Differential fatty acid profiles of *Chlorella kessleri* grown with organic materials

Yan Wang, a* Tao Chen b and Song Qin a

**Abstract**

**BACKGROUND:** Integration of oleaginous microalgae cultivation with wastewater treatment is considered a low-cost approach for manufacturing algae-based biodiesel. However, autotrophic microalgae cannot survive in organic wastewater where the effluent is usually turbid and sunlight cannot penetrate into the wastewater. Thus mixotrophic microalgae should be explored. The objective of this study was to investigate the potential of using mixotrophic *Chlorella kessleri* to produce fatty acids from organic materials.

**RESULTS:** Results revealed that mixotrophic *C. kessleri* fatty acids display much greater productions (up to 54.67% of cell dry weight) and more suitable compositions for biodiesel production (moderate carbon chain length mainly with C16 and C18, down to 1.17 \( \gamma / \text{mol of unsaturation degree} \)) than autotrophic ones. A suitable final pH (near neutral) after nitrate-depletion and a high organic carbon consumption seemed to be the key factors manipulating fatty acids production under whichever organic substrate tested.

**CONCLUSION:** These characteristics increase the acceptability in using mixotrophic *C. kessleri* as a potential easy-control candidate in biodiesel production. If fed with available organic effluent of wastewater as the nutrient supply, *C. kessleri* may have great potential for profitable biodiesel.

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**Keywords:** *Chlorella kessleri;* fatty acid; glucose; glycerol; mixotrophic

**INTRODUCTION**

The use of fossil fuels as energy is now widely accepted as unsustainable due to depleting resources and also due to the accumulation of greenhouse gas in the environment. 1, 2 Alternative fuels are receiving considerable attention. One of the promising alternatives is biodiesel (methyl esters of fatty acids, FAME). 3, 4 The current sources of commercial biodiesel include soybean oil, rapeseed oil, palm oil, corn oil, animal fat and waste cooking oil. However, wide-scale development of biodiesel from those resources cannot realistically satisfy the existing demand for transport fuels. In this regard, microalgae have emerged as promising feedstock owing to their widespread availability and higher oil yields. Moreover, mass production of microalgae requires significantly less land area than crop-based biodiesel and will also not compromise production of food, fodder and other products derived from crops. 1, 5

The current technology for manufacturing algae-based biodiesel, however, is still far from economical due to its high production cost. An investigation of cost extensive approaches for the algal biodiesel production is needed. One promising alternative seems to be the integration of microalgae cultivation with fish-farms, food processing facilities and wastewater treatment plants etc., offering the low-cost nutrient supply required for algal biomass cultivation. 6 This approach not only provides raw materials for the system but also converts waste into resources.

Microalgae commonly grow on sunlight and \( \text{CO}_2 \) in wastewater, removing the remaining nitrogen and phosphorus and heavy metal pollutants. 7, 8 Differently, in the wastewater with a high content of organic compounds, microalgae grow mainly under mixotrophic conditions because rich organic pollutants and high turbidity prevent microalgae growing under sunlight and \( \text{CO}_2 \). 9 Usually, anaerobic digestion (AD) by bacteria has been recommended to degrade organic waste materials. As a matter of fact, AD has limited capability to remove all the pollutants from the wastes and the AD effluent still heavily pollutes the local environment, while at the same time valuable nutrient resources are wasted with direct discharge. 10 Thus, mixotrophic oleaginous microalgae should be explored, as an auxiliary technology, to apply in the case where the effluent of wastewater is usually turbid and sunlight cannot reach the bulk of the wastewater.

*Chlorella kessleri* is a particular green microalga that is available in commercial applications for wastewater treatment purposes and has shown more tolerance than other microalgae species to chromium, copper, herbicide and hexachlorobenzene. 11, 12, 13, 14 Recently, *C. kessleri* was reported to be capable of mixotrophic growth to accumulate fatty acids when cultivated on centrate, which is generated by centrifuging activated sludge and contains

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both organic carbon and inorganic carbon. These suggest potential for integration of C. kessleri cultivation as biodiesel production feedstock with organic wastewater treatment. The objective of this work was to investigate the potential of using C. kessleri to produce fatty acids from other organic materials under mixotrophic conditions. Although very similar to other chlorella species, C. kessleri has its own distinct features and resulted different responses to various organic carbon sources.

