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Evaluation of 1,3-Propanediol Production from Crude Glycerol by *Citrobacter freundii* ATCC 8090

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1,3-Propanediol (1,3-PDO) is a bifunctional organic compound used for many synthesis reactions. This molecule can be obtained by chemical or biochemical route. The natural substrate for microbial production of 1,3-PDO is glycerol. So, a way to cheapen 1,3-PDO production process by biochemical route is to use glycerol from biodiesel production. As demand and production of biodiesel grow exponentially, the utilization of glycerol becomes an urgent topic. In the present work, it was evaluated *Citrobacter freundii* ATCC 8090 capacity to produce 1,3-PDO from crude glycerol. The glycerol used was from a pilot plant of PETROBRAS. The tests were carried out in rotary shaker at 250 rpm and 30 °C for 24 h under anaerobic conditions. The glycerol consume, 1,3-PDO production and byproducts were analyzed by high-performance liquid chromatography (HPLC). This strain was capable to consume crude glycerol and produce 1,3-PDO, reaching a concentration of 4.85 $g.L^{-1}$. The 1,3-PDO yield was approximately 29 % and biomass yield about 7 %, but acetic acid was also produced in high amounts, reaching 9.72 $g \cdot L^{-1}$, double of 1,3-PDO. Another organic acid was also produced, succinic acid, with a yield about 15 %. The results showed that *C. freundii* ATCC 8090 has capacity to consume glycerol from biodiesel production even its containing wastes (triglycerides, salts, methanol, catalyst). So, this strain has potential to be used in the bioprocesses of interest, 1,3-PDO production from crude glycerol.

1. Introduction

1,3-PDO is a bifunctional organic compound could potentially be used for many synthesis reactions, in particular as a monomer for polycondensations to produce polyesters, polyethers and polyurethanes (Biebl et al., 1999). 1,3-PDO also has a number of other interesting applications in addition to that of polymer constituent. It can give improved properties for solvents, adhesives, laminates, resins, detergents and cosmetic (Zeng and Biebl, 2002). This molecule can be obtained by chemical or biochemical route.

The natural substrate for microbial production of 1,3-PDO is glycerol. Glycerol is dehydrated to 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase. The product of dehydration reaction, 3-HPA, is reduced in 1,3-PDO by an NAD-dependent oxidoreductase (Papanikolaou et al., 2000). Since 50 % of entire cost of microbial production of 1,3-PDO is due to the price of raw materials, raw glycerol from biodiesel production processes may be an interesting renewable carbon source for microorganisms that produce 1,3-PDO (González-Pajuelo et al., 2004).

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Glycerol conversion to 1,3-PDO can be carried out by Clostridia as well as Enterobacteriaceae (Biebl et al., 1999). In the actual fermentation a number of other byproducts are formed, i.e., ethanol, lactic acid, succinic acid, and 2,3-butanediol, by the enterobacteria *Klebsiella pneumoniae*, *Citrobacter freundii* and *Enterobacter agglomerans*, butyric acid by *Clostridium butyricum*, and butanol by *Clostridium pasterianum* (Zeng and Biebl, 2002).

Biodiesel production increased exponentially in past several years. The principal byproduct of its production is glycerol, also known as glycerin. As the demand and production of biodiesel grows, the quantity of crude glycerol generated will be considerable, and the utilization of it will become an urgent topic (Thompson and He, 2008). The rest of crude glycerin consists of unconverted triglycerides, unconverted methanol, biodiesel, soaps and contamination. Therefore, this crude glycerol contains too many contaminants for a useful application in chemistry or pharmacy without treatment and the high purification cost of glycerin makes its applications, in pharmaceutical and chemical applications, limited (Amaral et al. 2009).

So, many bioprocesses using glycerol are being studied. In the present work, *Citrobacter freundii* ATCC 8090 was grown in medium containing crude glycerol from biodiesel production. The objective was to evaluate *C. freundii* ATCC 8090 capacity of crude glycerol consumption as principal carbon source and produce 1,3-propanediol. *C. freundii* ATCC 8090 was chosen to be a promising strain to convert crude glycerol to 1,3-PDO since literature reports 1,3-PDO production from pure glycerol by this strain (Barbirato et al., 1996).

