EFFECT OF PH AND TEMPERATURE ON FRUCTOSYLTRANSFERASE (FTase) PRODUCTION FROM *CANDIDA KRUSEI*

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SUPERVISOR DECLARATION

I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology)

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STUDENT DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. This thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

Signature

Name	Mohd Nazarni Bin Che Isa
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In The Name of Allah, Most Gracious, Most Merciful

Love special dedicated to…

Special inspiring and special encouraging of my lovely parent: Che Isa bin Che Mat and Faridah Binti Zakaria;

My siblings (Kak Lin, Kak era and Nurul),

and

also my truly best friends,

Those who has influenced my life on the right course

Thank you so much

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EFFECT OF PH AND TEMPERATURE ON FRUCTOSYLTRANSFERASE (FTase) PRODUCTION FROM *CANDIDA KRUSEI*

ABSTRACT

The relationship between consumer, food and health become one of important issues because of increasingly demand on healthier food. Based on functional properties of fructosyltransferases (FOS), the consumer demands have been increasing in order to response on that. In Malaysia, it is reported that the number of diabetic patients is increasing daily and it is one of the fatal diseases. A significant driving force in the 'functional food' market place is the quest by consumers to optimize their health through food. The enzyme source synthesis production can be divided into two classes which are from microorganism and plant. Where by plant, the production is restricted from any sources .In view of that, production of crude enzyme from micro fungi is studied in order to obtain high yield of FOS. This study is carried out using sucrose as a substrate in the fermentation process to produce crude enzyme from micro fungi, namely *Candida krusei*. In this study, an investigation on pH and temperature of enzyme was analysing ranging from 3.0-8.0 and 25-55^{o}C, respectively. The crude enzyme was analysed using UV-Vis U-1800 Spectrophotometer at wavelength of 540 nm. The protein concentration was determined using UV-Vis U-1800 Spectrophotometer at wavelength of 750 nm. By using an extracellular enzyme which is fructosyltansferase (FTase) from selected micro fungi, the food-grade FOS can be produced commercially from sucrose. As result indicates that the highest pH and temperature for activity of FTase from *Candida krusei* is pH5.5 and temperature 55^{o}C. The highest enzyme activity (148.501 IU/mL) was figured out at pH 5.5, 55^{o}C and the specific activity was observes as 10.638 IU/mg. As a conclusion, this research was done to produce other alternative way of producing crude FTase using selected microfungi that are less harmfull to use in a production of FOS in food health industries.

KESAN DARI PENGGUNAAN PH DAN SUHU TERHADAP PENGHASILAN FRUKTOSILTRANSFERASE (FTase) DARI *CANDIDA KRUSEI*

ABSTRAK

Hubungan yang terjalin antara pengguna, makanan dan juga kesihatan menjadi salah satu isu yang penting kerana meningkatnya permintaan terhadap makanan yang sihat. Berdasarkan kepada ciri-ciri dan fungsi yang ada pada fruktosiltransferase (FOS), permintaan pengguna terhadapnya telah meningkat. Di Malaysia, telah dilaporkan bahawa bilangan pesakit kencing manis semakin meningkat setiap hari dan ia merupakan salah satu penyakit yang membawa maut. Satu daya penggerak penting dalam pasaran "makanan berfungsi" adalah usaha yang dilakukan oleh pengguna untuk mengoptimumkan kesihatan mereka melalui pemilihan makanan yang betul. Sumber penghasilan sintesis enzim boleh dibahagikan kepada dua kelas iaitu mikroorganisma dan juga tumbuhan. Jika dari tumbuhan, pengeluaran enzim adalah terhad dari mana-mana sumber. Sehubungan itu, pengeluaran enzim mentah dari kulat mikro telah dikaji dalam usaha mendapatkan penghasilan yang tggi dalam FOS. Kajian ini telah dijalankan menggunakan sukrosa sebagai substrat dalam proses penapaian untuk menghasilkan enzim mentah dari kulat mikro, iaitu *Candida krusei*. Dalam kajian ini, siasatan telah dijalankan keatas kesan pH dan suhu enzim yang telah dianalisis masing-masing antara 3.0-8.0 dan 25-55ºC. Enzim mentah dan kepekatan protein telah dianalisa dengan menggunakan UV-Vis U-1800 Spektrofotometer pada panjang gelombang 540 nm dan 750 nm. Dengan menggunakan enzim extracellular fruktosiltransferase (FTase) daripada kulat mikro yang dipilih, gred makanan berasaskan FOS boleh dihasilkan secara komersial daripada sukrosa. Hasil keputusan menunjukkan bahawa pH dan suhu paling tinggi untuk aktiviti FTase enzim dari *Candida krusei* adalah pada pH 5.5 dan suhu 55ºC. Aktiviti enzim yang tertinggi (148.501 IU/mL) telah dicatatkan pada pH 5.5, 55ºC dan aktiviti tertentu telah diperhatikan pada nilai 10.638 IU/mg. Sebagai kesimpulannya, kajian ini dilakukan untuk menghasilkan cara alternative lain dalam menghasilakn FTase mentah menggunakan mikrofungi terpilih yang kurang berbahaya untuk digunakan dalam penghasilan FOS dalam industri makanan kesihatan.

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LIST OF SYMBOL

GF_2	1-kestose
GF_3	nystose
GF_4	1-β-fructofuranosylnystose
g/L	gram over liter
w/w	weight over weight
w/v	weight over volume
IU	1 unit of enzyme

LIST OF ABBREVIATION

US	United Stated
BSA	Bovine serum albumin
DNS	Dinitrosalicyclic acid
FFase	Fructofuranosidase
FFT	Fructan-1-fructosyltransferase
FOS	Fructooligosaccharides
FOSHU	Food of Specified Health Used
FTase	Fructosyltransferase
OD	Optical density
YEA	Yeast extract agar
SST	Sucrose 1-fructosyltransferase

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The global market of functional foods is estimated up to 33 billion U.S dollar. It was generated from large country such as United Stated, Japan and among European countries like Germany, France, the United Kingdom and Netherlands. Specific health related food category called FOSHU (Foods of Specified Health Use) in Japan is one another important market of functional foods in industries. According to Sangeetha *et al.*,(2005) said that an estimated market value of FOSHU labelled products was 2 billion U.S dollar in year 2000 with an estimated turnover of 14 billion US dollar. An estimated market value of FOSHU labelled products was 2

billion US dollar in year 2000 with an estimated turnover of 14 billion US dollar (Sangeetha *et al*., 2005) .

Yet, various forms of functional foods have already been introduced into the market in a large quantity which contains a number of characteristic functional ingredients such as include dietary fiber, oligosaccharides, sugar alcohols, peptides and proteins, prebiotics and probiotics, phytochemicals and antioxidants and polyunsaturated fatty acids (Sangeetha *et al.*, 2005).

Dietary carbohydrates have been caught a lot of attention especially carbohydrates, in particular, fructooligosaccharides (FOS). Their popularity has been increased as food ingredients because of the possible health beneficial associated with the consumption of these compounds and also being promoted as alternative sweeteners for diabetic formulations. According to Flamm *et al.*, (2001) average daily consumption of FOS has been estimated to be 1–4 g in US and 3–11 g in Europe. The most common sources of FOS are wheat, honey, onion, garlic and banana (Flamm *et al.*, 2001).

For the past two decades, research work has been carried out all over the world on the production, properties, analytical aspects and nutritional benefits of FOS. Many review articles describing the occurrence, preparation, properties and applications of FOS (Crittenden & Playne, 1996; Yun, 1996; Slavin, 1999) have been published. Flamm *et al.* (2001) have critically reviewed the composition and source of FOS, its physiological effects upon consumption, and its relation to the dietary fibre concept.

The production of fructooligosaccharides (FOS) has received particular attention in recent years because of their excellent biological and functional properties and necessities on the development of efficient enzymatic systems. FOS

posses extraordinary importance as functional food ingredients owing to their prebiotic properties (Sangeetha *et al.*, 2005).

FOS is composed of 1-Ketose (GF2), nytose (GF3) and F-fructofuranosyl (GF4) in which fructosyl units are linked at β-2,1 positions of sucrose. Neo-frustooligosaccharides (neo-FOS) with β-2,6 linkage between two fructose units (6F-FOS; 6-Ketose) or between fructose and a glucosyl group (6G-FOS; neo Kestose, neo nystose and neo-fructofuranosylnystose) have a structure different from that of FOS and might have better prebiotic properties and chemical stability compared to FOS (Chen *et al.*, 2010).

FOS production at industrial scale is commercially produced using two classes of enzymes either microbial fructosyltransferases or β-fructofuranosidases also called invertases. The enzyme source synthesis (FTase) can be divided into two classes which are from plants and microorganisms. FTase is produced intra- and extracellular by several fungi.

The enzymes source of FOS synthesis or known as fructosyl-transferring enzymes have been purified and characterized into two classes; one is higher plants such as asparagus roots, onion bulbs, Jerusalem artichoke; and the other one consists of different organisms which are bacteria and fungi such as *Aspergillus niger, Aspergillus japonicus, Bacillus macerans, Schwanniomyces occidentalis*, *Candida utilis* (Ghazi *et al.*, 2007) *Aureobasidium* sp., *Aspergillus* sp., *Arthrobacter* sp. and *Fusarium* sp. (Park *et al.*, 2001).

1.2 Problem Statement

Many bioproducts are enzymes derived from plant, animal or microorganisms and there is a great demand for their production. Fructosyltransferase is one of the enzymes that are being derived from microorganisms such as *Aspergillus niger*, *Aspergillus japonicus*, *Bacillus macerans*, *Schwanniomyces occidentalis*, *Candida utilis* and *Candida krusei* (Ghazi *et al.*, 2007).

Purification and preliminary characterization of this enzyme from various sources have been reported. However, until now the accumulated information on fructosyltransferase is rather confusing differing from one source to another, from one microorganism to another, even from one strain to another. The source of enzyme and the difficulty of purification no doubt influenced the results obtained and thus the conclusion drawn. The purification and the characterization of this enzyme are necessary steps to improve our understanding of its mode of action, the nature of the hydrolytic activity and to decide the type of enzyme in which it should be classified (L'Hocine *et al.*, 2000).

Most of enzyme produced from microbial such as bacteria and fungi are harmful to human health. Thus, this study was proposed to solve that problem by using less harmful microbial which is *Candida krusei* by doing study on the enzyme production. By using this microbial behaviour, it can improve the fructosyltransferase production by using nature materials which is less harmful, reduce the production cost and make it more environmental friendly.

This research was designed to determine the optimum condition of temperature and pH in fermentation broth to acquire the highest amount of

fructosyltranferase produce by chosen microbe. Thus, the target of this research to study the production of fructosyltransferase enzyme by using *Candida krusei*.

1.3 Research Objectives

The main objective of this research is to study the effect of pH on enzyme production from *Candida krusei* using sucrose as a substrate

The measurable objectives of this research are:

a) To determine the effect of incubation time of fructosyltransferase production using *Candida krusei*.
b) To study the effect of temperature on crude enzyme produced from *Candida krusei*
c) To study the effect of pH on crude enzyme produced from *Candida krusei*
d) To determine the activity and specific activity of enzyme produced from *Candida krusei*

1.4 Scope of Study

FTase produced from microfungi derived from sucrose is more preferable. In view of that, production of crude enzyme from microfungi is studied in order to obtain high yield of FTase. This study is carried out using sucrose as a substrate in the fermentation process to produce crude enzyme from microorganism. This study is beginning with the cultivation of the enzyme. The microorganism is maintained on

agar petri plate at 4^{o}C. The inoculums is developed by transferring the mycelia from a 3 days old agar petri plate into the inoculum medium consist of (g/l) 10 sucrose, 3 yeast extract at pH 5.5. The flask was incubated for 24 h at 30^{o}C on a rotary shaker.

