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Microalgal Lipid Production by Microalgae *Chlorella* sp. KKU-S2

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***Abstract*—**The objective of this work is to produce heterotrophic microalgal lipid in flask-batch fermentation. *Chlorella* sp. KKU-S2 supported maximum values of 0.374 g/L/d, 0.478 g lipid/g cells, and 0.112 g/L/d for volumetric lipid production rate, and specific yield of lipid, and specific rate of lipid production, respectively when culture was performed on BG-11 medium supplemented with 50g/L glucose. Among the carbon sources tested, maximum cell yield coefficient ($Y_{X/S}$, g/L), maximum specific yield of lipid ($Y_{P/X}$, g lipid/g cells) and volumetric lipid production rate (Q_P, g/L/d) were found of 0.728, 0.237, and 0.619, respectively, using sugarcane molasses as carbon source. The main 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.

***Keywords*—** Microalgal lipid, *Chlorella* sp. KKU-S2, kinetic parameters, biodiesel.

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I. Introduction

BIODIESEL is defined as a fuel comprised of mono-alkyl esters of long chain fatty acids derived from triacylglycerol (TAG), can be produced from renewable resources such as vegetable oil or animal fat, the first generation feedstock [1]. 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. Microalgae are seen as non-food feedstock promising candidates for the industrial production of biodiesel because of their advantages of higher photosynthetic efficiency, higher biomass production and faster growth compared to other energy crops [2]. In fact, 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 microalgal oils are mainly TAG which is the right kind of oil for producing biodiesel [2, 3]. At present, microalgal biomass production has been achieved by photoautotrophic cultivation by using solar energy and CO_2, and heterotrophic cultivation using organic carbon source. Heterotrophic cultivation of microalgae using organic carbon source offer several advantages over photoautotrophic cultivation including elimination of light, good control of cultivation process, high biomass and lipid content in cells [4, 5]. 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. The cost of lipid production currently is relatively high and therefore, it is prudent to search for inexpensive carbon sources, which are nutritionally rich enough to support the growth of the microorganism as well as the production of lipid such as agricultural product or agro-industry by product.

The Monod model is the most widely used and considered the basic equation of an unstructured model [6]. This model introduced the concept of growth-limiting substrate (S), relating the specific growth rate (μ) to the concentration of a single growth-limiting substrate via two parameters, the maximum specific growth rate (μ_{max}), and the Monod's constant or saturation constant (K_s). The growth rate has been shown by Monod to be related to the concentration of substrate medium by the equation:

$$\mu = \frac{\mu_{\max} S}{K_s + S} \tag{1}$$

With the linearization method, the specific growth rate is determined by calculating the difference in the natural log of the biomass concentrations over time, corresponding to the exponential growth phase was plotted:

$$\mu = \frac{\ln(X_t - X_0)}{t} \tag{2}$$

where X_0 is the biomass concentration (g/L) at the beginning of the exponential growth phase, Xt is the biomass concentration at time t.

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 [6].

In this work, the main aim is to investigate the effects of different glucose concentration and carbon sources on growth

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kinetics and lipid production of microalgae strains in batch heterotrophic fermentation.

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. Microalgae Strains and Culture condition

The green microalgae namely *Chlorella* sp. KKU-S2, *Chlorella* sp. KKU-W7, and *Chlorella* sp. KKU-W9 used, were isolated from freshwater taken from pond in the area of Khon Kaen province, northeastern region of Thailand. The seed culture was initially cultivated onto BG-11 medium supplemented with 20 g/L glucose at 30°C in an incubator shaker at a shaking speed of 150 rpm for 3 day in the dark. The BG-11 medium was consisted of (g/L): $NaNO_3$ 1.5, $K_2HPO_4.3H_2O$ 0.04, $MgSO_4.7H_2O$ 0.075, $CaCl_2.2H_2O$ 0.036, citric acid 0.006, ferric ammonium citrate 0.006, Na_2EDTA 0.001, Na_2CO_3 0.02, pH 7.0.

B. Screening of Microalgae Strains

For selection of suitable microalgae strains for lipid production, three microalgae strains were carried out in a 500-mL Erlenmeyer flask containing 200mL of BG-11 medium at 30°C in an incubator shaker at a shaking speed of 130 rpm, the pre-culture was cultivated onto BG-11 medium supplemented with 20g/L glucose with 10% (v/v) of seed culture.

