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ORIGINAL PAPER

Effects of cassava starch hydrolysate on cell growth and lipid accumulation of the heterotrophic microalgae *Chlorella protothecoides*

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Abstract Heterotrophic fermentation of microalgae has been shown to accumulate high amounts of microalgal lipids, which are regarded as one of the most promising feedstocks for sustainable biodiesel production. To increase the biomass and reduce the cost of microalgal culture, the purpose of this study was to evaluate the possibility of using cassava starch hydrolysate (CSH) instead of glucose as carbon source for heterotrophic culture of *Chlorella protothecoides* in flasks. First, the two-step enzymatic process of hydrolysis of cassava starch by α-amylase and glucoamylase was optimized; the conversion efficiency for cassava starch was up to 97.7%, and over 80% of CSH was glucose. Subsequently, we compared heterotrophic cultures of *C. protothecoiedes* using glucose or CSH as carbon source. The results demonstrated that when using CSH as the organic carbon source, the highest biomass and the maximum total lipid yield obtained were 15.8 and 4.19 g/L, representing increases of 42.3 and 27.7%, respectively, compared to using glucose as the organic carbon source. This suggests that CSH is a better carbon source than glucose for heterotrophic *Chlorella protothecoides*.

Keywords Cassava starch · Hydrolysis · Microalgae · Lipid · Biodiesel

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Introduction

Biodiesel refers to alkyl esters of fatty acids from vegetable oils or animal fats. Usually, the required characteristics of fatty acids for biodiesel are long-chain and unbranched fatty acids with 16–20 carbons; high stability; low water and volatiles content; a low amount of polymers containing sulfur and nitrogen elements, etc. [2]. Recently, biodiesel has received more attention due to the fact that it is a renewable, biodegradable and environmentally friendly fuel. Current sources of commercial biodiesel are primarily soybean oil, rapeseed oil, palm oil, corn oil, waste cooking oil and animal fat. However, these sources of biodiesel cannot realistically meet even a small fraction of the existing demand for transportation fuels [2]. Microalgae have become one of the most promising feedstocks for biodiesel production due to their widespread availability and high oil content. Moreover, microalgae, in contrast to traditional oilseed crops, can grow in ponds, waste water or fermentors, and thus avoid occupying land required for crops and forests [8].

A number of microalgal species, for example, *Botryococcus* species [6, 9], *Chlorella* species [4, 8, 17, 18], *Dunaliella* species [3, 14], *Nannochloris* species [13], *Neochloris* species [7, 15], and *Parietochloris* species [1], have been shown to have the capacity of accumulating large quantities of lipids in microalgal cells under appropriate conditions. *Chlorella* strains have great potential as a

resource for biodiesel production due to their relatively fast growth and easy cultivation. Recently, a novel approach—heterotrophic culture of *Chlorella protothecoides*—has been developed for high quality biodiesel production [18]. Compared to classical photoautotrophic culture, heterotrophic fermentation of *Chlorella* can reach higher biomass concentrations and accumulate much higher lipid contents [10]. However, a major challenge in heterotrophic culture of microalgae for biodiesel production is its relatively high cost.

One critical means of decreasing the cost of biodiesel production from heterotrophic fermentation of *Chlorella* is to reduce the cost of culture medium, which represents about 50% of the cost of heterotrophic culture of *Chlorella*. Further, glucose is estimated to account for around 80% of total medium costs [5]. Therefore, using low cost materials (such as starch or cellulose-hydrolyzed solution) instead of glucose is a good strategy. Recently, corn powder hydrolysate has been shown to be a realistic substitution for heterotrophic culture of *Chlorella protothecoides* [18]. At present, cassava is relatively cheap compared to corn, hence it could be an ideal carbon source for cultivating *Chlorella*. The objective of this study was to investigate the effects of cassava starch hydrolysate (CSH) on cell growth and lipid accumulation in heterotrophic *Chlorella protothecoides*, evaluating the possibility of replacing glucose with CSH as an organic carbon source for heterotrophic microalgae.

