Production of Biodiesel Feedstock by Integrated Growth of Isolated Oleaginous Yeast and Microalgae

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***Abstract*—The oleaginous microalgae and yeasts have been suggested as a promising biodiesel feedstock because of their potential for lipid production under various mode of cultivation. In this study, microbial biomass and lipid production to use as biodiesel feedstock by microalgae *Chlorella* sp. KKU-S2 and yeast *Torulasporaglobosa*YU5/2 via integrated cultivation technique using CO_2 emissions from yeast cultivation as inorganic carbon source for mixotrophic growth of microalgae were investigated. A maximum specific growth rate of *Chlorella* sp. KKU-S2 of 0.380(1/d) was obtained via integrated growth using CO_2 emissions from yeast *T.globosa*YU5/2 grown on molasses while specific growth rate of 0.219(1/d) was found via non-integrated growth. A high value of lipid productivity (Q_P, 0.338 g/L/d), specific product yield ($Y_{P/X}$, 0.202), biomass productivity (Q_X, 1.633g/L/d) of *Chlorella* sp. KKU-S2 were found by integrated growth with *T.globosa*YU5/2 cultivation on molasses, overall biomass and lipid yield of 17.71g/L and 2.89g/L was obtained, respectively. To our knowledge there are a few reports about the microbial biomass and microbial lipid production from isolated microalgae *Chlorella* sp. KKU-S2 and isolated yeast *T.globosa*YU5/2in an integrated technique by using CO_2 emissions from yeast cultivation.**

***Index Terms*—Microbial lipid, biodiesel feedstock, microalgae, oleaginous yeast, integrated cultivation.**

I. Introduction

Microbial oils, lipid produced from oleaginous microorganisms involving yeasts, moulds, and microalgae, which have ability to accumulate lipids over 20% of their biomass, are considered as the third generation of biodiesel feedstock due to some advantages such as short production period, higher biomass production and faster growth compared to other energy crops, easiness to scale up [1], [2]. Microalgae have the highest oil or lipid yield among various plant oils, and the lipid content of some microalgae has up to 80% and the compositions of microalgallipids are mainly triglyceride which is the right kind of oil for producing biodiesel [2].

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Microalgae may assume many types of metabolisms, such as photoautotrophic, heterotrophic and mixotrophic growths [3]. In photoautotrophic growth, the sole energy source for biomass production is light energy and the sole carbon source is inorganic compounds especially carbon dioxide (CO_2).

Compared to autotrophic cultivation, heterotrophic and mixotrophic cultivations allow some microalgae to provide high biomass and lipid productivity [4]-[6] Mixotrophic growth of microalgae have an edge over photoautotrophic growth as they have two energy sources as organic carbon source and light, they can simultaneously drive photoautotrophic and heterotrophic to utilize both inorganic (as CO_2) and organic carbon substrates, therefore, microalgae cultivated under mixotrophic culture synthesize compounds characteristic at high production rates of both photosynthetic and heterotrophic metabolisms of organic substrates are independent of each other [3], [7]. It was reported that only few microalgae can be cultivated mixotrophically such as *Haematococcuspluvialis*, *Chlorellaprotothecoides*, *Chlorella* sp. KKU-S2 [8], [9].

CO_2 as a nutrient represents one of the most costly components in the cultivation of microalgae. Therefore a system that couples a waste CO_2 source with the cultivation of CO_2 fixing microalgae can not only reduce cultivation costs but also mitigate or remove CO_2, greenhouse gas (GHG) as an environmental pollution. Waste CO_2 can be provided by using CO_2 emissions from agro-industrial plants such as the ethanol fermentation by yeast [10].

Oleaginous yeasts are capable of accumulating large amounts of cellular lipids and it also has a high growth rate and some strains can accumulate intracellular lipids to level exceeding 70% of their biomass under nutrient limitation condition [11]. Lipid production from yeast cultivation produces CO_2 which can be provided for mixotrophic growth of microalgae by using an integrated culture technique that incorporates both CO_2 consumption and microbial lipid production appear to be the best approach to enable industrial application of these new technologies for environmental benefit. Therefore, the main aim of this study is to investigate the production of microbial lipid via integrated growth of yeast *T. globosa*YU5/2 and microalgae *Chlorella* sp. KKU-S2.

II. Materials and Methods

A. Carbon Substrate, Microorganisms and Culture Conditions

The carbon substrates used in this study were sugarcane juice (S), sugarcane molasses (M) and sweet potato tubers collected from a local market in Khon Kaen province, Thailand. The sweet potato powder was mixed with

hydrochloric acid for final concentration of 2% (v/v), and then treated in water baht at 100°C for 20 min. After cooling, the liquid fraction as sweet potato hydrolysate (P) was separated by centrifugation to remove insoluble particles then neutralized by adding 5N NaOH and stored at 4°C prior to use.

