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Research article

Biosynthesis of single cell oils extracted from microbial cultures

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Abstract

In recent years, microorganisms have been receiving increased attention as sources of edible lipids, thus they are potential to become a source for producing novel single cell lipids (SCLs). Considerable research has been devoted to techniques for growth, extraction, and purification of high value lipids for their use as biofuels and biosurfactants as well as high-value metabolites for nutrition and health. The performance of SCL can be improved by modifying the fatty acids through transesterification using organic solvents in the availability of strong base or acid catalysts. The purpose of this study was to explore the potential of single cell oils extracted from fungal and algal strains through some preparation procedures, including incubation, oil extraction, esterification, transesterification and emulsification of oil containing microbial biomass indirectly. Transesterification using hexane-isopropanol produced a high fatty acid methyl ester content. The SCL that were extracted from molds (*Monascus purpureus, Monilia sitophyla, Aspergillus oryzae, Mucor javanicus, Fusarium oxysporum*) and algae (*Scenedesmus dimorphus, Chlorella vulgaris* and *Spirulina fusiformis* could produce SCL through direct transesterification by application of heat with a lot of anhydrous methanol and a sodium hydroxide as a catalyst which serves to increase reaction speed and yield. The SCL derived from the *F. oxysporum* and *M. javanicus* were predominated with palmitic acid (41% and 40%), while those were from *A. oryzae*, *M. purpureus* and *M. sitophyla* were predominated with linoleic acid (42%, 36,1% and 36%). The SCL derived from *C. vulgaris* and *S. fusiformis* dominated by linolenic acid (24% and 36%, respectively), while that was from *S. dimorphus* was dominated by linoleic acid (24%). Palmitic and linoleic acids were potentially esterified and transesterified in the manufacturing of methyl ester of fatty acids.

Key words: algae, mold, fermentation, single-cell lipid, fatty acid, transesterification.

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1. Introduction

Like all living cells, microorganisms contain lipids. It indicates a triacylglycerol type of lipid, similar to that found in plant and animal edible oils and fats and known as single cell lipid (SCL) [1,2]. Microorganisms product lipid for the essential functioning of

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cell membranes and other membranous structure [3,4]. Single cell lipid (SCL) has gained attention in recent years as a biochemical conversion option for the biotechnology industry. SCL is commonly defined as neutral storage lipids accumulated by eukaryotic microorganisms (e.g. yeast, mold, microalgae) and is often synonymous with lipid, oil, and triacylglycerol (TAG) and polyhydroxy-alkanoates (PHA) as the hallmark lipids accumulated in eukaryotes and prokaryotes, respectively. Lipids have many metabolic roles, for example, they act as storage materials in animals, plant and microbial cells and are also responsible for the structure of cell membranes, and protect the body against cold and other environmental influences, etc. One of their important physiological roles is that they are precursors of hormone-like compounds, which is performed mainly by polyunsaturated fatty acids [5,6]. These lipids are currently viewed as valuable potential biofuel sources, high-value compounds in the food and pharmaceutical industries, building blocks for biomaterials, potential tools to treat chronic diseases, and natural alternatives for the production of oleochemicals, among many other uses. Approximately 80% of the world's oil and fat need is derived from agricultural products, and the remainder coming from animal and marine sources. It is essential to find new sources for oil and protein supplement with concern to the nutritional problems accompanying the rapid growth of the world's population. In fear of the depletion of oil resources and the global warming, present biotechnological research has concentrated on the commercial exploitation of microorganisms for the production of fuels and chemical materials. Single cell lipids are potential candidates [7]. SCLs are lipids produced by oleaginous microorganisms, including bacteria, yeast, fungi, and algae that are capable of accumulating at least 20% of their dry cell mass as lipids [8]. Their production does not compete with the food supply, since it can utilize agro-industrial wastes and by-products [9], such as lignocellulosic biomass [10]. Other advantages of using SCLs rather than plant oils are that arable land is not required, environmental parameters (such as weather conditions) are irrelevant, and cultivation times are shorter than those for the crops [4,11]. Although the price of biodiesel from SCO is higher than first generation biodiesel, it is expected to decrease as the technology matures [12]. Furthermore, reduction of its cost can be achieved if zero or negative value waste substrates are employed as carbon and nitrogen sources [13] and if high valuable co-products produced concomitantly with SCL by the microorganisms are sold, such as carotenoids or polyunsaturated fatty acids [12,13]. Algal and fungal strains offer advantages over other oleaginous bacteria since lipid compositions and short cultivation times are more suitable for biofuel production than those obtained with bacteria and yeast [14] and there is very few reports on the standard method of choice for neither its extraction aiming at SCLs quantification [15,16] nor for process development. The aim of the present study is to extract and esterified lipid accumulation under the condition for optimal lipid yield using conventional algal and fungal strains.

