2011 International Conference on Food Engineering and Biotechnology
IPCBEE vol.9 (2011) © (2011)IACSIT Press, Singapore

Heterotrophic Growth of *Chlorella* sp. KKU-S2 for Lipid Production using Molasses as a Carbon Substrate

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Abstract. The kinetics of growth of the microalgae *Chlorella* sp. KKU-S2 has been investigated using molasses as carbon substrate and yeast extract as nitrogen source. At low concentration of nitrogen, maximum lipid production rate was observed while high nitrogen concentration (6.0 g/L) resulted in high both volumetric cell mass production rate (1.796 g/L/d) and specific growth rate (0.522 1/d). *Chlorella* sp. KKU-S2 supported maximum values of 0.220g/L/d, 0.203 g lipid/g cells, 0.881 g/L, and 0.093 g/L d for volumetric lipid production rate, and specific yield of lipid, lipid concentration, and specific rate of lipid production, respectively when culture was performed in culture medium supplemented with 30g/L molasses and 2.0 g/L yeast extract. The Monod's constant (K_S, g/L) and μ_{max} (1/d) of 1.283 and 0.435, were obtained, respectively.

Keywords: *Chlorella* sp. KKU-S2, heterotrophic growth, molasses, biodiesel.

1. Introduction

Biodiesel is defined as a fuel comprised of mono-alkyl esters of long-chain fatty acids from mostly plant oils, as a clean and renewable alternative to the traditional fossil fuel, has attracted more and more attention in recent years [1]. However, the consumption of a large amount of plant oils for biodiesel production would result in a shortage in edible oils and soar of food price. Nowadays, there has been an increasing interest in looking for new oil feedstock for biodiesel production. Among them, microbial oils, namely single cell oils (SCOs), lipids produced by the oleaginous microorganisms especially microalgae are now considered as promising candidates because of their advantages of higher photosynthetic efficiency, higher biomass production and faster growth compared to other energy crops [2, 3]. 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 triglyceride that can be applied to form biodiesel through transesterification [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]. However, to realize the large-scale production of biodiesel from microalgal oils, it was necessary to obtain a large amount of biomass and lipid content as well as the low cost of cultivation process. The microalgae *Chlorella* sp. KKU-S2 can grow heterotrophic cultivation and accumulates much higher production of lipids and higher growth rate using glucose as carbon substrate, 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 [5]. However, more economical carbon source should be employed to replace of glucose as substrate such as molasses, distillery slop, etc.

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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, $\mu = (\mu_{max}.S) / (K_s+S)$. Where, μ is the specific growth rate, μ_{max} is the maximum specific growth rate unlimited by low concentrations of the substrate, S is substrate concentration, K_S is the concentration of substrate that supports a rate equal to $\mu_{max}/2$. 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, $\mu = (\ln X_t - X_0)/t$. Where X_0 is the biomass concentration at the beginning of the exponential growth phase, X is the biomass concentration at time t. Straight lines were obtained with slopes equal to μ and intercepts equal to lag phase time, for each set of experiments carried out.

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].

The objective of this study is to investigate the effects of different concentration of nitrogen and molasses sources on growth kinetics of *Chlorella* sp. KKU-S2 and to develop a kinetic model incorporating substrate inhibition which would be used to assist the design and scale-up of the cultivation process.

2. Material and Methods

2.1. Microalgal strain

The freshwater microalgae *Chlorella* sp. KKU-S2 used in this study was isolated from freshwater taken from pond in the area of Khon Kaen province, Thailand [5]. The seed culture was performed on the Bristol's modified medium (20 g/L molasses) in an incubator shaker at 30°C and 150 rpm in the dark. 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, pH 7.0. Then, 10% (v/v) seed culture was inoculated into 250-mL Erlenmeyer flask containing 100mL Bristol's culture medium and batch heterotrophic growth was carried out in an incubator shaker at 30°C in the dark. To investigate the influence of initial nitrogen source and carbon source concentrations on the biomass and lipid production, different concentration of yeast extract and molasses were added into the culture medium.

