Producing of Microbial Oil by Mixed Culture of Microalgae and Oleaginous Yeast Using Sugarcane Molasses as Carbon Substrate

Thidarat Papone, Supaporn Kookkhunthod, Mutiyaporn Paungbut, and Ratanaporn Leesing

***Abstract*—The oleaginous microorganisms involving yeasts and microalgae have the potential to generate significant quantities of biomass and oil suitable for conversion to biodiesel. Compared to microbial oil production, little work has been performed for mixed culture of microalgae and yeast. In this work, microbial oil production from mono and mixed cultures of microalgae *Chlorella* sp. KKU-S2 and yeast *Toluraspora maleeae* Y30, *Toluraspora globosa* YU5/2 under mixotrophic cultivation using sugarcane molasses as carbon substrate was demonstrated. In monoculture, a biomass of 5.23g/L with lipid yield of 0.31g/L, 9.43g/L of biomass with lipid yield of 0.20g/L, 3.3g/L with lipid yield of 0.12g/L was obtained from *T. maleeae* Y30, *T. globosa* YU5/2 and *Chlorella* sp. KKU-S2, respectively. In mixed culture of microalgae *Chlorella* sp. KKU-S2 and *T. maleeae* Y30, a biomass of 5.47g/L and lipid yield of 0.25g/L were obtained. A biomass of 6.90g/L with lipid yield of 0.33g/L was obtained for a mixed culture of *T. globosa* YU5/2 with *Chlorella* sp. KKU-S2. Maximum process product yield ($Y_{P/S}$) of 0.03g/L and maximum volumetric lipid production rate (Q_P) of 0.041 were obtained in mixed culture of *T. globosa* YU5/2 with *Chlorella* sp. KKU-S2. The results obtained from this study shows that mixed culture of the oleaginous yeast with microalgae is a desirable cultivation process for enhance of microbial oil production.**

***Index Terms*—Microbial lipid, microalgae, oleaginous yeast, mixed culture.**

I. Introduction

Microbial oil produced from many oleaginous microorganisms involving yeasts, moulds, and microalgae are known as promising candidates for biodiesel production because of their advantages of higher biomass production and faster growth compared to other energy crops and their fatty acid compositions are similar to vegetable oils [1], [2]. Microalgae consume carbon dioxide (CO_2) which can reduces CO_2, greenhouse gas as an environmental pollution, therefore, biodiesel production from microalgae lipids is increasingly regarded as a more sustainable and feasible alternative to conventional biodiesel feedstock derived from terrestrial bio-energy crops [3], [4]. Microalgae can trap light energy as the energy source and assimilate CO_2 as the carbon source and organic substrates can also be utilized as the carbon and energy sources by many microalgae [5].

Microalgae may assume many types of metabolisms, such as photoautotrophic, heterotrophic, photoheterotrophic and mixotrophic growths [6]. Mixotrophic microalgae growths can simultaneously drive photoautotrophic and heterotrophic to utilize both CO_2 and organic carbon substrates, they assimilate organic compounds and CO_2 as a carbon source, and the CO_2 released by microalgae via respiration will be trapped and reused under phototrophic growth [7].

Lipid content of some microalgae such as *Scenedesmus* sp., *Chlorella* sp., *Neochloris oleoabundans*, *Spirulina maxima*, *Euglena gracilis* can achieve from 10% to 50% of total cell dry weight [6], [8], revealing the significant potential of biodiesel production. The cellular lipid content in microalgae reaches 75% in *Botryococcus braunii*, but is associated with a low productivity of biomass [1], [9]. Microalgae *Chlorella* sp. are widely available strains in the commercial applications as they presented high potentials as biodiesel producers due to their high growth rate, and their high lipid contents [2], [8]. However, the isolated microalgae *Chlorella* sp. KKU-S2 can grow under photoautotrophic, heterotrophic and mixotrophic conditions and their fatty acid components were palmitic acid, stearic acid, oleic acid and linoleic acid [10].

