PO_4 (%)	00.50	00.10	00.60	00.40
X_7	NaCl (%)	00.50	00.10	00.60	00.40
X_8	NH_4Cl (%)	00.75	00.25	01.00	00.50
X_9	pH of the medium	05.50	05.00	05.50	05.00
X_{10}	Inoculum (%)	15.00	05.00	20.00	10.00
X_{11}	Fermentation time (h)	96.00	48.00	120.00	72.00
X_{12}	Substrate concentration (%)	80.00	50.00	70.00	60.00
X_{13}	Enzyme concentration (ml)	01.00	00.50	00.75	00.25
X_{14}	Reaction time (h)	12.00	01.00	18.00	06.00
X_{15}	Temperature (°C)	60.00	50.00	55.00	45.00
X_{16}	pH of substrate	06.00	05.00	05.50	04.50

X_1-X_{16} – Variables screened, X_{17}-X_{19} – Dummy variables (Variables that are not assigned any values and are used to estimate experimental error).

3. c.1. 2. 5. Statistical Analysis:

The results of model validation experiment were evaluated using three statistical values (Ramesh, 2000):

(1) Root mean square deviation (RMSD)

(2) Standard deviation of difference (S_D) and

(3) The average percent error (E).

3. c. 1. 2. 6. Analytical procedure:

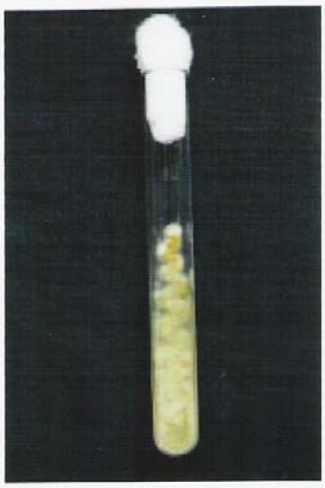
The FOS was analyzed as given in Section 3. a. 2. 5 and 3. a. 2. 6. The final FOS was expressed as the yield (% w/w) based on the initial sucrose concentration. The retention times of the individual FOS were compared with that of authentic standards of 1-kestose (GF_2), 1-nystose (GF_3) and 1-fructofuranosyl nystose (GF_4) for identification.

3. c. 1. 3. RESULTS AND DISCUSSION

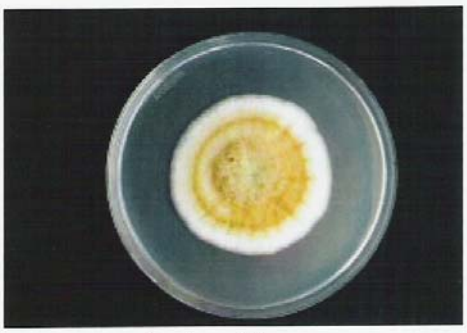
A. oryzae CFR 202, selected for this investigation, is an aerobic filamentous fungus. *A. oryzae* grows vegetatively as haploid multinucleate filaments designated hyphae or mycelia. Hyphae extend at the apical tips and multiply by branching, covering the surface of agar medium. The colony is initially white, when grown on solid medium, because of vegetative hyphal growth, then turns yellowish green as the conidia which bear spores are formed in large numbers. Figure 3. c. 1. 1. illustrates the growth of *A. oryzae* CFR 202 on slant and plate containing PDA medium and in the liquid medium used for FTase production. Scanning electron micrographs of *A. oryzae* CFR 202 pellets grown by submerged fermentation is shown in Figure 3. c. 1. 2. The surface of the pellets show swollen vesicles bearing specialized cells that produce asexual spores called conidiospores. Inspite of its close relatedness to the aflatoxin-producing members of the group, *A. oryzae* is not known to produce aflatoxins and is used in fermented food manufacturing. Figure 3. c. 1. 3. shows the thin layer chromatograph of *A. oryzae* CFR 202 culture fluid, showing the absence of aflatoxin. *A. oryzae* has been widely used for the industrial production of a variety of enzymes and also fermented foods. It is accepted as a microorganism having GRAS (Generally Regarded As Safe) status (Gomi, 1999).

Figure 3.c.1.1. *A. oryzae* CFR 202 grown on PDA (a) slant (b) plate and (c) medium for FTase production.

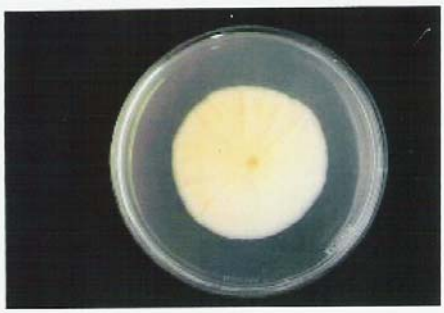
(a)



(b)



Surface View



Reverse View

(c)

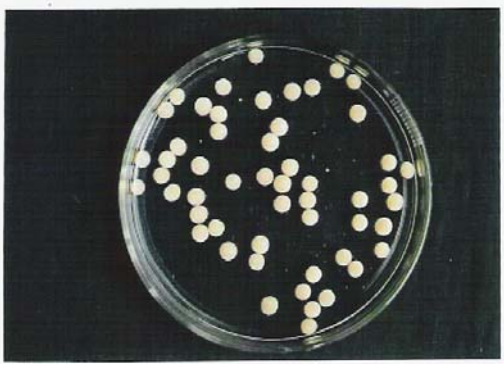
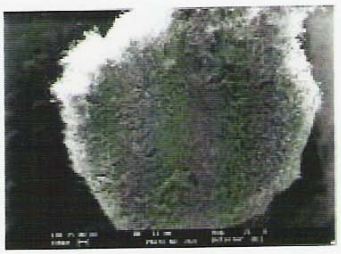


Figure 3.c.1.2. Scanning electron micrographs of *A. oryzae* CFR 202 (a) pellet (b) cross section of the pellet (c) surface view of the pellet (d) core of the pellet showing vegetative hyphae (e) enlarged view of the vesicles forming conidiospores.

(a) Magnification 22 x



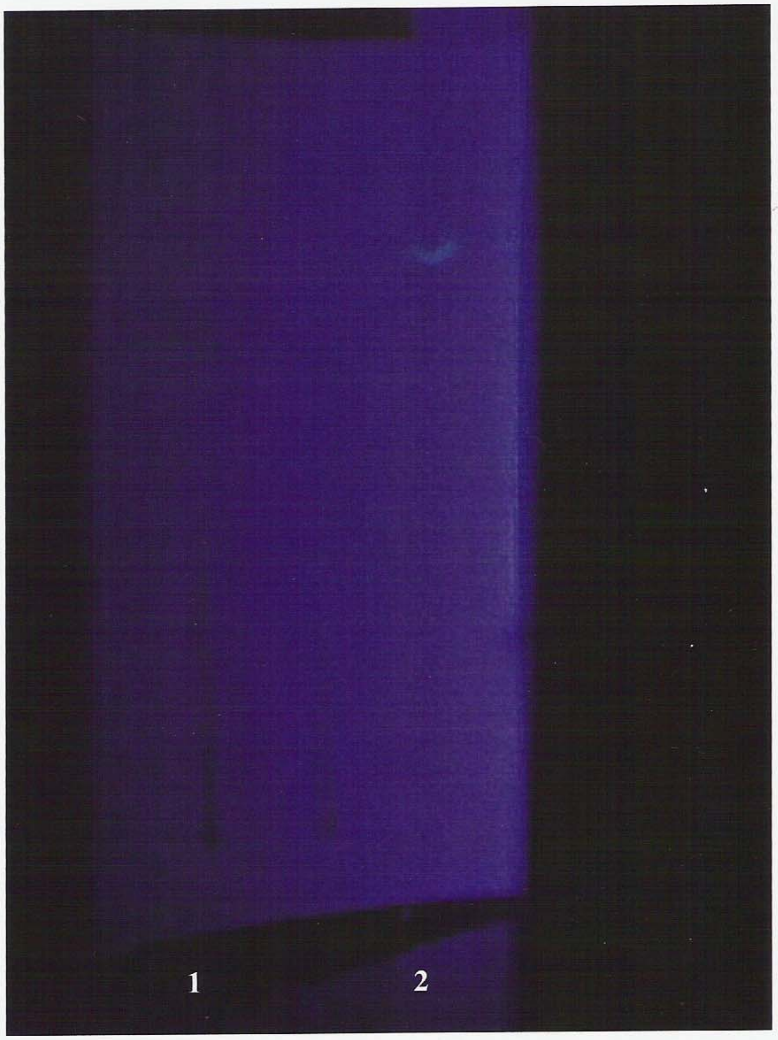
(b) Magnification 21 x

(c) Magnification 170 x

(d) Magnification 500 x

(e) Magnification 861 x

Figure 3.c.1.3. Thin layer chromatogram showing the distinct absence of aflatoxin in the culture fluid of *A. oryzae* CFR 202.



1. Culture fluid of *A.oryzae* CFR 202
2. Standard aflatoxin

3. c. 1. 3. 1. Effect of variables on FOS production

The effect of variables on FOS production is shown in Figure 3. c. 1. 4. This corresponds to FOS yields, which varied from 21.5 g/L to 435.68 g/L corresponding to 4.3 % to 54.46 % (w/w) of the initial sucrose respectively. In the first experiment, the highest yield of FOS was obtained in experiment no: 5 followed by experiment no: 10, 14, 16 and 7 in that order. Using statistical analysis, the variables that had a positive effect on FOS production are reaction time, fermentation time, pH of the reaction mixture, KH_2PO_4 and sucrose in the fermentation medium, pH of the fermentation medium and K_2HPO_4 in the fermentation medium in their decreasing order.

3. c. 1. 3. 2. Changes in pH, biomass, U_t and U_h:

The final pH of the medium, biomass, U_t and U_h of the crude enzyme (culture fluid) were monitored at the end of the respective fermentation time in each run. In the first set of experiments carried out for model development, the pH and biomass were maximum in trial no. 5 (Table 3. c. 1. 2). U_t was highest in trial no. 7. This trial showed highest U_h also. During model validation experiment, pH was highest in trial no. 5. Maximum biomass was found in run no.10. Highest U_t and U_h values were observed in run no. 15 (Table 3. c. 1. 2).

3. c. 1. 3. 3. Regression coefficients and t values

The regression model for the yield Y of FOS is given by the following equation

$$Y = B_o + \sum_{i=1}^{16} B_i X_i$$

where B_o is a constant and B_i are the respective regression coefficients and X_i represents the 16 variables in Table 3. c. 1. 1. The above model equation can be written as

$$\% \text{ Yield} = 28.773 + 4.69\,X_1 + 0.3884\,X_2 + 1.376\,X_3 - 0.316\,X_4 - 4.001\,X_5 + 5.344\,X_6 + 0.292\,X_7 + 2.489\,X_8 - 4.207\,X_9 + 3.085\,X_{10} + 9.286\,X_{11} - 0.553\,X_{12} + 3.213\,X_{13} + 19.121\,X_{14} - 0.615\,X_{15} + 6.533\,X_{16}.$$

Figure 3. c. 1. 4 FOS yield obtained in the 20 different trials of model development experiment using different combinations of media ingredients / reaction parameters.

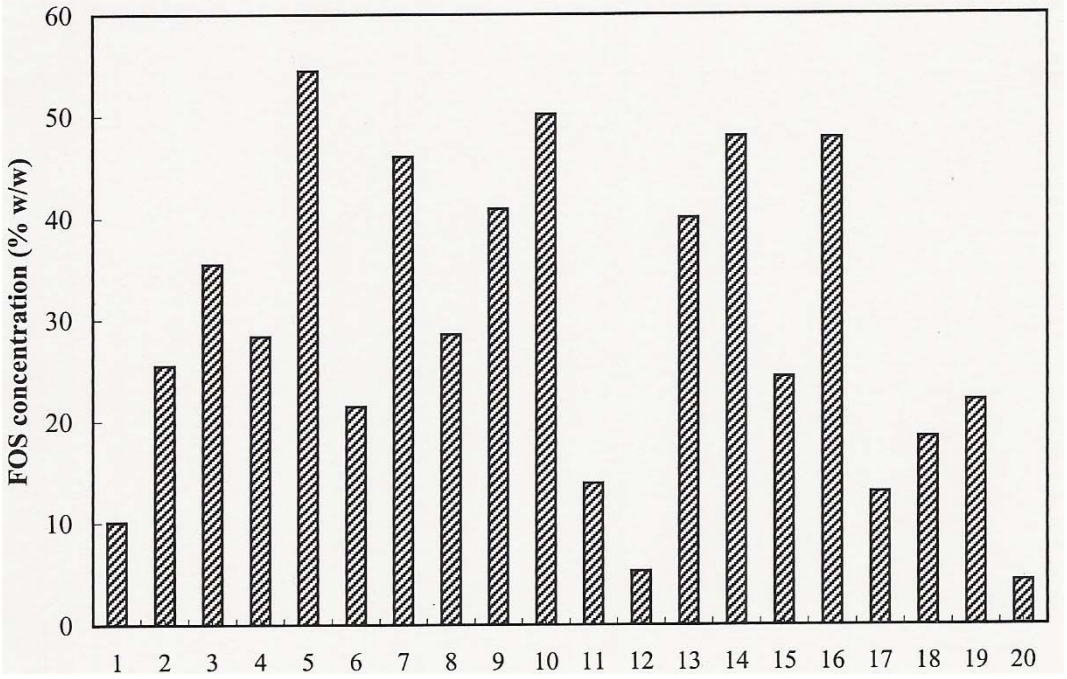


Table 3.c.1.2. Effect of the different combinations of media ingredients/parameters on final pH, biomass, U_t (transfructosylating activity) and U_h (hydrolytic activity) in model development and model validation experiments.

Model development					Model validation			
Exp No	Final pH	Biomass (g/L)	U_t (U/ml/min)	U_h (U/ml/min)	Final pH	Biomass (g/L)	U_t (U/ml/min)	U_h (U/ml/min)
1	5.08	08.01	16.50	1.25	5.40	15.05	24.98	0.10
2	5.85	13.65	54.02	1.22	6.01	21.08	42.95	0.05
3	5.21	05.03	45.83	1.26	5.36	08.56	38.46	0.10
4	5.45	13.08	65.48	1.52	5.86	21.31	66.49	0.11
5	6.42	15.10	63.73	1.35	6.34	17.06	54.01	0.05
6	5.64	06.87	44.28	1.32	5.62	13.69	40.48	0.05
7	5.19	07.28	94.48	1.49	5.75	19.08	64.29	0.07
8	5.22	05.69	58.96	1.12	5.86	12.69	62.62	0.09
9	5.17	07.70	78.04	1.23	5.66	14.63	66.11	0.12
10	5.97	14.31	55.17	1.27	6.11	27.78	49.93	0.06
11	5.21	07.20	19.59	1.23	5.36	14.87	33.19	0.18
12	4.43	11.20	48.30	0.92	6.57	21.80	50.20	0.09
13	5.59	10.36	68.19	1.25	5.49	14.24	74.12	0.16
14	5.64	13.79	88.80	1.53	6.01	18.92	67.92	0.14
15	5.63	05.33	40.28	1.23	5.48	09.19	85.41	0.18
16	5.51	04.43	66.63	1.31	5.55	12.64	69.63	0.17
17	5.84	07.29	32.97	1.33	6.38	15.68	37.94	0.06
18	5.52	05.52	18.20	1.29	5.88	10.04	19.97	0.06
19	5.37	08.55	29.96	1.45	5.65	18.54	20.42	0.17
20	5.64	05.67	05.30	0.92	5.88	12.35	17.52	0.13

The t-values for the 16 ingredients are presented in Table 3. c. 1. 3 which shows that reaction time, fermentation time, pH of the reaction mixture and KH_2PO_4 and sucrose in the fermentation medium have positive effect on the production of FOS. These five parameters have a threshold t-value close to 1.

Table 3.c.1.3. t-values of the variables in model development experiment.

Variables	Ingredient	t values
X_1	Sucrose (%)	0.95
X_2	Yeast extract (%)	0.78
X_3	Sodium nitrate (%)	0.27
X_4	Magnesium sulfate (%)	0.06
X_5	K_2HPO_4 (%)	0.81
X_6	KH_2PO_4 (%)	1.08
X_7	Sodium chloride (%)	0.05
X_8	Ammonium chloride (%)	0.50
X_9	pH of medium	0.85
X_{10}	Inoculum (%)	0.62
X_{11}	Fermentation time (h)	1.88
X_{12}	Substrate concentration (%)	0.11
X_{13}	Enzyme concentration (ml)	0.65
X_{14}	Reaction time (h)	3.87
X_{15}	Temperature (°C)	0.12
X_{16}	pH of reaction mixture	1.32

3. c. 1. 3. 4. Ingredients/ Parameters influencing FTase production

KH_2PO_4 and sucrose in the fermentation medium were found to have positive effect on FOS production (t-values of 1.08 and 0.95 respectively). This shows that sucrose is an important ingredient required for the expression of FTase. The effect of minerals on enzyme production has also been reported (Hayashi *et al*, 1993).

Fermentation time also had a positive effect (t-value of 1.88) on the production of FOS. This could be attributed to the specific property of the enzyme present in different microorganisms. In our experiments, it was observed that FOS yield was comparatively more when the fermentation was carried out for 96 h rather than stopping at the end of 48 h (Run no. 2, 4, 5, 6, 7, 10, 11, 13, 14 and 19 of model development experiment, Figure 3. c. 1. 4).

3. c. 1. 3. 5. Parameters influencing FOS production

The most positive effect of reaction time is indicated by the t-value of 3.87. The enzyme fructosyl transferase acts on sucrose in a disproportionate type reaction and hence the reaction time has a significant effect on yield of FOS. As the reaction proceeds, the higher oligomers formed (GF_3 and GF_4) also serve as the acceptor of fructose. Similar observation on the effect of reaction time has been reported earlier (Yun and Song, 1993; Barthomeuf and Pourrat, 1995) where the maximum FOS yield was obtained at the end of 24 h and 10 h of reaction respectively.

The pH of the reaction mixture also influenced the FOS yield (t-value of 1.32). This shows that pH played an important role during transfructosylation reaction. There have been reports on the optimum pH required for the FTase enzyme to be between 5 and 6.5 (Yun, 1996).

3. c. 1. 3. 6. Validation experiment:

The second set of experiments was carried out to compare the experimental values with the predicted values derived from the results of I^{st} experiment. The highest yield obtained in this experiment was 58 % (w/w) (Table 3. c. 1. 4), in which GF_2, GF_3, GF_4 and GF_5 represented 27 %, 25 %, 5.35 % and 0.5 % respectively. The residual sucrose and glucose constituted 14 % and 27 % of the total mixture respectively. The higher oligomer, GF_5 that was not produced in any of the earlier experiments, was found to be produced in trace amounts in this particular combination of nutrients (run no 10), which is an interesting finding. When variables with confidence levels above 80 % (reaction time and fermentation time) were considered, the results of 17 experiments out of 20 were close to the predicted values (Table 3. c. 1. 4). The statistical parameters indicate the goodness of fit of the model in predicting the values, wherein the RMSD value was 7.74 %, the S_D value was 7.90 % and the average percent error was only 0.033 %.

The highest FOS yield obtained in this experiment is 58 % w/w based on the initial sucrose concentration as against 25 % w/w in the initial screening experiments (Figure 3. a. 3). This corresponded to an increase of 33 % in FOS

yield. A 35 % increase in riboflavin yield by *Erymothecium ashbyii* UV mutant and a 73 % increase in α-galactosidase yield by *Aspergillus niger* MRSS 234 have been reported using this technique (Venugopal and Chandra, 2000; Srinivas *et al*, 1994).

3. c. 1. 4. CONCLUSIONS

An attempt has been made to carry out a set of 20 experiments, firstly for model development and another 20 experiments for validation of the results for the selection of parameters that significantly affect the yield of FOS. The study has brought out the important parameters influencing the production of FTase as well as FOS with the help of a single experimental design. KH_2PO_4 and sucrose in the fermentation medium and fermentation time were found to influence FTase production. In order to get higher titres of FTase, it was necessary to carry out fermentation beyond 96 h. FTase activity was found to be higher when the concentration of sucrose was low in the media. KH_2PO_4 concentration in the media was found to be more (0.5 – 0.6 %) in those trials which gave higher FOS yields. Hayashi *et al* (1993) have reported that sucrose at higher concentration in the media inhibited the growth of *Aureobasidium* sp. ATCC 20524 and decreased the amount of enzyme produced. They have also observed that optimum concentration of K_2HPO_4 for enzyme production by *Aureobasidium* sp. was 0.5 – 0.75 % w/v. pH of the reaction mixture and reaction time were found to have significant effect on FOS production. FOS production was more when the pH of the reaction mixture was in the range 5.5 – 6.0. It was also observed that FOS yield increased with the increase in reaction time. All the five above mentioned variables with t-values above 0.95 were selected for further optimization by response surface methodology, although only two variables (reaction time and fermentation time) were found to have confidence level above 80 %.

Table 3.c.1.4 Comparison of the experimental yield of FOS with the predicted yield obtained from model validation experiment.

Run no.	Predicted yield (% w/w)	Experimental yield (% w/w)
1	27.91	30.42
2	37.20	48.10
3	48.75	38.38
4	37.20	43.12
5	58.04	57.30
6	37.20	40.48
7	58.04	55.31
8	48.75	41.89
9	48.75	45.02
10	58.04	58.27
11	37.20	39.37
12	27.91	47.30
13	58.04	47.40
14	58.04	51.01
15	27.91	42.56
16	48.75	46.64
17	48.75	53.04
18	27.91	32.74
19	37.20	30.48
20	27.91	27.34

3. c. 2. OPTIMIZATION OF FOS PRODUCTION BY *A. ORYZAE* CFR 202 USING RESPONSE SURFACE METHODOLOGY

3. c. 2. 1. INTRODUCTION

Different approaches have been used in the development of predictive equations to describe the effects of various media ingredients and cultural factors on the production of metabolites. Response surface methodology (RSM) has been applied in biotechnological studies to optimize the medium components for the production of xanthan by *Xanthomonas campestris* (Roseiro *et al*, 1992), endoglucanase by *Trichoderma reesei* QM 9414 etc (Sinha and Panda, 1998).

In the previous section, use of Plackett Burman design to select the important media components and parameters influencing the production of FTase and FOS is detailed. A 33 % increase in FOS yield was obtained in the experiment in which sucrose and KH_2PO_4 in the media, fermentation time, reaction time and pH of the reaction mixture were identified as significant parameters influencing FTase and FOS production.

The aim of the present study was to determine the effects and interactions of sucrose, KH_2PO_4, fermentation time, reaction time and pH of the reaction mixture and their combinations on the production of FTase and subsequently the production of FOS. A Doehlert experimental Shell Design (Doehlert, 1970) was used to determine the synergistic combination of five of the above mentioned parameters required for the optimum production of FOS.

3. c. 2. 2. MATERIALS AND METHODS

Chemicals, Microorganism and Inoculum development were as described in Section 3. a. 2. 1 to 3. a. 2. 3.

3. c. 2. 2. 1. Fermentation

20 % (v/v) inoculum was transferred into the fermentation medium containing sucrose and KH_2PO_4 in different concentrations as given in the design. The fermentation time was also based on the design (Table 3. c. 2. 1).

Table 3.c.2.1. Coded and actual values of independent variables in the experimental shell design for FOS production

Coded	x_1	KH_2PO_4 (%)		-1	-0.5	0	0.5	1	
Actual				0.2	0.4	0.6	0.8	1	
Coded	x_2	Sucrose (%)	-0.8661	-0.584	-0.298	0 12	0.29 16	0.58 20	0.87 24
Actual									
Coded	x_3	Fermentation	-0.816	-0.61	-0.2	0	0.2	0.61	0.82
Actual		Time (h)	36	48	60	72	84	96	108
Coded	x_4	Reaction	-0.7911	-0.6334	-0.1588	0 12	0.16 16	0.63 20	0.79 24
Actual		Time (h)							
Coded	x_5	pH			-0.7755	0 5.5	0.78 6		
Actual									

All other media constituents and parameters were kept constant in all the experiments (Table 3. c. 2. 2) based on our previous experiments. Fermentation, harvesting of the cultures and monitoring of pH and dry cell weight are described in Section 3. a. 2. 4.

3. c. 2. 2. 2. FOS production

FOS production was carried out as described in Section 3. b. 2. 2. The pH of the reaction mixture and the duration of reaction were according to the design (Table 3. c. 2. 3).

Analytical procedure is as described in Section 3. a. 2.

Table 3.c.2.2. Values of the constant variables involved in the production of FOS

Ingredient/ Parameter	Values
Yeast extract (%)	0.80
$NaNO_3$ (%)	2.00
$MgSO_4$. $7H_2O$ (%)	0.03
K_2HPO_4 (%)	0.40
NaCl (%)	0.60
NH_4Cl (%)	1.00
PH	5.00
Inoculum concentration (%)	20.0
Substrate concentration (%)	60.0
Enzyme concentration (ml)	0.25
Temperature (°C)	55.0

3. c. 2. 2. 3. Experimental design and statistics

The variables chosen for the study were those selected based on a preliminary screening experiment using Plackett Burman design (Section 3. c. 1). Preliminary studies were carried out to determine specific levels of each parameter and a Doehlert experimental design was selected (Doehlert, 1970). This experimental matrix displayed a uniform distribution of the points within the experimental domain and allowed a number of distinct levels of each variable. Maximal number levels were assigned to the most important factors. The variables investigated were KH_2PO_4 concentration (x_1, five levels), sucrose (x_2, seven levels), fermentation time (x_3, seven levels), reaction time (x_4, seven levels) and pH of the reaction mixture (x_5, three levels). The total number of experiments for five factors was 34 with three repetitions at the center point (0,0,0,0,0).

3. c. 2. 2. 4. Data analysis

The multivariate model was developed using Corel Word Perfect Suite 8 (1997, Corel Corporation, Dublin, Ireland). Analyses were carried out using MS Excel (200, version 5.0, Microsoft Corporation, USA). The roots of the canonical equations were obtained using Poly bas program (Constantinides, 1987).

3. *c. 2. 2. 5. Validation of the model*

Predicted values were compared with the experimental values at the stationery points derived from the model. Validation was carried out with two sets of experiments. In the first set, the stationery points for the five significant parameters were derived based on the canonical analysis. In the second set of experiments, the five significant parameters were selected based on the contour plots to get maximum yields.

Table 3.c.2.3. Experimental design of five independent variables in coded and actual values

Run No	Variables									
	x_1		x_2		x_3		x_4		x_5	
	Coded	Actual	Coded	Actual	Coded	Actual	Coded	Actual	Coded	Actual
1	0.0	0.6	0.000	12.0	0.000	72.0	0.000	12.0	0.000	5.5
2	1.0	1.0	0.000	12.0	0.000	72.0	0.000	12.0	0.000	5.5
3	0.5	0.8	0.866	24.0	0.000	72.0	0.000	12.0	0.000	5.5
4	0.5	0.8	0.289	16.0	0.816	108.0	0.000	12.0	0.000	5.5
5	0.5	0.8	0.289	16.0	0.204	84.0	0.791	24.0	0.000	5.5
6	0.5	0.8	0.289	16.0	0.204	84.0	0.158	16.0	0.775	6.0
7	-1.0	0.2	0.000	12.0	0.000	72.0	0.000	12.0	0.000	5.5
8	-0.5	0.4	-0.866	1.0	0.000	72.0	0.000	12.0	0.000	5.5
9	-0.5	0.4	-0.289	8.0	-0.816	36.0	0.000	12.0	0.000	5.5
10	-0.5	0.4	-0.289	8.0	-0.204	60.0	-0.791	1.0	0.000	5.5
11	-0.5	0.4	-0.289	8.0	-0.204	60.0	-0.158	8.0	-0.775	5.0
12	0.5	0.8	-0.866	1.0	0.000	72.0	0.000	12.0	0.000	5.5
13	0.5	0.8	-0.289	8.0	-0.816	36.0	0.000	12.0	0.000	5.5
14	0.5	0.8	-0.289	8.0	-0.204	60.0	-0.791	1.0	0.000	5.5
15	0.5	0.8	-0.289	8.0	-0.204	60.0	-0.158	8.0	-0.775	5.0
16	0.0	0.6	0.577	20.0	-0.816	36.0	0.000	12.0	0.000	5.5
17	0.0	0.6	0.577	20.0	-0.204	60.0	-0.791	1.0	0.000	5.5
18	0.0	0.6	0.577	20.0	-0.204	60.0	-0.158	8.0	-0.775	5.0
19	0.0	0.6	0.000	12.0	0.612	96.0	-0.791	1.0	0.000	5.5
20	0.0	0.6	0.000	12.0	0.612	96.0	-0.158	8.0	-0.775	5.0
21	0.0	0.6	0.000	12.0	0.000	72.0	0.633	20.0	-0.775	5.0
22	-0.5	0.4	0.866	24.0	0.000	72.0	0.000	12.0	0.000	5.5
23	-0.5	0.4	0.289	16.0	0.816	108.0	0.000	12.0	0.000	5.5
24	-0.5	0.4	0.289	16.0	0.204	84.0	0.791	24.0	0.000	5.5
25	-0.5	0.4	0.289	16.0	0.204	84.0	0.158	16.0	0.775	6.0
26	0.0	0.6	-0.577	4.0	0.816	108.0	0.000	12.0	0.000	5.5
27	0.0	0.6	-0.577	4.0	0.204	84.0	0.791	24.0	0.000	5.5
28	0.0	0.6	-0.577	4.0	0.204	84.0	0.158	16.0	0.775	6.0
29	0.0	0.6	0.000	12.0	-0.612	48.0	0.791	24.0	0.000	5.5
30	0.0	0.6	0.000	12.0	-0.612	48.0	0.158	16.0	0.775	6.0
31	0.0	0.6	0.000	12.0	0.000	72.0	-0.633	4.0	0.775	6.0
32	0.0	0.6	0.000	12.0	0.000	72.0	0.000	12.0	0.000	5.5
33	0.0	0.6	0.000	12.0	0.000	72.0	0.000	12.0	0.000	5.5
34	0.0	0.6	0.000	12.0	0.000	72.0	0.000	12.0	0.000	5.5

3. c. 2. 3. RESULTS AND DISCUSSION

3. c. 2. 3. 1. Model Fitting

Table 3. c. 2. 1 gives the coded and actual values of the five variables. Table 3. c. 2. 3 shows the combinations of the five variables in 34 experiments. There were three replicates at the central coding condition (0,0,0,0,0), i.e., KH_2PO_4 at 0.6 %, sucrose at 12 %, fermentation time at 72 h, reaction time at 12 h and pH at 5.5. They were used to evaluate the reproducibility of the experiment. The polynomial equation based on the coded values obtained from the analysis of multiple regression was

$$y = 49.9475 - 0.73x_1 + 2.4711x_2 + 9.7713x_3 + 28.8359x_4 + 5.4481x_5 + 1.2725x_1^2 - 2.1132x_1x_2 + 0.4237x_1x_3 + 0.5111x_1x_4 + 2.7465x_1x_5 - 4.8278x_2^2 - 5.0516x_2x_3 - 0.5179x_2x_4 + 2.8065x_2x_5 - 6.5084x_3^2 + 12.4881x_3x_4 - 7.6001x_3x_5 - 28.6731x_4^2 - 11.2031x_4x_5 - 5.5517x_5^2 \longrightarrow \quad (1)$$

Based on the p values (> 0.5), the above equation was modified to have lesser number of variables, to improve the adequacy of the model (equation). Accordingly the revised equation was

$$y = 48.6042 + 2.4716\ x_2 + 9.7712\ x_3 + 28.8359\ x_4 + 5.4481\ x_5 - 4.6901\ x_3^2 - 13.896\ x_3x_4 - 27.6\ x_4^2 - 11.64\ x_4x_5 - 4.3881\ x_5^2 \longrightarrow \quad (2)$$

The ANOVA for the above model is shown in Table 3. c. 2. 4. The table indicates that the regression is significant (82.98) and that lack of fit is not significant (3.30) at $p < 0.01$. The lack of fit 'F' value is less than the $F_{\text{critical value}}$ (α = 0.01 at dof = 21 & 3) of 5.18. Hence, it can be concluded that the developed equation (model) adequately approximates the response surfaces and can be suitably used to predict the % FOS at any other values of the parameters within the experimental domain (Montgomery, 1991).

The pH, biomass and FOS yields (% w/w) were monitored in each experiment. The results are given in Table 3. c. 2. 5. The comparison of the residuals with the error variance (9.91 in Table 3. c. 2. 4) indicates that none of

the individual residual exceeds twice the square root of the residual variance. This further indicates an excellent adequacy of the regression model.

Table 3.c.2.4. Analysis of variance (ANOVA) for quadratic model and regression statistics

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-test	Significance (P value)
Regression	7401.41	9	822.38	82.98	6.98×10^{-16}
Residual	237.86	24	9.91		
Pure error	9.86	3	3.29		
Lack of fit	228.00	21	10.86	3.30	
Total	7639.27	33	231.49		

$F_{0.01, 21,3} = 5.18$

The student 't' distribution and the corresponding values, along with the parameter estimates are given in Table 3. c. 2. 6. P-values and student t-test determined the significance of each coefficient. Student t- test was employed to determine the knowledge of the error mean square that is essential in testing the significance of the estimated coefficient of the regression equation (Eq. 1). Student's t- test values can be obtained by dividing each coefficient by its standard error. A large t- value implies that the coefficient is much greater than its standard error. The p- values were used as a tool to check the significance of each of the coefficients. The p- values are necessary to understand the pattern of the mutual interactions between the test variables (Khuri and Cornell, 1987). The larger magnitude of the t- value and smaller p- value identifies the effect that appears to be very important (Lee *et al*, 1999).

Table 3.c.2.5. Final pH & biomass at specified fermentation time (h) and FOS yields by transfructosylation.

Run No	pH	Biomass (g/L)	% FOS (w/w)		Residuals
			Predicted	Experimental	
1	5.79	22.03	49.95	48.60	-1.35
2	6.03	21.43	50.49	49.56	-0.93
3	5.37	20.80	47.51	50.51	3.00
4	6.23	13.78	52.53	52.28	-0.25
5	5.97	17.32	54.31	54.43	0.12
6	6.00	15.57	54.84	52.89	-1.95
7	6.45	17.53	51.95	52.88	0.93
8	6.21	07.90	43.96	40.95	-3.01
9	4.90	06.71	35.88	36.13	0.25
10	5.91	14.00	04.01	03.89	-0.12
11	6.17	18.00	32.59	34.54	1.95
12	6.19	05.70	45.06	42.48	-2.58
13	5.09	07.49	35.42	38.06	2.64
14	5.82	14.00	03.40	03.18	-0.22
15	5.56	16.00	30.18	31.26	1.08
16	5.01	04.38	39.84	37.88	-1.96
17	5.44	36.00	05.57	03.83	-1.74
18	5.18	30.00	31.08	29.20	-1.88
19	6.52	15.71	18.79	16.65	-2.14
20	6.55	16.50	44.10	47.17	3.07
21	6.27	22.00	54.65	50.44	-4.21
22	5.71	22.10	50.07	52.64	2.57
23	6.31	15.43	53.52	50.88	-2.64
24	6.22	16.14	55.16	55.38	0.22
25	6.16	13.92	53.88	52.80	-1.08
26	6.13	11.28	52.93	54.89	1.96
27	6.03	11.06	52.32	54.06	1.74
28	6.16	10.36	49.78	51.66	1.88
29	5.25	9.30	52.44	54.58	2.14
30	4.76	13.24	49.70	46.63	-3.03
31	6.32	16.50	26.59	30.80	4.21
32	5.71	28.00	49.95	50.50	0.55
33	5.68	16.00	49.95	50.02	0.07
34	6.13	18.97	49.95	50.67	0.72

Table 3.c.2.6. Statistical analysis of the model coefficients

Variables	Coefficients	Standard error	t-test	p-value
Intercept	48.60	0.98	49.362	0.0000
X_2	02.47	1.28	01.923	0.0632
X_3	09.77	1.28	07.598	9.6×10^{-9}
X_4	28.83	1.28	22.448	0.0000
X_5	05.45	1.28	04.241	0.0002
X_3^2	-04.69	2.37	-01.979	0.0562
X_3x_4	-13.89	4.15	-03.347	0.0021
X_4^2	-27.60	2.23	-12.379	6×10^{-14}
X_4x_5	-11.64	4.28	-02.721	0.0103
X_5^2	-04.39	2.07	-02.114	0.0422

$R^2 = 0.96886$, $R_{adj}^2 = 0.95719$, $R = 0.98431$

In order to determine whether the quadratic model is significant, it is necessary to conduct an analysis of variance (ANOVA). The ANOVA for the quadratic model are presented in Table 3. c. 2. 4. It shows that the probability value for the multiple regression is very small ($p = 6.98 \times 10^{-16}$). Since the p value is less than 0.05, the model is excellent (Sharma *et al*, 2000). The ANOVA indicated that the model was adequate to represent the actual relationship between the response (FOS yields) and the significant variables. The coefficient of determination (R^2) is a measure for this criterion and can be calculated by dividing of the variation explained by model to total variation (Khuri and Cornell, 1987). The closer the value of R^2 is to unity, the better is the correlation between the observed and predicted values. In this study, the R^2 and the adjusted R^2 of the model were 0.96886 and 0.95719, respectively. A higher value of the correlation coefficient R (0.98431) justifies an excellent correlation between the independent variables (Box *et al*, 1978).

3. c. 2. 3. 2. Effects of parameters

It was observed that the first order main effects of fermentation time (x_3), reaction time (x_4), pH of the reaction mixture (x_5) and their second order main effect of x_4 (x_4^2) are highly significant as their respective p values are very small

($p < 0.001$). The second order main effects of x_3 and x_5 (x_3^2 and x_5^2) are less significant ($p < 0.1$). x_3, x_4 and x_5 have a positive influence while x_3^2, x_4^2, x_5^2 have a negative effect on FOS production. This means that these variables have a direct relationship with FOS production. Among the interactions, those between fermentation time and reaction time (x_3 and x_4) and reaction time and pH of the reaction mixture (x_4 and x_5) are modestly significant (Table 3. c. 2. 6). These interactions have a negative effect on FOS production.

3. c. 2. 3. 3. Optimization of FOS production

3. c. 2. 3. 3. 1. Canonical analysis

With the regression coefficients obtained, the stationary point of the fitted surface was computed. To characterize the stationary point, canonical analysis was performed on the polynomial equation to transform the fitted model to a new coordinate system with the origin at x_0. The axes of the system were then rotated until they were parallel to the principal axes of the response surface (Ma and Ooraikul, 1986).

This results in the fitted model

$$y = y_0 + \lambda_1 w_1^2 + \lambda_2 w_2^2 + \ldots\ldots\ldots + \lambda_k w_k^2 \longrightarrow (3)$$

where $\{w_i\}$ are the transformed independent variables and the $\{\lambda_i\}$ are the constants. Furthermore, the $\{\lambda_i\}$ are just the eigenvalues or characteristic roots of the polynomial equation in matrix notation. The nature of the response surface can be determined from the stationary point and the sign and magnitude of the $\{\lambda_i\}$. It could represent a point of maximum response, a point of minimum response or a saddle point. If the $\{\lambda_i\}$ are all positive, then x_0 is a point of minimum response; if $\{\lambda_i\}$ are all negative, then x_0 is a point of maximum response; and if $\{\lambda_i\}$ have different signs, x_0 is a saddle point. Furthermore the surface is steepest in the w_i direction for which $\{\lambda_i\}$ is greatest (Montgomery, 1991).

The canonical form of the equation demonstrating the nature of the response surface was

$$y = 58.9 - 10.49 w_1^2 + 1.43 w_2^2 - 2.775 w_3^2 - 1.7 w_4 2 - 30.74 w_5^2 \longrightarrow (4)$$

Here, the surface is steepest in the w_5 direction for which the coefficient $\lambda_5 = 30.74$. Since x_i have different signs, the stationary point is a saddle point. The

predicted yield together with the actual values of the variables of the stationary point is shown in Table 3. c. 2. 7.

3. c. 2. 3. 4. Validation experiments for verification of the model

Validation experiments were carried out to obtain the optimum and maximum yields possible. The first experiment was done using the conditions obtained at the stationary point, i.e. KH_2PO_4 – 0.72 %, Sucrose – 6.455 %, Fermentation time 107.30 h, Reaction time – 18 h and pH of the reaction mixture – 5.15. The observed FOS yield was 56.4 %, which closely agreed with the predicted maximum yield of 58.9 % (Table 3. c. 2. 7). The contour plot corresponding to the stationary point is shown in Figure 3. c. 2. 1.

Table 3.c.2.7. Predicted and experimental FOS yields in validation experiments based on contour plots and stationary points

Variables							
Stationary points							
Expt .no.	KH_2PO_4 (%)	Sucrose (%)	FT[a] (h)	RT[b] (h)	pH	FOS yield (% w/w) Predicted	Experimental [c]
	0.72	6.46	108	18	5.15	58.90	56.35 ± 0.15
Contour plots							
1	0.9	14.0	102	18	5.5	57.45	55.90 ± 1.14
2	0.9	10.0	90	18	5.5	58.40	57.26 ± 0.39
3	0.9	06.0	102	18	5.5	58.30	57.37 ± 0.90
4	0.9	06.0	102	18	5.0	59.20	55.67 ± 1.25
5	0.7	10.0	102	18	5.0	58.70	54.11 ± 1.92

[a] Fermentation Time
[b] Reaction Time
[c] Mean ± SD of 3 experiments

The contour plots and 3 - dimensional surfaces are the most useful approach to predict the response for different values of the test variables and identify the type of interactions between test variables (Box *et al*, 1978). The contour plots that give the variation of FOS yields with independent variables are shown in Figures 3. c. 2. 2 to 3. c. 2. 6. Each contour curve represents an infinite number of combinations of two test variables with the other three maintained constant. A contour plot of FOS yields as a function of the

fermentation time and reaction time at a fixed KH_2PO_4 concentration of 0.9 %, sucrose concentration of 14 % and reaction pH of 5.5 is presented in Figure 3. c. 2. 2. As can be seen, the FOS yields increased with increase in fermentation time and reaction time reaching above 55 % (w/w) at the end of 14 h of reaction. Figure 3. c. 2. 3 shows the FOS yields as a function of fermentation time and reaction time at a fixed KH_2PO_4 concentration of 0.9 % and sucrose concentration of 10 % and reaction pH of 5.5. Figure 3. c. 2. 4 shows the contour plot with FOS yields at a fixed KH_2PO_4 concentration of 0.9 % and sucrose concentration of 6 % and reaction pH of 5.5. Figure 3. c. 2. 5 shows FOS yields at a fixed KH_2PO_4 concentration of 0.9 % and sucrose concentration of 6 % and reaction pH of 5.0. Figure 3. c. 2. 6 shows FOS yields at a fixed KH_2PO_4 concentration of 0.7 % and sucrose concentration of 10 % and reaction pH of 5.0. All the figures show that FOS yield reach above 55 % at the end of 14 h of reaction, irrespective of the other parameters varied.

In the validation experiment, the adequacy of the model was also examined by additional independent experiments at the suggested optimum conditions. The values for the five parameters were taken from the contour plots which yielded values close to 60 % FOS, the maximum possible value for the production of FOS. The values of the variables along with the predicted and experimental yields obtained are given in Table 3. c. 2. 7. All the experiments were carried out in triplicates. The predicted and experimental values matched closely with each other. This indicates that the generated model adequately predicted the FOS yield. Thus, the optimum conditions for FOS production were successfully developed by Shell Design and RSM.

Based on the validation experiments, a “what - if” analysis was done, it was found that among the 16 parameters used in the experiment, sucrose, $MgSO_4$, KH_2PO_4, Fermentation time, Substrate concentration, Enzyme concentration, Temperature and pH of the reaction mixture affected the yield of FOS and the remaining 8 parameters had virtually no effect. Of the 8 significant parameters some showed positive (↑) effect where as others showed negative (↓) effect (Table 3. c. 2. 8).

Figure 3. c. 2. 1. Contour plot of fructooligosaccharide concentration: The effect of fermentation time and reaction time and their mutual interaction on production of fructooligosaccharides. Other variables are KH_2PO_4 – 0.72 %, Sucrose – 6.5 % and pH of reaction mixture – 5.15.

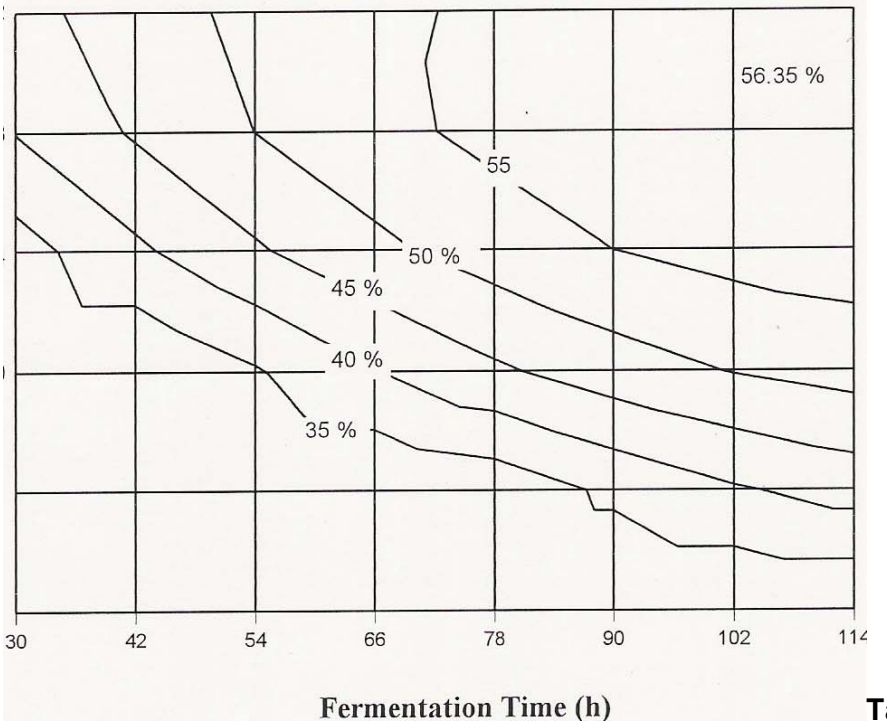


Table 3.c.2.8.

Figure 3. c. 2. 2. Contour plot of fructooligosaccharide concentration: The effect of fermentation time and reaction time and their mutual interaction on production of fructooligosaccharides. Other variables are KH_2PO_4 – 0.9 %, Sucrose – 14 % and pH of reaction mixture – 5.5.

