PRODUCTION OF TANNASE BY *Aspergillus niger* USING MANGROVE (*Rhizophora apiculata*) BARK UNDER SOLID SUBSTRATE FERMENTATION.

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percent
°C	Degree Celcius
cm	centimeter
g	gram
m	milli
mm	millimetre
mM	millimolar
mg	milligram
ml	millilitre
nm	nanometer
rpm	revolution per minute
µ	micro
µg	microgram
µl	micro litre
µm	micrometer
kD	kilo Dalton
K	Kilo
Kg	Kilogram
Rf	Relative mobility
M	Molar
U	Unit

v/ v	volume over volume
w/ v	weight over volume
w/ w	weight over weight
BSA	Bovine serum albumin
DEAE	Diethylamino ethyl-
DNS	Dinitrosalicylic
MEA	Malt extract agar
SDS	Sodium dodecyl sulphate
SDS- PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
SmF	Submerged fermentation
SSF	Solid substrate fermentation
TEM	Transmission electron microscope

PENGHASILAN ENZIM TANNASE DARIPADA *Aspergillus niger* DENGAN MENGGUNAKAN KULIT KAYU BAKAU (*Rhizophora apiculata*) SECARA FERMENTASI SUBSTRAT PEPEJAL

ABSTRAK

Kulit kayu *Rhizophora apiculata* merupakan bahan buangan industri arang kayu yang kaya dengan tannin dan digunakan sebagai substrat bagi penghasilan tannase melalui fermentasi substrat pepejal (SSF). Selepas perbaikan parameter fizikal and kimia di dalam sistem kelalang goncangan, didapati gabungan saiz zarah substrat 0.75 mm dan 2.00 mm, medium Czapek-Dox (pH7) sebagai agen pelembapan, kandungan kelembapan awal pada nisbah 1:1.5 (b/i), pengeraman pada suhu 30 ℃, pengadukan pada setiap 24 jam serta saiz inoculum sebanyak 1×10^6 spora/ml menunjukkan tannase mencapai 4.97 U/g substrat terfermentasi pada hari ke-6 tempoh pengkulturan. Selepas penambahan 5% asid tannik (b/b) dan 1% urea (b/b), tannase mencapai 39.34 U/g substrat terfermentasi selepas 6 hari pengkulturan. Aktiviti enzim meningkat sebanyak 1280.35% berbanding profil awal (2.85 U/g substrat terfermentasi). Sistem dulang (15.0 cm × 15.0 cm × 7.5 cm) digunakan untuk peningkatan skala bagi penghasilan enzim. Selepas perbaikan beberapa parameter utama, didapati ketebalan substrat sebanyak 0.6 cm (50 g substrat), kandungan kelembapan sebanyak 1:1.5 (b/i), pengadukan pada setiap 48 jam semasa waktu pengkulturan, saiz inokulum sebanyak 1

$\times 10^6$ spora/ml serta 6 hari masa pengkulturan menghasilkan tannase sebanyak 63.24 U/g substrat terfermentasi. Peningkatan skala selanjutnya dengan penggunaan 1 kg substrat pepejal melalui sistem dulang yang lebih besar (32 cm × 22 cm × 11 cm) menunjukkan tannase mencapai 127.13 U/g substrat terfermentasi pada hari ke-14 pengkulturan. Aktiviti tannase meningkat sebanyak 4360.70% berbanding dengan profil awal (2.85 U/g) dari sistem kelalang goncangan. Enzim kasar dipekatkan serta ditulenkan sebanyak 55 kali ganda dengan aktiviti spesifiknya 1.10, hasil sebanyak 0.02% berserta dengan berat molekul 55.92 kDa. Suhu optimum bagi aktiviti tannase kasar dan tulen ialah 70 ℃ dan stabil pada 30- 70 ℃ selama 1 jam. pH optimum bagi enzim kasar dan tulen masing-masing ialah pH 6 dan pH 5.5. Masing-masing stabil pada pH5.0 dan 5.5 kira-kira 1 jam. Asid tannik pada 0.4% (b/i) didapati merupakan substrat spesifik kepada tannase kasar and tulen. Ion logam seperti Al^{3+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, K^{+}, Li^{2+}, Mg^{2+}, Mn^{2+}, Na^{+} and Zn^{2+} didapati merencatkan aktiviti tannase kasar and tulen. Pemerhatian biodegradasi substrat yang dilakukan dengan mikroskop cahaya, SEM and TEM menunjukkan kesan biodegradasi oleh *Aspergillus niger* dan aktiviti tannase terhadap substrat terfermentasi.

PRODUCTION OF TANNASE BY *Aspergillus niger* USING MANGROVE (*Rhizophora apiculata*) BARK UNDER SOLID SUBSTRATE FERMENTATION.

