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TITLE: Screening the Microorganism for Lipid Accumulation on Glycerol a by-product from Biodiesel Production

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THESIS

SCREENING THE MICROORGANISM FOR LIPID ACCUMULATION ON GLYCEROL A BY-PRODUCT FROM BIODIESEL PRODUCTION

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Aksorn Riengsilchai 2014: Screening the Microorganism for Lipid Accumulation on Glycerol a by-product from Biodiesel Production. Master of Science (Chemistry), Major Field: Chemistry, Department of Chemistry. Thesis Advisor: Associate Professor Vittaya Punsuvon, Ph.D. 93 pages.

In this work, two kinds of oleaginous microorganisms: *M. isabellina* and *R. glutinis* were screened for their abilities to utilize glycerol, a by-product which is partially purified from palm oil-biodiesel production plant, as a carbon source for biomass and lipid productions. The optimization of lipid production of the most promising oleaginous strains was conducted using response surface methodology (RSM). A 5-level 2-factor central composite design (CCD) was used to build the statistical model. The optimum cultivation conditions for *M. isabellina* NBRC 105998 found in this study were: the glycerol concentration in the medium (44.14 g/L), the fungal colony for 2 piece fungal discs (7.48 x 10^6 spores), 7 mm diameter and incubated at 30°C for 12 days. This optimum conditions gave 45.13% of the lipid content. The optimum cultivation conditions for *R. glutinis* NBRC 1099 found in this study was: the glycerol concentration in the medium (34.14 g/L), the inoculum volume was 1.6 mL (4.5 x 10^7 cell) and incubated at 30°C for 24 hours. This optimum condition *R. glutinis* NBRC 1099 gave 43.65% of the lipid content. Furthermore, the biodiesel production potencies of the best lipid accumulation strains were also investigated. The percentage of fatty acid methyl ester of biodiesel produced from *M. isabellina* NBRC 105998 was 95.15% and the percentage of fatty acid methyl ester of biodiesel produced from *R. glutinis* NBRC 1099 was 88.36%. *M. isabellina* NBRC 105998 and *R. glutinis* NBRC 1099 provided high quantity of lipid contentfor biodiesel production. Therefore, the biodiesel produced by these oleaginous fungi and yeast strain, which can grow rapidly without any environmental or ethical issues, can be an excellent alternative source for *biodiesel production* in the future.

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Student's signature Thesis Advisor's signature

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Aksorn Riengsilchai

July 2014

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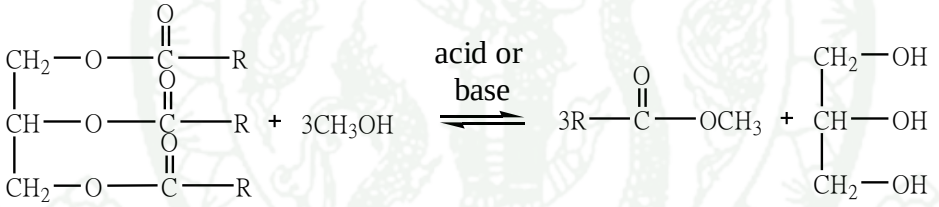
SCREENING THE MICROORGANISM FOR LIPID ACCUMULATION ON GLYCEROL A BY-PRODUCT FROM BIODIESEL PRODUCTION

INTRODUCTION

According to current environmental problems and depleting fossil fuel, searching for the renewable energy sources for substitution of the fossil energy is necessary and important. Many sources and methods have been suggested, however, biodiesel, an alternative fuel, has attracted considerable attention worldwide as renewable, biodegradable, and nontoxic fuel. Biodiesel is nonpetroleum-based fuel produced from vegetable oil or animal fat which molecule has been reduced into ethyl ester or methyl ester form via the transesterification reaction as shown in Equation 1.



(1)

Oil or fat Methanol Ester (Biodiesel) Glycerol

From this reaction, a major by-product glycerol was generated around 10% by weight. Since the global biodiesel industry has grown significantly, this caused the glycerol surplus as aftereffect. Despite of the wide applications of pure glycerol in food, pharmaceutical, cosmetics, and many other industries, the crude glycerol is still too costly to refine to a high purity, especially for small and medium biodiesel plants. Therefore the use of crude or partial purified glycerol as the carbon source for cultivating some oleaginous microorganisms is a promising for the utilization of an abundant glycerol waste and it could reduce the cost of the microbial oil productions as well. It was reported that some microorganisms had the ability to use glycerol as the carbon sources for oil accumulation (Meesters *et al.*, 1996). In the future, with the large-scale development of biodiesel, more and more by-product glycerol will be

produced, and the utilization of crude glycerol for the microbial oil production might be another interesting research field with much promising prospect.

Microbial oils or single cell oils, are produced by some oleaginous microorganisms, such as yeast, fungi, bacteria, and microalgae. The production of microbial oils has many advantages of short life cycle, less labor required, less affection by venue, season and climate, and easier to scale up. It has been demonstrated that these microbial oils can be used as feedstocks for biodiesel production, compared to other vegetable oils and animal fats (Li *et al*., 2008). Among the groups of oleaginous microorganisms, the yeast *R. glutinis* and the fungus *M. isabellina* have attracted considerable attention because they can accumulate large amount of the lipids up to 70% and 80% of their cellular dry weight, respectively (Czabany *et al.*, 2007; Chatzifragkou *et al.*, 2010). Furthermore, it was reported that these oleaginous microorganisms had the ability to grow and accumulate lipids when they were grown on glycerol (Meesters *et al*., 1996), also had short generation time with very minimal nutrient requirements.

Response surface methodology (RSM) is a useful statistical technique which has been applied in research to study complex variable processes. It employs multiple regression and correlation analyses as tools to assess the effects of two or more independent factors on the dependent variables. Furthermore, the central composite design (CCD) of response surface methodology has been successfully applied in the optimization of several biotechnological and chemical processes, e.g., the optimization of biodiesel production from many different kinds of fats and oils (Jeong *et. al.*, 2009).

In this work, two kinds of oleaginous microorganisms, *R. glutinis* and *M. isabellina*, were screened for their abilities to utilize glycerol, a by-product which is partially purified from palm oil-biodiesel production plant, as a carbon source for biomass and lipid production. The optimization of lipid production for the most promising oleaginous strains was conducted using response surface methodology (RSM). Furthermore, the biodiesel production potencies of the best lipid accumulation strains were also investigated.

OBJECTIVES

1. To screen the potential oleaginous microorganisms: three yeast strains of *R. glutinis* and three fungal strains of *M. isabellina* for the lipid accumulation on glycerol, a by-product from palm oil biodiesel plant.

2. To optimize the cultivation condition for the best lipid accumulation strains using RSM.

3. To determine the fatty acid profiles in the lipids produced by the best obtained strains.

4. To evaluate the potentials of biodiesel production from the best strains obtained produced lipids.

LITERATURE REVIEW

1. Biodiesel

Biodiesel, an alternative diesel fuel, is made from renewable biological sources such as vegetable oils and animal fats. It is biodegradable and nontoxic and it has low emission profiles so it is environmentally beneficial fuel (Krawczyk, 1996).

One hundred years ago, Rudolf Diesel tested vegetable oil as fuel for his engine. With the advent of cheap petroleum, appropriate crude oil fractions were refined to serve as fuel and diesel fuels and diesel engines evolved together. In the 1930s and 1940s vegetable oils were used as diesel fuels from time to time, but usually only in emergency situations. Recently, because of increases in crude oil prices, limited resources of fossil oil and environmental concerns there has been a renewed focus on vegetable oils and animal fats to make biodiesel fuels. Continued and increasing use of petroleum will intensify local air pollution and magnify the global warming problems caused by CO_2 (Shay, 1993). In a particular case, such as the emission of pollutants in the closed environments of underground mines, biodiesel fuel has the potential to reduce the level of pollutants and the level of potential or probable carcinogens (Krawczyk, 1996).

Fats and oils are primarily water-insoluble, hydrophobic substances in the plant and animal kingdom that are made up of one mole of glycerol and three moles of fatty acids. they are commonly referred to as triglycerides (Sonntag, 1979). Fatty acids vary in carbon chain length and in the number of unsaturated bonds (double bonds). The fatty acids found in vegetable oils are summarized in Table 1. Table 2 shows fatty acid profiles of *R. glutinis* cultivated on different carbon sources in comparison to plant seed oils commonly used for biodiesel production.

Table 1 Chemical properties of vegetable oil

Vegetable oil	Fatty acid composition (% w/w)								
	16:0	18:0	20:0	22:0	24:0	18:1	22:1	18:2	18:3
Corn	11.67	1.85	0.24	0.00	0.00	25.16	0.00	60.60	0.48
Cottonseed	28.33	0.89	0.00	0.00	0.00	13.27	0.00	57.51	0.00
Peanut	11.38	2.39	1.32	5.52	1.23	48.28	0.00	31.95	0.93
Rapeseed	3.49	0.85	0.00	0.00	0.00	64.40	0.00	22.30	8.23
Soybean	11.75	3.15	0.00	0.00	0.00	23.26	0.00	55.53	6.31
Sunflower	6.08	3.26	0.00	0.00	0.00	16.93	0.00	73.73	0.00

Source : Goering *et al.* (1982)

Table2 The fatty acid profile of *R. glutinis* cultivated on different carbon sources in comparison to plant seed oils commonly used for biodiesel production

Fatty acid composition, % by weight					Carbon source/ Oil source	References
C16:0	C18:0	C18:1	C18:2	C18:3		
20.37	10.33	47.88	7.31	0.85	Plam oil mill effluent	Saenge *et al.* (2011)
13.64	2.83	32.35	20.41	8.63	Glucose	Perrier *et al.* (1995)
16.01	21.86	18.05	15.91	1.76	Glycerol	Easterling *et al.* (2009)
18.70	13.20	43.8	12.7	5.50	Acid hydrate (Swithgrass)	Zhang *et al.* (2011)
18.74	1.16	66.96	4.57	n.m.	Lignocellulosic hydrolyzates	Dai *et al.* (2007)
13.90	5.75	46.54	26.57	3.37	Glucose	Schnieder *et al.* (2013)

Source : Schnieder *et al.* (2013)

Biodiesel is monoalkyl esters of long chain fatty acids derived from vegetable

oils and animal fats. The example of one such fatty acid (lauric acid) is shown in Figure 1. Many researchers have produced biodiesel by transesterification. Transesterification is the process that glycerides from animal fat or vegetable oil reacted with alcohol using alkali or acid catalyst which converted to ester or biodiesel and glycerol. The example of one such alkyl ester (methyl oleate) is shown in Figure 2. The process of transesterification affected by various factors such as catalyst type and concentration, molar ratio of alcohol to oil, reaction time, temperature, free fatty acid (FFA) content and moisture in oil. FFA content in the raw material oil must be reduced to less than 2% w/w by esterification reaction because FFA in the raw material oil can react with alkali catalyst to produce soap that prevents the separation of ester and glycerol. Esterification reaction used a short-chain alcohol with acid catalyst such as sulphuric acid or hydrochloric acid.

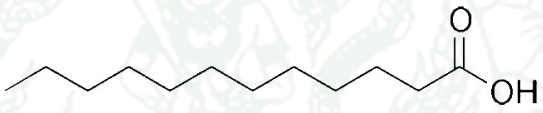


Figure 1 Chemical structure of a common fatty acid (lauric acid) found in oil.

Source : Atabani *et al.* (2013)

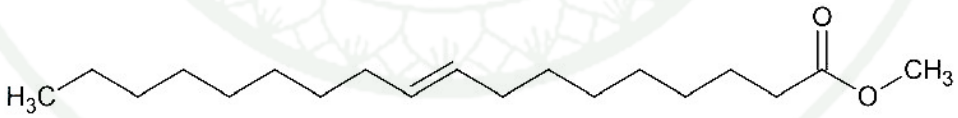


Figure 2 Chemical structure of a common methyl ester (methyl oleate) found in biodiesel.

Source : Atabani *et al.* (2013)

Bo and Zhoa. (2007) studied the biodiesel production method that featured acid-promoted direct methanolysis of cellular biomass of oleaginous yeasts and filamentous fungi. The process was optimized for tuning operation parameters, such as methanol dosage, catalyst concentration, reaction temperature and time. Up to 98%

yield was reached with reaction conditions of 70°C, under ambient pressure for 20 hours and a dried biomass to methanol ratio 1:20 (w/v) catalyzed by either 0.2 mol/L H_2SO_4 or 0.4 mol/L HCl. Cetane numbers for these products were estimated to range from 56 to 59. This integrated method was thus effective and technically attractive, as dried microbial biomass as feedstocks omited otherwise tedious and time-consuming oil extraction processes.

Qiang *et al.* (2008) studied the biodiesel that had become more attractive recently because of its environmental benefits and the fact that it was made from renewable resources. The cost of biodiesel, however, was the main hurdle to commercialization of the product. The used cooking oils were used as raw material, adaption of continuous transesterification process and recovery of high quality glycerol from biodiesel by-product were primary options to be considered to lower the cost of biodiesel. There were four primary ways to make biodiesel, direct used and blending, microemulsions, thermal cracking and transesterification. The most commonly used method was transesterification of vegetable oils and animal fats. The transesterification reaction was afected by molar ratio of glycerol to alcohol, catalysts, reaction temperature, reaction time and FFA and water content of oils.

2. Glycerol

Glycerol is the simplest trihydric alcohol. It is considered to be a derivative of propane and is called 1,2,3-propanetriol. It is colorless, viscous at room temperature, and odorless when pure, has a warm sweet taste and is neutral to indicators.

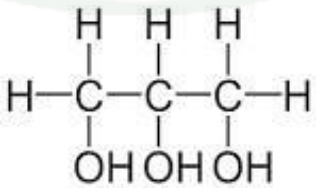


Figure 3 Structural formula of glycerol.

Its empirical formula $C_3H_80_3$ indicates the molecular weight 92.09, and its structural formula in Figure 3 shows two primary and one secondary hydroxyl groups. The hydroxyl hydrogen are replaceable by metals to form glyceroxides, by acid groups to form esters and by alkyl and aryl radicals to form ethers. Its chemical nature is that of the alcohols, but because of the multiple hydroxyl groups, it possesses possibilities for more than the usual number of reactions and derivatives. The primary hydroxyls are usually more reactive than the secondary group, and the first one to react does so more readily than the second.

Table 3 Physical properties of glycerol

Property	Value
Melting point (°C)	18.17
Boiling point (°C)	
0.53 kPa	14.90
1.33 kPa	166.10
13.33 kPa	222.20
101.3 kPa	290.00
Specific gravity, 25/25(°C)	1.26
Vapor pressure (Pa)	
50 °C	0.33
100 °C	526
150 °C	573
200 °C	6100
Viscosity (20 °C, mPa.s)	1449
Heat of vaporization (J/mol)	
55 °C	88.12
95 °C	76.02
Flash point (°C)	
Cleveland open cup	177
Pensky-Martens closed cup	199

Source : Knothe *et al*. (2005)

Glycerol had many applications, it was used for the production of food, cosmetics, paints, pharmaceutics, paper, textiles, leather and for the production of various chemicals (Wang *et al.*, 2001). It could be used as a stabilizing agent for storage of cells and proteins. Physiologically, glycerol was essential for the biosynthesis of membranes, since it was the backbone of glycerolipids. And for its function as a component of lipids and fats it was an abundant source of carbon and energy in nature. Glycerol could be used as a source of carbon and energy by many organisms. The initial step of glycerol utilization was its uptake into the cell. Albeit the small and uncharged molecule could diffuse through membranes without a transport system, many organisms possess glycerol transporters (Volker *et al.*, 2011).

