PRODUCTION OF POLYSACCHARIDES FROM WHITE MUSHROOMS (*Pleurotus florida*) BY SUBMERGED CULTURE FERMENTATION (SCF)

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PRODUCTION OF POLYSACCHARIDES FROM WHITE MUSHROOMS (*Pleurotus florida*) BY SUBMERGED CULTURE FERMENTATION (SCF)

FAZLIN BINTI MASRA

Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
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JANUARY 2014

SUPERVISOR'S DECLARATION

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

Signature	:
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Date	: JANUARY 2014

STUDENT'S DECLARATION

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

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Date : JANUARY 2014

Dedication

Dedicated especially to my beloved mother, siblings, lectures, friends and to ones who give me support and inspiration that made this work possible.

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In the name of Allah SWT, most Grateful and most Merciful,

Alhamdulillah, thank to Allah SWT for giving me strength and endurance in finishing my thesis. I had met various people that help me and contributed towards my understanding and giving me idea and strategies in this project development.

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ABSTRACT

P.florida is commonly available edible mushroom among different types of oyster mushrooms of the genus *Pleurotus*. The bioactive compound contain in the *P.florida* (polysaccharides and protein) have benefit to health include immunomodulatory, anti-cancer, and hypocholesterolemic effect. The objective of this research is to study the production of polysaccharides and biomass from white mushrooms (*P. florida*) by submerged culture fermentation (SCF) and determination of protein content by using Kjeldahl method. The effect of harvest time on the production of mycelial biomass, exopolysaccharides (EPS) and protein by *P.florida* was investigated in research. The optimal culture condition was 20 g/L glucose, 4 g/L yeast extract, 0.46 g/L KH2PO4, 1 g/L K2HPO4, and 0.5 g/L MgSO4 7 H2O at temperature 28oC and pH 8. The modes of reactor operation used in this cultivation of mushroom are shake flask and air lift bioreactor and Somogyi-Nelson reagent, Phenol-Sulphuric reagent and 4% Phenol reagent were used during the sample analysis. In this present study, the result implies that the highest biomass, polysaccharides and protein yield production change with the time of cultivation. This indicated that the maximum production of production day in producing biomass was at days 15 (2.464 g/L). However, the production polysaccharides and protein indicates the highest productivity on the same day (days 12) with 8.42 g/L and 28.69 % respectively. It was found that productivity in airlift bioreactor much higher than the productivity in the shake flask. Thus it can be concluded that that harvest time selection is very important factor to obtain maximal fungal production. This may due to the gentle agitation and aeration hydraulic in airlift bioreactor leads to the productivity of mycelia biomass, exopolysaccharides and protein more efficient than in shake flask.

ABSTRAK

P.florida adalah antara cendawan yang boleh dimakan diantara pelbagai jenis cendawan tiram daripada genus Pleurotus. Sebatian bioaktif yang terdapat di dalam *P.florida* (polisakarida dan protein) mempunyai manfaat untuk kesihatan termasuk immunomodulatori, anti-kanser, dan kesan hipokolesterolemik. Objektif bagi kajian ini ini ialah untuk mengkaji pengeluaran polisakarida dan biojisim daripada cendawan putih (*P. florida*) dengan melalui kaedah tenggelam penapaian (SCF) dan analisa kandungan protein pula menggunakan kaedah Kjeldahl. Kesan masa menuai kepada pengeluaran biomass mycelial, exopolysaccharides (EPS) dan protein dengan *P.florida* telah dikaji dalam penyelidikan ini. Kultur yang optimum adalah pada 20 g/L glukosa, 4 g/L ekstrak yis, 0.46 g/L KH_2PO_4, 1 g/L K_2HPO_4, dan 0.5g/L $MgSO_4$ 7 H_2O pada suhu 28 ^oC dan pH 8. Mod operasi reaktor yang digunakan dalam pengkulturan cendawan ini adalah kelalang dan bioreaktor angkat udara manakala Somogyi-Nelson reagen, Fenol-Sulphuric reagen dan 4% Fenol reagen telah digunakan semasa analisis sampel. Di dalam kajian ini, hasilnya menunjukkan bahawa biomas tertinggi, polisakarida dan protein berkaitrapat dengan masa penanaman. Ini menunjukkan bahawa pengeluaran maksimum hari pengeluaran dalam menghasilkan biomass adalah pada hari 15 (2,464 g/L). Walau bagaimanapun, polisakarida dan protein pengeluaran menunjukkan produktiviti tertinggi pada hari yang sama (hari 12) dengan 8.42 g/L dan 28.69%. Hasil kajian ini juga mendapati bahawa produktiviti pada mod penyelamatan udara bioreaktor lebih tinggi daripada produktiviti di kelalang. Oleh itu, ia dapat disimpulkan bahawa bahawa pilihan masa menuai adalah faktor penting untuk mendapatkan pengeluaran kulat maksimum dan kerana pergolakan lembut dan pengudaraan hidraulik dalam penyelamatan udara bioreaktor membuat produktiviti biomas mycelia, exopolysaccharides dan protein lebih baik daripada di dalam kelalang.

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LIST OF SYMBOL

°C	Degree Celsius
cm	Centimetre
%	Percentage
g	Gram
mg	Milligram
µg	Microgram
L	Litre
ml	Milliliter
V	volume

LIST OF ABBREVIATIONS

DW	Dry Weight
SSF	Solid State Fermentation
SCF	Submerged Culture Fermentation
EPS	Exopolysaccharides
PPS	Endopolysaccharide

1 INTRODUCTION

1.1 Introduction

Cancer is a leading cause of death worldwide. Based on the report by Ministry of Health Malaysia in 2006, an amount of 21,773 cancer incidence was reported per 1000,000 populations in peninsular Malaysia. It comprises of 9,974 males and 11,799 females. The five most common cancers among population of Peninsular Malaysia in 2006 were breast, cervix colorectal, nasopharynx, and lung. Mushrooms consist of bioactive compound such as polysaccharides and proteins as reported in Huang and Liu (2008). Recently, a number of bioactive molecules, including anti-cancer agents, have been identified from various mushrooms. These bioactive compounds are responsible in anti-cancer effects. Polysaccharides are the best known and most potent mushroom-derived substances with anti-cancer and immunomodulation properties (Daba and Ezeronye., 2003).

Polysaccharides especially β-glucan obtained from mushroom have been used for various purposes. Glucan with different active unit linkage such as (1→3), (1→6)-β-glucan and (1→3)-α-glucans constitute mushroom polysaccharides which perform immunomodultor activity as they are biological response modifiers (BRMs) (Rout *et al*., 2005). Exopolysaccharide are highly molecular weight polymers composed of sugar residue that secreted by microorganism. In both prokaryotic and eukaryotic microbial pathogens, polysaccharides are major cell wall components and act as a source of therapeutic agents which are able to modulate animal and human response and able to inhibit certain tumor growth.

The crude protein content of the common mushroom (*P. florida*) has been reported to be 19-38% on dry weight (DW) basis, but the measurement are complicated by the presence on non-protein nitrogen containing compounds. Protein content of mushroom is influenced by the compost composition, flush number, harvest time and strain (Braaksma *et. al*., 1995).

Submerged culture fermentation (SCF) is an alternative methodology to produce polysaccharides (bioactive compound) from mushrooms at higher yield for nutraceutical, pharmaceutical, and cosmetics industries. Modes of reactor operation that are the most amenable to mycelium production of mushroom is air lift bioreactor due to its gentle agitation and aeration hydraulics.

1.2 Objectives

The current medicine for anti-cancer that available in market pose several side effect and complications in clinical management of various form of cancer which highlights the urgent need for novel effective and less-toxic therapeutic approaches (Patel *et al*., 2012). A few of clinical trial have been conducted in identifying the benefit of using commercial medicine from mushroom extracts in cancer therapy it contains bioactive compounds like protein and polysaccharide. It has a function as anti-cancer agents. In addition, the production of polysaccharides and protein by submerged culture fermentation (SCF) is an alternative way and has advantage to reduce the cost and improve the productivity (Smiderle *et al*., 2011), as compared to the solid state fermentation (SSF), besides more precise control on a range of factors such as pH, moisture, light, and temperature (Lin and Sung, 2006). Hence, this application will be implemented in this present study.