2. Materials and Methods

Media and Microbial Cultures

Microbial cultures that were used including algae (*Chlorella vulgaris, Scenedesmus dimorphus, Spirulina fusiformis*), and molds (*Monascus purpureus, Monilia sitophyla,*

Aspergillus oryzae, Mucor javanicus, Fusarium oxysporum). Medium to cultivate molds containing 2% glucose, 0.5% yeast extract and 0.1% KH_2PO_4 in phosphate buffer pH 6.5 was sterilized by autoclaving at 121C for 15 min. Media were inoculated with 5% (v/v) starter culture at a density of 3.0-4.0 x107 cells/mL, i.e. after 24 h of incubation. The cultures were shaken at 150 rpm at room temperature for 4-5 days.

Algal Cultivating media

Algal cultures were grown on phosphat hydrogen media (PHM) [17] with an optimum light intensity at 25°C. The PHM medium was prepared by dissolving 1.0g KNO_3 and $MgSO_4.7H_2O$ as amount of 0.2g into distilled water, and then added with 1.0mL of trace metals solution and soil extract, respectively, and divided into three of 300 mL conical flasks containing 150 mL PHM media and then sterilized using autoclave at 121°C for 20 min. Into the sterilized media as much as 0.15 mL Fe and K_2HPO_4 solution were added respectively, prior to inoculation with 15mL algal culture and continuously stirred at 25°C and illuminated under fluorescent lights.

Algal Biomass and Lipid Extraction

Algal biomass were harvested by centrifugation. After accumulating the algal biomass in the centrifuge tubes, furthermore the biomass were suspended into distilled water and then resulting slurry could be stored for 1-2 weeks into a chiller or frozen after adding with a cryoprotectant to maintain integrity of cells during a process of freeze drying. The SCL was extracted from algal culture, according to the modified method of Lepage *et al.* [18]. Approximately 10 mL of hexane-isopropanol (3:2, v/v) was added to the freeze-dried biomass in a 45mL centrifuge tube, and then homogenized for 1 min and centrifuged at 5000 rpm for 10 min. The extraction process was repeated two times to its residue and to all obtaining supernatants were then remerged. An amount of 10 mL of 0.47M natrium sulphate was then added to the supernatant to breakdown emulsion. The top phase containing SCL was transferred to another tube and then evaporated to dryness using nitrogen gas over a water bath at 45°C. The dry weight of its residue was defined as the total weight of the SCL.

Fungal Single Cell Lipid Extraction

The fungal cultures that have incubated for 4-5 days were then filtered through Whatman filter paper No. 1 in order to separate their biomass and fluid. The biomass were then freeze dried at -40°C and 0.15 Mbar in order to obtain a constant weight. The fluid without biomass was then analyzed for determination of its COD and reducing sugar. The COD was determined according to the methods of APHA and the determination of reducing sugar was according to DNS method.

Lipase Production

Production of extracellular lipase from fungal cultures were performed in 250mL conical flasks containing 50 mL media consisting of 0.5% peptone, 0.3% yeast extract, 0.25% NaCl, 0.05% $MgSO_4$ and 3.0% coconut oil at pH 7.0. Medium sterilized and inoculated with 3.5 mL (4×10^8 CFU) and then incubated for 60 h at 34°C on a rotary shaker at 120rpm. At the end of incubation period, the culture media were centrifuged at 6987*g* for 10min and obtaining supernatants were treated with acetone (1:4, v/v) for 1 h at 4°C, and centrifuge at 6987*g* for 10min. Precipitates were dissolved into 50 mM phosphate buffer pH 5.0 prior to use as lipase preparation.

