SENRA Academic Publishers, Burnaby, British Columbia
Vol. 6, No. 3, pp. 2057-2062, October 2012
Online ISSN: 1920-3853; Print ISSN: 1715-9997

Canadian Journal of pure&applied sciences

STUDY OF VIABILITY OF 1,3-PROPANEDIOL PRODUCTION BY *CLOSTRIDIUM ACETOBUTYLICUM* ATCC 4259

FGD Ferreira, GM Vinhas, YMB Almeida and *Maria Alice Gomes de Andrade Lima
Department of Chemical Engineering, Federal University of Pernambuco, Recife, PE, Brazil

ABSTRACT

The conversion of glycerol to 1,3-propanediol (1,3-PD) by *Clostridium acetobutylicum* ATCC 4259 was studied in an anaerobic culture using commercial glycerol as the sole carbon source. The objective of this study was to determine the best concentration of glycerol used for the production of 1,3-propanediol and to study the cell growth kinetics of *Clostridium acetobutylicum* ATCC 4259 using fermentation of $10g.L^{-1}$ glycerol and to compare the experimental results with those obtained through the traditional mathematics model. The concentrations evaluated of glycerol were 10.0; 30.0 and $60.0g.L^{-1}$. The amount of glycerol consumed and the 1,3-PD produced were determined using chromatography. Cell growth kinetics was measured turbidimetrically (at 660nm). The best conversion rate of glycerol to 1,3-propanediol and biomass was $0.31g.g^{-1}$ and $0.21g.g^{-1}$, obtained from the fermentation of $10g.L^{-1}$ substrate. In this research, the Monod model presented a way to express the experimental data, showing the following values of kinetic constants, $0.024h^{-1}$ and $0.011g.L^{-1}$ to μ_{max} and K respectively.

Keywords: *Clostridium acetobutylicum* ATCC 4259, glycerol, 1,3-propanediol

INTRODUCTION

Glycerol is a low-cost renewable resource appearing in increasing quantities as the principal by-product from biodiesel production. Conversion of glycerol to various compounds with the aid of microbial cells has recently attracted much interest, which and has focused on the production of 1,3-propanediol (1,3-PD) (Papanikolau *et al*., 2004). 1,3-PD was first observed as a product of glycerol in 1881. It is one of the oldest known fermentation products, but little attention has been paid to this microbial pathway for over a century (Biebl *et al*., 1999). 1,3-PD, an emerging bulk chemical, is a monomer for producing plastics with special properties, such as biodegradability, and is the base of the new polyester PTT (polypropylene terephtalate), with better chemical and mechanical properties for the finished material compared to PET (polyethylene terephtalate) (Biebl *et al*., 1999; Deckwer, 1995).

In the past, 1,3-PD was produced only chemically by two methods: the hydration of acrolein or the hydroformylation of ethylene. Chemical synthesis, however, has many disadvantages – it requires high pressure, high temperature and catalysts. Consequently, the costs of 1,3-PD production are very high (Igari *et al*., 2000). An attractive alternative for chemical synthesis is the microbial conversion of raw materials to 1,3-PD. This method is easy and does not generate toxic by-products. Nevertheless, the major limitation of industrial microbial production of 1,3-PD is the relatively high cost of the typical substrate such as glucose. An economically attractive solution to this problem might be the use of crude glycerol (without prior purification) as a fermentative substrate (Nakamura *et al*., 2003; Mu *et al*., 2006).

