CHAPTER 1

1.0 INTRODUCTION

1.1 WASTE GENERATION AND MANAGEMENT

Municipal solid waste (MSW) generation and management is the current major issue faced by many developing nations, including Malaysia, as the waste generation is increasing tremendously every year. In year 2000, the global MSW generation was 12 billion metric tons, and this amount is expected to increase up to 18 billion metric tons in 2025 and 25 billion metric tons in 2050 (Tanaka et al., 2009). Currently, the annual global waste generation waste is about 7%, while the annual waste generation rate for Malaysia is 3% (Agamuthu, 2001). In 2004, approximately 18,000 metric tons of wastes were generated daily and this amount increased to 31,000 metric tons per day in 2009 with 1.3 kg per capita per day (Agamuthu et al., 2007; Agamuthu et al., 2009). The waste generation volume in 2009 was almost two times higher than the waste generated in 2004. Certain cities in Malaysia, especially Kuala Lumpur and Petaling Jaya, the generation rate increased 1.5 – 2.5 Kg capita\(^{-1}\) day\(^{-1}\) (Agamuthu et al., 2008). The annual waste generation in Malaysia is increased up to 11 million metric tons in 2009 (Fauziah, 2010).

The population growth rate, consumption pattern, economic development, income, urbanization and industrialization could be the major reasons for the drastic increase in high waste generation. Waste disposal in developed countries and transitory countries are always higher than in underdeveloped countries, as the latter nations
practiced better waste recycling (Agamuthu, 2001).

Excessive waste generation is a sign of danger to the environment and the living organisms in this globe. Excessive waste generation can directly contribute to environmental degradation, emission of greenhouse gasses and also is highly capable in causing global warming (Andersen et al., 2010). Besides, the cost associated with waste management is also skyrocketing as the technology has to be updated to suit the waste quality, quantity and composition (Latifah et al., 2009).

Greenhouse gases such as methane (CH\textsubscript{4}) and carbon dioxide (CO\textsubscript{2}) are the main gases produced in the landfill resulting from the decomposition and biodegradation of biomass (solid wastes), especially the organic components in the anaerobic condition. Tropical countries like Malaysia are blessed with sunshine and rain throughout the year. During the raining season, water gets entrapped in the piles of wastes, where anaerobic conditions are developed and persist. Degradation occurs almost everywhere, there is dead biomass (Abbasi and Abbasi, 2010). Besides, 90 % of the landfills in Malaysia are non-sanitary landfills which have no proper or lacking of landfill liners and gas pipes, which are highly pro for land and water source contamination (Agamuthu, 2010).

According to Andersen et al., (2010) CH\textsubscript{4} and NO\textsubscript{2} has higher global warming potential with 25 and 298 over 100-year time frame, respectively (Hansen and Agamuthu, 2008). Landfills are the major contributor for the emission of methane gases as it contributes 18 % of the total methane emission in the atmosphere which
will lead to global warming (Agamuthu, 2010). Changes in global temperature and changes in rainfall patterns are among the impacts of global warming, which can give a great pressure and threat to the environment and living things in this earth (Hansen, 2005). In year 2005, Malaysia was ranked as 67th largest per capita GHG generator (Agamuthu, 2010).

1.2 INDUSTRIAL WASTE GENERATION

Malaysia is a developing industrial country and it envisages being fully industrialized country by 2020 (Mohd Nasir et al., 2005). According to Central Intelligent Agency (CIA) (2011), as for 2009, the manufacturing sector in Malaysia contributes 41.6 % of the total Gross Domestic Product (GDP). Simultaneously, the numbers of industries are also increasing parallel with year. There was 19,141 industries in year 2003 and the number increased to 32,584 in 2007 (DoS, 2010). Food and beverages, furniture, textiles, plastics and petroleum, electrics and electronics, communication and telecommunication based companies are among the industries operating in Malaysia (Ahmad Fariz, 2008).

Industrial wastes represent 30 % of the total solid waste generated. In addition, this amount is expected to increase 4 % annually as the industrialization activity in Malaysia is increasing (Ahmad Fariz et al., 2008). During year 2006, 1,103,457 metric tones of scheduled wastes were generated and increased to 1,138,839 metric tones during 2007. Gypsum, dross, slag, clinker, oil and hydrocarbon, heavy metal sludge, mineral sludge and e-waste are among the scheduled wastes generated in
Malaysia (IWW, 2010).

Industrial wastes, which are categorized into hazardous and non-hazardous wastes, are treated in different manner. Usually more attention is paid for hazardous waste, since its impacts are more visible and less important paid towards organic wastes. As a result of it, organic wastes can be seen in landfills. Improper management of this organic solid waste could cause awful impacts to the environment and living organism.

However, using these low cost organic residues will increase the economic feasibility in producing above said valuable products. The increased pressure from society, media and government in reducing the impacts caused by improper management of organic residues, results in adopting technologies and procedures that can minimize the industrial residues either by reusing or recycling them. Besides producing energy from organic waste, either from direct or indirect method, it can also save or reduce the usage of non-renewable energy resources consumption and reduce the residues production as well as improving the sustainability of agribusiness (Santos et al., 2010).

Diverting these organic waste materials into proper channel, not only reduce the pollution caused by dumping these material and wastage, but it also can be used as a raw material for other processes. Sugar in these residues, act as a raw material in a number of biotechnological process (Juhasz et al., 2005). Besides, sustainable resource recovery and management can be implemented.
1.3 BREWERY SPENT GRAIN

Brewery spent grain (BSG) is the lignocellulosic waste generated during the beer production. BSG mainly consists of malted barley and its composition varies according to the beer types. In a typical Malaysian brewery, approximately 31,000 tons of BSG is being produced annually. Carlsberg Malaysia runs 10 brews per day and each brew produced about 8.6 tons of BSG. Therefore, approximately 86 tons of BSG are produced daily, if there are no interruptions in brewing process, such as machinery breakdown or conduct cleaning process.

In the brewing process, selected nutrient for the beer production will be extracted out leaving behind the organic insoluble protein and cell wall residues of husk pericarp and seed coat (Mussatto et al., 2006). Malaysian brewery claims that 100% of the BSG generated in their brewery is sent to local cattle farmers and there is no wastage. However, BSG sent to cattle farm is not fully used as feedstuff. Most of it is still been wasted and is eventually disposed into landfills. Besides, most breweries, especially in large towns are facing problems during the same period of the year, when plenty of other cattle feed is available and there is no demand for BSG. Moreover, microorganisms rapidly grow on wet BSG lowering its quality and market value (Silva et al., 2004).

BSG contains high nutritional value and it is suitable for many industrial applications. BSG can be used as a source of carbohydrate for bioethanol production (Jay et al., 2008), for the production of high fiber enriched bread (Stojceska and Ainsworth,
2008), as a feedstock supplements, especially for cattle (Mussatto et al., 2006) and is used to improve soil quality or used as a soil conditioner (Moyin-Jesu, 2007).

However, due to its physical characteristics such as high moisture content and odor, BSG is always underutilized, especially in food and beverages industries. BSG could not be kept longer due to its high moisture content, odor and high fermentable sugar content. Therefore, BSG can be easily contaminated and is liable to deteriorate due to microbial activity, since it provides a favorable condition for the microbial growth. BSG can be preserved either by deep freeze or in dried form. However, the preservation process involves high expenses, more work load and more energy. Therefore, to avoid unnecessary expenses and wastage, brewing industries are more prefer to discard the BSG as soon as the brewing cycle is over.

Dumping BSG into landfills or in any open dumps can cause various environmental disasters, since this substrate is rich in lignin and nutrition content. Conversely, converting these materials into value-added products would be an alternative to reduce the environmental impacts caused by this material. Specific mechanisms, processes or technologies can be employed for the conversion of BSG into some secondary metabolites such as enzymes, antibiotics, drugs, and acid.

1.4 SOLID STATE FERMENTATION

Solid state fermentation (SSF) is an example of the mechanism used in the conversion of organic substrates into value-added product. It is an established fermentation
process used for many industrial metabolite productions. This process usually employs organic wastes (agricultural or agro-based industrial waste), which are high in sugar content. So that, the sugar content in the substrate, will make the fermentation process become more economical and effective (Couto and Sanroman, 2005). Besides, it also provides a natural habitat for the fungal growth and metabolism, which will make the process occur as in nature (Singhania et al., 2009). Wide range of metabolites especially enzymes which mainly derived from mould origin can be produced through this process (Viniegra-Gonzalez et al., 2003).

1.5 FUNGAL METABOLISM AND ENZYME PRODUCTION

Nowadays, utilizing wide range of filamentous fungi in the production of industrial enzymes has been increased. Filamentous fungi are well recognized for the production of wide range of extracellular enzymes, since they are capable to adapt to severe environmental constrains (Shugaba et al., 2010). Filamentous fungi are capable to penetrate the woody components and convert the nutrients to valuable metabolites (Mohebby, 2005). Fungi, which is classified into three groups (i) soft-rot fungi (ii) brown-rot fungi and (iii) white-rot fungi have different capacity in wood degradation and their mode of attack varies based on the substrate component (Raberg et al., 2009; Blanchette et al., 1990; Charlie et al., 2001). However, most of the studies conducted were with white-rot basidiomycetes, since they act as selective and simultaneous fungi, where they degrade both lignin and cellulose components (Charlie et al., 2001).
Filamentous fungi are considered as the most suitable for SSF process, since their mycelium and hyphae can grow on both surface and penetrate the substrate and absorb the nutrients to produce metabolites (Santos et al., 2004). Besides, most of the fungi are classified as “Generally Regarded as Safe” (GRAS), where these fungi can be used to produce various products including for consumption purposes (Germano et al., 2003).

Recently, the usage of enzymes or bio-catalysts in many industrial applications has increased due to the awareness of the impacts caused by chemical usage. Food and beverages industries, paper and textiles industries and detergent companies, are among the industries using large amount of enzymes (Mulimani et al., 2000; Ramachandran et al., 2004). Enzymes also can be used in the wastewater treatments, where it can remove the pollutants; especially phenolic and non-phenolic components used in bioremediation and replace the usage of bleach in paper industries for bio-bleaching (Alam et al., 2005; Meza et al., 2005). Unlike the chemicals, enzymes only remove the pollutants from the environment by changing the harmful components into non-harmful.

In Malaysia bio-catalysts or enzyme production is emerging. Currently only few enzymes like tannase, xylanase, cellulase and phytase are produced locally, using mainly Aspergillus niger species, while the rest are imported from other countries (BIOTECHCORP, 2009). Annually, enzymes worth RM3.5 million (USD 1.15 million) are being imported for local consumption (Ibrahim, 2008). According to BIOTECHCORP, in 2008, Malaysia exported about 633 tons of repackaged enzymes.
throughout the region. However, 70% of the local market is dominated by foreign companies such as Novozymes and Danico. In 2008, industrial enzyme manufacturing facilities were established in Malaysia by Enzyme Technology Sdn. Bhd and Insect Biotech of Korea. This step would definitely will increase local enzyme production and reduce the dependency on imported enzymes.

In Malaysia, currently about 11 types of bio-catalysts are being isolated using various types of microorganisms utilizing fermentation technology. Fungi, such as *Aspergillus niger*, *Trichoderma* sp. *Trichoderma reesei*, *Fusarium* sp. and *Phanerochaete chrysoporium* are used to produce enzymes, including lipase, protease, cellulase, lignin degrading enzymes and tannase (BIOTECHCORP, 2009).

### 1.6 KINETICS IN SOLID STATE FERMENTATION

Mathematical models are important in optimizing the SSF process (Mitchell *et al.*, 2004), where it enables to discover the important parameters including the specific growth rate (µ), process yield, process productivity, heat generation, process control criteria, strategy for the production of particular products and also in scaling up process (Spier *et al.*, 2009). First order kinetics has been used to estimate the fungal specific growth rate, which can be used to estimate the maxima specific growth rate and fungal doubling time (Half-life time). Eventually, the correlation between fungal growth and enzyme activity can be determined.
1.7 PROBLEM STATEMENT

Annually, large amount of organic industrial waste is being generated by the food and beverage industries in Malaysia. However, due to the lack of specific definition for industrial organic waste management in Malaysia, these wastes are often end up in landfills without prior treatment. Organic materials from industries are highly liable for deteriorate due to microbial activities where the physical and chemical content of these materials are suitable the microbial growth.

Therefore, continuous dumping can cause the landfills are highly susceptible for various environmental problems, especially leachate contamination and toxicity in long term.

However, since these materials are high in nutritional values, it can be diverted into various applications. Various researches have been carried out to exploit the potential utilization of organic wastes in enzymes production. Yet, less researches has been carried out in Malaysia. At the same time Malaysia and other Asian countries are depending on western countries for chemicals and enzymes supplies which are expensive. By conducting more researches on enzyme production utilizing these organic wastes, which is not only economically feasible, but also could reduce the environmental impacts and also could help in creating a local market for enzymes in Asian region.
1.8 OBJECTIVES

* To characterize the organic by-products (BSG, SMC and SCB) used as substrate for enzyme production.
* To determine the effects of incubation period and temperature on enzyme production.
* To compare the effectiveness of chemicals in inducing enzyme activities
* To draw a correlation between fungal growth and enzyme activity by incorporating kinetic models.

1.9 HYPOTHESIS

1. Enzyme production is higher in BSG compared to SCB and SMC.
2. Chemical components in the substrates contribute in higher enzyme production.
3. Fungi shows optimum enzyme yield at 30°C during seven day incubation period.
4. Chemically supplemented BSG show higher enzyme activity.
CHAPTER 2

2.0 LITERATURE REVIEW

2.1 WASTE

According to Bai and Sutanto, (2002), the Environmental Public Health Act (EPHA, 1999) explained waste as “any substances or articles which required to be disposed of as being broken, worn out, contaminated or otherwise spoiled”. Waste is also defined as all the items that no longer have any use to people or any organization, which have to be discarded or already been discarded (Nguyen Ngoc and Schnitzer, 2009).

Waste is a by-product of human activities. Waste generation is relatively propositional to human activities and developments (Demirbas, 2011). Increase in population rate, changes in consumption pattern, economic development, changes in income, industrialization and urbanization processes cause the drastic changes in the increase of solid waste generation (Agamuthu, 2001) and geographical location, season of the year, legislation, public attitude and habits, enforcement of anti-litter laws, efficiency of solid waste management and cultural factors are among the factors that influence the solid waste generation in Malaysia (Abu Eusuf et al., 2007).

2.1.1 Waste Classification

Wastes can be classified into municipal solid waste (MSW), construction and demolition waste, institutional, commercial and industrial waste, agricultural waste,
medical or clinical waste, hazardous, radical and electronic waste and biodegradable waste (Latifah et al., 2009). It can be generated from residential, commercial, industrial, institutional constructions and also from demolition process and municipal services (Nguyen Ngoc and Schnitzer, 2009).

2.2 INDUSTRIALIZATION AND INDUSTRIAL WASTES IN MALAYSIA

In Malaysia, the number of established industries is increasing every year. According to DoS (2010), during 2003, approximately 19,141 companies were established throughout the country, including Sabah and Sarawak. However, the number doubled to 32,584 during 2007. There are many types of business established in Malaysia, including food product and beverages industries, tobacco product industries, textile industries, leather product manufactures, paper and paper product industries, chemical industries, rubber and plastic industries, metal industries and electrical machinery industries (DoS, 2010). Rapid development in industrialization process in Malaysia has resulted in the generation of high amount of industrial and hazardous waste (Mohd Nasir et al., 2005).

Generally industrial wastes can be classified into (i) hazardous and (ii) non-hazardous wastes (Nguyen Ngoc and Schnitzer, 2009). In Malaysia, industrial wastes are categorized as (i) solid wastes including wastes generated by manufacturing process or by product and (ii) toxic and hazardous wastes, including any matter prescribed to be scheduled waste, or any matter (solid, semi-solid, liquid, gas or vapor), which emitted or discharged to environment, exceeding the volume or composition permitted
under the Government Standard (Ahmad Fariz et al., 2008). In Malaysia, non-hazardous industrial wastes are often categorized as municipal solid waste, since lack of specific definition for it. However, hazardous or scheduled wastes are collected and send to Kualiti Alam for further treatment (Rahmah, 2001). Hazardous waste is classified based on four characteristics including (i) ignitability (ii) corrosivity (iii) reactivity and (iv) toxicity (Talinli et al., 2005).

Rapid industrialization contributes to tremendous increase in industrial waste generation. During 1950’s, industrial pollutants were disposed by diluting and dispersing. Currently end-of-pipe approach is mainly practiced as a treatment and disposal method in Malaysia. However, this method leads to illegal dumping (since facilities and factories have to follow the pollution standards established by Malaysian government) and consequently creating environmental degradation and social impacts. Many incidents of illegal dumping of hazardous waste were reported from year 1989 to 2003 in Malaysia (Table 2.1)

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Waste</th>
</tr>
</thead>
<tbody>
<tr>
<td>1989</td>
<td>Pantai Remis, Perak</td>
<td>1,500 tons of toxic waste</td>
</tr>
<tr>
<td>1993</td>
<td>Bkit Merah, Perak</td>
<td>Radioactive waste</td>
</tr>
<tr>
<td>1995</td>
<td>Pangkor Island, Perak</td>
<td>41 drums of toxic potassium cyanide</td>
</tr>
<tr>
<td>1995</td>
<td>Penang Island</td>
<td>28 drums of trichlorofluoromethane</td>
</tr>
<tr>
<td>2001</td>
<td>Ulu Tiram, Johor</td>
<td>1000 tons of metal ashes</td>
</tr>
<tr>
<td>2003</td>
<td>Ijok, Selangor</td>
<td>500 drums of paint sludge and glue</td>
</tr>
</tbody>
</table>

Concurrently, waste recovery results in reduction of manufacturing costs, increase the efficiency of resource utilization, promotes environmental friendly product, and also reduce the negative impacts on environment and human (Ahmad Fariz et al., 2008). Therefore, in 2007, Department of National Solid Waste Management (DNSWM) was established by Malaysian Government (as the amount of industrial waste was increasing and the complexity of waste management increased), for effective industrial solid waste management by practicing 3R activities and intermediate treatments to reduce waste generation (Abdul Nasir, 2007). Around 119 industrial solid waste recyclers and 122 hazardous waste recyclers were licensed to carry out waste recovery from industrial wastes and Nasir et al., (1998) estimated almost 70% of the total industrial solid wastes generated were recovered. Approximately 1.12 million tons of industrial hazardous wastes have been recovered within the period of 2000 to 2005 and the recovery is estimated at RM 4.48 billion (USD 1.21 billion). Plastics, paper, steel and glass are among the industrial solid waste been recovered (Ahmad Fariz et al., 2008).

However, recovery of organic wastes produced in food processing industries was neglected. Most of the organic wastes were dumped into landfills. Improper management of these organic wastes could contribute to environment degradation. According to Malaysia Green Pages (2006), organic wastes contribute 13% of total BOD pollution load from 1986 to 1990.
2.3 BREWERY SPENT GRAIN (BSG)

Brewery spent grain (BSG) (Plate 2.1) is the by-product produced in beer producing industry (Santos et al., 2003). It consists mainly of grain husks, pericarp, seed coat and other components (Figure 2.1) which are not converted into fermentable sugar during the mashing process. 80% of BSG is made up of cell wall materials, which is partially lignified and rich in arabinoxylan polysaccharides (Jay et al., 2008). BSG is the most abundant by-product, which represents 80-85% of the total waste generated in the brewing process (Mussatto et al., 2006).

![Figure 2.1: Schematic representation of a barley kernel used in brewing process (adapted from Lewis and Young, 1995).](image-url)
2.3.1 Brewing Industry and BSG Generation

Carlsberg Group has worldwide business, in 16 countries including Europe and Asian countries like Malaysia and China. In 2007, there were only 44 sites owned by this company. However the number of sites increased to 77 in 2008. Studies show that beer is the fifth most consumed beverages in the world, after tea, carbonate, milk and coffee. Every year beer is consumed at the rate of 23 litters per person (Filladeau et al., 2006). Approximately 4018 million liters of beer were produced in 2006 (Carlsberg Group Environmental Report 2005-2006), and this volume increased to 10.91 billion liters of beer in 2008 (Carlsberg Group Environmental Report 2009). Increasing number of sites contribute to large volume of beer production.

In Malaysia, the BSG generation rate was 14kg for every hectoliter of beer produced during 1995 (Agamuthu, 2001). Globally, in 2006, 16.5 kg of BSG was generated for
every hectoliter of beer produced. This amount reduced to 12.6 kg per hectoliter of beer produced in 2008 (Carlsberg Environmental report 2005/2006 and Carlsberg Group Environmental Report 2009). However, some studies showed that the BSG generation increased to 20kg for every hectoliter of beer produced in later years and the amount of malt used also increased from 240 tons in 2006 to 395.2 tons in 2008 (Jay et al., 2008) as the number of sites increased.

The Environment Agencies estimated that approximately over half million metric tones of BSG was generated in UK itself and about 3.5 million metric tones across Europe (Jay et al., 2008). However, for Malaysian Carlsberg only 31,000 metric tones of BSG being generated annually. For a typical Malaysian Carlsberg, it can run 10 brews per day and each brew generates approximately 8.6 metric tones of BSG. Therefore, 86 metric tones of BSG are generated per day. There are number of different form of grains being used for brewery depending on the beer types. Larger barley, black barley, roasted barley, caramel barley and maize (Plate 2.2- Plate 2.6) are among the grains used produce alcoholic drinks such as Skol, Danish Royal stout, Carlsberg Gold, Special Brew, Tuborg, Sandy, and Nutrimalt. The barley used in Carlsberg Malaysia is imported from Denmark and the malting process is conducted in Denmark to prevent the grain spoilage.
Plate 2.2: Roasted barley

Plate 2.3: Larger barley (the common malt used for all types of beer preparation)
Plate 2.4: Caramel barley

Plate 2.5: Black barley
2.3.2 Brewing Process and BSG Production

Beer production involves five steps, including malting, mashing, brewing (primary fermentation and maturation), clarification and packaging (Figure 2.2). The raw material used in beer production is barley grains. The barley grains are cleaned and grouped according to its size upon harvesting. The selected barley grains will undergo a dormancy period which takes about four to six weeks, before it channeled to malting process.

There are three stages in malting process; steeping, germination and drying/kilning. During the steeping process, the selected grains will be soaked into water tanks for two days at 5°C to 18°C and the water will be changed every six hours. This process will initiate the grain germination, where the water will enter the embryo through
micropyhle and the moisture content will increase from 42°C to 48°C. The dehydration process will initiate the germination. Then, these grains will be channeled into germination vessels. In this vessel, the grain will be turned frequently and the temperature will be maintained at 15°C to 21°C. This stage takes about 6-7 days before the drying process take over. During the drying or kilning process, the moisture content of germinated grains will be bring down to 4-5% while the temperature will be maintained at 40°C to 60°C to avoid microbial contamination. The malted barley will be stored for three to four weeks to reach homogeneity. For Malaysian brewery, the above mentioned processes (malting) were carried out in Denmark itself.

The next step in beer production is mashing process. This process will be carried out in a mash tun. The temperature of the tank will be increased simultaneously from 37°C to 78°C to promote the enzymatic hydrolysis of malt constituent and other components. The starch will be converted to maltose (fermentable sugar) and dextrin (non-fermentable sugar).

At this stage, protein will be partially degraded to polypeptides and amino acid components. At the end of the process, the un-degraded grains will settle down at the bottom of the tank, while the wort (sweet liquid) will be filtered out. The wort will be used for beer production. Un-degraded solid particles will be filtered out using Mash Filter (Meura 2001), will be discard as a brewery spent grain.

The brewing process is very selective, since it only removes the important nutrients for wort production, leaving washed, insoluble protein and cell wall residues of husk
pericarp and seed coat (Mussatto et al., 2006).

Overall, brewing process involves three chemical and biochemical reactions (mashing, boiling, fermentation and maturation) and three solid-liquid separations (wort separation, wort clarification and beer clarification) (Filladeau et al., 2006).
Figure 2.2: Brewing process. Source: Fillaudeau et al., 2006
2.3.3 BSG Composition

Barley is the main raw material in the brewing process as mentioned earlier. Variety of barley, harvesting time, characteristics of hops and adjuncts, malting and mashing condition and the brewing technology contributes for the composition and it varies (Santos et al., 2003; Mussatto et al., 2006). BSG materials are rich in feruloylated arabinoxylan polysaccharides (Hernanz et al., 2001).

BSG contains 17% cellulose (Valverde 1994), 28% non-cellulosic polysaccharides and 28 % lignin (Ng et al., 2007; Mussatto and Roberto 2005; Gregori et al., 2008) and 25 % protein (Santos et al., 2003; Agamuthu 2001; Bartolome and Cordoves 1999; Douwenga et al., 1988). BSG contains high moisture content up to 80% and is high in fiber content (70 %) (Gregori et al., 2008).

2.3.4 Storage and Handling of BSG

Due to high moisture content >80 %<, BSG is an unstable material to be kept long (Gregori et al., 2008). Besides, high fermentable sugar content in BSG made it easy to deteriorate (Santos et al., 2003), since it creates a favorable condition for microbial activity (Mussatto et al., 2006). Therefore, fresh, wet BSG is hard to store and keep for longer period.

In order to avoid the spoilage, BSG must be dried before stored. The drying process can be done in two steps: (i) pressing to obtain material less than 65% humidity and (ii) drying to reduced humidity less than 10%. Drying process will reduce the final volume of the product. Consequently the transportation cost and storage cost and
space can be reduced (Valverde, 1994). However, drying large amount of BSG requires substantial energy and denotes a great financial burden (Gregori et al., 2008). Stroem et al., (2009) supported an alternative for conventional air-drying by introducing superheated steam (SHS). SHS showed increased efficiency, reduced risk of fire and explosion, no odors or particulate emission and the BSG stickiness can be minimized. Besides, 60 to 76 % energy can be saved using this method (Stroem et al., 2009).

2.3.5 Application of BSG in Other Industries

Heretofore, BSG has been used as cattle feed, composted or disposed into landfill. In Malaysia, spent grain is used as cattle feed but only a small amount. The under-utilization is attributed to its high moisture content (causing transportation and storage difficulties), complex composition, low utilization as a feedstock compared to its generation, the stigma of being labeled a waste material (Khidzir et al., 2007) and potential for rapid spoilage (Douwenga et al., 1988). However, in Europe BSG is being used in many industrial applications due to its nutritional value.

BSG can be used in the production of high fiber enriched bread (Stojceska and Ainsworth, 2008). BSG is sold as feedstock supplements (International Finance Corporation, 2007; Mussatto et al., 2006) but at the same time it is used to improve soil quality and yield of egg plant (Moyin-Jesu, 2007), suitable for production of xylitol (Mussatto et al., 2005) and suitable in the cultivation of oyster mushrooms (Wang et al., 2001), as a source of fiber for consumption (Carvalheiro et al., 2004), able to increase the porosity and strength of bricks (Russ et al., 2005) and suitable
feed replacement in crayfish culture (Muzinic et al., 2004). BSG provides three times higher protein source and essential amino acids compared to rice bran, which can replaced as fish-feed (carp) (Kaur and Saxena 2004). Besides it nutritive values, BSG has the capability to be used as a sorbent for lead and cadmium in industrial waste water treatment (Low et al., 2000).