Assay for enzymatic activity

Lipase activity test carried out by titrimetric method. Olive oil as the amount of 1.0 mL in 100 mL conical flask was added subsequently with 0.5ml of 0.1M $CaCl_2$ and 4.5mL of 0.05M citrate buffer pH 6.0. The reaction mixture was incubated at 40ºC for 10 min and then 10% (v/v) lipase solution was added to each culture and incubated again at 40ºC on the rotary shaker at 120 rpm for 30 min. Furthermore, the reaction mixture was stopped with addition of 10 mL ethanol and acetone (1:1, v/v) and finally added with 3 drops of 1% phenolphthalein as an indicator prior to titration with 0.05 N NaOH untill its color change to pink. One unit of enzyme activity equals to 1.0 µmol of free fatty acids resulting from hydrolysis of substrate during the time of incubation.

Esterification of Single Cell Lipids

Approximately 0.5 mL of the SCL was placed onto a test tube and then added with 15 mL of 0.5M NaOH in methanol. The tube containing sample of SCL was sealed and then vortexed and heated for 5 min at 80°C and after cooling down at room temperature, it was then added with 10 mL of BF_3 in 30% methanol and furthermore votexed. The solution was reheated at a 85°C for 30 min and cooled down for a few minutes and then added with 5mL hexane and 15 mL saturated NaCl. After vortexing, the hexane layer was pipetted onto reaction tube and then added with 5g of unhydrate Na_2SO_4 and the obtaining SCL was then analyzed furthermore through GC. Transesterification reaction by application of lipase as biocatalyst to replace NaOH as chemical catalyst was carried out at 30°C on the rotary shaker at 200 rpm and then followed by similar procedure to the process of esterification by using the chemical catalyst.

3. Results and Discussion

Fungal Single Cell Lipid Extraction

Figure 1 showed the growth of fungal cultured (*M. sitophila, M. javanicus, F. oxysporum* and *A. oryzae*) grown on potato dextrose media for 2-3 days, however the culture of *M. purpureus* which was grown on bean-sprouts medium was slowest growing among of cultures, while M. *sitophyla* was growing fastest among of the cultures. All the fungal cultures showed optimally grown after cultivating for 2-3 days, thus it could be transferred into SCL production media. The molds could be very fastly in forming propagules while growing on the media, thus it should be more carefully handling while doing a transfer since it may contaminate to some other cultures.

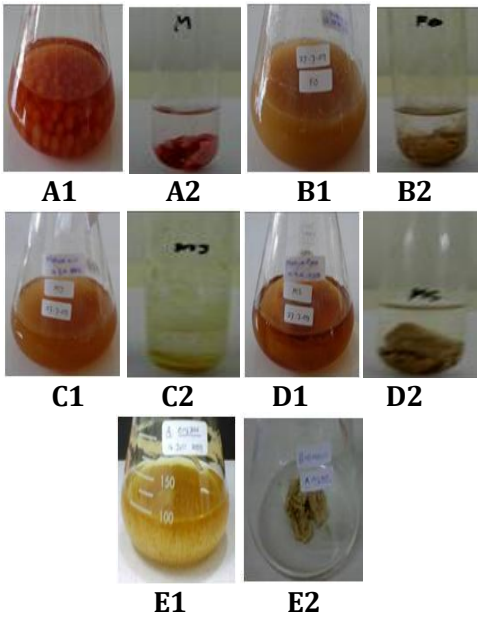


Figure 1. Fungal cultures of *M. purpureus* (A1-2), *F. oxysporum* (B1-2), *M. javanicus* (C1-2), *M. sitophyla* (D1-2), *A. oryzae* (E1-2) before and after extraction of their SCLs.

