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Efficient production of dihydroxyacetone from biodiesel-derived crude glycerol by newly isolated *Gluconobacter frateurii*

Yu-Peng Liu [a,1], Yang Sun [a,1], Cong Tan [b], Hua Li [a], Xiao-Juan Zheng [a], Kui-Qi Jin [a], Gang Wang [a,*]

[a] Institute of Bioengineering, School of Life Sciences, Henan University, Kaifeng 475004, PR China
[b] Changxing Pharmaceutical Co., Ltd., ChangXing 313100, PR China

HIGHLIGHTS

- Satisfactory dihydroxyacetone production by *Gluconobacter frateurii* on crude glycerol.
- High production of dihydroxyacetone (up to 125.8 g l^{-1}) from crude glycerol.
- Optimization of dihydroxyacetone fermentation process by *G. frateurii*.

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ABSTRACT

The efficient production of dihydroxyacetone (DHA) on biodiesel-derived glycerol based media was developed. A newly isolated strain, *Gluconobacter frateurii* CGMCC 5397, could convert crude glycerol to DHA with high yield and productivity. In shake-flask fermentation, the DHA concentration of 73.1 g l^{-1} was attained at 48 h using an optimum medium containing biodiesel-derived crude glycerol. When fed-batch fermentation was carried out in a 7-l stirred bioreactor with crude glycerol, the DHA concentration, productivity, and yield were 125.8 g l^{-1}, 2.6 g l^{-1} h^{-1}, and 90.5% at 48 h, respectively. This study suggests that the inexpensive biodiesel-derived crude glycerol could be utilized for efficient production of DHA by *G. frateurii*.

1. Introduction

Nowadays, fuel crisis has globally flounced the economy in every region, particularly the oil consuming countries due to the rapidly decreasing available global stocks. Due to this serious situation, biodiesel which comes from 100% renewable resources provides an alternative fuel option for future (Ayoub and Abdullah, 2012). Several sources for producing biodiesel have been studied such as rape seed, coal seed, palm oil, sunflower oil, waste cooking oil, soybean oil, etc. (Zhang et al., 2003). Glycerol is produced as a byproduct at levels of approximately 10% (w/w) of the total biodiesel generated (Johnson and Taconi, 2007). Worldwide, crude glycerol derived from biodiesel conversion has increased from 200,000 tons in 2004 to 1.224 million tons in 2008 (Yang et al., 2012). Therefore, it is of great importance for scientists to find new applications for crude glycerol. The crude glycerol is a relatively inexpensive raw material and has already been used for the production of a number of industrial important chemicals, such as ethanol (Oh et al., 2011), citric acid (Papanikolaou and Aggelis, 2003), docosahexaenoic acid (Chi et al., 2007), polyhydroxybutyrate (Naranjo et al., 2013; Vrana Špoljarić et al., 2013), erythritol (Rymowicz et al., 2009), 1,3-propanediol and 2,3-butanediol (Metsoviti et al., 2012). At the same time, the 1,3-dihydroxyacetone (DHA) is also a value-added product from crude glycerol.

DHA is a very important chemical product, and used extensively in the cosmetic industry for making artificial suntans (Brown, 2001; Levy, 1992; Nguyen and Kochevar, 2003). DHA has also been proposed to be involved in weight augmentation and fat loss, antioxidant activity, and increasing endurance capacity (Stanko et al., 1990), and it offers a safe and effective therapeutic option for recalcitrant vitiligo. Moreover, DHA serves as a variety of fine chemicals such as 1,2-propylene glycerol or lactic acid (Bicker et al., 2005; Hekmat et al., 2003).

The production of DHA from glycerol can be done either via chemical or microbial route. It has been reported that the microbial route of DHA production was more efficient as compared to that of chemical route (Mishra et al., 2008). Commercial synthesis of DHA is done more economically via microbial route due to the expensive safety measurements required in case of chemical route. It has recently been reported that the enzyme catalyzed chemical

* Corresponding author. Tel.: +86 378 3887799.
E-mail address: wlwsw2012@yahoo.cn (G. Wang).
[1] These authors contributed equally to this work.

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bioconversions are more acceptable by the pharmaceutical and chemical industries as a practical alternative to chemical synthesis methods due to the intractable synthetic problems involved with the chemical synthesis methods and also because of stringent environmental constraints.

Since Bertrand first observed production of DHA from glycerol through bacterial route in the year 1898, various microorganisms used for DHA production have been reported (Mishra et al., 2008). *Gluconobacter* strains produce DHA via incomplete oxidation of glycerol with the activity of membrane-bound glycerol dehydrogenase (Deppenmeier et al., 2002), and have been the most extensively used microorganisms in the present research for DHA production (Hu et al., 2010; Lu et al., 2012; Tkac et al., 2001; Wethmar and Deckwer, 1999).

In this article, we systematically reported, for the first time, the efficient production of DHA by newly isolated *Gluconobacter frateurii* from biodiesel-derived crude glycerol. The effects of initial glycerol concentration and complex nitrogen sources on cell growth and DHA production from biodiesel-derived glycerol were examined. Moreover, the crude glycerol-fed batch fermentation was also developed to enhance the production of DHA.