Oleaginous yeasts are capable of accumulating large amounts of cellular lipids and it also has a high growth rate and some oleaginous yeast strains such as *Rhodosporidium* sp., *Rhodotorula* sp. can accumulate cellular lipids to level exceeding 70% of their biomass [11]. The isolated yeast *Torulaspora maleeae* Y30 and *Torulaspora globosa* YU5/2 can accumulate high lipid yield and their fatty acids profiles were palmitic acid, stearic acid, and oleic acid that are comparable to vegetable oils [12], [13].

Mixed cultures are two or more preselected species of microorganism are synchronously cultivated within the same medium [14]. In the mixed culture of yeast and microalgae, under mixotrophic culture, microalgae could act as an oxygen generator for the yeast while the yeast provided CO_2 to microalgae and both carried out production of microbial oils [3], [4].

Currently, the cost of biodiesel produced from microbial oil is much higher than that of diesel derived from petroleum due to the lower culture process efficiency and higher cost of feedstock production is mainly contributed by microbial

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cultivation including biomass, lipid accumulation and extraction is essential step as feedstock takes up to 70% of the overall cost [15], [16]. Due to high production costs of microbial oil, the use of inexpensive carbon substrate containing sugar such as sugarcane molasses to cultivate the oleaginous microorganisms could reduce these costs. This study have proved an approach for the production of microbial oils by mixed culture of isolated microalgae and yeasts on sugarcane molasses, suggesting that they have potential of converting sugarcane molasses, a by-product of refining sugar factory to microbial oils under mixotrophic cultivation, and compare them with those under monoculture conditions.

II. Materials and Methods

A. Carbon Substrate, Microorganisms, Culture Conditions

The carbon substrate used in this study was sugarcane molasses collected from a local market in Khon Kaen province, Thailand. The pre-treated sugarcane molasses was mixed with sulfuric acid for final concentration of 1% (v/v). The mixture was treated in water baht at 100 ℃ for 20 min. After cooling, the liquid fraction as sugarcane molasses hydrolysate (SMH) was separated by centrifugation to remove insoluble particles. Then, reducing sugar was analyzed by DNS method and adjusted the reducing sugar to obtain desirable concentration for used as the carbon source.

The microalgae *Chlorella* sp. KKU-S2 was isolated from freshwater taken from pond in the area of Khon Kaen province, Thailand. The seed culture was pre-cultivated onto Bristol's medium supplemented with 20 g/L glucose and sugarcane molasses hydrolysate as carbon substrate at room temperature in an incubator shaker at a shaking speed of 150 rpm for 3 days under continuous illuminated from overhead by 80W cool-white fluorescent lamps. The Bristol's medium contained the following components (mg/L): $NaNO_3$ 250, K_2HPO_4 75, KH_2PO_4 175, $CaCl_2$ 25, NaCl 25, $MgSO_4{\cdot}7H_2O$ 75, $FeCl_2$ 0.3, $MnSO_4{\cdot}2H_2O$ 0.3, $ZnSO_4{\cdot}7H_2O$ 0.2, H_3BO_3 0.2, $CuSO_4{\cdot}5H_2O$ 0.06, and pH will be adjusted to 6.0 before sterilization.

The oleaginous yeasts *Torulaspora maleeae* Y30 and *Torulaspora globosa* YU5/2 used in this study were isolated from soil samples taken from forest in the area of Chaiyapoom and Udonthani provinces northeastern of Thailand. The seed cultures were cultivated onto Lipid accumulation (LA) medium supplemented with 20g/L glucose and sugarcane molasses hydrolysate as carbon substrate at room temperature in an incubator shaker at 150 rpm for 1 day. The LA medium was consisted of (g/L): $(NH_4)2SO_4$ 0.1, KH_2PO_4 0.4, $MgSO_4{\cdot}7H_2O$ 1.5, $ZnSO_4$ 0.0044, $CaCl_2$ 0.0025, $MnCl_2$ 0.0005, $CuSO_4$ 0.0003 and yeast extract 0.75 and pH is adjusted to 5.5 before sterilization.

