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Production and productivity of 1,3-propanediol from glycerol by *Klebsiella pneumoniae* GLC29

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ABSTRACT

Interest in the development of the bioproduction of 1,3-propanediol, an important chemical intermediate with various industrial applications, has increased in recent years. *Klebsiella pneumoniae* is one of the most studied and efficient bacteria for 1,3-propanediol production from glycerol. A new isolate of *K. pneumoniae* was investigated using response surface methodology by central composite design for the production of 1,3-propanediol using glycerol. The effects of pH, temperature, stirrer speed, and glycerol concentration on the production and productivity of 1,3-propanediol were examined. Considering both production and productivity, the best conditions for glycerol conversion in 1,3-propanediol are: a pH range of 6.9–7.1, a temperature between 33 and 38.5 °C, a stirrer speed of 110–180 rpm, and a glycerol concentration of 39–49 g l^{-1}. Batch fermentation carried out at a pH of 7.0, a temperature of 35 °C, a stirrer speed of 150 rpm, and a glycerol concentration of 40 g l^{-1} produced 20.4 g 1,3-propanediol l^{-1}, with a maximum volumetric productivity of 2.92 g l^{-1} h^{-1} and a yield of 0.51 g g^{-1}. The main byproducts were acetic acid (approximately 7.0 g l^{-1}) and formate (approximately 3.7 g l^{-1}). The newly isolated *K. pneumoniae* GLC29 showed potential for the conversion of glycerol into 1,3-propanediol, with high production and productivity.

1. Introduction

In recent years, many countries have adopted biofuels, such as biodiesel, an alternative diesel fuel produced from renewable sources. Their increased use is related to environmental pollution caused by the emission of greenhouse gases into the atmosphere mainly through the burning of fossil fuels. Fossil oil is a non-renewable resource, thus leading to price instability in the international market. Biodiesel is a mono-alkyl ester of long chain fatty acids produced from vegetable oils and animal fats. A transesterification reaction between the oil and an alcohol in the presence of a catalyst produces esters (biodiesel) and about 10% glycerol (w/w) as a byproduct [1]. Glycerol, a substance recognized as safe for human and animal health, is a chemical with widespread uses in several industrial sectors, like food, cosmetics and pharmaceutical industries, but the increasing biodiesel production worldwide is responsible for the generation of a glycerol surplus in the market. Crude glycerol from biodiesel refineries consists of glycerol, water, salts such as potassium, and residual alcohol [2,3]. Glycerol must be highly purified to be used in products such as drugs and food, and the high cost of purification makes this process impractical for the glycerol derived from biodiesel industries [4].

This glycerol surplus is an environmental problem for biorefineries since its disposal and/or storage generates economic costs, raising the final biodiesel price. Therefore, the search for new glycerol applications is one objective of researchers and industries. Possibilities for new glycerol utilization by chemicals [5] or industrial microbiology [1] have been reviewed. Among possible new applications in the chemical industry is the use of glycerol as a raw material for the production of various chemicals, including mesoxalic acid, 1,3-dicloropropanol, glyceryl ethers, glycerol carbonate, glyceryl esters, hydroxypyruvic acid, and polyglycerol [5]. Biotechnologically, glycerol can be converted by microorganisms in a number of valuable chemicals such as 1,3-propanediol, dihydroxyacetone, succinic, propionic, and citric acids, ethanol, pigments, polyhydroxyalkanoates, biosurfactants, 2,3-butanediol, amino acids, glyceric acids, D-xylulose, hydrogen, 3-hydroxypropionaldehyde, and fatty acids [1,6–16]. Microbial bioconversion of glycerol has a number of advantages over chemical synthesis, including a greater selectivity on the final products,

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efficiency, and a shorter reaction time. In general, chemical synthesis requires several intermediate steps before reaching the final product, specific catalysts, and extreme and well controlled conditions such as high temperatures and high pressures [5,9], which favor the biotechnological route of glycerol bioconversion [17].

