PRODUCTION OF FRUCTOSYLTRANSFERASE AND FRUCTOOLIGOSACCHARIDES BY *PENICILLIUM SIMPLICISSIMUM*

NORAZIAH ABU YAZID

UNIVERSITI SAINS MALAYSIA

2010

PRODUCTION OF FRUCTOSYLTRANSFERASE AND FRUCTOOLIGOSACCHARIDES BY *PENICILLIUM SIMPLICISSIMUM*

by

NORAZIAH ABU YAZID

Thesis submitted in fulfillment of the
requirements for the degree
of Master of Science

MAY 2010

ACKNOWLEDGEMENTS

In the name of Allah, the Most Beneficient and the Most Merciful. All praises to Allah the Almighty for giving me the strength, guidance and patience in completing this thesis.

My great appreciation goes to my supervisor and co-supervisor, Assc. Prof. Dr. Mashitah Mat Don, Professor Subhash Bhatia, and Assc. Prof. Shukri for their intellectual support, encouragement, and enthusiasm throughout my study.

My special acknowledgement goes to the Dean of Chemical Engineering School, Professor Abdul Latif Ahmad for his grateful support towards my postgraduate affairs. I would like to express my appreciation to administrative and technical staff for their valuable help.

The scholarship National Science Fund (NSF) from Ministry of Science, Technology and Innovation (MOSTI) throughout my study is gratefully acknowledged.

I would like to express my deepest gratitude to all my friends especially master and PhD student of Chemical Engineering school for their kindness and moral support during my research work. Thank you for the friendship and memories.

I feel indebted to my best friend, Tijah for her help in many ways and bittersweet memories during my study in USM. I also wish to thank Kak Min, who is always by my side, for her warm support and understanding.

Special thank goes to my family for their love and support. Without their love, I would not be able to deal with ups and downs during my study. Finally, my heartfelt gratitude goes to my beloved parent, Abu Yazid Mohd Jani and Rokiah Mohd Daud, for their love and inspiration. My graduation could have been not been possible without their warm support and understanding.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

3D	Three dimensional
ANOVA	Analysis of variance
CCD	Central composite design
CSTB	Conventional stirred tank bioreactor
De	Oxygen diffusivity
DEQ	Differential equation
DNS	Dinitrosalicylic
DO	Dissolved oxygen
DOE	Design of experiment
dof	Degree of freedom
DOT	Dissolved oxygen tension
DP	Degree of polymerization
Eq	Equation
FOS	Fructooligosaccharide
FTase	Fructosyltransferase
G	Glucose
GC-MS	Gas chromatography mass spectrometry
GF	Sucrose
GF_2	1-kestose
GF_3	Nystose
GF_4	1-fructofuranosylnystose
GI	Gastrointestinal
GRAS	Generally recognized as safe

h	Hour
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detector
HPLC	High performance liquid chromatography
KOH	Potassium hydroxide
L	Liter
LDL	Low density lipoprotein
L-M	Levenberg-Marquadrat
$MgSO_4$	Magnesium sulfate
mL	Mililiter
min	Minute
MSE	Means squared error
Na_2SO_4	Sodium sulfate
$NaBH_4$	Sodium borohydride
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
ODE	Ordinary differential equation
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
PDA	Potato dextrose agar
PID	Proportional integral derivatives
pO_2	Partial dissolved oxygen

RFBB	Rotating fibrous bed bioreactor
RID	Refractive index detector
RKF45	Runge-Kutta Fehlberg
RMSE	Square roots of the means square error
rpm	Revolutions per minute
RSM	Response surface methodology
SEM	Scanning electron microscopy
TLC	Thin layer chromatography
vvm	Volume per volume per minute

LIST OF SYMBOLS

a	Penetration depth	mm
A	Surface area of biofilm	m^2
α	Coefficient of growth	g/g
β	Coefficient of non-growth	g/g·h
C_{AL1}	Concentration of dissolved oxygen in liquid Bulk	mg/L
C_La	Bulk oxygen concentration in compartment (a)	mg/L
C_Lb	Bulk oxygen concentration in compartment (b)	mg/L
D_e	Effective diffusivity	m^2/s
δ	Biofilm thickness	mm
k_a	Adsorption rate constant	L/g·h
k_d	Desorption rate constant	1/h
k_d'	Desorption rate constant and mass transfer constant	1/h
k_m	External mass transfer rate constant	1/h
K_I	Inhibition constant	dimensionless
k_La	Volumetric mass transfer coefficient	1/min
K_P	Saturation constant	dimensionless
K_S	Product inhibition constant	mg/L
k_0	Volumetric reaction rate of the solute inside the biofilm	mg/L·h
m_S	Maintenance coefficient	g/g·h
μ_0	Specific growth rate	1/h
μ_{max}	Maximum specific growth rate	1/h
η_t	Length of actual data period	dimensionless
P	Product concentration	g/L
P_m	Maximum FOS production	g/L
P_0	Initial product concentration	g/L
q_p	Specific rate of product formation	1/h
Q_P	Volumetric rate of FOS	g/L·h
q_s	Specific rate of substrate utilization	1/h

R^2	Coefficient of determination	dimensionless
Re	Reynolds number	dimensionless
S	Substrate concentration	g/L
Sc	Schmidt number	dimensionless
Sh	Sherwood number	dimensionless
S_0	Solute concentration at the biofilm-bulk fluid interface	mg/L
t	Time	h
t_d	Doubling time	h
v	Oxygen uptake rate	mg/L·min
V	Volume	L
σ	Variance	dimensionless
V_{imb}	Wet biofilm volume	cm^3
x_1	Rate of aeration	vvm
x_2	Agitation speed	rpm
x_3	Initial sugarcane juice concentration	g/L
X	Cell biomass concentration	g/L
ΔX	Step change	dimensionless
X_0	Initial biomass concentration	g/L
X_f	Final cell biomass	g/L
X_m	Maximum biomass	g/L
X_S	Cell biomass on the surface of biofilm	g/L
Y_B	Response of growth yield	g/L
Y_E	Response of FOS yield using extracellular enzyme source	g/g
y_{exp}	Experimental data	dimensionless
Y_I	Response of FOS yield using intracellular enzyme source	g/g
y_i	Model data	dimensionless
$Y_{P/S}$	Product yield per sucrose consumed	g/g
$Y_{P/X}$	Product yield per biomass produced	g/g
$Y_{X/S}$	Biomass yield per sucrose consumed	g/g
$Y'_{X/S}$	Actual biomass yield per sucrose consumed	g/g