1.3 Research objective

To study the production of polysaccharides and biomass from white mushrooms (*Pleurotus florida*) by submerged culture fermentation (SCF) and determination of protein content by using Kjeldahl method.

1.4 Scope of research

In order to achieve the objectives, the following scopes have been identified:

i. To identify the effect of harvest time on polysaccharides production in shake flask.
ii. To identify the effect of harvest time on mycelium production in shake flask
iii. To identify the effect of harvest time on protein production in shake flask.
iv. To compare the productivity for polysaccharides, mycelium and protein in shake flask and airlift bioreactor at 28^{o}C (lee at al, 2009).

2 LITERATURE REVIEW

2.1 Mushroom cultivation and consumption

2.1.1 Mushroom production

Approximately 14,000 species of the 1.5 million fungi estimated in the world produce fruiting that are large enough to be considered as mushrooms. There are three categories of mushrooms available in industries which are edible mushrooms, medicinal mushrooms products, and wild mushrooms (Chang, 2006). In Far East, over 200 species have been collected from the wild and used for various traditional medical purposes. About 20 species have been cultivated on industrial scale and 35 species have been cultivated commercially. The most cultivated mushroom worldwide is *Agaricus bisporus*, followed by *Lentinula edodes*, *Pleurotus* spp. and *Flammulina velutipes* (Aida *et al*., 2009) & (Chang and Miles, 2004). These species require shorter growth time when compared to other edible mushrooms, they demand few environmental controls, and they can be cultivated in a simple and cheap way (Bonatti *et al*., 2004). Table 2.1 shows summarization world mushroom production taken from Food and Agriculture Organization of United Nations (2009). China was found be the biggest production of mushrooms as they produced more than 1.5 million tonnes in 2007. This was followed by United Stated of America as the second highest world production mushrooms. However, Indonesia and India producing least mushroom this maybe due to less demand of mushrooms in the country.

Table 2.1 Summarize World mushroom Production, 1961-2007; United Nations (Food and Agriculture Organization of United Nations, 2009).

Countries	Production (tonnes) 2007
China	15,68,523
United States of America	3,59,630
Netherlands	2,40,000
Indonesia	48,247
India	48,000
Other countries	59,297

2.1.2 *P. florida*

Pleurotus species are commonly called Oyster mushrooms. There are about 40 species of this mushroom. Oyster mushrooms now rank second among the important cultivated mushrooms in the world. *P.florida*, the oyster mushroom, is a common edible mushroom. It was first cultivated in Germany as a subsistence measure during World War I and is now *P.florida* is widely cultivated and consumed as food in Malaysia. The scientific classification of *P.florida* is stated as in Table 2.2.

Table 2.2 Classification of *P.florida* (http://en.wikipedia.org/wiki/Pleurotus)

Kingdom	Fungi
Phylum	*Basidiomycota*
Class	*Agaricomycetes*
Order	*Agaricales*
Family	*Pleurotaceae*
Genus	*Pleurotus*
Species	*P. florida*
Binomial name	*Pleurotus florida*

Figure 2.1: Fruit body of *P. florida.*

2.2 Medicinal and pharmaceutical properties of mushroom

2.2.1 Anti-cancer properties of mushrooms

Cancer is a leading cause of death worldwide. The current medicine for anti-cancer that available in market pose several side effect and complications in clinical management of various form of cancer which highlights the urgent need for novel effective and less-toxic therapeutic approaches (Patel *et. al*., 2012). A few of clinical trial have been conducted in identifying the benefit of using commercial medicine from mushroom extracts in cancer therapy. Mushrooms consist of bioactive compound such as polysaccharides, proteins, fats, ash, glycosides, alkaloids, volatile oils, tocopherols, phenolics, flavonoids, carotenoids, folates, ascorbic acid enzymes, and organic acids. These bioactive compunds, are responsible in anti-cancer effects. Recently, a number of bioactive molecules, including anti-cancer agents, have been identified from various mushrooms. Polysaccharides are the best known and most potent mushroom-derived substances with anti-cancer and immunomodulation properties. Figure 2.2 shows structure of anti-cancer compounds isolated from mushrooms.

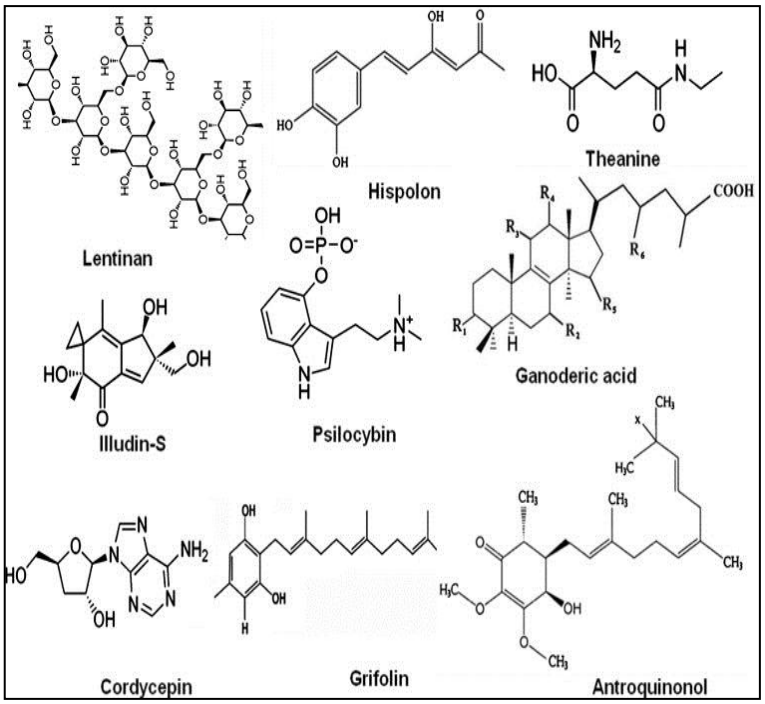

Figure 2.2 Structure of anti-cancer compounds for mushrooms (Patel *et. al*.,2012)

2.2.2 Structure and anti-cancer activities of polysaccharides

Polysaccharides belong to a structurally diverse class of macromolecules. Unlike proteins and nucleic acids, they contain repetitive structural features which are polymers of monosaccharide residues joined together by glycosidic linkages with a general chemical formula of $C_x(H2O)_y$. Research done by Maity, (2013) found that mushroom (*P. florida)* polysaccharides are present mostly as glucans with different types of glycosidic linkages, such as water-insoluble (1→3)(1→6)-β-D-glucans, water-soluble (1→6)-α-D-glucan, NaCl soluble (1→3)(1→6)-α, β-D-glucan a water-soluble heteroglycan are widely used as anti-cancer and immunostimulating agent. Table 2.3 shows the polysaccharides isolated from the parent strains. Polysaccharides play important roles in biological mechanisms such as infection, adhesion, immune response and signal transduction. In addition, mushroom polysaccharides exert their anti-cancer action through activation of immune response (Wasser and Weis, 1999) and these substances are regarded as biological response modifiers. Figure 2.3 shows the structure of repeating unit present in polysaccharides.

