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Microbial conversion of biodiesel byproduct glycerol to triacylglycerols by oleaginous yeast *Rhodosporidium toruloides* and the individual effect of some impurities on lipid production

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ABSTRACT

With the development of biodiesel industry, the byproduct glycerol will become a considerable resource as feedstock for production of many other chemicals. In present work, microbial conversion of crude glycerol to triacylglycerols (microbial lipid) was proposed and investigated using the oleaginous yeast *Rhodosporidium toruloides* (*R. toruloides*) by one-stage batch fermentation. Compared with glucose and refined glycerol, the crude glycerol could obtain significantly higher biomass concentration and lipid yield. The highest biomass concentration of *R. toruloides* obtained from crude glycerol in a 5 L fermenter reached 26.7 g/L with an intracellular lipid content of 70%. Further study was performed to investigate the individual effect of five representative compounds which were present in crude glycerol as impurities. It was found that within the general concentration ranges, only methanol displayed somewhat inhibitive effect, while others showed positive influence on lipid production. These results indicated that crude glycerol could be directly converted to triacylglycerols by *R. toruloides* without purification. Contrarily, certain amount of salt and soap could promote the accumulation of biomass and lipid.

1. Introduction

Biodiesel industry has become important due to the shortage of fossil oil and increasing awareness of environmental issue. For its sustainable development, however, it is primarily necessary to insure that triacylglycerol (TAG) can be produced continually without competing with food industry. Lipids derived from microorganisms, known as microbial lipid, can be fast synthesized and accumulated intracellularly, with fatty acids compositions resembling these of vegetable oils. Since the production of microbial lipid has many advantages over that of vegetable oils such as short life cycle and no need of agricultural land, it has attracted much interest in recent decades, and considered as a potential non-food feedstock for biodiesel production. Importantly, however, during microbial conversion, abundance of carbon source that can be effectively utilized by microorganisms is required. Traditionally this conversion process is based on starch glucose. Nevertheless high expense of glucose feedstock would significantly limit the industrialization of microbial lipid production. For this reason, renewable and affordable carbon source in abundance is favored.

Crude glycerol is a main byproduct of biodiesel production. As the production of biodiesel grows, it will be generated in a considerable quantity, and its utilization will become an urgent topic. Although refined glycerol could be a valuable product, the purification process is always costly and generally out of the range of economic feasibility for small to medium-sized plants [1]. In some European countries, crude glycerol is even simply treated as industrial wastewater [2]. Nowadays, trials are underway and researchers have already successfully converted crude glycerol not only to useful chemicals such as 1,3-propanediol [3], phytase [4], citric acid [3], but also single cell oil, which can further be transformed to biodiesel [3].

In present work, *Rhodosporidium toruloides* (*R. toruloides*) was employed as a promising candidate for prospective industrial application. It can accumulate intracellular lipid up to 60% of its cellular dry weight [5], which is mainly triacylglycerol, resembling that of oils from food crops in terms of fatty acid composition, and has been successfully converted to biodiesel [6]. Besides, *R. toruloides* has already been proved to be capable to grow on a broad range of substrates including but not limited to: sugars like glucose and xylose, lignocellulosic hydrolysate, and excess sludge hydrolysate [5,7]. Furthermore, it can simultaneously produce carotenoids as by-products, which may have some potential values (data not published yet). However, no related data on the conversion of glycerol to microbial lipid by this robust yeast are found in the

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literatures. Besides, for effective utilization of biodiesel byproduct, detailed knowledge is required to comprehensively evaluate its fermentability and also the effects of impurities. Therefore, the objective of present work is to find out whether the crude glycerol can be directly used as carbon source for microbial lipid production, and how the impurities present in crude glycerol affect the growth, lipid accumulation and as well as the lipid fatty acids composition of *R. toruloides*.

2. Materials and methods

2.1. Microorganism and chemicals

The oleaginous microorganism used in the experiments was *R. toruloides* AS2.1389, which was obtained from China General Microbiological Culture Collection Center (CGMCC). The yeast was maintained at 4 °C on yeast extract peptone dextrose (YEPD) slopes containing: 20 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, and 20 g/L agar. For preparing inocula, the organism was pre-cultured overnight in the medium containing 20 g/L glucose (or 15 g/L glycerol), 10 g/L peptone and 10 g/L yeast extract at 30 °C and 200 rpm in an air-bath shaker.

The chemicals and reagents in all the experiments were analytically pure and purchased locally. The crude glycerol samples were provided by Hunan Rivers Bioengineering Co., Ltd., Hunan, China.

