EFFECT OF SUBSTRATE CONCENTRATION ON PROFILE GROWTH OF *Candida tropicalis* IFO 0618

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ABSTRACT

This study presents effect of substrate concentration on the profile growth of *Candida tropicalis* IFO 0618. Different substrate concentration; D-Glucose were introduced to *Candida tropicalis* IFO 0618 in the Solid State Fermentation (SSF) process. The effect of substrate concentration on the profile growth of *Candida tropicalis* IFO 0618 was analyzed using UV-Vis Spectrophotometer and High Performance Liquid Chromatography (HPLC). The kinetic parameter (μ_{max}) was also calculated to describe the microbial growth. From the laboratory data, the specific growth rate (μ), maximum specific growth rate (μ_{max}) and half saturation coefficients (K_S) were determined using the Monod equation. The maximum observed growth rate (μ_{max}) for *Candida tropicalis* IFO 0618 was at the substrate concentration of 8.5 (g/g) with μ_{max} value of 1.9991 hr^{-1}, specific growth rate (μ) of 0.0821 hr^{-1} and half saturation coefficients (K_S) of 198.4663 g/l. Result from the High Performance Liquid Chromatography also shows that there was sorbitol produced at 8.5 (g/g) substrate concentrations.

ABSTRAK

Kajian ini membentangkan kesan kepekatan substrat ke atas pertumbuhan profil *Candida tropicalis* IFO 0618. Kepekatan substrat; D-Glukosa telah diperkenalkan kepada *Candida tropicalis* IFO 0618 di dalam Penapaian Berkeadaan Pepejal (SSF). Kesan kepekatan substrat ke atas pertumbuhan profil *Candida tropicalis* IFO 0618 telah dianalisa dengan menggunakan UV-Vis Spektrofotometer dan Kromatografi Cecair Prestasi Tinggi (HPLC). Parameter kinetik (μ_{max}) juga dikira untuk menunjukkan pertumbuhan mikrob. Daripada data makmal, kadar pertumbuhan spesifik (μ), kadar pertumbuhan spesifik maksimum (μ_{max}) dan pekali separa tepu (K_s) telah dikira dengan menggunakan persamaan Monod. Nilai tertinggi bagi kadar pertumbuhan maksimum (μ_{max}) dan kadar pertumbuhan spesifik (μ) untuk *Candida tropicalis* IFO 0618 adalah dilihat pada kepekatan 8.5 (g/g) dengan nilai 1.9991 jam^{-1} dan 0.0821 jam^{-1} dengan menggunakan nilai pekali separa tepu sebanyak 198.4663 g/l. Hasil daripada analisa Kromatografi Cecair Prestasi Tinggi (HPLC) juga telah menunjukkan bahawa terdapat sorbitol yang dihasilkan pada kepekatan substrat 8.5 (g/g).

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LIST OF SYMBOLS

A_w	Water activity
A	Asymptote
B	Number of bacteria
d	Dilution factor
K_S	Monod constant
N	Average number of colonies
n	Positive coefficient
S	Substrate
$[S]$	Substrate concentration
X	Cell concentration
Y_{XS}	Yield coefficient

Greek

$\Delta G°'$	Effective free energy change
$\Delta G°$	Change of standard free energy
τ_d	Doubling time
μ	Specific growth rate
μ_{obs}	Observed specific growth rate
μ_{max}	Maximum specific growth rate
λ	Lag phase time

LIST OF ABBREVIATIONS

Abs	Absorbance
ATCC	American Type Culture Collection
CFU	Colony Forming Unit
DCW	Dry Cell Weight
HPLC	High Performance Liquid Chromatography
OD	Optical Density
SSF	Solid State Fermentation
SmF	Submerged Fermentation
YPD	Yeast Peptone Dextrose