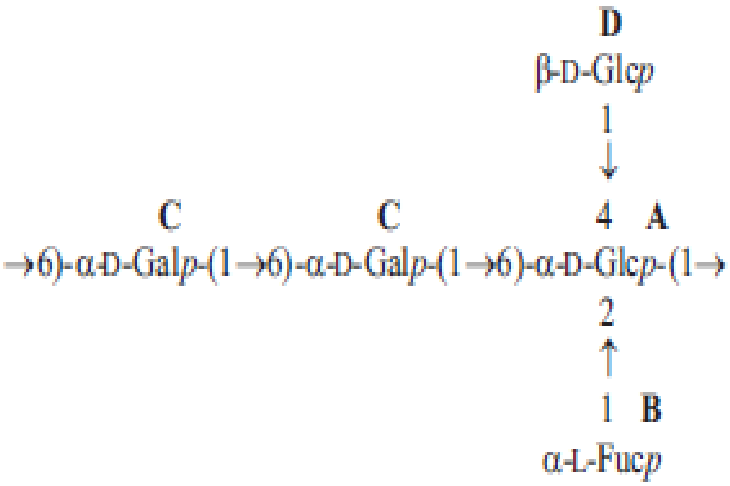

Figure 2.3 The structure of the repeating unit present in the polysaccharides (Maity et al., 2011).

Table 2.3 The polysaccharides isolated from the parent strains (Wasser., 2002).

Parent strain	**Polysaccharide**
P.florida	(1-6)-a-D-Glucan (from hot aqueous extract),
	(1-3), (1-6)-a-, b-D-Glucan (from NaCl soluble Fr. I)
	(1-3), (1-6)-b-D-Glucan (water insoluble)
	(1-3), (1-6)-b-D-Glucan (from cold alkaline extract)

2.2.3 Determination protein in mushrooms

Mushrooms have been considered as a source human healthy food for a long time ago (Chang and Miles., 1992). Mushrooms are rich in carbohydrate and protein and the protein elaborated by these fungi have shown several biological activities like immunomodulatory, anti viral, antiproliferative, antifungal and anti bacterial effects (Jonathan and Fasidi., 2003).

Protein content in mushrooms is influenced by the flush number, harvest time (Crissan and Sands., 1978), and compost composition (Bakowski *et al.,* 1986). Protein content is often expressed on dry weight (DW) basis, but DW is not constant and is reported to be variable between 7.4 to 13.7% (Weaver et al., 1977). There is a few of research report that the total protein content of harvested mushrooms in range between 19 to 39 % on

DW basis (Weaver et al., 1977). It also suggested that the crude protein content in mushrooms arises by the presence of nitrogen determination (Kjeldahl) and usually attributed to protein. The protein is then determined on the basis of total nitrogen content, a Kjeldahl factor of N x 4.38 is commonly used (Levai, 1989).

The Kjeldahl method process include the digestion of the sample by place the digestion tablets into the Kjeldahl tube. Next process involve was the distillation of the sample followed with the titration and the calculation of the crude protein with Kjeldahl factor of N x 4.38. (Riddellová, undated). Figure 2.4 shows the digestion method involves in the determination of protein content in *P.Florida.*

Figure 2.4: Digestion process in Kjeldahl method

2.3 Technique in mushroom cultivation

2.3.1 Solid State Fermentation (SSF)

Many different techniques and substrates have been successfully utilized for mushroom cultivation. For production of mushroom fruiting bodies, various forms of SSF are employed, whereas for mycelial biomass and polysaccharides production, submerged fermentation is preferable to produce a more uniform biomass and pharmaceutical products.

Solid-state fermentation (SSF) is defined as any fermentation process occurring in the absence or near absence of free liquid, employing an inert substrate (synthetic materials) or a natural substrate (organic materials) as a solid support (Pandey et. al., 2000 and Couto et. al., 2007). Figure 2.5 shows the solid state fermentation in *P.Florida* production. SSF is most appropriate for bioconversion of plant raw materials into value-added products, such as mushroom fruiting bodies, fodder, secondary metabolites, and enzymes. SSF has several advantages as compared with submerged cultivation; in particular, with small energy consumption, the nutrient medium is concentrated, and high volumetric productivity can be achieved in a smaller bioreactor. In addition, till now, the major obstacles for the commercial applications of SSF techniques have not been completely overcome. They are related to the design and operation of large-scale bioreactors due to problems concerned with the control of parameters such as pH, temperature, aeration and oxygen transfer, moisture, and agitation.

Figure 2.5: Solid State Fermentation (SSF) in mushroom cultivation.

2.3.2 Submerged culture fermentation (SCF)

In submerged culture fermentation (SCF) is different with SSF where it requires large energy expenditures to agitate nutrient medium and to supply oxygen. However, the submerged culture works as a homogeneous system, and the cultivation process control is easy using many on line sensors. In this case, a very wide range of products can be produced from a wide range of microorganisms with the best productivity, due to medium mixing and unlimited diffusion of nutrients. Moreover, although the downstream processing after submerged fermentation requires removal of large volumes of water and is more expensive, the product purification may be easier as compared to SSF. Hence, mushroom submerged cultivation has significant industrial potential, but its success on a commercial scale depends on cost compared with existing technology (Pandey. et. al., 2000). Figure 2.6 shows the submerged culture fermentation (SCF) in mushroom cultivation in the shake flask.

Figure 2.6: Submerged Culture Fermentation (SCF) in mushroom cultivation.

2.3.3 Submerged culture in airlift bioreactor

In mushrooms cultivation, the productivity in an airlift reactor was higher than that in the stirred-tank reactor and shake flask (Cho et al., 2006). In airlift fermenter, mixing is accomplished without any mechanical agitation. Airlift bioreactor is suitable to be used for tissue culture because tissue is shear sensitive and normal mixing is not possible. Since there is no agitation, sterility can easily be maintained (Najafpour, Undated). Research done by Kim et al., (2002) has mentioned that the mycelial biomass productions were 3 times and 2 times higher than those in shake cultures, respectively. Lee et al., (1998) have reported that maximum polysaccharide production and mycelial biomass of the same species of mushroom. This proved the effectiveness of mushroom cultivation in airlift bioreactor. Figure 2.7 shows the cultivation of mushrooms in airlift bioreactor.

Figure 2.7: Mushrooms cultivation in airlift bioreactor.

3 MATERIALS AND METHODS

3.1 Material used

3.1.1 Fungi (Mushrooms)

The raw material used in this study was *P. florida* (white mushroom). It was purchased from the local hypermarket. The mushrooms were transported to the laboratory and placed in the freezer with temperature 20ºC to reduce any protease activity before handling the experiment.

3.1.1 Chemicals

For the analysis of polysaccharides, Somogyi Nelson reagent, Arsenomolybdate reagent and 4% Phenol reagent was used. For the Somogyi-Nelson reagent, the copper sulphate pentahydrate, anhydrous sodium carbonate, sodium hydrogen carbonate and sodium tartarate tetahydrate were purchased from Sigma Aldrich (Malaysia). While for Arsenomolybdate reagent, ammonium molybdate, concentrated sulphuric acid and disodium hydrogen arsenate heptahydrate were also purchased from Sigma Aldrich (Malaysia). Besides, 4% Phenol reagent also purchased from Sigma Aldrich (Malaysia).

3.2 Experimental procedure

For the analysis of polysaccharides, Somogyi Nelson reagent, Arsenomolybdate reagent and 4% Phenol reagent was used. For the Somogyi-Nelson reagent, the copper sulphate pentahydrate, anhydrous sodium carbonate, sodium hydrogen carbonate and sodium tartarate tetahydrate were purchased from Sigma Aldrich (Malaysia). While for Arsenomolybdate reagent, ammonium molybdate, concentrated sulphuric acid and disodium hydrogen arsenate heptahydrate were also purchased from Sigma Aldrich (Malaysia). Besides, 4% Phenol reagent also purchased from Sigma Aldrich (Malaysia).

3.2.1 Somogyi-Nelson Reagent preparation

The first solution was prepared by mixed 4 g of copper sulphate pentahydrate and 16 g sodium hydrogen carbonate into 200 ml distilled water. Second solution prepared with 24 g anhydrous sodium carbonate and 12 g sodium tartarate tetahydrate diluted in 250 ml distilled water. Separately, prepare a solution of 180 g anhydrous sodium sulfate in 500 ml of boiling distilled water. The two solutions were combined in a volumetric flask and diluted the final solution to 1 liter (Winstad, 2001).