Under anaerobic conditions glycerol can be used as a substrate for growth by many microorganisms. In addition, it has been known for the past 50 years that glycerol can be converted by facultative anaerobic bacteria to 1,3-PD and other compounds (Deckwer, 1995). This is a typical product of glycerol fermentation and has not been found in anaerobic conversions of other organic substrates (Biebl *et al*., 1999). In most (but not all) microorganisms studied under anaerobic conditions, glycerol is metabolized through a dismulation process – two branches, reductive and oxidative. The reductive branch leads to 1,3-PD formation. This branch is initiated by glycerol dehydradation by enzyme glycerol dehydratase (GDHt), which produces 3-hydroxypropionaldehyde (3-HPA). Afterwards, 3-HPA is reduced to 1,3-PD dehydrogenase (1,3-PD DH). The second, oxidative branch begins with glycerol oxidation by NAD^+-dependent glycerol dehydrogenase (GDH), which dihydroxyacetone (DHA) and subsequently phosphorylated to yield dihydroxyacetone-phosphate (DHAP) and is then further metabolized to form such metabolites as CO_2, H_2, acetate, butyrate, lactate, ethanol, butanol or 2,3-butanediol (Abbad-Andaloussi *et al*., 1998;

*Corresponding author email: magal@ufpe.br

González-Pajuelo *et al*., 2006; Rivaldi *et al*., 2007; Celinska, 2010; Schutz and Radler, 1984). The biological production of 1,3-PD from glycerol has been demonstrated with several bacterial species, e.g., *Lactobacillus brevis*, *Lactobacillus buchnerii* (Solobov and smiley, 1960; Humphers, 1924), *Bacillus welchii* (Streekstra *et al*., 1987), *Citrobacter freunddi*, *Klebsiella pneumoniae* (Homann *et al*., 1990; Dabrock *et al*., 1992) *Clostridium pasteurianum* (Heyndrickx *et al*., 1991) and *Clostridium butyricum* (Biebl *et al*., 1992; Saint-Amans *et al*., 1994; Cornilot and Soucaille, 1996). Among these microorganisms, *Clostridium butyricum* is, to our knowledge, the best "natural producer" in terms of both the yield and titer of 1,3-PD produced (Cornillot and Soucaille, 1996). This understanding led to the possibility of converting other clostridia to 1,3-PD. Among the clostridia, *Clostridium acetobutylicum* is the microorganism of choice, as it has already been used for the industrial production of solvent (González-Pajuelo *et al*., 2005).

Kinetic models are tools used in engineering, applied to the prediction of microbial growth to analyze, design, improve and control the cultivation processes. Many unstructured models of bacterial cultures are described in the literature, but no work for *Clostridium acetbutylicum* ATCC 4259 (Gunzel *et al*., 1991) has been found. Some kinetic models have been proposed to describe the effects of substrate concentrations on the rate of biomass growth. The substrate is usually composed of carbohydrates that can provide carbon and energy sources for 1,3-propanediol produced by bacteria. Among them the classical Monod model (or Michaelis-Menten Model) (Eq.1) has been widely used. According to Monod, μ is a Michaelis-Menten function of the substrate concentration S.

$$\mu = \frac{\mu_{\max} \cdot S}{K + S} \quad (1)$$

A low saturation constant implies a longer duration of the exponential phase. In this phase, the growth rate is proportional to cell concentration X. The exponential phase can be written by first-order equation (Eq. 2).

$$\frac{dX}{dt} = \frac{\mu_{\max} \cdot S \cdot X}{K + S} \quad (2)$$

The rate of glycerol consumption without considering the product formed is described by Equation 3.

$$\frac{dS}{dt} = -\frac{\mu_{\max} \cdot S \cdot X}{Y_{X/S}\left(K + S\right)} \quad (3)$$

The parameters for the kinetic model were determined from the integration of equations (2) and (3) obtaining Equation 4.

$$X = X_0 + Y_{X/S}(S_0 - S) \quad (4)$$

Assuming the Eq. 4 in Eq. 3 we obtain,

$$\frac{K + S}{S\left[X_0 + Y_{X/S}(S_0 - S)\right]} = -\frac{\mu_{\max}}{Y_{X/S}} dt \quad (5)$$

