PRODUCTION OF FRUCTOSYLTRANFERASE BY *PENICILLIUM SIMPLICISSIMUM* IN SUBMERGED CULTURE

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by

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

AGLI	A-Galactoside
ANN	Artificial Neural Network
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CBS	Fungal Diversity Centre (Netherlands)
CCD	Central Composite Design
CFR	Code of Federal Regulation
DCW	Dry Cell Weight
DEQ	Differential Equations
DO	Dissolved oxygen
ES	Enzyme-Substrate Complex
FANN	Feed forward Neural Network
FFT	Fructan-1-fructosyltransferase
FL	Fuzzy Logic
FOS	Fructooligosaccharides
FRIM	Forest Research Institute Malaysia
FTase	Fructosyltransferase
HPLC	High Performance Liquid Chromatography
IAM	Institute of Molecular and Cellular Biosciences, University of Tokyo
KCCM	Korean Culture Center of Microorganisms
KFCC	Korean Federation of Culture Collection
KCTC	Korean Collection for Type Culture

MSE	Mean Square Error
MW	Molecular Weight
NAP	National Agricultural Policy Plan
NCIMB	National Culture of Industrial and Marine Bacteria
NRRL	ARS Culture Collection
PDA	Potato Dextrose Agar
RFBB	Rotating Fibrous Bed Bioreactor
RKF	Runge-Kutta-Fehlberg
RSM	Response Surface Methodology
SmF	Submerged Fermentation
SSE	Sum square error
SSF	Solid State Fermentation
SST	Sucrose:Sucrose Fructosyltransferase
TCA	Tricaboxylic Acid

LIST OF SYMBOLS

A	Arrhenius constant	$time^{-1}$
A	First factor or input variable- sucrose concentration	gL^{-1}
B	First factor or input variable- fermentation time	h
C	First factor or input variable- pH	dimensionless
C_A	Concentration of the reactant A	gL^{-1}
c_D	Molar concentration of inactive enzyme	M
c_E	Molar concentration of active enzyme	M
c_{E0}	Initial concentration of enzyme	M
D	First factor or input variable- inoculum size	% v/v
dX/dt	Growth rate	g/L h
E	First factor or input variable- rate of agitation	rpm
E_a	Activation energy	kJ/mol
E_d	Denaturation energy	kJ/mol
F	Fructose	gL^{-1}
G	Glucose	gL^{-1}
GF	Sucrose	gL^{-1}
GF_2	1-kestose	gL^{-1}
GF_3	Nystose	gL^{-1}
GF_4	1^F-fructofuranosyl nystose	gL^{-1}
h	Plancks constant	dimensionless
K_i	Inhibition substrate concentration	gL^{-1}
K_m	Michaelis constant	g/mL
K_S	Substrate constant	dimensionless

k_B	Boltmanns constant	dimensionless
k_{cat}	Catalytic constant	s^{-1}
k_d	Denaturation constants	dimensionless
m_s	Maintenance coefficient	g/g/h
N_A	Avogadro's number	mol^{-1}
P_0	Product concentration at t = 0	IU/mL
P_t	Product concentration at time t	IU/mL
Q_p	FTase formation parameters – IU Ftase produced/l/h	IU/l/h
q_p	FTase formation parameters – IU Ftase produced/g cells/h	IU/g cells/h
Q_S	Substrate consumption parameters – g substrate consumed/ g cells/h	g/l/h
q_S	Substrate consumption parameters – g substrate consumed/g cells/h	g/g cells/l/h
Q_x	Substrate consumption parameters – g cells formed/l/h	g cells/l/h
r_p	Volumetric rate of product formation	$kgm^{-3}s^{-1}$
r_S	Volumetric rate of substrate consumption	$kgm^{-3}s^{-1}$
r_X	Volumetric rate of biomass production	$kgm^{-3}s^{-1}$
R	Carbonyl of an aldose	gL^{-1}
R	Universal gas constant	$kJmol^{-1}K^{-1}$
S	Concentration of the limiting substrate	gL^{-1}
T	Absolute temperature	°C
Uh	Hydrolytic activity	U/mL
Ut	Transfructosylating activity	U/mL
$u_1(t)$	Aeration at time t	vvm
$u_2(t)$	Agitation at a time t	rpm

$u_3(t)$	Sugarcane juice conctration at time t	gL^{-1}
v	Enzyme reaction rate	$\mu mol.mL^{-1}.min^{-1}$
V_{max}	Maximum rate of reaction	IU/mL.min
X	Concentration of biomass	gL^{-1}
X_0	Initial biomass concentration	gL^{-1}
X_1	First factor or input variable-aeration rate	vvm
X_2	First factor or input variable-agitation speed	rpm
X_3	First factor or input variable-sucrose concentration	gL^{-1}
X_m	Maximum biomass concentration	gL^{-1}
Y_B	Biomass concentration	gL^{-1}
Y_{EXT}	Extracellular FTase activity	IU/mL
Y_{INT}	Intracellular FTase activity	IU/mL
$Y_{P/S}$	Yield of FTase	IU/g
$Y_{X/S}$	Biomass yield	g/g
y(t)	Predicted process output of FTase activity at time t	IU/mL

Greek symbols

α	Growth associated constant	dimensionless
β	Non-growth associated constant	dimensionless
ΔG^*	Gibbs free energy	kJ mol^{-1}
ΔH^*	Enthalpy of inactivation	kJ mol^{-1}
ΔS^*	Entropy of inactivation	J $mol^{-1}K$
τ	Half-life	time
μ_{max}	Maximum specific growth rate	h^{-1}
γ	Activity coefficient of sucrose	dimensionless

PENGHASILAN FRUKTOSILTRANSFERASE OLEH *PENICILLIUM SIMPLICISSIMUM* DI DALAM KULTUR TENGGELAM

ABSTRAK

Fruktosiltransferase (FTase) adalah enzim yang bertanggungjawab dalam penghasilan fruktooligosakarida (FOS). FOS ialah bahan penting di dalam industri kosmetik, agrokimia, farmaseutikal dan makanan. Permintaan yang tinggi terhadap FOS telah menjurus kepada pencarian mikroorganisma baru yang boleh menghasilkan FTase dan substratum yang berkos rendah. Sejumlah tujuh belas jenis kulat dari genus yang berbeza ((*Trametes, Pycnoporous, Lentinus, Schizophyllum, Penicillium, Aspergillus and Trichoderma*) telah diuji dari segi kebolehan mereka menghasilkan FTase. *Penicillium simplicissimum* menunjukkan tumbesaran sel pada 3.8 g/L dan aktiviti FTase tertinggi 506 IU/mL untuk ekstrasel dan 128 IU/mL untuk intrasel, masing-masing. Air tebu dikenalpasti memiliki kepekatan sukrosa tertinggi sebanyak 1.249×10^3 g/L berbanding dengan tujuh sumber gula tempatan yang lain.