Glycerol was the principal by-product of biodiesel production which was 10% of the product output or 1lb of glycerol for each gallon of biodiesel fuel. Crude glycerol derived from biodiesel production possesses very low value because of the impurities (Easterling *et al.*, 2009; Dasari, 2007; Papanikolaou and Aggelis, 2002). Therefore, using glycerol for the production of various chemicals and biofuel was advantageous both economically and environmentally (Moon *et al.*, 2010). There has been studied in the lipid production of microbial oil from oleaginous yeasts by using and crude glycerol obtained from biodiesel process (Papanikolaou *et al*., 2008).

3. Microorganism

Microbial oils, single cell oils (SCO) that had long been considered as alternative oil sources, specifically as regards lipids rarely found in the plant or animal. As regards polyunsaturated fatty acids (PUFAs) produced by microbial means, gamma linolenic acid (GLA) was of great pharmaceutical interest, due to its selective anticancer properties. Another attractive perspective that emerged recently refers to the use of SCO as the starting material for the production of biodiesel. Indeed, this approach represents a very interesting alternative to the use of plant oils for bio-diesel production because oleaginous microorganisms could transform a variety of substrates to SCO, which could then be transformed to biodiesel. The microorganisms which could accumulate more than 20% of their dry biomass as oil.

However, the production costs of SCO were still high, with serious efforts being made to reduce these costs by using waste materials as substrates (Stylianos *et al*., 2009).

Among waste materials, lignocellulose was of great importance because it was produced in enormous quantities annually, as a result of land cultivation. Xylose was the second most abundant sugar of lignocellulose biomass, being produced during the dilute acid hydrolysis treatment of this biomass. It was suggested that xylose and its polymer xylan could be used for SCO production by yeasts; this oil could then be used as a fuel. Although it was surmised that xylose may be more efficient than glucose in terms of oil yield, reports on the efficiency of xylose conversion to SCO were equivocal (Stylianos *et al*., 2009). Table 4 shows the oil content (% dry weight) of some microorganisms. Table 5 shows Lipid composition of some microorganisms.

Table 4 The oil content by different oleaginous yeasts and fungi

Microorganisms	Oil content (% dry weight)
Yeast	
Cryptococcus albidus	65
Rhodotorula glutinis	72
Fungal	
Mortierella isabellina	86
Mortierella vinacea	66

Table 5 Lipid composition of some microorganisms

Microorganisms	Lipid composition (% w/w)					
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Yeast	11-37	1-6	1-10	28-66	3-24	1-3
Fungal	7-23	1-6	2-6	19-81	8-40	4-42

Source: Xin *et al.* (2009)

3.1 The oleaginous fungi *M. isabellina*

M. isabellina was capable of accumulating the amount of lipids, up to 80% of cell biomass. *M. isabellina* could be cultivated on various of substrates, including monomer sugars (Chatzifragkou *et al.*, 2010), glycerol (Fakas *et al*., 2008), as well as lignocellulosic biomass hydrolysates. *M. isabellina* had the best performance on lipid production among five oleaginous fungi that growed on dilute sulfuric acid pretreated wheat straw (Zheng *et al*. 2012). These features, together with good tolerance to inhibitors derived from lignocellulosic materials (Zeng *et al*., 2012), suggested that *M. isabellina* could be a good candidate for lipid production from low cost renewable feedstock.

Application of *M. isabellina*

Xian *et al*. (2001) studied that *M. isabellina* could accumulate the large amounts of unsaturated fatty acids when it was grown in a medium of butanol (5 g/L) and yeast extract (10 g/L), incubate at 25 ˚C for 5 days. The result mycelial lipids accounted for 40.1% of the dry mycelia, while about 34% of butanol in the medium was converted. The mycelia lipids contained Four unsaturated fatty acids from mycelia lipids were palmitoleic (4.9%), oleic (54.1%), linoleic (10.4%) and linolenic (5.4%) acids. The effects of the culture conditions, such as cultivation temperature, initial pH of the medium and additional feeding butanol in the course of cultivation.

Seraphim *et al*. (2004) studied that the *M. isabellina* cultivated in nitrogen-limited media could be growth up to 35.9 g/L and high glucose with sugar was 100 g/L of concentrations in media. After nitrogen depletion, significant lipids quantities were accumulated inside the fungal (50–55% (w/w) lipids of dry biomass), resulting of single cell oil production of 18.1 g/l of culture medium. Total dry biomass and lipid yields were 0.34 and 0.17 g respectively. The microbial lipid produced contained c-linolenic acid (GLA) was 3.5±1.0% (w/w) of concentration, which corresponded to 16–19 mg (GLA/g of dry biomass) and a maximum concentration of 0.801 g (GLA/L of culture medium).

Gemma *et al.* (2009) studied that the filamentous fungal *Mucor circinelloides* as a potential feedstock for biodiesel production. These microbial lipids showed a high content (more than 85%) of saponification and a suitable fatty acid profile for biodiesel production. The effectiveness of three different solvent systems ($CHCl_3$:CH_3OH, $CHCl_3$:CH_3OH:H_2O and C_6H_{14}) for the lipid extraction process. Biodiesel was produced by the acid catalysed (esterification) following two different approaches: transformation of extracted microbial lipids and direct transformation of dry biomass. The reaction of BF_3, H_2SO_4 or HCl as acid catalysts at 65 ˚C for 8 hours, the direct process produced FAMEs with higher purities (more than 99%) than those from the two-step process (91.4 – 98.0%). In addition, the yield was also significantly higher in the direct transformation due to a more efficient lipid extraction when the acid catalyst was presented.

Stylianos *et al.* (2009) studied that the biochemical behavior two oleaginous *Mucorales* strains, namely *M. isabellina* ATHUM 2935 and *C. echinulata* ATHUM 4411, was studied when growth on xylose, raw glycerol and glucose under N_2 conditions. The difference in process of lipid accumulation as related to the carbon sources used was observed for both microorganisms. Although glucose containing media the production with total biomass of *M. isabellina* and *C. echinulata* were 27 g/L and 15 g/L respectively. Lipid accumulation was induced on xylose containing media of *M. isabellina* and *C. echinulata* were 65.5% and 57.7% w/w respectively. This fungus, when cultivated on xylose, produced 6.7 g/L of single cell oil and 1119 mg/L of GLA. Finally, the growth of both *C. echinulata* and *M. isabellina* on raw glycerol resulted in lower yields in terms of both biomass and lipids produced than the growth on xylose.

Dahui *et al.* (2012) studied that the corn fiber hydrolysate was used as feedstock for microbial lipids production using the oleaginous *M. isabellina* M2. First, we evaluated the effects of varying the HCl concentration, temperature, time and substrate concentration on corn fiber hydrolysis, then the effects of varying the concentrations of sugars, the nitrogen source, and culture time on lipid accumulation by *M. isabellina* M2. Under optimized corn fiber hydrolysis conditions, the sugars yield reached 83%. The microbial lipid content reached up to 50% when glucose,

xylose, mannose and arabinose were used as single carbon sources, and when the corn fiber hydrolysate was used, the optimum sugar concentration, nitrogen source and cultivation time (for batch cultivation under otherwise constant conditions) were 60 g/L, 2 g/L $(NH_4)_2SO$, and 6 days, respectively. In validation experiments under this set of culture conditions the biomass, lipid content and lipid yield reached 17.5 g/L, 50.5% and 8.84 g/L, respectively. Results of GC/MS analysis indicate that the fatty acid composition of the lipid was similar to that of vegetable oil, thus it is a promising material for biodiesel production.

Difeng *et al.* (2013) evaluated the culture conditions including nitrogen source, xylose concentration, and inoculum volume for the effect on cell growth and lipid production of an oleaginous fungus (*M. isabellina*). Yeast extract and ammonium sulfate were found to be the best amongst the organic and inorganic nitrogen sources tested. The highest cell biomass 28.8 g/L and lipid 18.5 g/L were obtained on a medium contents 100 g/L of xylose and 50.4 mM of nitrogen with a spore concentration of 108 mL^{-1}. Specifically, nitrogen concentration and inoculum volume were demonstrated to be important for obtaining a high lipid yield on xylose consumed of 0.182 g/g. The results suggest that *M. isabellina* holds great potential to be a candidate for biofuel production from xylose, the second most abundant sugar from lignocellulose.

3.2 The oleaginous yeast *R. glutinis*

R. glutinis was an oleaginous yeast which was able to activate non-esterified fatty acids for the synthesis of triacylglycerol. In *R. glutinis*, fatty acids were activated in an adenosine tri phosphate (ATP) dependent manner prior to being used. This had demonstrated that an enzyme, acyl–acyl carrier protein (ACP) plays a role in activating fatty acids for triacylglycerol biosynthesis. There was plenty evidence to suggest that this organism had the potential to be a source of fatty acids for the production of biodiesel. Oleaginous yeasts had the ability to grow and accumulate lipids when grown on glycerol. Oleaginous yeasts had short generation times, and very minimal nutrient requirements (Emily *et al.*,2009).

Application of *R. glutinis*

Emily *et al.* (2009) studied that the effects of different growth substrates on triacylglycerols (TAG) accumulation and fatty acid compositions produced by *R. glutinis*. The results showed that yeast 24 hours on medium containing with sugars as dextrose, xylose, glycerol, dextrose and xylose, xylose and glycerol, or dextrose and glycerol accumulated 16, 12, 25, 10, 21, and 34% TAG on a dry biomass, respectively. Lipids were extracted from yeast and transesterification to FAMEs. The results also showed that cells cultivated on glycerol alone had the highest unsaturated fatty acids at 53% while xylose had the lowest unsaturated fatty acid at 25%. *R. glutinis* could be cultivated on all sugars tested as single carbon substrates or in mixtures.

Bradley *et al.* (2011) studied microalgae as a potential biodiesel feedstock due to their high lipid productivity and potential for cultivation on marginal land. One of the challenges in utilizing microalgae to make biodiesel was the complexities of extracting the lipids using organic solvents followed by transesterification of the extracts to biodiesel. In their work, reaction conditions were optimized that allowed a single step extraction and conversion to biodiesel in high yield from microalgae. From the optimized conditions, it was demonstrated that quantitative conversion of triglycerides from several different microalgae and cyanobacteria could be achieved, including mixed microbial biomass collected from a municipal wastewater lagoon.

Chanika *et al.* (2011) studied crude glycerol was used as the sole carbon source for production of lipids and carotenoids by oleaginous yeast *R. glutinis* TISTR 5159. The addition of $(NH_4)_2SO_4$ as a nitrogen source and Tween 20 as a surfactant increased the accumulation of lipids and carotenoids. The optimum condition with response surface methodology (RSM) for biomass was glycerol concentration of 8.5% and C/N ratio of 60, while that for lipid content and carotenoids was glycerol concentration of 9.5% and C/N ratio of 85. The production of lipids and carotenoids were further improved in a stirred tank bioreactor with pH at 6.0 and aeration rate at 2 vvm. In fed-batch fermentation, the highest lipids production of 6.05 g/L with a cellular lipid content of 60.7% and carotenoids of 135.25 mg/L were obtained.

4. Response surface methodology (RSM)

RSM was a collection of mathematical and statistical techniques that were useful for the model building and analysis of problems in a response of interest variable. By design of experiments, the objective was to optimize a response (output variable) which was influenced by several independent variables (input variables).An experiment was a series of tests, called runs, in which changes were made in the input variables in order to identify the reasons for changes in the output response. The main idea of RSM was to replace a complicated response function with an approximate function by studying the relative significance of the effects of several variables supposed to have influence on the response of interest. The response could be presented graphically, either in the three-dimensional space or as response surface plots that help visualize the shape of the response surface. Response surface plot were curves of constant response in the x, y, z axis keeping all other variables fixed. But the two-dimensional was also preformed that was call contour plot (Myers and Montgometry, 1995). Each shape plot corresponded to a particular height of the response surface, as shown in Figure 4.

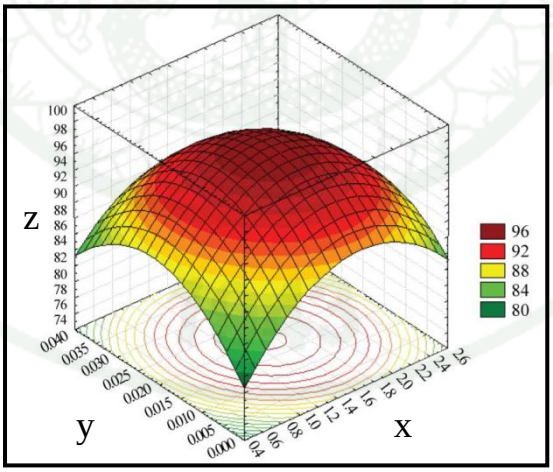


Figure 4 Three-dimensional response surface plot.

Source : Sridevi *et al.* (2011)

The process of RSM was to select the experimental design for building surface plot which were assigned variable and set the level of interest condition in experiment. The variable value could be separated in to two types. The first type was called uncode value which was the unit of measurement variable such as mole and minute. The second type was called code value which was an uncode value transformed to dimensionless. The experimental data made the suitable equation which was called model fitting equation.

The suitable equation was considered from the correlation coefficient (R) of determination that had R^2 value more than 0.70. In addition, the lack of fit test was another one variable which was used to consider the suitable of equation too. The equation was suitable when high R^2 value and lack of fit was significant. If the result gave low R^2 value and lack of fit was significant that mean the equation was not suitable. If the result give high R^2 value and lack of fit was significant that mean the equation was not suitable too. In these cases, they should be further performed validation test. They performed by running the reaction again and comparing the response of experimental value with predicted value. The condition for validation test will be different from conditions that use to generate the equation but it must be the value with the same range. If both values (experimental and predicted values) were similar, the equation was suitable. If the both values were not similar, the equation was not suitable. In this case, it must be return to set a new variable for running a reaction again. The new conditions would generate a new equation to fix correlation coefficient (R^2) and lack of fit value. For the optimization process, the suitable model was used to generate the response surface plot. Generally, the program creates the response surface plot as a function of two independent variable and response at one time. Thus, if we studied three variables such as A, B and C the response surface plot could generate A&B, A&C and B&C. The three plots of them were overlapped together that create the optimum region of each variable. Moreover, the reaction was done again by setting five conditions from the optimum region. Finally, the optimum condition was selected from those five conditions. The diagram of RSM process was shown in Figure 5.

The equation of first, second and third-order model of RSM are shown in Equations 2, 3 and 4 respectively.