Materials and methods

Hydrolysis of cassava starch

A two-step enzymatic hydrolysis procedure using commercially available α-amylase (5,400 U/g) and glucoamylase (10^5 U/g), both from Aoboxing Company, Beijing, China, was used to hydrolyze cassava starch obtained from a local supermarket. Firstly, 20 g cassava starch was mixed (1:4, w/v) with a buffer solution of citric acid and Na_2HPO_4, slurried in a thermostated water bath shaker (59°C) for 30 min and cooled to room temperature; $CaCl_2$ solution (0.01 mol/L) and α-amylase were introduced for liquefaction at 55°C and pH 5.6. After 30 min, the solution was boiled to deactivate the enzymes, cooled to room temperature and the pH was adjusted to 4.0. Glucoamylase was added to perform saccharification at 75°C for 4–8 h, then the enzyme was deactivated (boiling by microwave), and the solution was centrifuged and filtered to obtain CSH.

Algal strain, medium and culture conditions

The green microalga *Chlorella protothecoides* was purchased from the CSIRO Marine Laboratory, Hobart, Australia. The components of modified basal culture medium comprised (per liter) 3 g urea, 1.25 g KH_2PO_4, 1 g $MgSO_4{\cdot}7H_2O$, 0.5 g EDTA, 114.2 mg boron, 83.75 mg $CaCl_2$, 49.8 mg $FeSO_4{\cdot}7H_2O$, 38.2 mg $ZnSO_4{\cdot}7H_2O$, 14.4 mg $MnCl_2{\cdot}4H_2O$, 7.1 mg MoO_3, 15.7 mg $CuSO_4{\cdot}5H_2O$, and 4.9 mg $CoNO_3{\cdot}6H_2O$. To prevent the inhibitory effects of high nitrogen concentration on cell growth, 0.05 mol/L urea was selected. To compare cultures using glucose or CSH as carbon source, five levels of glucose concentration (10, 20, 30, 40 and 50 g/L) were used in the glucose-based medium; the same five glucose concentrations (10, 20, 30, 40 and 50 g/L) were also used in the CSH-based medium by adding appropriate volumes of CSH solution to the basal medium to achieve the necessary glucose concentration, for example, given a solution of CSH with 128.6 mg/mL glucose content, to prepare 100 mL CSH-based medium in which the glucose concentration is 10, 20, 30, 40, or 50 g/L, the corresponding amount of CSH solution needed was 7.8, 15.6, 23.4, 31.1, or 38.9 mL, respectively. Both glucose-based and CSH-based media were adjusted to pH 6.0 and autoclaved, alga (to 5%) was inoculated into an Erlenmeyer flask (250 mL, containing 100 mL medium) and cultivated for 240 h in an orbital shaker (160 rpm) at a temperature of 28°C and no illumination (dark conditions).

Analytical methods

Measurement of reducing sugars and glucose in CSH

The content of reducing sugars in CSH was determined by the 3,5-dinitrosalicylic acid method [11]. A standard curve was drawn by measuring the absorbance of known concentrations of glucose solutions at 540 nm and the concentration of reducing sugar was calculated. The glucose concentration in CSH was measured using an SBA-80 biosensor.

Analysis of carbohydrate composition in CSH

Carbohydrate profiles in CSH were analyzed by HPLC [12]. Samples (20 μL) were injected into a Sugar Pak column (6.5 × 300 mm) operating on Waters 600 Controller machine with a Waters 2414 RID detector; the working temperature was 90°C and the flow rate was 0.5 mL/min. The mobile phase consisted of EDTA-Ca^{2+} (0.05 g/L) and 0.01 mol/L oligosaccharide standards, purchased from Sigma (St. Louis, MO).

Biomass and residual glucose measurement

For biomass measurement, 5 mL culture fluid was transferred to a pre-weighed centrifuge tube and centrifuged at

3,000 rpm for 10 min. After rinsing three times with distilled water, the pellet was dried at 50°C to constant weight and cooled down to room temperature in a desiccator, then the dry cell weight was determined [16]. The residual glucose concentration in the culture fluid was measured using an SBA-80 biosensor.

