STRAIN IMPROVEMENT VIA RANDOM MUTATION FOR GLUCOAMYLASE PRODUCTION BY Aspergillus niger van Tieghem THROUGH SOLID STATE FERMENTATION

by

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS AND ABBREVIATIONS	FULL NAME
%	Percentage
x *g*	Times gravity
β	Beta
α	Alpha
°C	Degree Celcius
µg	Microgram
µL	Microliter
µm	Micromolar
U	Enzyme unit
U/ml	Enzyme unit in SmF
U/g	Enzyme unit in SSF
2D	Two dimensional
3D	Three dimensional
A	Adenine
ANOVA	Analysis of variance
AO	Acridine orange
C	Cytosine
CCD	Central composite design
Cd (II)	Cadmium ion
CO_2	Carbon dioxide
$C_3H_8O_3S$	Ethyl methane sulphonate
cm	Centimeter
Da	Dalton
EMS	Ethyl methane sulphonate
g	Gram
G	Guanine
GA	Glucoamylase
HCl	Hydrochloric acid

H_2O	Water
H_2O_2	Hydrogen peroxide
HNO_3	Nitric acid
kDa	Kilodalton
KH_2PO_4	Monopotassium phosphate
L	Litre
M	Molarity
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
ml	Millilitre
min	Minutes
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
$NaHCO_3$	Monosodium carbonate
NaOH	Sodium hydroxide
NH_2OH	Hydroxylamine
O_2	Oxygen
OD	Optical density
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
psi	Pound per square inch
RAPD	Randomly amplified polymorphic DNA
RPM	Rotation per minute
RSM	Response surface methodology
SEM	Scanning electron microscope
SmF	Submerged cultivation system
SSF	Solid state fermentation
T	Thymine
TBE	Tris Borate EDTA
UV	Ultra violet
V	Volt
w/v	Weight per volume
Zn (II)	Zinc ion

PENAMBAHBAIKAN STRAIN *Aspergillus niger* van Tieghem SECARA MUTASI RAWAK UNTUK PENGHASILAN ENZIM GLUKOAMILASE MELALUI FERMENTASI KEADAAN PEPEJAL

ABSTRAK

Enzim glukoamilase digunakan secara meluas untuk penukaran kanji kepada glukosa semasa proses sakarifikasi. *Aspergillus niger* van Tieghem lazimnya digunakan untuk penghasilan enzim glukoamilase melalui fermentasi kultur tenggelam dan fermentasi keadaan pepejal. Kajian telah dibuat untuk meningkatkan penghasilan glukoamilase oleh *Aspergillus niger* van Tieghem melalui mutasi rawak menggunakan agen mutasi kimia, etil metana sulfonat (EMS). Mutan ANC9 telah dikenalpasti sebagai mutan yang berpotensi kerana aktiviti enzim glukoamilase oleh ANC9 adalah masing-masing sebanyak 1.9 dan 1.6 kali ganda terhadap kanji masak dan kanji mentah melalui fermentasi kultur tenggelam menggunakan kanji masak sebagai substrat. Aktiviti enzim glukoamilase yang telah dihasilkan oleh mutan ANC9 melalui fermentasi keadaan pepejal terhadap kanji mentah dan kanji masak adalah sebanyak 44.81±1.2 U/g dan 101.50±2.2 U/g masing-masing berbanding dengan strain asal *A. niger* van Tieghem yang hanya menghasilkam enzim glukoamilase sebanyak 34.4±0.5 U/g terhadap kanji mentah dan 79.1±2.2 U/g terhadap kanji masak. Kajian untuk mengenalpasti kestabilan mutan menunjukkan enzim glukoamilase yang telah dihasilkan oleh mutan ANC9 untuk lima generasi secara berturut-turut adalah sama. EMS juga memberi kesan terhadap morfologi mutan selepas proses mutasi. Diameter spora konidia strain asal dan mutan ANC9 telah diukur dan diameter spora strain asal didapati lebih besar ($P<0.05$) berbanding dengan diameter spora strain mutan. Analisa capjari RAPD menggunakan OPG 05, OPG 07 dan OPG 19 menunjukan perbezaan dalam pola jaluran yang diperolehi

daripada strain asal dan strain mutan ANC9. Oleh itu mutan ANC9 adalah berbeza dari segi genetik daripada strain asal *A. niger* van Tieghem. Berdasarkan kajian awal, terdapat dua faktor yang penting untuk penghasilan enzim glukoamilase melalui fermentasi keadaan pepejal iaitu substrat dan bahan sokongan. Kaedah permukaan bergerakbalas menunjukkan bahawa keadaan optimum untuk penghasilan enzim glukoamilase ialah, 0.51 g kanji ubi kayu, 3.95 g sekam padi dan juga tempoh pengeraman selama 7 hari. Selepas proses pengoptimuman, akitiviti enzim glukoamilase yang dihasilkan oleh mutan ANC9 telah meningkat sebanyak 2.1 kali ganda terhadap kanji mentah dan 1.1 kali ganda terhadap kanji masak. Ketebalan substrat yang berbeza dalam penghasilan glukoamilase di dalam sistem dulang menunjukkan yang ketebalan substrat sebanyak 1 cm menghasilkan aktiviti glukoamilase tertinggi terhadap kanji masak (124.03±2.7 U/g) dan kanji mentah (98.35±2.3 U/g) berbanding ketebalan substrat 0.5 cm dan 2 cm di dalam sistem dulang. Penemuan penting dalam kajian ini adalah peningkatan dalam penghasilan enzim glukoamilase terhadap kanji mentah sebanyak dua kali ganda apabila keadaan optimum digunakan. Penghasilan enzim glukoamilase yang dapat menguraikan kanji mentah adalah sangat penting dalam industri pemprosesan kanji kerana ia boleh menjimatkan kos dan juga masa pemprosesan semasa proses sakarifikasi.