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum^{n-1} \sum_{i<j=1}^{n} b_{ij} x_{ij} \quad (2)$$

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum^{n-1} \sum_{i<j=1}^{n} b_{ij} x_{ij} \quad (3)$$

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n} b_{iii} x_i^3 \sum_{i<j}^{n-1} \sum_{j=1}^{n} b_{ij} x_{ij} + \sum_{i<j}^{n-2} \sum_{j<k}^{n-1} \sum_{k=1}^{n} b_{ijk} x_{ijk} \quad (4)$$

Where Y was response, b_0 was constant coefficient, b_i was linear term coefficient x_i, x_{ij} and x_{ijk} were independent variables, b_{ii} was cubic term coefficient, b_{ij} was cross-product coefficients for quadratic term, b_{ijk} was cross-product coefficients for cubic term.

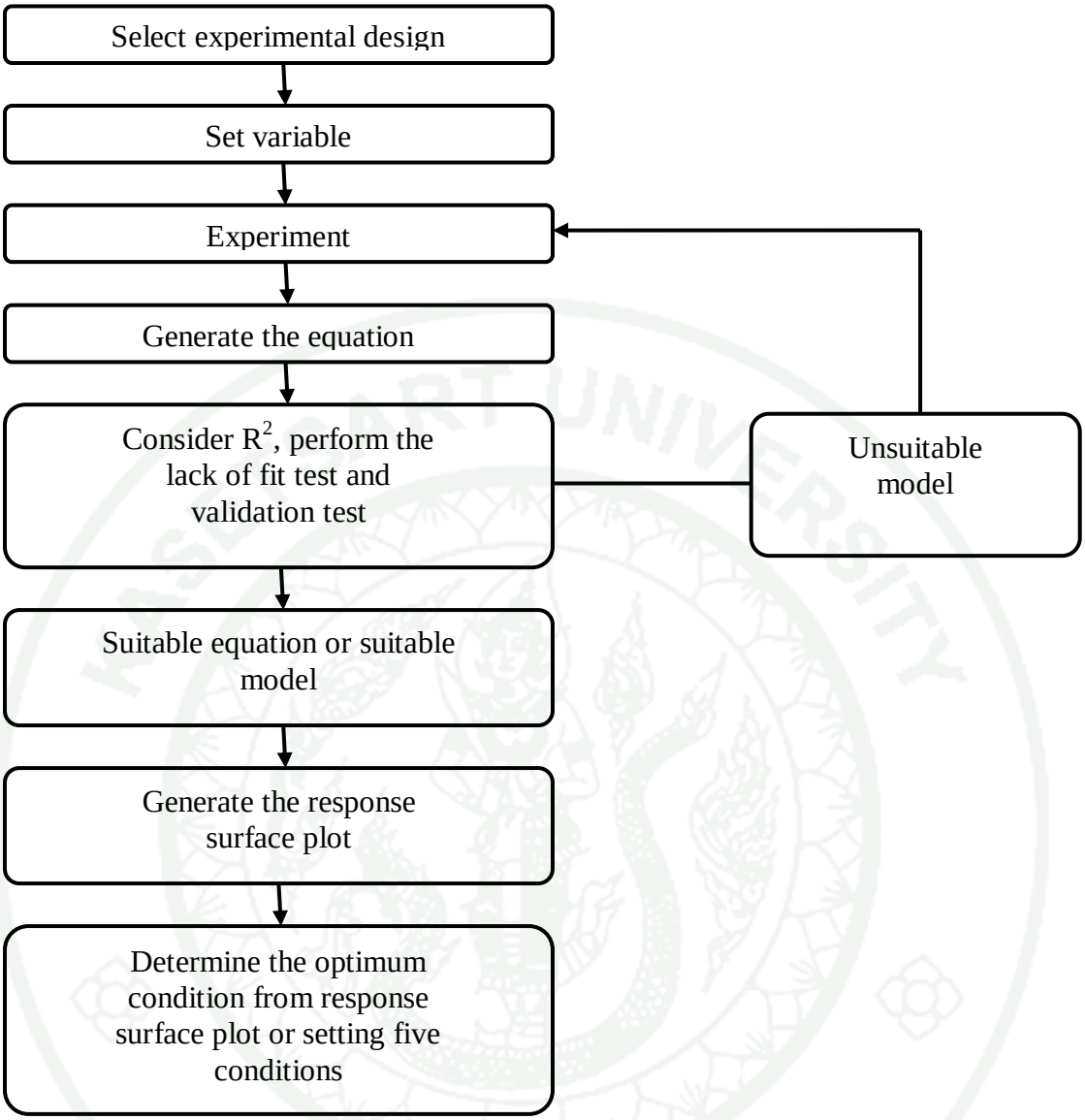


Figure 5 The diagram of RSM process.

Source : Wanida (2013)

The second order model was generally used to approximate the response once it was realized that the experiment was close to the optimum response region. Therefore, for the purpose of analysis of response surfaces, special designs were used to help the experimenter fit the second order model to response with the use of a minimum number of runs.

4.1 Advantage of the second order model

The second order model was widely used in response surface methodology for several reasons. These reasons were as follow: (Myers and Montgometry, 1995).

4.1.1 The second order model was very flexible. It can generate a various functional forms of response surface.

4.1.2 The second order was easy to estimate the coefficient parameter in the second order model.

4.1.3 The second order model could solve a real response surface problem in many researches.

4.2 Experimental design for the second order model (Tapanwong, 2012)

4.2.1 Experiment of 2^k factorial in completely randomized design (CRD) or randomized complete block design (RCD, RCBD, RBD) including cross product was used to generate the second order model. The factorial could be used to investigate several factors at the same time and also study interaction of factor. A 2^k factorial means that this factorial had k factors and 2 levels of each factor. The CRD was random treatment and manages to a unit of experiment.

4.2.2 The second order model was generated by model of 3^k factorial incompletely randomized design (CRD) or random complete block design (RCD, RCBD, RBD). A 3^k factorial means that this factorial had k factors and 3 levels of each factor.

4.2.3 The second order model was performed using experiment of fractional factorial in completely randomized design (CRD) or random complete block design (RCD, RCBD, RBD). The fractional factorial considered some treatment such as 2^{k-1} fractional factorial will consider a half of all treatments.

4.2.4 Rotatable design was designed the distance of every treatment from center point of geometry picture in the same distance. Normally, the treatment was made from different geometric pictures such as circle, sphere, square or cube. All of 2^k factorial was rotatable. A 2^k factorial meaned that this factorial had k factors and 2 levels of each factor. The coordination at the angle of geometry picture was used as a code level. The code level of two factors rotatable design was shown in Figure 6.

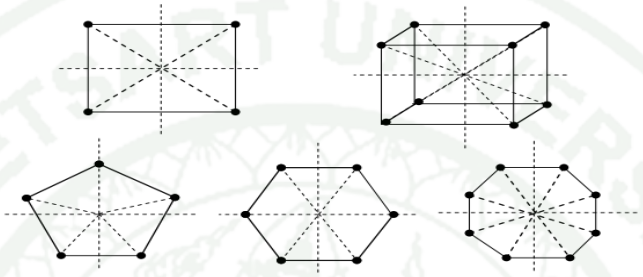


Figure 6 The code level of 2 factors for rotatable design.

Source : Wanida (2013)

4.2.5 Central composite design (CCD) was the experiment that was added treatment between the levels of factor to generate the high order of a model such as third-order model. Normally, CCD was duplicated at a center point of each factor to estimate the error of the experiment. The performance of CCD was to make 2^k factorial and then added the coordination with $\pm\alpha$ of code level. The treatment was finally randomized into a unit of experiment. The 2^k factorial of CCD was also been a rotatable design. The code levels of two and three factors CCD were shown in Figure 7.

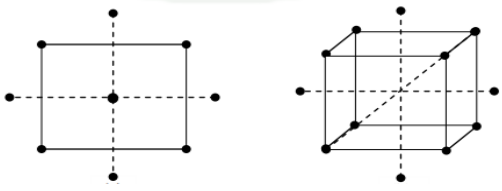


Figure 7 The code level of central composite design: (a) 2 factors, (b) 3 factors.

Source : Wanida (2013)

In some case of $\alpha = \pm 1$, each point of experiment was added on the face center of geometry picture which was known as face centered central composite design. This design reduce the number of each factor from a 5-level of CCD to 3 level of face-centered central composite design. The advantages of face-centered CCD were reduced the cost and time for performing the experiment. The code level of three factors for face-centered central composite design was shown in Figure 8.

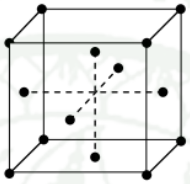


Figure 8 The code level of 3 factors for face-centered central composite design.

Source : Wanida (2013)

4.2.6 Box-Behnken design was one choice of 3^k factorial. The experimental designs were combination of 2^k factorial and incompletely block design (BIB) together. The code level of three factors Box-Behnken design was shown in Figure 9.

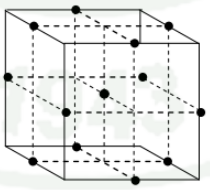


Figure 9 The code level of three factors for Box-Behnken design.

Source : Wanida (2013)

4.2.7 Mixture design was simple experiment to study more than two factors. It was suitable for developing process and normally used a three factor in

each performing experiment. The base of each axis had a value as zero and the top of each factor was value of one. The code level of mixture design was shown in Figure 10.

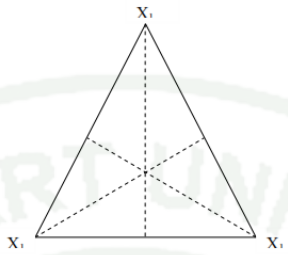


Figure 10 The trilinear coordinate system in Mixture design.

Source : Wanida (2013)

MATERIALS AND METHODS

1. Materials

1.1 Agar (Difco culture media, USA)
1.2 Ammonium sulfate (Univar, USA)
1.3 Calcium chloride dihydrate (Univar, USA)
1.4 Calcium nitrate monohydrate (Univar, USA)
1.5 Chloroform (Merck, Germany)
1.6 Copper(II) sulfate pentahydrate (Univar, USA)
1.7 37-Component FAME Mix (Sigma-Aldrich, USA)
1.8 Dextrose (Univar, USA)
1.9 Di-sodium hydrogen phosphate (Univar, USA)
1.10 Ethyl alcohol (Merck, Germany)
1.11 Glycerol (QReC, New Zealand)
1.12 Hexane (Merck, Germany)
1.13 Internal standard methyl heptadecanoate : C_{17} (Fluka, Switzerland)
1.14 Iron(III) chloride hexahydrate (Univar, USA)
1.15 Magnesium sulfate heptahydrate (Univar, USA)
1.16 Manganese(II) sulfate monohydrate (Univar, USA)
1.17 Methyl alcohol (Merck, Germany)
1.18 Peptone (Difco culture media, USA)
1.19 Peptone dextrose agar (Hi-media, India)
1.20 Potassium dihydrogen phosphate (Univar, USA)
1.21 Yeast extract (Hi-media, India)
1.22 Zinc sulfate heptahydrate (Univar, USA)

2. Equipments

2.1 Autoclave (Hirayama, Japan)
2.2 Centrifuge (Hethich Zentrifugen, EBA20, USA)
2.3 Fourier-Transform Infrared Spectrometer (FT-IR, EQUINX 55, Bruker)

2.4 Gas Chromatograph (GC) (Agilent, 6890N, USA)

2.5 Gas Chromatograph (GC) (Shimadzu, Japan)

2.6 Hot air oven (Binder, USA)

2.7 Hot plate (IKA, C-MAG HS7, Malaysia)

2.8 Suction pump (Millipore, MA01821, USA)

2.9 The laminar flow clean bench (Telstar, Bio-II-A, Spain)

2.10 Ultrasonic cleaner (Branson 2510 , Bransonic, USA)

2.11 UV-Spectrophotometer (Rayleigh, UV-9200, USA)

2.12 Vacuum oven (Yamato, ADP 200, Japan)

2.13 Statistica version 5.0 of RSM

3. Microorganism and glycerol sample

Microorganisms

Three yeast strains of *M. isabellina*: NBRC 7874, NBRC 7884, NBRC 105998 and three fungal strains of *R. glutinis*: NBRC 0695, NBRC 1099, NBRC 1501 were obtained from the Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan.

Glycerol

The glycerol sample, a by-product from palm oil-biodiesel production, was obtained from Patum vegetable oil Co., Ltd., Thailand. It was stored in a closed container at 20^{o}C until analysis.

4. Medium for microorganisms

4.1 Medium for *M. isabellina*

The stock culture medium for *M. isabellina* was cultured for 7 days on peptone dextrose agar medium (PDA); 39 g of PDA in 1 L of distilled water. And the test medium used in the experiments composed of (per 1 L distilled water): glycerol

sample from biodiesel production 30 g, yeast extract 1.0 g, KH_2PO_4 7.0 g, Na_2HPO_4 2.0 g, $MgSO_4 \cdot 7H2O$ 1.5 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, $FeCl_3 \cdot 6H_2O$ 0.08 g, $ZnSO_4 \cdot 7H_2O$ 0.001g $CuSO_4 \cdot 5H_2O$ 0.0001g, $Ca(NO_3)_3 \cdot H_2O$ 0.0001 g, $MnSO_4 \cdot 5H_2O$ 0.0001 g and $(NH_4)_2SO_4$ 0.5 g.

4.2 Medium for *R. glutinis*

The stock culture medium for *R. glutinis* was cultured for 48 hours on yeast peptone dextrose medium (YPD); per 1 L distilled water ; yeast extract 3 g, Peptone 5 g, Dextrose 10 g and Agar 15 g as a stock culture medium. And the test medium used in the experiments composed of (per 1 L distilled water): glycerol sample from biodiesel production 30 g, Yeast extract 1.0 g, KH_2PO_4 7.0 g, Na_2HPO_4 2.5 g, $MgSO_4 \cdot 7H_2O$ 1.5 g, $FeCl_3 \cdot 6H_2O$ 0.15 g, $ZnSO_4 \cdot 7H_2O$ 0.02 g, $MnSO_4 \cdot 5H_2O$ 0.06 g, $CaCl_2 \cdot 2H_2O$ 0.15g, $(NH_4)_2SO_4$ 0.5 g.

The medium was sterilized in autoclave at 121°C for 15 minutes.

5. Methods

5.1 Characterization of glycerol

Glycerol was characterized using standard test methods. Glycerol was determined for its glycerol content, ash content, water content (moisture), matter organic non-glycerol (MONG) and pH.

5.1.1 Glycerol content

Glycerol was analyzed by High performance liquid chromatography ((HPLC) Shimadzu, Japan), the system was equipped with a cation-exclusion column (Aminex HPX-87H ; 300 mm x 7.8 mm, 9 µm; Bio-Rad Chemical Divi-sion, USA) and with pulsed refractive index detector (Nor *et al.*, 2011). The mobile phase used 0.005 N H_2SO_4 solution was passed through the column at 0.6 ml/min.

5.1.2 Ash content

Ash content was analyzed according to the standard method (ISO 2098-1972) by slowly heating 2 g of glycerol on a hot plate to eliminate the free moisture and volatile matter. And then no further mist was generated, the glycerol was burnt in a muffle furnace at 750 ± 10 °C for 3 hours. The residue was cooled down to room temperature in dessicator and weighed (Mali *et al.*, 2013).

5.1.3 Water content

The water content was measured following the standard method ISO 2097-1972 by using the Karl Fisher titration (703 Ti Stand Metrohm, Switzerland).