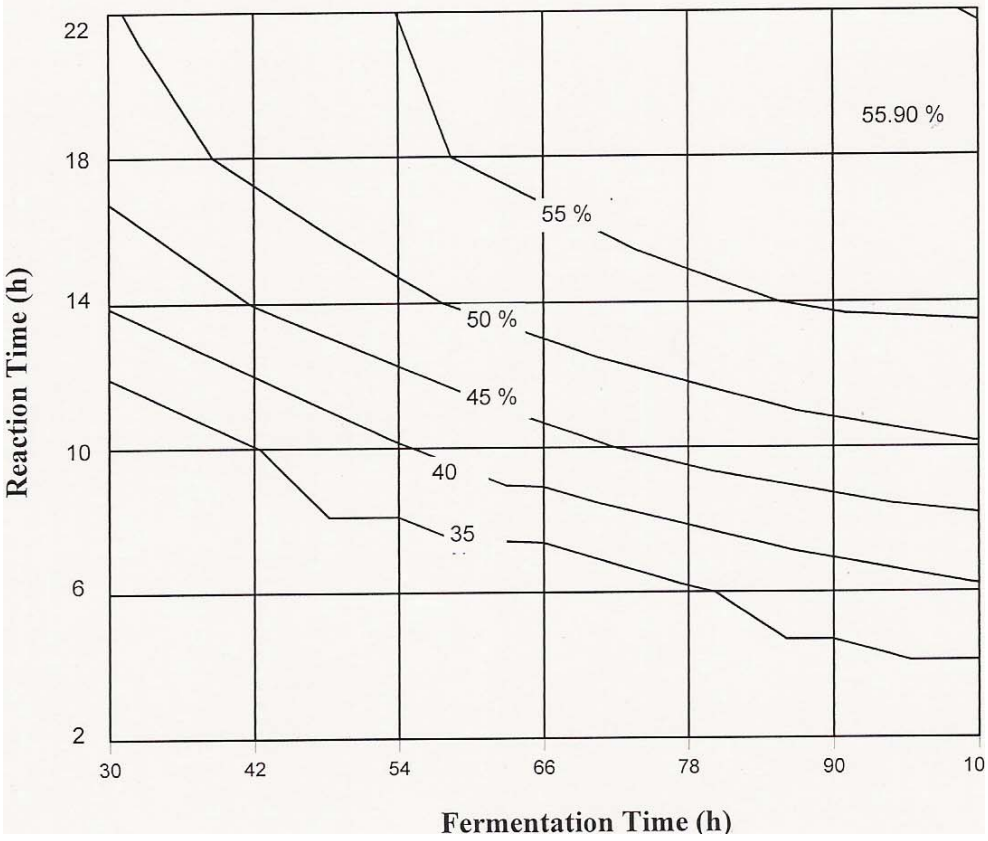


Figure 3. c. 2. 3. Contour plot of fructooligosaccharide concentration: The effect of fermentation time and reaction time and their mutual interaction on production of fructooligosaccharides. Other variables are KH_2PO_4 – 0.9 %, Sucrose – 10 % and pH of reaction mixture – 5.5.

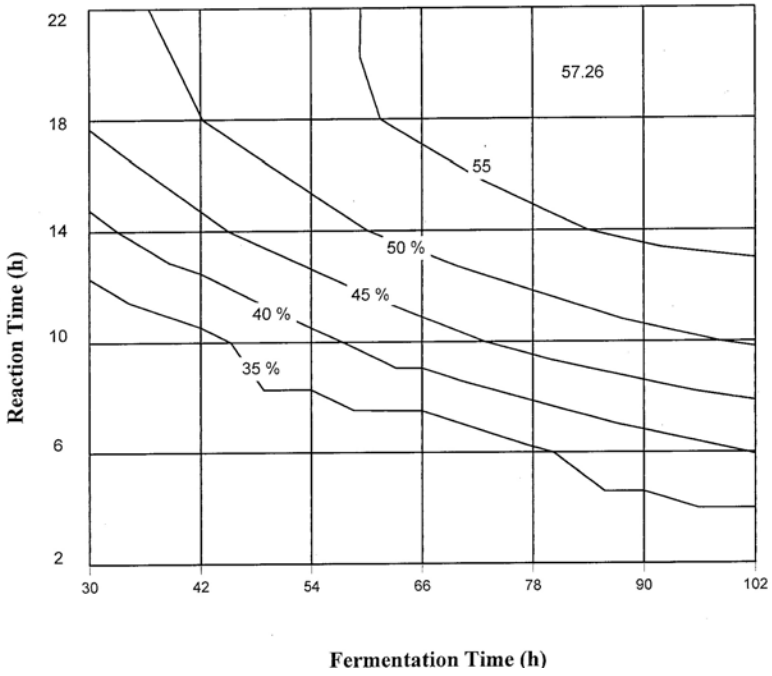


Figure 3. c. 2. 4. Contour plot of fructooligosaccharide concentration: The effect of fermentation time and reaction time and their mutual interaction on production of fructooligosaccharides. Other variables are KH2PO4 – 0.9 %, Sucrose – 6 % and pH of reaction mixture – 5.5.

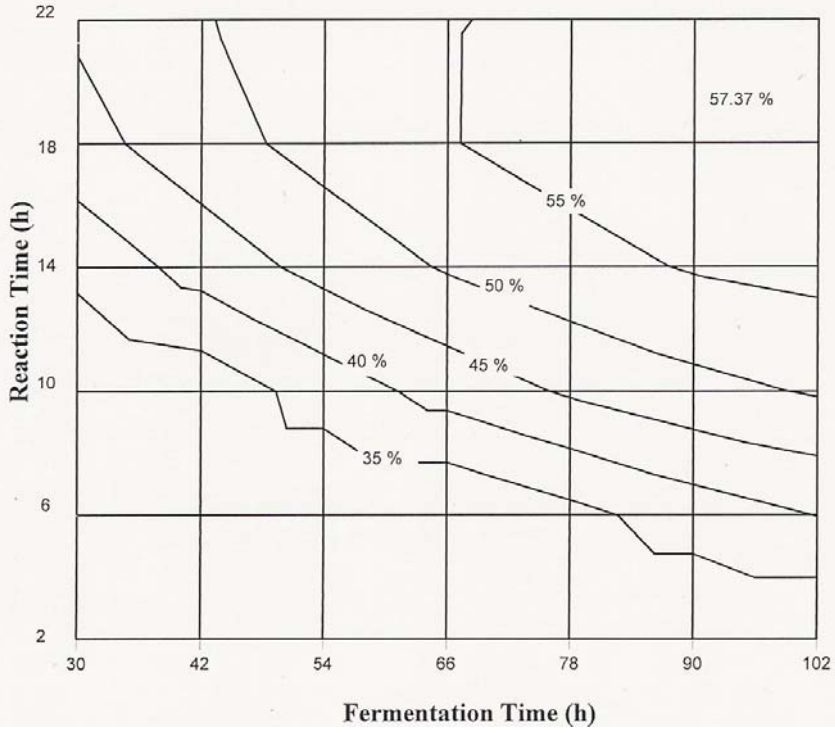


Figure 3. c. 2. 5. Contour plot of fructooligosaccharide concentration: The effect of fermentation time and reaction time and their mutual interaction on production of fructooligosaccharides. Other variables are KH_2PO_4 – 0.9 %, Sucrose – 6 % and pH of reaction mixture – 5.0.

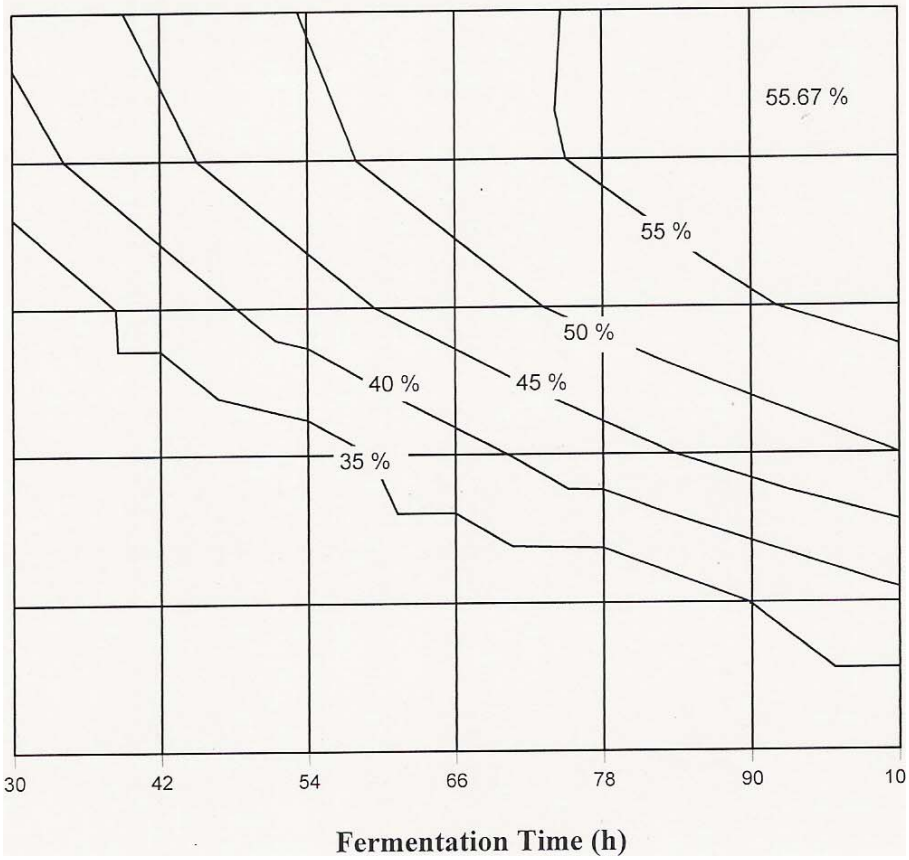
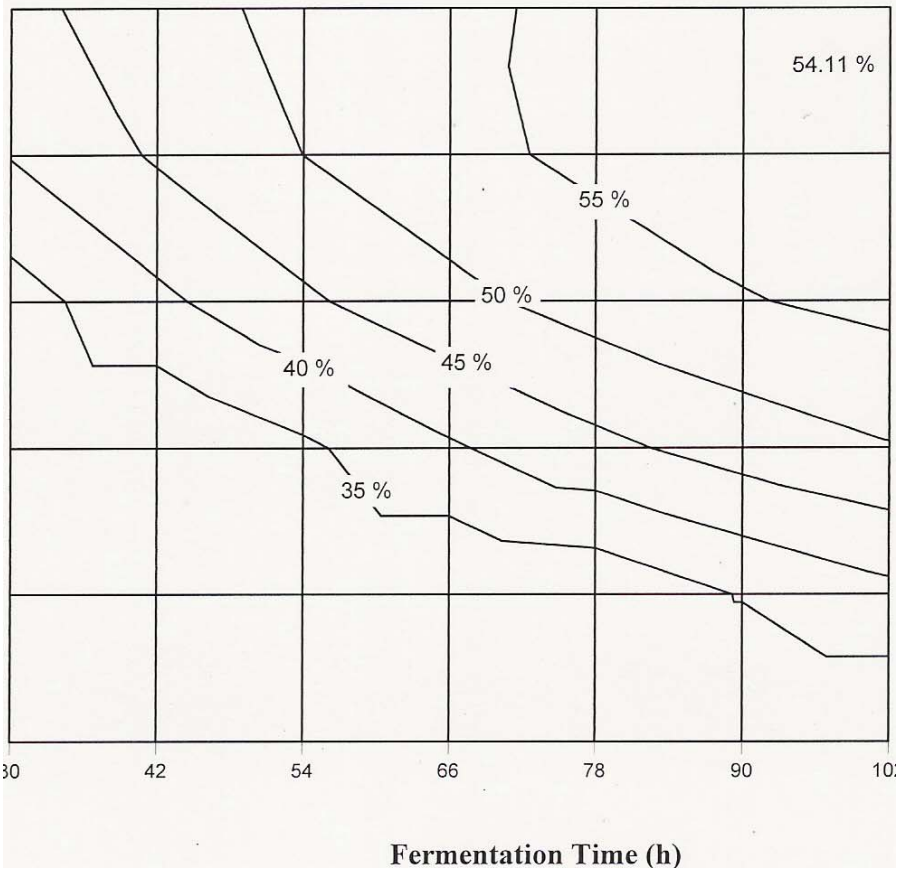


Figure 3. c. 2. 6. Contour plot of fructooligosaccharide concentration: The effect of fermentation time and reaction time and their mutual interaction on production of fructooligosaccharides. Other variables are KH_2PO_4 – 0.7 %, Sucrose – 10 % and pH of reaction mixture – 5.0.



Effect of various parameters on FOS yields based on 'what-if' analysis

Ingredient/Parameter	Effect
Sucrose	↓
$MgSO_4.7H_2O$	↑
KH_2PO_4	↑
Fermentation Time	↓
Substrate concentration	↓
Enzyme concentration	↓
Temperature	↑
pH of the reaction mixture	↑

↓ Negative effect; ↑ Positive effect

Response Surface Methodology was used as a tool to determine optimum values of media components and reaction parameters to attain maximum production of FOS using FTase enzyme from *A oryzae* CFR 202. This study demonstrated that RSM is an efficient experimental design when several variables affecting the process are evaluated simultaneously. Optimum values of these variables that would produce highest FOS yield were obtained with a minimum number of experiments. The effect of interaction between variables and reliability of the experiment could also be statistically evaluated.

3. c. 2. 4. CONCLUSIONS

The RSM methodology used, based on Doehlert experimental design consisted of 34 experiments with five variables, four repetitions at the center point. The results showed the effects and interactions of different parameters on FOS yields by FTase grown under submerged fermentation. A response equation has been obtained for the FOS yields. From this equation, it is possible to predict adequately the operation conditions required to obtain higher yields of FOS. It was found that the most effective parameters were fermentation time, reaction time and pH of the reaction mixture. These factors have a positive influence on FOS yields. Due to the disproportionate nature of reaction exhibited by FTase, the reaction time plays an important role in FOS production. Among the interactions, fermentation time – reaction time

interactions are significant ($p < 0.01$) and they have a negative influence on FOS production. According to these results, it was found that the maximum predicted FOS yield of 58.7 % (w/w) by the equation agrees well with the experimental value of 57.37 $\pm$ 0.9 % (w/w) FOS. This indicates that the generated model adequately predicted the FOS yields.

The optimized media and reaction conditions for the maximum yield of FTase and FOS is represented in Table. 3. c. 2. 9.

Table 3.c.2.9. Optimized values of media ingredients and reaction parameters for FTase and FOS production

Media ingredient	Optimum value (%)	Parameter	Optimum value
Sucrose	10.0	pH of media	5.0
Yeast extract	0.8	Fermentation time	90 h
$NaNO_3$	2.0	Substrate concentration	60 %
KH_2PO_4	0.9	Enzyme concentration	0.5 ml
K_2HPO_4	0.4	Reaction time	18 h
NaCl	0.6	Temperature	55 °C
NH_4Cl	1.0	PH	5.15
$MgSO_4$. 7 H_2O	0.03		
Inoculum	20		

3.d. FTase PRODUCTION BY *A. ORYZAE* CFR 202 DURING RECYCLING CELL CULTURE.

3. d. 1. INTRODUCTION

Preliminary studies on the screening of a few fungal strains for FTase activity resulted in the selection of *A. oryzae* CFR 202 as a potent strain based on high U_t/ U_h ratio. Important parameters influencing the production of FTase and FOS using this strain were studied using Plackett Burmann Design and their levels were optimized using Response Surface Methodology. Using this optimized media, *A. oryzae* CFR 202 produced compact, round pellets which were stable through out the fermentation period of 90 h. This is a common phenomenon observed when filamentous fungi are grown in submerged culture. These pellets consisting of compact masses of hyphae may change their morphology as growth proceeds. In an industrial fermentation, the formation of pellets is advantageous, since the filamentous form of fungus may wrap around the impeller and damage the agitator blades, and is often prone to block the spargers (Whitaker and Long, 1973). Also, pellet formation makes downstream processing easier in industrial fermentation.

The pellets of *A. oryzae* CFR 202 were so compact that they could be used for the next cycle of fermentation, provided they have the FTase activity. A cell recycling system can be designed to produce FOS by recycling the cells every 24 h to produce FTase and thereby FOS. The advantage of this system is that the pellets grown during fermentation can be efficiently reused and will save the use of fresh inoculum. Onishi and Tanaka (1998) have reported a system for galactooligosaccharide production using recycling cell culture of *Sterigmatomyces elviae* CBS8119.

3. d. 2. MATERIALS AND METHODS

All chemicals, microorganism and culture conditions were as described in Section 3. a. 2. 1 to 3 a. 2. 3. Fermentation medium, culture conditions and reaction conditions for FOS production were as given in Table 3.c.2.9. At the end of 48 h of fermentation, the pellets were separated by decanting the culture broth into a container under aseptic conditions and fresh media was added to the pellets. At the end of every 24 h next, the broth was decanted and fresh

media was added. Fermentation was continued upto 144 h. The decanted broth was checked for its pH, FTase activity and production of FOS under the reaction conditions specified. The scheme for recycling cell culture is given in Figure 3. d. 1.

3. d. 3. RESULTS AND DISCUSSION

FTase activity and pH were monitored in the culture fluid obtained at the end of each cycle of fermentation. The pH of the broth increased from 5.0 to 6.03 at the end of 48 h of fermentation. Afterwards, the pH increased to 6.18 and remained same through out the fermentation (Fig 3. d. 2). FTase activity was 7.8 $Uml^{-1}min^{-1}$ at the end of 48 h of fermentation. It reached a maximum value of 16. 5 $Uml^{-1}min^{-1}$ after the next two consecutive cycles and then decreased to 11. 9 $Uml^{-1}min^{-1}$ at the end of the sixth recycle. However, FOS yield was maximum at the end of the third recycle and was maintained at the same level even at the end of the sixth recycle (Figure 3. d. 3). The pellets could not be recycled further since it lost the compactness and started disintegrating after the sixth recycle.

Onishi and Tanaka (1998) have reported a similar system for the production of galactooligosaccharide using a recycling cell culture of *Sterigmatomyces elviae* CBS 8119 where they could maintain 60 % yield over the period of recycling. Similarly, the results have shown that an efficient cell recycling system could be developed for the continuous production of FTase by successfully reusing the pellets of *A. oryzae* CFR 202 over a considerable period.

Figure. 3.d.1. Schematic Diagram of Recycling Cell Culture

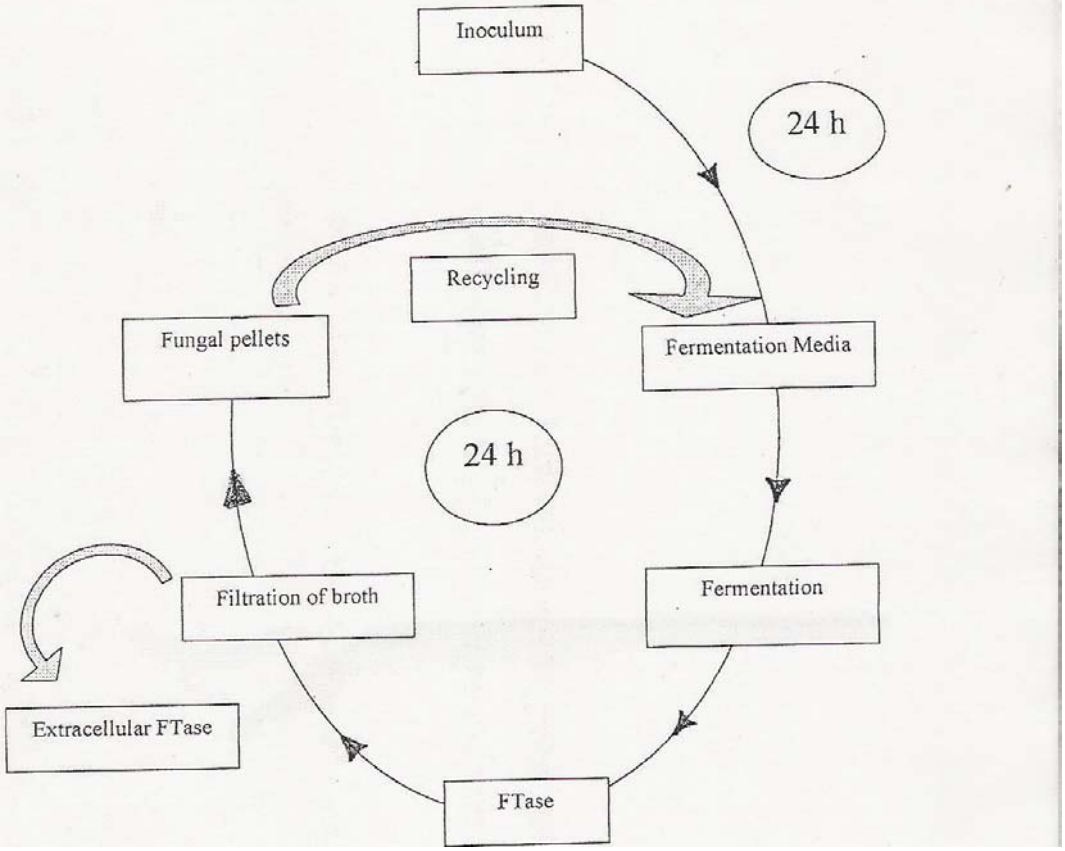


Figure 3.d.2. Changes in pH and FTase activity during recycling cell culture of *A.oryzae* CFR 202.

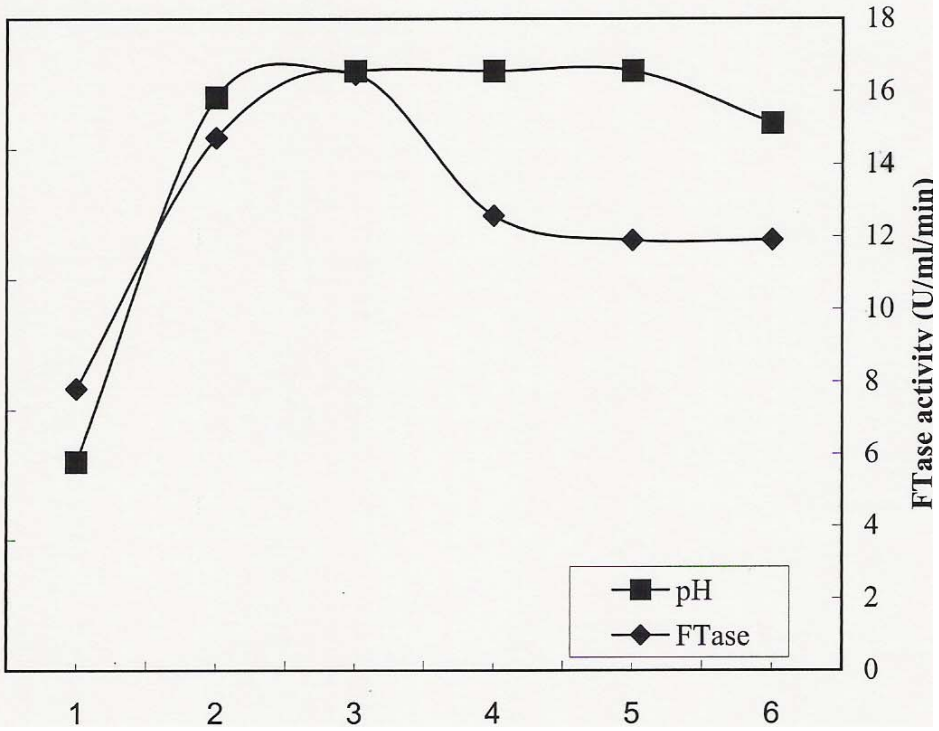
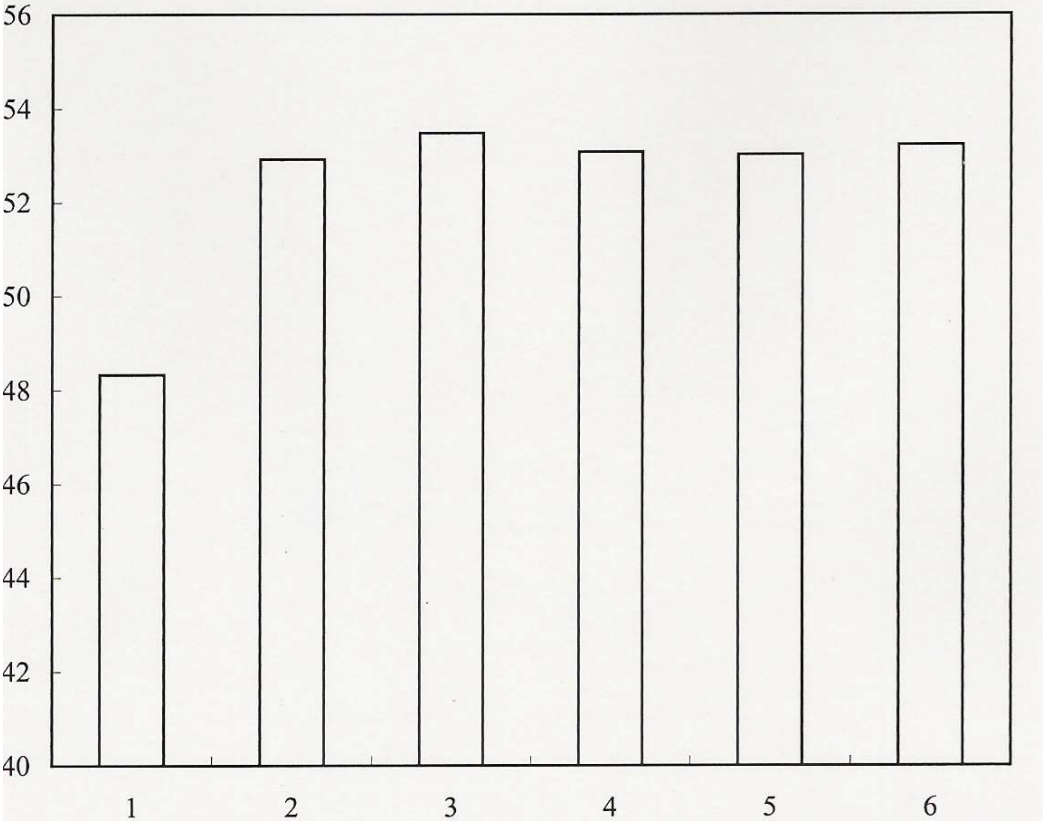


Figure 3.d.3. Production of FOS during recycling cell culture of *A. oryzae* CFR 202.



3. e. ALTERNATE SUBSTRATES FOR THE PRODUCTION OF FTase AND FOS.

3. e. 1. INTRODUCTION

Sucrose is widely used as carbon source in many fermentation processes. As mentioned in the previous chapters, growth of *A. oryzae* CFR 202 in sucrose containing medium has resulted in the production of FTase. Further, the production of FOS requires specifically sucrose as the substrate due to the reaction mechanism involved. In the present investigation, some alternate sources of sucrose like jaggery and sugarcane juice have been tried as substrates for FOS production. Jaggery has also been used as carbon source in the fermentation medium for FTase production. Jaggery, which is the concentrated form of sugarcane juice containing 75 – 85 % sucrose, is used as a sugar substitute in India. There are a few reports on the use of jaggery in fermentation processes. Vijayendra *et al* (2001) have reported the use of jaggery for pullulan production by *A. pullulans* CFR 77. Palmyra jaggery – the sugar syrup from palmyra palm – was used to produce citric acid using *A. niger* MTCC 281 (Ambati and Ayyanna, 2001).

3. e. 2. MATERIALS AND METHODS

All chemicals, microorganism and culture conditions were as described in Section 3. a. 2. 1 to 3 a. 2. 3. Jaggery was procured from the local market and sugarcane juice was from the local juice vendor. Fermentation medium, culture conditions and reaction conditions for FOS production were as given in Table 3.c.2.9. In the fermentation medium, 10 % (w/v) jaggery was used as carbon source. For FOS production, 60 % (w/v) jaggery was used as substrate. Another substrate used was sugarcane juice. 7.5 ml of sugarcane juice was mixed with 2.5 ml FTase. All the other conditions remained same as mentioned in Table 3.c.2.9.

3. e. 3. RESULTS AND DISCUSSION

In any fermentation process, the requirement of a carbon source in the growth medium is inevitable. The cost of any process depends partly on the nutrients present in the medium. A process can be made economical by substituting pure chemicals used in the media with unprocessed natural source

of the nutrient. In the present study, results obtained demonstrate that jaggery can be used as a carbon source in the fermentation medium for FTase production and it can be also be used as substrate for FOS production. Table 3. e. 1. depicts the yield of FOS obtained using jaggery and sugarcane juice for FTase and FOS production.

Table 3.e.1. FOS yields obtained using jaggery and sugarcane juice as substrates

Serial no.	Substrate for FTase	Substrate for FOS	° brix of substrate for FOS production	FOS yields (% w/w)
1	Sucrose	Sucrose	56	58.0
2	Sucrose	Jaggery	60	42.0
3	Jaggery	Sucrose	56	48.6
4	Jaggery	Jaggery	60	40.0
5	Sucrose	Sugarcane juice	18	24.0
6	Sucrose	Conc. Sugarcane juice	60	27.0

Although the yield of FOS is less compared to that when sucrose is used, the economy of the process advocates the use of jaggery and sugarcane juice in the process. This also provides value addition to these products resulting in the production of a high value product like FOS.

CHAPTER 4

PURIFICATION AND CHARACTERIZATION OF FTase

4. 1. INTRODUCTION

Purification and characterization of an enzyme are important steps to improve our understanding of its mode of action. As mentioned in Chapter 3, during screening of a few fungal strains, *A. oryzae* CFR 202 was selected as a potent producer of Fructosyl Transferase (FTase). This strain being a novel source of FTase, attempts have been made to purify and characterize the extracellular FTase obtained by submerged fermentation. The structure and number of FOS produced depends on the type of FTase produced by the microorganism. In this context, it is essential to purify and characterize the enzyme. There are many reports on the purification of FTase from fungal and bacterial sources (Patil and Patil, 1999; L' Hocine *et al*, 2000; Park *et al*, 2001). However, so far, there has been no reports on studies on FTase from *A. oryzae*. The present chapter discusses the purification and characterization of FTase obtained from *A. oryzae* CFR 202.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals

Ammonium sulphate, Sodium dihydrogen phosphate, Disodium hydrogen phosphate, Sodium chloride, Methanol, Acetic acid, Sodium thiosulphate, Formaldehyde, Silver nitrate, Sodium carbonate, Glycine, Sucrose, Sodium hydroxide, Copper sulphate, Sodium potassium tartrate were from Qualigens Fine Chemicals (Mumbai, India). DEAE Cellulose, Acrylamide, Bis- acrylamide, Ammonium persulphate, TEMED, Sodium Dodecyl Sulphate, Coomassie Brilliant Blue, Bromophenol Blue, Glycerol, Tris buffer, Triphenyl tetrazolium chloride, Folin's Ciocalteaeu reagent, Trifluroacetic acid were from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Sephadex G- 200, β-mercaptoethanol, Bovine Serum Albumin were from Sigma Chemical Company (St. Louis, USA). Iodoacetamide was from Hi media Laboratories (Mumbai, India). Glucose oxidase – Peroxidase kit was from Dr. Reddy's Laboratories, Hyderabad. The molecular weight marker kit for SDS – PAGE was from BDH Laboratory Supplies (Poole, England).

4. 2. 2. FTase production by Submerged Fermentation

FTase was produced by submerged fermentation using *A. oryzae* CFR 202. The inoculum and fermentation media composition was as given in Table 3. c. 2. 9. Media preparation and fermentation were as described in Section 3. c. 2. 2. 1. Enzyme assay was as described in Section 3. a. 2. Analysis of FOS was as given in Section 3 a. 2. 5 and 3. a. 2. 6. After 90 h of fermentation time, the fungal pellets were removed by filtration (Whatman No.2) and the filtrate was used as the crude enzyme for the purification process.

4. 2. 3. Characterization of crude enzyme

4.2.3.1. Optimum pH and temperature for FTase activity

The optimum pH for the enzyme activity was determined by incubating the enzyme at 55 °C with the substrate at different pH values. The substrate was prepared in different buffers such as citrate buffer (pH 3.0 – 5.0), sodium phosphate buffer (pH 6.0 – 8.0) and bicarbonate buffer (pH 9.0 & 10.0). After incubation for 1 h, the enzyme activity was determined as given in the previous section. Optimum temperature was determined by incubating the enzyme with the substrate at pH 5.5 at temperatures 30 °C, 40 °C, 50 °C, 60 °C and 70 °C for 1 h. The residual enzyme activity was determined as described above.

4. 2. 3. 2. Thermostability and pH stability of FTase

The enzyme solution was incubated at different temperatures (30 – 70 °C) for 24 h at pH 5.15. An aliquot was drawn every 1 h and the residual enzyme activity was determined. To determine pH stability, the crude enzyme was dispersed in 0.1 M buffer solution (1: 1 ratio) at pH 3.0 – 5.0 (citrate buffer), 6.0 – 8.0 (sodium phosphate buffer) and 9.0 & 10.0 (bicarbonate buffer) and incubated at room temperature for 24 h. The activity was determined after 24 h.

4. 2. 4. Estimation of protein concentration

Protein was measured by the method of Lowry *et al* (1951) using Bovine Serum Albumin (BSA) as standard. Absorbance at 280 nm was used for monitoring protein in column eluates.

4. 2. 5. Purification procedure

Purification of enzyme was carried out as described below. Unless otherwise mentioned, all steps were conducted at room temperature.

4. 2. 5. 1. Ammonium sulphate fractionation

Solid AR grade Ammonium Sulphate was added to the crude enzyme to 30 % saturation and incubated at 4 °C for 1 h. After centrifugation (10,000 rpm, 20 min), ammonium sulphate was added to the supernatant to bring it to 80 % saturation. After incubation at 4 °C for 1 h, it was again centrifuged. The precipitate after each step was redissolved in minimum volume of 0.1 M phosphate buffer (pH - 7.0). The enzyme activity and protein concentration were assayed in the supernatants and precipitates obtained after each step.

4. 2. 5. 2. Dialysis

The precipitate having maximum FTase activity was redissolved in 0.1 M phosphate buffer (pH –7.0) and dialyzed in a cellulose dialysis tubing of 12 kD cutoff against the same buffer at 20 mM concentration for 24 h with 3 changes of buffer after every 8 h to remove the traces of ammonium sulphate present.

4. 2. 5. 3. Ion exchange chromatography on DEAE Cellulose

The dialyzed enzyme solution was centrifuged (10,000 rpm, 20 min) and subjected to ion exchange chromatography. DEAE cellulose was activated by first washing with 0.1 N HCl and then with 0.1 M NaOH. After each wash, the pH of the resin was brought back to neutral by washing with distilled water. The resin was equilibrated at pH – 7.0 using 20 mM phosphate buffer (pH – 7.0). The resin was then packed into a glass column (18 x 3 cm) and washed with 2 bed volumes of the same buffer before loading the sample. The non- adsorbed proteins were eluted from the column using 2 bed volumes of the buffer and the adsorbed proteins were eluted with a linear gradient of sodium chloride (0.1–1M) in the same buffer at a flow rate of 10 ml h^{-1}. Enzyme activity and protein concentration were recorded in the fractions collected.

4. 2. 5. 4. Gel Filtration chromatography on Sephadex G – 200

The active fractions eluted from DEAE cellulose column were pooled and dialyzed against 20 mM phosphate buffer (pH – 7.0) for 24 h with 3 changes of buffer. The dialyzed sample was concentrated by lyophilization (Heto Drywinner DW3, Heto-Halten, Denmark) and dissolved in minimum volume of buffer. The sample was then loaded on Sephadex G – 200 column (95 x 1.75 cm) equilibrated with 20 mM phosphate buffer (pH – 7.0). The proteins were eluted using the same buffer at a flow rate of 10 ml h^{-1}. Enzyme activity and protein concentration were recorded in the fractions. Fractions with enzyme activity were pooled and concentrated by lyophilization.

4. 2. 5. 5. Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed under native and denaturing conditions according to Laemmli's procedure (Laemmli, 1970). For both native and SDS PAGE, 10 % acrylamide resolving gel was used.

4. 2. 5. 5.1. Protein Staining

The proteins in the gel were stained by silver staining method (Wray *et al*, 1981) as described below.

Solutions required:

1. Fixative solution (100 ml) containing 6 ml acetic acid, 25 ml methanol and 1.42 ml 0.2 M Formaldehyde.
2. 50 % ethanol.
3. 0.2 % (w/v) sodium thiosulphate.
4. 0.1 % silver nitrate solution containing 25 μL formaldehyde.
5. Developing solution: 6 g sodium carbonate, 75 μL formaldehyde and 1 mg sodium thiosulphate in 100 ml water.
6. Stop solution: 6 ml acetic acid and 25 ml methanol.

The gel was incubated in solution 1 for 20 minutes followed by solution 2 for 30 min with shaking. The gel was then pretreated with solution 3 for 1 min and then incubated for 20 min in solution 4. This was followed by developing in

solution 5 until bands were visible. Solution 6 was used to stop the development. After each step, the gel was washed with distilled water.

4. 2. 5. 5. 2. Activity Staining

FTase activity was detected in situ after native PAGE by the staining procedure of Gabriel and Wang (1969) as follows.

1. The gel was washed with water and incubated under enzyme assay conditions as described in Table 3. C. 2. 9.
2. It was then transferred in to 0.1 M iodoacetamide and kept for 5 min at room temperature followed by rinsing with water.
3. The gel was then immersed in freshly prepared 0.1 % Triphenyl Tetrazolium Chloride in 0.5 M Sodium hydroxide and heated in boiling water bath for 1 to 1.5 min.
4. The gel was then washed with water and stored in 7.5 % acetic acid.

4. 2. 5. 5. 3. Molecular weight determination by SDS – PAGE

SDS – PAGE was performed on a 10 % gel. A mixture of molecular weight markers containing Ovalbumin (42,700), Glutamate dehydrogenase (55,500), Ovotransferrin (77,000), Phosphorylase b (97,400) β-galactosidase (1,16,300) and Myosin (2,00,000) were used for determining the molecular weight of the purified FTase. The mobility of each protein was determined by calculating the distance moved by each protein and the distance moved by the dye. A graph was plotted with the mobility versus log molecular weight for the standards. From the mobility of the unknown, its molecular weight was obtained by intrapolation.

4. 2. 6. Analytical methods

4.2.6.1. Reverse phase HPLC

Reverse phase HPLC of the purified protein was carried out using a Shimadzu CLC – ODS column (25 cm x 5 mm; 5 μ) on LC 10 A system using a gradient of two solvents: A – 0.1 % Trifluroacetic acid (TFA) in water and B – 0.1 % TFA in 70 % acetonitrile. The gradient for separation consisted of 1 % B traversing to 100 % in 50 minutes at a flow rate of 1.0 $mlmin^{-1}$. The detection was carried out using Diode array detector at wavelengths 220 and 280 nm.

4. 2. 6. 2. Capillary Electrophoresis

Capillary Electrophoresis of purified FTase was carried out on a Prince Capillary electrophoresis system (Prince 560, Prince Technologies, The Netherlands) using untreated fused silica capillary (100 μm i.d., 100 cm length). The selected wavelength was 220 nm. Before the run, the capillary was rinsed with Tris glycine buffer (pH – 8.0). The samples were introduced in to the capillary using a low-pressure (0.5 psi) hydrodynamic injection time of 2 sec. The separation voltage was 20 kV and capillary temperature was 25 °C. The data acquisition and control were performed on a DAX software.

4. 2. 7. Characterization of purified FTase

4. 2. 7. 1. Optimum pH and temperature for FTase activity

The optimum pH and temperature for the enzyme activity was determined as described in Section 4. 2. 3. 1.

4. 2. 7. 2. Thermostability and pH stability of purified FTase

Thermostability and pH stability of purified FTase was determined by the procedure given in Section 4. 2. 3. 2.

4. 2. 7. 3. Effect of metal ions and chemicals on FTase activity

The effect of metal ions and chemicals was investigated by incubating the enzyme with sucrose in the presence of various salts of metals ions and additives at 1 mM and 1 % concentration respectively. The enzyme activity was assayed under the conditions given in Table 3. c. 2. 9.

4. 2. 8. FOS Production

FOS production was carried out under the conditions given in Table 3. c. 2. 9. An aliquot of the sample was taken after every 1 h and analyzed by HPLC as given in Section 3. a. 2. 5 and 3. a. 2. 6.

4. 3. RESULTS AND DISCUSSION

4. 3. 1. Enzyme Purification

FTase is the enzyme responsible for the production of FOS from sucrose. There are many reports in literature on purification of FTases from different microbial and plant sources. In the present investigation, conventional techniques were used for the purification of FTase. The results are presented below.

4. 3. 1. 1. Ammonium sulphate fractionation

Ammonium sulphate fractionation involves the principle of salting out of protein by subjecting it to high salt concentration. Precipitation by addition of neutral salts is probably the most commonly used method for fractionating proteins by precipitation. The precipitated protein is usually not denatured and activity is recovered upon redissolving the pellet. In addition, these salts can stabilize the proteins against denaturation or proteolysis (Harris, 1989). The crude FTase was first brought to 30 % saturation with ammonium sulphate. After centrifugation, it was observed that the precipitate does not contain enzyme activity and hence it was discarded. The supernatant was further brought to 80 % saturation. Analysis of the precipitate showed that it has resulted in 1.16 fold purification of FTase having a specific activity of 1.7 with 59 % recovery (Table 4.1). The precipitate was further dialyzed and subjected to DEAE cellulose column chromatography.

4. 3. 1. 2. Ion exchange chromatography

Ion exchange is the separation of proteins based on their charge and can be used to resolve proteins, which differ only marginally in their charged groups. Separation of proteins is achieved by their difference in equilibrium distribution between a buffered mobile phase and a stationary phase consisting of a matrix to which charged inorganic groups are attached. Preliminary studies on binding of FTase to DEAE cellulose equilibrated with buffers of different pH showed that the maximum binding of the enzyme occurred with the resin at pH – 7.0. Hence, the ammonium sulphate precipitated sample was subjected to ion exchange chromatography and unbound proteins were removed by washing with 2 bed volumes of 20 mM phosphate buffer of pH – 7.0. The adsorbed

proteins were eluted with the same buffer containing 0.1 M NaCl followed by 0.3, 0.5 and 1 M NaCl. The elution profile showed that a single active peak appeared (fraction no. 74 – 86) while washing with 0.5 M NaCl (Figure 4. 1). The active fractions were pooled. The pooled fractions had a specific activity of 155.2 with 106.3 fold purification and 26.76 % recovery of the enzyme (Table 4.1). The pooled fractions were dialyzed against the buffer and concentrated by lyophilization. The concentrated enzyme was then subjected to gel filtration chromatography.

4. 3. 1. 3. Gel filtration chromatography

Gel filtration chromatography is used for separating molecules of different sizes using a porous gel matrix. The smaller molecules enter the matrix and hence move slowly through the column; the larger molecules are excluded from the stationary phase and hence elute first from the column; molecules of intermediate size can enter but spend less time inside the matrix. Thus, the molecules are eluted depending on their decreasing size. In the present study, FTase was eluted as a single peak (fraction no. 8 to 12) as shown in Figure 4. 2 with a specific activity of 508.13 with 348.03 fold purification with 24.17 % yield. The overall scheme of purification of FTase is given in Table 4. 1.

4. 3. 1. 4. Criteria of homogeneity

The homogeneity of the purified enzyme was assessed by Native PAGE, HPLC and capillary electrophoresis. During electrophoresis on Native PAGE, the final purified enzyme yielded diffuse and broad band. Activity staining revealed an intense pink band showing the presence of high FTase activity (Figure 4. 3). When staining was carried out without incubating the gel under enzyme assay conditions, the pink band was absent. This proved that the band was due to the release of glucose in the presence of FTase activity. Capillary electrophoresis of the purified FTase showed a single peak (Figure 4. 4). Figure 4.5 shows the reverse phase HPLC elution profile of purified FTase.