All the cultures were incubated on a rotary shaker for 4-5 days prior to be harvested and prepared for the SCL extraction process. *M. purpureus* could relatively grow

well and faster than the other cultures in a liquid state medium. Its cultural medium immediately turned to reddish and its mycelium colony to form larger spherical pellets rather than other cultures. The culture quickly grew to cause its liquid media to be highly viscous and turned to murky brown. However, its viscosity was slightly lower than that of viscosity of the medium which was overgrown by *F. oxysporum*. Its liquid medium turned to slight reddish and to form globular yellowish white fungal colonies and the culture grew well and flourish. Respective fungal cultures exhibited different speed of growing and their performance, thus allowing them for difference in their SCL content (Figure 1). Differences in the structure can allow for differences LSTnya content. For the purpose of cell multiplication, 5 mL of liquid culture was inoculated into 45 mL of fresh media, and then homogenized to break that mold colonies into single cells are homogeneous. The cells are then extracted the fat so obtained LST. Cultures that have a homogeneous, then filtered using Whatman No. 1. A. oryzae cultures need not homogenized, but was immediately filtered using ordinary filter paper, because the culture of A. oryzae spores have great size that can be screened directly. For the purpose of multiplication, thus every 10% of parent cultures were inoculated into fresh medium and then homogenized to destroy their colonies to become a homogenous cell suspension. The crushed cells were then extracted in order to collect SCL by filtering the obtaining oily fluids using Whatman filter paper No. 1. The highest reducing sugar content shown by the culture of *F. oxysporum* (11.903ppm).

Algal Single Cell Cultivation

Algal cultures that used in this research were *Chlorella vulgaris*, *Scenedesmus dimorphus* and *Spirulina fusiformis*. Algal biomass that obtained from the cultures were wet and then freeze-dried to get a dry biomass by removing residual water content. The dried algal biomass were added with hexane-isopropanol solution and allowed to stand for one night (±12h) in order to extract algal SCL, thus the extraction process could take place optimally. The mixture was homogenized for 10-20 min, thus the lipid deposit inside algal cell were extracted out thus extraction process could run perfectly and the homogenized ones were then centrifuged at 10.000rpm for 10min. The treatment was repeated twice. All the obtained supernatants were remerged and added with 10mL of 0.47M sodium sulphate and then continuously processed as the same procedure dealing with preparation of fungal SCL as mentioned above. Figure 2 showed the result on determination of total algal SCL content through GC. The total SCL produced from the culture of *S. fusiformis* was 0.81g (9.55%) derived from 8.50g dry weight based of biomass of *S. fusiformis C. vulgaris*. SCL produced from the culture of *C. vulgaris* was 0.40g (9.90%) derived from 4.0g dry weight based of biomass of *C. vulgaris*. SCL produced from the culture of *S. dimorphus* was 0.53g (9.70%) derived from 5.5g dry weight based of biomass of *S. dimorphus* (*data not shown*).

Fungal Single Cell Lipid Extraction

The respective fungal cultures of *M. sitophila, M. javanicus, F. oxysporum* and *A. oryzae* and algal cultures of *Chlorella vulgaris*, *Scenedesmus dimorphus* and *Spirulina fusiformis* were furthermore homogenized and centrifuged at 10.000 rpm for 10 min to obtain their supernatants in order to determine their contents of

reducing sugar according to DNS method, while their precipitates (pellets) obtained by centrifugation were then freeze dried to remove its residual water contained in their biomass thus to obtain dry biomass with a constant weight. In order to extract the lipid contained in their biomass, the respective biomass obtained from all the cultures were dried and added with hexane-isopropanol (3:2, v/v). The mixture was then homogenized for 10-20 min and then centrifuged at 10.000 rpm for 10min.

