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Production of 1,3-Propanediol by *Clostridium butyricum* Growing on Biodiesel Derived Glycerol

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The continuous demand for alternative biofuels resulted in a significant rise of biodiesel production in last decade. As a consequence, high quantities of raw glycerol have been accumulated. The conversion of this abundant carbon source into value-added products using biotechnology consists in a significant opportunity for industry. Crude glycerol may be used in different processes, including bioconversion to 1,3-propanediol (1,3-PDO). 1,3-PDO is an important intermediate chemical for polymer synthesis. Some species are known to produce 1,3-PDO by fermentation of glycerol as *K. pneumoneae, E. agglomerans, C. freundii, C. acetobutylicum, C. butyricum, C. pasterianum, L. brevis* and *L. buchneri*. In this work, the objective was to produce 1,3- PDO by *Clostridium butyricum* NCIMB 8082 cultivated on biodiesel derived glycerol using batch and fed-batch strategies. The experiments were performed in the 1.0 L reactor, under anaerobic conditions, at 200 rpm with temperature (37ºC) and pH (7,0) control. In batch and fed-batch fermentations, initial glycerol concentration was 60 $g.L^{-1}$and 20 $g.L^{-1}$,respectively. In fed-batch fermentation, two feeds were performed containing 20 $g.L^{-1}$ each one. The glycerol consumption and product formation were analysed by high-performance liquid chromatography (HPLC). After approximately 13 hours of fermentation, a concentration of 32.18 $g.L^{-1}$ of 1,3-PDO in batch condition was reached.. In fed-batch condition, the 1,3-PDO final concentration was 29.83 $g.L^{-1}$ after 11 hours of fermentation. The productivity values were 2.38 and 2.55 $g.L^{-1}.h^{-1}$ in batch and fed-batch conditions, respectively.

1. Introduction

Current industrialization and decrease of petroleum stock have raised the worldwide need for energy generation deriving from various alternative and renewable resources (eq. biodiesel, biohydrogen and/or bioethanol) with biodiesel being considered as one of the most important renewable energy sources utilized (Saxena et al., 2009).

Crude glycerol is the main by-product of oil and fat transesterification in biodiesel industry which grew considerably during the last decade. Glycerol is therefore a cheap feedstock for chemicals production and also an interesting substrate for biotechnological processes. A number of value-added products can be produced from glycerol fermentation as hydrogen, ethanol, succinate and 1,3-propanediol (1,3-PDO).

1,3-PDO constitutes a specialty chemical, implemented as a monomer for the production of plastics of particular properties, such as polyesters, polyethers and polyurethanes (Wilke and Vorlop, 2004). Besides its utilization as a base unit for the synthesis of biodegradable plastics, PDO can present various interesting applications in the chemical industry; this chemical compound can be efficiently used as a polyglycol-type lubrificant, and its addition can significantly improve the properties in various solvent systems, adhesives, laminates, resins and cosmetology products (Paanikolaou, 2009). PDO is a product with an expanding market and a continuously increasing demand over 50,000 tons per year, which has attracted a great commercial interest because of its extensive use in the chemical industry (Szymanowska-Powalowska, 2014).

1,3-PDO is a biofunction organic compound mainly synthesized by chemical route from acrolein or ethylene oxide until few years ago. These chemical processes have been marred by issues such as dependence on valuable catalysers and severe reaction conditions (Ferreira, 2014). Therefore microbial production of 1,3-

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propanediol through fermentation process is increasingly becoming a reasonable alternative to the chemical process (Anand et al., 2011).
A wide range of microorganisms belonging to genera of *Klebsiella*, *Clostridium*, *Citrobacter*, *Enterobacter* and *Lactobacillus* are known to be natural producers of 1,3-PDO from glycerol (Biebl et al., 1991 and Zheng et al., 2009). Glycerol is dehydrated to 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase. The product of dehydratation reaction, 3-HPA, is reduced in 1,3-PDO by NAD-dependent oxidoreductase (Papanikolaou et al., 2000).
The literature report 1,3-PDO production using *Clostridium butyricum*, but there is few reports using *C.butyricum* NCIMB 8082 for 1,3-propanediol production. Thus, the objective of this study was produce 1,3-propanediol by *Clostridium butyricum* NCIMB 8082 cultivated on crude glycerol using batch and fed-batch strategies.