1.0 INTRODUCTION

1.0 Background of study

Fermentation can be defined as an anaerobic process in which energy can be released from glucose even with the absence of oxygen. Fermentation usually takes place in the yeast cells such as *Candida tropicalis*. *Candida tropicalis* is believed to represent 4% of the yeast that can be obtained from the sea sediments, seawater, mud flats, marine fish intestine, mangrove plants and marine algae and shrimp. Besides that, *Candida tropicalis* can also be cultured from various fruits and soil and can also be isolated from blood, urine and sputum of human (Basu et al, 2011). *Candida tropicalis* is classified as gram positive type of microorganism and when grow on YPD (Yeast-Peptone-Dextrose) agar the colonies of *Candida tropicalis* are white to cream colored, glabrous, smooth and yeast-like in appearance. Under microscopic morphology, *Candida tropicalis* is spherical to sub-spherical budding yeast-like cells with 3.0-5.5 x 4.0-9.0 μm in size.

In this research, the product that will be produced from the *Candida tropicalis* IFO 0618 fermentation is Sorbitol. Sorbitol is a low calorie sugar known as polyol (alcohol sugar) that is widely used in food industry, not only as sweetener but also as humectant (binding moisture), texturizer and softener (Cazeta et al., 2004). Sorbitol is very similar to glucose but it acts as a laxative by being absorbed very slowly into the blood. In addition, Sorbitol is a kind of product from the cellulose that is high demand in current food industry, cosmetic, pharmaceutical and paper good.

Solid State Fermentation (SSF) process and also Submerged Fermentation (SmF) process are the two types of fermentation processes that have potential in the production of Sorbitol by the fermentation of commercial glucose (D-Glucose) by using *Candida tropicalis* (IFO 0618). Solid State Fermentation (SSF) is defined as fermentation involving solids in absence (or nearly absence) of free water; however, substrate must possess enough moisture to support the growth and metabolism of the microorganism (Pandey, 2003). In

Solid State Fermentation (SSF), the water content of a solid mash in often varies between 40% and 80%.

In this research, Solid State Fermentation (SSF) was chosen over Submerged Fermentation (SmF) based on its advantages. Among the advantages of Solid State Fermentation (SSF) includes the types of bacteria (*Candida tropicalis*) that was used in this study was more suitable for the Solid State Fermentation (SSF) process compared to Submerged Fermentation (SmF). Besides that, only small volume of fermentation mash or reactor volume is needed. This will result in the lower capital and operating cost. Next, the product that is being produced is easy to separate compared to the Submerged Fermentation (SmF). It also stated that generally in the term of yield the product yield are mostly higher in SSF compared to the SmF (Pandey, 2003).

The major differences between these two types of fermentation processes are of the water content in the substrate. This explain why the products produced in the Submerged Fermentation (SmF) process much dilute and less stable compared to the products produce from the Solid State Fermentation (SSF) (Chandran et al., 2005) and because of that, large-scale of bioreactors are needed (Manpreet et al., 2005). In addition, in the Solid State Fermentation (SSF) process, the content less water but an important gas exists between the particles. This is important due to the poor thermal conductivity of the air compared to the water in the scale up process (Durand, 2003). Hence, the Solid State Fermentation (SSF) was chosen over Submerged Fermentation (SmF).

Candida tropicalis IFO 0618, Solid State Fermentation (SSF) and glucose (D-Glucose) are the main characters in this research. In this research the concentration of glucose (D-Glucose) will be varied by 7.0 g, 7.5 g, 8.0 g, 8.5 g and 9.0 g. An increase in the substrate concentration at initial stage will results in increase in the glucose consumption rate (Favela et al, 1997). The effect of each concentration of glucose on the growth of *Candida tropicalis* (IFO 0618) will be studied by analyzing the Monod Equation.

1.1 Motivation

Nowadays, the number of patients with diabetics had achieved a very alarming number. One of the factors of diabetics is the diet that is taken by the people worlds wide besides obesity and sedentary lifestyles (Vischer et al, 2009). Unstable diet with high content of sugars can causes diabetics. Surprisingly, Malaysia has one of the world's greatest numbers of diabetic cases among its population with 2.6 million registered patients (Adie, 2012). Untreated diabetics may cause chronic renal failure (Mauro et al, 2001).

