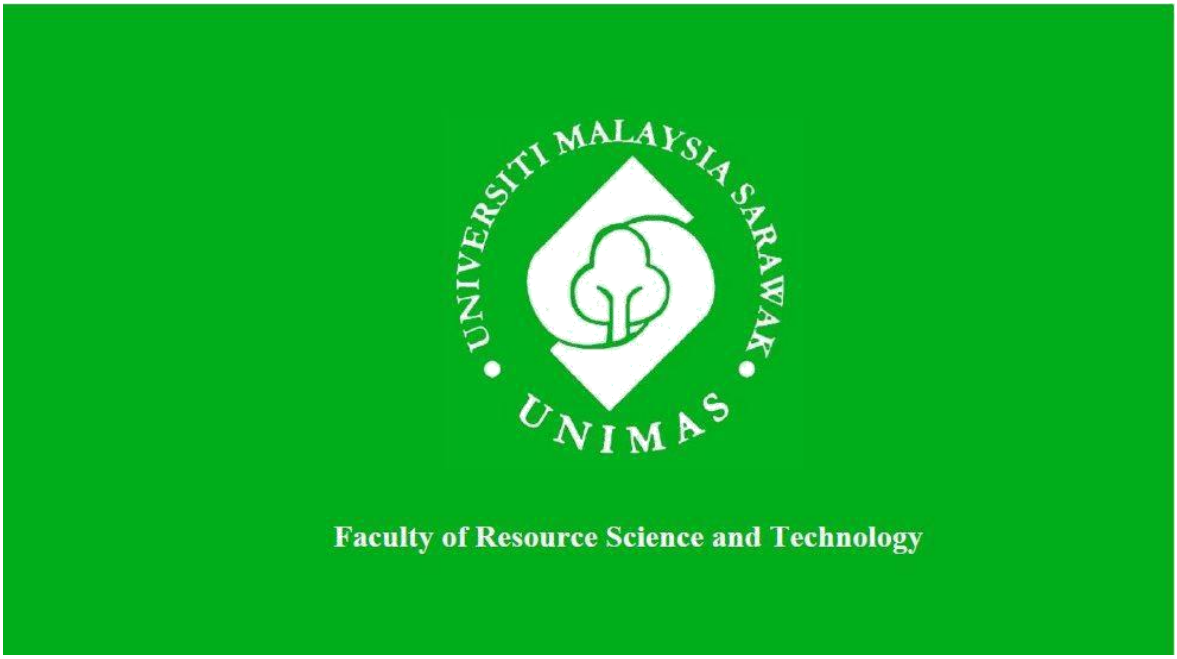

GROWTH OF *Rhizopus oligosporus* IN SAGO EFFLUENT AT DIFFERENT NITROGEN CONCENTRATION

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Bachelor of Science with Honours
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A report submitted in partial fulfillment of the Final Year Project (STF3015)

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DECLARATION

I hereby declare this Final Year Project Report entitled "**Growth of *Rhizopus oligosporus* in Sago Effluent at Different Nitrogen Concentration**" is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously submitted for any other degree at UNIMAS or other institutions.

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(SHAHIRAH SAFIN BINTI AMRAN)

JUNE 2015

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LIST OF ABBREVIATIONS

˚C	Degree Celsius
%	Percentage
h	Hour
min	Minute
g	Gram
L	Liter
mL	Milliliter
µm	Micrometer
g/L	Gram per liter
t/ha	Tons per hectares
SmF	Submerged Fermentation
SSF	Solid State Fermentation
COD	Chemical Oxygen Demand
BOD	Biological Oxygen Demand
TSS	Total Suspended Solid

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Growth of *Rhizopus oligosporus* in Sago Effluent at Different Nitrogen Concentration