PENGHASILAN FRUKTOSILTRANSFERASE DAN FRUKTOOLIGOSAKARIDA OLEH *PENICILLIUM SIMPLICISSIMUM*

ABSTRAK

Penghasilan fruktooligosakarida (FOS) sebagai pemanis alternatif daripada sumber asli telah mendapat tumpuan, dan sukrosa adalah merupakan sumber karbon yang biasa untuk penghasilannya. Untuk meminimumkan kos pengeluaran, kebolehsanaan penggunaan substratum kos rendah yang diperolehi daripada sumber pertanian tempatan seperti air tebu telah dipilih untuk menggantikan sukrosa asli. Beberapa kulat telah diuji kemampuan mereka menghasilkan fruktosiltransferase (FTase) and FOS. *Penicillium simplicissimum* telah didapati mempunyai kemampuan yang tinggi untuk menghasilkan FTase dan FOS dengan aktiviti enzim FTase yang tertinggi pada 5.54 X 10^2 IU/ml dan hasilan FOS per sukrosa gunaan pada 0.39 g/g, masing-masing.

Penapaian telah dijalankan di dalam mod kelompok menggunakan *P. simplicissimum* dan air tebu sebagai substratum untuk menghasilkan FOS di dalam kultur kelalang. Beberapa parameter telah dikaji menggunakan kaedah satu-faktor-pada-satu-masa dan menghasilkan misilia biojisim dan FOS yang paling tinggi iaitu 10.07 g/L dan 66.50 g/L, masing-masing. Oleh kerana penapaian oleh kulat berfilamen di dalam bioreaktor tangki teraduk menyukarkan, morfologi kulat telah dikawal dengan menyekatgerakkan miselium di dalam bioreaktor lapisan gentian berputar dan keputusan menunjukkan yang penghasilan misilia biojisim dan produktiviti FOS yang paling tinggi adalah pada 0.21 g/L·h dan 4.01 g/L·h, masing-masing.

Untuk kajian pengoptimuman, parameter-parameter bagi proses ini telah dikaji dengan menggunakan kaedah sambutan permukaan (RSM) di dalam kultur

kelalang dan bioreaktor. Dalam keadaan optimum yang diperolehi daripada RSM, penghasilan misilia biojisim dan FOS dari enzim FTase di luar dan dalam sel adalah 7.43 g/L, 0.83 g/g dan 0.75 g/g di dalam kultur kelalang, masing-masing. Dalam kajian bioreaktor menggunakan sistem misilia terampai bebas dan tersekatgerak, keputusan menunjukkan hasil yang memuaskan dengan pekali penentuan, R^2 untuk hasilan FOS bagi kedua-dua sistem adalah lebih tinggi daripada 0.9. Di bawah keadaan optimum, sistem sel tersekatgerak telah menunjukkan ramalan hasilan FOS yang paling tinggi pada 1.76 g/g.

Model kinetik tidak berstruktur seperti model Logistik untuk tumbesaran misilia, model Leudeking-Piret untuk penggunaan sukrosa, dan Leudeking-Piret digabungkan bersama model Logistik untuk penghasilan FOS telah dicadang dan disahkan bagi misilia *P. simplicissimum* yang terampai bebas di dalam bioreaktor tangki teraduk. Kesemua model menunjukkan penerangan yang bersesuaian semasa fasa pertumbuhan dengan R^2 lebih tinggi daripada 0.9. Penghalangan substratum dan produk ke atas pertumbuhan kulat telah juga dikaji dengan menggunakan model yang berbeza. Model bagi persaingan substratum dan penghalangan produk adalah amat berpadanan dengan data eksperimen dengan R^2=0.99 dan R^2=0.98, masing-masing.

Untuk sistem sel yang tersekatgerak, model untuk pertumbuhan sel ($X(t)$), penggunaan substratum ($S(t)$), dan pembentukan produk ($P(t)$) telah dibangunkan daripada model Logistik yang digabung bersama penjerapan (k_a), dan nyahjerapan (k_d) dengan pemindahan jisim (k_m) dengan mengambil kira kesan kelajuan pengadukan. Model menunjukkan lebih tinggi kelajuan pengadukan, lebih banyak miselium terpisah daripada matrik gentian dan mempengaruhi sintesis misilia, penggunaan sukrosa, dan penghasilan FOS. Dalam semua kes, model penyelakuan

amat berpadanan dengan pemerhatian eksperimen dengan ralat min kuasa dua (MSE) kurang daripada 10%.

PRODUCTION OF FRUCTOSYLTRANSFERASE AND FRUCTOOLIGOSACCHARIDES BY *PENICILLIUM SIMPLICISSIMUM*

ABSTRACT

Fructooligosaccharides (FOS) produced from natural resources as an alternative sweetener has received greater attention and sucrose is the common carbon source for its production. To minimize the production cost, feasibility of using low cost substrates derived from local agricultural resources such as sugarcane juice was chosen to replace the pure sucrose. Several fungi were screened for their ability to produce fructosyltransferase (FTase) and FOS. *Penicillium simplicissimum* was found to be a potent producer for FTase and FOS with the highest FTase activity at 5.54 X 10^2 IU/ml and FOS yield per sucrose consumed of 0.39 g/g, respectively.

Fermentation was undertaken in batch mode using *P. simplicissimum* and sugarcane juice as a substrate for the FOS production in shake flask culture. Several parameters were studied using one-factor-at-a-time method and resulted that the highest mycelial biomass and FOS production were at 10.07 g/L and 66.50 g/L, accordingly. Due to the troublesome of filamentous fungal fermentation by freely suspended mycelium, the morphology was controlled by mycelia immobilization in a rotating fibrous bed bioreactor and showed good result with the highest mycelia biomass and FOS productivity at 0.21 g/L·h and 4.01 g/L·h, respectively.

For optimization studies, the process parameters were further studied in shake flasks and bioreactor using response surface methodology (RSM). Under optimum conditions obtained from RSM in shake flasks culture, higher mycelial biomass were produced at 7.43 g/L and FOS yield using extra- and intracellular FTase source at 0.83 g/g and 0.75 g/g, respectively. In bioreactor studies, satisfactory results were obtained for the freely suspended and immobilized mycelia system with the

coefficient of determination, R^2 for FOS yield higher than 0.9. Under optimum conditions, the immobilized cells system predicted the highest FOS yield at 1.76 g/g.