The oleaginous yeasts *Torulasporaglobosa*YU5/2used in this study was isolated from soil samples taken from forest in the area of Chaiyapoom Province, Thailand [12]. Active cultures for inoculation were obtained in 500mL Erlenmeyer flasks with 200mL of growth medium containing 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract. The seed cultures were cultivated onto yeast medium supplemented with 20g/L glucose at 30 ℃ in rotary shaker at 150 rpm for 1 day. The yeast 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, initial pH was adjusted to 5.0-5.5.

The microalgae *Chlorella* sp. KKU-S2 used in this study was isolated from freshwater taken from pond in the area of Khon Kaen province, Thailand [7]. Active cultures for inoculation were obtained in 500mL Erlenmeyer flasks with 200mL of Bristol's medium supplemented with 20 g/L glucose. The seed culture was cultivated onto Bristol's medium containing 20g/L glucose at 30 ℃ in rotary shaker at 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, 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 initial pH was adjusted to 6.0-6.5.

B. Microbial Oil Production by Integrated Cultivation Technique for Lipid Production

Cultivation of each strain was performed in 4000mL Erlenmeyer flask with a working volume of 2000mL. Yeast *T. globosa*YU5/2 was cultivated onto LA medium containing of different types of carbon substrate as sugarcane juice (S), sugarcane molasses (M) and sweet potato hydrolysate (P) with 20g/L of reducing sugar and 1.0g/L yeast extract as nitrogen source at ambient temperature under continuous agitation. The microalgae *Chlorella* sp. KKU-S2 was cultivated onto Bristol's medium containing 20g/L glucose and 1.0g/L$NaNO_3$ as nitrogen source with 10% (v/v) seed culture of each strain and incubated at ambient temperature under continuous illumination by using 80W cool-white fluorescent lamps. The CO_2 produced by the yeast cultivation is split and connected directly into the surrounding microalgae flask for mixotrophic growth (Fig. 1). To comparison of growth and lipid production, cultivation of microalgae was investigated without the addition of CO_2 emissions from yeast *T. globosa*YU5/2 cultivation.

C. Analytical Methods

Cell growth of *Chlorella* sp. KKU-S2 and *T. globosa*YU5/2 were determined by optical density reading at 680 nm (OD_{680}) and 660 nm (OD_{660}), respectively. A standard curve was prepared by plotting dry cell weight (DCW) values (g/L) against corresponding optical density (OD_{680}) readings by using spectrophotometer.

A linear regression fit was obtained for dry cell weight (DCW) of *Chlorella* sp. KKU-S2 as a function of OD_{680}, $y = 1.9039x + 2.2149$, $R^2 = 0.9964$, and that linear regression fit of *T. globosa*YU5/2 as a function of OD_{660}, $y = 2.9886x+0.5627$, $R^2 = 0.9948$. There was a direct correlation between optical density and dry cell weight.

The culture broth was centrifuged at 5,000 rpm for 5 min then the supernatant was analyzed for reducing sugar concentration according to DNS method [13]. Cellular lipids were determined by the modified method of Kwon and Rhee [14]. Biomass and Lipid productivities were calculated.

Biomass productivity (Q_X, g/L/d) during the culture period was calculated; $Q_X = (X_t-X_0) / (t_t-t_0)$, where X_t was the biomass concentration (g/L) at the end of growth phase (t_t) and X_0 the initial biomass concentration (g/L) at t_0 (day).

Lipid productivity (Q_P, g/L/d) at the end of cultivation was calculated: $Q_P = (P_t-P_0) / (t_t-t_0)$, where P_t was the lipid yield (g/L) at the end of growth phase (t_t) and P_0 the initial lipid yield (g/L) at t_0 (day). Specific product yield ($Y_{P/X}$) was determined using relationship dP/dX.

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 $Y_{P/X}$.

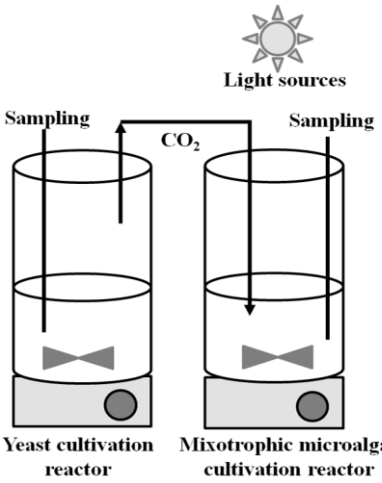


Fig. 1. Simplified schematic of yeast *T. globosa*YU5/2 cultivation and mixotrophic microalgae *Chlorella* sp. KKU-S2 cultivation for microbial biomass and lipid production, cultivated at ambient temperature under continuous illuminated with 80W cool-white fluorescent lamps.