STRAIN IMPROVEMENT VIA RANDOM MUTATION FOR GLUCOAMYLASE PRODUCTION BY *Aspergillus niger* van Tieghem THROUGH SOLID STATE FERMENTATION

ABSTRACT

Glucoamylase enzyme is widely utilized for conversion of starch into glucose during saccharification process. *Aspergillus niger* van Tieghem is commonly used for glucoamylase production in submerged cultivation system (SmF) and solid state fermentation (SSF). This research was carried out to enhance glucoamylase production by mutating *A. niger* van Tieghem using chemical mutagen, ethyl methane sulphonate (EMS) via random mutation. Mutant ANC9 was identified as a potential mutant which produced, 1.9 and 1.6 fold higher glucoamylase activity towards cooked starch and raw starch respectively in SmF using cooked starch as substrate. Glucoamylase activity towards raw starch and cooked starch in SSF by mutant ANC9 were 44.81±1.2 U/g and 101.50 ±2.2 U/g respectively compared to wild strain which was only able to produce 34.4±0.5 U/g towards cooked starch and 79.1±2.2 U/g towards raw starch. The stability studies of the mutant revealed that glucoamylase enzyme produced by mutant ANC9 retained almost similar enzyme activity for five successive generations. EMS exposure on *A. niger* van Tieghem affected the morphology of mutants besides affecting the ability of producing glucoamylase enzyme. Conidia spore diameter of wild and mutants were measured and the spore diameter of wild strain was significantly ($P < 0.05$) larger than mutant

strains. Mutant ANC9 was genetically different from wild strain of *A. niger* van Tieghem. It was proven through RAPD fingerprinting analysis using OPG 05, OPG 07 and OPG 19 which illustrated different banding patterns for wild strain and mutant ANC9. Based on preliminary studies, there were two significant factors (amount of substrate and amount of supporting material) affecting glucoamylase production in solid state fermentation (SSF). Response surface methodology (RSM) revealed that the optimum conditions for glucoamylase production were 0.51 g tapioca starch and 3.95 g rice husk for 7 days of incubation period. The improvement fold of glucoamylase activity by mutant ANC9 towards raw starch and cooked starch under optimum conditions were 2.1 and 1.1 respectively. Different bed height for glucoamylase production on tray system showed 1 cm bed height produced high glucoamylase activity towards cooked starch (124.03±2.7 U/g) and raw starch (98.35±2.1 U/g) compared to 0.5 cm and 2 cm bed height of tray system. The most significant finding of this research is the production of raw starch degrading glucoamylase. Glucoamylase activity towards raw starch under optimum conditions was double up which is crucial in starch processing industry to reduce production cost and time consumption during saccharification process.

1.0 INTRODUCTION

Glucoamylase (GA) or 1,4-α-D-glucan glucohydrolase; EC 3.2.1.3 is an exo acting enzyme, which releases D-glucose units sequentially from the non-reducing ends of starch and related poly- and oligosaccharides. Glucoamylase is a glycoprotein containing 5-20% carbohydrate, mainly mannose, glucose, galactose and glucosamine. The enzyme acts on a range of glucosidic linkages with the highest affinity to α-1,4-glucosidic bonds (Polakovic and Bryjak, 2004). Glucoamylase is widely used for saccharification of starch, production of syrups with various dextrinization degrees and also in the production of glucose. Among different moulds *Aspergillus* sp. and *Rhizopus* sp. are main glucoamylase producers in the industrial scale (Rajoka and Yasmeen, 2005). Fungal growth and morphology varies according to the culture conditions such as fermentation medium, oxygen, pH, inoculums size and hydro dynamic shear force (Koutinas *et al.*, 2002).

Conversion of starch into glucose, syrup and dextrin is important in the starch processing industry (Marshall, 1975). The hydrolysates produced are widely utilized as carbon source in the fermentation process and as sweeteners in the food and beverage processing industry. Starch acts as source of energy in the chemical industry and the glucose produced from the hydrolysis of starch can be converted into ethanol and antibiotic through fermentation process (Kennedy *et al.*, 1991). Tapioca starch which is classified as a source of raw product is abundantly found in Malaysia and one of the crucial raw materials in the fermentation processes. Therefore, enzymes such as amylase and glucoamylase

are needed in order to utilize this source in the fermentation process. Glucoamylase enzyme which has the ability to hydrolyze cooked and raw tapioca starch are crucial for industrial application.

Solid state fermentation (SSF) has been gaining momentum since it is a simple process which generates high yield. The term SSF denotes cultivation of microorganisms on solid, moist substrates in the absence of a free aqueous phase (Holker and Lenz, 2005). Submerged fermentation (SmF) is widely used in industries; however the cost of production in SmF is high and uneconomical. Therefore, SSF can be developed in order to substitute SmF. SSF has many advantages; it is simpler, requires lower capital, and has high productivity (Ellaiah *et al.*, 2002). Besides that, SSF reduces energy requirement and employs simpler fermentation media. SSF also consumes less water, generates low waste and have easier control against bacterial contamination (Kumar *et al.*, 2003). Instead of that, the scaling up of SSF system requires simple designation and does not require much automation in controlling the system. Generally, high yield of product can be obtained using SSF in comparison to SmF. Since the usage of water is limited in SSF, the disposal of fermented residue is easy and simple (Manpreet *et al.*, 2005). The sterility of SSF process is not so crucial since it is less susceptible for contamination due to absence of free flow of water.