ABSTRACT

Rhizophora apiculata bark is tannin rich waste material from charcoal industry, and was utilized as the solid substrate for the production of tannase in solid substrate fermentation system. Upon improvement of physical and chemical parameters via a simple shake flask system, combination of 0.75 mm and 2.00 mm of substrate particles, Czapek-Dox medium (pH7) as moisturizing agent, initial moisture content of 1:1.5 (w/v), incubation temperature at 30 °C, mixing at every 24 hours and inoculum size of 1×10^6 spores/ml showed tannase achieved 4.97 U/g of fermented substrate on the 6^{th} day of cultivation time. After supplementation of 5% (w/w) tannic acid and 1% (w/w) of urea, tannase achieved 39.34 U/g of fermented substrate after 6 days of cultivation. Enzyme activity increased 1280.35% compared to initial profile (2.85 U/g of fermented substrate). A tray system (15.0 cm × 15.0 cm × 7.5 cm) was carried out to scale-up the production of enzyme. After improvement of several key process parameters, substrate bed height of 0.6 cm (50 g of solid substrates), moisture content at 1:1.5 (w/v), mixing at every 48 hours during the cultivation period, inoculum size of 1×10^6 spores/ml and 6 days of cultivation time showed that tannase was 63. 24 U/g of fermented substrate. A further scale-up with 1

kg of solid substrates via a bigger tray system (32 cm × 22 cm × 11 cm) showed that tannase production achieved 127. 13 U/g of fermented substrate on the day 14 of cultivation. Enzyme activity increased 4360.70 % compared to initial profile (2.85 U/g) from shake flask system. Crude enzyme was concentrated and purified about 55 fold with specific activity of 1.10, recovery at 0.02 % and molecular weight of 55.92 kDa. Optimum reaction temperature for both crude and purified enzymes was 70 °C and stable at the range of 30-70 °C around 1 hour. Optimum pH for crude and purified enzyme was pH 6 and pH 5.5, respectively. They were stable at pH 5.0 and 5.5, respectively, around 1 hour. Tannic acid at 0.4% (w/v) was specific substrate for both crude and purified tannase. Metal ions of Al^{3+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, K^{+}, Li^{2+}, Mg^{2+}, Mn^{2+}, Na^{+} and Zn^{2+} inhibited the crude and purified tannase activities. Substrate degradation evaluation with the aid of light microscope, SEM and TEM showed the effect of biodegradation by *Aspergillus niger* and activity of tannase on fermented substrate.

CHAPTER 1 INTRODUCTION

1.1 Mangrove Forest in Malaysia

In the year 2003, mangrove forest in Malaysia was recorded a total area of 566,866 hectare, in which 17.6% is located in Peninsular Malaysia, 22.3% in Sarawak and 60% are located in Sabah (Loo, 2008; Oo, 2008). Forestry Department of Malaysia plays the role in managing the reserved mangrove forests to ensure the controlled production of woods. Oo (2008) reported that Malaysia is practicing clear-filling logging system. The replanting of mangrove trees is done by following the cycle of 20 – 30 years.

According to Malaysia Timber Council (2009), The Matang Mangrove Forest Reserve is the largest mangrove forest in Peninsular Malaysia. It slightly covers a total area of more than 40, 000 hectares. This mangrove forest which has been very well managed over the years has been able to successfully balance the preservation of the mangrove ecosystem in the country as well as the country's continuous demand for wood resources. Hence, Matang Mangrove Forest had been acknowledged as the best-managed mangrove forest in Malaysia.

For Matang mangrove forest, up to 73.6% have been classified as productive forest while 26.4% the non-productive or protected forest. The productive forests are meant and assigned by the ministry for the purpose of timber production to support the demand of wood sources. On the other hand, the Non-productive forests are meant for the conservation of bio-diversity, the alleviation of erosion, local community's needs and settlement, recreation, research and education (Loo, 2008).

In Matang forest, including rivers, various waterways form the important means of transportation. At the same time, these waterways also divide the forest reserve. As a consequence, about 85% of the Matang mangrove forests are tidal swamps which flooded naturally during high tide. The complex and fragile ecosystem of swamp forests play an utmost role in conserving the bio-diversity of the flora and fauna in the area (Malaysia Timber Council, 2009).

According to Afidah (2005), Bakau Minyak (*Rhizophora apiculata*) and Bakau Kurap (*Rhizophora mucronata*) are the two main mangrove species found in Matang forest. These mangrove plants are usually found along the riverbanks as well as in more tide-submerged areas. These two species of plants take up to 85% of the total forest area and hence they are also the major marketable species and high in commercial values. Besides, small channels frequently fetch in large quantities of Lenggadai (*Bruguiera parviflora*) propagules. These plants set in very fast in areas

that had been clear-felled, impairing the growth of bakau. Moreover, *Bruguiera cylindrica* (Seaward Berus Forest) is one more *Bruguiera* species that occurs mainly seawards. Since it is considered a standard lower than *Rhizophora* as a raw material for charcoal production, huge areas of this species stay unexploited in Matang Mangrove Forest.

1.2 Charcoal production in Matang

According to Oo (2008) and Loo (2008), since 1950, productive forests are clear-felled once the trees reach 30-year-old in Matang Mangroves Forest. Intensive planting is carried out every two years after final felling where it is necessary. The 30-year rotation provides the highest net return on capital value. The availability and productivity of the forest for final felling determine the quantity of greenwood that could be harvested for charcoal production and also the amount of charcoal processing kilns to be operated.

