Effect of Adding Cellulose on Lipid Accumulation in Cellulbiohydrolase and Delta-6 Desaturase Engineered *Mucor circinelloides* Strains

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**Abstract:** Despite microbial production of multifunctional gamma-linolenic acid (GLA) being an important and promising way, the high cost is still a major factor limiting industrial applications. Previously, we proposed a novel approach to promote the production of GLA from cellulose, a widely available and cheapest resource, by overexpression of the cellulbiohydrolase (CBH2) and delta-6 desaturase (D6) in *Mucor circinelloides*. In the present study, an in-depth analysis of growth and lipid accumulation in engineered *M. circinelloides* strains using cellulosic substrate was to be explored. When cultivated in modified Kendrick and Ratledge (K and R) medium supplemented with the ratio of 1:1 for glucose and carboxymethyl cellulose (CMC), the cellulbiohydrolase and delta-6 desaturase overexpressing strains led to increases in the biomass (up to 10.4 g/L) and lipid yield (up to 2.6 g/L) of 68% and 2.2-fold, respectively, compared with that of the control strain. The yield of GLA in the cellulbiohydrolase and delta-6 desaturase co-expression strains reached 415 mg/L, which was a remarkable increase of 3.1-fold compared to that of the control strain. These results proved that the introduction of CBH2 and D6 two key enzymes could significantly enhance the utilization of CMC and effectively convert cellulose into GLA by engineered *M. circinelloides*. This study provided a reference for the further potential application of the engineered *M. circinelloides* strains in the conversion of functional GLA from cellulose.

**Keywords:** \(\Gamma\)-Linolenic Acid, *Mucor circinelloides*, Cellulose, Cellulbiohydrolase, Delta-6 Desaturase

**Introduction**

Gamma-linolenic acid (GLA, 18:3) is an essential n-6 polyunsaturated fatty acid, that has anti-cancer, anti-tumor, anti-atherosclerosis, and hypoglycemic and lipid-lowering effects (Miyake et al., 2020; Ide, 2023; Paredes et al., 2023; Zhang et al., 2022). Because of its unique physiological impacts, GLA will be increasingly in demand as a source of nutraceuticals and medicines. However, the traditional production cost of GLA is relatively high. Microbial fermentation to produce GLA is an important and promising way. *Mucor circinelloides* was one of the first strains to be used for industrial production of GLA, but it was discontinued due to cost concerns (Andrade et al., 2014; Fazili et al., 2022; Zhang and Song, 2021; Zhang et al., 2023a). Therefore, it is feasible to reduce the cost of GLA biosynthesis by using cheap raw materials, such as cellulose, the widely available and cheapest resources.

In general, the use of cellulosic raw materials includes the process of cellulose degradation and its subsequent fermentation and biotransformation (Singhania et al., 2022). Cellulosic raw materials have been used in many fields and some products (such as ethanol) have been successfully scaled up to industrial production levels (Ismail et al., 2022; Plioni et al., 2022). However, the production of microbial lipids from cellulosic raw materials is still in its infancy. The commonly used microbial production of lipids from cellulose must first degrade cellulose into small molecules of available monosaccharides and then transform into lipids by
microbial fermentation. Most of the cellulose treatment processes use the traditional acid-base method, but there are a series of problems such as toxic by-product inhibition, cumbersome steps, high process cost, and serious pollution (Vadivel et al., 2017). At present, most research has focused on screening suitable strains to produce lipids from cellulotic feedstock hydrolysates. In the long run, the direct use of microorganisms that not only have the ability to produce cellulase to degrade cellulose but also can accumulate a high content of lipids, can realize the synchronization of cellulose-monosaccharide-lipids in one step and greatly reduce the production cost.

Since most oleaginous microorganisms cannot directly synthesize functional lipids from cellulose due to their lack of cellulases, the cellobiohydrolase (CBH2) from T. longibrachiatum was previously selected to be overexpressed in M. circinelloides for the efficient use of cellulose to accumulate lipid (Zhang et al., 2022). In order to further enhance the content of GLA, the homologous Delta-6 desaturase (D6) was also co-overexpressed in M. circinelloides, which finally led to an increase in the GLA content from cellulose (Zhang et al., 2022). Generally, in our preliminary study, multiple engineering strains with overexpression or co-expression of cellobiohydrolase and delta-6 desaturase were constructed through homologous recombination technology (Zhang et al., 2022). However, only a few studies have focused on the GLA synthesis from cellulotic substrates in this cellobiohydrolase and delta-6 desaturase-engineered M. circinelloides strains.

