ISOLATION OF OLEAGINOUS FUNGI FROM VARIOUS SOURCES IN KUCHING, SARAWAK

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ISOLATION OF OLEAGINOUS FUNGI FROM VARIOUS SOURCES IN KUCHING, SARAWAK

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A Final project report submitted in partially fulfillment of the Final Year Project II (STF 3015) Resource Biotechnology

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DECLARATION

I declare that the thesis entitled “Isolation of oleaginous fungi from various sources in Kuching, Sarawak” hereby is submitted for the Resource Biotechnology degree at the Universiti Malaysia Sarawak is my own work and that it has not been previously submitted anywhere for any award or any other university.

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<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>ddNTPs</td>
<td>Dideoxynucleotide triphosphate</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double-distilled water</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base pairs</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
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<tr>
<td>μl</td>
<td>Microlitre</td>
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<tr>
<td>min</td>
<td>Minutes</td>
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<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>RBDC agar</td>
<td>Rose Bengal Dichloran Chloramphenicol Agar</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>sec</td>
<td>Seconds</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>TAG</td>
<td>Triacylglycerols</td>
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Isolation of Oleaginous Fungi from Various Sources in Kuching, Sarawak.

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ABSTRACT

The purpose of this project was to isolate and identify oleaginous fungi from various sources in Kuching, Sarawak. Samplings of the oleaginous fungi were performed from food stalls in several markets, soil samples, sago effluent, and from sugar cane juices. Samplings were carried out using enrichment medium and Rose Bengal Dichloran Chloramphenicol Agar. The morphologies and sizes of the selected yeasts were observed under light microscope and Sudan IV was used to determine the lipid accumulation abilities in the fungi cells. In addition, the oleaginous fungi isolated from Rose Bengal Dichloran Chloramphenicol Agar underwent Polymerase Chain Reaction and the typing of fungi strains were identified using partial sequencing of D1/D2 region of 26S rDNA to establish their identities. The resultant sequences were compared with those available on the NCBI website database through the BlastN algorithm. From the BlastN analysis, two yeast species; Candida tropicalis and Candida krusei were identified from the twelve cultures.

Keywords: Oleaginous fungi, Rose Bengal Dichloran Chloramphenicol Agar (RBDC), Polymerase Chain Reaction (PCR), 26S rDNA, Sudan IV, Lipid accumulation.

ABSTRAK

Tujuan projek ini adalah untuk mengenal pasti kulat oleaginuous dari pelbagai sumber di Kuching, Sarawak. Persampelan kulat oleaginous diambil daripada gerai-gerai makanan di beberapa pasaran, sampel tanah, efluen sagu, dan jus tebu. Persampelan dijalankan menggunakan medium pengayaan dan agar Rose Bengal Dichloran Chloramphenicol. Morfologi dan saiz daripada yis yang dipilih telah diperhatikan menggunakan mikroscop serta Sudan IV digunakan untuk menentukan kebolehan pengumpulan lipid dalam sel-sel kulat. Di samping itu, kulat oleaginous diingkang dari agar Rose Bengal Dichloran Chloramphenicol telah menjalani proses Polymerase Chain Reaction dan kepelbagaian jenis spesies kulat dikenal pasti menggunakan penjukuan ranta D1/D2 26S rDNA diikuti oleh penjukuan DNA untuk mengenal pasti identiti mereka. Perbandingan urutan DNA yang dihasilkan boleh didapat di laman web NCBI melalui algoritma BlastN. Dari analisis BlastN, dua spesies yis iaitu; Candida tropicalis dan Candida krusei telah dikenal pasti daripada dua belas kultur.

Kata kunci: Kulat oleaginous, Rose Bengal Dichloran Chloramphenicol Agar (RBDC), Polymerase Chain Reaction (PCR), 26S rDNA, Sudan IV, Pengumpulan lipid.
The over-exploitation of non-renewable natural resources such as fossil fuel is causing worldwide petroleum depletion (Gonzalez, 2006). Therefore, alternative fuel to replace fossil fuel need to be found so that fossil fuels can be sustained for future use. One example of the alternative fuel is biodiesel which can act as both transportation fuel and to generate electricity (Walker, 2007).

According to Vertes et al. (2010), biodiesel is defined as transesterification of lipids with alcohol to produce alkyl esters. The advantages use of biodiesel is that it can be made from a variety of naturally occurring feedstock such as plant oil and animal fat (Wilson & Burgh, 2008). However, the production of biodiesel can be quite expensive when plant oil is used as raw materials. The alternative feedstock is oil from algae, but even this can be very technology intensive as well as being costly. This is because algae are photosynthetic organisms which require sunlight or light energy from electric sources for growth. On the other hand, they are other microorganisms such as fungi that do not need much light energy to growth and it also can produce lipid from various wastes for biodiesel production (Schaechter, 2012).