Unstructured kinetic models namely the Logistic model for mycelia growth, the Leudeking-Piret for sucrose consumption, and Leudeking-Piret incorporated with Logistic model for FOS yield were proposed and validated for the freely suspended mycelia of *P. simplicissimum* in stirred tank bioreactor. The models appeared to provide a reasonable description for each parameter during the growth phase with R^2 higher than 0.9. The inhibition of substrate and product on the growth of the tested fungus were also studied using different types of models. The competitive substrate and product inhibition models fitted well with the experimental data with R^2=0.99 and R^2=0.98, respectively.

For the immobilized cells system, the models for cell growth ($X(t)$), substrate consumption ($S(t)$), and product formation ($P(t)$) were developed from the Logistic model incorporated with adsorption (k_a), and desorption rate (k_d) with mass transfer (k_m) considering the effect of agitation speed. The model showed that as the agitation speed increased more mycelium detached from the fibrous matrix and affecting the synthesis of mycelium, sucrose consumption, and FOS production. In all cases, the models simulation matched well with the experimental observation with mean square error (MSE) less than 10%.

CHAPTER ONE

INTRODUCTION

1.0 Functional foods

Functional foods are used to enhance certain physiological functions in order to prevent or even to cure diseases (Roberfroid, 2000). The term 'functional food' was born in Japan. The Japanese were the first to observe that food could have a role beyond gastronomic pleasure and energy, and nutrient supply to the organisms (Lopez-Varela *et al*., 2002). According to Sangeetha *et al.*, (2005[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. In fact, in addition to their basic nutritional content and natural being it also contains a proper balance ingredient which will help to improve many aspects of human lives, including the prevention and treatment of illness and disease (Goldberg, 1994). It is important to highlight that functional food must be a food and not a drug. Beneficial effects should be obtained by consuming normal amounts of a functional food within the normal diet.

Food products are designed for taste, appearance, cost and convenience for consumer. The design of food products that confer a health benefit is 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 organization and industries involved in formulating foods

for health benefit into new areas of understanding. Functional foods drives the market place due to consumer needs to optimize their health through food.

In response to an increasing demand from consumer for healthier and calorie controlled foods, a number of so-called alternative sweeteners such as palatinose and various oligosaccharides including isomaltooligosaccharides, soybean-oligosaccharides, and fructooligosaccharides have emerged since 1980s (Yun, 1996). They are important primarily because of their functional properties rather than sweetness. A large number of functional foods have been introduced into the market. Many of them contained a number of characteristics functional properties that could control some diseases such as heart disease, cancer, stress, high cholesterol, weight control, osteoporosis and diabetes (Gilbert and Sloan, 1998).

Oligosaccharides are widely used in functional foods throughout the world for their health-promoting and technological properties. They are ingredients of the future that meet the needs of the food industry today, and are on the leading edge of the emerging trend toward functional foods (Niness, 1999).

1.1 Oligosaccharides

Oligosaccharides are usually defined as glycosides that contained between three and ten sugar moieties (Crittenden and Playne, 1996). A lot of attention is being paid to dietary carbohydrates, especially oligosaccharides, in particular, fructooligosaccharides (FOS). FOS from sucrose have been of increasing importance because of their favorable functionalities such as being low caloric, noncariogenic

and acting as a growth factor for beneficial microorganisms in the intestinal flora (Oku *et al*., 1984; Hidaka *et al.,* 1986; Yun, 1996). 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. Flamm *et al.*, (2001) reported that an average daily consumption of FOS has been estimated to be 1-4 g in United States and 3-11 g in Europe. The most common sources of FOS are wheat, honey, onion, garlic, and banana.

Oligosaccharides provide several manufacturing and health benefits, which was used as food ingredients. The specific physicochemical and physiological properties of food-grade oligosaccharide products varied depending on the type of mixture purchased (Crittenden and Playne, 1996). Accordingly, the most appropriate oligosaccharide for a particular food application will also vary. However, some properties are common to almost all oligosaccharide products.

Oligosaccharides are water soluble and mildly sweet, typically 0.3-0.6 times as sweet as sucrose (Oku, 1994). The sweetness of the oligosaccharide product is dependent on the chemical structure and molecular mass of the oligosaccharides present, and the levels of mono- and disaccharides in the mixture. Their relatively low sweetness is useful in food production when a bulking agent with reduced sweetness is desirable to enhance other food flavors. Compared with mono- and disaccharides, the higher molecular weight of oligosaccharides provides an increase viscosity, leading to improved body and mouthfeel. 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 (Crittenden and Playne, 1996). Oligosaccharides provide a high moisture-retaining capacity, preventing excessive drying, and a low water activity, which is convenient in controlling microbial contamination (Nakakuki, 1993).

Unlike starch and simple sugars, the currently available food-grade oligosaccharides are not utilized by mouth microflora to form acid or polyglucans. Hence, they are presently used as low-cariogenic sugar substitutes in confectionary, chewing gums, yoghurts and drinks. Many oligosaccharides are not digested by humans (Tomomatsu, 1994). This property makes them suitable for use in sweet, low-calorie diet foods, and for consumption by individuals with diabetes. Oligosaccharides can be used to mask the after tastes produced by some of intense sweeteners such as aspartame, phenylalanine or sucralose (Nakakuki, 1993). The indigestible quality of oligosaccharides means that they have effects similar to dietary fiber, and thus prevent constipation.

In recent years, the ability of many oligosaccharides to promote the proliferation of bifidobacteria in the colon has been recognized. These bacteria are believed to be beneficial to health and together with other health promoting microorganisms that are termed probiotics (Gibson and Roberfroid, 1995). Subsequently, oligosaccharides have recently been described as one of several 'prebiotics', which can stimulate the growth of beneficial microflora (Gibson *et al.*, 1994). Much of the present marketing and research on oligosaccharides is focusing on this functional property. Effective bifidogenic doses appear to vary among the different oligosaccharide types. However, most oligosaccharides have been

demonstrated to increase bifidobacteria numbers in the colon at doses of <15 g/d (Spiegel *et al*., 1994; Gibson *et al.*, 1994; Tannock, 1995; Playne, 1995).