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ABSTRACT

There are increasing concerns over the pollution created by sago effluent. Fortunately, biotechnological treatment of sago effluent wastewater can yield valuable products while also purifying the sago effluent. In this study, we reported the growth rate of *Rhizopus oligosporus* in sago effluent at 0, 25, 50 and 100 mM nitrogen concentration via submerged fermentation (SMF). The highest biomass production of the fungus was notable at 100 mM nitrogen concentration which was 84.47 g/L for wet biomass and 3.83 g/L for dry biomass followed by 50, 25 and 0 mM NO_3^-. These phenomena may be explained that a higher nitrogen concentration resulted in fast fungal cell growth leading to a higher biomass production. The phenol sulfuric test showed carbohydrate concentration fell as growth proceed. Reduction in phenol concentrations by day 6 reached 95% with nitrogen concentration of 100 mM. The study showed the total nitrate and nitrite in the sago effluent decreased gradually after 6 days of fermentation. In 100 mM NO_3^-, the nitrate and nitrite concentration was reduced from 0.984 to 0.271 g/L and 0.194 to 0.032 g/L, respectively. This result proved the promising applicability of *R. oligosporus* to treat and purify sago effluent via biological processes. The study have shown that the biomass produced possesses a high protein value and can be used as a better choice for high protein fungal biomass (HPFB) production using inexpensive energy sources like sago effluent.

Keywords: *Rhizopus oligosporus*, sago effluent, submerged fermentation (SmF), water treatment and high protein fungal biomass (HPFB).

ABSTRAK

Terdapat kebimbangan yang semakin meningkat ke atas pencemaran yang dicipta oleh efluen sagu. Mujurlah, rawatan bioteknologi efluen air sisa sagu boleh menghasilkan produk yang bernilai sementara juga menulenkan efluen sagu. Dalam kajian ini, kami melaporkan kadar pertumbuhan Rhizopus oligosporus dalam efluen sagu pada 0, 25, 50 dan 100 mM kepekatan nitrogen melalui penapaian tenggelam (SMF). Pengeluaran biomass tertinggi kulat adalah ketara pada 100 mM kepekatan nitrogen yang 84.47 g/L bagi biomas basah dan 3.83 g/L untuk biojisim kering diikuti dengan 50, 25 dan 0 mM NO_3^-. Fenomena ini boleh dijelaskan bahawa kepekatan nitrogen yang lebih tinggi menyebabkan pertumbuhan sel kulat cepat membawa kepada pengeluaran biomass yang lebih tinggi. The fenol ujian sulfurik menunjukkan kepekatan karbohidrat jatuh pertumbuhan meneruskan. Pengurangan dalam kepekatan fenol siang 6 sampai 95% dengan kepekatan nitrogen 100 mM. Kajian itu menunjukkan jumlah nitrat dan nitrit di dalam efluen sagu menurun secara beransur-ansur selepas 6 hari penapaian. Dalam 100 mM NO_3^-, nitrat dan nitrit kepekatan dikurangkan 0.984 kepada 0.271 g/L dan 0.194 kepada 0.032 g/L, masing-masing. Keputusan ini membuktikan kesesuaian yang menjanjikan R. oligosporus untuk merawat dan membersihkan efluen sagu melalui proses biologi. Kajian ini telah menunjukkan bahawa biomass yang dihasilkan mempunyai nilai protein yang tinggi dan boleh digunakan sebagai pilihan yang lebih baik untuk protein tinggi biomass kulat (HPFB) pengeluaran menggunakan sumber tenaga yang murah seperti efluen sagu.

Kata kunci: Rhizopus oligosporus, efluen sagu, fermentasi tenggelam, rawatan air dan biomass kulat tinggi protein.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Sago palm mainly thrives on peat soil, which is inappropriate for most other crops. It does not need any form of fertilization, apart from some cleaning at the ground throughout its first year of growth. Awg-Adeni *et al*. (2010) stated that sago is the highest starch producer compared to all other starch crops of the world such as corn, rice, wheat, and potato. Thus, sago palm is the best candidate as the main starch source for tropical country like Malaysia. Even though the initial waiting period is quite long and sometimes up to 8 years to harvest, but it is worth it since sago can produce starch approximately at 25 t/ha annually (Awg-Adeni *et al*., 2010). Unfortunately, the effluent that flows from sago processing plants into the rivers is potentially leading to river pollution due to the large amount of suspended solid and liquid and also from minor concentrations of starch and sugar residues.

