CHEMICAL ENGINEERING TRANSACTIONS
VOL. 38, 2014
A publication of AIDIC
The Italian Association of Chemical Engineering
www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Taj Keshavarz

ISBN 978-88-95608-29-7; **ISSN** 2283-9216
DOI: 10.3303/CET1438080

Evaluation of 1,3-Propanediol Production from Glycerine by *Clostridium Butyricum* NCIMB 8082

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1,3-Propanediol (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. 1,3-PDO also has a number of other interesting applications: solvents, adhesives, laminates, resins, detergents and cosmetic. This molecule can be obtained by chemical or biochemical route, but only the last one is operating industrially. It can be produced from renewable resources as glycerin using microorganisms. A number of microorganisms can ferment glycerol to 1,3-propanediol, but the most studied species are *Klebsiella pneumonia*, *Citrobacter freundii*, *Clostridium butyricum* and *Lactobacillus brevis*. This is an interesting route because the biodiesel production increases every year in Brazil, producing an excess of glycerin. The present work investigated the 1,3-PDO production capacity of *Clostridium butyricum* NCIMB 8082 using glycerin as substrate. Initially, this strain was grown in two different mediums from literature at 35 ºC and 250 rpm in 250 ml SCOOT flasks. The 1,3-PDO was produced in both conditions. In medium 1, the conversion into 1,3-PDO was 50 % and the productivity was 0.32 $g.L^{-1}.h^{-1}$. In medium 2, the conversion into 1,3-PDO was 56 % and the productivity was 0.21 $g.L^{-1}.h^{-1}$. These experiments showed that *C. butyricum* NCIMB 8082 is a good producer of 1,3-PDO. So, fed-batch fermentation was performed in a bioreactor 1 L with pH control and *Cl. butyricum* NCIMB 8082 showed to be a potential producer of 1,3-propanediol. The productivity of 1,3-propanediol was 1.29 $g.L^{-1}.h^{-1}$ and the yield was 0.56 $g.g^{-1}$.

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. 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 (Ferreira et al., 2012).

So far, 1,3-propanediol has been mainly synthesized by chemical route either from acrolein or ethylene oxide. These chemical processes has been marred by issues such as dependence on valuable catalysers and severe reaction conditions such as high temperature and pressure which in turn consume large amounts of energy resulting in the high cost of 1,3-propanediol production. Therefore, microbial production of 1,3-propanediol through fermentation processes is increasingly becoming a reasonable alternative to the chemical method (Anand et al. 2011).

Thus, There are three industrial routes for obtaining 1,3-propanediol: hydration of acrolein followed by hydrogenation, hydroformylation and hydrogenation of ethylene oxide and a fermentative route from glucose. However, currently only the biotechnological route is used for commercial purposes (Jagger, 2012).

Please cite this article as: Ferreira T., Saab V., Matos P., Ribeiro C.M., Coelho M.A., 2014, Evaluation of 1,3-propanediol production from glycerine by clostridium butyricum ncimb 8082, Chemical Engineering Transactions, 38, 475-480 DOI: 10.3303/CET1438080

The microbial production of 1,3-propanediol from glycerol has been demonstrated mainly by *Klebsiella pneumoniae*, *Citrobacter freundii*, *Clostridium pasteurianum*, and *Clostridium butyricum* (Saxena et al. 2009). 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).

Nevertheless, the euphoria once associated with biodiesel has given way to the disappointment. The reason for this lies in its price along with enormous generation of waste streams consisting mainly of glycerol. Therefore, it is very important to explore economical ways of utilizing this low-grade glycerol in order to defray the cost of biodiesel production in the growing global market. One of the innovative utilizations of crude glycerol is its microbial conversion to 1,3-propanediol (Anand et al. 2011).

The literature reports 1,3-propanediol production using *Clostridium butyricum*, but there is no report using the strain *Cl. butyricum* NCIMB 8082 for 1,3-propanediol production. So, the objective of the present work was to evaluate the ability of *Clostridium butyricum* NCIMB 8082 to grown on crude glycerol consumption and to produce 1,3-propanediol.

2. Materials and Methods

2.1 Strain and Culture Conditions

The bacterium used was *Clostridium butyricum* NCIMB 8082. This strain was obtained from National Collection of Industrial and Marine Bacteria (NCIMB). For culture activation was used Thioglycolate Fluid Medium and Reinforced Clostridial Medium.
Crude glycerol was obtained from pilot plant of biodiesel of by Petróleo Brasileiro S.A. (PETROBRAS).