3.2.2 Arsenomolybdate Reagent preparation

A solution of 25 g ammonium molybdate in 450 ml distilled water was prepared. The solution was added with stirring 21 ml concentrated sulphuric acid and 25 ml of distilled water containing 3 g disodium hydrogen arsenate heptahydrate. The solution was stirred continuously for 24 hours at 37ºC. The solution was stored in a 1 liter glass stoppered brown bottle and covered with aluminum foil to protect the solution from light (Winstad, 2001).

3.2.3 4% Phenol Reagent

40 g phenol was added in 1 litre distilled water (Winstad, 2001).

3.2.4 Tissue culture preparation

A piece of stem from fruit body *P.florida* was sterilely removed and placed in potato dextrose agar (PDA) plate. The sample was inoculated in the PDA agar for a few days in room temperature. After 10 days, mycelium grows from the tissue and colonized the agar. The tissue was then gently cut into small pieces (1 cm x 1 cm) before inoculated into the 500 ml shake flasks that contain mushroom complete medium (MCM).

3.2.5 Preparation of Mushroom Complete Media (MCM)

Add 20 g of glucose, 2 g yeast extract, 2 g meat peptone, 1 g K_2HPO_4, 0.46 g HK_2PO_4 and 0.5 g $MgSO_4.7H_2O$. After that, mix the chemicals with 1 liter distilled water. Then, divide the volume to 250 ml each and transfer to shake flask. Lastly, the media was autoclaved in 121ºC in 15 minutes (Winstad, 2001).

3.2.6 Submerged Culture Fermentation (SCF)

The samples were then left in 1 day in room temperature to grow before introduced into the shaker. The incubation was conducted on a rotary shaker at 25℃, 500 rpm for 6 days, 9 days, 12 days, 15 days and 18 days. For production in airlift bioreactor, the sample was fermented in shake flask first before being transferred in the reactor and was left in 7 days. The determinations of endopolysaccharides are by freeze dry of the biomass before weight the sample while the determination of exopolysaccharides has been done as below (Winstad, 2001).

3.3 Analysis

3.3.1 Determination of Reducing Sugar Using the Somogyi -Nelson Method

The samples were diluted first with the dilution factor 10 x. A portion of sample (1 ml) then transferred into 10 ml test tube. 1 ml of low-alkalinity copper reagent added and the tubes was heated in boiling water for 10 min. 1 ml of arsenomolybdate reagent was then added to the tubes and filled with the distilled water up to 5 ml. The sample was left in 15 minutes at room temperature. The solution transferred from the test tubes to cuvettes and the sugar measured at the Absorbance 500 by using spectrophotometer (Winstad, 2001).

3.3.2 Determination of reducing and non-reducing (total) sugar using the Phenol-Sulfuric Assay method

The samples were diluted first with dilution factor 10 x. Then, 1 ml of sample then transferred to 10 ml tube before 500 µl of 4% phenol was added. 25 ml of 90% sulphuric acid was then added into the sample and mixed well the sample with vortex. Then the solution transferred from the test tubes to cuvettes and the sugar measured at the Absorbance 490 by using spectrophotometer (Winstad, 2001).

3.3.3 Determination of Protein Using Kjeldahl Method

The protein content in the *P.florida* was analyzed in term of total nitrogen which is Kjeldahl method. The biomasses of all samples were dried first using freeze dryer. 0.2 mg of biomass were insert into the Kjeldahl tube and the Kjeldahl tablet were added. 10

ml of 97% sulfuric acid were added in each tube and the sample introduced to the digestion process with temperature of 60℃ in 5 hours. The sample then undergoes the distillation process by added 30 ml water, indicator and NaOH (40%) in each tube. Last stage of this determination was titration process where all the samples were titrated using HCl. (Braaksma *et. al.*, 1996)

4 RESULT AND DISCUSSION

4.1 Introduction

This chapter present the result achieved from this present study on the effect of time in the production of polysaccharides and protein. The cultivation of *P.Florida* was maintained in 500 ml Erlenmeyer flask containing 250 ml media (Mushroom Complete Media) MCM. The flask shaken by orbital shaker with speed 100 rpm and were maintained in room temperature 25℃. For the estimation of yield of biomass, the mycelia were freeze-dried for about 4-5 days up to constant weight and then the yields were measured. For the estimation of the yield of extracellular polysaccharide, the sample was analyzed by using Phenol Sulphuric Acid assay by measuring the total sugar. The absorbance of the sample solution after reaction was read and the data was collected at 490 nm by using UV-Vis spectrophotometer. The reducing sugar was analyzed using Somogyi-Nelson method and was read at 500 nm (Eric Fournier, 2001). Therefore the exopolysaccharides was obtained by subtraction of total sugar with reducing sugar. The freeze-dried mycelial biomass in different culture days (6, 9, 12, 15, and 18 days) were used to estimate the nitrogen content by using Kjedahl method. The protein content was calculated using Kjeldahl factor of 4.38 (Braaksma and Schaap 1996).

4.2 Standard curve for phenol-sulphuric acid

A standard curve of Phenol-Sulphuric was prepared as references for the absorbance value from UV-Vis spectrophotometer. The standard solution of sample was done successfully by diluting the sample first into 10 times dilution factor. Then, 500 µL of 4% phenol solution was added into the sample solution. The sample was then added with 25 mL of 90% sulphuric acid solution. The sample solution has been measured by absorbance at wavelength 490 nm through UV-Vis spectrophotometer. The readings of absorbance obtained through UV-Vis spectrophotometer have been tabulated in Table 4.1 below and the standard curve of Phenol-Sulphuric Acid was generated as in Figure 4.1 below.

Table 4.1: Absorbance value of Phenol-Sulphuric Acid

Glucose Concentration, µg/ml	Absorbance, 490 nm
0	0
20	0.23
40	0.56
60	0.76
80	0.88
100	1.06

A standard curve of Phenol-Sulphuric Acid Assay was generated as a reference for this study. The equation of y = mx + c in Figure 4.1 below was determined in order to show the accuracy of result. Since R^2 value is equal to 0.9978 which is near to 1, it can be concluded that this result is accepted and can be used as reference in this present study.