2.3.5.1 Enzyme Production

BSG is a hemicellulose rich industrial by-product, which has potential for multi-enzyme production. Intensive researches have been conducted to determine the feasibility of BSG in enzyme production. Solid state fermentation or solid state cultivation is one of the most common technologies used for enzyme production, utilizing BSG. Gregori et al., (2008) used BSG as substrate for the production laccase, manganese peroxidase and manganese-independent peroxidase using Pleurotus ostreatus. List of enzymes including endoglucanase, cellulbiohydrolase, β-D-glucosidase, xylanase, feruloyl esterase, acetyl esterase, β-D-xylosidase and α-L arabinofuranosidase were produced by fermenting BSG with Fusarium oxysporum (Xiros et al., 2008).

2.3.5.2 As A Food Ingredient and Human Nutrition

High nutrient content in BSG enables it to be consumed or used as an ingredient in the food production for human consumption. It is described as a good adjunct for human food (Santos et al., 2003; Gregori et al., 2008). BSG can be used in the production of high fiber enriched bread (Stojceska and Ainsworth 2008). High fiber breads containing BSG, which has been tested with rats showed to reduce plasma, total lipids
and cholesterols (Hassona, 1993). Besides, it is also been used in the production of flakes, whole wheat bread, biscuits and aperitif snacks. BSG been converted into flour to produce bread, muffin, cookies, mixed grain cereals, fruits and vegetable loaves, cakes, pancakes, tortillas, snacks, doughnuts and brownies (Mussatto et al., 2006). BSG flour can be produced by grinding and sifting the dry BSG and it can also be used in the formulated food (Santos et al., 2003).

2.3.5.3 Animal Nutrition

The high protein and fiber content in BSG is capable of promoting an increase in milk production without affecting the animal fertility. Besides fertility, it also did not affect the blood plasma concentration of glucose, total protein, albumin, urea, triglycerides, cholesterol, phospholipids, sodium, potassium and magnesium (Mussatto et al., 2006). Besides, BSG has also been examined as a replacement for rice bran in fish diet. The studies showed that, the fish weight gain by using 30% of BSG (Kaur and Saxsena 2004). Apart from grinding and sifting, acid pretreated BSG can be used as higher protein animal feed (Jay et al., 2008).

2.3.5.4 Energy and Charcoal Production

BSG can be used as source of biogas production through direct combustion or by fermentation process (Mussatto et al., 2006). Besides, it is also been used as a source of carbohydrate for bioethanol production (Jay et al., 2008).

2.3.5.5 Other Biotechnological Applications

BSG has wide biotechnological applications. BSG can be used as a substrate in the
solid state fermentation for the cultivation of microorganisms, especially fungi for fungal enzyme production. Wide varieties of enzymes can be produced through this process.

At the same time, BSG has tremendous application in improving the soil quality. Studies have been done by growing eggplant in soil fertilized with BSG (Moyin-Jesu 2007) and showed that BSG is a good fertilizer for eggplant. It is also suitable in the production of xylitol (Mussatto et al., 2006) and alcohol (Kopsahelis et al., 2007).

BSG also shows a high improvement in removing the heavy metals from industrial pollutants (Low et al., 2000). Also it is a good material for the cultivation of oyster mushrooms especially *Pleurotus ostreatus* (Wang et al., 2001) where it provides favorable growth condition for mushroom, such as high protein content, high moisture and appropriate physical properties (Gregori et al., 2008). Besides, BSG also has been used as a supplement for degrading of lubricant oil in soil (Abioye et al., 2009).

### 2.4 SUGARCANE BAGASSE (SCB) AND ITS GENERATION

Sugarcane bagasse (SCB) is the fibrous waste material that is left over after crushing the sugarcane stalk for extraction of its juice. It is a ligno-cellulosic residue (Pandey et al., 2000). Every 100 tones of sugarcane generate 4 tones of bagasse. In Malaysia, approximately 733, 500 tones of sugarcane were produced in 2007 resulting in the generation of approximately 30,000 tones of bagasse (DoA, 2009).

Asia is the main sugarcane producer, which generates 45 % of the total global generation (Nguyen Ngoc and Schnitzer 2009). Apart from Asia, sugarcane bagasse is
most abundant in Brazil. Annually 103 million tones of SCB were generated (Santos et al., 2008). South America is the second highest sugarcane producer with 35% of the total global production (Najafi et al., 2009). Compared to other industrial crops, sugarcane produced high yield. Every hector of land can yield 80 tones of sugarcane (Pandey et al., 2000).

2.4.1 Sugarcane bagasse Composition and Its Applications In Biotechnology

According to Pandey et al., (2000), SCB contains 50% cellulose, 25% hemicellulose and 25% lignin and very low ash content. While Kansoh et al., (1999) mentioned that SCB consists of 21.75% lignin, 23.43% hemicellulose, 73.97% holocellulose, 38.37% cellulose and 1.38% ash. SCB is low in protein and fat content but high level of cell wall component (Cellulose) (Cordova et al., 1998).

The possible utilization of SCB includes, the use as a fuel source to provide both heat energy and electricity and for the production of materials like paper, paper board, bagasse concrete, plastic, animal feed and soil amendment (Mesa et al., 2010). Besides, SCB has also been used in the production of single cell protein, ethanol, paper, enzymes and food additives (Santos et al., 2008). SCB is also used as a fuel for boilers, in electricity generation, pulp and paper production and product based fermentation. According to Cordova et al., (1998) almost 80-85% of total SCB produced been used as fuel. Besides it also used as a protein enriched cattle feed (Pandey et al., 2000).

SCB can be used as an alternative, low cost biomaterial for cell immobilization for
xylitol production. Xylitol is a sweetener with anti-carcinogenic properties, used widely in food industries and biomedical sectors. The current process used to produce xylitol is expensive, as it requires several steps of raw material purification. Using unpurified hemcellulosic hydrolysates (SCB) is cheaper and is a less technical option (Santos et al., 2008). Besides, SCB can also be used in the oil waste management system and prevent pollution caused by the oil. Trejo-Hernandez et al., (2007) used SCB as a solid support for the biodegradation of heavy crude oil Maya contaminant in Mexico.

SCB has been used as a solid substrate in the production of various types of enzymes through solid state fermentation technology using different fungi. Xylanase is produced using *Thermoascus aurantiacus* (Milagress et al., 2004), lipase using *Rhizomucor* sp. (Cordova et al., 1998), laccase enzyme using *Phcnoporus annabarinus* (Meza et al., 2005) and cellulose enzyme using *Trichoderma reesei* (Muthuvelayudham and Viruthagiri 2006).

**2.5 SPENT MUSHROOM COMPOST (SMC)**

Spent mushroom compost (SMC) material is a waste product from the production process of edible mushroom (white or button *Agarius* sp.), which is composed mainly of saw dust, wheat straw, field hay, corn cobs, cotton seed hulls, horse manure, chicken litter, gypsum and brewer’s grain (DEFRA, 2006; Mamiro and Royse, 2008). Usually the compost is known as a mushroom substrate while after harvesting the mushroom, the substrate is known as spent. Mushroom substrate formation involves
three stages including pre-wetting and mixing of substrates, mixing and bunkers and composting involves pasteurization and conditioning.

2.5.1 SMC Generation and Its Composition

SMC is the production material that remains after mushroom are harvested (AMI, 2005). The generation rate of SMC in UK alone reached 200,000 tons per annum. According to Ntougias et al., (2004), SMC quantities produced in Europe is estimated to exceed 2 million tons annually. While in US, particularly in Pennsylvania alone nearly 21.6 mil m³ of SMC (Davis and Kuhns, 2005) and in USA 36mil m³ (AMI, 2005) been produced annually. Every one kilogram of mushroom produced five kilogram of SMC (Finney et al., 2009). SMC contains 24 % protein, 15 % fiber and 6.5 % ash (Zhang et al., 1995) and 25 % lignin, 38 % cellulose and 19 % hemicelluloses (Jordan et al., 2008).

2.5.2 Applications of SMC

SMC has been used for land reclamation, tested as a substrate (growth medium) for peat and as a ruminant feed. In Ireland, almost 72 % of the SMC produced was used in the land applications, since it increased the dry matter production on grassland soil (Mullen and McMahon, 2001). After harvesting the mushroom, the substrate losses its nutrient value, especially the potential feed value, because during spawn-run stage it loss its organic matter. Therefore using SMC as an animal feed is not suitable (Zhang et al., 1995). On the other hand, Fazaeli and Talebian Masoodi, (2006) mentioned that, SMC is the suitable feed for sheep and environmental enrichment in intensive pig farms (Beattie et al., 2001).
SMC is also used as soil supplement to improve turf (Landsheue and McNitt, 2005) or potting material in agriculture, horticulture and environmental amelioration (Guo and Chorover, 2006) as a growth medium for plants (Romaine and Holcomb, 2001). Wischmann and Steinhart, (1997) used SMC as an enhancer in the degradation of a synthetic PAH/N-PAH mixture enriched in recalcitrant compounds. Besides, Trejo-Hernandez et al., (2001) also classified SMC as a potential source for laccase production which can be used as a cost effective waste management alternative for some phenolic compounds and they used SMC in degradation of crude oil to prevent oil contamination (Trejo-Hernandez et al., 2007).

SMC also often used for fuel generation purpose. SMC combusted in a bubbling fluidized bed to generate power with high efficiency (McCahey et al., 2003; Williams et al., 2001). The ash produced from SMC combustion with the combination of pulverized fuel ash is used to produce cement with high strength (Russell et al., 2005). SMC has also been used as bio-sorbent or heavy metals like cadmium, lead and chromium (Chen et al., 2005). A study conducted by Polat et al., (2009) showed, SMC can be used as an organic matter source for growth of greenhouse cucumber, where the utilization increased the total yield, fruits width, fruits length and increased nutrition content in the fruit.

SMC often considered as environmentally unfriendly, undesirable and represents solid waste disposal problem for mushroom growers. Therefore, majority of SCB is either dumped into landfills or used as land fertilizer (Finney et al., 2009). Improper SMC handling and management could cause environmental impacts. Field weathering of
SMC produced leachate which will harm the groundwater source, since leachate contains high concentration of dissolved organic matter and inorganic salts (Guo and Chorover, 2006) such as phosphorous and nitrates which cause eutrophication (Finney et al., 2009).

2.6 SOLID STATE FERMENTATION

Solid state fermentation (SSF) is a fermentation process, widely used in producing value-added products by promoting and stimulating fungal growth and metabolism on moistened insoluble solid substrates (Meza et al., 2005; Mulimani and Patil, 2000). Table 2.2 shows the history and development of SSF process. The process is often carried out either in the absence or near the absence of free flowing water (Osma et al., 2007; Botella et al., 2007) utilizing solid substrates. However, the substrates should posses enough moisture to sustain the microbial growth and metabolism (Pandey et al., 2000; Mahadik et al., 2002; Botella et al., 2007; Orzua et al., 2009). The sufficient moisture content will stimulate the fermentation to occur in nature (Osma et al., 2007).

Agricultural wastes (AW) or agricultural-industrial wastes (AIW) of organic nature are often used as substrate in SSF process. These wastes either act as an inert or non-inert substrate in this process. Inert substrates provide an attachment or physical support the microbial growth, while non-inert substrates not only provide physical support, but also providing nutrients (carbon source) for the microbial growth and its metabolism (Pandey, 2003; Couto et al., 2004).
Example of agricultural wastes and agricultural-industrial wastes are wheat bran, rice husk, rice bran, jackfruit seed powder, (Sandhya et al., 2005), defatted soybean cake (Germano et al., 2003), grape pomace, coffee pulp, sugarcane bagasse, apple pomace (Botella et al., 2007), barley bran, molasses bran, corncobs, wheat flakes, maize meals (Kunamneni et al., 2005), palm oil biomass (Alam et al., 2005), and kiwi fruit wastes (Rosales et al., 2005).

Table 2.2: History and Development of SSF (Adapted from Pandey, 1992)

<table>
<thead>
<tr>
<th>Period Development</th>
<th>Developments</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,600 BC*</td>
<td>Bread making by Egyptians</td>
</tr>
<tr>
<td>BC in Asia (recorded history 1,000 BP**)</td>
<td>Cheese making by Penicillium roqueforti</td>
</tr>
<tr>
<td>2,500 BP</td>
<td>Fish fermentation/preservation with sugar, starch, salts, etc Koji process</td>
</tr>
<tr>
<td>7th Century</td>
<td>Koji process from China to Japan by Buddhist priests</td>
</tr>
<tr>
<td>18th Century</td>
<td>Vinegar from pomace Gallic acid used in tanning, printing, etc</td>
</tr>
<tr>
<td>1860-1900</td>
<td>Sewage treatment</td>
</tr>
<tr>
<td>1900-1920</td>
<td>Fungal enzymes (mainly amylases), kojic acid</td>
</tr>
<tr>
<td>1920-1940</td>
<td>Fungal enzymes, gluconic acid, rotary drum fermenter, citric acid</td>
</tr>
<tr>
<td>1940-1950</td>
<td>Fantastic development in fermentation industry. Penicillin by SSF and SmF</td>
</tr>
<tr>
<td>1950-1960</td>
<td>Steroid transformation by fungal cultures</td>
</tr>
<tr>
<td>1960-1980</td>
<td>Production of mycotoxins, protein enriched feed</td>
</tr>
<tr>
<td>1980-present</td>
<td>Various other products like alcohol, gibberellic acid</td>
</tr>
</tbody>
</table>

*BC, before Christ **BP, before present

One of the prominent advantages in SSF is, it resembles the natural biodegradation process, since it provides the natural living condition for the fungal growth and utilize the organic waste. Besides, reutilization of the wastes from food processing and
agricultural industries offer many other advantages, including in economical and environmental point of view (Couto et al., 2004). SSF can be classified as a biological treatment of organic waste. Improper management of organic wastes can contribute to environmental impacts.

SSF is the most appropriate method for filamentous fungal cultivation and lignocellulolytic enzyme production. Figure 2.3 shows the SSF system, the binding between the solid particles and filamentous fungi and the presence of water molecules in between the substrate. SSF system provides growth conditions similar to natural habitat of these fungi due to which they may be more capable of producing certain enzymes with high productivity in comparison to submerged fermentation (Mishra and Kumar, 2007).

**Figure 2.3**: Features of Solid-state fermentation (SSF) system [Adapted from Moo- Young et al., 1983].
2.6.1 Applications of SSF

SSF technology has generated credibility in biotechnological industries due to its potential application in producing value added product from organic wastes. It has been employed in many applications, such as in food production and value-added product invention. Traditional foods, such as tempeh, cheese bread, idli and dosai (Mulimani and Patil, 2000) are made with this fermentation technology. Tempeh is a famous dish in Indonesia, made of fermented soybean, while idli and dosai are Indian traditional food.

Besides food preparation, SSF also been used in the production of fine chemicals and secondary metabolites from microbial sources for commercial purposes (Nagao et al., 2003). Enzymes, antibiotics, flavoring compounds (Kheng and Ibrahim, 2005), antibodies, surfactants, biocides (Couto and Sanroman, 2005), ethanol, biofuels, organic acid and amino acids (Prabhakar et al., 2005) are among the products produced utilizing SSF technology.

2.6.2 Advantages Over Submerged Fermentation

Both solid state fermentation (SSF) and submerge fermentation (SmF) are biological treatment for organic wastes. However, SSF technology offers distinct advantages over SmF technology in various aspects.

SSF is a simple technology with less process energy (lower energy demand) but high quality production. It involves low capital investments where, it requires no complex machinery and simple fermentation media (Mahadik et al., 2002). Substrates used in
this process are usually available throughout the year with low cost or no cost. The moistened substrates hold the capability of absorbing oxygen which will favor the microbial growth during the fermentation (Prabhakar *et al*., 2005). Lower wastewater production, better product recovery (Mulimani and Patil, 2000) and easier downstream process made this process more environmental friendly (Vandenberghe *et al*., 2000). Moreover, utilizing organic waste in their natural form helps in preventing environmental impacts caused by the accumulation of these residues (Milagres *et al*., 2004).

Low moisture content in SSF process helps in preventing contamination caused by unwanted microorganisms (Nagao *et al*., 2003). Therefore, it is easier to control contamination and spoilage of media in this process unlike in SmF. Besides, low moisture content also contributes for high concentrated enzyme production (Viniegra-Gonzalez *et al*., 2003).

The production cost of SSF process is relatively lower, since the substrates for this process is available throughout the year at low cost (Osma *et al*., 2007). While, SmF treatment involves high cost and it requires larger containers (Nagao *et al*., 2003). Many comparatives studies proved that SSF process is more economical than SmF process. Tengerdy (1996) compared the cellulase production in SmF and SSF system, which showed that production cost for SmF was about $ 20 per Kg of cellulase, while the production cost of cellulase in SSF system was only $ 0.2 per kg of cellulase. Besides, another comparative economic analysis carried out by Castillo *et al*., (2000) between SSF and SmF processes for the production of lipases by *Penicillium*
From the observation, SmF required a total capital investment 78% higher than the capital investment for SSF for 100m³ of lipases production. Besides, the product produced through SmF had a unitary cost 68% higher than market price. In another study conducted by Viniegra-Gonzalez et al., (2003), SSF generates higher enzyme titer compared to SmF using the same strain which is almost six fold or 488% higher than SmF (Fujian et al., 2001). SSF also has better treatment method for rubber biodegradation than SmF (Roy et al., 2006). Vintila et al., (2009) strongly recommended SSF process for industrial enzyme production.

Besides, SSF is also more economical since the substrate itself provides the carbon source for the process and there is no dependent on additional carbon source. SSF resembles a natural habitat for microbial growth, which favor the microbes to grow. SSF stimulate the living condition of many higher filamentous fungi including from ascomycetes, bisidiomycetes and deuteromycetes fungi (Holker and Lenz, 2005).

### 2.6.3 Factors Affecting Enzyme Production

Physiochemical and biochemical parameters such as particle size, initial moisture, pH, pre-treatment of substrate, relative humidity, incubation temperature, agitation and aeration, age and size of inoculum and supplementation contribute for higher enzyme production (Pandey, 2003).

#### 2.6.3.1 Substrate Selection

Organic wastes are often utilized as solid substrate in SSF process. Components of organic substrates especially agricultural based wastes, such as lignin and cellulose
can be converted into useful materials. Substrates occupied in SSF process must have
certain characteristics which can make them suitable for this process. Substrate must
be solid porous matrix where they can be biodegradable and should have larger
surface area per unit of volume. When the substrate is biodegradable, the downstream
process will be easier since, most of the components in the substrates will be
converted into other products. For example, the lignin component, which is toxicity
causing component in organic wastes, will be converted to lignin peroxidase enzyme
(Couto and Sanroman, 2005).

Larger surface area provides a platform for the microbial growth and metabolism.
Only short time-frame is needed for the microbes to degrade the substrate component
and convert them into value added product. Substrate should absorb water amounting
to one or several time its dry weight with high water activity. These characteristics
will allow high biochemical process to occur. Air mixture of oxygen with other gases
should be below the low pressure (Orzua et al., 2009). Besides, particle size, porosity
and chemical composition of substrates also play crucial role in the substrate selection
in SSF process (Osma et al., 2007).

2.6.3.2 Incubation Temperature

During SSF process, large amount of heat is generated which is directly proportional
to metabolic activity of microorganism. (Charlie et al., 2001; Hamidi-Esfahani et al.,
2004). The solid material used in this process should have low thermal
conductivities, so that the heat accumulation process could be slower. Sometimes, the
accumulation of heat during the process might be high. In this situation, the tendency
of denaturation of the product formed is high.

The heat accumulation in the fermentation bed can cause the temperature increase up to 20°C higher than the incubation temperature. This situation will affect the growth of microbes, pores formation and germination and also the product formation (Pandey, 2003). Besides, the lack of free water and low conductivity of solid particles can lead to heat gradient, if no mixing applied to the substrate during the fermentation process. Since not all microorganisms can tolerate the heat gradient, a non-isothermal condition can be created without aeration (Hamidi-Esfahani et al., 2004).

2.6.3.3 Incubation Period

Incubation period is also an important aspect in producing higher enzyme yield. Longer incubation period is required for hard and rough substrates. Besides, the inoculated fungi also need more time to adapt to the new environment. When the substrate is rough, the attachment period would be longer. Subsequently longer time is required to penetrate the substrate and break down the substrate components (Yamane et al., 2002).

2.6.3.4 Moisture Content

SSF process usually occurs in low moisture content. Therefore, only limited microorganisms can be occupied in this process. Besides, low moisture content in SSF would prevent the spoilage and contamination by unwanted microorganisms. In addition, high concentrated products will be produced under lower moisture level (Prabhakar et al., 2005).
High moisture content will decrease the porosity of substrate, which will prevent oxygen penetration and does not allow good diffusion of solutes or gas. Subsequently this would contribute for bacterial contamination. While, in very low moisture content, microbial growth is weak due to poor accessibility of nutrients (Pandey, 2003). At this situation, cell metabolism can be either slower or can be stopped (Gervais and Molin, 2003). In the substrate the water molecules are linked each other with other molecules such as sugars and enzymes (Mazur et al., 1981).

2.7 ENZYMES

Industrial enzymes are grouped into four categories, including (i) detergent enzymes (ii) technical enzymes (iii) food enzymes and (iv) feed enzymes (Aberer et al., 2002). While technical enzymes are divided into textile enzymes, leather enzymes, pulp and paper enzymes and fuel ethanol. According to BBC Research 2011, global market for industrial enzymes is estimated at $3.3 billion in 2010 and the amount is expected to reach $4.4 billion in year 2015 with 6 % compound annual growth rate (CAGR) over five years forecast period. Among that, food and beverage contributes $975 million in 2010 and expected to increase to $1.3billion in 2015. According to Strohl (1997), approximately $1.6 billion worth enzymes were produced in 1996, and 45 % of the total amount produced by food industries, 34 % detergent industries, 11% textile industries, 3 % leather industries and 1.2 % pulp and paper industries.

2.7.1 Malaysian Scenario

In Malaysia, enzymes has been widely used in many industrial applications such as in
edible oil and palm oil products, oleo chemical industries, detergent, baking, food and beverages industries and in manufacturing animal feed. Enzyme produced in Malaysia is not sufficient for these industries. Therefore, annually, Malaysian Government is importing enzymes worth RM 3.5 million (USD 1.15 million) from Netherland, Belgium and other countries (Ibrahim, 2008) to meet the local demand.

With the development of science and technology in Malaysia, Malaysian Biotechnology Corporation (BIOTECHCORP), under the Ministry of Science, Technology and Innovation (MOSTI) is establishing Bionexus status companies, which involve in the production of bio-catalyst. To date, two companies were established with a partnership with Korea. More than 11 types of enzyme are being produced through SSF and SmF process and approximately 633 tons of repackaged enzymes are being exported to other countries (BIOTECHCORP, 2009). Generating our own enzyme could reduce the dependency towards imported enzymes and also can allow competitive pricing of enzyme in the current market for industrial application (Ibrahim, 2008).

2.7.2 Microbial Enzyme Production and Its Application

The volume of enzymes produced using microorganisms is higher than plant and animal sources (Ibrahim, 2008), and the majority enzymes available today is manufactured from microorganism (Aberer et al., 2002). For example, enzymes produced by mesophilic fungi such as *Trichoderma* and *Aspergillus* spp. normally have a temperature optimum between 45°C and 60°C, which is lower than temperatures found during the more advanced stages of silage processing. Ideally,
enzymes must act rapidly during the crucial first stage of ensilage before they become thermally inactivated (Margesin and Schinner, 1994).

a) **Textile and Leather Industries**

Enzymes that used in detergent production, for pulp and paper application, textile manufacturing, and leather industries are classified as technical enzymes (Aberer *et al.*, 2002). Enzymes like amylase, cellulase, catalase and protease used in textile industries, while in protease used in leather industries. In Textile industries, amylase used to remove the starch films after the weaving process, followed by cellulase to enhance the appearance and feel of garment made with a variety of cellulosic materials including cotton, linen, lyocell and viscose. Catalase used to eliminate hydrogen peroxidase residual, while protease used to softening and preventing the wool from pilling and sandwashing sink.

b) **Beverage and dairy products industries**

Food enzymes are widely applied in baking industries, fruit juices manufacturing, in wine making and brewing as well as cheese production (Aberer *et al.*, 2002). In fruit juice production, pectinase used to increase the juice yield and also to improve the quality of clarification by removing the pectin. While in wine making process, pectinase used to maintain the color, clarity and organoleptic properties. In brewing industries, enzymes used to breakdown the polysaccharides in the cereals (malt) into fermentable sugars. In airy products, chymosin used to prepare cheese.
c) **Cosmetics and medicinal products**

Protease (papain and bromolaine) used to clean the skin and smother the skin by peeling of the dead or damaged skin (Lods et al., 2000). Lacto peroxidase is used in cosmetic industries to prevent the cosmetic formulation from bacterial attack (Bussmann, 1996), while proteolytic enzymes used to remove the protein films and other deposits from contact lens. Enzymes are also used for skin peeling, as a medication for digestive aid, wound cleaning, lysis of vein thromboses, acute therapy of myocardial infraction and as a support in certain type of leukemia. Technical enzymes also used for pharmaceutical chiral subs in chemical industries (Areber et al., 2002).

### 2.7.3 Laccase

Laccase (EC1.10.3.2) is a multi copper protein, contains four atoms per monomeric molecules (Leontievsky et al., 1997). It is also known as benzenediol:oxygen oxidoreductase (Arora and Gill, 2001) are either mono or multimeric copper-containing oxidases (Monole et al., 2008). This enzyme is capable of oxidizing both phenolic and non-phenolic compounds and aromatic amines by reducing oxygen molecules to water molecules (Couto et al., 2007; Meza et al., 2005; Arora and Gill 2001; Travares et al., 2009; Gomez et al., 2005). Apart from that, it is also capable of degrading lignin related compounds and high recalcitrant environmental pollutants (Osma et al., 2007).

Laccase can be found in many higher plants, some insects, bacteria and in many fungi (Leontievsky et al., 1997; Saito et al., 2003). Since laccase accomplished in oxidizing
non-phenolic aromatic compounds, it is widely used in pulp and paper industry, where it has been used to de-lignify wood pulp, decolorize and detoxify effluents from pulp processing plant, degrade toxic environmental pollutants such as benzoprene, dioxins and xenobiotics (Meza et al., 2005). Enzymes applied in dye degradation have low energy costs, are easy to control and have low impact on ecosystem (Travares et al., 2009). It also been used in wastewater treatment, bioremediation (Couto and Toca-Herrera, 2007), in the preparation of musts and wines and fruit juice stabilization (Arora and Gill, 2000), enzymatic conversion of chemical intermediates and production of useful chemicals from lignin (Minussi et al., 2007). Laccase production can be stimulated by inducers like phenolic substrates, alcohol and sulphones (Meza et al., 2005).