2. Materials and Methods

2.1 Strain and Culture Conditions

The bacterium used was *Citrobacter freundii* ATCC 8090. Culture medium used in this work was adapted from Barbirato et al. (1998): 20 $g \cdot L^{-1}$ crude glycerol, 14 $g \cdot L^{-1}$ K_2HPO_4, 6 $g \cdot L^{-1}$ KH_2PO_4, 3 $g \cdot L^{-1}$ $(NH_4)_2SO_4$, 0.2 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 12 $mg \cdot L^{-1}$ $CoCl_2 \cdot 6H_2O$, 0.2 $g \cdot L^{-1}$ yeast extract and 0.2 $g \cdot L^{-1}$ cysteine. Crude glycerol was obtained from pilot plant of biodiesel of by Petróleo Brasileiro S.A. (PETROBRAS).

2.2 Experimental Methodology

The strain was maintained at 4 °C on LB medium. For inoculum, cells were cultivated at 30 °C in a rotary skaker at 250 rpm using 500 mL anaerobic flasks containing 200 mL of 2 % LB medium. After 24 h of cultivation, these cells were centrifuged in sufficient amount to inoculate approximately 1.25 $mg \cdot mL^{-1}$ of cells (dry weight) on culture medium.

The experiment was performed under anaerobic conditions in flask of 500 mL with 200 mL of culture medium at 30 °C in rotary skaker at 250 rpm during 24 h. Samples were taken for cell growth analysis, glycerol consumption analysis and quantification of 1,3-PDO production and byproducts. The experiment was performed in duplicate.

2.4 Analytical Methods

2.4.1 Cell Growth Determination

Growth was followed by optical density measures at 580 nm and O.D. values were converted to cell dry weight per volume (mg $dw \cdot mL^{-1}$) using a factor previously determined.

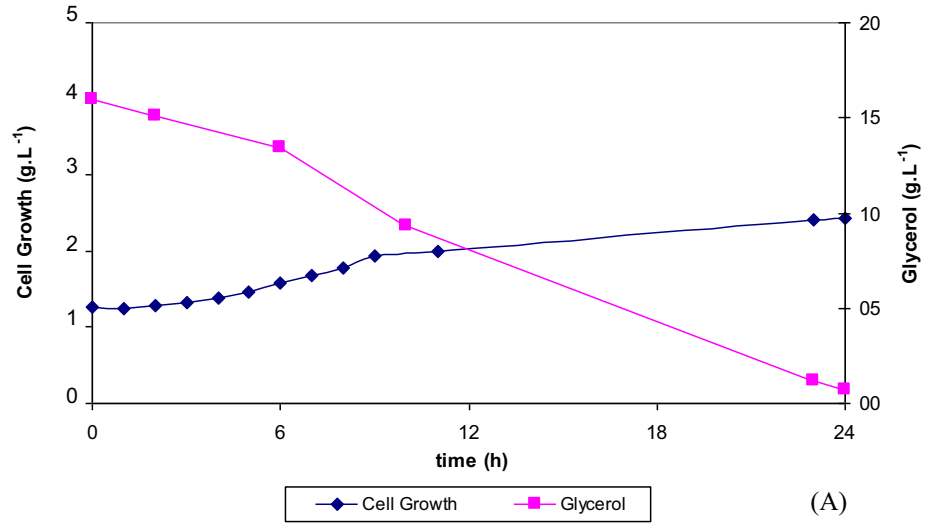
2.4.2 Analysis

1,3-PDO and byproducts as 2,3-butanediol and some organic acids (oxalic, succinic, citric, pyruvic, lactic and acetic) were analysed by high performance liquid chromatography (Waters ®). It was used column Aminex® HPX-87H, 300 x 7.8 mm (Bio-Rad Laboratories Ltd) and pre-column (Bio-Rad Laboratories Ltd), IR detector (Waters 2414), binary pump (Waters 1525), furnace and temperature controller module (Waters) chromatographic software: Breeze. The mobile phase was 5 mM H_2SO_4 at flow rate of 0.8 $mL \cdot min^{-1}$. Injection volume was 20 µL and temperature analysis 60 °C.

The initial and the final pH of culture medium were analyzed at a pH Digimed model DM-22.

3. Results and Discussion

Citrobacter freundii ATCC 8090 was capable to consume crude glycerol and to grow even though its impurities (Figure 1(A)). It is possible to visualize that almost all glycerol was consumed and final cell concentration was approximately 2.5 g·L^{-1}, the double of initial cell concentration. It is important to note that initial glycerol concentration was around 16 g·L^{-1} because crude glycerin used has about 80 % of purity.