2. Methods

2.1. Crude glycerol

Crude glycerol used in this work was obtained from palm oil-based biodiesel plant in Malaysia operated by Vance Bioenergy [composition: 80.5% (w/w) glycerol, 10.1% (w/w) water, 5.2% (w/w) sodium salts, 0.4% (w/w) potassium salts, 0.3% (w/w) other salts, 0.5% (w/w) methanol, 2% (w/w) other organics (esters, free fatty acids, soaps, etc.)]. Crude glycerol was used as carbon source in culture medium without purification. The purity of crude glycerol (80.5%, w/w) used in each experiment has been taken into consideration and the appropriate calculations were made, thus, in any case the initial concentrations of glycerol quoted refer to pure glycerol.

2.2. Isolation of crude glycerol-utilizing microorganism

Screening plates and fermentation screening medium were all prepared by crude glycerol in this study. Samples were collected at various locations in Kaifeng (PR China), that contained sewage, sludge, rotted fruits, soil, honey, etc. All samples were stored at 4 °C before isolation, ten grams of each sample was suspended in 90 ml of sterile distilled water and shaking for an hour at 30 °C with 100 rpm, respectively. Aliquots of the cultures (0.2 ml) were spread on screening agar plates (100 g l^{-1} glycerol, 15 g l^{-1} yeast extract, 3 g l^{-1} KH_2PO_4, 20 g l^{-1} agar, pH 6.0) and incubated at 30 °C for 3 days. Strains utilizing crude glycerol were selected from agar plates and pure cultures were obtained by slant culture. An overnight culture of isolated strains (2% inocula) was inoculated into 250 ml shake-flasks with 30 ml of fermentation screening medium (80 g l^{-1} glycerol, 15 g l^{-1} yeast extract, 3 g l^{-1} KH_2PO_4, pH 6.0) and the flasks were incubated at 220 rpm shaking under 30 °C for 48 h. The cultures were collected and subjected for further assay by measuring the concentration of DHA.

2.3. Microbial identification

The 16S rRNA gene was amplified from genomic DNA by PCR using the bacterial primers. The sequences of the primers used for amplification were 5′-AGAGTTTGATCATGGCTCAG-3′ (forward) and 5′-AAGGAGGTGATCCAGCCGCA-3′ (reverse), and the PCR product was purified and the sequence was determined by TaKaRa Biotechnology (Dalian) Co., Ltd. The sequence was aligned with reference sequences obtained from databases using ClustalW program. Pairwise evolutionary distances of them were calculated using Kimura's two-parameter model. A phylogenetic tree from distance matrices was constructed by the neighbor-joining method.

2.4. Physiological and biochemical characterization of strain HD924

To investigate the physiological and biochemical characteristics, standard techniques were performed, including Gram staining, the oxidase reaction, catalase, production of water-soluble brown pigment, production of DHA and 5-keto-D-gluconic acid, acid production from carbohydrates, the G + C (mol %) of DNA and etc.

2.5. Fermentation in shake-flasks

Cells were grown in 250 ml shake-flasks containing 30 ml medium prepared by crude glycerol. The seed medium containing per liter: 15 g glycerol, 15 g yeast extract, 3 g KH_2PO_4. The fermentation medium containing per liter: 100 g glycerol, 15 g yeast extract (or 24 g corn steep liquor), 3 g $CaCO_3$. The pH of the medium was adjusted to 6.0 with 2 mol l^{-1} NaOH, and the medium was heat sterilized (20 min at 121 °C). The seed medium was inoculated with 2 ml stock culture from −80 °C ultra-low temperature freezer and incubated at 30 °C for 16 h. For the fermentation experiments, the medium was inoculated with 5% of the seed culture and incubated at 220 rpm shaking under 30 °C for 48 h.

2.6. Fermentation in stirred bioreactors

Batch fermentation was carried out at 30 °C with 4 l medium in 7-l stirred bioreactors (BioFlo 4000, New Brunswick Scientific, Edison, NJ, USA). The fermentation medium was prepared by crude glycerol (or pure glycerol) and pH was controlled at 5.5 with 2 mol l^{-1} NaOH during the fermentation process. Air rate and agitation speed were controlled at 1.5 vvm and 350 rpm.

Fed-batch fermentation was carried out under the same conditions as batch fermentation except the initial glycerol concentration was 20 g l^{-1}. When the concentration of glycerol was lower than 5 g l^{-1}, a sterilized crude glycerol was fed into the stirred bioreactor using a peristaltic pump to maintain the glycerol concentration within 5–15 g l^{-1} during the fermentation process.

2.7. Analytical methods

After the desired incubation period, the culture was diluted with 0.2 mol l^{-1} HCl, and the cell concentration (OD_{560}) was determined by measuring the absorbance at 560 nm using a spectrophotometer (U-752).

DHA and glycerol were analyzed by high-performance liquid chromatography (HPLC). Fermentation samples were centrifuged (8000g, 10 min) at 4 °C, and the concentrations of DHA and glycerol in supernatants were quantified using a HPLC system (Waters, Milford, MA) equipped with a refractive index detector, an UV detector, and an Aminex HPX-87H column (300 × 7.8 mm, 9 μm; Bio-Rad Chemical Division, Richmond, Calif.). The mobile phase was 8 mmol l^{-1} H_2SO_4 solution at a flow rate of 0.5 ml/min, and the column was operated at 55 °C.

DHA yield was defined as the amount of DHA produced from each gram of glycerol consumed (expressed in percentage), and was normalized in accordance with the dilution factor of base or crude glycerol solution. The glycerol utilization was calculated as glycerol consumed/initial glycerol and expressed in percentage.