2. Materials and Methods

2.1 Microbial Culture, Culture Conditions and Crude Glycerol

The strain used was *Clostridium butyricum* NCIMB 8082. This bacteria was obtained from National Collection of Industrial and Marine Bacteria (NCIMB). For culture activation was used Reinforced Clostridial Medium (OXOID).
The crude glycerol was obtained from pilot plant of biodiesel of by Petroleo Brasileiro S.A, Brazil (PETROBRAS).

2.2 Experimental Methodology

2.2.1. Pre-culture

The strain was maintained in penicillin flasks at 4°C containing 50 mL of Reinforced Clostridial Medium. For the preparation of the pre-culture, these penicillin flasks containing the grown cells were inoculated in Schott flasks at 37°C, 150 rpm for 18 hours in anaerobic conditions.
The culture medium used to grow of the pre-culture was composed by (in 1L of distilled water) 2.0 g KH_2PO4, 0.5 g NH_4Cl, 0.15 g $MgSO_4$, 0.01 g $CaSO_4$, 0.75 mg $ZnCl_2$, 0.075 g $NaMoO_4$, 0.15 mg $CuCl_2$, 10 mg $FeSO_4$, 0.15 mg Na_2SeO_3, 0.5 g yeast extract, 20 g of glycerol.

2.2.2 1,3-PDO production performed in batch

All culture medium containing in Schott flasks (pre-culture) was transferred to a bioreactor containing 600 mL of medium as described in 2.2.1. The experiment was performed under anaerobic conditions in bioreactor (TecBioTecnal®) with useful capacity of 1 L at 37°C, 200 rpm and pH (7,0) control. Samples were obtained at different times to analyse the glycerol consumption, products formation and cell growth.

2.2.3 1,3-PDO production performed in fed-batch

The culture containing in Schott flasks was transferred to a reactor containing 600 mL of medium (composition described in 2.2.1).
The experiment was performed in bioreactor (TecBioTecnal®) with 1L of capacity at 37°C, 200 rpm and pH control (7.0). Two feeds were performed, each containing 20 $g.L^{-1}$ glycerol and 0,5 $g.L^{-1}$ yeast extract. Samples were obtained at different times to analyse the glycerol consumption, products formation and cell growth.

2.3 Analytical Methods

2.3.1. Cell Growth Determination

Growth cell was determined by optical density measures (D.O.) at 600 nm (Bel Photonics S2000). Absorbance values were converted to dry weight (g per liter) by a predetermined factor.

2.3.2. Analysis

1,3-PDO, glycerol and by-products such as acetic acid and butyric acid were analysed by high performance liquid chromatography (Shimadzu®). It was used column Aminex® HPX-87H, 300 x 7.8 mm (Bio-Rad Laboratories Ltd) and IR detector (Shimadzu®), binary pump (Shimadzu®), temperature controller module (Shimadzu®), chromatographic software: LabSolution (Shimadzu®).
The mobile phase was 5 mM H_2SO_4 at flow rate 0,8 $mL.min^{-1}$. Injection volume was 20 µL and temperature analysis at 60°C.

3. Results and Discussion

In previous studies (Ferreira et al., 2014), *Clostridium butyricum* NCIMB 8082 was capable to grow and to consume crude glycerol when it was cultivated at bioreactor.

The Figure 1 shows cell concentration, glycerol consumption and1,3-propanediol production in batch condition. Acetic acid and butyric acid were also produced. It is possible to observe that the strain was capable to consume 58.5 g.L-1 of glycerol present in the medium after approximately 13 hours of fermentation. The 1,3-PDO final concentration was 32.18 g.L-1. At the same fermentation time, the concentration of acetic acid and butyric acid were 5.64 g.L-1 and 6.44 g.L-1, respectively.