2.2 Experimental Methodology

2.2.1. Experiments performed in SCOOT flasks
The strain was maintained in penicillin flasks at 4 °C containing 50 mL of Reinforced Clostridial Medium with Thioglycolate Fluid Medium. For inoculum, these penicillin flasks containing the grown cells were incubated at 37 °C for 30 minutes in a rotary skaker at 250 rpm. After that, the cells were inoculated in SCOOT flasks containing 200 mL of 2 different media described in Table 1. In both mediums was purged N_2 before and after the inoculum. Samples were obtained at different times to analyse the glycerol consumption, products formed and cell growth.

Table 1: Composition of different media used.

Medium 1 (Chatzifragkou et al., 2011)	Medium 2 (Wilkens et al., 2012)
3.4 g.L^{-1} K_2HPO_4	
1.3 g.L^{-1} KH_2PO_4	2.71 g.L^{-1} KH_2PO_4
2.0 g.L^{-1} $(NH_4)_2SO_4$	2.69 g.L^{-1} NH_4Cl
0.2 g.L^{-1} $MgSO_4.7H_2O$	0.186 g.L^{-1} $MgSO_4.7H_2O$
0.02 g.L^{-1} $CaCl_2.2H_2O$	0.025 g.L^{-1} $CaSO_4.2H_2O$
2.0 g.L^{-1} $CaCO_3$	0.015 g.L^{-1} $FeSO_4.7H_2O$
1.0 g.L-1 yeast extract	5.0 g.L-1 yeast extract
20.0 g.L^{-1} glycerine	20.0 g.L^{-1} glycerine
2.0 mL.L^{-1} trace element solution[1]	3.0 mL.L^{-1} trace element solution[3]
1.0 mL.L^{-1} Fe solution[2]	pH = 7.0
pH = 7.0	

[1]70 mg.L^{-1} $ZnCl_2$; 0.1 g.L^{-1} $MnCl_2.4H_2O$; 60 mg.L^{-1} H_3BO_3; 0.2 g.L^{-1} $CoCl_2.2H_2O$; 20 mg.L^{-1} $CuCl_2.2H_2O$; 25 mg.L^{-1} $NiCl_2.4H_2O$; 35 mg.L^{-1} $Na_2MO_4.2H_2O$; 0.9 mL.L^{-1} HCl 37%.
[2]5.0 g.L^{-1} $FeSO_4.7H_2O$; 0.9 mL.L^{-1} HCl 37%.
[3]1.0 g.L^{-1} $FeCl_3.4H_2O$; 0.5 g.L^{-1} $MnCl_2.4H_2O$; 0.85 g.L^{-1} $CoCl_2.6H_2O$; 0.5 g.L^{-1} $CaCl_2.2H_2O$; 0.5 g.L^{-1} $ZnCl_2$; 0.1 g.L^{-1} $CuCl_2$; 0.05 g.L^{-1} H_3BO_3; 0.05 g.L^{-1} $Na_2MO_4.2H_2O$; 0.13 g.L^{-1} $NiCl_2.6H_2O$; 5.0 g.L^{-1} NaCl; 0.1 g.L^{-1} $Na_2SeO_3.5H_2O$.

2.2.2. Experiments performed in bioreactor

The strain was maintained in penicillin flasks at 4 °C containing 50 mL of Reinforced Clostridial Medium. For inoculum, these penicillin flasks containing the grown cells were incubated at 37 °C for 30 minutes in a rotary skaker at 250 rpm. After that, all culture medium containing in SCOOT flasks was transferred to a bioreactor containing 800 mL of Medium 1. This procedure was performed using purge of N_2. Samples were obtained at different times to analyse the glycerol consumption, products formed and cell growth.

The experiment was performed under anaerobic conditions in bioreactor with useful capacity of 1 L at 37 °C in rotary skaker at 200 rpm during 30 h with temperature and pH control. The feed medium was composed of glycerol (200 $g.L^{-1}$) and yeast extract (10 $g.L^{-1}$).

2.3 Analytical Methods

2.3.1 Cell Growth Determination

Growth was followed by optical density measures (D.O.) at 600 nm.

2.3.2 Analysis

1,3-PDO and by-products such as butanol, 2,3-butanediol, ethanol and some organic acids (butyric, 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·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

Clostridium butyricum NCIMB ATCC 8082 was capable to consume crude glycerol and to grow even though its impurities (Table 2). It is possible to visualize that much of glycerol was consumed after 30 hours even the low inoculum used. It is important to note that initial glycerol concentration varies due to the difficulty of sampling glycerin because of their viscosity. The crude glycerine used has about 80 % of purity.

Table 2: Results obtained in the experiments performed at SCOTT flasks using Medium 1 and Medium 2.