Integrating the Equation 5 we obtain the equation of a line,

$$Z = aU + b \quad (6)$$

With (7)

$$Z = \frac{\ln \frac{S}{S_0}}{t}$$

$$U = \frac{\ln\left[1 + \frac{Y_{X/S}}{X_0}(S_0 - S)\right]}{t} \quad (8)$$

$$a = 1 + \frac{X_0 + Y_{X/S} S_0}{Y_{X/S} K} \quad (9)$$

$$b = \frac{\mu_{\max}(X_0 + Y_{X/S} S_0)}{Y_{X/S} K} \quad (10)$$

The parameters of the model were estimated based on experimental data that could be adjusted according to Eq. 6, with $Y_{X/S}$ being determined according to Eq. 11.

$$Y_{X/S} = \frac{X - X_0}{S_0 - S} \quad (11)$$

Where X, X_0 represent the final and initial biomass concentration, respectively and S_0, S are initial and final glycerol concentration, respectively.

Once the parameters K and $\mu_{\max}$ were determined, these were replaced in Eq. 5 to obtain the values of S as a function of time and these on the other hand were replaced in Eq. 4 to get the values of X, using-Statistic 7.0 software.

We have investigated and compared the batch fermentation of glycerol in different concentrations (10.0; 30.0 and 60.0g.L^{-1}) of *Clostridium acetobutylicum* ATCC 4259 under similar culture conditions. The purpose of this study is to demonstrate the advantages and limitations encountered during anaerobic cultures with glycerol as a carbon source deriving from these 1,3-PD-producing bacteria in order to reveal the microorganism which has the most efficient abilities for industrial application.

MATERIALS AND METHODS

Microorganism and media

Clostridium acetobutylicum ATCC 4259 strain was kindly provided by Culture Collection Department of Antibiotics (DANTI/UFPE – Brazil). The microorganism was maintained in a Tioglicolate Fluid medium, in 10 mL anaerobic flasks, at 4ºC. Before any experiment, a heat-shock (80ºC/10 min) treatment was performed, in order to stimulate the germination of spores. Then, pre-cultures of the post-germination culture were performed in 100 mL anaerobic flasks filled with 10 mL of pre-culture medium, infused with nitrogen gas, and incubated at 35ºC for 18-20 hours. The pre-culture medium used in order to set the microorganism in exponential phase before inoculation had the following composition (per liter of distilled water; Gunzel *et al*., 1991): 3.4 g K_2HPO_4, 1.3 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$, 2.0 g $CaCO_3$, 20.0 g glycerol, 2.0 mL of trace element solution and 1.0 mL Fe solutions.

The composition of the Fe solution per liter was as follows: 5.0 g $FeSO_4.7H_2O$ and 4.0 mL HCl (37%). The trace element solution per liter consisted of the following: 70.0 mg $ZnCl_2$, 0.1 g $MnCl_2.4H_2O$, 60.0 mg H_3BO_3, 0.2 g $CoCl_2.2H_2O$, 20.0 mg $CuCl_2.2H_2O$, 25.0 mg $NiCl_2.6H_2O$, 35.0 mg $Na_2MoO_4.2H_2O$ and 0.9 mL HCl (37%). The fermentation medium used in all experiments contained (per liter of distilled water; Gunzel *et al*. 1991): 1.0 g K_2HPO_4, 0.5 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$, 2.0 g $CaCO_3$, 2.0 mL of trace element solution and 1.0 mL Fe solutions. As carbon source, pure glycerol (99.0% analytical grade, Sigma) was used.