After 24 h, the inoculum was transferred into 250 ml of fermentation medium consisting of (g/l) 30 sucrose, 5 yeast extract, 3 peptone, 0.5 magnesium sulphate heptahydrate and 0.5 kalium phosphate. The inoculum will undergo fermentation and transform into biomass cell. Then it will be separated to obtain the supernatant which is source of enzyme. The enzyme is assayed with different temperature, pH and incubation time range in order to determine the effect of it towards enzyme activity produced *by Candida krusei*. Then, the activities of crude enzymes and protein concentration is analysed by using correlation of glucose standard curve from UV-Vis Spectrophotometer for the enzymatic activities.

1.5 Rationale and Significance

Fructosyltransferase (FTase) enzymes were widely used as a biocatalyst in the production of fructooligosaccharides (FOS) in many food and beverage industries. There are a lot of sources with fructosyltransferase enzyme production in industries nowadays. These researches were conducted in order to improve the amount of fructosyltransferase which produce by chosen bacteria such as *Candida krusei.* Moreover, the rationale of this paper to determine the optimum conditions of fructosytransferase production by monitoring parameters such as time and pH used. As known, fructosyltransferase can be produced from chemical and microbial production. This microbial techniques were resulted with reduces the cost production

of fructooligosaccharides and increase the demand on manufacturing using this technique. It also was more feasible, economical, environmental friendly and convenient suit to enzyme and biotechnology field.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The production of fructooligosaccharide (FOS) has been an interest at the industrial level for the world market. Mainly, the microbial production of FOSs is enzymatic reaction has been selected rather than chemical synthesis due to acknowledge as being more feasible, economical, environmentally friendly and more convenient. According to research done by sangeetha et al. (2005) said that basically this method requires the two stage experimental work. The first stage refers to the production of enzyme which is fructosyltransferases (FTase) from microbes and finally FTase will be used for the second stage that is reaction with sucrose to produce FOS.

In this study, the enzymatic reaction will be remaining but the process of microbial production of FTase will be focused more as an objective. Besides, the fungal *Aspergillus niger* and the yeast of *Candida krusei* species were also has been introduces as the new sources of FTase production. These approaches will be studied and discussed in this research for the new technique beyond industrial application for FTase production that know is really important in human's health and life.

2.2 Sucrose

Sucrose is one of the organic compounds that are widely known as table sugar and sometimes called saccharide. It has physical properties such as white, odourless, crystalline powder with a sweet taste. The best features of sucrose commonly known as best in nutritional role. It is produced naturally by all plants as food by combining glucose and fructose molecules during the process of photosynthesis with the molecular formula $C_{12}H_{22}o_{11}$ (Klaus *et al.*, 2002).

The sucrose molecule falls into multiple biochemical classes. It is a carbohydrate, meaning that it's composed of carbon, hydrogen and oxygen. More specifically, it is a small, sweet-tasting carbohydrate. Sucrose is composed of two smaller sugar units, specifically glucose and fructose. Sucrose is quite common in nature, occurring in many fruits and other plant parts (Klaus *et al.*, 2002).

2.3 Oligosaccharides

Oligosaccharides are carbohydrates which have 3-10 simple sugars linked together. They are found naturally, at least in small amounts, in many plants. Plants with large amounts of oligosaccharides include chicory root, from which most commercial inulin is extracted, and so-called Jerusalem artichokes (the root of a member of the sunflower family). They are also found in onions (leeks and garlic), legumes, wheat, asparagus, jicama, and other plant foods (Maiorano *et al.*, 2008). Most of oligosaccharides have mildly sweet taste, and have certain other characteristics, such as the mouth feel they lend to food, that has drawn the interest of the food industry as a partial substitute for fat and sugars in some foods as well as improved texture. Because of this, more and more of the oligosaccharides in food synthetically produced. Recent interest has also been drawn to oligosaccharides from the nutritional community because of an important characteristic: the human digestive system has a hard time breaking down many of these carbohydrates (Laura Dolson, 2009).

2.4 Fructooligosaccharides (FOS)

Fructooligosaccharides (FOS) also known as oligofructose or oligofructan used as alternative sweetener. Fructo-oligosaccharides are made up of plant sugars

linked in chains. FOS exhibits sweetness levels between 30 and 50 % of sugar in commercially-prepared syrups. Its molecular structure of GF-Fn, (n = 1-9). They are most taken from asparagus, Jerusalem artichokes, and soybeans. It occurs naturally, and its commercial use emerged in the 1980s in response to consumer demand for healthier and calorie-reduced foods (Noraziah *et al.*, 2011). People use this sugar to make medicine and known as nutrition, health care, the health effects trinity of the twenty-first century, a new sugar source (Noraziah *et al.*, 2011).

FOS is an important composition of human diet. It has significant effect to improve bowel function, prevent constipation and diarrhea, lower blood lipids and the human immune system. It is because fructooligosaccharides having a wide variety of physiological functions and superior physical and chemical properties.

2.4.1 Physical and Chemical Properties

FOS are about half as sweet as sugar but with a fraction of the calorific value and as they were first developed as a low calorie, low cariogenic, sugar substitute. However, as awareness has grown of the differential effects that FOS has on the human microflora, it has become the standard bearer of ‘prebiotics’ as a new type of nutritional supplement. According to the Zhengzhou Friend Biological Engineering Co., Ltd stated that FOS was having a variety of physical and chemical properties and also the health indicator as shown in Table 2.1.

Table 2.1 Physical and chemical properties of FOS

Item	Indicators
Physical	
Solid (20 refractive count method)%≥	75
Total content%≥	50,70,90
Chemical	
PH Value	4.5-7.0
Arsenic (As)mg/kg≤	1.0
Lead(Pb)mg/kg≤	0.5

(Source: Zhengzhou Friend Biological Engineering Co., Ltd)

2.4.2 Functional Properties of FOS

Regular use of fructooligosaccharides can cause health effect to users. This has been proven through research study by Sangeetha *et al.,* (2005). Studies with inulin and FOS have shown reduction of chemically induced aberrant crypts and prevention of colon cancer. According to Pool-Zobel *et al.*,(2002) in rats, a prebiotics effect resulting in the proliferation of bifidobacteria (with major metabolites lactate or acetate) as well as of other bacteria could be responsible for the observed anticancer effects. According to Henryk & Marcel (2005), dietary treatment with inulin/oligofructose (15%) incorporated in the basal diets for experimental animals resulted in (a) reduction of the incidence of mammary tumors induced in Sprague Dawley rats by methylnitrosourea (b) inhibited the growth of transplantable malignant tumors in mice and (c) decrease the incidence of lung metastases of a malignant tumor implanted intramuscularly in mice. It is reported that the dietary

treatment with FOS/inulin significantly potentiated the effects of subtherapeutic doses of six different cytotoxic drugs commonly utilized in human cancer treatment (Sangeeta *et al.*, 2005).

2.4.3 Application of Fructooligosaccharides

There have been reported that FOS was used in many application such as medical, human defences system, and metabolism and also food formulations were using FOS as one of ingredient in the products. It makes fructooligosaccharides become as a target point in the many research nowadays even a long time ago because of their potential to become dietary carbohydrate that are also being promoted as alternatives sweetener for diabetic patience. Table 2.2 indicates some of the application involve with FOS.

Table 2.2 Application of FOS

Application	Description	References
Prebiotic	-Investigation have been performed that the prebiotic of FOS by studying the metabolism of two types of chicory fructooligosaccharides by *Bifidobacterium longum, B. infatis and B. angulatum.* Biomass production was highest with *B. infatis* (1.4 and 1.7 g dry wt/l) for it cultivation in a medium supplemented with fibruline instant as substrate	Durieux *et al.,* (2002)

Table 2.2 Application of FOS (continued)

Application	Description	References
Dietary fibre	-Dietary fibre consists of remnants of edible plant cell polysaccharides and associated substances resistant to hydrolysis by human alimentary enzyme which may benefit health through a wide range of physiological effects. FOS resists digestion and absorption in the stomach and small intestine of human, as shown by their full recovery at the end of the ileum of healthy or ileostomised volunteers.	Sangeetha et al., (2005)
	-Studies in patients with a conventional ileostomy have shown that mean excretion of FOS at the end of ileum was about 90% of the ingested dose	Cherbut (2002)
Mineral Absorption	Colonic fermentation of FOS leads to decrease in pH in the colon and this facilitates the absorption of minerals ions from intestine, mainly calcium and magnesium. This has been indicate by long term beneficial effects on bone health such as accumulation of bone mineral content in growing rats or prevention of bone loss in ovariectomised rats	Sangeetha et al., (2005)
Food Formulation	Inulin and Oligofructose are ingredients that are deliver a number of important nutritional benefits as well as contribute functional properties that enhance shelf life and taste profile of various food products like nutrition bars. In Ice cream production, FOS can be used with inulin to replace all the sugar and reduce the fat content and give excellent mouthfeel characteristics.	Sangeetha et al., (2005)

2.5 Frutosyltransferase (FTase)

Generally, enzyme is a high molecular-weight protein or protein like substance that acts on a substrate which is reactant molecule to transform it chemically at a greatly accelerated rate, normally 100 times above faster than the

uncatalyzed rate. Without enzymes, essential biological reactions would not take place at necessary to sustain life. Enzymes are usually present in small quantities and not consumed during the reaction occurred nor do they affect the chemical reaction equilibrium. Alternative pathway is provided by enzymes for the reaction to occur thereby requiring lower activation energy (Ghazi *et al*, 2007).

Fructosyltransferase (FTase) is an enzyme that catalyses the transformation of sucrose into fructooligosaccharides (FOS), which are important prebiotic compounds having a broad application in food and pharmaceutical industries. Fructosyltransferase catalyses the transfer of fructosyl moieties where a donor or acceptor of these moieties can be sucrose or fructooligosapcharides. In the industrial production of fructooligosaccharides, the cells with FTase activity are produced by aerobic cultivation of fungi such as *Aspergillus niger, Aspergillus japonicas,* or *Aureofusidium pullulans*. They are applied for the biocatalytic process in immobilized form (Afizan, 2009).

2.5.1 Mechanisms of Fructosyltransferase (FTase)

Fructosyltranferase (FTases) is the enzymes that responsible for microbial production of FOS. FTase produces FOS (GFn) from sucrose (GF) in a disproportionate mode, thereby forming 1-kestose (GF2) initially, then l-nystose (GF3), followed by 1- fructofuranosyl nystose (GF4) as explained by Yun (1996). Microbial FTase is derived from bacterial and fungal sources.

In the literature, different microbial sources of FTase are reported to produce FOS with different linkages to form 1-kestose, 6-kestose and neokestose in varying

yields based on initial sucrose concentration is also discussed. By reviewing Sangeetha *et al.*,(2005) the uses of immobilized enzymes and cells have led to the development of effective and economic methods for large-scale production of FOS. Forced flow membrane reactor systems, biocatalyst system with a bioreactor equipped with a microfiltration systems, have been used for production of high content FOS by removing the released glucose and unreacted sucrose from the reaction mixture resulting up to 98% (w/w) FOS. The use of mixed enzyme system of fructosyltransferase and glucose oxidase or glucose dehydrogenase could produce highly concentrated FOS up to 90 to 98% (w/w). Nano-filtration for removing glucose resulted in FOS of $90^{o}/_{o}$ (w/w) concentration. The purified enzyme was found to produce kestose and nystose unlike the crude enzyme which produced GF5 and GF6 oligosaccharides (Sangeetha *et al*., 2005).