Bagi kultur kelalang goncang, penghasilan FTase telah dijalankan dengan menggunakan teknik "satu-faktor-pada-satu-masa" dan kaedah sambutan permukaan (RSM) berganding dengan rekabentuk komposit berpusat (CCD) untuk mengoptimumkan parameter bagi proses itu. Keadaan optimum yang diperolehi adalah pada kepekatan air tebu 20 g/L, masa fermentasi 36 jam, saiz inokulum 15% (v/v) dan kelajuan pengadukan 150 psm dengan aktiviti FTase ekstrasel 118.86 IU/mL, aktiviti FTase intrasel 71.97 IU/mL dan biojisim sel 14.16 g/L, masing-masing.

Di dalam kajian bioreaktor, kesan kepekatan permulaan sukrosa di dalam air tebu, kelajuan pengadukan dan kadar pengudaraan ke atas pengoptimuman FTase dari *Penicillium simplicissimum* turut juga dikaji menggunakan kaedah statistik, RSM. Keputusan menunjukkan kadar pengudaraan 2 vvm, kelajuan pengadukan 200 psm dan kepekatan permulaan sukrosa di dalam air tebu 30 g/L menghasilkan aktiviti FTase yang maksimum pada 161.17 IU/mL. Kadar pertumbuhan spesifik yang maksimum (μ_{max}) 0.877 j^{-1} dan hasil biojisim per sukrosa digunakan 0.667 g/g turut diperolehi. Penggunaan Jaringan Saraf Tiruan (ANN) untuk meramal aktiviti FTase dan pertumbuhan *P. simplicissimum* turut ditekankan dalam kajian ini. Proses ramalan satu langkah-kehadapan melalui kaedah pensampelan semula ikat-but telah terbukti konsisten dengan data eksperimen dengan pekali penentuan R^2 0.999 dan 0.9937 untuk aktiviti FTase dan biojisim sel, masing-masing.

Model kinetik tidak berstruktur seperti model Logistik, model Monod, model Logistik digabungkan bersama model Leudeking-Piret telah dicadang dan disahkan. Didapati semua model adalah sesuai untuk menerangkan pertumbuhan sel, penggunaan subtratum dan penghasilan FTase pada kepekatan permulaan sukrosa di dalam air tebu pada julat 10-50 g/L di dalam kultur kelompok. Perencatan substratum keatas pertumbuhan *Penicillium simplicissimum* telah juga dikaji, dimana model saingan didapati berpadanan dengan data eksperimen dengan nilai R^2 0.864.

Enzim FTase turut diciri berdasarkan kestabilan suhu, kestabilan pH, suhu dan pH yang optimum. Didapati aktiviti maksimum FTase telah dicapai pada suhu 55°C dan pH 5.5. Plot Lineweaver-Burk memberi nilai K_m dan V_{max} 6.51 g/mL dan 6.39 IU/mL.min, masing-masing. Tiga model enzim kinetik telah dicadang dan

disahkan bagi menerangkan kelakuan aktiviti enzim dan kadar tindakbalas sama ada untuk sel yang terampai bebas atau sel yang tersekat-gerak. Untuk penyahaktifan FTase, model penyahaktifan enzim tertib pertama didapati berpadanan dengan data eksperimen dengan julat R^2 0.971 hingga 0.979. Model kadar tindakbalas enzim dalam fungsi suhu dan waktu tindakbalas telah dipilih berdasarkan padanannya dengan data eksperimen bagi julat suhu yang besar dengan R^2 0.982. Bagi perencatan enzim, model perencatan bersaing lebih berpadanan dengan data eksperimen dengan R^2 0.961.

PRODUCTION OF FRUCTOSYLTRANSFERASE (FTase) BY *PENICILLIUM SIMPLICISSIMUM* IN SUBMERGED CULTURE

ABSTRACT

Fructosyltransferase (FTase) is an enzyme responsible for the production of fructooligosaccharides (FOS). FOS is an important ingredient in the cosmetic, agrochemical, pharmaceutical and food industries. Recent increase in the demand of FOS has led to new search of FTase producing microorganism and a low cost substrate. Seventeen fungal isolates from different genera (*Trametes, Pycnoporous, Lentinus, Schizophyllum, Penicillium, Aspergillus and Trichoderma*) were evaluated for FTase production. *Penicillium simplicissimum* was found to attain the highest cell growth at 3.8 g/L and FTase activities of 506 IU/ml for extracellular and 128 IU/ml for intracellular, respectively. Sugarcane juice was found to have the highest sucrose concentration at 1.249×10^3 g/L among the seven sources of local sugar tested.

In shake flask culture, FTase production were carried out using "one-factor-at-a-time" method and a statistical design approach response surface methodology (RSM) coupled with central composite design (CCD). The optimum culture conditions was obtained at sugarcane juice concentration 20 g/L, fermentation time 36 h, pH 6, inoculum size 15% (v/v) and agitation speed 150 rpm with extra-cellular FTase activity of 118.86 IU/mL, intracellular activity of 71.97 IU/mL and biomass concentration of 14.16 g/L, respectively.

