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Microbial Oil Production by Monoculture and Mixed Cultures of Microalgae and Oleaginous Yeasts using Sugarcane Juice as Substrate

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Abstract**—**Monoculture and mixed cultures of microalgae and the oleaginous yeast for microbial oil productions were investigated using sugarcane juice as carbon substrate. The monoculture of yeast *Torulaspora maleeae* Y30, *Torulaspora globosa* YU5/2 grew faster than that of microalgae *Chlorella* sp. KKU-S2. In monoculture of *T. maleeae* Y30, a biomass of 8.267g/L with lipid yield of 0.920g/L were obtained, while 8.333g/L of biomass with lipid yield of 1.141g/L were obtained for monoculture of *T. globosa* YU5/2. A biomass of 1.933g/L with lipid yield of 0.052g/L was found for monoculture of *Chlorella* sp. KKU-S2. The biomass concentration in the mixed culture of the oleaginous yeast with microalgae increased faster and was higher compared with that in the monocultures. A biomass of 8.733g/L with lipid yield of 1.564g/L was obtained for a mixed culture of *T. maleeae* Y30 with *Chlorella* sp. KKU-S2, while 8.010g/L of biomass with lipid yield of 2.424g/L was found for mixed culture of *T. globosa* YU5/2 with *Chlorella* sp. KKU-S2. Maximum cell yield coefficient ($Y_{X/S}$, g/L) was found of 0.323 in monoculture of *Chlorella* sp. KKU-S2 but low level of both specific yield of lipid ($Y_{P/X}$, g lipid/g cells) of 0.027 and volumetric lipid production rate (Q_P, g/L/d) of 0.003 were observed. While, maximum $Y_{P/X}$ (0.303), Q_P (0.105) and maximum process product yield ($Y_{P/S}$, 0.061) were obtained in mixed culture of *T. globosa* YU5/2 with *Chlorella* sp. KKU-S2. The results obtained from the study shows that mixed culture of yeast with microalgae is a desirable cultivation process for microbial oil production.

Keywords**—**Microbial oil, *Chlorella* sp. KKU-S2, *Torulaspora maleeae* Y30, *Torulaspora globosa* YU5/2, mixed culture, biodiesel.

I. Introduction

CURRENT biodiesel production methods require the increasing amounts of arable land which compete with terrestrial fuel crops, heightening concern over food affordability. Microorganisms offer a unique alternative as it does not compete with agricultural food production. Microbial oils, lipid produced from many oleaginous microorganisms involving yeasts, moulds, and microalgae, which have ability to accumulate lipids over 20 % of their biomass, are seen as non-food feedstock promising candidates for the production of biodiesel because of their similar fatty acid composition to that of vegetable oils, the culture of these microbe species is affected neither by seasons nor by climates and can accumulate lipids within a short period of time as well as grow well on a variety of substrates [1, 2]. Microalgae have the potential to generate significant quantities of biomass and oil suitable for conversion to biodiesel.

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Microalgae may assume many types of metabolisms, such as photoautotrophic, heterotrophic, mixotrophic and photoheterotrophic growths [3]. Mixotrophic cultivation is when microalgae undergo photosynthesis and use both organic compounds and inorganic carbon (CO_2) as a carbon source for growth. Microalgae 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 cultivation [3, 4].

Many microalgal species can be induced to accumulate substantial contents of lipids. The cellular lipid content in microalgae reaches 75% in *Botryococcus braunii*, but is associated with a low productivity of biomass [5]. The microalgae *Chlorella* sp., especially *C. protothecoides* and *C. vulgaris* are two widely available microalgae strains in the commercial applications. They showed great potentials as future industrial biodiesel producers due to their high growth rate, and their high oil contents and they can be cultured both under photoautotrophic and heterotrophic growths. However, the locally microalgae *Chlorella* sp. KKU-S2 can grow under photoautotrophic, heterotrophic and mixotrophic conditions and accumulates much higher production of lipids, and the components of fatty acid from extracted lipid were palmitic acid, stearic acid, oleic acid and linoleic acid which similar to vegetable oils and suitable to biodiesel production [6, 7, 8].