C. Lipid Production by Chlorella sp. KKU-S2

To study of different glucose concentrations, the seed culture (10%, v/v) was inoculated into BG-11, supplemented with glucose to formulate a medium with an initial concentration of 30, 40, 50, 60, 70, 80 and 90 g/L.

For study the effect of different carbon sources, the seed culture (10%, v/v) was inoculated into BG-11 medium, supplemented with sugarcane molasses, distillery slop from sugarcane molasses-based ethanol plants, xylose, and pure glycerol. Carbon source concentration effect was studied using 25 g/L of total sugar available.

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 (1986) with modifications [7]. Lipid content was expressed as gram lipid per gram dry biomass. The fatty acid profile of the lipid was determined as fatty acid methyl esters (FAMEs) by the direct transesterification method with BF_3-methanol at 100°C for 45 min, reported by Lepage and Roy [8]. FAMEs samples were analyzed by gas chromatography (Shimadzu) equipped with a flame ionization detector (FID). The condition of GC analysis was as follows: FID 350 °C, N_2 carrier gas 40 mL/min, injection port temperature 230 °C, oven temperature 190 °C.

E. Determination of Growth Kinetic

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 is the slope determined by plotting the natural log of biomass versus time for each substrate concentration during the initial phase of exponential growth (equation 2) 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

A. Screening of Microalgae Strains

As a preliminary step, heterotrophic growth of microalgae was investigated for screening of high cellular lipid accumulation strains. Biomass, lipid production and residual sugar of *Chlorella* sp. KKU-S2, *Chlorella* sp. KKU-W7, and *Chlorella* sp. KKU-W9 with time in batch heterotrophic cultivation are presented in Fig. 1 and Table 1. A maximum biomass and lipid yield of 4.82 g/L and 1.30g/L with lipid content of 26.9%of cell dry weight (CDW) of *Chlorella* sp. KKU-S2 were obtained. *Chlorella* sp. KKU-W7 showed low growth under heterotrophic cultivation with a biomass of 1.24g/L with specific growth rate of 0.095d^{-1}. The maximum process product yield ($Y_{P/S}$, 0.07) was obtained from *Chlorella* sp. KKU-S2, followed by *Chlorella* sp. KKU-W9 ($Y_{P/S}$, 0.042), and *Chlorella* sp. KKU-W7 ($Y_{P/S}$, 0.028). The obtained result presented that *Chlorella* sp. KKU-S2 is a suitable strain for lipid production under heterotrophic cultivation.

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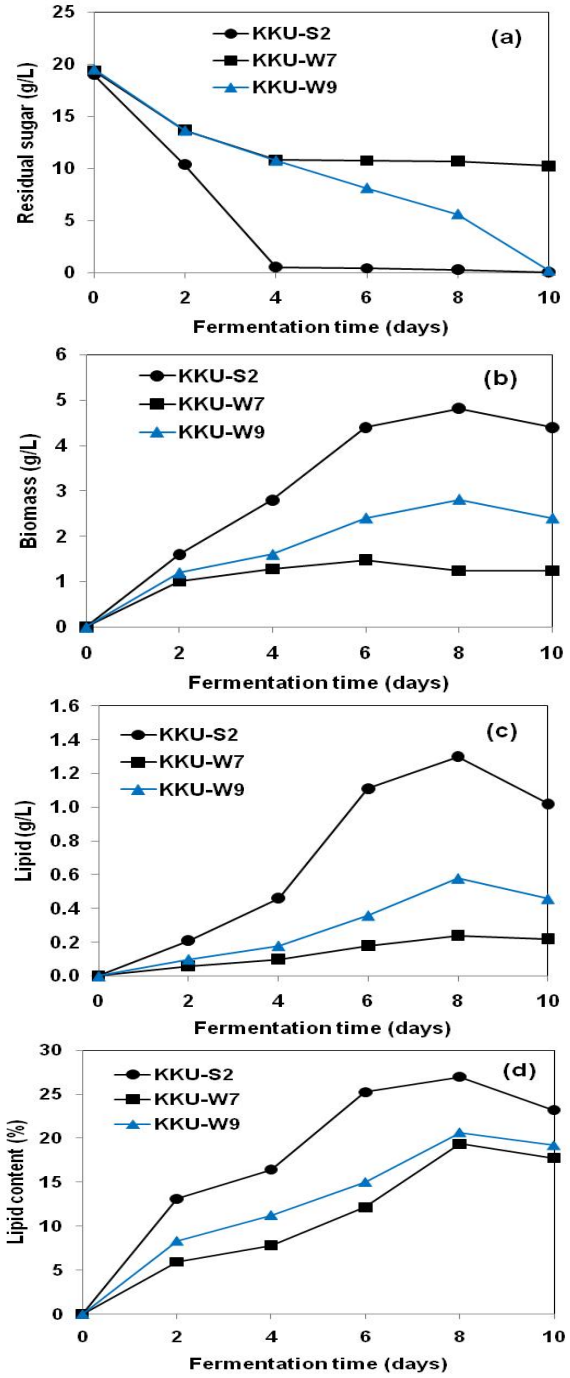