Oleaginous fungi are known for the production microbial oil, which can be accumulated up to 70% of intracellular lipid under stress condition. Under specific conditions, the oleaginous microorganisms can also grow at high rate with a short period of time to
produce high lipid content. Furthermore, these microorganisms can produce lipid content when grown on cheap sources of carbon (Mukhopadhyay et al., 2012). Therefore, this will lead to the production of cheap lipid for biodiesel production.

The habitats of the oleaginous fungi are oil-polluted environment and food such as meat, poultry product, and fruit (Rossi et al., 2011). In this study, sampling and isolation of oleaginous fungi from fruit stall, soil sample, sago effluent and sugar cane juices were carried out. Then, the morphology of the isolated oleaginous fungi was observed under the light microscope. Lipid accumulation assay was the carried out for selected fungi to determine their ability to produce lipids. For this Sudan IV was used for the confirmation of lipid presence. In addition, sequencing of the 26S rDNA polymerase chain reaction product was performed. Following this, BLAST analyses were done to identify the isolated fungi.

The aims of this project are to:

1. Isolate oleaginous fungi from the surface of fruit stall, soil samples, sago effluent and sugar cane juices.
2. Study the characteristic of oleaginous fungi under the light microscope.
3. Establish the ability of oleaginous fungi that are selected to accumulate lipid.
4. Identify oleaginous fungi by using D1/ D2 region of 26S rDNA sequencing analysis.
2.0 LITERATURE REVIEW

2.1. Biodiesel

Biodiesel is a derivation of monoalkyl esters of long chain fatty acids from renewable biolipids that is produced through transesterification of plant oils and animal fat with methanol in the present of catalyst to yield ethyl ester and glycerin (Demirbas, 2002). In general, biodiesel is produced mostly from agriculture co-products and byproduct such as plant oil natural oils and animal fat. The advantage of using biodiesel is its environmental friendliness because it is made from renewable resources. Besides that, Kirakosyan and Kaufman (2009) state that biodiesel is considered as cleaner-burning fuel than petrodiesel because biodiesel do not have sulfur content. Since biodiesel is a cleaner-burning fuel, it can increase the life of the fuel injection systems due to the clean engine combustion chamber as combustion becomes highly efficiency (Kirakosyan & Kaufman, 2009). Today, advanced technology uses microorganisms to produce biodiesel feedstock. The advantage of this technology is the fact that microorganisms grows much faster and are easier to handle than other conventional feedstocks. In the future, production of microbial lipid will become a major oil source in the transportation sector (Zhang & Hu, 2011).
2.2. Oleaginous fungi

Fungi are group of organisms that are classified in a separate kingdom from algae as they do not have chlorophyll. They mostly act as decomposers of plant materials and residues that will release the useful substances to other member of the ecosystem (Hine & Martin, 2005). In the ecosystem, fungi can act as mutualistic symbionts, decomposer, saprophytes, and parasites of other organisms. In general, majority of fungi are filamentous and unicellular yeast (Kavanagh, 2011).

Some fungal groups have the abilities to accumulate lipid. These fungi are called oleaginous fungi and they have been known from the 1970s (Rossi et al., 2011). Examples of oleaginous fungi are *Candida utilis*, *Lipomyces starkeyi*, *Trichosporon cutaneum*, and *Rhodotorula minuta* (Pan et al., 2009). Oleaginous fungi have the capability to synthesizes and accumulate high amounts of triacylglycerols (TAG) within their cells. This TAG can be easily converted to biodiesel through a process called transesterification via the conversion of TAG and methanol with potassium hydroxide as a catalyst (Socha & Sello, 2010). The advantage in using oleaginous fungi is their abilities to grow in extreme environments such as low temperature, and low oxygen availabilities (Butinar et al., 2007). Furthermore, their short live spans enables the production of high-value TAG to produce biodiesel in a short period of time at low a cost as compared with the traditional feedstock such as plant oils.
2.3. Lipid accumulation in fungi

Lipid accumulation in oleaginous fungi occurs when there is severe limitation of nitrogen in the growth medium while excess carbon source is available (Rossi et al., 2011). In the normal growth condition of fungi, nitrogen plays an important role for the synthesis of proteins and nucleic acids. Meanwhile, carbon flux distributes anabolic process to produce the organic compounds such as carbohydrates and lipids. Rossi et al. (2011) stated that when nitrogen is limited, growth rate slows down and the synthesis of proteins and nucleic acids stop. The excess carbons are then converted into storage polysaccharides in non-oleaginous organism whereas oleaginous organism utilized excess carbon source for lipid synthesis (Rossi et al., 2011). As a result, TAG accumulation occurred within intracellular bodies of oleaginous organism (Granger et al., 1993; Ratledge & Wynn, 2002). High amount of lipid accumulated in oleaginous fungi is dependent on the regulation biosynthetic pathway and the supply of precursors such as acetyl-CoA, malonyl-CoA, and the cofactor NADPH.