In the last decade there is a great attention on oleaginous yeasts because some of them are capable of accumulating large amounts of cellular lipids with characteristics similar to vegetable oil and it also has a high growth rate and can be cultured in a single medium with low cost substrate [9]. Some oleaginous yeast strains, such as *Rhodosporidium* sp., *Rhodotorula* sp., can accumulate intracellular lipids to level exceeding 70% of their biomass under nutrient limitation condition [10]. Our recent study has proved that locally oleaginous yeast *Torulaspora maleeae* Y30 and *Torulaspora globosa* YU5/2 can grow well and accumulate lipid efficiently not only on glucose but also on sugarcane molasses and three major constituent fatty acids of lipids were palmitic acid, stearic acid, and oleic acid that are comparable to vegetable oils [11, 12].

However, to expand this novel feedstock, research and development is needed in several domains, from the selection of suitable strains to the optimization of production process as well as the low cost of cultivation process to obtain a large amount of biomass and lipid productivity. Many methods and techniques, such as the use of bioreactors, the heterotrophic and mixotrophic cultures of microalgae, the use of the inexpensive carbon substrates such as industrial wastes and the mixed culture of microorganisms, have been developed to reduce the costs of microbial oil production. Of these techniques, the mixed culture of microorganisms is a

convenient solution for achieving the goals. Mixed cultures of microorganisms are common in natural ecological systems. They are often used for the treatment of agro-industrial wastes, as well as for the production of biomass and bioactive compounds. When using a mixed culture, two or more preselected species of microorganism are synchronously cultivated within the same medium, where these microorganisms can mutually exploit complementary metabolic activities to survive, grow, and reproduce [13]. 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 [4].

Although the fact that growth is prompted when microalgae and oleaginous yeast are mixed culture in the same medium was confirmed in other fields [13, 14], little information for microbial oil preparation is available on mixed cultures of microalgae and yeast being used as biodiesel feedstock. Here, we have developed an approach for the production of microbial oils by mixed culture of microalgae *Chlorella* sp. KKU-S2 and the oleaginous yeasts *T. maleeae* Y30, *T. globosa* YU5/2 using sugarcane juice as carbon substrate, and compare them with those under monoculture or pure conditions.

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 cell mass production rate (g cells/L/d)
q_P : Specific lipid production rate (g lipid /g cells/d)
q_S : Specific substrate consumption rate (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)

II. Materials and Methods

A. Microorganisms and Culture Conditions

The microalgae *Chlorella* sp. KKU-S2 isolated from freshwater taken from pond in the area of Khon Kaen province, northeastern Thailand [6], was used for microbial oil production. The seed culture was pre-cultivated onto Bristol's medium supplemented with 20 g/L glucose at 30°C in an incubator shaker at a shaking speed of 150 rpm for 3 days and 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, $MgSO_4.7H_2O$ 75, and FeCl2 0.3, $MnSO_4.2H_2O$ 0.3, $ZnSO_4$ $7H_2O$ 0.2, H_3BO_3 0.2, $CuSO_4.5H_2O$ 0.06, and pH was adjusted to 6.0 before sterilization.

The oleaginous yeasts *T. maleeae* Y30 and *T. 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 [11, 12]. The seed cultures were cultivated onto Lipid accumulation (LA) medium supplemented with 20g/L glucose at 30°C in an incubator shaker at a shaking speed of 150 rpm for 1 day. The LA medium was consisted of (g/L): $(NH_4)_2SO_4$ 0.1, KH_2PO_4 0.4, $MgSO_4.7H_2O$ 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 was adjusted to 5.0 before sterilization.

B. Raw Materials

The raw material used in this study was sugarcane juice collected from a local market in Khon Kaen province, Northeastern Thailand. The pre-treated sugarcane juice was mixed with sulfuric acid for final concentration of 2% (v/v). The mixture was treated in water baht at 100°C for 20 min. After cooling, the liquid fraction or sugarcane juice hydrolysate (SJH) was separated by centrifugation in order to remove insoluble particles and stored at 4°C prior to use.

C. Microbial Oil Production by Pure and Mixed Cultures

Batch cultivations were performed in 1000mL Erlenmeyer flasks, each containing 500mL of medium supplemented with treated sugarcane juice or SJH, flasks were inoculated with 10% (v/v) seed culture of yeast or microalgae and cultivated at 30°C in rotary shaker set to 150 rpm under continuous illumination by using 80W cool-white fluorescent lamps. The experiments were performed in form of pure culture of each *T. maleeae* Y30, *T. globosa* YU5/2 and *Chlorella* sp. KKU-S2, mixed culture of *Chlorella* sp. KKU-S2 with *T. maleeae* Y30 and mixed culture of *Chlorella* sp. KKU-S2 with *T. globosa* YU5/2.