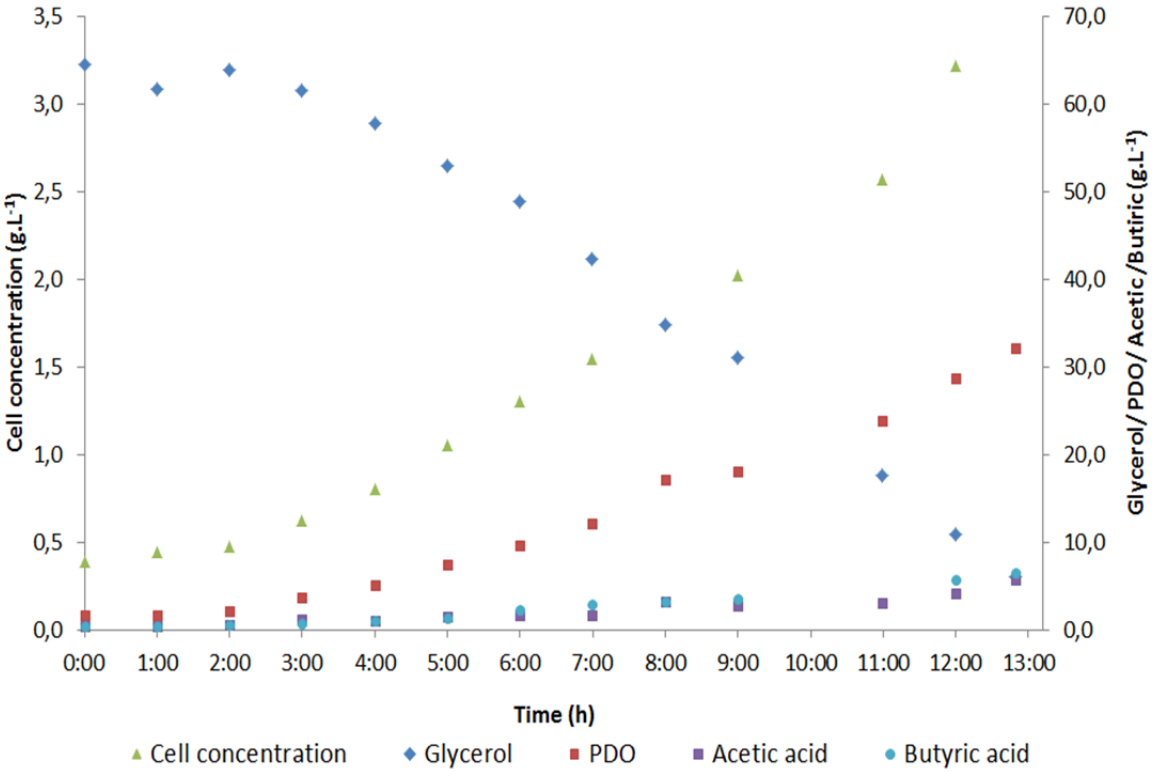

Figure 1: Cell concentration (g.L^{-1}), glycerol consumption, production 1,3-PDO, acetic acid and butyric acid (g.L^{-1}) obtained in experiment performed in batch.

The Figure 2 represents cell concentration, glycerol consumption and production of 1,3-propanediol, acetic acid and butyric acid performed in fed-batch. In fed-batch fermentation was performed two feeds containing 20 g.L^{-1} each one. After 11 hours of fermentation the concentration of 1,3-PDO reached 29.83 g.L^{-1} and the total glycerol consumption was 58.26 g.L^{-1}. It was produced 4,67g.L^{-1}of acetic acid and 5,72 g.L^{-1}of butyric acid.

On visual observation it was observed that the cells began to agglomerate when the glycerol concentration achieved values lower than 5.0g.L^{-1}. Thus, before of pellets formation, the bioreactor was fed with crude glycerol and yeast extract. It probably happened because of nutrients shortage.