2.2. Analytical methods

Duplicate samples were analyzed for cell dry weight, lipid, and residual glucose. The culture broth was centrifuged at 5,000g for 5 min. The supernatants were analyzed for reducing sugar concentration by dinitrosalicyclic acid (DNS) assay [7]. 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 method of Know and Rhee (1989) with modifications [8].

2.3. Determination of kinetic parameters

Volumetric product formation rate (Q_P, g/L/d) was determined from a plot between lipids concentration (g/L) and fermentation time, process product yield ($Y_{P/S}$, g lipid/g substrate) was determined from dP/dS, and specific product yield ($Y_{P/X}$, g lipid/g cells) was determined using relationship dP/dX, while volumetric rate of substrate consumption (Q_S, g substrate/L/d) was determined from a plot between substrate (g/L) present in the fermentation medium and time of fermentation. Volumetric cell mass production rate (Q_X, g cell/L/d) was determined from a plot of dry cells (g/L) versus time of fermentation (d). The specific growth rate (μ, 1/d) 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, g lipid/g cell/d) was a multiple of μ and $Y_{P/X}$. Then, the determined values of specific growth rate and substrate concentration determined are used to estimate the kinetics parameters, maximum specific growth rate (μ_{max}) and Monod's constant (K_s), with Hanes linear methods.

3. Results and Discussion

3.1. Time course of cell growth

Cell growth, lipid production and residual sugar of *Chlorella* sp. KKU-S2 with time in batch heterotrophic cultivation are presented in Fig 1. It is apparent that molasses referred to reducing residue sugar was used mainly for cell growth at the beginning of cell growth phase. Biomass, lipid production and consumed reducing sugar gradually increase and lipid yield reached the maximum of 19.05% of cellular dry weight (CDW) at day 4 of fermentation time. During the period between day 4 and 5, there showed an apparent decrease in biomass and lipid content was observed, suggesting that the absence of carbon source in the culture medium, cell could be used lipid as storage energy source to maintenance their growth led to decrease of cellular lipid content. The similar changes were observed in cellular lipid content of *Chlorella protothecoides*, after exhaustion of carbon source in the growth medium [4].

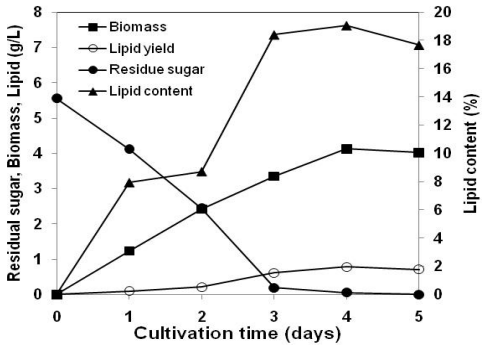

Fig. 1: Growth kinetic for biomass, lipid production and residue sugar of *Chlorella* sp. KKU-S2 on Bristol's medium supplemented with 20g/L molasses, 2.0g/L yeast extract in an incubator shaker at 150 rpm, 30°C for 5 days.

3.2. Effect of nitrogen concentration on growth kinetics

After 5 days of cultivation, higher nitrogen concentration led to an increase in biomass concentration, with the maximum biomass concentration of 7.18 g/L obtained by cultivation with yeast extract of 6.0 g/L (Fig 2.). In the experimental data, an increase in the yeast extract concentration of the culture medium led to a decrease in lipid content of cells. *Chlorella* sp. KKU-S2 had the highest total lipid content of 19.29% CDW by cultivation of an initial yeast extract of 2.0 g/L.