5.1.4 Matter organic non-glycerol (MONG)

The quantity of the matter organic non-glycerol (MONG) measured in terms of contaminants was calculated by the difference from a hundred of the previous three compositions (glycerol, water and ash content) (Mali *et al.*, 2013) by this Equation 5.

$$MONG = 100 - (A+B+C) \quad (5)$$

Where

A is the percentage content of glycerol

B is the percentage content of ash

C is the percentage content of water

5.1.5 Fourier Transform Infrared spectroscopy (FT-IR) analysis

Glycerol was analyzed by Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR: Bruker equinox 55). FTIR was used to analyze the characteristics of the glycerol.

5.1.6 pH measurement

The pH of a sample solution of 20% glycerol in water (v/v) was measured with a pH meter (Yong *et al.*, 2001).

5.2 Screening of the potential strains of microorganism for the lipid accumulation ability on glycerol from palm oil-biodiesel production

5.2.1 Selection of effective for *M. isabellina*

Three strains of oleaginous fungal *M. isabellina*; NBRC 7874, NBRC 7884 and NBRC 105998 were studied for the capability of lipid accumulation on the test medium (4.1). Each fungal strain was cultured in the stock culture medium (4.1) for 7 days. Fungal was placed in test medium (fungal disc 7 mm.). Three replicate cultures of each strain of *M. isabellina* were incubated at 30°C on a shaker set at 180 rpm. Lipid accumulation in each fungal biomass was extracted to determine the accumulation efficiency at 4, 8, 12 and 16 days.

5.2.2 Selection of effective for *R. Glutinis*

Three strains of oleaginous yeast *R. glutinis*; NBRC 0695, NBRC 1099 and NBRC 1501 were studied for the capability of lipid accumulation on the test medium (4.2). Each yeast strain was cultured in the stock culture medium (4.2) for 48 hours. The concentrated yeast cells were used to inoculate on the test medium. The medium was inoculated to a cell density of 0.5 mg/mL which was correlated to an absorbance of 0.5 at 650 nm. Three replicate cultures of each strain of *R. glutinis* were incubated at 30°C on a shaker set at 180 rpm. Lipid accumulation in each yeast biomass was extracted to determine the accumulation efficiency at 12, 24, 36, 48 and 96 hours.

5.3 Lipid extraction

The lipid accumulation in *M. isabellina* and *R. glutinis* biomass were extracted with a 2:1 (v/v) chloroform : methanol according to Folch *et al.* (1957) with

slight modification. The mixture solution (biomass and chloroform : methanol) was shook for 20 minutes with vortex and sonicated for 10 minutes with Ultrasonic cleaner. The mixture solution was separated with 0.45 um of syringe filter (3 times for extracted lipids). The solution and residue were heated to evaporate solvent in oven at 65 ^{o}C for 24 hours. The extracted lipids were weighted and lipid content per 100 g of cell dry weight was calculated.

5.4 Optimization of cultivation condition for the best oil producing strain obtained by response surface methodology (RSM)

5.4.1 Response surface methodology (RSM) for *M. isabellina*

Response surface methodology (RSM), using a 5-level 2-factor central composite design (CCD), was performed. There were 13 experiments involving the two investigated variables of the glycerol concentration (15.86 - 44.14 (g/L)) and the inoculums size (2 – 8 (disc)). Factors and their respective levels are summarized in Table 6

Table 6 Experimental ranges of the two independent variables used in RSM for *M. isabellina*

Variables	Symbol coded	Code level				
		-1.414 (-α)	-1	0	+1	+1.414 (+α)
glycerol concentration (g/L)	X_1	15.86	20	30	40	44.14
inoculums size (disc)*,**	X_2	2	3	5	7	8

* = 1.87 x 10^6 spores/disc, ** disc. I.D. = 7 mm

Thirteen of cultivation conditions for optimization the best oil producing strain of *M. isabellina* using central composite design were shown in Table 7.

Table 7 Experimental design of lipid production yield from *M. isabellina* for CCD

Run	Coded		X_1 (Glycerol concentration (g/L))	X_2 (inoculums size (disc))
	X_1	X_2		
1	-1	-1	20	3
2	+1	-1	40	3
3	-1	+1	20	7
4	+1	+1	40	7
5	-1.414	0	15.86	5
6	1.414	0	44.14	5
7	0	-1.414	30	2
8	0	1.414	30	8
9	0	0	30	5
10	0	0	30	5
11	0	0	30	5
12	0	0	30	5
13	0	0	30	5

5.4.2 Response surface methodology (RSM) for *R. Glutinis*

Response surface methodology (RSM), using a 5-level 2-factor central composite design (CCD), was performed. There were 13 experiments involving the two investigated variables of the glycerol concentration (5.86 - 34.14 (g/L)) and the inoculums size (1.6 - 4.4 (mL)). Factors and their respective levels are summarized in Table 8.

Table 8 Experimental ranges of the two independent variables used in RSM for *R. glutinis*

Variables	symbol	Code level				
		-1.414 (-α)	-1	0	+1	+1.414 (+α)
glycerol concentration (g/L)	X_1	5.86	10	20	30	34.14
inoculums size (mL)*	X_2	1.6	2	3	4	4.4

* = The spores is 2.84 x 10^7 cell/mL

Thirteen of cultivation conditions for optimization of the best oil producing strain of *R. glutinis* using central composite design were shown in Table 9.

Table 9 Experimental design of lipid production yield from *R. glutinis* for CCD

Run	Coded		X_1 (Glycerol concentration (g/L))	X_2 (Inoculum size (% *,**))
	X_1	X_2		
1	-1	-1	10	4
2	+1	-1	30	4
3	-1	+1	10	8
4	+1	+1	30	8
5	-1.414	0	5.86	6
6	1.414	0	34.14	6
7	0	-1.414	20	3.2
8	0	1.414	20	8.8
9	0	0	20	6
10	0	0	20	6
11	0	0	20	6

Table 9 (Continued)

Run	Coded		X_1 (Glycerol concentration (g/L))	X_2 (Inoculum size (% *,**))
	X_1	X_2		
12	0	0	20	6
13	0	0	20	6

* = 2.84 x 10^7 cell/ml, ** v/v

5.5 Fatty acid determination

The fatty acid profile of the lipid samples (the best oil producing strains) were estimated by these processes. Firstly, the lipid sample was hydrolyzed under alkali condition (Saponification reaction, (5.5.1)). Secondly, the fatty acids in the lipid were converted into methyl esters (FAMEs, (5.5.2)). The fatty acid methyl esters was identified and separated by gas chromatrography (GC).

5.5.1 Saponification of lipid sample

The extracted lipid from microbial biomass (30 mg) was refluxed with a 90% methanolic NaOH (5 ml) for 90 minutes. The solution was cooled, then distilled water (10 ml) was added and the mixture is extracted thoroughly with n-hexane (10 x 3 ml). Finally, the sample solution was evaporated to dryness under a steam of nitrogen.

5.5.2 The procedure of making methylation of the fatty acids

The obtained sample from 5.3 (30 mg) was refluxed with a 2% sulfuric acid in methanol (5 ml) for 90 minutes. The solution was cooled, then distilled water (0.5 ml) was added and the required esters are extracted with hexane (5 x 3 ml). Finally, the sample solution was evaporated to dryness on a rotary evaporator and then FAMEs weight was determined.

5.5.3 Analysis of fatty acid methyl esters by gas chromatograph

The fatty acid methyl esters (FAMEs) were analyzed by gas chromatography (GC) fitted with a flame ionization detector (FID) (Shimadzu, Japan). A fused capillary column BPX70 (0.32 mm i.d. × 30 m, 0.25 µm film thickness; SGE, Australia) was used. GC-FID was performed using the following conditions: carrier gas He; flow rate 1.5 ml/min; injection temperature 250°C; oven temperature programmed from 210°C (9 min hold) to 240°C at 20°C/min (6 min hold); detector temperature 280°C. Individual fatty acids were identified by comparing them with the retention time of authentic fatty acid standards obtained from Sigma Co., USA.

5.6 Biodiesel production

Biodiesel production from yeast and fungi was initiated by the addition of lipid from microbial biomass to methanol containing 2% v/v of sulfuric acid (H_2SO_4) at a ratio of 5 ml of methanol for 50 mg of lipid from microbial biomass. Solution was maintained at 60°C for 6 hours with oven. After the established reaction time the mixture was placed in separation funnel. Phase separation was then accomplished by adding hexane. The upper phase consisted of methanol and H_2SO_4 while the lower phase mainly consisted of fatty acid methyl ester (FAME), triacylglycerol (TAG), and other lipids partitioned with hexane.

5.7 Fatty acid methyl ester content (%FAME) analysis

The content of fatty acid methyl ester was measured via EN 14103 standard. Analysis was conducted on gas chromatography (Agilent Technologies) equipped with a flame ionization detector and a 30 m x 0.32 mm (i.d.) fused siloxane capillary column (DB-WAX 127-7012, 0.25 µm film thickness). Oven temperature was maintained at 110˚C for 5 minutes, and then increased to 200˚C at 5˚C/minutes. Injector and detector temperature were 210˚C and 250˚C, respectively. Helium was the carrier gas at a split ratio of 30:1 and a constant flow rate of 1.0 ml/min. Fatty acid

peaks were identified by comparing retention times with known fatty acid methyl ester standards (Sigma-Aldrich Chemie). The quantification of each fatty acid was performed by internal normalization method. The fatty acid methyl ester content was calculated via Equation 6

$$\%FAME = \frac{(\sum A) - A_{EI}}{A_{EI}} \times \frac{C_{EI} \times V_{EI}}{W} \times 100 \quad (6)$$

Where

ΣA is the sum of all areas under the chromatogram from C8 to C24

A is the area under the chromatogram of C17:0

CEI is the concentration of C17:0 (mg/mL)

VEI is the amount of C17:0 used (μL)

W is the weight of product (mg)

RESULTS AND DISCUSSION

1. The study of the glycerol sample composition.

According to characterization result of glycerol sample showed in Table 10 and Figure 11 the glycerol sample was high purity (the quantity of glycerol sample was 93.97% (w/w)), moisture content was 1.10% (w/w), ash content was 0.02% (w/w), and matter organic non-glycerol (MONG) component showed the reduction to 4.91% (w/w).

Table 10 The chemical compositions of glycerol sample

Components	Percentage *
Glycerol	93.97
Water	1.10
Ash	0.02
Matter organic non-glycerol (MONG)	4.91

* based on the glycerol sample weight

The HPLC chromatogram results were shown in Figure 11. Summary of the glycerol sample was high purity (93.97% (w/w)) with relation time was 13.105 minutes and area peaks was 1385117 uRIU.

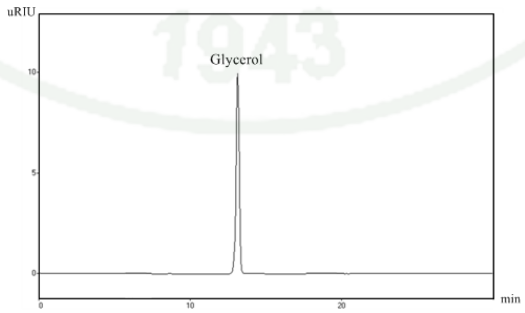


Figure 11 HPLC chromatogram of glycerol sample derived from biodiesel production

Fourier Transform Infrared Spectrophotometer was used to confirm glycerol structure. FT-IR spectrums of commercial glycerol and glycerol sample derived from biodiesel were showed in Figure 12 and Table 12.

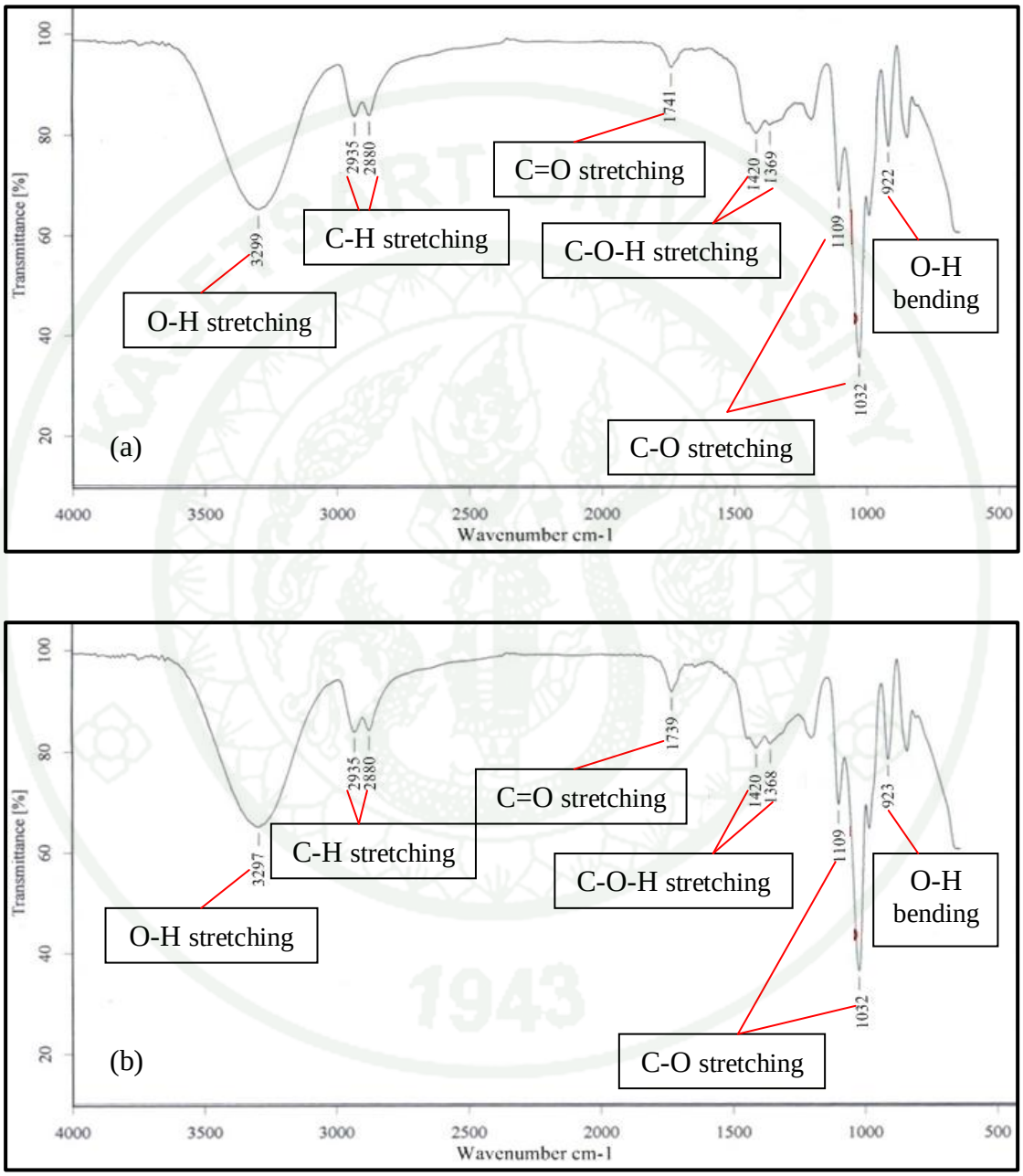


Figure 12 FT-IR spectra of (a) commercial glycerol and (b) glycerol sample derived from biodiesel production

Table 11 IR interpretation of commercial glycerol and glycerol sample

Functional group		Wave number (cm^{-1})	
		Commercial glycerol	Glycerol sample
O-H	stretching	3299	3297
C-H	stretching	2935 to 2880	2935 to 2880
C=O	stretching	1741	1739
C-O-H	bending	1369 to 1420	1368 to 1420
C-O	stretching	1109 to 1032	1109 to 1032
O-H	bending	922	923

The FTIR spectra results were shown in Figure 12. Summary of characteristic bands found in commercial glycerol are summarized in Table 11. The functional group of commercial glycerol illustrated in Figure 12(a) including O-H stretching at 3299 cm^{-1}, C-H stretching at 2935 to 2880 cm^{-1}, C-O-H bending at 1420 to 1369 cm^{-1}, O-H bending at 922 cm^{-1} and C-O stretching from 1109 to 1032 cm^{-1}. And the FTIR spectrum of glycerol sample derived from biodiesel production was illustrated in Figure 12(b). The functional group of glycerol sample was O-H stretching at 3297 cm^{-1}, C-H stretching at 2935 to 2880 cm^{-1} , C-O-H bending at 1420 to 1368 cm^{-1}, O-H bending at 923 cm^{-1} and C-O stretching from 1109 to 1032 cm^{-1}. The small peak of commercial glycerol and glycerol sample (1741 cm^{-1} and 1739 cm^{-1}, respectively) were indicated the presence of C=O compound of ester.