The reaction mechanism of the FTase is depending on the type of source of the enzyme. Most of the microbial FTases have a high regiospecificity which selectively transfer the fructofuranosyl moiety of sucrose to the 1-OH furanoside of the other sucrose molecule (self-transfer), forming 1-kestose family of fructooligosaccharides (*O-β*-D-(fructofuranosyl)n-(2 $\longrightarrow$ 1)-α-Dsucrose, n<4). The proposed reaction mechanism is as follows:

Sucrose (GF) + sucrose (GF) $\longrightarrow$ 1-kestose (GF2) + glucose (GF) (2.1)

1-kestose (GF2) + 1-kestose (GF2) $\longrightarrow$ nystose (GF3) + sucrose (GF) (2.2)

Overall, GFn + GFn $\longrightarrow$ GFn-1 + GFn+1 (2.5)

According to this mechanism, equation 2.1 explained that 1F-fructofuranosyl nystose (GF4) forms by the following reaction step: GF3 + GF3 $\longrightarrow$ GF2 + GF4. In addition,

1F-fructofuranosyl nystose can also form by utilizing nystose and sucrose as describe in equation 2.2 (GF3 + GF ⟶ G + GF4). These two reaction mechanisms differ according to enzyme source (van Balken *et al*., 1991; Yun, 1996). Provided that the fructooligosaccharide (GF4) produced by the new FTase is 1F-fructofuranosyl nystose, 1-kestose and nystose should be accumulated to form GF4 throughout the reaction.

2.5.2 Bacterial FTase

FOS producing enzymes are very rare in bacterial strains but there have been reported that inulinases are produce by bacterial stains. Sangeetha *et al*., (2005) was said that a transfructosylating enzyrme, which produces FOS from sucrose, has been isolated from Bacillus *macerans* EG-6 which unlike other. Several microorganisms capable of producing FTase have been screened. Table 2.3 shows a list of microorganisms reported to produce FTase enzyme.

Table 2.3 List of microorganisms able to produce FTase enzyme

Source	Referrence
Fungal	
Aweobasuduim pullulans	Yun, 1996
Aureobasidium sp	Yun, 1996
Aspergillus iaponica	Yun, 1996
Aspergillus niger	Yun, 1996
Aspergillus sydowi	Yun, 1996
Calviceps purutria	Yun, 1996
Penicilium frequent ant s	Yun, 1996
Penicilium spinolosum	Yun, 1996
P hytophtora parasitica	Yun, 1996
Scopulmiopsis br evicauli s	Yun, 1996
Sac chm omice s c er evi s ae	Yun, 1996
Penicilium citrinum	Hatashi et al., 2000

Table 2.3 List of microorganisms able to produce FTase enzyme (continued)

Source	Reference
Plant	
Agave Americana	Yun, 1996
Agave vers crrz	Yun, 1996
Asparagus ffieinalis	Yun, 1996
Alliurn cepa	Yun, 1996
Chicorium intybus	Yun, 1996
Crinum longifoliurn	Yun, 1996
Sugor beet leoves	Yun, 1996
Heliantlrus toberosus	Yun, 1996
Lycoris radiqte	Yun, 1996
Taraxicum oficinale	Yun, 1996
Bacterial	
Artrobacter sps	Yun, 1996
Bacilus macerans	Park et al., 2001

(Source: Sangeetha *et al.,* 2005)

FTases, produced selectively GF5 and GF6 fructooligosaccharide. The final yield of FOS was reported to be 33% (w/w) when 50 % (w/w) sucrose was used as substrate (Park *et al.,* 2001). The ethanol producing bacteria *Zymomonas mobilis* has been reported to produce a levansucrase capable of producing FOS and levan. Referring to Beker *et al*.,(2002) ,the extracellular levansucrase that precipitated along with levan after ethanol treatment of culture fluid has been used as a biocatalyst for FOS production in sugar syrup. The yield of FOS was found to be 24 to 32% (w/w), which constituted a mixture of 1- kestose 6-kestose, neokestose and nystose. Glucose content was found to increase during all 24 h of reaction. The presence of ethanol (7.0%) in sucrose syrup limited the enzyme's FOS forming activity to 24% (w/w) during the first 24 h of incubation. Fructan syrup produced from sucrose using levan-levansucrase sediment as biocatalyst was reported to have satisfactory taste, reduced

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

Lactobacillus reutri strain 121 reviewed by Schwab *et al.,* (2007) has been reported to produce 10 g,/L, FOS (95% (w/w) kestose and 5 % (w/w) nystose in the supernatants when grown in sucrose containing medium. FTase isolated from the stain when incubated with sucrose, produced FOS as well as inulin. After 17 h of incubation with sucrose, 5.1 g/L FOS and 0.8 g/L inulin were synthesized. With the increasing demand for FOS in the functional foods market, there is always scope for discovery of novel sources of FTase (Sangeetha *et al*., 2005).

2.5.3 Fungal Ftase

Several fungal strains especially of *Aspergillus* sp., are known to produce extracellular or intracellular FTase. *Aspergillus niger* AS 0023 has been reported to produce an intracellular FTase which yielded 54% (w/w) FOS using 50% (w/w) sucrose as substate (L'Hocine *et al*., 2000). Purification and partial characterization of fructosyl fransferase and invertase from the cells of *Penicillium citrinum* have been reported (L'Hocine *et al*., 2000) to produce a syrup containing neofructooligosaccharides wherein the efficiency of FOS production with more than 55% using 70% sucrose as substrate. Production of FOS from sucrose catalyzed by β-Fructofuranosidase (FTase) was achieved by Chien *et al*. (2001) with the use of mycelia as *Aspergillus japonicus* immobilized in gluten. One gram of mycelia-immobilized particles having a cell content of 20% (w/w) was incubated with 100 ml sucrose solution with an initial concentration of 400 g/mL. After a reaction period of

5 h, the FOS yield was 61 percent of the total sugars. The reaction velocity increased with the cell content in the gluten matrix and a maximum value was obtained when the cell content was as high as 20% (w/w) (Sangeetha *et al*., 2005).

Researchers have reported *Aspergillus oryzae* as a novel source of extracellular FTase (Sangeetha et a1.,2003). The cultural conditions and reaction parameters have been standardized get FOS yield of 58% (w/v) (Sangeetha *et al.,* 2002). Culture fluid, cells and culture broth homogenate of *A. oryzae* CFR 202 and *A. pullulans* CFR 77 have also been used for FOS production to get up to 60% FOS (Sangeetha *et al*.,2004). Table 2.4 shows the summary of the FOS yields obtained using FTase from various microorganisms.

Table 2.4 FOS yields obtained using FTase from various microorganisms

Source	Substrate (g/L sucrose)	Yield (Percent)	Reference
Aspergillus niger AS 0023	500	54	L'Hocine *et al.* (2002)
Penicillium citrinum	700	55	Hayashi *et al*. (2002)
Aspergillus japonicas	400	61	Chien *et al. 2001*
Aspergillus oryzae CFR 202	600	58	Sangeetha *et al* (2002)
Aureobasidium pullulans CFR77	550	60	Sangeetha *et al* (2004a)
Bacillus mncerans EG-6	500	33	Park *et al.* (2001)
Zymomonas mobilis	500 – 600	24 – 32	Beker *et al*. (2002)

2.5.4 Fermentative Method of FTase production

There are two methods that can be used to produce FTase production which are submerged fermentation (SmF) and solid state fermentation (SSF). Production of enzymes by SSF has potential advantages over SmF with respect to simplicity in the operation, high productivity fermentation, less favourable for growth of contaminants and concentrated product formation. SSF requires less space capital and operating costs, simpler equipment and the downstream processing is easier compared to SmF. In addition, it permits the use of agro-industrial residues as substrates, which are converted into bulk chemicals and fine products with high commercial value. There are many reports on FTase production by SmF using various microorganisms. Sangeetha *et al.* (2000) have discussed FTase production by SSF in detail. However, SSF has not been attempted for FTase production except for the SmF report using apple pomace as substrate (Sangeetha *et al.*, 2005).

Researcher have screened an array of substrates like cereal bran, corn products, by-products of coffee and tea processing industries, sugar cane bagasse and cassava bagasse for the production of FTase by *A. oryzae* under SSF conditions. In addition, alternate sources of sucrose like jiggery and sugarcane juice were used for the production of FOS using FTase from *A. oryzae*. This has resulted in value addition of the agricultural by-products to prepare a high value product like FOS (Sageetha *et al*., 2005).

2.6 Features of *Candida krusei*

According to Samaranayake *et al.* (1994) said that in contrast to a majority of other *Candida sp.* which are ovoid in shape, the cells of *Candida krusei* are generally elongated and have the appearances of "long grain rice". A feature which the *Candida krusei* measures 2.2 -5.6 x 4.3 -15.2 µm with wide variation in the length and breadth of the isolates. The macrostructure of *Candida krusei* shows in Figure 2.1.

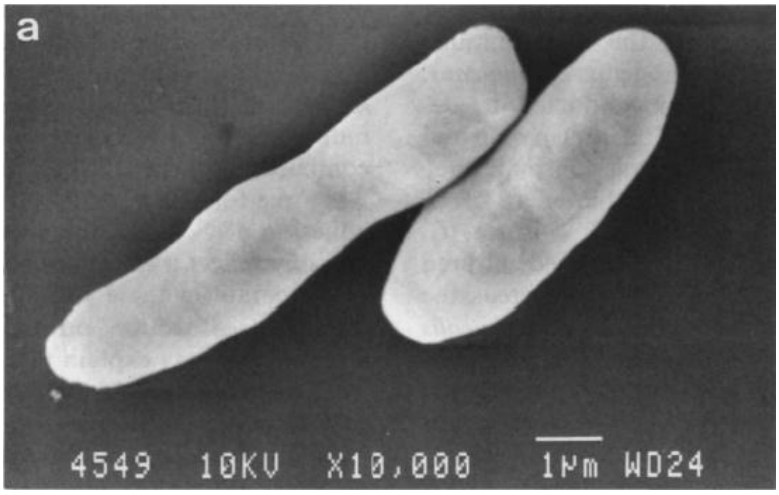

Figure 2.1 Macrostructure of *Candida krusei*

(Source: Samaranayake, 1994)

2.6.1 Application of *Candida krusei*

There is nearly 150 asporogeneous yeast species presently classified in the genus *Candida*. Of these, *C. albicans, C. tropicalis,* and *C. glabrata* comprise more than 80 percent of clinical Candida isolates while others such as *C. krusei, C.*

parapsilosis, C. guil;iermondii and *C. kefyr* are isolated sporadically and are thought to be less virulent. The yeast belonging to the genus *Candida* were first discovered by Langenbeck in 1839 from buccal aphtae in a patient with typhus, but the suggestion that *C. krusei* may cause disease in man was proposed by Castellani' more than 75 years later. Since then, this organism has been generally recognised as a commensal in warm-blooded animals with very low pathogenicity and virulence (Samaranayake *et al.*, 1994).

Candida krusei is a rare type of *Candida* that is mainly found in immunocompromised human hosts. There have been reports that *Candida krusei* infections have been associated with high mortality rate, particularly among hospitalized patients. Its prevalence in hospital environments has been associated with the prophylactic use of antifungals. Like *Candida parapsilosis, Candida krusei* has a high natural resistance to standard anti-fungal treatments.

According to Medical Microbial Journal reported by Samaranayake *et al.,* (1994) *Candida krusei* is an emerging fungal pathogen found primarily in immunocompromised patients. Intrinsic resistance to fluconazole and decreasing susceptibility to other anti-fungal agents are problematic. When colonization occurs, lymphoblastic leukemia who despite being treated prophylactically with fluconazole, developed disseminated *C. krusei.*

2.7 Characterization of enzyme

There are several factors that are used to characterize the enzyme production which produced from microbial such as effect of temperature, pH, substrate concentration, fermentation time, incubation time, protein concentration, protein purity and also determination of kinetic parameters. In this study, the effect of pH and temperature was characterized in order to study about FTase enzyme produced from *Candida krusei*.

2.7.1 The effect of pH

The productivity and efficiency of FTase enzyme produced are significance in sucrose transfructosylation at pH condition that is used in fermentation. Different pH applied for working condition can give effect on productivity and efficiency of enzyme because it can have an affect on the state of ionization of acidic or basic amino acid. If the state of ionization of amino acids in a protein is altered, then then ionic bonds that help to determine the 3-D shape of the protein can be altered.

Previous studies indicate in their research that most of efficiency and productivity of enzyme was analyse using combination effect of pH and acetate buffer (L'Hocine *et al.,* 2000). The pH and acetate buffer on FTase produced from *Candida sp*. were studied at various pH range. The result showed that the suitable pH

range for FTase production and enzyme activity was pH 5.0 - 6.0 respectively (Ghazi *et al.,* 2007).