In bioreactor studies, the effect of initial sugarcane juice concentration, agitation speed and rate of aeration for the optimization of FTase from *Penicillium*

simplicissimum were also investigated using a statistical tool, RSM. Results showed that aeration rate of 2 vvm, agitation speed of 200 rpm and initial sugarcane juice concentration of 30 g/L had a maximum extracellular FTase activity of 161.17 IU/mL. Under such condition, maximum specific growth rate (μ_{max}) of 0.877 h^{-1} and biomass yield to sucrose consumed (Y_{XS}) of 0.667 g/g were obtained. The prediction of FTase activity and *P.simplicissimum* growth using Artificial Neural Networks (ANN) were also highlighted in this study. One step-ahead prediction process through bootstrap resampling method have proved to be consistent with the experimental data with R^2 of 0.9903 and 0.9937 for FTase activity and biomass concentration, respectively.

An unstructured kinetic models namely the Logistic, the Monod, the Logistic incorporating Leudeking-Piret-like equation were proposed and validated. The models were suitable to describe biomass growth, substrate utilization and FTase production at different initial sugarcane juice concentration ranged from 10-50 g/L in batch culture. The inhibition of substrate on the growth of the *P.simplicissimum* was also studied. The data fit the competitive model with R^2 of 0.864.

The FTase enzyme was also characterized with respect to thermostability, pH stability, optimum temperature and pH. The maximum activities were observed at 55°C and pH 5.5. The Lineweaver-Burk plots gave the K_m and V_{max} values of 6.51 g/mL and 6.39 IU/mL.min, respectively. Three enzyme kinetic models have been proposed and validated to explain the behaviour of enzyme activity and reaction rates. For deactivation of the FTase, first order enzyme deactivation model satisfactorily fit the experimental data with R^2 range from 0.971 to 0.979. Enzymatic

reaction rates model as a function of temperature and reaction time has been selected as the model is able to fit the experimental data correctly in wide range of temperature with R^2 of 0.982. In inhibition of the enzyme, a competitive inhibition model fitted the experimental data better than non-competitive and un-competitive with R^2 of 0.961.

CHAPTER 1

INTRODUCTION

1.1 The Demand of Enzyme in Industry

Enzymes are among the most important products obtained for human needs through microbial sources. A large number of industrial processes in the areas of environmental and food biotechnology utilize enzymes at some stage or the other. Current developments in biotechnology are yielding new applications for enzymes (Pandey *et al.*, 2009).

Enzymes are highly efficient catalysts from biological sources, which catalysed synthetic and degradative reactions of living organisms. It was first reported in the second half of the nineteenth century (Koeller and Wong, 2001). Since then its usage has increased manifolds in various industries. In the last three decades with rapid strides in the field of biotechnology, especially in the fields of genetic and protein engineering, there has been many exciting research works involving enzymes with the development of new commercially important industrial processes. Enzymes are used industrially because of their high catalytic power, specific mode of action, stereo-specificity, eco-friendly use and reduced energy requirements (Kirk *et al.*, 2002).

All types of living organism, where metabolic reactions occur produce enzymes. A wide range of sources is used for the production of commercial enzymes. Out of the total enzymes being used industrially, over half are extracted from fungi

and yeast. One third are obtained from bacterial systems, and the remaining from animal (8%) and plant (4%) sources (Marwaha and Arora, 2000).

1.2 Industrial Production of Fructosyltransferase (FTase)

Fructosyltransferase (FTase) are the enzymes responsible for the microbial production of fructooligosaccharide (FOS) (Sangeetha *et al.*, 2005b). FOS are functional food ingredients with prebiotic properties, and recent increase in the use of oligosaccharides in the food industry has led to the search for „new" microorganisms and enzymes for the production of oligosaccharides (Maugeri and Hernalsteens, 2007).

The search for „new" enzymes for oligosaccharides production, using either microbial screening or molecular engineering, became necessary as a result of the increasing number of applications of oligosaccharides in the cosmetic, agrochemical, pharmaceutical and food industries (Clarkson *et al.*, 2001). A lot of attention is being paid to dietary carbohydrates especially oligosaccharides, in particular FOS. Average daily consumption of FOS has been estimated to be 1 - 4 g in US and 3 - 11 g in Europe (Sangeetha *et al.*, 2005b). The production of FOS using enzymes originated from plants was low and their mass production quite limited by seasonal conditions; therefore industrial production depends on fungal enzymes from either *Aureobasidium* sp. or *A.niger* (Yun, 1996).

Actually, enzymes with the potential for achieving a high yield of FOS production were found in the late 1980s and early 1990s. In 1984, Meiji Seika Co. in Japan was the first succeeded in the commercial production of FOSs by *A.niger*

enzyme (FTase) and verified their excellent functional properties. A decade later, Cheil Foods & Chemicals Co. in Korea succeeded in FTase industrial production by using immobilized cells of *A.pullulans* (Yun, 1996).

Hidaka *et al.* (1987) studied *A.niger* enzymes, which then fully characterized the enzyme and virtually developed it into industrial production of FOSs syrup. Smith *et al.* (1982) and Jung *et al.* (1987) also reported on FTase preparation with high activity using the black yeast *A. pullulans*. Hayashi *et al.* (1990) investigated another FOS production process by using *Aureobasidium* sp. This enzyme can compete with other industrial FOS-producing enzymes due to a considerably high enzyme activity. Later in 1991, Van Balken *et al.* reported another FTase which showed higher activity from *Aspergillus phoenicis* which showed great potential of the enzyme at industrial level.

Currently, the main industrial FOS producer is the fructosyltransferase (FTase) from *Aspergillus* (Sangeetha *et al.*, 2005a; Benito *et al.*, 2007; Ghazi *et al.*, 2007). In spite of the utilization of FTase in the industrial production of FOS and numerous scientific investigations, the only commercially available source of FTase is Pectinex SP-L, a pectinolytic and cellulolytic preparation designated for fruit juice processing (Antosova *et al.*, 2008).

1.3 Problem Statement

Increase demand for FOS as functional food ingredients with prebiotic properties has led to the search of new microorganism, carbon source and optimum process conditions for FTase production. In order to use FTase as biocatalyst in the

production of FOS as food additive, it becomes a necessary objective to reduce the production costs. Carbohydrate source is an essential constituent in the cultivation media especially in formation of cell constituent. However, the use of commercial sucrose as carbon source is a bit expensive and thus, the alternative sucrose source from local sugar was considered. The selected sugar must not only of low cost and has high sucrose content but it also need a capability to act as a substrate in producing higher FTase activity and cell biomass.