1.2 Problem statements

Nowadays, there are many diseases emerged and most of them are due to the food that has been consumed. Focus on the dietary of Malaysian that led to obesity (BMI>30) and related health problems, including coronary heart disease and diabetics, that are without question a public health concern (Nestle, 2003). Accordingly, fructooligosaccharide (FOS) is one of the ingredients that offer such benefits for reducing lipoprotein cholesterol ratio (Lee *et. al.,* 1994) and body fat content (Chin *et. al.,* 1992) especially for people whom are obese. Also, it is suitable for consumption of diabetic patient due to the non-caloric and non-cariogenic as a sweetener.

Though FOS is present in plant sources like garlic, wheat, barley, onion, rye, banana, and asparagus, their concentrations are low and their mass production is limited by seasonal conditions and harvesting time (Flamm *et al*., 2001). Hence, microbial production by the action of fungal fructosyl transferase (FTase) on sucrose is more feasible at industrial level. Microbial production of FOS provided a cost effective and convenient alternative to chemical synthesis (Prapulla *et al.*, 2000). Enzymes derived from microorganisms like *Aspergillus phoenicis*, *A. japonicus, A. niger, Fusarium oxysporum, Scopulariopsis brevicaulis, Penicillium frequentens, Penicillium rugulosum, Aureobasidium pullulans* and *Arthrobacter* sp. have been reported to produce FOS from sucrose (Sangeetha *et al*., 2005[a]). In terms of ease of

manipulation, fungal fermentation does not need to be fed like animal, or no need to be planted and harvested like plant; it is just by simply inoculating medium with starter seeds and harvests the product after some periods of time. Moreover, it is independent of climate and the mass production is not complicated because the environmental condition can be controlled in a reactor.

FOS consists of sucrose molecules to which 1, 2 or 3 additional fructose units were added by β-(2-1)-glycosidic linkage to the fructose units of sucrose (Clevenger *et al.*, 1988; Sangeetha *et al.,* 2005[a]). They are manufactured either from sucrose by transfructosylation or from inulin by controlled enzymatic hydrolysis (Crittenden and Playne, 1996). Since the sugarcane industries in Malaysia produce a large amount of sucrose syrups, a wide range of applications could be exploited. However, its utilization as a substrate (carbon source) in batch and continuous fermentations by micro- and macro- fungi are less reported.

However, filamentous fungal fermentation is a complex process as compared to bacterial and yeast fermentations. Fungal morphology affects broth rheology which leads to numerous problems in gas dispersion, mass and heat transfer, and mixing in a conventional stirred tank bioreactor (Schugerl *et al.,* 1983; Xu and Yang, 2007). The diversity and change in morphology during the fermentation are difficult to control and often cause severe problems in operation (Papagianni, 2004). Therefore, controlling the fungal morphology is required to obtain higher production rate and good performance. Various cell immobilization methods to overcome the problem in fungal fermentation have been studied (Vajia *et al*., 1982; Eikmeyer *et al*., 1984; Roukas, 1991; Tay and Yang, 2002; Xu and Yang, 2007).

However, neither systematic study on its kinetics and modeling of FOS production by *P. simplicissimum* has been reported, nor there has been any description on how adsorption-desorption of the immobilized *P. simplicissimum* mycelial onto the fibrous matrix and its mass transfer in a rotating fibrous bed bioreactor (RFBB) are related over a batch culture time course. Consequently, a fermentation kinetic analysis over a broad range of environmental conditions need to be carried out for the development of better strategies for the optimization of fermentation process, either for the immobilized or freely suspended *P. simplicissimum* mycelia system. It is anticipated that such an approach would provide means of accessing the importance of such effect to microbial growth, substrate consumption and inhibition, and FOS production, and will allow an estimation of the values of the kinetic constants involved in each of the experiment.

1.3 Research objectives

The main objective of this project is to provide an alternative method for fructooligosaccharides (FOS) production utilizing microbial-base enzymes of *P. simplicissimum* using sugarcane juice as a substrate.

The measurable objectives are:

- To determine the proximate composition of different types of sugar and selecting the best fructooligosaccharides (FOS) producing fungus.
- To study the effect of different culture variables (pH, substrate concentration, inoculum size, aeration, agitation, fermentation time) on

the production of fructooligosaccharides (FOS) in shake flasks culture and in a bioreactor.

- To optimize the fructooligosaccharides (FOS) production using design of experiments (DOE).
- To propose and validate various unstructured models with respect to cell growth, substrate consumption and inhibition, and fructooligosaccharides (FOS) production either for freely suspended and immobilized mycelia system in a bioreactor.

1.4 Scope of study

FOS has been a popular dietary supplement worldwide for its prebiotic and functional effects. Though FOS can be produced by plant, it is preferable to be produced commercially from sucrose by microbial enzymes having transfructosylating activity. In view of the demand of FOS derived from sucrose by microorganism, this study was carried out using sugarcane juice as a substrate as it contains higher concentration of sucrose. However, various types of sugar (honey, sugarcane juice, palm sugar, molasses, granulated table sugar, brown sugar, jaggery) were also analyzed for sucrose, glucose and fructose.

It was reported that several fungi have an ability to produce transfructosylating activities such as *Aspergillus* sp., *Fusarium* sp., and *Aureobasidium* sp., but few of them have potential for industrial application due to their low transfructosylating activities (Yun, 1996). Thus, selection of FOS producing fungus was carried out using fungi from various genera such as

Penicillium sp., *Aspergillus* sp., *Trichoderma* sp., *Trametes* sp., *Lentinus* sp., *Pycnoporous* sp., and *Scyzhophyllum* sp. in shake flask culture. Fungus that shows the best transfructosylating activity and FOS production was then selected for further fermentation experiments.

Initially, process optimization of various culture conditions using one-factor-at-a-time method on the production of FOS including initial substrate concentration, incubation temperature, initial pH, and inoculum size were studied in order to obtain the optimum conditions for producing higher FOS in shake flasks culture. Then, optimization using freely suspended and immobilized mycelia systems at various aeration rate, agitation speed, and initial substrate concentration in a bioreactor was also investigated.

Process optimization using a statistical approach with selected independent parameters for either in shake flasks culture and bioreactor were also discussed. Response surface methodology (RSM) coupled with central composite design (CCD) were used in this study.