Malaysia Timber Council (2009) reported that RM27.2 million was the estimated annual value of charcoal for year 2000-2009. Charcoal is hence becomes the primary economic timber product from Matang Mangrove. In Matang, around 1.6m long of mangrove poles or billets will be placed inside each kiln for 28 days for charcoal production. The final product, charcoal is then left for a week in the kiln to cool before being taken out. Around 10.5 tonnes of charcoal will be obtained from each burn. The

entire amount of charcoal produced from this industry in Matang, is exported to Japan. Besides of using charcoal as a source of fuel, charcoal is also further processed into other useful products such as cigarette filters, shoe soles, soap, and water filters.

1.3 Research Objectives

Debarking is a must prior the charcoal making process. This is because barks of the mangrove logs contain high amount of water and therefore interfering the heating process in kilns. The removal barks are wastes of the charcoal making industry. These wastes will either left rotten in the field or burn off by the workers.

Nevertheless, due to the acidic nature of this bark, their disposal is a problem for the processing industries as its toxicity leads to serious environmental burdens.

Studies showed that high content of raw tannins ranges from 20- 40% (w/w) could be extracted from the barks of *Rhizophora apiculata* mangrove based on the 3 days of solid-liquid extraction with 70% (v/v) aqueous acetone (Yeong, 2003; Oo, 2008; Afidah, 2005). Barks *of Rhizophora apiculata* mangrove are reported as a good source of tannins because they are low in cost and abundantly available from the environment, especially the charcoal manufacturing plant from Matang, Perak. (Oo, 2008).

In order to use the barks and turn it into some value added products, SSF system is the choice. Even though tannase production by submerged fermentation has been studied extensively but tannase production via SSF system is scarce and none ever reported from Malaysia. Therefore, this research "converting waste to wealth" was designed with these objectives;

i. To study the usage of *Rhizophora apiculata* bark as a substrate for the production of tannase by *Aspergillus niger* under SSF.

ii. To improve physical parameters (cultural conditions) and chemical parameters (medium compositions) for maximal tannase yield in a shake flask system and shallow tray system – a scaling-up process.

iii. To purify and characterize tannase.

CHAPTER 2 LITERATURE REVIEW

2.1 Solid substrate fermentation

According to Standbury *et al.* (1995), fermentation carried the meaning of "to boil". It is the term derived from Latin verb *fervere*, which was actually describing the appearance of yeast action on malted grains or fruits extracts. The boiling appearance is due to the production of carbon dioxide bubbles which caused by the anaerobic catabolism of sugars present in the extract.

Five major groups of commercially important fermentations were categorized, namely:

- "Fermentation which produce microbial cells or biomass as products"
- "Fermentation which produce microbial enzymes as products"
- "Fermentation which produce microbial metabolites as products"
- "Fermentation which produce recombinant products"
- "Fermentation which modify a compound that is added to the fermentation process (Transformation process)"

In recent years, solid state (substrate) fermentation (SSF) has shown much promise in the development of several bioprocesses and products (Pandey *et al*., 2000). Solid substrate fermentation is generally defined as the growth of microorganisms, often fungi,

on solid moist substrates, or in other words, on solid substrates in systems with a continuous gas phase and no free flowing of water (Nagel, 2002). According to Pandey *et al*. (2004), water activity of the substrate, which is known as a_w, has a profound impact on SSF. Water activity is defined as the relative humidity of the gaseous atmosphere in equilibrium with the substrate. Pure water has water activity as 1.00. Therefore, SSF denotes the cultivation of microorganisms on solid, moist substrates, at the average water activity below 1.00 (Holker and Lenz, 2005). This also indirectly telling that, SSF only can be carried out by a limited number of microorganisms, mainly fungi, although some bacteria have also been used (Couto and Sanromán, 2006).

Most of the people have considered *solid state* and *solid substrate* fermentation essentially one and the same, but actually they are different. Krishna (2005) has critically reviewed the definitions of both solid state and solid substrate fermentation. According to this review, *solid substrate* fermentation includes those processes in which the substrate itself acts as the carbon source, and occurring in the absence or near-absence of free water, whereas *solid state* fermentation is defined as any fermentation process occurring in the absence or near-absence of free water, employing a natural substrate or on an inert substrate as solid support. Pandey *et al*. (2004) also reported that fermentation processes in which substrate itself takes part in biological process, i.e. used by the microorganisms, should be specifically termed as "solid substrate fermentation" while solid state fermentation should refer to a process specifically in which the solid substrate acts as support only and the liquid growth or fermentation medium, containing a soluble carbon source is impregnated on this. There are two types of SSF systems which have been distinguished depending upon the nature of the solid phase used. The

first type of SSF system involves cultivation on a natural material and it is the most commonly used system. On the other hand, the second type involves cultivation on an inert support impregnated with a liquid medium and it is less frequently used compared to the first one (Krishna, 2005).