2.7.2 The effect of Temperature

The temperature of a system is a measure to some extent of the kinetic energy of the molecules in the system. Thus, the lower the kinetic energy, the lower the temperature of the system was and, likewise, the higher the kinetic energy, the greater the temperature of the system. Increases in the temperature of a system results from increases in the kinetic energy of the system. This has several effects on the rates of reactions.

Firstly, more energetic collisions occur in the system. By referring to Cook and Cleland (2008) said that when molecules collide, the kinetic energy of the molecules can be converted into chemical potential energy of the molecules. If the chemical potential energy of the molecules becomes great enough, the activation energy of an exergonic reaction can be achieved and a change in chemical state will result. Thus the greater the kinetic energy of the molecules in a system, the greater is the resulting chemical potential energy when two molecules collide. As the temperature of a system is increased it is possible that more molecules per unit time will reach the activation energy. Thus the rate of the reaction may increase.

Secondly, the number of collisions per unit time will increase. In order to convert substrate into product, enzymes must collide with and bind to the substrate at the active site. Increasing the temperature of a system will increase the number of collisions of enzyme and substrate per unit time. Thus, within limits, the rates of the reaction will increase. Thirdly, the heat of the molecules in the system will increase. As the temperature of the system is increased, the internal energy of the molecules in the system will increase. Some of this heat may be converted into chemical potential energy. If this chemical potential energy is increase to some amount, some of the weak bonds that determine the three dimensional shape of the active proteins maybe broken. This could lead to a thermal denaturation of the protein and thus inactivate the protein. Thus too much heat can cause the rate of an enzyme catalysed reaction to decrease because the enzyme or substrate becomes denatured and inactive (Cook and Cleland, 2008).

Different enzyme produced by different microbial given different enzyme activity at optimum temperature. According to Chavez *et al.*, (1997) in the research done by him on *Candida sp.* shows that the effect of temperature on sucrose hydrolysis by *Candida utilis* was assayed in the temperature range from 40 – 80^{o}C and optimum activity was detected at 55^{o}C.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Equipments

The chemicals and equipment used in this study is of analytical grades, and they are summarizes in Table 3.1 and Table 3.2.

Table 3.1 List of equipment used

Equipment	Brand	Principal Used
Rotary shaker	Infors Ht	Fermentation
Refrigerated centrifuge	Eppendorf	Fermentation
Shaking water bath	BS-21	Enzyme assay
UV-Vis Spectrophotometer	U-1800	Analysis
Electrophoresis	Genei	Analysis
Peristaltic pump	Watson and Marlow	Purification

Table 3.2 List of chemical used

Chemical	Supplier	Principal used
Sucrose	Sigma, Aldrich	Fermentation
Yeast extract	Sigma, Aldrich	Fermentation
KH_2PO_4(monosodium phosphate)	Sigma, Aldrich	Fermentation
K_2HPO_4(Disodium phosphate)	Sigma, Aldrich	Fermentation
$Mg.SO_4.7H_2O$ (magnesium sulphate heptahydrate)	Sigma, Aldrich	Fermentation
Sodium Hydroxide(NaOH)	Sigma, Aldrich	Analysis
Sodium Citrate Dihydrate	Sigma, Aldrich	Analysis
Acetonitrile	Sigma, Aldrich	Analysis
Ammonium sulphate	Sigma, Aldrich	Analysis
Potassium sodium tartrate	Sigma, Aldrich	Analysis

3.2 Overview of Methodology

Figure 3.0 summarizes the overview of this study.

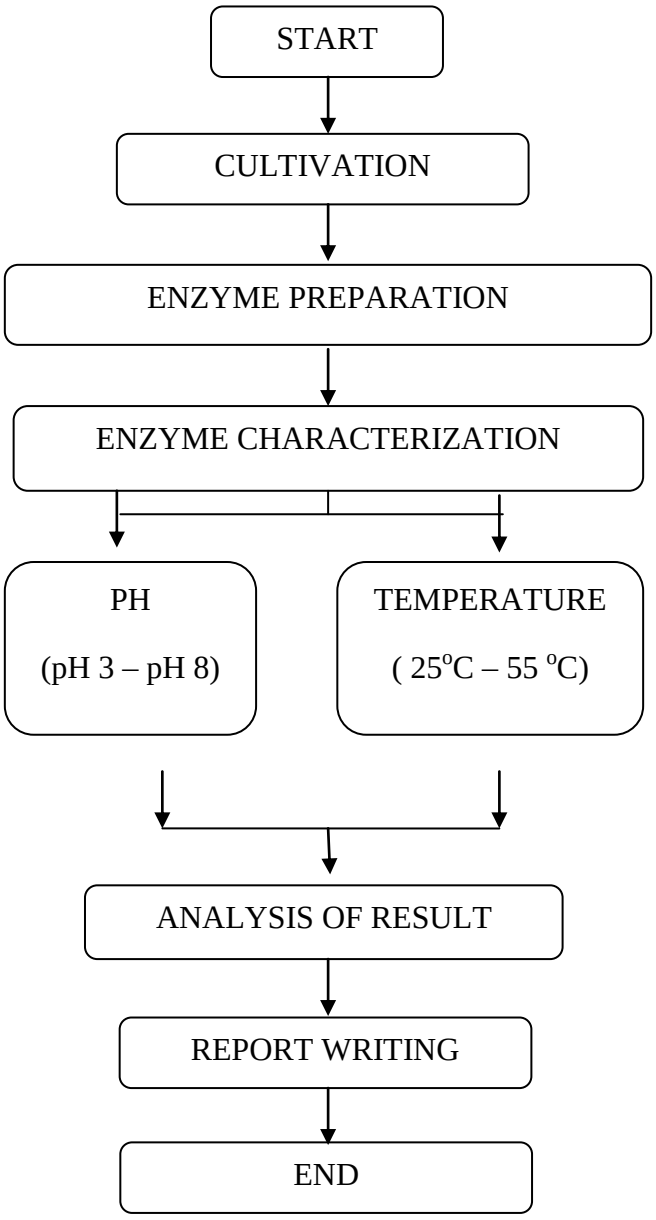

Figure 3.0 Overview of methodology

3.3 Culture Method

3.3.1 Agar Plate Culture

Candida krusei was aseptically transferred onto a plate containing sterile yeast extract agar (YEA). The strain was subculture once by previous study and incubated at 30 °C for 3 days or 72 hours as illustrated as Figure 3.1. Then, the stock cultures were sealed and stored in refrigerator at 4 °C until further required usage.

Figure 3.1 Agar plate straining of *Candida krusei*

3.3.2 Inoculum Preparation

The 3-days old strains were then transferred into inoculum medium. The inoculum medium consists of 1% sucrose and 0.2% yeast extract at pH 5.5. The mycelia mat was formed by punched onto the agar plate using a sterile test tube to obtain a round disk of 0.5 cm in diameter. About two disks were put into 100 mL of

inoculum medium in 250 mL shake flask. Then, the flask was incubated for 24 h at 30°C on rotary shaker at 250 rpm as shown in Figure 3.2 (Noraziah Abu Yazid, 2010). In order to get same growth rate in all shake flasks, the inoculum must be standardized which is the number of cell must be equal by using cell counting.

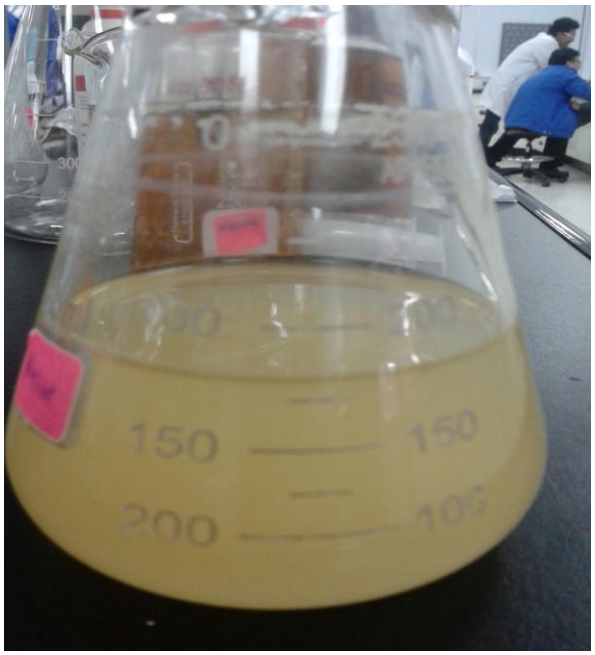

Figure 3.2 Inoculum of *Candida krusei*

3.3.3 Fermentation of *Candida krusei*

A 24-hour old inoculum was transferred into 250 mL, pH 5.5 of fermentation medium consists of 30% (w/v) sucrose, 3% (w/v) peptone, 5% (w/v) yeast extract, 0.5% $MgSO_4.7H_2O$ and 0.5% KH_2PO_4. By using incubator shaker at speed 220 rpm, the culture was incubated for 24h at 30±1 °C. Figure 3.3 shown incubator shaker use to fermentate of *Candida krusei* as Figure 3.4 at condition as mentioned earlier.

Figure 3.3 Incubator shaker Infors Ht

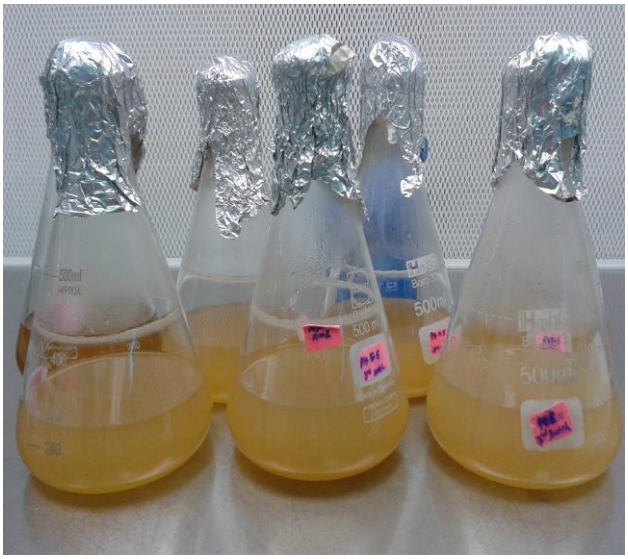

Figure 3.4 Fermentation of *Candida krusei* in 250 mL of shake flask at pH 5.5, temperature 30^{o}C, 220 rpm.

3.4 Data Analysis

3.4.1 Determination of FTase activity

At the end of incubation, 30 mL of the culture broth was centrifuged by refrigerated centrifuge for 15 minutes at 10 000 rpm and at 4°C for the extracellular enzyme source. The supernatant was taken as crude extracellular Ftase. The activity of the enzyme was then determined. One unit (1U) defined as the amount of enzyme activity required to produce 1 μmol of glucose/min (Shin *et al.,* 2003). By incubating 0.5 mL of enzyme with 1.5 mL of sucrose, the Ftase activity of the enzyme preparation was assayed as demonstrated in Figure 3.6. This is done in 0.1M citrate buffer (pH 5.0) at 55°C for 1 h in shaking water bath (Figure 3.5) to let the transfructosylation process to occur. The reaction was stopped by keeping the reaction mixture in boiling water for 15 min (Lateef *et al.,* 2006). Ftase activity was determined as the amount of enzyme required to liberate 1 μmol of glucose under specified condition (Lateef *et al.,* 2006). To calculate the enzyme activity based on the sugar production, standard glucose calibration curve was plotted. The enzyme reaction was terminated by dipping the reaction mixture in boiling water bath or at 100°C for 15 minutes (L'Hocine *et al.,* 2000).