III. Results and Discussion

Batch cultivation of yeast *T. globosa*YU5/2 was cultivated on culture medium supplemented with different types of carbon substrate as sugarcane juice (S), sugarcane molasses (M) and sweet potato hydrolysate (P) with initial reducing sugar of 20g/L. After cultivation for 6 days, a biomass and lipid yield reached the maximum of 8.08g/L with biomass productivity of 1.347g/L/d and 0.970g/L with lipid productivity of 0.153g/L/d were obtained from yeast *T. globosa*YU5/2 grown on sugarcane juice (S) (Fig. 2 and Table I). Waste CO_2 produced by the cultivation of yeast *T. globosa*YU5/2 during lipid production, is connected directly into the surrounding microalgae flask and combined with ambient air for mixotrophic growth of *Chlorella* sp. KKU-S2.

As shown in Fig. 3, there are significant different of optical density (OD_{680}) changes observed in the growth of microalgae during cell growth with different sources of CO_2, higher value of OD_{680} of 22.80 was obtained by cultivation of microalgae by integrated growth with couple-CO_2 emissions from yeast cultivation on molasses for 6days than that of the cultivation by integrated with yeast cultivation on sugarcane juice and sweet potato hydrolysate. The OD_{680} of 8.65 was obtained by non-integrated growth of *Chlorella* sp. KKU-S2.

A maximum biomass and lipid yield of *Chlorella* sp. KKU-S2 was obtained via integrated growth using CO_2 emissions from yeast grown on molasses (KKU-S2 (M)), a biomass of 9.80g/L with biomass productivity of 1.633g/L/d and lipid yield of 1.980g/L with lipid productivity 0.633g/L/d, while a biomass of 3.72g/L with biomass productivity of was found via non-integrated growth of *Chlorella* sp. KKU-S2 (Table I).

In case of integrated cultivation process, overall lipid yield of 2.89 g/L was obtained as 0.910g/L of *T. globosa*YU5/2 and 1.980g/L of *Chlorella* sp. KKU-S2, while only 0.410g/L of lipid yield was found from *Chlorella* sp. KKU-S2 via non-integrated growth technique. The integration of the mixotrophic microalgae cultivation systems into an existing yeast cultivation system is made economically feasible by the production of two new revenue streams as microbial lipid from microalgae and oleaginous yeast for used as biodiesel feedstock and the capture of CO_2 emissions from the yeast cultivation stage [7], [10].

TABLE I: KINETIC PARAMETERS OF INTEGRATED GROWTH OF *T. GLOBOSA* YU5/2 ON LIPID ACCUMULATION MEDIUM SUPPLEMENTED WITH MOLASSES (M), SWEET POTATO HYDROLYSATE (P) AND SUGARCANE JUICE (S) AND CHLORELLA SP. KKU+S2 ON BRISTOL'S MEDIUM SUPPLEMENTED WITH GLUCOSE AT DAY 6 OF CULTIVATION TIME AT AMBIENT TEMPERATURE, 150 RPM.

Culture conditions	Kinetic parameters						
	X (g/L)	P (g/L)	Q_X (g/L d)	Q_P (g/L d)	μ (1/d)	$Y_{P/X}$	q_P
T. globosa YU5/2 (YU52 M)	7.91	0.910	1.318	0.152	0.345	0.115	0.040
T. globosa YU5/2 (YU52 P)	8.08	0.970	1.347	0.162	0.348	0.146	0.051
T. globosa YU5/2 (YU52 S)	3.81	0.410	0.635	0.068	0.223	0.108	0.024
Chlorella sp. KKU-S2-integrated with YU52 (M)	9.80	1.980	1.633	0.330	0.380	0.202	0.077
Chlorella sp. KKU-S2-integrated with YU52 (P)	5.33	0.780	0.888	0.130	0.279	0.120	0.033
Chlorella sp. KKU-S2-integrated with YU52 (S)	5.85	0.920	0.975	0.153	0.294	0.157	0.046
Chlorella sp. KKU-S2 (non-integrated, control)	3.72	0.410	0.620	0.068	0.219	0.110	0.024