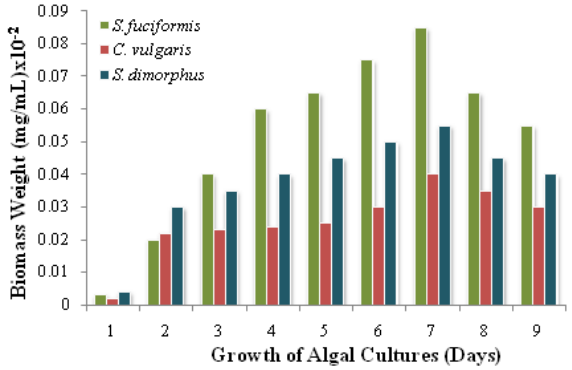


Figure 2. Profile of Algal culture growth on SCL producing media.

The obtained supernatant was then evaporated at 60°C as boiling point of hexane, and thus by evaporating hexane the SCL was still remained on evaporator flask until the remaining volume of fluid containing SCL went to approximately 10 mL when the evaporation process was completely terminated. Figure 3 showed the result on determination of fungal SCL content, while Figure 4 showed the result of the determination of algal SCL content through Gas Chromatography (GC).

Assay for Lipase Producing Cultures

Figure 5, 6 and 7, respectively showed the results of qualitative and quantitative assay for several lipase-producing cultures on BYPTA media [19] containing trybutirin as their inducer. Positive reaction indicated with the existence of a clear zone surround their colonies, whereas a negative reaction did not indicate any clear zone surround their colonies on the assay media, thus to detect the presence of free fatty acids released by hydrolysis of oil was demonstrated by the present of clear zone surround the testing colony.

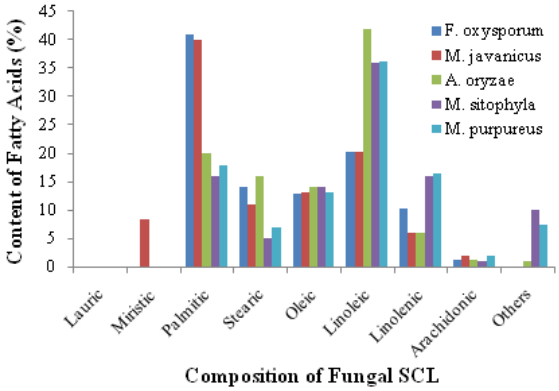


Figure 3. Determination of yield and composition of fungal single cells lipids.

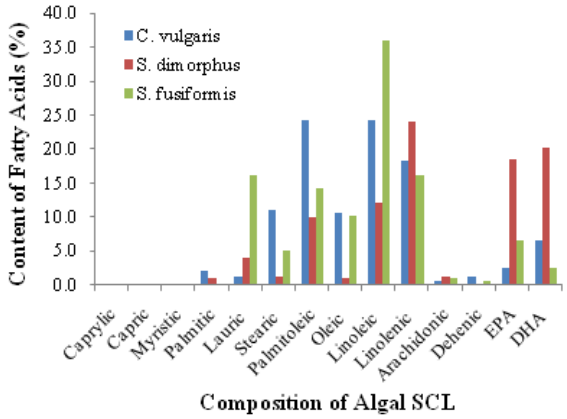
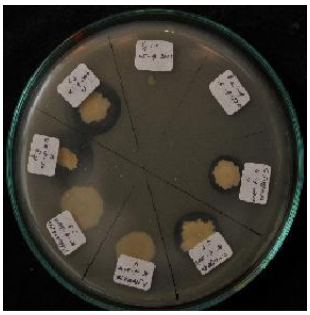


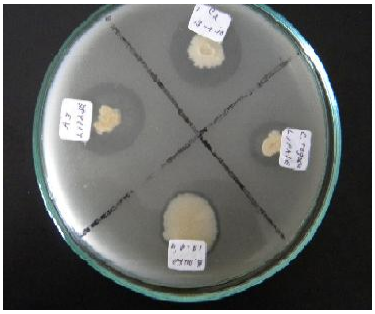
Figure 4. Determination of yield and composition of algal single cells lipids.

