MOLECULAR IDENTIFICATION OF *SCHIZOPHYLLUM COMMUNE* AND ITS PRODUCTION OF SCHIZOPHYLLAN

By

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ABSTRACT

MOLECULAR IDENTIFICATION OF *SCHIZOPHYLLUM COMMUNE* AND ITS PRODUCTION OF SCHIZOPHYLLAN

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Schizophyllan is a polysaccharide produced from the fungus, Basidiomycete, *Schizophyllum commune*. The fungus was identified through molecular identification of internal transcriber spacer (ITS) and 18S rDNA region. ITS was identified using ITS-1 and ITS-4 primers while 18S rDNA was identified using EF4 and EF3 primers. By blasting all sequences in NCBI, it was found that all sequences matched with the DNA sequence for *Schizophyllum commune* in the Gene bank which the fungus species is capable in producing schizophyllan. The production of schizophyllan was carried out by growing the fungus in a semi synthetic medium for 20 days. Dry cell weight, determination of glucose determination and analysis of schizophyllan were carried out throughout the 20 days of fermentation. From the results obtained, it showed that at day 4, the fungus entered the log phase where the dry cell weight started to increase rapidly and while the concentration of glucose reduced in the medium. This was because the fungus utilized glucose and converting them into schizophyllan which in turn increasing the product concentration. The growth of the fungus was able to grow continuously even though the glucose

content in the fermentation medium had depleted. This might be due to the utilization of its own product, schizophyllan as the alternative carbon source when glucose was insufficient for its survival. When the schizophyllan was depleting, the growth also started to decrease and eventually entered the death phase. As a conclusion, the fungus *Schizophyllum commune* was identified through NCBI BLAST by amplifying and sequencing the ITS region and 18S rDNA region. The fungus was able to produce the polysaccharide, schizophyllan from glucose as its primary carbon source and when glucose was depleted, the fungus can change to the utilization of its own product, schizophyllan as the alternate carbon source for survival purposes.

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Last but not least, I am very grateful for the moral support and encouragement given by my family and friends in completing this project.

DECLARATION

I hereby declare that the project report is based on my original work except for quotation and citations which have been duly acknowledge. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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CHERYL SIM YAN-YI

APPROVAL SHEET

The project report entitled "**MOLECULAR IDENTIFICATION OF FUNGUS AND ITS PRODUCTION OF SCHIZOPHYLLAN**" was prepare by CHERYL SIM YAN-YI was submitted as partial fulfilment of the requirement for the degree of Bachelor of Science (Hons) Biotechnology at Universiti Tunku Abdul Rahman.

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Date: _____

PERMISSION SHEET

It is hereby certified that **CHERYL SIM YAN-YI** (ID No: **10ADB03286**) has completed this final year project entitled "MOLECULAR IDENTIFICATION AND ITS PRODUCTION OF SCHIZOPHYLLAN" under supervision of Dr. Lisa Ong Gaik Ai from the Department of Biological Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public

Yours truly,

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(CHERYL SIM YAN-YI)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

β-glucan	Beta glucan
BLAST	Basic Local Allignment Search Tool
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic
dNTP	Deoxyribonucleotide
EDTA	Edetic acid
EMBL-EBI	European Molecular Biology Laboratory - European Bioinformatics Institute
ETS	External transcriber spacer
ITS	Internal transcriber spacer
KH_2PO_4	Potassium dihydrogen phosphate
$MgCl_2$	Magnesium chloride
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate heptahydrate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Center of Biotechnology Information
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PKT	PhileKorea Technology
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribobucleic acid
SDS	Sodium dodecyl sulphate
sp.	Species
TBE	Tris-Borate-EDTA
TE	Tris-EDTA

Tris-HCl	Tris hydrochloride
A	Absorbance
bp	base pair
˚C	Degree celcius
g	Gram
g/l	Gram per litter
kb	Kilobase
µl	Microliter
µg/ml	Microgram per millilitre
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
N	Normal
nm	Nanometer
pmol	picomole
rpm	Revolutions per minute
sec	Second
U/µl	Unit per microliter
× g	Gravity

CHAPTER 1

INTRODUCTION

There are five kingdoms in the world: Monera, Protista, Plantae, Animalia and Fungi. Animalia, Fungi and Plantae are three distinct kingdom of eukaryotes. What makes fungi different is their lifestyles which are different although they might have some similar basic genetics structure as animals and plants. Many people mistakenly grouped fungi under plants. This was due to the reason that some of them assumed during evolution, fungi had lost its function for photosynthesis and thought that if they were not animals then they must be plants (Moore and Frazer, 2002c).

There are a few phyla in the diversities of kingdom fungi. Chytridomycota, Zygomycota, Ascomycota and Basidiomycota are the four phyla of true fungi (Moore and Frazer, 2002a). Basidiomycota also known as the sister group to Ascomycota where both are combined in the subkingdom of dikarya (Moore, Robson and Trinci, 2011). Basidium, where its meiocyte is produced at the end of the hyphal filament is the common morphological character that is delimitating the group (Moore and Frazer, 2002a). Plant pathogens that cause smut and rust diseases like ectomycorrhizal species which are important to the forest ecosystem and saprotrophic species like the white-rot fungi where it decays the lignin of litters, woods or dung and also most frequently found

mushroom fungi are all included in Basidiomycota (Moore, Robson and Trinci, 2011).

One of the fungus species under phylum Basidiomycota is *Schizophyllum commune*. Species *Schizophyllum commune* came from the domain Eukarya as it possesses a nucleus; kingdom Fungi as it shares the characteristic with other phyla of fungi; phylum of Basidomycota as it produces fruiting body in club shapes, haploid basidiospores and dikaryon as its domain life stage; class of Agaricomycetes; order of Agaricales which is known as gilled fungi which is below the mushroom cap, family of Schizophyllaceae and genus *Schizophyllum* which means split gills (Maki, 2008).

There are 3 main purposes of studying fungus. Firstly, the roles of the fungi itself in the nature are very important in maintaining the life of earth. Secondly, fungi have a lot of potentials and functions that are useful in the industry nowadays and also in the future. Lastly, fungi provide us with easily studied model organisms as they most likely to be the oldest evolutionary line of eukaryotes.

In 1719, Dillenius firstly found that *Schizophyllum commune* could produce homoglucan that was called as schizophyllan. Schizophyllan is a jelly-like slimy material which is non-ionic, soluble in water and a neutral extracellular polysaccharide produced by the basidiomycete fungus known

as *Schizophyllum commune* (Zhang, et al., 2013). Schizophyllan can produce biopolymer from carbon source such as glucose. Schizophyllan is a homoglucan with molecular weight range of 6 to 12×10^6 g/mol (Kumari, Survase and Singhal, 2008). It is also a β (1→3)-D-glucan polysaccharide with β (1→6)-branched lateral glucose residues (Kony, et al., 2007) as shown in Figure 1.

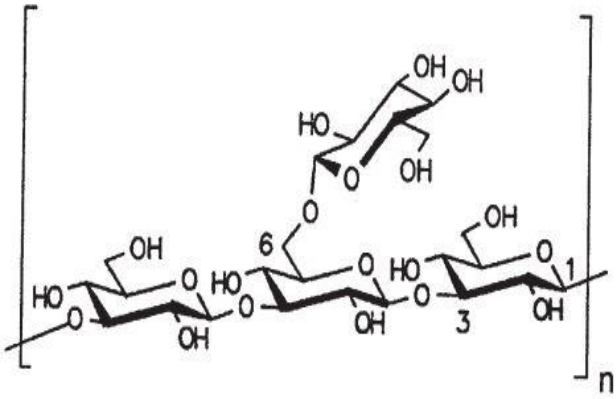


Figure 1.0: Primary molecular structure of schizophyllan.
(Source: Rau, 1999)

Previous researchers have also reported the industrial value of schizophyllan. *Schizophyllum commune* has great importance in the pharmaceutical and food industries, since it can produce metabolites which are essential in the production of industrial products (Reyes, Brabl and Rau, 2009).

The objectives of this research were to:

i) identify the fungal species through molecular identification of the 18S rDNA and ITS regions of the fungus.

ii) produce the schizophyllan by the locally isolated fungus through submerged fermentation.

iii) determine the dry cell weight (DCW) of the fungus, the production of schizophyllan and the utilization of the glucose by the fungus.

CHAPTER 2

LITERATURE REVIEW

2.1 Characteristic and Distribution of *Schizophyllum commune.*

Schizophyllum commune belongs to phylum Basidiomycota and subphylum Agaricomycotina which can be easily identified. It has small white colour fruiting bodies which are lack of stems. The way they attached themselves on the dead wood of deciduous trees were similar to those small bracket mushrooms as shown in Figure 2.1 (Kuo, 2003). The fungus is a non-sporulating endophytic fungus (Guo, 2010) and also possesses a metabolic ability to break down lignin where the cellulose remains when the fungus grows on the dead woods (Yadav and Tyagi, 2005). *Schizophyllum commune* has gills on its underside, instead of pores or a simple, flat surface which differentiate them from those bracket fungi. On close review, the gills turned out to be merely folds in the under surface and they were very distinctively split (Kuo, 2003). The split of gills are useful in the adaptation of changing environment. According to Else Vellinga research in 2003, he found that *Schizophyllum commune* that were grown on the stumps and dead branches were opened up and soft during the rainy season as shown in Figure 2.2(a) below and they turned back to their hard and white stage when there was sunshine again as shown in Figure 2.2(b) below (Vellinga, 2013).

Figure 2.1: *Schizophyllum commune* growing on dead woods (Kuo, 2003).

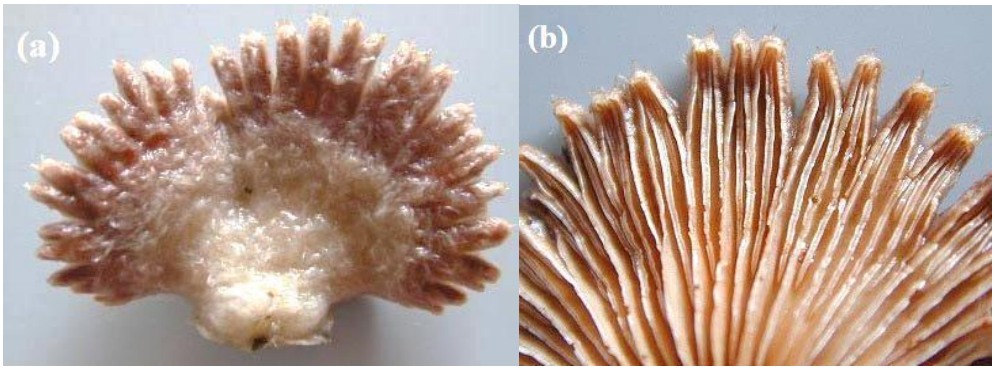


Figure 2.2: Condition of the underside of *Schizophyllum commune* during rainy sand sunny days (a) Fungus opened up and soft. (b) Fungus in hard and white stage (Vellinga, 2013).

Schizophyllum commune is very common and widely spread throughout all over the world in Africa, America, Asia, Australia and Europe. They are found usually on wide range of timber and many other plant based substrate (Nature, n.d.).

2.2 Molecular Identification

The most important challenge in identifying fungus is the design of an appropriate polymerase chain reaction (PCR) primer specifically for fungal

DNA at the same time minimizing the co-amplification of similar target DNA from other source besides fungus (Anderson, Campbell and Prossers, 2003). Usually rDNA were widely used for the identification of fungal isolates variation (Maheshwari, 2011) and also the constructing phylogenetic trees (Deacon, 2006). The eukaryotic DNA that corresponds to the rDNA regions consists of a cluster of 50 to a few hundred copies of repeated transcription unit where each of them consists of 18S-5.8S-28S rRNA arranged in that order (Moore and Frazer, 2002d) as shown in Figure 2.3 below.

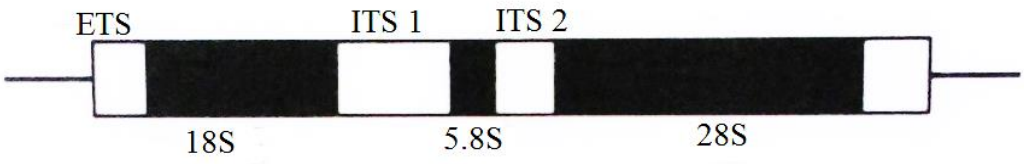


Figure 2.3: Organization of the fungi rDNA gene (Deacon, 2006).

rDNA composed of tandemly repeated unit where each unit composed of 18S, 5.8S and 28S rDNA. However, in between the conserved rDNA regions are the quickly evolving regions which are useful for identifying the relationship within more closely linked groups which are among species or between isolates of one species of fungi (Moore and Frazer, 2002d). The internal transcribed spacer (ITS) and external transcribed spacer (ETS) are the 2 noncoding regions which exist in each repeat can provide a source of DNA polymorphism (Maheshwari, 2011).

ITS universal primers were designed specifically to amplify fungal ITS regions which is within the transcription units of DNA flanking 5.8S rRNA while the

co-amplification of plant or other eukaryotic DNA is minimized. Even though a serious factor in designing PCR primers to fungal 18S rDNA gene is their specificity towards the target fungal DNA, attaining that specificity may eventually make the view to be bias that we attained by trying to avoid the amplification of non-fungal DNA. The reason was due to some regions of fungal 18S rDNA gene sequence shared great similarity with other eukaryotes. Moreover, 18S rDNA genes sequence is commonly known to have the capability of resolving taxonomic groups to the level of genus. However, the taxonomic resolution of fungal 18S rDNA and ITS sequence were limited by the existing availability of information held in the database (Anderson, Campbell and Prossers, 2003).

2.2.1 18S rDNA

Due to the numerous established universal fungal primers were based on the unique and well conserved regions of the 18S rDNA, this gene was selected to identify the fungus genus and species. This increases the probability of getting the PCR product from most of the fungi for sequencing purpose. For example, hybridization of *Eco*RI-digested genomic DNA of the fungus *Trichophyton rubrum* can detect polymorphism with a probe PCR amplified from the 18S rDNA (Moore and Frazer, 2002b). Next, the searching of similarities were more convenient due to the huge number of 18S rDNA sequences in GenBank (Gontia-Mishra, et al., 2013) and also helps the probe specificity evaluation before the start of extensive laboratory screening. In addition, this region actually evolved quite slowly compared to the ITS region which varies more

(Deacon, 2006), thus, more suitable for finding consensus conserved regions within the fungus. Lastly, the repetitive nature of rDNA, over 100 copies of which usually exists per fungal genome makes the rDNA-based detection more sensitive than detection system based on single copy genes (Wu, et al., 2003). A phylogeny tree of the true fungi can be built based on the 18S rDNA gene sequence (Moore and Frazer, 2002c). According to a research by Anderson, Cambell and Prossers in year 2003, to amplify the 18S rDNA region, primer pairs such as EF4 forward primer and EF3 reverse primer can be used for identification and also phylogenetic analysis of the fungus. The approximate PCR product size was about 1.4 kb to 1.5 kb (Anderson, Campbell and Prossers, 2003).