Total lipid measurement

To determine the total lipid content in algal cells, dried algal cells were blended with 0.5 mL distilled water and 3 mL chloroform/methanol (2:1), shaken for 20 min and centrifuged (10,000 rpm) for 10 min, collecting the chloroform phase, and repeating the same process five times. All the chloroform phases were collected together, evaporated and dried to constant weight under vacuum conditions, and finally weighed to give the total lipid content.

Fatty acids analysis

Fatty acids in algal cells were extracted with 1 mL NaOH–CH_3OH from 20 mg dried algal cells. The mixture was shaken for 10 min at 75°C in a thermostated water bath, cooled to room temperature, 2 mL boron trifluoride–methanol (1:2) was added, and shaken again for 10 min at 75°C in a thermostated water bath, cooled to room temperature, 0.3 mL saturated salt solution was added, leading to the layered solution. Finally, 2 mL hexane was introduced and centrifuged, the upper-layer fatty acids layer was subject to GC–MS analysis on an Agilent HP 6890N machine equipped with a 5975 inert mass selective detector and G1540N-210 FID detector. A capillary column (Agilent DB-23; 30 m × 250 μm × 0.25 μm) was used, the carrier gas was helium at a flow rate of 1 mL/min, the split ratio was 20:1, the injection volume was 1 μL, the solvent delay time was 3 min, the injection temperature was 250°C and the flame ionization detector (FID) temperature was 270°C. The initial oven temperature was at 130°C for 1 min, increased to 200°C at a rate of 5°C/min and held at this temperature for 5 min. Fatty acids were identified by autoscan and direct comparison of their mass spectral pattern and retention index with the NIST 05 mass spectral database. The quantities of individual fatty acids were estimated from the peak area on the FID chromatogram using nonadecanoic acid (C19:0) as the internal standard.

Results and discussion

Cassava starch hydrolysis

Many factors, including substrate concentration, type and concentration of the enzyme used, and other process conditions like temperature, pH, the stirring rate, etc., influence the degree of cassava starch hydrolysis. In order to achieve an appropriate liquefaction of cassava starch, we first investigated the effects of the concentration of α-amylase and the hydrolysis time on the degree of hydrolysis; the ratio of substrate concentration to buffer was set at 1:4 (w/v). The results indicate that the dextrose equivalent (DE) of the reducing sugars in CSH increased linearly with increased enzyme activity within 30 min of enzymatic analysis. Naturally, lower enzyme concentrations over a longer time of enzyme reaction could achieve the same conversion. However, it is noted that the longer application of high temperature (needed for starch gelatinization) will consume more electrical energy and could also lead to slight enzyme inactivation.

Next, we determined the optimal degree of liquefaction of cassava starch suspension required for subsequent saccharification. To achieve this goal, the same concentration of glucoamylase was added to the liquefied cassava starch suspensions with various degrees of liquefaction (corresponding to different DE values) for 4 h of saccharification. The results show that, after the actions of liquefying and saccharifying enzymes, the maximal DE value was obtained when the post-liquefaction DE value was around 17%. Therefore, saccharification was performed at this DE value of liquefaction.

Furthermore, we tried to determine the optimal concentration of glucoamylase, the important saccharifying enzyme essential for complete conversion of starch to glucose. Under the same post-liquefaction DE value (up to 17%) with 200 U α-amylase for 30 min, the effects of different amounts of glucoamylase (5,040, 7,560 and 10,080 U) on the final DE values of CSH are presented in Fig. 1. The results demonstrate that both the amount of enzyme and the hydrolysis time have significant influences on DE values

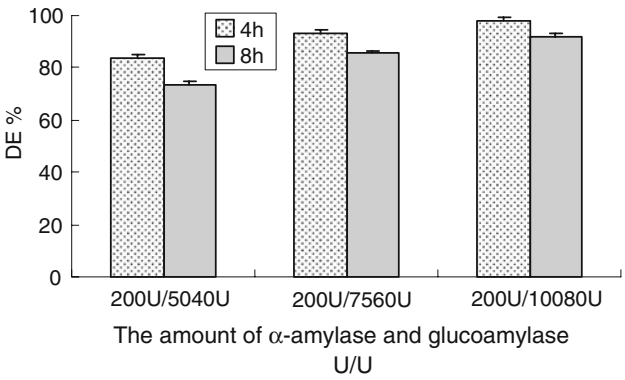

Fig. 1 Influence of the amount of glucoamylase on the dextrose equivalent (DE) value of hydrolysates. Process conditions for α-amylase: the ratio of substrate concentration to buffer 1:4 (w/v); 55°C; pH 5.6; *A* = 200 U; 30 min. Process conditions for glucoamylase: 75°C; pH 4.0; *A* = 5,040, 7,560 or 10,080 U; 4 and 8 h