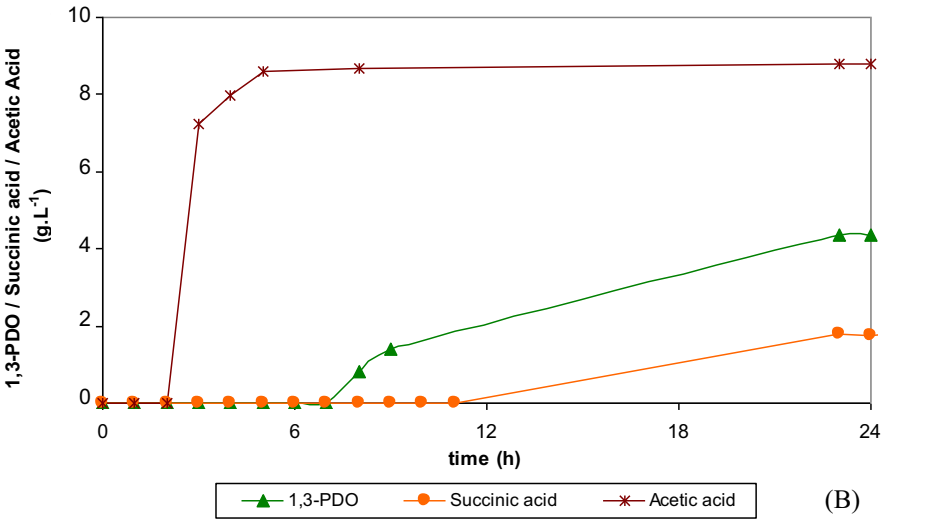


Figure 1: (A) Cell concentration, glycerol consumption, (B) production of 1,3-PDO, acetic acid and succinic acid in experiment with Citrobacter freundii *ATCC 8090*

The strain studied was capable to produce 1,3-propanediol, reaching the concentration of 4.35 g·L^{-1} 1,3-PDO. Other compounds were also produced as succinic acid and acetic acid. Acetic acid was produced at 8.8 g·L^{-1}, the double of 1,3-PDO, and succinic acid reached only 1·82 g.L^{-1}. Figure 1(B) presents the concentrations of 1,3-PDO, acetic acid and succinic acid produced during 24 h of experiment.

Table 1 shows the yields obtained for each product. It is possible to verify that adding all yields it will take a value greater than 100 % because this strain probably consumed others nutrients as triglycerides present in crude glycerol.

Table 1: Yield of biomass and products obtained in experiment with Citrobacter freundii *ATCC 8090*

$Y_{X/S}$	$Y_{P/S}$ 1,3-PDO	$Y_{P/S}$ Acetic acid	$Y_{P/S}$ Succinic acid
8 %	28 %	57 %	12 %

The yield of 1,3-PDO depends on the combination and stoichiometry of reductive and oxidative pathways. It has been shown that the combination of 1,3-PDO generation with acetic acid as sole byproduct of oxidative pathways results in maximum yield of 1,3-PDO (Zeng and Biebl, 2002). It occurs because acetic acid is the unique by-production that does not consume the $NADH.H^+$ formed in glycolysis, increasing the $NADH.H^+$ pool for 1,3-PDO production as shown in Figure 2 (Biebl et al., 1999).