A wide variety of microorganisms were found to have the ability to produce FTase. However, the problem exist was to select the microorganisms that not only have the ability to produce FTase but at the same time must have the highest cell biomass. Selection of a particular strain remains a tedious task, especially when commercially competent enzyme yields are to be achieved. Fungus was selected as the tested organism in this study due to several considerations. Eventhough FTase also can be produced from plant sources, microorganisms are generally preferred as source of industrial enzymes because their production cost is lower than the enzyme originated from plants and is quite limited by seasonal conditions (Yun *et al.,* 1992). Furthermore, microbial enzyme contents are more predictable, easy availability of raw materials for cultivations, bigger in size and more thermostable than plants (Yun, 1996; Sangeetha *et al.*, 2005b). Meanwhile, bacterial FTase showed low self-transfructosylating activity and narrow acceptor specificity compared to fungal FTase (Kim *et al.,* 1998; Nam *et al.*, 2000).

In order to produce higher FTase, the culture condition need to be upgraded and many attempts were made by fellow researchers to select the parameters that

significantly affect the yield of FTase. Reducing the costs of enzyme production by optimization of the fermentation process parameters is the goal of industrial application. By applying one-factor-at-a-time methods the process become more time consuming and does not bring out the effect of interaction of various parameters (Elibol, 1999). This conventional method is also laborious and less capable of reaching the true optimum. To overcome this difficulty and to increase the productivity, the use of statistical procedures (with the help of experimental design) will be suggested. Response Surface Methodology (RSM) was used to evaluate the important parameter that influences the production of FTase in shake flask culture and bioreactor studies.

A long term process stability of an enzyme preparation is a common prerequisite of successful large-scale operation of a biocatalytic process (Madlova *et al.,* 2000). These indicate the necessities of the characterization of FTase enzyme by focusing on its thermostability and pH stability as very little is known about the biochemical and mechanistic properties of FTase nowadays. Characterization of enzyme is a necessary step in order to understand its mode of action, the nature of the hydrolytic activity and to decide the type of enzyme in which it should be classified (Kluepfel *et al.,* 1992). However, until now the accumulated information on FTase is rather confusing from one source to another, from one microorganism to another, even from one strain to another (L'Hocine *et al.*, 2000). Studies on the effect of a wide range of pH and temperature have resulted in evaluating the consequence of subjecting FTase production to extreme conditions in food processing (Sangeetha *et al.,* 2005a). With proper choice of the process conditions, transfructosylation can be carried out at such temperature that hardly has any or no infection of the reaction

mixture can occur any more. The maximum transfructosylating temperatures desirable for obtaining favorable viscosity of the highly concentrated sugar solution. Enzyme kinetics was included in this study for its importance in order to describe the enzyme‟s biochemical properties (L‟Hocine *et al.*, 2000).

1.4 Research Objectives

This study addresses an alternative method for the FTase production by selected fungi in shake flask culture and in a bioreactor:

The measurable objectives are:

- To identify the FTase producing fungus in shake flask culture
- To optimize the fermentation conditions of FTase production by *P.simplicissimum* using “one-factor-at-a-time” method and statistical design approach in shake-flask culture and in a bioreactor.
- To propose and validate the kinetics model for microbial growth, substrate consumption and inhibition and FTase production by *P. simplicissimum* in a batch culture.
- To characterize, propose and validate the FTase kinetic model freely suspended *P. simplicissimum* cells

1.5 Scope of Study

With an increased demand of FOS as functional food, scope exists for identification of newer strains capable of producing FTase. In order to produce FTase as a commercial enzyme, the use of low cost substrate (sucrose) is inevitable. The first part of this study will cover the determination of different sources of sugar. This is followed by the identification of FTase producing strain either from macro or

micro-fungi. The strain that produced FTase with the highest activity will be selected for further studies.

Shake flask experiment for FTase production was carried out using one-factor-at-a-time technique. The concentration of sucrose, fermentation time, pH, inoculum size and rate of agitation effect were studied one by one in a selected range determined from the literature review. In order to improve the production of FTase, a response surface methodology (RSM) based on central composite design was employed. The same parameters were chosen as the input factors while biomass and enzyme activity were the responses. The interactions among these various parameters were also discussed.

For bioreactor studies, the effects of aeration rate, agitation speed and initial sugarcane juice concentration were studied in batch fermentation. An optimization procedure using RSM was developed to determine the optional combination of parameters for maximal production of FTase in a bioreactor. Artificial neural network (ANN) was also used to predict the production of FTase. Unstructured mathematical model related on microbial growth, product formation, substrate consumption and inhibition of FTase production by *P.simplicissimum* were also proposed and validated.

Characterization of FTase includes the enzyme thermostability, pH stability and the determination of the kinetic parameters (K_m and V_{max}) for FTase enzyme. The kinetic parameters were then computed by conventional linearization of the Michaelis-Menten kinetics. Further studies on thermodynamic kinetic parameters,

activation energy and deactivation energy were also carried out. Mathematical model that described the enzyme reaction rates, enzyme deactivation and inhibited enzyme kinetics were also proposed and validated.

1.6 Organization of the Thesis

This thesis is divided into five chapters as follow:

Chapter 1 describes the demand of enzyme in industry and industrial production of fructosyltransferase (FTase) enzyme. This chapter focused on the problem statement, scope of study and the objectives of the study.

Chapter 2 describes the literature review from other researchers and methods applied in the present days for the industrial production of FTase. This section covers an overview of sugarcane industry in Malaysia, production process of FTase, fermentation and enzyme kinetics in FTase production. The details for design of experiment using response surface methodology and prediction of FTase production using neural networks were also highlighted.

Chapter 3 describes the materials, methods and analysis required for the fermentation process of *P. simplicissimum* either in shake flask or a bioreactor.

Chapter 4 presents the experimental results together with the data analysis of various operating condition and process parameters for FTase production. The kinetics and optimization of fermentation process using Response Surface Methodology have also been discussed through this section. Characterization of

enzyme was highlighted. The kinetics and modeling for fermentation process and enzyme kinetics in batch systems were also discussed.