Table 4. 1: Purification of FTase produced by *Aspergillus oryzae* CFR 202 by submerged fermentation

Purification step	Total volume (ml)	Enzyme activity (U/ml/min)	Total activity (U)	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg protein)	Purification fold	Yield %
Crude	**200**	**12.93**	**2586.00**	**8.872**	**1774.40**	**1.46**	**---**	**100.00**
$(NH_4)_2SO_4$ precipitation	**3**	**513.62**	**1540.86**	**302.130**	**906.39**	**1.70**	**1.16**	**59.58**
IEC	**20**	**34.61**	**692.20**	**0.223**	**4.46**	**155.20**	**106.30**	**26.76**
GFC	**10**	**62.50**	**625.00**	**0.123**	**1.23**	**508.13**	**348.03**	**24.17**

IEC – Ion exchange chromatography; GFC – Gel Filtration chromatography

Figure 4. 1. DEAE cellulose column chromatogram of ammonium sulphate fraction of FTase produced by *A. oryzae* CFR 202

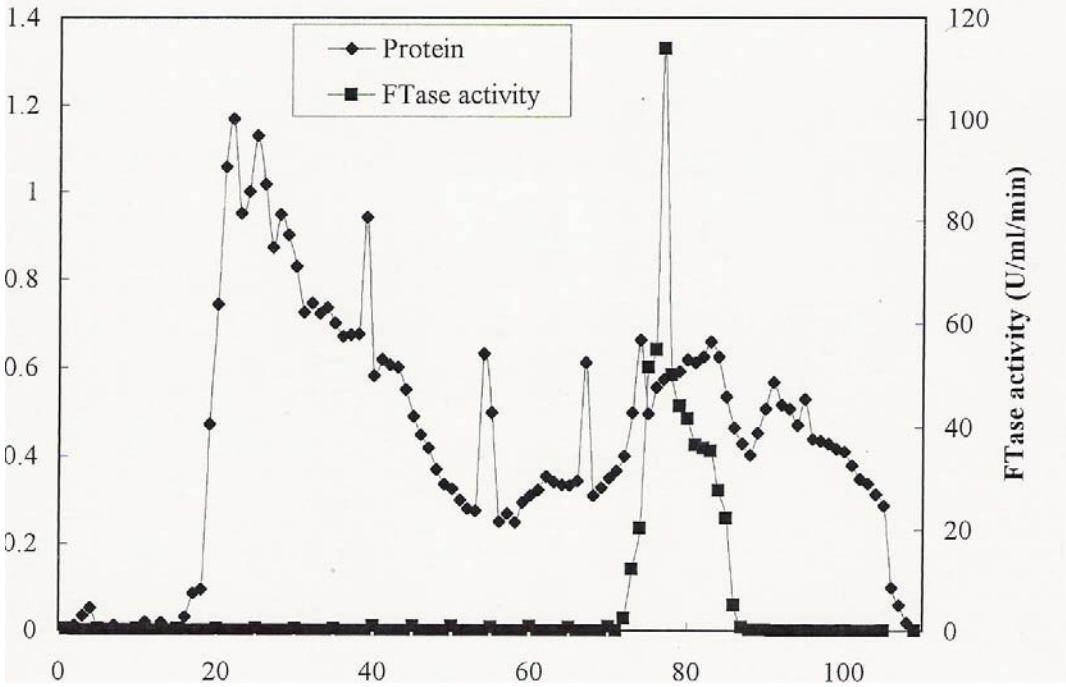


Figure 4. 2. Sephadex G - 200 column chromatogram of active fraction of DEAE cellulose column chromatography

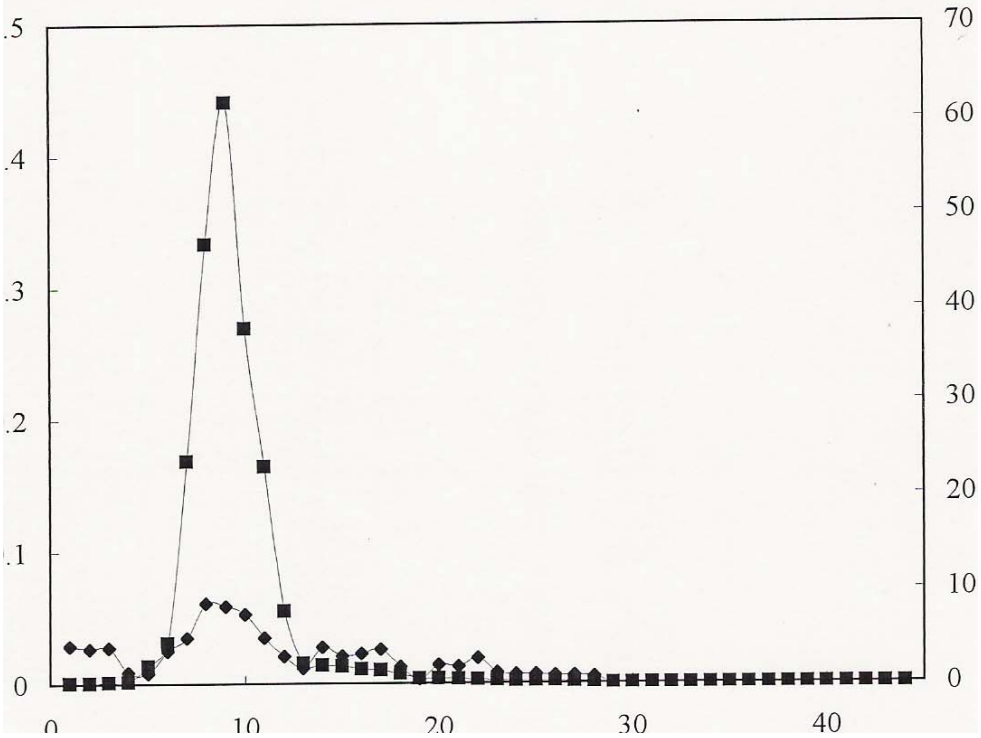
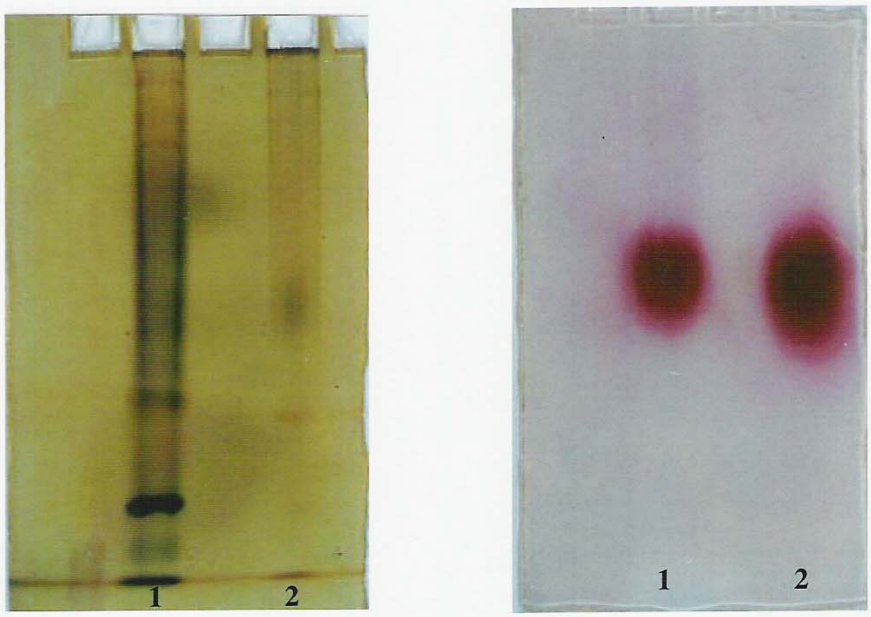


Figure 4.3. Native PAGE of purified FTase (a) Silver staining (b) Activity staining.



(a) (b)

1. Crude FTase
2. Purified FTase

Figure 4.4. Capillary electropherogram of purified FTase

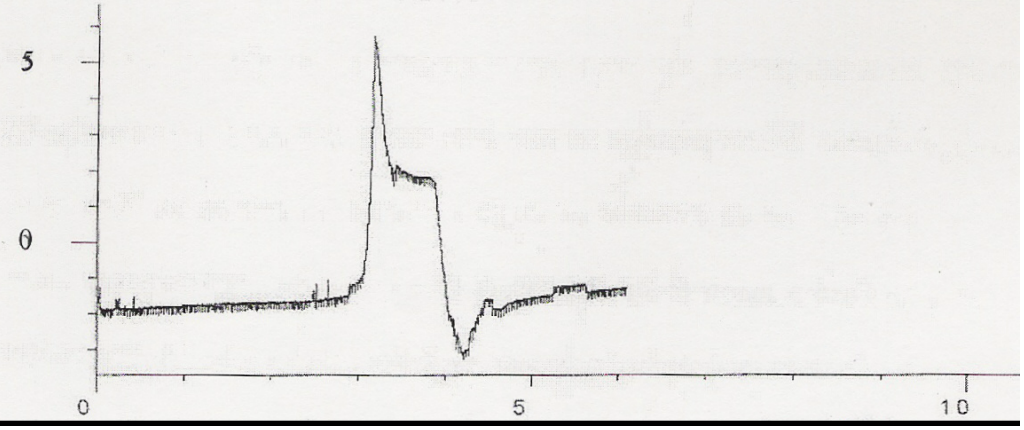
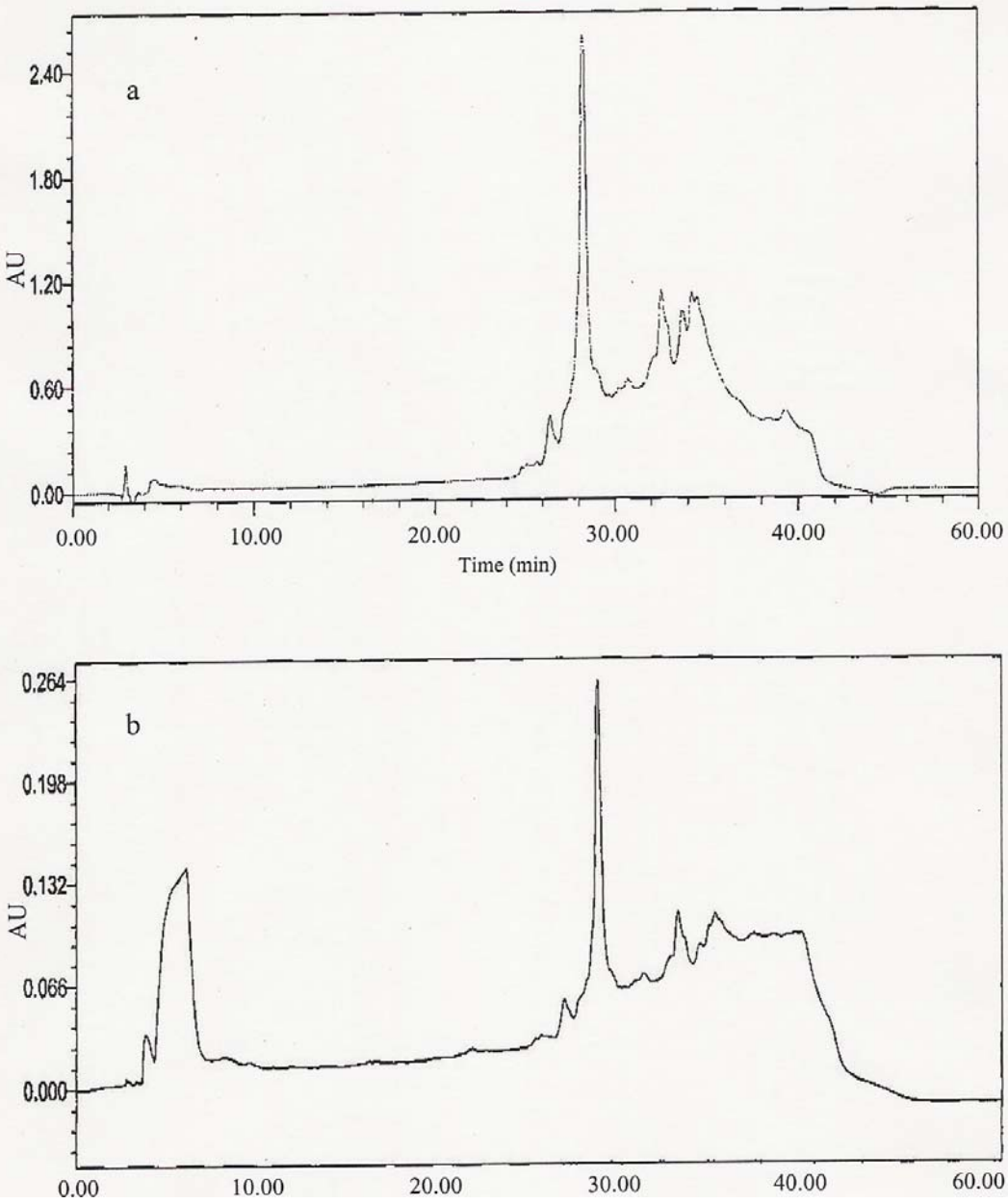


Figure 4.5. Reverse phase HPLC elution profile of purified FTase at (a) 220 nm and (b) 280 nm



4. 3. 1. 5. Molecular weight determination

The apparent molecular weight of the purified FTase was determined by SDS PAGE. Silver staining revealed two bands, one corresponding to 97.4 kDa and another to 69.0 kDa (Figure 4. 6). Figure 4. 7 shows the calibration graph using molecular weight standards on SDS PAGE.

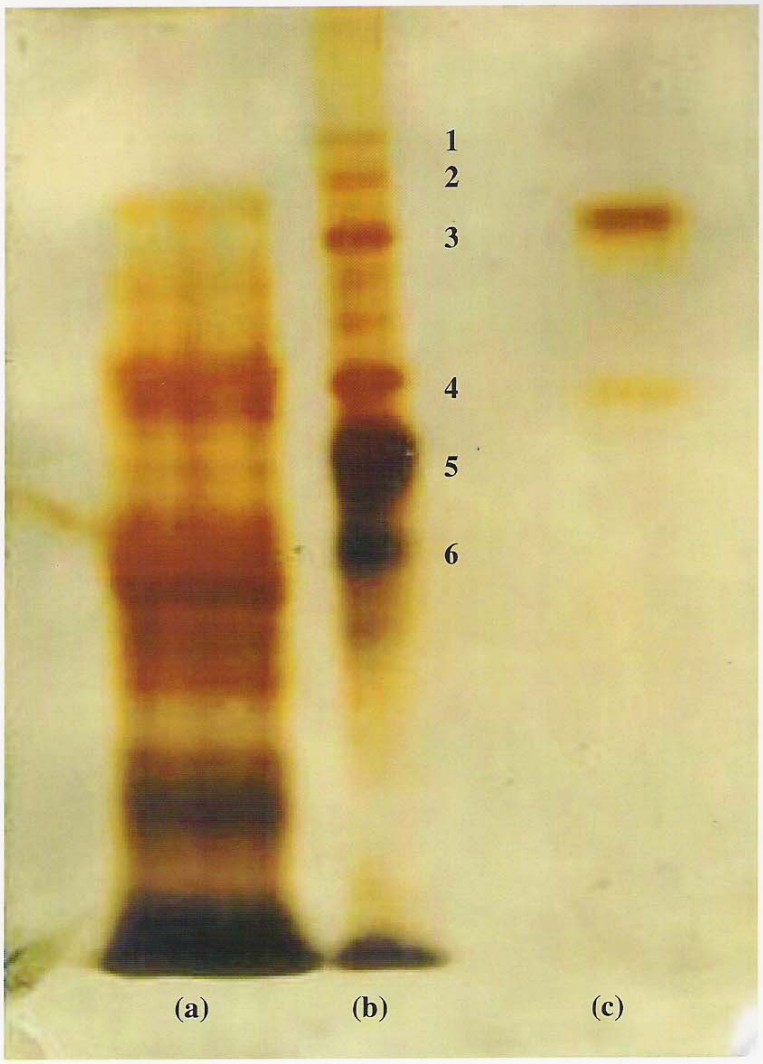
FTase from various sources differ in their molecular weights. Many microbial FTases were reported to be monomeric having molecular weight of around 45 to 52 kDa including *Fusarium* sp. (Murayama and Onodera, 1979), *A. niger* ATCC 20611 (Hirayama *et al*, 1989), *Arthrobacter* sp. K- 1 (Fujita *et al*, 1990), *A. sydowi* (Muramatsu and Nakakuki, 1995) and *Microbacterium* sp. AL-210 (Cha *et al*, 2001) enzymes. FTase purified from *B. macerans* had molecular weight of 66 kDa (Park *et al*, 2001). The purified FTase of *Streptococcus salivarius* ATCC 25975 had a molecular weight of 125.4 kDa on SDS PAGE, 180.6 kDa by gel filtration and 102 kDa on native PAGE (Song and Jacques, 1999). However, there are also reports suggesting the polymeric nature of FTase. Native PAGE of FTase from *A. niger* AS0023 showed two bands of 600 kDa and 309 kDa. But, SDS PAGE yielded one band with 168 kDa confirming its polymeric structure (L' Hocine *et al* 2000). The molecular weight of FOS producing enzyme purified from *A. niger* ATCC 20611 was 340 kDa by gel filtration and 100 kDa by SDS PAGE suggesting the presence of sub unit structure of the enzyme (Hirayama *et al* (1989). FTase purified from *A. oryzae* CFR 202 in the present study had molecular weight within the range reported so far.

4. 3. 2. Characterization of FTase

4.3.2.1. Temperature and pH stability

Crude FTase retained 80 % activity at 30 and 40 °C up to 1 h and after 9 h, 55 % activity was found to be retained. At 50 °C, the enzyme lost 50 % activity by the end of 2 h, whereas at 60 °C, the enzyme retained only 10 % activity by the end of 1 h (Figure 4. 8). The enzyme was stable at pH 4 and 5 and it retained 85 % activity at pH 6 and 70 % activity at pH 7 (Figure 4. 9). Purified FTase was stable at 30 and 40 °C and over a pH range of 4.0 to 6.0 retaining 80 % of their activity (Figures 4. 8 & 4. 9).

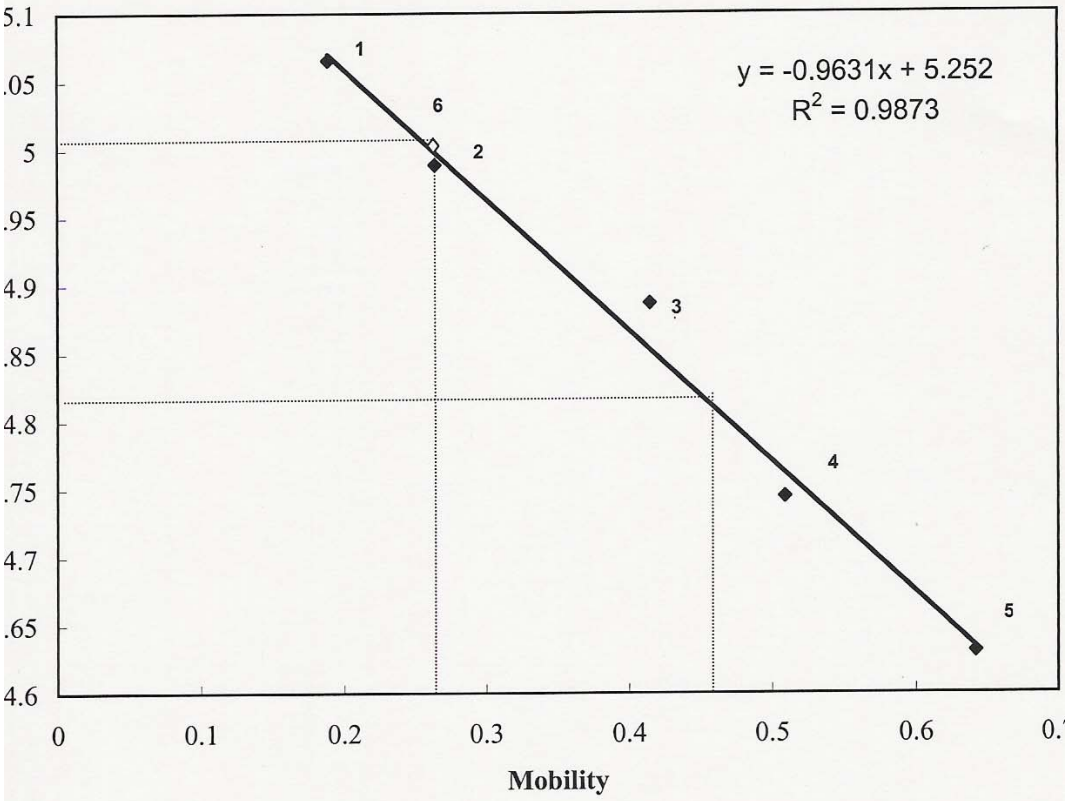
Figure 4.6. SDS PAGE of purified FTase detected by silver staining.



a) Crude FTase ; b) Standard Protein Markers ; c) Purified FTase

1) Myosin - 200 kD
2) β-galactosidase - 116.3 kD
3) Phosphorylase b - 97.4 kD
4) Ovotransferrin - 77.0 kD
5) Glutamate dehydrogenase - 55.5 kD
6) Ovalbumin - 42.7 kD

Figure 4.7. Estimation of molecular weight of purified FTase by SDS PAGE



SDS PAGE Standard Proteins

1 - β - galactosidase
2 - Phosphorylase b
3 - Ovotransferrin
4 - Glutamate dehydrogenase
5 - Ovalbumin
6 - Purified FTase

Figure 4.8. Effect of temperature on stability of crude and purified FTase

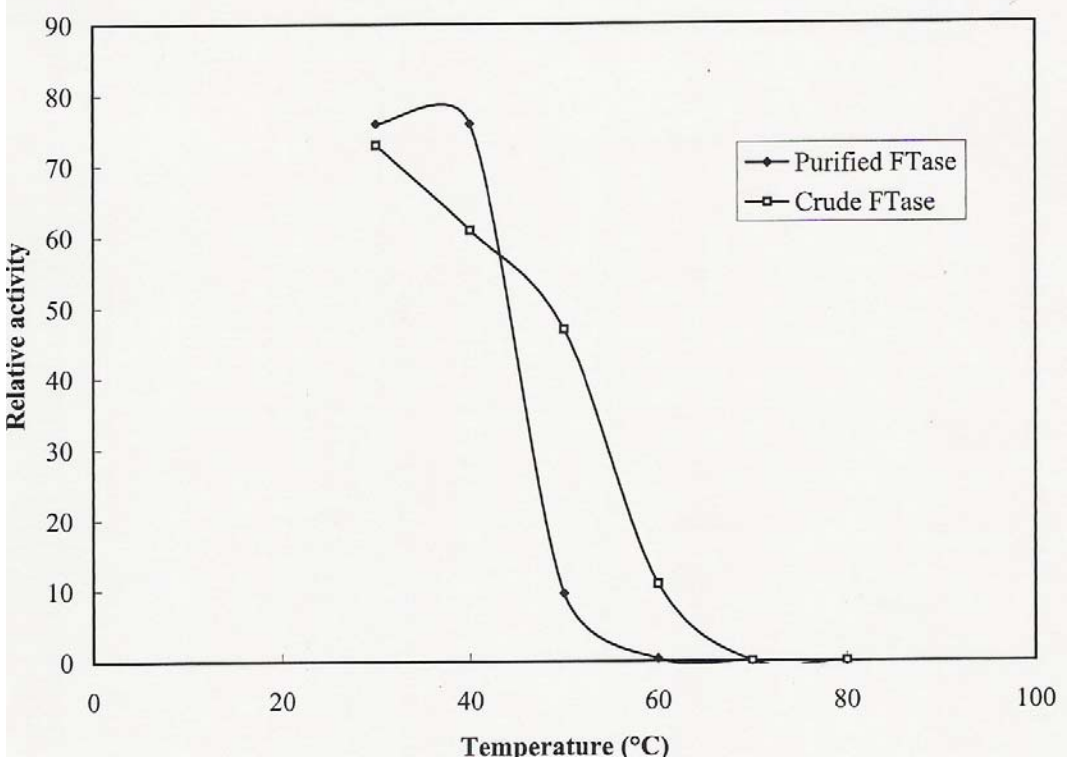
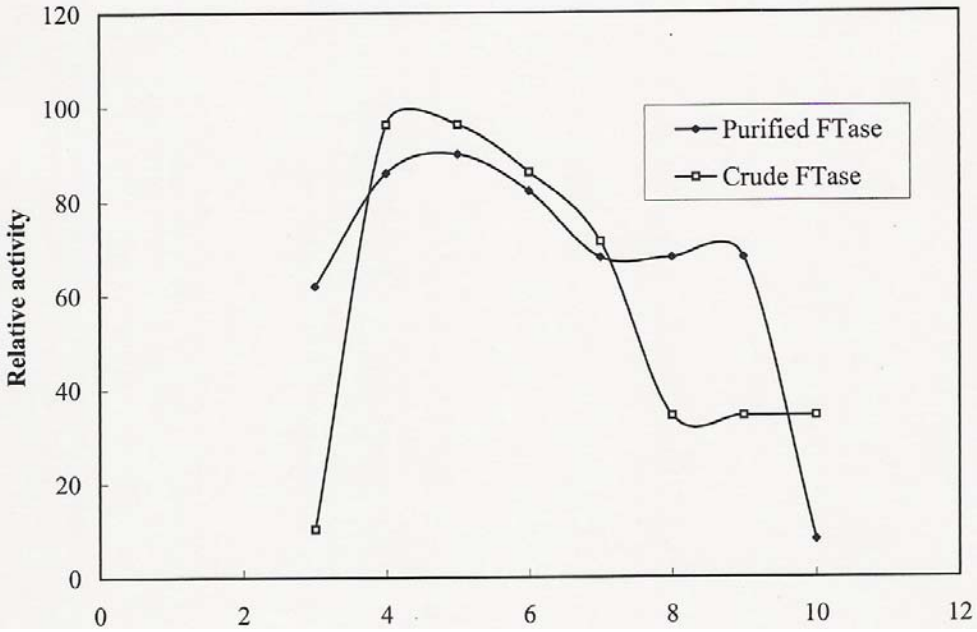


Figure 4.9. Effect of pH on stability of crude and purified FTase



Temperature and pH stability of FTases varies from one microorganism to another. Most of the FTases produced by *B. macerans* (Park *et al*, 2001), *A. niger* AS0023 (L' Hocine *et al*, 2000), *Microbacterium* sp. AL-210 (Cha *et al*, 2001) and *Bacillus subtilis* (Peter *et al*, 1989) were stable upto 50 °C supporting the results obtained in the present study. However, there are reports on some FTases stable upto 60 and 75 °C also (Hirayama *et al*, 1989; Muramatsu and Nakakuki, 1995). Most of the enzymes are stable within a limited range of pH. FTase from *B. macerans* was stable from pH 5 – 7 (Park *et al*, 2001) whereas the enzyme from *Microbacterium* was stable at a pH range 7.0–8.0 (Cha *et al*, 2001). There are also reports on enzymes that exhibit stability over a wide range of pH. The enzyme from *A. niger* AS0023 was stable from pH 4.5 to 11 (L' Hocine *et al* 2000). FTase from *A. niger* and β- fructofuranosidase from *A. sydowi* was stable from pH 4.5 – 10.0 (Hirayama *et al*, 1989; Muramatsu and Nakakuki, 1995).

4.3.2.2. Optimum temperature and pH for activity

The optimum temperature for activity was 60 °C, whereas at 50 °C and 70 °C, the enzyme retained 90 and 80 % activity respectively (Figure 4. 10). The optimum activity was at pH 7.0 and exhibited 97 % activity at pH 5.0 and 6.0 (Figure 4. 11). The optimum pH and temperature for FTases from various sources varied from 5.0 to 7.0 and 40 – 60 °C respectively.

FTase from *A. pullulans* (Patil and Patil 1999), *B. macerans* (Park *et al*, 2001), *A. niger* AS 0023 (L' Hocine *et al* 2000), *A. niger* (Hirayama *et al,* 1989) and *A. sydowi* (Muramatsu and Nakakuki, 1995) have maximum activity at temperature ranging from 50 to 60 °C. This is in concurrence with the result obtained in the present study. Some bacterial enzymes produced by *Microbacterium, S. salivarius* and *A. diazotrophicus* were reported to have lower temperature optima ranging from 37 – 40 °C (Cha *et al*, 2001; Song and Jacques, 1999 and Tambara *et al*, 1999). Optimum pH for FTase from *A. pullulans* was reported to be 4.0 (Patil and Patil, 1999). FTase from *Bacillus sp.* has optimum pH at 5 (Peter *et al*, 1989; Park *et al,* 2001). The optimum pH of FTase from *A. niger, A. diazotrophicus* and *A. sydowi* was from 5.0 to 6.0 (Hirayama *et al*, 1989; Muramatsu and Nakakuki, 1995; Tambara *et al*, 1999; L'

Hocine *et al*, 2000). Some bacterial enzymes from *Microbacterium* and *Streptococcus salivarius* ATCC 25975 were reported to have maximum activity at pH – 6.0 and 7.0 (Song and Jacques, 1999; Cha *et al*, 2001).

4. 3. 2. 3. Effect of metal ions, detergents, additives and inhibitors on FTase activity

Among the various metal ions used, Mn^{2+} ions decreased the activity by 10 % whereas, Ca^{2+}, Ag^{2+} and Mg^{2+} decreased the activity by 30, 20 and 18 %, respectively. Detergents like SDS and Tween 80 and additives like glycerol and PEG did not inhibit the activity but, mercaptoethanol inhibited the activity by 75 % (Table 4. 2). Literature reports suggest that most of the FTases were inhibited by mercury and silver ions. FTase from *A. niger* AS0023 was inhibited by mercury and silver ions (L' Hocine *et al*, 2000). Mercury was found to inhibit FTase activity in *A. niger* (Hirayama *et al*, 1989). In *Microbacterium,* $FeCl_2$, $AgNO_3$ and 2-Mercaptoethanol inhibited the enzyme activity. Peter *et al* (1989) have isolated an extracellular fructosyl transferase from *B. subtilis* which was unaffected by anionic, cationic or non-ionic detergents. Zn^{2+} and Hg^{2+} inhibited 44 % and 100 % of the activity. The purified FTase of *Streptococcus salivarius* ATCC 25975 was activated by Ca^{2+}, but inhibited by the metal ions Cu^{2+}, Zn^{2+}, Hg^{2+} and Fe^{3+} (Song and Jacques, 1999).

Table 4.2. Effect of metal ions, additives, detergents and inhibitors on FTase activity

Additive	Relative activity (%)
Metal ions (1mM)	
Fe^{2+}	**85**
Ag^{2+}	**80**
Mg^{2+}	**82**
Mn^{2+}	**90**
Zn^{2+}	**87**
Cu^{2+}	**87**
Ca^{2+}	**71**
Detergents (1 %)	
SDS	**103**
Tween 80	**88**
Additives (1 %)	
Glycerol	**94**
PEG	**91**
Inhibitors (1 %)	
Mercaptoethanol	**24**

Figure 4.10. Effect of temperature on activity of crude and purified FTase

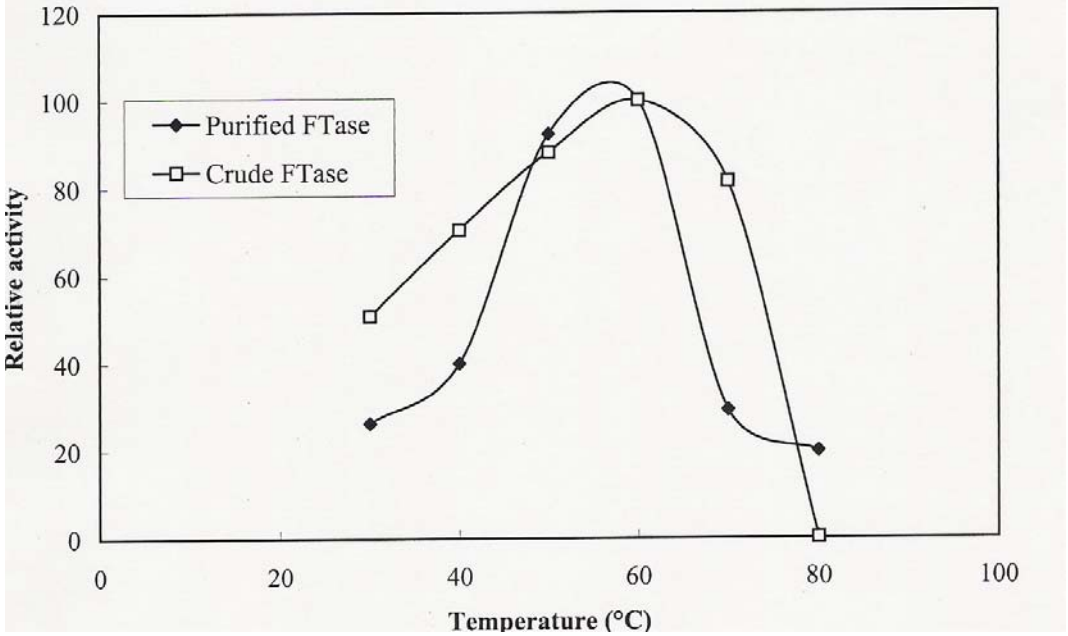
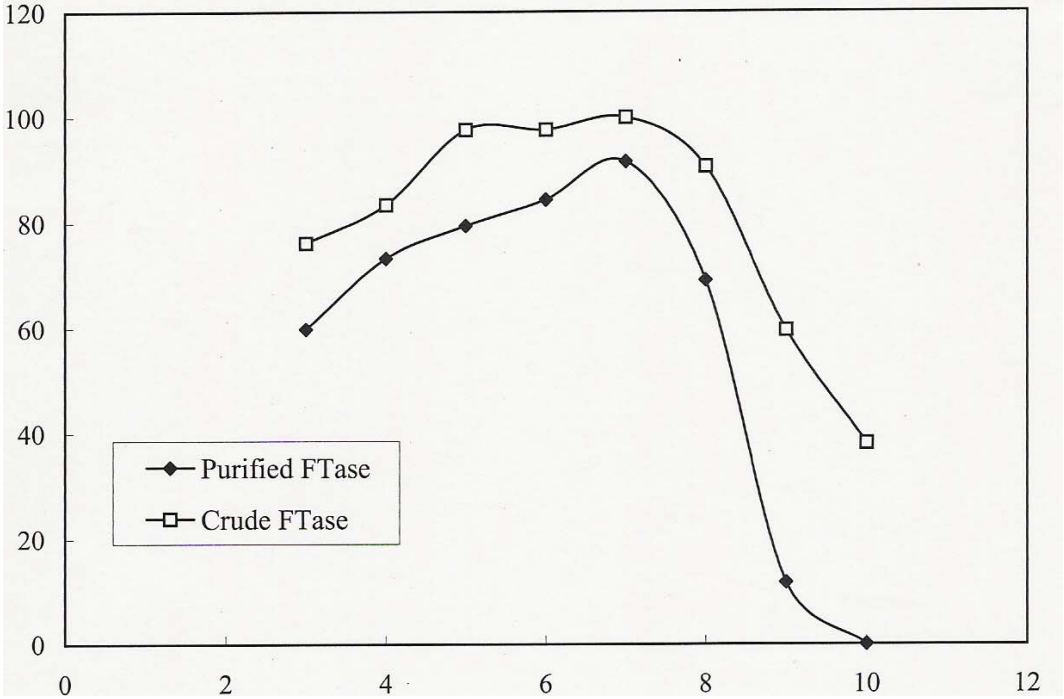


Figure 4.11. Effect of pH on activity of crude and purified FTase

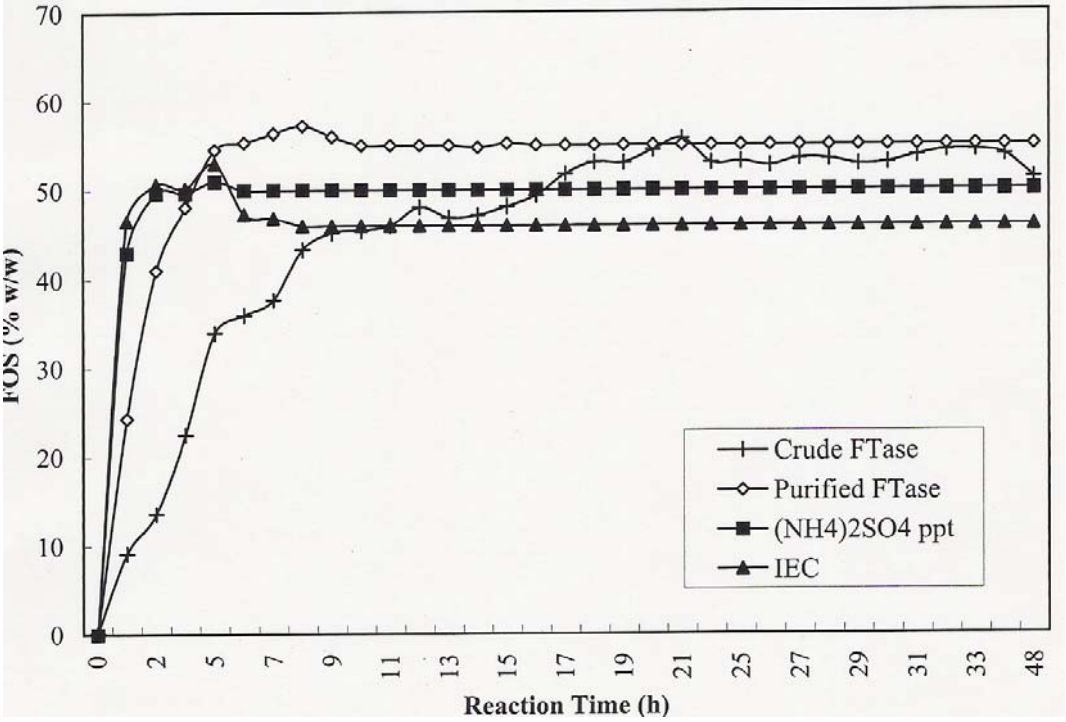


4. 3. 3. Production of FOS

FOS production carried out using crude FTase produced maximum FOS yields after 18 h of reaction with sucrose whereas after purification the maximum yields could be achieved after 4 h of reaction itself (Figure 4. 12). This could be attributed to the increase in fold of purification and the high specific activity of the purified FTase (Table 4. 1).

In conclusion, FTase produced by *A. oryzae* CFR 202 was characterized as a moderately large molecule of 97 kDa molecular weight that is stable upto 40 °C and at pH 4.0 to 6.0. The optimum pH and temperature of this enzyme are 7.0 and 60 °C respectively, but it was not affected much by metal ions. Eventhough purification of FTase considerably reduced the reaction time to get maximum FOS yields, for mass production of FOS, it is economical to use crude FTase.

Fig 4.12. Time course of FOS production using FTase at various stages of purification



CHAPTER 5
FTase PRODUCTION BY SOLID STATE FERMENTATION

5. 1. INTRODUCTION

Solid State Fermentation (SSF) has been defined as a process in which microorganisms are grown on solid substrates in the absence of free water. (Lagemaat and Pyle, 2001). SSF processes involve the growth of microorganisms, especially fungi, on moist solid substrates, and have considerable economical potential for the preparation of products for the food, feed, pharmaceutical and agricultural industries.

SSF systems can be classified into two types depending on the nature of the solid substrate used. The first and most commonly used system involving the growth on a natural material is referred to as 'cultivation on natural substrates'. The second system, which is not so frequently used, involves the growth on an inert support impregnated with a liquid medium. The first system uses natural materials that serve both as a support and as a nutrient source. These materials are typically starch – or (ligno)- cellulose – based agricultural products or agro- industrial sources such as grains and grain by - products, cassava, potato, beans and sugar beet pulp. The solid support of the second system, which can be of natural origin, serves only as an anchor point for the microorganism. The substrates used include hemp, perlite, polyurethane foam, sugarcane bagasse and vermiculite (Ooijkaas *et al*, 2000).

SSF holds tremendous potential for the production of enzymes. It is of special interest in those processes where the crude fermented product may be used directly as the enzyme source. Agro-industrial residues are generally considered the best substrates for the SSF processes, especially for enzyme production. Some of the substrates that have been used include sugarcane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soy hull, corncobs, banana waste, tea waste, cassava waste, apple pomace etc, to name a few. Wheat bran however, is the most commonly used substrate.

Major factors that affect microbial synthesis of enzymes in a SSF system include: selection of a suitable substrate and microorganism, pretreatment of the substrate, particle size of the substrate, water content and water activity

(a_w) of the substrate, relative humidity, type and size of the inoculum, control of temperature of fermenting matter / removal of metabolic heat, period of cultivation, maintenance of uniformity in the environment of SSF system and the gaseous atmosphere (oxygen consumption and CO_2 release) (Pandey *et al*, 1999). Production of enzymes by solid state fermentation has potential advantages over the submerged state fermentation with respect to simplicity in operation, high productivity fermentation, less favourable for growth of contaminants and concentrated product formation (Balasubramaniem *et al*, 2001).

SSF can be used to convert the agro-industrial residues to value added products by using them as substrates for the growth of microorganisms. Filamentous fungi have the best capability to grow on solid substrates in the absence of free water. Production of α - galactosidase from *Aspergillus oryzae* has been studied by SSF using wheat bran, rice, soybean, soyflour and mixture of all these substrates (Annunziato *et al*, 1986). The use of SSF for pectinase production has been proposed using different solid agricultural and agro-industrial residues as substrates such as wheat bran, soy bran, apple pomace, cran berry and straw berry pomace, beet pulp, coffee pulp and husk, cocoa, lemon and orange peel and sugar cane bagasse (Martins *et al*, 2002).

SSF has been considered as a useful tool for biomass energy conservation, solid waste treatment and production of value added molecules such as enzymes, organic acids and biologically active secondary metabolites. The use of agro-industrial residues in bioprocesses on the one hand provides alternative substrates, and on the other hand helps in solving pollution problems, which may be caused by their disposal. Many new avenues have opened for their utilization, mainly in the area of enzyme and fermentation technology. With the advent of biotechnological innovations, it has been observed that the minimal amount of water present during SSF allows the production of metabolites in a more concentrated form, making the downstream processing less time consuming and less expensive. The low moisture content also minimizes problems with bacterial contamination during fermentation.

5. 2. MATERIALS AND METHODS

5. 2. 1. Microorganism

The microorganism used in this study is *Aspergillus oryzae* CFR 202. Growth and maintenance of the strain is as mentioned in Section 3. a. 2. 2.

5. 2. 2. Solid Substrates and Preparation of fermentation media

The solid substrates used were (a) cereal bran like wheat bran, rice bran and oat bran; (b) corn products like corn cob, corn bran, corn germ, corn meal and corn grits; (c) coffee and tea processing by - products like coffee husk, coffee pulp, spent coffee and spent tea; (d) sugarcane bagasse and (e) cassava bagasse (tippi).

(a) The various cereal bran were obtained from the local market.

(b) The corn products were obtained after milling from the Pilot Maize Mill of the Institute.

(c) The coffee and tea processing byproducts were obtained from PPSFT Department, of the Institute.

(d) Sugarcane bagasse was obtained from the local market. Cassava bagasse (tippi) was obtained from Selvakumar Sago Industries, Salem, Tamilnadu.

The moisture content of the substrates were determined by AOAC (AOAC, 1984) method by drying them to constant weight in a hot air oven at 110 °C (The Andhra Scientific company Ltd., Andhra Pradesh, India). SSF was carried out in 250 ml conical flasks containing 10 g of substrates moistened with water in order to give a final moisture content of 55 – 60 %. The flasks were sterilized at 121 °C for 1 h.

5. 2. 3. Inoculum preparation and Solid State Fermentation

The inoculum was prepared as described in Section 3. a. 2. 3. The substrates were inoculated with 20 % (v/w) inoculum. The contents of the flasks were mixed uniformly and the flasks were incubated in slanting position at 30 ± 1 °C in an incubator (KITCO, C. U. Kamath & Co., New Delhi). Fermentation was carried out up to 120 h.

5. 2. 4. Effect of moisture content of corn products on FOS production

FTase production was carried out by growing the organism on different corn products with varying levels of moisture. The different moisture contents tried were 12 ± 3 %, 30 ± 3 %, 41 ± 3 %, 51 ± 3 % and 57 ± 3 %. Fermentation was carried out for 96 h as described in Section 5. 2. 3. pH of the Moldy Substrate Extract (MSE) was recorded after fermentation time.

5. 2. 5. Effect of supplementation of substrates with nitrogen, complete synthetic media and rice bran

The effect of supplementation of substrates like coffee and tea processing by - products, sugarcane bagasse and tippi was studied by mixing the substrates with various nitrogen sources like ammonium sulfate, urea and yeast extract to give 1 % Nitrogen, complete synthetic media used for Submerged Fermentation (Table. 3. c. 2. 9) and rice bran in the proportion of 30 %.

5. 2. 6. Fructosyl Transferase assay, FOS production and analytical procedure

At the end of specified fermentation time, the substrates in each flask were mixed with 50 ml water and the flasks were agitated at 250 rpm on a rotary shaker (Emenvee Rotary Shaker, 48N3, Pune, India) for 90 min. The MSE was filtered using filter paper (Whatman No. 2) and the filtrate was used as FTase. The pH of the filtrate was recorded. Assay of FTase was carried out as described in Section 3. a. 2. 5 using 60 % sucrose as substrate. Enzyme activity was determined based on glucose released after the reaction. Glucose released was measured using Glucose kit (GOD/ POD, Dr. Reddy's Laboratories Ltd., Hyderabad). FOS production was also carried out as described in Section 3. b. 2. 2. and analysis of the products was as given in Section 3. a. 2. 6.

5. 2. 7. Characterization of FTase

5. 2. 7. 1. Optimum pH and temperature for enzyme activity

The optimum pH and temperature for crude FTase activity was determined as described in Section 4. 2. 3. 1.

5. 2. 7. 2. Thermostability of the enzyme

Thermostability and pH stability of the enzyme were determined by the procedure given in Sections 4. 2. 3. 2.

5. 2. 7. 3. pH stability of the enzyme

The crude enzyme was dispersed in 0.1 M buffer solution (1:1 ratio) at pH 3.0 – 5.0 (citrate buffer), 6.0 – 8.0 (sodium phosphate buffer) and 9.0 & 10.0 (bicarbonate buffer) and incubated at room temperature for 24 h. The activity was determined after 24 h.

5. 2. 8. Production of FOS

FOS production was carried out as described in Section 4. 2. 8.

5. 3. RESULTS AND DISCUSSION

5. 3. 1. FOS production with FTase obtained by SSF using agro-industrial by - products

SSF is a culture system that has been used in several countries since antiquity. One of the major advantages that SSF offers is in the utilization of agro-industrial residues. In the present study, the use of various agricultural by - products have been explored for the production of FTase. All the substrates were used for fermentation after determining the moisture content. Table 5. 1 summarizes the moisture contents of the substrates used for fermentation.

5. 3. 1. a. Cereal bran

India is one of the leading producers of cereals and cereal bran is an easily available cheap commodity, which finds use only as poultry feed and fuel in the rural areas. Cereal bran, especially wheat bran and rice bran, being rich sources of carbon and nitrogen can thus serve as a suitable substrate for the growth of microorganisms by SSF. The proximate composition of the different types of bran is given in Table 5. 2.

Table 5.1. Moisture content of different solid substrates used for FTase production by *A. oryzae* CFR 202

Substrates	Moisture content (%)
Wheat bran	14.0
Rice bran	20.0
Oat bran	06.5
Corn germ	12.0
Corn meal	14.0
Corn cob	08.5
Corn grits	15.0
Corn bran	12.0
Coffee pulp	11.1
Coffee husk	08.0
Spent coffee	06.3
Spent tea	07.2
Sugar cane bagasse	04 .0
Cassava Fibrous residue	13 .0

A. oryzae CFR 202 was grown on wheat bran and rice bran with 60 % moisture and oat bran with 53 % moisture. The fungus showed profuse growth when cultivated on rice bran, wheat bran and oat bran. The FTase activity was analyzed over a period of 120 h. When wheat bran was used as substrate, pH of the extracts was found to increase from 5.0 at 48 h of fermentation to 7.5 at 120 h fermentation. In the case of rice bran, the pH varied from 6.3 to 8.0 whereas in the case of oat bran the pH varied from 4.8 – 6.0 (Figure 5.1). FTase produced using rice bran as substrate had maximum activity (22.6 Uml $^{-1}$min $^{-1}$) followed by FTase produced using wheat bran (17 Uml $^{-1}$min $^{-1}$) and oat bran (13.3 Uml $^{-1}$min $^{-1}$) (Figure 5. 2). When SSF was carried out on rice bran, the % FOS produced by the FTase was found to increase with fermentation time and maximum yield was obtained using FTase produced at the end of 120 h of fermentation. In the case of wheat bran FTase, maximum yield of FOS was obtained using FTase at the end of the 48 h fermentation after which FOS yields were decreasing. In the case of oat bran, maximum yield of FOS was

obtained with FTase produced at the end of 96 h. The results given in Figure 5. 3 shows that FTase obtained by SSF on rice bran resulted in the maximum yield of FOS (51.7 %) whereas, FTase from wheat bran produced 49.5 % FOS and FTase from oat bran produced 27 % FOS.

Table 5.2. Composition of cereal bran (% w/w) (Kahlon and Chow, 2000).

		Dietary fibre				
Substrate	**Moisture**	**Total**	**Insoluble**	**Soluble**	**Fat**	**Nitrogen**
Rice bran	5.4	27.0	24.5	2.5	23.7	2.5
Oat bran	8.9	19.5	14.5	7.5	7.5	2.9
Wheat bran	14.6	52.2	48.4	3.8	3.8	2.7
Corn bran	4.9	86.5	86.3	1.4	1.4	0.5

The difference in FOS yields may be attributed to the particle size of the substrates as well as the nutrient availability in the substrates. Smaller substrate particles provide larger surface area for microbial attack and thus are a desirable factor for more product formation. This may be the reason for rice bran being a better substrate than wheat bran for FTase production. However, too small a substrate particle size may result in substrate accumulation, which may interfere with microbial respiration / aeration, and therefore result in poor growth. Oat bran being smaller in size, resulted in clumping even with 50 % moisture and hence had less FTase activity. In contrast, larger particles provide better respiration / aeration efficiency (due to increased inter particle space). This substantiates that an optimum particle size is required for a particular process, which is provided by wheat bran and rice bran.