B. Microbial Oil Production by Mono and Mixed Cultures

Batch cultivations were performed in 500mL Erlenmeyer flasks, each containing 300mL of medium contained 20g/L sugarcane molasses hydrolysate as carbon substrate. The experiments were performed in form of monoculture of each *T. maleeae* Y30, *T. globosa* YU5/2, *Chlorella* sp. KKU-S2 and mixed culture of *Chlorella* sp. KKU-S2 with *T. maleeae* Y30, *Chlorella* sp. KKU-S2 with *T. globosa* YU5/2. The seed cultures were 5% yeast and 5% microalgae (v/v) for mixed culture and 10% (v/v) of each strain for monoculture, cultivated at room temperature in an incubator rotary shaker at a shaking speed of 150 rpm under continuous illumination by using 80W cool-white fluorescent lamps for mixotrophic growth of microalgae and mixed culture of yeast and microalgae.

C. Analytical Methods

Duplicate samples were analyzed for cell dry weight, harvested biomass was washed twice with distilled water and then dried at 90 ℃ to constant weight. The biomass was measured gravimetrically. The total lipids were determined by the modified method of Kwon and Rhee [17]. Lipid content was expressed as gram lipid per gram dry biomass, while residual glucose concentration from supernatant was analyzed according to DNS method [18].

Volumetric lipid production rate (Q_P) was determined from a plot between lipids (g/L) and fermentation time, process product yield ($Y_{P/S}$) was determined from dP/dS, and specific product yield ($Y_{P/X}$) was determined using relationship dP/dX, while volumetric rate of substrate consumption (Q_S) was determined from a plot between substrate (g/L) present in the fermentation medium and fermentation time.

Volumetric cell mass production rate (Q_X) was determined from a plot of dry cells (g/L) versus time of fermentation (d). The specific growth rate (μ) is the slope determined by plotting the natural log of biomass versus time for each substrate concentration during the initial phase of exponential growth before the substrate concentration decreases significantly while specific rate of lipid production (qP) was a multiple of μ and $Y_{P/X}$.

D. Abbreviations

P: Lipid concentration (g/L), Q_P: Volumetric lipid production rate (g/L/d), Q_S: Volumetric substrate consumption rate (g substrate/L/d), Q_X: Volumetric biomass production rate (g cells/L/d), q_P: Specific rate of lipid production (g lipid /g cells/d), q_S: Specific rate of substrate consumption (g substrate/g cells/ d), S: Substrate concentration (g/L), X: Cell mass concentration (g/L), $Y_{P/S}$: Process product yield (g lipid/g substrate), $Y_{P/X}$: Specific yield of lipid (g lipid/g cells), $Y_{X/S}$: Cell yield coefficient (g cells/g substrate), μ: Specific growth rate coefficient (1/d).

III. Results and Discussion

Comparison of microbial cultivations by monoculture and mixed cultures of oleaginous yeast *T. maleeae* Y30, *T. globosa* YU5/2, microalgae *Chlorella* sp. KKU-S2 using sugarcane molasses as carbon substrate by batch cultivations were investigated. Reducing sugar, pH of medium, biomass and lipid production of monoculture and mixed cultures are presented in Fig. 1 and Table I. SMH referred to reducing sugar, Fig. 1(a) apparent that oleaginous yeast can use SMH faster than microalgae. It is presented that reducing sugar was used mainly for cell growth at the beginning of the cultivation time.