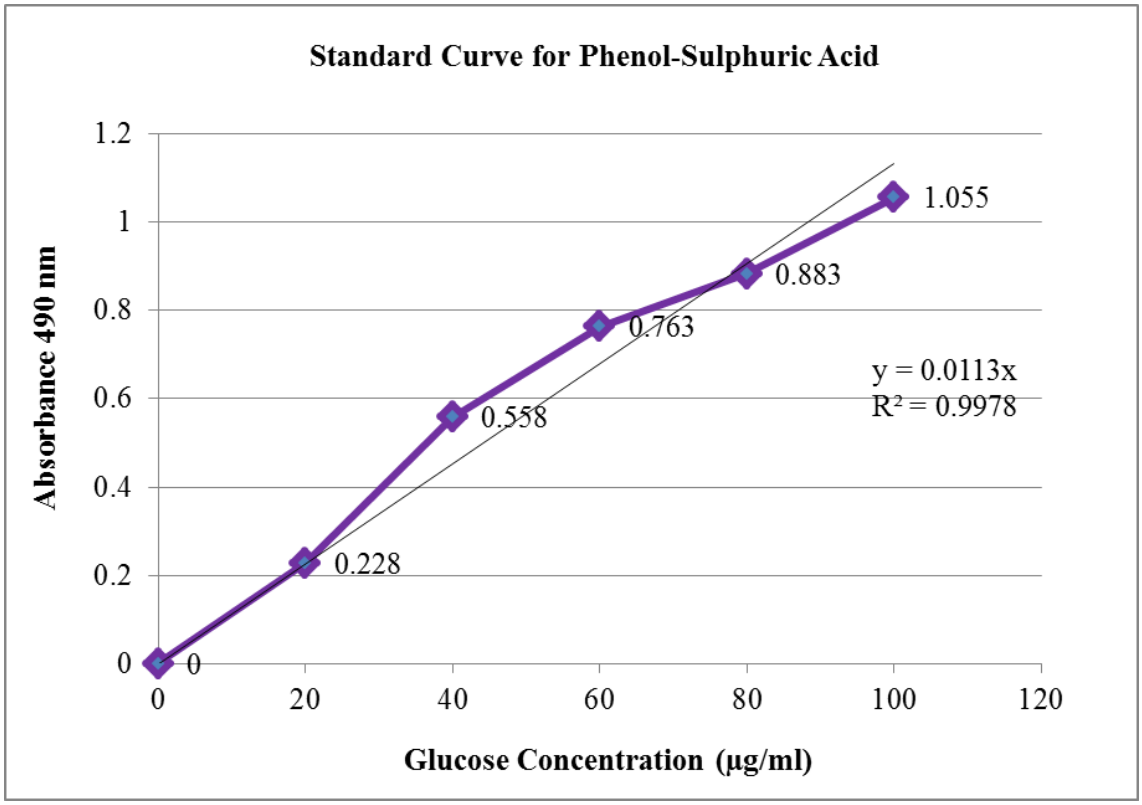

Figure 4.1: Standard curve for Phenol-Sulphuric Acid

4.3 Standard curve for Somogyi-Nelson

A standard curve of Somogyi-Nelson was prepared as references for the absorbance value from UV-Vis spectrophotometer. The standard solution of sample was done successfully by diluting the sample first into 10 times dilution factor. Then, 1 mL of low-alkalinity copper reagent was added into the sample solution. The sample was boiled in 10 minutes and 1 mL of Arsenomolybdate reagent was added into the sample solution. The solution was top up up to 5 mL with distilled water. The sample was being left at room temperature in 15 minutes. Then, the sample solution has been measured by absorbance at wavelength 500nm through UV-Vis spectrophotometer. The readings of absorbance obtained through UV-Vis spectrophotometer have been tabulated in Table 4.2 below and the standard curve of Somogyi-Nelson was generated as in Figure 4.2 below.

Table 4.2: Absorbance value of Somogyi-Nelson

Glucose Concentration, µg/ml	Absorbance, 500 nm
0	0
20	0.16
40	0.35
60	0.52
80	0.70
100	0.86

A standard curve of Somogyi-Nelson was generated as a reference for this study. The equation of $y = mx + c$ in Figure 4.2 below was determined in order to show the accuracy of result. Since R^2 value is equal to 0.9995 which is near to 1, it can be concluded that this result is accepted and can be used as reference in this present study.

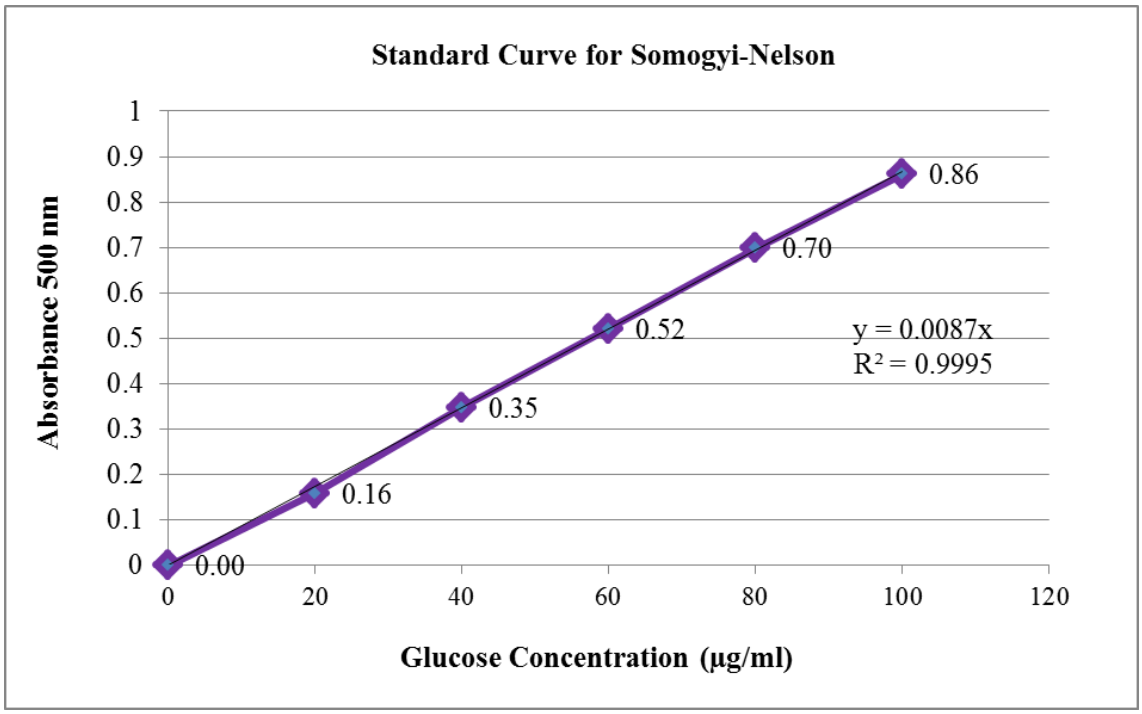

Figure 4.2: Standard curve for Somogyi-Nelson

4.4 Mycelium biomass productions in P.florida (shake flask)

Figure 4.3 indicated the fermentation curve of *P.Florida* species harvested in different day (6, 9, 12, 15, and 18). The highest production of mycelium biomass was obtained at day 15 with dry weight of 2.46 g/L, and the productivity of 0.66 g/L.day as tabulated in Table 4.3. It can be observed in Figure 4.1 that the mycelium biomass exponentially increased from day 6 to 15 days, until it reaches the optimum condition at 15 days. After that, the production was depleted at 18 days. The mycelial biomass was collected in days 15 promotes double production compared with the biomass harvested in days 18, that produced 1.16 g/L dry weight. Lower productivity was also recorded for the 18 days at 0.26 g/L.day compared to the 15 days, as shown in Table 4.3. This is possibly because of all nutrient in media was almost consumed and enhanced production of mycelial by days 15. In comparison with the work by Rosado et al. (2003), their result show that the optimal growth fermentation time for the production of biomass from *P.Florida* was at 9 days incubation.

When comparing to different types of mushroom, Regina Maria (2008) reported that the production of mycelial biomass for species *P.Ostreatus* reached optimum production in 8 days (15.0 g/L). Their production were decreased to 9.0 (g/L) after the 10 days of cultivation. Lee et al. (2007) also reported that the mycelial biomass for species *G.*

applanatum reached stationary phase after 8 days in culture. Their death phase was after 12 days in culture.

Contents of endopolysaccharides (PPS) in the cell increased as culture period increased (Lee et al., 2007). Overall, it is though that the pattern of mycelial biomass production in this present study was quite similar to endopolysaccharides production as reported by Lee et al. (2007). Their trend is that mycelia biomass was increased until it reached an optimum period, and then slightly decreases after that. The optimal period for the production of cell biomass, PPS from *G. applanatum* appears to be dependent on its growth kinetic.

