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A study on lipid production of the mixotrophic microalgae *Phaeodactylum tricornutum* on various carbon sources

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This study is aimed to investigate the potential use of various carbon sources (glucose, starch, and acetate sodium) for culturing *Phaeodactylum tricornutum* with higher microalgae oil production in mixotrophic batch cultures. Results showed *P. tricornutum* could grow mixotrophically with those organic substrate in a tested range from 0.5 to 5.0 g/l. Glucose and acetate sodium exerted significant promotion on growth of *P. tricornutum*, while starch did not exhibit this enhancement. The presence of carbon sources in medium could significantly increase the lipid content, especially with glucose. The maximum lipid productivities in mixotrophic cultures with glucose, starch and acetate in medium were 0.053, 0.023 and 0.020 g/l·day, respectively. These values were respectively 4.6-, 2.0-, and 1.7-fold of those obtained in the corresponding photoautotrophic control cultures. For the composition of fatty acids in microalgae oil, there was no significant sensitivity to the variation of the organic carbon sources. The data in this study suggests that *P. tricomutum* could provide high lipid productivity combined of high oil content and biomass in mixotrophic culture, which offers a promise to be one of the sources of biodiesel.

Key words: Lipid productivity, mixotrophic growth, organic carbon sources, *Phaeodactylum tricornutum*.

INTRODUCTION

Microalgae with high oil productivities offers great promise to be one of the sources of biodiesel that substitutes fossil diesel due to transforming carbon dioxide to potential biofuels by driving-force of sunlight (Chisti, 2007; Pienkos and Darzins, 2009). Though hundreds of microalgal strains capable of producing high content of lipid have been screened (Huntley and Redalje, 2007), the classical photoautotrophic culture is difficult to reach a high density of microalgae biomass since the limited light penetration in broth (Chen and Johns, 1991, 1995). Moreover, it is hard to realize an ideal process of producing lipid at the highest productivity resulted from a combination of high oil content and high rates of biomass production.

To overcome the limitation of light penetration, many microalgae that are able to grow heterotrophically by using organic carbon sources such as sugars or organic acids without light were studied widely (Miao and Wu, 2006; Wei et al., 2009). Furthermore, a mixotrophic metabolism was found to exist in some microalgae. That is, they can fix CO\(_2\) and utilize organic carbon sources simultaneously when exposed to light, while a number of microalgal species were studied to explain the relationship of photosynthetic and respiratory metabolism in mixotrophic culture conditions and drew opposite conclusions (Zhang et al., 1998; García et al., 2000; Ip et al., 2004; García et al., 2005; García et al., 2006; Andrade and Costa, 2007; Liu et al., 2009; Heredia-Arroyo et al., 2010).

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Phaeodactylum tricornutum, a model diatom for which the complete genome information is available (Bowler et al., 2008), showed high growth rates under optimum conditions and was studied as a potential source of polyunsaturated fatty acids (PUFAs) (Yongmanitchai and Ward, 1991). Its common oil content under photo-autotrophic conditions is 20-30% (dry wt) (Chisti, 2007). On mixotrophic culture of P. tricornutum, the addition of organic carbon glycerol led to a remarkable increasing of the respiration rate (Liu et al., 2009). So far, a number of preliminary studies on P. tricornutum demonstrated that a higher biomass concentration could potentially be achieved by mixotrophic culture (García et al., 2000; García et al., 2005; García et al., 2006; Liu et al., 2009). Namely, even with same oil content, the oil production of P. tricornutum could be promoted by mixotrophic cultivation for biomass rise.

Besides quantity of lipid in microalgae, the quality of microalgal oils also varied with environmental factors. Composition of fatty acids in microalgal oils and corresponding fatty acid methyl esters (FAMES) determined their acceptability for use in biodiesel production, especially the extent of unsaturation of microalgal oil (Chisti, 2007). However, few studies have reported the effects of carbon source on the lipid content and fatty acids composition of lipid in P. tricornutum (García et al., 2000; García et al., 2005; García et al., 2006). In view of the limited amount of knowledge on the potential of mixotrophic growth of P. tricornutum, this research set out to investigate different organic carbon sources and analyze the impact of their varied concentrations on the growth, lipid production and fatty acids composition of the microalgae in batch culture under mixotrophic conditions to accumulate lipids for biodiesel production.