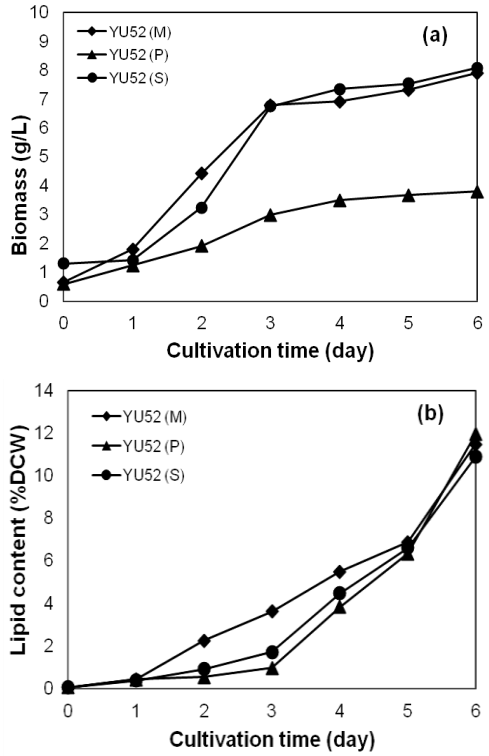
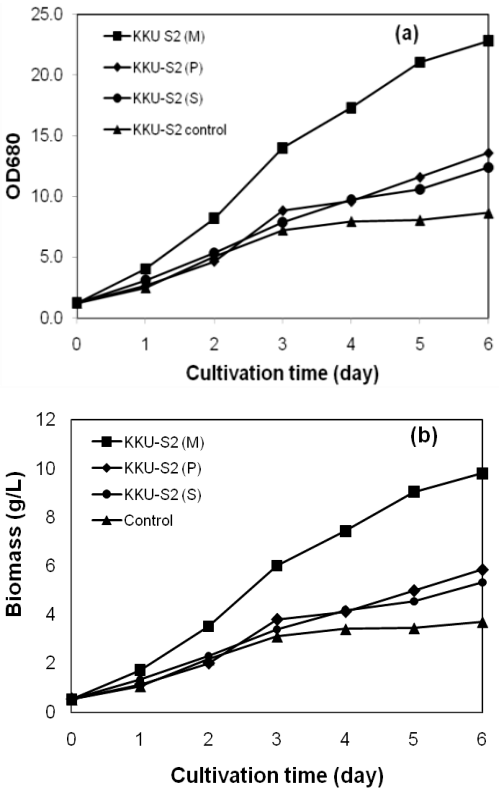


Fig. 2. Biomass yield (a) and lipid yield (b) during cultivation of *T.globosa*YU5/2 on lipid accumulation medium supplemented with molasses (M), sweet potato hydrolysate (P) and sugarcane juice (S) as carbon substrate with 20g/L reducing sugar, incubated at ambient temperature, 6 days.

There are significant different of lipid productivity (Q_P), specific product yields ($Y_{P/X}$), biomass productivity (Q_X) and specific rate of lipid production (q_P) by using integrated growth techniques. A high value of all parameters was found when using CO_2 emissions from yeast cultivation for supported the growth and lipid production of microalgae *Chlorella* sp. KKU-S2. Similarly, *Scenedesmusobliquus* and *Chlorella kessleri* showed a particularly high potential for bio-fixation of CO_2 [15].



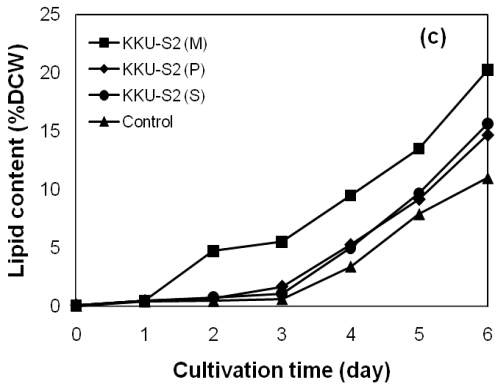


Fig. 3. Optical density (OD680) (a), biomass yield (b) and lipid yield (c) of *Chlorella* sp. KKU-S2 on Bristol's medium containing 20g/L glucose under mixotrophic cultivation via integrated growth by using CO_2 emitted from *T. globosa*YU5/2 cultivation with molasses (KKU-S2(M)), sweet potato hydrolysate (KKU-S2(P)) and sugarcane juice (KKU-S2(S)) as carbon substrate and non-integrated growth without CO_2 coupled from CO_2 emitted from yeast (control), incubated at ambient temperature, 6 days.

IV. Conclusions

In conclusion, we present an integrated growth and microbial oil production of yeast and microalgae. To our knowledge this is the unique report about the microbial lipid production from isolated microalgae *Chlorella* sp. KKU-S2 under mixotrophic growth and oleaginous yeast *T. globosa*YU5/2in an integrated technique to improve the biomass and lipid productivity by using CO_2 emissions from yeast cultivation suggesting to reduce cultivation costs and also remove and value-added of CO_2, greenhouse gas, this process could be so called that environmental friendly process. This cultivation method will open new perspectives in the production of microbial biomass and microbial oil which could be used as potential feedstock for biodiesel production. In further works, conversion of oil contained-microbial biomass to biodiesel as fatty acid methyl ester (FAME) by direct transesterification, characteristics and quality of FAME will be investigated.

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