Figure 5.1. pH profile during the production of FTase by *A. oryzae* CFR 202 grown on cereal bran

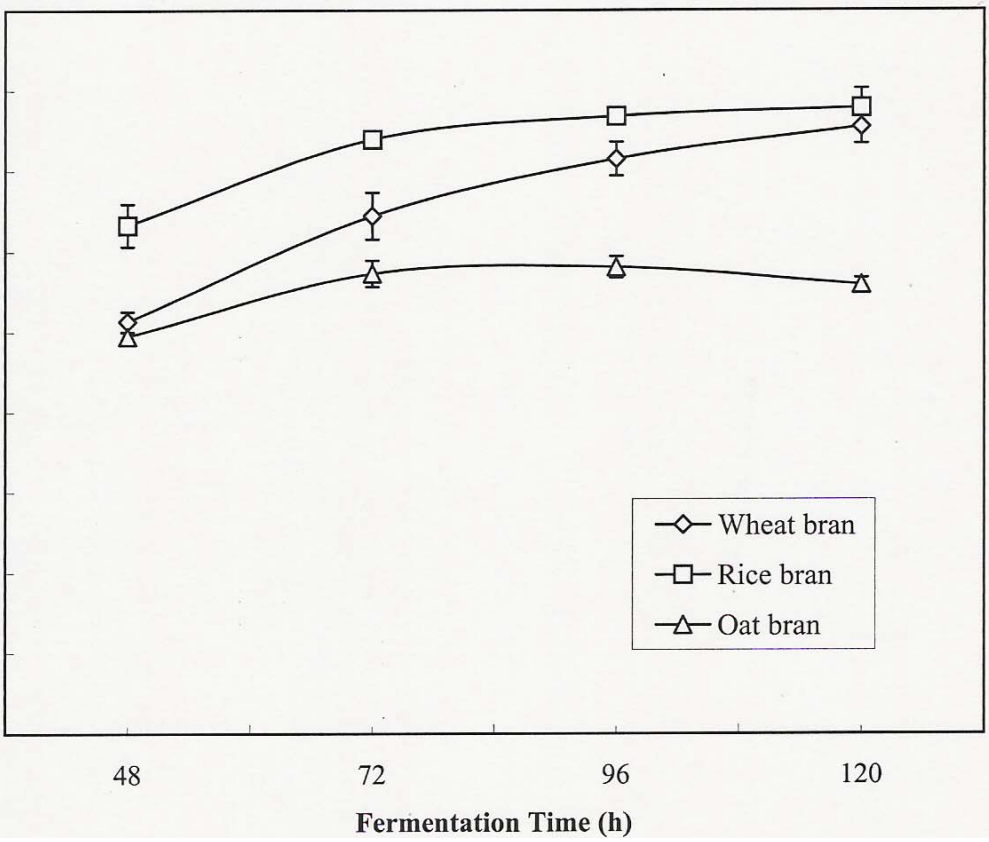


Figure 5. 2. FTase production by *A. oryzae* CFR 202 grown on cereal bran

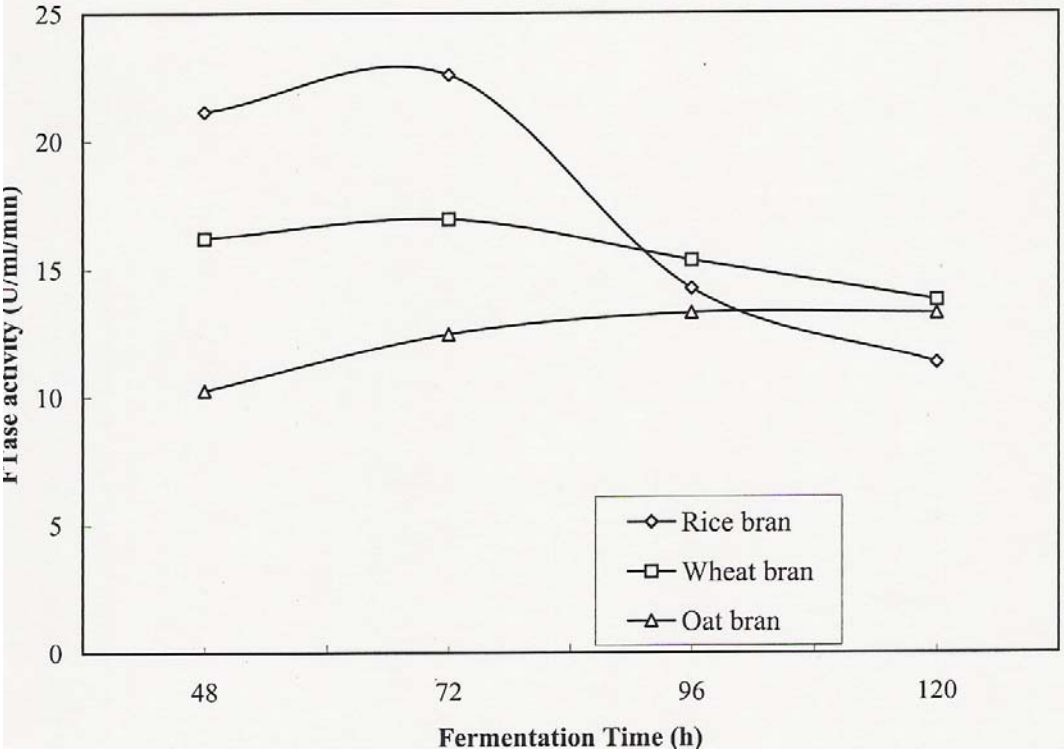
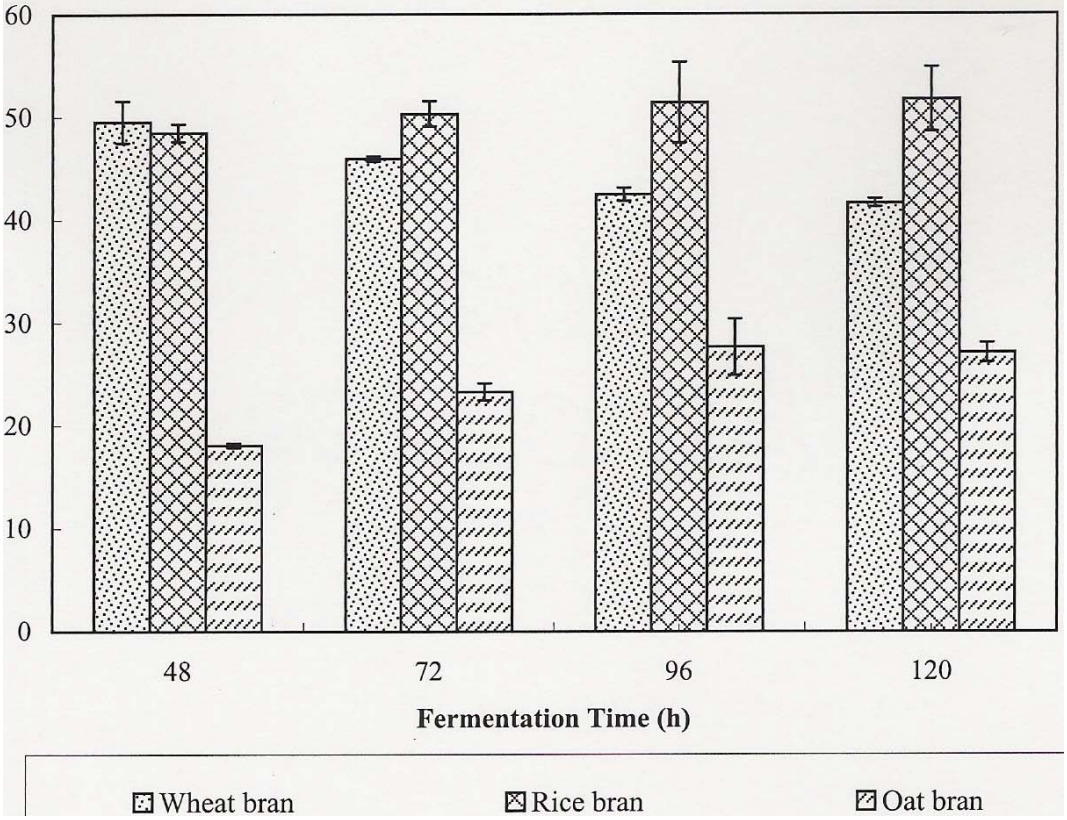


Figure 5.3. FOS yields obtained using FTase produced by *A. oryzae* CFR 202 grown on cereal bran



5. 3. 1. b. Corn products

Corn is an agronomically versatile crop grown in many countries all over the world. Its annual world production is around 600 million tonnes and it ranks second in production among cereals after wheat. In India, corn ranks fourth in production after rice, wheat and sorghum with a production of about 10 million tonnes per annum. Fifty percent of maize produced is used for food purposes, 40 % for feed purposes and 5 % each for seed and industrial applications. Dry milling of corn results in the separation of germ from grits, the process in which bran and meal are by – products. Table 4. 3 gives the composition of various corn products. Corn meal and grits find use as food products whereas corn germ serves as a rich source of oil. But, by – products like corn bran and corn cob are not utilized for any purpose except as fuel. In the present section, all the corn products have been explored as substrates for the growth of *A. oryzae* CFR 202 to produce FTase.

Table 5.3. Composition of corn products (Brockington, 1970)

Substrates	Carbohydrate	Protein	Lipid	Fiber	Ash
Corn Germ	20.0	18.8	34.5	---	10.1
Corn bran	7.6	3.7	1.0	---	0.8
Corn grits	78.1	8.7	0.9	0.4	0.4
Corn meal	68.8	28.0	---	---	---

Corn cob has 41.2 % cellulose, 36 % hemicellulose, 3.1 % pectins and 6.1 % lignins (Bagby and Widstrom, 1987). FTase production was different when different corn products were used as substrates for SSF. Growth of *A. oryzae* CFR 202 was found to be good when corn germ and corn meal were used as substrates but the growth was poor on corn bran, corn grits and corn cob. Simultaneously, FTase obtained from these substrates showed similar trend (Figure 5. 4). FTase obtained by the SSF of corn germ had maximum activity at the end of 48 h (17.3 U ml $^{-1}$min $^{-1}$) after which it decreased to 13 U ml $^{-1}$min $^{-1}$ at the end of 120 h of fermentation time. This may be attributed to the high carbohydrate content and protein content present in the germ which provides the required nutrients for the growth of the organism as well as the production of the enzyme. Corn meal was the second best substrate for FTase

production. But unlike in germ, FTase activity increased with fermentation time and reached a maximum of 8 U ml $^{-1}$min $^{-1}$ at the end of 96 h after which it decreased. This may be due to the clumping of the substrate due to their small particle size which may inturn affect the oxygen availability to the organism. Other corn products resulted in still lower titres of FTase (2 – 6 U ml $^{-1}$min $^{-1}$).

pH of the MSE was found to be in the range of 4.5 – 7.0 in the case of corn cob, corn meal and corn germ; where as in the case of corn bran and corn grits, it was found to vary from 3.5 to 5.5 (Figure 5. 5). FOS production showed the same trend as FTase production. Using FTase obtained by SSF of corn germ, FOS production was found to be around 49 % at the end of 48 h, beyond which it decreased to 43 % using FTase at the end of 120 h of fermentation. FTase obtained by SSF of corn meal produced 36 % FOS at the end of 96 h of fermentation whereas FTase obtained by SSF on corn grits, corn bran and corn cob produced only 2 to 15 % FOS (Figure 5. 6).

5. 3.1. c. Effect of moisture contents of corn products on FOS production

SSF processes are distinct from SmF, since microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture contents. Thus it is crucial to provide optimized water content and it is equally important to control the water activity of the fermenting substrate, because the availability of water in lower or higher concentrations affects microbial activity adversely. High substrate moisture results in decreased substrate porosity, which in turn prevents oxygen availability (Pandey, 1992).

Water activity (a_w) is an indicator of water availability and is a critical factor in SSF of different kinds of substrates. The effect of water availability on enzyme production in SSF has been studied by many researchers. The low level of water activity of the solid substrate has a significant effect on the physiological activity of microorganisms and enzyme production (Antier *et al*, 1993). Low a_w values have been reported to decrease or to increase extracellular enzyme production. Decrease in a_w of growing cultures of fungi

and yeast leads to modifications in the phospholipid fatty acid saturation and

Figure 5. 4. FTase production by *A. oryzae* CFR 202 grown on corn products

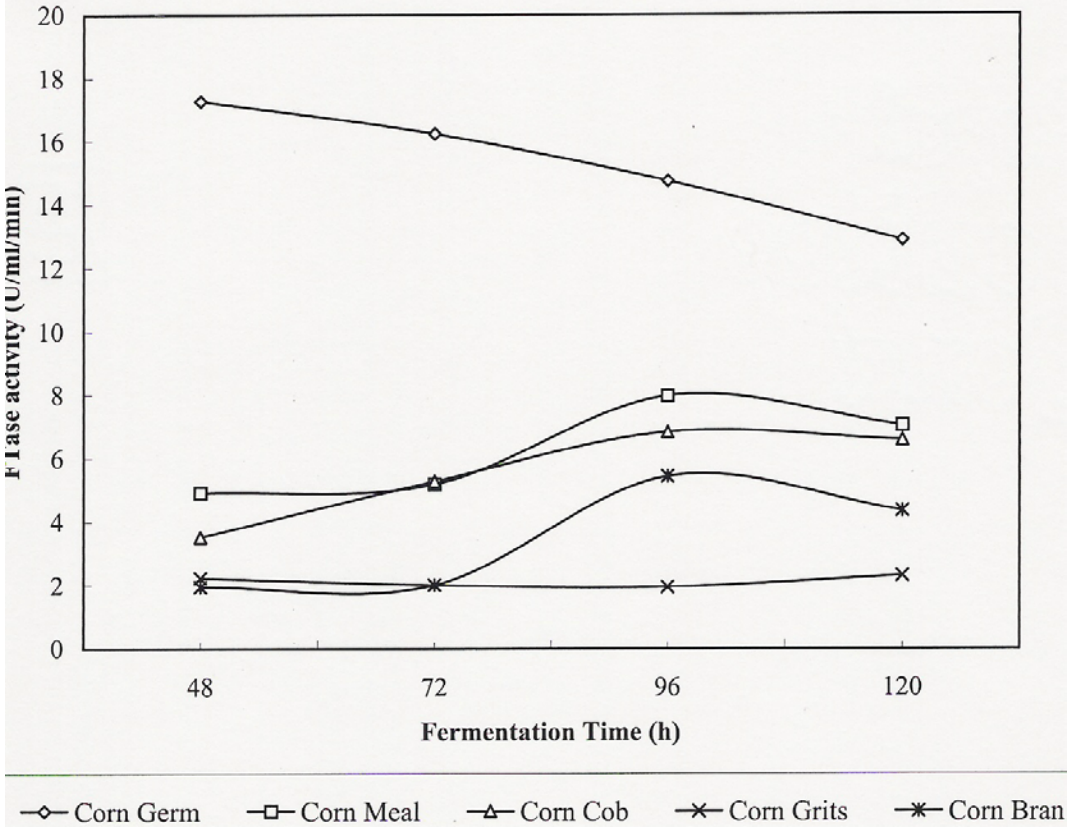


Figure 5. 5. pH profile during the production of FTase by *A. oryzae* CFR 202 grown on corn products

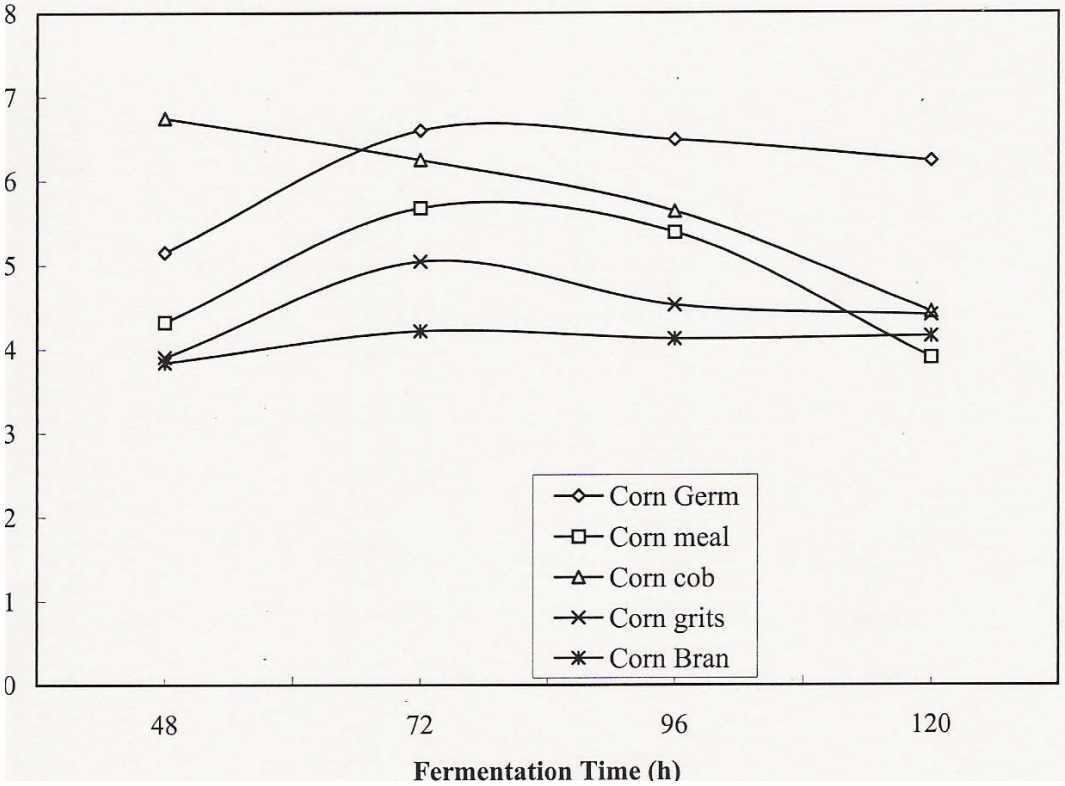
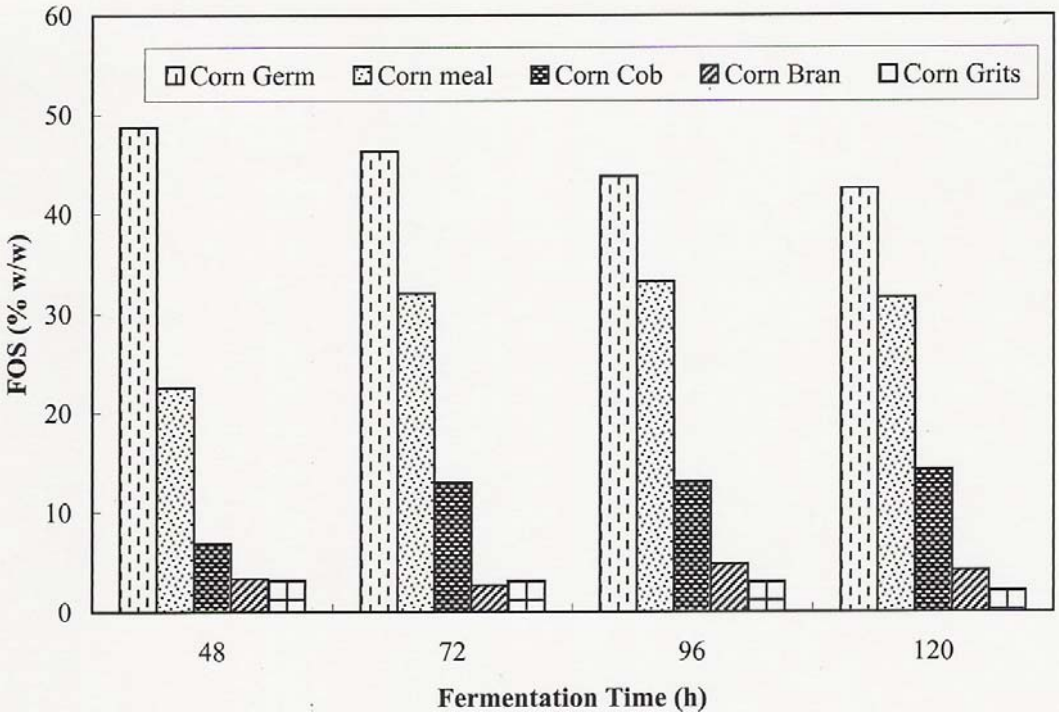


Figure 5. 6. FOS yields obtained using FTase produced by *A. oryzae* CFR 202 grown on corn products

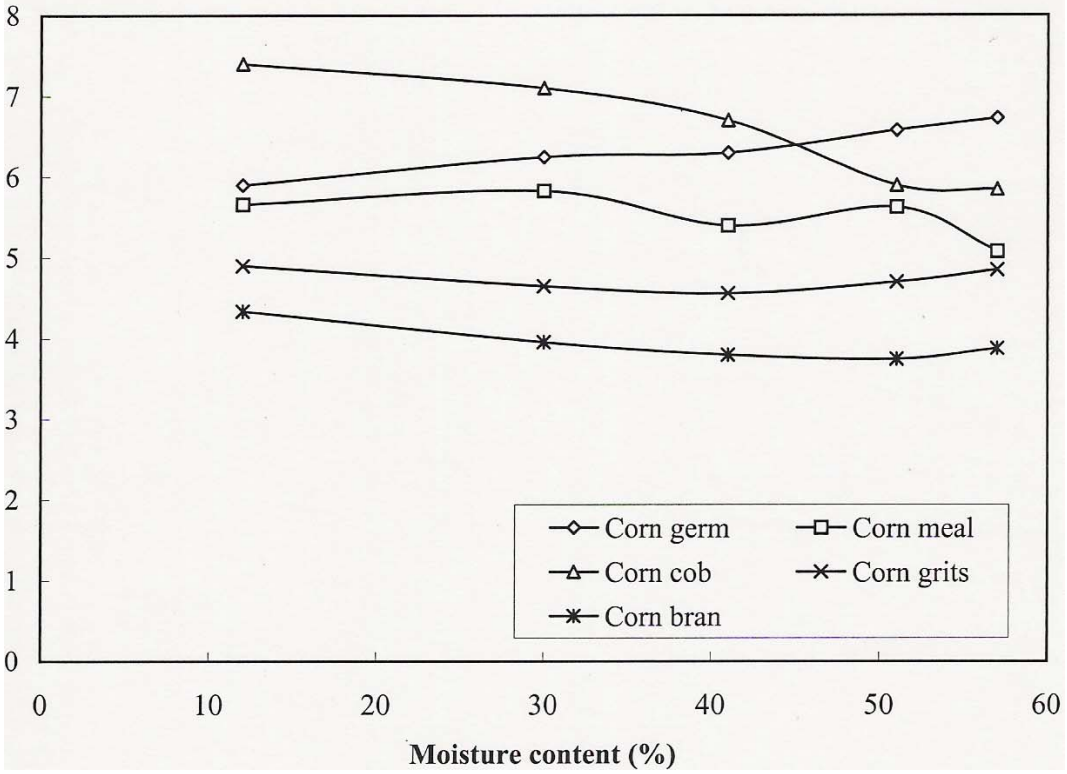


consequently to reduction in the membrane fluidity and permeability. Hence, the nutrients can be accumulated in the culture medium, probably due to membrane transport limitations (Acuna – Arguelles *et al*, 1994).

The amount of moistening agent was shown to influence the physiochemical properties of the solids and productivity of SSF (Nampoothiri and Pandey, 1996; Nigam and Singh, 1994; Gonzalez *et al*, 1988). Water content in the substrate has been reported to influence the water availability, oxygen diffusion through the substrate and penetration of mycelia for effective utilization of substrates (Ramana Murthy *et al*, 1993; Raimbault and Alazard, 1980). Effect of water content has been studied on the production of enzymes like xylanase (Archana and Satyanarayana, 1997), protease (Battaglino *et al*, 1991), pectinases (Acuna- Arguelles *et al*, 1994) and glucoamylase (Pandey *et al*, 1994).

In the present study, after the specified fermentation time, the pH of the MSE was recorded. In corn germ extract, the pH varied with increase in moisture content from 5.9 to 6.7. In corn meal extract, the pH increased from 5.6 to 5.8 and then decreased to 5.1. In corn cob extract, the pH decreased with increase in moisture content from 7.4 to 5.9. When corn grits were used, the pH of the extract remained almost stable in the range of 4.6 ± 0.3 whereas with corn bran, pH decreased from 4.34 to 3.8. The results are illustrated in Figure 5. 7. Moisture content did not have significant effect on FOS production. It was observed that pH of the extract has a significant effect on maintaining high FOS production. FTase activity was found more only when the pH of the extract was in the range of 5 – 7. At acidic pH, the enzyme activity was less and consequently FOS produced was also less. Varying the moisture content in corn products showed very little difference in FOS yield. In corn germ and corn meal, 51 % moisture content resulted in maximum FOS yields of 49 % and 39 % respectively. In corn cob, 57 % moisture content resulted in maximum FOS yield of 7.6 % whereas in corn grits and corn bran, 30 % moisture was favourable for FOS production (6.3 % and 2.7 % respectively) (Figure 5. 8).

Figure 5. 7. pH profile during the production of FTase by *A. oryzae* CFR 202 grown on corn products with varying moisture content



The results are in concurrence with those reported by Acuña-Arguelles *et al* (1994) wherein moisture contents between 40 % and 55 % resulted in an optimum water availability, substrate swelling and oxygen diffusion effects, favouring pectinase formation by *A. niger*. Similar results were reported by Raimbault and Alazard (1980) wherein enzyme production by *A. niger* was high in media with 50 – 55 % moisture contents and poor with 70 % and 25 % moisture contents wherein the porosity and oxygen diffusion of the medium were reduced.

5. 3. 1. d. Coffee and tea processing byproducts

India produces on an average, more than one and a half lakh tonnes of coffee annually. Processing of coffee berries result in several by – products like coffee pulp and coffee husk (parchment) which find use only as organic manure (Alwar and Ramaiah, 1986). The composition of dried coffee pulp is as follows:

Table 5.4 Composition (%) of dried coffee pulp (Gathuo *et al*, 1991).

Crude fibre	Crude protein	Ash	Tannins	Pectic substances	Sugars
21.4	10.1	1.5	7.8	6.5	14.4

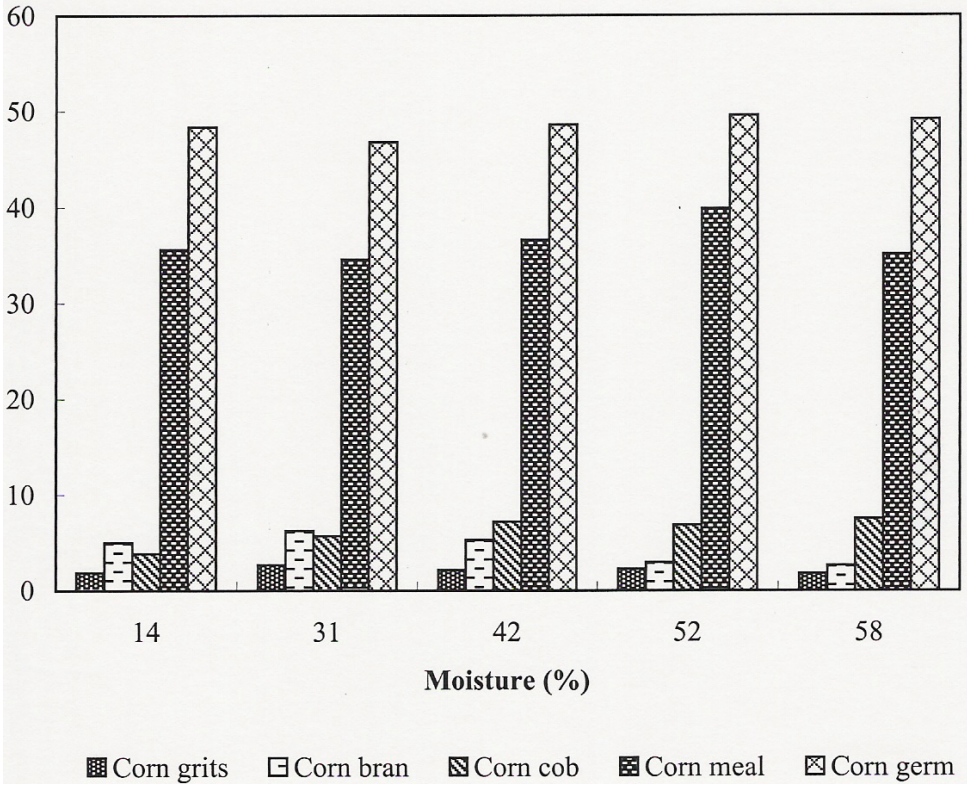
Of the total sugars, 10 – 15 % is fructose, 2.8 – 3.2 % sucrose and 1.9 – 2.4 % galactose. Therefore, it serves as a good substrate for growth of moulds. Due to the presence of fermentable sugars, the coffee pulp forms a major source of the pollution of rivers and lakes located near the coffee processing sites as well as environment. This can be avoided by using it as a substrate for production of value added microbial metabolites by SSF system (Boccas *et al*, 1994).

Coffee husk is another residue, which is generated, in large quantities during the dry processing of coffee berries. Table 5. 5 gives the composition of coffee husk.

Table 5.5. Composition (%) of coffee husk (Elias, 1979)

Moisture	Dry matter	Crude fat	Ash	Protein
7.6	92.8	0.6	0.5	0.95

Figure 5. 8. Effect of moisture content of corn products on FOS yields obtained using FTase produced by *A. oryzae* CFR 202



Due to the presence of anti - nutritional factors such as caffeine and tannins, coffee husk does not find any potential application and its disposal is a serious problem in coffee producing countries (Martinez carrera *et al*, 1988). Attempts have been made to use it for the cultivation of edible fungi and microbial production of enzymes (Boccas *et al*, 1994) or organic acids. Coffee husk supplemented with glucose has been used for the production of fruity flavours by *Ceratocystis fimbriata.* It is reported that coffee husk supplemented with glucose, resulted in the increased production of flavours (Soares *et al*, 2000).

Spent coffee and spent tea are waste generated after preparation of the beverage. Due to their granular nature, they serve as suitable substrate for the growth of moulds providing adequate anchorage for the mycelia. Spent coffee consists of 7-8 % moisture, 10 – 12 % protein, 35 – 44 % fibre and 0.25 – 1 % ash (Adams and Dougan, 1987).

Among the coffee and tea processing by – products used in the present study, *A. oryzae* CFR 202 was found to grow profusely on spent coffee and spent tea, but growth was poor on coffee husk and coffee pulp. Figure 5. 9 shows the change in pH during the growth of *A. oryzae* CFR 202 on coffee and tea processing byproducts moistened with water and supplemented with nitrogen sources and complete synthetic media. With all the substrates, FTase activity was less when water was used as moistening agent, the activity increased when complete media was used as supplement but, maximum activity was obtained when the substrate was supplemented with yeast extract (Figure 5. 10). The maximum FTase activity obtained was 6.6 ml $^{-1}$min $^{-1}$ using spent tea, 5.5 U ml $^{-1}$min $^{-1}$ using spent coffee, 2.4 U ml $^{-1}$min $^{-1}$ using coffee pulp and 1.96 U ml $^{-1}$min $^{-1}$ using coffee husk. FOS production was very less with water as wetting agent whereas, supplementation with yeast extract and complete synthetic media increased FOS yields considerably (Figure 5. 11). The maximum FOS yields obtained by FTase produced using coffee pulp, coffee husk, spent tea and spent coffee were 15 %, 20 %, 30 % and 33 % respectively.

Figure 5. 9. pH profile of FTase produced by *A. oryzae* CFR 202 grown on coffee and tea processing by - products with supplementation

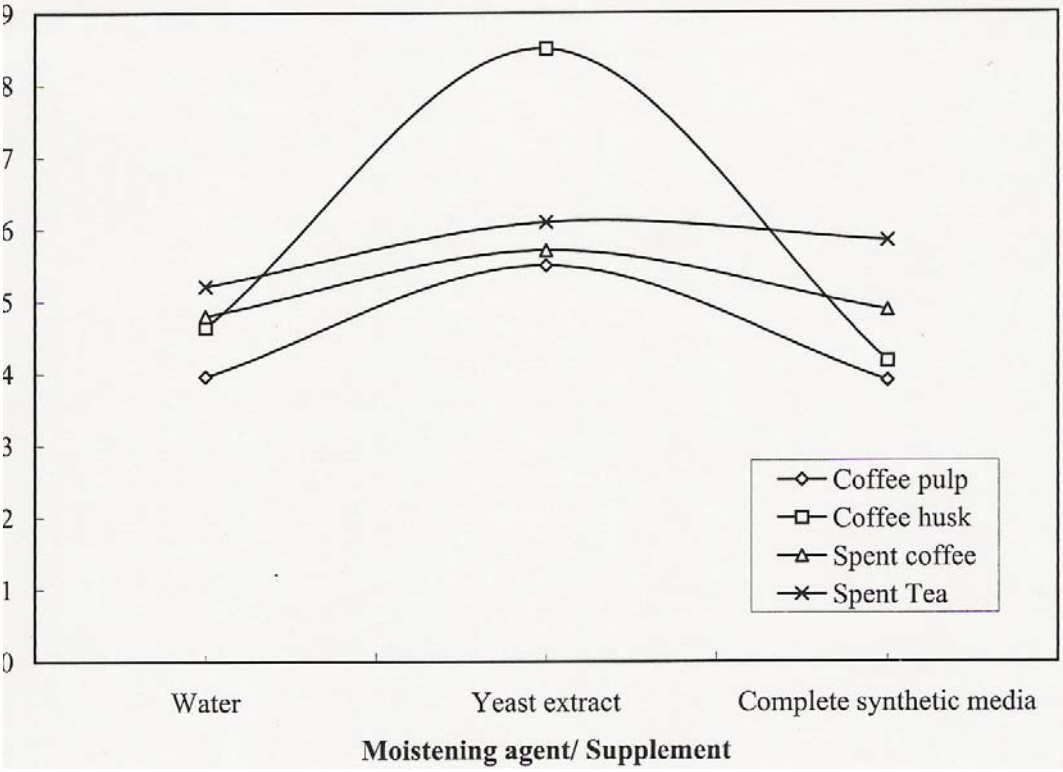


Figure 5. 10. Activity profile of FTase produced by *A. oryzae* CFR 202 grown on coffee and tea processing by - products with supplementation

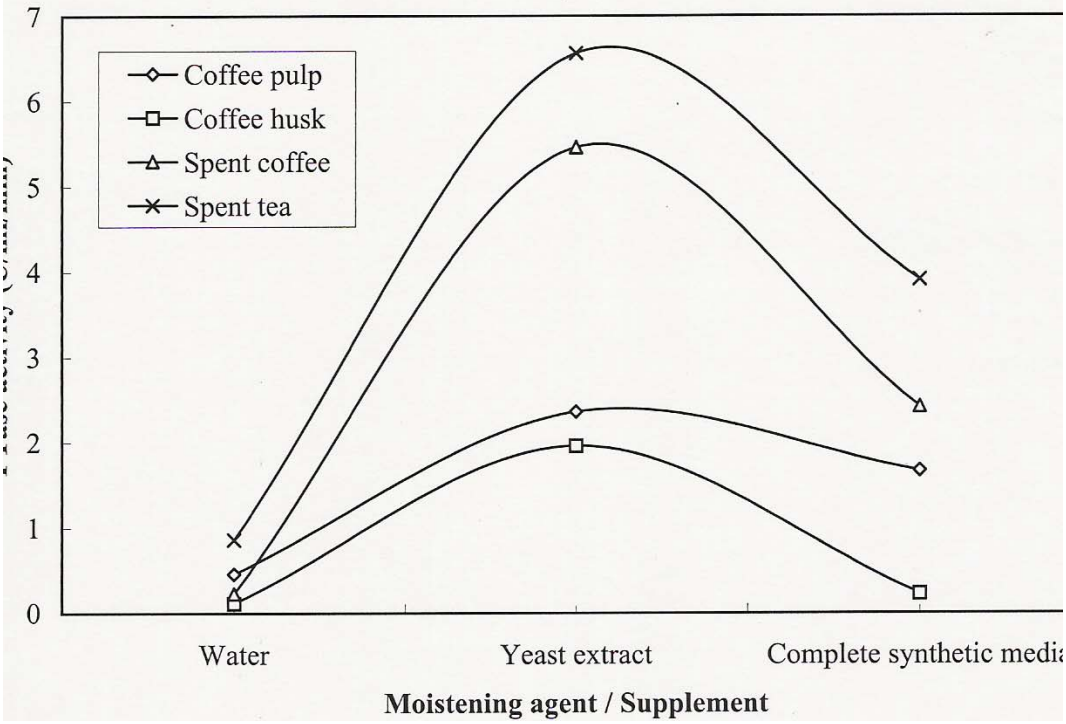
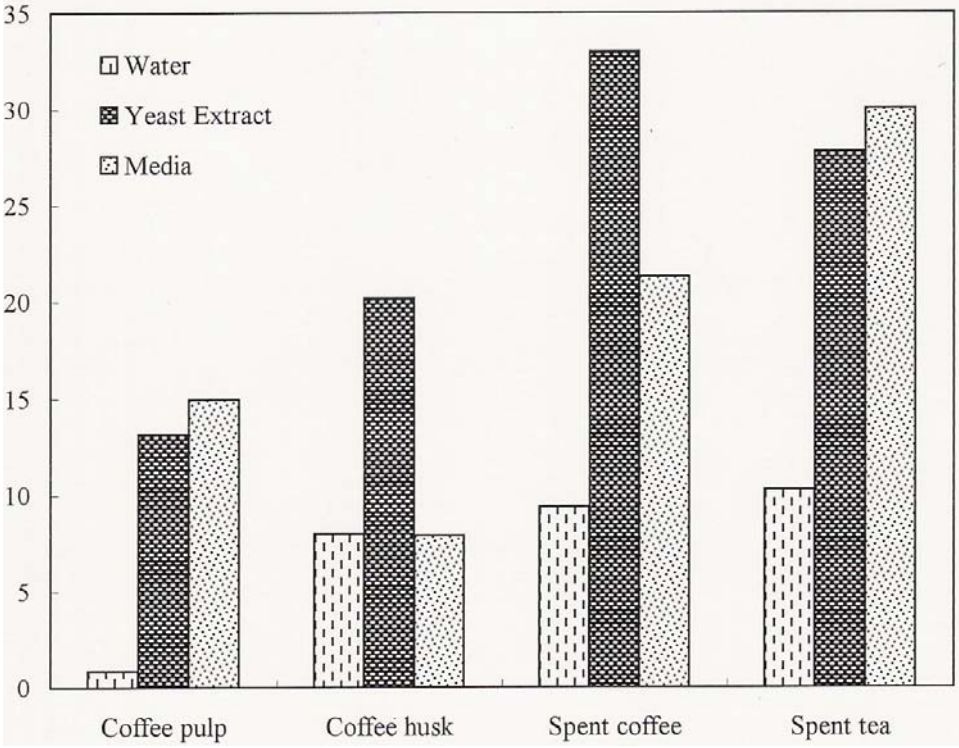


Figure 5. 11. FOS yields obtained using FTase produced by *A. oryzae* CFR 202 grown on coffee and tea processing by - products with supplementation



The solid substrate not only supplies the nutrients to the microbial cultures but also serves as an anchorage for the cells. However, some of the nutrients may be available in sub optimal concentrations, or even absent in the substrates. In such cases, it becomes necessary to supplement them externally with nutrients. In the present study, it is obvious that the substrates are deficient in nitrogen source and the supplementation with a complex nitrogen source like yeast extract results in an increase in the FTase activity considerably. Since coffee pulp and coffee husk are rich in cellulose, they do not facilitate good growth and FTase production where as spent coffee and spent tea with less cellulose content were good for FTase production and thereby FOS production. This could be attributed to their granular nature, which facilitates easy penetration by mycelia.

5. 3. 1. e. Sugarcane bagasse

Agricultural by – products like cereal bran, corn products, coffee and tea processing by – products described above have been used for economical production of value added products like enzymes. However, natural substrates have a major disadvantage that the carbon source constitutes part of their structure. During the growth of the microorganism, the solid medium is degraded, and therefore, the geometric and physical characteristic of the medium changes. Consequently, heat and mass transfer can be reduced. This disadvantage can be overcome by the use of an inert support with a more or less constant physical structure throughout the process enabling improved control of heat and mass transfer. An additional advantage that SSF on inert supports has compared with SSF on natural substrates is its less complicated product recovery. The extracellular products of interest can be easily extracted from the inert support, therefore products can be obtained with fewer impurities compared with natural substrates, and the support can be reused (Ooijkaas *et al*, 2000). In this context, sugarcane bagasse serves as a good inert support for SSF.

The composition of sugarcane bagasse is as follows: Cellulose – 50 %, hemicellulose – 25 %, lignin – 25 % and ash- 2.4 % (Pandey *et al*, 2000). Advances in industrial biotechnology offer potential opportunities for economic

utilization of sugarcane bagasse. Sugarcane bagasse, which is a complex material, is the major by-product of the sugarcane industry. Due to its abundant availability, it can serve as an ideal substrate for microbial processes for the production of value-added products. Attempts have been made to produce protein-enriched animal feed, enzymes, amino acids, organic acids and compounds of pharmaceutical importance, using bagasse as solid substrate (Pandey *et al*, 2000). Because of its low ash content (2.4 %), bagasse offers numerous advantages in comparison to other crop residues such as rice straw and wheat straw, which have 17.5 % and 11.0 % ash contents, respectively, for usage in bioconversion processes using microbial cultures.

Sugarcane bagasse has been successfully applied to penicillin production (Barrios Gonzalez *et al*, 1993). It has also been used as carbon source for the production of protein- enriched animal feed by SSF using Basidiomycetes (Nigam, 1990). Pandey *et al* (1999) have discussed the bioconversion processes involving agro-industrial residues such as bagasse for their effective utilization to produce value – added products like enzymes, especially cellulases. Mixed culture of two different microorganisms and pretreatment of bagasse has shown improved production of enzymes (Gupte and Madamwar, 1997). Bagasse has been used as an inert support impregnated with nutrients for the production of value added products like glutamic acid (Nampoothiri and Pandey, 1996), polygalacturonase (Acuna-Arguelles *et al*, 1994) and pigments (Chiu and Chan, 1992).

In the present study, dried sugarcane bagasse did not support the growth of *A. oryzae* CFR 202 but growth was very good on wet bagasse. Although growth was there, there was no FTase production and hence no FOS production. The pH of the MSE was very less (3.5 – 4.0) supporting the absence of FTase activity (Figure 5. 12). FTase production was not successful with sugar cane bagasse unless it was supplemented with yeast extract, complete synthetic media, rice bran etc. Yeast extract was the best supplement which clearly explains that the deficiency of nitrogen in the substrates could be the reason for not favouring the production of FTase. Supplementation with rice bran improved the mass transfer and aeration by increasing the space between

the particles. Upon supplementation with yeast extract solution, media and rice bran, *A. oryzae* CFR 202 grown on bagasse produced FTase and the yield of FOS was around 25 – 30 % (Figure 5. 13). The maximum FOS yield obtained using FTase produced by growing on bagasse supplemented with yeast extract was 45 %. Supplementing with rice bran resulted in FTase that produced 20 % FOS whereas supplementation with complete synthetic media resulted in FTase that produced only 14 % FOS. This was found to be less compared to 51 % FOS obtained using rice bran alone as substrate and 56 % obtained by SmF using the complete synthetic media.

5. 3. 1. f. Cassava Bagasse (Tippi)

Cassava bagasse (tippi) is the fibrous waste residue obtained in the manufacture of starch and sago from Cassava and it poses serious disposal problems as it can be used only as fuel after drying. Being an abundant source of starch (60 – 70 % on dry weight basis) tippi has excellent prospects as a substrate for SSF to produce many value – added products. Tippi is reported to be composed of 13 % moisture, 62 % starch, 13 % crude fibre, 0.6 % ash, 0.4 % reducing sugars, 2 % pentosan and 8 % other polysaccharides in g/100g basis (Ghildyal and Lonsane, 1990). Bioconversion of cassava bagasse could be economically useful in certain cases, e.g. for the production of enzymes, organic acids, feed, etc. Production of microbial enzymes could be an area to be exploited using cassava bagasse. When compared to sugarcane bagasse, cassava offers advantages, as it does not require any pretreatment and can be easily attacked by microorganisms. Microorganisms, which use starch as the substrate for growth and activity, have generally been preferred for bioconversion processes using cassava bagasse because of its high starch content. A few yeasts and fungi have been used for growth on cassava bagasse. However, filamentous fungi have been most widely employed. High water retention capacity (85–90 %) also makes it an ideal substrate for SSF processes. SSF has been carried out for aflatoxin production using cassava bagasse (Barrios Gonzalez *et al*, 1990). As illustrated in Figure 5.13, SSF on tippi supplemented with rice bran resulted in FTase capable of producing 35 % FOS whereas supplementation with yeast extract and complete synthetic media resulted in 27 % and 19 % FOS respectively.