2.2.2 ITS

ITS region between the 18S and 28S rDNA is considered as a more variable region within the species and strains (Alam, et al., 2010). It has been widely used to distinguish between species (Deacon, 2006). ITS sequences were repetitive, polymorphic and ideal candidates as molecular markers (Moore and Frazer, 2002b). ITS region has 4 advantages over the other regions. First, ITS has multiple copy, thus low quantity of starting material needed for effective amplification. Second, ITS has well-conserved fungi specific priming sites which were directly adjacent to the multiple highly variable regions. Third, there were many accessible sequences that were already ready for evaluation. It significantly facilitates the identification of unknown samples. Lastly, ITS correlates well with morphologically defined species in many groups (Prey,

Kennedy and Burns, 2008). Although ITS regions have functioned through the processing of primary rRNA transcripts but at times they are also called non-functional sequences as this region are unrestricted to mutate without adverse selection pressure (Moore and Frazer, 2002d). ITS-1 and ITS-4 universal primers are frequently used for the identification of fungus. ITS-1 forward primer and ITS-4 reverse primer were chosen to amplify the ITS 1 and ITS 2 region as shown in Figure 2.4 below. The PCR product size was about 500 bp to 800 bp (Anderson, Cambell and Prossers, 2003).

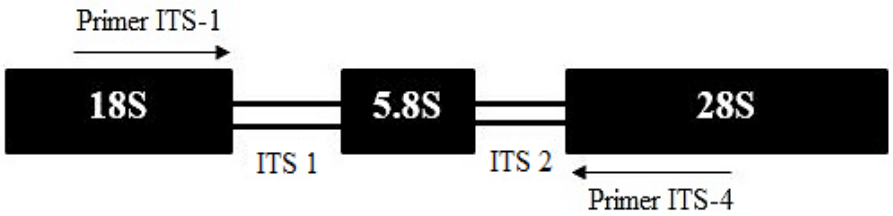


Figure 2.4: Forward primer ITS-1 and reverse primer ITS-4 showing the binding position on the rDNA gene in a fungal genome (Babu, Saikia and Aurora, 2010)

2.3 Production and analysis of Schizophyllan

The biopolymer schizophyllan production can be produced from the fungus *Schizophyllum commune* because the biopolymer is non-pathogenic to human and it can be easily cultured in the lab (Yadav and Tyagi, 2005). Schizophyllan can be extracted from fruit body, mycelium and fermentation broth. The extraction process of schizophyllan from fruiting body and mycelium has several disadvantages such as time consuming, complicated process and high cost during the separation (Shu and Hsu, 2011). Since the polysaccharide schizophyllan can produced and excreted into the fermentation broth, the

tedious hyphal disruption can be omitted (Yadav and Tyagi, 2005). Thus, more attention was focused on exo-schizophyllan in the fermentation broth (Shu and Hsu, 2011). Exo-polysaccharides were high-molecular-weight polymers that were synthesized by the microorganism and secreted into the surrounding environment (Zhang and Bobo, 2011).

In bioreactor cultivation, the agitator created shear stress which could reduce pellet formation at the same time encouraging the release of schizophyllan. However, shear stress that is too high can cause damage to the hyphae and its product itself which can lead to the cell fragments that obstruct the cell separation throughout the following downstream processing (Rau, 2004). Besides shear stress, optimum oxygen supply is another factor to improve the schizophyllan production (Rau, 1999). Therefore, the mixing and mass transferring of the pseudoplastic fluid and the schizophyllan release form the cell wall have to be compromised with the applied agitation speed at the same time generating low shear stress on the fungus and its product (Rau, 2004).

In this research, mycelium was culture to obtain the metabolite by using submerged fermentation method. Submerged fermentation is a method used for the production of microorganisms including mushroom mycelium in synthetic defined medium, complex medium or on a variety of waste substrates without the stage of sporulation of the microorganisms during the fermentation process (Zhang and Bobo, 2011). Besides that, mushroom mycelium has comparable nutritive value, medicinal value and industrial value compare to mushroom

fruiting body and also a shorter cultivation period. It is a valuable commodity to be explored (Zhang and Bobo, 2011). Since mycelium of the fungus can get tangled at the propeller in a bioreactor, thus, a seed tank is more suitable to be used during the fermentation process. However, installation and operation of a seed tank which is more suitable for fungus would be very costly. Hence, isolation of schizophyllan can also be done in a shake flask.

Schizophyllan will not be produced from the fungus if it was grown under normal condition such as in PDA or PDB. The polysaccharide schizophyllan only will be produced in the presence of suitable carbon source such as glucose and soluble starch in the submerged fermentation (Yadav and Tyagi, 2005). Although glucose and mannitol had been reported as good substrates for vegetative growth (Adejoye, et al., 2007) but due to glucose was easier to be obtained, glucose was chosen for the project experiment. Semi-synthetic production medium [in (g/L): Glucose – 30.0, yeast extract – 1.0, $MgSO_4 \cdot 7H_2O$ – 0.5, KH_2PO_4 – 1.0] as reported by Rau, et al. (1992) can produce maximum yield of schizophyllan. Besides that, another journal published by Kumari, Survase and Singhal, 2008 also used the semi synthetic medium as suggested by Rau, et al. (1992) for the production of schizophyllan. Thus, this medium composition was selected for the following experiment. When the carbon source became limiting, the fungus could switched the utilization of glucan as carbon source due to its ability to produce ß-glucanases which is a ß-glucan degrading enzyme (Reyes, Brabl and Rau, 2009). The production of ß-glucanases can degrade and metabolize the schizophyllan which somehow increased the glucose concentration a little together with a

decreased in specific viscosity of the ß-glucan as the cultivation continues after the glucose was depleted (Rau, 2004).

Coconut water can be used as a medium for the production of schizophyllan. The performance of *Schizophyllum commune* grown in coconut water was determined to be in parallel with the performance in semi-synthetic medium. The peak production of schizophyllan was obtained a day earlier than using semi-synthetic medium. Matured coconut water contained primarily sugar either in reducing or non-reducing form and minerals with fats and proteins as minor constituent. The better performance of the fungus to produce schizophyllan derived from matured coconut (Reyes, Brabl and Rau, 2009). However, the composition of coconut water can vary from one coconut with another coconut. This can affect the accuracy of the data obtained for the production of schizophyllan. Thus, semi-synthetic media [in (g/L): Glucose – 30.0, yeast extract – 1.0, $MgSO_4 \cdot 7H_2O$ – 0.5, KH_2PO_4 – 1.0] was still preferred to be used as the medium (Rau, et al, 1992).

According to another research done by Shu and Hsu (2011) which was the fermentation of *Schizophyllum commune* using rise hull hydrolysate with or without removal of toxic was done to compare the product, schizophyllan yield. It was found that for rice hull hydrolysate that did not detoxified with activated charcoal have some inhibitors such as furfural and sodium present which affected its growth and hence giving a lower production yield. However for the fermentation with detoxified rice hull hydrolysate, the product yield was

higher as the inhibitor had been removed. This research also found out that acetic acid could reduced the activity of the inhibitor especially at high salt concnetration condition (Shu and Hsu, 2011).

Schizophyllan can be precipitated from the culture medium with organic solvent in an aqueous solution. The organic solvent that is miscible in water at all concentration is either ethanol or isopropanol which are normally be used as it will minimize the dielectric constant which in turn decreased the solubility of the polysaccharide (Yadav and Tyagi, 2005).

For glucose determination, the dinitrosalicylic acid (DNS) reagent developed by Sumner and co-worker for the determination of reducing sugar was composed of dinitrosalicylic acid, Rochelle salt, phenol, sodium bisulfite, and sodium hydroxide. According to the authors of the test, Rochelle salt was introduced to prevent the reagent from dissolving oxygen while phenol was used to increase the amount of colour produced. Bisulfite was used to stabilize the colour obtained in the presence of the phenol and the alkali was required for the reducing action of glucose on dinitrosalicylic acid (Miller, 1959).

2.4 Application of Schizophyllan

Schizophyllum commune was a model organism for developmental biology, population genetics, transmission genetics and many other different quantitative genetics study on growth rate (Alam, et al., 2010). Its product,

Schizophyllan has numerous potential applications, such as thickener for cosmetic lotions and can act as oxygen-impermeable films for food preservation (Teoh and Don, 2012). In Japan, schizophyllan was used in the manufacturing of airtight film to package fruits and vegetables (Yadav and Tyagi, 2005).

Besides that, schizophyllan also can enhance the anticancer therapies (Ooi and Liu, 2007), antitumor agent and also enhances the effect of vaccines (Teoh and Don, 2012) as it is a macrophage stimulant which is not mitogenic to lymphocytes (Sugawara, Lee and Wong, 1984). Schizophyllan also can acts as a biological response modifier and a non-specific stimulator of the immune system by degrading glucan (Rau, 1999). The most important key points of antitumor and immunomodulating effects of mushroom polysaccharides are the prevention of oncogenesis by oral consumption of mushrooms or their preparations also known as cancer-preventing activity, direct antitumor activity to induce the apoptosis of tumor cells also known as direct tumor inhibitory activity and tumor metastasis also known as immunoenhancing activity (Zhang and Bobo, 2011). Schizophyllan has also been tested for the use in enhanced petroleum recovery (Sutivisedsak, et al., 2013). It was conventionally produced by submerged culture fermentation using glucose as a carbon source (Nongnuch, et al., 2012).

CHAPTER 3

MATERIALS AND METHODS

3.1 Maintenance of culture and seed culture preparation

A disk of 5 mm mycelia inoculum from the culture collection was placed into the middle of the potato dextrose agar (PDA) as shown in Figure 3.1 below. The culture was then incubated at 30 ℃ for 6 days in an incubator oven (Adejoye, et al., 2007).

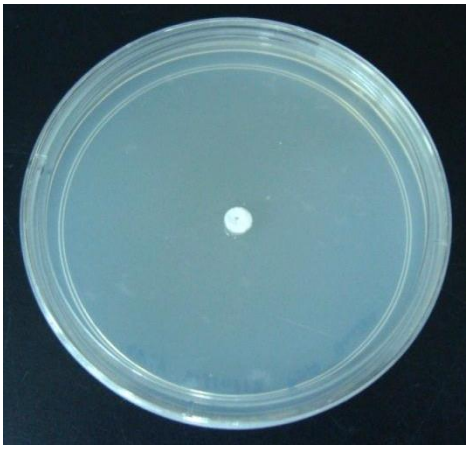


Figure 3.1: Fungus culture in the middle of PDA.

3.2 Molecular Identification

3.2.1 DNA Extraction

A sterile toothpick was used to take a small lump of mycelia from the PDA and transferred to a 1.5 ml of microcentrifuge tube. Lysis buffer (500 µl) containing 400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl and 1% sodium dodecyl sulphate, 1.25 µl of proteinase K (50 µg/ml) and 150

µl of potassium acetate mix were added into the same microcentrifuge tube and vortex briefly. After that, the microcentrifuge tube was centrifuged at 12,000 rpm for 1 minute and the supernatant was transferred to a new 1.5 ml microcentrifuge tube. It was then centrifuged again at 12,000 rpm for 1 minute. An equal volume of isopropanol was added and inverted briefly to precipitate the DNA. After that, it was centrifuged at 12,000 rpm for 2 minutes and the supernatant was discarded. The pellet obtained was washed with 300 µl of 70% ethanol and centrifuged at 10,000 rpm for 1 minute. The supernatant was discarded and the pellet was allowed to air dried. After that, the pellet was dissolved in 50 µl of TE buffer and 2 µl of RNAse A (10 mg/ml) were added. The microcentrifuge tube was then incubated at 65 ℃ water bath for 1 hour. Gel electrophoresis was carried out on a 0.8% TBE agarose gel to check the presence of DNA. Absorbance of 260 nm / 280 nm which is the purity of DNA was measured and recorded with the Thermo Scientific Nano Drop 2000 UV-Vis Spectrophotometer (Liu, et al., 2000).

3.2.2 DNA PCR Amplification

The 18S rDNA was amplified using the forward primers EF4 (5’ GGAAGGGRTGTATTTATTAG 3’) and reverse primer EF3 (5’ TCCTCTAAATGACCAAGTTTG 3’) (Anderson, Campbell and Prossers, 2003) while the ITS region was amplified with forward primers ITS-1 (5’ TCCGTAGGTGAACCTGCG 3’) and reverse primer ITS-4 (5’ TCCTCCGCTTATTGATATGC 3’). PCR was carried out in a total volume of 20 µL containing 2 µl of 10× Taq buffer, 2 µl of 25 mM $MgCl_2$, 0.32 µl of 10

mM of dNTP, 0.2 µl of 100 pmol of each primers (ITS-1 and ITS-4 or EF4 and EF3), 0.15 µl of 5 U/µl Taq polymerase, 2 µL of genomic DNA and 13.13 µl of sterile distilled water. One reaction mixture containing sterile distilled water which replaced the DNA template was used as the contamination control. For PCR in the thermocycler (Biometra Tpersonal), the following profile were used: initial denaturation stage for 5 min at 94 ℃, 35 cycles of amplification consist of denaturation for 30 sec at 94 ℃, then follow by 30 sec annealing at 55 ℃, and extension for 30 sec at 72 ℃; finally was the final extension at 72 ℃ for 5 min. The PCR products were then verified by running gel electrophoresis with 1 kb of DNA ladder (PKT, Korea) used as marker. TBE agarose gel (1 %) was then visualized under ultraviolet light after stained with ethidium bromide for 5 minutes (Buzina, et al., 2001).