MATERIALS AND METHODS

Algae and cultivation

Samples of P. tricornutum were collected from a salted lake and isolated by the South-center University for Nationalities in China. The inocula were grown in 100-ml conical flasks with 50 ml of media in aseptic conditions without aeration. The medium used was modified from f/2 medium (Guillard and Ryther, 1962).

Static cultures of mixotrophy were carried out with an axenic condition in triplicate in 500-ml conical flasks containing 250 ml of the culture medium with inoculation in linear growth phase prepared above. The organic carbon sources (sodium acetate, soluble starch, and glucose, all purchased as reagent's purity) in the culture medium were separately sterilized by filtration through 0.2 μm pore membranes as well as others were sterilized in an autoclave at 121°C for 20 min. Batch runs were carried out with various carbon sources at concentrations varied within 0.5 and 5.0 g/l according to García et al. (2000, 2005). Controls of photoautotrophic culture were included in each case. Light was continuously supplied (Philips TLD 36W/54 fluorescent lamp) at an intensity of 165 μE m⁻² s⁻¹ measured at the flasks’ surface. The initial pH value was adjusted to 8.0 and temperature was set at 20 ± 0.5°C. The cultivation lasted for 10 days and then be harvested for measurement.

Measurement of growth

Biomass concentration (dry-weight of cell powder in the culture medium, g/l) was estimated by an equation which was periodic corroborated with dry-weight determinations:

\[
DCW\ (g/l) = 0.392x-0.009,
\]

where \(x\) was absorbance measured at 625 nm by an UV-vis spectrophotometer (UV-1750, Shimadzu).

The specific growth rate was calculated by the equation:

\[
\mu\ (day^{-1}) = \frac{1}{t}\ln\left(\frac{X_m}{X_0}\right),
\]

where \(X_m\) and \(X_0\) (g/l) were the concentrations of biomass at the end and beginning of the exponential phase, \(t\) (day) was the time between the two measurements.

Measurement of lipid production

The cell suspensions were collected and centrifuged at 6000 g for 10 min, and then the pellets were freeze-dried and stored at 20°C before analysis. Determination of total lipids was determined using the method of Bligh and Dyer (Iverson et al., 2001). The content of the microalgae lipids was calculated by the equation:

\[
C_l\ (g/g) = \frac{W_L}{W_A},
\]

where \(W_L\) and \(W_A\) were weights of the extracted lipids and dry algae biomass, respectively.

The lipid productivity was calculated by the equation:

\[
P_{lipid}\ (g\cdot l^{-1}\cdot day^{-1}) = \frac{C_l \times DCW}{t},
\]

where \(C_l\) (g/g) was the concentration of lipids at the end of the batch run, \(DCW\) (g/l) was the concentration of biomass, and \(t\) (day) was the duration of the run.

Fatty acid analysis

The fatty acid of algae cells was analyzed by gas chromatography followed Slover and Lanza (1979). Three milligram of freeze-dried sample was put into a capped test tube and extracted with 2 ml of CHCl₃–CH₃OH (V/V = 2/1) by ultrasound for three times under N₂ protection. The extracted oil was saponified with 2 ml of saturated KOH–CH₃OH solution at 75°C for 10 min and then submitted to 1 ml boron trifluoride methanol solution at 75°C for another 30 min. After the suspension cooled, 2 ml of hexane and 2 ml of saturated NaCl solution were added in and the upper clear liquid fluid was pipetted out for analysis. All operations should be under low-light and protection of nitrogen. Samples were analyzed by a gas chromatograph HP6890A equipped with a flame ionization detector. The injector temperature was set to 250°C, and separation was achieved on a HP-5 capillary column (30.0 m × 0.320 μm × 0.25 μm, 50% phenyl Methyl Siloxane). The flow of carrier gas (N₂) was
1 ml/min, and 1 μl of methyl ester solution of sample was injected under split mode (split ratio = 1:30) for each analysis. The column temperature program was as follows: an initial temperature was 150°C, raised to 210°C at a rate of 10°C /min and then increased to 250°C at a rate of 2°C/min, the final column temperature was kept at 250°C for 25 min. Identification of components were proceeded by comparing their retention time and fragmentation pattern with a Supelco™ 37-Component FAME Mix (Sigma, USA). An established standard of heptadecanoic acid (Sigma, USA) was added as an internal standard constant weight for measurement.

Statistical treatment

Results were presented with average values of three independent experiments and their standard deviation. The statistical analysis was performed by GraphPad Prism 5 (Graph Pad Software, Inc., USA). One-way analysis of variance and Tukey’s multiple comparison test in the software were chosen for the treatment comparisons for each data set. All used statistics were based on a confidence level of 95%, and p values < 0.05 were considered statistically significant.