Amirul and co-workers (1996) have isolated *A. niger* van Tieghem from tapioca processing factory, Sg. Siput, Perak, Malaysia. In order to improve the glucoamylase production using SSF, wild strain of *A. niger* van Tieghem had been mutated using ethylmethane sulphonate (EMS).

Selection of suitable strain for the production of glucoamylase is a tedious process. Strain improvement is an alternative method to increase the enzyme production. Industrial potential of fungal enzymes has stimulated research in the development of methods to improve strains, since the production level of enzymes in naturally occurring strains is sometimes low for commercial exploitation. Usually mutation can be caused by the combination ultraviolet (UV) irradiation or other chemicals such as nitrosoguanidine, EMS and some other mutagen. In this research EMS as sole mutagen was chosen to mutate *A. niger* van Tieghem randomly since it has been known as an effective mutagen for fungus as well as bacterial cells (Rakariyatham *et al.*, 2006). In most cases, mutation is harmful. Mutation can be useful in some stipulation in order to make the strain more adaptable to the surrounding environment. Mutagenesis steps applied in this research involve finding the suitable exposure time for mutation and screening of best mutants which produce high glucoamylase enzyme.

Optimization of process parameters is important to obtain high enzyme production. Usually parameters such as incubation period, type and amount of substrates and supporting materials, inoculums size and concentration of mineral medium greatly influence the enzyme production. It is crucial to optimize the parameters for glucoamylase production by mutant strain of *A. niger* van Tieghem. Generally, agriculture wastes in Malaysia such as rice husk, bean sprout, sugarcane waste and tapioca waste are utilized as supporting material in

SSF according to the concept of 'waste to wealth'. Besides ensuring sustainability of agricultural industry, these agro waste materials are found abundantly in Malaysia and classified as cheap carbon sources. Therefore, it is a smart choice to employ these wastes for glucoamylase production.

Optimization of process parameters is carried out using statistical design. Basically, there are two steps (general factorial design and two level factorial design) carried out in statistical design before proceeding to response surface methodology (RSM) to discover the optimum condition for glucoamylase production. RSM can be used to find the interaction between various parameters and to determine those giving significant effect on glucoamylase production. The present study is aimed to achieve the following objectives:

1) to improve strain of *A. niger* van Tieghem through random mutation using chemical mutagen, EMS for glucoamylase production
2) to identify important parameters that affect glucoamylase production in solid state fermentation by mutant strain of *A. niger* van Tighem
3) to optimize glucoamylase production in solid state fermentation by mutant strain of *A. niger* van Tieghem using response surface methodology

2.0 LITERATURE REVIEW

2.1 Glucoamylase

Glucoamylase enzyme (1,4-α-D-glucan glucohydrolase) is an exo acting enzyme which releases D-glucose unit from non-reducing end of the starch. Hydrolysis rate of α-1,6 residue is slower in the process of dextrin conversion to glucose. Glucoamylase enzyme is stable at pH less than 9 and at temperature lower than 50°C (Amirul *et al.*, 1996). Optimum glucoamylase activity is in the range of 40°C to 60°C and the pHs are between 4.5 to 5.0. Besides that, glucoamylase also exhibits high debranching activity by hydrolyzing β-dextrin and glycogen into glucose unit (Fogarty, 1983).

Glucoamylase is produced by various microorganisms including fungi, yeast and bacteria (Ellaiah *et al.*, 2002). However, glucoamylase synthesized actively by various fungi such as *A. foetidus, A. niger, A. oryzae, A. terreus, Mucor rouxians, Mucor javanicus, Neurospora crassa, Rhizopus delmar, Rhizopus oryzae* (Pandey *et al.*, 2000). *A. niger* able to synthesize glucoamylase enzyme which possesses optimum temperature at 50°C to 60°C (Norouzian *et al.*, 2000) and optimum pH at 4.5 to 5.0 (Miah and Ueda, 1997). Optimum temperature for glucoamylase produced by filamentous fungi such as *Trichoderma reesi* is at 70°C (Aquino *et al.*, 2001). Molecular weight of glucoamylase enzyme from fungi is in the range of 48 to 90 kDa. However, molecular weight of the glucoamylase produced by *A. niger* is 125 kDa (Suresh *et al.*, 1999).

Carbohydrate structure in glucoamylase is bounded by glycosidic bond to L-serine and L-theonine (Pazur *et al.*, 1980). Most of the starch processing industry is focusing on glucoamylase produced by *A. niger* and *R. oryzae* because the enzyme produced by these fungi is more stable at high temperature and has good activity near neutral pH. Glucoamylase enzyme shows better 1,6-glucosidase activity at high temperature (65°C) and low pH (4.5) which is crucial for commercial application (Frandsen *et al.*, 1999).