One of the main products obtained through microbial bioconversion of glycerol is 1,3-propanediol (1,3-PDO), a polyol with applications in the cosmetics, food, lubricant, and pharmaceutical industries [18–21]. The development of polypropylene terephthalate (PPT), a thermoplastic with superior physicochemical properties to those of polyethylene terephthalate (PET), used in the production of fabrics, carpets, and engineered plastics has created a new demand for 1,3-propanediol. Interest in the commercial production of 1,3-PDO through either chemical synthesis or microbial conversion of glycerol has increased in recent years. Chemically, 1,3-PDO is produced through two different routes. One of them uses acrolein (2-propenal) as a raw material, which is hydrated to 3-hydroxypropionic acid, which is then hydrogenated in the presence of a catalyst to 1,3-PDO. Another route involves the hydroformylation of ethylene oxide with CO and H under high pressure in the presence of a catalyst and a solvent [18]. This reaction produces a dioxane that is hydrogenated to 1,3-PDO. These methods are expensive, create pollutants, [22] and are dependent on crude oil.

Bioconversion of glycerol into 1,3-propanediol has been demonstrated only in bacteria, such as *Citrobacter freundii*, *Klebsiella pneumoniae*, *Clostridium pasteurianum*, *Clostridium butyricum*, and *Enterobacter agglomerans* [1,18,23,24]. One of the most studied and efficient bacteria for 1,3-propanediol production is *K. pneumoniae* [18,24]. A number works have focused on improving the production and productivity of 1,3-propanediol using glycerol. Yang et al. [25], in fed-batch fermentation at a pH of 7.0, a temperature of 37 °C, and microaerobiosis, using mutants of *K. oxytoca* deficient in lactate formation and sucrose as a co-substrate, obtained 83.56 g 1,3-PDO l^{-1}, with a yield of 0.62 mol mol^{-1}, and a productivity of 1.61 g l^{-1} h^{-1}, with 60.11 g l^{-1} of 2,3-butanediol (2,3-BDO) produced in parallel with 1,3-PDO. Seo et al. [26] obtained mutants deficient in the oxidative pathway, however, the production of 1,3-PDO was not improved, probably due to a redox imbalance. Zhu et al. [27] cloned the *yqh*D gene encoding 1,3-propanediol oxidoreductase isoenzyme (PDORI) from *Escherichia coli* in *K. pneumoniae.* The overexpression of PDORI led to a higher 1,3-PDO production, reaching 67.6 g l^{-1}. In addition, the concentration of the toxic intermediate 3-hydroxypropionaldehyde was reduced by 22.4% when compared to the original strain. Huang et al. [15] examined the effects of an over-expressed aldehyde dehydrogenase gene on the simultaneous production of 3-hydroxypropionic acid and 1,3-PDO by *K. pneumoniae*, obtaining 24.4 g l^{-1} and 49.3 g l^{-1}, respectively. A high production of 1,3-PDO using co-substrates is recorded by Oh et al. [28] using *K. pneumoniae* mutant deficient in carbon catabolite repression. Under optimized conditions, the concentration of 1,3-PDO from glycerol was 81.2 g l^{-1} using molasses as a co-substrate. Rossi et al. [20] reported concentrations up to 23.80 g 1,3-PDO l^{-1} in batch fermentations under controlled pH, while in fed-batch cultivations the 1,3-PDO production was 36.86 g l^{-1} using a new strain of *K. pneumoniae*. Sattayasamitsathit et al. [29] applied a statistical optimization for the simultaneous production of 1,3-PDO and 2,3-BDO using crude glycerol by a new *K. pneumoniae* isolate. They reported a yield of 24.98 g 1,3-PDO l^{-1} and 9.54 g 2,3-BDO l^{-1}. A high production of 1,3-PDO by a D-lactate deficient mutant of *K. pneumoniae* was reported by Xu et al. [30], obtaining 102.06 g 1,3-PDO l^{-1} from aerobic fed-batch fermentation. The same approach was tested by Durgapal et al. [31], which was used to construct a *K. pneumoniae* mutant for lactate formation. In glycerol fed-batch fermentation, the mutant strain produced 58.0 g 1,3-PDO l^{-1} with a yield of 0.35 g g^{-1} and 2,3-BDO as the main byproduct (26.6 g l^{-1}).