In Malaysia, sago effluent is abundantly generated from numerous sago mills and discharged every day as wastewater into the river (Getha *et al*., 1998). Generally, a sago mill produces about 25 tons dried sago daily. As 20 L wastewater per kg of starch formed, it will generate around 500 tons effluent that contain 25 tons fibers and 15 tons starch. Sago effluent contains enormous amount of organic material that can possibly cause water pollution. Since the fibers degrade slowly, it will reduce water quality as the amount of suspended solid in the water keeps increasing. Both fiber and wastewater can be treated by the fungal cultivation via submerged fermentation (Jin *et al*., 1999). The focus of this research is the treatment process of sago effluent by *R. oligosporus* by reducing the Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), and Total Suspended Solid (TSS) in order to minimize the polluting effect to the environment.

The use of fungi to treat waste water as a substrate is the right option since it can produce a wide range of enzymes and fine biochemical (Gopalakrishnan *et al*., 2012). Besides that, fungi are more effective in metabolizing carbohydrate like starch as compared to bacteria (Gopalakrishnan *et al*., 2012). It is convenient to use *R. oligosporus* for production of high protein fungal biomass (HPFB) when cultured in cheap waste material. The rapid growth and high protein content have made the fungi the best candidates in the production of protein rich food (Jaganmohan *et al*., 2013). Besides that, non-pathogenic *R. oligosporus* has a lack demand for any specific growth factors to produce biomass in a relatively large quantities.

This cultivation process is very beneficial as it can convert the organic substances in waste water into harvestable fungal biomass that later can be used as animal feed and possibly in human foods. At the beginning, yeast was used for human foodstuffs but the growing shortage of essential amino acid for protein synthesis in animals has stimulated the research of HPFB production in animal feedstock (Jaganmohan *et al*., 2013). This protein-enriched feed can safely be fed to the poultry and fish as the researchers have found the supplementation of methionine to the animal feedstock helps to promote growth performance by increasing protein synthesis and enhancing feed efficiency of the animal (Nasseri *et al*., 2011).

Nowadays, SMF is widely used in the production of HPFB for human and animal foods. Large scale fermentation of SMF could help to improve conversion efficiency and consequently increase yield. This industrial biotechnology application may reduce the total cost of the final product (Zhang *et al*., 2008). In the current research, an attempt has been made to synthesise HPFB with *R. oligosporus* through SMF by using sago effluent as inexpensive energy sources.

The main purpose of this project is to study the growth of *R. oligosporus* in sago effluent at different nitrogen concentration. The specific objectives of this study are:

1. To optimize the growth rate of *R. oligosporus* in sago effluent (0, 25, 50 and 100 mM NO_3^-).
2. To determine the optimum nitrogen concentration for the growth of *R. oligosporus* in sago effluent.
3. To determine the ability of *R. oligosporus* to treat sago effluent via submerged fermentation.

1.2 Problem statement

Sago effluent contains enormous amount of organic material that can possibly cause water pollution. In order to reduce this problem, further research is conducted on how to remove organic material from sago effluent by using a biological process such as submerged fermentation. *R. oligosporus* was cultured in the sago effluent for up to 6 days. As the culture was harvested, the product can be used as a protein source and organic health supplement while at the same time purifying the sago effluent. This research suggested the biological process will reduce BOD, COD, and TSS of the sago.

CHAPTER 2

LITERATURE REVIEW

2.1 *Rhizopus oligosporus*

Rhizopus oligosporus is a zygomycete filamentous fungus that belongs to the *Mucoraceae* family (Takaya *et al*., 1998). The sporangial size and the number of spores per sporangia differed between strains. Specifically, the sporangial diameters of the *R. oligosporus* is in the range of 40 to 120 µm that had approximately 100 spores (Jennessen *et al*., 2008). The sporangiophores of the fungus are unbranched, short and very irregular in shape which show no stripes under any conditions of growth.