	Medium 1					Medium 2				
Time (h)	$O.D._{600nm}$	Glycerol ($g.L^{-1}$)	PDO ($g.L^{-1}$)	Acetate ($g.L^{-1}$)	Succinate ($g.L^{-1}$)	$O.D._{600nm}$	Glycerol ($g.L^{-1}$)	PDO ($g.L^{-1}$)	Acetate ($g.L^{-1}$)	Succinate ($g.L^{-1}$)
0	0.367	16.3	0.00	0.00	0.00	0.261	19.0	000	0.00	0.00
4	0.298	16.1	0.00	0.24	0.05	0.244	18.9	0,00	0.07	0.07
8	0.275	11.3	1.05	1.40	0.43	0.198	17.9	0.41	0.26	0.26
24	3.900	6.0	5.25	1.23	0.36	0.800	16.7	0.65	0.11	0.11
28	3.960	5.6	5.37	1.21	0.37	1.300	15.2	2.57	0.06	0.06
30	3.960	4.7	5.45	1.38	0.34	1.300	10.8	4.56	0.07	0.07

The cell growth was higher in Medium 1 than in Medium 2. The glycerol consumption and 1,3-propanediol production were also higher in Medium 1. After 24 hours of fermentation, remained 5.6 $g.L^{-1}$ of glycerol in Medium 1 and 16.7 $g.L^{-1}$ in Medium 2. At the same time, 1,3-propanediol concentration was 5.25 $g.L^{-1}$ in Medium 1 and only 0.65 $g.L^{-1}$ in Medium 2. After 30 hours of fermentation, still remain significant amounts of glycerol. It probably happened because of pH reduction that inhibited cell metabolism.

The final pH was lower in Medium 1, that showed the highest production of acetate and succinate.

Despite Medium 2 have provided a greater 1,3-propanediol yield (56 %) compared with Medium 1 (50 %), this one showed highest 1,3-propanediol productivity (0.33 $g.L^{-1}.h^{-1}$).

The 1,3-propanediol productivity in Medium 2 was only 0.21 $g.L^{-1}.h^{-1}$. So, the Medium 1 was chosen to performer the experiment in bioreactor with pH control for the purpose of evaluate the strain ability to produce 1,3-propandiol.

The Figure 1 shows the dates of cell growth, glycerol consumption, 1,3-propanediol production and pH of feed-batch performed at bioreactor. Even at low cell inoculum it was obtained a high 1,3-propanediol

production in a few hours. It is possible to note that all glycerol was consumed in 6 hours, producing 9.40 $g.L^{-1}$ of 1,3-propanediol.

The cells began to agglomerate near six hours of fermentation, forming pellets. It probably happened because of nutrients shortage. After that, the bioreactor was fed with glycerine and yeast extract. So, the pellets disappeared because the cells dispersed again in the medium.
Clostridium butyrium NCIMB 8082 was capable to continue consuming quickly the glycerol and after 10 hours of fermentation the 1,3-propanediol concentration reached 14.0 $g.L^{-1}$. The productivity was 1.29 $g.L^{-1}.h^{-1}$ and the yield was 0.56 $g.g^{-1}$. This productivity values are reported in the literature but only for *Clostridium butyricum* genetically modified.
Chatzifragkou *et al.* (2011) performed fed-batch fermentation using a strain of *Cl. butyricum*. The final 1,3-propanediol concentration was 67.9 $g.L^{-1}$ and the yield was 0.55 $g.g^{-1}$. However these results was obtained after 96 hours of fermentation, so the productivity was 0.70 $g.L^{-1}.h^{-1}$, much lower than that obtained in this work using the same medium composition.
Wilkens *et al.* (2012) also 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 they used a *Cl. butyricum* genetically modified and a culture medium with more nutrients than the one used in the present work.