Purified GA enzyme from *A. niger* contains two types of isozyme, mainly GAI and GAII (Meagher *et al.*, 1989). These enzymes act towards glycosidic bond and they have higher affinity towards α-1,4 glycosidic bond. The ability of enzyme to hydrolyze both α-1,4 and α-1,6 glycosidic bond causes the perfect degradation of starch to glucose which is important in syrup producing industry (Lloyd and Nelso, 1984). Moreover, both GAI and GAII are differing in some characteristics. Molecular weight of GAI is 74,000 Da and it has isoelectric point at pH 3.95 with optimum pH of 4.2. GAI also stable at pH in between 4.0 to 7.0 and optimum temperature at 60°C. Conversely, molecular weight of GAII is 96,000 Da and it has isoelectric point at pH 3.8 with optimum pH of 4.5. In addition to this, GAII stable at pH in between 4.0 to 7.0 and optimum temperature at 65°C (Amirul *et al.*, 1996). GAII enzyme has higher affinity towards cooked starch and raw starch in comparison with GAI except for dextrin. In addition to this, GAII easily adsorbs into raw starch, and the hydrolysis rate is faster than GAI (Hayashida *et al.*, 1982).

Hydrolysis mechanism of glucoamylase involves proton transfer to the glycosidic oxygen of the scissile bond from general acid catalyst; formation of an oxacarbenium ion and a nucleophilic attack of water assisted by general base catalyst (Sauer *et al.*, 2000). Glu79 and Glu400 in GA from *A.niger* are general acid and base catalysts and the hydrolysis step involves these catalysts. The acid-base will donate hydrogen to the glycosidic oxygen and the catalytic-base will guide the nucleophilic attack by a water molecule on the carbon-1 of the glucose moiety (Norouzian *et al.*, 2005).

2.2 Starch

Starch is a polysaccharide abundantly found in plant and widely used for the production of glucose, fructose and maltose syrup in food industry (Roy and Gupta, 2004). Starch in cereal crops such as barley, sorghum, rice, maize, wheat, tubers and seeds are rich in grains. In addition to this, the sugar formed can be further fermented for the production of bioethanol and some other products (Giordano *et al.*, 2000). Generally, starch is in the native form need to be modified in order to apply in paper, food and textile industry. Besides that, physical modification such as shear, moisture, heat and radiation are gaining attention due to the by- products production and presence of chemical reagents in starch (Zavareze and Dias, 2011)

Molecules of starch granules are compressed to form polycrystalline with inter and intra bond. Due to this property, starch is insoluble in cold water and has good retention against chemical agent and enzyme treatment (Hamilton *et al.*, 1999).

The structure and packaging of starch granules varies expansively between plant species. Besides the structural differences, sources of starch are also different in the amylase, amylopectin, protein and lipid content (Dona *et al.*, 2010).

Starch is composed of the linear and branched D-glucose homopolymer, amylose and amylopectin. Amylose is a linear molecule with D-glucose unit which are held together with α-1,4 bond while amylopectin is branched polymer with α-1,6 bond. But, both polymers are varying in terms of their physical characteristics (Amirul *et al.*, 1996). Most of the natural starch consists of 20% amylose and 80% amylopectin. Interestingly, amylopectin comprises the major component of starch (about 70-80%) which strongly influences the physiochemical and culinary characteristic of starch (Dona *et al.*, 2010). Starch granules are synthesized in a broad array of plant tissues and within many plant species. Starch granules vary between one another in terms of size (1-100 μm in diameter), shape (round, lenticular, polygonal), size distribution (uni or bi-modal), association as individual (simple) or granule cluster (compound) and composition (α-glucan, lipid, moisture, protein and mineral content) as it reflects the botanical basis as indicated in Table 2.1 (Tester *et al.*, 2004).

Table 2.1: Characteristic of starch granules obtained from different botanical sources (Tester *et al.*, 2004).

Starch	Type	Shape	Distribution	Size (μm)
Barley	Cereal	Lenticular/ spherical	Bimodal	15-25, 2-5
Maize	Cereal	Spherical/ polyhedral	Unimodal	2-30
Millet	Cereal	Polyhedral	Unimodal	4-12
Oat	Cereal	Polyhedral	Unimodal	(3-10 single), 80 (compound)
Pea	Legume	Rentiform (single)	Unimodal	5-10
Potato	Tuber	Lenticular	Unimodal	5-100
Rice	Cereal	Polyhedral	Unimodal	3-8 (Single)
				150 Compound
Sorghum	Cereal	Spherical	Unimodel	5-20
Tapioca	Root	Spherical/ lenticular	Unimodel	5-25
Wheat	Cereal	Lenticular	Bimodel	15-35
			Spherical	2-10
			(B type)	

2.2.1 Tapioca starch

Tapioca (*Manihot esculenta* Crantz) or also known as cassava is a widely utilized starch in our country. Tapioca starch is used as source of food or raw materials in many industries such as production of monosodium glutamate (Chan *et al.*, 1983). Most of the granules are round in shape with size between 5-20 μm. The lipid and phosphorus content in tapioca starch is low while amylose content is in between 20-27% (Charlotte, 2004). Gelatinization temperature of tapioca starch is low in comparison to other starches determined by Richkard and co-workers (1991) which are mainly in the range of 62-73°C.