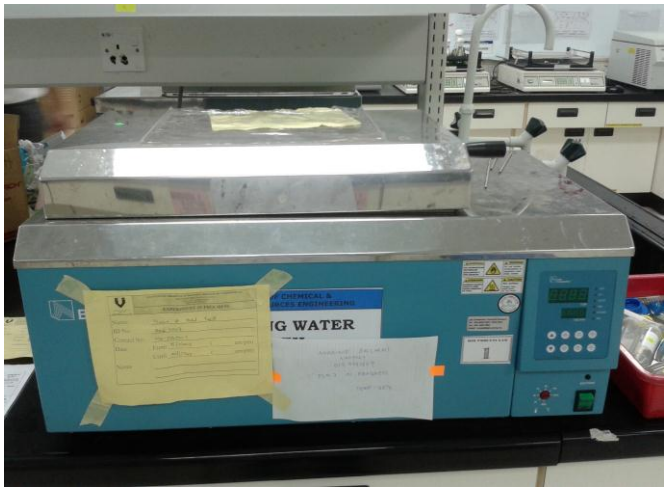

Figure 3.5 Shaking water bath BS-21

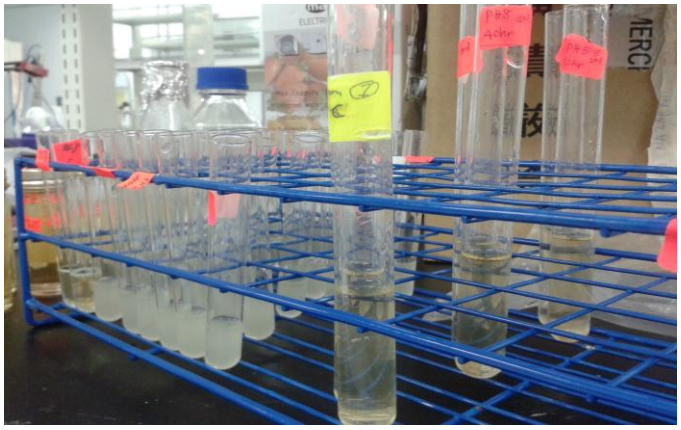

Figure 3.6 Mixture of enzyme assayed at 0.5 ml crude enzyme, 1.5 ml glucose (60 %) and 1.0 ml of 0.1M citrate buffer at pH 5.0 before incubated at 55^{o}C

3.4.2 Determination of sucrose consumption

By using dinitrosalicyclic acid (DNS) method for glucose analysis, sucrose consumption can be determined. 3 mL of DNS reagent was added into each test tube and dipped into boiling water for 5 minutes; hence 1 mL of potassium sodium tartrate (Rochelle salt) solution was added to stabilize the solution in order to develop the red brown color which represented the presence of reducing sugar (Miller, 1959) as shown in Figure 3.7. The samples were then measured by using UV-Vis

Spectrophotometer at 540 nm. The standard calibration curve (Appendix A.1) was generated in order to correlate the absorbance to the sucrose concentration.

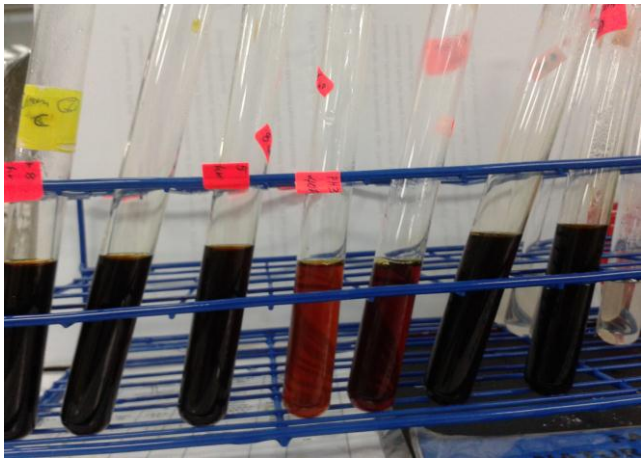

Figure 3.7: Determination of glucose using DNS method at incubation period 1 hour and different pH range at temperature 55^{o}C

3.4.3 Determination of Biomass

The samples were withdrawn from the fermentation broth at every 8 hours interval for 48 hours. The sample was centrifuged at 10 000 rpm for 15 minutes at 4°C. This was done to separate the biomass and the supernatant. The biomass was filtered using Whatmann No. 1 filter paper and washed several times using distilled water in order to remove media residues. The washed biomass was dried at 80°C for 24 h to get the constant biomass weight.

3.4.4 Determination of protein concentration

Protein concentration measurement was carried out according to Lowry's method. Bovine serum albumin (BSA) was used as a standard. BSA standard was prepared at concentration of 200, 500, 1000, 1500 and 2000 µg/ml. 1 ml of Lowry

reagent was added into 0.2 ml of each concentration of BSA and the solution was mixed well and leaved at room temperature for 10 minutes. The solution then was added with 0.1 ml of 1.0 N Folin-Ciocalteu reagent and leaved at room temperature after mixed well. The optical density of the mixture was analyses using UV-Vis Spectrophotometers at wavelength 750 nm against blank after 30 minutes leaved at room temperature. The calibration curve (OD vs concentration) for each BSA (Appendix A.2) concentration was plotted. The sample enzyme concentration was measured using the same procedure by replacing 0.2 ml of BSA solution with 0.2 ml of sample.

3.4.5 Determination of specific activity

Calculating specific activity requires performing of two assayed: a total enzyme assayed like Lowry's method (Appendix A.3) used and an assayed specific only for FTase of interest. The specific activity of Ftase enzyme was analyses by using correlation between enzyme activities of the Ftase in units/mL over concentration of enzyme produced detect in the solution as dhown in Equation (3.1)

$$\text{Specific activity} = \frac{[\text{activity of the Ftase enzyme in Units/mL}]}{[\text{mg of Ftase enzyme /mL}]} \quad (3.1)$$

CHAPTER 4

RESULT AND DISCUSSION

This chapter discuss on the results of the experiment that was done in order to produce fructosyltransferase (FTase) from *Candida krusei*. Fermentation was conducted at temperature 30^{O}C and pH 5.5 for 48 hours in 250 mL shake flask with 10% (v/v) of inoculum. For analysis, UV-Visible U-1800 Spectrophotometer (Hitachi) was used to check the concentration of glucose (540 nm) and protein (750 nm) concentration respectively.

4.1 Glucose calibration curve

Glucose calibration curve was used to determine the concentration of glucose in an unknown sample by comparing the unknown to a set of standard samples of known concentration. By using the UV-VIS U-1800 Spectrophotometer (Hitachi), the absorbance values obtained were plotted against the concentration of glucose. The following Equation (4.1) was obtained.

$$y = 11.535X \tag{4.1}$$

with coefficient $R^2 = 0.9765$ where y is equal to the optical density (absorbance) value while x is equal to the concentration of glucose (g/L).

Figure 4.1 shows the calibration curve of glucose for this experiment. The graph was plotted with optical density versus concentration of glucose. The graph exhibited that the optical density (nm) increased when the glucose concentration (g/L) was increased respectively. From the observation during the reaction between sample and DNS reagent used, it shows that the dark red colour was appear in the high concentration of sample standard compared to the lowest concentration. Generally; DNS colour was yellowish prior the reaction occurred.

After it was incubated with 100^{o}C in the water bath, it began to change colours from yellowish brown to red black colours for the high concentration. It means that, as the concentration of glucose is increase the colour will be darker. The result obtained also was followed the correlation made up using Figure 4.1 to find the glucose concentration in the reaction between crude enzyme, substrate and buffer.

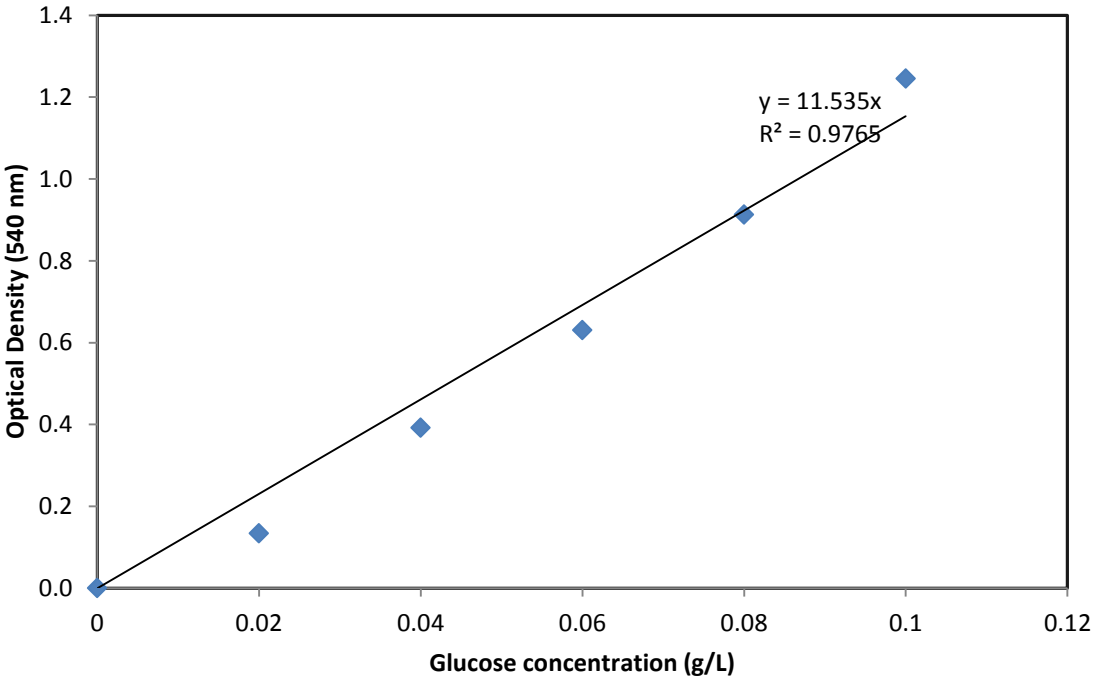

Figure 4.1 The calibration curve of glucose in 250mL of shake flask, 220 rpm, 55^{o}C,pH 5.5 and 10 g/L concentration.

4.2 Growth profile

The growth profile for *Candida krusei* strain was done in order to observe the bacteria growth in sucrose fermentation. Table 4.1 and Figure 4.2 show the profile growth of biomass fermentation from *Candida krus*ei.

Table 4.1 The profile growth of biomass

Time (Hour)	Biomass (g)
8	0.0192
16	0.0338
24	0.0532
32	0.0855
40	0.1132
48	0.1178

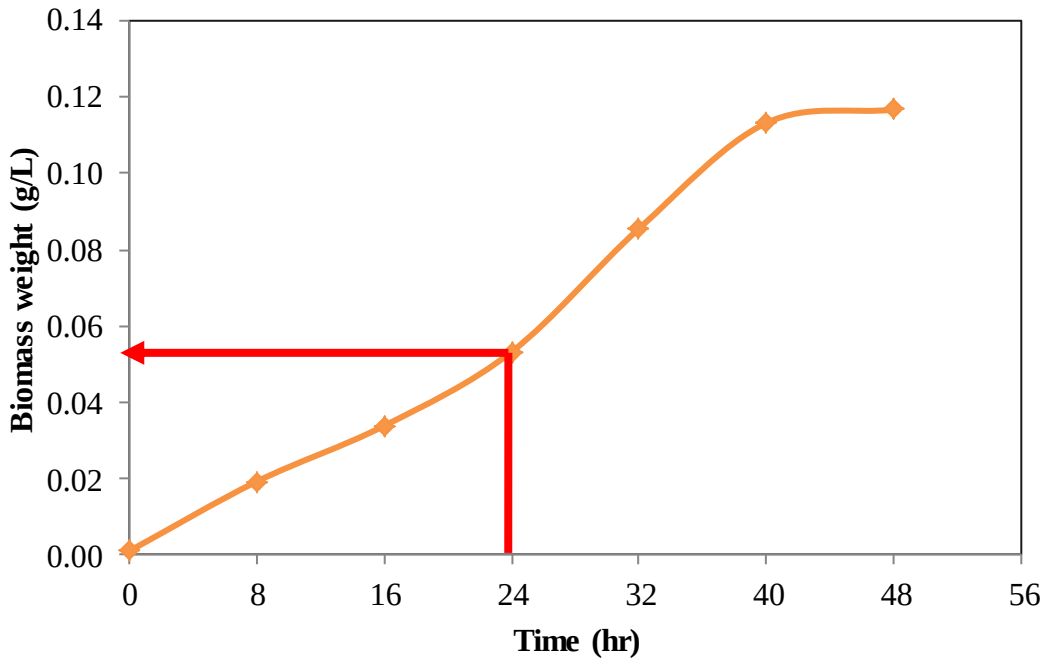

Figure 4.2 Graph of biomass profile growth of *Candida krusei* in 250 mL of shake flask, 220 rpm, 30 ^{o}C , pH 5.5 and 10 g/L of sucrose.