SSF have been used since antiquity, though mostly for food fermentation and production of a few types of enzymes. Pandey *et al*. (2004) reviewed that the recorded history of SSF was described in Asia before the birth of Christ on cheese making using *Penicillium roquefortii*. Ancient Egypt was reported to make bread by SSF (Sato and Sudo, 1999). The use of soy sauce koji has been reported in China in the years of 1000 BC and the koji making process migrated to Japan by Buddhists in seventh century. During these periods, several fermented food such as tempeh, miso to name a few were reported in many South-East Asian countries. During 18^{th} century, apple pomace was used to make vinegar and gallic acid was used for the tanning of leather via SSF (Pandey *et al*., 2004).

History indicates that SSF was lost in oblivion in western countries after 1940 due to the emergence of submerged fermentation technology (Singhania *et al*., 2009). Pandey (2003) reported that SSF research always continued in progress even though in isolated pockets. In 1950-1960, steroids were reported to be transformed by using fungal cultures. During 1960- 1970, the trends of SSF still keep going on and on. Mycotoxins production via SSF was reported during the milestone of 1960- 1970. This led significant impact on cancer research (Pandey *et al*., 2004). Other than that, the production of protein enriched cattle feed, which utilized agro-industrial residues was the next major activity reported.

It is hence offers a unique process development for value- addition of these otherwise low cost residues, which is to some extent, a type of environment pollutants. Since then, there is a continuous increment in the extension of SSF arena (Singhania *et al*., 2009).

The period of 1980's and more especially after 1985, worldwide resurgence of SSF was seen (Pandey *et al*., 2004). Production of microbial products, such as secondary metabolites production (Robinson *et al*., 2001), enzymes (Pandey *et al*., 1999), development of bioprocess and products (Pandey *et al*., 2000), development of bioreactors (Mitchell *et al*., 2000) to name a few had appeared on fundamental aspects of SSF.

Raimbault (1998) reviewed that SSF has been applied to large-scale industries processes mainly in Japan presently. For example, traditional koji which is made in small wooden and bamboo trays, has now changed gradually to more sophisticated fixed room fermentations, rotating drum processes and automated stainless steel chambers or trays with microprocessors, electronic sensors and servomechanical stirring, loading as well as discharging. Besides, outside Japan, medium scale production of enzymes, such as pectinases is reported in India. Koji fermentation has been adapted to local conditions in United States and other Western countries, including Cuba. Blue cheese production has also been modernized in France with control of environment conditions and improvements on the mechanical conditioning of cheeses production of mould spores. Composting, which is always developed as small scale mushroom production has also been improved, modernized and scaled up in United States and European Countries. Various firms in United States and European Countries produce mushroom spawn by

cultivating *Agaricus*, *Pleurotus* or Shii- take aseptically on sterile grains under SSF system.

The main applications of SSF processes in various economic sectors are summarized in Table 2.1.

Most of the recent research activities on SSF are done as a possible alternative for conventional submerged fermentations (SmF), which are the main process in pharmaceutical and food industries in industrialized nations. Some basic major differences between SSF and SmF are listed in Table 2.2.

Table 2.1: Applications of SSF in economic sectors

Sector	Application	Examples
Agro-food industry	Traditional food fermentations.	Koji, sake, ragi, tempeh, rae, fermented cheeses.
	Mushroom production and spawn.	*Agaricus*, *Pleurotus*, Shii-take.
	Bioconversion of by-products	Sugar, pulp, bagass, coffee pulp, silage composting, detoxication
	Food additives	Aroma compounds, dyestuffs, essential fats and organic acids.
Environmental control	Biocontrol, bioinsecticides, bioremediation and biodegradation of hazardous compounds.	Pesticides, caffeinated residues, polychlorinated biphenyls (PCBs)
	Plant hormones	Gibberellins
Industrial fermentation	Enzymes production	Amylases, cellulases, proteases, pectinases, xylanases.
	Antibiotic production	Penicillin, probiotics
	Organic acids production	Citric acid, fumaric acid, lactic acid, gallic acid.
	Biofuel	Ethanol production
	Miscellaneous compounds	Pigments, biosurfactants, vitamins, xanthum gum.

(Raimbault, 1998; Pérez-Guerra *et al*., 2003)

Table 2.2: Major basic differences between solid substrate fermentation and submerged fermentation.