D. Analytical Methods

Duplicate samples were analyzed for cell dry weight, and residual glucose. The culture broth (5 mL) was centrifuged at 5,000 rpm for 5 min. The supernatant was analyzed for glucose concentration according to DNS method [15]. Harvested biomass was washed twice with 5 mL of distilled water and then dried at 90°C to constant weight. The biomass was determined gravimetrically. The total lipids were determined by the modified method of Know and Rhee [16].

E. Determination of Growth Kinetic

In fermentation, variables which are of great relevance to the economic evaluation of biotechnological processes are the cell yield on a substrate ($Y_{X/S}$), specific growth rate (μ), volumetric substrate consumption rate (Q_S), specific substrate consumption rate (q_s), product yield based on substrate ($Y_{P/S}$), specific product yield ($Y_{P/X}$) and volumetric product formation rate (Q_P). All these kinetic parameters have major technological importance in up scaling the fermentation process [17]. 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 (q_P) was a multiple of μ and $Y_{P/X}$.

III. Results and Discussion

The production of microbial oil via pure or monoculture and mixed cultures of the oleaginous yeasts *T. maleeae* Y30, *T. globosa* YU5/2 and microalgae *Chlorella* sp. KKU-S2 using SJH as carbon substrate by batch cultivations were investigated. Biomass, lipid yield and residue sugar of monoculture and mixed cultures are presented Fig.1 and Table 1. It is apparent that SJH referred to reducing sugar was used mainly for cell growth at the beginning of cell growth phase. The monoculture of *T. maleeae* Y30, *T. globosa* YU5/2 grew faster than that of microalgae *Chlorella* sp. KKU-S2.

In monoculture of yeast *T. maleeae* Y30, a biomass of 8.267g/L with specific growth rate of 0.352(1/d) and lipid yield of 0.920g/L were obtained, while 8.333g/L of biomass with specific growth rate of 0.353 (1/d) and lipid yield of 1.141g/L were obtained for monoculture of *T. globosa* YU5/2. A biomass of 1.933g/L with specific growth rate of 0.110(1/d) and lipid yield of 0.052g/L were found for monoculture of *Chlorella* sp. KKU-S2.

The biomass concentration in the mixed culture of the oleaginous yeast with microalgae *Chlorella* sp. KKU-S2 increased faster and was higher compared with that in the monocultures. A biomass of 8.733g/L with specific growth rate (μ) of 0.361 (1/d) and lipid yield of 1.564g/L were obtained for a mixed culture of yeast *T. maleeae* Y30 with *Chlorella* sp. KKU-S2, while 8.010g/L of biomass with specific growth rate of 0.347(1/d) and lipid yield of 2.424g/L were found for mixed culture of yeast *T. globosa* YU5/2 with microalgae *Chlorella* sp. KKU-S2.

Maximum cell yield coefficient ($Y_{X/S}$, g/L) was found of 0.323 in monoculture of microalgae *Chlorella* sp. KKU-S2 but with low level of both specific yield of lipid ($Y_{P/X}$, g lipid/g cells) of 0.027 and volumetric lipid production rate (Q_P, g/L/d) of 0.003 were observed. While, maximum $Y_{P/X}$, g lipid/g cells) and Q_P of 0.303 and 0.105 were obtained, respectively, in mixed culture of yeast *T. globosa* YU5/2 with microalgae *Chlorella* sp. KKU-S2.

The maximum process product yield ($Y_{P/S}$, 0.061) was obtained from mixed culture of yeast *T. globosa* YU5/2 with microalgae *Chlorella* sp. KKU-S2, followed by mixed culture of yeast *T. maleeae* Y30 with microalgae *Chlorella* sp. KKU-S2 ($Y_{P/S}$, 0.039), pure culture of *T. globosa* YU5/2 ($Y_{P/S}$, 0.034), pure culture of *T. maleeae* Y30 ($Y_{P/S}$, 0.026), and pure culture of *Chlorella* sp. KKU-S2 ($Y_{P/S}$, 0.009).