2.2.2 Wheat starch

Wheat starch granules can be found in large size (ranging from 20-35 μm) and also small size in the range of 2-8 μm. Amylose content of wheat starch are about 20-30% as in tapioca starch (Charlotte, 2004). Gelatinization temperature of wheat starch is in the range of 53-64°C. Hydrolysis of wheat starch will produce wheat syrup and solid glucose (dextrose) from the reaction as mentioned below:

$$(C_6H_{10}O_5)_n + nH_2O \xrightarrow{\text{Enzyme, heat}} nC_6H_{12}O_6$$

2.2.3 Rice starch

Most of the rice starch is processed to rice and other bakery products. But, small portion of it is also processed for animal feed and other products. Granule of rice starch is the most smallest than all other starches. The size of the rice starch is in the range of 3-8 μm. Granule of rice starch is geometric in shape with smooth surface. Amylose content of rice starch is in the range of 75-85% (Charlotte, 2004).

2.2.4 Corn starch

Corn starch contains about 25% of amylose and 75% of amylopectin. Maximum gelatinization temperature of corn starch is 65.4°C (Charlotte, 2004). Some characteristic of corn starch depend on the condition of the place where the corn grows. Amylose content of corn starch is normally in the range of 16.9% to 21.3%. Whereas swelling power of corn starch is in the range of 13.7 to 20.7 g/g (Singh and Soni, 2001).

2.2.5 Gelatinization of starch

The starch consumed by animals are mostly uncooked (not gelatinised) while most of the starch containing food consumed by human are cooked or gelatinized starch. Starch can be gelatinized by heating in hot water which will eventually improve chemical reactivity towards amylolytic enzyme (Singh and Soni, 2001).

There are several stages involve during the cooking of the starch including glass transition, gelatinization, swelling, pasting and retrogradation (Dona *et al.*, 2010). Starch form suspension when mechanically dispersed in cold water due to its insolubility. Hence, heating of starch in excess water will cause the formation of homogenous starch water mixtures. During glass transition stage, the temperature used affects the amorphous region of crystalline starch polymer hence starch will lose its glassy and brittle properties.

During gelatinization process, starch granule expands when it is progressively heated in the presence of excéss water. During heating, the granules hydrate progressively and the double helices undo as hydrogen bonds are ruptured. In addition, the crystalline regions are converted to amorphous regions as a consequence of ruptured hydrogen bonds. Furthermore, granules continue to imbibe water and ultimately the granules swell until the granular form is lost and they tend towards gelatinization (~4% solids) and/or solubilization (≤4% solids) (Tester *et al.*, 2006).

Normally gelatinization takes place around 60°C. Cooking starch above the mentioned temperature will cause it to absorb considerable amount of water and lose its semi crystalline property (Miyazawa *et al.*, 2006). Interestingly, some starch completely solubilize when heated at 100°C. At the same time the starch suspension is widely autoclaved for the formation of homogenous starch besides degrading the starch polymer (Gidley *et al.*, 2010). Gelatinized starch will swell to multiple times

from their original volume. Swelling of the starch is caused by the leaching of amylose molecules and lipids bound in the granules while amylopectin contributes for the uptake of water during swelling yet the starch which has potential to swell contain more amylopectin composition (Bogracheya *et al.*, 2001).

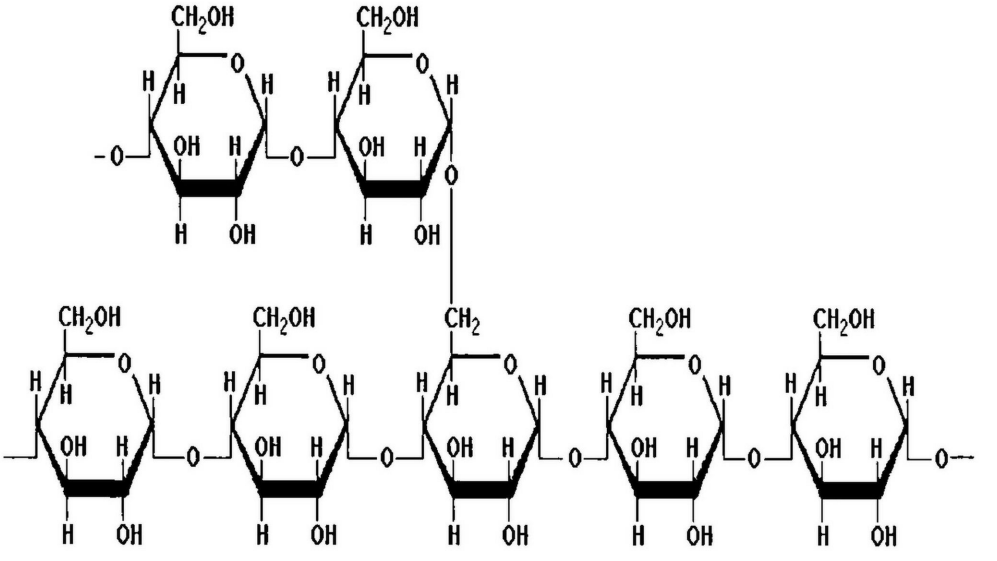
Gelatinized starch is more susceptible for the digestion by α-amylase compared to its native form. The rate of hydrolysis of gelatinized starch depends on the amylose/amylopectin ratio and also the amylose complexes with lipids. The main determinants of starch behavior are the changes occur during gelatinization (Zavareze and Dias., 2011).

2.2.6 Amylose and amylopectin structure

Amylose and amylopectin have different structures and properties ((Figure 2.1). Amylose is relatively long, linear α-glucan containing around 90% (α-1-4)- and (α-1-6) linkages and differs in size and structure depending on botanical origin. Amylose has molecular weight of approximately $1x10^5$ – $1x10^6$ kDa (Mua and Jackson, 1997). Each chain contains ~200-700 glucose residues equivalent to a molecular weight of 32400-113400 Da (Morrison and Karkalas, 1990).

