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Microbial Oil Production by Mixed Culture of Microalgae *Chlorella* sp. KKU-S2 and Yeast *Torulaspora maleeae* Y30

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***Abstract*—**Compared to oil production from microorganisms, little work has been performed for mixed culture of microalgae and yeast. In this article it is aimed to show high oil accumulation potential of mixed culture of microalgae *Chlorella* sp. KKU-S2 and oleaginous yeast *Torulaspora maleeae* Y30 using sugarcane molasses as substrate. The monoculture of *T. maleeae* Y30 grew faster than that of microalgae *Chlorella* sp. KKU-S2. In monoculture of yeast, a biomass of 6.4g/L with specific growth rate (μ) of 0.265 (1/d) and lipid yield of 0.466g/L were obtained, while 2.53g/L of biomass with μ of 0.133 (1/d) and lipid yield of 0.132g/L were obtained for monoculture of *Chlorella* sp. KKU-S2. The biomass concentration in the mixed culture of *T. maleeae* Y30 with *Chlorella* sp. KKU-S2 increased faster and was higher compared with that in the monoculture and mixed culture of microalgae. In mixed culture of microalgae *Chlorella* sp. KKU-S2 and *C. vulgaris* TISTR8580, a biomass of 3.47g/L and lipid yield of 0.123 g/L were obtained. In mixed culture of *T. maleeae* Y30 with *Chlorella* sp. KKU-S2, a maximum biomass of 7.33 g/L and lipid yield of 0.808g/L were obtained. Maximum cell yield coefficient ($Y_{X/S}$, 0.229g/L), specific yield of lipid ($Y_{P/X}$, 0.11g lipid/g cells) and volumetric lipid production rate (Q_P, 0.115 g/L/d) were obtained in mixed culture of yeast and microalgae. Clearly, *T. maleeae* Y30 and *Chlorella* sp. KKU-S2 use sugarcane molasses as organic nutrients efficiently in mixed culture under mixotrophic growth. The biomass productivity and lipid yield are notably enhanced in comparison with monoculture.

***Keywords*—**Microbial oil, *Chlorella* sp. KKU-S2, *Chlorella vulgaris*, *Torulaspora maleeae* Y30, mixed culture, biodiesel.

I. Introduction

NOWADAYS, there has been an increasing interest in looking for new oil feedstock for biodiesel production especially non-food feedstock to avoid the food-fuel conflict. Microbial oils production through fermentation using oleaginous microorganisms involving yeasts, moulds, and microalgae, can be an alternative way to the traditional oil crop-based practice and are seen as non-food feedstock promising candidates for the industrial production of biodiesel because of their advantages of higher biomass production and faster growth compared to other energy crops [1]. Microalgae have the highest oil or lipid yield among various crop oils, and the compositions of oils are mainly triglyceride which is the right kind of oil for producing biodiesel [2]. Microalgae may assume many types of metabolisms, such as photoautotrophic, heterotrophic, mixotrophic and photoheterotrophic growths [3].

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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]. The most common microalgae such as *Chlorella*, *Nannochloropsis* sp. possess oil levels between 20% and 50%, along with interesting productivities; *Chlorella* appears in particular to be a good option for biodiesel production. Our study has proved that *Chlorella* sp. KKU-S2 can grow under hetertrophic and mixotrophic cultivations 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 for biodiesel production [4, 5].

In the last decade there is a great attention on oleaginous yeasts because some of them are capable of accumulating large amounts of lipids in their cells and the majority of these lipids are comparable to vegetable oils [6]. The locally isolated yeast *Torulaspora maleeae* Y30 is a kind of oleaginous yeast and our recent study has proved that *T. maleeae* Y30 can grow well and accumulate lipid efficiently not only on glucose but also on sugarcane molasses and three major constituent fatty acids of *T. maleeae* Y30 were palmitic acid, stearic acid, and oleic acid that are comparable to vegetable oils and could be used as potential feedstock for biodiesel production [7].

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. Four stages are involved in microalgal biodiesel production system include cultivation, dewatering, extraction and transesterification. Each of the stages requires high energy thus contributes in high production cost [8]. 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 [9]. 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 lipids [3]. Little information for microbial oil preparation is available on mixed cultures of microalgae and yeast being used as biodiesel feedstock. Therefore, the objective of this study is to investigate the growth performance and microbial oils production of mixed culture of oleaginous microorganisms, yeast and microalgae using sugarcane molasses as carbon substrate, the by-product of sugary refinery and compare them with those under monoculture conditions. To our knowledge this is the first report about the lipid accumulating properties of locally microalgae *Chlorella* sp. KKU-S2 and the oleaginous yeast *T. maleeae* Y30 cells in mixed culture using sugarcane molasses as carbon substrate.