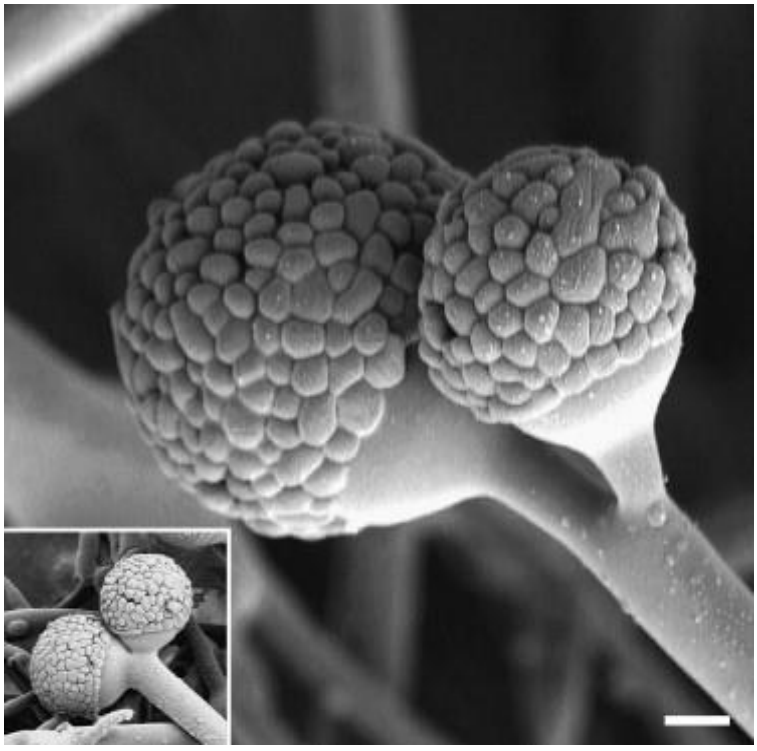

Figure 1: Morphology of *R. oligosporus* as seen under LT-SEM (Jennessen *et al*., 2008).

This fungus is widely used to produce a fermented Indonesian soybean product, tempeh since ancient times (Rusmin & Ko, 1974). Throughout the incubation process, the soybeans are bound with *R. oligosporus* together with white mycelium to form a cake and various

enzymes. The enzymes that are released by the fungus will make the protein-rich product more digestible and palatable to human. Moreover, the enzymes synthesised will hydrolyse the lipid, starch, and protein that subsequently change the taste, aroma and the texture of the tempeh (Handoyo, 2006). The fungus grows quickly at the temperature between 34 and 35 ˚C (Miszkiewicz *et al*., 2004).

There are many other applications of *R. oligosporus* including production of industrial enzymes and as well as treatment of wastewater (Jennessen *et al*., 2008). There are many advantages using the fungi over the bacteria in the wastewater treatment since it is low in costs due to the use of raw material and it does not need specific nutrients. Most fungi, including *R. oligosporus* can be tolerated to low pH environment and it is easy to separate the pellet biomass from the fermentation broth (Huang *et al*., 2005).

R. oligosporus also has the ability to grow in sago effluent and thus produce biomass which had potential as an aquaculture (Jin *et al*., 1999). In addition, *R. oligosporus* are less susceptible to contamination by other microorganism. Awg-Adeni *et al*. (2010) have undertaken a study on the bioconversion of sago residue into value added products such as animal feed, enzyme, fermentable sugar and adsorbent. Other studies by Getha *et al*. (1998) have shown that the biomass is harmless and safe to Artemia and can be right supplement for prawn feed. According to Miszkiewicz *et al*. (2004), *R. oligosporus* is a generally regarded as safe (GRAS) organism.

2.2 Sago effluent

Sago residue contains solid and liquid materials such as pith residue, bark, and wastewater that are produced in large quantities as a by-product from the sago starch processing mill especially in Sarawak, Malaysia (Getha *et al*., 1998). The large amount of organic material in sago effluent has a very high concentration of Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), and Total Suspended Solid (TSS). Previous study by Rashid *et al*. (2010) reported the BOD, COD, and TSS in primary effluent could be in the range from 910 mg/L to 1300 mg/L, 780 mg/L to 5130 mg/L, and 19 mg/L to 20000 mg/L respectively. Presently, these residues are discharged into nearby rivers together with wastewater, resulted in serious contamination of the rivers.