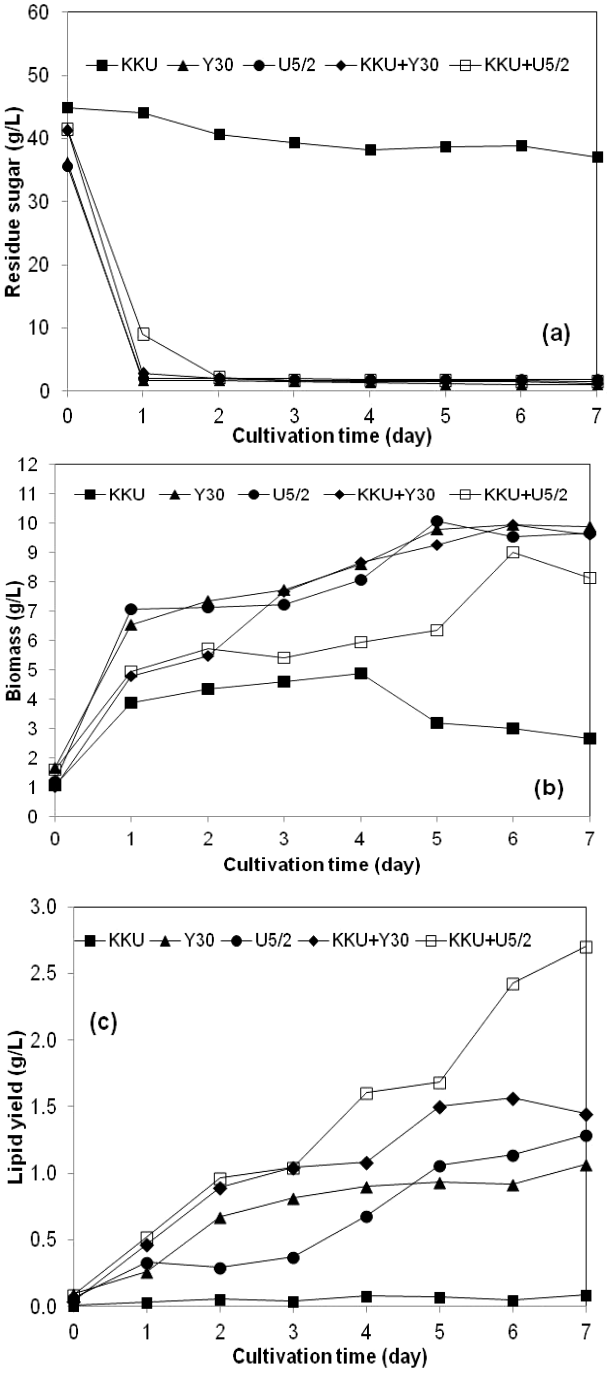

Fig. 1 Residue sugar (a), biomass concentration (b), lipid yield (c) during cultivation of pure and mixed cultures of *T. maleeae* Y30 (Y30), *T. globosa* YU5/2 (U5/2), *Chlorella* sp. KKU-S2 (KKU) using SJH as carbon substrate at 30°C, for 7 days

It is possible that the microalgae may function as an O_2 producer in the mixed culture and enhance the growth of yeast and the yeast produced CO_2 that could be used by the microalgae under photoautotrophic cultivation. In the mixed culture, the metabolic reactions of both CO_2 release and uptake were combined and complementary [18].

TABLE I
COMPARATIVE FERMENTATION KINETIC PARAMETERS OF BATCH CULTIVATION OF THE OLEAGINOUS YEASTS AND MICROALGAE ON CULTURE MEDIUM USING SUGARCANE JUICE AS CARBON SUBSTRATE AT 30°C

Kinetic parameters	Microbial cultures				
	Y30[1]	U5/2[2]	KKU[3]	KKU+Y30[4]	KKU+U5/2[5]
X	8.267	8.333	1.933	8.733	8.010
P	0.920	1.141	0.052	1.564	2.424
μ	0.352	0.353	0.110	0.361	0.347
Q_S	5.875	5.621	0.996	6.648	6.636
Q_P	0.153	0.190	0.009	0.261	0.404
Q_X	1.378	1.389	0.322	1.456	1.335
$Y_{X/S}$	0.235	0.247	0.323	0.219	0.201
$Y_{P/X}$	0.111	0.137	0.027	0.179	0.303
$Y_{P/S}$	0.026	0.034	0.009	0.039	0.061
q_S	0.711	0.675	0.515	0.761	0.829
q_P	0.039	0.048	0.003	0.065	0.105

[1] *Pure culture of T. maleeae* Y30,
[2] *Pure culture of T. globosa* YU5/2,
[3] *Pure culture of Chlorella* sp. KKU-S2
[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