3.2.3 Purification of DNA

The desired DNA were then purified using the PKT Xprep Gel Purification Kit (PKT, Korea) protocol. Two 1.5 ml microcentrifuge tube were weighted and the agarose gel with desired bands of ITS and EF DNA fragments were excised with a clean cutter blade and transferred into each new 1.5 ml microcentrifuge tube respectively. The microcentrifuge tubes with the excised DNA fragment were weighted again. After that, 3 volumes of the XPGP buffer were added into each tube containing the desired DNA fragment and vortexed. The DNA samples were then incubated at 55 ℃ for 15 minutes and vortexed every 3 minutes until the gel slide dissolved completely in the buffer. While each tube was allowed to cool down, a XPGP column was placed into a

collection tube. A maximum of 850 µl of sample mixture from each tube was transferred into a XPGP column, respectively and centrifuged for 1 minute at 10,000 ×g. The flow-through in each of the tube was then discarded. This step was then repeated for the remaining sample mixture. Next, 750 µl of wash buffer with ethanol added was then added into each XPGP column and centrifuged for 1 minute at 10,000 ×g. The flow through for each sample was the discarded. To totally dry the column, 3 minutes of centrifugation at 10,000 ×g was performed. Next, each XPGP column was placed into an elution tube. Elution buffer (40 µl) was added into each tube subsequently to the membrane center of the XPGP column and allowed to stand for 2 minutes. Each column was then centrifuged for 1 minute at 10,000 ×g to elute the DNA. Lastly, 1 µl of purified ITS DNA was allowed to run on 1.7 % agarose gel and 1 µl of purified EF DNA on 1 % agarose gel to check the present of DNA after purification process (Anon., n.d.).

3.2.4 DNA Sequencing

The purified PCR products were then sent to Medigene Sdn. Bhd. for sequencing. GenBank basic local alignment tools (BLAST) was used to aid in species identification of the fungus.

3.3 Production of Schizophyllan

Composition of semi synthetic production medium as shown in the Table 3.1 below were prepared for the production of schizophyllan (Rau, et al, 1992).

Table 3.1 Composition of semi synthetic production medium.

Reagent	Manufacturer	Concentration (g/L)
Glucose	Amresco	30.0
Yeast Extract	Scharlau	1.0
$MgSO_4 \cdot 7H_2O$	HmbG	0.5
KH_2PO_4	Qrec	1.0

Three disks of mycelia inoculum (5mm) were inoculated into each of the 100 ml Erlenmeyer flask that containing semi synthetic production medium as shown in Figure 3.2. After that, the flasks were incubated at 30 ℃ at 150 rpm in a rotary shaker incubator. Samples were collected every 24 hours for 20 days of fermentation.

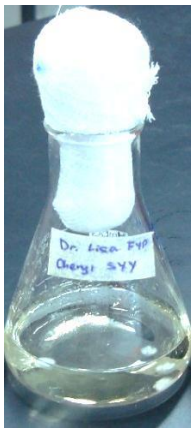


Figure 3.2: Submerge culture of fungus.

The content in the flask was used to analyse the biomass and estimated schizophyllan production and also glucose concentration (Kumari, Survase and Singhal, 2008). The samples were centrifuged for the first time for 20 min at 10,000 ×g. Cell pellet was then used for the dry cell weight determination and

the supernatant was used for glucose determination and analysis of schizophyllan.

3.3.1 Dry cell weight determination

The pellet obtained from the first centrifugation was washed with distilled water and filtered through the Whatman's filter paper with the Buchner funnel using the vacuum pump. After that, the filter paper was dried in the oven at 65 ℃ until constant weight was obtained (Kumari, Survase and Singhal, 2008).

3.3.2 Analysis of Schizophyllan

Isopropanol (15 ml) was mixed with 5 ml of supernatant obtained from the first centrifugation. This was then placed at 4 ℃ for 24 hours (1 day) to allow complete precipitation of the schizophyllan. After 24 hours, the sample was centrifuged at 4 ℃, 13,000 rpm for 10 minutes to separate the precipitate from isopropanol. The isopropanol was discarded and then vacuum dried the pellet for 24 hours (1day) in an oven set to 40 ℃ until constant weight was obtained (Reyes, Brabl and Rau, 2009).

3.3.3 Glucose determination by dinitrosalicylic acid (DNS) method

The glucose concentration was determined by using DNS method as shown in Appendix A described by Kvasnicova (2007), where 1 ml of supernatant from the first centrifugation was added with 1 ml of DNS reagent and drops of 1 N

NaOH. Then the test tubes were placed in a boiling water bath (100 ℃) for 5 min. After 5 minutes, the solution was allowed to cool under running tap water. Distilled water (10 ml) was added to make up the volume to 12 ml and left the suspension for 20 min in room temperature. The absorbance reading were taken at 540 nm by using a spectrophotometer. The concentration of glucose was determined based on the glucose standard curve which was prepared by repeating the DNS method except the 1 ml of supernatant was replaced with 1 ml of different glucose concentration which were 0.2 g/l, 0.4 g/l, 0.6 g/l, 0.8 g/l and 1.0 g/l.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Maintenance of culture and seed culture

A plate of a white colour cotton like fungus culture growing on a petri dish of potato dextrose agar (PDA) as shown in Figure 4.1. The white colour mycelium grew outward from center and eventually covers the whole agar plate when incubated at 30 ˚C for 6 days. Growing the fungus in PDA will not produce schizophyllan as the polysaccharide schizophyllan only will be produce in the presence of suitable carbon source such as glucose in the submerged fermentation as done in the experiment (Yadav and Tyagi, 2005).

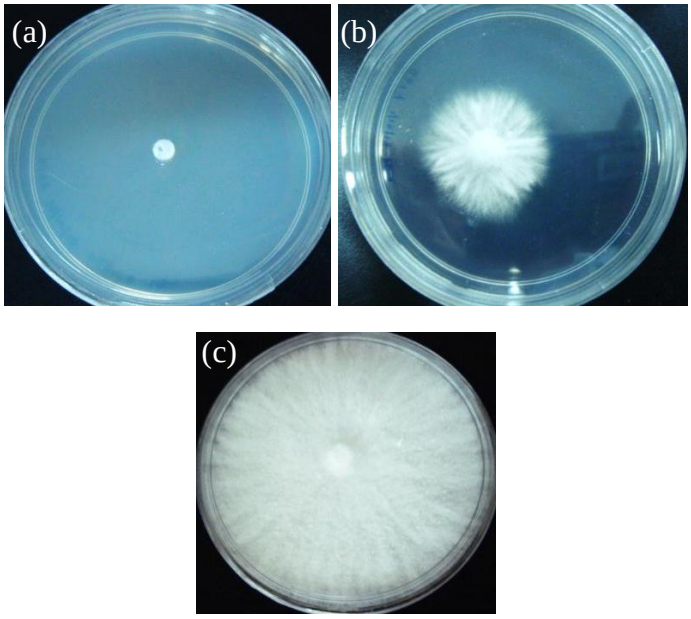


Figure 4.1: Growth progress of sample fungus on PDA plate from day 0 to day 6. (a) Fungus on PDA at day 0. (b) Fungus on PDA at day 3. (c) Fungus at day 6.

The source of the fungus growing on the PDA which were from the rotten oil palm fruit and bagasse as shown in Figure 4.2 were somehow similar with those growing naturally on those dead woods which was found in other journal as shown in Figure 2.1. By comparing the characteristic of the fungus as shown in Figure 4.2 with the one found on fallen timber as shown in Figure 2.1, they were quite similar to each other. The fungus showed white colour fruiting body without stem, attached themselves like small bracket fungi and has split gills on its underside (Kuo, 2003). The fungus was able to grow on a wide range of condition as the split gills has the ability to help them adapted in different environment (Vellinga, 2013).

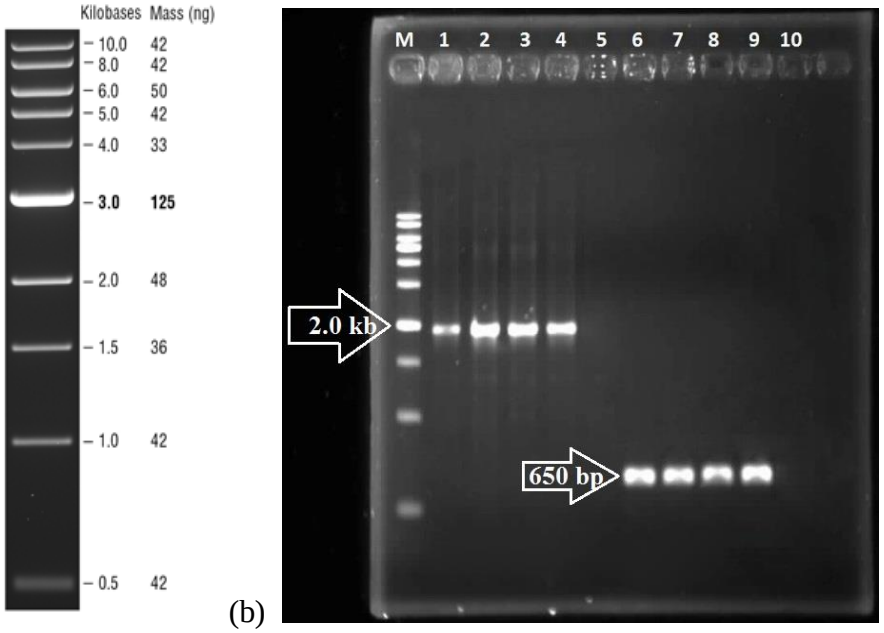
Figure 4.2: *Schizophyllum commune* growing on different sources.
(a) *Schizophyllum commune* growing on rotten oil palm fruit.
(b) *Schizophyllum commune* growing on bagasse (Kam, 2013).

4.2 Molecular Identification

Molecular identification is a common method that can achieve rapid and accurate detection and identification of fungus from the culture (Meyer, et al., 2009). The method as suggested by Liu, et al. (2000) was able to successfully isolate the fungus DNA from the mycelium.

4.2.1 DNA PCR Amplification

After isolation of fungus DNA, PCR was done for 2 different target regions. They are the ITS region and also the 18S rDNA region. 2 different types PCR products were allowed to run gel electrophoresis to indicate the size of the PCR products as shown in Figure 4.3. Bands of 18S rDNA PCR product showed DNA having size about 2.0 kb and bands of ITS PCR product showed DNA having size about 650 bp. The ITS region was generally shorter than 18S rDNA region. The purpose to amplify two different regions was to double confirm the fungus species identified from the culture collection.

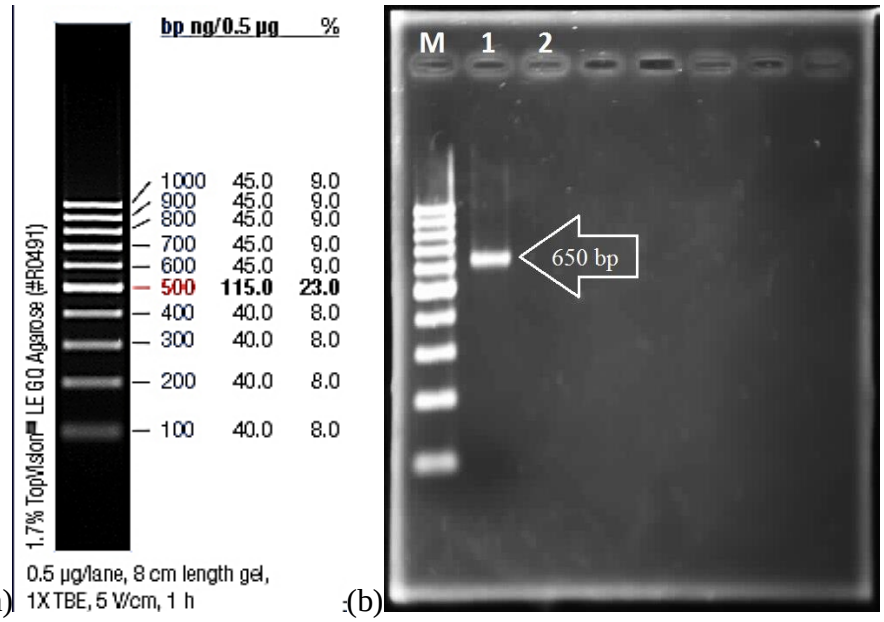


(a) (b)

Figure 4.3: TBE agarose gel (1 %) with 2 different PCR product of fungal ITS and 18s rDNA region. (a) PKT 1 kb DNA ladder. (b) Agarose gel with 2 different PCR products. Lane M – PKT 1 kb DNA ladder, Lane 1 to 4 – bands of 18S rDNA PCR product, Lane 5 – blank for 18S rDNA PCR product, Lane 6 to 9 – bands of ITS PCR product, Lane 10 – blank for ITS PCR product.

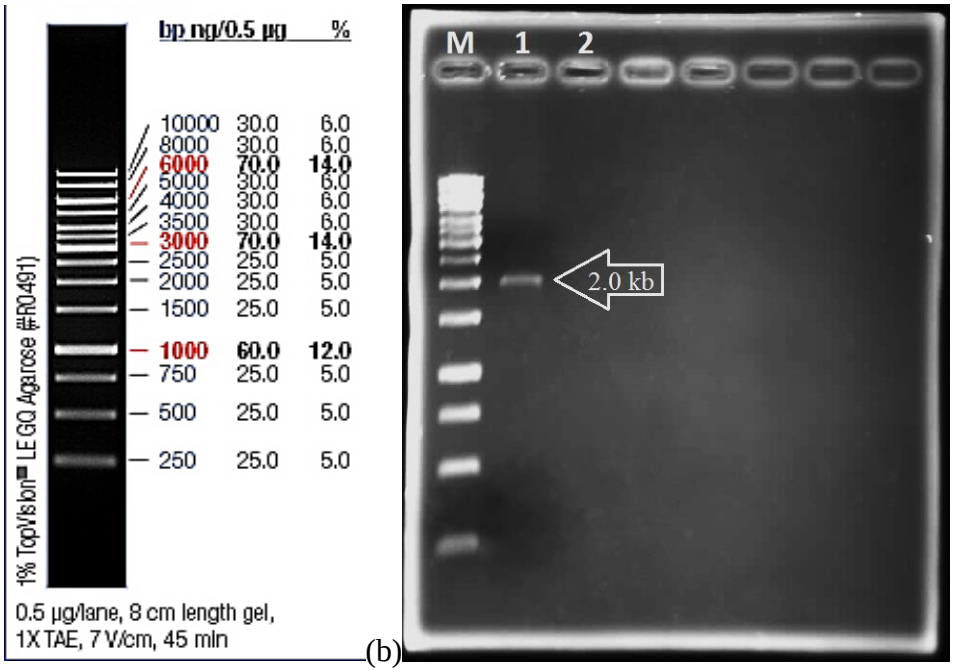
4.2.2 DNA Purification

After the conformation of PCR products, the next steps was purification of the PCR products by using the PKT Xprep Gel Purification Kit protocol (PKT, Korea). The result as shown in Figure 4.4 for PCR products of ITS region which have size about 650 bp, nucleic acid concentration of 26.5 ng/µl and purity ($A_{260/280}$) of 1.78. Figure 4.5 showed PCR products of 18S rDNA region which have size about 2 kb, nucleic acid concentration of 9.5 ng/µl, purity ($A_{260/280}$) of 1.79. The intensity of the bands varies which indicate different amount of DNA present. The intensity of the purified ITS PCR product band as shown in Figure 4.3 shows was brighter than the intensity of the purified 18S rDNA PCR product band as shown in Figure 4.4 which showed that higher amount of PCR product obtained for ITS compare to 18S rDNA.



