Conversion of Fermented Rice Noodle Wastewater to Microbial Lipid by Mixed Culture of Microalgae and Yeast

Mutiyaporn Puangbut, Suthasinee Rattanachan, Thidarat Papone, and Ratanaporn Leesing

***Abstract*—Microbial lipids are known as the third generation of biodiesel feedstock. Compared to microbial lipids production, little work has been performed for mixed culture of the oleaginous microalgae and yeasts. In this study, mixed culture of microalgae *Chlorella* sp. KKU-S2 and yeast *Toluraspora maleeae* Y30 using fermented rice noodle wastewater hydrolysate (FRNWH) as carbon substrate were investigated under mixotrophic growth for 6 days. Comparison of growth on FRNWH using yeast extract as nitrogen source, 2.71g/L biomass with lipid yield of 117.3mg/L, 2.32g/L biomass with lipid yield of 72mg/L, 2.02g/L biomass with lipid yield of 150.4mg/L were obtained from monoculture of *T. maleeae* Y30, *Chlorella* sp. KKU-S2 and mixed culture of both strains, respectively. Effect of nitrogen source on growth and lipid yield of mixed culture was investigated. Meat extract supported the maximum biomass of 5.32g/L with biomass productivity of 0.89g/L/d and specific growth rate of 0.28 (1/d), while urea supported the maximum lipid yield of 199.0 mg/L with lipid productivity of 33.17mg/L/d. To our knowledge this is the unique report about the microbial lipid production from mixed culture of isolated microalgae and yeast using fermented rice noodle wastewater as carbon substrate under mixotrophic growth.**

***Index Terms*—Biodiesel feedstock, microbial lipid, mixed culture, mixotrophic growth.**

I. Introduction

Microbial oils, lipid produced from many oleaginous microorganisms involving yeasts, moulds, and microalgae, are known as the third generation of biodiesel feedstock because of their similar fatty acid composition to that of vegetable oils and offer a unique alternative as it does not compete with agricultural food production [1]. Microalgae may assume many types of metabolisms, such as photoautotrophic, heterotrophic and mixotrophic growths [2]. Photoautotrophic growth assimilate CO_2 and light as carbon and energy sources, respectively, while, heterotrophic growth of microalgae involves the utilization of organic compounds as sole carbon and energy sources under dark condition that could get rid of the dependence on light [3]. Mixotrophic growths of microalgae have an edge over photoautotrophic cultures as they have two energy sources as organic carbon source and light, they can simultaneously drive photoautotrophic and heterotrophic to utilize both inorganic (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 [4], [5].

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 [1], [6]. 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 [7].

Oleaginous yeasts are capable of accumulating large amounts of cellular lipids and it also has a high growth rate and can be cultured in a single medium with low cost substrate and some oleaginous yeast strains can accumulate intracellular lipids to level exceeding 70% of dry biomass [8]. The isolated yeast *Torulaspora maleeae* Y30 can accumulate high lipid yield and their fatty acids profiles were palmitic acid, stearic acid, and oleic acid that are comparable to vegetable oils [9].

Mixed cultures of microorganisms are common in natural ecological systems. When using a mixed culture, two or more selected species of microorganisms are synchronously cultivated within the same medium, where these microorganisms can mutually exploit complementary metabolic activities to survive, grow, and reproduce [10]. In the mixed culture of yeast and microalgae, under mixotrophic culture, microalgae could act as an oxygen generator from photosynthetic growth for the yeast while the yeast provided CO_2 to microalgae and both carried out production of microbial oils [11], [12].

The high cost of microbial-derived biodiesel production is mainly contributed by microbial cultivation and downstream process is essential step as feedstock takes up to 70% of the overall cost [13], [14]. Glucose is most commonly organic carbon substrate used for sustaining microalgae growing under heterotrophic and mixotrophic cultures of several microalgal species reaching high biomass and lipids productivity [15]-[17], thus the use of this substrate result to high production costs of microbial lipid, then, the cost-effective carbon substrate containing sugar is needed for producing of microbial lipids. Fermented rice noodle wastewater from the typically local factory containing high

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concentration of organic carbon as sugar and starch has been generated during production process and was discharged to receiving water without treatment in many cases [18]. If microalgae could grow in fermented rice noodle wastewater, the organic carbon in wastewater would be reused and the cost of microalgae cultivation would be reduced. Therefore, the aim of this study is to produce microbial lipid from mixed culture of microalgae and yeast by using fermented rice noodle wastewater as carbon substrate and to confirm that mixed culture of both microorganisms could significantly enhance biomass and lipid production.