Figure 5. 12. pH profile during the production of FTase by *A. oryzae* CFR 202 grown on sugarcane bagasse and tippi

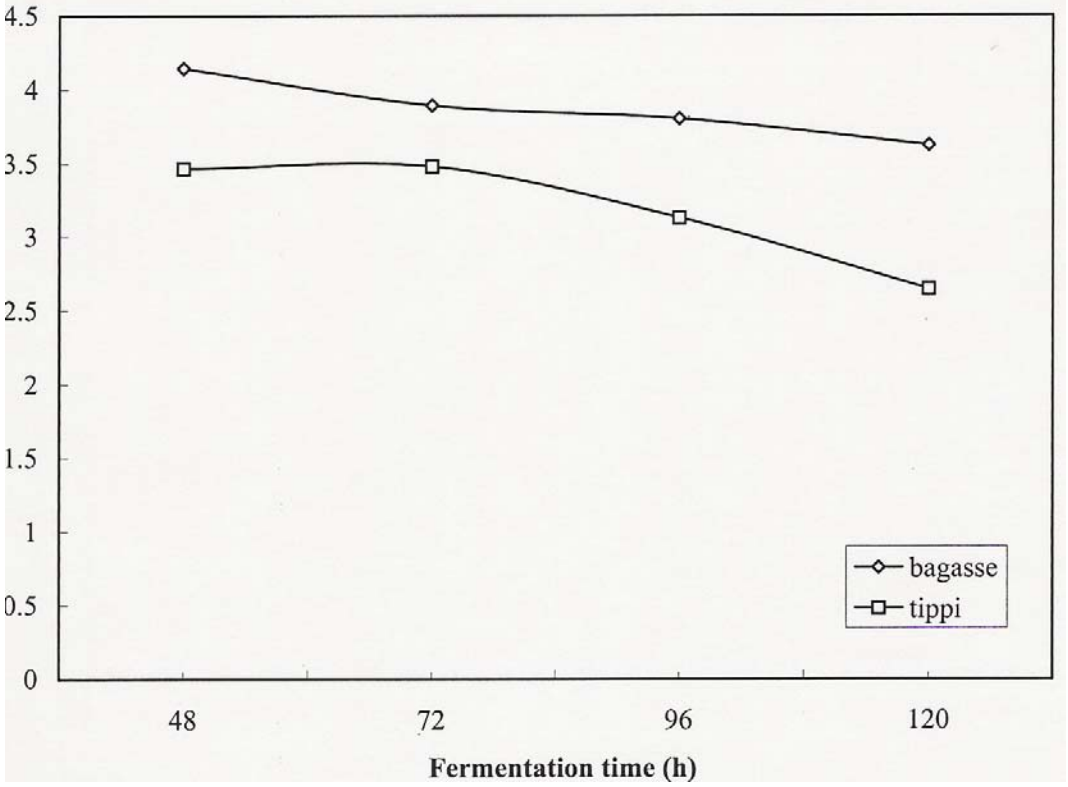
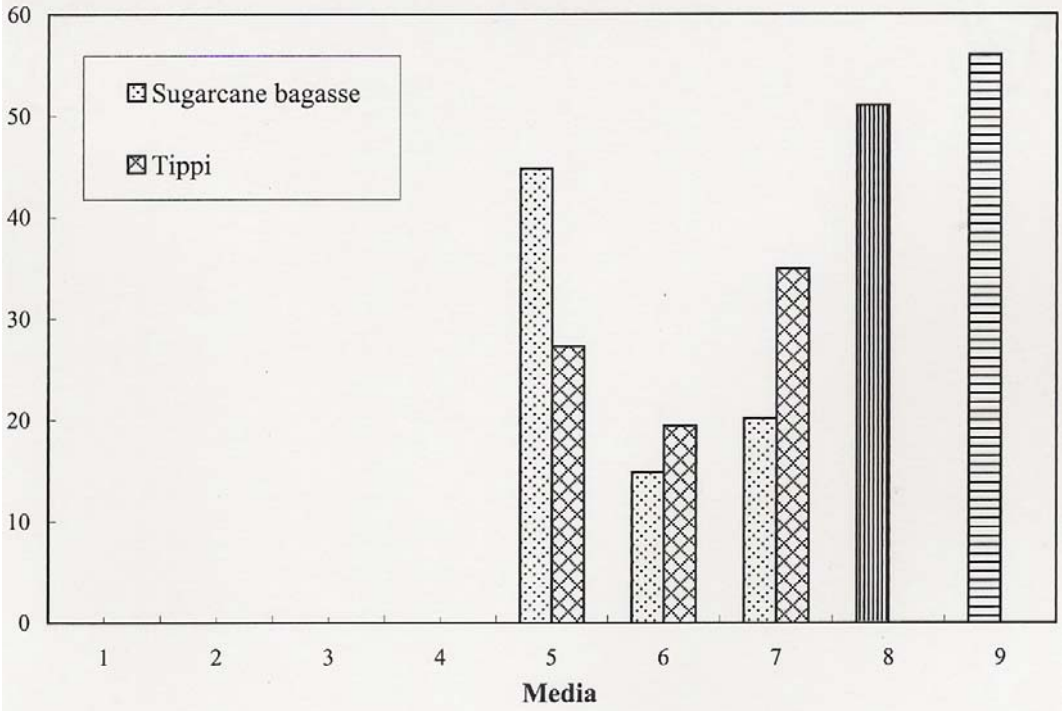


Figure 5. 13. FOS yields obtained using FTase produced by *A. oryzae* CFR 202 grown on sugarcane bagasse and tippi



1 - Bagasse alone
2 - Tippi alone
3 - Bagasse/ Tippi + Ammonium sulfate
4 - Bagasse/ Tippi + Urea
5 - Bagasse/ Tippi + Yeast extract
6 - Bagasse/ Tippi + Complete synthetic media
7 - Bagasse/ Tippi + 30 % rice bran
8 - Rice bran alone
9 - Complete synthetic media (SmF)

5. 3. 2. Characterization of FTase

Characterization of FTase obtained by SSF of wheat bran was carried out to find out the optimum temperature, pH, thermal stability and pH stability. As illustrated in Figure 5.14, FTase was found to be stable at 30 °C and 40 °C retaining 91 % of its activity after 2 h of incubation. Above 40 °C the enzyme was found to lose 80 % of its activity. The optimum temperature for the activity of FTase was found to be 60 °C (Figure 5. 15). FTase was stable from pH 5 to 7 and 100 % activity was retained at pH 7.0 where as the activity was 75 % and 88 % at pH 5 and 6 respectively (Figure 5. 16). The optimum pH for FTase activity was 6.0 where as 84 % activity and 77 % activity were retained at pH 5.0 and 7.0 respectively (Figure 5. 17).

A large number of reports have described the optimum pH and temperature of microbial FTase between 5 – 6.5 and 50 – 60 °C respectively (Yun, 1996). There have been reports suggesting the optimal temperature values to be higher for the enzymes produced by SSF than those produced by SmF. Enzymes produced by SSF are reported to be more thermostable than those obtained by SmF (Alazard and Raimbault, 1981; Deschamps and Huet, 1985). The results obtained in the present study also were similar suggesting that FTase obtained by SSF was more stable than FTase obtained by SmF (Chapter 4).

5. 3. 3. FOS production

When FOS production was carried out for 18 h, it was found that the maximum FOS yield (52 %) was obtained by the end of 8 h of reaction. Sucrose concentration decreased steadily in the initial 5 h from 60 % to 16 % and was 11 % at the end of 18 h of reaction. Glucose level steadily increased upto 8 h and then gradually increased throughout the reaction to reach 42 % at the end of 18 h. GF_2 concentration increased to 38 % at the end of 4 h after which it decreased, because it acts as the acceptor for the production of GF_3. GF_3 concentration was maximum (21 %) at the end of 15 h and GF_4 concentration was maximum (6 %) at the end of 19 h. Trace amounts of GF_5 (1 %) was also found to be produced at the end of 18 h of reaction. (Figure 5. 18).

Figure 5. 14. Effect of temperature on stability of FTase

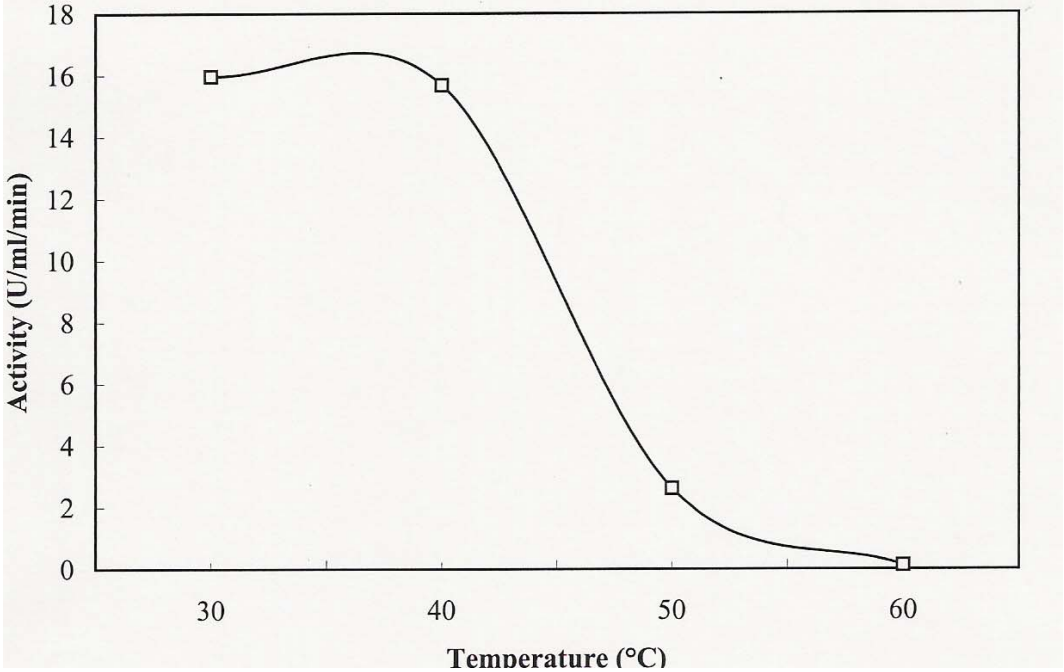


Figure 5. 15. Effect of temperature on FTase activity

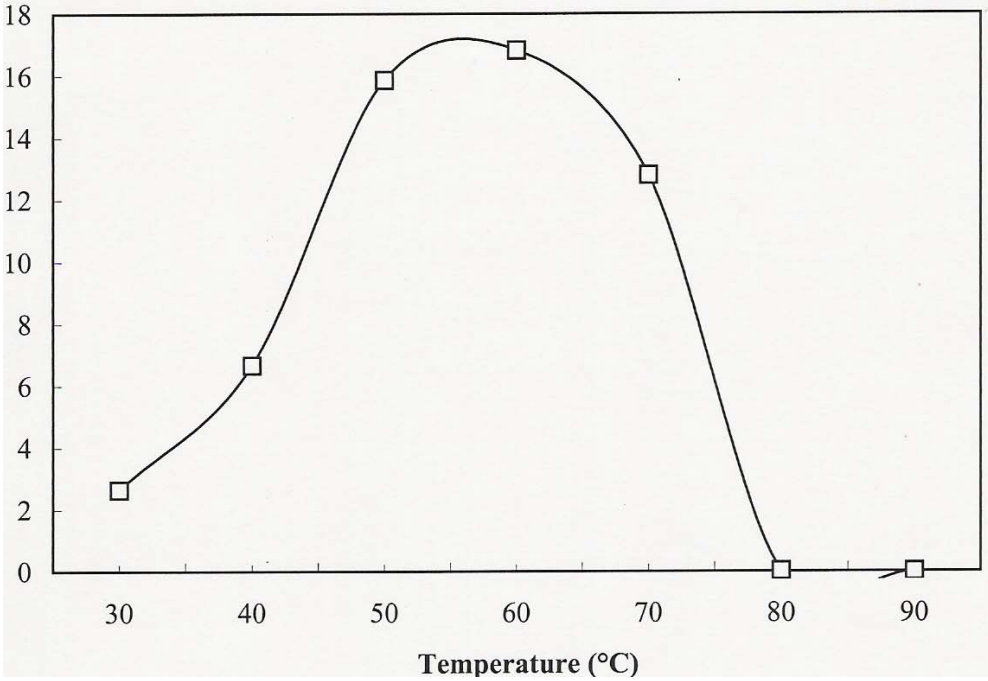


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Figure 5. 16. Effect of pH on stability of FTase

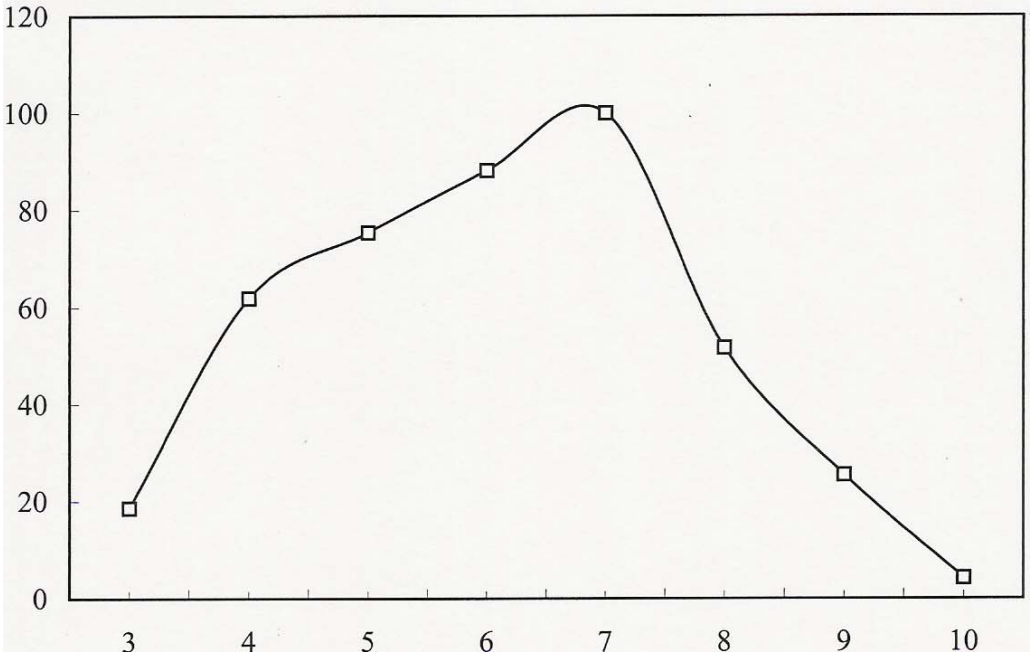


Figure 5. 17. Effect of pH on FTase activity

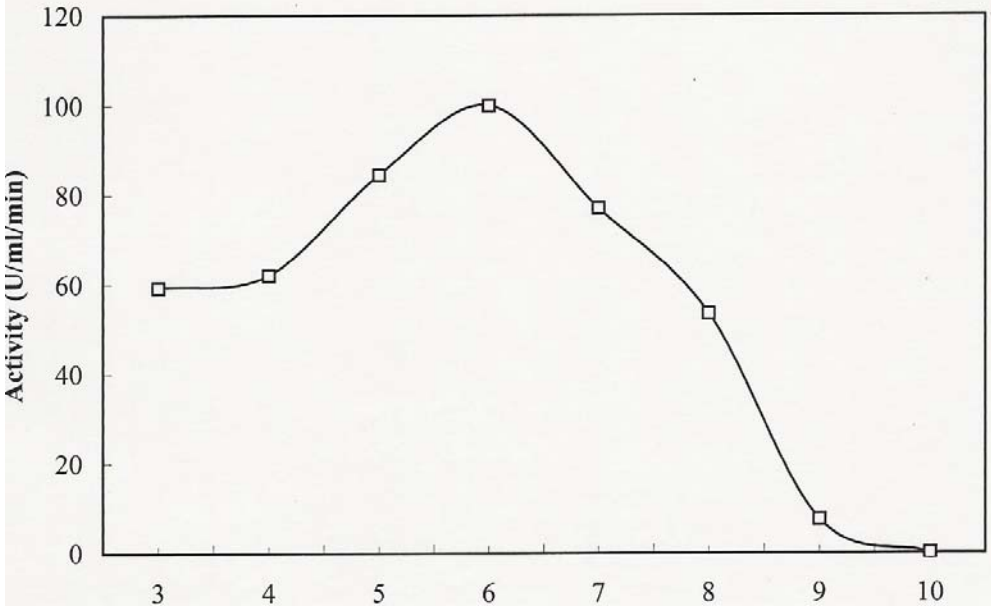
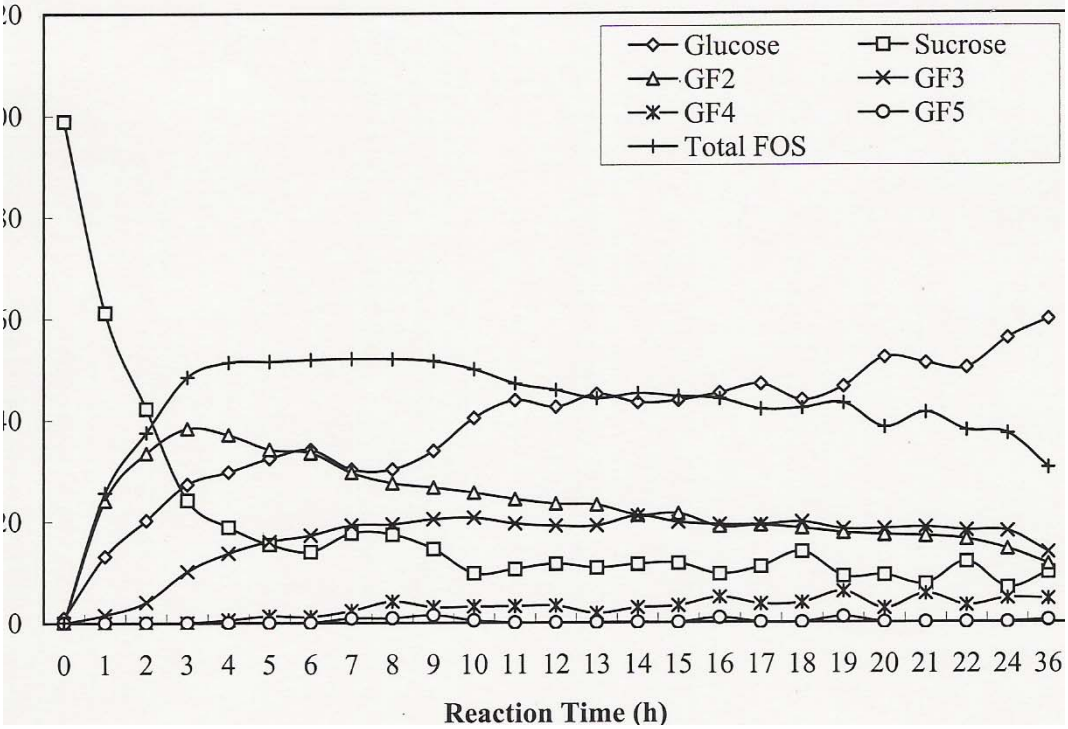


Figure 5. 18. Time course of FOS production using FTase produced by *A. oryzae* CFR 202 grown on wheat bran



5. 4. CONCLUSIONS

There have been many reports on the production of FTase by submerged fermentation, but there are no reports on the use of SSF for FTase production except for the use of apple pomace as substrate for FTase production. The extracellular FTase produced by *A. foetidus* NRRL 337 grown on commercial apple pomace was extracted by blending the pomace with water. The enzyme was capable of converting sucrose into syrup containing more than 50 % kestose. The production efficiencies ranged from 26 – 47 % depending on sucrose concentration and the reaction time (Hang *et al*, 1995). In this context, the present investigation has focussed on the use of a variety of agricultural by – products as substrates for the production of FTase by *A. oryzae* CFR 202. A variety of substrates like cereal bran to corn products, coffee and tea processing by - products, sugarcane bagasse and cassava fibrous residue have been used in the present study. All the substrates except sugarcane bagasse and tippi supported good growth of *A. oryzae* CFR 202 and concomitant production of FTase. Maximum activity was found to be present in FTase obtained from SSF of rice bran, wheat bran and corn germ. Even those substrates, which did not favour production of FTase, when supplemented with complex nitrogen source like yeast extract, supported good growth of *A. oryzae* CFR 202 and production of FTase. Addition of rice bran in the proportion of 30 % of the substrate resulted in interparticle spacing with a possible increase in mass transfer and oxygen transfer. This contributes to the value addition of those by – products, which are otherwise disposed adding to environmental pollution.

Characterization of FTase produced by SSF has shown that it is more stable compared to that produced by SmF. It was also capable of producing the maximum yield of FOS (52 %) within 8 h of reaction, unlike the enzyme produced by SmF, which requires 18 h for producing maximum FOS. The present investigation has thus shown that SSF opens a new avenue for the production of FTase and subsequently FOS using agricultural by - products.

CHAPTER 6
PREBIOTIC STUDIES ON FOS

6. 1. INTRODUCTION

A prebiotic is '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, that can improve the host health' (Gibson and Roberfroid, 1995). For a good ingredient to be classified as a prebiotic, it must meet the following criteria:

1) not be hydrolyzed or absorbed in the upper part of the gastrointestinal tract
2) be a selective substrate for one or a limited number of potentially beneficial bacteria commensal to the colon, like bifidobacteria and lactobacilli
3) be able to alter the colonic microflora towards a potentially more healthy composition.

Any food ingredient that enters the large intestine can serve as a prebiotic. Hence, non- digestible oligosaccharides including fructooligosaccharides can serve as prebiotics. Because of their prebiotic properties, they have recently received much attention as functional food ingredients. The present chapter discusses the effect of FOS on growth of some prebiotic bacteria like Lactic acid bacteria and acid production during its fermentation.

6.2. MATERIALS AND METHODS

6.2.1 Microorganisms and culture conditions

Three strains of Lactobacilli – *L. acidophilus, L. bulgaricus and B. coagulans* were used in the present study. All the strains were maintained on Lactobacillus MRS (LMRS) agar at 4 °C. Inoculum was prepared by transferring a loopful of culture to 5 ml LMRS broth and incubating at 37 ± 1 °C (Remi Orbital Shaking incubator, CIS 24, Remi Equipments, Mumbai) for 24 h under static conditions. 1 % inoculum was transferred into the basal MRS media (without carbon source) consisting of 1 % peptone, 1 % beef extract, 0.5 % yeast extract, 0.1 % Tween 80, 0.2 % ammonium citrate, 0.5 % sodium acetate, 0.01 % magnesium sulphate, 0.005 % manganese sulphate and 0.2 % dipotassium phosphate. Fermentation studies were carried out in basal MRS media supplemented with various carbon sources at 2 % concentration. FOS produced using FTase from *A. oryzae* CFR 202 was used in the present study.

Glucose and a commercially available FOS syrup (Miwon Oligosugar) were used as positive control. One set of experiment was also carried out without any carbon source. The flasks were incubated at 37 ± 1 °C (Remi Incubator Shaker CIS 24, Remi Instruments, Mumbai) under static conditions.

6. 2. 2. Analytical procedures

Sampling was done every hour and cell concentration was determined by measuring the optical density (O. D) at 660 nm using a spectrophotometer (Spectronic Genesys 5, Spectronic Instruments, New York) with the media as blank. For dry cell weight determination, the culture broth was centrifuged at 5000 rpm for 15 min (Remi Centrifuge R4C, Remi Equipments, Mumbai). The pellet was then washed with distilled water and dried to constant weight at 110 °C. The supernatant was used for estimation of acids produced during fermentation. Quantitative analysis of the acids was done using HPLC (LC –6 A, Shimadzu, Japan) with a UV spectrophotometric detector SPD 6A using a Supelcogel C –610 H column (30 cm x 7.8 mm i. d., Supelco Inc, Pennsylvania, USA) at 210 nm. The retention times of the acids were compared with that of standards for identification. The analysis was carried out at room temperature with 0.1 % o- phosphoric acid as mobile phase at a flow rate of 0.5 ml min^{-1}.

6.3. RESULTS AND DISCUSSION

All the strains grew well on all the media except the one without carbon source. Table 6.1 shows the dry cell weight of various strains of bacteria grown in different media.

Figures 6.1, 6.2 and 6.3 show the growth curves of *L. acidophilus, L. bulgaricus and B. coagulans* in different media. Although all the strains preferred glucose for growth, they grew well in FOS containing media also. This may be because glucose is an easily utilizable carbon source in any fermentation process. Growth was better compared to that in the media containing oligosugar. Similar results were reported in a study carried out by Perrin *et al* (2001) on fermentation of FOS by *Bifidobacterium infantis* ATCC 15697 wherein glucose was the best substrate for growth and biomass

production. In a study with chicory FOS, Wada (1990) has also reported that

Figure 6.1. Growth of *L. acidophilus* in MRS broth in the presence and absence of carbon source

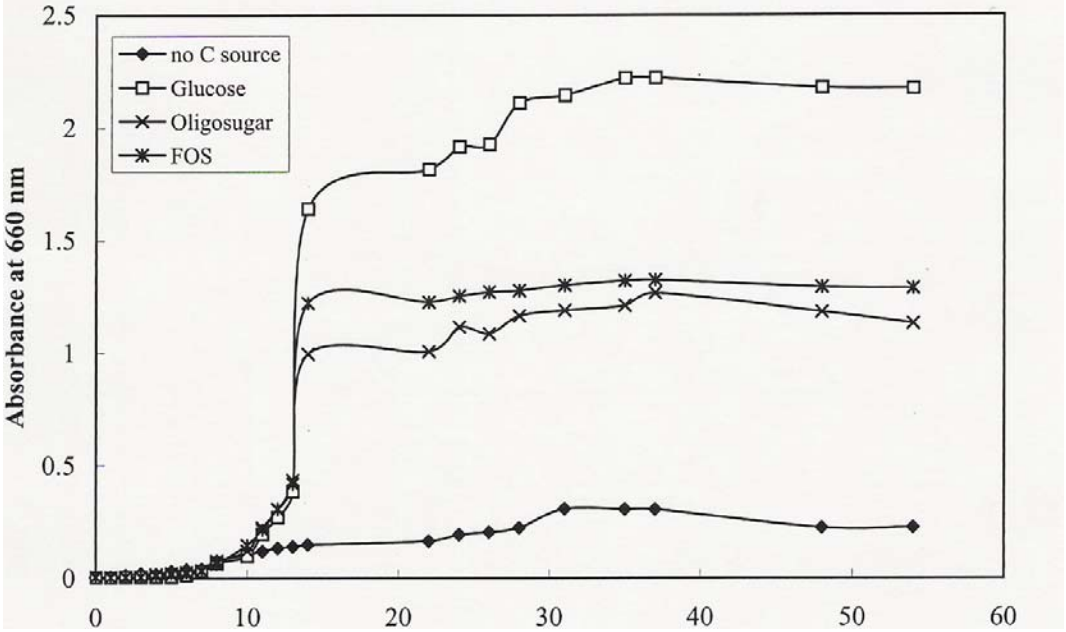


Figure 6.2. Growth of *L. bulgaricus* in MRS broth in the presence and absence of carbon source

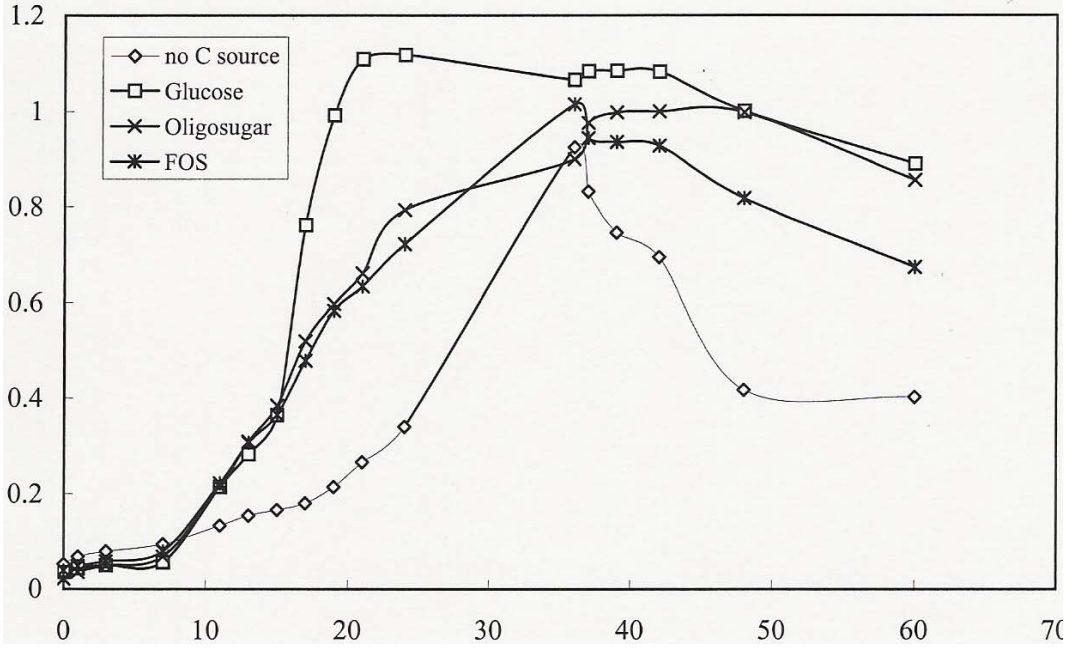
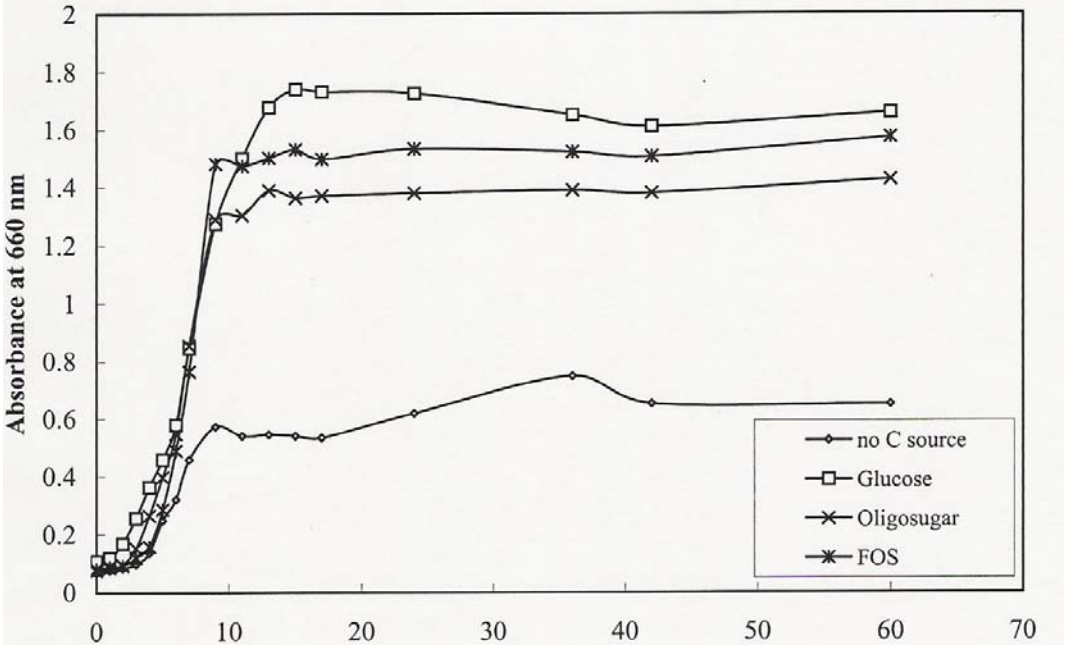


Figure 6. 3. Growth of *B . coagulans* in MRS broth in the presence and absence of carbon source



the utilization of FOS was lower compared to glucose in the media when both were used as carbon source. However, Kaplan and Hutkins (2000) have reported that growth rates of *Lactobacilli* and *Bifidobacteria* strains were same in media containing FOS as well as glucose.

There was a noticeable reduction in pH during the fermentation, which indicated acid production. Figures 6.4, 6.5 and 6.6 show the drop in pH during the growth of *L. acidophilus, L. bulgaricus and B. coagulans* in different media. During the growth of *B. coagulans* in glucose media, the pH dropped from an initial value of 6.7 to 4.3 by the end of 60 h. In FOS and oligosugar containing media, pH was 5.0 at the end of fermentation. During the growth of *L. bulgaricus*, pH dropped from 6.7 to 5.6 in glucose media and to 5.9 in FOS containing media. In the case of *L. acidophilus*, drop in pH was more in glucose media (from 6.7 to 4.0) than in FOS containing media (from 6.7 to 5.1).

Table 6.1. Dry cell weight of various strains of *Lactobacilli* grown in MRS broth in the presence or absence of carbon source

	Dry cell weight (g/L)			
Strain	**No C source**	**Glucose media**	**Oligosugar media**	**FOS media**
L. acidophilus	0.13 ± 0.004	2.2 ± 0.006	1.2 ± 0.003	1.9 ± 0.001
L. bulgaricus	0.947 ± 0.165	1.413 ± 0.047	2.193 ± 0.052	1.640 ± 0.059
B. coagulans	0.687 ± 0.034	1.20 ± 0.123	1.373 ± 0.104	1.646 ± 0.169

The production of short chain fatty acids during growth of *Lactobacilli* was analyzed by HPLC. Figure 6.7 shows a typical HPLC profile for acids detected in the fermented broth. The acids detected included lactic acid, acetic acid, propionic acid and butyric acid. Figures 6.8, 6.9 and 6.10 present the profile of acid production using *L. acidophilus, L. bulgaricus and B. coagulans* in different media respectively. Wada (1990) has reported that *in vitro* fermentation of chicory FOS by human colonic bacteria such as bifidobacteria produced lactate and acetate resulting in a marked decrease in culture medium pH. The results presented in the chapter shows that FOS produced using

FTase from *A. oryzae* CFR 202 can facilitate the growth of probiotic microorganisms like Lactic acid bacteria and hence may result in beneficial effect in colon. This can also be used in the preparation of 'synbiotics' wherein the probiotic property of Lactic acid bacteria and prebiotic property of FOS can be combined.

Figure 6.4. pH profile during the growth of *L. acidophilus* in MRS broth in the presence and absence of carbon source

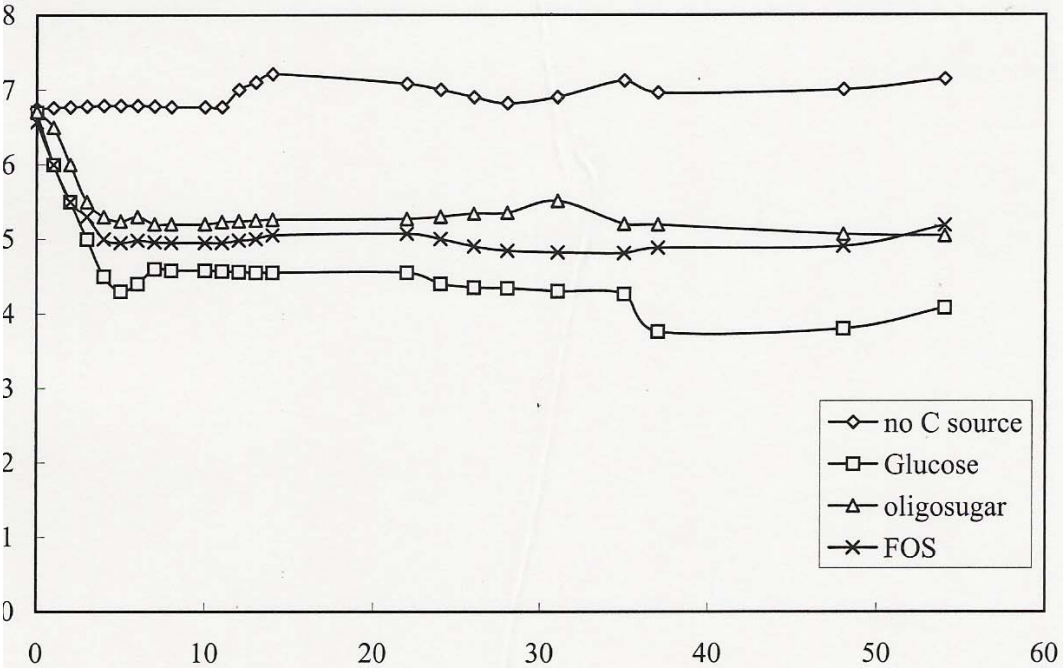


Figure 6.5. pH profile during the growth of *L. bulgaricus* in MRS broth in the presence and absence of carbon source

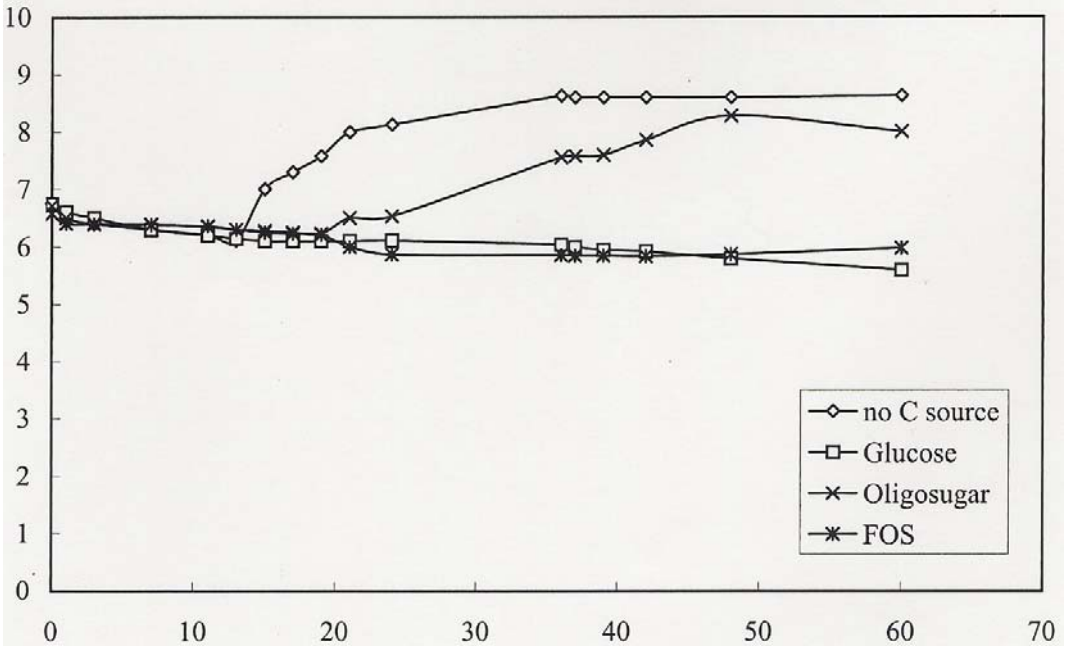
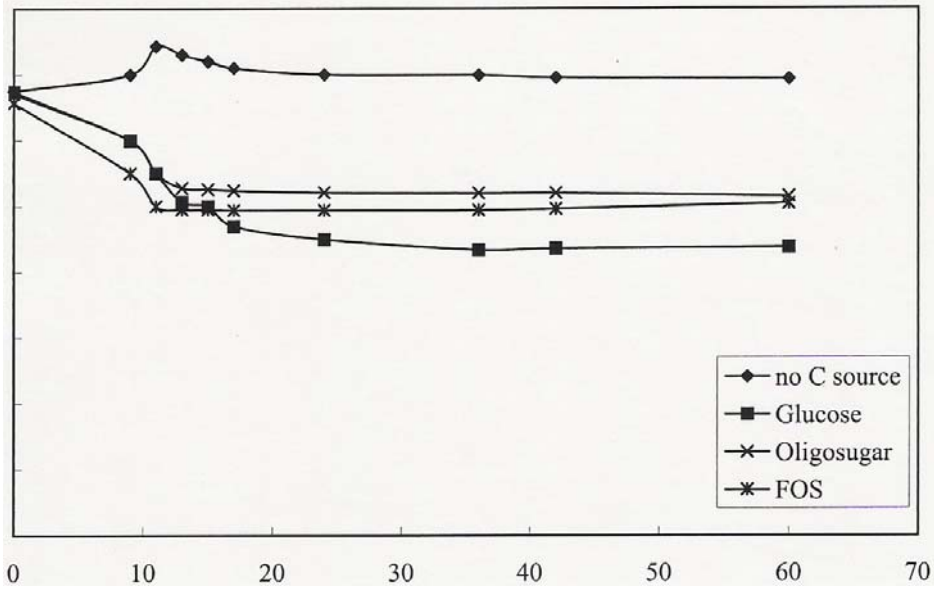


Figure 6.6. pH profile during the growth of *B. coagulans* in MRS broth in the presence and absence of carbon source



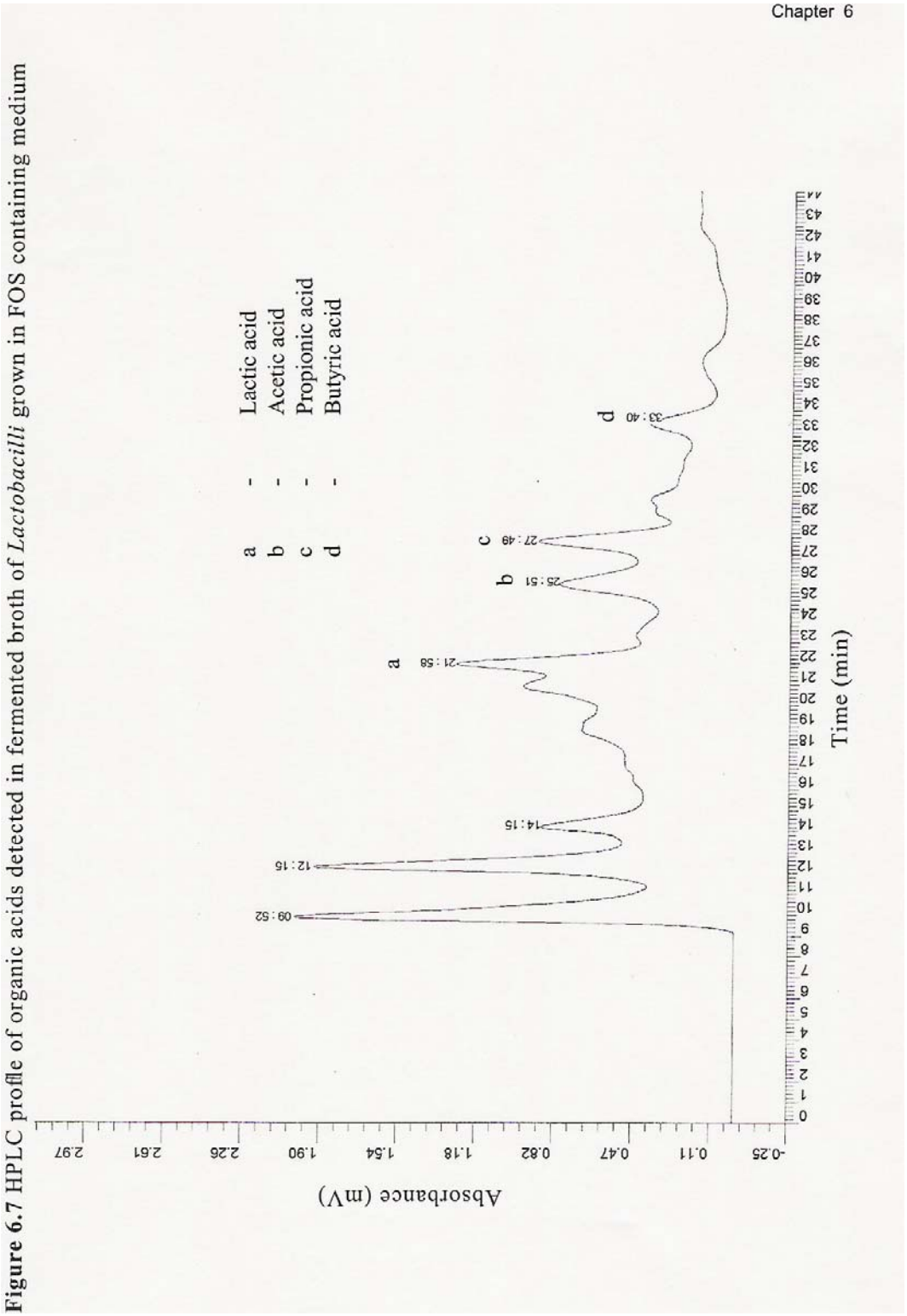


Figure 6.7 HPLC profile of organic acids detected in fermented broth of *Lactobacilli* grown in FOS containing medium

Figure 6.8. Organic acid production during the growth of *L. acidophilus* in MRS broth in the presence of different carbon sources

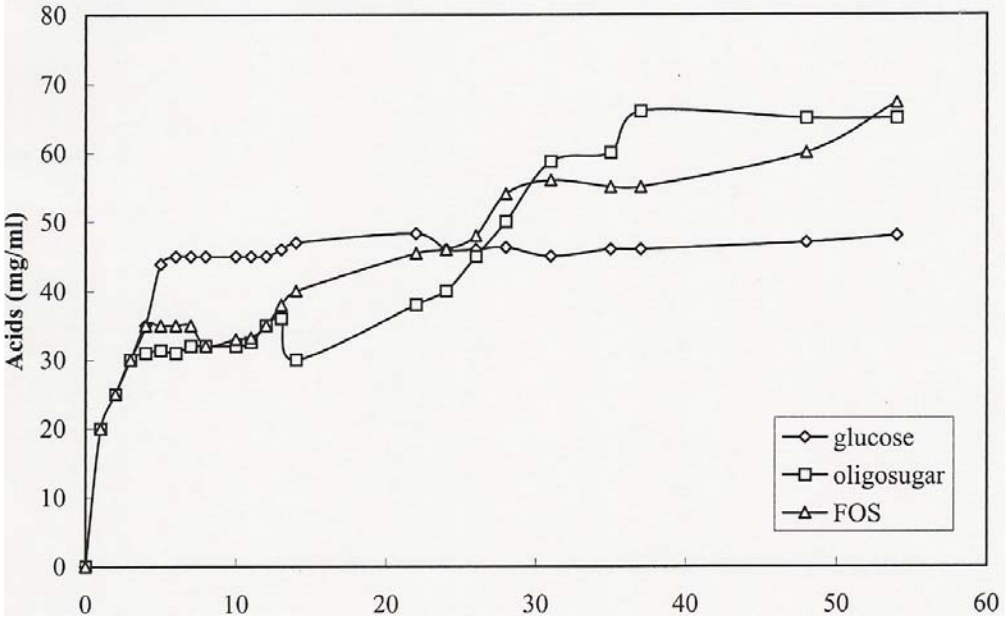


Figure 6.9. Organic acid production during the growth of *L. bulgaricus* in MRS broth in the presence of different carbon sources

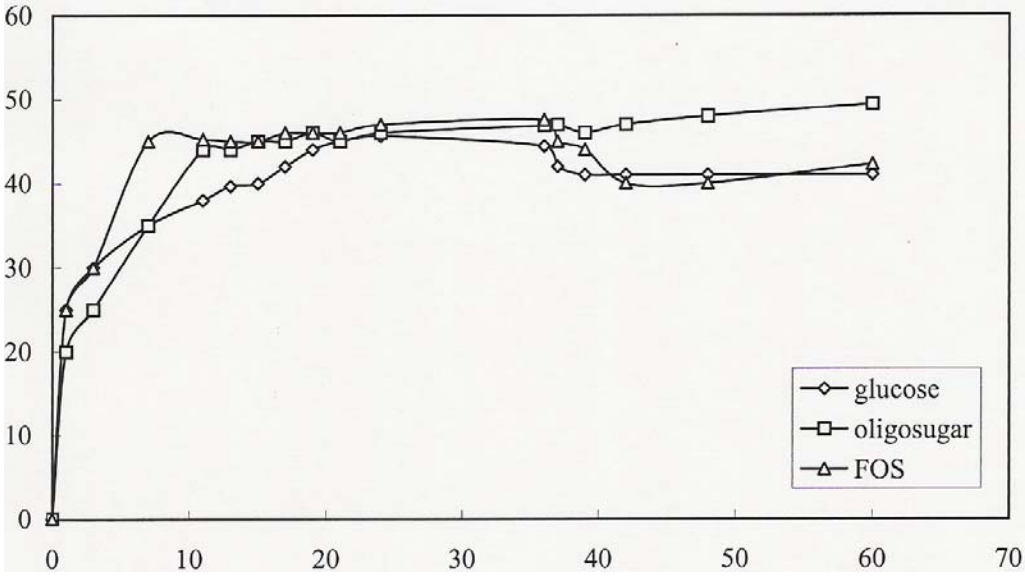
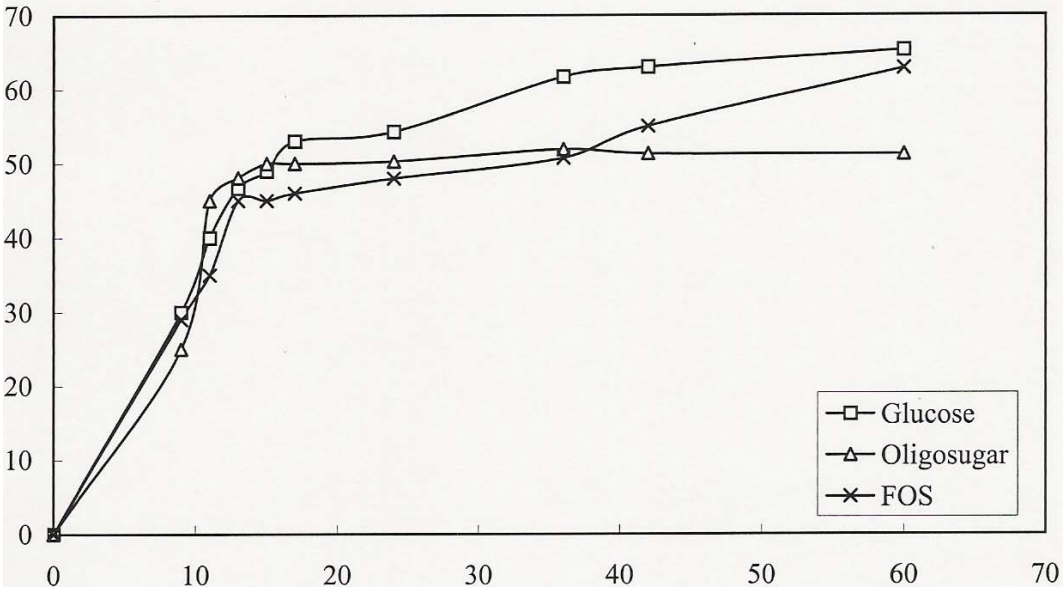


Figure 6.10. Organic acid production during the growth of *B. coagulans* in MRS broth in the presence of different carbon sources



CHAPTER 7

a. SCALE UP STUDIES ON FTase AND FOS

b. SPRAY DRYING OF FTase AND FOS

9. a. SCALE UP STUDIES ON FTase AND FOS

7. a. 1. INTRODUCTION

The conversion of a laboratory procedure to an industrial process is termed scale up. Scale up is necessary to implement a new process in the plant. Success in scale up is evaluated based on maximal yield for minimal operating cost and time. The production of FTase using *A. oryzae* CFR 202 was scaled up in two stages to 3 L and 15 L levels using the optimized media and culture conditions described in Chapter 3. A reactor of 10 L capacity was designed for the large scale production of FOS under controlled temperature conditions. The present chapter discusses the results obtained during the scale up studies for the production of FTase and FOS.