The one with diabetics can still consume sugar but, the volume must be controlled and under doctors surveillance. Aspartame, Saccharin and Sucralose are the example of artificial sweeteners that always being taken by the diabetics. However, these artificial sweeteners have side effects. Aspartame is said to cause headache, nausea and even weight gain. Sucralose on the other hand, is said can cause stomach cramps and skin irritation (FitDay.com)

The main issue in this research is to produce a type of sweeteners that can be consumed by the diabetics, bio-based products, has no side effects and low in cost. Sorbitol, a type of sugar alcohol that is suitable with the criteria mentioned above. This sweetener can be produced by the fermentation process. In this study, Sorbitol will be produced by the fermentation process of glucose (D-Glucose) by *Candida tropicalis* in Solid State Fermentation (SSF) process.

1.2 Problem statement

Application of fermentation has been recommended in order to produce sorbitol in large scale using green technology. There are two methods of fermentation which are Submerged Fermentation (SmF) and Solid State Fermentation (SSF). As Submerged Fermentation (SmF) has more drawbacks compared to Solid State Fermentation (SSF) as difficult to separate, low conversion yield and long incubation time, researchers nowadays prefer to use Solid State Fermentation (SSF). However, to produce sorbitol in high conversion yield in Solid State Fermentation (SSF), the optimum microbial growth rate in an aqueous

environment to the concentration of the limiting nutrients should be identified. Thus, in this study, the growth of *Candida tropicalis* was determined by different concentration of glucose in Solid State Fermentation (SSF) process using Monod Equation.

1.3 Objective

The objective in this research is to study the kinetic parameter (μ_{max}) by *Candida tropicalis* during Solid State Fermentation (SSF) process.

1.4 Scope of study

In order to achieve the objective of this research, several scope of study has been identified:

1) Monod Equation. This equation is one of the best-known kinetic models describing the microbial growth, which shows the functional relationship between the specific growth rate and an essential substrate concentration. According to the collision theory for microbial growth, increase in substance concentration in bulk solution resulted in increase in microbial growth. The range of substrate concentration studied is between between $0x10^{-3}$ to $25x10^{-3}$ mol/L (Liu, 2006).

2) Solid State Fermentation (SSF) process was chosen over Submerged Fermentation (SmF) due to the SSF produced high yield of product compared to Submerged Fermentation (SmF).

3) *Candida tropicalis* which is a type of yeast was used in this study. Yeast is suitable for Solid State Fermentation because of its ability to survive under the low moisture content in the range from 40% to 80% (Pandey, 2003).

4) The production of Sorbitol was analyzed by measuring the Optical Density (OD) of samples for each glucose concentration by UV-Vis Spectrophotometer (Hitachi U-1800 Spectrophotometer) and also HPLC (Agilent Technologies 1100 Series).

2.0 LITERATURE REVIEW

2.1 Types of microorganisms

In this research the type of the microorganism used was the *Candida tropicalis*. This yeast can be isolated either from the environmental or from human being. Environmentally, *Candida species* usually can be isolated either from soil or water that is rich with decomposing plant cells such as fruit shop dumps and leather industry (Sulman and Rehman, 2013). In addition, *Candida tropicalis* also can be isolated from leaves and flowers. On the other hand, *Candida tropicalis* can be found on the human skin, blood, urine, sputum and also mucus membrane of human being (Basu et al., 2011). Table 2.1 shows the prevalence of *Candida* species that are isolated from blood, urine and sputum.