In the present study, an in-depth survey of GLA accumulation by cellobiohydrolase and delta-6 desaturase overexpressing M. circinelloides strains using cellulotic substrate was to be pursued. The differences in cell biomass, lipid content, lipid composition, and GLA yield in engineered M. circinelloides strains were compared when grown on the base medium with cellulose. In addition, the effects of adding different proportions of cellulose on the growth, lipid accumulation, and GLA synthesis of engineered M. circinelloides strains were also analyzed. This study provided a reference for the further potential use of the engineered M. circinelloides strains in the conversion of functional GLA from cellulose.

Materials and Methods

Strains and Media

The recombinant strains Mc-2075 (control strain with empty plasmid), Mc-C2 (cellobiohydrolase overexpression strain), Mc-D6 (delta-6 desaturase overexpression strain), Mc-C2TD6 and Mc-C2PD6 (cellobiohydrolase and delta-6 desaturase coexpression strains with different linkers) were previously constructed and stocked in our laboratory (Zhang et al., 2022). The strains used in this study are summarized in Table 1.

<table>
<thead>
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<th>Table 1: The strains used in this study</th>
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<tr>
<td>Strains</td>
</tr>
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<td>Mc-D6</td>
</tr>
<tr>
<td>Mc-C2TD6</td>
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<tr>
<td>Mc-C2PD6</td>
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</table>

Keeping other components unchanged, the modified Kendrick and Ratledge (K and R) mediums (Kendrick and Ratledge, 1992) with glucose and different proportions of carboxymethyl cellulose (CMC) as carbon sources were prepared. For seed growth, the modified K and R medium consisted of (g/L): glucose with different proportions of CMC 30, ammonium tartrate 2.0, KH₂PO₄ 7.0, and MgSO₄·7H₂O 1.5, Na₂HPO₄ 2.0, yeast extract 1.5, CaCl₂·2H₂O 0.1, CuSO₄·5H₂O 0.0001, ZnSO₄·7H₂O 0.001, MnSO₄·5H₂O 0.0001, Co(NO₃)₂·6H₂O 0.0001, FeCl₃·6H₂O 0.008 and agar 20 for solid medium. The nitrogen-limited medium for lipid accumulation is consisting of the following (g/L): glucose with different proportions of CMC 50, ammonium tartrate 2.0, KH₂PO₄ 7.0, MgSO₄·7H₂O 1.5, Na₂HPO₄ 2.0, yeast extract 1.5, CaCl₂·2H₂O 0.1, CuSO₄·5H₂O 0.0001, ZnSO₄·7H₂O 0.001, MnSO₄·5H₂O 0.0001, Co(NO₃)₂·6H₂O 0.0001 and FeCl₃·6H₂O 0.008 (Kendrick and Ratledge, 1992).

The reagents used in the fermentation culture were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All of them were analytical-grade standard products unless otherwise stated.

Culture Conditions

Solid Culture

To investigate the effects of cellulose addition on the growth of engineered M. circinelloides strains, solid culture was adopted. Fresh spore suspensions with a concentration of 20~30μL (10 μL) were inoculated and coated on seed solid medium with different proportions of CMC and placed in a constant temperature incubator under 30°C for culture 24-48 h to observe the growth of strains.

Liquid Culture

The liquid culture was adopted to verify the growth and lipid production of engineered M. circinelloides strains at the optimal ratio of glucose: CMC. The spore’s suspension (approx. 10⁷ spores/mL) of the constructed strains was cultured in K&R medium with shaking at 150 rpm at 28°C for 24 h. The above cultures were then inoculated at 10% (v/v) into a 2 L fermenter (BioFlo 110, New Brunswick Scientific Co., Ltd) of the modified K and R medium supplemented with CMC. The fermenter was controlled at 28°C, stirred at 600 rpm, and aerated
at 0.5 v/v min⁻¹. The pH was kept at 6.0 by automatically adding 2 mol/L HCl or 4 mol/L NaOH. The following indicators were detected by regular sampling during the culture process.