($P < 0.05$). The conversion efficiency within 4 h is better than that within 8 h, and the two-step enzymatic hydrolysis process is more efficient than the one-step enzymatic hydrolysis process. The highest DE value (97.7%) was reached with the combined action of 200 U α-amylase and 10,080 U glucoamylase after 4 h. However, a similar conversion (DE = 93.43%) was also achieved after 4 h of two-step enzymatic hydrolysis with 200 U α-amylase and 7,560 U glucoamylase, suggesting that the effective conversion of cassava starch can be realized with a lower amount of enzyme.

Finally, the carbohydrate composition of CSH was analyzed by HPLC. By comparing retention times with those of oligosaccharides standards, the main peaks 1–3 were determined as maltotriose, maltose and glucose, respectively. By integrating the peak area, the compositional ratio among the three main components is glucose:maltose:maltotriose = 25:5:1, i.e., their relative percentage in the final hydrolysate is 80.65, 16.13 and 3.23%, respectively. This indicates that CSH, with more than 80% of glucose content, could be a good carbon source for subsequent heterotrophic culture experiments. In a word, a satisfactory conversion rate for cassava starch (up to 97.7%) was achieved with the two enzymes used in this study.

Comparison of glucose versus CSH as carbon source for heterotrophic *Chlorella*

In the culture (240 h) with glucose as carbon source, increased glucose concentration resulted in increased biomass but a decreased content of total lipid (Fig. 2a). At 10 g/L glucose, the total lipid content reached the highest value (34.5%), while biomass was lowest; at 50 g/L glucose, biomass reached a maximum of 11.1 g/L, but the total lipid content was only 26%. This means that a lower concentration of glucose is best for accumulation of total lipid, but may decrease biomass. Overall, 40 g/L glucose is a good choice for both biomass (10.7 g/L) and total lipid content (30.7%), with the highest total lipid yield: biomass (10.7 g/L) × total lipid content (30.7%) = 3.28 g/L.

On the other hand, in the culture (240 h) using CSH as carbon source, when the glucose concentration increased from 10 to 50 g/L, the total lipid content basically decreases from 28.5 to 22%, while biomass first increases and then decreases, with the maximum biomass of 15.8 g/L being achieved at 30 g/L glucose in CSH (Fig. 2b). Thus, higher or lower glucose concentration in CSH will inhibit the growth of *Chlorella*. Considering both biomass and lipid content, 30 g/L glucose in CSH should be selected for heterotrophic culture of *Chlorella*, giving the highest biomass (15.8 g/L) and a moderate total lipid content (26.5%) with maximal total lipid yield: biomass (15.8 g/L) × total lipid content (26.5%) = 4.19 g/L.

Time-course profiles (240 h) of cell growth and glucose consumption using glucose (40 g/L) or CSH (glucose