II. Materials and Methods

A. Carbon Substrate and Microorganisms

The carbon substrate used in this study was fermented rice noodles wastewater (FRNW) collected from a local factory in Khon Kaen province, Thailand. The pre-treated FRNW was mixed with sulfuric acid for final concentration of 1% (v/v), pH 3.0-4.0. The mixture was treated in water baht at 100°C for 40 min, the liquid fraction as fermented rice noodle wastewater hydrolysate (FRNWH) was separated by centrifugation to remove insoluble particles and neutralized by adding 5N NaOH, then stored at 4°C prior to use. The reducing sugar of FRNWH was analyzed by DNS method and adjusted the reducing sugar to obtain desirable concentration for used as the carbon substrate.

The microalgae *Chlorella* sp. KKU-S2 used in this study was isolated from freshwater [7]. Active microalgae cultures for inoculation were obtained in 250mL Erlenmeyer flasks with 100mL of Bristol's medium supplemented with 20 g/L glucose. The seed culture was grown in Bristol's medium supplemented with glucose or FRNWH as carbon substrate at 30 ℃ in an incubator rotary shaker at 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, $MgSO_4.7H_2O$ 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.

The oleaginous yeast *Toluraspora maleeae* Y30 used in this study was isolated from forest soil samples [9]. Active yeast cultures for inoculation were obtained in 250mL Erlenmeyer flasks with 100mL of growth medium containing 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract. The seed culture was cultivated in lipid accumulation (LA) medium supplemented with glucose or FRNWH as carbon substrate at 30 ℃ in an incubator rotary shaker at 150 rpm for 2 days. 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 initial pH was adjusted to 5.0-5.5.

B. Lipid Production

Batch cultures were performed in 250mL Erlenmeyer flasks, each containing 100mL of medium supplemented with 15g/L FRNWH (refer to reducing sugar) or glucose, initial inoculums were 3% yeast and 7% microalgae (v/v). The flasks were placed in an incubator rotary shaker at 150 rpm at 30 ℃ under continuous illuminated from overhead by 80W cool-white fluorescent lamps for 6 days. The experiments were performed in form of monoculture of each strains and mixed culture of *Chlorella* sp. KKU-S2 with *T. maleeae* Y30. Lipid production of mixed culture while growth occurs on FRNWH was tested with different types of nitrogen sources. Periodic samples were taken from the flasks to determine the cell biomass and lipid yields, which were then used to calculate the biomass and lipid productivities.

C. Measurement of Cell Growth, Residual Sugar and Lipid Yields

The culture broth was centrifuged at 5,000 rpm for 5 min then the supernatant was analyzed for residual glucose concentration according to DNS method [19]. Harvested biomass was washed twice with distilled water and then dried at 90 ℃ to constant weight. The biomass was determined gravimetrically. Cellular lipids were determined by the modified method of Kwon and Rhee [20].

Biomass productivity (g/L/d) during the culture period was calculated from the Eq. (1), where X_t was the biomass yield (g/L) at the end of growth phase (t_t) and X_0 the initial biomass yield (g/L) at t_0 (day),

$$\text{Biomass productivity} = (X_t\text{-}X_0) / (t_t\text{-}t_0) \quad (1)$$

Lipid productivity (QX, g/L/d) at the end of cultivation was calculated from the Eq. (2), 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),

$$\text{Lipid productivity} = (P_t\text{-}P_0)/ (t_t\text{-}t_0) \quad (2)$$

Process product yield ($Y_{P/S}$) at the end of cultivation was calculated from Eq. (3), where P was lipid yield (g/L) and S (g/L) was consumed carbon substrate,

$$Y_{P/S} = \text{lipid yield/consumed carbon substrate} \quad (3)$$

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 $Y_{P/X}$. The specific product yield ($Y_{P/X}$) was determined using relationship dP/dX, while specific cell yield ($Y_{X/S}$) was determined using relationship dX/dS.

D. Abbreviations

P: Lipid concentration (mg/L), Q_P: Volumetric lipid productivity (mg/L/d), Q_X: Volumetric biomass productivity (g cells/L/d), q_p: Specific rate of lipid production (mg lipid /g cells/d), μ: Specific growth rate coefficient (1/d).

q_s: specific rate of substrate consumption (g substrate/g cells/d), X: Biomass concentration (g/L), $Y_{P/S}$: Process product yield (mg lipid/g substrate), $Y_{P/X}$: Specific product (lipid) yield (mg lipid/g cells), $Y_{X/S}$: specific ell yield (g cells/g substrate).