Chapter 5 presents the conclusion and recommendation for the improvement in future studies.

CHAPTER 2

LITERATURE REVIEW

2.1 Sugarcane Industries in Malaysia

Sugarcane is the main sugar-producing crop in the world and has long been grown in tropical and subtropical regions (Galloway, 2005). Over three-fourths of sugar produced is from sugarcane, with the remainder produced from sugar beet (Cordeiro *et al.,* 2000). Malaysia's domestic sugar production is small and static at around 105, 000 – 110, 000 tonnes, raw value and represents less than 10% consumption (Gudoshnikov *et al.*, 2004).

The sugar industry in Malaysia is characterized by rapidly increasing direct domestic consumption supported by an equally fast growing food processing industry, and on the supply side by a small domestic production base that is unlikely to expand (Greenfield, 1997). Southeast Asia regions including Malaysia were characterized by high levels of sugarcane production. At the same time the market for freely traded sugar is large and deep compared with other agricultural commodities (Landiyanto and Wardaya, 2005).

Production is concentrated in the Northwest extremity of Peninsular Malaysia in the states of Perlis and Kedah. This area has a distinct dry season needed for cost-efficient sugarcane production (Greenfield, 1997). In recent years the sugarcane harvested area has averaged between 20 000 and 24 000 hectares (Table 2.1). Most of the cane areas is under the management of three sugarcane plantations, two in the

State of Perlis and one in the state of Kedah, with smallholders contributing only about 15 percent of the total (Greenfield, 1997).

Table 2.1: Malaysia sugarcane area, yield and production (Greenfield, 1997)

Year	Harvested area	Yield	Production
	'000 Ha	*Mt / Ha*	*'000 Mt*
1976	25	35	870
1977	20	50	1 000
1978	21	45	963
1979	20	50	1 005
1980	18	40	720
1981	17	40	680
1982	20	50	985
1983	21	50	1 025
1984	22	50	1 100
1985	22	49.1	1 080
1986	23	53	1 219
1987	17	71	1 207
1988	19	60	1 140
1989	19	64	1 216
1990	20	65	1 300
1991	20	61	1 220
1992	20	66	1 320
1993	23	68	1 547
1994	23	68	1 541
1995	24	68	1 601
1996	24	68.1	1 600

Domestic consumption of sugar in Malaysia has increased rapidly in recent years. However, the country's buoyant economy has also led to a particularly strong growth in the food processing industry. Ice cream, chocolates, sweetened condensed milk, and soft drinks are some of the items that have created new demand for sugar. On a per caput basis, the level of sugar consumption in Malaysia at about 50 kilograms (raw equivalent) is among the highest of the region (Greenfield, 1997).

The National Agricultural Policy Plan (NAP) for the period 1992 to 2010 gives minimal attention to sugar compared with oil palm and fruits and vegetables.

Apart from encouraging improvement in the productivity of existing areas and milling efficiency, the Malaysian Government is reportedly not anxious to foster expansion of sugarcane cultivation in the country (Greenfield, 1997).

The competitive sugar world market is pushing sugarcane research to identify new markets for other sugarcane products. Sugarcane as a biofactory is the new term that brings together the technologies for production of high-value materials such as functional foods and nutraceuticals, biopolymers and enzymes in sugarcane (Cordeiro *et al.*, 2000).

2.2 Fructosyltransferase (FTase) Production

Fructosyltransferase (FTase) is enzyme that catalyze the transfer of the fructosyl residue from sucrose to another sucrose molecule or growing fructose chain. FTase is present in bacteria, fungi and plant, where they are implicated in the biosynthesis of fructans (levan, inulin and fructooligosaccharides) (Fernandez *et al.*, 2007). According to Henry and Darbyshire (1980), FTase is the enzyme that catalyzes the initial reaction by transferring a fructosyl group from a sucrose donor to a sucrose acceptor to produce trisaccharide and glucose.

2.2.1 Occurrence

The enzyme source for FOS synthesis can be divided into two classes; one is plant such as sugar beet (Allen and Bacon, 1956), asparagus (Shiomi *et al.*,1976; 1979 and Shiomi, 1982), onion (Henry and Darbyshire, 1980), Jerusalem artichoke (Bacon and Edelman, 1951) and the other from bacterial and fungal origins such as *Aspergillus* sp. (Muramatsu *et al.*, 1988; Hidaka *et al.*, 1987; Kida *et al.*, 1988),

Aureobasidium sp. (Hayashi *et al.*, 1990; Lee *et al.* 1992; Yun *et al.*, 1992; Yun *et al.*, 1997), *Arthrobacter* sp. (Fujita *et al.,* 1990) and *Fusarium* sp (Maruyama and Onodera, 1979; Gupta and Bhatia, 1982; Patel *et al.,* 1994).

Based on previous studies, FTase have been purified and characterized from higher plants, such as asparagus, onion, Jerusalem artichoke and etc (Ghazi *et al.,* 2007). Plant FTase evolved from vacuolar invertase, a process likely connected with the independent adaptation of unrelated families to cold and arid environment (Banguela and Hernandez, 2006).The occurrence of FTase has also been reported in other plant sources such as *Claviceps purpurea, Phytophora parasitica, Streptococcus mutans* and *Fusarium oxysporum* (Lee *et al.,* 1992). This study indicated that FTase is associated with carbohydrate metabolism in plants and microorganism. A series of fructose oligomers and polymers derived from sucrose occur in many higher plants as reserve carbohydrates (Yun, 1996).

Allen and Bacon (1956) found transfructosylation activity from the enzymes derived from the leaves of the sugar beet (*Betavulgaris* L.). In 1968, Edelman and Jefford discovered the enzyme that transfer terminal fructosyl residue from trisaccharide to sucrose in Jerusalem arthichoke (*Heliantus tuberosus*). Onions and asparagus are also important sources of FTase. Shiomi (1982) extensively studied the FTase extracted from asparagus roots (*Asparagus officinalis* L.). Asparagus oligosaccharides are produced by cooperative enzymatic reactions with at least three kinds of FTase: sucrose 1-fructosyltransferase, 6^G-fructosyltransferase and 1^F-fructosyltransferase (Shiomi, 1982). It was found that the general properties resembled those of the Jerusalem artichoke but its substrate specificity differed. The

naturally occurring oligosaccharides were also found in agave (*Agave vera cruz)* consists of 1-kestose, neokestose, 6-kestose and their derivatives (Satyanarayana, 1976).