Kinetic studies of cell growth, substrate consumption, and FOS production using freely suspended and immobilized mycelia system in a conventional stirred tank bioreactor (CSTB) and rotating fibrous bed bioreactor were carried out to evaluate the fermentation characteristics. For freely suspended mycelia system, three models were proposed namely the Monod and the Logistic equations for mycelia growth, the Leudeking Piret equation for substrate consumption and FOS production. The inhibition of substrate on growth of mycelia and FOS production were also

studied. Several inhibition kinetic models were used such as Aiba, Andrew, Tessier, competitive and noncompetitive substrate inhibition models. The models were then tested and the results obtained for each parameter was compared. For the immobilized mycelia system using rotating fibrous bed bioreactor (RFBB), the models were developed by incorporating the Logistic equation with adsorption, desorption, and mass transfer parameters for cell growth, substrate consumption, and FOS production. The validity of each models for both freely suspended and immobilized mycelia system were confirmed by judging the coefficient of determination, R^2 value and means square error (MSE). In order to better understand and control the oxygen diffusion of the immobilized mycelia in the RFBB, oxygen diffusion model was also used to estimate the critical thickness of the immobilized fungal mycelia attached onto the fibrous matrix.

1.5 Organization of thesis

There are five chapters in this thesis and each chapter describes the sequence of this research.

Chapter 1 emphasizes on the term functional foods derived from oligosaccharides and its market trends throughout the world. Problems statement, research objectives, scopes of research, and thesis organization were also highlighted.

Chapter 2 covers the history, chemical structure, properties and application of FOS. Fermentation of FOS in batch mode utilizing freely suspended mycelia and

immobilized mycelia cultured in conventional and rotating fibrous bed bioreactor (RFBB) were also discussed. Fermentation kinetics and modeling studies, and optimization of culture conditions using design of experiment (DOE) were highlighted in detail.

Chapter 3 refers to the material and methods describing the experimental procedures for the production of FOS using freely suspended mycelia and immobilized mycelia system in batch mode. This chapter also covers the analytical methods, kinetics and modeling, and optimization of fermentation parameters using response surface methodology.

Chapter 4 presents the fermentation results of FOS by *P. simplicissimum* in batch mode using immobilized and freely suspended mycelia either in shake flask culture or in a bioreactor. Process parameter optimization of FOS in shake flask and bioreactor were obtained from the Response Surface Methodology (RSM) coupled with central composite design (CCD). The kinetics and modeling for the fermentation process were also presented.

Chapter 5 summarizes the overall findings based on the results obtained in the previous chapter. Recommendations for future research are also given in the chapter.

CHAPTER TWO

LITERATURE REVIEW

2.1 Fructooligosaccharides (FOS)

2.1.1 History of fructooligosaccharide (FOS) production

Fructooligosaccharides (FOS) also called oligofructose or oligofructan, is a class of oligosaccharides used as an artificial or alternative sweetener. FOS usage emerged in the 1980s in response to consumer demand for healthier and calorie-reduced foods (Yun, 1996). The term oligosaccharide refers to a short chain of sugar molecules where in the case FOS, is really just a bunch of fructose molecules that connected to each other chemically (Krol and Grzlak, 2006).

FOS is known as functional food and has been a popular dietary supplement in Japan for many years, even before 1990, when the Japanese government installed a “Functionalized Food Study Committee” of 22 experts to start regulates functional foods which contained the categories of fortified foods (O’Donnell, 2009). These foods, in turn, could improve the health of the Japanese people. Functionalized foods were based on the concept that foods had three purposes (Stark and Madar, 1994). Their primary function was as a source of nutrition to maintain life. Their secondary function was to provide sensory appeal. Lastly, some foods had a tertiary, "body-regulating," function to prevent, cure, or assist in the recuperation from disease. In a broad sense, "functional foods" were foods that possessed all three functions, while

"functionalized foods" were more narrowly defined as products specifically aimed at foods' tertiary purpose (Farr, 1997).

Now FOS becoming increasingly popular in Western cultures for its prebiotic effects. FOS served as a substrate for microflora in the large intestine and increasing the overall gastrointestinal tract (GI Tract) health. It has also been touted as a supplement for preventing yeast infections (Roberfroid, 1993).

Several studies have found that FOS and inulin promote calcium absorption in both the animal and human guts (Zafar and Weaver, 2004). The intestinal microflora in the lower gut could ferment FOS, which resulted in a reduced pH. FOS can be considered as a small dietary fiber with low calorific value. The fermentation of FOS results in the production of gasses and acids. The latter provided some energy to the body (Houdijk *et al.,* 2002).

FOS was first introduced into the market as foodstuffs by Meiji Seika Co. in Japan during 1984. Japan has the largest commercial market where its market volume amounted to over 4000 metric tons in 1990 (Yun, 1996). Although FOS has not been marketed in the U.S. and Europe, several companies have been trying to get the Generally Recognized As Safe (GRAS) status for using FOS with plain, unsweetened dairy products (Yun, 1996). It is evident that from some consumption data the consumer interest in low-calorie food products was increasing (Stamp, 1990). Thus, indicating that the consumption of FOS in the sugar market is expected to rise. Many petitions for FOS usage in foods are currently in-progress in Japan, Korea, and some European countries.

2.1.2 Chemical structure and physicochemical properties of fructooligosaccharides (FOS)

2.1.2 (a) Chemical structure of fructooligosaccharides (FOS)

FOS is defined by the IUB-IUPAC Joint Commision on Biochemical Nomenclature and the AOAC as fructose oligosaccharide containing 2-10 monosaccharide residues connected by glycosidic linkages (IUB-IUPAC Joint Commision on Biochemical Nomenclature, 1982). Generally, FOS is understood as inulin type oligosaccharides of D-fructose attached by β-(2→1) linkages between the fructose molecules that carry a D-glucosyl residue at the end of the chain (Yun, 1996). These linkages prevent FOS from being digested like a typical carbohydrate and are responsible for its reduced caloric value and dietary fiber effects (Niness, 1999). They constitute a series of homologous oligosaccharides derived from sucrose usually represented by the formula GF_n as depicted in Figure 2.1. The structures of FOS synthesized in cell free enzyme systems are essentially identical to those produced by whole cells systems.