Solid substrate fermentation (SSF)	Submerged fermentation (SmF)
Culture medium is not free- flowing.	Culture medium is always free- flowing.
Depth of the medium is usually shallow (except in case of bioreactor).	Medium depth is greater.
Substrate used is water insoluble form.	Normally substrate used is water soluble form (except in few cases where one of these maybe water- insoluble)
Single water- insoluble substrate provides carbon, nitrogen, minerals and energy.	Different water-soluble sources of each are usually employed.
Solid substrate absorbs water, becomes moist and nutrient uptake is from such moist solids.	Nutrients are dissolved in water and their uptake is from dissolved state thru water.
Gradient in nutrient concentration with the progress of fermentation is a common feature.	Nutrients are uniformly distributed in the medium at any given time during entire course of fermentation.
Water availability is just sufficient to sustain optimum growth and metabolism of the culture and therefore water constitutes a lower proportion of medium volume.	Water availability is abundant without any standardization for its requirement and hence constitutes a major proportion of medium volume.
Culture system consists of three phases, namely solid, liquid and gaseous.	Culture system consists of two phases, namely liquid and gaseous. (In some cases, solid phase is also involved, but the concentration of solid is much lower compared to liquid phase and hence resulting in its suspension in the medium.)
Liquid phase in the medium is discontinuous.	Continuity in the liquid phase is a common feature.
Culture system is not aseptic beyond sterilization step with reliance on good-housekeeping.	The whole system is always under aseptic conditions unless otherwise required for economic reasons and has negligible effect on productivity.
Rigorous control of parameters during fermentation is not required (except for heat removal, oxygen supply and moisture).	Rigorous control of all the parameters during fermentation is a must.
Inoculum ratio is always larger.	Inoculum ratio is quite low unless dictated by the process.

Table 2.2: Continued

Solid substrate fermentation (SSF)	Submerged fermentation (SmF)
Oxygen for growth and metabolism is derived largely from gaseous phase and to a lesser extend from that dissolved in water associated with solid substrate.	Oxygen uptake is solely from that dissolved in liquid medium.
Aeration provides oxygen and removes metabolic heat as well as gaseous products.	Aeration just provides oxygen and removes gaseous products. Cooling water is passed through jacket or coil to remove heat.
Agitation of the medium may or may not be involved in the system.	Agitation of the medium is always essential and necessary for imparting uniformity except in the case of anaerobic fermentation.
Fungal growth happened with the hyphae penetrate deep into the solid substrate particles.	Fungal mycelial cells grow in the form of individual mycelium and form mycelial pellets which are uniformly distributed in agitated culture medium.
Yeast cells or bacterial cells grow by adhering themselves on solid substrate particles.	Yeasts and bacterial cells are uniformly distributed throughout the body of liquid in agitated culture.
Fermented medium at harvest is in moist solid form and contains products in highly concentrated form.	Fermented medium at harvest is in liquid state and contains products in relatively low concentrations.
pH control was done by buffering the solid substrates.	pH control is relatively easy by adjusting the pH of culture medium.

(Mitchell and Lonsane, 1992; Raimbault, 1998; Sato and Sudo, 1999)

2.2 Advantages of solid substrate fermentation over submerged fermentation

The advantages of SSF over SmF have been summarized by many authors. Doelle *et al.* (1992), Sato and Sudo (1999), Pérez-Guerra *et al.* (2003) and Hölker *et al.* (2004) have reported that SSF is relatively resistant to bacterial contamination as bacterial growth is restricted by low water activity and because of the low moisture content of solid substrate. Hence, the serious contamination on the solid substrate is rarely happen. The volumetric loading of substrate is much higher than SmF because the moisture content of solid substrate is lower. Sato and Sudo (1999) also stated that it is simple to treat fermented residues. Because of the low moisture content, the fermented residue can be dried and used as animal feed or fertilizer. SSF requires much less solvent and recovery cost compared to SmF to extract the products upon fermentation process. Moreover, it is also have been reported that compared with SmF, SSF can give greater yields, better product characteristics, lower energy consumption, a lesser environment impact of process and differential expression of metabolites (Gelmi *et al*., 2000; Couto and Sanromán, 2006). Ooijkaas *et al.* (2000) also reported that there is lower catabolite repression of enzyme production under SSF. Initial investigations at Biocon, more than 15 years ago, comparing minor metabolite (additionally co-expressed enzyme side activities) profiles of similar enzymes produced by SSF and equivalent enzymes produced by SmF led to the conclusion that commercially available solid state produced enzymes were richer in side activities compared to commercially available SmF enzymes, when normalized to the same main activity (Suryanarayan, 2003). In addition, Hölker and Lenz (2005) had done a critical review on biotechnological advantages of

SSF. They classified those advantages in several categories, namely biological features, ecological features, engineering features and from the viewpoint of economic as well.

2.3 Tannase

Tannase or tannin acyl hydrolase (E. C. 3. 1.1. 20) is an enzyme found accidentally by Tieghem in year 1867 according to Aguilar *et al*. (2007). This enzyme was discovered in an experiment where formation of gallic acid into an aqueous solution of tannin taken place. Two fungal species which were grown in this experiment were later identified as *Penicillium glaucum* and *Aspergilus niger* (Lekha and Lonsane, 1997).

Tannase, an inducible enzyme (Mahendran *et al*., 2006), able to catalyze the breakdown of hydrolysable tannins (Aguilar *et al*., 2007) and releases gallic acids and glucose (Figure 2.1; Lekha and Lonsane, 1997). Tannase acts on the depside (Figure 2.2) and ester linkages in hydrolysable tannins (Mondal *et al*., 2001a).