Amylopectin is larger molecule than amylose with molecular weight of $1x10^7$ – $1x10^9$ Da and heavily branched structure built from about 95% (α-1-4) and 5% (α-1-6) linkages (Morrison and Karkalas, 1990).

(a)



(b)

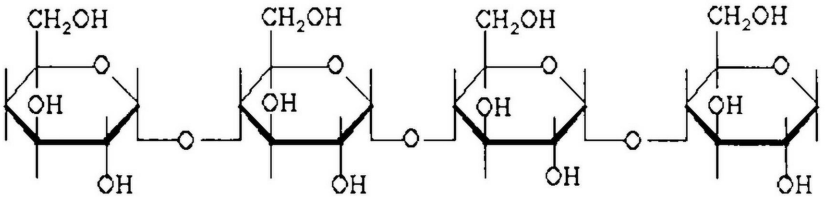


Figure 2.1: Stucture of (a) amylopectin and (b) amylose (Tester and Karkalas, 2002)

Similar as amylose molecular size, shape, structure, and polydisperity of the molecule vary with botanical origin (Table 2.2). Conversely, there is a great additional variation with respect to the unit chain length and branching patterns. Amylopectin unit chains are relatively shorter compared to amylose molecules with a broad distribution profile (Tester *et al.*, 2004). They are typically ~18-25 units long on average although the range is extended (19-31) if high amylose starches are also included (Jane *et al.*, 1999). In addition to this, both amylopectin and amylase can form double helices which may in turn associate to form crystalline domains. In most starches these are confined to the amylopectin component (Tester *et al.*, 2004).

Table 2.2: Differences between physical characteristic of amylose and amylopectin (Fredriksson *et al.*, 1998)

Characteristic	Amylose	Amylopectin
Structure	Linear (α-1,4)	Branched (α-1,6)
Stability in the solution	Not stable	Stable
Iodin colour	Blue	Grey
Digestin, β-amylase	100%	60%
Polymerization degree	1500-6000	$3x10^5$- $3x10^6$

2.2.7 Enzymatic hydrolysis of starch

In starch processing industry starch can be converted into sugar, syrup and dextrin. Glucose is a crucial product of starch hydrolysis since it can be converted into commercial product such as ethanol, antibiotic and sorbitol. Therefore, starch is classified as source of energy and raw product in chemical industry (Ibrahim, 1994).

Hydrolysis process involves two stages; thermal and enzymatic liquefaction followed by enzymatic saccharification. Meanwhile, third stage hydrolysis process begins with melting of starch, followed by liquefaction and enzymatic saccharification (Veen *et al.*, 2006). During saccharification process, starch will be gelatinized in order to open its structure for enzymatic reaction (Sigh and Soni, 2001). Gelatinized starch undergoes liquefaction process using heat stable α-amylase at high temperature and followed by the action of glucoamylase enzyme for saccharification process at temperature around 50-60°C (Sigh and Soni, 2001). The whole process requires high energy and therefore, estimated cost of production is high.

Consumption of natural source and starch hydrolysis using one stage at gelatinization temperature is expected in order to reduce energy consumption and cost of production (Kelly *et al.*, 1995). The research carried by Ueda (1981) stated that, utilization of glucoamylase from *A.awamori* can degrade raw starch completely to glucose without leaving any dextrin in one-step saccharification process. Amylose

enzyme can perform better on gelatinized starch than on raw starch. There are many research findings reported that, the best amylase enzyme producer for starch degradation are fungi such as *Aspergillus* sp., *Rhizopus* sp. and *Cortivium rolfsi* (Goyal *et al.*, 2005).

2.2.8 Saccharification process

Saccharification process always carried out using glucoamylase enzyme from *Aspergillus niger* for glucose production (Pandey, 1995). Glucoamylase enzyme hydrolyzes α-1,4 bond of starch to glucose then catalyses condensation process on α-1,6 amylopectin (Meagher and Reilly, 1989). The saccharification process of starch is shown in Figure 2.2. In the beginning of saccharification process, formation of dextrin is high. The concentration of dextrin reduces at the end of the saccharification process. High concentration of dextrose in the beginning stage causes the branching of dextrose to isomaltose and other saccharides. Therefore, up to one point, the concentration of dextrose will reduce (Olsen, 1995). Purification of end product is crucial in order to obtain glucose rather than other co-product of starch saccharification. After the glucose isolated from other co-products, screening of glucose is carried out to ensure the real application of glucose in variety of industry especially food and medicine producing industries.