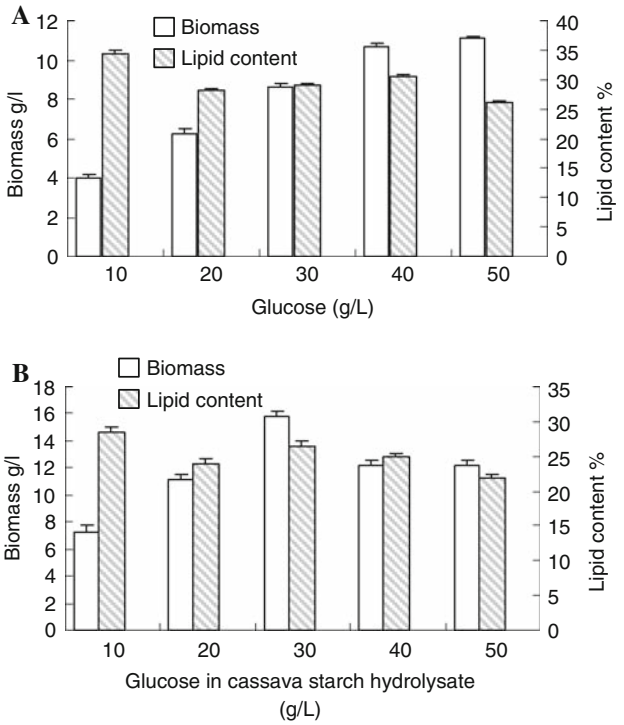

Fig. 2 Biomass and total lipid content of heterotrophic *Chlorella protothecoides* using **a** glucose or **b** cassava starch hydrolysate as carbon source

concentration is 30 g/L) as carbon source, i.e., the concentrations of each substrate giving optimal total lipid yields, are shown in Fig. 3a and b, respectively.

Furthermore, fatty acid composition was analyzed by GC–MS. Specific fatty acid compositions in glucose-based or CSH-based cultures are summarized in Tables 1 and 2, respectively. The results show that nine types of saturated and unsaturated fatty acids, with carbon chain lengths from C16 to C18 exist in the cells of heterotrophic *Chlorella*, which is ideal for biodiesel production. C16:0, C16:2, C18:1, C18:2 and C18:3 are the main fatty acids; in particular, the content of C18 fatty acid amounts to 58–72% of the total fatty acids in microalgal cells, regardless of whether glucose or CSH is used as carbon source. However, it was noted that the maximum total fatty acid yield in glucose-based cultures (achieved at 40 g/L glucose) was 1.39 g/L, while the maximum total fatty acid yield for CSH-based cultures (achieved at 30 g/L glucose in CSH) was 1.69 g/L.

Taken together, using CSH as an organic carbon source, the highest biomass is increased by 42.3% [(15.8 − 11.1)/11.1 × 100%], the maximum total lipid yield is increased by 27.7% [(4.19 − 3.28)/3.28 × 100%], and the maximum total fatty acids yield is increased by 21.6% [(1.69 − 1.39)/1.39 × 100%], compared to using glucose as an organic carbon source. This means that more effective accumulation of total lipid and fatty acids in microalgal cells can be achieved by using CSH instead of glucose as organic carbon

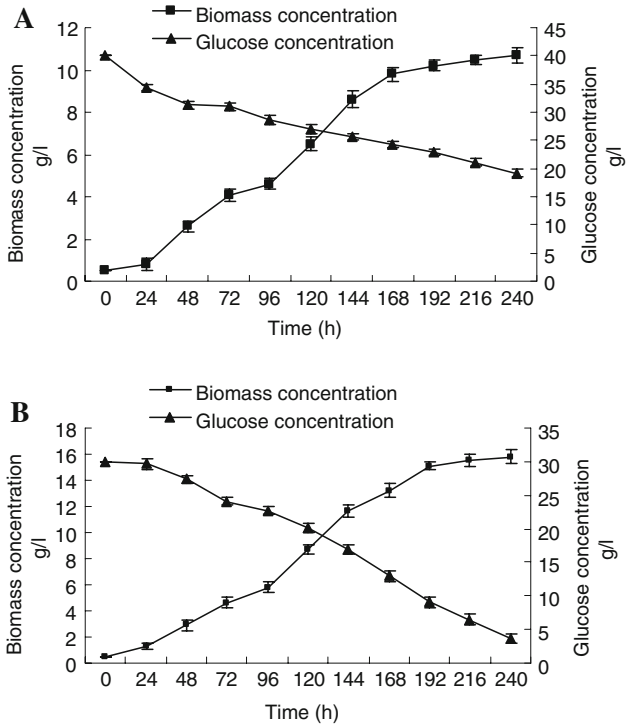

Fig. 3 Time-course profiles of cell growth and glucose consumption using **a** glucose (40 g/L) or **b** CSH (glucose concentration in CSH is 30 g/L), i.e., the concentrations of each substrate giving optimal total lipid yields, as carbon source

source. Besides, cassava starch is less costly than glucose (of course, enzyme, labor and energy costs for processing cassava starch into CSH should also be considered), which would be an important advantage in industrial processes. Thus, CSH is a superior carbon source to glucose for heterotrophic *C. protothecoides*.