Aguilar *et al.* (2007) reviewed that tannase hydrolyses tannic acid completely to gallic acid and glucose through 2, 3, 4, 6, - tetragalloyl glucose and another two kinds of monogalloyl glucose. This is supported by the facts that gallic acid of methyl. *m*. digallate is liberated first and the same products can be detected too in the hydrolysate of 1, 2, 3, 4, 6, -pentagalloyl glucose.

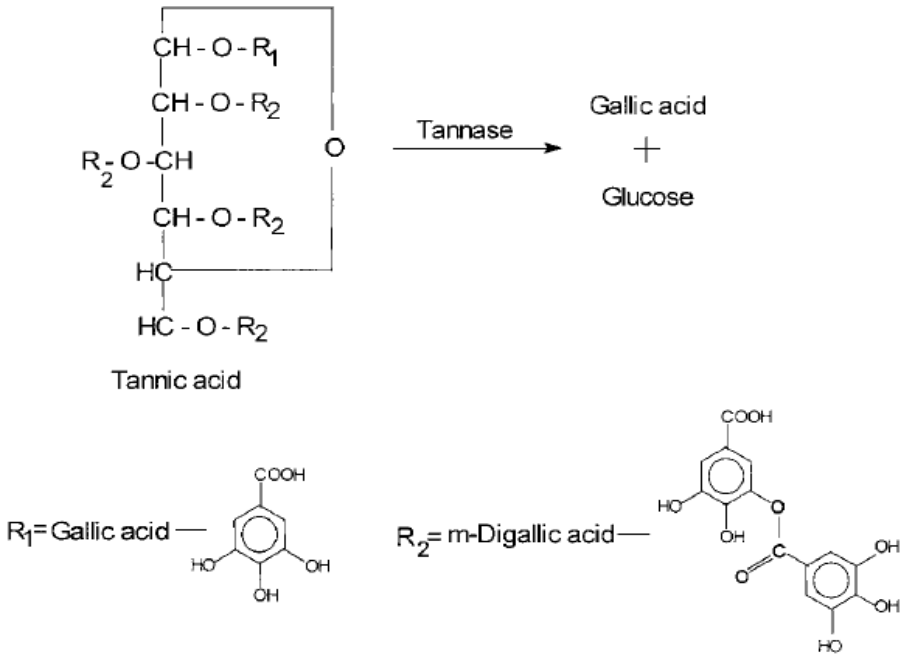

Figure 2.1: The hydrolysis of tannic acid by tannase into gallic acid and glucose.

(Adopted from Mondal *et al*., 2001a)

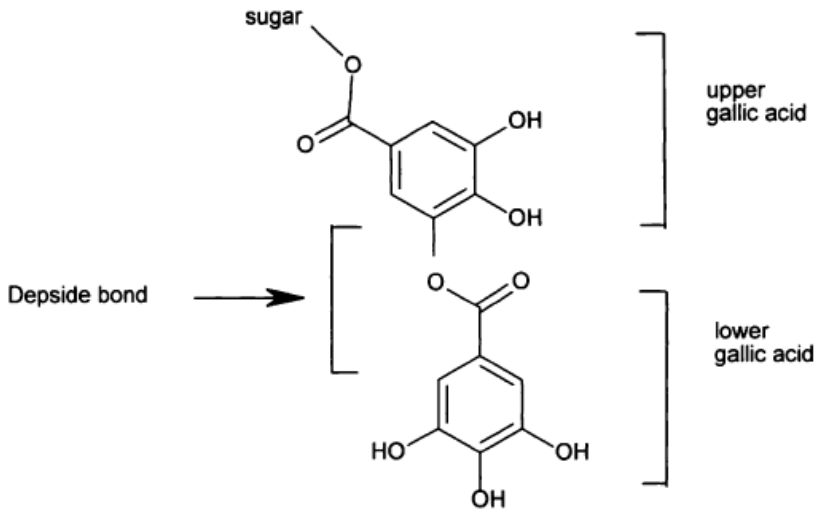

Figure 2.2: The depside bond

(Adopted from Mueller- Harvey, 2001)

According to the report of Albertse (2002), in the mechanism of action of tannase, some criteria are important to ensure the true enzyme-substrate complex to be formed. First of all, although the acid should be gallic acid, there should not be any restriction on the structure of an alcohol composing a substrate ester. Besides, a true enzyme- substrate (ES) complex might be prevented from forming due to the reaction of any phenolic hydroxyl on the binding site of the enzyme. Last but not least, an ester bond does not link to the enzyme by itself because an ester compound is not hydrolyzed by or inhibits the enzyme unless it has phenolic hydroxyls.

2.3.1 Tannase substrates

Spencer *et al.* (1988) defined tannins as naturally occurring water soluble polyphenol with varying molecular weights, which also differ from most other natural phenolic compounds in the ability in precipitating proteins from a solution.

Tannins are plant secondary metabolites as they do not play a part in plant metabolic pathway. They are considered second large group of plant polyphenolics after lignin (Bhat *et al.*, 1998). Tannins can be found abundantly in the plant kingdom such as pteridophytes, gymnosperms and angiosperms and widespread in leaves, fruits, bark, wood, needles, heartwood, grasses, seeds and even flowers of vascular plants (Bhat *et al*., 1998; Belmares *et al*., 2004; Aguilar *et al*., 2007). Tannins have molecular weight of higher than 500 and reaching values above 20000 kDa (Belmares *et al*., 2004).