Figure 2: Sago processing mill in Mukah, Sarawak

A typical sago processing mill consumes around 1000 logs daily, generating at least 400 tons of slurry effluent that contains approximately 5% solids. The discharged sago effluent definitely affected the fish and other aquatic organism in the river (Singhal *et al*., 2008). A study by Nagarajan and Suresh (2005) proved that sago effluent reduces the fish performance by damaging the tissue of Indian carp. Without proper treatment and visible enforcement from the relevant authorities on wastewater disposal, sago effluent is inevitably led to serious water pollution (Awg-Adeni *et al*., 2010).

Various studies have been conducted on the treatment of sago effluent using a biological conversion process. Sago effluent has a relatively a very high carbon to nitrogen ratio (105: 0.12) which is very suitable for fermentation process for the production of high protein fungal biomass (Singhal *et al*., 2008). Phang *et al*. (2000) indicated that High Rate Algal Pond (HRAP) can treat sago effluent while supporting the growth and production of *Spirulina platensis*.

Based on study by Vikineswary *et al*. (1997), *Rhodopseudomonas palustris* strain B1 produces high biomass concentration after 96 hours of growth in anaerobic-light culture system that consequently reduces the Chemical Oxygen Demand (COD) of the sago effluent. Banu *et al*. (2006) verified that fermentation process can reduce COD level up to 91% and at the same time decrease the total solid from 67% in the sago waste. All of these studies were carried out as an alternatives for the waste water treatment to prevent pollution.

2.3 Submerged Fermentation (SmF)

Fermentation is the biological process that converts complex substrates into simple compounds by numerous microorganisms such as fungi and bacteria (Subramaniyam & Vimala, 2012). The process involves mass cultivation of cells that are carried out in bioreactors which provide efficient mass transfer that consequently give the best conditions for the microorganisms to grow. Nowadays, fermentation techniques have been broadly and commonly used for the production of highly beneficial substances to individuals and industry. There are two types of fermentation that is suitable for this study which are Solid State Fermentation (SSF) and Submerged Fermentation (SmF). Generally, these techniques are classified based on the type of substrate used during fermentation process.

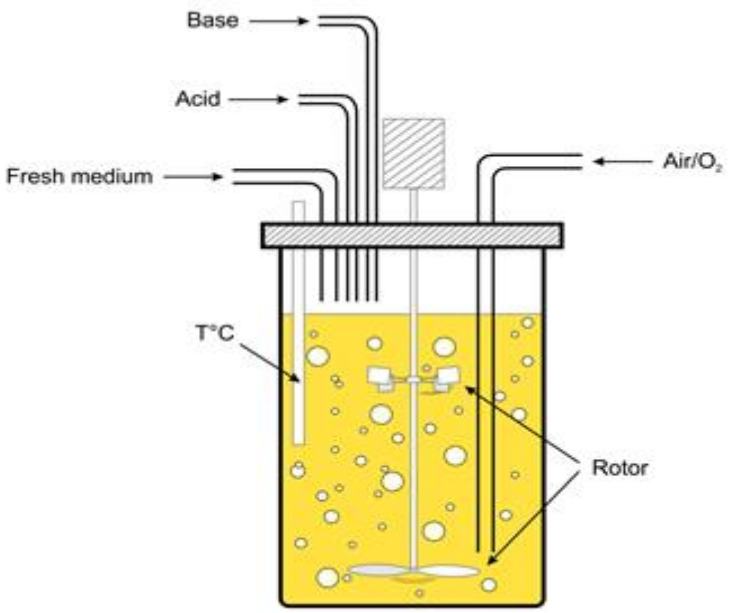

Figure 3: Fed-batch fermentation in submerged cultures. (Retrieved from http://www.tankonyvtar.hu/)

SmF or also known as liquid fermentation usually uses free flowing liquid substrate like broths and molasses. In this fermentation technique, the bioactive compounds that are produced will be secreted into the fermentation broth. Generally, nutrients need to be constantly supplemented since the substrates are utilized quite fast in this fermentation technique. Some common substrates that are used in SmF consist of fruit and vegetable juices, liquid media, soluble sugars, molasses, and sewage water (Zoppas *et al.*, 2013). Utilization of low cost agricultural and agro-residual residue such as sago effluent as substrates can lower down the capital and operating costs (Zoppas *et al*., 2013). According to Subramaniyam and Vimala (2012), SmF is best suited for fermentation techniques involving microorganisms that require high moisture content.