Quantitative test on lipase activity from selected cultures were *P. Fluorescens, C. rugosa*, *Actinomycetes* and *B. subtilis* showed the highest activity, respectively, at pH 8.0 to 8.5, (2.2 to 3.9 U/mmol) and temperatures between 55-60°C (3.6 to 4.8 U/mmol). Another results showed that the

source and volume of lipase enzyme affected transesterification process (*data not shown*). These results suggested that the microbial lipase could work effectively as biocatalyst for transesterification process resulting in the hydrolysis of tributyrin into free fatty acids that was used for synthesis of methyl esters of fatty acid.



(A)



(B)

Figure 5. Qualitative test on lipase-producing cultures (A) and selected culture used as biocatalyst for transesterification of SCL (B).

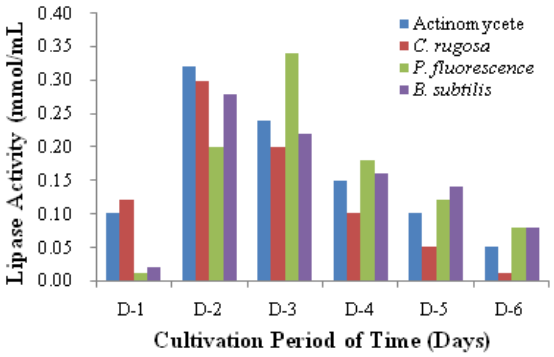


Figure 6. Profile of microbial growth on lipase-producing media.

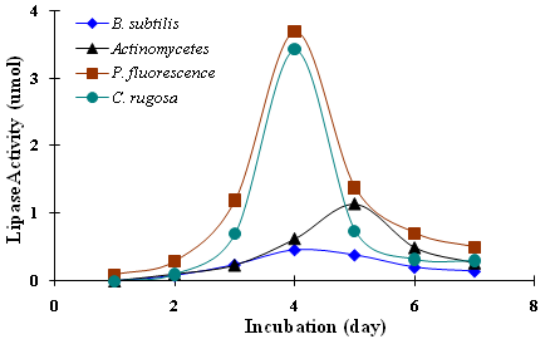


Figure 7. Lipase-producing cultures used as biocatalyst for transesterification of SCL.

Transesterification of fungal and Algal Single Cell Lipids

Table 1 and 2 showed the results of the transesterification reaction of algal SCLs that were extracted from the culture of *C. vulgaris*, *S. dimorphus*, and *S. fusiformis* with 10% methanol and transesterified by application of chemical catalyst (Table 1) and by application of lipase as biocatalyst from cultures of *P. fluorescence* and *C. rugosa* during 24h of incubation period of time. Qualitatively, the occurrence of transesterification reactions shown by the formation of fatty acid ester had a higher polarity and solubility as to compare with control that was not given alcohol as additional solvent. Reaction mixture showed a change in a better solubility properties, characterized by high levels of unsaturated fatty acid rather than saturated fatty acid product as the result of enzymatic hydrolysis of triglycerides were 23, 24 and 18% SCL (*C. vulgaris*), and 12, 16 and 24% SCL (*S. dimorphus*) and 14, 28 and 16% SCL (*S. fusiformis*). Synthesis of methyl ester of fatty acids from algal SCL was influenced by the temperature of incubation and concentration of lipase was added into reaction mixtures. Conversion increased with increasing of temperature up to 55°C close to the boiling point of reaction mixture. Approximately 90% of fatty acids ester could be synthesized through the

addition of lipase into the reaction mixture containing SCL and methanol at ratio 1:4, at 55°C for 24-48 h. In the transesterification reaction, the addition of lipase can increase FFA levels as much as 20-25%. But with the addition of alcohol as solvent, the decline in FFA levels reached 25-30%, even up to 35% on the SCL methanolysis substrate with lipase.

Table 1. Esterification of SCL using Chemical Catalyst.

Retention Time (min)	Carbon Chain	Trivial Name	Content (%)
1.05	C 8	Caprilic acid	6.33
1.61	C10	Capric acid	5.95
2.12	C12-0	Lauric acid	0.02
2.98	C12-1	Lauroleinic acid	34.92
3.96	C14	Myristic acid	0.03
5.4	C16-0	Palmitic acid	21.46
6.93	C16-1	Palmitoleinic acid	0.04
8.9	C18	Stearic acid	14.11
11.18	C22-0	Behenic acid	0.02
14.16	C22-1	Erucic acid	17.12

Table 2. Transesterification of SCL using Lipase as Biocatalyst.