(a) (b)

Figure 4.4: TBE agarose gel (1.7 %) showing the purified ITS PCR DNA product. (a) GeneRuler 100 bp DNA Ladder. (b) Agarose gel with purified ITS DNA. Lane M – GeneRuler 100 bp DNA Ladder. Lane 1 – Purified ITS DNA. Lane 2 – Blank.



(a) (b)

Figure 4.5: TBE agarose gel (1 %) showing the purified 18S rDNA PCR product. (a) GeneRuler 1 kb DNA Ladder. (b) Agarose gel with purified 18S rDNA. Lane M – GeneRuler 1 kb DNA Ladder. Lane 1 – Purified 18S rDNA. Lane 2 – Blank.

4.2.3 ITS Sequencing Analysis and Species Identification

The purified product were then sent for sequencing and the overlaps of the ITS primer sequence have an identity of 99 % and 0 % of gaps as shown in Figure 4.6. The identity refers to the extent where 2 sequence having the same residue at the same position in an alignment (Fassler and Cooper, 2011). 99 % identity of the overlaps means that both forward and reverse sequence were complement to each other almost perfectly.

Figure 4.6: Figure shows the overlapping of the PCR products of the ITS using NCBI BLAST software.

According to the NCBI BLAST database, each primer sequence were used to identify the species of the fungus. Based on the sequence using ITS-1 and ITS-4 universal primers, (Ferrer, et al., 2001) it showed the corresponding fungus species was *Schizophyllum commune* as shown in Figure 4.7 and Figure 4.8.

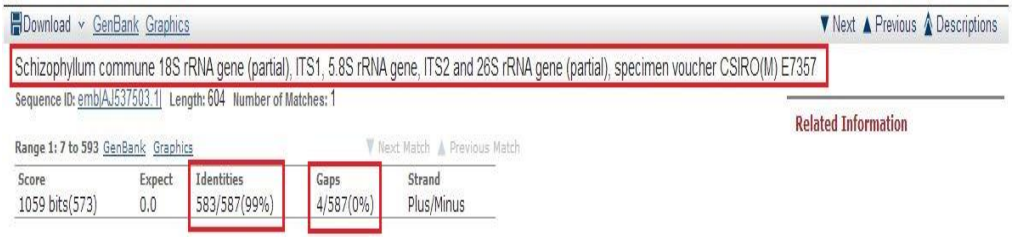


Figure 4.7: DNA Sequence using ITS-1 forward primer shows 99 % identity and 0 % gaps for fungus *Schizophyllum commune.*

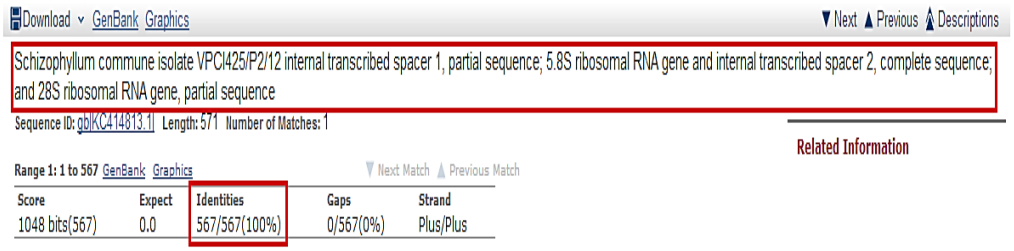


Figure 4.8: DNA Sequence using ITS-4 reverse primer shows 99 % identity and 0 % gaps for fungus *Schizophyllum commune.*

The whole ITS region which consist of ITS1 5.8S rDNA and ITS2 region were amplified with forward primer, ITS-1 and reverse primer, ITS-4. The size obtained was about 650 bp as shown in Figure 4.3. From the ITS sequence obtained as shown in Figure 4.6 and Figure 4.7, both sequence shows 99 % identity with *Schizophyllum commune*.

According to a research done in 2010, the molecular identification of fungus which was also done by amplifying the ITS region using the ITS-1 and ITS-4 primers revealed the length of the sequences were ranging from 538 bp to 561 bp. The fungus being identified was also *Schizophyllum commune* (Alam, et al., 2010). Another journal published regarding the PCR amplification of the ITS region of the oomycetes fungus and other filamentous fungus such as *Pythium sp.*, *Saprolegnia sp.*, *Aspergillus niger sp.* and *Penicillum sp.* also using ITS-1 and ITS-4 primers showed that the length of the sequence obtained was about 800 bp to 1 kb (Prabha, et al., 2013). A journal reported in 2012, stated that the identification of the fungus isolates which was confirmed by the sequence obtained for the ITS region showed 100 % identity with *Schizophyllum commune* (Chowdhary, et al., 2013).

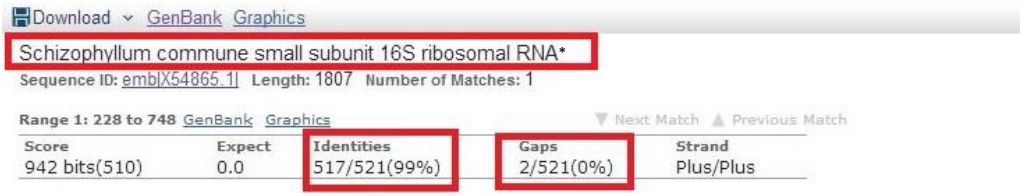
Based on the lineage report of the ITS region, it showed majority of the class of fungus identified using ITS-1 and ITS-4 primer were basidiomycetes and fewer of class ascomycetes and oomycytes as shown in Appendix E.

Therefore, the ITS region amplified with ITS-1 and ITS-4 primers in the experiment identified the fungus from the culture collection was *Schizophyllum commune.*

4.2.4 18S rDNA Sequence Analysis and Species Identification

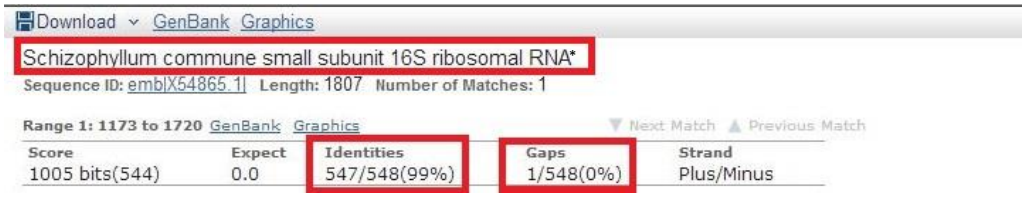
The sequence for 18S rDNA could not be overlapped probably due to the DNA template concentration sent for sequencing was not high enough or the quality of preparation was low. The yield might be containing incomplete removal of proteins, salts or other contaminated component which was not fully removed that can inhibit the sequencing reaction enzyme before sending out for sequencing (Eurofins Genomics, 2014).

Although both sequence was not able to overlap to each other but when both sequence were still analyse with BLAST, both sequence showed 99 % identity and 0 % gaps to *Schizophyllum commune* as shown in Figure 4.9 and Figure 4.10. The sequences were still able to identify the fungus probably due to the gene specific primer were used instead of the universal primer which might share some similarities with other eukaryotic species (Gontia-Mishra, et al., 2013).



*Published as 18S rRNA as in the journal Evolutionary relationships with the fungi: Analyses of nuclear small subunit rRNA sequences (Bruns, et al., 1992).

Figure 4.9: DNA Sequence using EF4 forward primer shows 99 % identity and 0 % gaps for fungus *Schizophyllum commune.*



*Published as 18S rRNA as in the journal Evolutionary relationships with the fungi: Analyses of nuclear small subunit rRNA sequences (Bruns, et al., 1992).

Figure 4.10: DNA Sequence using EF3 reverse primer shows 100 % identity and 0 % gaps for fungus *Schizophyllum commune.*

Under the description, it stated '*Schizophyllum commune* small subunit 16S ribosomal RNA' which it was suspected to be a mistake done by the author who submitted this because according to the original paper published as found in EMBL-EBI, the journal was referring to 18S rRNA for the investigation of evolutionary relationship of fungi (Bruns, et al., 1992). The mistake was confirmed by Richard Gibson from European Nucleotide Archive through an email received. He clarified that the original paper had clearly published as 18S rRNA gene. Hence the record was sent to the updated queue for correction which will take about several weeks to be visible on the public nucleotide browser as shown in the email in Appendix F (Richard, 2014).

There were a few research done on the PCR amplification of 18S rDNA region where some were using EF3 and EF4 primers while some used other primers for the PCR amplification. EF3 and EF4 were the gene specific primers used specifically to amplify the 18S rDNA region (Gontia-Mishra, et al., 2013). Some other journal published used universal primers such as NS1, NS3, NS4, NS5, NS6 and NS8 for the identification of 18S rDNA. Even though the 18S rDNA were highly conserved regions, but there were still some gene sequence which shared similarities with other eukaryotes. Thus, gene specific primers were better to be used in this experiment as they have the bias characteristic which mainly allowed the identification majority of basidiomycete (Anderson, Campbell and Prossers, 2003). A journal published in 2004 actually used NS1 which is a universal primer for 18S rDNA and ITS-4 which is a universal primer for the ITS region for the amplification of both 18S rDNA and ITS region to screen for the soil filamentous fungus (Plaza, et al., 2004).

According to the lineage report as shown in in Appendix G, the sequences using the primers EF3 and EF4 showed majority of the class of fungus identified were basidiomycete. This was probably due to the primer bias (Anderson, Campbell and Prossers, 2003).

Therefore, the 18S rDNA region amplified with EF4 and EF3 primers in the experiment identified the fungus from the culture collection was *Schizophyllum commune.*

4.3 Production of Schizophyllan

4.3.1 Submerge Fermentation Cultures

Schizophyllan was allowed to grow in a semi synthetic medium as suggested by Rau, et al. (1992) as shown in Figure 4.11 for 20 days to study the growth profile of the fungus. The concentration of fungus increased and the medium in the flask however got lesser and became more viscous as it reached day 20.

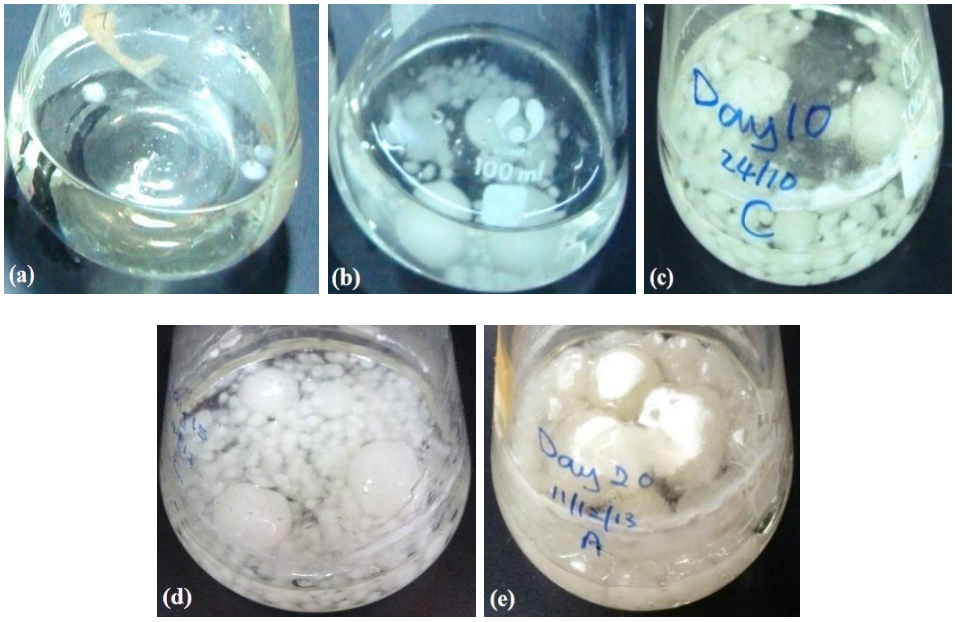


Figure 4.11: Growth progress of sample fungus in semi synthetic medium from day 0 to day 20. (a) Day 0. (b) Day 5. (c) Day 10. (d) Day 15. (e) Day 20.

A journal published by Shu and Hsu, (2011), a study on the production of schizophyllan was done using rice hull hydrolysate with toxins removed by activated charcoal and also without toxins removed. Based on the result shown, it also compared the product concentration, dry cell weight and also glucose concentration all in a graph (Shu and Hsu, 2011).

Another journal published in 2008 which was using Respond Surface Method to optimized the medium composition concentration for the production of schizophyllan compared the three data on a graph (Kumari, Survase and Singhal, 2008). According to a journal by Kumari, Survase and Singhal, 2008, the production of the polysaccharide schizophyllan was optimum by using the semi synthetic medium as suggested by Rau, et al, (1992). Hence, in the experiment, the fungus was assumed that it grew optimally in the same semi synthetic medium as suggested by the two journal published.

4.3.2 Dry Cell Weight (DCW)

Growth profile of the fungus was analysed form the dry cell weight. DCW was taken by obtaining the difference between the weights of the dried pellet divided by the sample volume. Appendix B shows the DCW raw data obtained throughout the 20 days of culture.

A graph of dry cell weight against day was plotted to observe the growth profile of the fungus throughout the 20 days as shown in Figure 4.12. There were the 4 phases of growth was determined. Lag phase begun from day 0 until day 4. After that, from day 4 until day 13 was the log phase where the growth was at its optimum stage. Stationary phase lasted for the next 4 days which was from day 14 until day 17 and after than the fungus gradually enter the death phase where the growth of the fungus declined in the last 3 days.