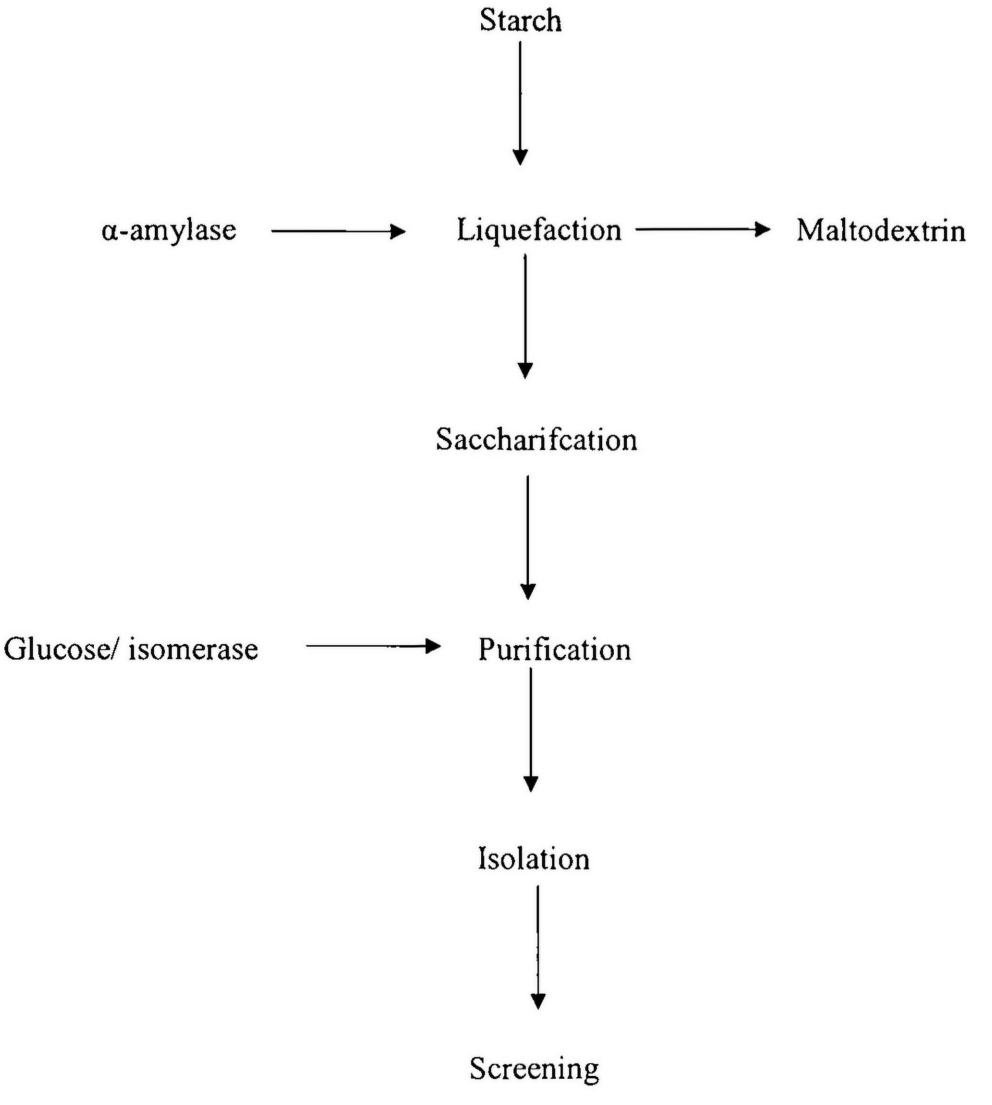


Figure 2.2: Enzymatic process of starch conversion (Olsen, 1995).

There are limitations in the mathematical modeling of starch hydrolysis. First of all, starch consists of a mixture of highly branched amylopectin, linear chain of amylose and intermediate structures. These polymers are differing in respect to glucopyranosyl linkages, molecular weight distribution and hydrolysis rate (Gupta *et al.*, 2004). As the hydrolysis of starch by glucoamylase proceeds by a stepwise splitting of glucose units from non reducing end, the molar concentration of substrate does not essentially change until the late phase of reaction (Polakovic and Bryjak, 2004). Besides that, another limitation is the presence of a wide array of linear and branched dextrins in the reaction mixture, during the starch saccharification.

2.3 Solid state fermentation

SSF has been employed since ancient time for the production of traditional foods and also some metabolites. SSF begins with the making of bread and cheese by Egyptians during 2600 $_{B.C}$. It is then developed for koji process from China to Japan by Buddhist priest (Rodriguez *et al.*, 1999). SSF is used until 18th century for food processing and the fermented food produced in SSF is easily digested, less toxic and the smell and taste can be improved (Pandey *et al.*, 2000). Later, SSF is developed to compose and prepare livestock food (Ooijkaas *et al.*, 2000). Advancement in biotechnology has attracted interest in developing SSF. Steroid transformation using fungal cultures in SSF is first reported during 1950-1960. Thus, begin the development of SSF for the production of mycotoxins and protein rich feed which utilizes agro-industrial residue (Manpreet *et al.*, 2005).

SSF is defined as a fermentation process occurring in the absence of free flow of water (Rodriguez and Sanroman, 2005). SSF has shown some unique applications for the production of enzyme by filamentous fungi. There are many important aspects in SSF. The most concern issues in the fermentation process are selection of microorganism and substrate, optimum process parameters and also purification of the end product. Hence, for yeast and fungus, SSF is more suitable since these species are well adapted to grow on low water activity (Manpreet *et al.*, 2005). Yet, some findings have reported the application of bacteria on SSF for metabolite production after some modifications have been carried out.

2.3.1 Developments of SSF technology

Development of SSF is held up in western countries due to the development of submerged cultivation system (SmF) which substitutes the role of SSF in 1940. Demand for the penicillin during second world war has made the SmF developed extensively and established for the production of metabolites from microorganisms (Selvakumar and Pandey, 1999). Besides that, presence of mycotoxin from fungus has been the major drawback for the development of SSF in later years. Mycotoxin is the secondary metabolite from fungus which is poisonous and harmful for human and animals. Mycotoxin usually spreads through touch or by scenting the spores of fungus which can produce mycotoxin (Ferreira *et al.*, 1999). Therefore, SSF was only concentrated for the production of food for livestock which is rich in protein content. Normally, substrates used for this purpose are from agricultural wastes.

