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Production of fumaric acid from biodiesel-derived crude glycerol by *Rhizopus arrhizus*

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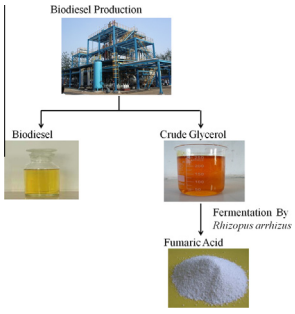
HIGHLIGHTS

- *R. arrhizus* can assimilate crude glycerol and convert it into fumaric acid.
- The *R. arrhizus* pellet can be formed when growing on crude glycerol medium.
- The co-fermentation of glycerol and glucose was studied to produce fumaric acid.
- By the co-fermentation, the production cost of fumaric acid was reduced by 14%.

GRAPHICAL ABSTRACT



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ABSTRACT

This work investigated the capability of *Rhizopus arrhizus* to assimilate biodiesel-derived crude glycerol and convert it into fumaric acid. After optimizing the initial glycerol concentration, spore inoculum and yeast extract concentration, smaller pellets (0.7 mm) and higher biomass (3.11 g/L) were obtained when *R. arrhizus* grew on crude glycerol. It was found that crude glycerol was more suitable than glucose for smaller *R. arrhizus* pellet forming. When 80 g/L crude glycerol was used as carbon source, the fumaric acid production of 4.37 g/L was obtained at 192 h. With a highest concentration of 22.81 g/L achieved in the co-fermentation of crude glycerol (40 g/L) and glucose (40 g/L) at 144 h, the fumaric acid production was enhanced by 553.6%, compared to the fermentation using glycerol (80 g/L) as sole carbon source. Moreover, the production cost of fumaric acid in co-fermentation was reduced by approximately 14% compared to glucose fermentation.

1. Introduction

Crude glycerol is the primary by-product in biodiesel industry. Currently, extravagant production of biodiesel is severely causing the excess of crude glycerol. Such abundant supplies of crude glycerol have greatly disturbed the market for traditional glycerol in terms of price and created a significant environmental problem at the same time because of the difficulty in disposal (Oh et al., 2011). Pure glycerol can present numerous applications in the cosmetic, paint, automotive, food, pharmaceutical, pulp and paper, leather and textile industries (Wang et al., 2001). However, crude glycerol needs further purification in order to be used in such industrial applications. Due to the complicated procedures and

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high costs, purification of crude glycerol is infeasible on the economy (Johnson and Taconi, 2007; Prielipp and Keller, 1956). Thus, utilizing crude glycerol as a feedstock to produce higher added-value products through microbial fermentation is a promising application. It can not only confront with the ongoing increment of glycerol waste streams, but also improve the economics of biodiesel industry (Singhabhandhu and Tezuka, 2010; Saxena et al., 2009). Crude glycerol has been exploited as a carbon source for the production of added-value compounds, such as 1,3-propanediol (Hiremath et al., 2011), ethanol (Wu et al., 2011), succinic acid (Lee et al., 2001) and trehalose (Ruhal et al., 2011).

In this work, crude glycerol was targeted for production of fumaric acid through fermentation. Fumaric acid is a four-carbon unsaturated dicarboxylic acid which has many potential industrial applications, ranging from the manufacture of synthetic resins and biodegradable polymers to the production of intermediates for chemical synthesis (Roa Engel et al., 2008). Currently, fumaric acid is produced in two ways including chemical conversion from maleic anhydride and biological conversion by fungi (Zhou et al., 2011). However, as petroleum is becoming scarcer, it has attracted increasing attentions on producing fumaric acid by fermentation (Roa Engel et al., 2008, 2011). Gangl et al. (1990) pointed out that the high cost of raw materials is one of the greatest obstacles restraining the industrialization of fumaric acid fermentation. According to former studies, most of the commonly used feedstocks for fumaric acid production, such as purified sugars, starchy materials and cellulosic material, encounter various issues that impeding them from being an ideal raw material. Purified sugars are expensive for industrial utilization, and using sugars and starchy materials as feedstocks can directly compete with humans for food and break the equilibrium of supply and demand of grain (Tollefson, 2008; Aho, 2007). Furthermore, cellulosic material could not be directly used for fermentation. It needs to be processed by acid or cellulase in order to release fermentable sugars (Huang et al., 2009). The pretreatment processes are complicated and costly. Hence, searching for economic carbon source is crucial.

Rhizopus arrhizus has been reported as an excellent fumaric acid producer (Roa Engel et al., 2008), as well as being capable of producing a high level of fumaric acid when growing on sugar or starchy materials. However, growing the *R. arrhizus* on biodiesel-derived crude glycerol has not been investigated yet, and only a few works have been performed to evaluate fumaric acid production from crude glycerol. Therefore, the objective of this work was to investigate the potential of using crude glycerol to culture *R. arrhizus* and produce fumaric acid.