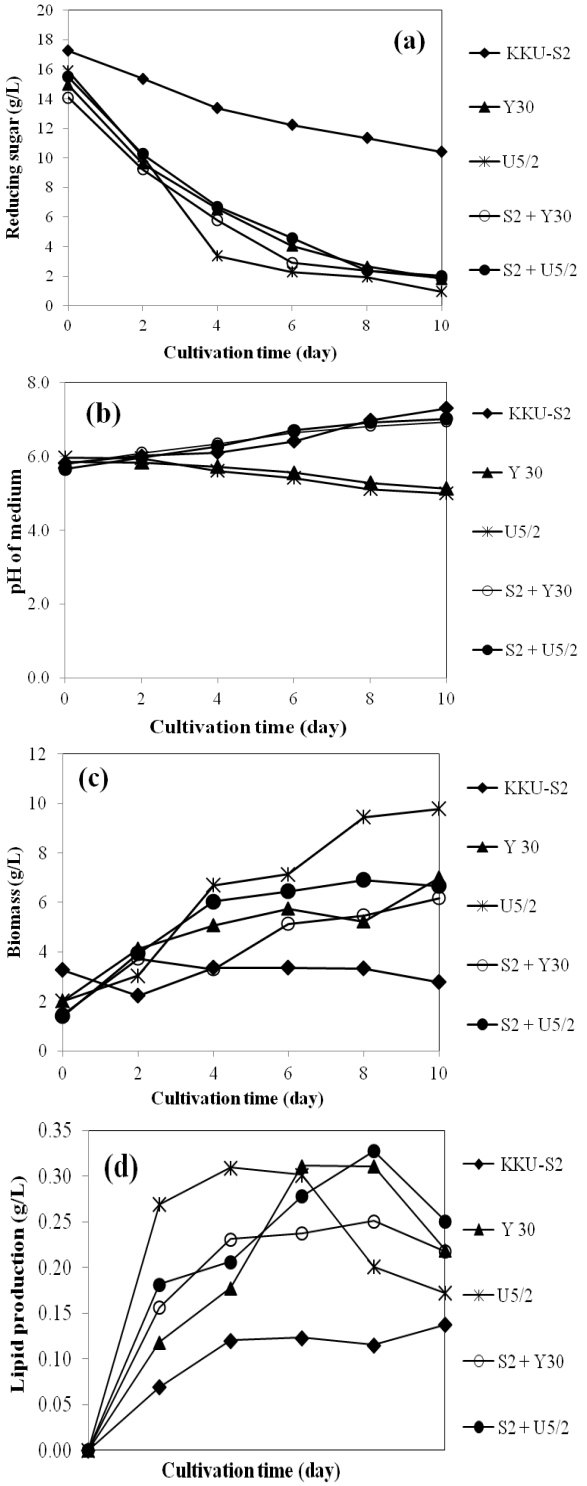

Fig. 1. Residue sugar (a), pH of medium (b), biomass concentration (c) and lipid production (d) during cultivation of monoculture of *T. maleeae* Y30 (Y30), *T. globosa* YU5/2 (U5/2), *Chlorella* sp. KKU-S2 (KKU-S2) and mixed cultures of *Chlorella* sp. KKU-S2 with *T. maleeae* Y30 (S2+Y30), *Chlorella* sp. KKU-S2 with *T. globosa* YU5/2 (S2+U5/2) using SMH as carbon substrate for 10 days.

After cultivation of microalgae, pH of medium increased from 5.5 to 7.0 for mixed culture and from 5.8 up to 7.3 for monoculture. When CO_2 dissolves in water at neutral pH, bicarbonate (HCO_3^-) formed. During photosynthesis activity by microalgae, HCO_3^- is converted to CO_2 and hydroxide ion (OH^-). Therefore, when CO_2 is consumed by microalgae, the OH^- is formed, and the pH becomes more alkaline [19].

In monoculture, *T. maleeae* Y30, *T. globosa* YU5/2 grew faster than that microalgae *Chlorella* sp. KKU-S2. The biomass of 5.23g/L with specific growth rate of 0.206 (1/d) and lipid production of 0.31g/L were obtained from *T. maleeae* Y30, while 9.43g/L of biomass with specific growth rate of 0.28 (1/d) and lipid production of 0.2g/L were obtained for monoculture of *T. globosa* YU5/2. A biomass of 3.33g/L with specific growth rate of 0.15 (1/d) and lipid production of 0.12g/L were found from *Chlorella* sp. KKU-S2.

TABLE I: FERMENTATION KINETIC PARAMETERS OF BATCH MIXOTROPHIC CULTIVATION OF THE MONOCULTURE AND MIXED CULTURE OF *T. MALEEAE* Y30, *T. GLOBOSA* YU5/2 AND MICROALGAE *CHLORELLA* SP. KKU-S2 ON THE CULTURE MEDIUM USING SUGARCANE MOLASSES AS CARBON SUBSTRATE AT ROOM TEMPERATURE, 150 RPM UNDER CONTINUOUS ILLUMINATION BY USING 80W COOL-WHITE FLUORESCENT LAMPS FOR 10 DAYS