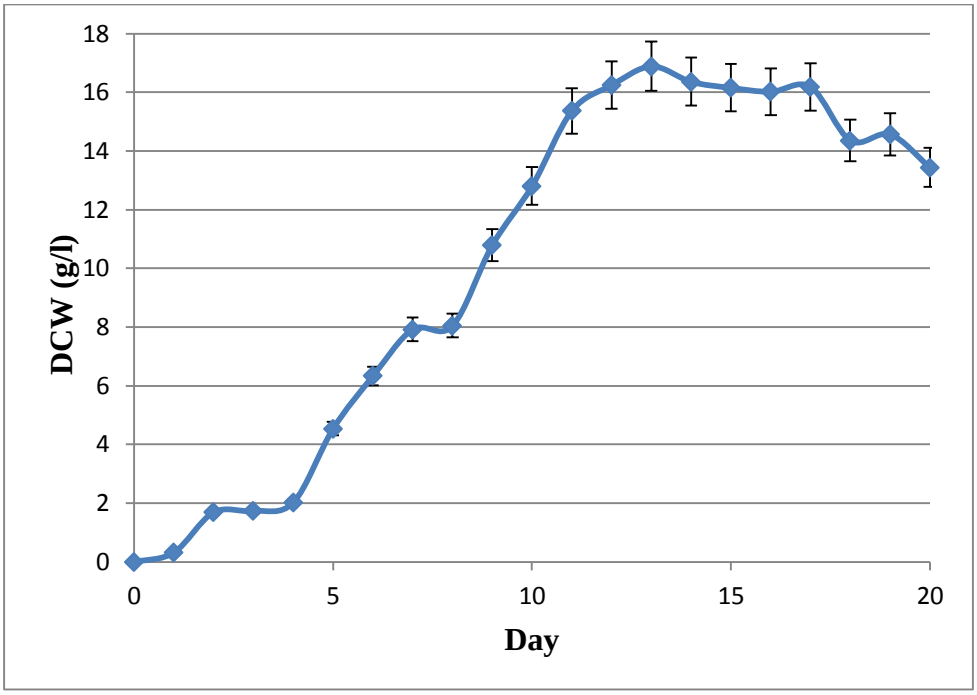


Figure 4.12: Graph of dry cell weight against days of fermentation.

4.3.3 Analysis of Schizophyllan production

Analysis of schizophyllan is the measure of the estimated product, schizophyllan concentration produced in the medium as shown in Appendix C. Since schizophyllan is a non-ionic compound and water soluble polysaccharide, therefore it would not be soluble in organic solvent such as isopropanol (Yadav and Tyagi, 2005). According to a section of the book titled Microbial Production and Processing of the Polysaccharide Schizophyllan, it stated that using organic solvent such as ethanol or isopropanol can decrease the dielectric constant and also decrease the solubility of the polysaccharide from the culture medium. Thus, the schizophyllan was estimated to be precipitated out from the fermentation medium with isopropanol (Yadav and Tyagi, 2005).

A graph of estimated product concentration against day was plotted as shown in Figure 4.13. The graph increase slowly from day 0 until day 4 and increases rapidly for the next 7 day which was until day 11. For the rest of the day the amount of schizophyllan decreases gradually.

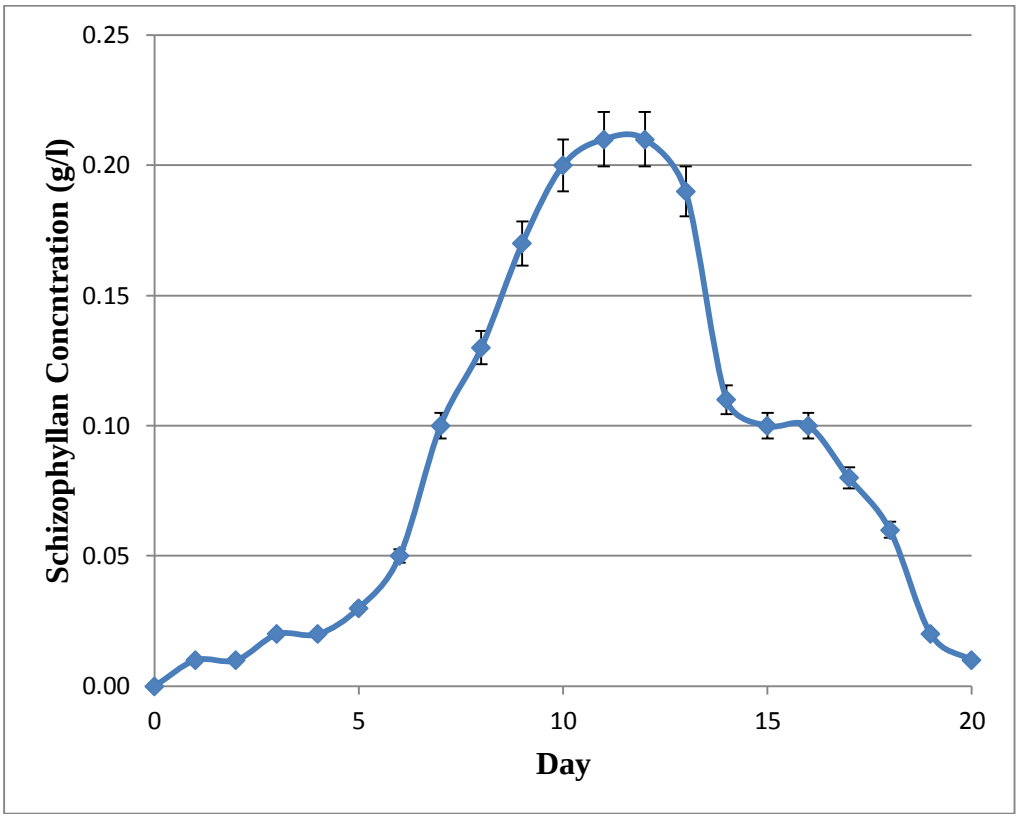


Figure 4.13: Graph of estimation of schizophyllan concentration against days of fermentation.

According to a published journal, the production of schizophyllan was estimated based on the precipitation by isopropanol and allowed to dry until constant weight obtained (Kumari, et al., 2008). Another journal regarding the production of schizophyllan in the culture medium made from coconut water also used the similar method that is by precipitating the polysaccharide with

isopropanol to estimate the schizophyllan production (Reyes, Brabl and Rau, 2009).

4.3.4 Glucose determination

A standard curve of glucose determination aided the measure of the actual glucose concentration remained in the culture medium as the fungus utilised glucose for survival and produced schizophyllan. The absorbance readings at 540nm for the concentration of glucose for standard curve were tabulated in Appendix D.

A graph of standard curve was plotted as shown in Figure 4.14 to allow the determination of the actual glucose concentration in the medium based on the absorbance reading at 540nm. The graph showed a linear straight line passing through the origin.

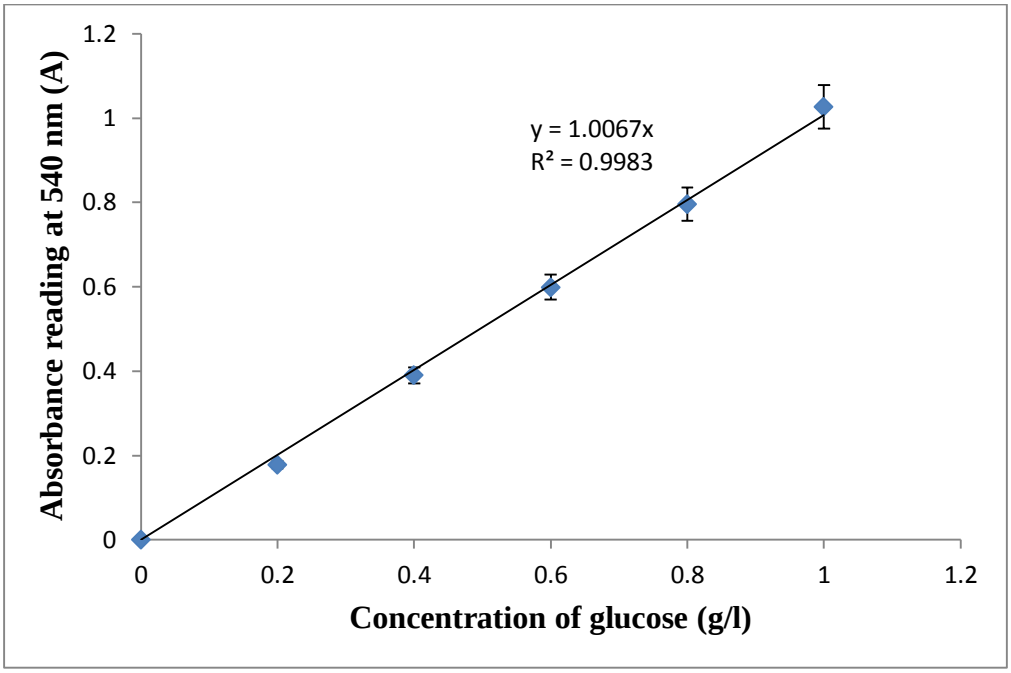


Figure 4.14: Standard curve for glucose determination.

Based on the absorbance reading obtained from the experiment, the actual glucose concentration was determined through the standard curve in Figure 4.14 and was tabulated in Appendix D.

A graph of glucose concentration against days of fermentation was plotted as shown in Figure 4.15. From the graph, the glucose concentration in the medium reduced starting from day 4 and decreased drastically until day 10 of fermentation and gradually depleted.

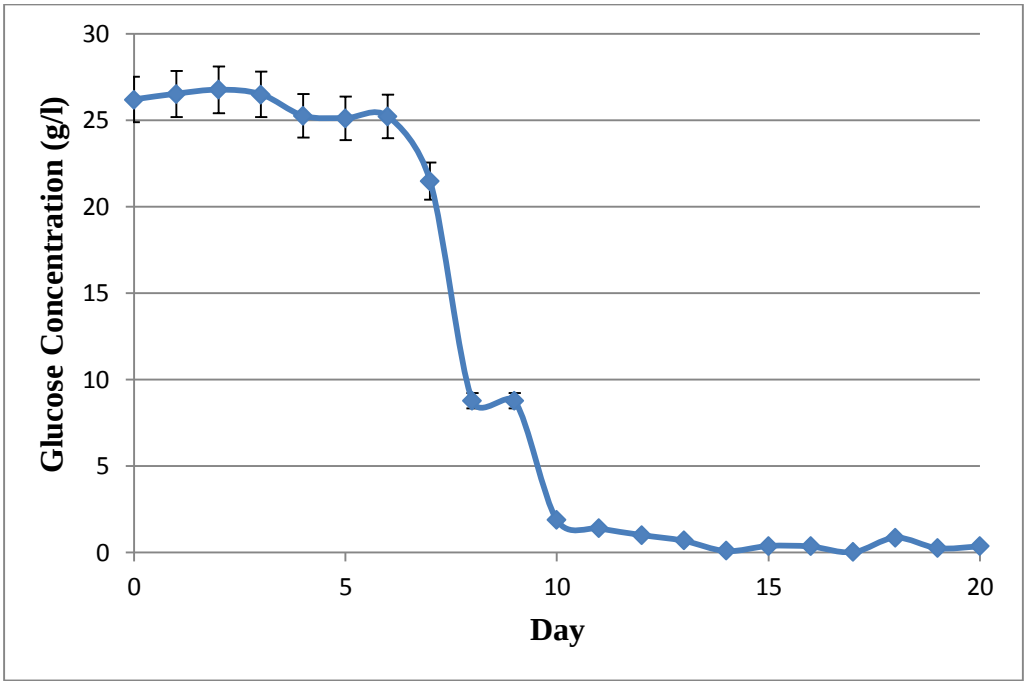


Figure 4.15: Graph of actual glucose concentration against day.

A graph combining all three analysis which were the dry cell weight analysis, estimated schizophyllan concentration and glucose concentration are as shown in Figure 4.16. In the lag phase that was starting from day 0 to day 4, the dry cell weight increased slowly as the growth of the fungus grows slowly, adapting itself in the semi synthetic medium. Starting from day 4, the dry cell weight begun to increase rapidly as the fungus utilized the glucose for the production of schizophyllan and for survival purposes. A journal published by Kumari, Survase and Singhal, (2008) found that glucose was utilized by the fungus *Schizophyllum commune* as its primary carbon source for the production of schizophyllan.

When the fungus had fully adapted in the semi synthetic medium, the growth of the cell entered the log phase of the growth profile from day 4 to day 13. In this log phase, the utilization of glucose by the fungus was rapid as this was

shown in the graph where glucose decrease very quickly from day 4 to day 10 and the estimated product, schizophyllan produced also increased quickly from day 4 to day 12. From day 10 to day 13, the glucose concentration in the culture medium started to deplete to a concentration near to zero and the production of schizophyllan also started to slow down as shown in day 10 to day 12 and gradually decreased from day 12 onwards.

The growth of the fungus entered the stationary phase from day 13 to day 17. Although the carbon source glucose concentration had depleted by day 14, the growth of the fungus was able to maintain until day 17. The fungus was able to prolong its survival a few more days even though the carbon source was depleting. This was possible because the fungus was suspected for utilizing the alternative carbon source which was the products, schizophyllan itself for their survival purpose. This was suspected at day 13 where the estimated product concentration begun decreasing when the dry cell weight enter the stationary phase until day 17. A journal published by Reyes, et al. (2009) supported the statement that when the glucose carbon source initially utilized by the fungus, *Schizophyllum commune* and became depleted, the fungus was able to utilize its own product, schizophyllan as an alternative carbon source to prolong its survivability for a few more days. The fungus was able to utilize its own product, schizophyllan because of the production of ß-glucanases from the *Schizophyllum commune* itself which is a ß-glucan degrading enzyme when the primary carbon source had depleted (Reyes, Brabl and Rau, 2009).

From day 17, when the estimated product, schizophyllan concentration continued to decrease and became limited, the fungus was suspected that it has no other carbon source to sustain its growth anymore and hence it entered the death phase where the dry cell weight started to decreased as the estimated schizophyllan was depleting. The decreased of the estimated product concentration showed that the fungus had utilized them to sustain its growth was actually possible. If the fungus did not produce ß-glucan degrading enzyme which can utilize the product, the level of the schizophyllan would had remain at a relatively constant concentration even though the fungus entered the death phase of growth (Sutivisedsak, et al., 2013).