III. Results and Discussion

A. Effect of Carbon Source on Growth and Lipid Production from Mono- and Mixed Culture

So far, glucose, cassava starch and Jerusalem artichoke

hydrolysate have been used as the carbon substrate for lipid production by oleaginous microorganisms [21], [22]. Fermented rice noodle wastewater containing sugar and starch was successfully applied for the cultivation of yeast and mixotrophic growth of microalgae. Thus, affect of carbon source on growth and lipid production of mixed cultures of yeast *T. maleeae* Y30 and microalgae *Chlorella* sp. KKU-S2 using yeast extract as nitrogen source were preliminary investigated.

As shown in Fig. 1 and Table I, the biomass and lipid yield of mixed culture grow on glucose was higher than that of mixed culture grow on FRNWH. It was observed that biomass of monoculture of yeast and microalgae were higher than that of mixed culture when cultivated on medium supplemented with FRNWH as carbon substrate. The monoculture of *T. maleeae* Y30 grew faster than that of microalgae *Chlorella* sp. KKU-S2.

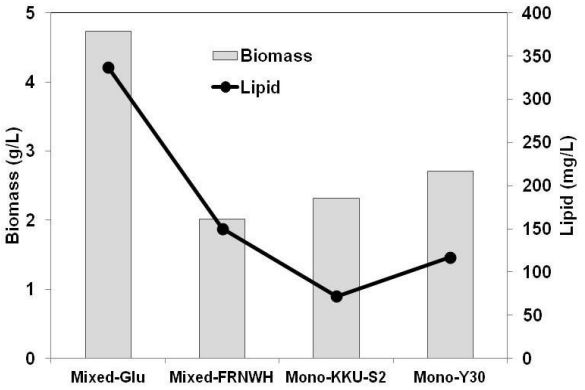

Fig. 1. Biomass and lipid yield during growth of mixed culture and monoculture of *T. maleeae* Y30 and *Chlorella* sp. KKU-S2 on culture medium with different culture modes, incubated at 30°C, 150 rpm for 6 days; Mixed-Glu: mixed culture on glucose, Mixed-FRNWH: mixed culture on FRNWH, Mono-KKU-2: monoculture of microalgae-FRNWH, Mono-Y30: monoculture of yeast-FRNWH.

TABLE I: FERMENTATION KINETIC PARAMETERS OF MIXED CULTURE OF *CHLORELLA* SP. KKU-S2 AND *T. MALEEAE* Y30 ON CULTURE MEDIUM SUPPLEMENTED WITH GLUCOSE AND FERMENTED RICE NOODLE WASTEWATER HYDROLYSATE AT 30°C IN AN INCUBATOR SHAKER AT 150 RPM UNDER CONTINUOUS ILLUMINATION BY USING 80W COOL-WHITE FLUORESCENT LAMPS FOR 6 DAYS

Kinetic parameters	Growth condition–carbon substrate			
	Mixed culture-Glucose	Mixed culture-FRNWH	Monoculture KKU-S2-FRNWH	Monoculture Y30-FRNWH
X	4.73	2.02	2.32	2.71
P	337.0	150.4	72.0	117.3
Q_X	0.79	0.34	0.39	0.45
Q_P	56.17	25.06	12.01	19.54
$Y_{X/S}$	0.26	0.28	0.26	0.28
$Y_{P/S}$	18.25	20.57	8.14	11.97
$Y_{P/X}$	71.34	74.60	31.11	43.26
μ	0.26	0.12	0.14	0.17
q_P	18.46	8.71	4.35	7.19
q_S	0.652	0.605	0.637	0.603

X (g/L); P (mg/L); Q_X (g/L/d); Q_P (mg/L/d); $Y_{X/S}$ (g cells/g substrate); $Y_{P/S}$ (mg lipid/g substrate); $Y_{P/X}$ (mg lipid/g cells); q_P (mg lipid /g cells/d); q_S (g substrate/g cells/ d); μ (1/d).

It was observed that pH of medium increased from 5.0 to 7.1 for monoculture of microalgae *Chlorella* sp. KKU-S2 and from 5.5 up to 7.2 for the mixed culture of *Chlorella* sp. KKU-S2 with yeast *T. maleeae* Y30, due to the bicarbonate (HCO_3^-) is formed when CO_2 dissolves in water at neutral pH. 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 hydroxide ion formed, and the pH becomes more alkaline [17].

In monoculture of yeast *T. maleeae* Y30, a biomass of 2.71g/L with specific growth rate of 0.17(1/d) and lipid yield of 117.3mg/L were obtained. A biomass of 2.32g/L with specific growth rate of 0.14(1/d) and lipid yield of 72mg/L were found for monoculture of *Chlorella* sp. KKU-S2. However, the lipid yield of mixed culture was higher than that of monoculture of yeast and microalgae using both glucose and FRNWH as carbon substrate. The obtained result in this study, FRNWH sounded potential carbon substrate for cell growth and cellular lipid accumulation.