Table 2-1: Prevalence of Candida Species (extracted from Basu et al, 2011)

Name of sample	No. of sample	No. of isolates (%)	No. of different types of isolates		
			C. tropicalis (%)	*C. albicans* (%)	*C. glabrata* (%)
Blood	120	10 (8.33)	5 (50)	3 (30)	2 (20)
Urine	115	13 (11.30)	5 (38.5)	5 (38.5)	3 (23)
Sputum	110	12 (10.90)	5 (41.60)	4 (33.40)	3 (25)
All samples	345	35 (10.15)	15 (42.85)	12 (34.40)	8 (22.85)

There are about 154 species of *Candida* that have been discovered by scientist. However, only six of the *Candida* species that is frequently isolated. They are *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei* and *Candida lusitaniae*. Among all the top abundant isolated species, *Candida tropicalis* is said to be one of the most frequently isolated non-albicans *Candida* species for study.

Taxonomy by definition means the branch of science that emphasized the classification, especially organisms (oxforddictionaries.com). Taxonomically, *Candida tropicalis* can be briefly described as in Table 2.2. *Candida tropicalis* also is classified as yeast cells that possessed hyphae and pseudo-hyphae (AvianBiotech.com).

Table 2.2: Taxonomic Classification of *Candida tropicalis* IFO 0618

Taxonomic Classification	
Kingdom	Fungi
Phylum	Ascomycota
Subphylum	Ascomycotina
Class	Ascomycetes
Order	Saccharomycetales
Family	Saccharomycetaceae
Genus	Candida

Generally after Scanning Electron Microscope Morphology was done on *Candida tropicalis*, the colonies of the cells appear to be large oval shaped bodies. The outer coating of the cell was said to be thin and become thinner once it reach the apex of the colonies. The cells within the colonies also appear smooth and rounded and they are interconnected between cells (Basu et al,.2011). In the term of size, the average diameter of the cells is approximately three to six micro meters in diameter. *Candida tropicalis* is a gram-positive type of cells and can grows within 24 hours on most fungal media (AvianBiotech.com). In addition, the morphology as well as the biochemical traits of *Candida tropicalis* is briefly described in the Table 2.3.

Table 2-3: The morphology and biochemical properties of *Candida tropicalis* (Sulman and Rehman, 2013)

Characters	*C.tropicalis*
Colony shape	Round
Size	0.1-0.36 mm
Color	Cream
Texture	Smooth
Margin	Entire
Elevation	Raised
Type	Budding
Starch hydrolysis	-
Ester production	+
Citrate utilization	-
Tolerance of 1% acetic acid	-
Acid production from glucose	-
Production of ammonia from urea	+
Nitrate reduction	+
Sugar fermentation	+
(Glucose, Sucrose, Maltose)	+
	+

(+) positive: (-) negative

Based on the research done, *Candida tropicalis* was streaked on the Yeast Peptone Dextrose (YPD) agar and also in the Yeast Peptone Dextrose (YPD) broth. Applying the nature of yeast, *Candida tropicalis* was able to grow rapidly in 24 hours durations and become mature in three days. *Candida tropicalis* if grown on the Yeast Peptone Dextrose (YPD) agar will appears as cream coloured with a little mycelia border.

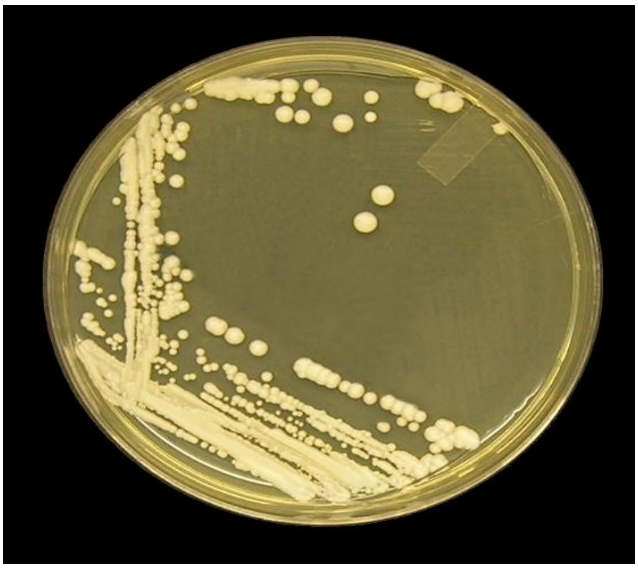

Figure 2-1: *Candida tropicalis* on YPD Agar after incubated for 24 hours at 30°C