MATERIALS AND METHODS

Microalga and growth conditions

The microalga Chlorella kessleri (CGMCC No. 4917) was maintained at 4 °C on an agar slant containing Kuhl medium. Liquid Kuhl medium was inoculated with cells from slants and the alga was grown in flasks at 26 ± 1 °C with orbital shaking at 150 rpm and continual fluorescence light of 150 µmol m−2 s−1 at the flask surface. The cells at the end of the linear growth phase (5 days old) were used as inoculum. An inoculum of 10% (by volume) was inoculated into each 250 mL Erlenmeyer flask containing 100 mL medium. All media were adjusted to pH 6.5 then were autoclaved at 121 °C for 20 min. Different concentrations of glucose (0–300 mmol L−1), glycerol (0–600 mmol L−1), or ethanol (0–100 mmol L−1) were added to the flasks. All the cultures were without an external supply of CO2 and developed over the same growth time (3 weeks).

Determination of algal dry biomass

Algal dry biomass was measured by filtration of selected culture volumes (5–40 mL, depending on culture cell density) through a preweighed WhatmanGF/C glass fibre filter, and dried at 80 °C to constant weight.

Determination of medium pH and nitrate concentration

Medium pH was measured on samples using a standard pH meter. Concentration of nitrate was measured by ion chromatography (IC) using an Ionpac AS23+AG23 Anion-Exchange Column (Dionex) with a 4.5 mmol L−1 Na2CO3 + 0.8 mmol L−1 NaHCO3 eluent set at a flow rate of 1.0 mL min−1. Suppressor was Dionex ASRS300 4 mm. Detection was performed using a conductivity detector.

Fatty acids analysis

Fatty acid methyl esters (FAMEs) were prepared by acid transesterification. Briefly, lyophilized cells were incubated with a solvent mixture of toluene and 1% sulphuric acid in methanol (1 : 2, v/v) overnight at 50 °C to produce FAMEs that were then extracted with hexane. The FAMEs were analyzed using an Agilent 7890 capillary gas chromatograph equipped with a flame ionization detector (FID) and a DB-23 capillary column (30 m × 0.25 mm × 0.25 µm). Nitrogen was used as carrier gas. Temperature programming consisted of 5 min at 140 °C and subsequently increasing to 240 °C at 2.5 °C min−1. The injector was kept as 250 °C with an injection volume of 1 µL under split mode (5 : 1). The FID temperature was set at 260 °C. FAMEs were identified by chromatographic comparison with authentic standards (Sigma). The quantities of individual FAMEs were estimated from the peak areas on the chromatogram using Nonadecanoic acid (Sigma) as the internal standard.

RESULTS AND DISCUSSION

Effect of organic carbon source on cell dry biomass production

The initial glucose concentrations used were 0 mmol L−1 (control), 1, 5, 10, 50, 100, 200 and 300 mmol L−1. As the concentration of glucose increased, the color of the cultures turned from green to yellow. As shown in Fig. 1, higher final dry biomass concentration was obtained with input of higher initial glucose concentration. The greatest cell dry biomass was obtained when the glucose concentration was 300 mmol L−1, reaching 13.00 g L−1. This value was about 7-fold higher than in the autotrophically grown control and is among the highest reported for a microalga in batch cultures.

The initial glycerol concentrations used were 0 mmol L−1 (control), 1, 10, 50, 100, 200, 300 and 600 mmol L−1. For optimal growth of C. kessleri, a glycerol concentration of 200 mmol L−1 is required, increasing cell dry biomass 3-fold more compared with autotrophic culture (Fig. 2). At glycerol concentrations < or > 200 mmol L−1, the cell dry biomass decreased but was still higher than that obtained in the autotrophically grown control. Similar results were observed in other microalgae species under mixotrophic conditions. For example, in a culture media supplemented with 100 mmol L−1 glycerol and 165 µmol photons m−2 s−1, Phaeodactylum tricornutum increased its growth by 74% compared with autotrophic culture. In C. protothecoides, the biomass concentration reached 23–45 g L−1 using a crude glycerol medium.