2.7.4 Amylase

Amylase (EC 3.2.1.1) or α-amylase, β-amylase, glucoamylase or endo-1,4-α-D-glucan glycohydrolase (Kunamneni et al., 2005) is an extracellular endo enzyme, but responsible for the hydrolysis of starch, glycogen and other related polysaccharides (Qader et al., 2006). Hydrolysis occur with random cleaves of 1,4-α-D-glucosidic linkages between adjacent glucose units (Ramachandran et al., 2004; and Gangadharan et al., 2008).

α-amylase from Bacillus licheniformis was the first enzyme of industrial application. It was used in the production of transgenic plants. Amylase can be obtained from plants, animals and microorganisms. It has potential application in food industry (cake production), bread making and baking, preparation of digestive aids, fruit juices,
saccharification of starch, textile and paper industry, detergent preparation, alcoholic beverages, sweeteners, fuel ethanol, fermentation process and to remove spot in dry cleaning (Mulimani et al., 2000; Qader et al., 2006; Ramachandran et al., 2004; Kunamneni et al., 2005; Gangadharan et al., 2008).

2.7.5 Cellulase

Cellulase is the key enzyme in degrading the cellulose components by hydrolyzing the β-1,4-glucosidic linkages to produce primary products such as glucose, cellobiose and cello-oligosaccharides (Schulein, 2000; Sukumaran et al., 2005). It is made up of three main components. (i) endo-1,4-β-D-glucanases (EC 3.2.1.4) which is responsible to cleave the internal glucosidic bonds, by creating free chain ends in cellulose and attacking the region of low crystallinity (ii) exo-1,4-β-D-glucan cellulobiohydrolases (EC 3.2.1.91) responsible for cleaving cellulobiosyl units from the ends of cellulose chains by removing the cellobiase units from the free chain ends by degrading the molecules and (iii) 1,4-β-D-glucosidase (EC 3.2.1.21), responsible for cleaving glucose units for cello-oligosaccharides by hydrolyzing the cellobiase (Milala et al., 2008; Mussatto et al., 2008; Singhania et al., 2010; Jorgensen et al., 2003).

Cellulase is the most abundant carbon source from plant materials. Currently cellulases are ranked as third largest industrial enzyme worldwide by dollar volume, due to its wide industrial applications (Singhania et al., 2010). It has promising application in ethanol, single cell protein, bioremediation, waste water treatment and textile industry (Alam et al., 2005). Cellulase and hemicellulase are developed for
textile, animal foods and paper pulp processing (Milala et al., 2008).

Wide variety of microorganism involved in cellulase production, including aerobic and anaerobic microorganisms (Chung-Yi et al., 2009; Kumar et al., 2003), white rot and soft rot fungi (Tanaka et al., 2009; Shrestha et al., 2009; Lo et al., 2010) and anaerobic fungi (Dashtban et al., 2009; Ljungdahl, 2008). *Trichoderma, Penicillium, Fusarum, Humicola* and *Phanerochaete* are among the filamentous fungi used widely in producing industrial cellulases (Lo et al., 2010; de Siqueira et al., 2010; Mathew et al., 2008; Bak et al., 2009). According to Gusakov et al. (2007), the most available commercial cellulases are produced from *Trichoderma reesei* and *Aspergillus niger*.

### 2.7.6 Xylanase

Xylanase (E C 3.2.1.8) is capable in hydrolyzing xylan to xylo oligosaccharides. Xylan usually presents in the hemicellulose of plant cell wall (Walton and Brown 1999). Xylanase can be produce by a variety of microorganisms including bacteria, yeast and filamentous fungi. Among these microorganisms, *Trichoderma, Aspergillus, Penicillium* and *Fusarium* species showed high xylanase production (Azin et al., 2007).

Xylanase play crucial role in many industrial applications such as food and animal feed industry, pulp and paper industry and it has a worldwide market around $ 200 million annually (Nagamalleswararao et al., 2008). In the food industry, especially in bread making industry, xylanase is important in improving the texture, increasing the loaf volume and shelf life of the bread (Dobrev et al., 2007). Besides, it also improves
the nutritive value and nutrient digestibility in ruminant and poultries food (Botella et al., 2007).

In pulp and paper industry, xylanase has been used to remove the lignin content. Xylanase can reduce and replace the usage of chlorine in this industry. Thus the environmental impacts caused by chlorine-base bleaching agents will be reduced (Christopher et al., 2005; Milagres et al., 2004). Besides, the effectiveness of xylanase treatment in increasing the brightness of pulp is better than chlorine (Muthezhilan et al., 2007). According to Katapodis et al., (2007), the usage of chlorine has been reduced 20-30 % in pre-bleaching of kraft pulps. Xylanase also been used in the bioconversion of lignocelluloses and hemicelluloses materials into fermentable sugar and ethanol for fuel production (Farani de Souza et al., 2001; Katapodis et al., 2007). Apart from this, xylanase is also used in brewing industry, in juices and wines clarification (Kheng and Ibrahim, 2005).

2.8 FUNGI

Fungi are classified as a kingdom, divided into one subkingdom, seven phyla and ten subphyla. Approximately 1.5 million fungal species are identified. These fungi are living in various habitats, including fresh water and sea, soil, litter, decaying remains of plant and animal, in dung, in living plants and animals (Hawksworth, 1991). However, some fungi are able to grow in extreme conditions such as psychrophiles (extreme of cold), thermophiles or thermotolerent (extreme of heat), xerophiles (low water content) and osmophiles (high osmotic pressure). Fungi kingdom covers
various ecological backgrounds with different life cycle strategies and morphologies ranging from single cell to large mushrooms. Fungi are divided into four groups:

1. **Zygomycetes** – Reproduce sexually by producing spores and zygospores
2. **Ascomycetes** – Reproduce sexually by producing spores and ascospores
3. **Basidiomycetes** – Reproduce sexually by producing spores and basiodiospores
4. **Mitosporic fungi** – No sexually produced spores.

Fungi are classified as heterotrophs where they act as saprotrops, as parasites of plants and animals and as mutualistic symbionts of many phototropic organisms. Most of the fungi grow as a cylindrical, thread-like structures called as hyphae. It can grow 5-6 \( \mu \)m in diameter and it grows by extension at the tip. Branching is formed as the new tips emerge along the existing hyphae. Branching generate the formation of mycelium. Mycelium is an interconnected network of hyphae. Hyphae can be separated by septa which contains spores. Unlike plants and bacteria, fungal wall (hyphae) is made up of chitin, a structure that gives strength to the wall. Many fungal species has specialized hyphal structure, which involves in nutrient uptake from living host or organic matter by penetrating them.

### 2.8.1 Fungal Classification and Its Characteristics

Fungal classification includes soft-rot, brown-rot and white-rot fungi. The fungal activity in a wood can be observed by looking at the reduction in the wood density, its strength and the aesthetic properties. Usually the fungal attack on the wood is based on enzymatic system. The enzyme will penetrate the cell walls of wood and will alter
the chemistry of wood by breaking down the cell wall polymers into constituents, which will be taken up by fungal hyphae later (Mohebby, 2005).

1. **Soft-rot Fungi**

Soft-rot fungi consist of Ascomycota group fungi and it undergoes asexual reproduction stages. It can be found commonly in the dry environment (Blanchette et al., 2004), in above-ground condition (Raberg et al., 2009) and on wood in damp environments. Soft-rot fungi also can be found in the place where brown-rot and white-rot inhibit Daniel (2003). There are two types of decaying pattern can be observed in soft-rot fungi. In type I attack, cavity chains will be developed by attacking the polysaccharides in secondary cell walls, while type II attack pattern involves the erosion and attack of cell from the cell wall of lumen towards to the middle of lamella and remove the polymers at similar rate (Daniel and Nilsson, 1998). Soft-rot fungi are only able to degrade the cellulose component in the woody particle and they are incapable in the lignin component. It causes the losses of woody properties in the early stage of attack (Raberg et al., 2009).

2. **Brown-rot Fungi**

Brown-rot fungi belong to the wood rotting basidiomycetes (Kirk and Highley, 1973). It is differ than white-rot fungi, where it is unable to degrade lignin component in the woody particle and it has different mode of cellulose degradation (Blanchette et al., 1990). Brown-rot fungi able to secrete hemicellulose and low molecular weight agents including iron, hydrogen peroxidase, oxalate and cellulose by degrading the cellulose components in woods (Xu and Goodell, 2001). According to Cohen et al., (2005),
brown-rot fungi are selectively degrading carbohydrate in lignocellulosic materials. However, in crystalline cellulose, the fungal growth is very slow compared to cellulose degradation in wood. However, Machuca and Ferraz (2001) reported that these fungi are able to release reducing sugar from crystalline cellulose. In a study conducted by Jae-Won et al., (2008), three species of brown-rot fungi used in enzymatic saccharification of lignocellulosic biomass to produce valuable enzymes like endoglucanase, β-glucosidase, xylanase and cellobiohydrolase.

3. White-rot Fungi

White-rot fungi consist of basidiomyce and ascomycete fungi; have been used widely in lignin removal process and in degrading the pollutants having similar structure related to lignin (Robertson et al., 2008). This fungus has the ability to degrade polycyclic aromatic hydrocarbons (PAHs), chlorinated phenols, polychlorinated biphenyl, dioxins, pesticides, explosives and dyes (Levin et al., 2008). It is also very efficient microorganism in the deconstruction, depolymerization and mineralization of biomass (Dinis et al., 2009; Mishra and Kumar 2007). Most of white-rot fungi degrade both cellulose and lignin components will leave a light white, fibrous residue. Some fungi in this group have selective delignification, where it attacks the lignin component first before it attacks the cellulose component (Charlie et al., 2001).

White-rot fungi usually used to pre-treat the biomass before the pyrolysis process. During the pre-treatment the fungi will convert the complex constituent (lignin) of biomass to simpler structure. Thus, this conversion will make the pyrolysis process faster under mild condition, consuming less energy and cause less environmental
contaminations (Yang et al., 2010). White-rot fungi grouped as selective and simultaneous fungi, based on their mode of attack. Selective fungi degrade hemicellulose and lignin components. While simultaneous or non-selective fungi will remove the lignin, hemicellulose and cellulose component at similar rate. Therefore, the cell wall will be homogenously decayed (Blanchette et al., 1990).

2.8.2 Fungal Growth and Its Contribution

2.8.2.1 Fermentation technology – Enzyme production via Solid state fermentation

Fungi have been considered to be the organisms most adapted to SSF since the fungal hyphae can grow on particle surfaces and penetrate into the inter particle spaces and thereby colonizing solid substrates (Santos et al., 2004). They are also capable in degrading organic components, subsequently convert and release some valuable components. This ability made them suitable to be used in SSF. Filamentous fungi have the ability to degrade and remove the lignin content and other aromatic components in the substrate (Cabaleiro et al., 2002; Chi et al., 2007).

Generally enzymes produced in SSF using fungi are extracellular enzymes and the enzyme produced are safer than the enzymes produced using bacteria. Most of the fungi are recognized as Generally Regarded as Safe (GRAS) (Germano et al., 2003). Besides fungal spore produced by SSF process show higher stability, are more resistant to drying and exhibit higher germination rates for extended periods of time after freeze-drying than do spores in SmF (Holker and Lenz, 2005).
2.8.2.2 Soil Immobilization and Mineralization

Fungi plays crucial role in maintaining the soil fertility by conducting immobilizing and mineralization. They are the most efficient decomposers, decomposing the dead plant residues (mineralization) and immobilize the plant nutrients. Fungi are more efficient in assimilating and storing the nutrient in soil compared to bacteria. Chemical composition in fungal cell wall also contributes for higher C: N ratio. Fungal cell wall made of polymers of chitin and melanin are resistant to degradation. Therefore, fungi need more carbon (C) source for its growth, so they absorb it from the organic soil (Wichern and Hafeel, 2004).

2.8.2.3 Fungi in Paper Production

Fungi are utilized to remove free and esterified steroid ketones that prevent the formation of detrimental pitch in Eucalyptus Wood (Martinez-Inogito et al., 2001). *Phlebia radita* was used to remove almost 70% sterols with minimum wood loss (1-4 %). Fungal originate enzymes are widely used in the bio-bleaching of pulps replacing conventional bleaching chemicals to improve the brightness, breaking length, burst index and manufacturing yield of paper (Jimenez et al., 1997). Therefore, pollutants and toxics created by the conventional chemical bleaches can be reduced. Besides, the combination of fungal mycelium and conventional fibers will strengthen the paper quality. The production of high gloss papers with acceptable writing and printing qualities contains 10 % of fungal mycelium (Yamanak et al., 1992). While in transparent papers with printing and writing properties contains 90 % of fungal mycelia (Van Horn and Shema, 1957).
Fungi are always classified as a destroyer, to as they cause harm to environment and human health, as well as creating impacts on economics. Fungi are well known as decomposers. They are capable in degrading papers, cardboards, construction materials, either building materials or packaging materials. Several fungi, including *Penicillium citrinium*, *Aspergillus niger*, *Trichoderma reesei* and *Aurebasidium pullulans* are the most common fungi involved in paper degradation (Jerusik, 2010). According to his survey, almost 40% of houses in North America have fungal contamination. Besides, the rapid growth of this fungi cause health problems, such as asthma and pulmonary problems. Art work and historical documents also can be affected due to the production of fungal pigments (Szczepanowska and Lovett, 1992).

2.8.3 EFFECT OF ENVIRONMENT ON FUNGAL GROWTH

2.8.3.1 Oxygen

Oxygen is one the most vital criteria in the fungal growth. Most of the fungi are aerobics and they depend on the oxygen (Mitchell *et al.*, 2001). However, under low oxygen tension, some fungi are capable in using nitrate as an oxidant, which at the end can cause toxicity and nitrate formation. For example, *Fusarium* sp. are able to grow under low oxygen tension, where its mitochondria performs bacteria-like de-nitration (Mitchell *et al.*, 2001). In SSF process, larger volume of substrates can creates problem in oxygen distribution throughout the fermentation process.

2.8.3.2 Temperature

The optimum temperature for most of the fungi is 30°C to 40°C. However, some fungi
can grow in an extreme temperature. Some fungi can grow at few degrees above the freezing point of water. Fungi are grouped as psychrotolerent if it can grow at freezing point or little below than freezing point. While, psychrophilic fungi cannot grow above 20°C (Carlile et al., 2001).

Fungi that grow above 40°C are usually classified as decomposers and they widely found in decomposition vegetation (compost). *Mucor pusillus*, *Chaetomium thermophile* and *Theromoascus auranticus* among the fungi that play important role in composting which can grow at temperature more than 40°C. Thermotolerant fungi capable grow at temperature 50°C or more, while thermophilic fungi incapable to grow below 20°C (Carlile et al., 2001).

Temperature influences the primary and secondary metabolism in fungi. High temperature will increase the nutritional requirement for the fungi. For example, when *Saccharomyces cerevisiae* incubated at 30°C, it does not require any additional nutrient, but when the temperature increased to 39°C, it requires panthotenic acid for its growth (Carlile et al., 2001).

Besides, for some fungus small changes in temperature can stimulate high growth. Also, when the temperature is above the mesophilic range, the mesophiles growth will be restricted and the growth will be prevented (Neville and Webster, 1994).

**2.8.4 Aspergillus niger**

*Aspergilli* is a large and diverse genus (Ascomycota) comprising some well-known
fungi with some commercial value and medically significant molds. For example; *A. niger* and *A. oryzae* used in the production of value-added products with substantial commercial value, *A. parasitius* and *A. fumigatus* are medically significant pathogens, *A. terrues* is used as a source of cholesterol lowering agent and *A. nidulans* used as a model in genetic research, for cellular function studies (Lubertozzi and Keasling, 2009). *Aspergilli* grow well at 10-50°C, pH 2-11 and at osmomolarity near to pure water, up to 34% salt.

*Aspergillus niger* is well known fungus in this genus causes black mold diseases on certain types of fruits and vegetables including grapes, onions and peanuts. Yet, it is also capable in producing 19 types of extracellular enzymes and numerous types of value-added products including citric acid and alcohol (Orzua et al., 2009) using agricultural wastes. This is because *A. niger* is capable in breaking down polysaccharides and protein into sugars and amino acid to produce extracellular enzymes (Charlie et al., 2001).

Citric acid production started since 1923, using *A. niger* as a principal source in the fermentation process. Annually 300K tones of citric acid been produced and it has been widely used in the preparation of soft drinks, food and pharmaceutical products (Charlie et al., 2001). Food industry is the largest consumer of citric acid with 70% of the production, followed by 12% by pharmaceutical industry and 18% for other purposes (Vandenberghhe et al., 2000).

*Aspergillus niger* also been used in the production of many useful enzymes
(glucoamylase, pectinases and α-galactosidase) are also been produced by fermenting this fungi. Apart from producing enzymes, this fungi also been used in the production of sake, soy source, shoyu, amazake and mirin (Ramachandran et al., 2004). *Aspergillus niger* has been tested to reduce the Biochemical oxygen Demand (BOD) level in spent grain liquor in brewing industry. Spent liquor is a great issue in brewing industry since it contributes 30-60% of total BOD. *Aspergillus niger* showed an excellent BOD reduction from 22500 mg/L to 900 mg/L, which is about 96% reduction compared to yeast and mushrooms which gives 20 to 45% BOD reduction. Therefore, *A. niger* has great economic value in waste disposal (Hang et al., 1975).

### 2.8.5 *Fusarium* sp.

*Fusarium* genus is clustered as filamentous fungi, is well known plant pathogen. It contains conidiophores, which is often branched and it produces large pasty masses of spores from phialides. Phialides are either cylindrical, simple or branched with small collarette at the apex. There are two types of spores can be found in this fungi. (i) Macrospores, which is produced from phialides on un-branched or branched conidiophores. The spores are curved, with two or more celled, thick walls, smooth and canoe shaped. While (ii) microconidia are usually long or short simple conidiophores. This spore is typically unicellular hyaline, ovoid to ellipsoid and arranged in balls or chains. *Fusarium* sp. has high growth rate and the colonies has flat to wooly or cottony structure. It appears either in white, yellow, read, violet, pink or purple. There are more than 20 species in this genus (De Hoog et al., 2000).

This genus commonly found in soil, dead or living plants and grains in tropical and
subtropical area which is often associate with humidifiers (De Hoog et al., 2000). Some inhibit in soil in the cold climate while some are telemorphic state (Larone, 1995). It is also a widespread pathogen on maize and small grains cereals including wheat, barley, oat, rye and triticale. *Fusarium* is a common plant pathogen causing plant diseases, including root, stem and ear rot, vascular wilt and fruit rot (Nicholson et al., 2003). Therefore, it will decrease the yield and diminish the quality and value of grains.

*Fusarium* species produced a number of mycotoxins. Triehothecanes, fumonisins and zearalenone are among the main mycotoxin classes produced by this species. *Fusarium graminearum* causes infection in barley grains which causes the root rot and seedling blight (Priest and Campbell, 2003), while *F. culmorum* is the important pathogen of wheat causing seedling blight, root rot, and head blight, which is also known as scab (Wagacha and Muthomi, 2007). Another species in this genus, namely *F. oxysporum* is the most common species in this genus causes vascular wilt in a wide variety of crops.

*Fusarium* sp. is capable in producing various secondary metabolites. Some of these metabolites associate with cellular toxicity, effects on growth and development of animal and cancer in human and agricultural and food safety. However, *Fusarium* is also been used for harmless secondary metabolites production. *Fusarium* was described as a good cellulase and xylanase producer using lignocellulosic residues as the main carbon source in solid state fermentation (Panagiotou et al., 2003; Panagiotou et al., 2005). *Fusarium* also used for ethanol production by hydrolyzing
brewery spent grain (Xiros et al., 2008) and straw (Panagiotou et al., 2005). Roncero et al., (2003) used *Fusarium* species as a model for studying virulence in soil borne plant pathogens.

2.8.6 *Penicillium chrysogenum*

*Penicillium* sp. is filamentous Ascomycota fungi and classified as *Penicillium* genus. This species usually has branched conidiospores and the conidia (spores) are usually round and unicellular. *Penicillium* sp. cell walls are made of glucan. Besides, they have smaller hyphae and smaller peripheral growth zones. Generally the fungal spores have hydrophobic space (Bancerz et al., 2005). *Penicillium* species are usually classified as pathogen.

*Penicillium* sp. is the most common causes of fungal spoilage of fruits and vegetables. *P. expansum* attacks fruits especially apples and this fungi is classified as the most aggressive, because it is quite durable and it can live for longer period. *P. digitatum* produces ethylene which will accelerate the fruit ripening, *P. italicum* causes slimy rot and produces blue-green conidia and *P. verrucosum* grow on cereal products. *P. chrysogenum* is cable to grow in low water activities and low relative humidity and they are good in colonizing acrylic based paint finishes (Adan and Samson 1994) and wood building materials (Hunter and Lea 1995).

*Penicillium* sp. is not merely harmful fungus. It also has many useful contributions for industrial applications. For example, *P. roquefortii* and *P. camambertii* are used to make blue cheese and some other fungi in the genus are responsible in preventing
fungal decay. Some of these fungi also been used in secondary metabolites production (Tournas 2005).

*P. chrysogenum* is the member of this genus, classified as psychrotropic microorganism. This species reproduce through forming dry chains of spore from brush-shaped conidiospores. *P. chrysogenum* conidiospore are blue-green in color (Shen *et al.*, 2003). Bancerz *et al.*, (2005) mentioned that *P. chrysogenum* is the best lipase producer among other fungi in arctic tundra. They have high enzymatic activity and also have the ability to produce α-amylase. Whereas, Okafor *et al.*, (2007) mentioned that *P. chrysogenum* is capable of degrading lignocellulosic components and they can grow effectively and produce extra-cellular protein using the lignocellulosic components. He conducted a study using this fungi to test the xylanase production using wood wastes such as saw dust, wheat bean and sugarcane pulp. Higher xylanase was discovered in wheat bran compared to other materials. Another study was conducted by Germano *et al.*, (2003) to look at the *P. chrysogenum* capability in producing protease using defatted soybean cake via solid state fermentation technology.

Countless studies were conducted to test the ability of *P. chrysogenum* in producing various value-added products. Samson *et al.*, (1997) mentioned that *P. chrysogenum* produce secondary metabolites like penicillin, requefortine C, mekeagrin, chrysogine, xanthocillins, and sorbicillin. Penicillin and xanthocillin produced from this fungus used in the treatment of pulp mill waste. Enzymes such as polyamine oxidase, phosphor-gluconate dehydrogenase and glucose oxidase also can be produced from this
fungus (De Hoog et al., 2000). Engineered *P. chrysogenum* can be used to produce carbamoylated cephalosporin which can be used as the synthos for semi-synthetic cephalosporins (Harris et al., 2009) and *P. chrysogenum* also can be used in the production of proteinaceous chrysolysin which is used to promote inflammatory response (Donohue et al., 2005). Besides, *P. chrysogenum* also tested in the production of non-protein nitrogen (NPN) and volatile components (Benito et al., 2005; Martin et al., 2011).

### 2.8.7 *Schizophyllum commune*

*Schizophyllum commune* is a well-known filamentous homobasidiomycete used as a primary model organism to investigate the mating pattern in basidiomycete fungi. This fungal species has shown tremendous application in various processes. *Schizophyllum commune* is a good wood degrading basidiomycete (Oku et al., 1993). Therefore, it is capable in producing value-added metabolites by degrading the woody components. Oku et al., (1993) used this fungus to produce xylanase, while Kawagoe et al., (1997) used this fungus to produce L-malic acid. Beside, studies were conducted to test the capability of this fungus in producing cheese-like food that contains 0.58% β-D-glucan, by producing lactate dehydrogenase and milk-clotting enzyme. This food has shown preventive effects against cancer. *Schizophyllum commune* is rich in fiber, protein and vitamins like thiamin and riboflavin (Okamura-Matsui et al., 2001).

### 2.8.8 *Trichoderma* sp.

*Trichoderma* sp. is a filamentous ascomyetes widely used in many industrial
applications such as in the production of wide range of extracellular enzymes by decomposing and decaying agricultural wastes (Azin et al., 2007). *Trichoderma* sp. has high secretory capacity and inducible promoting characteristics (Mach and Zeilinger, 2003). This species are the most reported to degrade cellulose and other components in cell wall (Alam et al., 2005) and most efficient cellulose protein producer (Muthuvelayudham and Viruthagiri, 2006). *Trichoderma* is a well-known and promising bio-control agent (BCAs), which has been used to control wide range of plant pathogens (Guo et al., 2002).

Apart from that, *Trichoderma* sp. also been used in seed treatment which will stimulate the plant growth. Zheng and Shetty, (1998) grown *Trichoderma* strains on the apple pomace-based substrate to enhance the emergence of seedling vigour in pea plant. Porras et al., (2007) evaluate the effectiveness of *Trichoderma* species in enhancing strawberry yield. The results showed that *Trichoderma* has influenced the yield by increasing the root weight and root colonization which protect and enhanced the plant growth.

In another study conducted by Ousley et al., (1994), *Trichoderma* was used as a dry fermenter biomass to study the prospects in promoting bedding plant growth. He found out that, *Trichoderma* isolates able to increase either the number of flower, weight of flowers, shoot fresh weight or shot dry weight of petunia, marigold and verbena under different cultural conditions.
2.9 SOLID STATE FERMENTATION AND KINETICS

In early commercial fermentation, the main criterion of process performance was the final product yield and substrate conversion. However, the technology developments have aroused the worldwide research interest towards the time factors, such as productivity and the average rate of product formation (Mitchell et al., 2004). The proper description of biological activity in SSF process is the essential step in developing this process (Smiths et al., 1996). Therefore, mathematical modeling was incorporated to determine the growth profile of microorganism in the process.

Mathematical modeling plays crucial role in SSF bioreactors, where it involves in the optimization of design and operation (Mitchell et al., 2004). These mathematical modeling can be used to describe the kinetics of microbial growth and how this is affected by the environmental conditions. Study of kinetics enables the discovery of various important parameters in the SSF process, including specific growth rate, process yield, process productivity, heat generation, process control criteria, strategy to increase the production of particular products and also industrial scale consideration (Spier et al., 2009). Kinetics can be described by various simple equations. Logistic, exponential, linear, deceleration or fast acceleration/slow decelerations are among the equations used for estimating the growth profiles (Sangsurasak et al., 1996; Viccini et al., 2003). In the early development of kinetics, single phase equations such as linear, logistic and exponential was used before the two-phase equation introduced to describe fast-acceleration and slow deceleration (Ikasari and Mitchell 2000).
Kinetic model used for optimizing and scale-up in SSF bioreactors. While according to Heijnen and Romein (1995) kinetic equation which describe the specific growth rate ($\mu$) of microorganism as a function of the condition of substrate are crucial for understanding and describing many phenomena in biotechnological process. Monod equation is the most common equation used for this purpose. Kinetic models also used to describe the overall performance of reactors and the mass and heat transfer within and between various phases of bioreactors (Mitchell et al., 2004).