Aguilar *et al*. (2007) reported that tannins can be classified into four major groups, namely gallotannins, ellagitannins, complex tannins and condensed tannins (Figure 2.3). Gallotannins are hydrolysable tannic acids because it is easily hydrolyzed under mild acid or alkaline condition, either in hot water or enzymatically (López- Ríos, 1984). Ellagitannins are more stable than gallotannins while complex and condensed tannins are considered not to be easily hydrolysable because of the flavonoid building blocks which made up the complex (Ramirez- Coronel *et al*., 2004).

In short, hydrolysable tannins, which are the substrate of tannase, are constituted by several molecules of organic acids such as ellagic acid, digallic acids, chebulic acids and gallic acids, esterified to a molecule of glucose. Besides, molecules with core of quinic acid instead of glucose have been also considered as hydrolysable tannins (Figure 2.4; Mueller- Harvey, 2001).

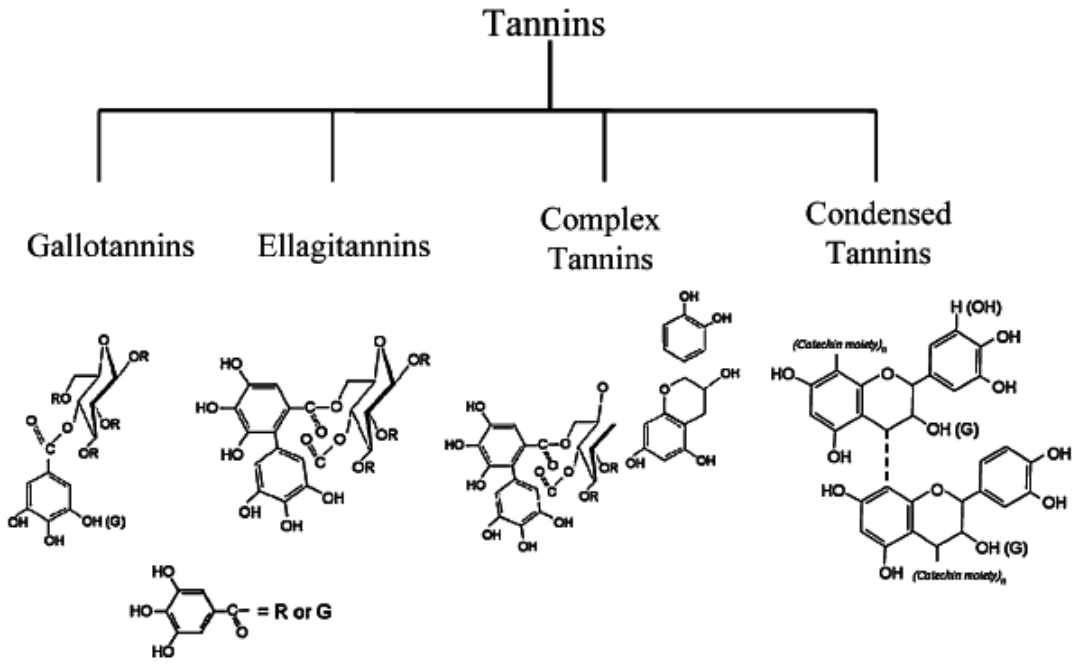

Figure 2.3: Sub groups of tannins

Adopted from Aguilar *et al*. (2007)