2. Screening of the potential strains of *M. isabellina* and *R. glutinis* for the lipid accumulation ability on glycerol from palm oil biodiesel production

According to the selection of microorganism species, the species which had the capability to produce and collect lipid were shown as follows.

2.1 Lipid production by the oleaginous fungal *M. isabellina*

Three *M. isabellina* strains were cultured in the test medium (The medium containing glycerol at the concentration of 30 g/L and fungi colony 5 disc.)

and incubated at 30°C on a shaker set at 180 rpm. The fungal biomass of each strain was isolated and the lipid accumulation in fungal mass was extracted at 4, 8, 12 and 16 days. Afterwards, the oil was extracted by using mixed solutions of $CHCI_3$:CH_3OH (2:1). The data of fungal mass (weight) and the lipid content accumulated in each fungal strain at different period of time are given in Table 12.

Table 12 Cell mass and lipid content accumulations of *M. isabellina* when grown at 30 °C with agitation (180 rpm) for 4, 8, 12 and 16 days

strains	time (days)	biomass (g/L)	lipid content(%)*
NBRC 7874	4	0.22	14.43
	8	0.45	20.86
	12	0.43	20.59
	16	0.45	20.70
NBRC 7884	4	0.44	15.25
	8	0.58	16.14
	12	0.63	24.61
	16	0.66	24.98
NBRC 105998	4	0.49	18.68
	8	0.67	32.26
	12	0.71	35.02
	16	0.73	35.01

* compared with biomass

Table 12 represented the increasing of the total biomass of *M .isabellina* according to the extension of the growth duration. When being compared in the duration of 16 days, the strains NBRC 105998 had the most total biomass which the weight was 0.73 g/L. Followed by the strains NBRC 7884 which the weight was 0.66 g/L and the strains, NBRC 7874 which had the less total biomass at 0.45g/L respectively.

The result of the lipid content from *M. isabellina* which derived from NBRC 7874 showed that there was the most lipid content (20.86%) at 8 days and was stable continuously. In addition, the lipid content of NBRC 7884 was intended to increase, the most lipid content was found (24.98%) at 16 days and was stable continuously. Lastly, it was found that the strains NBRC 105998 contained the most lipid content (35.02%) at 12 days. The study also reported that *M. isabellina* NBRC 105998 had the ability to accumulate the lipid among three strains.

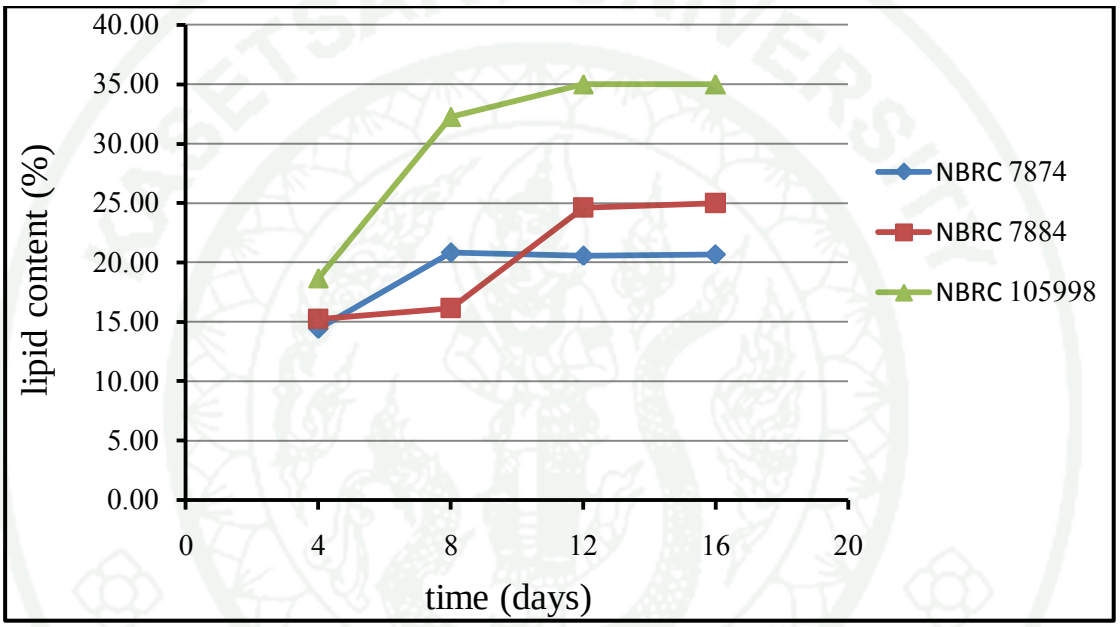


Figure 13 Lipid content (%) of *M. isabellina* 3 strains (NBRC 7874, NBRC 7884 and NBRC 105998) for 4, 8, 12 and 16 days.

Figure 13 presented the lipid content produced by 3 species of *M. isabellina* (The medium containing glycerol at the concentration of 30 g/L and fungi colony 5 disc.) and incubated at 30°C on a shaker set at 180 rpm. From the research, all these three strains had the good capability to produce and collect the lipid content. It was also found that the strains that had the most ability to produce and collect lipid content were NBRC 105998 (35.02%) at 12 days.

2.2 Lipid production by the oleaginous yeast *R. glutinis*

Three *R. glutinis* strains were cultured in the test medium by using glycerol at the concentration of 30 g/L and incubated at 30°C on a shaker set at 180 rpm. The yeast biomass of each strain was isolated and the lipid accumulation in yeast mass was extracted at 12, 24, 36, 48 and 96 hours. After that, the oil was extracted by using mixed solutions of $CHCI_3$:CH_3OH (2:1). The data of yeast mass (weight) and the lipid content accumulation in each yeast strain at different period of time are given in Table 13.

Table 13 Cell mass and lipid content accumulations of *R. glutinis* when grown at 30 °C with agitation (180 rpm) for 12, 24, 36, 48 and 96 hours

strains	time (hr)	biomass (g/L)	lipid content(%)*
NBRC 0695	12	0.05	7.60
	24	0.08	12.48
	36	0.14	23.27
	48	0.12	23.67
	96	0.23	22.54
NBRC 1099	12	0.16	37.12
	24	0.17	40.80
	36	0.37	21.33
	48	0.45	10.38
	96	0.84	8.88
NBRC 1501	12	0.16	28.70
	24	0.20	32.46
	36	0.46	14.49
	48	0.54	14.08
	96	0.82	14.23

* compared with biomass

Table 13 presented the total biomass of *R. glutinis* that tended to increase according to the increasing period of time. When being compared, NBRC 1099 were able to produce the most biomass for 0.84 g/L while NBRC 1501 and NBRC 0695 were able to produce biomass for 0.82 g/L and 0.23 g/L at 96 hours, respectively.

Regarding to the result from the study of lipid content produced from *R. glutinis*, NBRC 0695 showed that the most lipid content was produced in 48 hours for 23.67% then it was reduced slowly. Moreover, NBRC 1501 contained the most lipid content in 24 hours for 32.46%. Lastly, NBRC 1099 contained the most ability to produce lipid content in 24 hours for 40.80%. According to the result, NBRC 1099 was the best species that can accumulate lipid among three strains.

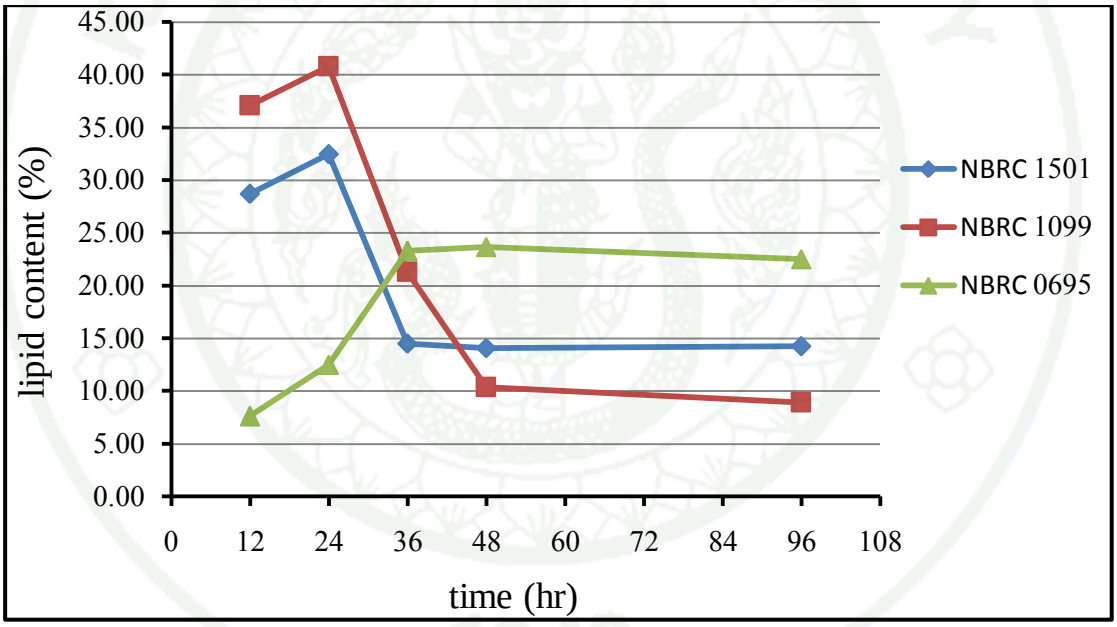


Figure 14 Lipid content (%) of *R. glutinis* 3 strains (NBRC 0695, NBRC 1501 and NBRC 1099) for 12, 24, 36, 48 and 96 hours.

Figure 14 showed lipid content in percentage that was produced by three species of *R. glutinis* (The medium containing glycerol at the concentration of 30 g/L and inoculums volume 2 ml) and incubated at 30°C on a shaker set at 180 rpm. The result showed that all these three species had good capability in lipid collection which was NBRC 1099 in 24 hours while the most percentage of the lipid contents was 40.80%.

3. The quantity analysis of the remaining and the using of glycerol in growing process of *M. isabellina* and *R. glutinis*

3.1 The quantities of the remaining and used glycerol in growing process of *M. isabellina* of three strains at 4, 8, 12 and 16 days.

The quantity analysis of the remaining and the using glycerol concentration 30 g/L in growing process of *M. isabellina* of three strains (NBRC 7874, NBRC 7884 and NBRC 105998) in the main medium at 4, 8, 12 and 16 days were presented in Table 14. In addition, there are comparing graph of the remaining glycerol content (%) of three strains. The show on Figure 15.

Table 14 The content of the remaining and used glycerol in growing process of *M. isabellina* of three strains at 4, 8, 12 and 16 days

Strains	Time (days)	The remaining of glycerol (g/L)	The remaining of glycerol (%)	
			remaining	using
NBRC 7874	4	24.82	82.73	17.27
	8	19.76	65.89	34.11
	12	19.51	65.03	34.97
	16	14.69	48.99	51.01
NBRC 7884	4	23.35	77.85	22.15
	8	20.66	68.86	31.14
	12	17.89	59.65	40.35
	16	12.32	41.07	58.93
NBRC 105998	4	18.61	62.04	37.96
	8	14.05	46.84	53.16
	12	3.93	13.10	86.90
	16	2.45	8.17	91.83

Table 14 represented the content of the remains and the used glycerol in growing process of three strains of *M. isabellina* (The medium containing glycerol

at the concentration of 30 g/L and fungi colony 5 disc.) and incubated at 30°C on a shaker set at 180 rpm at 4, 8, 12 and 16 days. The three species had more the capability in using glycerol when the period of time was increased. When comparing the result of strains selection, the strains having the best ability in producing and collecting the lipid was NBRC 105998. It had the best ability in collecting the lipid at 12 days. At this state, the microorganism could use the glycerol for 86.90%. Furthermore, it's capability in using glycerol was the best among others.

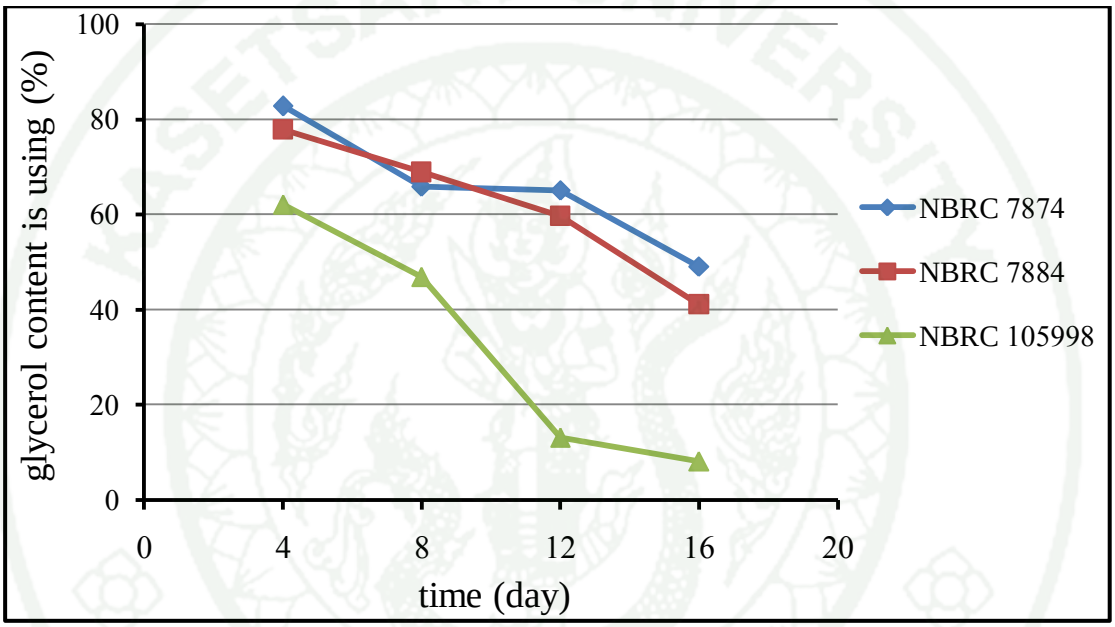


Figure 15 The content of the remaining and using glycerol in growing process of *M. isabellina* of three strains (NBRC 7874, NBRC 7884 and NBRC 105998) for 4,8,12 and 16 days.