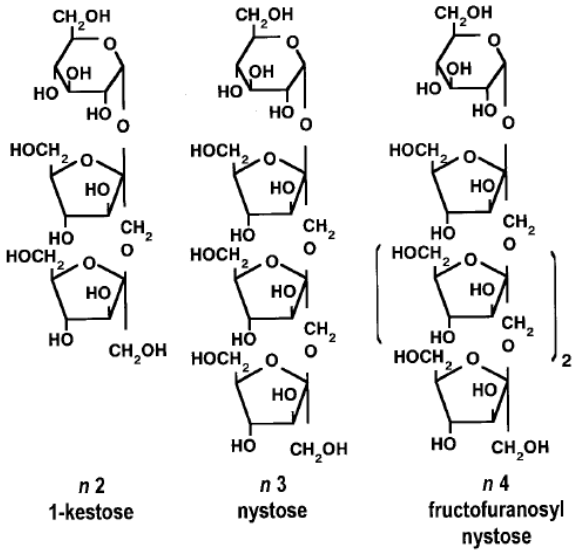

Figure 2.1 Chemical structures of fructooligosaccharides (Ohta *et al*., 1998).

A research group of Meiji Seika Co., is the first commercial producer of FOS, introduced the chemical structure of FOS produced from *Aspergillus niger* fructosyltransferase (Meiji Seika, 1984). The chemical structure of FOS produced by *Aureobasidium* fructosyltransferase was also identified by methylation, GLC, GC-MS, and NMR analysis (Hayashi *et al*., 1989). These two representatives FOS are now widely known to be oligosaccharides containing 1-kestose, nystose, and 1-fructofuranosyl nystose. Structural analysis is important in the study of FOS because the degree of polymerization and linkages of FOS varied with the enzyme sources, but they still carried essentially the same nutritional benefits (Yun, 1996).

2.1.2 (b) Physicochemical properties of fructooligosaccharide (FOS)

The extensive data on the physicochemical properties are scarcely available even though many articles have been published. According to Gross (1962), the specific rotation ($[\alpha]_D^{20}$) and melting temperature of 1-kestose are +28.5 ˚C and 199-200 ˚C, respectively. It forms fine white crystals rapidly. The relative sweetness of 1-kestose, nystose, and 1-fructofuranosyl nystose to 10% sucrose solution are 31%, 22%, and 16%, respectively (Yun, 1996). Niness (1999) reported that FOS is actually more soluble than sucrose and provides ~30-50% of the sweetness of table sugar. The low sweetness makes them suitable as bulking agents and as flavor enhancers. FOS is highly hygroscopic where it is difficult to keep the lyophilized products stable under atmospheric conditions for prolonged periods. The viscosity of FOS solution is relatively higher than sucrose when at the same concentration and the thermal stability is also higher than sucrose that provides leading to improve body and mouthfeel (Voragen, 1998). Furthermore, FOS is also highly stable in the normal pH

range for food (4.0-7.0) and at refrigerated temperatures over one year. According to Voragen (1998), in a 10% solution of pH 3.5 less than 10% is hydrolysed after heat treatments of 10 sec at 145 ˚C, 5 min at 45 ˚C, 60 min at 70 ˚C, and after two days at 30 ˚C less than 5% is hydrolysed. FOS contribute body to dairy products and humectant to soft baked goods, depresses the freezing point in frozen deserts, provides crispness to low fat cookies, and act as a binder in nutritional or granola bars, in much the same way as sugar, but with the added benefits of fewer calories, fiber enrichment and other nutritional properties (Roberfroid, 1993). While there have been few published studies comparing the physicochemical properties of FOS from sucrose, there is a strong indication that FOS resembles sucrose in many properties such as solubility, freezing and boiling points, and crystal data.

2.1.3 Sources and application of fructooligosacharides (FOS)

2.1.3 (a) Sources of fructooligosaccharides (FOS)

Predominately, FOS has been produced by the action of transfrutosylation activity by the enzyme fructosyltransferase from many plants and microorganisms. FOS is common to edible parts of a variety plants like onion, Jerusalem artichoke, chicory roots, leek, garlic, banana, rye, yacon, and salsify (Table 2.1). A series of fructose oligomers and polymers derived from sucrose occur in many higher plants as reserve carbohydrates. For most of these sources, concentrations range between 0.3% and 6% of fresh weight; for chicory roots and salsify these values are between 5% and 10% while in Jerusalem artichoke and yacon they can go up to 20% (Voragen, 1998). Allen and Bacon (1956) found that transfructosylation activity from the enzyme derived from the leaves of the sugar beet and were led to the

conclusion that in the presence of sucrose, the products of transfer were mainly 1-kestose with some neokestose.

Table 2.1 Fructooligosaccharide-synthetic enzymes from various plants

Source	Reference
Agave americana (agave)	Yun (1996)
Agave vera cruze (agave)	Satyanarayana (1976)
Asparagus officinalis (asparagus root)	Yun (1996)
Allium cepa (onion bulbs)	Henry and Darbyshire (1980)
Cichorium intybus (chicory)	Yun (1996)
Crinum longifolium	Yun (1996)
Sugar beet leaves	Allen and Bacon (1956)
Helianthus tuberosus (Jerusalem artichoke)	Edelman and Dickerson (1966)
Lactuca sativa (lettuce)	Yun (1996)
Lycoris radiata (monocot)	Yun (1996)
Taraxacum officinale	Yun (1996)

Furthermore, an enzyme which transfers the terminal fructosyl residue from the trisaccharide to sucrose to reform a donor molecule was discovered in the Jerusalem artichoke (Edelman and Dickerson, 1966). Onion and asparagus are also important sources of fructosyltranferases (Shiomi *et al*., 1976; Henry and Darbyshire, 1980). According to Shiomi and Izawa (1980), asparagus oligosaccharides are

produced by cooperative enzymatic reaction with at least three kinds of fructosyltransferase that are sucrose 1-fructosyltranferase, 6-fructosyltransferase, and 1-fructosyltransferase. Satyanarayana (1976) reported that the naturally occurring oligosaccharides in *Agave vera cruze* consists of 1-kestose, neokestose, 6-kestose, and their derivatives. These oligosaccharides arise not only by transfructosylation reactions but by the stepwise hydrolysis of the higher oligosaccharides and fructans catalyzed by the inherent hydrolytic activity of the enzyme. However, the production yield of FOS using enzymes originated from plants is low and the mass production of this enzyme is quite limited by seasonal conditions, therefore industrial production depends chiefly on microbial enzymes.