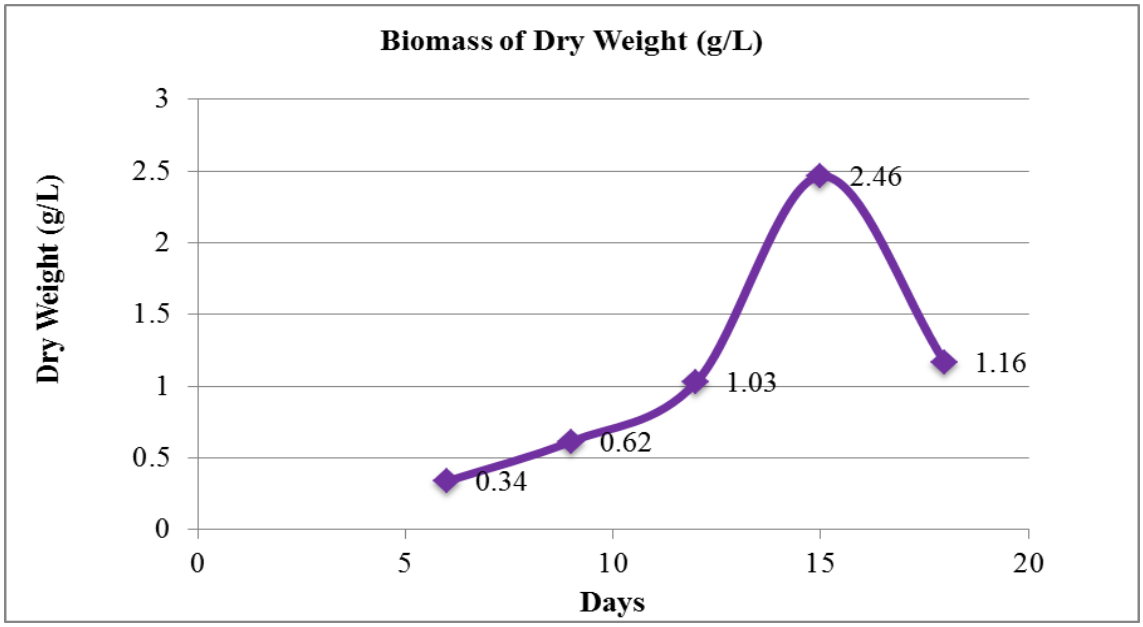

Figure 4.3: Fermentation curve of *P.Florida*

Table 4.3: Dry weight of mycelia biomass in shake flask and its productivity

Days	Dry weight (g/L)	Productivity (g/L.day^{-1})
6	0.34	0.23
9	0.62	0.27
12	1.03	0.34
15	2.46	0.66
18	1.16	0.26

4.5 Polysaccharides productions in P.florida (shake flask)

To maximize the production of polysaccharides, a time-course study was performed on the yield of polysaccharides. Figure 4.4 indicates the fermentation curve of production exopolysaccharides (EPS) from *P.Florida* species in the shake flask that was harvested in different day (6, 9, 12, 15, and 18). The production of EPS initially at days 6 was 6.97 g/L. Then the production was slightly increased up to 7.27 g/L. The EPS production reaches the optimum level up to 8.42 g/L at days 12. After that, the production was declined until days 18 down to 0.96 g/L. By refer table 4.4, the highest production of EPS indicates at days 12 with productivity 1.25 g/L.day. In comparison with productivity at days 18 accounts only at 0.05 g/L.day which is 25 times lower than productivity in days 12.

When comparing different type of mushrooms, J.J. Cheng *et al.,* (2008) reported that, the trend of production polysaccharides form *Fomitopsis pinicola,* the time course study of the polysaccharide yield showed that at 35 days of culture the highest value of 0.38 ± 0.01 g/l was achieved). The production after days 35 were decreased. Therefore, based on the growth curves of the polysaccharide yield, the days 35 culture was chosen to produce highest polysaccharides. In addition, *P.Florida* polysaccharides yield trend is comparable with another different type of mushroom. From the work done by Hong Gao *et al.,* (2006) with mushroom *Agaricus brasiliensis.* The production of polysaccharides shows increasing in trend from hours 12 with 0.05 (g/L) to hours 60 with 0.18 (g/L). After that, the production polysaccharides exponentially decreased down to 0.12 (g/L) at hours 84.

From all the results implies that the highest polysaccharide yield production change with the time of cultivation. Harvest time selection is also an important factor to obtain maximal fungal production (Cheng *et al.,* 2008).

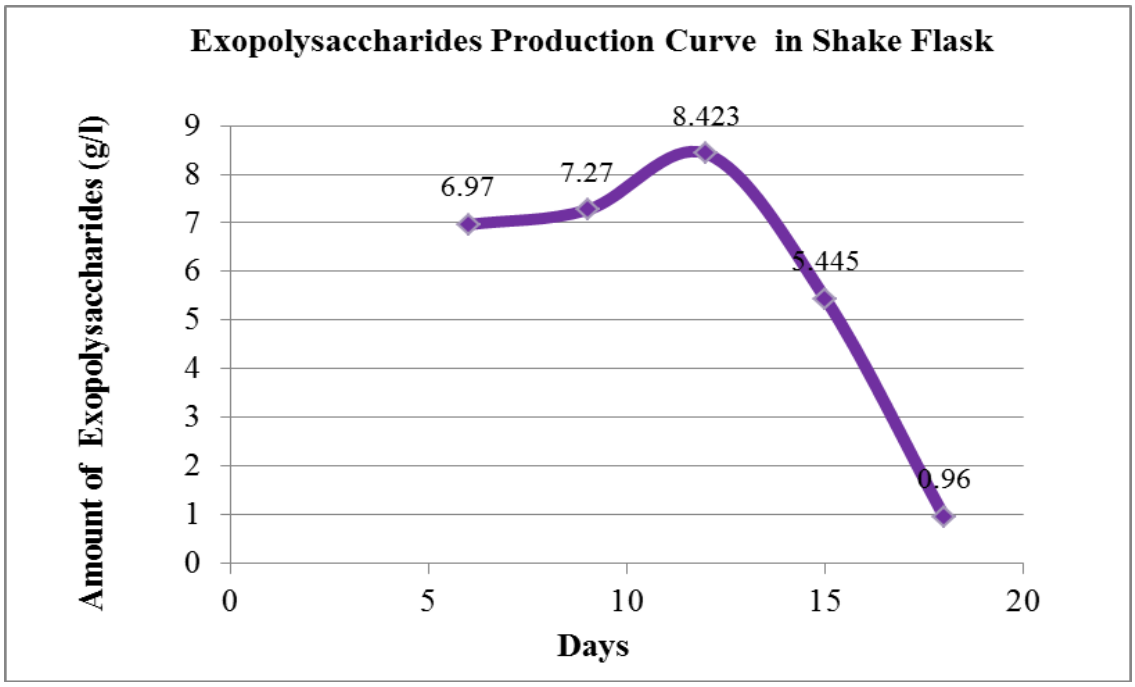

Figure 4.4: Exopolysaccharides production curve in shake flask

Table 4.4: Exopolysaccharides production in shake flask and its productivity

Days	Exopolysaccharide (g/l)	Productivity (g/L.day^{-1})
6	6.97	0.81
9	7.27	0.70
12	8.42	1.25
15	5.45	0.36
18	0.96	0.05

4.6 Protein production in P.florida (shake flask)

Figure 4.5 indicates the range of nitrogen content percentage (%) on different harvested days in mycelia biomass of *P.Florida*. It can be seen from Table 4.5 that the production of nitrogen was gradually increased from days 6 (9.80 g) up to days 12 (13.44 g). Then, the production was decreased from days 15 (10.92 g) to days 18 (8.40 g). From the result tabulated at Table 4.4, days 12 of culture shows the highest nitrogen content with 6.55% compared with other harvest days. In contrast, the mycelia biomass harvested at days 18 contributes the lowest percentage of nitrogen, which is at 3.70 %.

The protein amount can be calculated by conversion factor obtained from the nitrogen content result. It was found that the yield of protein was at 28.69%, by the calculation from the highest production on nitrogen in *P.Florida*. These protein values are within the range of 19-38% and are comparable to the work done by Braaksma & Schaap (1996). Braaksma & Schaap (1996) has reported in their study that sample in form of lyophilized mushroom powder of *Agaricus bisporus* yielded up to 28.0% of protein content.

Protein content is often expressed on dry weight (DW) basis, but DW is not constant and is reported that the values is varied from 7.4% to 13.7% (Weaver et al., 1977). It was reported by Weaver et. al (1977) that the total protein content of harvested mushrooms was in range from 19 % to 39% on DW basis. Protein is determined on the basis of total nitrogen content, Kjeldahl factor of N x 4.38 (FAO 1972), and is commonly used elsewhere (Levai 1989). The correction of the Kjeldahl factor from 6.25 to 4.38, which used in this present study, is not based on strict analytical data, but more on the common agreement as practices by other researcher (Braaksma and Schaap 1996).