The initial ethanol concentrations used were 0 mmol L−1 (control), 1, 5, 10, 50, and 100 mmol L−1. As shown in Fig. 3, ethanol supported cell growth under all observed conditions, especially in the cultures at ethanol concentrations of 50 mmol

Figure 1. Effect of gradient concentrations of glucose on cell dry biomass (g L−1) in C. kessleri under mixotrophic conditions. Data are mean values ± SD of three independent measurements.
It is well known that the hexose uptake protein gene (hup1), corresponding to the hexose/H+ symport system protein, is activated when autotrophically grown C. kessleri cells switch to heterotrophic growth in the presence of glucose. This might be possible under the present mixotrophic conditions. Some microalgal species, such as Prymnesium parvum and Dunaliella tertiolecta, are unable to assimilate glucose even though they possess the enzymes necessary for its metabolism.

It is worth noting that the concentration of glucose required for optimal growth in C. kessleri is very different from those reported in other microalgae. For example, to promote cellular growth of C. vulgaris and Scenedesmus acutus, the initial concentration of glucose should be limited to 50 mmol L\(^{-1}\) and 5 mmol L\(^{-1}\), respectively. For optimal growth of C. saccharophila, a concentration of glucose of 15 mmol L\(^{-1}\) is required and inhibition occurred at concentrations >140 mmol L\(^{-1}\); inhibition of C. sorokiniana occurs at concentrations >30 mmol L\(^{-1}\). In trials with Nitzschia laevis, yields decreased as concentration increased from 5 to 200 mmol L\(^{-1}\). C. protothecoides has been cultivated at concentrations as high as 470 mmol L\(^{-1}\) to obtain an optimal yield of biomass. G. sulphuraria grown with high concentrations of glucose or fructose up to 900 mmol L\(^{-1}\) continued to thrive, but higher concentrations inhibited growth. It is suggested that glucose uptake depends mainly on the species of microalgae that is used. The concentration of glucose required for optimal metabolic growth may be related to specific combinations of factors, with the microalgal species as the main factor and cultivation and environmental conditions as secondary factors. Consequently, each combination of factors may lead to different consumption levels. So it seems logical that the optimal concentrations of glucose for different microalgal growth are too scattered to reach a definite conclusion.

**Effect of organic carbon source on cell total fatty acids accumulation**

Over the range of glucose tested, nitrate-starvation occurred under all observed conditions. Higher addition of glucose caused the greatest fatty acids accumulation, especially in cultures with relatively suitable final pH (near neutral) after nitrate depletion. As shown in Fig. 4, in cultures with initial glucose concentration < 10 mmol L\(^{-1}\), the values of final pH showed no significant difference (P > 0.05) between treatments (Fig. 4(B)). Accordingly, their total fatty acids accumulations were similar to each other (Fig. 4(A)). On the contrary, in cultures with initial glucose concentration > 50 mmol L\(^{-1}\), elevated initial glucose concentration led to less medium final pH, combined with more cellular fatty acids accumulation. The greatest total fatty acids production, 54.67% by cell dry weight basis or 7.11 g L\(^{-1}\) by culture volume basis, was obtained when the glucose concentration was 300 mmol L\(^{-1}\), which was about 5 and 37-fold, respectively, higher than in the autotrophically grown control. This value is among the highest reported for Chlorella species in batch cultures. For example, Tan and Johns\(^{26}\) cultivated C. saccharophila with 14 mmol L\(^{-1}\) glucose and found that the lipid content doubled in response to heterotrophic growth when compared with photoautotrophic growth. Miao and Wu\(^{30}\) reported that the content of lipids from heterotrophic C. protothecoides cells was able to reach 55% of the cell dry weight, which was 4 times greater than that obtained from autotrophic cells.