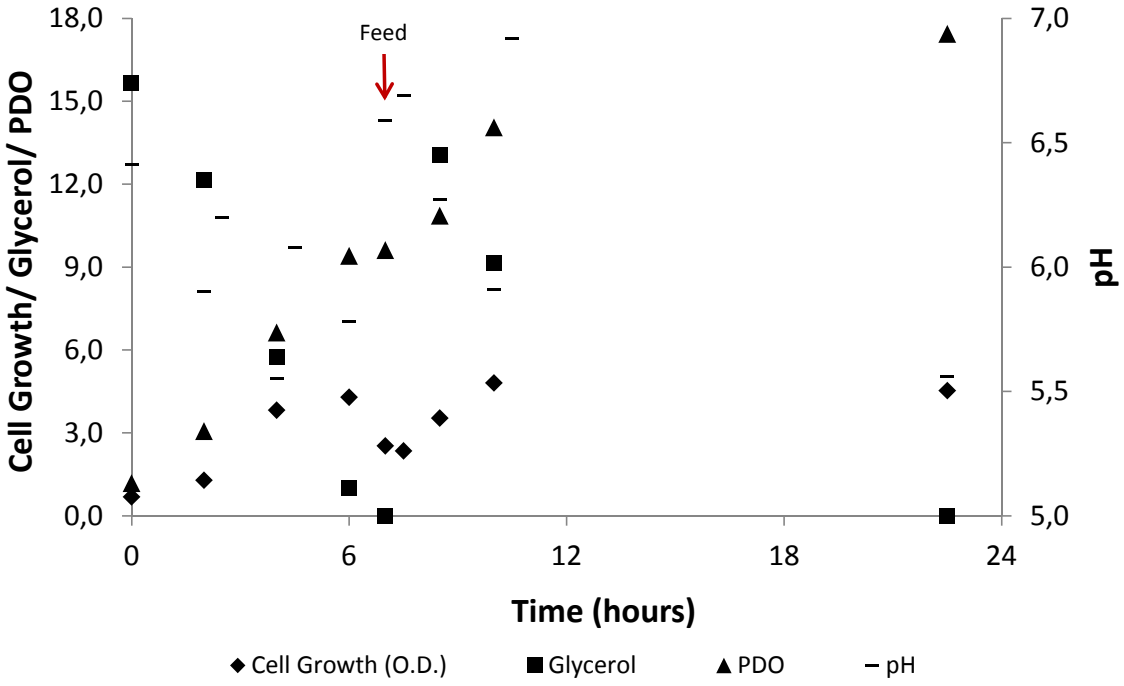

Figure 1: Cell growth (O.D.), glycerol consumption, 1,3-propanediol (PDO) production and pH from feed-batch performed with Cl. butyricum at bioreactor.

It is important to observe that the final 1,3-propanediol concentration (17.5 $g.L^{-1}$) may have been obtained before 22 hours of fermentation, but no sample was obtained during this period. The pH was not controlled during this period too. This fact may have contributed to reduced conversion into 1,3-propanediol because pH control is very important to improve 1,3-propanediol. Biebl et al. (1999) 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.
Many studies indicate the possibility of converting glycerol to 1,3-propanediol industrially. However, until recently, the majority of studies cited in literature dealing with the production of 1,3-propanediol, utilized pure glycerol as the sole carbon source. Only a relative small number of reports mentioned the valorization of raw, biodiesel-derived waste glycerol as substrate for 1,3-propanediol production (Barbirato et al., 1998; Gonza´lez-Pajuelo et al., 2004; Papanikolaou et al., 2004; Petitdemange et al., 1995).

Petitdemange et al. (1995) showed that several strains of *Cl. butyricum* were unable to grow on raw glycerol deriving from trans-esterification of rapeseed oil. Gonza´lez-Pajuelo et al. (2004) reported that raw glycerol can induce a growth inhibition of 86% on *Cl. butyricum* VPI 3266, when found in the fermentation medium in initial concentrations of 100 g/L. However, in the same work, it was stated that the effect of raw glycerol was minimal on batch and fed-batch cultures. In other study, the application of an analytical model showed that high inlet substrate concentrations positively affected the biosynthesis of butyric and acetic acids while biomass yield decreased due to the microbial metabolism being directed towards the pathway of organic acids biosynthesis (and hence carbon losses as CO_2) instead of that of biochemical anabolic reactions of biomass formation (Papanikolaou et al., 2004).
It is known that in order for a successful industrial production of PDO to be performed, very high final PDO concentrations (e.g., 80 g/L or even significantly higher ones) should be achieved in order to ensure a satisfactory downstream processing from the fermentation broth. These concentrations, although toxic for several PDO-producing strains, can be achieved only when large amounts of glycerol (e.g., 150 g/L or higher) can be consumed in batch or fed-batch mode (Chatzifragkou et al, 2010).
Further research is needed to find what the maximum glycerine concentration that *Cl. butyricum* NCIMB 8082 supports and the impact of glycerine impurities in metabolism of this strain, as has been done for *Cl. butyricum* VPI 1718 in previous works (Chatzifragkou et al, 2010). After knowing the maximum glycerine concentration that will not inhibit the strain, it is necessary to optimize the conditions of 1,3-propanediol production in fed-batch, which seems to be the best strategy for 1,3-propanediol.

4. Conclusions

The results obtained in this work showed that *Clostridium butyricum* NCIMB 8082 is an excellent strain to be studied for the 1,3-propanediol production. This strain was capable to produce 1,3-propanediol with a productivity of 1.29 $g.L^{-1}.h^{-1}$ and the yield was 0.56 $g.g^{-1}$. This productivity values are reported in the literature but only for *Clostridium butyricum* genetically modified. Furthermore, in the present study was used crude glycerol, a by-product of biodiesel production, which has impurities such as methanol, basic catalyst, salt, unconverted glycerides. The results showed that *Cl. butyricum* NCIMB 8082 was able to grow in crude glycerol using this residue as only carbon source for 1,3-propanediol production.
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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