3.2 The quantities of the remaining and using of glycerol in growing process of *R. glutinis* of three strains at 12, 24, 36, 48 and 96 hours

The content of the remaining and the using glycerol in growing process of *R. glutinis* of three strains (NBRC 0695, NBRC 1099, and NBRC 1501) at 12, 24, 36, 48 and 96 hours were presented in Table 15. Moreover, there are also comparing graph of the remaining glycerol quantity (%) of three strains as shown in Figure 16.

Table 15 The quantities of the remaining and using of glycerol in growing process of *R. glutinis* of three strains at 12, 24, 36, 48 and 96 hours

Strains	Time (hr)	The remaining of glycerol (g/L)	The remaining of glycerol (%)	
			The remaining	using
NBRC 0695	12	29.29	97.65	2.35
	24	28.86	96.22	3.78
	36	28.56	95.20	4.80
	48	26.65	88.82	11.18
	96	26.48	88.25	11.75
NBRC 1099	12	27.29	90.98	9.02
	24	26.04	86.81	13.19
	36	25.01	83.35	16.65
	48	22.62	75.38	24.62
	96	12.96	43.20	56.80
NBRC 1501	12	27.16	90.53	9.47
	24	26.96	89.88	10.12
	36	21.61	72.03	27.97
	48	17.13	57.10	42.90
	96	15.49	51.62	48.38

The content of the remaining and the used glycerol in growing process of *R. glutinis* of three strains (The medium containing glycerol at the concentration of 30 g/L and inoculums volume 2 ml) at 12, 24, 36, 48 and 96 hours, the three strains had the ability in using glycerol.

NBRC 1099 had the most capability to collect lipid at 24 hours. At this state, the microorganism could used glycerol for 13.19% when it was compared to the result of the strains selection of *R. glutinis* which had the most capability in producing and collecting the lipid contents.

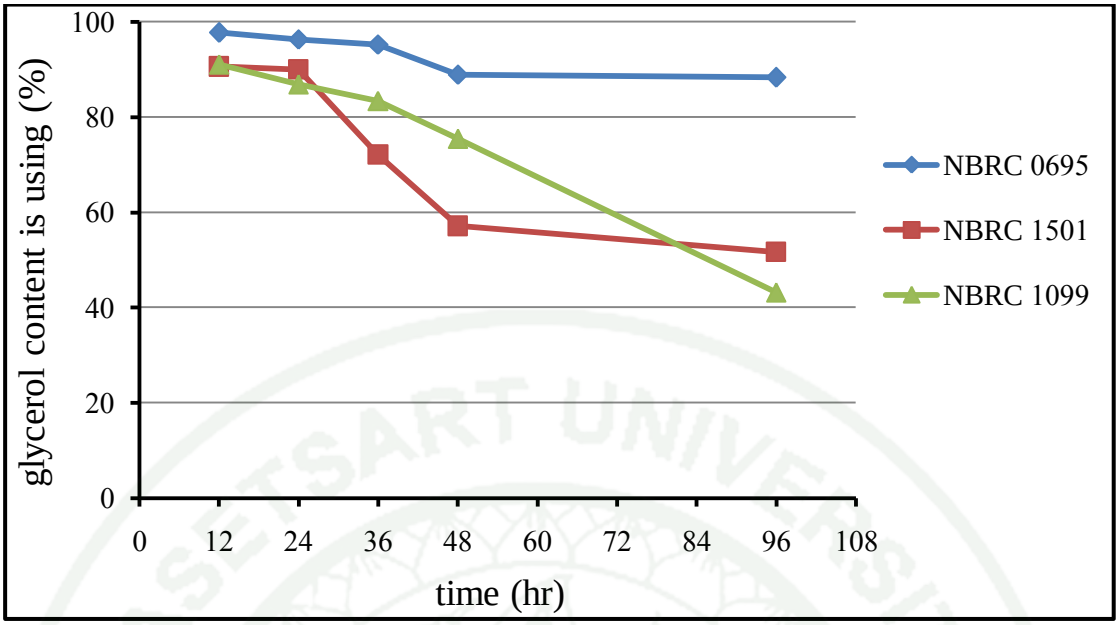


Figure 16 The quantities of the remaining and using of glycerol in growing process of *R.glutinis* of three strains (NBRC 0695, NBRC 1099 and NBRC 1501) at 12, 24, 36, 48 and 96 hours.

4. The suitable state in growing microorganism strains which had the capability in producing and collecting lipid the best

The optimum cultivation conditions for the best lipid product obtained from *M. isabellina* and *R. glutinis* were evaluated by using Response Surface Methodology (RSM). A 5-level 2-factor of Central Composite Design (CCD) was employed for the optimization of the culture conditions and the best capability to produce and collect the lipid content. A second-order polynomial equation was used to suite the predicted model to the experimental data. The development of the design matrices and analysis of variance (ANOVA) for the RSM model were conducted.

4.1 The suitable state in growing *M. isabellina* NBRC 105998

The analysis result of the produced lipid content in different states as experiment plan of CCD of *M. isabellina* NBRC 105998 and the result of 13

experimental were statistically analyzed by using multiple regression analysis (SPSS) as shown in Table 16.

Table 16 Experimental design and experimental predicted values of lipid production yield for CCD from *M. isabellina* NBRC 105998

Run no.	Code		Y [Lipid content (%)]	
	X_1	X_2	Experimental values ****	Predicted values
1	-1	-1	22.84	22.54
2	+1	-1	45.86	43.31
3	-1	+1	17.01	18.27
4	+1	+1	37.66	36.67
5	-1.414	0	13.49	12.55
6	1.414	0	38.00	40.24
7	0	-1.414	36.11	37.86
8	0	1.414	30.60	30.14
9	0	0	35.62	34.16
10	0	0	35.03	34.16
11	0	0	34.42	34.16
12	0	0	33.10	34.16
13	0	0	32.61	34.16

* average value from experimental 3 times

The effect of combination of glycerol concentration and initial of fungal on the lipid accumulation of *M. isabellina* (NBRC 105998) was shown in Table 17.

Table 17 Analysis of variance (ANOVA) for the quadratic model of the lipid content production yield from *M. isabellina* NBRC 105998

Parameters	Coefficients	p-value
Constant	34.156	0.000
X_1	9.791	0.000
X_2	-2.729	0.005
X_1X_2	-0.592	0.549
X_1X_1	-3.883	0.001
X_2X_2	-0.077	0.917

R-squared (R^2) = 0.956

Table 17 presented the concentration of glycerol (X_1) and the value of initial microorganism (X_2) had a significantly influence on the lipid content (%) that was produced from *M. isabellina* NBRC 105998 due to the "p" value was less than 0.01 ($p<0.01$). When the relationship between the quantity of the produced lipid and the factors (the glycerol concentration and the value of initial microorganism) are considered, the form of multiple regressions equation could be generated. Moreover, RSM and contour plot were showed at Equation 7. R^2 value was 0.956 which could indicated that the multiple regressions that were used in predicting the produced lipid content (%) were reliable.

$$Y = 34.156 + 9.791X_1 - 2.729X_2 - 0.592X_1X_2 - 3.883X_1^2 - 0.077X_2^2 \quad (7)$$

Where

Y represents lipid content (%)

X_1 represents glycerol concentration (g/L)

X_2 represents initial fungi (fungal disc, piece)

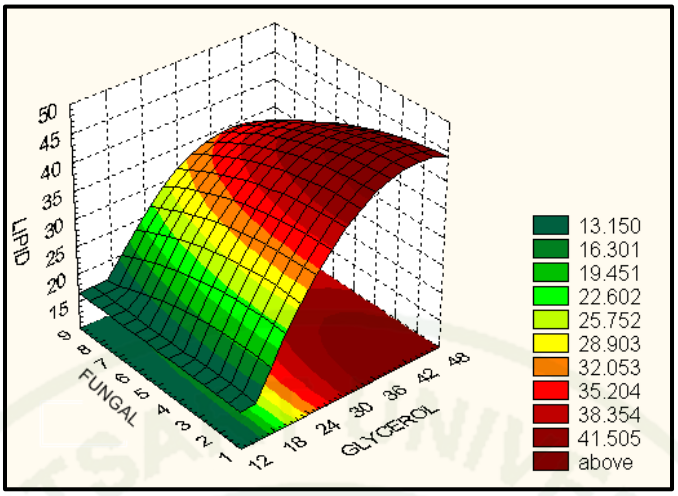


Figure 17 Response surface and contour plot of the combined effects of glycerol concentration and amount fungal on the lipid production from *M. isabellina* NBRC 105998

From Equation 7, the prediction of lipid content could be made by using the values from different states in experiment to substitute in the equation 7. The method could predict the produced lipid content in every different state.

Figure 17 showed the graph analysis of the relation of 2 factors with the contour plot and the prediction of the lipid content yield from *M. isabellina* NBRC 105998. It was found that the cultivation condition was at glycerol concentrate of 34-38 g/L. In addition, the fungal used was the fungal colony of *M. isabellina* for 1-3 fungal discs ($1.87x10^6$-$5.61x10^6$ spores). *M. isabellina* was incubation at 30 ℃ for 12 days. It was found that at this state *M. isabellina* NBRC 10599 could produce the lipid for 41-45% of biomass (the values from the experiment) which correlated to the prediction of Equation 7 that was 40-45% of biomass.

The optimum cultivation conditions for *M. isabellina* NBRC 105998 obtained from this study was found when the media were set as follows; the glycerol concentration in the medium of 44.14 g/L, the fungal colony of *M. isabellina* for 2 piece fungal discs, 7 mm diameter ($3.74x10^6$ spores) and incubation time at 30℃ for 12 days. This optimum condition gave 45.13% (the values from the experiment) of the lipid content which correlated to the prediction of Equation 7 that was 45.21%.

4.2 The suitable state in growing *R. glutinis* species NBRC 1099

The analysis result of the produced lipid content in different states as experimental plan of CCD of *R. glutinis* NBRC 1099 and the result of 13 experimental were statistically analyzed by using multiple regression analysis (SPSS) as shown in Table 18.

Table 18 Experimental design and experimental predicted values of lipid production yield for CCD from *R. glutinis* NBRC 1099

Run no.	Code		Y [Lipid content (%)]	
	X_1	X_2	Experimental Values****	Predicted values
1	-1	-1	17.73	17.60
2	+1	-1	34.86	35.80
3	-1	+1	19.58	17.93
4	+1	+1	25.77	25.18
5	-1.414	0	13.25	14.36
6	1.414	0	32.74	32.35
7	0	-1.414	29.27	28.54
8	0	1.414	19.82	21.26
9	0	0	24.21	23.64
10	0	0	23.46	23.64
11	0	0	23.67	23.64
12	0	0	23.16	23.64
13	0	0	23.69	23.64

* average value from experimental three times

The effect of combination of glycerol concentration and initial of fungi on the lipid accumulation of *R. glutinis* (NBRC 1099) was shown in Table 18. Based

on these data, the following equation, which predicts lipid accumulation in the linear regression model, is expressed as in Equation 8.

Table 19 Analysis of variance (ANOVA) for the quadratic model of the lipid content production yield from *R. glutinis* NBRC 1099

Parameters	Coefficients	p-value
Constant	23.636	0.000
X_1	6.361	0.000
X_2	-2.575	0.000
X_1X_2	-2.737	0.002
X_1X_1	-0.141	0.750
X_2X_2	0.632	0.175

R-squared (R^2) = 0.965

From Table 19, the glycerol concentration (X_1) and the value of initial microorganism (X_2) had an influence on the lipid content (%) produced from *M. isabellina* NBRC 105998 significantly due to the "p" value was less than 0.01 ($p<0.01$). Meanwhile, the relationship between the quantity of the produced lipid accumulation with the factors (glycerol concentration and the value of initial microorganism) was considered, they could be made in the form of multiple regressions as in Equation 8. RMS and contour plot were shown in Figure 19. R^2 value was 0.956 which could indicate that the multiple regressions used in predicting the produced lipid content (%) were reliable.

$$Y_1 = 23.636 + 6.361\ X_1 - 2.575\ X_2 - 2.737\ X_1\ X_2 - 0.141\ X_1^2 + 0.632\ X_2^2 \quad (8)$$

Where Y represents lipid content (%)
X_1 represents glycerol concentration (g/L)
X_2 represents inoculum volume (mL)

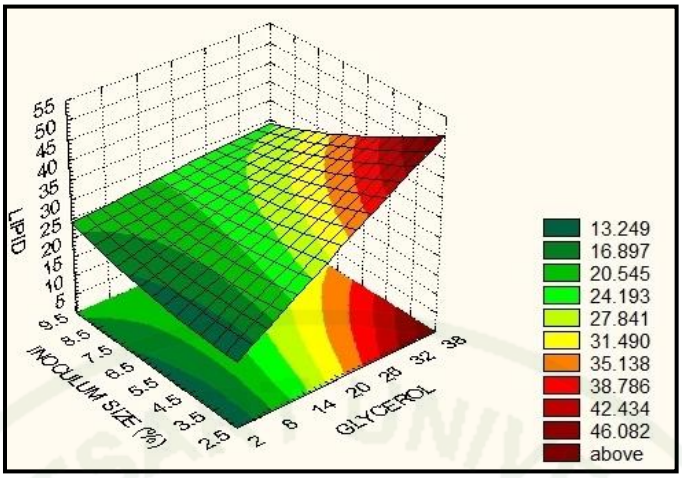


Figure 18 Response surface and contour plot of the combined effects of glycerol concentration and inoculum volume on the lipid production by *R. glutinis* NBRC 1099

The prediction of lipid content could be made by using the values from different states in experiment to substitute in Equation 8. The method could predict the produced lipid content in every different state.

Figure 18 shows the graph analysis of the relationship of 2 factors with the contour plot and the prediction of the lipid content yield from *R. glutinis* NBRC 1099. The study found the cultivative conditions was at 34-38g/L of glycerol concentration. In addition, the yeast was the inoculum volume of *R. glutinis* for 2.5-3.5% (3.98×10^7 - 5.11×10^7 cells). *R. glutinis* was incubation at temperature 30 ℃ for 24 hours. At this state, *R. glutinis* NBRC 1099 could produce the lipid for 41-45% of biomass (the values were retrieved from the experiment). The result correlated to the prediction of Equation 8 (43-47% of biomass).