In SmF process, the growth profile can be constructed on the basis of optical density (OD) or using the dry weight of biomass which obtained after the filtration of the samples. However, these methods or the direct measurement methods cannot be used to estimate the biomass in SSF process, since the microbial cells remain attached with the substrate and it is difficult to quantify them separately (Viccini et al., 2003; Mitchell et al., 2004; Machado et al., 2009; Pandey et al., 2008). Mycelium of the filamentous fungi used in the SSF process, spreads over, penetrate the substrate and intimately bound to the solid matrix (Zamani et al., 2008; Roche et al., 1993). Due to the strong connection between fungus and solid matrix (Scotti et al., 2001) and heterogeneous characteristics of SSF process, which considered as the principal difficulties encounter when kinetics accomplishment is attained in SSF process Mazutti et al., 2010; Machado et al., 2009).

Since, it is impossible for direct measurement of biomass in SSF, kinetic study must either be done in artificial system that mimic SF or based on indirect methods (chemical methods) of biomass estimation (Aidoo et al., 1981). Indirect method can
be done either by measuring (i) cell constituents such as chitin (Zamani et al., 2008; Roche et al., 1993; Scotti et al., 2001; Wagner, 1979; Sharma et al., 1977), ergosterol (Robertson et al., 2008), nucleic acid and protein (ii) biological activities such as ATP (Durand et al., 1988), enzymatic activity (Lestan and Lamar, 1996), respiration rate (Terebiznik and Pilosof, 1999; Mazutti et al., 2010) and immunological activity or (iii) nutrient consumption (Desgranges et al., 1991; Wojciechowski et al., 2005; Roche et al., 1993; Pandey et al., 2008; Chen et al., 2010).

2.9.1 Glucosamine Estimation for Fungal Biomass Determination

Glucosamine (C₆H₁₃NO₅) is an amino acid, the building units of polysaccharide chitosan and chitin. Chitin is a poly-N-acetylglucosamine with monomers connected by α, 1-4 links in a straight chain.. Cell wall of Aspergillus nidulas contains 19.1% chitin on dry weight basis (Zonneveld, 1971). Chitin is the most frequently occurring polymer in most of the fungal walls but not of higher plants (Warnock, 1971). Hydrolysis of glycosidic bonds between monomers, which can remove the acetyl group, can be used to measure the glucosamine or N-acetyl glucosamine in fungal cell wall (Zamani et al., 2008).

Glucosamine present at different seed as glucosamine may cause the difficulties in measuring the glucosamine. However, it does not interfere with the determination of total glucosamine, since the amount present is known to characteristics of species (Terebiznik and Pilosof, 1999) and it remain constant throughout the fermentation.
CHAPTER 3

3.0 METHODOLOGY

3.1 SAMPLE COLLECTION

Brewery spent grain (BSG) was collected from the brew house of Carlsberg Brewery (Malaysia) Berhad., which is located in Persiaran Selangor, Shah Alam (Selangor, Malaysia). BSG was collected immediately after the mashing process and before the spent sent to silo. The timing of collection was made to ensure that only fresh BSG was collected. Sugarcane bagasse (SCB) was collected from the night market at Petaling Jaya, while spent mushroom compost (SMC) was collected from a mushroom plantation in Tanjung Sepat.

For further analysis, SCB was dried and grinded into smaller particles and SMC was separated from the compost plastic and dried. Grinding was not required for BSG and SMC since the particle size is less than 1mm. Fresh BSG was used for analysis.

3.2 PHYSICAL AND CHEMICAL PROPERTIES ANALYSIS

BSG, SMC and SCB samples were used to study the physical and chemical properties.

3.2.1 Physical properties

a) Moisture content analysis

Moisture content (%) was determined by subtracting the weight of dried samples
(BSG, SCB and SMC) from the weight of fresh samples.

A crucible was weighed (BEL Engineering - Mark 500). The weight of the crucible was noted as $W_1$. A fixed mass of fresh BSG was weighed and placed into the same weighed crucible (noted as $W_2$). Three sets of fresh samples and crucibles were prepared in this manner.

The fresh samples and its crucible were placed in an oven (Memmert ULM 600) at 50 ± 2°C. Each subsequent day, at approximately the same time of day, the fresh samples and its crucible were removed from the oven, left at room temperature (approximately 27 ± 3°C) for ten minutes and weighed. This new weight was noted as $W_3$.

The procedure was repeated once a day for ten days. The average $W_3$ of the final eight days were taken into consideration to determine the moisture content (％).

To calculate the moisture content, the mass of dried fresh samples (average $W_3 - W_1$) was used.

\[
\text{Moisture content (％) = } \frac{W_2 - \text{average } W_3 - W_1}{W_2} \times 100\%
\]

Results are presented as the mean moisture content of four replicates, with standard deviation.
b) **pH analysis**

The pH of the substrate was measured using pH meter (Hanna HI8424) by diluting the substrate in distilled water. Each substrates (BSG, SMC and SCB) were measured for three times to calculate the average pH of sample. All results were presented in mean pH of three samples.

c) **Particle size**

Samples were sieved to determine the particle size.

### 3.2.2 Chemical properties

Chemical properties analyses were conducted for all three samples.

a) **Acid Detergent fiber determination (ADF)**

Acid detergent fiber analysis was conducted for all the three samples to measure lignin and cellulose content.

An acid detergent solution (ADS) was prepared by dissolving 29.00g of cetyl tri-methylammonium bromide in one liter of sulfuric acid (H$_2$SO$_4$).

A crucible was weighed and its weight was noted as $W_1$.

One gram of fresh samples was weighed on the previously weighed crucible (combined mass noted as $W_2$). The sample was completely poured into a 250 ml conical flask and mixed with 50 ml of ADS. The mixture was refluxed (Witeg,
Germany) at 90 ± 5°C for 60 minutes.

The mixture was then passed through a filter 55mm, (Advantech Qualitative Filter, Japan). The liquid portion of the mixture was discarded while the solid portion was rinsed with distilled water (90°C) and acetone (4°C) in tandem twice. The solid remnant was replaced into its crucible and dried in an oven (Shellab 1375 FX, USA) at 105°C for 12 hours.

Finally, the solid residue and its crucible were removed from the oven, left at room temperature for ten minutes and weighed (combined mass noted as W₃).

Acid Digestion Fiber (%) =

\[
\frac{(W₂ - W₁) - (W₃ - W₁) \times 100}{(W₂ - W₁)}
\]

Above procedures were repeated for all the three samples (BSG, SMC and SCB).

b) Soluble protein determination

A mass of 50 g of fresh samples (BSG, SMC and SCB) was weighed into 500 ml conical flasks separately. A volume of 400 ml of distilled water was poured into each flask. The flasks were mixed by shaking at 180 rpm for 60 minutes at 30°C. After removal, the flasks were left to settle at room temperature. Physical separation by centrifugation was done at 4000 rpm for 20 minutes at 30°C. The supernatant was used for further analysis.
The assay for measuring soluble proteins was adapted from Bradford (1976). A stock dye reagent was prepared by mixing 0.10g of Coomassie brilliant blue G-250 with 50 ml of ethanol (95% v/v) and phosphoric acid (85% w/v). This mixture was slowly poured into 200ml of distilled water. The stock dye reagent was stored in an amber bottle at 4°C.

Before use, the stock dye reagent was diluted four-fold. For the analysis, 0.1 ml of the sample to be studied and 0.1ml of distilled water was mixed with 5.0 ml of the dye reagent. The sample was allowed to equilibrate for two minutes.

The change in color was measured by absorbance, which was read at 595 nm. For standardization purposes, bovine serum albumin was used and soluble proteins were calculated. The activity was expressed in mg/ml.

Soluble Protein (%) =

\[
\frac{\text{Absorbance of sample} - (\text{C-intercept value})}{(\text{M-gradient value})} \times \frac{1}{0.2} \times \frac{50000\text{mg}}{200\text{ml}}
\]

c)  **Lignin content determination**

For the determination of lignin, three solutions were prepared. The first was a saturated potassium permanganate solution (SPPS), prepared by mixing 50.00 g of potassium permanganate with one liter of distilled water. The solution was stirred thoroughly. The SPPS was stored in an amber bottle at 4°C.
The second was a buffer mixture consisting of two solutions:

a. Solution $\alpha$ was prepared by mixing six grams of ferric nitrate and 0.15 g of argentum nitrate into 100 ml of distilled water.

b. Solution $\beta$ was prepared by dissolving five grams of potassium acetate in 500 ml of glacial acetic acid. This was mixed with 400 ml of tertiary butyl alcohol.

The buffer was prepared by slowly mixing solutions $\alpha$ and $\beta$, and stirred with a glass rod.

A combined solution of SPPS and buffer mixture was prepared by slowly mixing the two fore-mentioned solutions in a ratio of 2 : 1. The combined solution was stirred thoroughly and stored in an amber bottle at 4°C.

The third solution was a de-mineralizing solution that was prepared by mixing 50.00 g oxalic acid (dihydrate) with seven milliliters of ethanol, 50 ml of hydrochloric acid (12 N) and 250 ml of distilled water.

A crucible was weighed and its weight was noted as $W_1$. One gram of fresh sample was weighed on the previously weighed crucible (combined mass noted as $W_2$). The sample was completely transferred into a 250 ml conical flask and mixed with 25 ml of the combined solution. The mixture was left at room temperature for 90 minutes. The mixture was monitored - if the mixture turned brown, it was gently decanted (with the volume noted), discarded and immediately replaced.
After 90 minutes, the liquid portion was slowly decanted and discarded. A volume of 25 ml of the de-mineralizing solution was slowly poured onto the solid portion. Next, the de-mineralizing solution was discarded while the solid portion was rinsed with 80% ethanol and acetone (4°C) in tandem twice. The solid remnant was placed on its crucible and weighed (combined mass noted as W₃) and dried in an oven at 105°C for 12 hours.

Finally, the solid remnant and its crucible were removed from the oven, left at room temperature for ten minutes and weighed (noted as W₃).

Lignin (%) =

\[
\frac{(W₃ - W₁) - \text{Weight of Acid Digestion Fiber}}{(W₂ - W₁)} \times 100\%
\]

**d) Cellulose content determination**

Cellulose was calculated as below:

Cellulose (%) =

\[
\frac{\text{Weight of Lignin} - \text{Weight of Acid Digestion Fiber}}{(W₂ - W₁)} \times 100\%
\]

**e) Starch determination**

Method 948.02 of AOAC International (2006) was used to analyze starch content. The following solutions were prepared:
a. Iodine-potassium iodide (KI) solution was prepared by mixing 7.5 g of iodine (I₂) and 7.5 g of KI with 250 ml of distilled water.

b. An alcohol-sodium chloride solution was prepared by mixing 350 ml of 95 % ethanol with 80 ml of distilled water and 50 ml of a 20 % sodium chloride (NaCl) solution. The mixture was made to 500 ml by adding in distilled water.

c. An alcohol-sodium hydroxide (0.25 M) solution was prepared by mixing 350 ml of 95 % ethanol with 100 ml of distilled water and 25 ml of 5 M NaOH. The mixture was made to 500 ml by adding distilled water.

d. A 0.7 M hydrochloric acid solution was prepared by mixing 60 ml of HCl with 1 liter of distilled water.

e. A somogyi phosphate sugar reagent was prepared as follows:

   i. Anhydrous sodium-hydrogen phosphate (Na₂HPO₄, 56 g) and 80 g of potassium sodium tartarate (KNaC₄H₄O₆·4H₂O) were mixed in one liter of distilled water and 200 ml NaOH (1 M). Next, 160 ml of a 10 % CuSO₄·5H₂O solution was poured into the mixture.

   ii. A mass of 360 g of anhydrous sodium sulphate (Na₂SO₄) was dissolved the mixture previously prepared. The mixture was transferred into a two liter volumetric flask.
A volume of 200 mL potassium iodide (KIO₃) solution (0.0167 M, 3.5667 g/L) was added.

(f) A sodium thiosulfate standard solution (0.0055 M) was prepared by mixing 2.73 g of Na₂S₂O₃·5H₂O in two liters of distilled water.

(g) A KI solution (2.5 %) was prepared by mixing 2.5 g of KI with 97.5 g of distilled water.

(h) The starch indicator was prepared by mixing 1.5 g of soluble starch with 5 ml of distilled water. This mixture was added into approximately 300 ml of boiling distilled water. Gentle stirring was applied.

A mass of 1.0 g of BSG was weighed and completely poured into a test tube. Five milliliters of distilled water was added. Thorough mixing was done in the test tube which was heated in a water bath (approximately 100°C) for 15 minutes to gelatinize the starch.

At the appropriate time, the tube was removed and allowed adequate cooling. Next, 5 ml of 60 % perchloric acid (HClO₄) was rapidly mixed into the solution.

Using a glass stirring rod, the BSG was manually pressed against the inner part of the tube. This was repeated every three minutes for 30 minutes. Next, the mixture was decanted into a volumetric flask that was filled with 100ml of distilled water. Then, 3
ml of 5% uranyl acetate solution was added to precipitate protein.

The mixture was centrifuged (Heraeus Christ, Germany) at 3000 rpm for 15 minutes. A volume of 10 ml of supernatant was removed and delivered into a clean test tube. The following was added: 0.1 g of kieselguhr, 5 ml of 20% NaCl solution and 2 ml of iodine-potassium iodide solution. The mixture was thoroughly mixed and left for 12 hours.

Next, 5 ml of the alcohol-NaCl solution was mixed into the solution. The mixture was centrifuged at 3000 rpm for 15 minutes. The supernatant was gently removed and 2 ml alcohol-NaOH solution was added to the precipitate. The mixture was gently tapped and shaken until the precipitate lost its blue color.

The inner walls of the test tube were washed with 5 ml of the alcohol-NaCl solution. The mixture was centrifuged to liberate the starch, and 5 ml of alcohol-NaCl solution was added. Next, 2 ml of 0.7 M HCl solution was added to precipitate the starch. The mixture was placed in a water bath at approximately 100°C for 150 minutes and later allowed to cool.

After adequate cooling, the mixture was poured into a 25 ml volumetric flask. Next, two drops of phenol red, two drops of 1 M NaOH and two drops of 0.05 M oxalic acid were added. Distilled water was also added until the volume was made to 25 ml. Five milliliters was removed and mixed with 5 ml of the Somogyi reagent. The mixture was heated in a water bath at approximately 100°C for 15 minutes. At the appropriate time,
tubes were removed from the water bath and left to cool.

Next, 1 ml 2.5% KI solution and 3ml of 0.75M H$_2$SO$_4$ was mixed into the solution to facilitate Cu$_2$O dissolution. The solution was titrated with 0.0055M Na$_2$S$_2$O$_3$, with the starch indicator added just before the end point was reached.

\[
\text{Starch, } \% = \left\{ \frac{50 \text{ (ml blank - ml test solution)}}{\text{mg test portion}} \times \frac{0.90}{\text{mg test portion}} \times \frac{\text{M}}{0.005} \times \frac{G}{100} \right\}
\]

Where;

50 = dilution factor

0.90 = factor glucose to starch

M = actual molarity Na$_2$S$_2$O$_3$ solution

G = mg glucose equivalent to 1 mL 0.005M Na$_2$S$_2$O$_3$.

f) Ash determination

For the determination of ash, the solid residue from the analysis of lignin was placed in a previously weighed crucible and baked in a furnace (Wisetherm FH – 05, Korea) at 450°C for eight hours. After adequate cooling, the ash and its crucible were removed from the furnace and weighed (noted as $W_3$).

\[
\text{Ash } (\%) = \frac{W_3}{(W_2 - W_1)} \times 100
\]
g) Chemical elements determination

For elemental analysis of BSG, method 200.7 of the United States Environmental Protection Agency (1994) was used.

A 50% nitric acid (HNO$_3$) solution was prepared by slowly pouring 500ml of concentrated nitric acid into 400ml of distilled water. The solution was then made to one liter. Preparation was done in a fume cupboard.

A 20% hydrochloric acid (HCl) solution was prepared by slowly pouring 200ml of hydrochloric acid into 400ml of distilled water. The solution was then made to one liter with distilled water. Gentle mixing was applied.

One gram of BSG was weighed into a 500 ml beaker. A volume of four mL of 50% HNO$_3$ and ten mL of 10% HCl was poured into the beaker. A watch glass was used to cover the beaker opening and the mixture was refluxed (Witeg, Germany) at 90 ± 5$^\circ$C for 30 minutes, after which, the mixture was allowed to sufficiently cool.

Next, the mixture was poured into a 100 ml volumetric flask. Distilled water was added to make the complete volume. The mixture was left overnight to allow all suspended matter to settle.

Inductively coupled plasma-atomic emission spectrometry (ICP-AES) was used to determine metals and some nonmetals in solution. The following elements were analyzed: Copper (Cu), Cobalt (Co), Calcium (Ca), Iron (Fe), Manganese (Mn),
Magnesium (Mg), Sodium (Na), Selenium (Se), Silica (Si), Phosphorus (P) and Sulphur (S).

3.2.3 Total Carbon and Nitrogen Determination

a) Total Carbon

All three samples were analyzed for total carbon analysis. The methods of Schumacher (2002), based on the loss-on-ignition (LOI) method of determining total organic carbon were used.

A crucible was weighed and its weight was noted as $W_1$. One gram of fresh was weighed on the previously weighed crucible (combined mass noted as $W_2$).

The sample and its crucible were then baked in a furnace at 400°C for 12 hours. At the appropriate time, the solid remnants and its crucible were removed from the oven, left at room temperature for adequate cooling and weighed (noted as $W_3$).

Total Carbon (%) =

\[
\frac{(W_3 - W_1)}{(W_2 - W_1)} \times 100\%
\]

b) Total Nitrogen

The methods of Hiller et al. (1948), based on the Kjeldahl method of nitrogen analysis were used.
A mercuric sulfate solution was prepared by slowly pouring 12ml of concentrated 
H₂SO₄ into 100ml of distilled water. Next, 10g of red mercuric oxide was weighed 
and slowly dissolved in the H₂SO₄ solution.

One gram of sample was weighed into a glass tube. Next, 0.5ml of the mercuric 
sulfate solution, 0.5g of potassium sulfate, one milliliter of concentrated sulfuric acid 
and four pieces of aluminum oxide granules were added.

The tube was placed into a glass beaker and refluxed (Witeg, Germany) at 90 ± 5°C 
for 30 minutes; then at 60 ± 5°C for 60 minutes or until the mixture turned clear. After 
this point, the mixture was refluxed for a further 30 minutes at the same temperature, 
after which the mixture was allowed to sufficiently cool.

The mixture was decanted into a distillation flask and two milliliters of distilled water 
was used to rinse the inner walls of the flask. Next, 0.2g zinc was added into the 
distillation flask. Then, 5ml of sodium hydroxide (NaOH, 10N) was poured onto the 
inner-sides of the distilling flask. A 10ml volume of 0.01428N H₂SO₄ was added into 
a distilling flask and distillation was allowed to take place.

A volume of 0.8ml of 0.1% alizarin red solution was added into the distilled mixture 
and the mixture was mixed gently. The mixture was titrated with 0.01428N NaOH 
until the mixture became clear. The volume of 0.01428N NaOH used to titrate the 
mixture was noted. Total nitrogen was calculated and expressed as a percentage of the 
mass of sample used in the analysis.
Blank analysis incorporating one gram of distilled water instead of sample was made. The methods were repeated as above. The volume of 0.01428N NaOH used to titrate the mixture was noted.

All results are presented as the mean results of four replicates, with standard deviation.

Total Nitrogen (mg) = [0.2 × [(volume of 0.01428N H₂SO₄ used to blank distillate) - (volume of 0.01428N H₂SO₄ used to titrate sample distillate)]

Total Nitrogen (%) = Total Nitrogen (mg) / 0.001 × 100

c) Total protein determination

Total protein was calculated based on Hiller et al. (1948) and AOAC International methods 930.29 and 991.20, as below:

Total Nitrogen (mg) = [0.2 × [(volume of 0.01428N H₂SO₄ used to blank distillate) - (volume of 0.01428N H₂SO₄ used to titrate sample distillate)]

Total Nitrogen (%) = [Total Nitrogen (mg) / 0.001 × 100]

Total Protein (%) = [Total Nitrogen (%)] × 6.38
3.3 FUNGAL PREPARATION AND MAINTAINANCE

Five species of fungi were studied for their enzyme activity. The following fungi were selected for this study due to their ability to grow and degrade the biomass components easily and able to produce the secondary metabolites.

a. *Aspergillus niger*

b. *Fusarium* sp.

c. *Penicillium chrysogenum*

d. *Trichoderma* sp.

e. *Schizophyllum commune*

Each fungus was inoculated on potato dextrose agar (PDA, Oxoid, England) and incubated at 30°C. PDA was prepared by mixing 19.5 g of PDA powder in 500ml of distilled water and gently heated to 65°C with stirring (Heidolph D–91126). The dissolved PDA was sterilized at 121°C for 20 minutes and left to cool. The cooled PDA was carefully poured into Petri dishes and left to solidify.

Inoculation of fungi was conducted according to a standard procedure. Fungus slants were obtained from stock cultures maintained in the Mycology Laboratory, Faculty of Science, University of Malaya. With the sterile wire loop, a fungus from a PDA slant was inoculated on to a fresh PDA plate. Six plates were inoculated for each fungus. The plates were incubated for seven days at 32 ± 2°C (Memmert B 80).
3.4 SOLID STATE FERMENTATION

BSG (fresh), SCB (dried and grinded) and SMC (dried) were used in solid state fermentation (SSF) process. The moisture content was maintained at 75±2 % moisture content and pH was maintained 4 to 5. Fermentation flasks were prepared by sterilizing 25g of substrates at 121°C for 25 minutes. The mouth of each flask was covered with a cotton stopper.

Seven-day old cultures of fungi growing on PDA plates were used as the inocula for SSF. With the sterile wire loop, four mycelial disks, each measuring approximately 1cm diameter, were removed from the PDA plate and inoculated directly into each fermentation flask containing the cooled, sterile substrates. Negative controls were made by leaving sterile spent grain flasks un-inoculated but covered with a cotton stopper. Four flasks were inoculated for each fungus and four negative control flasks were prepared for each substrate. All flasks were incubated at 32 ± 2°C for seven days (Figure 3.1).
Plate 3.1: Solid state fermentation of BSG takes place in Incubator Chamber

3.5 ENZYME EXTRACT AND ENZYME ASSAY

3.5.1 Enzyme extraction

A volume of 400ml of distilled water was poured into each SSF flask. The flasks were shaking at 180 rpm for 60 minutes at 30°C (New Brunswick Scientific Series 25 Incubator Shaker, USA) to mix the fermented culture with water. After removal, the flasks were left to settle at room temperature. Physical separation by filtration was done by using vacuum pump at room temperature. The supernatant of each flask were used for enzyme assays. All results are presented as the mean results of four replicates, with standard deviation.
3.5.2 Enzymes assay

a) Laccase activity determination

Laccase assay was based on Singh et al. (2002) and Saito et al. (2003). First, 0.1ml of the enzyme extract was mixed with 2.9ml syringaldazine (20µM, diluted in 50mM, sodium phosphate). Ten minutes later. The change in color was measured by absorbance at 525nm after 10 minutes. The enzyme activity was calculated based on the Eq 3.1.

One unit of enzyme activity was defined as the enzyme producing one unit change in absorbance/minute/g of substrate. The activity was expressed in U/g.

Laccase Activity (U/g) =

\[
\frac{0.033\text{ml}}{0.1\text{ml}} \times \frac{400\text{ml}}{25\text{g}} \times \frac{\text{Absorbance of sample} \times 10 \text{ minutes}}{\text{Absorbance of sample} \times 10 \text{ minutes}}
\]

Where;

0.033 = dilution factor (0.1ml / 3ml)

0.1 = Volume of Enzyme

b) Lignin peroxidase activity determination

Lignin peroxidase was assayed based on Singh et al. (2002) and Arora and Gill (2001). A volume of 500µl of the enzyme extract was mixed with 1ml sodium tartarate buffer (125mM, pH 3), 500µl of veratryl alcohol (10mM) and 500µl of hydrogen peroxide
(2mM). Change in color was measured by absorbance, which was read at 310nm after one minute.

For standardization purposes, veratryl alcohol was used and the enzyme activity was calculated. One unit of enzyme activity was defined as 1µmole of veratryldehyde released/minute/g of substrate. The activity was expressed in U/ml.

Lignin Peroxidase Activity (U/g) = Eq 3.2

\[
\text{Absorbance of sample} + 0.208 = 0.2 \times 1 \times 1000\mu\text{g} \times 1\mu\text{mole} \times 400\text{ml} \\
\text{Absorbance of sample} = 0.0485 \\
0.5\text{ml} \times 1\text{ minute} \times 1\text{mg} \times 168.19 \times 25\text{g} \\
\frac{0.0485}{0.2} = \text{Dilution factor (0.5ml / 0.2ml)}
\]

c) Amylase activity determination

α - amylase was assayed based on Bernfeld (1955). First, 150µl of the enzyme extract was mixed with 200µl of 1% soluble starch (prepared in 0.1M phosphate buffer). The mixture was incubated at 37°C for 25 minutes. Then, 400µl of 3, 5 - dinitrosalicylic acid was mixed in and the mixture was placed in a water bath at 60°C for 5 minutes and later allowed to cool. Next, 8ml of distilled water was added. Change in color was measured by absorbance at 489nm.

\[
\text{α - Amylase Activity (U/g)} = \text{Eq 3.3}
\]

\[
\text{Absorbance of sample} + 0.607 = 0.018\text{ml} \times 1 \times 1000\mu\text{g} \times 1\mu\text{mole} \times 400\text{ml} \\
\text{Absorbance of sample} + 0.208 = 0.15\text{ml} \times 25\text{ min} \times 1\text{mg} \times 180.16\mu\text{g} \times 25\text{g} \\
0.018 = \text{Dilution factor} = ((0.15\text{ml} / (0.15 + 0.20 +0.40 + 8.0\text{ml})))
\]
For standardization purposes, D-glucose was used and the enzyme activity was calculated. One unit of enzyme activity was defined as 1µmole of reducing sugar (glucose) released/minute/g of substrate. The activity was expressed in U/ml.

d) **Cellulase activity determination**

The assay of CMCase was based on Yamane *et al.* (2002) and Silva *et al.* (2004). First, 400µl ml of the enzyme extract was mixed with 1.6ml of a 1% carboxymethylcellulose solution (prepared with a 40mM sodium acetate buffer solution). The mixture was incubated at 37°C for 25 minutes. Then, 400µl of 3, 5 - dinitrosalicylic acid was added and the mixture placed in a water bath at 60°C for 5 minutes and later allowed to cool. Next, 8ml distilled water was added. Change in color was measured by absorbance, which was read at 489nm.