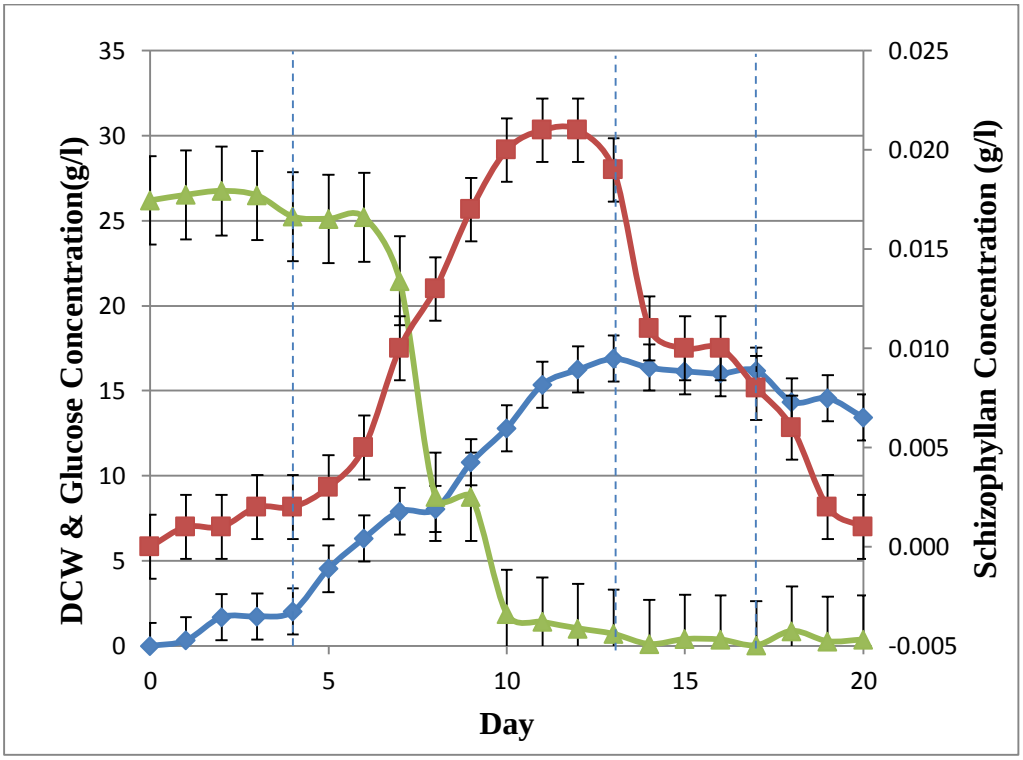


Figure 4.16: Combination graph of average DCW, estimated schizophyllan concentration and glucose concentration against day. - Estimated schizophyllan concentration (g/l) (g). - Dry cell weight (g/l). - Glucose concentration (g/l).

Carbon source such as glucose were used by the fungus as an energy source and also for the synthesis of schizophyllan. Besides that, glucose were an important component for the fungus to build the cellular material (Kumari, et al., 2008). According to all these researches done, as long as there were carbon source such as glucose present in the medium used, schizophyllan can be produced from the fungus *Schizophyllum commune*.

4.4 Further Study

Growing the fungus on different condition allows physiochemical study of the fungus. Different condition such as different temperature, pH and carbon source can be tested to see their effect on the fungus, *Schizophyllum commune* growth.

Fluorescent microassay for 1,3-β-glucan quantification can be done to quantify the amount of schizophyllan more specifically. This microassay is based on the binding of aniline blue dye with the 1,3-β-glucan of schizophyllan specifically. The binding of aniline blue to the glucan will give fluorescent which the fluorescent intensity can be detected by fluorescent spectrophotometer.

The study of the structure of the schizophyllan could also be done by using a Fourier Transform Infrared spectroscopy (FTIR). This spectroscopy is a great tool used for the determination of the organic group present in the schizophyllan itself. By obtaining the spectrum of the polysaccharide, it is

possible to determine the organic groups that were present in the polysaccharide.

Lastly, solid state fermentation of *Schizophyllum commune* can be done to compare between solid state and submerged fermentation. Phytase production usually could be produce through solid state fermentation. The growth profile can be determine and compare base on the dry cell weight and product formation in solid state fermentation.

CHAPTER 5

CONCLUSION

As a conclusion, molecular identification based on the fungus Internal Transcriber Spacer region and 18S rDNA region was achieved. Fungus ITS region has been widely used for the molecular identification as this region was unique to differentiate between fungus species. The primers ITS-1 and ITS-4 were the universal primers for the ITS region. This ITS region which was also flanking 5.8S rRNAs reduces the co-amplification of plant or other eukaryotic DNA. Although 18S rDNA was a highly conserved region but it still might share similarities with other eukaryotic gene sequence. Hence, the gene specific primers EF4 and EF3 was used to confirm the fungal species. Hence, both ITS and 18S rDNA region of the fungus identified were confirmed to be fungus *Schizophyllum commune*. The ITS sequence was also published and deposited into the GeneBank with an accession number of KJ608191.

Next, the production of schizophyllan was able to produce in the mixture of chemical composition of the semi synthetic medium as suggested. The schizophyllan was possible to be extracted by precipitating with isopropanol. The growth profile, estimated schizophyllan concentration and also glucose concentration was determined in this study. The fungus begun with a lag phase from day 0 to day 4 where it grew slowly while adapting themselves in the

medium and entered the log phase until day 13 where the growth of the fungus was rapid as it utilized glucose rapidly for the production of schizophyllan. After that it entered the stationary phase where the dry cell weight remain relatively constant until day 17 and finally entered the death phase. As the glucose was depleted, the fungus actually switched to the utilization of the alternative carbon source which was the schizophyllan to sustain its growth until the product was depleted. The fungus entered the death phase when both glucose and schizophyllan have depleted as no more carbon source available to sustain its survival.

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APPENDIX A

Dinitrosalicylic acid (DNS) preparation

The composition of DNS is as shown in the table below. Distilled water, dinitrosalicylic acid and NaOH were gently dissolved in water bath at 80 ℃ until a clear solution was obtained. After that, Rochelle salt (sodium potassium tartrate), phenol (Anon., n.d.) melted at 60 ℃ and of sodium meta bisulphate were added. After dissolving the all the ingredients, the solution was filtered and stored at room temperature in an amber bottle to avoid photo oxidation (Anon., n.d.).

Reagent	**Amount**
Distilled water	100 ml
Dinitrosalicylic acid	0.749 g
NaOH	1.377 g
Sodium potassium tartrate	21.186 g
Phenol	0.2 g
Sodium meta bisulphate	0.586 g

APPENDIX B

Dry Cell Weight

Day	DCW (g/l)			Average DCW (g/l)	Actual DCW (g/l)
	A	B	C		
0	0.0700	0.0300	0.1200	0.073	0.000
1	0.3400	0.3775	0.4775	0.398	0.325
2	1.4949	2.6700	1.1375	1.767	1.694
3	1.5763	2.6893	1.1633	1.810	1.737
4	2.0305	3.0012	1.2706	2.101	2.028
5	5.8725	4.0875	3.8700	4.610	4.537
6	6.6925	4.5250	7.9975	6.405	6.332
7	9.1725	6.8250	7.9850	7.994	7.921
8	5.7250	10.4925	8.1400	8.119	8.046
9	8.0950	10.9100	13.5875	10.864	10.791
10	13.5875	12.6900	12.3625	12.880	12.807
11	15.9075	14.4225	15.9900	15.440	15.367
12	15.1575	15.5575	18.2425	16.319	16.246
13	16.3225	17.5450	17.0175	16.962	16.888
14	17.1975	15.8650	16.2425	16.435	16.362
15	15.5250	16.4175	16.7450	16.229	16.156
16	15.8400	16.4300	16.0225	16.098	16.024
17	15.7750	16.0875	16.9225	16.262	16.188
18	15.6250	16.3800	11.2925	14.433	14.359
19	15.9675	15.7400	12.2225	14.643	14.570
20	12.9425	13.6825	13.9200	13.515	13.442

APPENDIX C

Estimated Schizophyllan Concentration

Day	Product Weight (g)			Average Product Weight (g)	Actual Product Weight (g)	Concentration of product (g/l)
	A	B	C			
0	0.0045	0.0055	0.0068	0.006	0.000	0.000
1	0.0065	0.0073	0.0058	0.007	0.001	0.010
2	0.0071	0.0089	0.0063	0.007	0.001	0.010
3	0.0082	0.0093	0.0076	0.008	0.002	0.020
4	0.0102	0.0070	0.0077	0.008	0.002	0.020
5	0.0107	0.0079	0.0098	0.009	0.003	0.030
6	0.0128	0.0086	0.0126	0.011	0.005	0.050
7	0.0161	0.0161	0.0152	0.016	0.010	0.100
8	0.0180	0.0232	0.0155	0.019	0.013	0.130
9	0.0196	0.0224	0.0270	0.023	0.017	0.170
10	0.0313	0.0244	0.0231	0.026	0.020	0.200
11	0.0238	0.0237	0.0334	0.027	0.021	0.210
12	0.0216	0.0357	0.0229	0.027	0.021	0.210
13	0.0217	0.0234	0.0294	0.025	0.019	0.190
14	0.0087	0.0206	0.0223	0.017	0.011	0.110
15	0.0104	0.0132	0.0232	0.016	0.010	0.100
16	0.0127	0.0176	0.0186	0.016	0.010	0.100
17	0.0160	0.0159	0.0115	0.014	0.008	0.080
18	0.0021	0.0219	0.0121	0.012	0.006	0.060
19	0.0015	0.0117	0.0116	0.008	0.002	0.020
20	0.0076	0.0053	0.0085	0.007	0.001	0.010

APPENDIX D

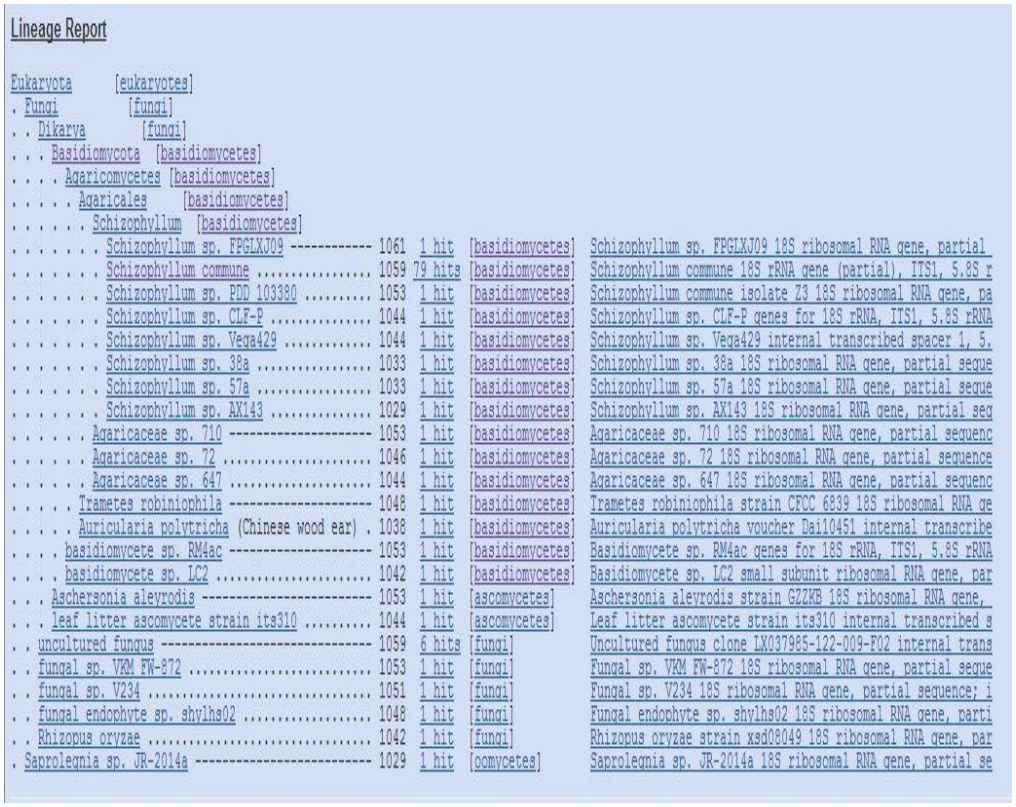
Glucose Determination

Standard curve for glucose determination	
Concentration of glucose (g/l)	Absorbance reading at 540 nm (A)
0	0.000
0.2	0.178
0.4	0.390
0.6	0.599
0.8	0.796
1.0	1.027

Day	Actual Glucose Concentration			Average Actual Glucose Concentration (g/l)
	A	B	C	
0	26.125	24.933	26.522	25.860
1	26.572	24.486	28.509	26.522
2	24.287	27.168	28.807	26.754
3	28.459	28.211	22.797	26.489
4	24.883	26.771	24.089	25.248
5	23.294	25.231	26.820	25.115
6	26.473	30.744	22.400	26.539
7	20.165	21.357	22.897	21.473
8	15.198	14.354	20.562	16.705
9	13.410	6.308	6.606	8.775
10	1.674	1.876	2.105	1.885
11	1.008	1.967	1.259	1.411
12	1.442	0.965	0.687	1.031
13	1.112	0.615	0.374	0.700
14	0.242	0.062	0.016	0.107
15	0.431	0.303	0.467	0.400
16	0.047	0.532	0.525	0.368
17	0.074	0.011	0.028	0.038
18	0.000	0.280	2.360	0.880
19	0.213	0.605	0.000	0.273
20	0.096	0.104	0.932	0.377

APPENDIX E

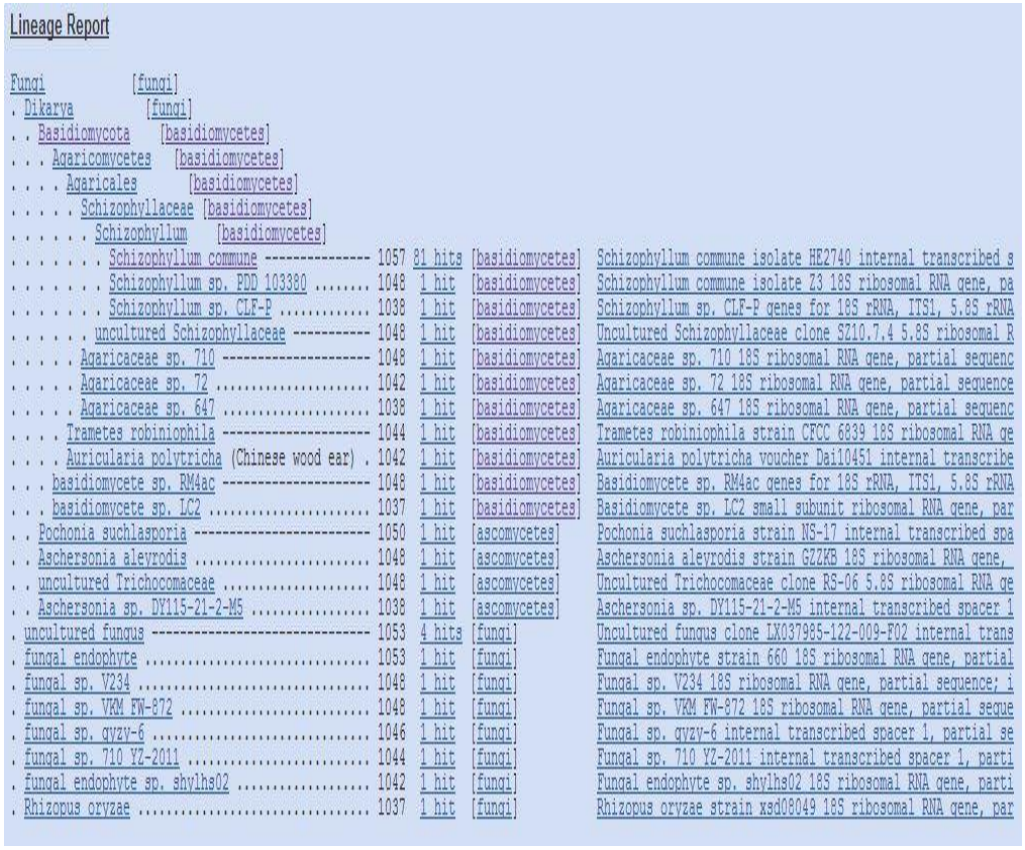
Linage report for ITS region using ITS-1 primer