Retention Time (min)	Carbon Chain	Trivial Name	Content (%)
0.92	C 8	Caprilic acid	7.64
1.36	C10	Capric acid	7.14
1.78	C12-0	Lauric acid	0.02
2.57	C12-1	Lauroleinic acid	39.72
3.42	C14	Myristic acid	0.03
4.86	C16	Palmitic acid	20.51
8.35	C18	Stearic acid	11.3
13.47	C22	Behenic acid	13.63

Transesterification reactions can be analyzed based on the comparison of the number of hydroxyl groups on the substrate before and after the enzymatic reaction, thus the volume of lipase were added effect to the decrease of free fatty acids.

Transesterification reaction catalyzed by the enzymatic potential to increase the content of unsaturated fatty acids, but instead reduce the saturated fatty acid content varies. These results indicate that the enzyme activity to a significant increase or decrease in FFA content significantly. Changes in fatty acid composition and content contained in the SCL is not optimal, thus it still can be increased given the high fatty acid content in the SCL were not fully converted properly. To improve the enzymatic transesterification more effectively and efficiently, required optimization of enzymes from different sources of microbial culture resources, especially from thermophilic and alkalotolerant groups, as well as the optimum conditions of incubation and type of organic solvent, thus the whole content of fatty acids contained in the substrate can be converted into fatty acid esters optimally. Transesterification is the formation of esters from carboxylic acids and alcohols and both are united in releasing a molecule of water in the process using methanol and suitable biocatalyst are commonly selected in industrial practice [20]. The transesterification process is the final stage in the synthesis of methyl ester of fatty acids. SCL obtained from the extraction of microbial lipids are esterified to become methyl ester and glycerol. The methyl ester obtained from the transesterification process is referred to methyl ester of fatty acids. The methyl ester of fatty acids is defined as mono-alkyl ester of long chain fatty acids contained in vegetable oils or animal fats as well as SCL. The methyl ester

of fatty acids may be obtained by transesterification of free fatty acids, which depends on the quality of the SCL used as a raw material [21]. However, before continuing to the stage of transesterification, water and biocatalysts used must be removed first in order to facilitate the process [22]. The methyl ester of fatty acids may be used as an alternative biofuel to substitute diesel oil from petroleum and also can be utilized as stove biokerosene.

Conclusions

Algal and fungal cultures can be potentially used as a source for the synthesis of methyl esters of fatty acids through esterification and transesterification of single cell lipid (SCL) derived from the microbial cultures, including *Monascus purpureus, Monilia sitophyla, Aspergillus oryzae, Mucor javanicus, Fusarium oxysporum, Scenedesmus dimorphus, Chlorella vulgaris* and *Spirulina fusiformis*. The SCL extracted from *F. oxysporum* and *M. javanicus* dominated by palmitic acid (41% and 40%, respectively), while the SCL derived from *A. oryzae*, *M. purpureus* and *M. sitophyla* dominated by linoleic acid (42%, 36.1% and 36 %, respectively). The SCL extracted from *C. vulgaris* and *S. fusiformis* dominated by linolenic acid (24% and 36%, respectively), while that was from *S. dimorphus* was dominated by linoleic acid (24%). Palmitic and linoleic acids were potentially esterified and transesterified in the manufacturing of methyl ester of fatty acids. In transesterification, a large amount of lipase and methanol might be used to produce more methyl esters as the final product.