7. a. 2. MATERIALS AND METHODS

7. a. 2. 1. Scale up of FTase production

Scale up of FTase was carried out in 3 L (Laboratory Fermentor Model LF – 10, Murhopye Scientific Company, Mysore, India) and 15 L (Chemap Fermentor, Chemap AG, Mannedorf, Switzerland) fermentors. Preparation of inoculum media and fermentation media were as mentioned in Section 3. c. 2. 2. 1. The inoculum (20 %) was pumped into the fermentation media using dosing pump. Fermentation was carried out with agitation at 150 – 200 rpm and aeration of 1 vvm for 120 h. Sampling was done at every 12 h. The broth was filtered using Whatman No. 2. filterpaper and the pH of the filtrate was recorded. FTase activity was assayed in the filtrate and it was used for FOS production. FTase assay was carried out as given in Section 3. a. 2 and FOS production was as given in Section 4. 2. 8. Activity was calculated as given in 3. a. 2 and FOS analysis was carried out as mentioned in 3. b. 2. 2. Figure 7.a.1 shows the 15 L fermentor used for the scale up of FTase production.

7. a. 2. 2. Scale up of FOS production

FOS production was scaled up using a specially developed reactor. The reactor consisted of a steel vessel of 10 L capacity with a heating coil that is connected to a temperature controller to maintain the temperature at 55 $\pm$ 3 °C.

The vessel was equipped with a motor driven stirrer controlled by a speed

Figure 7.a.1. Fermentor (15 L) used for scale up of FTase production.

regulator (Remi Equipments, Mumbai) to facilitate uniform mixing of the sample. 9 L of the substrate (60 % sucrose) was incubated with 1 L of FTase in the reactor at 55 °C for 18 h. Sampling was done at every 6 h and reaction was carried out up to 24 h. The reaction was terminated by raising the temperature to 80 °C for 15 minutes in the same reactor. The reactor used for scale up of FOS production is depicted in Figure 7.a.2.

7. a. 3. RESULTS AND DISCUSSION

7. a. 3. 1. Scale up of FTase production

A. oryzae CFR 202 exhibited profuse growth both at 3L and 15 L levels. During 3L fermentor trial, the pH of the broth was found to increase from 4.8 to 6.2 at the end of 106 h of fermentation beyond which it decreased to 5.3 at the end of 144 h. FTase activity steadily increased throughout fermentation starting from 1.85 U ml $^{-1}$min $^{-1}$ to 7.5 U ml $^{-1}$min $^{-1}$ at the end of 144 h (Figure 7.a.3). FOS produced using the FTase also increased from 3.1 % to 54 % at the end of 120 h after which it was reduced to 39 % at the end of 144 h (Figure 7.a.4).

In 15 L fermentor, pH of the broth decreased from an initial value of 5.3 to 4.7 at the end of 24 h of fermentation after which it increased steadily to 6.4 at the end of 96 h of fermentation. FTase activity steadily increased from 5.42 to 19.5 U ml $^{-1}$min $^{-1}$ at the end of 60 h of fermentation (Figure 7.a.5), remained constant at 20 $\pm$ 0.5 U ml $^{-1}$min $^{-1}$ till 84 h and slightly reduced. FOS produced using the FTase also increased correspondingly starting from 5.26 % to 53 % using FTase at the end of 60 h of fermentation (Figure 7.a.6). The observed trend in FOS production is characteristic of any fermentation process, wherein the microorganism has the maximum potential for product formation once maximum growth is attained.

7. a. 3. 2. Scale up of FOS production

Preliminary studies were carried out to check the efficiency of FOS production at various concentrations of sucrose. The results showed that FOS concentration increased with increase in sucrose concentration up to 60 %. 60 % sucrose was found most suitable for FOS production (Figure 7.a.7) agreeing

with the results obtained during optimization studies using RSM reported in

Figure 7.a.2. Reactor (10 L) designed for FOS production.

a) With Stirrer

b) With Motor driven agitator

Figure 7.a. 3. Activity profile of FTase produced by *A. oryzae* CFR 202 in 3 L fermentor

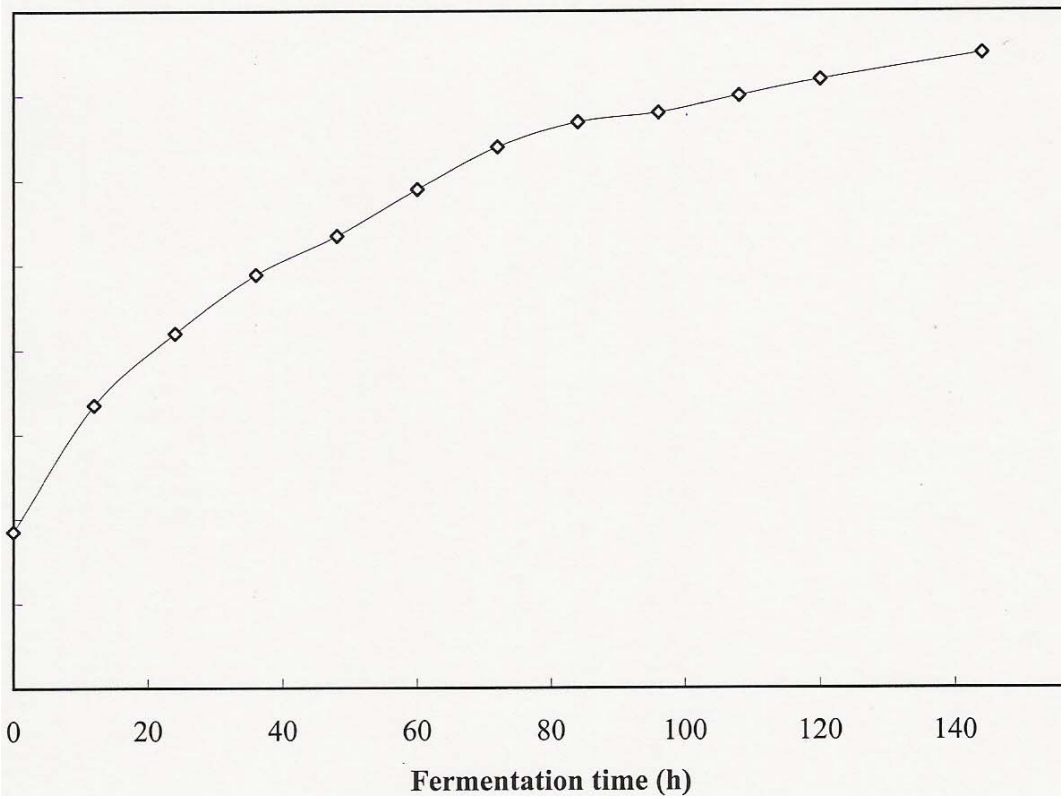


Figure 7. a. 4. FOS production using FTase produced by *A. oryzae* CFR 202 in 3 L fermentor

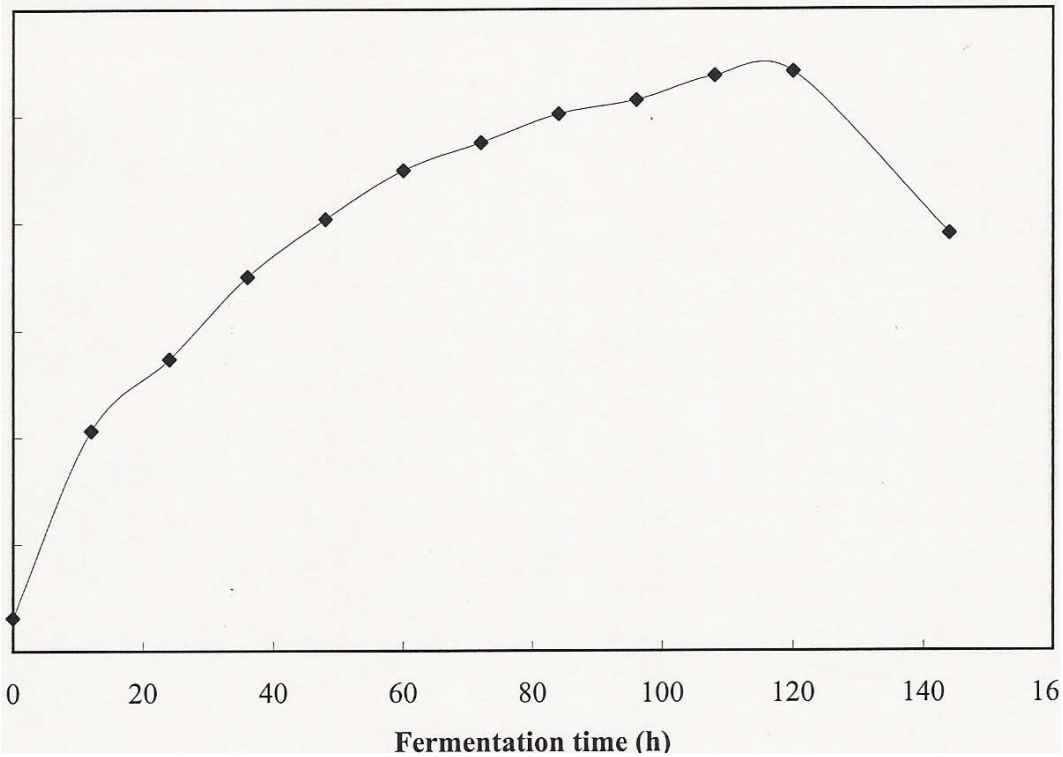


Figure 7. a.5. Activity profile of FTase produced by *A. oryzae* CFR 202 grown in 15 L fermentor

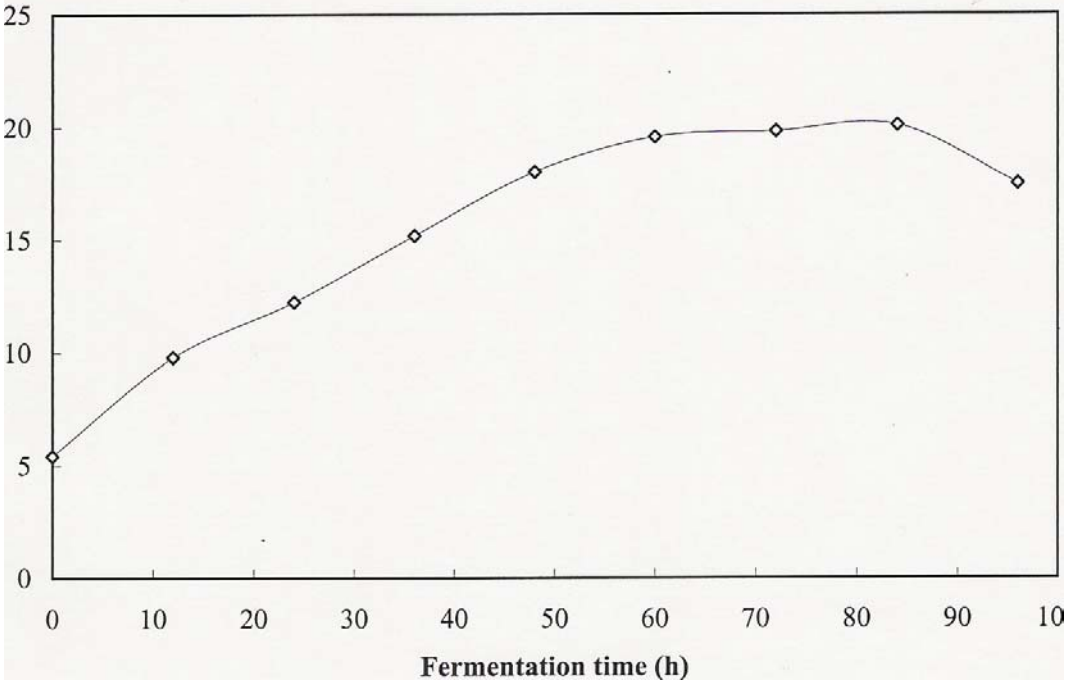
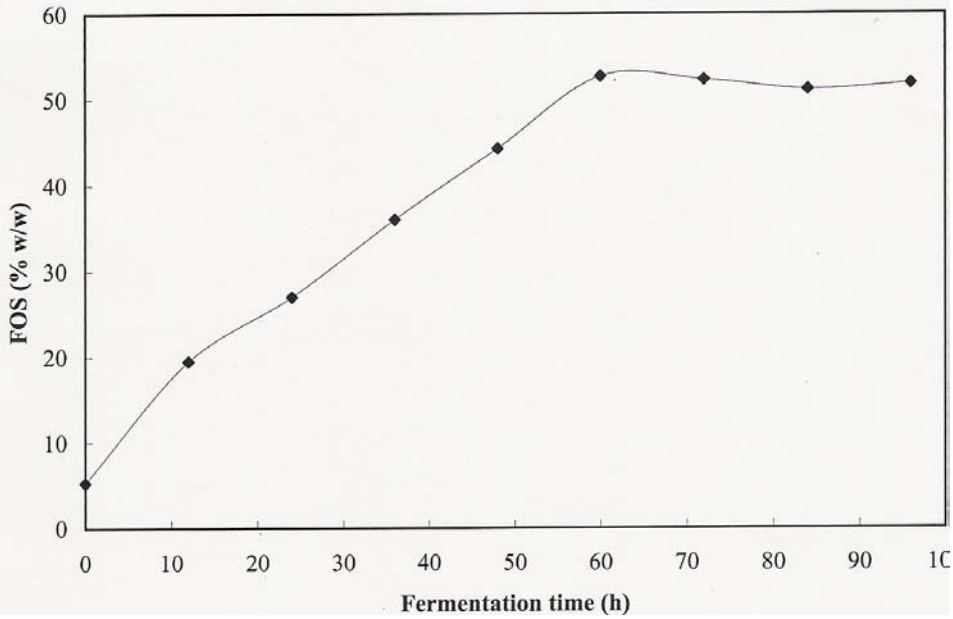


Figure 7. a. 6. FOS production using FTase produced by *A. oryzae* CFR 202 in 15 L fermentor



Section 3.c. 2. Studies were also carried out to minimize the levels of enzyme required for maximum FOS production. Results indicated that use of 10 – 25 % (v/v) FTase resulted in the maximum FOS production and hence the lowest level of enzyme was chosen for scale up of FOS production (Figure 7.a.8). Large-scale production of FOS resulted in 52 % (w/w) yield of FOS at the end of 18 h of reaction.

Results obtained in the present study have indicated that the process for FTase production could be scaled up efficiently. A considerable reduction in the fermentation time from 90 h in shake flask to 60 h in scaled up process was achieved. A maximum yield of 52 – 56 % of FOS was obtained using the FTase produced at the end of 60 h of fermentation in scale up trials. The present study resulted in the design of a simple reactor for the efficient production of FOS at 10 L scale. Scope exists for further scale up.

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Figure 7.a.7. Effect of substrate (sucrose) concentration on FOS production

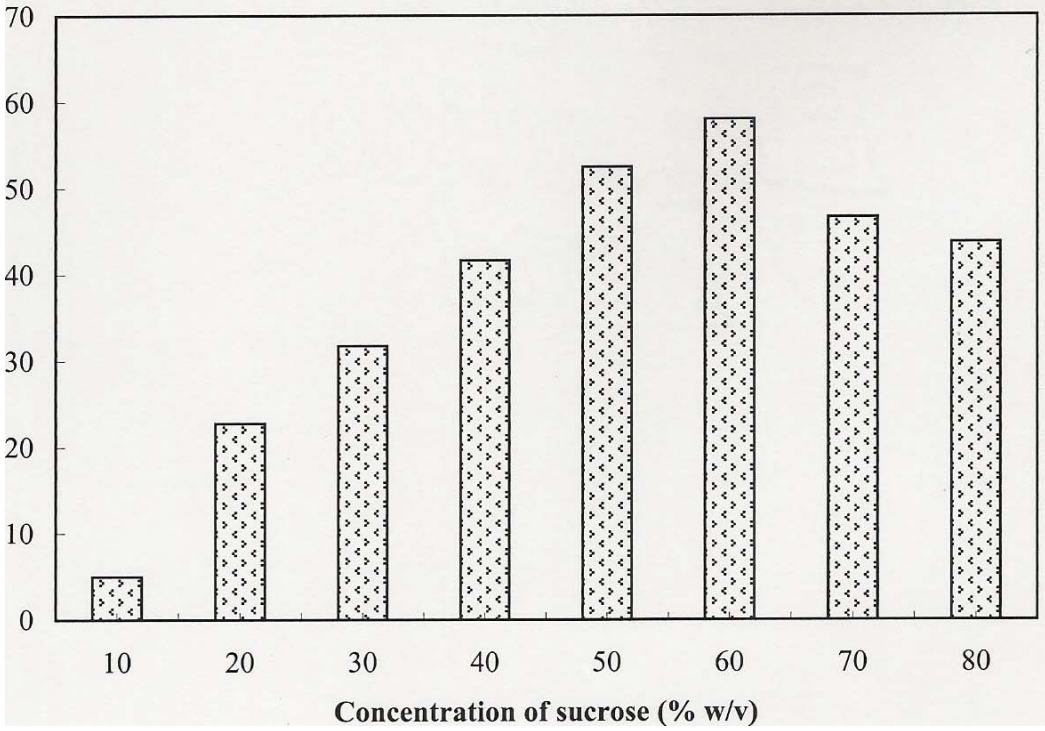
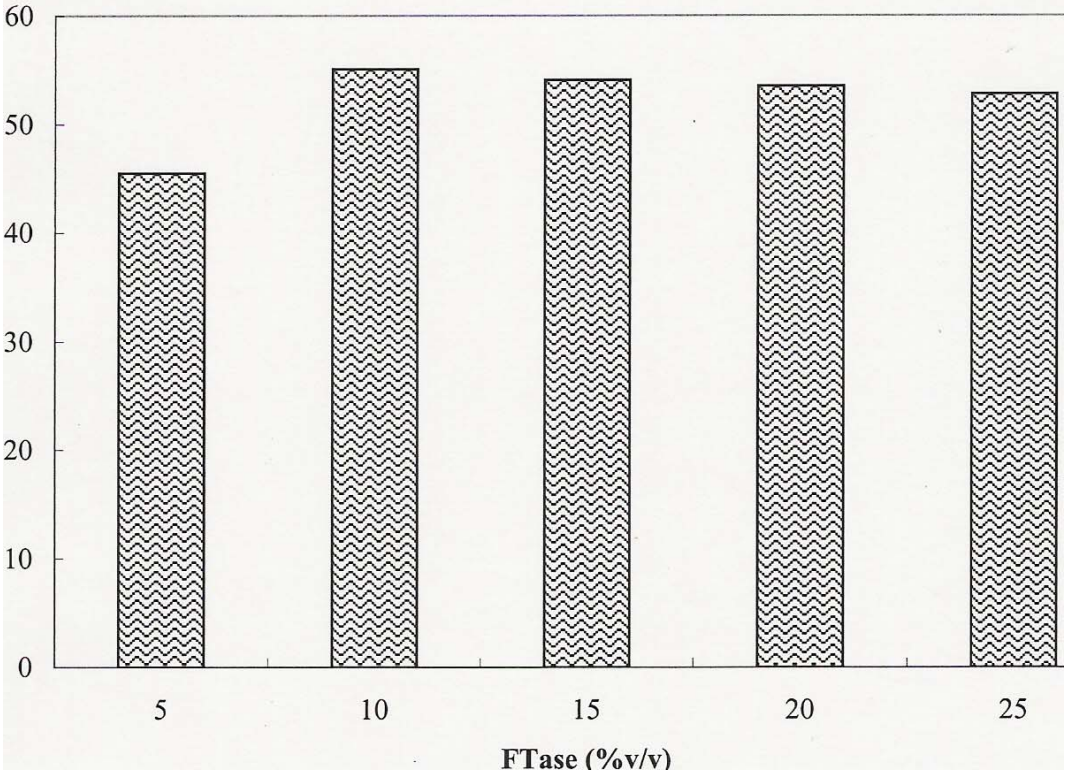


Figure 7.a.8. Effect of different concentrations of FTase on FOS production



7. b. SPRAY DRYING OF FTase AND FOS

7. b. 1. INTRODUCTION

The application and the importance of enzymes in modern technology is increasing rapidly. One of the major drawbacks of these enzymes however, is their aptitude to deactivation, caused not only by extremes of temperature, pH and the like during processing, but also occurring spontaneously on prolonged storage under otherwise normal conditions. Often applied methods known in the art to diminish this problem include the use of a variety of additives claimed to stabilize enzymes in solution or the conversion of the enzyme solution to a dry formulation by means of freeze drying, spray drying or other techniques suitable for this purpose. The conversion of an enzyme in solution to a dry form is often obligatory when the application so demands (eg. convenient mixing with other dry components). Although drying in itself is a valuable tool in the improvement of the enzyme storage stability, the process step itself often causes a substantial loss of activity and the final product is still susceptible to inactivation. This activity loss during storage or processing is strongly dependent on moisture content of the preparation and this therefore has to be most stringently controlled to maintain the so valued product stability. This also includes a severe reduction of choice in the compounds suited for addition to the final enzyme product for the sake of standardization or other purposes. In a variety of cases, the dry enzyme is intended for applications in which the enzyme has to be incorporated in a product in which the moisture content cannot be so strictly controlled. In these cases the enzyme stability is then severely reduced.

Spray drying is a convective drying technique that uses hot air to transfer heat and remove the water evaporated. Since it is a short time process, even heat sensitive enzymes can be processed with the addition of suitable stabilizers. The process involves three phases consisting of spray formation, drying and air-powder separation. The effect of spray drying has been studied on several enzymes like α-amylase, peroxidase, glucose oxidase and alkaline phosphatase. Addition of stabilizing solutes to labile enzymes is a common way of protecting them during preparation, drying and storage. A wide

variety of solutes like sugars, polyols, amino acids, methylamines and salts are effective in minimizing thermal denaturation (Pilosof and Terebiznik, 2000). The effect of maltodextrin on β-galactosidase activity retention during drying has been studied (Yamamato and Sano, 1992). Several other enzymes have been spray dried to improve their stability and enzyme activity using inorganic salts, hydrophilic silica and salts of free fatty acids (Barendse *et al*, 1998; Harz *et al*, 1999; Bewert *et al*, 1994; Henriksen *et al*, 2002). The present section describes the results of the studies on the preparation of spray dried FTase and FOS powder using additives like maltodextrin.

7. b. 2. MATERIALS AND METHODS

7. b. 2. 1. Chemicals

Maltodextrin DE 14 was from Hi Media Laboratories (Mumbai, India). Tricalcium phosphate was from SD Fine –Chem Ltd (Mumbai, India).

7. b. 2. 2. FTase and FOS preparation

FTase enzyme was prepared as described in Section 7. a. 2. 1. FOS was prepared as given in Section 7. a. 2. 2.

7. b. 2. 3. Spray drying of FTase

Maltodextrin was added to FTase enzyme at different concentrations (5 %, 15 % and 25 %) with thorough mixing on a magnetic stirrer to make a uniform suspension. The brix of the samples were measured using a hand held refractometer (Erma, Tokyo, Japan). It was then used for spray drying after filtering using cheesecloth to remove any suspended particles that may block the spraying nozzles. Spray drying was done at inlet temperature of 125 °C, outlet temperature of 80 °C at a flow rate of 65 ml min^{-1} in a 2L water evaporation capacity spray drier (BE 1216, Bowen Engineering Inc., Somerville, New Jersey, USA).

7. b. 2. 4. Spray drying of FOS

FOS syrup was mixed with 10 % maltodextrin, 2 % tricalcium phosphate or 10 % maltodextrin and 1 % Tricalcium phosphate. The brix of the samples was measured using a hand held refractometer (Erma, Tokyo, Japan). It was

then used for spray drying after filtering using cheesecloth to remove any suspended particles that may block the spraying nozzles. Spray drying was done at inlet temperature of 130 °C, outlet temperature of 90 °C at a flow rate of 60 ml min^{-1}.

7. b. 2. 5. FTase activity and FOS production

FTase activity was assayed and FOS production was carried out after each step of powder formation, ie. before adding maltodextrin, after adding maltodextrin and after spray drying. The powder obtained was reconstituted with different volumes of water and used as enzyme sample. A time course study was also carried out on FOS production using different concentrations of FTase powder with 5, 15 and 25 % maltodextrin. Analysis of reaction products was carried out using HPLC as mentioned in Section 3. a. 2. 5 and 3. a. 2. 6.

7. b. 2. 6. Characterization of FTase and FOS powder

7. b. 2. 6. 1. Moisture content

A known amount of the sample was weighed and dried in a hot air oven (The Andhra Scientific Company Ltd, Andhra Pradesh) at 110 °C to constant weight.

7. b. 2. 6. 2. Solubility

Cold water solubility of the samples were determined by the method of Eastman and Moore (1984). Distilled water (100 ml) was precisely measured and transferred into a beaker. Sample (1g) was weighed and added into the beaker and mixed thoroughly using a magnetic stirrer (Remi Equipments, Mumbai). The suspension was then transferred to a 250 ml centrifuge bottle and centrifuged at 1200 x g for 15 min. A 25 ml aliquot of the supernatant was transferred to a tared petri dish and dried in an oven at 110 °C for 4 h. The cold water solubility was calculated as follows:

$$\text{CWS (\%)} = \frac{\text{grams of solids in supernatant} \times 4}{\text{Grams of sample}} \times 100\ \%$$

7. b. 2. 6. 3. Ash

The samples were weighed into an ignited pre-weighed silica dish and gently heated in a muffle furnace until the sample is black and dry and there is no danger of loss by foaming and overflowing. The sample is then ignited in a muffle furnace (MR 170, W Germany) at 600 ^{o}C to constant weight. The sample is cooled before weighing.

7. b. 2. 6. 4. Microbial count

Microbial count in the sample was analyzed by plating the samples on nutrient agar and potato dextrose agar plates.

7. b. 2. 7. Scanning Electron Microscopy

FOS powder samples were coated with thin layer of gold using Polaron SEM coating system and viewed using a Scanning Electron Microscope.

7. b. 2. 8. FOS production using FTase powder

FOS production was carried out using FTase powder spray dried with various concentrations of maltodextrin (5, 15 and 25 %). Reaction was allowed to continue for 24 h under the assay conditions given in Table 3. c. 2. 9. Sampling was done at every 6 h and FOS produced was analyzed as given in Section 3. a. 2. 5. And 3. a. 2. 6.

7. b. 3. RESULTS AND DISCUSSION

7. b. 3. 1. Spray drying of FTase

Spray drying is a well-known technique in stabilizing compounds in powder form. There are several reports on the use of this technique for preserving enzymes. Eventhough FTase production from microorganisms and FOS production using the enzyme is well known, there have been no reports on availability of FTase in powder form. Hence, attempts have been made to prepare powder form of FTase using maltodextrin as additive.

Table 7.b.1 presents the FTase activity and FOS yield obtained after spray drying FTase with various concentrations of maltodextrin. 15 % Maltodextrin was ideal compared to 5 and 25 % as seen from the results. This

may be attributed to the loss of activity in FTase with 5 % maltodextrin since it is more prone to heat during spray drying. FTase with 25 % maltodextrin has less activity due to high concentration of maltodextrin.

Studies were carried out on the stability of FTase powder at room temperature as well as at refrigerated conditions. The results are presented in Figures 7.b.1, 7.b.2 and 7.b.3. FOS yields obtained using the spray dried FTase with 5 % maltodextrin stored at room and low temperatures followed the same pattern with maximum yield of 40 % at the end of 12 h of reaction.

Table 7.b.1. Activity and FOS yield obtained using spray dried FTase powder

Sl. No.	Additive used	FTase activity (U/g/min)	FOS (%)
1	Control FTase	31.66	47.9
2	5 % Maltodextrin	18.07	35.3
3	15 % Maltodextrin	26.15	40.7
4	25 % Maltodextrin	20.48	46.4

The FTase activity after 2 weeks was 22 and 25 Ug^{-1} for the enzyme stored at room and low temperatures respectively. This marginal difference did not affect the FOS yields. FTase with 15 % Maltodextrin stored at room temperature and low temperature gave maximum FOS (45 %) after 6 h of reaction. The FTase activity was 16.7 and 23.74 Ug^{-1} for the enzyme at room temperature and low temperature respectively. FTase with 25 % Maltodextrin has given maximum FOS after 18 h of reaction when stored at low temperature and room temperature. The FTase activity was 19.66 and 19.96 for enzyme stored at room temperature and low temperature respectively.

7. b. 3. 2. FOS production using FTase powder

Figures 7.b.4, 7.b.5 and 7.b.6 shows the time course of FOS production using FTase powder spray dried with different concentrations (5 %, 15 % and 25 %) of maltodextrin. As seen from the figures, FOS production was maximum at the end of 12 h of reaction after which it reduced.

Figure 7.b.1. FOS yields obtained using spray dried FTase (5 % Maltodextrin) after 15 days of storage

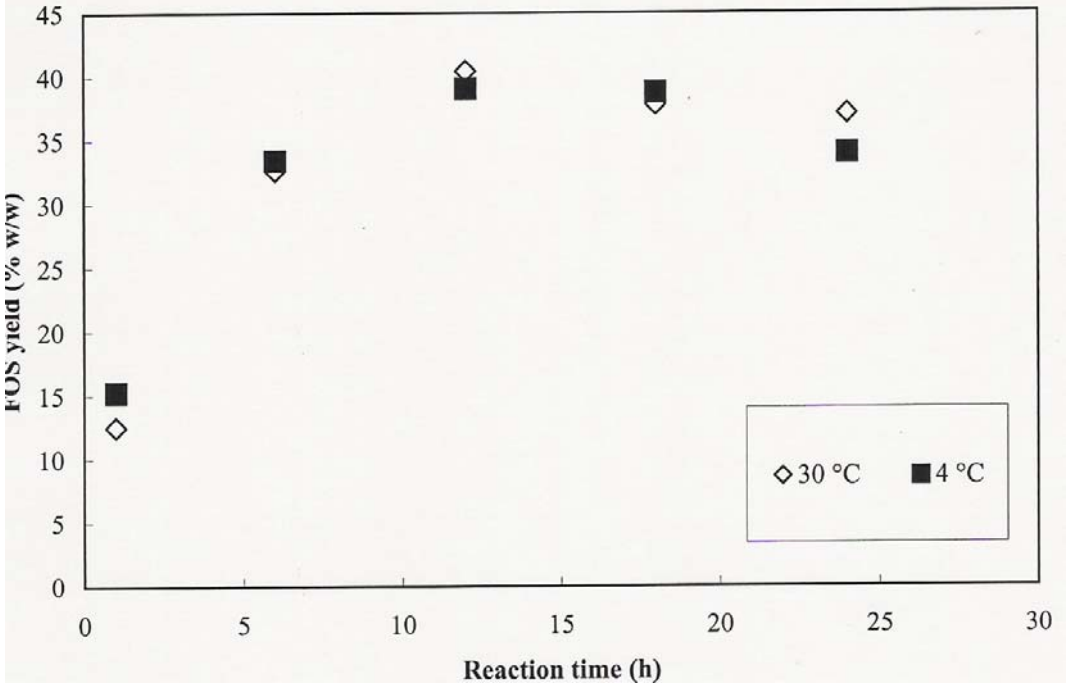


Figure 7.b.2. FOS yields obtained using spray dried FTase (15 % Maltodextrin) after 15 days of storage

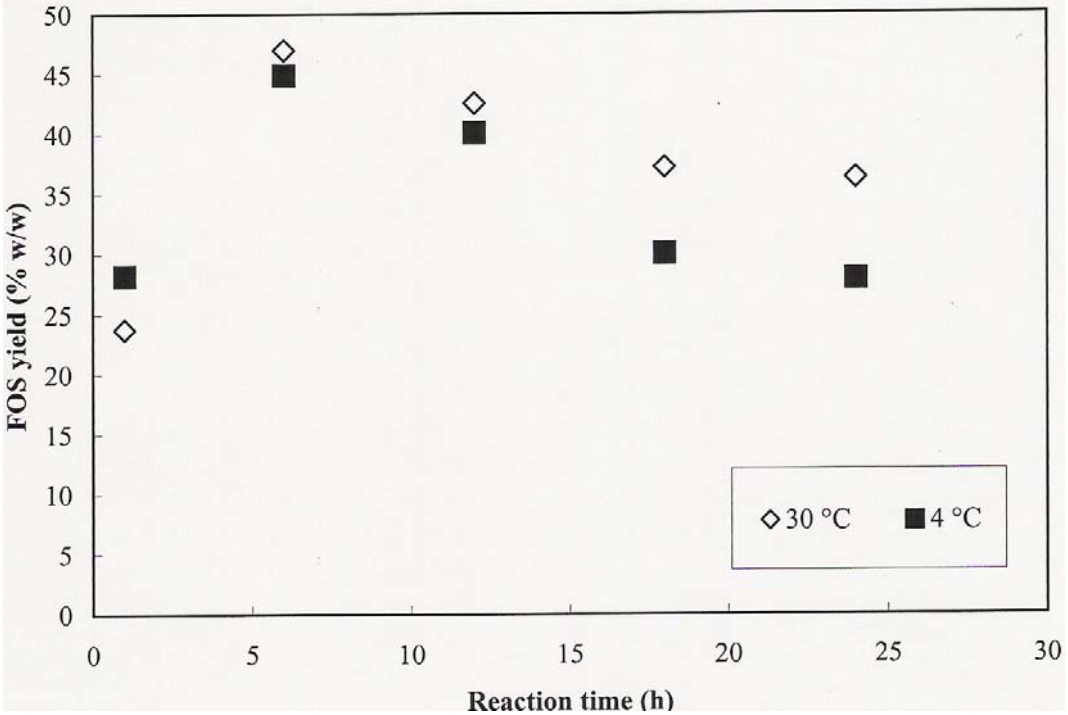
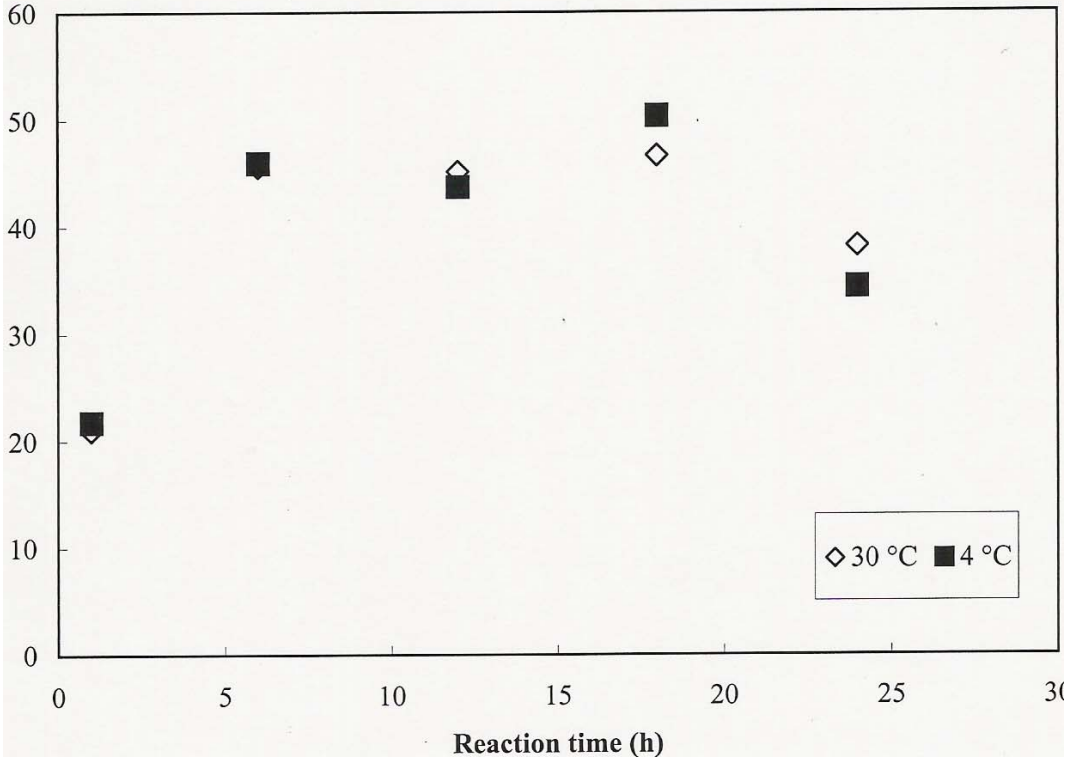


Figure 7.b.3.FOS yields obtained using spray dried FTase (25 % Maltodextrin) after 15 days of storage



7. b. 3. 3. Spray drying of FOS

FOS is available as syrup or as tablets. The reason for its non-availability in powder form is its highly hygroscopic nature. Even though it is highly hygroscopic, chicory derived FOS is marketed in powder form. In the present investigation, attempts have been made to stabilize FOS in powder form using additives like maltodextrin and anticaking agents like tricalcium phosphate. Table 7.b.2. presents the total soluble solids before spray drying and final FOS content of the samples after spray drying.

Table 7.b.2. Total soluble solids and FOS content (% w/w of powder) in spray dried FOS powder (reconstituted as 11 mg/ml)

Sl. No.	**Total Soluble Solids (° Brix)**	**Additive used**	**% FOS**
1	56	----------	78.77
2	60	10 % Maltodextrin	62.9
3	52	2 % Tricalcium phosphate	96.4
4	58	10 % Maltodextrin and 2 % tricalcium phosphate	50.9

Table 7.b.3. presents the characteristics of FTase and FOS powder.

Table 7.b.3 Characteristics of FTase and FOS powder

Characteristic	**FTase**	**FOS**
Appearance	Pale yellow powder	Creamish powder
Dry matter (%)	96.4 – 96.6	98.6 – 98.8
Solubility (%)		
Cold	97 – 98	95 – 96
Hot	-----	100
Ash (%)	11-15	0.4 - 1
Bacterial count (cfu / g)	310 – 780	Nil
Mold count (cfu / g)	130 – 1260	Nil

Figure 7. b.4. FOS production using different concentrations of spray dried FTase (5 % Maltodextrin)

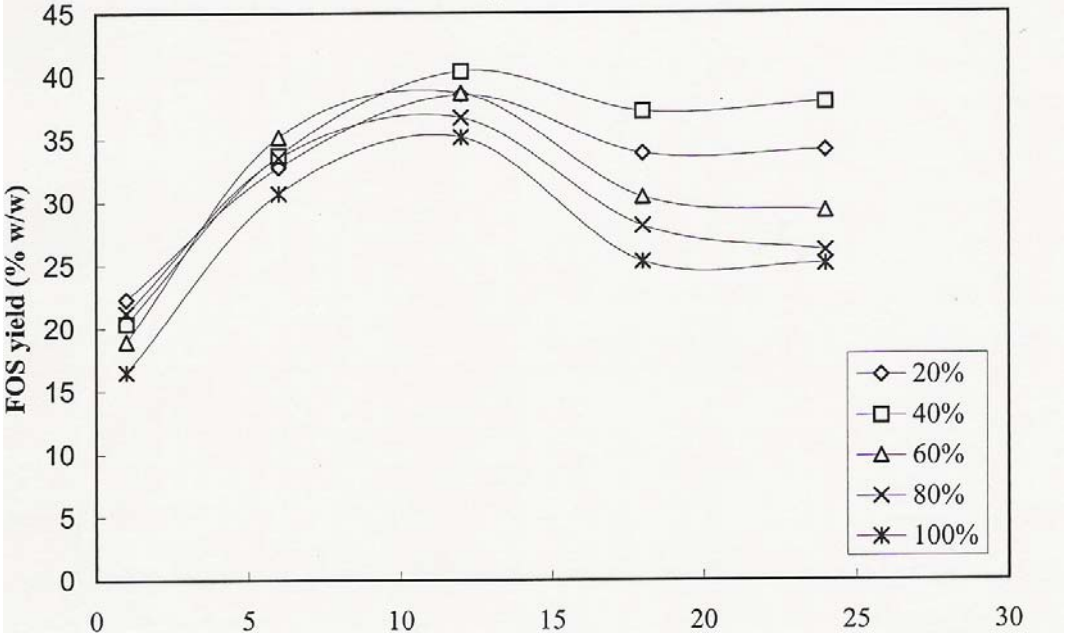


Figure 7. b.5. FOS production using different concentrations of spray dried FTase (15 % Maltodextrin)

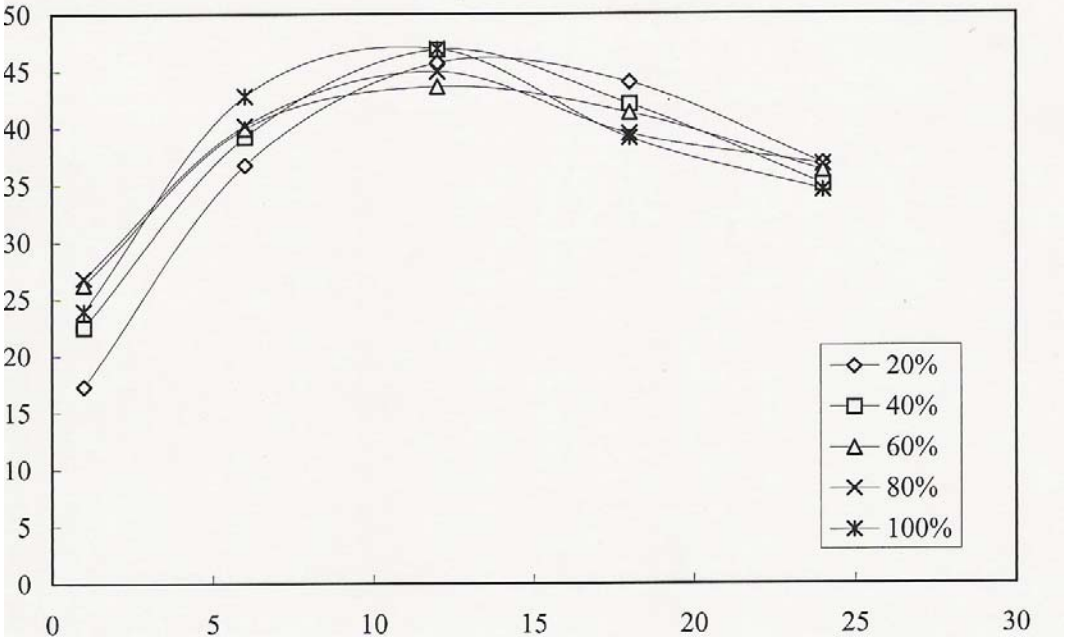
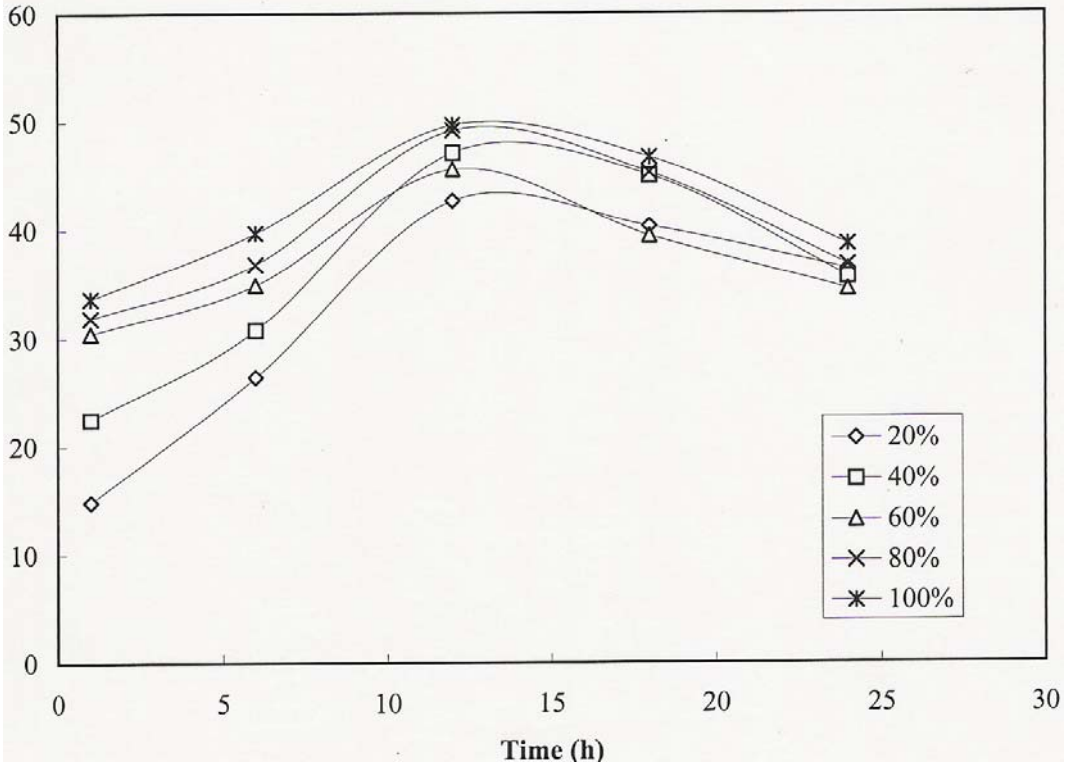


Figure 7.b.6. FOS production using different concentrations of spray dried FTase (25 % Maltodextrin)



FOS powder had very little ash content, where as FTase powder had high ash content, which may be attributed to the protein content and salts present in the media. Microbial count was nil in FOS powder assuring its safety for consumption. The microbial counts of the FTase samples were well within safe limits. Figure 7.b.7 shows the FOS powder. Scanning electron micrographs of FOS powder with and without maltodextrin is showed in Figure 7.b.8.