For standardization purposes, D-glucose was also used and the enzyme activity was calculated. One unit of enzyme activity was defined as 1µmole of reducing sugar (glucose) released/minute/g of substrate. The activity was expressed in U/ml.

\[
\text{CMCase Activity (U/g)} = \text{Eq 3.4}
\]

<table>
<thead>
<tr>
<th>Absorbance of sample + 0.5289</th>
<th>0.0038ml</th>
<th>0.0038ml</th>
<th>0.009</th>
<th>0.4ml</th>
<th>25 min</th>
<th>1mg</th>
<th>180.16µg</th>
<th>25g</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0038 ml = Dilution factor (0.4 ml / (1.6 + 0.4 + 0.4 + 8.0))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

e) **Xylanase activity determination**

The assay of xylanase was based on Bailey *et al*., (1992). First, 200µl of the enzyme extract was mixed with 1.8ml of a 1% oat xylan solution (prepared in 0.05M sodium
citrate buffer, pH 6.5). The mixture was incubated at 50°C for 5 minutes. Next, 3 ml of 3, 5 - dinitrosalicylic acid was added and the mixture placed in a water bath at 60°C for 15 minutes and later allowed to cool. Change in color was measured by absorbance, which was read at 540 nm.

For standardization purposes, xylose was used and the enzyme activity was calculated. One unit of enzyme activity was defined as 1 μmole of reducing sugar (xylose) released/minute/g of substrate. The activity was expressed in U/ml.

Xylanase Activity (U/g) = Eq (3.5)

\[
\text{Absorbance of sample} + 0.8347 \times 0.04 \text{ml} \times 1000 \mu g \times 1 \mu m o l e \times 400 \text{ml} \\
\frac{0.1435}{0.2 \text{ ml}} \times 25 \text{ min} \times 1 \text{ mg} \times 150.13 \mu g \times 25 \text{ g} \\
0.04 \text{ ml} = \text{Dilution factor} (0.2 \text{ ml} / (0.2 + 1.8 + 3.0))
\]

3.6 SOLID STATE FERMENTATION – OPTIMIZATION OF ENZYME PRODUCTION

Only autoclaved BSG sample was used in the optimization analysis. SSF process was conducted by inoculating five fungal species separately on autoclaved BSG samples to determine the activity of five types of enzymes. Sample preparation for SSF process is similar as described in 3.4, except the sample used in this optimization analysis (except 3.6.3) was only autoclaved BSG.
3.6.1 Effects of incubation period
The fermentation process was conducted, considering the incubation period. The inoculated substrates were incubated for one to seven days at 32±2°C. The crude enzyme was extracted every day using the above mentioned method.

3.6.2 Effects of incubation temperature
The inoculated flasks were incubated at different temperature (20°C, 25°C, 30°C, 35°C, and 40°C) for seven days. The fermented substrates (after seven days) were used to extract the crude enzyme by adding distilled water and filter it later. All crudes were undergone various assays for different enzymes.

3.6.3 Effect of mixed substrates
Sample preparation:
Other organic materials such as SCB and SMC were mixed with BSG at different percentage (10% - 50%). Fermentation flasks containing mixed substrates were autoclaved at 121°C for 20 minutes. Four mycellial discs were inoculated into each flask and incubated at 32±2°C for seven days. After seven days of incubation period, the crude was extracted using filtration method. The crude was undergone different assays to determine the enzyme activity. Results were presented as the mean enzyme activity of three replicates, with standard deviation

3.6.4 Effects of chemical supplementation
Four types of chemicals namely, Cobalt chloride (CoCl₂), Copper sulphate (CuSO₄), Manganase sulphate (MnSO₄) and Iron sulphate (FeSO₄) were supplemented to the
autoclaved BSG at different concentrations (0.2%, 0.4%, 0.6%, 0.8% and 1.0%). 2ml of chemical at different concentration was mixed into fermentation flask separately before the fungal inoculation. For the chemical supplementation analysis, only *A.niger* and *S.commune* were used. Fugal selection was based on the genus. Moisture was maintained at 75%.

Fermentation flasks containing autoclaved BSG with chemicals at different concentrations were incubated for seven days at 32±2°C after fungal inoculation. The crude was extracted and assayed after seven day incubation period. Results are presented as the mean enzyme activity of three replicates, with standard deviation.

### 3.7 KINETICS IN SOLID STATE FERMENTATION

Kinetic modal (Monod) was incorporated into solid state fermentation by determining the fungal growth on autoclaved BSG and enzyme titration.

\[
X = X_0 \cdot e^{kt}
\]

- Eq (3.6)

Solid state fermentation was conducted as described in 3.4 using only autoclaved BSG samples. Fungal biomass was estimated by estimating the glucosamine content in the fermented samples (daily samples Day1 - Day 7) using Chen *et al.*, 2010 method. The glucosamine value (*Xo*), temperature (k) (20°C to 40°C) and time (t) (incubation period) were incorporated into the Equation 3.6 to estimate the total.
biomass generation (X) for all incubation period. The temperature (k) was standardized at 30°C and the time (t) varies from 24 hours to 168 hours with 24 hours interval.

Graphs were plotted on Glucosamine concentration (mg/g dw) versus incubation period for each fungi to estimated regression correlation ($r^2$). This regression correlation was used to estimate the specific growth rate ($\mu$) of the fungi. Equation 3.7 was used to estimate the doubling time of the each fungi based on the Glucosamine Content graph.

$$Td = \ln 2 \frac{\mu_{max}}{\mu_{max}}$$  \hspace{1cm} \text{– Eq (3.7)}

Doubling time is estimated to determine the correlation between fungal growth and enzyme titration.

### 3.7.1 Biomass Estimation

Autoclaved BSG was inoculated with five types of fungi separately and incubated at 30°C from one to seven days. Four replicates were prepared for each incubation day for each fungi including control (without fungal inoculation). Four fermentation flasks (replicates) inoculated with each fungal species were sampled at 24hrs interval. Five gram of fermented medium from each flasks (BSG +fungi) were dried (Chen et al., 2010). Biomass estimation was done by hydrolyzing glucosamine from fungal chitin.

A volume of 20mg of dried sample (which was sampled at every 24 hours) was
incubated with 2ml of H$_2$SO$_4$ (72%) in a test tube and incubate in a rotary shaker (130rpm) for 60 minutes at 25°C. Then the sample was diluted with 3ml of sterile distilled water and autoclaved at 121°C for 2 hours. The hydrolyzate was neutralized at pH 7.0 with 10M and then 0.5M NaOH and diluted to 100ml (Chen et al., 2010).

The hydrolyzed glucosamine was assayed by calorimetric method described by Tsuji et al., (1969) and modified by Ride and Drysdale (1972).

(i) 1ml diluted hydrolyzed was mixed with 1ml of NaNO$_2$ (5%) and 1ml of KHSO$_4$ (5%) in a centrifuge tube.
(ii) After shaking occasionally for 15 minutes, sample was centrifuged at 3500rpm for 5minutes
(iii) 2ml supernatant was mixed with 0.67ml of NH$_4$SO$_3$NH$_2$ (12.5%) and shaken for 3 minutes.
(iv) 0.67 ml of 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH; 0.5% -prepared daily) was added to the sample and boiled for 3 minutes and immediately cooled to room temperature.
(v) 0.67 ml of FeCl$_3$ (0.5% - prepared within 3 days) added to the sample and keep for 30 minutes
(vi) After 30 minutes of standing, the absorbance at 650nm was measured spectrophotometrically.
(vii) Glucosamine content was calculated as milligram per gram of fungal biomass according to standard curve.
CHAPTER 4

4.0 RESULTS AND DISCUSSION

4.1 PHYSICAL AND CHEMICAL PROPERTIES OF SUBSTRATES

4.1.1 Physical properties of substrate

(i) Brewery spent grain (BSG)

BSG was sampled immediately after the wort separation (a process involves the separation of undigested solid particles (BSG) from the wort (sweet liquid) which will be used in the beer production) in brewing process. Fresh BSG contained high moisture content and high temperature at 73% and 80°C respectively. During the brewing process, barley grains were mashed at high temperature ranged from 58°C to 78°C to promote enzymatic hydrolysis (Mussatto et al., 2006; Serena and Bach Knudsen, 2007).

Table 4.1 represents the physical properties of BSG which were examined immediately after sampling. BSG has irregular shape which was less than 1mm. During the brewing process the barley grains were soaked in water at specific temperature to initiate the enzymatic hydrolysis to extract the sugar from the grains. The grains became soft which allows enzymatic hydrolysis to occur fast. Therefore, the soft grains became irregular when physical pressure was introduced during the mashing process.

After the mashing process, BSG was separated from the wort by pressing and filtering. Besides, BSG particle size also depends on the machine used during the filtering.
process. Jay et al., (2008), used different size of BSG particles, ranging from less than 53µm to more than 250µm, which was collected from different machines to compare the enzyme production. BSG particles were clumped together and formed spent cake due to high moisture content (Plate 4.1). However, the dried BSG substrate (Plate 4.2) forms free particles. The color of the substrates was also observed to be different. Dried BSG was dark in color, while fresh BSG was light in color.

**Table 4.1: Physical and Chemical properties of substrates tested in SSF**

<table>
<thead>
<tr>
<th></th>
<th>BSG</th>
<th>SCB</th>
<th>SMC</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture content (%)</td>
<td>73</td>
<td>87</td>
<td>63</td>
<td>Drying</td>
</tr>
<tr>
<td>pH</td>
<td>5.49</td>
<td>4.77</td>
<td>5.54</td>
<td>pH meter</td>
</tr>
<tr>
<td>Particle Size (mm)</td>
<td>&lt;1mm</td>
<td>&gt;1mm</td>
<td>&lt;1mm</td>
<td>Sieving</td>
</tr>
<tr>
<td>Total Carbon (%)</td>
<td>28.8</td>
<td>16</td>
<td>16.2</td>
<td>LOI</td>
</tr>
<tr>
<td>Total Nitrogen (%)</td>
<td>0.9</td>
<td>1.8</td>
<td>0.69</td>
<td>Kjeldahl</td>
</tr>
<tr>
<td>Soluble Protein (g/100g)</td>
<td>0.5</td>
<td>4.5</td>
<td>4.8</td>
<td>Bradford 1976</td>
</tr>
<tr>
<td>Lignin (%)</td>
<td>28</td>
<td>46</td>
<td>25</td>
<td>ADF</td>
</tr>
<tr>
<td>Cellulose (%)</td>
<td>17</td>
<td>24.5</td>
<td>38</td>
<td>ADF</td>
</tr>
<tr>
<td>Hemicelluloses (%)</td>
<td>29</td>
<td>19.95</td>
<td>19</td>
<td>ADF</td>
</tr>
<tr>
<td>Starch</td>
<td>ND&lt;0.1</td>
<td>ND&lt;0.1</td>
<td>ND&lt;0.1</td>
<td>AOAC 2006</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>4.30</td>
<td>4.23</td>
<td>4.50</td>
<td>Combustion</td>
</tr>
<tr>
<td>Others (%)</td>
<td>26</td>
<td>9.55</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>
Plate 4.1: Fresh BSG forming spent cake

BSG substrate contains high C/N ratio (32:1) and less protein content. However, some studies showed that BSG contains high concentration of protein component (Bartolome and Cordoves 1999; Douwenga et al., 1988).
Fresh SCB collected from night market contained high moisture content and is at room temperature. SCB is made of fibrous spongy pith which has high water holding capacity, and that explains the higher moisture content (83%) observed in SCB (Table 4.1). Besides, physical observation also showed SCB to be watery and the juice can be extracted if a strong physical pressure is introduced (Plate 4.3). The size of SCB and moisture content varies, depending on the efficiency of the juice extraction machine. Most of the SCB comes out as a long crushed stalk (bigger particle). Therefore, the SCB had to be ground to obtain smaller particles (Plate 4.4).
**Plate 4.3**: Fresh SCB forming long stalks

**Plate 4.4**: SCB in powder form
(iii) **Spent mushroom compost (SMC)**

SMC forms a spent cake covered with fungal mycelium (Plate 4.5). The moisture content of SMC is very low (63%) compared to other substrates. SMC is also very light. The initial weight of SMC bag is approximately 2 kg each. The weight of the SMC bags decreased gradually as the harvesting repetition increased. This is due to the loss of nutrients and water content in SMC. Nutrients were utilized for the mushroom growth and mushroom can be harvested five to six times from the same bag before it can be discarded.

**Plate 4.5: Spent Mushroom Compost**
4.1.2 Chemical Properties of Substrates

Table 4.1 shows the chemical properties of the three substrates used in this study. All three by products (BSG, SCB and SMC) are organic wastes from industrial processes. Therefore, these materials contain high cell wall components such as lignin, cellulose and hemicelluloses components. SCB contains 46% of lignin, 24.5% of cellulose and 20.0% of hemicelluloses. SMC contains 25% lignin, 38% cellulose and 19% hemicellulose. BSG contains 28% lignin, 17% cellulose and 26% hemicelluloses. This result was similar with Jordan et al., (2008).

BSG contains lower protein content (0.5g/100g) compared to SCB (4.5g/100g) and SMC (4.8g/100g). While the ash content in all three substrates were almost equal (4.23-4.5g/100g). BSG contains higher C:N ratio, 35:1, followed by SMC 23:1 and SCB 9:1. According to Xiros et al., (2008), BSG contains 11.5% of lignin, 40% cellulose, 11.9% hemicellulose, 3.2% ash and 13% total protein. While Couto and Sanroman, (2005) reported that SCB contains 14% lignin, 33% cellulose and 22% hemicelluloses. Jay et al., (2008), explained that the variation in the substrate’s chemical composition due to the substrate particle size and the process itself. It is also supported by Santos et al., (2003) where, BSG composition may vary with barley the variety, time of harvest, characteristics of hops and other adjuncts added, and brewery technology.
Table 4.2: Elemental content in BSG, SCB and SMC

<table>
<thead>
<tr>
<th>Elements (ppm)</th>
<th>Substrates used in SSF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BSG</td>
</tr>
<tr>
<td>Copper (Cu)</td>
<td>ND&lt;0.01</td>
</tr>
<tr>
<td>Cobalt (Co)</td>
<td>ND&lt;0.01</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
<td>0.03</td>
</tr>
<tr>
<td>Iron (Fe)</td>
<td>0.01</td>
</tr>
<tr>
<td>Manganese (Mn)</td>
<td>0.008</td>
</tr>
<tr>
<td>Magnesium (Mg)</td>
<td>0.009</td>
</tr>
<tr>
<td>Sodium (Na)</td>
<td>0.01</td>
</tr>
<tr>
<td>Phosphorus (P)</td>
<td>400</td>
</tr>
<tr>
<td>Sulphur (S)</td>
<td>ND&lt;0.01</td>
</tr>
<tr>
<td>Aluminum (Al)</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Table 4.2 shows the elements contained in each substrate. SCB contains high Ca content (0.9 ppm), Fe (0.1ppm) and P content (480ppm) compared to other substrates. SMC contains higher amount of Mn (0.01ppm), Mg (0.01ppm) and Al (0.57ppm). All substrates contain almost similar amount of Na and BSG and SMC contains same amount of P (400ppm). No Cu, Co and S were found in any of these substrates. According to Mussatto et al., (2006) BSG contains very low (lower than 0.5%) concentration of Mg, Mn, P, K, Na and S.

4.2 FUNGAL MAINTENANCE

The five types of fungi used in this study inoculated onto PDA plates are shown in Plate 4.6 (a-f). Fungal growth on PDA can be observed from the second day onwards after inoculation. At the end of incubation (on seventh day) the PDA plates were fully
covered with fungal mycelium and fungal spores. The growth of spore-forming fungi (Aspergillus niger and Penicillium chrysogenum) is faster than non-spore forming fungi (Schizophyllum commune and Fusarium sp).

From the physical observation, fungi formed a network of mycelium, covered the agar plates and also penetrated the medium, forming branches of mycelium. This is because, filamentous fungi have the ability to grow away from the initial point, such that hyphae at the edge of a colony constantly encounter fresh nutrients (Mishra and Kumar, 2007; Mohebby, 2005; Raberg et al., 2009).
Plate 4.6: Five types of fungi used in SSF were grown on PDA. (a) *Aspergillus niger* (b) *Schizophyllum commune* (c) *Penicillium chrysogenum* (d) *Trichoderma* sp. (e) *Fusarium* sp.

4.3 SOLID STATE FERMENTATION

The growth of fungi on the substrates is obvious on the second day onwards (Plate 4.7)
and Plate 4.8). This could be due to the fact that, the fungi need time to adapt to the environment (substrate) and start to utilize the nutrients content within the substrate. The fungi started to decay the plant materials and convert them into secondary metabolites. All the fungi showed progressive growth, and at the end of the fermentation process, the substrate was fully covered with fungal mycelium which can be observed in Plate 4.7. Filamentous fungi are capable of growing in a complex solid matrix even in the absence of free-flowing water (Orzua et al., 2009). Under the natural condition, fungus contact with substrate surface for nutrient uptake, hyphal apical growth and enzyme secretion. Besides, filamentous fungi can grow up to significant extend in the absence of free water (Gao et al., 2009). After the fermentation, the substrates will be left to settle down (Plate 4.9) and the extracts were (Plate 4.10) been used for various assays.

4.4 ENZYME ACTIVITY COMPARISON IN DIFFERENT SUBSTRATE

(i) Brewery spent grain (BSG)

Among the five types of enzymes tested, laccase activity were observed at highest followed by lignin peroxidase, xylanase, cellulase and amylase activity. *Penicillium chrysogenum* grown on BSG showed the higher laccase activity (15,413.33 U/g) and higher lignin peroxidase activity (2024.94 U/g). While, *A. niger* grown on this substrate gave higher amylase activity (4.42 U/g) and higher xylanase activity (82.85 U/g) and *Trichoderma* sp showed maximum cellulase activity at 36.49 U/g.
Plate 4.7: *A. niger* growing on BSG

Plate 4.8: *S. commune* is growing on the substrate
Plate 4.9: Substrate left to settle down before the extraction process start

Plate 4.10: The crude extract for enzyme assay
A study conducted by Hamed et al., (2008) with three strains of *Penicilium* sp. evidenced these strains could produce higher laccase activity. Among the enzymes tested for BSG, amylase activity was observed lowest. Less starch and lower protein content (0.5g/100g substrate) in this substrate could be the cause for lower amylase activity. This is also supported by Forssell et al., (2008), where chemical analysis of BSG showed only 1.55% of starch (traces) found in this substrate, since the starch components has been extracted for beer production.

(ii) **Sugarcane bagasse (SCB)**

SCB fermented by *A. niger* showed highest laccase activity at 6223 U/g, which was 728% higher than control and 54% to 183% higher than laccase activity observed in other fungi. Unlike in BSG, *P. chrysogenum* in SCB showed the least laccase activity at 2200 U/g. However, Vinoth Kumar et al., (2010) showed that *P. chrysogenum* is capable of generating high level of Penicillin (18kg/kg) with 50% of SCB. Lignin peroxidase activity found to be the second highest, after laccase activity. Lignin peroxidase activity in SCB fermented by all fungi ranged from 811 U/g to 1152.26 U/g. Among the fungi grown on SCB, *Trichoderma* sp. produced the highest lignin peroxidase activity. Xylanase activity and cellulase activity in SCB was almost 30 to 50 times lower than lignin peroxidase activity and the enzyme activity ranged between 33.40 U/g – 38.76 U/g for xylanase and 20.91 U/g to 26.80 U/g for cellulase activity. According to Alriksson et al., (2009), SCB served as a good medium for the growth of recombinant *A. niger* where higher cellulase activity was found (125.97 U/ml) by this organism. However, he also mentioned that, the enzyme activity level observed depends on the strain used.
Besides, *Aspergillus* strains grown on SCB are also capable of producing high level of citric acid, yet the yield and biomass growth was not the same for each strain (Mahin *et al.*, 2008). Amylase activity showed the least activity in SCB, where all the fungi used, generates amylase activity in the range (3.25 U/g to 4.24U/g). This is supported by Mohan and Vijay-Raj, (2009), where less amylase activity was found in *Penicillium* sp.NIOM-02 grown on SCB, compared to wheat and corn flour. On the other hand, Rajagopalan and Krishnan, (2008) study showed that *Bacillus subtilis* strain KCC103 produced maximum amylase activity (67.4 U/ml) in media containing SCB hydrolysate with 1% reducing sugar (w/v).

The enzyme activity observed in this study was relatively lower compared to other researchers. Two possible explanations can be derived for the variation in the enzyme activity in this substrate. Firstly, SCB used in other studies, undergone a pre-treatment before employed in fermentation. Different types of pre-treatment (alkaline, acid or thermal treatment) were conducted to increase the affinity of biocatalyst to the lignocellulosic material (Santos *et al.*, 2008) which will increase the enzyme production (Vinoth Kumar *et al.*, 2010). Usage of salt solution could be the second reason for higher enzyme activity observed in other studies. Salt solutions, such as NaOH (Santos *et al.*, 2008) and Vogel’s salt solution (Milagres *et al.*, 2004) were used to increase the moisture content of the substrate. While, in the present study, sterile distilled water was used to increase the moisture content. Salt solution used, can increase the nutrient content in the substrate, which provides a favourable condition for good fungal growth and higher enzyme production.
(iii) **Spent mushroom compost (SMC)**

Laccase activity in SMC ranged at 3047 U/g to 4211 U/g, for five fungi used, except *Trichoderma* sp. which showed highest laccase activity (6568.07 U/g). *Schizophyllum commune* showed the maximum lignin peroxidase activity (1490.61 U/g). The lignin peroxidase activity in other fungi grown on this substrate ranged at 1031.87 U/g to 1193.33 U/g. *Aspergillus niger* showed higher amylase (7.00 U/g) and higher xylanase (61.95 U/g) activity in SMC. SMC fermented by *Fusarium* sp. showed high cellulase activity at 29.16 U/g. According to Zhang *et al.*, (1995), SMC has not been used as a sole carbon source for enzyme production. In his study, he mixed SMC with maize powder, soybean cake, wheat bran and ammonia sulphate with *A. niger* to produce amino acids. This is due to the fact that, SMC losses half of its weight, decreased nitrogen level below 1.5 % and high level of porosity (Polat *et al.*, 2009), which make this substrate not suitable as sole carbon source for enzyme production.

### 4.4.1 General Discussion on Comparison of Enzyme Activity on Different Substrate

Enzyme activity comparison between three substrates (BSG, SMC and SCB) was conducted to compare the ability of substrates in producing higher enzyme activity at similar conditions. Eventually this can be used to determine the most suitable substrate for SSF process.

Among the fungi tested, *Trichoderma* sp. in SMC (Figure 4.1) and *A. niger* in SCB showed higher laccase activity at 6568.07 U/g and 6223.33 U/g respectively. However, *Trichoderma* cultivated on BSG showed highest laccase activity at 15413.33 U/g,
which is 102 % and 323 % higher than laccase activity observed in SMC and SCB respectively. While for *P. chrysogenum* in BSG, which showed the highest laccase activity also showed 266 % higher than SMC and 600 % higher than SCB.

The variation in the laccase activity among the substrates might be due to the differences in the physical and chemical properties of the substrates. According to Gomez *et al.*, (2005), high cellulose component in the substrate lead to high laccase activity. In that case, in this study, the SMC should have given higher laccase activity followed by SCB and BSG. In contrast, BSG gave the higher laccase activity. This is because; generally cellulose component in the organic substrates (SMC and SCB) was covered by thick lignin and hemicellulose components which safeguard the cellulose component from fungal attack. Fungi have to attack the lignin and hemicellulose components before digesting the cellulose.
In this case, BSG has less lignin component compared to SCB. Therefore, fungal mycelium reached the cellulose faster and enzymatic conversions occurred faster, in BSG compared to other substrates. In a study conducted by Couto et al., (2002), barley bran was the better substrate for higher laccase production compared to chestnut shell using *Trametes versicolor*. Conversely, Moldes et al., (2003) mentioned that higher lignin content is best for laccase production by using *Trametes hirsuta* in SSF. At the same time the fungal strain used in this study also has to be considered. Generally, basidiomycete fungi has different mode of cellulose degradation (Blanchette et al., 1990). Some fungi are selective, while some are unable to degrade certain carbohydrate components in the woody substrates (Cohen et al., 2005;
Robertson et al., 2008).

Among the substrates tested, BSG showed higher lignin peroxidase activity followed by SMC and SCB (Figure 4.2). *Penicillium chrysogenum* in BSG showed highest lignin peroxidase activity at 2024.94 U/g, followed by *Fusarium* (1576.49 U/g) and *A. niger* (1565.44 U/g). *Schizophyllum commune* showed higher lignin peroxidase activity in SMC (1490.61 U/g) and *Trichoderma* showed higher lignin peroxidase activity in SCB (1152.26 U/g). *Penicillium chrysogenum* which showed highest lignin peroxidase activity in BSG is 73 % to 118 % higher than SMC and SCB.

Xylanase activity comparison study between the substrates showed, BSG generated higher xylanase activity compared to SMC and SCB (Figure 4.3). Xylanase activity in BSG ranged from 40.52 U/g to 82.85 U/g. Xylanase activity was observed lowest in SCB substrate, which ranged 36.03 U/g to 38.76 U/g. *Aspergillus niger* which showed higher xylanase activity in both BSG and SMC substrates, showed lower xylanase activity in SMC. However, in a study conducted by Acuna-Arguelles et al., (1994), SCB fermented by *A. niger* demonstrated as a potential substrate for higher xylanase activity, when the fermentation is conducted at optimal condition.

Suitable C:N ratio played crucial role in the xylanase production. Based on Maciel et al., (2008), low level of N in SCB is unable to support good fungal growth which, eventually contributed to the lower xylanase production. This was also supported by Park et al., (2002), low N in rice straw gave lower xylanase production.
Figure 4.2: Lignin peroxidase activity comparison between BSG, SCB and SMC

Milagres et al., (2004) suggested introducing air flow during fermentation has significant impact in increasing xylanase activity. In his studies, air flow introduced into the medium bed (SCB), produced 22 times higher xylanase activity compared to static environment. Air flow will increase the porosity of the substrate, which will eventually increase the efficient oxygen transfer and lead to good fungal growth and metabolism. Consequently higher enzyme activity can be observed.
BSG exhibit higher cellulase activity compared to SMC and SCB (Figure 4.4). Cellulase activity observed in SMC and SCB were 5 %–49 % and 13 % - 59 % lower than the cellulase activity observed in BSG. Cellulase activity in BSG fermented by *Trichoderma* exhibit higher activity (36.49 U/g) followed by *Fusarium* (33.21 U/g) and *S. commune* (32.69 U/g). *Trichoderma* which showed higher cellulase activity in BSG, exhibit lower cellulase activity in SMC and SCB, which is 38 % and 49 % lower than in BSG.