References

1. Fakas S, Makri A, Mavromati M, Tselepi M and Aggelis G. Bioresource Technol. 2009; 100: 6118-6120.
2. Fidler N, Koletzko B and Sauerwald TU. Zb. Biotehni. ke fak. Univ. v Ljubljani. Kmetijstvo. Zootehnika. 1999; 74:37-45.
3. Muhid F, Wan Nawi WNN, Abdul Kader AJ, Wan Yusoff WM, Abdul Hamid A. J. Biological Sciences. 2008; 8(4):80-85.
4. Somashekar D, Venkateshwaran G, Sambaiah S and Lokesh BR. Process Biochem. 2002; 38: 1719-1724.
5. Sprecher H. Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochimica Biophysica Acta 2000; 1486:219-231.
6. Akpinar-Bayizit A and Ozcan-Yilsay T. Peyniraltı suyundan mikrobiyel yağ üretimi (in Turkish), Akademik Gıda 2004; 2: 15-17.
7. Liu B, Zhao ZK. Biodiesel production by direct methanolysis of oleaginous microbial biomass. J. Chem. Technol. Biotechnol. 2007; 82:775–780.
8. Ratledge, C. Single cell oil. Enzyme Microb. Technol. 1982; 4:58–60.
9. Papanikolaou S and Aggelis G. Lipids of oleaginous yeasts. Part II: Technology and potential applications. Eur. J. Lipid Sci. Technol. 2011; 113:1052–1073.
10. Yen HW and Zhang ZY. Effects of dissolved oxygen level on cell growth and total lipid accumulation in cultivation of Rhodotorula glutinis. J. Biosci. Bioeng. 2011; 112:71–74.
11. Mercer P and Armenta RE. Developments in oil extraction from microalgae. Eur. J. Lipid Sci. Technol. 2011; 113:539–547.
12. Jin M, Slininger PJ, Dien BS, Waghmode S, Moser BR, Orjuela A, Souza LC and Balan V. Microbial lipid-based lignocellulosic biorefinery: Feasibility and challenges. Trends Biotechnol. 2015; 33: 43–54.
13. Christophe G, Kumar V, Nouaille R, Gaudet G, Fontanille P, Pandey A, Soccol CR and Larroche C. Recent developments in microbial oils production: A possible alternative to vegetable oils for biodiesel

without competition with human food? Braz. Arch. Biol. Technol. 2012; 55:29–46.
14. Li Q, Du W and Liu D. Appl. Microbiol. Biotechnol. 2008; 80:749-754.
15. Pedersen TA. Lipid formation in Cryptococcus terricolus 3. Extraction and purification of lipids. Acta Chem. Scand. 1962; 16:374–382.
16. Jacob Z. Yeast lipids—Extraction, quality analysis, and acceptability. Crit. Rev. Biotechnol. 1992; 12:463–491.
17. Chrismadha T and Nofdianto. Experiment culture of algae as a production base of natural food for the purpose of inland aquaculture. Research Center for Limnology, Bogor, 1993; 37-42.
18. Lepage G, Collet S, Bougle D, Kien LC, Lepage D, Dallaire L, Darling P and Roy CC. The Composition of preterm milk in relation to the degree of prematurity. American Journal of Clinical Nutrition 1984; 40(5):1042-1049.
19. Mourey A and Kilbertus G. Simple Media Containing stabilized Tributyrin for Demonstrating Lipolytic Bacteria in Foods and soils. Journal of Applied Microbiology 1975; 40(1):47-51.
20. Soerawidjaja T. Fondasi-Fondasi Ilmiah dan Keteknikan dari Teknologi Pembuatan Biodiesel. Seminar Nasional "Biodiesel Sebagai Energi Alternatif Masa Depan" UGM, Yogyakarta 2006. (in Indonesian).
21. Destianna M, Agustina Z dan Nazef SP. Intensifikasi Proses Produksi Biodiesel. Karya Ilmiah Mahasiswa ITB Bidang Energi. ITB: Bandung. http://pub.bhaktiganesha.or.id/itb77/files/Penelitian%20mahasiswa%20ITB/BIODIESEL.pdf. 2007. (in Indonesian).
22. Joelianingsih, Tambunan AH, Nabetani H, Sagara Y and Abdullah K. Perkembangan Proses Pembuatan Biodiesel Sebagai Bahan Bakar Nabati. Jurnal Keteknikan Pertanian 2006; 20(3):205-216. (in Indonesian).