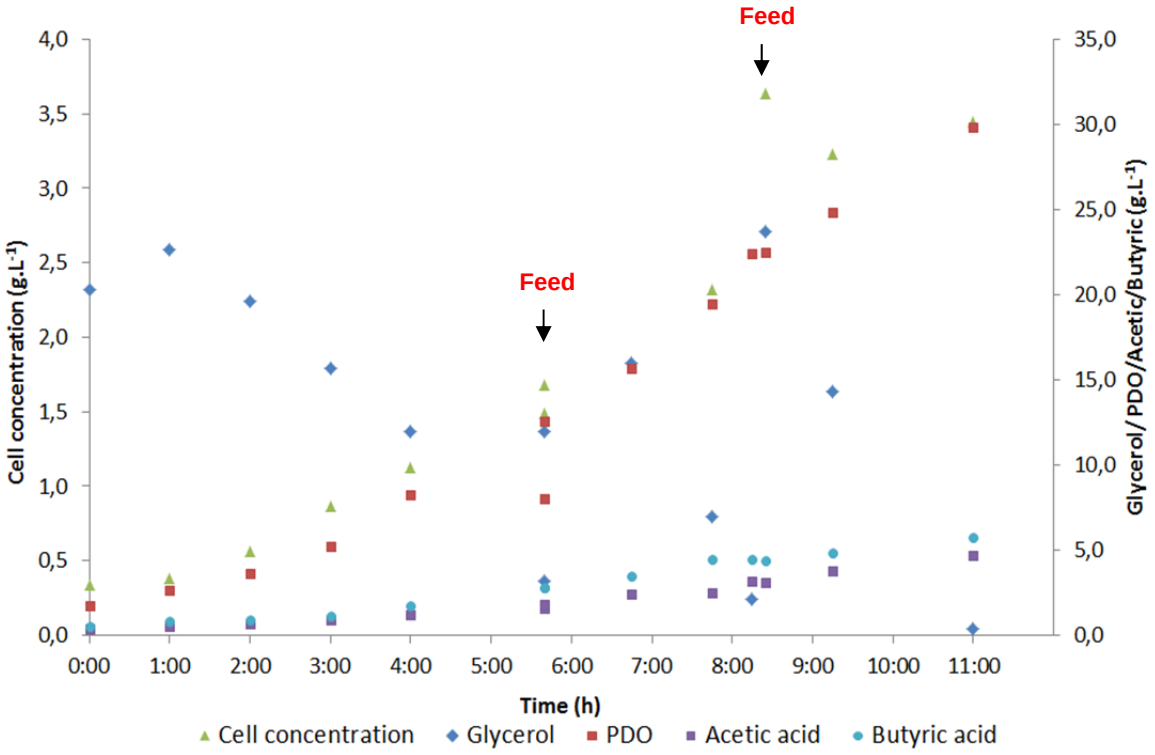

Figure 2: Cell concentration ($g.L^{-1}$), glycerol consumption, 1,3-PDO production, acetic acid and butyric acid ($g.L^{-1}$) obtained in experiment performed in fed-batch.

The Table 1 shows the kinetic parameters obtained in batch e fed-batch performed using *Clostridium butyricum* NCIMB 8082 in crude glycerol. It is possible to observe that yield ($Y_{1,3\ PDO}$) was 0.52 $g.g^{-1}$ in batch and 0.48 $g.g^{-1}$ in fed-batch fermentation. For the productivity was observed for batch and fed-batch, 2.38 $g.L^{-1}.h^{-1}$ and 2.55 $g.L^{-1}.h^{-1}$, respectively.

Table 1: Kinetic parameters obtained during batch and fed-batch fermentations of Clostridium butyricum NCIMB 8082 in crude glycerol.