Furthermore, the structure of the colony may also be pasty, smooth and a little bit wrinkled upon matured. In Yeast Peptone Dextrose (YPD) broth on the other side, *Candida tropicalis* grow and will produce white thin surface film and also air bubbles. The broth that was initially clear will eventually turn cloudy after 24 hours of incubation. Figure 2.1 shows that the picture of *Candida tropicalis* that grown on the Yeast Peptone Dextrose (YPD) agar.

2.2 D-Glucose as the carbon source

In this research, the substrate used was D-glucose. Glucose or known as D-glucose is a simple monosaccharide found in plants. Glucose is produced by the plants during photosynthesis process. According to the Whitmarsh et al. (1995), photosynthesis is a physico-chemical process by which plant, algae and photosynthetic bacteria uses light energy to synthesis the organic compounds. In the photosynthesis process, oxygen and glucose will be produced.

$6CO_2 + 12H_2O + \text{Light Energy} \rightarrow C_6H_{12}O_6 + 6O2 + 6H_2O.$ (2.1)

In order for the glucose molecule to be used in the photosynthesis process or to be used by the cells for cellular respiration, a larger molecule which is known as cellulose has to undergo degradation process. This degradation process will eventually convert the complex chain of cellulose into much simpler compound called glucose that involving a series of enzymatic reaction (Moat et al, 2002). Figure 2.2 shows the enzymatic degradation of cellulose to glucose.