Microbial fructosyltransferase (FTase) are derived from bacterial and fungal sources. Several microorganisms capable of producing FTase have been screened and tabulated in Table 2.2. *Fusarium oxysporum* has been reported to produce enzyme source functioning transfructosylation activity during cultivation in the sucrose medium (Maruyama and Onodera, 1979; Gupta and Bhatia, 1982, Patel *et al*., 1994). Hidaka *et al*., (1988) reported that they fully characterized an enzyme from *Aspergillus niger* and virtually developed it into industrial production of FOS syrup. By using *A. niger* enzyme, the maximum FOS conversion reached 55-60% (w/w) based on total sugars. Furthermore, Hayashi *et al*., (1990) reported another FOS producing enzyme by *Aureobasidium* sp., where this enzyme can compete with other industrial FOS producing enzymes due to its considerably high enzyme activity.

Table 2.2 Fructooligosaccharides producing microorganisms

Microorganism	Reference
Fungal source	
Aureobasidium pullulans	Jung *et al*., (1989)
Aureobasidium sp.	Hayashi *et al*., (1989)
Aspergillus niger	Hidaka *et al.* (1988)
Aspergillus sydowi	Yun (1996)
Aspergillus foetidus	Wang and Rakshit (2000)
Aspergillus japonicus	Hayashi *et al*., (1992[a])
Aspergillus oryzae	Mabel *et al*., (2008)
Aspergillus phoenicis	van Balken *et al*., (1991)
Aspergillus aculeatus	Ghazi *et al*., (2005)
Calviceps purpurea	Yun (1996)
Fusarium oxysporum	Gupta and Bhatia (1982), Patel *et al*., (1994)
Penicillium frequentans	Yun (1996)
Penicillium spinulosum	Yun (1996)
Penicillium simplicissimum	Present study (2009)
Penicillium citrinum	Hayashi *et al. (*2000)
Penicillium rugulosum	Barthomeuf and Pourrat (1995)
Phytophthora parasitica	Yun (1996)
Scopulariopsis brevicaulis	Takeda *et al.* (1994)
Saccharomyces cerevisiae	Yun (1996)
Sporotrichum thermophile	Katapodis *et al*., (2004)
Bacterial source	
Arthrobacter sp.	Yun (1996)
Bacillus macerans	Park *et al. (*2001)
Zymomonas mobilis	Crittenden and Playne (2002)
Lactobacillus plantarum	Saulnier *et al.,* (2007)
Bacillus subtilis	Euzenat *et al.,* (1997)

Jung *et al*., (1987) in their studies also stated that the black yeast, *Aureobasidium pullulans* produced fructosyltransferase with higher activity. Van Balken *et al*., (1991) stated that fructosyltransferase from *Aspergillus phoenicis* showed higher activity which then produces FOS at 60% (w/w) yield. This enzyme

also has the potential for an industrial application. However, according Jung *et al.* (1989) this enzyme did not produce 1-fructofuranosyl nystose and is inhibited not only by glucose but by 1-kestose and nystose, unlike the enzyme of *A. pullulans*. Takeda *et al.,* (1994), for their part later mentioned that a new fungal strain, *Scopulariopsis brevicaulis* has the ability of selective by produced 1-kestose, a major component of FOS, and the production activity was found to be located only intracellularly unlike other FOS-producing organisms which exhibit both intra- and extracellular production.

2.1.3 (b) Advantages and application of fructooligosaccharides (FOS)

FOS has a number of interesting functional and nutritional properties that make them important in food ingredients. FOS has been used in many countries to replace fat or sugar and reduce the calories of foods such as ice cream, dairy products, confections, and baked goods (Niness, 1999). FOS has lower caloric values than typical carbohydrates due to the β → (2-1) bonds linking the fructose molecules. These bonds render them non-digestible by human intestinal enzymes. Thus, FOS passed through the mouth, stomach, and small intestine without being metabolized (Roberfroid, 1993). Due to the non-digestibility of FOS, it was found to be suitable for consumption by diabetics patient. In this context, low calorie sweeteners like FOS has special significance because of its beneficial effects on lowering blood glucose. FOS has been claimed to lower fasting glycemia and serum total cholesterol concentrations, possibly via effects of short chain fatty acids produced during fermentation (Sangeetha *et al*., 2005[a]). Besides that, there were no influence on

serum glucose, no stimulation of insulin secretion and no influence on glucagons secretion.

FOS is actually storage carbohydrates found in a number of vegetables, fruits, and whole grains. It resists 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 (Cherbut, 2002). FOS influences intestinal function by increasing stool frequency, particularly in constipated patients, increasing stool weight as much as 2 g/g of FOS ingested and decreasing fecal pH, which has been linked to suppression of the production of putrefactive substances in the colon (Hidaka *et al*., 1988; Roberfroid, 1993; Gibson *et al*., 1994). Furthermore, it was reported that FOS decreased serum triglycerides and blood cholesterol levels in hypercholesterolemic patients (Gibson and Roberfroid, 1995).

The best known nutritional effect of FOS is its action to stimulate bifidobacteria growth in the intestine. Since FOS is not hydrolyzed by the human digestive enzymes, it undergoes fermentation in the colon and encouraged the growth of beneficial bacteria in the colon. Hence, it discourages the growth of potentially putrefactive microorganisms in the colon resulting in a healthy gut environment (Sangeetha *et al*., 2005[a]). FOS has been termed prebiotics because it is a non-digestible food ingredient that selectively stimulates growth of potentially health stimulating intestinal bacteria that is known as probiotics (Gibson *et al*., 1994). The combinations of pre-and probiotics have synergistic effects, referred to as symbiotic, because in addition to the action of prebiotics that promotes the growth of existing strains of beneficial bacteria in the colon. The symbiotic health concept is being used

by many dairy drink and yogurt manufacturers (Ebringer *et al*., 2008). Colonic fermentation of FOS leads to decrease in pH in the colon and this facilitated the absorption of mineral ions from the intestine, mainly calcium and magnesium (Delzenne *et al*., 1995). Other researchers have observed an increased capacity of calcium transporters (calbindin) in the colon (Ohta *et al*., 1998). According to Sangeetha *et al.*, (2005[a]), the addition of 5% FOS prevented bone loss significantly in the femur and lumbar vertebra in the presence of dietary calcium (1%). 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 (Ahrens and Schrezenmeir, 2002).