It is worth noting that, in other research, a high lipid content in heterotrophic cultivation of *C. protothecoides* in flasks was reported by Xu et al. [18], i.e., 54.7 and 55.3% with glucose and corn powder hydrolysate, respectively, as substrate, but the corresponding biomass was only 3.74 and 3.92 g/L, hence the lipid yield was 2.05 and 2.17 g/L, respectively. Our lipid yield using glucose as carbon source is 1.5- to 1.6-fold more than these lipid yields, and when using CSH as carbon source our lipid yield is 1.9- to 2.0-fold greater. These differences could be caused by different culture conditions, e.g., nitrogen source (glycine [18] vs. urea); most importantly, although the same species of *C. protothecoides* was used, the algal strains from the two different laboratories differ, suggesting that the effect of carbon/nitrogen source on cell growth and lipid accumulation in heterotrophic microalga might be not only species-specific but also strain-specific. For an algal species to be ideal for biodiesel production, both high lipid content and high biomass are needed, because higher lipid content but lower biomass could result in lower lipid yield, thus limiting industrial application. In fact, some algal species are known to contain a large amount of intracellular lipids, e.g., the lipid content in cells of the marine microalga *Dunaliella tertiolecta* [14] can be up to 70%, and even as much as 80% in cells of the freshwater species *Neochloris oleoabundans* [15], but because their biomass concentrations are only about 1–3 g/L, significant improvement would be required.

Conclusion

The present study showed that a two-step enzymatic hydrolysis of cassava starch is more efficient than a one-step enzymatic hydrolysis process. The highest DE value (97.7%) was reached with the combined action of 200 U α-amylase and 10,080 U glucoamylase after 4 h. The primary carbohydrates in CSH analyzed by HPLC were determined as maltotriose, maltose and glucose. By integrating the peak area, the compositional ratio was glucose:

Table 1 Fatty acid compositions and yields in heterotrophic *Chlorella protothecoides* using glucose as carbon source

Relative content of fatty acid (%)	Glucose (g/L)				
	10	20	30	40	50
C16:0	27.27 ± 0.64	22.71 ± 0.74	20.54 ± 0.19	19.44 ± 0.53	20.44 ± 0.32
C16:1 (D9)	2.20 ± 0.46	2.90 ± 0.05	3.06 ± 0.07	3.43 ± 0.46	3.00 ± 0.04
C16:2 (D7, 10)	10.16 ± 0.14	7.88 ± 0.14	7.70 ± 0.18	7.25 ± 0.20	7.14 ± 0.14
C16:3 (D7, 10, 13)	1.24 ± 0.14	0.91 ± 0.07	0.97 ± 0.02	1.11 ± 0.20	1.23 ± 0.06
C17:0	0.96 ± 0.08	0.44 ± 0.04	0.47 ± 0.01	0.69 ± 0.38	0.54 ± 0.02
C18:0	0.57 ± 0.05	3.30 ± 0.04	3.96 ± 0.03	4.40 ± 0.77	3.80 ± 0.20
C18:1 (D9)	8.62 ± 0.85	24.82 ± 0.91	27.11 ± 0.88	29.46 ± 0.73	27.45 ± 1.27
C18:2 (D9, 12)	39.87 ± 0.74	31.08 ± 0.46	30.17 ± 0.60	27.91 ± 1.26	28.90 ± 0.58
C18:3 (D9, 12, 15)	9.18 ± 0.61	5.96 ± 0.24	6.00 ± 0.09	6.29 ± 0.66	7.49 ± 0.43
TFA/DW (%)[a]	7.02 ± 0.19	9.13 ± 0.13	10.93 ± 0.10	12.96 ± 0.26	11.35 ± 0.15
TFA/TL (%)[b]	20.34 ± 0.55	32.61 ± 0.46	37.68 ± 0.34	42.49 ± 0.85	43.65 ± 0.58
Yield of TFA (g/L)	0.28 ± 0.01	0.57 ± 0.01	0.7 ± 0.01	1.39 ± 0.01	1.26 ± 0.01