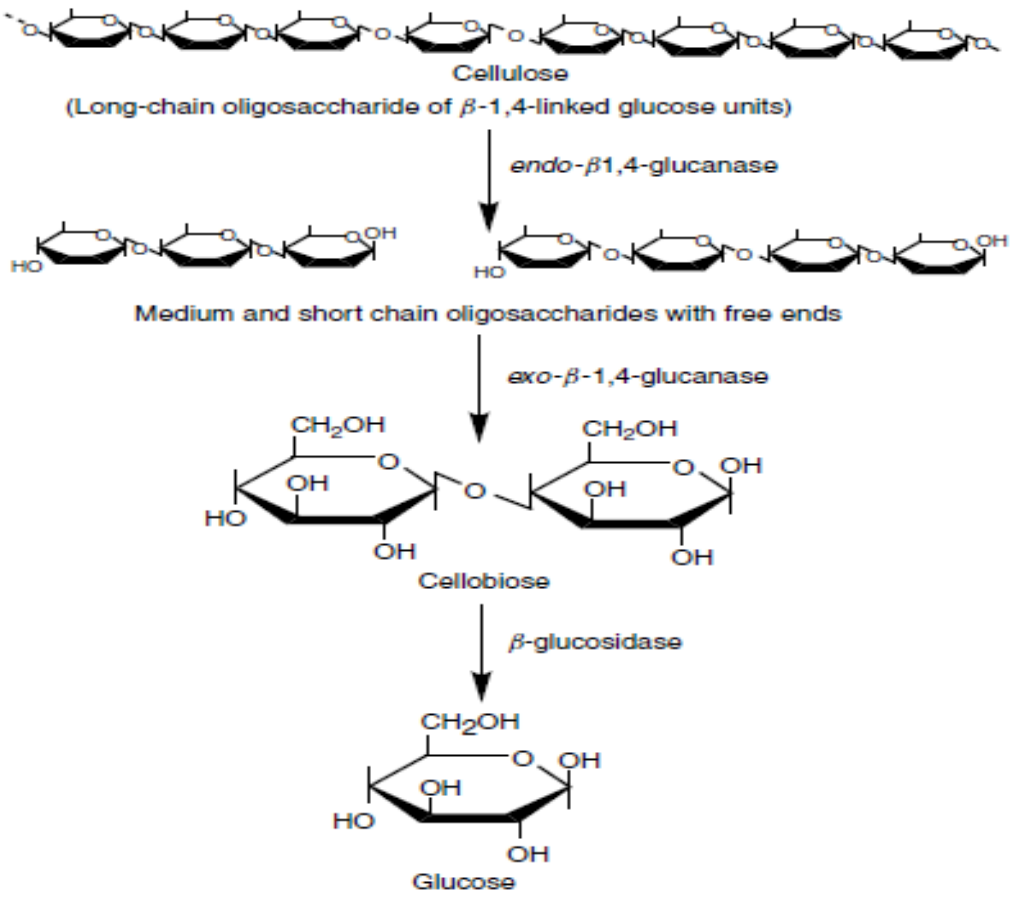

Figure 2-2: Enzymatic degredation of cellulose (Moat et al.,2002)

Since glucose can be easily found in the cellulose or hemicellulose of the plants, raw materials such as rice straw, saw dust, sugar cane bagasse, corncobs, soybeans and wheat can be a good hemicellulose source (Walther et al., 2001). Furthermore, by using these types of raw materials can save a lot of cost since the price is cheap and it is renewable source. However, these raw materials have to undergo pre-treatment prior to the study.

Table 2.4, Table 2.5 and Table 2.6 will show the composition of the glucose in hydrolysates in the raw materials.

Table 2-4: Compositions of the hydrolysates in sugar cane bagasse (Pessoa et al., 1996)

pH	1.0
TRS (g/l)	25.4
Xylose (g/l)	18.5
Glucose (g/l)	5.1
Furfural (g/l)	2.0
Hydroxymethyl-furfural (g/l)	0.08
Acetic acid (g/l)	3.7

Table 2-5: Composition of the hydrolysates in rice straw (Mussatto and Roberto, 2004)

Components	Hydrolysate	
	Raw	Detoxified
Glucose (g/l)	15.26	15.09
Xylose (g/l)	91.15	91.01
Arabinose (g/l)	18.34	18.80
Acetic acid (g/l)	1.518	1.263
Furfural (g/l)	0.025	0.007
HMF (g/l)	0.248	0.075
Lignin derivatives (absorbance at 280 nm)	0.410	0.294

Table 2-6: Composition of hydrolysates in various raw materials (Walther et al.,2001)

Hydrolysate	Xylose	Glucose	Arabinose	Galactose	Mannose	Reference
Sugar cane bagasse	58	16	26	-	-	Chen and Gong (1985)
	75	14	11	-	-	Roberto et al. (1995)
Rice straw	67	21	12	-	-	Roberto et al. (1995)
Hardwood	70	14	5	5	5	Perego et al. (1990)
	62	16	4	8	9	Jeffries and Screenath (1988)
	27	11	5	14	43	Olsson and Hahn-Hàgerdahl (1993)
Corn fiber	16	71	11	2	-	Saha et al. (1998)
	31	41	25	4	-	Hespell et al. (1997)
Isolated corn fiber xylan	26-60	16-37	24-46	-	-	Hespell et al. (1997)

Based on the composition shown in Table 2.4, Table 2.5 and also Table 2.6, there are two compounds that have the abilities to produce sugar alcohol. Glucose or D-Glucose has the tendency to produce sorbitol while Xylose or D-xylose has the tendency to produce xylitol. However, in this research the main character is the glucose or D-Glucose in which has the tendency to produce sorbitol. Figure 2.3 and Figure 2.4 shows the structure of D-Glucose and also Sorbitol while Figure 2.5 and Figure 2.6 show the structure of D-Xylose and Xylitol.