A specific converting factor has to be used for the calculation of crude protein in mushroom by the Kjeldahl method. This is due to the high proportion of non-protein nitrogen, mainly in chitin. Meanwhile, the factor of 4.16 was recommended by Bauer-Petrovska (2001), who observed a mean proportion of 33.4%, of non-protein (from total nitrogen) in the above-mentioned numerous samples. Barros et al. (2008) also used the converting factor of 4.38.

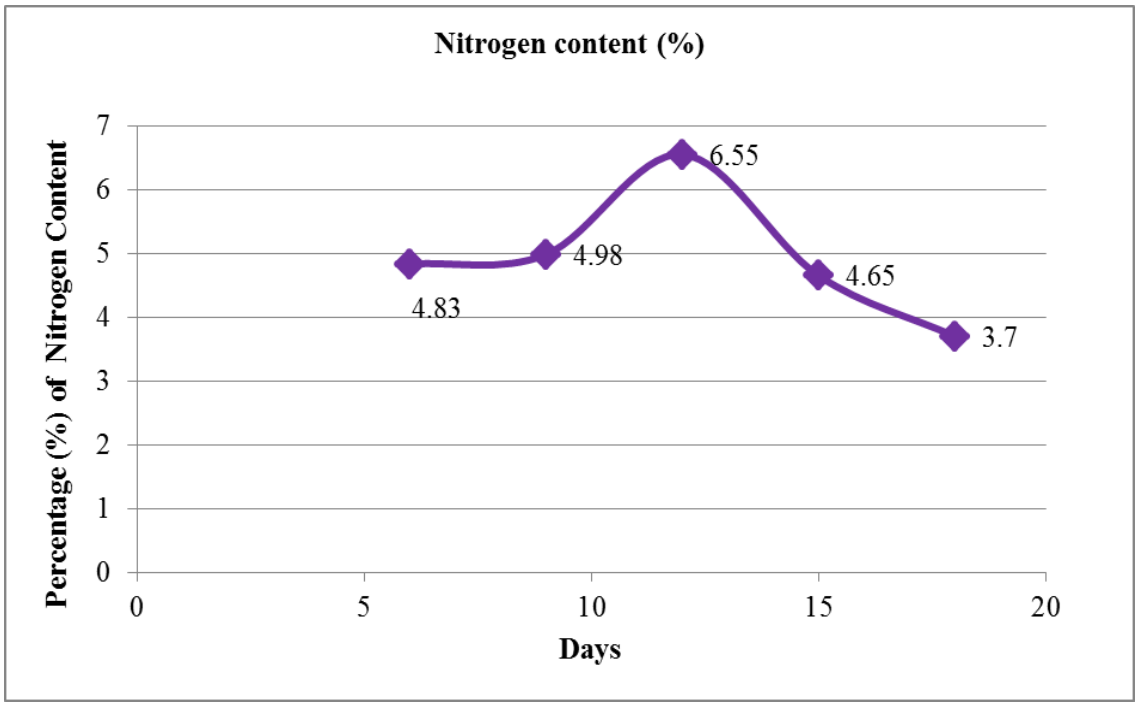

Table 4.5: The nitrogen content (%) in the mycelium of *P.florida*

Table 4.5: Quantity of Nitrogen in mycelium *P.Florida* in the shake flask

Days	Dry Weight (g)	N sample (g)	%N	Protein content (% N X 4.38)
6	0.20	9.80	4.83	21.15
9	0.23	11.55	4.98	21.81
12	0.21	13.44	6.55	28.69
15	0.24	10.92	4.65	20.37
18	0.23	8.40	3.7	16.21

4.7 Mycelium biomass and polysaccharides productions in P.florida (air lift bioreactor)

Table 4.6 indicates the biomass, exopolysaccharides and protein production in airlift bioreactor and shake flask at days 6. From the table, it can be seen that the amount of dry weight mycelia biomass in airlift bioreactor at days 6 was 3.13 g/L. It indicates 9 times larger than the production in the shake flask with 0.34 g/L. While for the exopolysaccharide production in airlift bioreactor show the 3 times higher than shake flask with amount of production up to 21.15 g/L compared to the production in shake flask which is about 6.97 g/L. However, the production of protein in airlift bioreactor shows slightly higher in protein content (21.98%) compared shake flask with only 21.16%. From this comparison, it can be seen that the productivity mycelia biomass, exopolysaccharides and protein in airlift bioreactor much higher than the production in shake flask. The reasons behind these phenomena are, due to its gentle agitation and aeration hydraulic make the productivity of mycelia biomass, exopolysaccharides and protein in airlift bioreactor more efficient than in shake flask (Najafpuor, 2007).

Table 4.6: Dry weight of mycelia biomass and polysaccharides in airlift bioreactor.

Fermentation Modes	**Days of culture**	**Mycelial Biomass (g/L)**	**Exopolysaccharides (g/L)**	**Protein content (% N X 4.38)**
Airlift Bioreactor	6	3.13	21.15	21.98
Shake Flask	6	0.34	6.97	21.16

5 CONCLUSION

5.1 Conclusion

In this present study, the production of polysaccharides and mycelium from white mushroom (*P. florida*) by submerged culture fermentation (SCF) and determination of protein content by using Kjeldahl method was successfully done. The harvest time effect of production of polysaccharides, biomass and protein in *P. florida* was investigated.

From the experimental work done in shake flask from *P. florida* under the maximum condition at temperature 25℃ with speed 500 rpm, the result shows that the production of biomass, polysaccharides and protein are related with the harvest time. It was found that, the maximum production day in producing biomass were at days 15 with dry weight of 2.464 g/L. While the production polysaccharides were found highest at days 12 with 8.42 g/L. The production of protein indicates the highest productivity at the same day as polysaccharides (days 12) with 28.69% crude protein. From all the results implies that the highest biomass, polysaccharides and protein yield production change with the time of cultivation. Thus it can be concluded that harvest time selection is an important factor to obtain maximal fungal production (Cheng *et al.,* 2008).

The comparison of production biomass, polysaccharides and protein in airlift bioreactor and shake flask was done in this research. The result shows that the productivity in airlift bioreactor much higher than the productivity in the shake flask. It can be seen that the amount of dry weight mycelia biomass in airlift bioreactor at days 6 was 3.13 g/L that is 9 times larger than the production in the shake flask with only 0.34 g/L. While for the polysaccharide production in airlift bioreactor shows 3 times higher (21.15 g/L) than in the shake flask (6.97 g/L). However, the production of protein in airlift bioreactor shows only slightly higher in protein content (21.98 %) compared shake flask with only 21.16%. Due to the gentle agitation and aeration hydraulic in airlift bioreactor make the productivity of mycelia biomass, exopolysaccharides and protein more efficient than in shake flask (Najafpuor, 2007).

5.2 Future work

To improve this research project accuracy, several recommendations has been proposed during the study. Firstly, the production of mycelial biomass, polysaccharides and protein in airlift bioreactor can be maximized by increase the duration fermentation day by refer the maximum production harvest time in shake flask.

Secondly, in handling the experiment for airlift bioreactor, the stopper in the reactor always opened due to high pressure in the reactor. These will be contributing to contamination on the sample due to exposure to the surrounding. These problem can be avoid by reduce the gauze used in the filter tube. By doing this, the air flows are easier going in and out.

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APPENDICES

A-1 Selection of standard curve using Mushroom Complete Medium (MCM) by using control

Sample	OD : 500nm Somogyi-Nelson	OD : 490nm Phenol Sulphuric	Total sugar (mg/ml)	Reducing sugar (mg/ml)	Exo polysaccharide (mg/ml)
MCM	0.27	0.51	22.70	17.31	5.4

The amount of exopolysaccharide is in small amount (5.4 mg/ml) and it is acceptable to use for analysis.