B. Effect of Nitrogen Source on Growth and Lipid Production

It has been reported that different nitrogen sources had varied influence on microbial lipid production. Therefore, effects of different nitrogen sources on growth and cellular lipid production of mixed culture of *Chlorella* sp. KKU-S2 and *T. maleeae* Y30 were tested using 15g/L reducing sugar of FRNWH as carbon substrate.

As shown in Fig. 2 and Table II, among the nitrogen sources tested, meat extract supported the maximum biomass of 5.32g/L with biomass productivity (Q_X) of 0.89g/L/d while urea supported the maximum lipid yield of 199.0 mg/L with lipid productivity (Q_P) of 33.17mg/L/d. However, Wu et al. (2014) showed that potassium nitrate was the suitable nitrogen source for both cell growth and lipid accumulation of *Monoraphidium* sp. SB2 under the photoautotrophic conditions [23].

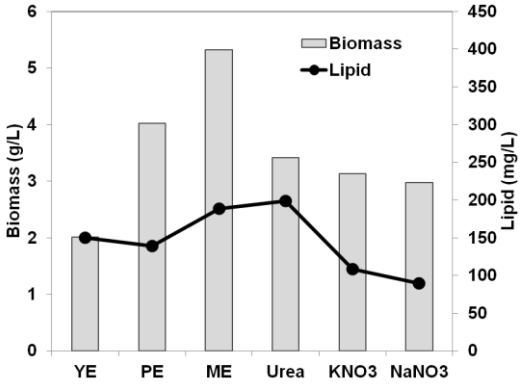

Fig. 2. Biomass and lipid yield of mixed culture of *T. maleeae* Y30 and *Chlorella* sp. KKU-S2 on culture medium containing 15g/L reducing sugar of FRNWH with different types of nitrogen sources, incubated at 30°C, 150 rpm for 6 days under continuous illuminated from overhead by 80W cool-white fluorescent lamps; YE: yeast extract; PT: peptone; ME: meat extract.

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 result presented that mixed culture of yeast with microalgae could utilized FRNWH as carbon substrate for cell growth and lipid accumulation, suggesting that the costs of microbial lipids production would be reduced and mixed culture technique is a desirable cultivation process for microbial oil production in wastewater.

TABLE II: FERMENTATION KINETIC PARAMETERS OF MIXED CULTURE OF *CHLORELLA* SP. KKU-S2 AND *T. MALEEAE* Y30 ON CULTURE MEDIUM CONTAINING 15G/L REDUCING SUGAR OF FERMENTED RICE NOODLE WASTEWATER HYDROLYSATE WITH DIFFERENT NITROGEN SOURCES, AT 30°C IN AN INCUBATOR SHAKER AT 150 RPM UNDER CONTINUOUS ILLUMINATION BY USING 80W COOL-WHITE FLUORESCENT LAMPS, 6 DAYS

Kinetic parameters	Nitrogen sources					
	YE	PE	ME	Urea	KNO_3	$NaNO_3$
X	2.02	4.02	5.32	3.42	3.13	2.98
P	150.4	139.2	188.9	199.0	108.4	89.4
Q_X	0.34	0.67	0.89	0.57	0.52	0.50
Q_P	25.06	23.20	31.48	33.17	18.07	14.90
$Y_{X/S}$	0.28	0.56	0.58	0.54	0.85	0.55
$Y_{P/S}$	20.57	19.33	20.75	31.65	29.47	16.52
$Y_{P/X}$	74.60	34.59	35.52	58.20	34.59	30.03
μ	0.12	0.23	0.28	0.20	0.19	0.18
q_P	8.71	8.03	9.89	11.93	6.58	5.46
q_S	0.605	0.298	0.285	0.307	0.196	0.303

X (g/L); P (mg/L); Q_X (g/L/d); Q_P (mg/L/d); $Y_{X/S}$ (g cells/g substrate); $Y_{P/S}$ (mg lipid/g substrate); $Y_{P/X}$ (mg lipid/g cells); q_P (mg lipid /g cells/d); q_S (g substrate/g cells/ d)

IV. CONCLUSIONS

In conclusion, the biomass and lipid yields from mixed culture of *Chlorella* sp. KKU-S2 and *T. maleeae* Y30 are notably enhanced in comparison with monoculture. To our knowledge this is the unique report about the production of microbial lipid from mixed culture of yeast and microalgae using FRNWH as carbon substrate. In further works, optimizing of culture condition for increasing of biomass and lipid yield will be investigated by using statistical method such as respond surface methodology and then completed with conversion of microbial biomass to biodiesel fuel by direct transesterification.

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