Batch fermentation

Trials were conducted in flasks, with a working volume of

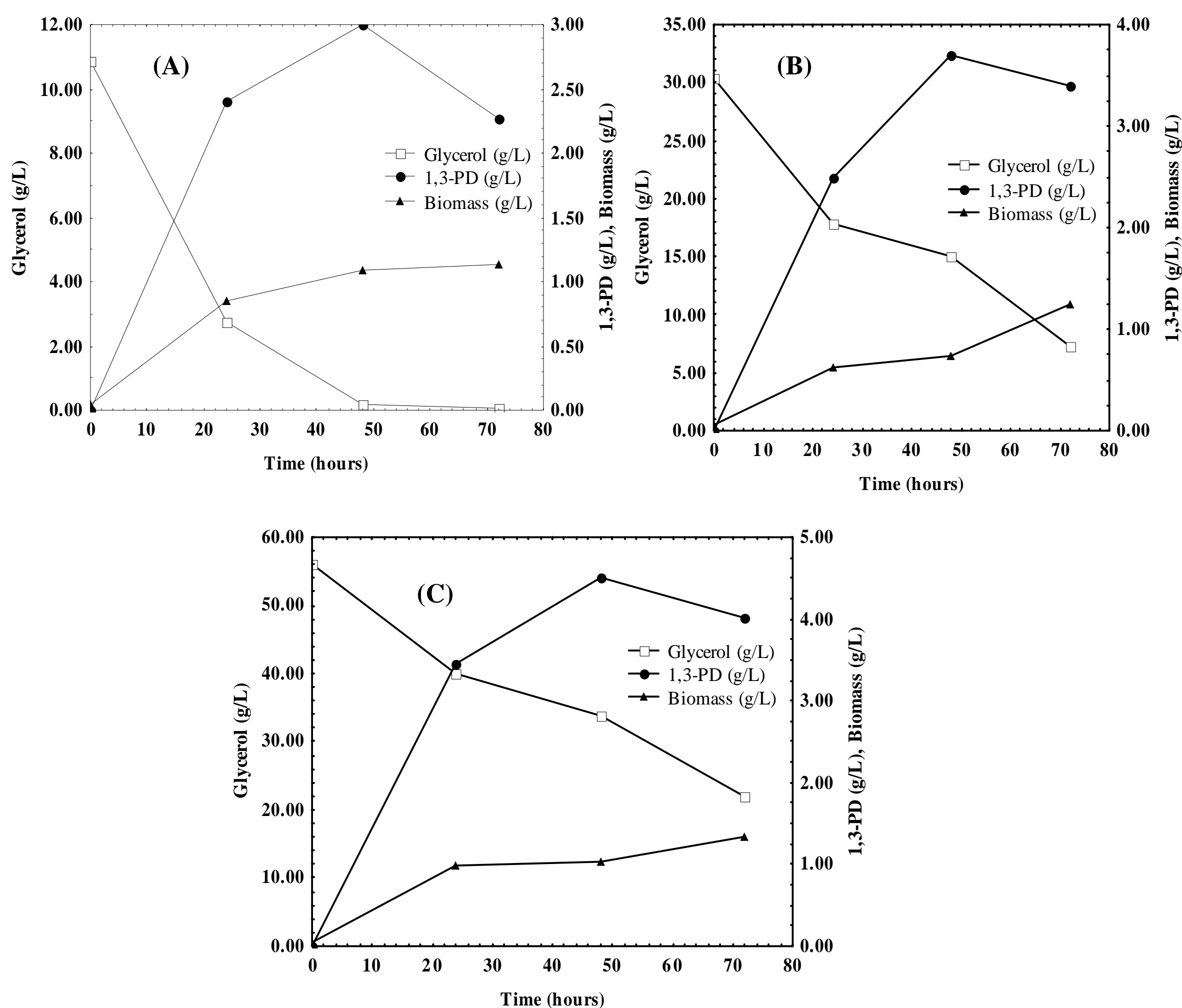

Fig. 1. Batch fermentation of (A) $10.0 g.L^{-1}$, (B) $30.0 g.L^{-1}$ and (C) $60.0 g.L^{-1}$ glycerol by *Clostridium acetobutylicum* ATCC 4259 at 35°C by 72 hours.

Table 1. Batch cultures of *Clostridium acetobutylicum* ATCC 4259 on commercial glycerol in synthetic medium.