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 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)

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 [4, 5], and *C. vulgaris* TISTR8580 obtained from the Thailand Institute of Scientific and Technological Research (TISTR) were used for microbial oil production. The seed culture was pre-cultivated in 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 was consisted of (mg/L): $NaNO_3$ 250, K_2HPO_4 75, KH_2PO_4 175, $CaCl_2$ 25, NaCl 25, $MgSO_4.7H_2O$ 75, $FeCl_2$ 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 yeast *T. maleeae* Y30 used in this study was isolated from soil samples taken from forest in the area of Chulabhorn Dam, Chaiyapoom Province Northeastern of Thailand [7]. *T. maleeae* Y30 was maintained on YM agar slant. 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 molasses collected from a local market in Khon Kaen province, Northeastern Thailand. The pre-treated sugarcane molasses 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 was separated by centrifugation in order to remove insoluble particles and stored at 4°C prior to use.

C. Microbial Oil Production

Batch cultivations were performed in 500mL Erlenmeyer flasks, each containing 200mL of medium supplemented with treated sugarcane molasses, 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 monoculture of each *T. maleeae* Y30 and *Chlorella* sp. KKU-S2, mixed culture of *T. maleeae* Y30 with *Chlorella* sp. KKU-S2 and mixed culture of *Chlorella* sp. KKU-S2 with *C. vulgaris* TISTR8580. All the experiments were carried out in duplicate.

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. 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 [10].

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 [11]. 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 while specific rate of lipid production (q_P) was a multiple of μ and $Y_{P/X}$.

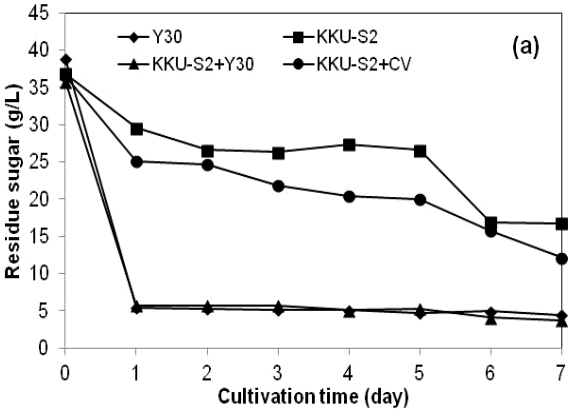
III. Results and Discussion

The comparison of microbial oil production by monoculture and mixed cultures of oleaginous yeast *T. maleeae* Y30, microalgae *Chlorella* sp. KKU-S2 and *C. vulgaris* TISTR8580 using sugarcane molasses as carbon substrate by batch cultivations were investigated. Biomass, lipid yield, residue sugar and pH of medium of pure and mixed cultures are

presented Fig.1 and Table 1. It is apparent that sugarcane molasses referred to reducing residue sugar was used mainly for cell growth at the beginning of cell growth phase. The monoculture of *T. maleeae* Y30 grew faster than that of microalgae *Chlorella* sp. KKU-S2. In monoculture of yeast, a biomass of 6.4g/L with specific growth rate of 0.265 (1/d) and lipid yield of 0.466g/L were obtained, while 2.53g/L of biomass with specific growth rate of 0.133 (1/d) and lipid yield of 0.132g/L were obtained for monoculture of microalgae *Chlorella* sp. KKU-S2.

The biomass concentration in the mixed culture of *T. maleeae* Y30 with *Chlorella* sp. KKU-S2 increased faster and was higher compared with that in the monocultures and mixed culture of microalgae *Chlorella* sp. KKU-S2 with *C. vulgaris* TISTR8580. In mixed culture of microalgae *Chlorella* sp. KKU-S2 and *C. vulgaris* TISTR8580, a biomass of 3.47g/L and lipid yield of 0.123 g/L with specific growth rate of 0.178 (1/d) were obtained. In mixed culture of *T. maleeae* Y30 with *Chlorella* sp. KKU-S2, a maximum biomass and lipid yield of 7.33 g/L and 0.808g/L with specific growth rate (μ, 1/d) of 0.285 were obtained. Cultivation of monoculture of *Chlorella* sp. KKU-S2 showed low growth with a biomass of 2.53g/L with specific growth rate (μ, 1/d) of 0.133.

Maximum cell yield coefficient ($Y_{X/S}$, g/L) was found of 0.229 in mixed culture of yeast with microalgae. The maximum specific yield of lipid ($Y_{P/X}$, g lipid/gcells), volumetric lipid production rate (Q_P, g/L/d) of 0.11 and 0.115 were obtained, respectively. 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 [12]. Photosynthesis generates oxygen. Dissolved oxygen (DO) levels much greater than the air saturation values inhibit photosynthesis [13]. Furthermore, a high concentration of DO in combination with intense light produces photooxidative damage to microalgal cells [14]. To prevent inhibition and damage, the maximum tolerable DO level should not generally exceed about 40% of air saturation value [13]. It is therefore desirable to remove CO_2 from the yeast fermentation broth and O_2 from the photosynthetic microalgae broth via mixed culture of these two species.