[a] Total fatty acids/dry cell weight × 100%

[b] Total fatty acids/total lipids × 100%

Table 2 Fatty acid compositions and yields in heterotrophic *C. protothecoides* using cassava starch hydrolysate (CSH) as carbon source

Relative content of fatty acid (%)	Glucose in cassava starch hydrolysate (g/L)				
	10	20	30	40	50
C16:0	24.78 ± 0.83	22.45 ± 1.88	21.53 ± 0.42	17.93 ± 0.90	16.87 ± 1.92
C16:1 (D9)	1.37 ± 0.25	3.29 ± 0.04	3.40 ± 0.29	2.37 ± 0.06	2.54 ± 0.18
C16:2 (D7, 10)	11.94 ± 0.39	11.18 ± 0.01	11.02 ± 0.67	6.30 ± 0.27	6.31 ± 0.25
C16:3 (D7, 10, 13)	1.47 ± 0.22	0.83 ± 0.22	0.92 ± 0.13	1.70 ± 0.03	1.38 ± 0.09
C17:0	0.89 ± 0.16	0.57 ± 0.10	0.45 ± 0.03	0.54 ± 0.04	0.57 ± 0.13
C18:0	0.53 ± 0.06	0.44 ± 0.06	0.70 ± 0.13	0.91 ± 0.09	0.99 ± 0.03
C18:1 (D9)	8.12 ± 1.26	19.84 ± 2.60	24.65 ± 1.48	34.06 ± 1.06	37.16 ± 2.67
C18:2 (D9, 12)	40.92 ± 0.40	35.25 ± 0.86	31.37 ± 1.35	27.26 ± 0.61	26.36 ± 0.72
C18:3 (D9, 12, 15)	9.98 ± 1.02	6.15 ± 1.24	5.96 ± 0.59	9.06 ± 0.09	7.83 ± 0.43
TFA/DW (%)[a]	7.31 ± 0.21	8.13 ± 0.35	10.72 ± 0.25	11.02 ± 0.16	9.98 ± 0.32
TFA/TL (%)[b]	25.65 ± 0.74	33.88 ± 1.46	40.45 ± 0.94	44.08 ± 0.64	45.36 ± 1.45
Yield of TFA (g/L)	0.53 ± 0.01	0.90 ± 0.02	1.69 ± 0.01	1.34 ± 0.01	1.22 ± 0.02

[a] Total fatty acids/dry cell weight × 100%

[b] Total fatty acids/total lipids × 100%

maltose:maltotriose = 25:5:1, i.e., their relative percentages in the final hydrolysate were 80.65, 16.13 and 3.23%, respectively. This indicates that CSH, with a glucose content of more than 80%, could be a good carbon source for heterotrophic culture of *Chlorella*.

Subsequently, a comparative study of heterotrophic *C. protothecoides* using glucose or CSH as carbon source was performed. The results demonstrated that, using CSH as organic carbon source, the highest biomass increased by 42.3%, maximum total lipid yield increased 27.7%, and maximum total fatty acid yield increased 21.6%, compared to using glucose as organic carbon source. Furthermore, cassava starch is less costly than glucose (of course, the enzyme, labor and energy costs for processing cassava starch into CSH should also be considered), which would be an important advantage in industrial processes. Therefore, CSH is a good carbon source, and superior to glucose, for heterotrophic *C. protothecoides*.

Moreover, the fatty acid composition determined by GC–MS showed that nine types of saturated and unsaturated fatty acids of carbon chain length from C16 to C18 exist in heterotrophic *Chlorella* cells, with C16:0, C16:2, C18:1, C18:2 and C18:3 being the main fatty acids. Specifically, C18 fatty acids amounted to 58–72% of the total fatty acids in microalgal cells, revealing that microalgal lipids from heterotrophic *C. protothecoides* are ideal for biodiesel production.

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