RESULTS

Effect of organic carbon sources on growth

The graph of the specific growth rate of P. tricornutum in Figure 1 illustrated that glucose and sodium acetate significantly stimulated the growth at concentrations below 1.0 g/l (p < 0.05) and above 1.0 g/l (p < 0.05), respectively. The starch in the medium did not influence the specific growth rate with concentrations below 1.0 g/l but inhibited significantly above 2.0 g/l (p < 0.05).

As shown in Figure 1, glucose was the best among the three tested organic carbon sources. It is obvious that the biomass increased with 1.0 g/l of glucose and 0.5 g/l of sodium acetate (p < 0.05). The biomass analysis also revealed that the algae did not promote growth at all starch concentrations. The maximum biomass concentration obtained with glucose as the carbon source was 1.16 ± 0.21 g/l, which was approximately 1.74 times of that obtained with photoautotrophic controls (0.66 ± 0.06 g/l). With other two carbon sources, the highest biomass concentrations were 0.83 ± 0.01 g/l at 2.0 g/l of starch and 0.89 ± 0.11 g/l at 0.5 g/l of sodium acetate, respectively.

Effect of organic carbon sources on lipid content and lipid productivity

Figure 2 shows that the lipid content of the microalgae tending to increase at all tested concentrations of the three organic carbon sources significantly (p < 0.01). With the concentration of glucose increased from 0.5 to 2.0 g/l, the lipid content of cells was more than 0.40 g/g as well as the lipid productivity was more than 0.038 g/l·d. The highest values of the lipid content and lipid productivity with glucose in media were approximately 2.8 times (at 2.0 g/l) and 4.6 times (at 1.0 g/l) compared to control. As the content of glucose increased to 5.0 g/l, the total lipid content and lipid productivity were only 0.29 g/g and 0.017 g/l·day, though they were a little higher than the

Figure 2. Variation of lipid content and lipid productivity for each organic carbon source concentration in mixotrophically cultivated *P. tricornutum* cells (the connecting lines between symbols are just for distinguishing them conveniently).

Effect of organic carbon sources on fatty acids composition

Furthermore, fatty acid profiles were determined for all cultures with the three tested organic carbon sources and the results are presented in Figure 3. The results showed that four types of saturated and eight types of unsaturated fatty acids with carbon chain lengths ranging from C16 to C22 were detected in the cells of *P. tricornutum*. Among those, C16:1 (palmitoleic acid), C16:0 (hexadecylic acid) and C20:5n3 (eicosapentaenoic acid, EPA) were the main fatty acids. In particular, the content of C16 fatty acid amounted to 67-76% of the total fatty acids in all samples. However, fatty acids composition was not sensitive to variations of the tested organic carbon sources here.

DISCUSSION

Glucose, starch and sodium acetate were tested as organic carbon sources for mixotrophic growth of *P. tricornutum* in this study. Among them the maximum biomass productivity was 1.16 g/l and was obtained by 1.0 g/l of glucose, which was 1.74 times greater than control (Figure 1). But a growth inhibitory was exhibited with 5.0 g/l of glucose addition. Effects of a wide range of externally supplied carbon compounds on the growth of *P. tricornutum* in mixotrophic conditions were studied previously. García et al. (2005) reported a stimulatory effect on growth of *P. tricornutum* by glucose, but no inhibition exhibited with higher concentrations of glucose. They found with 0.05 M (approximately 9.0 g/l) of glucose in the culture supplement, a maximum biomass concentration was 1.48 times of photoautotrophic control. But the use of acetate showed a negative effect by slowing down the growth and reducing the biomass concentration. Contrarily, the stimulatory effect on growth was observed at all sodium acetate concentrations from 0.5 to 5.0 g/l in this study, while only an increase of biomass concentration exerted at 0.5 g/l of acetate. In Liu’s report (2009) the biomass concentrations were significantly increased by addition of glucose and acetate with 1.21 times and 1.28 times of control obtained in photoautotrophic culture, which are well in agreement with the results in this study. As for starch, it didn’t stimulate the cells growth for all the concentrations tested, and a significant inhibitory effect was observed above 2.0 g/l of starch. But García et al. (2005) have clearly identified a stimulated growth for all starch concentrations with a same experimental design in this study. However, Fabregas et al. (1997) demonstrated that the productivity of *P. tricornutum* increased 2.4 times higher than the control (0.18 g/g and 0.0117 g/l/day). This means that a lower concentration of glucose would facilitate the lipid production of the microalgae. For lipid productivity with starch and sodium acetate in media, no significant difference could be observed compared to the control except 2.0 g/l of them ($p < 0.01$), which were 0.024 and 0.017 g/l/day, respectively.
autotrophic culture with soluble fraction of raw potato supplement that including starch, but they attributed the high productivity obtained by the presence of the nitrogen species existed in the soluble fraction of potato.