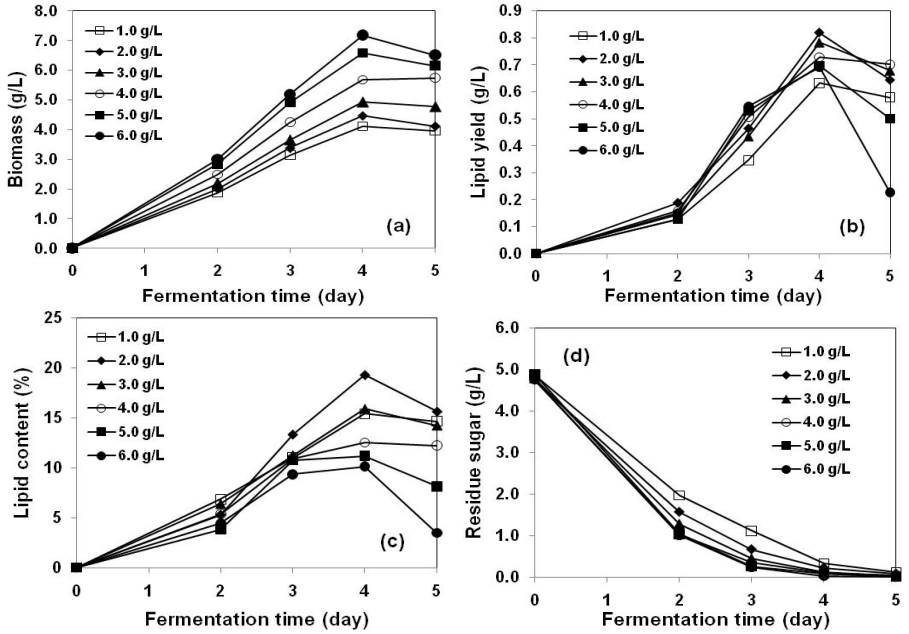

Fig. 2: Effect of different yeast extract concentration on biomass concentration (a), lipid yield (b), lipid content (c), and residual sugar (d) during fermentation of Chlorella sp. KKU-S2 on Bristol's medium supplemented with 20 g/L molasses.

The growth rate and lipid productivity of *Chlorella* sp. KKU-S2 were strongly related to nitrogen concentration. Kinetic and yield parameters were calculated and the results are presented in Table 1. As can be seen, all parameter values were dependent on the nitrogen concentration. The increase in nitrogen concentration resulted in an increase in specific growth rate and $Y_{X/S}$ values, and a decrease in lipid concentration (P). The maximum lipid production rate (Q_P, g/L/d) was obtained at 0.198 when initial yeast extract concentration was 2.0 g/L with specific growth rate (1/d) of 0.508. Maximum biomass concentration (X, g/L) of 7.18 g/L was obtained using 6.0 g/L yeast extract. The specific growth rate (μ, d^{-1}) obtained in these case was higher (0.552 d^{-1}) than that obtained for the initial nitrogen concentration of 1.0 (0.479 d^{-1}), 2.0 (0.508 d^{-1}), 3.0 (0.510 d^{-1}), 4.0 (0.536 d^{-1}) and 5.0 (0.547 d^{-1}) g/L, respectively. Volumetric cell mass production rate (Q_X, g/L/d) of 1.027, 1.117, 1.232, 1.421, 1.645 and 1.796 were obtained using 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 g/L of yeast extract concentration. It was found that lipid production rate decreased as yeast extract concentration increased from 2.0 to 6.0 g/L. Consequently, initial yeast extract concentration of 2.0 g/L, was considered to be appropriated to achieve high lipid production rate and high process product yields ($Y_{P/S}$). The growth rate and lipid accumulation of *Chlorella* sp. KKU-S2 were strongly related to nitrogen concentration. Generally, the results confirmed that high nitrogen concentration supported maximum profiles of cell mass production rate, and low nitrogen concentration supported maximum lipid production.

Table 1. Kinetic parameters of Chlorella sp. KKU-S2 on Bristol's medium supplemented with 20 g/L molasses (pH 7.0) with different yeast extract concentration.

Yeast extract (g/L)	Kinetic parameters										
	X (g/L)	P (g/L)	LP* (%)	μ (1/d)	$Y_{X/S}$	$Y_{P/S}$	$Y_{P/X}$	Q_X	Q_P	q_S	q_P
1.0	4.11	0.632	15.37	0.479	0.906	0.139	0.154	1.027	0.158	0.276	0.074
2.0	4.47	0.819	18.34	0.508	0.988	0.175	0.177	1.117	0.198	0.253	0.090
3.0	4.93	0.782	15.87	0.510	1.056	0.170	0.161	1.232	0.196	0.237	0.082
4.0	5.68	0.728	12.81	0.536	1.218	0.156	0.128	1.421	0.182	0.205	0.069
5.0	6.58	0.696	10.58	0.547	1.368	0.145	0.106	1.645	0.174	0.183	0.058
6.0	7.18	0.691	9.61	0.552	1.517	0.146	0.096	1.796	0.173	0.165	0.053