A-2 Analysis of Phenol-Sulphuric Acid from sample cultured in shake flask

Days	OD 490nm (replicate)			Average	Y=A/0.0113 (µg/ml)	Y=[(A/0.0113) x (Df/1000)] (mg/ml)
	1	2	3			
6	0.52	0.55	0.55	0.54	47.61	23.81
9	0.52	0.52	0.65	0.56	49.82	24.91
12	0.54	0.53	0.52	0.52	46.33	23.16
15	0.41	0.44	0.38	0.42	37.52	18.76
18	0.42	0.46	1.01	0.44	38.76	19.38

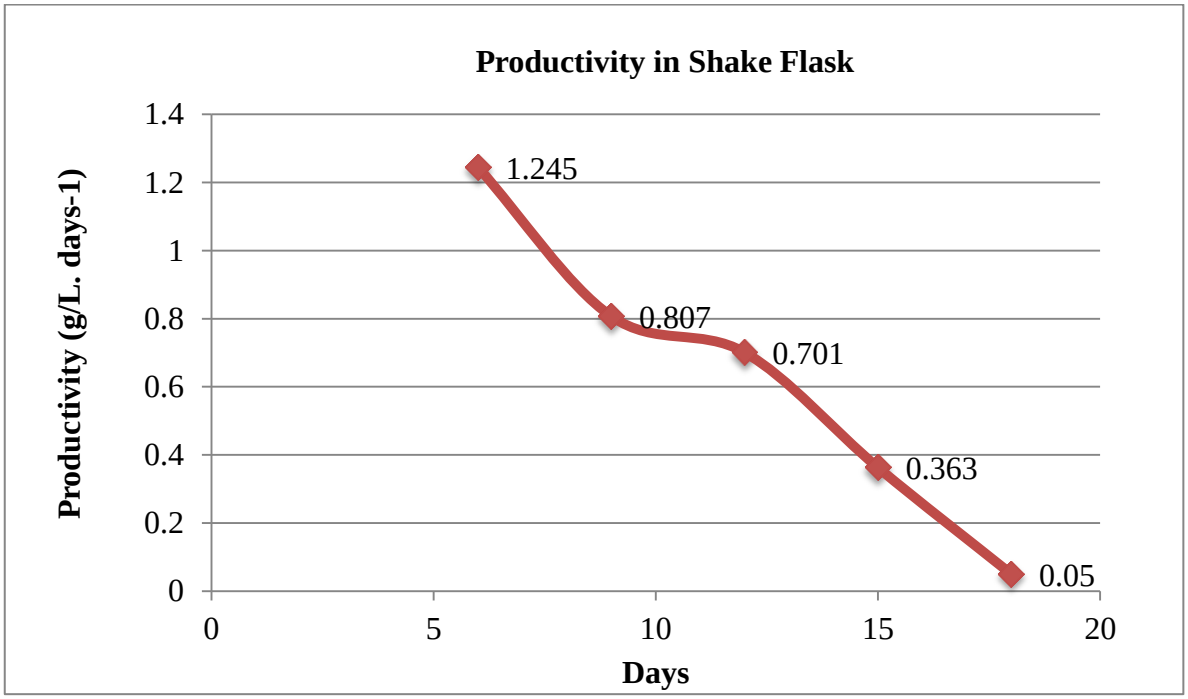
A-3 Analysis of Somogyi-Nelson from sample cultured in shake flask

Days	OD 500nm (replicate)			Average	Y=A/0.0087 (µg/ml)	Y=[(A/0.0087) x (Df/1000)] (mg/ml)
	1	2	3			
6	0.27	0.29	0.29	0.28	32.68	16.34
9	0.30	0.30	0.32	0.31	35.29	17.64
12	0.57	0.26	0.26	0.26	29.48	14.74
15	0.22	0.23	0.25	0.23	26.63	13.32
18	0.33	0.44	0.31	0.32	36.84	18.42

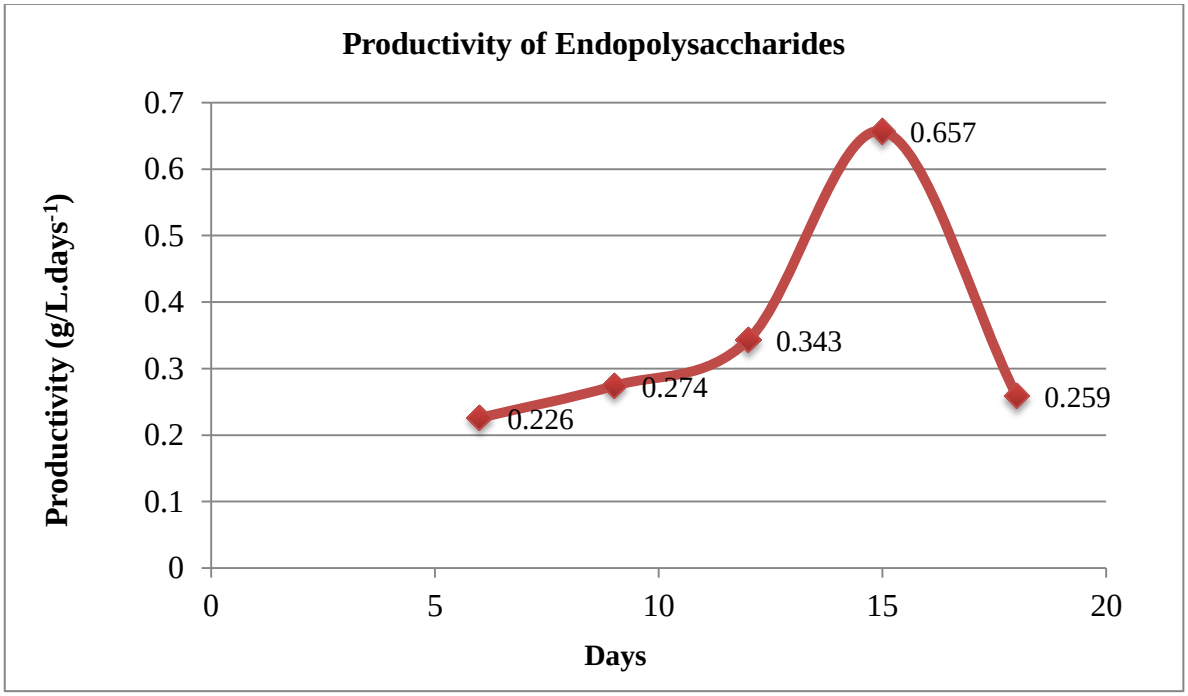
A-4 Analysis of exopolysaccharides and productivity from sample cultured in shake flask

Days	Total sugar (mg/ml)	Reducing sugar (mg/ml)	Exopolysaccharide (mg/ml)	Productivity ($g/L.day^{-1}$)
6	23.81	16.34	7.47	1.25
9	24.91	17.64	7.27	0.81
12	23.16	14.74	8.42	0.70
15	18.76	13.32	5.44	0.36
18	19.38	18.42	0.96	0.05

A-5 Exopolysaccharides productivity in shake flask



A-6 Mycelial biomass productivity in shake flask



A-7 Analysis of Phenol Sulphuric from sample cultured in airlift bioreactor (5L)

Days of culture	Replicate	OD (490nm)	Average	Y=A/0.0113 (µg/ml)	Y=[(A/0.0113) x (Df/1000)] (mg/ml)
6	1	0.53	0.53	46.67	23.33
	2	0.44			
	3	0.57			

A-8 Analysis of Somogyi-Nelson from sample cultured in airlift bioreactor (5L)

Days of culture	Replicate	OD (500nm)	Average	Y=A/0.0087 (µg/ml)	Y=[(A/0.0087) x (Df/1000)] (mg/ml)
6	1	0.39	0.37	42.30	21.15
	2	0.36			
	3	0.35			

A-9 KJELDAHL METHOD