2.2.2 Mechanism of FTase Synthesis

The reaction mechanism of enzyme synthesis depends on the source of enzyme, but most of the microbial enzymes may catalyze the reactions of a readily reversible primary step and a subsequent irreversible step as in Equation (2.1):

$$\begin{aligned} &F-R+E \leftrightarrow F-E+R \\ &F-E+acceptor \rightarrow F-acceptor+E \end{aligned} \qquad (2.1)$$

where F is fructose, E is fructosyltransferase and R represents a carbonyl of an aldose. According to this mechanism, one molecule of sucrose serves as a donor and another acts as an acceptor for GF_2 (1-kestose) synthesis, releasing one molecule of glucose, for the production of GF_3 (nystose) and the GF_2 which acts as an acceptor (Yun, 1996).

The mechanism of the FTase synthesis was first reported in plant sources. Yun (1996) also reported that agave enzymes catalyzed the transfructosylation reaction to induce higher FOS formation, in which synthesis of FOS from sucrose took place as follows:

$$GF + FTase \rightarrow F-FTase+G \qquad (2.2)$$

$$F-FTase+GF \rightarrow GF_2+FTase \qquad (2.3)$$

In this mechanism, it is notable that glucose, not fructose, acts as the acceptor of the fructose molecule from sucrose. GF_2, GF_3 and GF_4 cannot act as donors of the fructosyl moiety for the synthesis of higher oligosaccharides.

Dickerson (1972) proposed the reaction mechanism of *C.purpurea* enzyme which produce mainly neokestose-based oligosaccharides. The suggested mechanism is summarized as follows:

$$F2 \rightarrow 1G + F2 \rightarrow 1G \rightarrow F2 \rightarrow 6GI \leftarrow 2F + G \qquad (2.4)$$

$$F2 \rightarrow 1G + F2 \rightarrow 6GI \leftarrow 2F \rightarrow F2 \rightarrow 1F2 \rightarrow 6G1 \leftarrow 2F + G \qquad (2.5)$$

where numbers indicate the position of carbonyl carbon atoms and arrows represent the direction of glycosidic linkage (e.g., F2 + 1G refers to sucrose). In addition to the above two synthetic reactions, the hydrolyzing reactions also occurs. A hydrolysate like F2 and 6G acts again as fructose donor and acceptor for the synthesis of neokestose and its tetraoligomer.

According to Yun (1996), fructan metabolism in Jerusalem artichoke (*H.tuberosus*) is established by two enzymes: sucrose:sucrose fructosyltransferase (SST) and β(2→1)fructan:β(2→1)fructan-1-fructosyltransferase (FFT). Firstly, the SST converts sucrose into glucose and an oligofructoside while further higher polymers are consecutively synthesized by FFT. The overall reaction mechanism was expressed by Edelman and Jefford (1968) as follows:

$$GF + GF \rightarrow GF - F + G \qquad \text{by SST} \qquad (2.6)$$

$$GF-F_n + GF-F_m \quad GF-F_{n-1} + GF-F_{m+1} \qquad \text{by FFT} \qquad (2.7)$$

where GF is a sucrosyl group and n is the number of extrasucrosyl fructose residues.

Gupta and Bhatia (1982) proposed a model for the fructosyltransferase in *F. oxysporum***.** They suggested that fructose is transferred from the donor site to the fructosylated nucleotide bridge and this, in turn, transfers the fructose moiety to the sucrose at the acceptor site to form GF_2. GF_4 was the highest glucofructosan, suggesting that the acceptor site is perhaps just big enough to accommodate up to GF_4.

Later, the mechanism of FTase synthesis derived from microbial sources was proposed. Jung *et al.* (1987) proposed a mathematical model for the mode of action of fructosyltransferase derived from *A. pullulans*. The enzyme reaction mechanism (Figure 2.1) can be expressed as follows:

$$GF_n + GF_n \rightarrow GF_{n-1} + GF_{n+1} \qquad n = 1-3 \qquad (2.8)$$

According to this mechanism, the enzyme acts on sucrose in a disproportionation type reaction where one molecule of sucrose serves as a donor and another acts as an acceptor.

In summary, most of the microbial fructosyltransferases may catalyze the reactions of a readily reversible primary step and a subsequent irreversible step as been described by Yun (1996). Possibly, the aldoside part of the substrate molecule is replaced by an enzyme linked group, and partial decomposition of this FOS precursor to aldose and ketose may furnish the energy necessary for FOS synthesis.

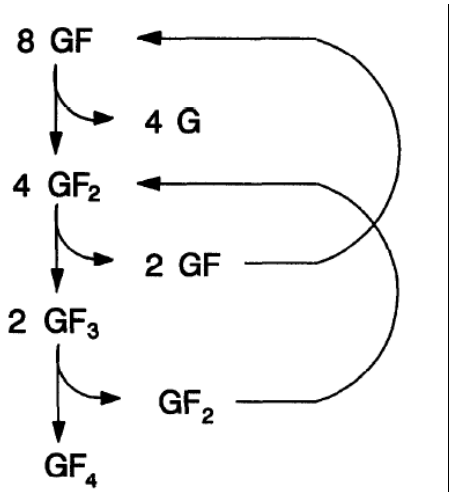

Figure 2.1: Network of reaction mechanism for the production of FOS from sucrose catalyzed by FTase derived from *A.pullulans*: G, GF, GF_2, GF_3 and GF_4 means glucose, sucrose, 1-kestose, nystose and 1^F-fructofuranosyl nystose (Yun, 1996)

2.2.3 Sources of Microbial FTase

A large number of microorganisms, including bacteria, yeast and fungi produce different groups of enzymes. Selection of a particular strain, however, remains a tedious task, especially when commercially competent enzyme yields are to be achieved (Pandey *et al.,* 2009). In addition to the conventional applications in food and fermentation industries, microbial enzymes have attained significant role in biotransformations involving organic solvent media, mainly for bioactive compounds achieved (Pandey *et al.,* 2009).