Figure 4.2 indicates the growth profile of *Candida krusei* obtained within 48 hr fermentation period when bacteria are cultivated in batch culture using sucrose as a substrate, nutrient concentration declined and concentration of waste increase since there is no fresh medium is provided during fermentation. The growth of bacteria was plotted as biomass produce over fermentation time which is the number of biomass weight versus the fermentation time. The resulting curve above has three distinct phases which are lag (from 0 till 8 hours), exponential (from 8 to 40 hours) and stationary phases (from 40 to 48 hours).

From Figure 4.2, the lag phase for *Candida krusei* was at 0 to 8 hours. At this phase, the bacteria started to adapt to medium and synthesis new components such as refilled spent materials. After the cells have adapted to the new environment, the cell division occurred at increasing frequency until the maximum growth rate reached.

The exponential phase occurred when the strains' population was uniform in terms of chemical and physical properties. This phase took place at 8 to 40 hours of the microbial growth. At this stage supposedly the cells were dividing and doubling in number at regular intervals. In this study, *Candida krusei* growth started to increase actively at 24 hours.

During 40 – 48 hours, the total number of biomass cells remains constant because of metabolically active cell stop dividing or reproductive. This phase is known as stationary phase. The possible reasons bacteria turned into stationary phase because of nutrient limitation or limited oxygen availability. The other possible reason was decreasing size of bacteria. This profile was done before start the production fermentation in order to determine an active time for the microbe to growth.

Based on the graph illustrated, it shows that μ_{max} and $\mu_{1/2}$ of this profile growth are 40 hours and 24 hours respectively. Thus, an active time for the microbe to be growth was at 24 hours. From this result obtained, the fermentation of *Candida krusei* was carried out at 24 hours, 220 rpm, 30^{o}C, pH 5.5 and 10 g/L of sucrose as substrate.

4.3 Effect of incubation time on enzyme activity

The effect of incubation time on enzyme activity was conducted in order to get the optimum enzyme activity. The incubation time was manipulated at different time allocated at 1, 6, 12, 24 and 36 hours at different pH ranging from pH 3 to pH 8 and different temperature at 25°C to 55°C on the water bath respectively. From the observation, as time taken was longer, lesser activity of an enzyme was obtained as shown in Figure 4.3.

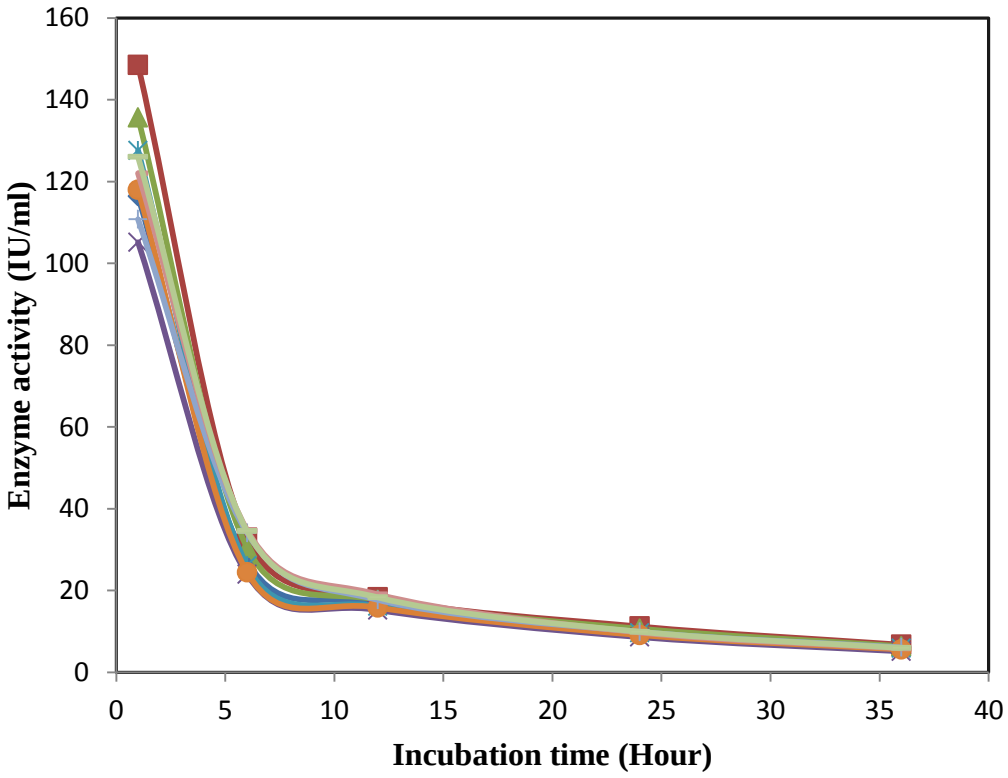

Figure 4.3 Effect of incubation time on enzyme activity at different incubation temperature. pH (T=55), pH 5.5 (T=55), pH 8 (T=55), pH 3 (T=25), pH 5.5 (T=25), pH 8(T=25), pH 3 (T=40), pH 5.5 (T=40), pH 8 (T=40)

The highest activity (148.501 IU/mL) of enzyme was occurred at 1 hour incubation time rather than 6, 12, 24 and 36 hour respectively. Based on the Figure 4.3, also indicated that enzyme activity was highest at temperature 55^{o}C at pH 5.5 at 1 hour to 36 hour incubation time rather than other temperature. This result means that fructosyltransferase enzyme produced was highly active in 1 hour incubation period compared to the other time frame. Longer time of incubation was taken, more enzymes loss their active site to transfer a substrate to product. As been mentioned by Chavez *et al.,* (1997) reported that enzyme activity was decreased gradually along the incubation time occur at different temperature and pH involved.

4.4 Effect of temperature on enzyme activity

Effect of temperature on enzyme activity was carried out in a water bath BS-21 at different temperature range from 25^{o}C, 40^{o}C and 55^{o}C respectively. The temperature parameter range was generated using design of experimental at constant pH range from pH 3 until pH 8 and 1 hour incubation time respectively. The upcoming result will be demonstrated as the highest enzyme activity generated by applying highest temperature during incubation. The Figure 4.4 illustrates the effect of temperature applied on the enzyme activity at constant pH and incubation time.

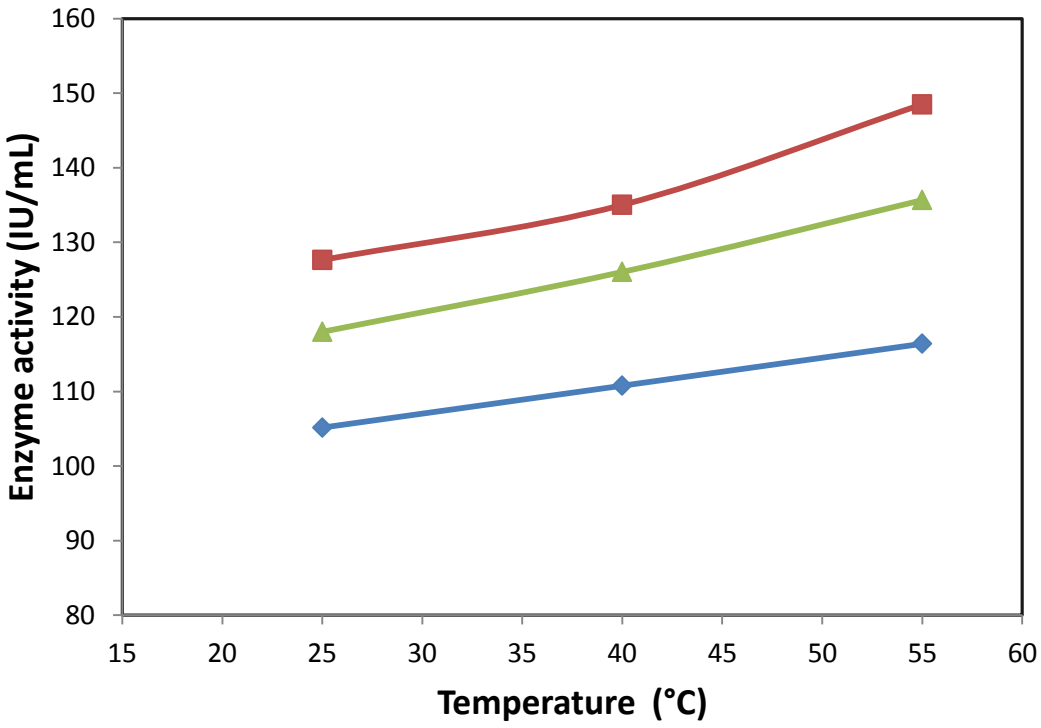

Figure 4.4 The effect of temperature towards enzyme activity at different pH. This graph obtained from reaction between 0.5 ml FTase enzyme with 1.5 ml glucose and 0.1 M citrate buffer at 1 hour incubation time. pH 3, pH 5.5, pH8

According to Figure 4.4, the result shown that highest temperature gives highest enzyme activity (148.501 IU/mL) at different pH 5.5 values. This graph was illustrates at a constant incubation time which is 1 hour because FTase enzyme was shown having a highest enzyme activity at 1 hour incubation time rather than other time. From Figure 4.4, it can be seen that pH 5.5 having optimum enzyme activity with increasing temperature gradually from 25^{o}C to 55^{o}C compared to pH5, pH 8 and pH 3. It also indicate that the pattern of the increased on enzyme activity at pH 5.5 was same with the pH 5 and pH 8, except for pH 3 only having slow response on enzyme activity. It is because an enzyme cannot be active in an acidic condition rather than neutral and alkaline condition. This phenomenon similarly as reported by

Ghazi *et al.,* (2007) that no significant activity was founded at pH values below than 3.5 and above 9.5.

From Figure 4.4, it can be said that FTase produced was having optimum enzyme activity at temperature 55^{o}C and pH 5.5 with 1 hour incubation time. This result was followed the previous research done by Chen *et al.,* (2010) that reported the optimum temperature for enzyme activity at pH 5.5 was 55^{o}C which was accordance with the optimum temperature of other species of yeast such as *S.occidentalis* (45- 55 ^{o}C). According to L'Hocine *et al.,* (2000) stated that the optimum temperature on enzyme activity of FTase was in the range of 50 -55^{o}C by using the standard method at their optimum pH.

4.5 Effect of pH on enzyme activity

The fructosyltransferase enzyme produced by *Candida krusei* was used in order to determine the enzyme activity towards the effect of different pH. The effect of pH on the enzyme activity was determined by using enzyme assay buffer ranging from pH 3.0 to pH 8.0. The expected result should be figured out as increasing in the enzyme activity by increased the pH value at constant temperature and incubation time applied. Figure 4.5 described the effect of temperature towards the enzyme activity at different pH of enzyme at constant incubation time which is 1 hour (most active time).