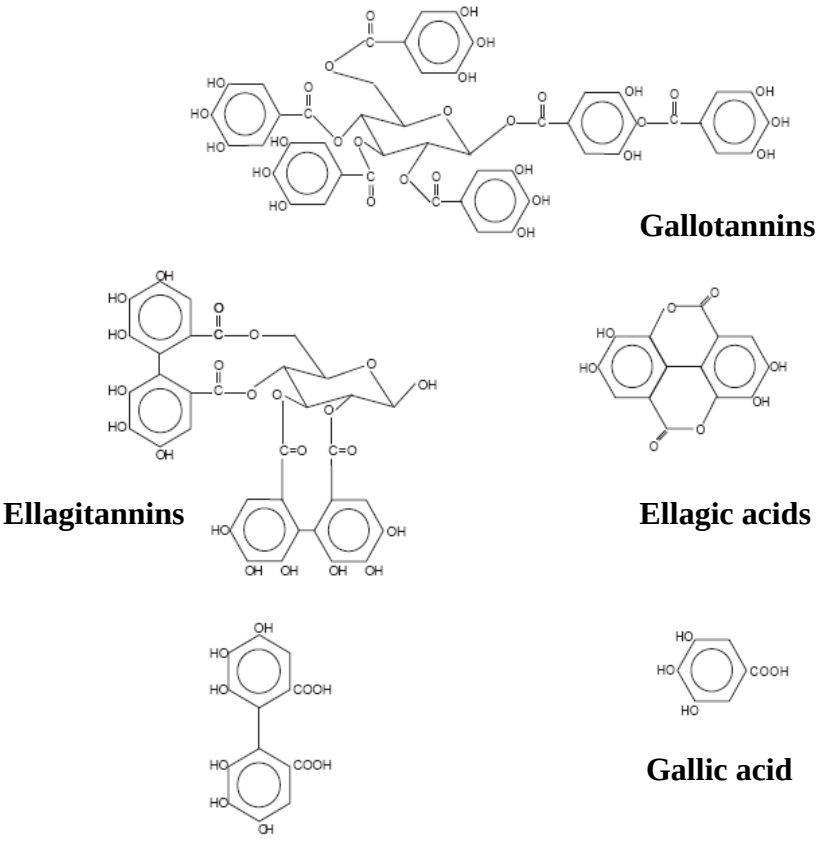

Figure 2.4: Hydrolysable tannins and their constituents.

(Adopted from Belmares *et al*., 2004)

2.4 Tannase production by solid substrate fermentation (SSF)

Tannase had been reported to be produced under solid substrate or submerged fermentations by a number of researchers. Tannase production by SSF had been reported to exhibit some interesting advantages over tannase produced via SmF system. For examples, Lekha and Lonsanse (1994) reported that there was a higher titles production of extracellular tannase compared to tannase by SmF. Extracellular tannase was produced up to 5.5 times higher than tannase from SmF. Aguilar and the coworkers (1999) reported that maximum intracellular tannase activity was expressed 18 times more in SSF than in SmF, while the extracellular activity was expressed 2.5 times higher in SSF that in SmF. Furthermore, it was also found that tannase produced under SSF shows stability towards wide range of pH and temperature (Lekha and Lonsane, 1994; Ramírez- Coronel *et al*., 2003).

Before carry out industrial scale fermentation, the mechanistic understanding of the parameters involved in tannase production must be studied first. Therefore, a laboratory scale of fermentation would be carried out. This is done by using an Erlenmeyer flask, the simple shake flask system which resemble the smallest fermenter that cost effective and suitable for the initial stage of study. Both physical and chemical parameters influencing tannase production can be studied in this simple shake flask system. Then, all the optimized parameters were incorporated in the scale-up studies. For SSF, scale-up studies in a laboratory are carried out by using tray system. It was reported that, many different types of trays such as wood, plastic or metals were used by

the researchers in a laboratory (Krishna, 2005). Trays are always arranged one above the other with adequate gap in between.

2.4.1 Physical factors governing the production of tannase

Physical parameters such as size of substrate particles, types of moisturizing agent, ratio of initial moisture content, initial pH of moisturizing agent, incubation temperature, mixing frequency and size of inoculum play utmost role in influencing the production of tannase.

For SSF, all solid substrates share a common feature, which is the basic macromolecular structure that confers the properties of solid to the substrate and hence generally plays the role in providing carbon source and energy to the SSF system (Raimbault, 1998). Krishna (2005) reported that the size of substrate particles is important as it is related to substrate characterization and system capacity to interchange with microbial growth and heat and mass transfer during SSF process. Besides, it also affects the surface area to volume ratio of the particle. Hence, solid substrate used in SSF system is normally prepared and pre-treated prior the fermentation process. This is done to convert the raw material into a form of substrate which is ready and suitable to be used in SSF. The necessary steps of preparation might be size reduction by chopping, rasping or grinding, physical or chemical treatments on substrate to increase the substrate availability level by the fungus.

Kumar *et al*. (2007) dried ber, jamun and amla leaves at 60°C and powdered them before use as substrate in SSF for tannase production. Treviño-Cueto *et al*. (2007) dried *Larrea tridentata Cov*. leaves at 55°C and finely ground them into 50 µm particle size. Mukherjee and Banerjee (2006) dried and powdered myrobalan and teri pod cover with a grinder prior the SSF study to produce tannase. The particle size of the substrate used was about 0.1- 0. 5 mm.

Generally, microbial growth is affected by initial moisture content in a SSF system (Sato and Sudo, 1999). Since there is no free water in solid substrate of SSF, moisture plays a significant role (Pandey *et al*., 2004). Krishna (2005) reported that as a general acceptance criterion, the water requirement of microorganisms should be defined in terms of the water activity (a_w) rather than water content of the substrate used in SSF. a_w represents the availability of water for reaction in the solid substrate and it is highly dependent upon water binding properties of the substrate. The water activity of solid substrate can decrease during SSF as a result of dehydration of the solid substrate and accumulation of solutes in the substrate. Reduced water activity has a marked effect on microbial growth in SSF system as it extends the lag phase, decreases the specific growth rate and hence, results in low amount of biomass production.

pH is yet another important process parameter and it may change in response to metabolic activities. For tannase production, secretion of organic acid, such as gallic acid will cause the dropping of pH in the SSF system. However, the nature of different types of solid substrate used might have a strong influence on pH kinetics, due to the buffering effect of lignocellulosic materials (Raimbault, 1998). Composition of