In this study, the optimum cultivative condition for *R. glutinis* NBRC 1099 obtained when the medium were set as follows; the glycerol concentration of 34.14 g/L, the inoculum volume of *R. glutinis* of 3.2% (4.5×10^7 cells) and incubation time at 30℃ for 24 hours. This optimum condition gave 43.65 % (the values were retrieved from the experiment) of the lipid content which correlated to the prediction of Equation 8 (41.44%)

5. The comparison of the lipid production potential from *M. isabellina* NBRC 105998 and *R. glutinis* NBRC 1099 using raw glycerol and pure glycerol as a source of carbon substrates

The study aimed to compare the produced lipid content from *M. isabellina* NBRC 105998 (glycerol concentration was 44.14 g/L and fungi colony was 2 disc. with shook at 180 rpm and incubate at 30°C for 12 days.) and *R. glutinis* NBRC 1099 (glycerol concentration was 34.14 g/L and inoculums volume was 1.6 ml with shook at 180 rpm and incubate at 30°C for 24 hours.) when using the glycerol from the biodiesel process and the pure glycerol as source of carbon substrates. The research used the state of incubate processing from no.4 which presented the produced lipid content and the used glycerol concentration in the medium (percentage) with Table 20.

Table 20 Lipid content (%) in process and using of glycerol content for *M. isabellina* NBRC 105998 and *R. glutinis* NBRC 1099

microorganism	Glycerol sample		Commercial glycerol	
	Lipid content (%)	Glycerol concentration (%)	Lipid content (%)	Glycerol concentration (%)
R. glutinis NBRC 1099	43.69	14.36	42.13	9.57
M. isabellina NBRC 105998	43.83	40.59	42.12	30.21

Table 20 stated that *M. isabellina* NBRC 105998 (glycerol concentration was 44.14 g/L and fungi colony was 2 disc. with shook at 180 rpm and incubate at 30°C for 12 days.) and *R. glutinis* NBRC 1099 (glycerol concentration was 34.14 g/L and inoculums volume was 1.6 ml with shook at 180 rpm and incubate at 30°C for 24 hours.) were grown with glycerol from the biodiesel process at the cultivative condition from no.4.1- 4.2 respectively and it also had the capability in producing

lipid as good as growing by pure glycerol. Furthermore, the research analyzed the quantity of the remaining of glycerol by HPLC and it was found that *M. isabellina* NBRC 105998 and *R. glutinis* NBRC 1099 had more capability to use glycerol from the biodiesel process than the pure glycerol (commercial glycerol) because MONG content (fatty acid) was effectively for using of glycerol content for both microoganisms. Consequently, the glycerol from the biodiesel process of palm oil was suitable as the source of a carbon substrate in order to substitute the pure glycerol which the price was higher.

6. The analysis of the fatty acid composition of extracted lipids from *M. isabellina* NBRC 105998 and *R. glutinis* NBRC 1099 which were grown in the suitable states

The lipid produced from this condition (*M. isabellina* NBRC 105998 (no. 4.1) and *R. glutinis* NBRC 1099 (no. 4.2)) was further analyzed for its fatty acid compositions by GC-FID. The GC chromatogram and relative percentage of the fatty acid compositions in the lipid sample.

6.1 The fatty acid composition from *M. isabellina* NBRC 105998

Six fatty acids were indentified in the lipid produced from *M. isabellina* NBRC 105998 under the optimum condition. The major fatty acids were stearic acid (57.28%), palmitic acid (19.93%), oleic acid (13.15%) and the small amounts of linolenic acid (2.77%), linoleic acid (5.80%) and myristic acid (1.07%). The results showed that the lipid produced similar as Xin *et al.*, 2009. The results showed that the lipid produced from *M. isabellina* NBRC 105998 were mostly composed of the long chain saturated fatty acid (78.28%), which had a suitable property to be used as an alternative feedstock for biodiesel production.

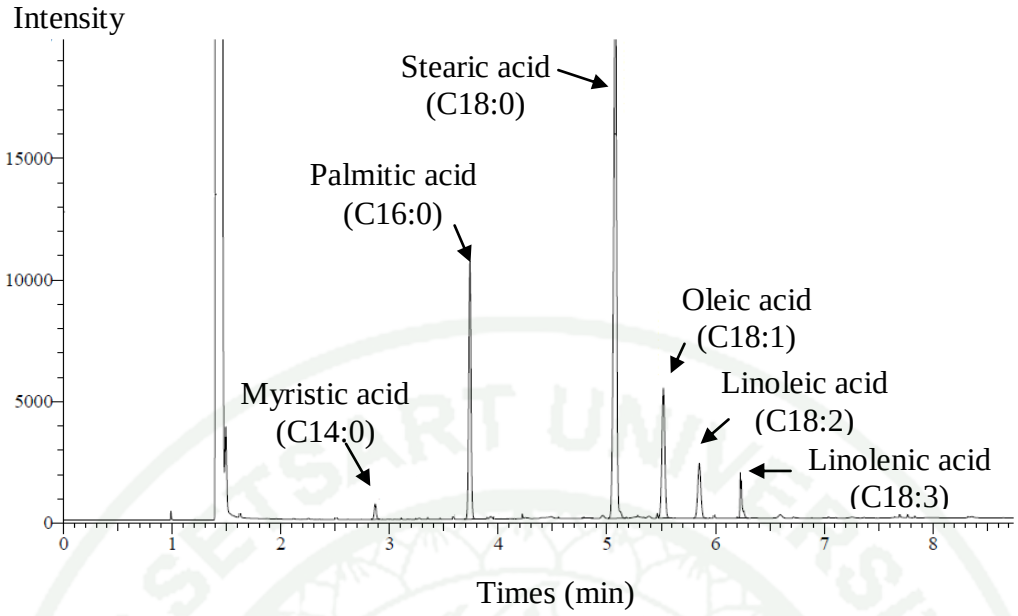


Figure 19 GC chromatogram of fatty acid compositions in the lipid produced from *M. isabellina* NBRC 105998 when grown under optimum condition.

Table 21 Relative percentage of the fatty acid compositions in the lipid extracted from *M. isabellina* NBRC 105998 when grown under the optimum condition

Fatty acid	Relative (%)
Myristic acid (C14:0)	1.07
Palmitic acid (C16:0)	19.93
Stearic acid (C18:0)	57.28
Oleic acid (C18:1)	13.15
Linoleic acid (C18:2)	5.80
Linolenic acid (C18:3)	2.77

6.2 The fatty acid composition from *R. glutinis* NBRC 1099

Six fatty acids were indentified in the lipid produced from *R. glutinis* NBRC 1099 under the optimum condition. The major fatty acids found were stearic acid (54.59%), palmitic acid (19.64%), oleic acid (19.44%) and the small amounts of linolenic acid (3.56%), linoleic acid (2.04%) and myristic acid (0.72%). The results

showed that the lipid produced similar as Xin *et al.*, 2009. The results showed that the lipid produced from *R. glutinis* NBRC 1099 was mostly composed of the long chain saturated fatty acid (74.95%) which had a suitable property to be used as an alternative feedstock for biodiesel production.

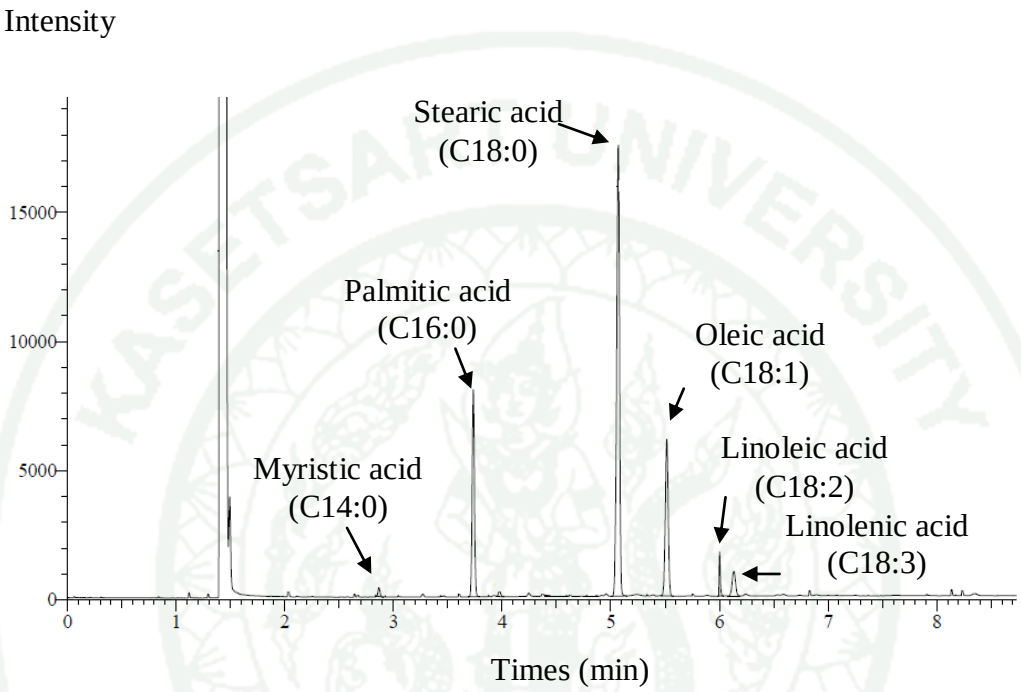


Figure 20 GC chromatogram of fatty acid compositions in the lipid produced from *R. glutinis* NBRC 1099 when grown under optimum condition

Table 22 Relative percentage of the fatty acid compositions in the lipid extracted from *R. glutinis* NBRC 1099 when grown under the optimum condition

Fatty acid	Relative (%)
Myristic acid (C14:0)	0.72
Palmitic acid (C16:0)	19.64
Stearic acid (C18:0)	54.59
Oleic acid (C18:1)	19.44
Linoleic acid (C18:2)	2.04
Linolenic acid (C18:3)	3.56

7. Fatty acid methyl ester content (%FAME) analysis of *M. isabellina*

NBRC 105998 and *R. glutinis* NBRC 1099 by gas chromatography

7.1 Fatty acid methyl ester content (% FAME) analysis of *M. isabellina* NBRC 105998

The fatty acid methyl ester content (% FAME) analysis of *M. isabellina* NBRC 105998 was analyzed by gas chromatography when grown under the optimum condition (glycerol concentration was 44.14 g/L and fungi colony was 2 disc. with shook at 180 rpm and incubate at 30^{o}C for 12 days.). FAME analysis of *M. isabellina* NBRC 105998 was 95.15% analyzed from the peak area of 124329.87. The results of FAME content in lipid were nearby the same as EN 14214 standard of minimum was 96.5% that worked by Gemma *et al.* (2009). Yield of biodiesel from fungi lipid was 84.56%. The results are shown in Table 23.

Table 23 Yield and fatty acid methyl ester value of biodiesel from *M. isabellina* NBRC 105998 when grown under the optimum condition

M. isabellina NBRC 105998	Value
% yield	84.56
% FAME	95.15

7.2 Fatty acid methyl ester content (% FAME) analysis of *R. glutinis* NBRC 1099

The fatty acid methyl ester content (% FAME) analysis of *R. glutinis* NBRC 1099 was analyzed by gas chromatography (GC) when grown under the optimum condition (glycerol concentration was 34.14 g/L and inoculums volume was 1.6 ml with shook at 180 rpm and incubate at 30^{o}C for 24 hours). FAME analysis of *R. glutinis* NBRC 1099 was 88.36% analyzed from the peak area of 30216.10. The results of FAME content in lipid were lower than EN 14214 standard of minimum was 96.5% that worked by Gemma *et al.* (2009).Yield of biodiesel from yeast lipid was 5.53%. The results are shown in Table 24.

Table 24 Yield and fatty acid methyl ester value of biodiesel from *R. glutinis* NBRC 1099 when grown under the optimum condition

M. isabellina NBRC 105998	Value
% yield	5.53
% FAME	88.36

CONCLUSIONS

Two groups of oleaginous microorganisms: the fungus *M. isabellina* and the yeast *R. glutinis* were screened for their abilities to utilize a by-product glycerol from palm oil-biodiesel production, as a carbon source for the biomass and lipid productions, also the utilization of their produced biomass as feedstocks for biodiesel production had been conducted. All the study steps and their results could be concluded as:

1. The compositional analysis of glycerol sample, a by-product which was partially purified from palm oil-biodiesel production showed that the glycerol sample had relatively high purity (93.97 %) with low contaminant levels (0.02% ash, 1.10% water, 4.91% MONG).

2. The screening of potential strains for the biomass and lipid productions on a by-product glycerol from palm oil biodiesel plant had been done on two groups of oleaginous microorganisms: three fungal strains of *M. isabellina* (NBRC 7874, NBRC 7884, NBRC 105998) and three yeast strains of *R. glutinis* (NBRC 0695, NBRC 1099, NBRC 1501). The results found that *M. isabellina* NBRC 105998 and *R. glutinis* NBRC 1099 were identified as the best lipid accumulation strains among the yeast and fungal strains tested, respectively. These two potent microbial strains could accumulate the lipid reached, 35.02% based on cell dry weight with a total biomass of 0.73 g/L at 12 days for *M. isabellina* NBRC 105998 and 40.80% with a total biomass of 0.82 g/L at 24 hours for *R. glutinis* NBRC 1099 when using the concentration of glycerol in the medium at 30 g/L.

3. The optimization of cultivation conditions for the best oil producing strains (*R. glutinis* NBRC 1099 and *M. isabellina* NBRC 105998) were evaluated using response surface methodology (RSM). A 5-level 2-factor central composite design (CCD) was used to build the statistical model. The optimum cultivation conditions for *R. glutinis* NBRC 1099 and *M. isabellina* NBRC 105998 were: the glycerol concentration in the medium [34.14 g/L and 44.14 g/L], the inoculum size [1.6 (v/v) and 2 fungal discs (1.87 x 10^6 spores/disc)], incubation time [24 hours and 12 days]

and incubated both strains at 30 °C. These optimum conditions of the lipid production from both strains gave 41-45% of the lipid contents.

4. The effect of two different carbon sources (glycerol sample and commercial glycerol) on lipid accumulations by the best oil producing strains (*R. glutinis* NBRC 1099 and *M. isabellina* NBRC 105998) were evaluated. The results found that these two microbial strains showed higher consumption ability of glycerol sample than commercial glycerol, and also gave the higher lipid yields when using glycerol sample as a carbon source.

5. The fatty acid profiles in the lipids produced by the best fungal and yeast strains obtained under optimum condition were determined. Six fatty acids were identified in the lipids produced from both strains. The major fatty acids in the lipids were stearic acid (57.28% and 54.59%), palmitic acid (19.93% and 19.64%), oleic acid (13.15% and 19.44%) and the small amounts of linolenic acid [C18:3] (2.77% and 3.56%), linoleic acid [C18:2] (5.80% and 2.04% and) and myristic acid (1.07% and 0.72%) for *M. isabellina* NBRC 105998 and *R. glutinis* NBRC 1099, respectively. These analysis results showed that the lipid produced from these oleaginous microbial strains were mostly composed of the long chain saturated fatty acid (78.28% and 74.95%) which were similar to the main vegetable oils used in biodiesel production, e.g. palm oil. Thus, their physicochemical characteristics might also be expected to be resemblance.

6. The percentage of fatty acid methyl ester of biodiesel produced from *M. isabellina* NBRC 105998 was 95.15% and the percentage of fatty acid methyl ester of biodiesel produced from *R. glutinis* NBRC 1099 was 88.36%. *M. isabellina* NBRC 105998 and *R. glutinis* NBRC 1099 are high quantity for biodiesel production. Therefore, the biodiesel produced by these oleaginous fungi and yeast strain, which can grow rapidly without any environmental or ethical issues, can be an excellent alternative source for biodiesel production in the future.