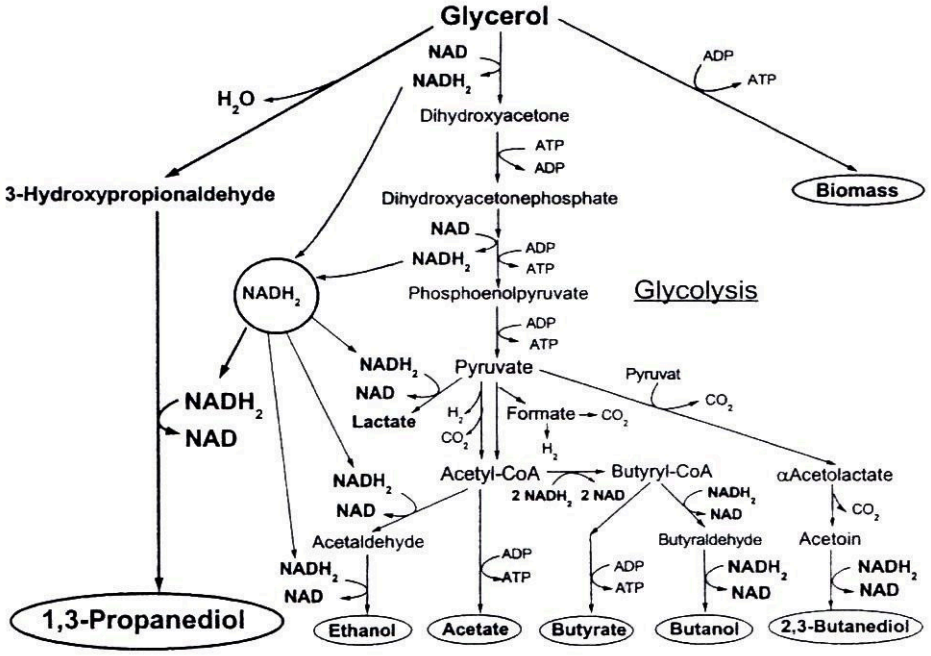


Figure 2: Metabolic pathways of glycerol metabolism (Zeng and Biebl, 2002)

If only 1,3-PDO and acetic acid are formed, the yield of 1,3-PDO is 67 % (mol/mol) and for each acetic acid molecule formed is obtained two 1,3-PDO molecules (Equation 1). However, all byproducts are associated with a loss in 1,3-PDO relative to acetic acid (Zeng and Biebl, 2002).

$$3\ CH3OH\text{-}CHOH\text{-}CH2OH \rightarrow CH3COOH + CO2 + H2 + 2\ CH2OH\text{-}CH2\text{-}CH2OH \quad (1)$$

Some byproducts affect 1,3-PDO yield more than others because do not contribute to the $NADH.H^+$ pool at all (Figure 2). One of them is succinic acid that was formed in this experiment what can justify the reduction in 1,3-PDO yield.

A parameter very important that can affect 1,3-PDO yield is pH. The initial pH of culture medium was 7.05, but pH reduced considerably after 24 h of experiment, reaching 5.25. This result is probably due to organic acids production.

Biebl et al. (1998) produced 1,3-PDO from glycerol by *Klebsiella pneumonia* and observed the largest increase in 1,3-PDO at pH 7.0. It has been shown in this investigation that, under conditions of low pH, the electron supply for 1,3-PDO formation can be entirely provided from the fermentation route to others products.
Forsberg (1987) verified that growing *Clostridium butylicum* B593 in a chemostat culture at pH 6.5, 61 % of the glycerol fermented was converted to 1,3-PDO. When the pH was decreased to 4.9, growth and 1,3-propanediol production were substantially reduced.
So, it is possible that high production of acetic acid at the beginning of the experiment contributes to pH reduction and its affected 1,3-PDO production.
Another way to increase the pool of $NADH.H^+$ for 1,3-PDO production is to increase the amount of cysteine in culture medium. Because low-pK_a cysteine are selectively oxidized (Rhee et al., 2000). So, the cysteine reduces NADH to $NADH.H^+$, increasing the availability of NAD reduced form. However, this amino acid is not an inexpensive reducing agent.
The potential for the electrocatalytic oxidation of cysteine is closely related to the Co^{III}/Co^{II} couple in acidic media and to the Co^{II}/Co^{I} couple in basic media (Maree and Nyokong, 2000). So, it is also necessary to optimize the concentration of cobalt ion in the culture medium. Furthermore, the catalytic currents and the oxidation potential for cysteine are dependent on the pH of the solution, the potential becoming less positive as the pH increases and the catalytic currents decreasing with increase in pH, for the same concentration of cysteine (Maree and Nyokong, 2000). This is another reason that shows the need to control the pH during 1,3-PDO production.

4. Conclusion

The results showed that *Citrobacter freundii* ATCC 8090 has capacity to consume the glycerol from biodiesel production even its containing wastes (triglycerides, salts, methanol, catalyst). *C. freundii* ATCC 8090 was able convert glycerol to 1,3-propanodiol. After 24 h of experiment, the 1,3-PDO concentration was 4.35 $g \cdot L^{-1}$, but this concentration may have been reached before. The 1,3-PDO yield was 28 %, but the major product was acetic acid (8.8 $g \cdot L^{-1}$; $Y_{P/S}$ = 57 %), that was double of 1,3-PDO. Another organic acid was also produced, succinic acid (1.82 $g \cdot L^{-1}$), with yield about 12 %.
So, *C. Freundii* ATCC 8090 showed to be a potential producer of 1,3-propanediol from crude glycerol. However, it is necessary to carry out new experiments with pH control and optimize some parameters in attempt to increase the yield and productivity of 1,3-PDO.

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