2. Methods

2.1. Pre-treatment of crude glycerol

The crude glycerol was obtained from the biodiesel production which was prepared from base catalyzed trans-esterification reaction of soya bean oil, with KOH as base catalyst (Jiangsu Kate New Energy Co., Ltd, China). Crude glycerol was dilute with deionized water at a ratio of 3:1 (v/v) to reduce its viscosity. The pH was adjusted to about 4 with 85% phosphoric acid. Then the acid-treated glycerol was centrifuged at 5000 rpm, followed by collection of the middle layer (glycerol and water). Methanol was removed by atmospheric distillation at 75 °C. After the distillation, a filtration was carried out to remove the crystallized phosphate salts.

After the aforementioned pre-treatment, the compositions of crude glycerol obtained were 80.1% (w/w) glycerol, 13.4% (w/w) water, 3.7% (w/w) phosphate salts, 1.0% (w/w) free-fatty acids and 1.8% (w/w) other compositions.

2.2. Microorganism and culture medium

R. arrhizus RH-07-13, a mutant of *R. arrhizus* was used in this research (Wen et al., 2013). The strain was cultured on potato-dextrose agar (PDA) by periodical transfers following incubation at 30 °C for 7 days and storing at 4 °C (Gu et al., 2013). The compositions of the seed medium were (per liter): 25 g glycerol, 1.5 g yeast extract, 1.5 g urea, 0.6 g KH_2PO_4, 1.0 g $MgSO_4{\cdot}7H_2O$, 0.0176 g $ZnSO_4{\cdot}7H_2O$, and 0.0005 g $FeSO_4{\cdot}7H_2O$, with pH 3.5. The compositions of another seed medium using glucose as carbon source were the same as the former, except that the glycerol was replaced by glucose. The compositions of the fermentation medium with glycerol as sole carbon source were (per liter): 80 g glycerol, 0.3 g peptone, 1.55 g KH_2PO_4, 1.0 g $MgSO_4{\cdot}7H_2O$, 0.025 g $ZnSO_4{\cdot}7H_2O$, 0.0005 g $FeSO_4{\cdot}7H_2O$, and 40 g $CaCO_3$. The pH was adjusted to 5.5 with 1 mol/L KOH. In co-fermentation of glycerol and glucose, glycerol was replaced as the carbon source by 40 g glycerol and 40 g glucose, and the other components of medium were the same as the fermentation medium with glycerol as sole carbon source. All media were sterilized by autoclaving at 116 °C for 25 min.

2.3. Spore inoculum preparation

Spores of *R. arrhizus* growing on PDA slants were washed with sterilized water and shaked for 35 min with glass beads to obtain the spore suspension. After being filtered twice with sterile lens paper, spore inoculums concentration was counted with a hemacytometer under a microscope. The spore inoculum concentration of 4.8×10^7 per milliliter was needed.

2.4. Seed culture conditions

The fermentation process of fumaric acid production by *R. arrhizus* fits non-growth-coupled model (Zhen et al., 2009). The first stage of this process was the seed culture, and the second stage was the fumaric acid production (fermentation stage). In the seed culture stage, for the *R. arrhizus* pellet formation, spores inoculum was inoculated 1% (v/v) into 250 mL Erlenmeyer flasks containing 100 mL of seed medium and cultivated at 30 °C, 165 rpm in rotary shakers for 24 h. The conditions of the liquid volume and shaking speed were chosen based on the former studies (Gu et al., 2013; Wen et al., 2013) and a series of optimization experiments (data not shown). After 24 h growth, *R. arrhizus* with small pellet morphology was obtained.

2.5. Fermentation conditions for fumaric acid production

In the fermentation stage for fumaric acid production, 20% (v/v) of *R. arrhizus* pellets were transferred into another 250 mL Erlenmeyer flask containing 50 mL of fermentation medium and cultured at 30 °C, 200 rpm in rotary shakers for 8 days. All experiments were duplicated and averaged data are reported.

2.6. Analytical methods

Moisture content was measured by an automatic titrator ZDJ-400 (Xianqu Weifeng, Beijing, China). Phosphate salts was analyzed by colorimetric methods (Berenblum and Chain, 1938). Free-fatty acids were analyzed by a gas chromatograph (Liu et al., 2013a). Fumaric acid, glucose and glycerol concentrations were quantified by a high-performance liquid chromatograph (HPLC) with a Bio-Rad Aminex HPX-87 H ion exclusion column with a refractive index detector and UV detector at 210 nm. The column was eluted with 0.005 M H_2SO_4 at a column temperature of 50 °C and a flow rate of 0.6 mL/min.