In batch fermentation of oleaginous fungi, there consist of two phase; growth phase and lipogenic phase which are shown is Figure 2.1. Rossi et al., (2011) stated that the production of lipid-free biomass occurs during the growth phase whereas lipid production occurs during the lipogenic phase. During the growth phase, the oleaginous fungi growth rapidly and therefore the growth is balanced and lipid-free biomass is mostly produced. Meanwhile, during the lipogenic phase, concentration of nitrogen become limited lead to decreasing of both growth rate and carbon flow toward biomass generation. Furthermore, the lipid production is triggered in lipogenic phase.
Time Course of Batch Fermentation

Figure 2.1: Graph of time course of a batch fermentation of oleaginous fungi.

2.4. Sudan IV lipid staining

Sudan IV is a lysochrome or fat soluble dye used in lipid staining, triglycerides demonstration in frozen food, and also lipoprotein staining in paraffin sections. Dorland (2011) stated that the dark red Sudan IV is also known as Scarlet Red or Scharlach R because the appearance of positive result is reddish-orange when the lipid is present in the medium along with 70% ethanol. Chemically, Sudan IV is not soluble in water but soluble in lipids. Sudan IV does not involve any chemical bonding but the color present the function of the solubility of the dye in the lipid.
2.5. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a powerful method for amplifying particular segments of DNA through a three-step process in multiple cycles (Saiki et al., 1985). The three steps cycling process are denaturation, annealing and elongation. In the first step of the PCR, the double-stranded DNA template is denatured at temperature to 94 °C for 15 seconds. In the denaturation process, the single-stranded DNA template is produced from separation of two intertwined strands of DNA. The single-stranded DNA template is then annealing with sequence-specific primers followed by the addition of a thermostable DNA polymerase, *Taq* DNA polymerase, at the temperature of about 65 °C for another 15 seconds (Chien et al., 1976; Kaledin et al., 1980; Longley et al., 1990; Lawyer et al., 1993; Lyamichev et al., 1993). The *Taq* DNA polymerase is an enzyme for extending the annealed primers at 72 °C for 5 minutes. The new product of PCR then becomes an additional template for subsequent cycles of amplification. These three steps are repeated in cycles for 25 to 30 times which can amplify $3 \times 10^5$ target molecules and also increase amount of target DNA concentration of $10^5$ to $10^9$ times the original (Innis & Gelfand, 1990).

2.6. Gel electrophoresis

Gel electrophoresis is a process which allows DNA fragment to be separated according to the size of the fragments. This is a very important technique in the study of DNA, because this technique allowed the researcher to identify the number of base pair of DNA and also the size of the DNA (Lee, 2009). During gel electrophoresis, the DNA in placed in the negative end of the electrophoresis gel to allow it to move, because DNA are negatively
charged, and this allows the DNA fragments to move from the negative end to the positive end of the gel (Clark & Pazdernik, 2012). There are several types of gel to be used in electrophoresis namely agarose gel and polyacrylamide gel. The most popular gel used for gel electrophoresis is the agarose gel. The advantage of using agarose gel as the gel matrix in gel electrophoresis is that, it is easier to be prepared and it is non-toxic before the ethidium bromide is added into the agarose gel. In the other hand, polyacrylamide is a neurotoxin to human, which require careful handling (Greene & Pohanish, 2005). The polyacrylamide are able to separate the fragment which is in small size of DNA fragment in the range between 1 bp to 500 bp, while the agarose gel able to separate DNA fragment from size between 200 bp to 50,000 bp. The use of gel matrix should be depending on the size of DNA used during electrophoresis. The factors that have important effects on the mobility of DNA fragments in agarose gels are the molecular size of the DNA, agarose concentration, the conformation of agarose, the applied voltage, electrophoresis buffer, type of agarose, and the present of ethidium bromide in the gel (Vennison, 2009).