Besides lowering the cost of production, SSF also reduces the risk of contamination from agro waste (Pandey *et al.*, 2000). However, scientists are started to pay attention again on SSF since few years ago. Many researches and patents about SSF are produced in the development of fermentor or bioreactor, modeling of microorganism, production of fermented food, production of primary and secondary metabolite and also bioprocess involving biofiltration, bioproduction of paper, and recovery process (Pandey *et al.*, 2000).

SSF is started to be exploited for the production of single cell protein from agro waste since the year of 2000. Besides that, feedstock food produced from starch materials, ethanol production from sugar cane and tapioca and also production of enzyme, organic acid, biogas, antibiotic and surfactant are extensively produced using SSF (Raghavarao *et al.*, 2002). The main reason SSF getting back its attention is due to the competition to produce metabolites from microbes with low cost of production which can be achieved using SSF. The products obtained from SSF are in higher amount and more concentrated compared to SmF. Gonzalez co-researchers (2002), proved that production of pectinase, tannase and invertase through SSF are almost double in comparison to the production through SmF.

Recently, SSF are widely utilized for bioremediation purposes. Major chemical pollutants such as hazardous chemical and synthetic dyes such as congo red and azo dye are decolorized by employing various SSF bioreactors (Singhania *et al.*, 2009).

2.3.2 Advantages and challenges of SSF

SSF appears to have some advantages in comparison to SmF. It is reported that, the enzymes produced through SSF are richer in side activities compared to the enzymes produced via SmF when normalized to the same main activity (Suryanarayan, 2003). The enzyme titres produced through SSF are slightly higher compared to those produced in SmF. The experiments carried out by Rodriguez and Sanroman (2005) indicated that β-glucanase enzyme which is useful in brewing industry has 17% xylanase when produced through SSF. In contrast, the xylanase content is only 12% of β-glucanase when the enzyme is produced through SmF. In addition to this, production cost of SSF is low and economical since agricultural wastes are utilized for the enzyme production.

The sterility of the culture medium is not an important issue in SSF since the risk of contamination is very low due to the low water activity (Krishna, 1999). However, there are some challenges in SSF especially in the scale up and purification process. The monitoring process of heat and mass transfer in SSF is a difficult task. However, researches are going on in order to monitor these two factors and some other parameters via online monitoring system (Singhania *et al.*, 2009). The biomass estimation is another bottleneck in SSF. Hence, some procedures such as determination of glucosamine, ergosterol, protein, DNA and dry weight changes are widely utilized although they have some weakness in their own ways (Singhania *et al.*, 2009). Furthermore, end product isolation in SSF is tough and more costly when naturally occurring raw materials are utilized. This is because while extracting the product after fermentation, several water soluble components from the substrate

may leach together and mix with the end product which will cause difficulties during the purification process (Manpreet *et al.*, 2005).

There are some restrictions on scaling up process and bioreactor design for SSF. The major drawback in scale up of SSF is mass and heat transfer. There is no information regarding mass and heat transfer characterization up to date (Prabhakar *et al.*, 2005). In addition to this, limitation in the bioreactor design is also due to the wide variety of matrices used in the SSF which vary in terms of composition, size and mechanical property. Otherwise in SmF, all the media can be considered as liquid which will facilitate the bioreactor design (Durand, 2003).

2.3.3 Bioreactor design for SSF

Recently, researchers are starting to pay attention on scaling up SSF by focusing on bioreactor design. Good understanding of SSF over the period leads to the design and scale up of SSF bioreactors. SSF process can be operated in batches, fed batches or continuous modes (Prabhakar *et al.*, 2005). There are both laboratory scale bioreactors that consume from few grams up to few kilograms of dry solid medium, and pilot and industrial scale bioreactors that utilize several kilograms to tons of solid medium. There are few criteria that need to be considered in bioreactors design, such as the morphology of the fungus and its resistance towards mechanical agitation and also necessity for sterilization (Durand, 2003).

Generally petri dishes, wide mouth Erlenmeyer flasks or jars are utilized for laboratory scale bioreactors. In these bioreactors, only temperature can be regulated without forced aeration and agitation. These types of bioreactors are useful for screening of substrates or microorganism in the first step of the development process (Durand, 2003). However, it does not permit sampling during fermentation. In addition to this, a bioreactor known as rotating drum has been discovered for laboratory scale, which can be agitated throughout the fermentation time. This type of bioreactor will facilitate the contact between the drum wall and substrate which will enhance the oxygen supply to microorganism (Luciana *et al.*, 2003).

There are some bioreactors used for pilot and industrial scale. However, they cannot be commercialized because as the volume of substrate medium increase, it will restrict heat transfer which will affect productivity. SSF bioreactors without forced aeration are largely used in industry. It mimics the tray system whereby number of trays are increased and incubated in stalked on one another with few centimeters of gap at fixed temperature (Durand, 2003). It is difficult to ensure the sterility of this culture except if it is stored in the sterile room. Besides this, SSF bioreactors, known as bioreactors with unmixed forced aeration is also used for industrial applications. In this bioreactor, filtered air can flow through a sieve which supports the substrate. This bioreactor can be sterilized *in situ* using steam generated by water bath. There are few large scale reactors in use and still in the process of improving the property.