Microorganisms are generally preferred to plant and animal as source of industrial enzymes because their production cost is low. Enzyme contents are more predictable and controllable, easy availability of raw materials with constant composition for their cultivation. Different microbial sources of FTase reported in literature to produce FOS with different linkages to form 1-kestose, 6-kestose and neokestose in varying yields based on initial sucrose concentration (Sangeetha *et al.*, 2005b). It was also stated that microbial FTases are derived from bacterial and fungal

sources. In general, the enzymes (FTase) derived from microorganisms are bigger in size and more stable temperature-wise than those from plants (Yun, 1996).

2.2.3 (a) Bacterial FTase

Both Gram-positive and Gram-negative bacteria were used in the synthesis of the FTase enzyme. Fructosyltransferase from related genera is rather similar in amino acid sequence, but average of sequence identity between the enzyme from Gram-positive and Gram-negative bacteria is only 23%. As an addition, fructosyltransferase from several Gram-positive species required the presence of Ca^{2+} for optimum activity. It was also reported that all known bacterial FTase are extracellular or cell-bound proteins, although they follow different secretion routes (Banguela and Hernandez, 2006).

Bacillus subtilis is well known for its ability to produce an inducible and extracellular levansucrase. This enzyme (E.C. 2.4.1.10) is also recognized as a fructosyl transferase, but its products from sucrose are levan, a β (2→6) linked fructose homopolymer with some β (2→1) branching points (Le Gorrec *et.al.,* 2002). The authors had studied two strains, *B. subtilis* NCIMB 11871 and *B. subtilis* 11872. However, they found that both strain did not show higher FTase production as compared to the other two enzymatic activities, sucrase and polymerase.

Previously, Euzenat *et al.* (1997) also had studied the production of levansucrase from *Bacillus subtilis* C4. Results showed that *B.subtilis* levansucrase was much less specific in its catalysis of linkage formation than *A.niger* FTase, and the inulin production system of chicory root. In the experiment, only 32% mass yield was obtained based on consumed sucrose.

A new FTase produced by a new isolate of aerobic *Bacillus macerans* EG-6 showed mainly hydrolyzing activity at 10 g sucrose/L while transfructosylating activity were observed at 100 g sucrose/L (Kim *et al.*, 1998). However, it was found that fungus FTase (derived from *Aureobasidium* and *Aspergillus*) have broad spectra in substrate specificity compared to bacterial FTase, *Bacillus macerans*.

In 2000, Nam *et al.* had continued the work of Kim *et al.* (1998) by producing FTase from the strain *Bacillus macerans* EG-6. The optimal FTase concentration was 0.6 units per g sucrose. Among bacterial FTases, the β-fructofuranosidase I enzyme from *Arthrobacter* sp. K-1 showed a broad transfructosylating activity from sucrose to various acceptors such as mono and oligosaccharides, sugar alcohols, and saccharide derivatives, even though the self-transfructosylating activity was lower than that of fungal enzymes. The new FTase from *B. macerans* EG-6 used in this study did not display the self-transfructosylating activity in the presence of acceptor saccharides, while it showed narrow acceptor specificity, compared to the FTases from fungi and *Arthrobacter* sp.

Rozen *et al.* (2004) notified that *Streptococcus mutans* produced FTase enzyme, which synthesized fructan polymers from sucrose. FTase is produced from a single *S. mutans ftf* gene and synthesized large amount of inulin-type fructan polymers from sucrose and *Streptococcus salivarius* produced copious amount of levan-type fructans. The molecular weight (MW) of *S.mutans* was 87.6 kDa. Van Hijum *et al.* (2002) had reported the isolation and characterization of an FTF-encoding gene from *Lactobacillus reuteri* strain 121. Previously, it was reported that *Lactobacillus reuteri* strain 121 cultivated on medium containing sucrose produced both a glucan and a fructan polymer.

2.2.3 (b) Yeast FTase

Smith *et al.* (1982) had produced FTase enzyme from the black yeast *Aureobasidium pullulans*. The authors stated that any strain of *A. pullulans* is capable of producing FTase enzyme can be employed in the process. Suitable strains of this yeast include NRRL 3937, ATCC 12535, NRL 1673, NRRL Y 2311, NRRL YB 3892, ATCC 15223 and NRRL 3861. A particularly suitable strain is ATCC 9348. The yeast culture is maintained or preserved on agar slants with periodic transfer to maintain viability. In this process, over 80% of FTase enzyme is present in the broth.

According to Van Dooren *et al.* (1989), FTase can also be produced from a culture of the yeast *Pullularia pullulans*. The author pointed out that the cultures of yeast are usually more difficult to be processed further than cultures of fungi, and the cultures of yeast in particular are more difficult to be filtered. The thermal, stability of enzyme preparations derived from yeasts is lower, and at rising temperature the activity of such an enzyme preparation decreased sooner than the activity of enzyme preparations derived from fungi.

2.2.3 (c) Fungal FTase

Fungal species that produced fructans are basically included in the genera *Aspergillus, Aureobasidium, Penicillium, Fusarium, Pestalotiopsis, Myrothecium, Trichoderma* and *Phytophtora* (Banguela and Hernandez, 2006). The FTase enzymes from fungi catalyzed a self-transfructosylating reaction from sucrose at high sucrose concentration and produced a mixture of fructooligosaccharides, such as 1-kestose and nystose (Nam *et al.*, 2000).

Jung *et al.* (1987) has investigated the conditions for the production of intracellular FTase from a mutant derived from *Aureobasidium pullulans* KFCC 10245. The organism was maintained on agar slants containing stock medium (raw sugar 50 g/L; yeast extract 2 g/L; pH 5.5) at 4°C and subcultured biweekly. The inoculum was then transferred into the fermentation medium and cultured at 28°C for 4 days at180 rpm. The culture broth was centrifuged and the supernatant was used for the determination of extracellular enzyme activity. The centrifuged culture broth is treated with cell wall lysis enzyme Kitalase and the lysate were used for the determination of intracellular enzyme activity.