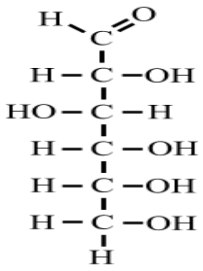

Figure 2-3: Structure of D-Glucose

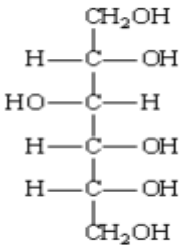

Figure 2-4: Structure of Sorbitol

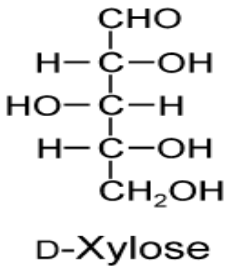

Figure 2-5: Structure of D-Xylose

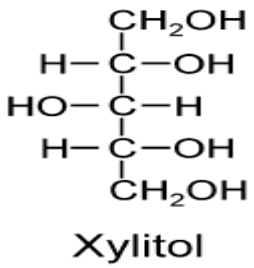

Figure 2-6: Structure of Xylitol

Besides study the kinetic growth of the *Candida tropicalis*, this research also aim to investigate the yield of sorbitol via the fermentation process. Sorbitol or D-Sorbitol; also known as D-glucitol (Akinterinwa et al., 2008) is six-carbon sugar alcohol which is the stereoisomer of mannitol nowadays, has vast application in the industry. In order to produce sorbitol, the metabolic pathway of glucose in *Candida tropicalis* is studied. Figure 2.7 shows the pathway of the sorbitol production with glucose as the carbon source.