Kinetic parameters	Microbial Cultures				
	KKU-S2[1]	Y30[2]	U5/2[3]	KKU-S2 + Y30[4]	KKU-S2 + U5/2[5]
X	3.33	5.23	9.43	5.47	6.90
P	0.12	0.31	0.20	0.25	0.33
$Y_{X/S}$	0.56	0.43	0.68	0.47	0.53
$Y_{P/S}$	0.02	0.03	0.01	0.02	0.03
$Y_{P/X}$	0.03	0.06	0.02	0.05	0.05
Q_S	0.74	1.54	1.74	1.46	1.64
Q_X	0.42	0.65	1.18	0.68	0.86
Q_P	0.014	0.039	0.025	0.031	0.041
q_S	1.77	2.35	1.48	2.14	1.90
q_P	0.03	0.06	0.02	0.05	0.05
μ	0.15	0.21	0.28	0.21	0.24

[1] *Monoculture of Chlorella* sp. KKU-S2,
[2] *Monoculture of T. maleeae* Y30,
[3] *Monoculture of T. globosa* YU5/2,
[4] *Mixed culture of Chlorella* sp. KKU-S2 with *T. maleeae* Y30,
[5] *Mixed culture of Chlorella* sp. KKU-S2 with *T. globosa* YU5/2

The obtained result in mixed culture showed that mixed culture of microalgae *Chlorella* sp. KKU-S2 and *T. globosa* YU5/2 grew faster than that of *Chlorella* sp. KKU-S2 and *T. maleeae* Y30. A high biomass of 6.90g/L with volumetric biomass production rate of 0.86g/L/d and specific growth rate of 0.24 (1/d) and lipid production of 0.33 g/L were obtained from mixed culture of *Chlorella* sp. KKU-S2 and *T. globosa* YU5/2. While, a biomass of 6.17g/L with volumetric biomass production rate of 0.68g/L/d and specific growth rate of 0.21 (1/d), lipid production of 0.25g/L were found for a mixed culture of *Chlorella* sp. KKU-S2 with *T. maleeae* Y30.

In case of monoculture of *T. globosa* YU5/2, high concentration of biomass was found. It is possible to describe that amount of initial seed culture can affected on cell growth. Due to yeasts grew faster than that of microalgae, the seed culture of monoculture of yeast (10%, v/v) that have large amount than yeast in seed culture of the mixed culture which have 5% of yeast and 5% microalgae of seed culture. Hence, monoculture of yeast *T. globosa* YU5/2 obtained a higher biomass when compared to mixed culture.

Maximum cell yield coefficient ($Y_{X/S}$, g/L) was found of 0.68 in monoculture of *T. globosa* YU5/2 but low level of both specific yield of lipid ($Y_{P/X}$, g lipid/g cells) of 0.02 and volumetric lipid production rate (Q_P, g/L/d) of 0.025 were observed. Maximum lipid production (P) of 0.33g/L, lipid

yield of 0.03g/L and maximum volumetric lipid production rate (Q_P) of 0.041 were obtained in mixed culture of *T. globosa* YU5/2 with *Chlorella* sp. KKU-S2. In the mixed culture, the microalgae may role as an O_2 producer and the yeast produced CO_2 that could be used by the microalgae, the metabolic reactions of both CO_2 release and uptake were combined and complementary [20].

The data obtained in the experimental results agreed with the data reported by Cheirsilp *et al.*, (2011), the highest biomass of 4.63g/L and lipid production of 2.88 g/L were obtained after 5 days of cultivation when using molasses as carbon substrate from the mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* [21].

IV. Conclusions

In conclusion, this mixed culture of the oleaginous yeast *T. globosa* YU5/2 and microalgae *Chlorella* sp. KKU-S2 strategy led to significant improvements in growth, biomass concentration and lipid production. The experimental obtained results presented that microbial oil production from mixed culture can be performed with low cost production process to biodiesel feedstock preparing using a by-products sugarcane molasses as carbon substrate under mixotrophic cultivation.

In further works, factor affecting on growth and lipid production such as initial of seed culture ratio of microalgae and yeast, concentration of sugarcane molasses, nitrogen source, cultivation type such as fed-batch fermentation, even up-scaling of microbial oil production will be considered through mixed culture of microalgae with yeast and completed with the production of biodiesel from microbial oil by indirect and direct transesterification.

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