**Strain Growth and Biomass Determination**

Three different directions were selected to read the growth diameter of the colony and calculate the colony area of the petri dish under different culture times. Cell dry weight (CDW) was obtained by the differential weight method. The mycelium was harvested at different times, filtered, and washed three times with distilled water, and then freeze-dried for 48 h. The biomass of each sample was gravimetrically determined.

**Determination of Glucose and Ammonium Concentrations**

Glucose content was measured by the glucose oxidase-peroxidase (GOD) method with a glucose measurement kit (Rongsheng Co. Ltd, China). The depletion of ammonium was determined by indophenol blue spectrophotometric method (Zhang et al., 2022).

**Determination of Lipid Content and Fatty Acid Composition**

The total lipids were extracted referring to the Folch method with minor modifications as follows: Approximately 20 mg of dried mycelium was taken with chloroform/methanol (2:1, v/v) and methylated with 10% HCl/methanol (v/v) at 60°C for 3 h. Pentadecanoic acid (C15:0, Sigma) was added to the freeze-dried cells as an internal standard. Fatty acid methyl esters were separated by n-hexane and analyzed by gas chromatography (GC) equipped with a 30 m ×0.32 mm DB-waxetr column with a film thickness of 0.25 µm. The program was set as follows: 120°C for 3 min, rising to 200°C at a rate of 5°C per min, and then rising to 220°C at a rate of 4°C per min and holding for 2 min (Wang et al., 2022; Zhang et al., 2017; 2022).

**Statistical Analysis**

SPSS Statistics 22 software was used for statistical analysis. All data were expressed as means ± S.D. The mean value and the standard error of the mean value were calculated from three independent experiments. Student’s t-test was used for differences between test means and p<0.05 was considered statistically significant.

**Results and Discussion**

**Effect of Cellulose Addition on the Growth of Engineered M. circinelloides Strains**

Carbon sources are very important for strain growth and metabolism, so the utilization of cheap and widespread carbon sources such as cellulose is a prerequisite for reducing GLA production cost (Lynd et al., 2002; Zhang and Song, 2021; Zhang et al., 2022; 2023b). Previously, the cultivation of engineered *M. circinelloides* strains on the modified K and R medium supplemented with microcrystalline cellulose was preliminarily verified (Zhang et al., 2022). In this study, another cellulosic substrate, carboxymethyl cellulose (CMC) was selected as an additive carbon because it is a kind of water-soluble cellulose ether obtained by chemical modification of natural cellulose (Kanikireddy et al., 2020). Remaining the other nutrients in the medium unchanged, a complex of glucose and CMC with different ratios were respectively used as carbon sources to investigate the effects of cellulose addition on the growth of engineered *M. circinelloides* strains and the results were shown in Fig. 1. As can be seen from Fig. 1a, except for CMC as the sole carbon source, there was no significant difference in the growth of each engineered strain when glucose and CMC were mixed with different proportions at the beginning of 24 h culture. However, when cultured to 48 h, the growth of the strains with different proportions of glucose and CMC as carbon sources showed significant differences (Fig. 1b). With the increasing proportion of CMC, the colony diameter of the strains gradually decreased. Especially when CMC was completely used as the carbon source, the growth of the strains was the worst. This is consistent with our previous result that the engineered strains did not grow well in the medium containing only microcrystalline cellulose as the carbon source (Zhang et al., 2022). This indicated that a small number of strains could not secrete enough cellulases to degrade cellulose and provide available sugars for strain growth. When using a combined carbon source of glucose and CMC with different ratios, the growth of strains is much better than only using CMC as the carbon source, but lower than the growth of that using glucose alone. Based on the growth results of the different mixing ratios of glucose and CMC (Fig. 1), the ratio of 1:1 for glucose and CMC was adopted for the growth and lipid accumulation of engineered *M. circinelloides* strains in the following study.