FOS has been found to reduce genotoxic enzymes associated with increasing bifidobacteria (Bouhnik *et al*., 1996). Two mechanisms have been proposed to explain the effect of prebiotics on the development of cancer. Firstly, production of protective metabolites where butyrate is a common fermentation end product and is known to stimulate apoptosis in colonic cancer lines and it is also the preferred fuel for healthy colonocytes (Prasad, 1980). Secondly, it is due to the shift of colonic metabolism away from benign end products (Manning and Gibson, 2004). It is thought that lactic acid bacteria have inhibitory effects on several bacteria that produced carcinogenic enzymes and are themselves non-producers. Moreover, prebiotics may indirectly modify the activities of enzymes produced by the lactic acid bacteria that are involved in carcinogenesis (Reddy, 1998).

Furthermore, 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 (Sangeetha *et al*., 2005[a]). There is interest in the food industry in developing functional foods to modulate blood lipids such as cholesterol and triglycerides. Colonic fermentation of FOS results in the synthesis of short chain fatty acids, which influenced the lipid metabolism in human beings (Delzenne *et al.*, 2002). FOS is able to counteract triglyceride metabolism disorder occurring through dietary manipulation in animals and sometimes independently on lipogenesis modulation.

According to Roberfroid and Slavin (2000), short chain fructans have been shown to lower serum total and low density lipoprotein (LDL) cholesterol in non-insulin dependent diabetic patients, but not in healthy subjects. There is evidence that FOS decrease the primary synthesis of triglycerides by the liver, which this occurs is not fully understood but the effect appears to be exerted at transcriptional level (Delzenne and Kok, 1999). The functionalities and application of FOS can be summarized in Table 2.3.

Table 2.3 Products that currently included FOS and nutritional functionalities of FOS

Industrial product	Properties and examples
1) Dessert	FOS used as the sole sweetening agent and reduces calories by 34% compared with sucrose. (jellies, puddings and light jam)
2) Ice cream	FOS can be used with inulin to replace all the sugar, reduce the fat content and give great taste. Freezing point depression is less than with sucrose; hence the texture can be harder
3) Confectionery products	Hard candies, gums, and marshmallows can be made while significantly reducing calorie values (candy, cookies, biscuits, breakfast cereals, chocolate, and sweets)
4) Meat products	(fish paste, tofu, sausage, meatballs)
5) Infant and old aged food	Food special for hospitalized people that contain less calories. Also addition of FOS can develop immunization for infant (milk, jellies, porridge, biscuits)
6) Bread and pastries	non-calories and low fat cake

Nutritional functionalities

Prevention of dental caries
Safe in diabetics
Proliferation of bifidobacteria and reduction of detrimental bacteria
Reduction of toxic metabolites and detrimental enzymes
Prevention of pathogenic and autogenous diarrhea
Prevention of constipation
Protection of liver function
Reduction of serum cholesterol
Reduction of blood pressure
Anticancer effect
Production of nutrients

2.1.4 Fructooligosaccharides synthesis mechanism

FOS can be synthesized by two different processes which result in slightly different end products (Figure 2.2). In the first method, FOS is produced from the disaccharide sucrose using the transfructosylation activity of the enzyme β-fructofuranosidase or fructosyltransferase (FTase). The FOS formed in this process contains between two and four β (2→1) linked fructosyl units linked to a terminal α-D-glucose residue. These are named 1-kestose, 1-nystose, and 1-fructofuranosylnystose. Glucose and small amounts of fructose formed as by-products in the reaction, as well as unreacted sucrose, are removed from the oligosaccharide mixture using chromatographic procedures to produce FOS of higher purity.

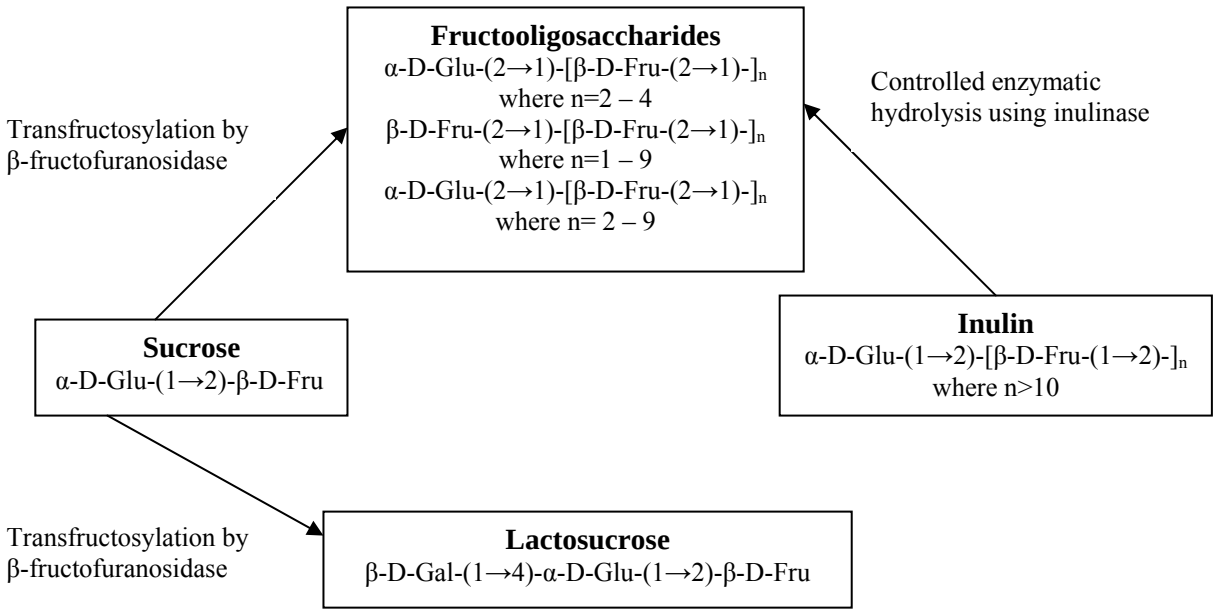

Figure 2.2 Production of FOS from sucrose or inulin (Crittenden and Playne, 1996)