Fig. 1 Residue sugar (a), biomass concentration (b), lipid yield (c), and lipid content (d) during heterotrophic growth of *Chlorella* sp. KKU-S2, *Chlorella* sp. KKU-W7, and *Chlorella* sp. KKU-W9 on BG-11 medium supplemented with 20g/L glucose at 30°C, for 10 days.

TABLE I
COMPARATIVE FERMENTATIVE KINETIC PARAMETERS OF THREE MICROALGAE STRAINS ON BG-11 MEDIUM SUPPLEMETED WITH 20G/L GLUCOSE AT 30°C

Kinetic parameters	**Microalgae strains**		
	Chlorella sp. KKU-S2	*Chlorella* sp. KKU-W7	*Chlorella* sp. KKU-W9
X	4.82	1.24	2.81
P	1.30	0.24	0.58
LC* (%)	26.9	19.4	20.6
μ	0.198	0.095	0.129
Q_S	2.336	1.086	1.734
Q_X	0.603	0.155	0.351
Q_P	0.163	0.030	0.073
$Y_{X/S}$	0.258	0.143	0.203
$Y_{P/X}$	0.270	0.194	0.206
$Y_{P/S}$	0.070	0.028	0.042
q_S	0.053	0.018	0.027
q_P	0.485	0.876	0.617

*LC: lipid content

B. Lipid Production by Chlorella sp. KKU-S2

Based on preliminary screening we selected *Chlorella* sp. KKU-S2 for study the effect of glucose concentration on growth kinetic. Batch cultures were investigated to determine the suitable glucose concentration of the initial medium. As shown in Table 2. The cellular lipid accumulation was quite low at low level of glucose concentration, then showed an increase when glucose concentration increased. Lipid production of *Chlorella* sp. KKU-S2 reached the maximum of 6.25 g/L with 47.8%CDW at 50 g/L glucose were obtained.

The increase in glucose concentration resulted in a decrease in cell yield coefficient values ($Y_{X/S}$), and an increase in lipid concentration (P). Maximum cell yield coefficient ($Y_{X/S}$, g/L) was found of 0.225 using 30 g/L glucose, whereas maximum specific yield of lipid ($Y_{P/X}$, g lipid/g cells) and volumetric lipid production rate (Q_P , g/L/d) of 0.478 and 0.374 were obtained using 50g/L glucose. Further increase in glucose beyond 60g/L resulted in a slight drop in lipid concentration and biomass, suggesting that a considerable glucose inhibitory effect had occurred.

However, the comparison of process product yield ($Y_{P/S}$) in batch fermentation at high substrate concentration, it was obvious that increase of glucose concentration resulting in decrease of this kinetic parameter, suggesting to difficult for up scaling of lipid production by microalgae due to high substrate consumption rate and high concentration of glucose with lower level of nitrogen source could be effect the cell growth, because nitrogen source supported the cell growth, thus, depleted of nitrogen may result to low biomass. 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. Xiong et al (2008) reported that cell density of *Chlorella protothecoides* achieved was 16.8

g/L in 184 h by performing fed-batch culture with lipid content of 50.3% CDW using glucose as carbon source [9].