**Growth and Lipid Accumulation of Engineered M. circinelloides Strains from Cellulose**

Based on the above results, the cell growth and lipid production in engineered *M. circinelloides* strains were investigated when cultivated with the ratio of 1:1 for glucose and CMC in the modified K and R medium (Fig. 2). Since nitrogen limitation is a prerequisite for lipid accumulation (Ratledge and Wynn, 2002; Zhang et al., 2023a) and a low nitrogen source concentration was used in the modified medium. As shown in Fig. 2a, ammonium was completely exhausted at about 12 h in all of the engineered *M. circinelloides* strains. Similarly, the glucose contents in the engineered *M. circinelloides*
strains decreased gradually with the extension of fermentation time. However, the glucose consumption in the cellobiohydrolase overexpression strains (Mc-C2, Mc-C2TD6, and Mc-C2PD6) slowed down and it was not completely consumed until 60 h, while that of other cellobiohydrolase deficient strains (Mc-D6 and Mc-2075) was depleted after only 36 h. This was consistent with the results of our previous study (Zhang et al., 2022) and the reason was probably because the lead of cellobiohydrolase contributed to the efficient degradation of CMC thus providing more carbon sources for the strains. It could also be inferred that the foreign cellobiohydrolase and the internal cellulases in M. circinelloides can act synergistically on CMC.

It can be seen from Fig. 2b that the biomass in the cellobiohydrolase overexpression strains increased rapidly during the first 72 h and then the growth entered into a stable period. The cellobiohydrolase overexpression strains have similar amounts of biomass (up to 10.4 g/L at 72 h), which were improved by 68% (for Mc-C2), 59% (for Mc-C2TD6) and 64% (for Mc-C2PD6), respectively, compared to that of the control strain (Mc-2075), (Fig. 2b). Due to the inability to obtain sufficient carbon sources, other strains lacking cellobiohydrolase grew slowly and stopped growing after 48 h, which resulted in a low biomass.

Figure 2c, the lipid accumulation patterns in engineered M. circinelloides strains were similar: The lipid content increased rapidly in the first 48 h and then it slowed down and leveled off. The maximum lipid contents of the engineered strains (except for Mc-D6) were improved by 17-34% when compared with that of the control strain (Fig. 2c). It should be noted that the lipid yields of the engineered strains (excluding Mc-D6) were also dramatically improved, which were enhanced 2.0-fold for Mc-C2, 2.1-fold for Mc-C2TD6 and 2.2-fold for Mc-C2PD6, respectively (Fig. 2d). Thus, it could be concluded that the strains containing cellobiohydrolase could effectively decompose CMC to provide more carbon sources for the growth and lipid production.

GLA Accumulation of Engineered M. circinelloides Strains from Cellulose

Generally, GLA biosynthesis requires a series of fatty acid desaturases to introduce double bonds at the 9, 12, and 6 carbon atoms of the stearic acid chain (Nykiforuk et al., 2012; Ratledge, 2005; Wang et al., 2022; Zhang et al., 2017). The final step in GLA formation is catalyzed by delta-6 fatty acid desaturase, which is the rate-limiting enzyme and a crucial regulatory point of the GLA biosynthetic pathway (Wang et al., 2023; Zhang et al., 2017; 2022). The fatty acid profiles of engineered M. circinelloides strains using CMC are shown in Table 2. Except that Mc-C2 had similar GLA content with that of the control strain, the intracellular GLA contents of other recombinant strains with overexpression of delta-6 desaturase were improved by 42-47% compared to that of the control strain, which indicated that delta-6 desaturase indeed played an important role in the synthesis of GLA. Many previous studies and ours have confirmed that the introduction of delta-6 desaturase has a crucial effect on improving GLA content.

Combined with the biomass and total lipid data of the engineered strains, the final GLA yields showed that the cellobiohydrolase and delta-6 desaturase coexpression strains had an advantage in producing GLA from CMC. When compared with the control strain Mc-2075, the GLA yields of Mc-C2TD6 and Mc-C2PD6 were significantly increased by 3.0- and 3.1-fold, respectively, which were much higher than that of the single gene overexpression strains for their inability to effectively utilize CMC (for Mc-D6) or enhance the synthesis of GLA (for Mc-C2). This is consistent with our previous findings that the engineered M. circinelloides strains possess the ability to decompose cellulose for GLA production (Zhang et al., 2022).

Fig. 1: Effect of adding different ratios of CMC on the colony diameters of engineered M. circinelloides strains. The ratios of glucose: CMC (w/w) were 1:0, 3:1, 1:1, 1:3 and 0:1: (a) The colony diameter of 24 h; (b) The colony diameter of 48 h values
Fig. 2: Cell growth and lipid accumulation of engineered *M. circinelloides* strains. Strains were cultured in a 1.5 L fermenter with modified K and R medium containing the ratio of glucose: CMC as 1:1 during 96 h at pH 6.0 and 28°C, with aeration at 2.0 v/v min⁻¹ and stirring at 600 rpm. The samples were taken from the fermenter at the specified times; (a) Glucose (solid marks) and ammonium (empty marks) concentration; (b) Cell dry weight; (c) Lipid content of cell dry weight; (d) Lipid yield. The value was the mean of three independent experiments. The error bar represented the standard error of the mean.