Figure 7.b.7. Spray dried FOS powder.

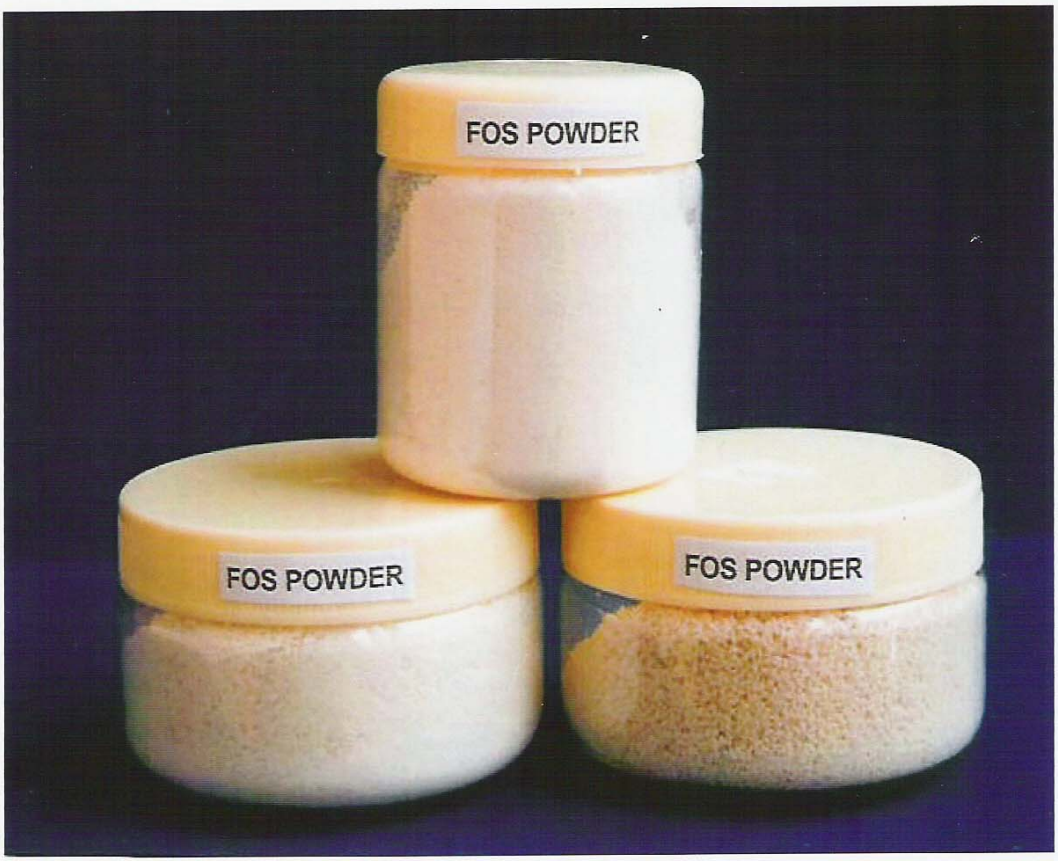
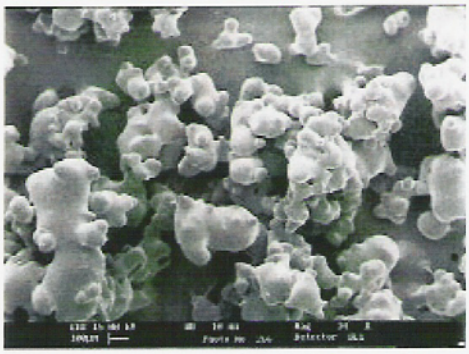
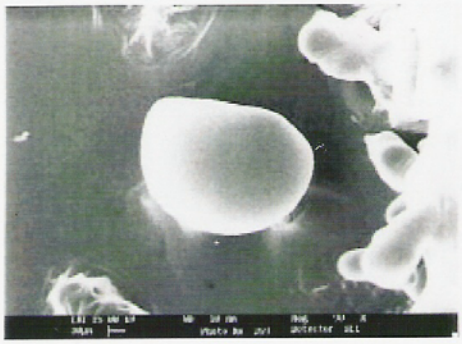


Figure 7.b.8. Scanning electron micrographs of FOS powder (a) without maltodextrin (b) with 10 % maltodextrin and 1 % Tricalcium phosphate.

Magnification - 20x

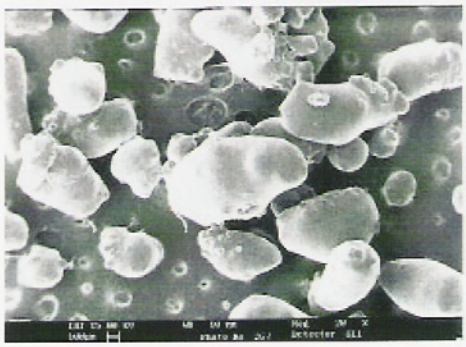


Magnification - 75x

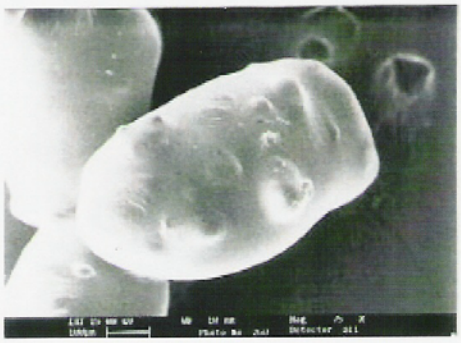


(a) without maltodextrin

Magnification - 31x



Magnification - 98x



(b) with 10 % maltodextrin and 1 % Tricalcium phosphate.

CHAPTER 8

a. FOS BASED PRODUCTS

b. CHARACTERIZATION OF FOS

8. a. FOS BASED PRODUCTS

8. a. 1. INTRODUCTION

The physicochemical characteristics of a food ingredient have significant impact on its versatility. The ideal ingredient should be sufficiently soluble to be used in various products on an industrial scale. Similarly, viscosity, surface tension, and pH are important for designing manufacturing processes or assessing ingredient compatabilities. The melting point of a food ingredient is a characteristic, which can influence the nature of the products in which it can be used to determine the appropriate storage conditions. Other physicochemical characteristics can be important when considering control of food manufacturing operations. This chapter presents the applicability of FOS in preparing various products like spread, honey - like product and beverage concentrates and assesses its implications from the product development perspective. The physicochemical characteristics of FOS are also discussed in the chapter.

FOS possesses a number of beneficial properties that makes it a versatile ingredient to be used in various food products. Due to its prebiotic properties, it can be used in various milk products and products aimed at maintaining a healthy gut environment. Due to its low calorific value and non-digestible nature, it can be used in food products targeting diabetics. Confectioneries can take advantage of its non – cariogenicity and products for children can be prepared.

In the present research work, attempts have been made to prepare spread, honey - like product and beverage concentrate using FOS produced by FTase from *A. oryzae* CFR 202. Spreads are one of the most versatile products within the food industry. Such products maybe used for several applications, from tasty toppings for bagels and ice cream to flavourful fillings for a variety of baked goods. Large and small manufacturers prepare these products. Since most of the spreads are based on fats like butter, the development of a low calorific, zero fat spread presents lot of scope to food industry.

Honey has been recognized for centuries as a special dietary treat. It can be processed for direct consumption or used as an ingredient in various processed food products. Because of its superior nutritional value and unique flavour characteristics, the cost of natural bee honey is much greater than other sweetening commodities. The three major components of honey are fructose, glucose and water averaging 38.2, 31.3 and 17.2 % respectively (Doner, 1977). According to available statistics, the world production of honey is estimated at about 500 million kg, of which India contributes only about 1.2 percent or 6 million kg (Phadke, 1967). It has been reported that honey of floral origin contains significant amounts of contaminants called pyrrolizidine alkaloids, which are threat to human health (Edgar *et al*, 2002). To eliminate this particular threat a honey - like product with additional health benefits was prepared.

With ever-increasing demand for soft beverages and fruit based beverages, both carbonated and still are available in the market in plenty. India offers itself a potential market for these products. The soft beverages are generally aerated or carbonated and offer themselves for quenching thirst and also as a source of energy. However, the nutritional quality of these beverages is very low. The soft beverage is carbonated and is sweetened with the natural sweetener, namely, sugar or a permitted synthetic sweetener. Carbonation adds 'Zip' and 'Sparkle' to the beverage not added by any other ingredient. Sweetened carbonated beverage, contains permitted colour and added flavours, contains no fruit juice and contains class II preservatives. This is one of the largest food industries in the world. Due to large competition in this field, manufacturers continuously look for innovations to market their products. FOS can be used as an ingredient in beverages to provide beneficial effects to the modern health conscious society. This can add on to the concept of nutraceutical beverages that continues to expand as one of the fastest growing niche markets.

8. a. 2 MATERIALS AND METHODS

8.a. 2.1. Chemicals and materials

Chemicals like Sulphuric acid and Phenol were from SD Fine Chem. Ltd. (Mumbai, India). Phenolphthalein was from Sisco Research Laboratories Pvt.

Ltd. (Mumbai, India), Dinitrosalicylic acid (DNS) was from Loba Chemie Pvt. Ltd, Mumbai. Commercial honey samples (processed and unprocessed) were procured from the local market.

8.a.2.2. Colour

Colour of the samples was measured using a Hunterlab Colour Measuring system (Labscan XE, Hunter Associates Laboratory Inc, Reston, USA). L*a*b* colour space was measured. L* indicates lightness or brightness of the sample, a* indicates red for positive value and green for negative value, b* indicates yellow for positive value and blue for negative value.

8.a.2.3. Specific gravity

Specific gravity was measured using specific gravity bottle (Riviera Glass Pvt. Ltd, Mumbai)

8.a.2.4. Polarization

The polarization of the samples was measured by using a Perkin Elmer 243 polarimeter (Perkin Elmer and Co., W Germany).

8.a.2.5. Moisture content

The refractive index of the sample was determined at a constant temperature using a refractometer (Rx 5000, Atago Co. Ltd., Tokyo, Japan). The moisture content (% w/w) was calculated based on the reading using the conversion table (IS 4941-1994).

8.a.2.6. Total Soluble solids

Total soluble solids were determined using a hand held refractometer (Erma, Tokyo, Japan)

8.a.2.7. Total and reducing sugars

Total sugars were measured by Phenol Sulphuric acid method (Dubois *et al*, 1956). Reducing sugars were measured using DNS method (Miller, 1959).

8.a.2.8. Acidity

Honey (10 g) was weighed and dissolved in 75 ml distilled water. The test sample was titrated against carbonate free 0.1 N sodium hydroxide solution using 4 –5 drops of neutralized phenolphthalein indicator (1 % w/v in ethanol) to get the end point colour (pink), which persisted for 10 sec. The result is expressed as milliequivalents acid kg^{-1} honey and is calculated as follows:

Acidity = weight of the sample x number of ml of 0.1 N NaOH used in the neutralization of honey

8.a.2.9. Ash

Honey was weighed accurately into an ignited pre-weighed silica dish (Vitreosil, England) and gently heated in a muffle furnace until the sample is black and dry and there is no danger of loss by foaming and overflowing. The sample is then ignited in a muffle furnace (MR 170, W Germany) at 600 ^{o}C to constant weight. The sample is cooled before weighing.

8.a.2.10. Protein

Total protein was determined by Kjeldhal method (AOAC, 1980).

8.a.2.11. Viscosity

Viscosity was measured using a viscometer (RI:3:M, Rheology International, Shannon, Ireland) at room temperature using spindle ASTM 6. The shear rate was 50 and 75 rpm and the viscosity was expressed in Pascal Second.

8.a.2.12. Texture measurement

Texture measurement was carried out using a Universal Texture Measuring System (LR5K, Lloyd Instruments, Hampshire, UK) fitted with a 5mm cylindrical plunger. Spread samples at 5 °C were placed in containers with the plunger set to penetrate each sample for a distance of 10 mm at a speed of 5 mm min^{-1}. The plunger was withdrawn at the same speed.

8.a.2.13. HPLC

Sugars and FOS were measured using HPLC as given in Section 3. a. 2.

8.a.2.14. Sensory analysis

Descriptors used for sensory analysis of honey samples were developed during initial sessions in which different samples of honey, varying in quality were presented to the panelists. The panelists were asked to describe the samples with as many spontaneous descriptive terms as they found applicable. Attributes representing superior quality (flowery and fruity) as well as those representing poor qualities (waxy and chemical) were identified. The common descriptors chosen by at least one third of the panel were compiled along with some significant descriptors found in the literature and selected for the formulation of scorecard.

The panel consisted of 25 people in the age group of 22 – 60 years, comprising both females and males, who regularly participated in sensory evaluation. The panelists comprised mainly the staff and students of the Institute, who had experience in odour and flavour profiling of a number of food products. The panelists were familiarized with different odour and flavour notes. The method of intensity scaling used was Quantitative Descriptive Analysis (QDA) (Stone *et al*, 1974). The scorecard consisted of 10 points QDA scale. The panelists were asked to mark the perceived intensity of the attribute by giving points.

Testing was performed in a laboratory with lighting equal to daylight. 25 ml of the honey sample, at room temperature (30 ± 1 °C) was served in 50 ml beakers labeled with codes. Water was used for palate cleansing in between the samples.

8.a.2.15. Product preparation

8.a.2.15.1. Spread

FOS was prepared as given in Section 7. a. 2. 2. The sample was then concentrated by keeping in a boiling water bath for 2 – 4 h with constant stirring. The product was then cooled for 30 – 40 min to set. 40 % maltodextrin or starch powder was also added to the FOS syrup before concentration. The FOS syrup with 50 ° brix total soluble solids was concentrated to get 75 – 80 ° brix. Care was taken to avoid scorching or caramelizing.

8.a.2.15.2. Honey

Honey - like product was prepared by keeping the FOS syrup in a boiling water bath for 1 h with constant stirring to attain the required viscosity. The product was then cooled at room temperature for 15 – 30 min. Care was taken to avoid scorching or caramelizing.

8. a. 2.15. 3. Beverage concentrate

FOS with 60 ° brix was added with small amount of sucrose to enhance the sweetness, cola, lime- lemon or orange flavours to improve the acceptability, and diluted with chilled carbonated water to prepare the beverage. The cola flavour concentrate consisted of 0.4 g caramel, 0.14 g phosphoric acid, 0.02 g caffeine, 0.02 ml cola flavour blend, 0.028 g sodium benzoate and 21 g sugar. The concentrate for orange flavour consisting of 0.06 g citric acid, 0.014 g sodium benzoate, 0.6 ml orange emulsion, 0.03 g tri sodium citrate and 26 g sugar. To add to the sweetness of the product, sometimes, the extract of an intensive sweetener like Stevia was also added. About 45 ml of the concentrate containing FOS was transferred into clean 200 ml marked glass bottles. The volume of 200 ml was made up with carbonated water, crown corked and stored at chilled condition.

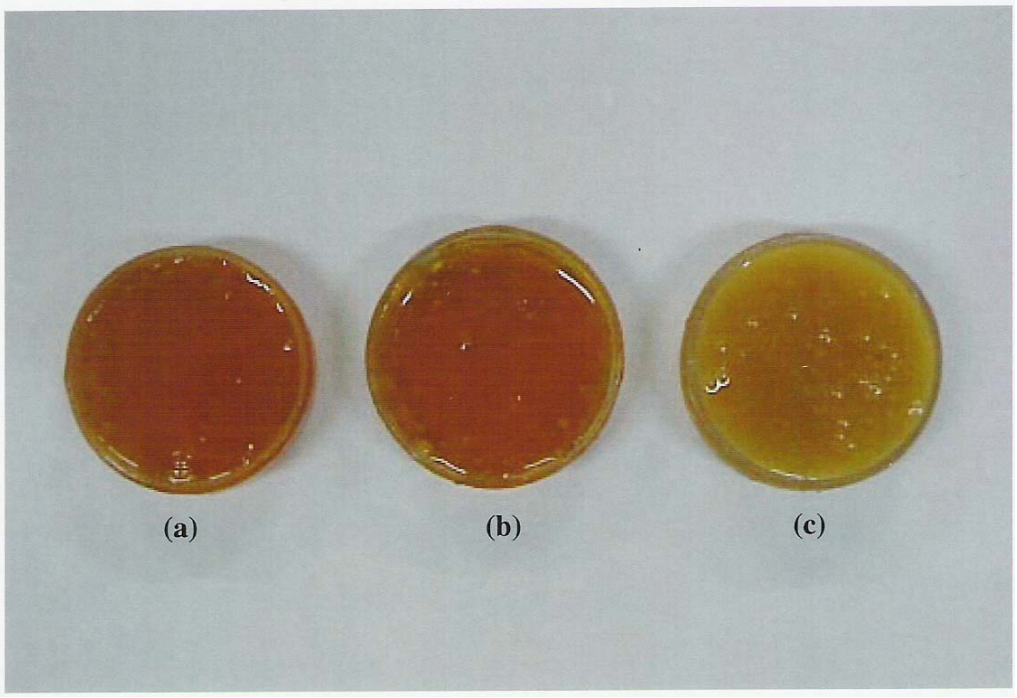
8. a. 3. RESULTS AND DISCUSSION

In the present study, FOS produced using FTase from *A. oryzae* CFR 202 was used to prepare various products of use in food industry. The products prepared included a spread, honey - like product and beverage concentrates. The spread varied in colour depending on the presence or absence of additives like maltodextrin and starch (Figure 8.a.1). Table 8.a.1 presents the results obtained during colour measurement of spread samples.

Table 8.a.1. Colour measurement of FOS based spread samples

Sample	**L**	**A**	**b**	**D_E**
FOS alone	16.02	1.65	5.80	74.89
FOS + Maltodextrin	33.35	4.65	15.82	59.58
FOS + Starch	34.45	6.14	16.77	58.94

Figure 8.a.1. FOS based spread (a) FOS alone (b) FOS with maltodextrin (c) FOS with starch



The final total soluble solids were 78 – 79 ° brix and the FOS content was 50 % in the spread. There was no major change in the texture of the spread with addition of starch and maltodextrin. The maximum load values for 10 mm depth was 0.107 - 0.112 for the three samples tested.

Another product prepared was honey - like product. The physicochemical characteristics of the product in comparison with commercial honey samples are given in the Table 8.a.2. Refractive index, Brix, Specific gravity, protein and pH of the product was comparable with processed commercial honey. Sucrose content, acidity and moisture content were close to the unprocessed honey. Viscosity value was in between that of processed and unprocessed samples where as ash content was higher than both. High ash content may be due to the presence of the residual enzyme. Another important feature is that FOS honey has very less amount of reducing sugars compared to commercial honey. This makes it a suitable ingredient of products aimed at diabetics. Sensory evaluation rated the sample in between processed and unprocessed samples. Apiary honeys vary widely in their colours from light yellow to dark red with all the intermediate shades. Colour of the product based on FOS was close to unprocessed honey.

Almost all the honey samples are laevorotatory. The average reducing sugar content in apiary honeys are 3.5 %. High fructose content and low non-reducing sugars may be one of the reasons for the high negative polarization of foreign honeys. FOS based honey - like product had positive polarization due to low free fructose content and high non- reducing sugar content. Moisture content of honey has an average value of 21 %. This is less than the limit of 22 % moisture beyond which honey samples are liable to fermentation. The moisture content of the FOS based honey - like product was 22 %, which is acceptable. However, the moisture content can be reduced by further concentrating the syrup, without affecting the other properties.

Table 8.a.2 Physicochemical characteristics of honey - like product in comparison with commercial honey samples

Physicochemical characteristic	Unprocessed Honey	Processed commercial honey	FOS based Honey	Literature report * (Phadke, 1967)	Remarks
Refractive index	1.47	1.48	1.48	-------	Comparable
Brix (%)	72.24	79.51	76.99	77.57	Comparable
Specific gravity	1.35	1.42	1.39	1.40	Comparable
Protein (%)	0.55	0.55	0.72	0.56	Comparable
Colour Yellowness index	10.40	6.27	10.84	13.93	Comparable
Reducing Sugars (%)	86.8	88.5	32.0	70.2	Less, advantageous
Non reducing sugars (%)	13.2	11.5	67.6	3.37	More, Advantageous
Sucrose	13.2	11.5	13.0	-----	Comparable
(FOS)	-----	-----	54.6	-----	Nutraceutical component
Ash (%)	0.19	0.21	0.50	0.19	More, Due to minerals in buffer and media
pH	4.2	4.6	5.4	------	Can be suitably modified by altering the processing conditions
Acidity (% as citric acid)	0.23	0.14	0.21	3.19	Comparable
Moisture (%)	19.4	12.0	22.6	20.89	Comparable
Viscosity (Pa.S)	3.9	12.1	5.56	-----	Comparable
Sensory evaluation	8.75	6.90	7.08	-----	Better than processed honey and comparable to unprocessed honey

Honey consists essentially of different sugars, predominantly glucose and fructose. But, the honey - like product presented in this chapter has no free fructose and less glucose. In addition, it has high concentration of FOS (50 – 55 %), which has beneficial properties like low calorific value, non – cariogenicity and bifidogenicity. Figure 8.a.2. presents the FOS based honey - like product.

Sensory analysis

Quantitative descriptive analysis was carried out and the overall acceptability was estimated on a hedonic scale of 10. Honey samples were evaluated for attributes like colour, body, aroma (flowery, fruity, waxy, jaggery – like, acidic, caramelized, fermented) and taste (sweet, sour, salty). Fig 8.a.3. shows the results obtained during sensory analysis. FOS based honey - like product was rated more acceptable than processed honey and less acceptable compared to unprocessed honey.

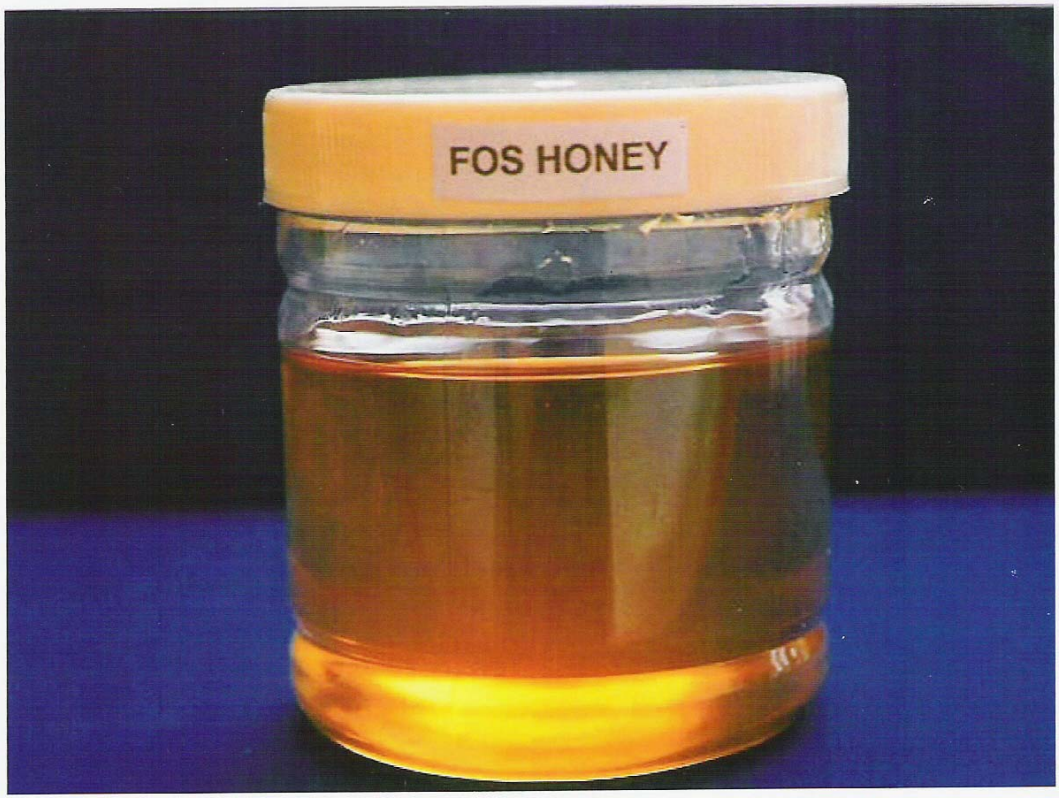
FOS content in the beverage samples was analyzed by HPLC. The RDA level of FOS in a food product is 5 g day^{-1}. The beverage samples were analyzed and found to contain upto 4 g of FOS. The results are presented in Table 8.a.3.

Table 8. a.3. FOS levels in beverage concentrates with added flavours

	Plain	Lime Lemon	Cola	Orange
FOS (g/200 ml)	4.60	4.82	3.81	4.86
FOS + Stevia (g/200 ml)	4.60	3.47	3.94	3.86

The beverage was tasted by a panel of trained scientists and evaluated for taste, aroma and overall acceptability on a 10 point hedonic scale. Samples were found to be acceptable and rated above 7 in all respects. The beverages prepared using FOS are shown in Figure 8.a.4.

Figure 8.a.2. FOS based honey – like product



Fgure 8.a.3. Quantitative descriptive analysis of FOS based honey - like product and commercial honey.

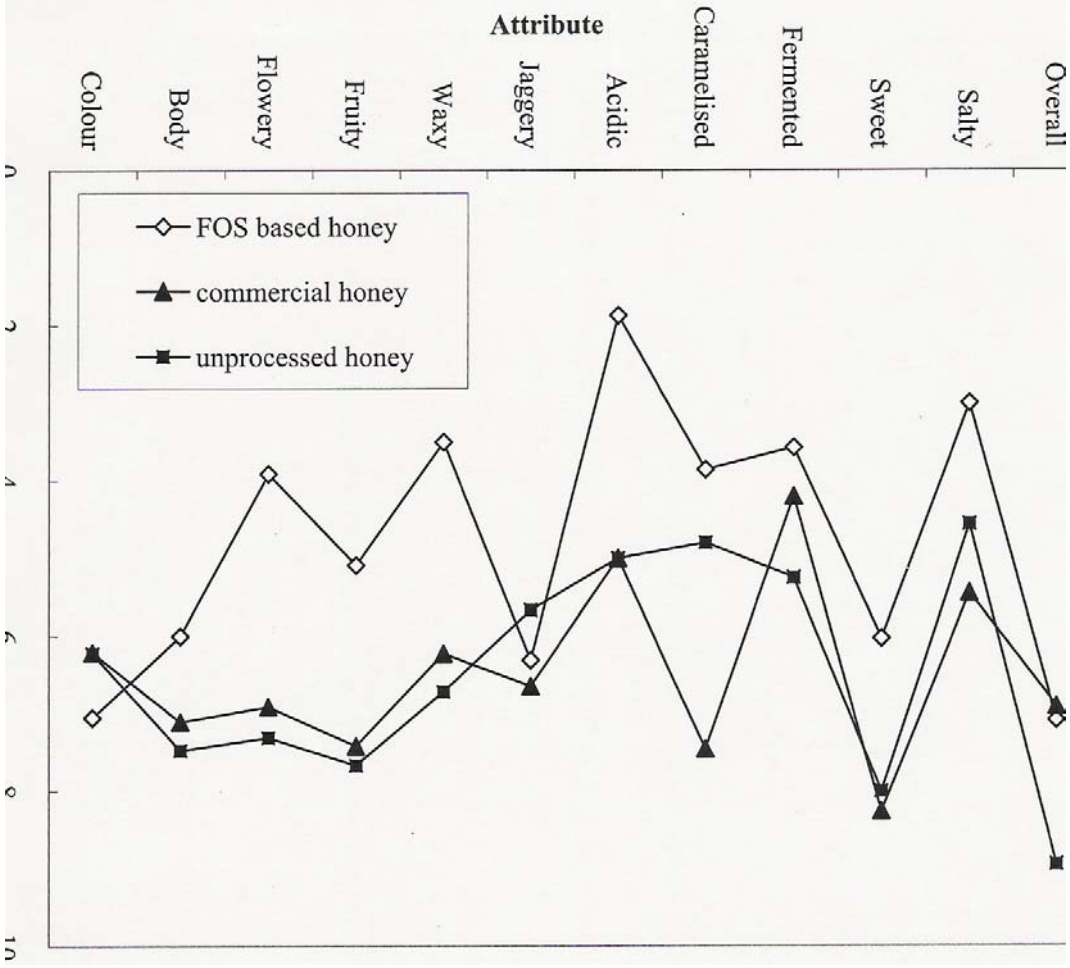


Figure 8.a.4. FOS based beverages with added flavours

(a) (b) (c)

a) Cola

b) Lime lemon

c) Orange

8. b. CHARACTERIZATION OF FOS

8. b. 1. INTRODUCTION

FOS produced using FTase from *A. oryzae* CFR 202 is composed of a mixture of three oligomers – GF_2, GF_3 and GF_4 along with the residual substrate (sucrose) and glucose released during the reaction. Removing sucrose and glucose from the mixture will increase the commercial value of the product. In this study, attempts have been made to purify the individual oligosaccharides by column chromatography.

8. b. 2. MATERIALS AND METHODS

8.b.2.1. Purification of FOS by column chromatography

1 g of lyophilized FOS was dissolved in 2 ml water, centrifuged and loaded to a Biogel P – 2 (BioRad Laboratories, California, USA) Gel Filtration column (100 x 1 cm). Elution was carried out using triple distilled water at a flow rate of 5 ml h^{-1} and 1 ml fractions were collected. The fractions were analyzed by HPLC as mentioned in Section. 3. a. 2.

8.b.2.2. Structural Determination of FOS by NMR

^{1}H and ^{13}C NMR spectra were recorded at 500 MHz on a Bruker DRX 500 MHz spectrometer (500.13 MHz proton and 125 MHz carbon frequencies). Proton and carbon 90° pulse width were 12.25 and 10.5 μs respectively. About 50 mg of the sample dissolved in D_2O was used for recording the spectra. Chemical shifts were expressed in ppm relative to internal TMS.

Two Dimensional Heteronuclear Multiple Quantum Coherence Transfer Spectra (2D-HMQCT) were recorded in magnitude mode with the sinusoidal shaped z gradients of strength 25.7, 15.42 and 20.56 G/cm in the ratio of 5:3:4 applied for a duration of 1 ms each with a gradient recovery delay of 100 μs to defocus unwanted coherences. The t_1 was incremented in 256 steps. The size of the computer memory used to accumulate the 2 D data was 4 K. The spectra were processed using unshifted and π /4 shifted sine bell window function in F_1 and F_2 dimensions respectively.

8.b.2.3. LC – MS analysis of purified oligomers of FOS

LC – MS was performed on a Bruker Esquire 3000 Mass Spectrometer (Bruker Instruments, Germany) connected to an Agilent 1100 HPLC system (Hewlett Packard Company, Pennsylvania) fitted with an Exsil Amino column (25 cm x 4.6 mm, 5 μ) at 25 °C with 80 % acetonitrile as mobile phase. Electrospray ionization (ESI) of the sample was carried out in the positive ionization mode at 51.0 psi nebulizer pressure, 10 L min^{-1} dry gas flow rate at 360 °C dry gas temperature. The detector used was an ion trap analyzer.

8. b. 3. RESULTS AND DISCUSSION

Studies about proton and carbon chemical alignments of kestose have already been reported (Calub *et al*, 1990; Liu *et al*, 1991). Three kestoses are known so far. They are 1-kestose [(O-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl –(2→1)-α-D-glucopyranoside], 6-kestose [(O-β-D-fructofuranosyl-(2→6)-β-D-fructofuranosyl –(2→1)-α-D-glucopyranoside] and neokestose [(O-β-D-fructofuranosyl-(2→6)-α-D-fructofuranosyl –(1→2)-glucopyranoside]. Commercial FOS is sold as sugar mixture because of the difficulty and the cost of purification. FOS used in the present study is the reaction product resulting from 18 h of enzymatic synthesis from sucrose using FTase. The sample was analyzed for structure by recording 2D - HMQCT spectra. The chemical shift values of ^{13}C NMR spectra of FOS are shown in Table 8.b.1. Most of the assignments were based on those of Fujita *et al* (1994), Barthomeuf *et al* (1997) and Hayashi *et al* (1989, 2000). The spectra clearly showed the presence of free glucose, a small amount of free fructose, unreacted sucrose, GF_2 and GF_3. The presence of GF_2 and GF_3 were clearly detected from both ^1H proton and ^{13}C spectral correlation observed in 2D-HMQCT. In GF_2 and GF_3, the linkage between Glucose and Fructose is 1→2 and α for the 1-linked glycosidic linkage and β for the 1→2 linked glycosidic linkage of fructose. The data clearly showed that 1-kestose [O-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl –(2→1)-α-D-glucopyranoside – inulin type] is the major product detected for GF_2. In GF_3 also, the 6-linkage was not detected in any fructose unit. Compared to GF_3, the concentration of GF_2 was found to be higher correlating with the HPLC data (Chapter 3.b, Figure 3.b.4).

The NMR spectra showed less intense peaks, which merged with the noise probably indicating small amounts of GF_4.

Table 8.b.1. ^{13}C chemical shifts (ppm) for FOS produced using FTase from *A. oryzae* CFR 202 *.

	Chemical Shifts (ppm)				
Carbon atom	**Glucose**	**Fructose**	**Sucrose**	**GF_2**	**GF_3**
α-D Glucopyranoside					
C-1	96.3 (β) 92.9 (α)	64.35	92.5	92.9	92.6
C-2	72.8	------	71.4	70.1	70.0
C-3	74.3	76.2	72.8	72.8	73.0
C-4	69.6	75.3	70.0	71.9	71.8
C-5	73.0 (α) 76.3 (β)	81.0	73.2	71.4	71.5
C-6	60.8	63.7	60.6	60.6	60.6
β-D Fructofuranoside					
C-1’			62.6	61.8	61.4
C-2’			104.0	103.6	103.6
C-3’			78.0	77.2	77.3
C-4’			74.4	74.9	74.8
C-5’			81.1	81.7	81.6
C-6’			62.7	61.2	61.2
β-D Fructofuranoside					
C-1”				61.1	60.9
C-2”				103.4	103.6
C-3”				77.1	77.1
C-4”				74.3	74.5
C-5”				81.5	81.5
C-6”				62.5	61.8
β-D Fructofuranoside					
C-1”’					60.85
C-2”’					103.4
C-3”’					76.8
C-4”’					74.3
C-5”’					81.4
C-6”’					62.6

* Some of the $C_2 – C_5$ signals of fructose units mentioned are interchangeable.

The free glucose was present both in α- and β-forms as observed by the anomeric C-1 signals (96.3 ppm for β and 92.8 ppm for α). Unreacted sucrose was found to be present in substantial amount (12 % by HPLC). In GF_2 and GF_3, the anomeric glucose linkage was observed at 92.8 ppm indicating that the linkage is α. Correspondingly, the C-2’, C-2” and C-2”’ signals observed for

fructose were all observed around 103 ppm indicating β-linkage. The C-1 and C-6 signals of fructose were in the range 60-9 to 61.8 ppm. All the fructose signals in both GF_2 and GF_3 were detected by NMR. From 2D-HMQCT, a few proton signals could also be detected (Table 8.b.2). Thus, NMR analysis conclusively proved the presence of GF_2 and GF_3 in FOS prepared using FTase produced by *A. oryzae* CFR 202. Figures 8. b.1, 8.b.2, and 8.b.3 shows the ^{1}H and ^{13}C NMR spectra of standard kestose, nystose and fructofuranosyl nystose respectively. 2 DHMQCT spectra of the standards are given in Figures 8.b.4, 8.b.5 and 8.b.6. Figure 8.b.7 shows the ^{1}H and ^{13}C NMR spectra of FOS produced using FTase from *A. oryzae* CFR 202. 2 - D HMQCT spectra of the FOS mixture is given in Figure 8.b.8.

The oligomers present in FOS mixture were purified using column chromatography. The different fractions containing pure kestose, nystose and fructofuranosyl nystose were analyzed by mass spectrometry. All the masses are presented as sodium adducts. Figures 8.b.9 shows the mass spectrum where the signal of mass 527.1 corresponds to kestose and the signal of mass 365.1 corresponds to sucrose where as the minor signal of mass 203 corresponds to glucose. The mass spectrum of nystose represented in Figure 8.b.10 shows a major signal of m/z 689.1 and Figure 8.b.11 shows the mass spectrum of fructofuranosyl nystose corresponding to m/z 851.1. The actual masses of kestose, nystose and fructofuranosyl nystose were 504, 666 and 828 respectively.

Table 8.b.2. ^{1}H chemical shifts (ppm) for FOS produced using FTase from *A. oryzae* CFR 202.

	Chemical Shifts (ppm)			
	Glucose (free)	**Fructose (free)**	**GF_2**	**GF_3**
α-D Glucopyranoside				
H-1	5.24 (α) 4.65 (β)	3.72	5.43	5.41
H-2	3.78		3.49	3.42
H-3		4.23	3.75	3.72
H-4				
H-5	3.52		3.56	3.57
H-6	3.69	3.74	3.83	
β-D Fructofuranoside				
H-1’				
H-2’				
H-3’			3.48	3.43
H-4’				
H-5’			3.88	3.81
H-6’				3.86
β-D Fructofuranoside				
H-1”				3.76
H-2”				
H-3”			4.2	4.21
H-4”			4.05	
H-5”				
H-6”				
β-D Fructofuranoside				
H-1”’				
H-2”’				
H-3”’				
H-4”’				
H-5”’				
H-6”’				3.9

Figure 8.b.1. (a) ^{1}H and (b) ^{13}C NMR spectra of standard 1-kestose recorded in D_2O

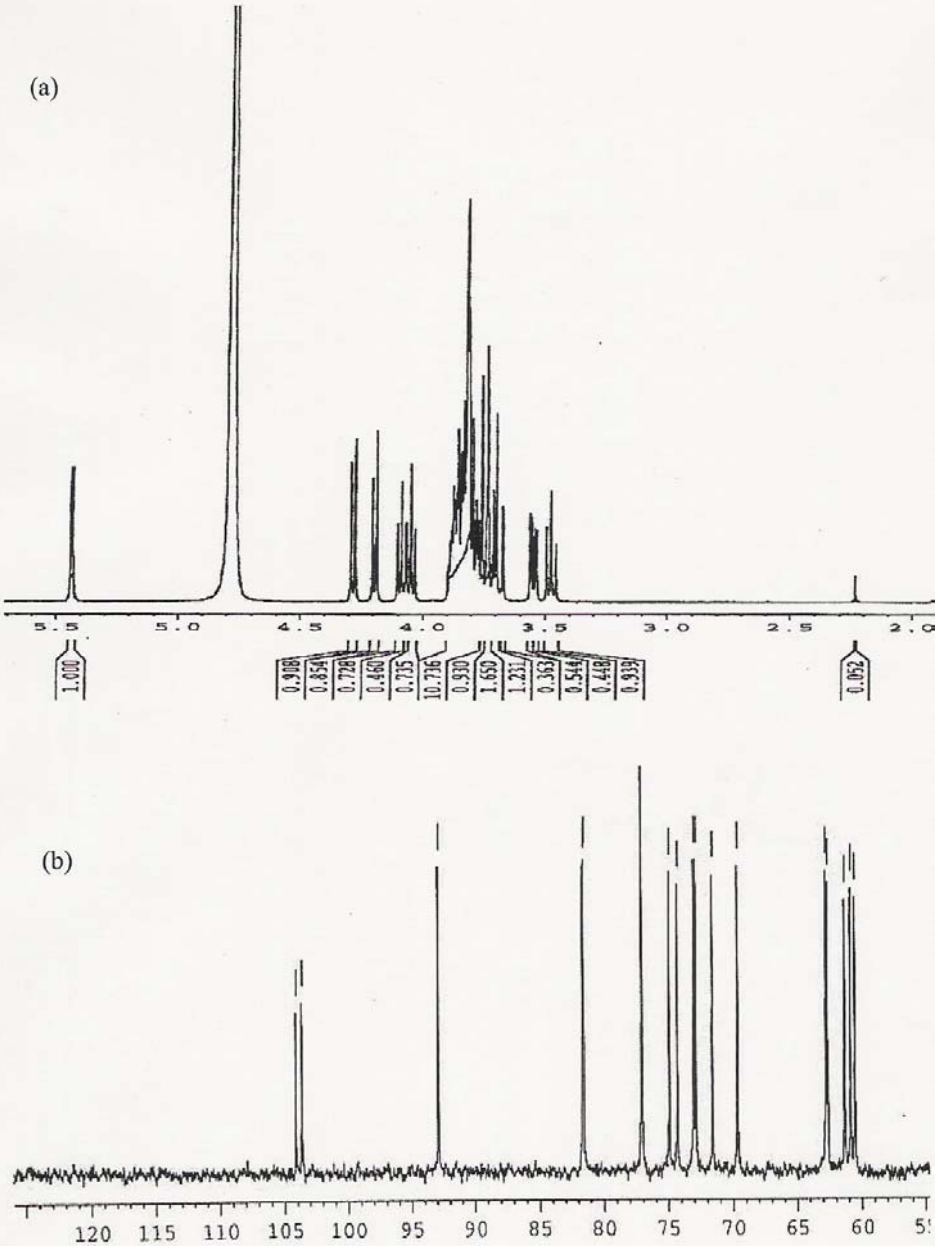


Figure 8.b.2. (a) ^{1}H and (b) ^{13}C NMR spectra of standard nystose recorded in D_2O

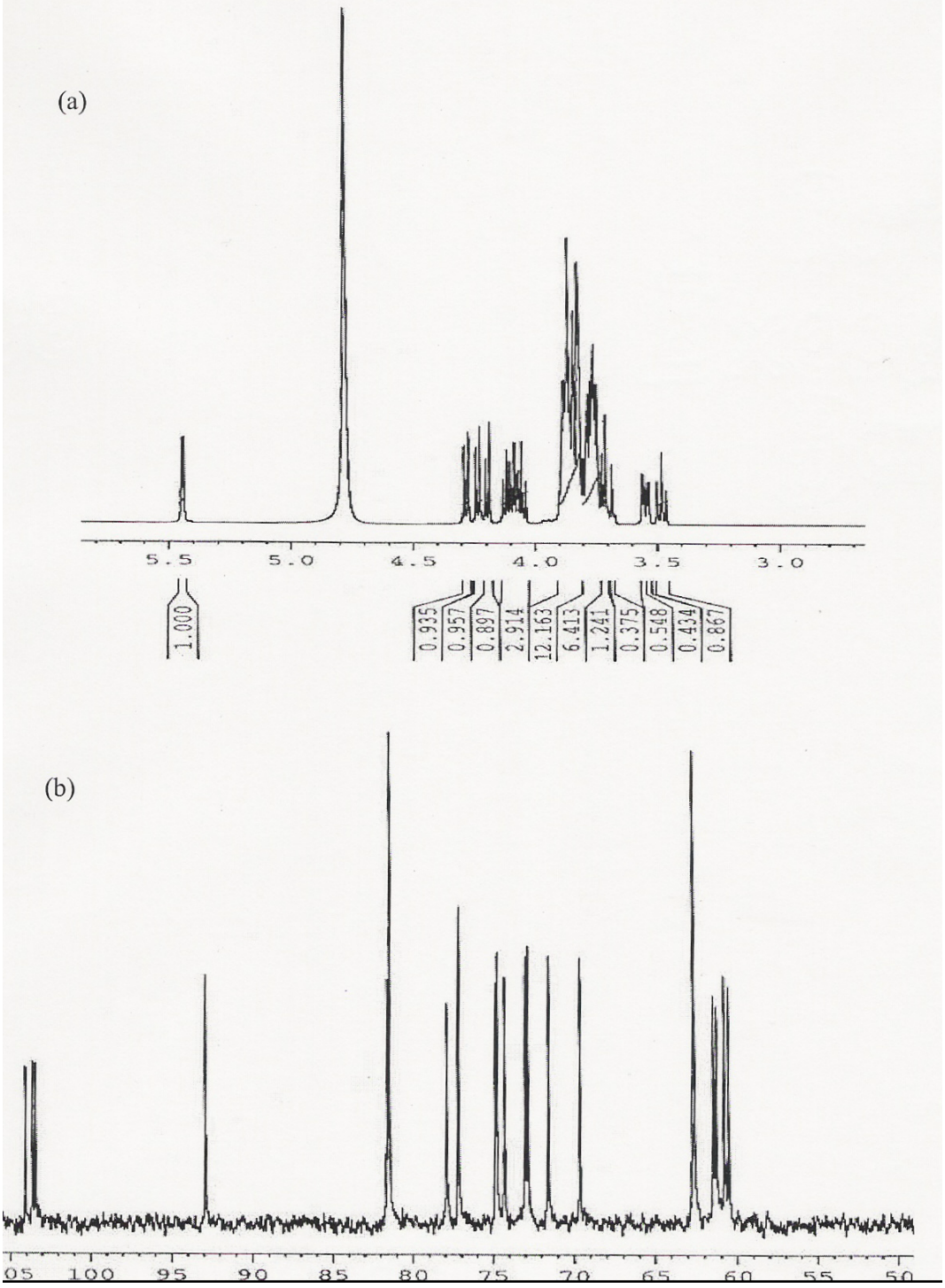


Figure 8.b.3. (a) ^{1}H and (b) ^{13}C NMR spectra of standard fructofuranosyl nystose recorded in D