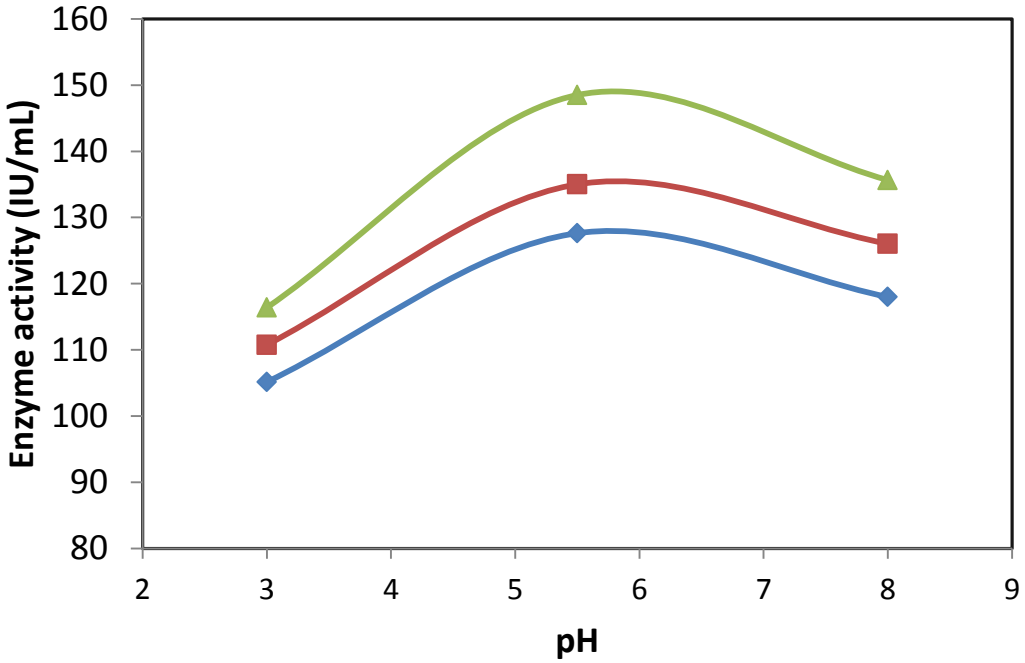

Figure 4.5: The effect of pH on enzyme activity. This result was obtained from correlation on reaction between 0.5 ml FTase enzyme with 1.5 ml glucose and 0.1 M citrate buffer at 1 hour incubation time. T=25^{o}C, T=40 ^{o}C, T=55 ^{o}C

According to Figure 4.5, it shown that as pH ranging from pH 3 to pH 8 was increased, the enzyme activity of FTase also was increased gradually until pH 5.5. While the enzyme activity of FTase at pH 8 was declined respectively for the entire temperature constant at same incubation time occur. The highest enzyme activity was observed at pH 5.0 until pH 5.5 with optimum enzyme activity (148.501 IU/mL) at pH 5.5. This graph also illustrated that incubation temperature of 55^{o}C at 1 hour time incubation was given the highest enzyme activity 148.501 IU/mL at optimum pH which is pH 5.5. While the moderate and the lowest temperature at optimum pH of enzyme activity was at temperature 40^{o}C and 25^{o}C respectively.

This result was fully supported by Chavez *et al.*, (1997) with resulted that the enzyme displayed high activity over abroad pH range (5 - 6) with optimum activity was at pH 5.5. Similarly as research done by L'Hocine *et al.*, (2000) reported that Ftase enzyme was highly active at pH 5.8 at temperature 50^{o}C to 55^{o}C.

4.6 Determination of protein concentration

One way to determine the protein concentration of a given protein containing solution that has unknown protein concentration is to compare the unknown solution with a set of protein solutions of known concentration. The absorbance associated with a set of protein solutions of known concentrations is called a protein standard curve. The first step in the determination of the concentration of the unknown solution is to prepare a standard curve using known concentrations of protein. A protein that is frequently used for standard curves is Bovine Serum Albumin (BSA). BSA is desirable because it is commercially available as a dried powder that is easily weighed with a laboratory balance.

A series of concentration was developed to obtained standard curve (Appendix A.2). Lowry's reagent (Appendix A.3) and Folin-Ciocalteu reagent was used as a reagent to react with the BSA. Folin-Ciocalteu reagent functioning as a reagent that selectively binds a dye on the certains amino acids found in most protein. Then, the mixture was analysed using UV-Vis U-1800 spectrophotometer (Hitachi) at wavelength 750 nm. After standard curve was developed, the crude

FTase enzyme produced at different pH and temperature was analysed using UV-Vis spectrophotometer at the same wavelength. The result correlation between the absorbance (nm) vs. concentration (mg/mL) was shown in the Appendix A.3. Figure 4.6 demonstrated the protein concentration of crude enzyme at different pH and temperature allocated.

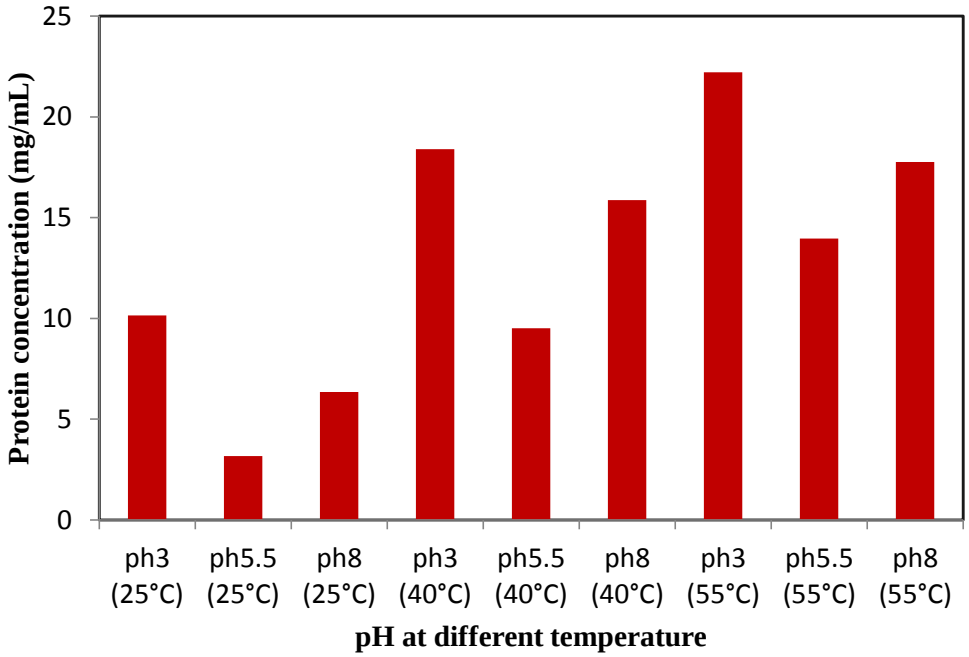

Figure 4.6 Correlation between protein concentration (mg/mL) of enzyme at different pH (pH 3- pH8), temperature (25^{o}C – 55^{o}C), and 1 hours incubation time

From the observation, it can be seen that when Folin-Ciocalteu was added to the mixture containing Lowry's reagent and BSA, the yellow-brown colours of Folin-Ciocalteu was slowly changes to blue. The more blue colour in sample indicates that the more protein was occurred at that point. The result obtained show that the highest protein produced was at pH 3 at all the temperature allocated rather than the other pH observed. This result means the more protein present in the mixture, and therefore

the more of the amino acids in crude FTase are to bind the dye, the more blue colour appears in the solution as mentioned by Lisa (2008).

4.7 Specific activity of enzyme

Specific activity is a convenient way of expressing the purity of a particular protein. It is calculated as the amount (or units) of the protein of interest divided by the total amount of all proteins in a sample. When a protein is very pure, all the protein in the preparations is the protein of interest and the specific activity is high. When the protein of interest is contaminated by other proteins, the specific activity is lower. The more contaminants developed, the lower the specific activity. The correlation between enzyme activity and specific activity of FTase from *Candida krusei* was plotted as describe in Figure 4.7.

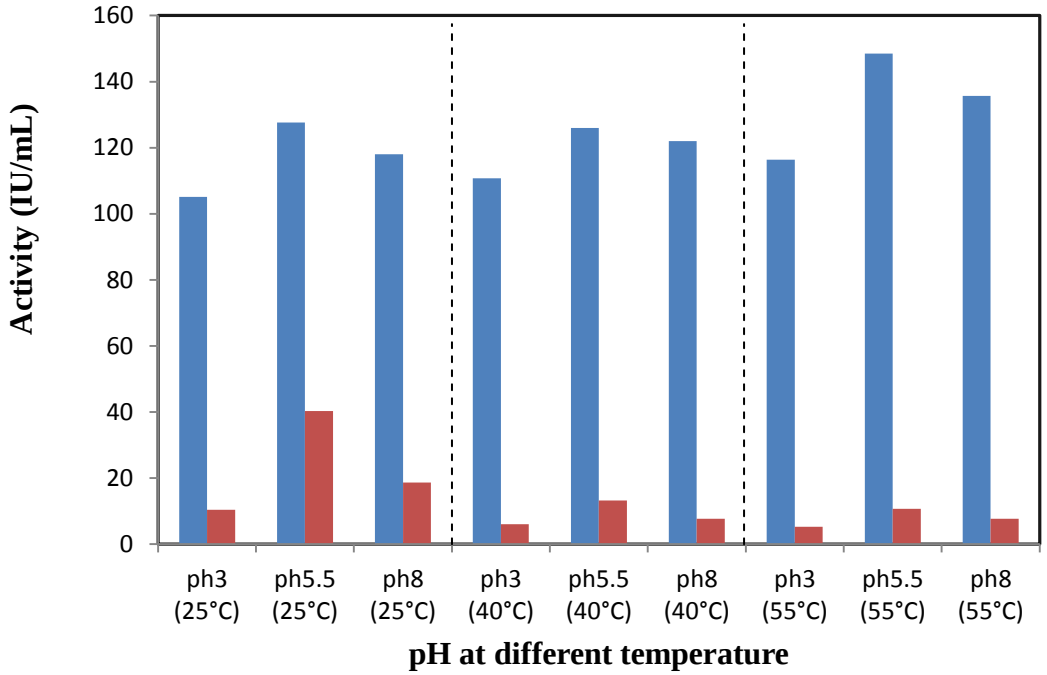

Figure 4.7 Correlation between ■ enzyme activity (IU/mL) and ■ specific activity (mg/mL) at different pH (pH 3- pH8), temperature (25^{o}C – 55^{o}C), and 1 hours incubation time

According to Figure 4.6, it illustrated that as FTase enzyme activity (IU/mL) was increased, the specific activity (mg/mL) of FTase was demonstrated declined along the pH ranging from pH 3 until pH 8 at different temperature applied. The lowest enzyme activity (105.155 IU/mL) measured was at pH 3 and temperature of 25^{o}C with the amount of specific activity was calculated at 10.358 IU/mg. While at the highest enzyme activity (148.501 IU/mL) was figured out at pH 5.5, 55^{o}C and the specific activity was observes as 10.638 IU/mg. This result indicates that specific activities are inversely proportional with the enzyme activity.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The main objective of this research is to study the effect of temperature and pH on FTase enzyme produced from *Candida krusei* using sucrose as a substrate was achieved. From the result obtained, we can conclude that Ftase enzyme favor less acidic condition which is pH 5.5. The study resulted the highest activity of crude enzyme (148.501 IU/mL) was obtained at pH 5.5, 55^{o}C and 1 hour incubation period. While the specific activity of crude enzyme at pH 5.5, 55^{o}C and 1 hour incubation time was at 10.638 IU/mg. These results can be concluded as the specific enzyme activity higher, the enzyme activity of FTase was higher while the protein concentration was lower respectively.

5.2 Recommendation

Several numbers of recommendations are being proposed in order to produce high FTase enzymatic activity to produce high yield of FOS are being listed as below:

1) Determination of other parameter.

Parameters that have been studied in this experiment are effect of pH, temperature and incubation time towards the enzyme activity. For the future study, additional parameter can be added such as substrate concentration, cultivation time, inhibition of other material towards enzyme activity and agitation speed in order to understand the enzyme characteristic or to find other way to improve the enzyme activity.