2.2. Fermentation process

Batch fermentation experiments with crude glycerol as carbon substrate were conducted in either flasks or 5-L fermenters. In flask fermentation, the experiments were conducted in 500 mL flasks with 100 mL liquid medium containing 50 g/L glycerol (for crude glycerol, the addition amount was decided by its exact glycerol concentration which was tested by HPLC), 0.1 g/L $(NH_4)_2SO_4$, 0.75 g/L yeast extract, 0.4 g/L KH_2PO_4, and 1.5 g/L $MgSO_4{\cdot}7H_2O$. The pH value of the liquid medium was adjusted to 6.0. Followed by sterilization at 115 °C for 15 min, 10 mL inocula were added to the medium, and the culture was conducted at 30 °C in an air-bath shaker at 200 rpm for 5–6 days. Before inoculation, the biomass concentration of the inocula was carefully controlled at 0.5–0.6 g/L. Each culture was performed by duplicate test.

For culture in 5-L fermenters, the experiments were conducted with 4 L culture volume. The initial glycerol concentration was 60 g/L and other nutrients concentrations were the same as those in flask culture. pH was controlled at 6.0 by feeding 40 wt% NaOH. Dissolved oxygen was controlled at 20–30% saturation by automatic adjustment of stirring speed with 2 vvm aeration. For comparison, glucose was also used as carbon source at the same initial concentration.

2.3. Analytical methods

The quantitative analysis of methanol, glucose, and glycerol was performed with a Shimadzu10AVP HPLC system (Shimadzu Corp., Japan) equipped with a RID-10A refractive index detector, Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, USA) at 65 °C with 5 mM H_2SO_4 as the eluent at a flow rate of 0.8 mL/min. The estimation of glyceride was performed with a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) with an ELSD-LT II low temperature-evaporative light scattering detector. Before analysis, 2 μL sample and 1 mL acetone were mixed thoroughly, and 20 μL of the aforementioned mixture was injected. A C18 column (5 μm, 250 mm × 4.6 mm) (Dikma Technology, PLATISIL ODS, China) and a gradient elution program by acetonitrile and dichloromethane at 1.5 mL/min was employed, respectively. The column temperature was 40 °C. The drift pipe temperature was 70 °C, and the nitrogen pressure was 320 kPa. The ash content of crude glycerol was determined by burning the glycerol sample in muffle furnace until a constant weight was reached.

The yeast biomass concentration was expressed as the dry weight concentration of cell biomass (C_B, g/L). After the fermentation broth was centrifuged, the wet cells were washed with deioned water and dried at 105 °C to a constant weight. The dry weight concentration of cell biomass was then calculated according to following equation:

$$C_B\ (\mathrm{g/L}) = \frac{\text{dry weight of cell biomass (g)}}{\text{volume of fermentation broth (L)}}$$

Total crude intracellular lipid was extracted by acid-heating procedure with mixture of chloroform and methanol as extractant [8]. Crude lipid content was expressed as gram lipid per gram of dry biomass. For determination of fatty acid compositions, the crude lipid was first converted to fatty acid methyl ester (FAME) with excess methanol in tert-butanol system under the catalysis of excess loading of immobilized lipase (NS 435 and NS 40044, produced by Novozymes) [9]. The obtained FAME was then analyzed with a GC-14B gas chromatography (Shimadzu, Japan) equipped with a CP-FFAP CB capillary column (25 m × 0.32 m × 0.30 μm) produced by Agilent. Heptadecanoic acid methyl ester was used as internal standard. The column temperature was kept at 180 °C for 0.5 min and heated to 250 °C at 10 °C/min, and then maintained for 6 min. The temperatures of the injector and detector were set at 245 °C and 260 °C, respectively.

For describing the efficiency of the microbial lipid production, several response variables, namely, lipid concentration (C_L), lipid yield (Y_L), and lipid content (C_{nL}) are defined as follows, respectively:

$$C_L\ (\mathrm{g/L}) = \frac{\text{weight of lipid (g)}}{\text{volume of fermentation broth (L)}}$$

$$Y_L\,(\mathrm{g/100\ \ g\ carbon\ substrate}) = \frac{\text{weight of lipid (g)}}{\text{weight of carbon substrate consumed (g)}} \times 100$$

$$C_{nL}\,(\%) = \frac{\text{weight of lipid (g)}}{\text{weight of cell biomass (g)}} \times 100\%$$

3. Results and discussion

3.1. Lipid production by R. toruloides in flask fermentation

Generally, in the biodiesel industry, about 0.1 t of crude glycerol is produced for every 1 t of biodiesel. Some soap and alcohol (usually methanol) together with small amount of fat soluble substance are also present in the glycerol phase depending on the feedstock and production processes. Two types of crude glycerol samples used in present work were analyzed, and their main components are shown in Table 1.

Glycerol A contained 85% glycerol, a small amount of glyceride and fatty acid methyl ester (FAME). Notably, after burning the sample to a constant weight, the content of ash residue reached 6.5%, most of which was soluble in water. The ash generally consisted of sodium salts from the catalyst (e.g. sodium hydroxide) of transesterification reaction. Therefore, ash content can be considered as the total percentage of salt and soap components in the crude glycerol sample.

Glycerol B was originated from lipase-catalyzed transesterification of palm oil. It contained 32.97% glycerol, a small amount of glyceride and fatty acid methyl ester, and a little ash. However, no