TABLE II
COMPARATIVE FERMENTATIVE KINETIC PARAMETERS OF CHLORELLA SP. KKU-S2 ON BG-11 MEDIUM SUPPLEMETED WITH DIFFERENT GLUCOSE CONCENTRATION AT 30°C

Kinetic parameters	Glucose concentration (g/L)						
	30	40	50	60	70	80	90
X	5.70	6.00	6.25	6.08	5.78	5.12	4.28
P	1.97	2.38	2.99	2.62	2.22	1.80	1.18
LC*	34.6	39.7	47.8	43.1	38.4	35.2	27.6
μ	0.211	0.226	0.235	0.226	0.211	0.209	0.198
Q_S	3.172	4.266	5.219	5.540	5.750	6.027	6.079
Q_X	0.713	0.750	0.781	0.760	0.723	0.640	0.535
Q_P	0.246	0.298	0.374	0.328	0.278	0.225	0.148
$Y_{X/S}$	0.225	0.175	0.150	0.137	0.126	0.106	0.088
$Y_{P/X}$	0.346	0.397	0.478	0.431	0.384	0.352	0.276
$Y_{P/S}$	0.078	0.070	0.072	0.059	0.048	0.037	0.024
q_S	0.556	0.711	0.835	0.911	0.995	1.177	1.420
q_P	0.073	0.090	0.112	0.097	0.081	0.073	0.055

*LC : lipid content (%)

GC analysis showed that the lipid extracted from heterotrophic cell of *Chlorella* sp. KKU-S2 using glucose as carbon source, mainly contained palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2), which is similar to that of vegetable oils, and the unsaturated fatty acids and saturated fatty acid amount to about 36.1% and 63.9% of total fatty acid, respectively. Based on these compositional data, microalgal lipids from *Chlorella* sp. KKU-S2 can be used as feedstock for biodiesel production.

C. Effects of Various Carbon Sources

In order to reduce the cost of oil production from microalgae under heterotrophic cultivation, low cost carbon source or alternative carbon source could be used to replace pure glucose. After cultivation of *Chlorella* sp. KKU-S2 on BG-11 medium supplemented with sugarcane molasses, distillery slop, xylose, and pure glycerol as a carbon substrate at 30°C for 8 days, *Chlorella* sp. KKU-S2 grew on several types of carbon source (Table 3). Among the carbon sources tested, sugarcane molasses supported the maximum biomass of 4.81 g/L with lipid content of 32.8 %CDW, followed by glycerol of 4.51 g/L with lipid content of 24.8%CDW. Maximum cell yield coefficient ($Y_{X/S}$, g/L), maximum specific yield of lipid ($Y_{P/X}$, g lipid/g cells) and volumetric lipid production rate (Q_P, g/L/d) were found of 0.356, 0.328, and 0.196, respectively, using molasses as carbon source.

The obtained result suggested that oil production from *Chlorella* sp. KKU-S2 can be performed with lower cost production process especially using distillery slop as carbon source, wastewater from ethanol production plant, not only suitable for the production of oil but also useful to wastewater treatment. In addition, agricultural residues could be used as available carbon source due to *Chlorella* sp. KKU-S2 can accumulated xylose, one type of fermentable sugar from agricultural residues. Thus, to realize the industrial production of biodiesel from microalgal oils, it was necessary to obtain a large amount of biomass and quantity, quality and lipid productivity from suitable strains under optimized culture conditions with low cost of cultivation process.

TABLE III
COMPARATIVE FERMENTATIVE KINETIC PARAMETERS OF CHLORELLA SP. KKU-S2 ON BG-11 MEDIUM SUPPLEMETED WITH DIFFERENT CARBON SOURCES AT 30°C

Kinetic parameters	Carbon sources			
	Sugarcane molasses	Distillery slop	Xylose	Glycerol
X	4.81	2.02	2.12	4.51
P	1.58	0.48	0.34	1.12
LC*	32.8	23.8	16.0	24.8
μ	0.196	0.088	0.094	0.188
Q_S	1.688	1.025	0.888	1.513
Q_X	0.601	0.253	0.265	0.564
Q_P	0.198	0.060	0.043	0.140
$Y_{X/S}$	0.356	0.246	0.299	0.373
$Y_{P/X}$	0.328	0.238	0.160	0.248
$Y_{P/S}$	0.117	0.059	0.048	0.093
q_S	0.351	0.507	0.419	0.335
q_P	0.064	0.021	0.015	0.047

*LC : lipid content (%)

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