Table 2: Fatty acid composition in engineered *M. circinelloides* strains cultured with CMC

<table>
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<tr>
<th>Strains</th>
<th>Time (h)</th>
<th>C14:0</th>
<th>C16:0</th>
<th>C16:1</th>
<th>C18:0</th>
<th>C18:1</th>
<th>C18:2</th>
<th>C18:3 (GLA)</th>
<th>% mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mc-2075</td>
<td>12</td>
<td>2.04±0.10</td>
<td>22.12±1.11</td>
<td>2.71±0.13</td>
<td>6.54±0.31</td>
<td>29.97±1.48</td>
<td>15.72±0.78</td>
<td>20.90±1.04</td>
<td>5.94±0.30</td>
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<tr>
<td></td>
<td>24</td>
<td>1.32±0.04</td>
<td>20.14±0.98</td>
<td>1.85±0.10</td>
<td>7.12±0.40</td>
<td>32.33±1.26</td>
<td>15.59±0.69</td>
<td>16.62±0.83</td>
<td>27.67±1.25</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.45±0.06</td>
<td>22.65±1.21</td>
<td>1.82±0.07</td>
<td>7.63±0.25</td>
<td>36.88±1.43</td>
<td>14.79±0.45</td>
<td>14.78±0.56</td>
<td>140.96±4.23</td>
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<tr>
<td></td>
<td>72</td>
<td>1.65±0.08</td>
<td>22.62±1.07</td>
<td>2.29±0.14</td>
<td>6.38±0.33</td>
<td>38.86±1.55</td>
<td>14.03±0.74</td>
<td>12.74±0.63</td>
<td>148.98±5.47</td>
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<td></td>
<td>96</td>
<td>1.90±0.12</td>
<td>23.63±1.47</td>
<td>2.72±0.12</td>
<td>5.44±0.17</td>
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<td>15.61±0.78</td>
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<td>242.93±6.24</td>
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strains were cultured in a 1.5 L fermenter with modified K&R medium containing CMC for 96 h. The fatty acid composition was displayed at different points in time. The values are means ± standard deviations of three independent experiments.

Conclusion

GLA is an important polyunsaturated fatty acid with nutritional and therapeutic applications. The oleaginous fungus *M. circinelloides* was one of the first strains to be used for industrial production of GLA, but it was discontinued due to cost concerns. Making full use of abundant cellulose resources for GLA biosynthesis is an effective way to reduce the cost. In the present study, the effects of adding different proportions of carboxymethyl cellulose on the growth, lipid accumulation, and GLA synthesis of engineered *M. circinelloides* strains were investigated in detail. When cultivated in a modified K&R medium supplemented with the ratio of 1:1 for glucose and cellulose (CMC), the maximum biomass, lipid content, and lipid yield of engineered *M. circinelloides* strains achieved 10.4 g/L, 25.4%, and 2.6 g/L, respectively, which were 68, 34% and 2.2-fold higher than that of the control strain, respectively. It was worth noting that the maximum GLA yield of the celllobiohydrolase and delta-6 desaturase coexpression strains reached 415 mg/L, which was 3.1-fold higher than that of the control strain. This study proved the ability of engineered *M. circinelloides* strains to produce GLA from CMC. It has also manifested the potential use of the constructed strains as biocatalysts in cellulosic biorefinery. This study is expected to achieve a one-step efficient conversion of cellulosic raw materials to functional lipids and play a positive role in promoting the sustainable development of the biological industry.

Acknowledgment

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Author’s Contributions

Yao Zhang: Involved in the study conception, experimental design, figures and tables’ arrangement, data analysis, result interpretation, manuscript written, and reviewed of the final manuscript.

Yaxia Wang: Carried out the experiments and collected data.

Qing Liu: Participated in the experimental research.

Yuanda Song: Conceived the study and reviewed the original manuscript.

Ethics

All authors read and approved the final version of this manuscript. There are not any ethical issues to declare that could arise after the publication of this manuscript.

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