Lineage Report

Eukaryota [eukaryotes]				
. Fungi [fungi]				
. . Dikarya [fungi]				
. . . Basidiomycota [basidiomycetes]				
. . . . Agaricomycetes [basidiomycetes]				
. . . . . Agaricales [basidiomycetes]				
. . . . . Schizophyllum [basidiomycetes]				
. . . . . Schizophyllum sp. FPGLXJ09 ----------	1061	1 hit	[basidiomycetes]	Schizophyllum sp. FPGLXJ09 18S ribosomal RNA gene, partial
. . . . . Schizophyllum commune	1059	79 hits	[basidiomycetes]	Schizophyllum commune 18S rRNA gene (partial), ITS1, 5.8S r
. . . . . Schizophyllum sp. PDD 103380	1053	1 hit	[basidiomycetes]	Schizophyllum commune isolate Z3 18S ribosomal RNA gene, pa
. . . . . Schizophyllum sp. CLF-P	1044	1 hit	[basidiomycetes]	Schizophyllum sp. CLF-P genes for 18S rRNA, ITS1, 5.8S rRNA
. . . . . Schizophyllum sp. Vega429	1044	1 hit	[basidiomycetes]	Schizophyllum sp. Vega429 internal transcribed spacer 1, 5.
. . . . . Schizophyllum sp. 38a	1033	1 hit	[basidiomycetes]	Schizophyllum sp. 38a 18S ribosomal RNA gene, partial seque
. . . . . Schizophyllum sp. 57a	1033	1 hit	[basidiomycetes]	Schizophyllum sp. 57a 18S ribosomal RNA gene, partial seque
. . . . . Schizophyllum sp. AX143	1029	1 hit	[basidiomycetes]	Schizophyllum sp. AX143 18S ribosomal RNA gene, partial seq
. . . . . Agaricaceae sp. 710 ----------	1053	1 hit	[basidiomycetes]	Agaricaceae sp. 710 18S ribosomal RNA gene, partial sequenc
. . . . . Agaricaceae sp. 72	1046	1 hit	[basidiomycetes]	Agaricaceae sp. 72 18S ribosomal RNA gene, partial sequence
. . . . . Agaricaceae sp. 647	1044	1 hit	[basidiomycetes]	Agaricaceae sp. 647 18S ribosomal RNA gene, partial sequenc
. . . . . Trametes robiniophila ----------	1048	1 hit	[basidiomycetes]	Trametes robiniophila strain CFCC 6839 18S ribosomal RNA ge
. . . . . Auricularia polytricha (Chinese wood ear) .	1038	1 hit	[basidiomycetes]	Auricularia polytricha voucher Dai10451 internal transcribe
. . . . basidiomycete sp. RM4ac ----------	1053	1 hit	[basidiomycetes]	Basidiomycete sp. RM4ac genes for 18S rRNA, ITS1, 5.8S rRNA
. . . . basidiomycete sp. LC2	1042	1 hit	[basidiomycetes]	Basidiomycete sp. LC2 small subunit ribosomal RNA gene, par
. . . Aschersonia aleyrodis ----------	1053	1 hit	[ascomycetes]	Aschersonia aleyrodis strain GZZKB 18S ribosomal RNA gene,
. . . leaf litter ascomycete strain its310	1044	1 hit	[ascomycetes]	Leaf litter ascomycete strain its310 internal transcribed s
. . uncultured fungus ----------	1059	6 hits	[fungi]	Uncultured fungus clone LX037985-122-009-F02 internal trans
. . fungal sp. VKM FW-872	1053	1 hit	[fungi]	Fungal sp. VKM FW-872 18S ribosomal RNA gene, partial seque
. . fungal sp. V234	1051	1 hit	[fungi]	Fungal sp. V234 18S ribosomal RNA gene, partial sequence; i
. . fungal endophyte sp. shylhs02	1048	1 hit	[fungi]	Fungal endophyte sp. shylhs02 18S ribosomal RNA gene, parti
. . Rhizopus oryzae	1042	1 hit	[fungi]	Rhizopus oryzae strain xsd08049 18S ribosomal RNA gene, par
. Saprolegnia sp. JR-2014a ----------	1029	1 hit	[oomycetes]	Saprolegnia sp. JR-2014a 18S ribosomal RNA gene, partial se

Lineage report of the ITS region using ITS-4 primer.



Lineage Report

Fungi [fungi]				
. Dikarya [fungi]				
. . Basidiomycota [basidiomycetes]				
. . . Agaricomycetes [basidiomycetes]				
. . . . Agaricales [basidiomycetes]				
. . . . . Schizophyllaceae [basidiomycetes]				
. . . . . Schizophyllum [basidiomycetes]				
. . . . . Schizophyllum commune	1057	81 hits	[basidiomycetes]	Schizophyllum commune isolate HE2740 internal transcribed s
. . . . . Schizophyllum sp. PDD 103380	1048	1 hit	[basidiomycetes]	Schizophyllum commune isolate Z3 18S ribosomal RNA gene, pa
. . . . . Schizophyllum sp. CLF-P	1038	1 hit	[basidiomycetes]	Schizophyllum sp. CLF-P genes for 18S rRNA, ITS1, 5.8S rRNA
. . . . . uncultured Schizophyllaceae	1048	1 hit	[basidiomycetes]	Uncultured Schizophyllaceae clone SZ10.7.4 5.8S ribosomal R
. . . . . Agaricaceae sp. 710	1048	1 hit	[basidiomycetes]	Agaricaceae sp. 710 18S ribosomal RNA gene, partial sequenc
. . . . . Agaricaceae sp. 72	1042	1 hit	[basidiomycetes]	Agaricaceae sp. 72 18S ribosomal RNA gene, partial sequence
. . . . . Agaricaceae sp. 647	1038	1 hit	[basidiomycetes]	Agaricaceae sp. 647 18S ribosomal RNA gene, partial sequenc
. . . . Trametes robiniophila	1044	1 hit	[basidiomycetes]	Trametes robiniophila strain CFCC 6839 18S ribosomal RNA ge
. . . . Auricularia polytricha (Chinese wood ear) .	1042	1 hit	[basidiomycetes]	Auricularia polytricha voucher Dai10451 internal transcribe
. . . basidiomycete sp. RM4ac	1048	1 hit	[basidiomycetes]	Basidiomycete sp. RM4ac genes for 18S rRNA, ITS1, 5.8S rRNA
. . . basidiomycete sp. LC2	1037	1 hit	[basidiomycetes]	Basidiomycete sp. LC2 small subunit ribosomal RNA gene, par
. . Pochonia suchlasporia	1050	1 hit	[ascomycetes]	Pochonia suchlasporia strain NS-17 internal transcribed spa
. . Aschersonia aleyrodis	1048	1 hit	[ascomycetes]	Aschersonia aleyrodis strain GZZKB 18S ribosomal RNA gene,
. . uncultured Trichocomaceae	1048	1 hit	[ascomycetes]	Uncultured Trichocomaceae clone RS-06 5.8S ribosomal RNA ge
. . Aschersonia sp. DY115-21-2-M5	1038	1 hit	[ascomycetes]	Aschersonia sp. DY115-21-2-M5 internal transcribed spacer 1
. uncultured fungus	1053	4 hits	[fungi]	Uncultured fungus clone LX037985-122-009-F02 internal trans
. fungal endophyte	1053	1 hit	[fungi]	Fungal endophyte strain 660 18S ribosomal RNA gene, partial
. fungal sp. V234	1048	1 hit	[fungi]	Fungal sp. V234 18S ribosomal RNA gene, partial sequence; i
. fungal sp. VKM FW-872	1048	1 hit	[fungi]	Fungal sp. VKM FW-872 18S ribosomal RNA gene, partial seque
. fungal sp. gyzy-6	1046	1 hit	[fungi]	Fungal sp. gyzy-6 internal transcribed spacer 1, partial se
. fungal sp. 710 YZ-2011	1044	1 hit	[fungi]	Fungal sp. 710 YZ-2011 internal transcribed spacer 1, parti
. fungal endophyte sp. shylhs02	1042	1 hit	[fungi]	Fungal endophyte sp. shylhs02 18S ribosomal RNA gene, parti
. Rhizopus oryzae	1037	1 hit	[fungi]	Rhizopus oryzae strain xsd08049 18S ribosomal RNA gene, par

APPENDIX F

An email received as an evidence regarding query to ENA concerning the sequence in GeneBank accession number X54865 (Richard, 2014).

Re: ENA browser ref: X54865 (SUB#867994)

datasubs@ebi.ac.uk (datasubs@ebi.ac.uk) Add to contacts 11/4/2014
To: cherylsyy_92@hotmail.com

From: **datasubs@ebi.ac.uk**
Sent: Friday, 11 Apr, 2014 8: 25 AM
To: cherylsyy_92@hotmail.com

Dear Cheryl Sim Yan-Yi,

Regarding your query to ENA concerning X54865, I have taken a look at the original paper and can see that it is clearly published as 18S rRNA gene. Since it is so clearly published, I will not require the author's confirmation for an edit. I will now send this record to our updates queue for correction. Please note that the changes may take at least several weeks before they are visible on the public nucleotide browsers (ENA, GenBank, DDBJ etc.).

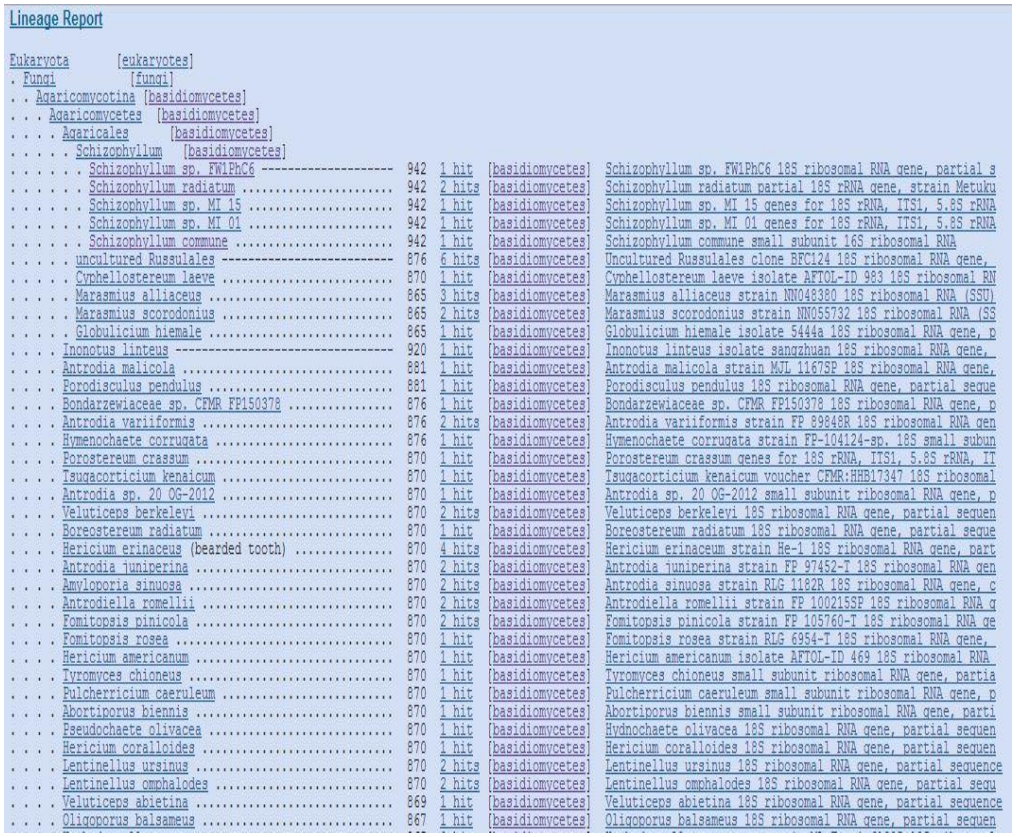
Thank you once again for informing us of this error.