Later in 2004, Shin *et al.* also had studied the production of the FTase enzyme by *Aureobasidium pullulans* cells. The specific intracellular enzyme activity was the highest with the strain KCCM 12017, and the enzyme production closely coupled to growth. Three different species of *A.pullulans* were grown in batch cultures to compare their abilities to produce enzyme. The strains KCCM 12017 and KCTC 6353 had higher enzyme production rates as compared with *A.pullulans* KCTC 6789. When the strain KCCM 12017 and KCTC 6353 were compared for their final cell concentration, the strain KCCM 12017 has much lower cell concentration. The study also found that both the intra- and extracellular enzyme production were closely coupled to growth as the enzyme activity exceeded more than 60 IU/mL and 50 IU/mL, respectively.

Cell immobilization of *Aureobasidium pullulans* by calcium alginate was done by Shin *et al.* (2004b). The beads were then placed at -15°C for 6 - 24 h to induce freeze-dehydration. At this temperature, they found out that there was no

change in the enzyme activity or cell morphology. They also discovered that the relative enzyme activity with the dehydrated beads was only 35%.

Van Dooren *et al.* (1989) found that FTase enzyme preparation with favorable properties can be prepared by cultivating fungus *Aspergillus phoenicis* CBS 294 in a culture medium suitable for fungal cultures and recovering the mycelium from the culture medium. The authors reported that *A.phoenicis* can be cultivated on any other suitable substrate in any suitable manner and the formation of FTase is promoted by sucrose in the culture media. The production of FTase from *A. phoenicis* CBS 294.80 mycelium has been established in 1991 by Van Balken *et al.* where fresh sucrose solution was added after each run.

In 1995, Hang and Woodams had studied the FTase activity of commercial enzyme preparations used in fruit juice processing and its potential for the production of FOS from sucrose. Of twenty-two commercial fungal enzyme used in fruit juice processing, Pectinex Ultra-SPL was found to posses the highest activity of FTase (44.8 IU/mL). The reaction mixture contained the enzyme solution, 0.6 M sucrose, and 0.01M sodium acetate buffer (pH 5.6) in a total volume of 2 mL. The reaction was conducted at 40°C for 15 h. In the same year, Barthomeuf and Pourrat (1995) have reported the mixed enzyme system using crude FTase from a new strain of *Penicillium rigolosum* and glycosidase. Under optimized conditions, they were able to obtain a yield of 80% FOS.

Fructosyltransferase (FTase) production by *Aspergillus oryzae* CFR 202 was carried out by solid state fermentation (SSF), using various agricultural by products like cereal bran, corn products, sugarcane bagasse, cassava bagasse (tippi) and by-

products of coffee and tea processing (Sangeetha *et al.,* 2004b). Screening of few fungal strains for FTase activity resulted in the selection of *Aspergillus oryzae* CFR 202 as a potent strain based on high U_t/U_h ratio (U_t stands for transfructosylating activity and U_h for hydrolytic activity). The pellets of *A. oryzae* CFR 202 obtained after 48 h of fermentation were supplemented with fresh media after every 24 h. FTase activity was maintained in the range of 15±2 U/mL/min up to six recycles (Sangeetha *et al.,* 2005c). In this study, they designed a cell recycling system where the pellets produced being reuse for the next cycle of fermentation. Aside from the reuse of the biomass, this system also offers an advantage of no need for the supplementation of the media during recycling.

Han *et al.* (2005) had produced FTase from *Penicillium citrinum* KCTC 10225BP of soil origin. The authors have finally identified a novel microorganism (*P. citrinum)* which is capable of producing FTase having a high sucrose hydrolysis titer. The sucrose hydrolysis titer of FTase prepared from the *P. citrinum* was found to be 1.5 units/1g sucrose on average, which is better than any other fungal FTase. Enzyme derived from *Aspergillus* for example needed 5 units of the enzyme to degrade 1 g of sucrose and 7 units of enzyme derived from *Aspergillus awamori* to degrade 1 g of sucrose.

Fungal FTase has been proved for its ability to produce microbial FTase that have the higher enzyme activity compared to bacterial FTase and yeast FTase. Fungal FTase have advantage over broader spectra in substrate specificity and higher self-transfructosylating activity than bacterial FTase. Furthermore, fungal FTase can be cultivated on any suitable substrate in any suitable manner (Van Dooren *et al.* 1989; Kim *et al.* 1998; Nam *et al.* 2000). As for yeast FTase, its being found out that

cultures of yeast are more difficult to be filtered and the thermal stability is much lower than fungal FTase (Van Dooren *et al.* 1989; Smith *et al.* 1982)

2.2.4 *Penicillium simplicissimum*

Fungi play such an important role in human society, in which it could be readily argued that they are the most important biotechnologically useful organism. Traditional technologies that employ fungi include the production and flavouring of foods, and production of biochemicals such as citric acid and antibiotics like penicillin. Certain filamentous fungi have also traditionally been used to improve the flavour of cheese, while other are used in Asian cultures to produce food such as sufu, tempeh and miso (Wainwright, 1992). *Penicillium* species are widespread and cosmopolitan. They are frequently referred to as green or blue moulds and are often found contaminating citrus fruits or causing decay on refrigerated cheese and other foodstuffs.

Penicillium simplicissimum, a strain isolated from soil, had showed high galactosyltransferase activity when incubated in highly concentrated lactose solution (Cruz *et al.,* 1999). Luonteri *et al.* (1998) previously reported that substrate specificities of three α-galactosidase of this strain (AGLI, AGLII AND AGLIII) were determined using various isolated galactose containing oligosaccharides and polymeric galacto(gluco)mannans. In fact, this species had also been used to study the effect of glucose, ammonium, nitrate or phosphate limitation on the excretion of tricaboxylic acid (TCA) cycle intermediates in continuous system (Gallmetzer and Burgsteller, 2002). In terms of lead and copper biosorption, *Penicillium simplicissimum* was immobilized on loofa sponge in batch experiments (Li *et al.,* 2008).