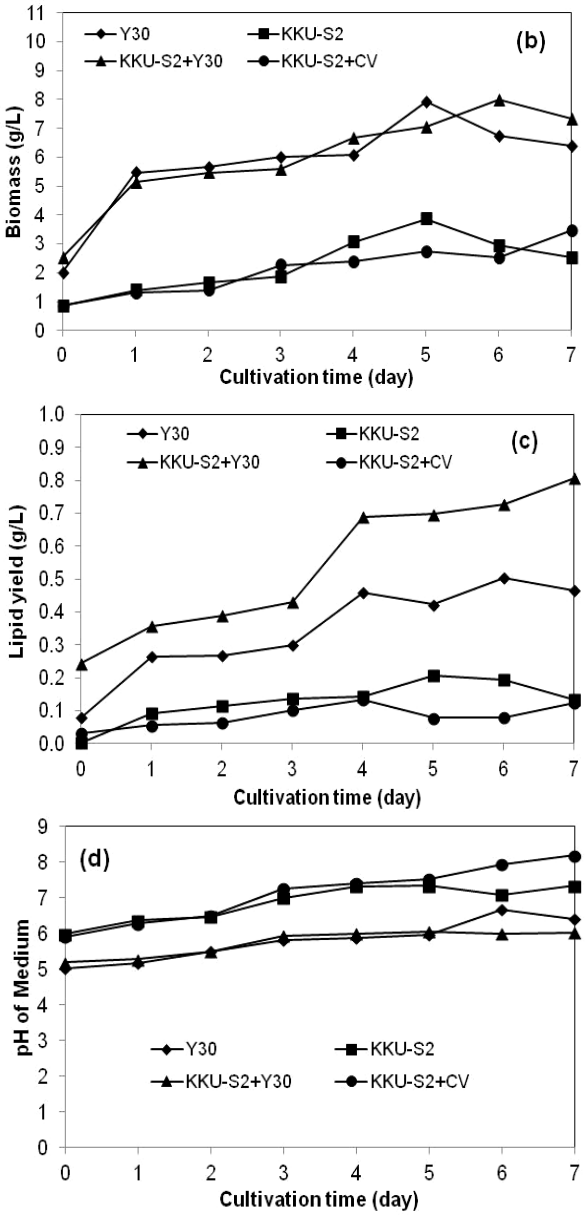


Fig. 1 Residue sugar (a), biomass concentration (b), lipid yield (c) and pH of medium (d) during cultivation of pure and mixed cultures of *T. maleeae* Y30 (Y30), *Chlorella* sp. KKU-S2 (KKU-S2) and *C. vulgaris* TISTR8580 (CV) using sugarcane molasses as carbon substrate at 30°C, for 7 days

Dong and Zhao reported that 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 alleviated. 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 [15]. The cultivation of microalgae, pH of medium increased from 5.9 up to 8.1 for mixed culture and from 5.9 up to 7.4 for monoculture. When CO_2 dissolves in water at neutral pH, bicarbonate (HCO^{3-}) is

TABLE I
COMPARATIVE FERMENTATION KINETIC PARAMETERS OF BATCH CULTIVATION OF YEAST AND MICROALGAE USING SUGARCANE MOLASSES AS CARBON SUBSTRATE

Kinetic parameters	Microbial cultures			
	Y30[1]	KKU-S2[2]	Y30+ KKU-S2[3]	KKU-S2 +CV[4]
X	6.40	2.53	7.33	3.47
P	0.466	0.132	0.808	0.123
μ	0.265	0.133	0.285	0.178
Q_S	4.916	2.870	4.581	3.487
Q_X	0.914	0.361	1.048	0.495
Q_P	0.067	0.019	0.115	0.018
$Y_{X/S}$	0.186	0.126	0.229	0.142
$Y_{P/X}$	0.073	0.052	0.110	0.036
$Y_{P/S}$	0.014	0.007	0.025	0.005
q_S	0.768	1.134	0.625	1.006
q_P	0.019	0.007	0.031	0.006

[1] *Monoculture of T. maleeae* Y30,
[2] *Monoculture of Chlorella* sp. KKU-S2,
[3] *Mixed culture of T. maleeae* Y30 with *Chlorella* sp. KKU-S2,
[4] *Mixed culture of Chlorella* sp. KKU-S2 with *C. vulgaris* TISTR8580

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 [16].

The maximum process product yield ($Y_{P/S}$, 0.025) was obtained from mixed culture of yeast *T. maleeae* Y30 with microalgae *Chlorella* sp. KKU-S2, followed by *T. maleeae* Y30 ($Y_{P/S}$, 0.014), *Chlorella* sp. KKU-S2 ($Y_{P/S}$, 0.007), and mixed culture of microalgae *Chlorella* sp. KKU-S2 with *C. vulgaris* TISTR8580 ($Y_{P/S}$, 0.005). 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 lipid production by 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. Fed-batch fermentation modes have been widely applied for microbial lipid production [17]. The experimental obtained results suggested that microbial oil production from mixed culture can be performed with lower cost production process using sugarcane molasses as carbon substrate. In conclusion, this mixed culture of the oleaginous yeast *T. maleeae* Y30 and microalgae *Chlorella* sp. KKU-S2 strategy led to significant improvements in growth, biomass concentration and lipid yield, which means that more biomass will be achieved in a given time when compared with the biomass achieved in monocultures. In further works, up-scaling of microbial oil production will be investigated via mixed culture of microalgae with yeast and completed with the production of biodiesel from microbial oil via direct and indirect transesterification methods.

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