Fermentation time (hours)	C10[a]		C30[b]		C60[c]	
	$Y_{X/S}$[d]	$Y_{P/S}$[e]	$Y_{X/S}$[d]	$Y_{P/S}$[e]	$Y_{X/S}$[d]	$Y_{P/S}$[e]
0	0	0	0	0	0	0
24	0.211	0.107	0.149	0.047	0.118	0.038
48	0.294	0.110	0.203	0.050	0.201	0.044
72	0.305	0.210	0.246	0.055	0.214	0.058

[a] Glycerol concentration 10g.L^{-1}. [b] Glycerol concentration 30 g.L^{-1}. [c] Glycerol concentration 60 g.L^{-1}.
[d] Biomass (*X*) yield g.g^{-1} glycerol (*S*) consumed. [e] 1,3-propanediol (*P*) yield g.g^{-1} glycerol (*S*) consumed.

500 mL and initial substrate concentration of 10.0, 30.0 and 60.0 g/L. In all cases, the culture medium was inoculated with 10% (v/v) of cell suspension in an exponential growth phase, while anaerobiosis was secured by sparging the media with pure N_2 prior to inoculation. Culture conditions were as follows: incubation temperature at 35ºC for 72 hours.

Analytical methods

Samples of 5.0 mL were withdrawn every 24 hours during batch fermentation. Biomass production (X, g.L^{-1}) was determined both turbidimetrically by means of optical density – OD at 660 nm using a Spectrum Meter SP – 2000UV Spectrophotometer and by cell dry weight – CDW (after cells dehydration at 100ºC/24 hours), so that a correlation between OD and CDW could be directly established, the equation of which is given below (Eq. 12):

$$\text{CDW (g.L}^{-1}) = 0.4619 + 0.0042,\ r^2 = 0.995 \qquad (12)$$

Glycerol (S, g.L^{-1}), 1,3-propanediol (P, g.L^{-1}) were determined by HPLC (Hewlett-Packard- Agillent), with Bio-Rad Aminex HPX-87H column and RI detection. Operating conditions were as follows: mobile phase, 0.5mM H_2SO_4, flow rate 0.5mL.min^{-1}, temperature 50ºC and injection sample 20μL.

RESULTS AND DISCUSSION

Batch cultures were made with pure glycerol employed as the sole carbon source. The microbial growth and 1,3-PD production carried out batch fermentations using different concentrations of glycerol by *Clostridium acetobutylicum* ATCC 4259 as presented in figure 1. Analyzing figures 1A, 1B and 1C, it can be observed that cell growth does not show the lag phase. The curves in figures 1A, 1B e 1C followed the similar patterns of in biomass, glycerol consumed and 1,3-PD produced.

Glycerol consumption was around 99% (Fig. 1A), indicating that the microorganism is capable of glycerol consumption, while 1,3-PD production corresponding 24,7 g.L^{-1} at S_0 = 10 g.L^{-1}. The conversion yield was 0,21g.g^{-1} and biomass conversion yield was 0,305g.g^{-1} in 72 hours (Table 1). The main reason for the low yield and productivity of glycerol fermentation to of 1,3-PD is that bioprocesses had been carried out at physiological temperature, atmospheric pressure and mostly in batch operation mode (Zeng *et al*., 2002).

This was achieved by the optimization of the process conditions along with the application of fedbatch and continuous operation mode with cell recycling or with immobilized cells (Saxena *et al*., 2009; Zeng *et al*., 2002).

Kinetic models describing the growth of *Clostridium acetobutylicum* ATCC 4259

With the aim of achieving further information about this fermentation process, a kinetic study was carried out. To determine the kinetic parameters (Table 2) a glycerol concentration (10g.L^{-1}) was chosen, considering the best condition for 1,3-PD production and biomass cell, as shown in figure 1A and table 1. The parameters of the model, K and $\mu_{\max}$ were estimated and applied to experimental data using Equations 5 to 10. All results are presented in table 2, with correlation coefficients for glycerol and biomass r^2 = 0,998 for the initial glycerol concentration 10g.L^{-1}.

Table 2. Kinetic parameters of batch fermentation of glycerol by *Clostridium acetobutylicum* ATCC 4259 on commercial glycerol in synthetic medium.