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APPENDICES

Appendix A

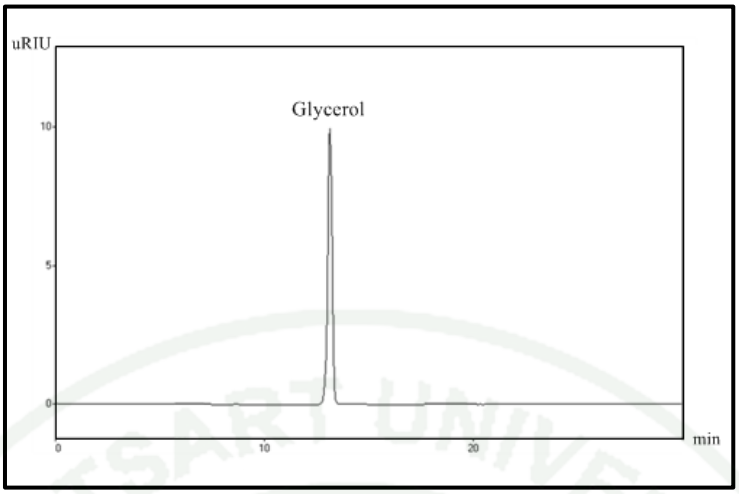
Commercial glycerol and crude glycerol

Appendix Figure A1 Commercial glycerol

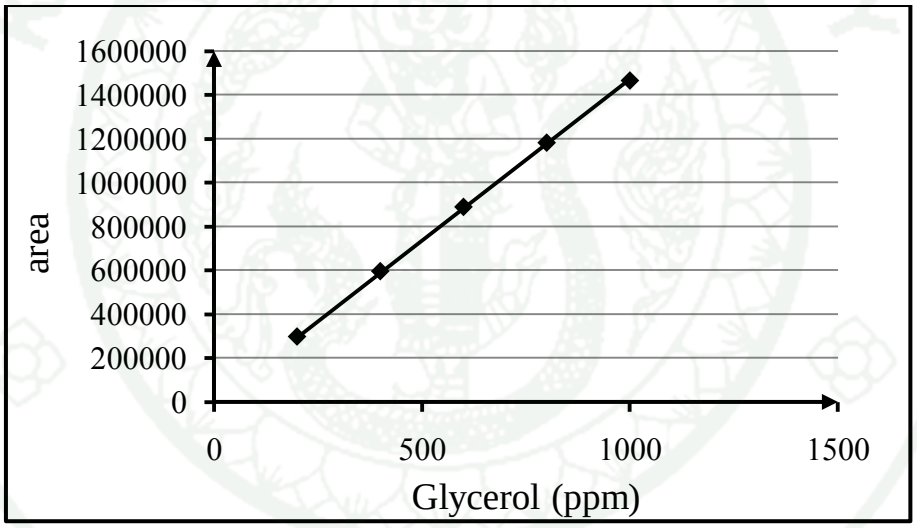
Appendix Figure A2 Crude glycerol from Patum Vegetable oil Co., Ltd.

Appendix B

HPLC chromatogram and calculation of standard for commercial glycerol



Appendix Figure B1 HPLC chromatogram of commercial glycerol



Appendix Figure B2 Standard calibration curves of commercial glycerol

Equation of linear standard calibration curves

$$Y = 1474X$$

R^2 is Pearson Coefficient of Determination

$$R^2 = 0.999$$

Appendix C

Calculation of matter organic non-glycerol (MONG) for glycerol sample

Appendix Table C1 The chemical compositions of glycerol sample

Components	Percentage (w/w)*
Glycerol	93.97
Water	1.10
Ash	0.02
Matter organic non-glycerol	4.91

* based on the glycerol sample weight

Equation of matter organic non-glycerol

$$MONG = 100 - (A+B+C)$$

Where

A is percentage content of glycerol sample

B is percentage content of ash

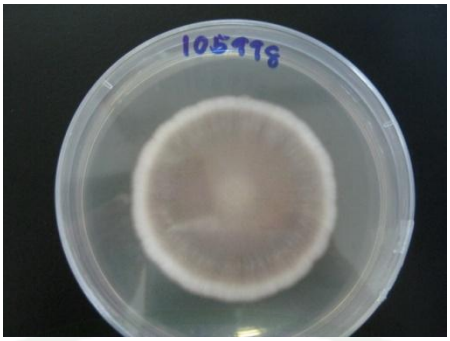
C is percentage content of water

$$MONG = 100 - (93.97+1.10+0.02)$$

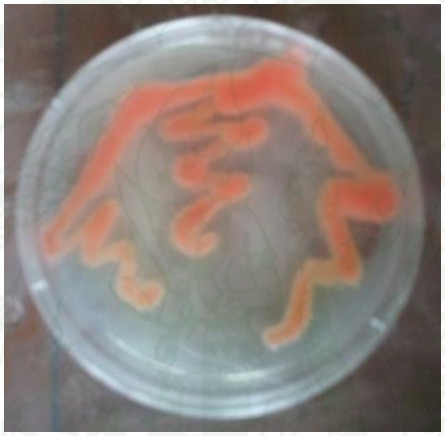
$$MONG = 4.91$$

Appendix D

Oleaginuos microorganisms



Appendix Figure D1 *Mortierella isabellina*



Appendix Figure D2 *Rhodotorula glutinis*

Appendix E

Preparation of Stock culture medium for oleaginuos microorganisms

Appendix E

1. Stock culture medium for oleaginuos microorganisms

1.1 Peptone Dextrose Agar (PDA) for *M. Isabellina*

- PDA per 1 L distilled water; Peptone Dextrose Agar (PDA) 39 g.

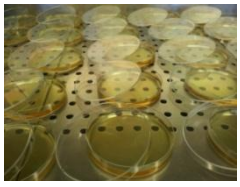
1.2 Yeast Peptone Dextrose medium (YPD) for *R. glutinis.*

- YPD per 1 L distilled water; Yeast extract 3 g, Peptone 5 g, Dextrose 10 g and Agar 15 g.

Appendix Figure E1 Stock culture medium

2. Stock culture medium was sterilized by using autoclave for 15 minutes at the temperature of 121 ℃.

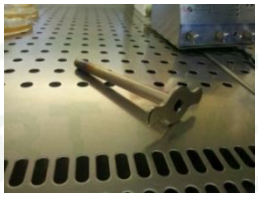
3. The 20 ml of each obtained stock culture medium was poured in Petri dish and left for cooling until to be solid jelly.



Appendix Figure E2 Stock culture medium

4. Placed microorganisms on stock culture media plate.

4.1 *M. isabellina* was cultured in the stock culture medium for 7 days and incubated at 30°C on PDA with crock no.3 (fungal disc 7 mm.).



Appendix Figure E3 Crock no.3 (disc. I.D. = 7 mm) for *M. isabellina*

4.2 *R. glutinis* was cultured in the stock culture medium for 48 hours and incubated at 30°C on YPD with loop.

Appendix Figure E4 Loop for *R. glutinis*

Appendix F

Test medium for oleaginuos microorganisms

Test medium for oleaginuos microorganisms

1. Test medium for *M. isabellina.*

The test medium used in the experiments composed of (per 1 L distilled water): glycerol sample from biodiesel production 30 g, Yeast extract 1 g, KH_2PO_4 7.0 g, Na_2HPO_4 2.0 g, $MgSO_4 \cdot 7H2O$ 1.5 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, $FeCl_3 \cdot 6H_2O$ 0.08 g, $ZnSO_4 \cdot 7H_2O$ 0.001g $CuSO_4 \cdot 5H_2O$ 0.0001g, $Ca(NO_3)_3 \cdot H_2O$ 0.0001 g, $MnSO_4 \cdot 5H_2O$ 0.0001 g and $(NH_4)_2SO_4$ 0.5 g.

2. Test medium for *R. glutinis.*

The test medium used in the experiments composed of (per 1 L distilled water): glycerol sample from biodiesel production 30 g, Yeast extract 1 g, KH_2PO_4 7 g, Na_2HPO_4 2.5 g, $MgSO_4 \cdot 7H_2O$ 1.5 g, $FeCl_3 \cdot 6H_2O$ 0.15 g, $ZnSO_4 \cdot 7H_2O$ 0.02 g, $MnSO_4 \cdot 5H_2O$ 0.06 g, $CaCl_2 \cdot 2H_2O$ 0.15g, $(NH_4)_2SO_4$ 0.5 g.

Appendix Figure F1 Test medium for oleaginuos microorganisms

3. Test medium was sterilized by using autoclave for 15 minutes at the temperature of 121 °C.

Appendix G

Lipid extraction

Appendix G1 Lipid extraction for *M. isabellina*

1. Culture broth was filtered with suction pump.

Appendix Figure G1 Step of culture broth was filtered

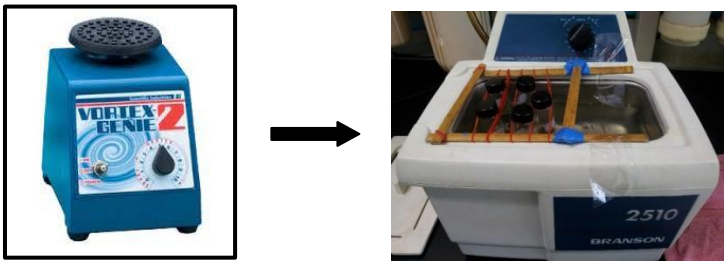
2. *M. isabellina* biomass was dried in vacuum oven at 85 °C for 48 hours.

Appendix Figure G2 Vacuum oven

3. *M. isabellina* biomass was mashed with mortar.

4. *M. isabellina* biomass was extracted with a 2:1 (v/v) chloroform: methanol.

5. The mixture solution was extracted with vortex and sonicated for 20 and 10 minutes, respectively.



Appendix Figure G3 Step of extracted with vortex and sonicated.

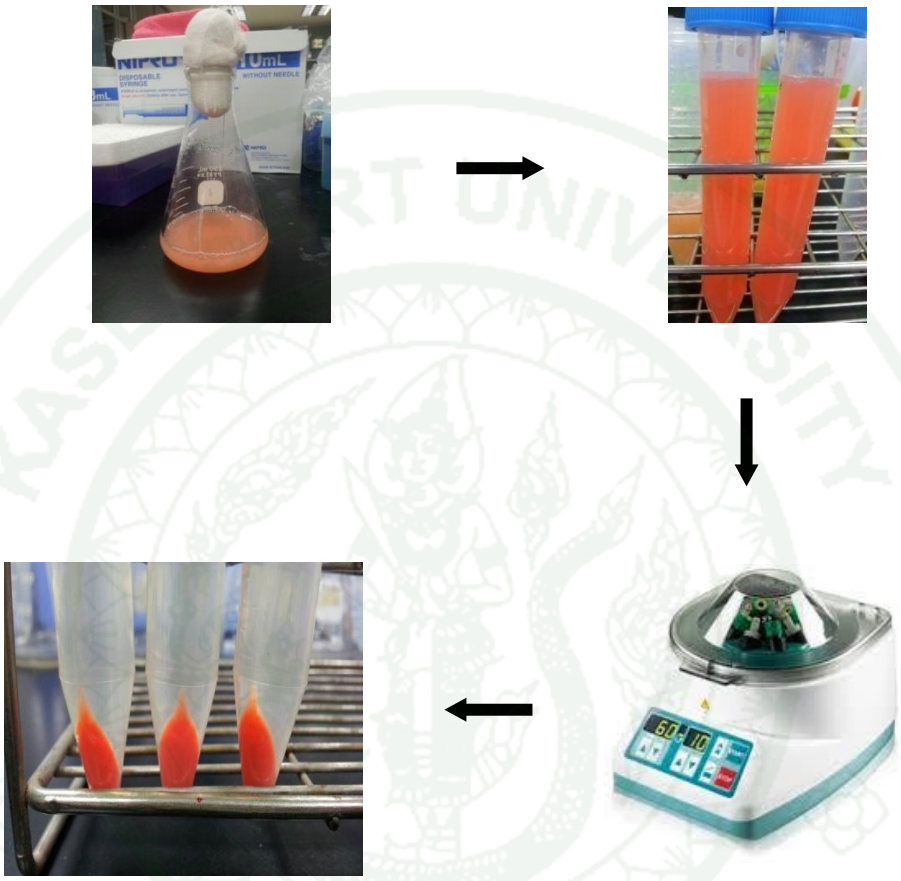
6. Solution was filtered with syringe filter (0.45 um).

Appendix Figure G4 Filtered with syringe filter (0.45 um) of *M. isabellina*

7. Solution and residue were baked in vacuum oven at 65 °C for 24 hours.

Appendix G2 Lipid extraction for *R. glutinis*

1. Culture broth was centrifuged 6,000 rpm for 3 minutes.



Appendix Figure G5 Step of culture broth was centrifuged

2. *R. glutinis* biomass was dried in vacuum oven at 85 °C for 48 hours.

3. *R. glutinis* biomass was mashed with mortar.

4. *R. glutinis* biomass was extracted with a 2:1 (v/v) chloroform: methanol.

5. The mixture solution was extracted with vortex and sonicated for 20 and 10 minutes, respectively.

6. Solution was filtered with syringe filter (0.45 um).

Appendix Figure G6 Filtered with syringe filter (0.45 um) of *R. glutinis*

7. Solution and residue were baked in vacuum oven at 65 °C for 24 hours.

Appendix H

Calculation of central composite design

Calculation of central composite design

The central composite design was carried out with increasing two experimental point along each coordinate axis to opposite side of the origin and at a distance equal to the semi diagonal of hypercube of the factorial design and new extreme value as low and high for each factor added in the central composite design model. The calculation was show in equation below.

$$\alpha = [2^K]^{1/4}$$

Appendix I

Biodiesel production

Biodiesel production

1. Lipid of microoganism weighed about 50 mg into screw capped glass tube. Add to methanol containing 2% v/v of sulfuric acid (H_2SO_4) at a ratio of 5 ml of methanol. Maintained at 60°C for 6 hours with oven.

2. After completely reaction, a solution was cool down and added by 2 ml of hexane and shaken for 5 times. The upper layer was fatty acid and transferred to 30 ml screw capped glass tube.

3. Rinsed fatty acid with 2 ml of distilled water for 3 times and transferred of upper layer to 30 ml screw capped glass tube. This fatty acid was evaporated to dryness using N_2.

4. The fatty acid was determined for fatty acid composition by gas chromatography (GC).

Appendix Figure I1 Biodiesel of lipid from microorganism

Appendix Table I1 The condition of the GC (Agilent Technologies) Mold splitless analysis of fatty acids in oil fat

Injector	Splitless		
Sample size	1 µL		
Inlet	Track		
Column temperature program			Run time (mim)
Initial temperature	80 ℃	Hold 1 min	1.0
Rate 1	10 ℃/min to 250 ℃	Hold 2 min	20.0
Post run	50	Hold 0 min	20
Detector temperature	270 ℃	-	-
Carrier gas flow rate	He 3 mL/mim	Measured at 50 ℃	-

CURRICULUM VITAE

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