Nannochloropsis oculata exhibited increases in biomass and lipid content when the CO_2 concentration supplied was increased [19]; similarly, *Chlorella kessleri* showed a particularly high potential for bio-fixation of CO_2 [20]. When oleaginous microorganisms are grown with an excess of carbon (inorganic and organic carbon) and limited quantity of nitrogen referred to low level of carbon to nitrogen (C/N) ratios, they may accumulate high concentration of cellular lipid. Cultivation of oleaginous microorganisms, with low nitrogen in the medium, results to the decrease of the activity of nicotinamide adenine dinucleotide isocitrate dehydrogenase (NADIDH) then the tricarboxylic acid cycle is repressed, metabolism pathway altered and protein synthesis stopped and lipid accumulation is activated [21]. Photosynthesis generates oxygen and dissolved oxygen levels much greater than the air saturation values inhibit photosynthesis [22]. Furthermore, a high concentration of dissolved oxygen in combination with intense light produces photooxidative damage to microalgal cells [23]. It is therefore desirable to remove CO_2 from the yeast fermentation broth and O_2 from the photosynthetic microalgae broth. The promotion effect on growth in mixed cultures can be attributed to sufficient *in situ* O_2 and CO_2 transitions, since the microalgae acted as an oxygen generator for the oleaginous yeast, while the oleaginous yeast produces CO_2 for the microalgae. As a result, the stresses caused by CO_2 on the yeast and O_2 on the microalgae were eliminated. Thus, the growth conditions were optimized for both species. Additionally, this sufficient *in situ* transition may maintain an O_2/CO_2 balance that enhances the photosynthesis of the microalgae [24]. The obtained result presented that mixed culture of yeast with microalgae is a desirable cultivation process for microbial oil production under mixotrophic cultivation. However, the process product yield ($Y_{P/S}$) obtained in batch fermentation by mixed culture of yeast with microalgae quite low, suggesting to difficult for up scaling of microbial lipid production by the microalgae due to high substrate consumption rate. To solve these phenomena, further fed-batch fermentation should investigated with initial nitrogen-rich medium to obtain high biomass or high cell density at the early stage of cell growth, then high concentration of carbon source will feed onto culture medium for stimulate the cellular lipid accumulation. Xiong et al. [25] reported that cell density of *Chlorella protothecoides* achieved was 16.8 g/L in a 5-L bioreactor for 184 h of cultivation time by performing fed-batch culture with lipid content of 50.3%cell dry weight using glucose as carbon source. Fed-batch fermentation modes have been widely applied for microbial lipid production. Although the research on the production of biodiesel from microbial oil is enormous, this system appears not economically viable in the current environment because it provides higher costs when comparing with conventional fuels but there are many methods to improve the low cost of microbial oil production processes. For example, the more economic carbon source should be employed to take the place of pure glucose or sucrose as substrate such as sweet potato, Jerusalem artichoke, sweet sorghum or other non-food agricultural products, agro-industrial waste residues, i.e. distillery slop [12, 26]. In addition, potential and realistic progress in transforming of lignocellulosic materials to fermentable carbon sources might provide an optimal way to reduce the cost of microbial oils production. Process engineering that leads to a higher lipid production rate and cellular lipid content may also contribute in this regard. Thus, to realize the large-scale production of biodiesel from microbial oils, it was necessary to obtain a large amount of biomass and lipid yield via optimal fermentation process such as mix cultivation, fed-batch fermentation as well as the low cost of cultivation process. The following conclusions can be drawn from this study; the oleaginous yeast *T. globosa* YU5/2 and microalgae *Chlorella* sp. KKU-S2 use sugarcane juice as organic nutrients efficiently in mixed culture under mixotrophic growth. The biomass productivity and lipid yield are notably enhanced in comparison with monoculture. To our knowledge this is the unique report about the production of microbial oil from *T. globosa* YU5/2 and *Chlorella* sp. KKU-S2 via mixed culture using sugarcane juice as substrate. In further works, increasing of biomass and lipid yield will be investigated in a 10L reactor via mix cultivation of mixotrophic microalgae with yeast fermentation using inexpensive raw materials by batch and fed-batch cultivation modes and fatty acids profile of mixed microbial lipid of yeast with microalgae will be studied, then completed with the production of biodiesel from microbial oil via one-step and two-step transesterification reactions.

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