Just as the responses of C. kessleri on glucose, over the range of glycerol tested, nitrate-starvation occurred under all observed conditions. Higher addition of glycerol caused the
greatest fatty acids accumulation, especially in cultures with relatively suitable final pH (near neutral) after nitrate depletion. As shown in Fig. 5, in cultures with initial glycerol concentration < 50 mmol L\(^{-1}\), the values of final pH were all at a relatively high level (Fig. 5(B)). Accordingly, their total fatty acids accumulation exhibited little change compared with the autotrophically grown control (Fig. 5(A)). On the contrary, in cultures with initial ethanol concentration > 100 mmol L\(^{-1}\), all the treatments exhibited relatively lower (near neutral) medium final pH. The highest total fatty acids production occurred in the cultures with optimal relatively lower (near neutral) medium final pH. The highest total fatty acids production was obtained at 50 mmol L\(^{-1}\) ethanol, with 0.58 g L\(^{-1}\), 3-fold higher than the control. Such an increase is mainly attributed to the increase in cell dry biomass induced by ethanol. It is interesting to note that such a small increase in total fatty acids production by ethanol also correlates with relatively suitable final pH after nitrate depletion, just as the responses for glucose and glycerol observed in the present study. Ethanol caused nitrate-starvation under all observed conditions. In cultures with initial ethanol concentration < 10 mmol L\(^{-1}\), the values of final pH showed no significant difference (\(P > 0.05\)) between treatments (Fig. 6(B)). Accordingly, their total fatty acids accumulations were similar (Fig. 6(A)). On the contrary, in cultures with initial ethanol concentration > 50 mmol L\(^{-1}\), elevated initial ethanol concentration led to less medium final pH, combined with more cellular fatty acids accumulation.

Although ethanol showed obvious positive results for cell dry biomass of \(C.\) kessleri, only slight changes in cell total fatty acids accumulation were observed with changes in the concentration of ethanol. As shown in Fig. 6(A), with initial ethanol concentration < 10 mmol L\(^{-1}\), the total fatty acids showed a similar level to, or even less than the autotrophically grown control. At more than 50 mmol L\(^{-1}\) of ethanol supplemented, the total fatty acids slightly increased. Expressed on a dry biomass basis, the greatest total fatty acids production was obtained at 100 mmol L\(^{-1}\) ethanol, with 12.99%. This value is only 1-fold as high as the control culture without supplemented ethanol. Expressed on a culture volume basis, the highest total fatty acids accumulation was obtained at 50 mmol L\(^{-1}\) ethanol, with 0.58 g L\(^{-1}\), 3-fold higher than the control. Such an increase is mainly attributed to the increase in cell dry biomass induced by ethanol. It is interesting to note that such a small increase in total fatty acids production by ethanol also correlates with relatively suitable final pH after nitrate depletion, just as the responses for glucose and glycerol observed in the present study. Ethanol caused nitrate-starvation under all observed conditions. In cultures with initial ethanol concentration < 10 mmol L\(^{-1}\), the values of final pH showed no significant difference (\(P > 0.05\)) between treatments (Fig. 6(B)). Accordingly, their total fatty acids accumulations were similar (Fig. 6(A)). On the contrary, in cultures with initial ethanol concentration > 50 mmol L\(^{-1}\), elevated initial ethanol concentration led to less medium final pH, combined with more cellular fatty acids accumulation.

Taken together, exogenous organic substrates displayed significant positive results in total fatty acids production with declining order: glucose > glycerol > ethanol. Glucose supported the highest total fatty acids production at 300 mmol L\(^{-1}\), with 54.67% or 7.11 g L\(^{-1}\), glycerol at 200 mmol L\(^{-1}\), with 21.19% or 1.32 g L\(^{-1}\), and ethanol at 100 mmol L\(^{-1}\), with 12.99% or at 50 mmol L\(^{-1}\), with 0.58 g L\(^{-1}\). Despite the different degrees, each highest accumulation of total fatty acids seemed to be determined by the combined response of high consumption of exogenous organic carbon sources and a suitable final pH (near neutral) after
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Figure 6. Effect of gradient concentrations of ethanol on production of total fatty acids in C. kessleri (A, mg g⁻¹, g L⁻¹) and final pH value in the medium (B) under mixotrophic conditions. Data are mean values ± SD of three independent measurements.