Best wishes,

Richard Gibson
European Nucleotide Archive

APPENDIX G

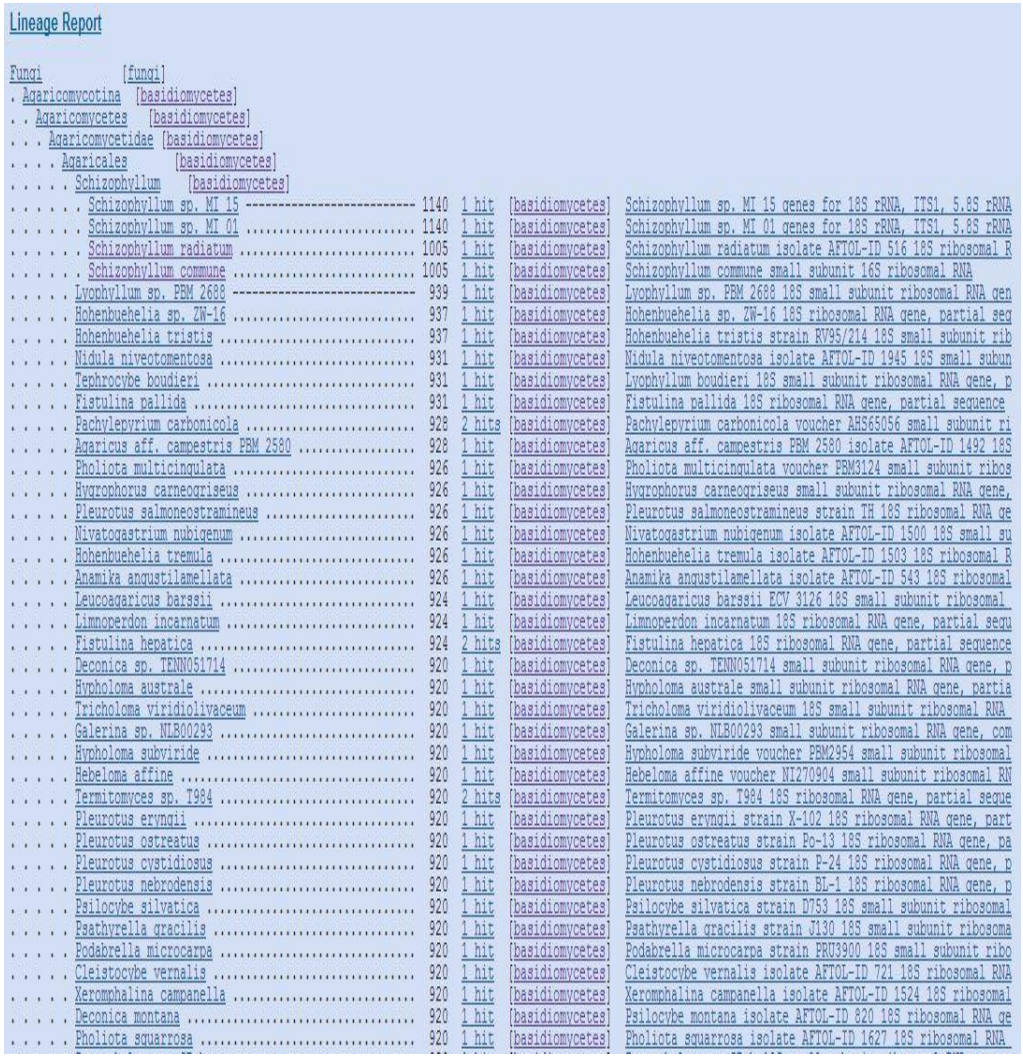
Lineage report of 18S rDNA region using EF4 primer



Lineage Report

Eukaryota [eukaryotes]				
. Fungi [fungi]				
. . Agaricomycotina [basidiomycetes]				
. . . Agaricomycetes [basidiomycetes]				
. . . . Agaricales [basidiomycetes]				
. . . . . Schizophyllum [basidiomycetes]				
. . . . . . Schizophyllum sp. FW1PhC6	942	1 hit	[basidiomycetes]	Schizophyllum sp. FW1PhC6 18S ribosomal RNA gene, partial s
. . . . . . Schizophyllum radiatum	942	2 hits	[basidiomycetes]	Schizophyllum radiatum partial 18S rRNA gene, strain Metuku
. . . . . . Schizophyllum sp. MI 15	942	1 hit	[basidiomycetes]	Schizophyllum sp. MI 15 genes for 18S rRNA, ITS1, 5.8S rRNA
. . . . . . Schizophyllum sp. MI 01	942	1 hit	[basidiomycetes]	Schizophyllum sp. MI 01 genes for 18S rRNA, ITS1, 5.8S rRNA
. . . . . . Schizophyllum commune	942	1 hit	[basidiomycetes]	Schizophyllum commune small subunit 16S ribosomal RNA
. . . . . uncultured Russulales	876	6 hits	[basidiomycetes]	Uncultured Russulales clone BFC124 18S ribosomal RNA gene,
. . . . . Cyphellostereum laeve	870	1 hit	[basidiomycetes]	Cyphellostereum laeve isolate AFTOL-ID 983 18S ribosomal RN
. . . . . Marasmius alliaceus	865	3 hits	[basidiomycetes]	Marasmius alliaceus strain NN048380 18S ribosomal RNA (SSU)
. . . . . Marasmius scorodonius	865	2 hits	[basidiomycetes]	Marasmius scorodonius strain NN055732 18S ribosomal RNA (SS
. . . . . Globulicium hiemale	865	1 hit	[basidiomycetes]	Globulicium hiemale isolate 5444a 18S ribosomal RNA gene, p
. . . . Inonotus linteus	920	1 hit	[basidiomycetes]	Inonotus linteus isolate sangzhuan 18S ribosomal RNA gene,
. . . . Antrodia malicola	881	1 hit	[basidiomycetes]	Antrodia malicola strain MJL 11675P 18S ribosomal RNA gene,
. . . . Porodisculus pendulus	881	1 hit	[basidiomycetes]	Porodisculus pendulus 18S ribosomal RNA gene, partial seque
. . . . Bondarzewiaceae sp. CFMR FP150378	876	1 hit	[basidiomycetes]	Bondarzewiaceae sp. CFMR FP150378 18S ribosomal RNA gene, p
. . . . Antrodia variiformis	876	2 hits	[basidiomycetes]	Antrodia variiformis strain FP 89848R 18S ribosomal RNA gen
. . . . Hymenochaete corrugata	876	1 hit	[basidiomycetes]	Hymenochaete corrugata strain FP-104124-sp. 18S small subun
. . . . Porostereum crassum	870	1 hit	[basidiomycetes]	Porostereum crassum genes for 18S rRNA, ITS1, 5.8S rRNA, IT
. . . . Tsugacorticium kenaicum	870	1 hit	[basidiomycetes]	Tsugacorticium kenaicum voucher CFMR:HHB17347 18S ribosomal
. . . . Antrodia sp. 20 OG-2012	870	1 hit	[basidiomycetes]	Antrodia sp. 20 OG-2012 small subunit ribosomal RNA gene, p
. . . . Veluticeps berkeleyi	870	2 hits	[basidiomycetes]	Veluticeps berkeleyi 18S ribosomal RNA gene, partial sequen
. . . . Boreostereum radiatum	870	1 hit	[basidiomycetes]	Boreostereum radiatum 18S ribosomal RNA gene, partial seque
. . . . Hericium erinaceus (bearded tooth)	870	4 hits	[basidiomycetes]	Hericium erinaceum strain He-1 18S ribosomal RNA gene, part
. . . . Antrodia juniperina	870	2 hits	[basidiomycetes]	Antrodia juniperina strain FP 97452-T 18S ribosomal RNA gen
. . . . Amyloporia sinuosa	870	2 hits	[basidiomycetes]	Antrodia sinuosa strain RLG 1182R 18S ribosomal RNA gene, c
. . . . Antrodiella romellii	870	2 hits	[basidiomycetes]	Antrodiella romellii strain FP 100215SP 18S ribosomal RNA g
. . . . Fomitopsis pinicola	870	2 hits	[basidiomycetes]	Fomitopsis pinicola strain FP 105760-T 18S ribosomal RNA ge
. . . . Fomitopsis rosea	870	1 hit	[basidiomycetes]	Fomitopsis rosea strain RLG 6954-T 18S ribosomal RNA gene,
. . . . Hericium americanum	870	1 hit	[basidiomycetes]	Hericium americanum isolate AFTOL-ID 469 18S ribosomal RNA
. . . . Tyromyces chioneus	870	1 hit	[basidiomycetes]	Tyromyces chioneus small subunit ribosomal RNA gene, partia
. . . . Pulcherricium caeruleum	870	1 hit	[basidiomycetes]	Pulcherricium caeruleum small subunit ribosomal RNA gene, p
. . . . Abortiporus biennis	870	1 hit	[basidiomycetes]	Abortiporus biennis small subunit ribosomal RNA gene, parti
. . . . Pseudochaete olivacea	870	1 hit	[basidiomycetes]	Hydnochaete olivacea 18S ribosomal RNA gene, partial sequen
. . . . Hericium coralloides	870	1 hit	[basidiomycetes]	Hericium coralloides 18S ribosomal RNA gene, partial sequen
. . . . Lentinellus ursinus	870	2 hits	[basidiomycetes]	Lentinellus ursinus 18S ribosomal RNA gene, partial sequence
. . . . Lentinellus omphalodes	870	2 hits	[basidiomycetes]	Lentinellus omphalodes 18S ribosomal RNA gene, partial sequ
. . . . Veluticeps abietina	869	1 hit	[basidiomycetes]	Veluticeps abietina 18S ribosomal RNA gene, partial sequence
. . . . Oligoporus balsameus	867	1 hit	[basidiomycetes]	Oligoporus balsameus 18S ribosomal RNA gene, partial sequen

Lineage report of the 18S rDNA region using EF3 primer.



Lineage Report

Fungi [fungi]				
. Agaricomycotina [basidiomycetes]				
. . Agaricomycetes [basidiomycetes]				
. . . Agaricomycetidae [basidiomycetes]				
. . . . Agaricales [basidiomycetes]				
. . . . . Schizophyllum [basidiomycetes]				
. . . . . Schizophyllum sp. MI 15	1140	1 hit	[basidiomycetes]	Schizophyllum sp. MI 15 genes for 18S rRNA, ITS1, 5.8S rRNA
. . . . . Schizophyllum sp. MI 01	1140	1 hit	[basidiomycetes]	Schizophyllum sp. MI 01 genes for 18S rRNA, ITS1, 5.8S rRNA
. . . . . Schizophyllum radiatum	1005	1 hit	[basidiomycetes]	Schizophyllum radiatum isolate AFTOL-ID 516 18S ribosomal R
. . . . . Schizophyllum commune	1005	1 hit	[basidiomycetes]	Schizophyllum commune small subunit 16S ribosomal RNA
. . . . . Lyophyllum sp. PBM 2688	939	1 hit	[basidiomycetes]	Lyophyllum sp. PBM 2688 18S small subunit ribosomal RNA gen
. . . . . Hohenbuehelia sp. ZW-16	937	1 hit	[basidiomycetes]	Hohenbuehelia sp. ZW-16 18S ribosomal RNA gene, partial seq
. . . . . Hohenbuehelia tristis	937	1 hit	[basidiomycetes]	Hohenbuehelia tristis strain RV95/214 18S small subunit rib
. . . . . Nidula niveotomentosa	931	1 hit	[basidiomycetes]	Nidula niveotomentosa isolate AFTOL-ID 1945 18S small subun
. . . . . Tephrocybe boudieri	931	1 hit	[basidiomycetes]	Lyophyllum boudieri 18S small subunit ribosomal RNA gene, p
. . . . . Fistulina pallida	931	1 hit	[basidiomycetes]	Fistulina pallida 18S ribosomal RNA gene, partial sequence
. . . . . Pachylepyrium carbonicola	928	2 hits	[basidiomycetes]	Pachylepyrium carbonicola voucher AH565056 small subunit ri
. . . . . Agaricus aff. campestris PBM 2580	928	1 hit	[basidiomycetes]	Agaricus aff. campestris PBM 2580 isolate AFTOL-ID 1492 18S
. . . . . Pholiota multicingulata	926	1 hit	[basidiomycetes]	Pholiota multicingulata voucher PBM3124 small subunit ribos
. . . . . Hygrophorus carneogriseus	926	1 hit	[basidiomycetes]	Hygrophorus carneogriseus small subunit ribosomal RNA gene,
. . . . . Pleurotus salmoneostramineus	926	1 hit	[basidiomycetes]	Pleurotus salmoneostramineus strain TH 18S ribosomal RNA ge
. . . . . Nivatogastrium nubigenum	926	1 hit	[basidiomycetes]	Nivatogastrium nubigenum isolate AFTOL-ID 1500 18S small su
. . . . . Hohenbuehelia tremula	926	1 hit	[basidiomycetes]	Hohenbuehelia tremula isolate AFTOL-ID 1503 18S ribosomal R
. . . . . Anamika angustilamellata	926	1 hit	[basidiomycetes]	Anamika angustilamellata isolate AFTOL-ID 543 18S ribosomal
. . . . . Leucoagaricus barssii	924	1 hit	[basidiomycetes]	Leucoagaricus barssii ECV 3126 18S small subunit ribosomal
. . . . . Limnoperdon incarnatum	924	1 hit	[basidiomycetes]	Limnoperdon incarnatum 18S ribosomal RNA gene, partial sequ
. . . . . Fistulina hepatica	924	2 hits	[basidiomycetes]	Fistulina hepatica 18S ribosomal RNA gene, partial sequence
. . . . . Deconica sp. TENN051714	920	1 hit	[basidiomycetes]	Deconica sp. TENN051714 small subunit ribosomal RNA gene, p
. . . . . Hypholoma australe	920	1 hit	[basidiomycetes]	Hypholoma australe small subunit ribosomal RNA gene, partia
. . . . . Tricholoma viridiolivaceum	920	1 hit	[basidiomycetes]	Tricholoma viridiolivaceum 18S small subunit ribosomal RNA
. . . . . Galerina sp. NLB00293	920	1 hit	[basidiomycetes]	Galerina sp. NLB00293 small subunit ribosomal RNA gene, com
. . . . . Hypholoma subviride	920	1 hit	[basidiomycetes]	Hypholoma subviride voucher PBM2954 small subunit ribosomal
. . . . . Hebeloma affine	920	1 hit	[basidiomycetes]	Hebeloma affine voucher NI270904 small subunit ribosomal RN
. . . . . Termitomyces sp. T984	920	2 hits	[basidiomycetes]	Termitomyces sp. T984 18S ribosomal RNA gene, partial seque
. . . . . Pleurotus eryngii	920	1 hit	[basidiomycetes]	Pleurotus eryngii strain X-102 18S ribosomal RNA gene, part
. . . . . Pleurotus ostreatus	920	1 hit	[basidiomycetes]	Pleurotus ostreatus strain Po-13 18S ribosomal RNA gene, pa
. . . . . Pleurotus cystidiosus	920	1 hit	[basidiomycetes]	Pleurotus cystidiosus strain P-24 18S ribosomal RNA gene, p
. . . . . Pleurotus nebrodensis	920	1 hit	[basidiomycetes]	Pleurotus nebrodensis strain BL-1 18S ribosomal RNA gene, p
. . . . . Psilocybe silvatica	920	1 hit	[basidiomycetes]	Psilocybe silvatica strain D753 18S small subunit ribosomal
. . . . . Psathyrella gracilis	920	1 hit	[basidiomycetes]	Psathyrella gracilis strain J130 18S small subunit ribosoma
. . . . . Podabrella microcarpa	920	1 hit	[basidiomycetes]	Podabrella microcarpa strain PRU3900 18S small subunit ribo
. . . . . Cleistocybe vernalis	920	1 hit	[basidiomycetes]	Cleistocybe vernalis isolate AFTOL-ID 721 18S ribosomal RNA
. . . . . Xeromphalina campanella	920	1 hit	[basidiomycetes]	Xeromphalina campanella isolate AFTOL-ID 1524 18S ribosomal
. . . . . Deconica montana	920	1 hit	[basidiomycetes]	Psilocybe montana isolate AFTOL-ID 820 18S ribosomal RNA ge
. . . . . Pholiota squarrosa	920	1 hit	[basidiomycetes]	Pholiota squarrosa isolate AFTOL-ID 1627 18S ribosomal RNA

LIST OF PUBLICATION

International DNA Database Publication

- Sim C.Y.Y., Kam Y.C., Ong, L.G.A. (2014). *Schizophyllum commune* isolates UTAR A1, internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence. (GeneBank Accession Number **KJ608191**)