Glucose is the most commonly used carbon source for mixotrophic and heterotrophic cultures of microalgal species (Chen and Johns, 1991, 1995). Here the prominent promotion of glucose on *P. tricornutum* growth might because glucose owns more energy content per mol compared with other substrates. For instance, glucose produces ~2.8 kJ/mol of energy compared to ~0.8 kJ/mol for acetate (Boyle and Morgan, 2009). Microalgal cells may require a lag period (an acclimation period) to develop the specific transport systems necessary before uptake carbon sources except glucose (Perez-Garcia et al., 2011). The growth reduction of
P. tricornutum with starch in this study gave a proof of that, while the final biomass concentration did not decrease compared with autotrophic culture. A model of acquisition of dissolved inorganic carbon and primary metabolic pathways of carbon was presented based on the genome sequences of P. tricornutum (Kroth et al., 2008). However, simultaneous utilization of organic carbon sources and CO₂ under mixotrophic culture needs further research.

Other two species of microalgae, Chlorella protothecoides and Scenedesmus obliquus, were revealed to give highest lipid productivity of 0.054 g/l·day when grown with 1% of glucose (Liang et al., 2009) and 36.62 mg/l·day in 1.5% glucose-supplemented medium (Mandal and Mallick, 2009), respectively. But no large increase of lipid content on their dry cell weight basis could be investigated, which induced that the rise in lipid yield of them was due to the increased biomass. To study the cultivation of Chlorella protothecoides in mixotrophic and autotrophic cultures, a similar result was reported by Heredia-Arroyo et al. (2010). The lipid productivity of P. tricornutum in the present study reached highest value (0.053 g/l·day, Figure 2) at 1 g/L of glucose. The lipid content (weight of total lipids per gram of dry algae cells) increased significantly in carbon-supplemented medium compared with control. So it can be drawn that the enhancement of lipid productivity was induced by the increase of total lipid content and biomass of P. tricornutum simultaneously.

Whatever the carbon source varied, no significant change in the composition of fatty acids of P. tricornutum was observed in this study. With a similar phenomenon in studying the microalga Pavlova lutheri, Guilhauneuf et al. (2009) suggested that desaturation activity can be sustained with a supply of organic carbon under mixotrophic batch cultures. But Garcia et al. (2006) found the EPA level of P. tricornutum increased 2-fold in glycerol-fed cultures compared to the control. The microalgal oils from P. tricornutum contains mainly monounsaturated fatty acids (51-62%) and saturated fatty acids (approximately 27%), which advocates its high oxidative stability. And its linolenic acid (C18:3) content of 2-4% (weight/weight) seems not to exceed the limitation of 12% (mol/mol) in the European Standard EN 14214, which limited the content of linolenic acid methyl ester in biodiesel for vehicle use (Knothe, 2006). Special attention should be taken to the C20:5n3 (EPA, approximately 14%), which is susceptible to oxidation during storage. European biodiesel standards (EN 14214 and 14213) limit the contents of fatty acid methyl esters with four and more double bonds to a maximum of 1% (mol/mol). This implies a requirement of additional treatment such as partial catalytic hydrogenation of the oil (Dijkstra, 2006), which would not be a significant limitation for production of biodiesel (Chisti, 2007).

Mixotrophic growth is in which CO₂ and organic carbon is assimilated simultaneously and could overcome the light limitations imposed by pure photoautotrophic culture (Miao et al., 2006; Wei et al., 2009). Conflict results of growth with glucose and acetate, enhancing or reducing, were reported by many papers with various microalgae (Heifetz et al., 2000; Sanchez et al., 2001; Kang et al., 2004; Heredia-Arroyo et al., 2010). The data in this study suggest that P. tricornutum could yield more biomass and lipids by utilizing organic carbon sources in mixotrophic culture, especially with a suitable concentration of glucose in medium.

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REFERENCES