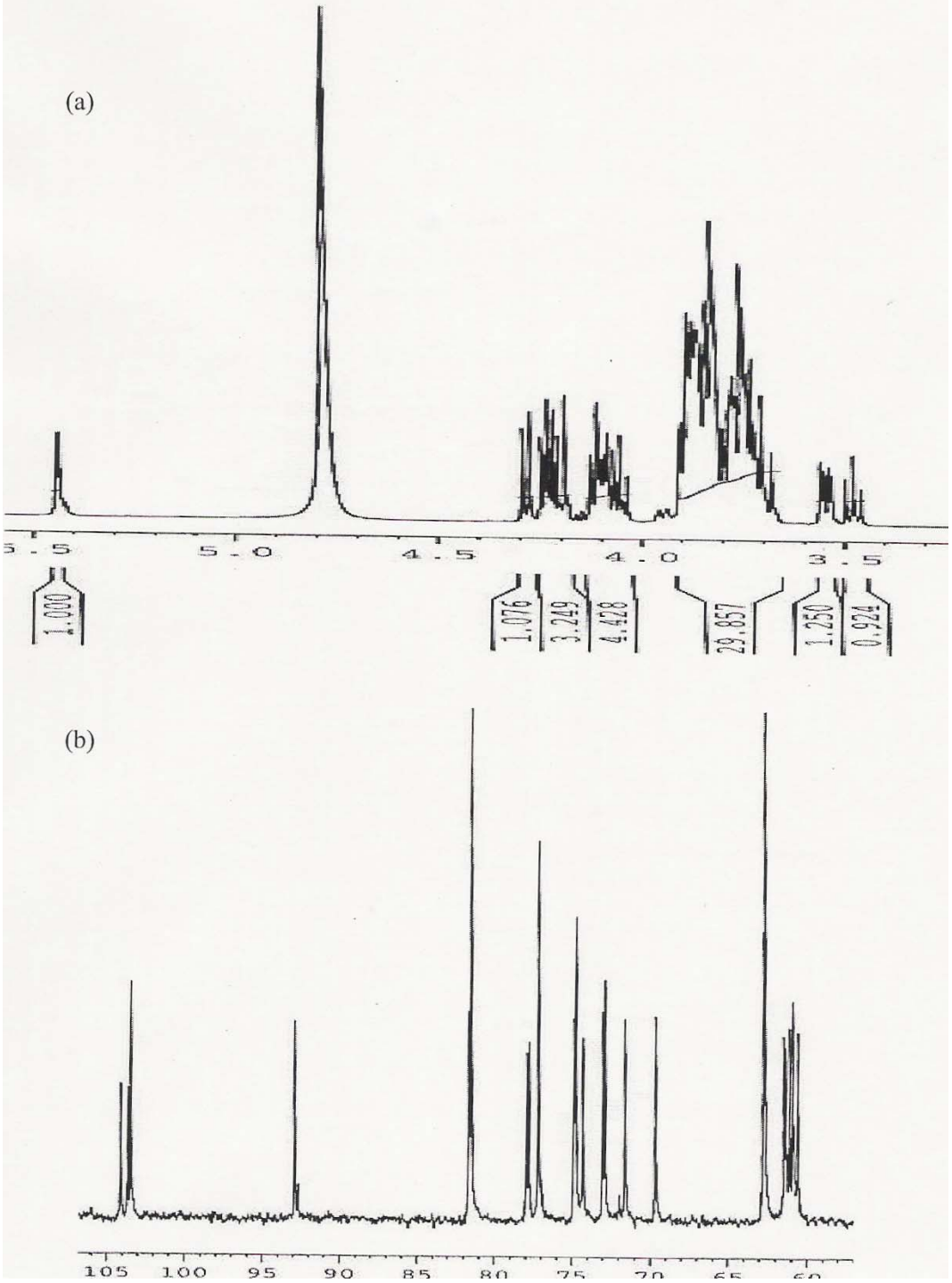


Figure 8.b.4. 2D HMQCT spectrum of standard 1-kestose recorded in D_2O

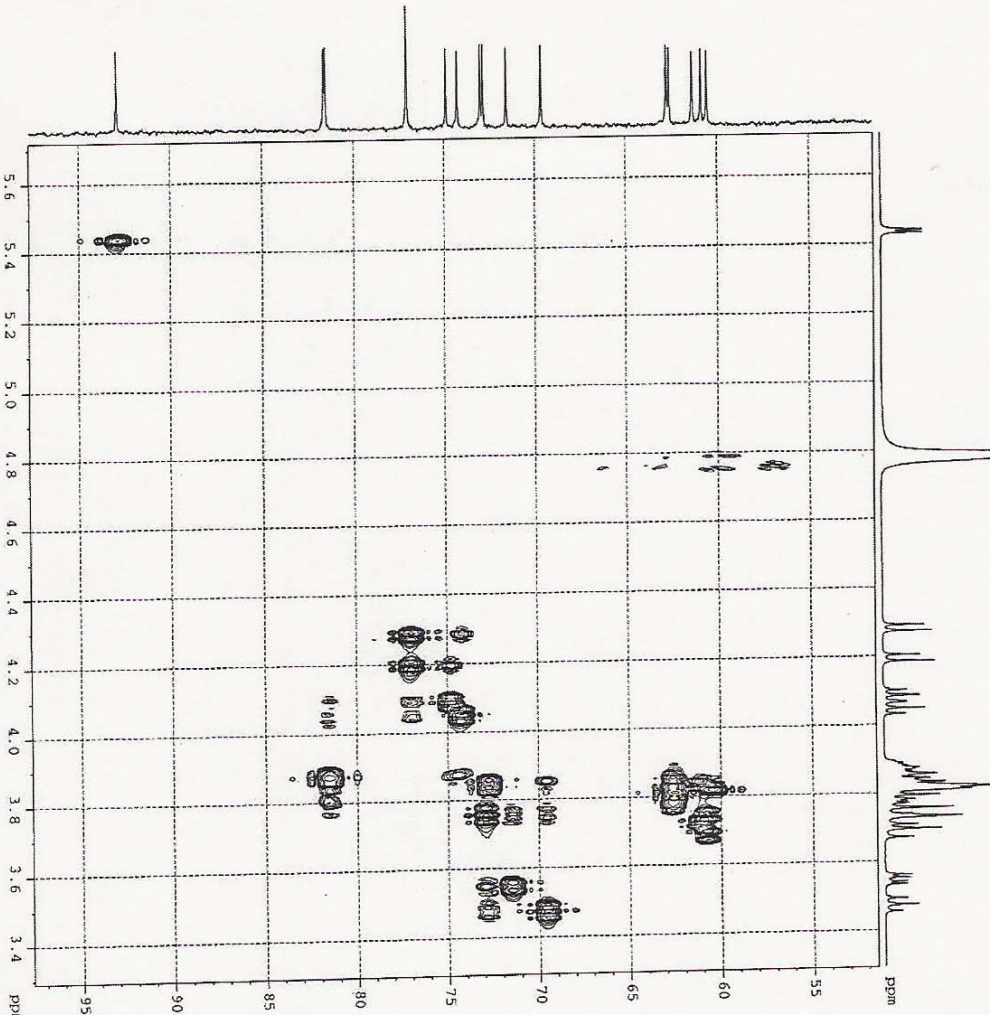


Figure 8.b.5. 2D-HMQCT spectrum of standard nystose recorded in D_2O

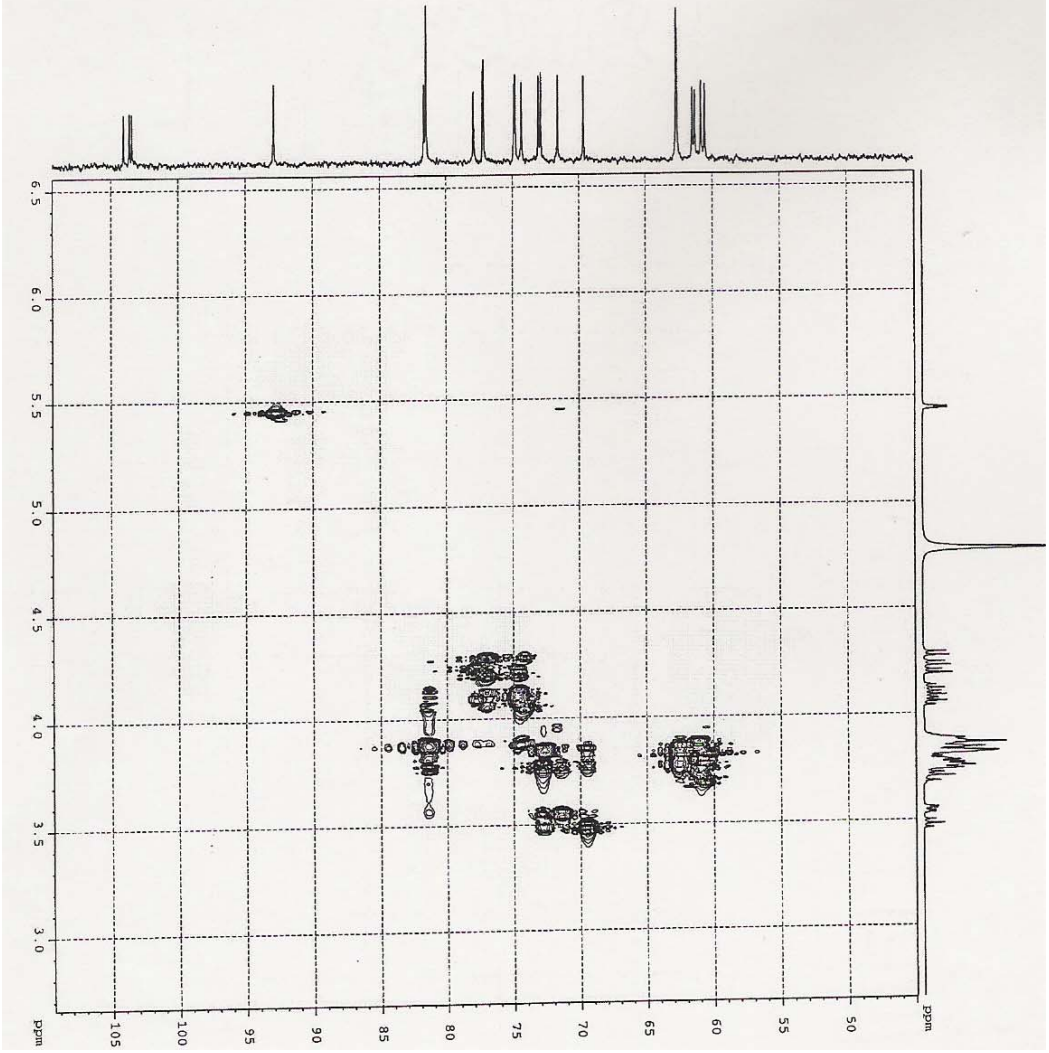


Figure 8.b.6. 2D-HMQCT spectrum of standard fructofuranosyl nystose recorded in D

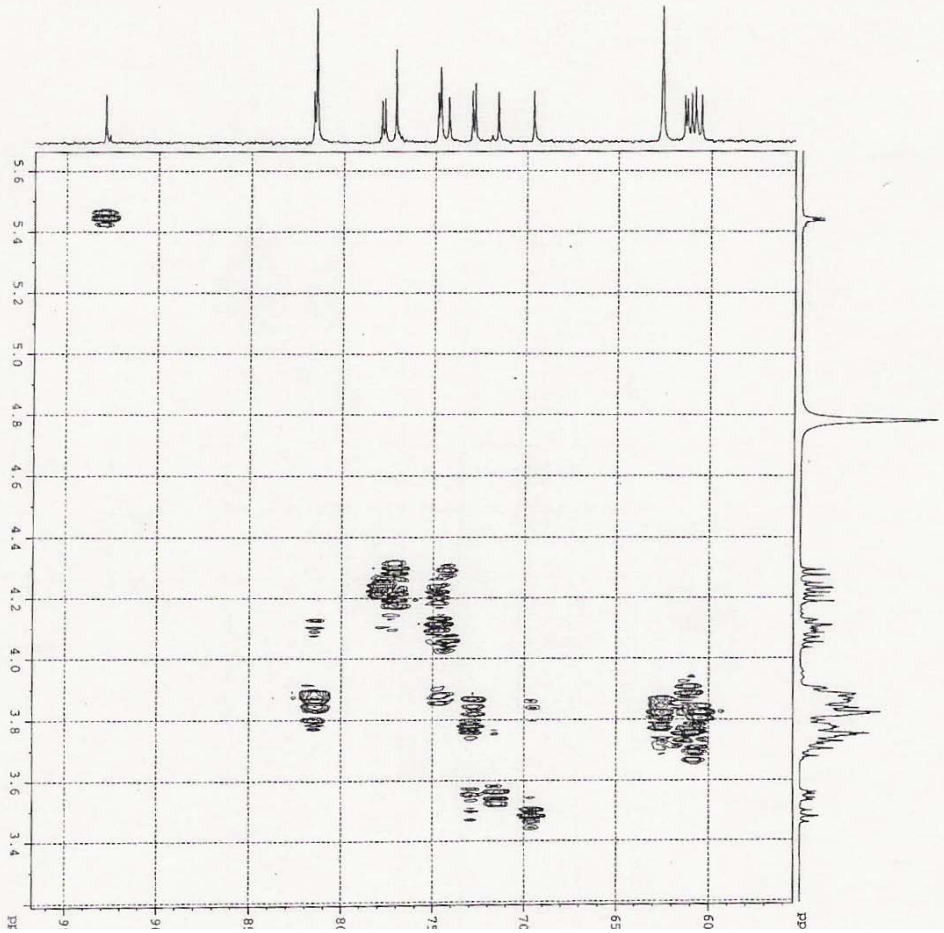


Figure 8.b.7. (a) ^{1}H (b) ^{13}C NMR spectra of FOS (in D_2O) produced using FTase from *A.oryzae* CFR 202

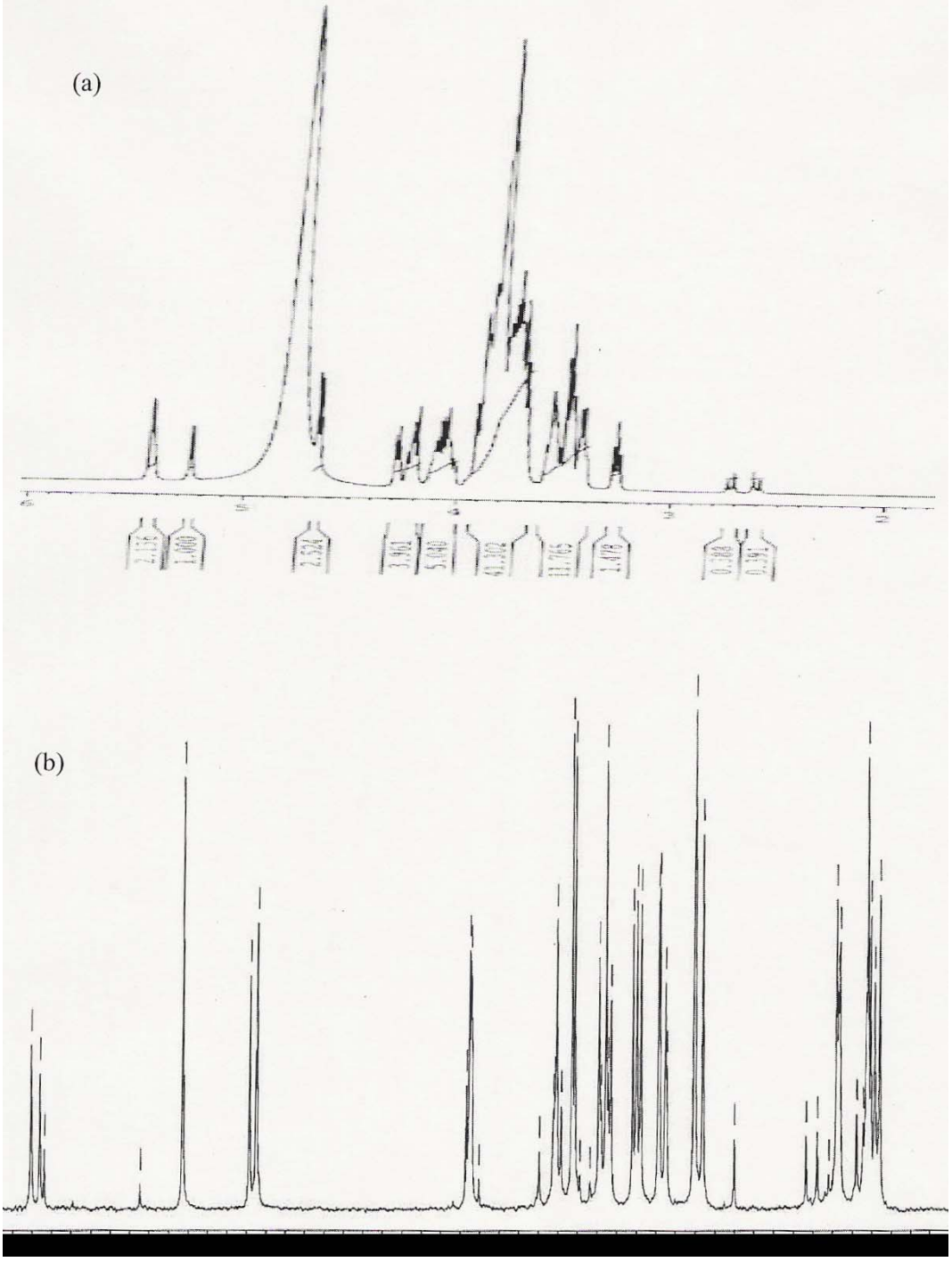


Figure 8.b.8. 2D-HMQCT spectrum of FOS (in D_2O) produced using FTase from *A.oryzae* CFR 202

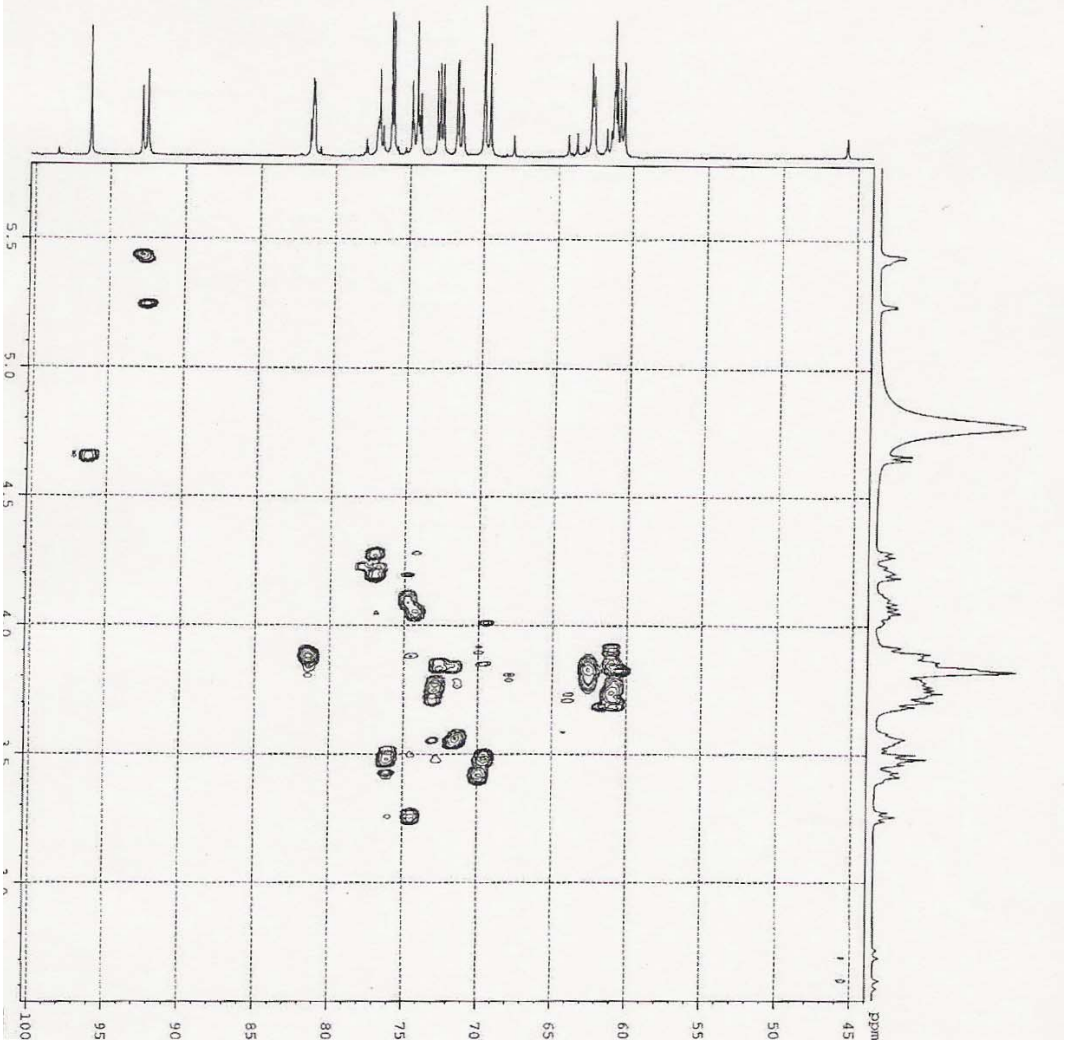


Figure 8.b.9. HPLC elution profile of purified 1-kestose.
Inset - Mass spectrum of 1-kestose

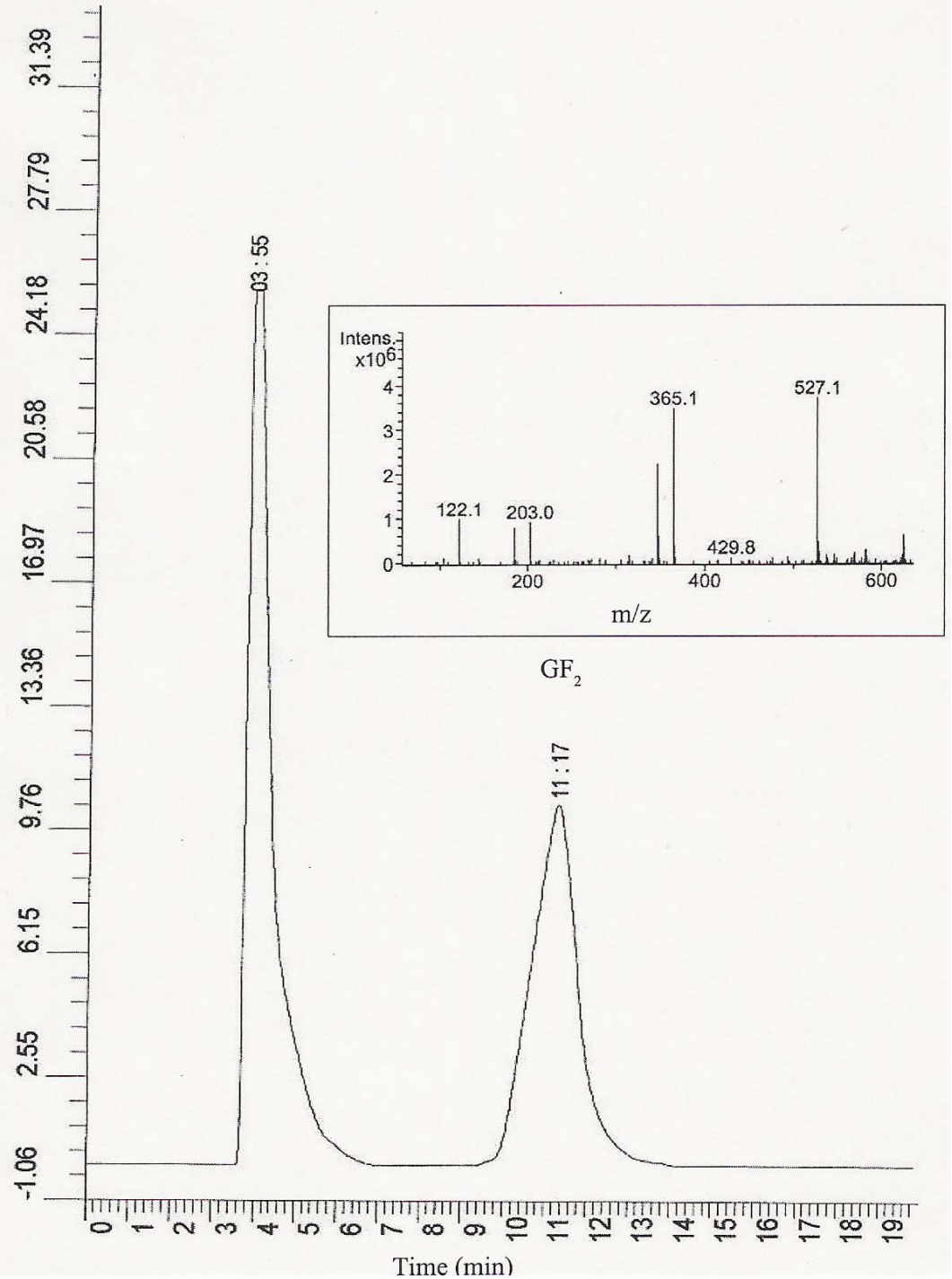


Figure 8.b.10. HPLC elution profile of purified nystose.
Inset - Mass spectrum of nystose

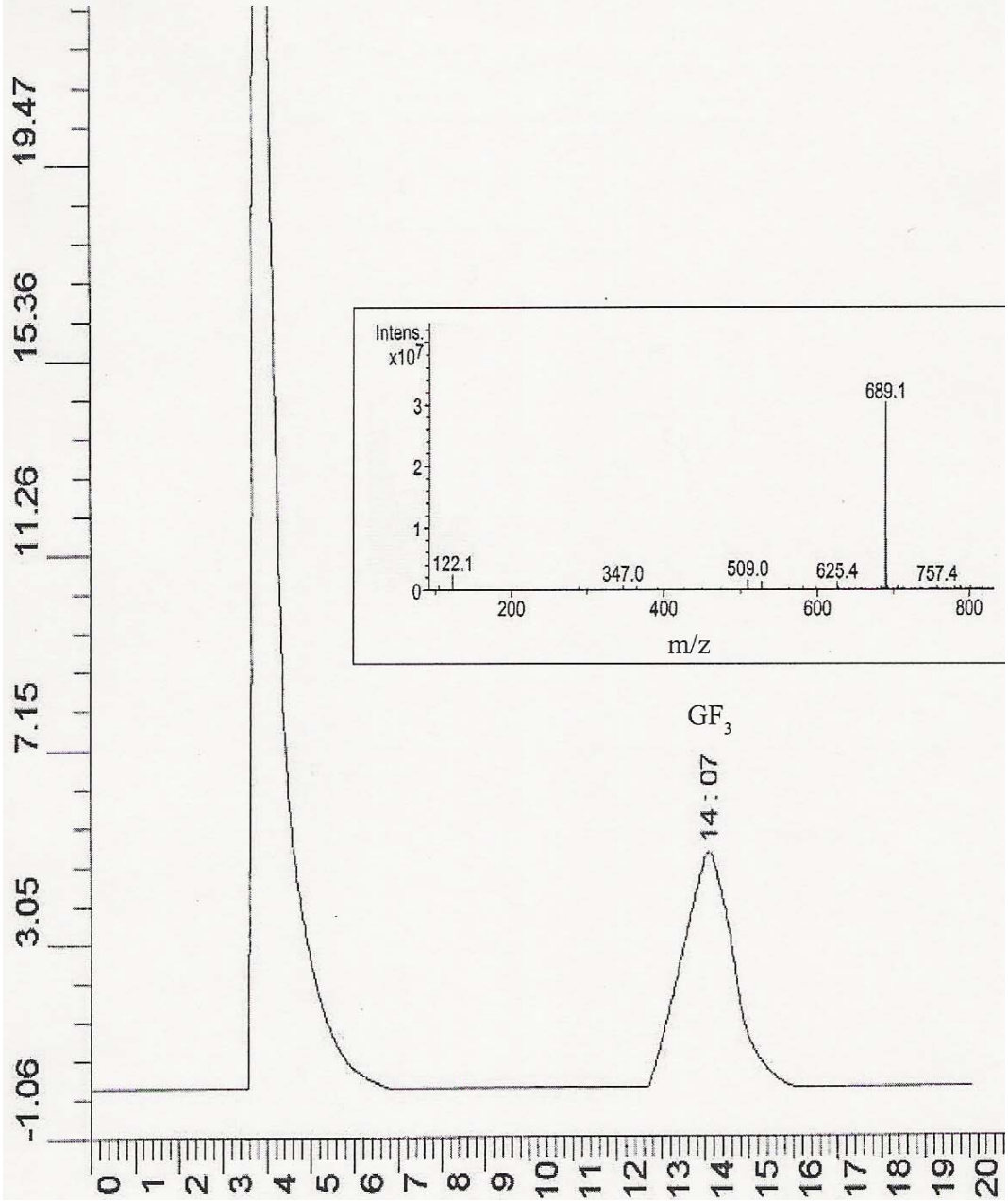
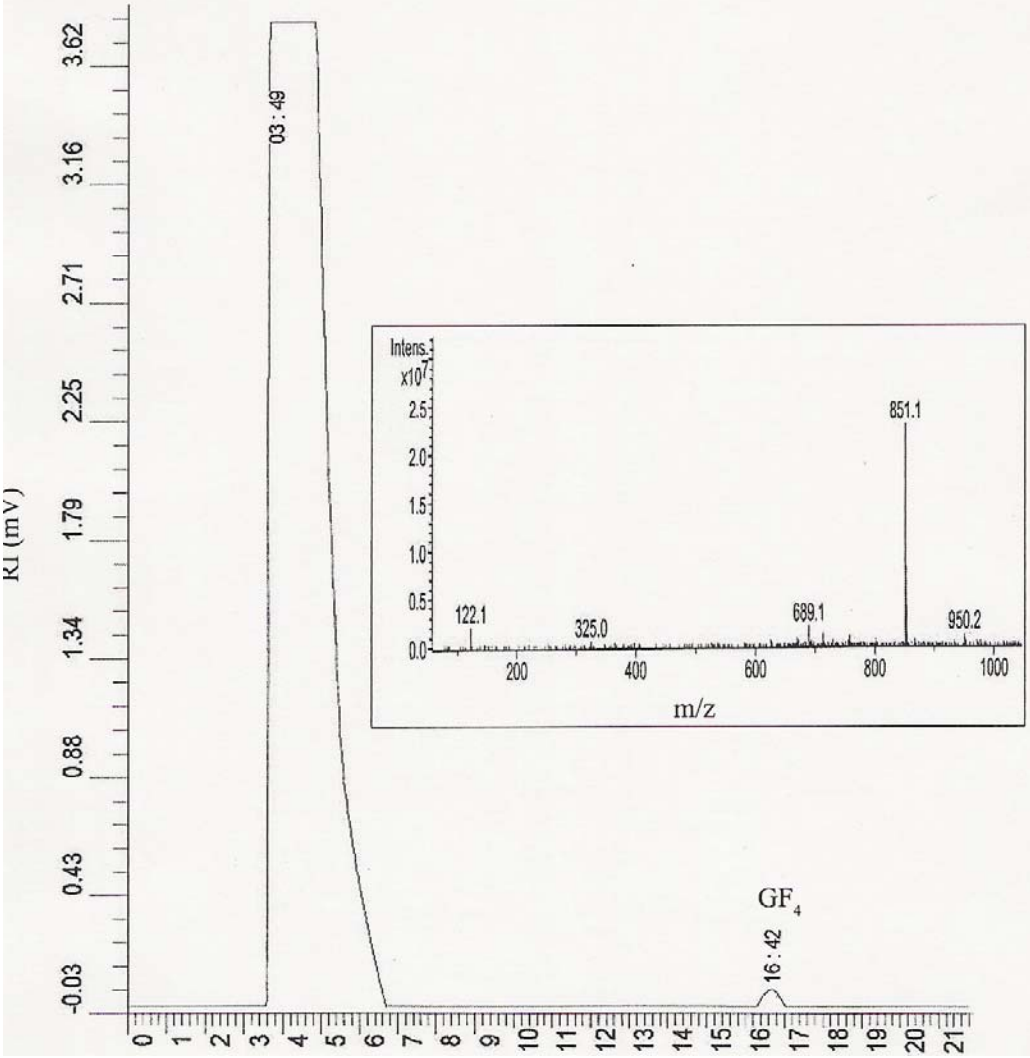


Figure 8.b.11. HPLC elution profile of purified fructofuranosyl nystose
Inset - Mass spectrum of fructofuranosyl nystose



SUMMARY

9. SUMMARY

There is a strong relationship between our health and the food we eat. The design of food products that confer a health benefit is a relatively new trend, and recognizes the growing acceptance of the role of diet in disease prevention, treatment and well being. Functional foods, designer foods, pharma foods and nutraceuticals are synonyms for foods that can prevent and treat diseases. Functional foods in addition to their basic nutritional content and natural being, will contain the proper balance of ingredients which will help to improve many aspects of human lives, including the prevention and treatment of illness and disease. Oligosaccharides are very well recognized as 'functional food ingredients' because of their positive effects on human health. Of all the oligosaccharides known so far, Fructooligosaccharides (FOS) have attracted special attention. FOS is a common name for fructose oligomers that are mainly composed of 1- Kestose (GF_2), 1 - Nystose (GF_3) and 1^F – Fructofuranosyl nystose (GF_4) in which fructosyl units (F) are bound at the β - 2, 1 position of sucrose (GF). FOS can be produced by the transfructosylation activity of enzymes designated as Fructosyl Transferases (FTase) (EC. 2. 4. 1. 9) present in plants and microorganisms. The yield of FOS produced using enzymes originated from plants is low and mass production of enzyme is limited by seasonal conditions. Therefore, industrial production depends chiefly on microbial enzymes. The present investigation therefore focuses on the various aspects of FOS production using microbial FTase with special emphasis on the selected stain – *Aspergillus oryzae* CFR 202.

A brief outline of the investigation is given below.

Chapter 1 gives an introduction on FOS – its occurrence, structure, properties, mode of preparation, functional properties, application and market demand.

Chapter 2 deals with the literature survey relating to recent developments in the area of microbial production of fructooligosaccharides.

Chapter 3 comprises of the screening and selection of microorganisms capable of FTase production by Submerged Fermentation and FOS production

using FTase from *A. oryzae* CFR 202 and *A. pullulans* CFR 77, selection and optimization of significant nutritional and cultural parameters involved in FTase and FOS production, recycling cell culture for FTase production by the selected microorganism (*A. oryzae* CFR 202) under the optimized conditions and the use of alternate substrates for FTase and FOS production.

Chapter 4 gives detailed account of the purification and characterization of FTase produced by *A. oryzae* CFR 202 by Submerged Fermentation.

Chapter 5 presents the results on the use of different agro - industrial byproducts for FTase production by *A. oryzae* CFR 202 under Solid State Fermentation and characterization of the crude enzyme produced thereby.

Chapter 6 deals with *in vitro* studies on prebiotic effects of FOS.

Chapter 7 deals with scale up studies for FTase production in 3 L and 15 L fermentors for FTase production by *A. oryzae* CFR 202 and 10 L level for FOS production. The chapter also discusses the preparation of FTase and FOS powder by spray drying.

Chapter 8 focuses on the applicability of FOS in preparing various food products like spread, honey and beverages, studies on the physicochemical properties of FOS, its purification by column chromatography and analysis by various methods like HPLC, LC - MS and NMR.

Chapter 9 gives the summary of the present research work and the recommendations for further work.

A few fungal strains were screened for FTase activity and *Aureobasidium pullulans* CFR 77, *Aspergillus oryzae* CFR 202 and *Aspergillus flavus* CFR 203 were found to be with maximum titres of FTase activity. Based on high U_t/U_h ratio, which is important for good transfructosylation activity, *A. oryzae* CFR 202 was selected as the best source for further studies. *A. niger*, *Penicillium* sp. and *M. miehei* exhibited comparatively less FTase activity and

hence are not suitable for economical FOS production. Studies were carried out using culture fluid, cells and culture broth homogenate of *A. oryzae* CFR 202 and *A. pullulans* CFR 77 as source of FTase using 55 and 80 % sucrose as substrate.

Selection of important parameters influencing FTase and FOS production was done using Plackett Burman design by carrying out a set of 20 experiments, firstly for model development and another 20 experiments for validation of the results. The study brought out the important parameters influencing the production of FTase as well as FOS with the help of a single experimental design. KH_2PO_4 and sucrose in the fermentation medium and fermentation time influenced FTase production. In order to get higher titres of FTase, it was necessary to carry out fermentation beyond 96 h. FTase activity was found to be higher when the concentration of sucrose was low in the media. KH_2PO_4 concentration in the media was found to be more (0.5 – 0.6 %) in those trials which gave higher FOS yields. pH of the reaction mixture and reaction time were found to have significant effect on FOS production. FOS production was more when the pH of the reaction mixture was in the range 5.5 – 6.0. It was also observed that FOS yield increased with the increase in reaction time. All the five above mentioned variables with t-values above 0.95 were selected for further optimization by response surface methodology.

The RSM methodology, based on Doehlert experimental design consisted of 34 experiments with five variables, four repetitions at the centre point. The results showed the effects and interactions of different parameters on FOS yields by FTase grown under submerged fermentation. A response equation has been obtained for the FOS yields. From this equation, it is possible to predict the operation conditions required to obtain higher yields of FOS. It was found that the most effective parameters were fermentation time, reaction time and pH of the reaction mixture. These factors have a positive influence on FOS yields. Due to the disproportionate nature of reaction exhibited by FTase, the reaction time plays an important role in FOS production. Among the interactions, fermentation time – reaction time interactions are significant ($p < 0.01$) and they have a negative influence on

FOS production. According to these results, it was found that the maximum predicted FOS yield of 58.7 % (w/w) by the equation agrees well with the experimental value of 57.37 ± 0.9 % (w/w) FOS. This indicates that the generated model adequately predicted the FOS yields.

An efficient cell recycling system was developed for the continuous production of FTase by successfully reusing the pellets of *A. oryzae* CFR 202 over a considerable period. During recycling, FTase activity was 7.8 $UmL^{-1}min^{-1}$ at the end of 48 h of fermentation. It reached a maximum value of 16. 5 U $mL^{-1}min^{-1}$ after the next two consecutive cycles and then decreased to 11. 9 U $mL^{-1}min^{-1}$ at the end of the sixth recycle. However, FOS yield was maximum at the end of the third recycle (53.2 ± 0.2 %) and remained constant even at the end of the sixth recycle. The pellets could not be recycled further since it lost the compactness and started disintegrating after the sixth recycle. However, if cell integrity can be maintained, further recycle is possible.

Alternate sources of sucrose like jaggery and sugarcane juice were used for FTase and FOS production. Using jaggery as carbon source for FTase production and substrate for FOS production, it was possible to obtain 40 % FOS. When the same FTase was used for FOS production with sucrose as substrate, it was possible to obtain 48 % FOS. When sucrose was used as carbon source for FTase production and jaggery was used as substrate for FOS production, the FOS yield was 42 %. Using sugarcane juice as substrate for FOS production, it was possible to obtain 24 – 27 % FOS. This has resulted in value addition of jaggery and sugarcane juice to produce high value product like FOS.

Purification of FTase resulted in understanding and characterizing FTase produced by *A. oryzae* CFR and obtaining the product (FOS) rapidly. Efforts have been made to purify the FTase enzyme produced by *A. oryzae* CFR 202 to get high specific activity and fold of purification. Theoretically, purified enzyme should result in higher yields of the product. However, the peculiar reaction mechanism involved in FOS production does not lead to

higher yields than the theoretical maximum of 56 – 58 % due to inhibition caused by accumulation of glucose. Nevertheless, the time involved in obtaining maximum FOS yield has been considerably reduced by using the purified FTase in comparison to the crude FTase. The enzyme was characterized as a moderately large molecule of 97 kDa molecular weight that is stable upto 40 °C and at pH 4.0 to 6.0. The optimum pH and temperature of this enzyme are 7.0 and 60 °C respectively, but it was not affected much by metal ions. Eventhough purification of FTase considerably reduced the reaction time to get maximum FOS yields, for mass production of FOS, it is economical to use crude FTase.

There have been many reports on the production of FTase by submerged fermentation, but there are no reports on the use of SSF for FTase production except for the use of apple pomace as substrate for FTase production. The present investigation has focussed on the use of a variety of agricultural by – products as substrates for the production of FTase by *A. oryzae* CFR 202. A variety of substrates like cereal bran to corn products, coffee and tea processing by - products, sugarcane bagasse and cassava fibrous residue (tippi) have been used in the present study. All the substrates except sugarcane bagasse and tippi supported good growth of *A. oryzae* CFR 202 and concomitant production of FTase. Maximum activity was found to be present in FTase obtained from SSF of rice bran, wheat bran and corn germ. Even those substrates, which did not favour production of FTase, when supplemented with complex nitrogen source like yeast extract, supported good growth of *A. oryzae* CFR 202 and production of FTase. Addition of rice bran in the proportion of 30 % of the substrate resulted in interparticle spacing with a possible increase in mass transfer and oxygen transfer. This contributes to the value addition of those by – products, which are otherwise disposed adding to environmental pollution. Characterization of FTase produced by SSF has shown that it is more stable compared to that produced by SmF. It was also capable of producing the maximum yield of FOS (52 %) within 8 h of reaction, unlike the enzyme produced by SmF, which requires 18 h for producing maximum FOS. The present investigation has thus shown that SSF opens a

new avenue for the production of FTase and subsequently FOS using agricultural by - products.

The prebiotic effect of FOS was studied by growing Lactobacilli strains in media containing FOS. All the three strains grew well in FOS containing media and produced short chain fatty acids like lactate, acetate, propionate and butyrate thereby reducing the pH of the media. This promotes the use of FOS as an ingredient in foods aimed at maintaining gut health.

FTase production was successfully scaled up to 15 L fermentor level using the optimized conditions at shake flask level. Fermentation time was reduced to 60 h compared to 90 h in shake flask studies. FOS yield of 53 % could be obtained using this FTase. FOS production was also scaled up to 10 L level using a specially designed reactor. FTase powder was prepared by spray drying liquid FTase with various concentrations of maltodextrin (5, 15 and 25 % maltodextrin). Storage studies showed that there was no loss of activity even after 15 days at room temperature. FOS powder was also prepared by spray drying with additives like 10 % maltodextrin and 1 % Tricalcium phosphate. Characterization of both the powders were carried out and microbial analysis showed that in both the samples microbial count was well within safety limits.

Various products like spread, honey like product and beverage concentrate were prepared using FOS. Characterization of the products was done. Honey like product had several advantages over commercial honey like low reducing sugar content, which finds application in formulating foods aimed at diabetics. The product when subjected to sensory evaluation was rated between processed and unprocessed commercial honey samples. Beverages prepared with FOS had approximately 4 g / 200 mL bottles, which is close to the RDA of FOS – 5 g / day.

FOS consisting of a mixture of oligomers was purified by Biogel P-2 column chromatography to get pure oligomers – kestose, nystose and fructofuranosyl nystose. NMR and Mass spectra confirmed the structure and molecular weight of the oligomers.

Recommendations for future work

1. Work may be carried out to improve the yield of FOS - 56 %.
2. Scope exists for the detailed study of FTase enzyme.
3. Efforts may be made to remove glucose and sucrose from the FOS mixture to get high content FOS.
4. Animal studies can be carried out to confirm the health effects of FOS.

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List of publications

Published

1. Sangeetha PT, Ramesh MN and Prapulla SG. Influence of media components and reaction parameters on the production of fructosyl transferase and fructooligosaccharides. *Sciences Des Aliments*, **22**, 2002. 277-287.

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1. Sangeetha PT, Ramesh MN and Prapulla SG. Microbial production of fructooligosacharides. *Asian Journal of Microbiology, Biotechnology and Environmental Sciences* (2003).

2. Sangeetha PT, Ramesh MN and Prapulla SG. Production of fructooligosaccharides by fructosyl transferase from A*spergillus oryzae* CFR 202 and *Aureobasidium pullulans* CFR 77. *Process Biochemistry* (2003)

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1. Sangeetha PT, Ramesh MN and Prapulla SG. Production of fructosyl transferase by *Aspergillus oryzae* CFR 202 in solid state fermentation using agricultural by-products. *Applied Microbiology and Biotechnology*.

2. Sangeetha PT, Ramesh MN and Prapulla SG. Fructooligosaccharide production using fructosyl transferase obtained from recycling culture of *Aspergillus oryzae* CFR 202. *Process Biochemistry*.

To be communicated

1. Sangeetha PT, Ramesh MN and Prapulla SG. Maximization of fructooligosaccharide production by two stage continuous process and its scale up.

Under preparation

1. Sangeetha PT and Prapulla SG. Purification and characterization of fructosyl transferase from *Aspergillus oryzae* CFR 202.

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3. Sangeetha PT, Ramesh MN and Prapulla SG. Honey - like product by fermentation.

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6. Sangeetha PT and Prapulla SG. Effect of fructooligosaccharide on the growth of lactic acid bacteria.

7. Sangeetha PT, Ramesh MN, Ramalakshmi K and Prapulla SG. Production of nutraceutical beverage by fermentation

List of patents

Filed:

1. Prapulla SG, Sangeetha PT and Ramesh MN. An improved process for the production of Fructooligosaccharides (411/DEL/2001)

2. Prapulla SG, Sangeetha PT and Ramesh MN. A process for the production of Fructooligosaccharides (439/DEL/2001)

3. Prapulla SG, Sangeetha PT and Ramesh MN. A process for the production of Fructooligosaccharides using corn products (66/DEL/2002)

4. Prapulla SG, Sangeetha PT and Ramesh MN. A process for the production of Fructooligosaccharides using cereal bran (163/DEL/2002)

5. Prapulla SG, Sangeetha PT and Ramesh MN. A process for the preparation of prebiotic bread spread (344/DEL/2002).

6. Sangeetha PT, Ramesh MN and Prapulla SG. A process for the production of fructooligosaccharides using jaggery (87 / DEL/ 2003)

7. Sangeetha PT, Ramesh MN and Prapulla SG. A process for the utilization of coffee and tea processing by-products for the production of fructooligosaccharides(FOS) (NF 598 / 2002)

8. Sangeetha PT, Ramesh MN and Prapulla SG. A process for the preparation of honey like product (92 / DEL/ 2003)

Submitted to CSIR:

1. Sangeetha PT, Ramesh MN and Prapulla SG. A process for the production of Fructooligosaccharides using Fructosyl Transferase obtained from recycling culture of *Aspergillus oryzae* CFR 202.

2. Ramesh MN, Shivakumara M, Sangeetha PT and Prapulla SG. Fructooligosaccharide powder

3. Ramesh MN, Ramalakshmi K, Sangeetha PT and Prapulla SG. Beverage concentrate based on fructooligosaccharides

Submitted to Patent Cell, CFTRI

1. Ramesh MN, Shivakumara M, Sangeetha PT and Prapulla SG. Fructosyl Transferase enzyme powder