In a study conducted by Xia and Chen (1999), *Trichoderma* sp. grown on 70 % of wheat substrate showed higher cellulase activity. Surface area of the substrate could
be a possible explanation for the deviation in enzyme activity. A comparative study conducted by Reddy et al., (2003) fermenting banana leaves and pseudo stems with *P. ostreatus* and *P. sajor-caju*, showed banana leaves, which has larger surface area produced higher cellulolytic enzymes. Substrate with smaller particle size, provide larger surface area for fungal growth and enzyme production (Pandey, 1991). Therefore BSG in this study, which has smaller particle size, provides larger surface area and produce higher enzyme activity.

**Figure 4.4:** Cellulase activity comparison in BSG, SCB and SMC
Amylase activity (Figure 4.5) in all three substrates was apparently similar and lower compared to other enzyme activity. Among the fungi tested, *A. niger* showed higher amylase activity in SMC (6.99 U/g) and BSG (4.43 U/g), while *Trichoderma* sp. showed higher amylase activity in SCB. This result might be due to the higher protein content in the substrate.

According to Zhang *et al.*, (1995) spent mushroom compost contains high level of protein up to 28 %, while the protein content in BSG is slightly lower with 20 %. Smaller substrate particle would provide large surface area for microbial metabolism, which is one of the desirable factors in enzyme production. However, too small
substrate particle will lead to substrate agglomeration, which may interfere with microbial respiration/aeration (oxygen transfer), and result in poor cellular growth (Pandey et al., 2000).

During the brewing process, grain kernels were mashed to initiate enzymatic hydrolysis and to extract the sugar and the mashed grains become small particles (<1 mm). However, the size of BSG particles was not considered as too small for SSF process, since the fungi grow well (no oxygen transfer limitation) and yield higher enzyme activity. On the other hand Botella et al., (2007) mentioned in his study that, the particle size did not significantly affect the enzyme production. During the mushroom cultivation, most of the nutrient in the compost was used up for mushroom growth. Besides, the nutrient depletion was higher, since each bag is harvested for five times.

Therefore, the compost losses half of its weight, lowered moisture content, decreased N level (below 1.5 %), which gives lower C/N ratio and high level of porosity (Polat et al., 2009). This could be the reason for low enzyme production in SMC. Lower C/N ratio in the SMC gives low level of enzyme activity, as the carbon and nitrogen source is involved in fungal growth (Pandey et al., 2008). However, the C/N ratio in SMC (24:1) is higher than SCB (9:1), which could be used to explain the lower enzyme activity observed in SCB compared to SMC. Chemical properties of the substrates also play a major role in enzyme activity.

Oasma et al., (2007) reported that particle size, porosity and chemical composition of
the substrates play crucial role in higher enzyme production. In an earlier study conducted by Gomez et al., (2005) barley bran had high porosity and roughness which allowed a better attachment for fungal growth, that lead to higher enzyme activity. Apart from these factors mentioned above, moisture content also played a role in producing more enzymes in SSF process where it involved microbial growth and product yield. In this research, the moisture content of the substrates was maintained at the initial moisture to keep the substrate natural condition.

In a study conducted by Sandhya et al., (2005), any substrate with 55 % moisture could yield higher protease enzyme using P. chrysogenum. Initial moisture of substrate influenced the biosynthesis and secretion of enzymes. A study conducted by Gervais and Molin (2003), 55 % and 75 % of moisture required by Trichoderma longibrachiatus and Aspergillus tereus respectively for higher enzyme production. While 65 % moisture content is optimum for grape pomace to produce higher enzyme in SSF process (Botella et al., 2007). According to Pandey et al., (2008), 30-80 % moisture content is optimum for SSF process. Higher moisture content will alter the particle structure and create a gummy structure while make the oxygen transfer became harder. While lower moisture content will reduce the diffusion of nutrients and create higher water tension. These two events eventually create boundaries for fungal growth which is directly related to the enzyme production in the system. However, this depends on the nature of the substrate as well.

Some studies also suggested that pretreatment should be carried out to increase the enzyme activity, especially on cellulase production. Romero et al., (1999) proved that
treated BSG showed higher cellulase production and Kansoh et al., (1999) also showed treated bagasse showed higher cellulase production (5.0 U/ml) compared to untreated bagasse (2.44 U/ml). Conducting substrate pretreatment will reduce the cellulose crystalinity and remove the lignin and hemicellulloses component, which prevent the fungi from reaching the cellulose component in the substrate (Santos et al., 2008). Madamwar et al., (1989) suggested alkaline pretreatment would be a good option for higher cellulase activity.

4.5 EFFECT OF INCUBATION PERIOD ON ENZYME ACTIVITY

Autoclaved BSG was used in this experiment. Aspergillus niger, Fusarium sp., P. chrysogenum, S. commune and Trichoderma sp. were inoculated into sterilized BSG flasks separately. Activity of amylase, cellulase, laccase, lignin peroxidase and xylanase was determined at the interval of 24 hours to determine the optimum fermentation period for each enzyme and fungi.

4.5.1 Aspergillus niger

Brewery spent grain cultivated with A. niger showed peak activity on 7th day of incubation period for all the five enzymes tested. The activity showed declining on 10th day fermentation. Among the enzymes tested, laccase showed highest activity followed by lignin peroxidase, xylanase, cellulase and amylase on 7th day of incubation.

Laccase activity (Figure 4.6) showed a drastic increase from 1st day (3382.22 U/g) to
7\textsuperscript{th} day (9337.78 U/g) with 176.1 \% increase. However, the activity decreased up to 85 \% on 10\textsuperscript{th} day fermentation (1353.78 U/g). On the other side, cellulase showed very slow increase, from 26.86 U/g on 1\textsuperscript{st} day to 30.44 U/g on 7\textsuperscript{th} day with only 13.3 \% increase. However, \textit{A. niger} showed linear correlation from day 1 to day 6 with correlation value $R^2 = 0.9696$.

![Figure 4.6: Laccase and lignin peroxidase activity in BSG fermented by \textit{A. niger} at various incubation period](image)

Amylase and lignin peroxidase showed 33.43 \% and 30.37 \% increase respectively compared from 1\textsuperscript{st} day to 7\textsuperscript{th} day fermentation. Amylase activity (Figure 4.7) in BSG fermented by \textit{A. niger} showed strong linear correlation ($R^2 = 0.9088$) with the incubation period. Xylanase activity was observed start to peak from 2\textsuperscript{nd} day (54.06 U/g) onwards and reach the maximum on 7\textsuperscript{th} day (82.85 U/g). However the activity dropped 3 folds on 10\textsuperscript{th} day fermentation. Xylanase activity in \textit{A. niger} was observed
linear increase from day 1 to day 4 ($R^2 = 0.9771$), decreased at day 5 and increase again from day 5 to day 7 ($R^2 = 0.9797$) and fall again after day 7.

![Graph](image.png)

**Figure 4.7**: Amylase, Cellulase and Xylanase activity in BSG fermented by *A. niger* at various incubation period

### 4.5.2 *Fusarium* sp.

Brewery spent grain fermented by *Fusarium* sp. showed all enzymes were peaked at 7th day incubation period. Laccase activity (Figure 4.8) showed highest activity on 7th day and the increase was observed drastic with 534.55% increase. *Fusarium* sp. showed a steady linear increase in laccase activity with the incubation period (day1-7) with the correlation value $R^2 = 0.9675$ before the activity drop after day 7. For lignin peroxidase, the activity was observed increasing steadily with the incubation period from 739.14 U/g on 1st day to 1576.49 U/g on 7th day with 113.29 % increase.
Figure 4.8: Laccase and lignin peroxidase activity in BSG fermented by *Fusarium* sp. at various incubation period

Xylanase activity (Figure 4.9) was observed increasing steadily and shoots ups from 5\textsuperscript{th} days onwards and peaks on 7\textsuperscript{th} day (58.24 U/g). However, the activity dropped 48.9% on 10\textsuperscript{th} day. *Fusarium* sp. showed strong linear correlation with incubation period with the correlation value $R^2 = 0.9817$ from day one to day seven.

While amylase activity showed very slow increase from 1\textsuperscript{st} day (3.58 U/g) to 7\textsuperscript{th} day (4.61 U/g) with 28.77 % increase and the activity dropped 17.14 % on 10\textsuperscript{th} day. Therefore, the correlation value for amylase activity in BSG fermented by *Fusarium* sp. was slightly lower $R^2 = 0.8727$. 
4.5.3 *Penicillium chrysogenum*

Among the enzymes tested on BSG fermented by *P. chrysogenum*, laccase showed highest activity on 7th day with 15413.33 U/g (Figure 4.10). Laccase activity also showed rapid increase from 1st day (5128.89 U/g) to 7th day with 200.52 % increase. However, the activity decreased to 6377.78 U/g on 10th day with 58.62 % reduction. *Penicillium chrysogenum*, showed slightly lower correlation $R^2 = 0.8618$ with day (1-7) of fermentation period. However, a strong linear correlation $R^2 = 0.9991$ was observed from day 1-4 of fermentation, where activity increased from 5128.89 U/g to 7680.00 U/g.
Figure 4.10: Laccase and lignin peroxidase activity in BSG fermented by *P. chrysogenum* at various incubation period

Amylase and cellulase activity (Figure 4.11) showed slow increase from the 1\textsuperscript{st} day and showed the peak on the 7\textsuperscript{th} day. There is no linear correlation was found in *P. chrysogenum* with the incubation period (from 1\textsuperscript{st} day to 7\textsuperscript{th} day) for amylase activity. However, *P. chrysogenum* showed a strong linear growth from day one to day three with the correlation value $R^2 = 0.9998$.

*Penicillium chrysogenum* showed drastic increase in xylanase activity from day 3 to day 5 with correlation value $R^2 = 0.980$. Xylanase activity was observed peak on 7\textsuperscript{th} day (38.72 U/g).
Figure 4.11: Amylase, Cellulase and Xylanase activity in BSG fermented by *P. chrysogenum* at various incubation period

### 4.5.4 *Schizophyllum commune*

Amylase activity (Figure 4.12) in BSG cultivated by *S. commune* showed slow increase from 1\(^{st}\) day (3.63 U/g) to 3\(^{rd}\) day (4.62 U/g) and start to decrease from 4\(^{th}\) day onwards (3.86 U/g to 3.67 U/g). Amylase activity was observed optimum on 3\(^{rd}\) in BSG fermented by *S. commune* compared to other fungi which showed 7\(^{th}\) day as the optimum incubation period for amylase production. There is no linear correlation been found in *S. commune* with the incubation period (from 1\(^{st}\) day to 7\(^{th}\) day) for amylase. However, *S. commune* showed a strong linear growth from day one to day three with the correlation value $R^2 = 0.9998$. 
Figure 4.12: Amylase, Cellulase and Xylanase activity in BSG fermented by *S. commune* at various incubation period

The same situation was also observed for cellulase activity in BSG fermented by *S. commune*. Cellulase activity increased slowly from 1<sup>st</sup> day (27.01 U/g) to 35.11 U/g to 4<sup>th</sup> day and slowly decreased after 4<sup>th</sup> day.

Xylanase showed 57% increase on 7<sup>th</sup> day (14 U/g) from 1<sup>st</sup> day (26 U/g) and declined to 34% on 10<sup>th</sup> day (27 U/g) compared to 7<sup>th</sup> day. *Schizophyllum commune* showed drastic increase in xylanase activity from day 3 to day 5 with correlation value $R^2 = 0.916$.

While for laccase, lignin peroxidase and xylanase, the activity was increasing from 1<sup>st</sup>
day to 7th day of fermentation period and decline after that. Laccase activity (Figure 4.13) in BSG fermented by *S. commune* showed 545% increase on 7th day (13,048 U/g) compared to the 1st day of fermentation (2022 U/g). *Schizophyllum commune* showed a steady linear increase in laccase activity with the incubation period (day 1-7) with the correlation value $R^2=0.9504$ before the activity drop after day 7. While for lignin peroxidase, the activity was observed 94% higher on 7th day (1449 U/g) compared to 1st day (747 U/g).

Figure 4.13: Laccase and lignin peroxidase activity in BSG fermented by *S. commune* over an incubation period

4.5.5 *Trichoderma* sp.

Amylase activity in BSG fermented by *Trichoderma* sp. showed higher activity on 7th
day (4.35 U/g) of fermentation period which is 24% higher than the 1st day (Figure 4.14). The activity was observed slightly decreased to 4.14 U/g on 10th day of fermentation period. *Trichoderma* sp. showed strong linear correlation of amylase activity with incubation period (from day 1 to day 7) with correlation value $R^2 = 0.9429$.

Cellulase activity in BSG fermented by *Trichoderma* sp. was observed at 24.32 U/g on the 1st day and increased to 37 U/g on 7th day, which is 52% higher than the 1st day. The activity was observed slightly decreased to 30.98 U/g on the 10th day of fermentation. Cellulase activity in *Trichoderma* sp. is proportional to the incubation period where it draw a strong linear correlation (from day1 to day 7) with the $R^2 = 0.977$.

![Figure 4.14: Amylase, Cellulase and Xylanase activity in BSG fermented by *Trichoderma* sp. at various incubation period](image)

Figure 4.14: Amylase, Cellulase and Xylanase activity in BSG fermented by *Trichoderma* sp. at various incubation period
Laccase activity in *Trichoderma* sp. was observed the highest among the enzymes tested (Figure 4.15). *Trichoderma* sp. showed peak activity on the 6th day of fermentation period. Laccase activity increased 260.26 % compared from the 1st day (3697.78 U/g) to 6th day (13848.89 U/g). The activity was observed declining after 6th day. *Trichoderma* sp. showed a steady linear increase in laccase activity with the incubation period (day 1-7) with the correlation value R^2 = 0.9407.

*Trichoderma* sp. showed a linear increase for xylanase activity from day 3 to day 7 (R^2 = 0.9424). Maximum activity was observed on the 7th day (57.16 U/g) and the activity decline 47 % on 10th day.

**Figure 4.15**: Laccase and lignin peroxidase activity in BSG fermented by *Trichoderma* sp. at various incubation period.
4.5.6 Discussion on the Effect of Incubation Period on Enzyme Activity

BSG fermented by all the fungi showed a proportional increase with incubation period. At the beginning of the fermentation, the enzyme activity (amylase, cellulase, laccase, lignin peroxidase and xylanase) was found lower, however, as the incubation period increased, the enzymes were started to increase as well. A slight fluctuation of enzyme activity at the beginning of process might due to fungal adaptation and growth. This was explained by Romero et al., (1999), where, during the fungal growth, lower enzyme activity will be obtained until the fungi reached the stationery phase where a sudden increase of enzyme activity will be observed. At the lower enzyme activity, fungi are predicted to decay the substrate and start to utilize the nutrient. According to Yamane et al., (2002), the decay of substrate and the increase of metabolites are propositional to the fungal growth time. While, according to Mussatto et al., (2006) the high sugar content and favorable moisture content encourage the microorganism to use the substrates and decay the materials.

This situation can be observed in all the fungi used in this study for all the enzyme activity. For example P. chrysogenum showed a very slow increase in laccase activity from day one to day six (5128.89 U/g to 8737.78 U/g) and a speed increase was observed at day 7 where the activity increased up to 15,413.33 U/g. The same situation can be observed in other fungi for all enzymes. Fungi need time to adapt to the substrate and environment and start to utilize the nutrients. Nutrient consumption also plays a role in the correlation between enzyme activity and fungal growth. Since, in this study, no additional nutrients were supplemented, the possibility of nutrient depletion is high. Nutrient depletion will stop the fungal growth, which eventually
will influence the enzyme activity as well. Since no nutrients are available, fungi cannot grow and conduct its metabolism (Murthy et al., 2009). Consequently, fungi will die out of nutrient, which will lead to a reduction in the enzyme activity.

There could also be another possibility, where, when the nutrients are depleted during the stationary phase, the fungi could have started to produce secondary metabolites which results in lower enzyme production (Ramachandran et al., 2004 and Sabu et al., 2005). Ramesh and Lonsane (1987) also mentioned that low enzyme activity beyond the optimum period, maybe because of the denaturation of enzymes which caused by the interaction of the enzymes with other components in the substrate. Studies by other researchers showed variation in the optimum incubation period for higher enzyme activities by various fungi. This is due to the nature and condition of fungi and substrate used for enzyme productions. Besides, Sandhya et al., (2005) mentioned that each fungal stain has own special condition for maximum enzyme production.

Among the fungi tested, *P. chrysogenum* showed highest lignin peroxidase activity (2024.94 U/g), followed by *Fusarium* sp. (1576.49 U/g), *A. niger* (1565.44 U/g), *Trichoderma* sp. (1456.6 U/g) and *S. commune* (1449.32 U/g). Lignin peroxidase activity in BSG fermented by *P. chrysogenum* was 28.45%-74.18% higher than in BSG fermented by other fungi.

Statistical analysis using SPSS (General Linear Model) was conducted to strengthen the lignin peroxidase results mentioned above. Turkey, Scheffe and Post Hoc tests were done at 95% Confidence Interval (CI). From the analysis, the following was
observed.

1. There was a significant difference in lignin peroxidase activity between *P. chrysogenum* and other fungi $F(5, 144)=227.006$ $p<0.05$

2. There was also a significant difference in lignin peroxidase activity between day 7 and other incubation periods $F(7,144)=417.696$, $p<0.05$

3. There was an interaction between fungi and incubation period, with more enzyme activity associated with *P. chrysogenum* in at seventh day fermentation compared to other fungi $F (35, 144) =34.28$, $p<0.05$

From the ANOVA result, the conclusion that could be derived is that both fungi and incubation period gave an impact on lignin peroxidase activity, since the significant value $p<0.55$. Among the fungi tested *P. chrysogenum* gave largest impact, based on the multiple comparisons done with this statistics. According to Fujian *et al*., (2001), lignin peroxidase activity was observed peak at 120 hrs in steam-exploded straw. While in Robinson *et al*., (2001), higher lignin peroxidase was discovered at $8^{th}$ day for *C. versicolor* and $9^{th}$ day for *P. ostreatus* in N-limiting Kirk’s medium. Ganesh Kumar *et al*., (2006) lignin peroxidase activity peaked at $7^{th}$ day of fermentation by *P. chrysosporium* in lignocellulosic material and Kanmani *et al*., (2009) found maximum activity on $28^{th}$ day with coir waste. Substrate pretreatment could be the reason for obtaining maximum activity earlier then others. High loss of lignin attribute to higher lignin peroxidase activity (Gupte *et al*., 2007). Lignin degradation is accompanied with the libration of $H_2O_2$ mediated by lignin peroxidase. The smaller substrate particle contributes in speeding up the lignin degradation, which eventually contribute
for higher lignin peroxidase production. As the fermentation period increased (after 7th day) the lignin peroxidase activity by all the fungi showed decreasing. This is due to the decrease of lignin component in the substrate. According to Ganesh Kumar et al., (2006) as the lignin degradation decrease, the lignin peroxidase activity also decreased.

*Penicillium chrysogenum* showed higher laccase activity (13,977 U/g), followed by *Trichoderma* sp. and *S. commune* with activity at 13,977.73 U/g and 13,266.67 U/g respectively. Results were strengthening with the statistical analysis done with ANOVA in SPSS with General Linear Model. The results showed that, laccase activity in *Trichoderma* is significantly different with other fungi F (5, 144) = 404.93, p<0.05. Seven day incubation period gave a significant F (7,144) = 448.19, p<0.05 compared to other incubation periods. Finally there is an interaction between fungi and incubation period with more laccase activity associated with *Trichoderma* sp at day 7 compared to other fungi F (35, 144) = 34.48, p<0.05.

Stajic et al., (2006) discovered higher laccase activity after 10th day with *P. ostreatus* and *P. pulmonarius* on grapevine sawdust. While Patel et al., (2009) showed maximum laccase activity in wheat straw on 8th day using *P. ostreatus* as well. On the other hand higher laccase was observed on 28th day with coir waste (Kanmani et al., 2009), on 18th day in barley straw and barley bran (Couto et al., 2002) and 20th day in banana peel (Osma et al., 2007). Gupte et al., (2007), higher laccase activity observed at the beginning of fermentation as the lignin degradation begun. However, as the lignin degradation increase and remain constant, the laccase activity decreased.
Besides, cellulose content in the substrates also can be act as an activator for laccase activity (Srinivasan et al., 1995). Increasing in laccase activity indicates the establishment of fungal mycelium on the substrate, nutrient utilization and conversion. While the drop of activity indicates maximal growth was achieved, nutrient depletion, secondary metabolites production and fungal death (Reddy et al., 2003). Highest xylanase activity was observed in A. niger (82.85 U/g) followed by Fusarium sp. (58.24 U/g) and Trichoderma sp. (57.16 U/g) on the 7th day of fermentation.

In a study conducted by Okafor et al., (2007), P. chrysogenum showed higher xylanase activity at 4th day of fermentation in wheat bran. While A. niger also showed maximum xylanase activity after 4th day in palm kernel cake (Sabu et al., 2005). Xylanase activity in all the fungi showed decrease after day seven. White rot fungi has high ability in lignin degradation which is coupled with high amount of xylanase production. High hemicelluloses content also contribute for higher xylanase production (Kanmani et al., 2009). Higher laccase activity was observed on 6th day in wheat straw and wheat bran by Muthezhilan et al., (2007) and Azin et al., (2007) respectively and on 2nd day in rice bran (Virupakshi et al., 2005).

Maximum, xylanase activity observed are varied at different lignocellulosic materials. The variation of sugar composition and the lignin content of the substrate give different level of xylanase activity (Virupakshi et al., 2005). As the incubation period increase, the xylanase activity also decrease. This is due to the reduction of nutrient level in the medium, inhibition and denaturation of enzymes (Sabu et al., 2005 and Okafor et al., 2007).
Trichoderma sp. showed highest cellulase activity (37 U/g), followed by Fusarium sp. (33.21 U/g) and S. commune (32.67 U/g). While A. niger (30.44 U/g) and P. chrysogenum (30.49 U/g) showed lowest cellulase activity among the fungi tested. All fungi showed higher cellulase activity on the 7th day except for S. commune (35.11 U/g) on 4th day and A. niger (31.01 U/g) on 5th day. According to Raimbault, (1980), T. harzianum just need four days for maximum cellulase activity in SCB. While A. niger in wheat bran showed maximum activity on 4th day (Jecu 2000) and 3rd day in maize (Milala et al., 2008). Berka et al., (1992) A. niger is a good cellulase producer.

As the incubation period increased, the cellulase activity was observed decreasing. This is maybe due to the decrease of moisture content in the substrate, which eventually leads to lower cellulase activity (Milala et al., 2008). Other studies also discover same situation, where enzyme activity propositional to incubation period (Jecu 2000; Raimbault, 1980 and Kanmani et al., 2009). Cellulase activity in corn cob was maximum on 5th day (Xia and Chen 1999) and 28th day on coir was (Kanmani et al., 2009). Cellulase production was essential for efficient degradation of cellulose.

Aspergillus niger showed higher maximum amylase activity (4.64 U/g) at seven day incubation period, followed by Fusarium sp. (4.61 U/g), Trichoderma sp. (4.35 U/g), P. chrysogenum (4.10 U/g) and S. commune (3.81 U/g). All fungi showed maximum amylase activity at day seven. Higher amylase activity (291 U/g) observed using Gibberella fujikuroi (Mulimani et al., 2000), (262 U/g) in T. lanuginosus with wheat bran (Kunamneni et al., 2005) and A. niger in wheat bran (Chimata et al., 2010) on 5th day. While, a study conducted by Ramachandran et al., (2004) showed very high
amylase activity (1827 U/g) was obtained at 3\textsuperscript{rd} day of fermentation using coconut oil cake (COC) with \textit{A. oryzae}. This activity is far higher than the amylase activity observed in this study with \textit{A. niger}. However, this can be explained by looking at the substrate used in the SSF process. COC used in this study is high in starchy component which eventually lead to higher amylase production. While the starchy component or specifically sugar component in BSG has been removed during the brewing process which gave low amylase activity in this study.

Based on the results from ANOVA in SPSS software, using the General Linear Model, \textit{Fusarium} sp showed significant difference in amylase activity compared to other fungi \(F(5, 144) = 1981.002, p<0.05\) and incubating at day seven also gave a significant different in amylase activity compared to other incubation periods \(F(7,144) = 2400.164, p<0.05\). Therefore there is an interaction between fungi and incubation period with more enzyme activity (amylase) was observed with \textit{Fusarium} at 7\textsuperscript{th} day compared to other fungi. However, when the amylase activity compared with other enzymes in BSG, it was observed very low. \textit{P. chrysogenum} which gave highest amylase activity (4.63 U/g) showed 9337.78 U/g of laccase activity. This is due to the fact that, the concentration of starchy components in BSG is very low compared to cellulose and lignin component. This is because, during the malting process, \(\alpha\)-amylase as well as \(\beta\)-glucanase are synthesized (enzymatic hydrolysis), which eventually break down the starch and \(\beta\)-glucan. This is the reason for low concentration of starch in BSG (Serene and Batch Knudsen 2007).
4.6 EFFECT OF INCUBATION TEMPERATURE ON ENZYME ACTIVITY

Each fungal species has its own ability in adapting to different temperature and produce different level of enzyme. Five incubation period ranged from 20°C to 40°C was used to test the optimum temperature that yield higher enzyme.

4.6.1 Laccase and Lignin Peroxidase Activity

Figures 4.16 to 4.20 represent the effect of incubation temperature in enzyme production. Almost all the fungi showed maximum enzyme activity at 30°C except for amylase. *Penicillium chrysogenum* showed highest lignin peroxidase (Figure 4.16) activity and laccase (Figure 4.17) activity at 30°C with 2022 U/g and 16,404 U/g respectively. *Penicillium chrysogenum* showed almost 176% to 458% higher laccase activity and 67% to 162 % higher lignin peroxidase activity at 30°C compared to other incubation temperatures (20°C, 25°C, 35°C and 40°C). At higher temperature, the spore germination, growth, product formation and sporulation been affected (Chen et al., 2005). Therefore low enzyme activity can be observed at higher temperature.

*Penicillium chrysogenum* showed higher laccase and lignin peroxidase activity since it is more capable of utilizing the plant component than the other fungi. *Trichoderma* sp and *S. commune* also showed higher laccase activity after *P. chrysogenum*, 13,211.11 U/g and 13,552.2 U/g respectively. While *Fusarium* sp. showed higher lignin peroxidase activity after *P. chrysogenum* (1713.92 U/g). According to Fujian et al., (2001), the optimum temperature for lignin peroxidase production was 39°C for steam-exploded straw. Based on the results from statistical analysis, ANOVA in SPSS
Lignin peroxidase activity:

(i) A significant difference was obtained in lignin peroxidase activity between \(P.\ chrysogenum\) and other fungi \(F(5, 90) = 7.537, p<0.05\). 

(ii) Also a significant difference between 30\(^\circ\)C and other incubation temperature \(F(4,90) = 333.552, p<0.05\)

(iii) There was an interaction between fungi and incubation temperature, with more lignin peroxidase activity observed in \(P.\ chrysogenum\) at 30\(^\circ\)C compared to other fungi \(F(20, 90) = 11.33, p < 0.05\).