Effect of organic carbon source on the fatty acids composition

Compositional analysis of the fatty acids collected from control and each best organic carbon concentration culture are shown in Table 1. Results show that the fatty acid composition in C. kessleri was predominated by C14:0, C16:0, C16:1, C16:2, C16:3, C18:0, C18:1, C18:2, and C18:3, not dependent on the type of supplemented organic carbon source. C16 and C18 altogether accounted for more than 95% of total fatty acids. This composition was also the same as those reported for other Chlorella species including autotrophic C. kessleri. Fatty acids are primary metabolites of acetyl CoA pathway, which is genetically determined, evolutionarily very old, and therefore conservative. Therefore, it seems logical that C. kessleri shows similar qualitative composition of fatty acids to other Chlorella species.

With change in the nature of supplemented organic carbon sources, mixotrophic C. kessleri cells displayed different values of both the percentage of individual fatty acid and the degree of fatty acids unsaturation. As shown in Table 1, in the control culture, the percentages of C16 and C18 in total fatty acids were 36.41% and 63.03%, respectively, while saturated fatty acids were only at 15.85% of total fatty acids and the degree of fatty acids unsaturation was at 1.87 \( \mu \text{mol} \). When ethanol was supplemented, saturated fatty acids increased accompanied with decreased unsaturated ones and the degree of fatty acids unsaturation decreased to 1.69 \( \mu \text{mol} \), despite similar quantitative composition of C16 (35.10%) and C18 (63.68%). When glycerol was added, more C16 (49.81%) and less C18 (46.10%) accumulated than in the control. Moreover, saturated fatty acids significantly increased to 51.15% of total fatty acids and unsaturated ones decreased with a very low level of fatty acids unsaturation at 1.17 \( \mu \text{mol} \). When glucose was used, less C16 (25.11%) and more C18 (73.51%) accumulated than in the control, while the degree of fatty acids unsaturation decreased to 1.47 \( \mu \text{mol} \).

It is well known that unsaturation degree and carbon chain length of fatty acids are key factors to determine the properties of biodiesel (e.g. cetane number, viscosity, cold flow, oxidative stability, and iodine value). Present results revealed that mixotrophic C. kessleri cells accumulated predominantly C16 and C18, which altogether accounted for more than 95% of total fatty acids. Moreover, they contained no polyunsaturated fatty acids with four or more double bonds and exhibited low unsaturation levels of fatty acids. Such composition and structure of fatty acid, together with the much higher accumulation of total fatty acids, increase the acceptability of using mixotrophic C. kessleri as future industrial biodiesel producer. If fed with available organic effluent of wastewater as the nutrient supply, C. kessleri may have the potential for profitable biodiesel.

CONCLUSION

Compared with autotrophic fatty acids, mixotrophic C. kessleri fatty acids display much greater production and more suitable composition for biodiesel production. For the different types of organic substrate tested, each highest accumulation of total fatty acids seemed to be determined by the combined response of high consumption of exogenous organic carbon sources and a suitable final pH (near neutral) after nitrate-depletion. Glucose is the preferred organic carbon source for C. kessleri growth and fatty acids accumulation, supporting the highest total fatty acids production at 300 mM with the value of 54.67% or 7.11 g l⁻¹. These results indicate the potential of this microalga strain as an easy-control candidate in biodiesel production. If fed with available organic effluent of wastewater as the nutrient supply, C. kessleri may provide profitable biodiesel.
Table 1. Compositional analysis of C. kessleri fatty acids collected from control and each best organic carbon concentration culture