One of the advantages of this technique is that purification of products is easier compared to SSF. Environmental factor such as temperature, pH, oxygen levels, water activity and concentrations of nutrients affect fungal growth and product formation (Zoppas *et al*., 2013). In submerged stirred fermentation, environmental control is relatively simple because of the solution of nutrients and products in the liquid phase. Main advantages in SmF are related to mixing, heat exchange, oxygen transfer, moisture control and gradients of pH, nutrient and product as a consequence of homogeneity of the culture (Chisti *et al*., 1999). SmF provides efficient mass transfers between the microbes and the environment. Optimal mass transfers in the SmF can be achieved by having a good mixing of the broth and good aeration to ensure the microbes are exposed to oxygen and nutrients.

2.4 Ammonium Nitrate

Ammonium nitrate is a chemical compound with the chemical formula NH_4NO_3. It is composed of salt of ammonia and nitric acid. Ammonium nitrate appears in a white crystalline and it is also colourless in room temperature. The melting point of ammonium nitrate is 337 ˚F or 169.6 ˚C (Daintith, 2008). The compound is rhombohedral in shape and highly soluble in water (Daintith, 2008).

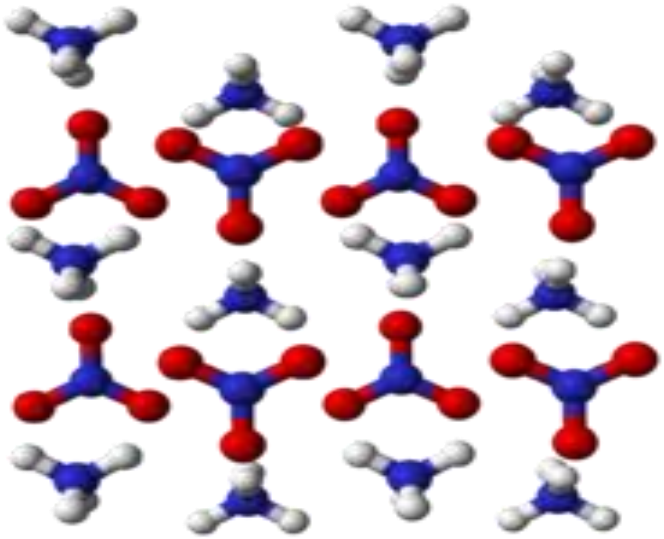

Figure 4: Rhombohedral shape of ammonium nitrate. (Sources: http://www.ammoniumnitrate.org/)

Generally, nitrogen source is one of the most plentiful substance in the fermentation media after carbon source. Nitrogen source can also be used as the energy source in certain organisms. Most of non-photosynthetic bacteria and fungi like *R. oligosporus* assimilate nitrogen from nitrate (Todaro & Vogel, 2014). Nitrogen is used for the anabolic synthesis of nitrogen-containing cellular substances for example purines, amino acids, RNA, and DNA. Unlike yeasts and bacteria which have problems utilizing nitrogen in this form, algae and fungi can use ammonium nitrate as nitrogen sources.

Various studies have been conducted using ammonium nitrate as a nitrogen sources in the fermentation of fungi. Based on study by Kavanagh (2011), ammonium nitrate has been used as a nitrogen sources for citric acid production by *Aspergillus niger*. It has been reported that the presence of nitrogen in the fermentation medium has profound effect on the production of citric acid (Kavanagh, 2011). Other studies by Lalitha *et al*. (2012) have proved that ammonium nitrate can be incorporated with any carbon sources to give high yield in the production of amylase by *Trichoderma viride*. A balanced fungal medium should contain about ten times more carbon than nitrogen (10: 1) to ensure high production of fungal proteins during cultivation (Gopalakrishnan *et al*., 2012).