Kinetic parameters	Batch	Fed-batch
Time (h)	12.8	11.0
X ($g.L^{-1}$)	3.21	3.44
S ($g.L^{-1}$)	58.42	58.26
P ($g.L^{-1}$)	32.18	29.83
$Y_{1,3\text{-PDO}}$ ($g.g^{-1}$)	0.52	0.48
Q ($g.L^{-1}.h^{-1}$)	2.38	2.55

X – biomass, S– glycerol consumed, P–1,3-PDO produced , Y 1,3 PDO – 1,3 PDO produced per glycerol consumed, Q – 1,3-PDO productivity

There are many reports on various methods of cultivation the *C. butyricum* for the synthesis of 1,3-PDO from crude glycerol. The table 2 demonstrate the experimental results of *C. butyricum* obtained by others authors in literature.

The results shows that the highest 1,3-PDO concentration was achieved by Wilkens et al (2012). The authors using a *C. butyricum* genetically modified, performed fed-batch fermentations in 1 L and obtained 76.2 $g.L^{-1}$ of 1,3-propanediol with a productivity of 2.3 $g.L^{-1}.h^{-1}$ after 32.5 hours of fermentation, but with more nutrients than the one used in the present work.

The second highest 1,3-PDO production presented in Table 2 was 63.4 $g.L^{-1}$, achieved by means of batch cultivation. Barbirato et al (1998) study on the synthesis of 1,3-propanediol from crude glycerol in batch

fermentation using *C. butyricum* CNCM1211. The productivity obtained was 1.68 $g.L^{-1}.h^{-1}$., significantly lower than that obtained in this work.
Szymanowska-Powalowska (2014) also studied on the synthesis of 1,3-propanediol from crude glycerol by *C. butyricum* DSP1. The final concentration of 1,3-PDO was 62.0 $g.L^{-1}$ in repeated batch and the maximum productivity, obtained during the second cycle, reached 1.68 $g.L^{-1}.h^{-1}$.
It is possible to note in Table 2 that the most promising result occurs when working in fed-batch. This is interesting, whereas according to some literature data a 20 $g.L^{-1}$ glycerol concentration is considered optimal for the growth and metabolism of Clostridium bacteria.

Table 2: Experimental results of C. butyricum strains production 1,3-propanediol under various fermentation configuration.

Fermentation type	Strain	Type glycerol	PDO ($g.L^{-1}$)	Q ($g.L^{-1}.h^{-1}$)	Reference
Repeated batch	*C. butyricum* DSP1	Crude	62.0	1.68	Szymanowska-Powalowska (2014)
Fed-batch	*C. butyricum* DSP1	Crude	54.2	1.55	Szymanowska-Powalowska (2014)
Fed-batch	*C. butyricum* AKR102a	Crude	76.2	2.30	Wilkens et al (2012)
Batch	*C. butyricum* F2b	Crude	47.1	1.12	Papanikolaou et al (2008)
Batch	*C. butyricum* CNCM1211	Crude	63.4	1.85	Barbirato et al (1998)
Continuous one-stage	*C. butyricum* F2b	Crude	48.1	0.96	Papanikolaou et al (2000)
Continuous two-stage	*C. butyricum* F2b	Crude	43.5	1.33	Papanikolaou et al (2008)

4. Conclusions

Clostridium butyricum NCIMB 8082 was able to grow in crude glycerol using this substrate as unique carbon source. After approximately 13 hours of fermentation the 1,3-PDO production reached 32,18 $g.L^{-1}$, the yield was 0.52 $g.g^{-1}$ and the productivity was 2.38 $g.L^{-1}.h^{-1}$ in batch. In fed-batch conditions the 1,3-PDO production was 29,83 $g.L^{-1}$, the yield was 0.48 $g.g^{-1}$ and the productivity was 2.55 $g.L^{-1}.h^{-1}$ after 11 hours of fermentation. It is possible to observe that the kinetic parameters were a little better when the experiment was performed in fed-batch condition. It happens probably because of the inhibition that occurs in high glycerol concentration.
The strain used in this study showed to be a good 1,3-PDO producer. The productivities obtained in this work were high comparing with other strains reported in literature.
This research shows potential for 1,3-propanediol production without the use of genetic modification tools, which makes the handling of industrial-scale process easier and more economical.

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