**Figure 4.16:** Lignin peroxidase activity of various fungi grown on BSG at different incubation period
Laccase activity

(i) Significant difference in Lac activity between *P. chrysogenum* and other fungi $F(5, 90) = 90.849, p < 0.05$

(ii) Significant difference in Lac activity between 30°C and other incubation temperature $F(4, 90) = 1246.065, p < 0.05$ and

(iii) There is an interaction between fungi (*P. chrysogenum*) and incubation temperature (30°C), $F(20, 90) = 38.683, p < 0.05$.

According to Vasudev et al., (2005), highest laccase activity was observed at 30°C for *Cyathus bulleri*, while Patel et al., (2009) found highest laccase activity at 28°C for *Pleurotus ostreatus* and no considerable activity observed at 20, 40 and 50°C. This is due to the respiration process occurred during the fermentation process. The respiration process increase the temperature in fermentation medium, where at high temperature, fungus could not further the metabolism process. Besides, at high temperature, the substrate tends to lose its moisture content and become dry. This will become an obstacle for the fungal growth and metabolism, which eventually leads to lower enzyme production (Niladevi et al., 2007 and Patel et al., 2009). The optimum temperature for laccase activity is 25°C to 30°C (Pointing, 2001 and Arora et al., 2002). However, Farnet et al., (2000) mentioned that the optimum temperature for laccase activity is differing greatly, depending on the strain used. While Zadrazil et al., (1999) showed that temperature more than 30°C cause the ligninolytic activity which leads to the reduction of enzyme activity (Adejoye et al., 2007).
Figure 4.17: Laccase activity of various fungi grown on BSG at different incubation periods

4.6.2 Xylanase Activity

Aspergillus niger showed highest xylanase activity (105.33 U/g) at 35°C, which is 19% to 265% higher than 20°C, 25°C, and 30°C (Figure 4.18). However, xylanase activity at 35°C (105.33 U/g) and at 40°C (105.06 U/g) is almost similar. This is because A. niger is capable of growing at higher temperatures up to 50°C. According to Virupakshi et al., (2005), Bacillus sp. produced higher xylanase activity at 50°C using rice bran as a substrate in SSF. Besides A. niger, P. chrysogenum and S. commune also showed higher xylanase activity at 35°C (67.24 U/g and 46.60 U/g respectively). However, according to Adejoye et al., (2007), the optimum temperature for S. commune is 25°C, where it grows best at this temperature with higher enzyme
yield. At 30°C, the growth was considerable and at 40°C, the growth was minimal. While other fungi showed higher xylanase activity at 30°C. *Penicillium chrysogenum* showed second higher xylanase activity.

![Figure 4.18: Xylanase activity of various fungi grown on BSG at different incubation period](image)

Statistics showed that there is significant difference in xylanase activity between *A. niger* and other fungi $F(5, 90) = 231.898$, $p < 0.05$, and significant difference between 35°C and other incubation period, $F (4, 90) = 404.884$, $p < 0.05$. Also there is an interaction between fungi and incubation temperature $F (20, 90) = 49.937$, $p < 0.05$. 
4.6.3 Cellulase Activity

*Penicillium chrysogenum* showed slightly higher cellulase activity (40.06 U/g) at 35°C compared to other fungi at the same temperature (Figure 4.19). While the other fungi showed maxima amylase activity at 30°C. *Aspergillus niger* grown on wheat bran showed higher cellulase activity at 30°C and the activity decreased as the temperature increased (Jecu 2000). Higher cellulase activity was observed at 25°C in soybean hull using *Phanerochaete* (Jha et al., 1995). Elshafei et al., (1990) and Duff et al., (1987) also showed the optimum temperature for cellulase production was 25°C. The enzyme activity increase as far as the microorganism can sustain the increase of temperature. Beyond the capability of the fungi, the temperature tends to act as a deactivation factor for enzyme production.

![Figure 4.19: Cellulase activity of various fungi grown on BSG at different incubation period](image)

Figure 4.19: Cellulase activity of various fungi grown on BSG at different incubation period
Cellulase activity in BSG fermented by various fungi at 30°C ranged from 31.60 U/g to 39.00 U/g. Statistical analysis strengthen the results where it shows a significant difference between *P. chrysogenum* and other fungi $F(5,90) = 19.496, p < 0.05$, and a significant difference between 30°C and other incubation temperature $F(4, 90) = 278.221, p < 0.05$. Therefore there was an interaction between fungi (*P. chrysogenum*) and incubation temperature (30°C) $F(20, 90) = 21.061, p < 0.05$, where, more cellulase activity was observed at this interaction compared to other fungi. However, *P. chrysogenum* showed lowered penicillin production at 30°C.

According to Vinoth Kumar *et al.*, (2010), at 30°C the biomass of *P. chrysogenum* increased, lowered the moisture content and yield lower penicillin. Besides, this organism also showed slow growth at lower temperature and the optimum temperature for *P. chrysogenum* for higher penicillin is at 26°C.

### 4.6.4 Amylase Activity

All the fungi showed maximum amylase activity at 35°C (Figure 4.20). Among the fungi, *A. niger* and *P. chrysogenum* showed the highest amylase activity, 9.17 U/g, which is almost double than the amylase activity observed at 30°C in both fungi. General Linear Model in ANOVA, showed that there was a significant difference in amylase activity between *A. niger* and other fungi $F(5,90) = 4.412, p < 0.05$, and significant difference between 35°C and other incubation temperature $F(4, 90) = 952.913, p < 0.05$. An interaction was observed between fungi and incubation temperature, with more activity associated with *A. niger* at 35°C compared to other fungi $F(20, 90) = 11.33, P < 0.05$.  

142
Sindhu et al., (2009) also agreed that 35°C was the optimum temperature for *Penicillium janthinellum* to produce higher amylase activity and the activity decline after 45°C. While Chimata et al., (2010), showed an *Aspergillus* strain showed maximum amylase activity observed at 30°C when used wheat bran as a substrate. On the other hand, Ellaiah et al., (2002), mentioned the optimal temperature for amylase activity was 50°C. This is also supported by Kunamneni et al., (2005), where higher amylase activity was observed at 50°C (263 U/g) when *T. lanuginosus* organism was used in SSF process. Different strains showed maximum amylase activity at different temperature, when range of different substrates utilized. Highest enzymes activity not only considers the temperature of the fermentation process, but also considering the
strain and substrate used in the process, since different organism has different optimizing level.

4.6.5 Discussion on the Effect of incubation temperature on enzyme activity

Almost all the filamentous fungi can grow over a wide range of temperature from 20°C to 55°C and the optimum temperature for fungal growth could be different from product formation (Kansoh et al., 1999 and Gowthaman et al., 2001). Fungi used in this study can grow from 20°C to 40°C. However, they have different affinity towards different enzyme production. For example, all fungi showed higher lignin peroxidase and laccase activity at 30°C and high amylase activity at 35°C.

While Ellaiyah et al., (2002) showed that the optimal temperature for amylase activity was 50°C, which gives 263 U/g. Many studies proved 30°C is the best temperature for enzyme synthesis in SSF process (Alva et al., 2007; Mulimani et al., 2000). Besides, Ano et al., (2009) also mentioned, SSF process should be maintained at or below 30°C, so that the heat accumulated as the temperature increase would affect the enzyme yield. Increasing the incubation temperature will also affect the fungal growth which was almost 3-fold less at 45°C than at 30°C (Ramachandran et al., 2004).

Adding to this, Pandey, (1990) mentioned that large amount of heat is generated in SSF directly propositional to the metabolites activities of microorganism. At the initial stage of fermentation, the temperature is same at all location of the bed. However, as the fermentation progresses, librating metabolic activity is not equally disperse due to poor thermal conductivity of substrate (Chen et al., 2005). Therefore a temperature
gradient is formed, which will eventually affect the further fungal growth and enzyme activity.

4.7 EFFECT OF MIXED SUBSTRATE ON ENZYME ACTIVITY

Lignin peroxidase, laccase, xylanase, cellulase and amylase activity was studied in the fermentation medium containing BSG with different percentage (10 % - 50 %) of SMC and SCB. The enzyme activity observed in mixed substrate was also compared with the enzyme activity observed in single substrates.

4.7.1 Lignin peroxidase activity

*Penicillium chrysogenum* and *Trichoderma* sp. generated higher lignin peroxidase activity (Figure 4.21) in BSG with SCB combination, which is 15-85 % higher than BSG alone. BSG with 10 % - 40 % SCB fermented with *P. chrysogenum* showed higher lignin peroxidase activity (1037.61 U/g – 1066.17 U/g) which was 15 % - 20 % higher than BSG alone and 11 % - 14 % higher than SCB alone. While for BSG with SMC combination, *Trichoderma* strain showed highest lignin peroxidase activity which is 95 %-127 % higher than BSG alone and 6 % to 14 % higher than SMC alone (Figure 4.22). *Shizophyllum commune* also showed higher lignin peroxidase activity (1042.26 U/g to 1044.62 U/g) and showed the maxima at 7:3 ratios of BSG and SMC, which is 46.6 % higher than the activity observed in dried BSG. However, this activity is still lower (27.5 %) than activity observed in SMC alone. While *A. niger* showed higher activity at 9:1 and 8:2 ratio, (BSG:SMC)
which is 42 % - 46 % higher than BSG alone. Again, it is still lower than the activity observed in SMC alone. *Aspergillus niger* in 35 % of SMC showed highest amino acid production (Zhang *et al*., 1995).

Lignin peroxidase activity in BSG supplemented with SMC is higher than BSG alone, since the activity was compared with dried BSG. Dried BSG showed lower enzyme activity compared to fresh BSG. Beside, for the organic supplementation, dried BSG was mixed with different percentage of other substrates (dried also).

**Figure 4.21**: Lignin peroxidase activity of various fungi grown on BSG mixed with different percentage of SCB
Therefore, the enzyme activity was lower than the fresh substrate. When lignin peroxidase activity compared between BSG supplemented with SMC and BSG supplemented with SCB, higher activity was observed in BSG supplemented with 30% of SMC fermented by *Trichoderma* sp.

![Figure 4.22](image)

**Figure 4.22:** Lignin peroxidase activity of various fungi grown on BSG mixed with different percentage of SMC

Besides, *P. chrysogenum*, *S. commune*, *Fusarium* sp. and *Trichoderma* sp showed higher lignin peroxidase activity in BSG supplemented with 10% to 50% of SMC and *A. niger* showed higher activity in BSG supplemented with 10% of SMC. Overall, the lignin peroxidase activity observed in BSG supplemented with SCB showed lower
than lignin peroxidase activity observed in BSG supplemented with SMC. A study conducted by Fujian et al., (2001), higher lignin peroxidase activity was obtained from the combination of steam-exploded straw with wheat bran with ration 5:2. Lignin peroxidase activity at this combination was higher compared to wheat straw and steam-exploded straw alone.

### 4.7.2 Laccase activity

In BSG supplemented with SCB (Figure 4.23), *P. chrysogenum* showed higher laccase (4720 U/g) activity in the combination of 30 % of SCB and 70 % of BSG, which are 49 % higher than BSG alone and 144.5 % higher than SCB alone. Laccase activity is slightly decreases as the percentage of SCB increased in fermentation medium. However, the decrease is not that noticeable. While in *P. chrysogenum*, highest laccase activity (4720 U/g) was recorded at 30 % SCB supplementation; however the activity decreases to 2166.67 U/g at 50 % of SCB supplementation. *Schizophyllum commune* also showed higher laccase activity (3606.67 U/g) on 30 % SCB supplementation.

While, *Fusarium* showed an increase in laccase activity as the percentage of SCB increased into the fermentation medium. Whereas in BSG supplemented with different percentage of SCB, *Trichoderma* sp. showed almost similar activity. However, the activity observed in all fungi with SCB supplementation, except for *P. chrysogenum* (3:7 ratio supplementation) showed lower than the activity observed in BSG alone and SCB alone. Adding to this, *P. chrysogenum* in 50 % of SCB supplemented with carbon source yield higher penicillin compared to 100 % SCB.
(Vinoth Kumar et al., 2010). However, laccase activity in the mixture of kiwi peeling alone (100 %) yield higher activity (almost 2.5 fold) compared to the mixture of kiwi pulp and kiwi peeling (Rosales et al., 2005).

**Figure 4.23**: Laccase activity of various fungi grown on BSG mixed with different percentage of SCB

*Trichoderma* strain showed higher laccase activity (12.5 % to 45 %) higher than activity in BSG supplemented with 10 % to 50 % SMC compared to BSG alone (Figure 4.24). However, this activity is still lower compared to laccase activity observed in SMC alone (6568.07U/g). *Aspergillus niger*, *Fusarium* sp. *S. commune* and *P. chrysogenum* showed lower laccase activity compared to dried BSG alone and SMC alone.
4.7.3 Xylanase activity

Among the fungi tested, *A. niger* showed the highest xylanase activity (53.38 U/g and 56.99 U/g) at 10 % and 20 % of SCB supplementation (Figure 4.25). This activity is only 2 % to 3 % higher than the activity observed in BSG alone and 50 % to 75 % higher than the activity observed in SCB alone. *Trichoderma* sp. showed the least xylanase activity, which is ranged from 27.20 U/g to 37.40 U/g.
However, *Trichoderma* showed an increase in xylanase activity as the percentage of SCB increased in the medium. Besides, *S. commune* also showed an increase in xylanase activity as the percentage of SCB increased with relatively low increase. However, overall xylanase activity observed in SCB supplementation was lower than the xylanase activity observed in BSG alone.

For BSG supplemented with SMC, *A. niger* showed higher xylanase activity (Figure 4.26). Xylanase activity in BSG with SMC, fermented by *A. niger* increased from 50.62 U/g (10 %) to 76.28 U/g (40 %). The activity was optimum at 40 % supplementation and then the activity started to decrease to 53.72 U/g at 50 % of
supplementation. Whereas, other fungi showed smaller enzyme increasing pattern at all the percentage of SMC.

**Figure 4.26:** Xylanase activity of various fungi grown on BSG mixed with different percentage of SMC

In a study conducted by Azin et al., (2007) combination of wheat bran and wheat straw with the ration 7:3 gave highest xylanase production, up to 14.9% increase. Insufficient N level in the mixture of substrate could be the reason for lower enzyme activity, where little hyphae accumulation can be observed during the fermentation process.

The lower N level could inhibit the formation of hyphal ramification (Rossi et al.,
2003). However, Maziero (1990) large amount of N level will reduce the lignin degradation in the SCB, which ultimately contribute for higher enzyme production. A study conducted by Rossi et al., (2003) showed, there is no effect on mixing the SCB and molasses for higher enzyme activity. However, sawdust with 20 % rice bran showed better fungal growth and higher enzyme activity (Teixeira 1996). While Song et al., (1987), better mycelia growth was observed at 30 g molasses alone compared to substrate mixture. Wheat bran with the combination of citrus pulp with the ration 5:5 showed highest xylanase activity. However, wheat bran with other combinations, including maize barn, rice husk, rice bran and gluten did not support good enzyme titer (Kaur and Sathiyanarayana 2004).

4.7.4 Cellulase activity

Overall cellulase activity (Figure 4.27) in BSG supplemented with different percentage of SCB fermented by all fungi showed similar. Aspergillus niger showed slightly higher cellulase activity (22.89 U/g and 22.29 U/g at 30 % and 50 % of SCB supplementation. Fusarium, and P.chrysogenum also showed higher cellulase activity (24.63 U/g, and 24.16 U/g respectively in 20 % of SCB supplementation.

In BSG supplemented with SMC (Figure 4.28), Trichoderma sp. showed higher cellulase activity in 10 % of SMC supplementation (34.74 U/g). This activity is 40 % and 42 % higher than the activity observed in SMC and BSG respectively. While, S. commune, A. niger and P. chrysogenum showed higher cellulase activity in 10 % of SMC. However, they are lower than BSG and SMC and activity in these fungi decreasing as the percentage of SMC increased. A study conducted by Cha et al.,
(2010), cellulase and xylanase activity in at 100 % rice bran was higher than in the combination of rice bran and soybean meal. This is due to gas exchange capability in the substrate.

Figure 4.27: Cellulase activity of various fungi grown on BSG mixed with different percentage of SCB

Larger particle size of substrate allows higher oxygen and lower CO\textsubscript{2} level in the fermentation bed. When substrates with different particle size mixed, two possible situations can be created, either with higher O\textsubscript{2} lever or higher CO\textsubscript{2} level. Presence of O\textsubscript{2} will stimulate the fungal growth, while CO\textsubscript{2} will inhibit the fungal growth (Raimbault, 1998).
The substrate (BSG or SCB or SMC) loses its exchange capability when another portion of lignocellulosic materials (carbon source) added, due to less empty spaces between the substrate particles. This ultimately will reduce the degrading activity of the fungus and the capability of gas exchange of the substrate and reducing mycelial growth rate (Rossi et al., 2003). Capacity of gas exchange of the substrate impaired with lack of nutrients.

This could be a possible explanation for the observation in this study, where lower enzyme activity observed in the combination of substrate compared to single substrate.
Besides, C/N level in the mixed substrate could be a reason for lower enzyme activity. When large amount of rice bran added into bagasse, the C/N ratio of the medium decreased. The mycelial growth also decreased significantly with increasing proposition of rice bran, where it inhibit the fungal growth (Rossi et al., 2003). SCB used in this study contain very low C/N ratio (8.9:1).

4.7.5 Amylase activity

Amylase activity (Figure 4.29) in BSG with different percentage of SCB is relatively very low since and it is just slightly higher than the control. Trichoderma sp. showed higher amylase activity (3.76 U/g) at 30% of SCB.

![Figure 4.29: Amylase activity of various fungi grown on BSG mixed with different percentage of SCB](image-url)
While in BSG supplemented with SMC (Figure 4.30), *P. chrysogenum* and *S. commune* showed slightly higher amylase activity at 10% of supplementation with 1 % - 10 % and 0.5 % to 11 % higher than BSG and SMC respectively. A study conducted by Mrudula and Kokila (2010), combination of wheat bran and gingerly oil cake (GIOC) showed great amylase activity (3120 U/g DBB) however, some combination such as GIOC and groundnut oil cake gave very poor enzyme activity. The organic supplementation (both SCB and SMC) not much helped in increasing the enzyme yield, as the enzyme activity in single substrate was much higher then the supplementation. The physical and chemical properties of SCB and SMC could attribute to this situation. SCB has high moisture content (85 %).

Figure 4.30: Amylase activity of various fungi grown on BSG mixed with different percentage of SMC
According to Orzua et al., (2009) the optimum moisture content for SSF is 30-80 %.
According to Botella et al. (2007), low enzyme activities are expected at high substrate moisture levels. This could be attributed to the decreased porosity, alteration in particle structure, gummy texture, lower oxygen transfer or increased formation of aerial hyphae. Lower moisture levels lead to reduced diffusion of the nutrients in the solid substrate, lower degree of swelling and higher water tension.

Apart from that, smaller substrate particle would provide large surface area for microbial metabolism, which is a desirable factor in enzyme production. However, too small substrate particle will lead to substrate agglomeration, which may interfere with microbial respiration/aeration, and result in poor cellular growth (Pandey et al., 2000).

The used compost (SMC) losses half of its weight, decreases nitrogen level to below 1.5 %, and has high level of porosity (Polat et al., 2009). Since it losses it weigh, the moisture level become low. Thus, this gives an unfavorable condition for the fungus to habitat there and tent to die with insufficient moisture for the cell growth and metabolic biosynthesis (Milagres et al., 2004). This gives the low enzyme production in BSG supplemented with SMC. Osma et al (2007) reported that particle size, porosity and chemical composition of the substrates play crucial role in higher enzyme production.

According to Gregori et al., (2008), BSG favors the growth of oyster mushroom due to the protein content, moisture content and physical properties such as size, volume weight, specific density, porosity and water holding capacity. This factor contributes
to high enzyme yield in BSG compare to the other substrates. SCB has low water holding capacity compared to other substrates. Besides, the shapes of solid particle, hydrophilic and hydrophobic nature of solid particles are the crucial factor for the adherence of fungus to solid substrate (Mulimani et al., 2000).

4.8 Enzyme activity comparison in BSG supplemented with chemicals

Trace elements have effect on inducing enzyme activity. Therefore four types of chemicals (CoCl$_2$, CuSO$_4$, MnSO$_4$ and FeSO$_4$) at different concentration used to measure the effectiveness of these chemicals in inducing the enzyme activities. High commercial valued ascomycete (A. niger) (Lubertozzi and Keasling, 2009) and good wood degrading basidiomycete (S. commune) (Oku et al., 1993) were cultivated on chemically supplemented BSG. Enzyme activities in BSG fermented by different fungal phylum were compared in the presence of chemicals.

4.8.1 Enzyme activity in BSG cultivated with A. niger

4.8.1.1 Amylase activity

Amylase activity in BSG fermented by A. niger showed 272 % to 433 % increase in the presence of chemicals compared to without chemicals (Figure 4.31). Among the chemicals used, CoCl$_2$ showed higher amylase activity at all the concentrations (16.09 U/g – 21.16 U/g) and the highest was observed at 10 g/l (1.0 % concentration). CuSO$_4$ and MnSO$_4$ showed highest amylase activity at 1.0 % concentration (10 g/l) at 17.07 U/g and 17.33 U/g respectively. According to a study conducted by Mrudula and Kokila, (2010), the presence of CoCl$_2$ and MnSO$_4$ showed higher amylase activity
compared to without these chemicals. This is possibly due to the least inhibitory effect found in ion Mn$^{2+}$ (Deng and Tabatabai, 1995).

![Figure 4.31: Amylase, cellulase and xylanase activity in chemically supplemented BSG, fermented by A. niger](image)

Amylase activity in the presence of FeSO$_4$ was increased gradually as the concentration of this chemical increased from 2 g/l (14.77 U/g) to 8 g/l (19.06 U/g) and drop at 10 g/l (15.74 U/g). Similar result was observed in a study conducted by Shankaranand and Lonsam, (1994), where citric acid production was propositional to iron concentration up to a limit. This is because iron in trace amounts; serve as growth factor in the early stages of fermentation however, excess amount of this chemical can
inhibit the fungal growth (Noyes, 1969) and eventually can reduce the enzyme production and activity.

4.8.1.2 Cellulase activity

Cellulase activity in chemically supplemented BSG, fermented by *A. niger* ranged from 32.87 U/g to 42.78 U/g. Cellulase activity under the influence of these chemicals showed 10% to 46% increase compared to without chemical induction. CoCl₂ showed 13% to 46% higher cellulase activity, followed by FeSO₄ and MnSO₄ (13% to 35%) and CuSO₄ at 10% to 21% increase. Among the chemicals used, COCl₂ at 0.8%, showed highest cellulase activity at 42.78 U/g, followed by 2 g/l FeSO₄ and 4 g/l MnSO₄ at 39.88 U/g respectively. For MnSO₄ and FeSO₄, higher cellulase activity was observed at lower concentration. As the concentration of these chemicals increased, the cellulase activity was observed decreased. Trace amount of chemicals support the enzyme activity, however, excess amount will inhibit the fungal growth and enzyme activity (Kapoor et al., 1982). While for CoCl₂ and CuSO₄, cellulase activity was propositional to the chemical concentration. Overall in this study, the cellulase activity in the presence of chemicals had induced the activity when compared to without chemicals. However, a study conducted by Cha et al., (2010), showed that there was no effect on cellulase activity in the presence of chemicals (92.4 U/ml) compared to without chemicals (93.4 U/ml) when rice bran was cultivated with *A. niger*.

Statistical analysis showed there was a significant difference between fungi *F* (1,120) = 987.035, *p* < 0.05 and between 10 g/l of MnSO₄ and other chemical concentrations
Xylanase activity in chemically supplemented BSG cultivated with A. niger, ranged from 92 U/g to 126 U/g, which is 69% to 130% higher than without chemicals. Xylanase activity in CoCl$_2$ was higher at lower concentration (2 g/l and 4 g/l), and started to decrease from 6 g/l and 8 g/l at 98 U/g and 97.7 U/g respectively. However, xylanase activity shoots up to 125.9 U/g when the concentration was increased to 10 g/l. Similar condition was also observed under the influence of MnSO$_4$. However, for FeSO$_4$, xylanase activity was increasing slowly as the concentration was increased from 2 g/l to 8 g/l (97.7 U/g to 125.2 U/g) and decrease drastically to 99.8 U/g at 10 g/l concentration. Among the chemicals tested, 10 g/l CoCl$_2$ and 8 g/l FeSO$_4$ showed highest xylanase activity at 125.89 U/g and 125.23 U/g respectively.

Statistical analysis showed there was a significant difference between fungi $F$ (1,120) = 1790.830, $p < 0.05$ and between 8 g/l of FeSO$_4$ and other chemical concentrations $F$ (19, 120) = 9.9869, $p < 0.05$. Also an interaction was observed between fungi and chemical component in laccase activity $F$ (19,120) = 6.428, $p < 0.05$.

**4.8.1.4 Laccase Activity**

Unlike other enzymes, A. niger showed lower laccase activity in BSG supplemented by chemicals compared to BSG without chemical supplementation (Figure 4.32). Laccase activity in chemically induced BSG ranged from 2106.7 U/g to 5800 U/g.
which is 40% to 77% lower than laccase activity in the absence of chemicals (9327 U/g). However, among the chemicals tested, 0.8% (8 g/l) of CoCl₂ showed highest laccase activity. Laccase activity is compared among the chemicals used, only CoCl₂ showed gradual increase from 2600 U/g at 2 g/l to 5800 U/g at 8 g/l. Laccase activity in other chemicals was decreasing as the concentration of the chemical increased.

![Graph showing enzyme activity](image)

**Figure 4.32**: Laccase and lignin peroxidase activity in chemically supplemented BSG, fermented by *A. niger*

In most cases, ion Cu²⁺ which is the micronutrient considered as a good laccase inducer (Collins and Dobson, 1997; Palmieri *et al.*, 2000; Baldrain and Gabriel, 2002). However, in this study, BSG with CuSO₄, fermented by *A. niger* showed lower laccase
activity compared to BSG without this chemical.

According to Gomez et al., (2005), presence of CuSO$_4$ in barley bran cultivated with *Corilopis rigida* showed 2-fold higher laccase activity compared to without this chemical. While Tychanowic et al., (2006) showed 25 mM CuSO$_4$ showed 8-fold higher laccase activity while 40 mM of CuSO$_4$ showed lower laccase activity compared to control (without CuSO$_4$).

No fungal growth was observed at high 40 mM, thus no enzyme activity. In another study, *P. ostreatus* showed eight fold higher laccase activity in the presence of 1 mM CuSO$_4$ (Baldrian and Gabriel 2002). Study conducted by Patel et al., (2009) showed, presence of 0.28 mM CuSO$_4$ showed almost four times higher laccase activity (14,189 U/g) compared to without this trace elements.