* LP represents Lipid content (%CDW)

3.3. Effect of molasses concentration on growth kinetics

To study of molasses concentration on cell growth and lipid production, the concentration of molasses of 10, 15, 20, 25, 30, 35, 40 and 45 g/L with 2.0 g/L yeast extract were investigated. As shown in Table 2, no significant difference in cell growth biomass using different molasses concentration. Whereas, the cellular lipid accumulation was quite low at low level of molasses concentration, then showed an increase when molasses concentration increased. Lipid production of *Chlorella* sp. KKU-S2 reached the maximum of 0.881 g/L with cellular lipid content of 20.65%CDW at 30 g/L glucose were obtained. Apparently, an increase in molasses concentration resulted in a decrease in cell yield coefficient values ($Y_{X/S}$). Cellular lipid accumulation was quite low with lipid content of 16.73% CDW at molasses concentration of 10g/L, then showed a gradually increase when molasses concentration increased from 15 to 30 g/L, and reached the maximum of 20.65 %CDW at 30g/L molasses with maximum volumetric lipid production rate (Q_P, g/L d) was obtained. Further increase in molasses concentration beyond 30g/L resulted in a slight drop in lipid concentration, suggesting that a considerable molasses as carbon source inhibitory effect had occurred. Indeed, carbon source concentration has been found to be the major impact factor for oil accumulation by the oleaginous microorganisms [9].

The determined values of specific growth rate and molasses concentration determined are used to estimate the kinetics parameters, maximum specific growth rate (μ_{max}) and Monod's constant or halt saturation constant (K_s), with Hanes linear methods. With fitted by linear regression of Hanes plot (y =2.301x-0.9913, R^2=0.9913), μ_{max} of 0.435 (1/d), and K_s of 1.283 g/L were obtained. Faster growth of *Chlorella* sp. KKU-S2 was observed with low value of K_S using molasses as carbon substrate.

However, the comparison of process product yield ($Y_{P/S}$) in batch fermentation at high substrate concentration, it was obvious that increase of molasses concentration resulting in decrease of this kinetic parameter, suggesting to difficult for up scaling of lipid production by the oleaginous organisms due to high substrate consumption rate and high concentration of carbon source 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. Xiong et al. [10] 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%CDW using glucose as carbon source.

Table2. Comparative fermentation kinetic parameters of Chlorella sp. KKU-S2 for lipid production on Bristol's medium supplemented with 2.0 g/L yeast extract (pH 7.0) in the presence of different molasses concentration in 250mL flask at 30°C.

Kinetic parameters	Molasses concentration (g/L)							
	10	15	20	25	30	35	40	45
X (g/L)	3.15	4.20	4.12	4.30	4.35	4.27	4.25	4.35
P (g/L)	0.654	0.631	0.726	0.843	0.881	0.781	0.790	0.743
LP* (%)	16.73	18.34	19.05	20.20	20.65	19.18	18.60	17.08
μ (1/d)	0.406	0.479	0.501	0.491	0.459	0.448	0.444	0.440
$Y_{X/S}$	0.833	0.945	0.750	0.738	0.667	0.635	0.615	0.547
$Y_{P/S}$	0.173	0.142	0.132	0.131	0.150	0.116	0.114	0.093
$Y_{P/X}$	0.208	0.150	0.176	0.196	0.203	0.183	0.186	0.171
Q_X	0.787	1.050	1.031	1.075	1.087	1.067	1.062	1.087
Q_P	0.164	0.158	0.181	0.211	0.220	0.195	0.198	0.186
q_S	0.300	0.265	0.333	0.375	0.339	0.394	0.407	0.457
q_P	0.084	0.072	0.088	0.096	0.093	0.082	0.083	0.075

* LP represents Lipid content (%CDW)

4. Acknowledgements

This work was financial supported by Khon Kaen University, Khon Kaen, Thailand.

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