2.7. 26S rDNA (D1/ D2 region) sequencing analysis

The 26S rDNA (D1/D2 region) sequencing is a popular molecular method for the identification and typing of fungi strains (Maoura et al., 2005). The advantage of sequencing the domain D1/D2 region subunit 26S rDNA is identification of fungi species and also permits phylogenetic analysis. DNA sequences data from fungal isolates have not been registered in data libraries can be identified from molecular phylogenetic analyses by using 26S rDNA sequences (Sugita & Nishikawa, 2003). According to Sugita and Nishikawa (2003), 26S rDNA data have been used in identifying 666 out of 685 fungi
DNA sequencing is a process whereby the nucleotide bases are read in order to determine the four bases: adenine, guanine, cytosine, and thymine in a strand of template DNA. In 1970s, two kind of traditional methods for sequencing the DNA were Sanger sequencing and Maxam and Gilbert sequencing method (Blackburn et al., 2006). According to Sanger and Coulson (1975), the Sanger sequencing method uses DNA polymerase to making a copy of a single-stranded DNA. Meanwhile, Maxam and Gilbert sequencing method is introduced by Allan Maxam and Walter Gilbert in 1977, uses chemical degradation method whereby the chemicals are used to break the DNA preferentially at each of the four nucleotide bases (Maxam & Gilbert, 1977). Both methods rely upon sequencing only one strand at a time.

Currently, there are two kinds of DNA sequencing method which are chain-terminator sequencing and dye-terminator sequencing (Hutchison, 2007). Chain-terminator
sequencing uses a single-stranded DNA template, a DNA primer, a DNA polymerase, a modified nucleotides (ddNTPs) as chain terminator for DNA strand elongation, and radioactively labeled nucleotides. Template DNA is the single-stranded DNA to be sequenced. In the first step of DNA sequencing, the short oligonucleotide act as primer that anneal on each of the template strands for the synthesis of a new DNA strand that will be complimentary to the template DNA. Hutchison (2007) state that the DNA sample is divided into four separate sequencing reactions which contain all four of deoxynucleotides (dATP, dGTP, dCTP, and dTTP) and the DNA polymerase. Next, one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, and ddTTP) are added into each reaction. Lacking a 3’-OH group play an importance role in the formation of a phosphodiester bond between two nucleotides. As a result, terminating DNA strand extension and DNA fragment to occur. The new synthesized and labeled DNA fragments are denatured and then running in polyacrylamide gel with four individual lanes (lanes A, T, G, C). The DNA sequence of polyacrylamide gel can be read by using autoradiography or UV light. On the other hand, the dye-terminator sequencing is a variant of Sanger sequencing whereby each of the four dideoxynucleotide chain terminators (ddNTPs) was labeled with a different fluorescent dye. This method was also known as the next generation sequencing because first report of automation DNA sequencing already be done in 1986. In addition, dye-terminator sequencing can be sequence the template DNA above 1000 nucleotides bases (Huson & Reinert, 2007).
2.9. Basic local alignment search tool (BLAST)

Basic local alignment search tool (BLAST) is an algorithm for comparing primary biological sequence information such as amino acid sequences of different protein or nucleotides of DNA sequences (Altschul et al., 1990). BLAST is available on the World Wide Web through a large server at National Center for Biotechnology Information (NCBI). By using this site, BLAST aligns a query sequence against the subject sequenced in the database. There BLAST programs which can distinguished the type of the query sequenced such as DNA or protein with the type of the subject database. For example, BLASTP, BLASTN, BLASTX, TBLASTN, and TBLASTX. BLASTP used to compare an amino acid query sequences against a protein sequenced database. BLASTN is used for compares a nucleotide query sequence against a nucleotide query sequence database. BLASTX compares the six frame conceptual translation products of a nucleotide query sequence in both strands against a protein sequences database. Meanwhile, TBLASTN compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames in both strands. Finally, TBLASTX compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database (Altschul et al., 1994). The results of BLAST analyses are obtained in the form of ranked list followed by a series of sequence alignments, statistical and analysis.
3.0 MATERIALS AND METHODS

3.1. Materials

3.1.1. Materials for sample collection, enrichment media and isolation.

1. 70% Ethanol (Hamburg, Germany)
2. 10% Dextrose anhydrous (Daejung, Korea)
3. Chloramphenicol (0.1 g/l) (Duchefa, Netherlands)
4. Crystal violet
5. Glycerol (Daejung, Korea)
6. Light microscope (Olympus BX51, Japan)
7. Luria broth (Sigma, USA)
8. Luria broth agar (Conda, Spain)
9. Rose-Bengal Dichloran Chloramphenicol Agar (RBDC) (Oxoid, England)
10. Sterile cotton bud
11. Sudan IV (Sigma, USA)
12. Yeast malt broth (Difco, USA)
13. Mo Bio PowerSoil® Isolation Kit (Mo Bio Laboratory, USA)
14. UltraClean® PCR Clean-Up Kit (Mo Bio Laboratory, USA)