According to Patel et al., (2009), high laccase production in the presence CuSO$_4$ is due to the defense mechanism against the oxidative stress, where laccase involves in the synthesis of pigments to prevent the uptake of metals. While the high concentration will cause toxicity for the microbial cells which will eventually will prevent fungal growth and reduce the laccase production, which is called as inhibitory effects (Patel et al., 2009 and Tychanowic et al., 2006).

In a study conducted by Mishra and Kumar (2007) showed laccase activity without CuSO$_4$ is much higher compared to with CuSO$_4$. However, adding the CuSO$_4$ on the day 3 of fermentation yield higher laccase compared to the first day of fermentation.
Less cell growth was observed when Cu$^{2+}$ was incorporating at early stage. While incorporating this chemical at third day enhance to fungal growth and enzyme production. This is maybe; fungi are already adapting to the substrate environment and ready to receive the supplement. In this case, CuSO$_4$ act as micronutrient on laccase production, rather than inducer.

Statistical analysis showed there was a significant difference between fungi $F(1,120) = 582.550$, $p < 0.05$ and between 6 g/l of MnSO$_4$ and other chemical concentrations $F(19, 120) = 20.999$, $p < 0.05$. Also an interaction was observed between fungi and chemical component in laccase activity $F(19,120) = 19.223$, $p < 0.05$.

4.8.1.5 Lignin Peroxidase Activity

BSG fermented by A. niger showed 61 % to 105 % higher lignin peroxidase activity in the presence of four types of chemicals at different percentage compared to without chemical influence. Among the chemicals tested, 4 g/l CuSO$_4$ and 2 g/l CoCl$_2$ showed higher lignin peroxidase activity at 1513 U/g and 1505 U/g respectively. However, for CoCl$_2$ and CuSO$_4$, the enzyme activity was observed decreasing as the concentration of these chemical increased. Same condition was also observed in BSG supplemented with FeSO$_4$ and MnSO$_4$. 
4.8.2 Enzyme activity in BSG cultivated with *S. commune*

4.8.2.1 Amylase Activity

Amylase activity in BSG cultivated with *S. commune* (Figure 4.33) in the presence of chemicals ranged from 11.73 U/g to 15.31 U/g, which is 219 % to 285 % higher than amylase activity observed in BSG without chemical supplementation. Among the chemicals tested, MnSO$_4$ at 6 g/l and 8 g/l showed maximum amylase activity at 15.31 U/g and 15.03 U/g respectively.

![Figure 4.33: Amylase, cellulase and xylanase activity in chemically supplemented BSG fermented with *S. commune*](image-url)
According to Kadrekar and Ramasarma, (1990) Mn$^{2+}$ favors the amylase activity, where higher amylase activity can be observed in the presence of this chemical. Amylase activity in the presence of CoCl$_2$, increased gradually as the concentration increased from 2 g/l (12.78 U/g) to 10 g/l (14.14 U/g). However, for CuSO$_4$ and FeSO$_4$, a slight decrease in enzyme activity was observed as the concentration of these chemicals increased.

Based on the result from statistical analysis, using ANOVA, there was a significant difference observed among the fungi used $F(1,120) = 744.826$, $p < 0.05$ and significant difference between 10 g/l of CoCl$_2$ with other chemical concentrations $F(19,120) = 10.525$, $p < 0.05$. There was an interaction between fungi and chemical concentration $F(19, 120) = 9.111p < 0.05$.

4.8.2.2 Cellulase Activity

MnSO$_2$ at 6 g/l showed maximum cellulase activity (58.85 U/g) in BSG fermented by S. commune (Figure 4.33). The activity observed was 136% higher than the activity observed in BSG fermented by S. commune without chemical supplementation. This was supported by the study conducted by Sinegani and Emtiazi (2006), where Mn$^{2+}$ showed increase in cellulase activity. CuSO$_4$ and MnSO$_4$ showed cellulase activity increase as the concentration of these chemicals increased. On the other hand, for FeSO$_4$, cellulase activity decreased from 48.23 U/g to 40.60 U/g as the concentration increased from 2 g/l to 10 g/l. Sinegani and Emtiazi, (2006) also proved that Fe$^{3+}$, Fe$^{2+}$ and Co$^{2+}$ showed inhibitory effect on cellulase activity. Generally, cellulase activity in the presence of all the chemicals showed 54 % to 136 % increase compared
to BSG without chemical supplementation.

4.8.2.3 Xylanase Activity

Four types of chemicals with different concentrations have induced the xylanase activity for BSG, fermented by *S. commune*. Xylanase activity with *S. commune* ranged from 133 U/g to 143 U/g, which is 146 % to165 % higher than the activity observed in BSG without chemical supplementation.

4.8.2.4 Laccase activity

6 g/l of MnSO$_4$ showed highest laccase activity (10,040 U/g) in the BSG fermented with *S. commune* (Figure 4.34) which is almost three fold higher than the activity observed in BSG without chemical supplementation. Among the other chemicals tested, 8 g/l and 10 g/l of CuSO$_4$ showed higher laccase activity at 7680 U/g and 7906 U/g respectively. MnSO$_4$, CuSO$_4$ and FeSO$_4$ at 2 g/l concentration showed lower enzyme activity compared to cellulase activity studied in BSG without chemical supplementation.

4.8.2.5 Lignin Peroxidase Activity

BSG supplemented by 2 g/l to 6 g/l of MnSO$_4$, fermented by *S. commune* showed similar activity ranged from 1336 U/g to 1357 U/g. Later the activity decreased to 12410U/g, when the concentration increased to 10 g/l.
Lignin peroxidase activity in BSG fermented by *S. commune* (Figure 4.34) in the presence of chemicals showed 38% to 90% increase compared to BSG without chemicals. In general lignin peroxidase activity fermented by *S. commune* in chemically supplemented BSG ranged from 1194 U/g to 2167 U/g. 4 g/l of CuSO$_4$ showed the highest lignin peroxidase activity at 2168 U/g. However, the activity decreased as the chemical concentration increased. Similarly, lignin peroxidase activity was observed decreased as the concentration of MnSO$_4$, CoCl$_2$ and FeSO$_4$ increased. Overall *S. commune* showed higher lignin peroxidase activity than *A. niger* in the presence of chemicals. Particularly, at 4 g/l in *S. commune* (2168 U/g) was 43% higher than 4 g/l CuSO$_4$ in *A. niger* (1513 U/g).
Statistical analysis showed there was no main effect of fungi on enzyme activity $F(1, 120) = 0.168$, $p > 0.05$. However, there is a significant difference in lignin peroxidase activity between $4 \text{ g/l}$ of CuSO$_4$ and other chemical concentrations $F(19, 120) = 21.243$, $p < 0.05$. Also an interaction was observed between fungi and chemical component in laccase activity $F(19, 120) = 10.489$, $p < 0.05$.

4.9 SSF KINETICS

4.9.1 Fungal Biomass Determination

Fungal biomass estimation is the crucial key for determining the kinetics in solid state fermentation. However, since direct biomass estimation is impossible in SSF, indirect biomass estimation (glucosamine) was used to estimate the fungal biomass. Fungal biomass was determined through estimating the glucosamine content in the autoclaved BSG substrate fermented with five types of fungi and compared with standard prepared with glucosamine hydrochloride (Oxoid).

The calibration curve (Figure 4.35) of glucosamine was constructed by taking the absorption maxima at different concentration of glucosamine to determine a suitable relationship for estimating the fungal glucosamine in SSF process. The evidence of a linear relationship was observed, and its correlation is shown in the equation: $y = 0.0179x + 0.0674 \ (R^2 = 0.9929)$. The biomass of all the fungi from the beginning to the end of fermentation process was calculated with the formula: $\text{BDw} = [(\text{glucosamine concentration} – 0.067)] / 0.0179$ represented in (Figure 4.35). This was used to estimate the biomass content which shown in Figure 4.36 to Figure 4.40.
Figure 4.35: Standard curve of Glucosamine content which used for glucosamine estimation in SSF.

Figure 4.35 to Figure 4.40 shows the glucosamine content in five types of fungi used in this process which was measured from day one to day seven of the fermentation period. All the fungi (*A. niger*, *Fusarium* sp., *P. chrysogenum*, *S. commune* and *Trichoderma* sp.) showed strong linear correlation of glucosamine content along the incubation period. This indicates that, the fungi are growing by creating more hypha and mycelium (amount of chitin content in the fungal cell increasing) as the incubation period increased.
Figure 4.36: Increase in fungal biomass (expressed as glucosamine equivalents mg/g) of *A. niger* at various time intervals

Figure 4.37: Increase in fungal biomass (expressed as glucosamine equivalents mg/g) of *P. chrysogenum* at various time intervals
Figure 4.38: Increase in fungal biomass (expressed as glucosamine equivalents mg/g) of *Fusarium* sp. at various time intervals

Figure 4.39: Increase in fungal biomass (expressed as glucosamine equivalents mg/g) of *S. commune* at various time intervals
Specific growth rate (SRG) ($\mu$) is an important parameter in kinetic modal. First-order Kinetics (Eq 1) used to determine specific growth rate.

Where $X$ is the biomass concentration (mg/g dw (biomass dry weight)), $X_o$ is the initial biomass concentration, $K$ is the specific growth rate constant and $t$ is time.
First-order Kinetics used to determine the rate of fungal growth in the solid state fermentation using BSG as the substrate. Monod model also used to estimate the fungal doubling time value (Eq 2). Since the specific growth rate is constant during the whole phase and maximum ($\mu_{\text{max}} = \mu$).

Where $T_d$ is doubling time, $\mu_{\text{max}}$ is maxima specific growth rate of fungi. Table 4.4 showed the specific growth rate, $R^2$ value, doubling time or half-life of five fungi used in this study.

**Table 4.3: Kinetic parameters for the fungi used in SSF process**

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Specific rate ($\mu$)</th>
<th>$R^2$ value</th>
<th>Doubling time (Td) or Half-life (Hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus niger</td>
<td>0.238</td>
<td>0.9919</td>
<td>69.84</td>
</tr>
<tr>
<td>Fusarium sp.</td>
<td>0.260</td>
<td>0.9883</td>
<td>64.08</td>
</tr>
<tr>
<td>Penicillium chrysogenum</td>
<td>0.310</td>
<td>0.9912</td>
<td>53.76</td>
</tr>
<tr>
<td>Schizophyllum commune</td>
<td>0.288</td>
<td>0.9851</td>
<td>57.84</td>
</tr>
<tr>
<td>Trichoderma sp.</td>
<td>0.272</td>
<td>0.9853</td>
<td>61.20</td>
</tr>
</tbody>
</table>

Doubling time is the time required for biomass to duplicate. In this case, *P. chrysogenum* required the least time (2.2 day) to replicate compared to other fungi. *Aspergillus niger* required the longest doubling time (almost 3 days) compared to other fungi. A correlation can be observed, when the specific growth rate of fungi compared with the doubling time required.

Fungi with higher SGR value need lesser doubling time than fungi with lower SGR value. For example, *Penicillium chrysogenum* with SGR 3.1 per day needs 2.2 day to
duplicates, while *A. niger* with less SGR value (2.38 per day) needs 3 days for duplicate. Overall doubling time shows that, almost all the fungi started to duplicate after second day of incubation, which is also can be proven when the enzyme activity compared with incubation period.

### 4.9.2 Correlation between fungal growth and enzyme activity

A correlation analysis between fungal growth on the autoclaved BSG and enzyme activity (laccase and lignin peroxidase) was carried out by plotting correlation regression graph (glucosamine content versus enzyme activity) and the correlation value $R^2$ values presented in Table 4.5.

<table>
<thead>
<tr>
<th></th>
<th>Laccase</th>
<th>Lignin peroxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. niger</em></td>
<td>0.9566</td>
<td>0.9219</td>
</tr>
<tr>
<td><em>Fusarium</em> sp.</td>
<td>0.9756</td>
<td>0.9136</td>
</tr>
<tr>
<td><em>Penicillium crysogenum</em></td>
<td>0.9672</td>
<td>0.5053</td>
</tr>
<tr>
<td><em>Scizophyllum commune</em></td>
<td>0.8051</td>
<td>0.9275</td>
</tr>
<tr>
<td><em>Trichoderma</em> sp.</td>
<td>0.8910</td>
<td>0.5191</td>
</tr>
</tbody>
</table>

*Aspergillus niger* and *Fusarium* sp. growth showed strong positive linear correlation with laccase ($R^2=0.9566$ and 0.9756 respectively) and lignin peroxidase activity ($R^2=0.9219$ and 0.9136 respectively), which indicates that, enzyme activity proportional to fungal growth. A study conducted by Wood (1979) showed the growth of *Agaricus* sp (mycelium) directly proportional to the activity of extracellular laccase. It also can be conclude as the fungal biomass increase, the enzyme activity also increase.
The increase in enzyme activity and biomass indicates the production of mycelial body and sporulation increase. However, the biomass yield is not same in the entire organism (Mahin et al., 2008) which shows the variation in the correlation value. According to Spier et al., (2009), biomass increase as the enzyme activity increase and this two factors showed a fairly propositional relationship. For *P. chrysogenum* and *Trichoderma* sp. a positive correlation was found with laccase activity with correlation value $R^2 = 0.9672$ and $R^2 = 0.8910$ respectively. However, Xavier et al., (2001) mentioned that the media that supports the fungal growth (high biomass) not necessarily will support higher enzyme yield.

This proved in the lignin peroxidase activity, where even though *Penicillium chrysogenum* showed weak correlation ($R^2 = 0.5053$), but it showed highest lignin peroxidase activity. The capacity of sugar utilization in fungi also varies depending on the isolates. Different isolates of *Aspergillus* species showed different level of citric acid production (Mahin et al., 2008).

In some cases, the substrate used in the fermentation only helped in fungal growth and not in enzyme production (Gutierrez-Correa et al., 1999). This could be the reason for lower $R^2$ value (which indicates non-linear correlation or no correlation) in laccase and lignin peroxidase activity by *P.chrysogenum* even though the fungal growth showed strong linear correlation with incubation period.

Besides, if the enzyme activity of this fungus over an incubation period showed that, very small increase was found. SRG can prove the above statement, where the SRG
value is lower than other fungi. The doubling time for this fungi is also longer (almost 3 days), which may cause the poor utilization of nutrients in substrate, which lead to poor enzyme production. The enzyme increase throughout the fermentation (from day 1 to day 8) was not noticeable. Thus no correlation or non-linear correlation was found in this fungus. According to Carvalho et al., 2006, type of microorganism and the cultivation condition plays role in correlation between fungal growth and enzyme activity.

4.10 SEM

Scanning Electron Microscope (SEM) was used to study the morphological event of fungal growth of S. commune and A. niger in the fermentation medium compared with BSG without fungal inoculation. Plate 4.11 shows the SEM picture of BSG with and without fungal inoculation.

Plate 4.11a and plate 4.11b showed the substrate BSG without fungal inoculation while Plate 4.11c and Plate 4.11d BSG with S. commune and Plate 4.11e and Plate 4.11f BSG with A. niger.

Both fungi were observed grew well attached to the BSG substrate, which is because of the hydrophobicity of the substrate which eases the attachment of the fungus to the substrate. Besides, the chemical properties of BSG, such as cellulose, lignin and hemicellulose (carbohydrate) react as the good non-inert substrate for fungal growth.
Plate 4.11: (a-b) BSG without fungal inoculation, (c-d) BSG fermented with *S. commune* and (e-f) BSG fermented with *A. niger*.
Osma et al., (2007) mentioned in his studies, that the substrate with high hydrophobicity and high in nutrients provides good attachment place for the fungi. *S. commune* growth showed denser than *A. niger* growth on the substrate. More spores can be observed in substrate fermented with *A. niger*. According to Nagao et al., (2003) fungi on the substrate form aggregated particles with the surface were covered by glue-like materials (mycelium).
CHAPTER 5

5.0 GENERAL DISCUSSION

In this study, BSG showed good potential in producing higher enzyme activity, but not SCB and SMC. However, in other studies conducted by Khosravi-Darani and Zoghi, (2008) and Gottschalk et al., (2010), SCB showed good ability in producing higher enzyme activity. This could be due to the substrate pre-treated with acid and alkali treatments. SCB used in this study contain thick lignin layer (46%). Thick lignin layer prevent fungal attack and slow the fermentation process. Therefore, pre-treatment is required to create more carbohydrate bonds (Lee et al., 1999) and increase the hemicellulose solubilization and convert them into fermentable sugar. Pre-treatment will modify the physical and chemical composition of the substrate and will support fungal growth and enzyme production (Santos et al., 2008; Vinoth Kumar et al., 2010; Milagres et al., 2004). However, BSG used in this research, which consist of 28% lignin showed higher enzyme activity without pre-treatment and this was supported by Jay et al., (2008). However, Xiros et al., (2008) suggested alkali pre-treated BSG showed higher enzyme activity than un-treated BSG.

The five fungi species used in this study showed different level of enzyme activity on different incubation duration as discussed in chapter four. Variation in optimum incubation period for enzyme activity can be explained under the three models below based on the combination of substrate, fungal strain and enzyme. First model is when the same substrate is utilized for one enzyme production by different fungal strains (Robinson et al., 2001; Stajic et al., 2006). Second model is when the same substrate
is fermented by the same fungal strain for different enzymes production (Chimata et al., 2010; Jecu, 2000; Muthezhilan et al., 2007; Ellaiah et al., 2002) and the third option is when the same fungal strain is used to produce the same enzyme, but utilizing different substrates (Ramachandran et al., 2004; Jecu, 2000; Milala et al., 2008). *S. commune* used in this research showed shorter optimum incubation duration for amylase and cellulase activity compared to other fungi. Some fungal species exhibit peak activity within short duration (Fujian et al., 2001; Panagiotou et al., 2003; Sabu et al., 2005) while others required longer fermentation period to reach optimum activity (Kanmani et al., 2009; Osma et al., 2007; Couto et al., 2002). This is because the incubation period depends on the fungal growth rate and fungal metabolism rate.

Enzyme activity in this study varies with substrate’s initial moisture content. Adequate initial moisture (30 %-80 %) allows the biosynthesis and secretion of enzymes (Pandey et al., 2008). Most of the fungal species can grow well at 70% initial moisture (Botella et al., 2007; Latifian et al., 2007; Sharma et al., 2008; Shojaosadati et al., 1999). SCB used in this study contains high moisture content and showed lower water holding capacity. Therefore, it created unfavorable condition for fungal growth and showed lower enzyme activity compared to BSG.

BSG and SMC combination at 9:1, 4:1 and 7:3 showed better enzyme activities compared to BSG with SCB combination. However, not all combinations of SMC and SCB showed increase in enzyme activity and this was proved by Dogaris et al., (2009) and Kaur and Sathiyanarayana, (2004). For example study conducted by Azin
et al., (2007) showed that the combination of wheat bran (WB) and wheat straw (WS) at 7:3 ratio, showed higher xylanase activity, while Dogaris et al., (2009) showed 1:5 ratio of these same substrates gave higher endoglucanase (cellulase) activity, and at 2:5 produce lignin peroxidise (Fujian et al., 2001), while none of the combinations helped in production of exoglucanase activity except for 100% WB (Dogaris et al., 2009). Beside, the fungal strains used in these studies also contribute in the variation of enzyme activities. However, enzyme activity was observed higher in BSG alone when compared with mixed substrates. Many researchers also support this statement, where higher enzyme activity observed in single substrate than mixed substrates (Cha et al., 2010; Wong et al., 1987; Rossi et al., 2003; Rosales et al., 2005; Dogaris et al., 2009). The main reason in the enzyme production under different substrates combination could be due to the substrate properties.

Amylase activity was observed at 227% to 433% increase in BSG supplemented with MnSO$_4$. According to Kadrekar and Ramasarma, (1990) and Sinegani and Emtiazi, (2006) Mn$^{2+}$ion favors the amylase and cellulase activity and this was also supported by Mrudula and Kokila, (2010) study. In this study, BSG supplemented with CuSO$_4$ did not enhance the laccase activity. Similar results have been reported by Patel et al., (2009), Tychanowic et al., (2006) and Mishra and Kumar, (2007). However, contradictory results for laccase enhancement were observed in the studies conducted by (Collins and Dobson, 1997), Palmieri et al., (2000) and, Baldrain and Gabriel, (2002) using different substrates. Cellulase activity in BSG supplemented FeSO$_4$ decreased as the concentration of this chemical increased as observed in Shankaranand and Lonsam, (1994). However, laccase activity was observed
increasing as the concentration of FeSO₄ increase. Similar result can be observed in La Nauze, (1966). In certain conditions, Fe²⁺ ion serve as fungal growth factor (Noyes, 1969). However, excess iron could affect the fungal growth by exhibiting inhibitory effects and affect the enzyme activity (Kapoor et al., 1982).

All fungal growth showed strong linear correlation with laccase and lignin peroxidise activity (R² = 0.5053 to 0.9756).

Fungal growth showed strong linear correlation with enzyme activity with the correlation ranged from r² = 0.80 to 0.97. Fungal doubling time (Td) was calculated based on the fungal biomass. Among the fungi, *P. chrysogenum* and *S. commune* showed shorter Td, 54 hours and 58 hours respectively. Fungi with shorter Td, implies higher growth rate. *P. chrysogenum* and *S. commune* (fungal biomass) also showed strong correlation value with lignin peroxidase (R² = 0.9816, and R² = 0.9275) and laccase activity (R² = 0.9672 and R² =0.8051). In this case, the biomass increase was proportional with enzyme activity and this was supported by Spier et al., (2009), where biomass increase will contribute in increasing enzyme activity. The biomass yield is not same in the entire organism (Mahin et al., 2008). In some cases, the media which support the fungal growth, not necessarily will support the enzyme activity (Xavier et al., 2001; Gutierrez-Correa et al., 1999).
CHAPTER 6

6.0 CONCLUSION

Among the three substrates tested (BSG, SMC and SCB), higher enzyme activity was observed in autoclaved BSG for all the five types of enzymes tested. Moisture content, particle size, cellulase content, hemicellulase content and lignin content were also considered as influencing factors for higher enzyme activity among the substrates.

Enzyme activities were studied for BSG in combination with SMC showed higher activity at different ratios compared to BSG in combination with SCB. However, enzyme activity was observed higher in BSG alone compared to any combination of these materials. Therefore, using BSG as single substrate for enzyme production would be the better option than mixed substrates.

Activities of five types of enzymes were studied in this research. Among the enzymes tested, laccase activity was the highest followed by lignin peroxidase, xylanase, cellulase and amylase by all five types of fungi. Five types of fungi were used in this fermentation and the enzyme yield varies depending on enzymes studied. \textit{P. chrysogenum} showed greater laccase and lignin peroxidase activity, \textit{A. niger} produced higher xylanase and amylase activity while \textit{Trichoderma} showed higher cellulase activity.

Based on Table 6.1, the following conclusions were made for this research

1. \textit{Penicillium chrysogenum} is the best laccase and lignin peroxidase
producer for autoclaved BSG fermented for seven days at 30°C.

2. *Trichoderma* is the best cellulase producer for BSG and SMC fermented for seven day at 30°C. While *Fusarium* is best for SCB.

3. *Aspergillus niger* is best amylase and xylanase producer for all the three substrates at seven day incubation.

Enzyme activity was optimized for various incubation duration and incubation temperature. Seven day fermentation was the optimal duration for all the fungi in term of enzyme activity. All the fungi showed optimal enzyme activity at 30°C except for amylase, which was higher at 35°C.

**Table 6.1:** Summary of best enzyme producer (Fungi) at different condition

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>BSG</th>
<th>SMC</th>
<th>SCB</th>
<th>Incubation period (BSG) (7th day)</th>
<th>Incubation Temperature (BSG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase</td>
<td>A. niger &amp; Fusarium</td>
<td>A. niger</td>
<td>Trichoderma</td>
<td>A. niger, Fusarium, Trichoderma &amp; P. chrysogenum</td>
<td>P. chrysogenum (35°C)</td>
</tr>
<tr>
<td>Cellulase</td>
<td>Trichoderma</td>
<td>Fusarium</td>
<td>Trichoderma</td>
<td>Trichoderma</td>
<td>Fusarium &amp; Trichoderma (30°C)</td>
</tr>
<tr>
<td>Laccase</td>
<td><em>P. chrysogenum, S. commune, Trichoderma</em></td>
<td>Trichoderma</td>
<td>A. niger</td>
<td><em>P. chrysogenum</em></td>
<td><em>P. chrysogenum</em> (30°C)</td>
</tr>
<tr>
<td>Lignin peroxidase</td>
<td><em>P. chrysogenum</em></td>
<td>S. commune</td>
<td>Trichoderma</td>
<td><em>P. chrysogenum</em></td>
<td><em>P. chrysogenum</em> (30°C)</td>
</tr>
<tr>
<td>Xylanase</td>
<td>A. niger</td>
<td>A. niger</td>
<td>A. niger</td>
<td>A. niger</td>
<td>A. niger (35°C)</td>
</tr>
</tbody>
</table>

BSG was supplemented with four types of chemicals (CoCl₂, CuSO₄, MnSO₄ and FeSO₄) at the concentration varies from 2 g/l to 10 g/l. All the enzyme activities
increased in the presence of these chemicals. Among the enzyme activities, amylase showed great increase (208% - 357%), when fermented by either *A. niger* and *S. commune*.

Table 6.2 explains the chemical concentrations which yield higher enzyme activities for the five enzymes tested. Based on the table, there is more than one optimum chemical concentration for higher enzyme activity. 10 g/l CoCl$_2$ induce for higher amylase and laccase activity in BSG fermented with *A. niger* and *S. commune* respectively. CuSO$_4$ induced cellulase and xylanase activity in BSG fermented by *S. commune*. While MnSO$_4$ helped in increasing xylanase activity. Generally, *S. commune* showed higher enzyme activity in chemical supplemented BSG compared to *A. niger*.

**Table 6.2: Summary of effect of the chemicals and fungi for higher enzyme production**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Fungi</th>
<th>Chemical</th>
<th>Concentration (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase</td>
<td><em>A. niger</em></td>
<td>CoCl$_2$</td>
<td>10, 2 and 4</td>
</tr>
<tr>
<td>Cellulase</td>
<td><em>S. commune</em></td>
<td>CuSO$_4$</td>
<td>6 &amp; 10</td>
</tr>
<tr>
<td>Laccase</td>
<td><em>S. commune</em></td>
<td>CoCl$_2$ &amp; FeSO$_4$</td>
<td>10 &amp; 8</td>
</tr>
<tr>
<td>Lignin peroxidase</td>
<td><em>S. commune</em></td>
<td>MnSO$_4$</td>
<td>6</td>
</tr>
<tr>
<td>Xylanase</td>
<td><em>S. commune</em></td>
<td>CuSO$_4$</td>
<td>4</td>
</tr>
</tbody>
</table>

Kinetic model was used to estimate the fungal biomass, which was required to estimate the doubling time of each fungus in the fermentation medium. A correlation was drawn between fungal growth (biomass yield), enzyme activity and duration of
fermentation. There was a strong linear correlation ($R^2=0.5035 - R^2 = 0.97$) between fungal growth and enzyme activity for all the fungi. The shorter the doubling time, the higher is the fungal growth.