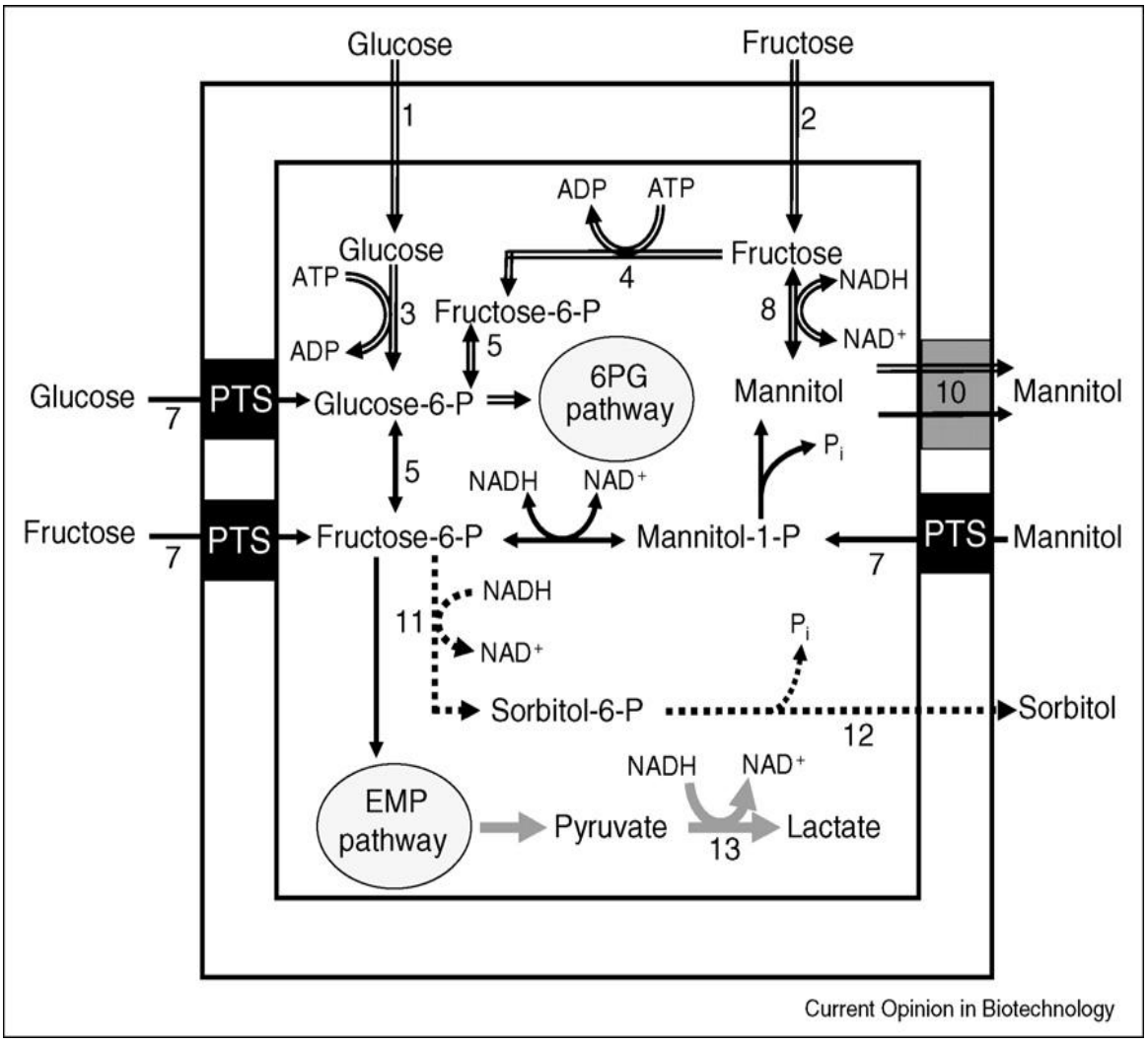

Figure 2-7: The pathway of sorbitol production (Akinterinwa et al., 2008) . Enzymes – 1: glucose permease; 2: fructose permease; 3: glucokinase; 4: fructokinase; 5:phosphoglucose isomerase; 6: mannitol-1-phosphate dehydrogenase; 7: PEP – dependent PTS; 8: mannitol dehydrogenase; 9: mannitol -1- phosphatase*; 10: unspecified hexitol transport; 11: sorbitol -6-phosphate dehydrogenase; 12: mechanism unspecified*; 13: lactate dehydrogenase. PTS, phosphotransferase system; 6PG, 6-phosphogluconate; EMP, Embden-Meyerhof – Parnas. *Reactions 9+10 and reaction 12 may occur via PTS.

When using xylose as the carbon source in the production of ethanol, xylitol will be produced in the fermentation process when the oxygen is set as the limiting condition. Besides that, the time consume for the utilization of xylose is lag up to 24 hours. (Farooq et al, 2000). However, if the D-glucose is used, the production of ethanol is four time faster than xylose. Hence, D-glucose was chosen as the substrate in the Solid State Fermentation (SSF) process instead of xylose.

2.3 Yeast Peptone Dextrose (YPD) Medium

Compared to bacteria, cultivation of yeast usually is much simple, economical and also in shorter time with a doubling time in rich medium if approximately up to 90 minutes (Bergman, 2001). Furthermore, yeast can grow either in liquid medium or on the surface of a solid agar plate. Generally, yeast cells have the ability to grow on minimal medium contain dextrose (glucose) as the carbon source and mineral salts that supply phosphorus, nitrogen and also trace elements that are needed by the yeast cells. However, yeast cells have the tendency to grow faster in the presence of rich medium that contains yeast extract and peptone as the reagents. For the selective isolation of fungi and isolation and identification of *Candida tropicalis* purpose, usually the BD Sabouraud GC Agar and CHROMagar Candida Medium (Biplate) are used.

Sabouraud Agar which is low in pH and high glucose concentration is more suitable for fungi. Fungi that can grow under this condition will eventually grow on this agar. In Sabouraud GC Agar the nitrogen source is peptone and the glucose will be the energy source to grow the fungi. CHROMagar Candida Medium on the other hand is a selective and differential medium for isolation and identification of Candida species including *Candida albicans*, *Candida tropicalis* and *Candida krusei*. On this agar the colonies of *Candida albicans* will appear light to medium green while colonies *of Candida tropicalis* will appear blue-greenish to metallic blue and *Candida krusei* colonies will appear light rose with a whitish border due to the inclusion of chromogenic substrate in the medium (Becton and Dickinson, 2003). Table 2.7 shows the strains that grow on both Sabouraud Agar and CHROMagar Candida Medium for incubation hours of 20 to 48 hours at 35 °C.