In this work, response surface methodology was used to determine the interaction effect of four independent variables (pH, temperature, stirrer speed, and glycerol concentration) on 1,3-propanediol production and productivity by a new *K. pneumoniae* isolate.

2. Materials and methods

2.1. Isolation and identification of microorganisms

For the isolation of microorganisms that use glycerol as their only carbon and energy source, Erlenmeyer flasks containing an enrichment minimal medium (g l^{-1} of deionized water: $NH_4H_2PO_4$ 1.0, K_2HPO_4 1.0, $MgSO_4$ 0.2, NaCl 5.0, glycerol 20) were directly inoculated with different natural samples (soil, decaying plant, leaves, mosses, etc.) and incubated at 30 °C and 100 rpm on a rotatory shaker. Pure cultures were obtained by inoculating Petri dishes containing the same enrichment medium. The potential of different isolates were determined by evaluating glycerol bioconversion in high value-added products (data not shown). The isolate GLC29 showed high potential for glycerol fermentation and was identified as a *K. pneumoniae* strain using the Enterobacteriaceae identification kit API 20E (Biomerieux, France). The new isolate GLC29 was characterized for ethanol tolerance, growth pH, osmotolerance, and carbon sources used. Cultures were maintained refrigerated in agar slants containing minimal medium, with periodical transference to new media; long time maintenance was performed in cryotubes containing glycerol 40% (v/v) at −20 °C.

2.2. Glycerol fermentation

For the preparation of the inoculum, the bacterium *K. pneumoniae* GLC29 from agar slants was grown in 500 ml Erlenmeyer flasks containing 200 ml of autoclaved (121 °C/15 min) minimal medium (g l^{-1} of deionized water: $NH_4H_2PO_4$ 5.0, K_2HPO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.2; NaCl 1.0, yeast extract 1.0, glycerol 20) and 200 μl of trace element solution (g l^{-1} of deionized water: EDTA 0.5, $CaCl_2{\cdot}2H_2O$ 0.5, $CoCl_2{\cdot}6H_2O$ 0.16, $MoNH_4{\cdot}4H_2O$ 0.1, $CuSO_4{\cdot}5H_2O$ 0.16, $FeSO_4{\cdot}7H_2O$ 0.5, $MnSO_4{\cdot}H_2O$ 0.5, $ZnSO_4{\cdot}7H_2O$ 0.22, $NiCl_2{\cdot}6H_2O$ 0.03, H_3BO_3 0.12). Seed culture flasks were incubated overnight on a rotatory shaker at 30 °C and 100 rpm.

The fermentation medium was the same as for seed inocula. Glycerol was added in different concentrations, and the pH was adjusted with NaOH, in agreement with each experiment (Table 1). Batch fermentations were carried out on a 2 l reactor containing 500 ml final working volume, with a 5% (v/v) inoculum. The pH was maintained through the automatic addition of 5 M NaOH, and the temperature was controlled by a water bath with microprocessor-based temperature control. The fermentation medium was stirred magnetically with cylindrical stir bars (12 mm × 55 mm). Each fermentation experiment was run in duplicate or triplicate.

2.3. Analytical methods

Fermentation samples were withdrawn periodically for growth monitoring and to determine residual substrates and metabolites. Samples for HPLC were centrifuged (approximately 12,000 × *g*/8 min), and the supernatant was frozen for posterior analysis. Cell growth was monitored at 600 nm (OD_{600}) on a Bel Photonics SP-220 UV/vis spectrophotometer. A standard curve was constructed relating OD_{600} to cell dry weight (CDW). The pH was recorded for fermentation monitoring (pH rises after carbon source depletion), and 5 M NaOH consumption was recorded for the correction of dilution due to base addition in the reactors. Residual substrate and metabolites (succinate, lactate, formate,