<table>
<thead>
<tr>
<th>Fatty acid (% total fatty acids, w/w)</th>
<th>Control (0 mM)</th>
<th>Ethanol (50 mM)</th>
<th>Glycerol (200 mM)</th>
<th>Glucose (300 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>0.56 ± 0.01</td>
<td>1.21 ± 0.02</td>
<td>4.09 ± 0.15</td>
<td>1.38 ± 0.06</td>
</tr>
<tr>
<td>C16:0</td>
<td>14.65 ± 0.50</td>
<td>21.07 ± 1.10</td>
<td>41.17 ± 1.80</td>
<td>22.17 ± 1.95</td>
</tr>
<tr>
<td>C16:1</td>
<td>10.71 ± 0.30</td>
<td>7.08 ± 0.35</td>
<td>3.03 ± 0.12</td>
<td>1.50 ± 0.08</td>
</tr>
<tr>
<td>C16:2</td>
<td>9.06 ± 0.20</td>
<td>5.25 ± 0.15</td>
<td>3.59 ± 0.10</td>
<td>0.51 ± 0.02</td>
</tr>
<tr>
<td>C16:3</td>
<td>2.00 ± 0.08</td>
<td>1.70 ± 0.10</td>
<td>2.01 ± 0.09</td>
<td>0.93 ± 0.03</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.65 ± 0.01</td>
<td>2.21 ± 0.10</td>
<td>5.89 ± 0.18</td>
<td>8.77 ± 0.10</td>
</tr>
<tr>
<td>C18:1</td>
<td>2.63 ± 0.08</td>
<td>3.75 ± 0.06</td>
<td>3.71 ± 0.10</td>
<td>16.16 ± 0.50</td>
</tr>
<tr>
<td>C18:2</td>
<td>30.01 ± 1.10</td>
<td>30.16 ± 0.95</td>
<td>12.30 ± 0.50</td>
<td>19.96 ± 0.80</td>
</tr>
<tr>
<td>C18:3</td>
<td>29.75 ± 0.90</td>
<td>27.56 ± 1.10</td>
<td>24.20 ± 0.80</td>
<td>28.62 ± 0.90</td>
</tr>
<tr>
<td>C16 fatty acids</td>
<td>36.41 ± 1.08</td>
<td>35.11 ± 1.70</td>
<td>49.81 ± 2.11</td>
<td>25.11 ± 2.08</td>
</tr>
<tr>
<td>C18 fatty acids</td>
<td>63.03 ± 2.09</td>
<td>63.68 ± 2.21</td>
<td>46.10 ± 1.58</td>
<td>73.51 ± 2.30</td>
</tr>
<tr>
<td>Saturated fatty acids</td>
<td>15.85 ± 0.52</td>
<td>24.49 ± 1.22</td>
<td>51.15 ± 2.13</td>
<td>32.31 ± 2.11</td>
</tr>
<tr>
<td>Mono-unsaturated fatty acids</td>
<td>13.34 ± 0.38</td>
<td>10.84 ± 0.41</td>
<td>6.74 ± 0.22</td>
<td>17.67 ± 0.58</td>
</tr>
<tr>
<td>Poly-unsaturated fatty acids</td>
<td>70.81 ± 2.28</td>
<td>64.67 ± 2.30</td>
<td>42.11 ± 1.49</td>
<td>50.02 ± 1.75</td>
</tr>
<tr>
<td>Degree of fatty acids unsaturation (V/mol)</td>
<td>1.87 ± 0.06</td>
<td>1.69 ± 0.06</td>
<td>1.17 ± 0.04</td>
<td>1.47 ± 0.05</td>
</tr>
<tr>
<td>Total fatty acids (g l⁻¹)</td>
<td>0.19 ± 0.02</td>
<td>0.58 ± 0.03</td>
<td>1.32 ± 0.12</td>
<td>7.11 ± 0.13</td>
</tr>
<tr>
<td>Total fatty acids (% dry weight)</td>
<td>10.20 ± 0.20</td>
<td>12.54 ± 0.30</td>
<td>21.19 ± 0.30</td>
<td>54.67 ± 0.55</td>
</tr>
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</table>

ACKNOWLEDGEMENTS
This work was supported by National Natural Science Foundation of China (No. 50904051), Science and Technology Planning Project of Yantai, China (No. 2010247), and open fund of Shandong Oriental Ocean Sci-Tech Co., Ltd (No. 200803).

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