2) Purification method of enzyme

This study only focuses on the effect of temperature and pH on crude FTase enzyme only. Further study should be applied on purification of crude FTase enzyme to be compared with the crude enzyme which one is having high enzymatic activity and specific activity observed.

3) Using other microbial organism to produce enzyme

Candida krusei has been used in this study to produce FTase. There are many more microbial organisms such as *Aureobasidium pullulan, Penicillium simplicissimum* and *Arthrobacter* sp. can produce FTase. Different microbial organism produce different yield of FTase and by using different microbial organism, we can determine the microorganism that produce high yield of enzyme.

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APPENDIX A

APPENDIX A.1

Figure A.1 illustrated the standard calibration curve of glucose that was analysed using UV-Vis U-1800 spectrophotometer (Hitachi) at wavelength of 540 nm.

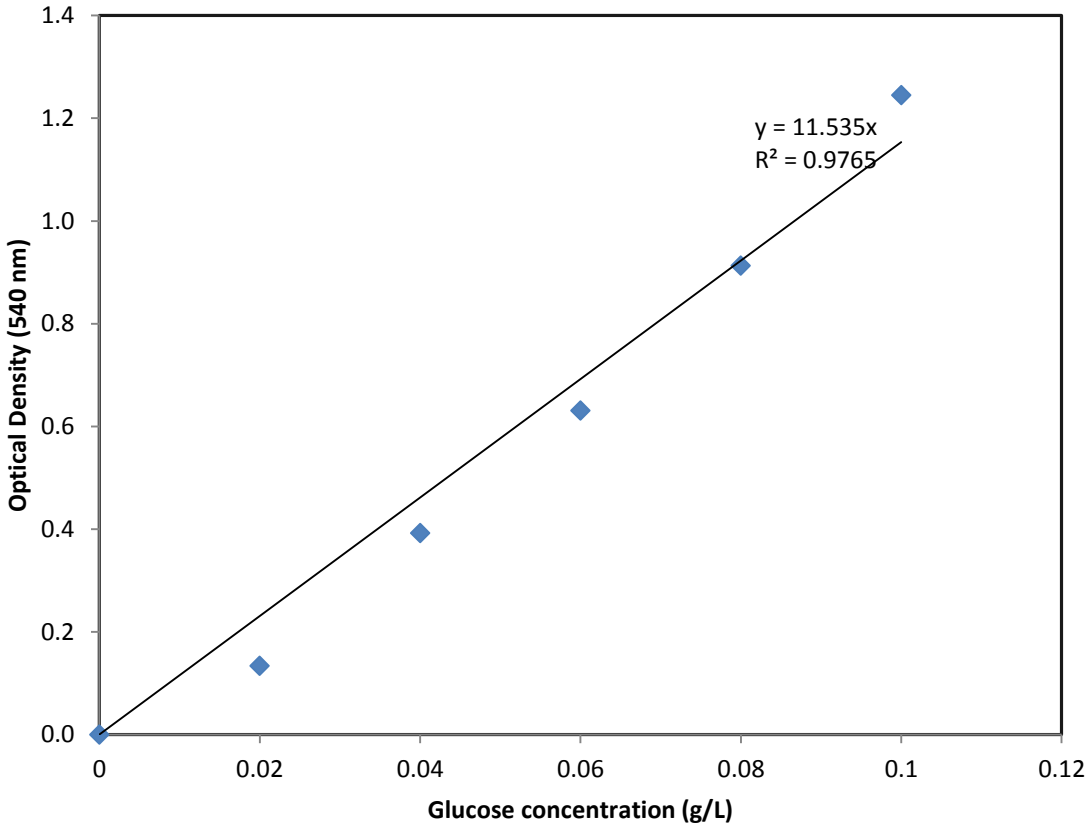

Figure A.1 Graph correlation between optical density (540 nm) and glucose concentration (g/L) for standard calibration curve of glucose

APPENDIX A.2

The standard calibration curve of Bovine Serum albumin (BSA) was demonstrated in Figure A.2 using correlation between optical density at 750 nm and BSA concentration that was analysed using UV-Vis U-1800 spectrophotometer (Hitachi).

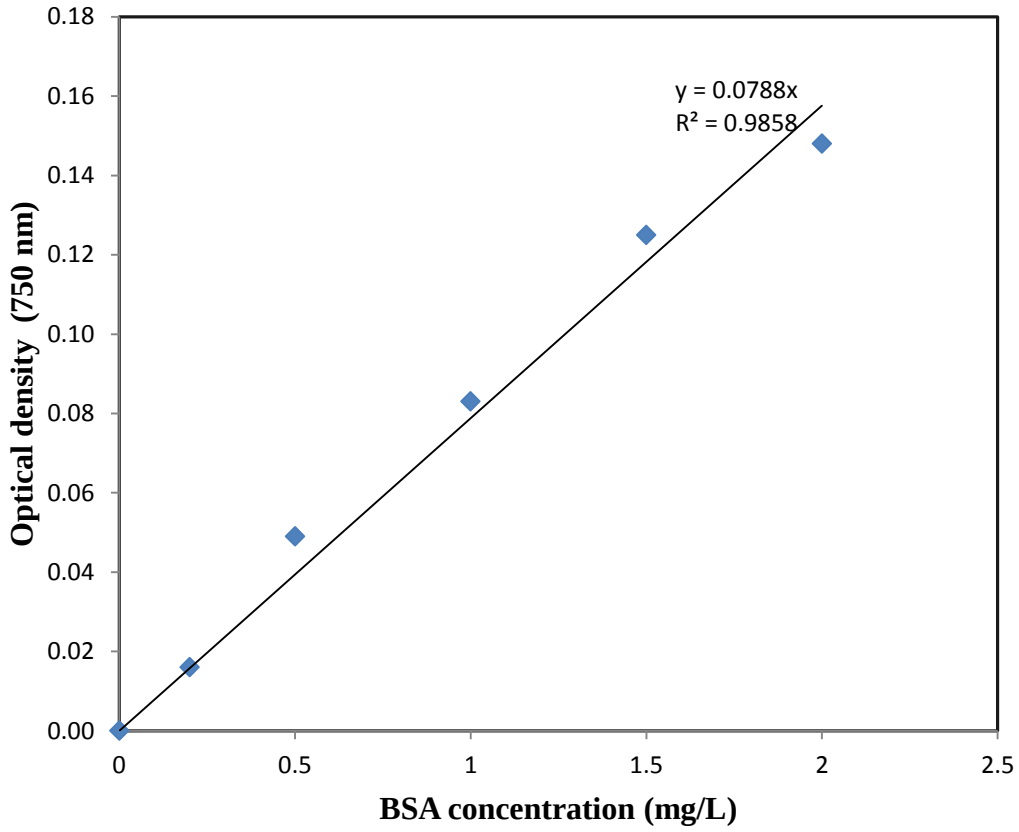

Figure A.2 Graph correlation between optical density at 750 nm and BSA concentration (mg/mL) for standard calibration curve of Bovine Serum Albumin (BSA)

APPENDIX A.3

Table A.1 shows the Lowry's procedure for preparing the Lowry's reagent and solution to be used in determining protein concentration on crude enzyme prepared.

Table A.1 Lowry's method procedure (Lowry's reagent)

Reagents for Modified Lowry's method Reagent	Preparation procedure	Notes
A	Dissolve 20 g of sodium carbonate and 4 g of sodium hydroxide in 1 L distilled water	Keep refrigerated
B	Dissolve 2.5 g of copper sulfate ($CuSO_4.5H_2O$) and 5 g of sodium citrate in 1 L distilled water	Wrap the bottle with aluminium foil to avoid discolorization and keep refrigerated
Lowry solution	Mix reagent A and B in a 50:1 ratio	Prepare only when needed and keep refrigerated
Folin-Ciocalteu (stock 2.0 N)	Dilute the stock with distilled water in 1:1 ratio	Prepare only when needed

(Bioprocess engineering lab manual, 2012)

APPENDIX B

Table B.1, B.2, B.3 demonstrated the result collected of glucose concentration and enzymatic activity of crude FTase at different incubation period for temperature of 25ºC until 55ºC.

Table B.1 Result of glucose concentration and enzymatic activity at different incubation period for 25ºC

Temperature	pH	Incubation time (H)	Glucose conc. (g/L)	Enzyme activity (IU)
		1	0.5678	105.155
		6	0.7759	23.9475
25	3	12	0.9883	15.2515
		24	1.1183	8.6291
		36	1.0013	5.1507
		1	0.6892	127.6309
		6	0.8453	26.0881
25	5.5	12	1.0489	16.1879
		24	1.2527	9.6659
		36	1.1443	5.8865
		1	0.6372	117.9984
		6	0.7932	24.4826
25	8	12	1.0273	15.8535
		24	1.1834	9.1308
		36	1.0923	5.6189

Table B.2 Result of glucose concentration and enzymatic activity at different incubation period for 40ºC

Temperature	pH	Incubation time (H)	Glucose conc. (g/mL)	Enzyme activity (IU/mL)
		1	0.5982	110.7739
		6	1.1053	34.1152
40	3	12	1.1487	17.7265
		24	1.2831	9.9001
		36	1.1877	6.1095
		1	0.6588	122.0119
		6	1.1270	34.7841
40	5.5	12	1.2354	19.0644
		24	1.2657	19.06436
		36	1.1400	9.7663
		1	0.6805	126.0255
		6	1.1183	34.5165
40	8	12	1.1877	18.3285
		24	1.2874	9.9335
		36	1.1617	5.9757

Table B.3 Result of glucose concentration and enzymatic activity at different incubation period for 55ºC

temperature	pH	Incubation time (H)	glucose conc. (g/mL)	enzyme activity (IU/mL)
		1	0.6285	116.3929
		6	0.8799	27.1584
55	3	12	1.1183	17.2583
		24	1.3047	10.06732
		36	1.1964	6.1541
		1	0.8019	148.5013
		6	1.0663	32.9111
55	5.5	12	1.1877	18.3285
		24	1.4521	11.2045
		36	1.3134	6.7562
		1	0.7325	135.658
		6	0.9883	30.5030
55	8	12	1.1487	17.7265
		24	1.3827	10.6694
		36	1.2440	6.3994

Table B.4 illustrated the data for the biomass growth at different fermentation period allocated.

Table B.4 Biomass weight (g/L) at different fermentation time (Hr)

Fermentation time (Hr)	Biomass (g/L)
0	0.001
8	0.0192
16	0.0338
24	0.0532
32	0.0855
40	0.1132
48	0.1167

Table B.5 and Table B.6 shows the data result for effect of enzymatic activity towards different applied temperature and pH at 1 hour incubation period.

Table B.5 Effect of enzymatic activity towards temperature at pH and 1 hour incubation constant

Temperature	pH		
	ph3	ph5.5	ph8
25	105.1550	127.6308	117.9984
40	110.7739	135.0119	126.0255

Table B.6 Effect of enzymatic activity towards temperature at pH and 1 hour incubation constant

pH	Temperature		
	25	40	55
3	105.155	110.7739	116.3929
5.5	127.6309	135.0119	148.5013
8	117.9984	126.025462	135.658

Table B.7 indicates the data collection about concentration of protein in crude FTase enzyme production and also specific activity of FTase enzyme at 1 hour incubation time allocated.

Table B.7 Correlation data between protein concentration and enzyme activity at different pH and temperature applied

pH at different temperature	Protein concentration (IU/mg)	Enzyme activity (IU/mL)	Specific activity (mg/mL)
ph3 (25°C)	10.1523	105.1550	10.3578
ph5.5 (25°C)	3.1726	127.6309	40.2293
ph8 (25°C)	6.3452	117.9984	18.5965
ph3 (40°C)	18.4010	110.7739	6.0199
ph5.5 (40°C)	9.5178	126.0255	13.2411
ph8 (40°C)	15.8629	122.0119	7.6916
ph3 (55°C)	22.2081	116.3929	5.2410
ph5.5 (55°C)	13.9594	148.5013	10.6381
ph8 (55°C)	17.7665	135.6579	7.6356