Parameters	Values
S_0 (g.L^{-1})	10.00
X_0 (g.L^{-1})	0.0516
$Y_{X/S}$ (g.g^{-1})	0.305
$\mu_{\max}$ (h^{-1})	0.024
K (g.L^{-1})	0.011
r^2	0.998

S_0: Initial glycerol concentration.
X_0: Initial biomass concentration.
$Y_{X/S}$: Conversion factor of glycerol on biomass.
μ_{max}: Specifies the maximum speed of cell growth.
K: Monod saturation constant.
r^2: correlation coefficient.

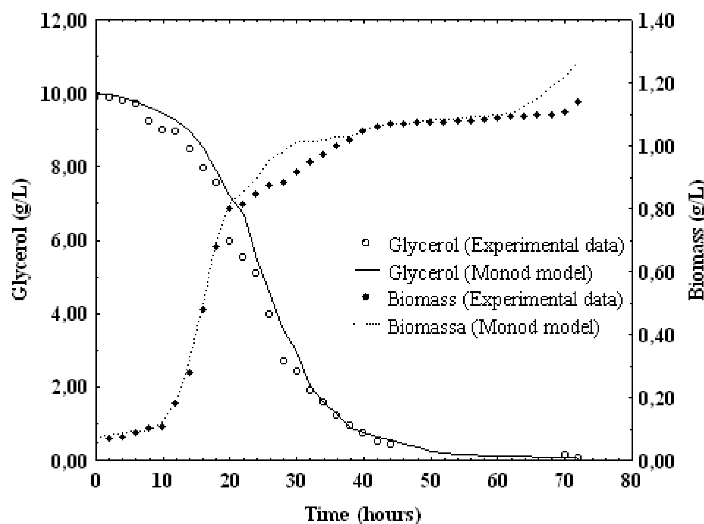

Fig. 2. Comparison between the values of biomass and glycerol calculated by the Monod model (solid lines) and experimental data (symbols) for *Clostridium acetobutylicum* ATCC 4259 in culture medium with $10g.L^{-1}$ glycerol.

Figure 2 shows the values of biomass and glycerol calculated using the Monod model and the experimental data, indicating that the model has adapted to the conditions used for glycerol ($10g.L^{-1}$) fermentation by *Clostridium acetobutylicum* ATCC 4259. As shown in figure 2, microbial growth was sufficient, accompanied by the complete consumption of substrate.

The kinetics of *Clostridium acetobutylicum* ATCC 4259 on glycerol (Fig. 2) showed a prolonged lag phase (10 hours), not accompanied by a significant substrate up-take. Then cell growth with simultaneous production of metabolites took place. The maximum specific rate (μ_{max}) of the *Clostridium acetobutylicum* ATCC 4259, evaluated in the exponential growth phase from the experimental data, was $0.024\ h^{-1}$.

CONCLUSION

In the present study, we found that *Clostridium acetobutylicum* ATCC 4259 could produce 1,3-propanediol under anaerobic conditions with different concentrations of glycerol. Although the anaerobic fermentation of glycerol and the simultaneous biosynthesis of 1,3-propanediol has been studied by numerous authors in batch, fed-batch and continuous cultures (Abbad-Andaloussi *et al*., 1998; González-Pajuelo *et al*., 2005; Papanikolau and Aggelis, 2003; Chatzifragkou *et al*., 2011), the majority of the investigations have been conducted with pure glycerol employed as the sole carbon source. During the present study in a typical batch culture, the strain presented significant cell growth with simultaneous production of 1,3-propanediol ($Y_{P/S}$ equal to $0.21g.g^{-1}$), using $10g.L^{-1}$ of glycerol, whereas a direct relationship between cell growth and the fermentation product was observed. In other batch cultures carried out in the literature (studies conducted with *Clostridium acetobutylicum* ATCC (pSPD5), DG1 (pSPD5), PJC4BK (pTLP)) (Papanikolau and Aggelis 2003) the production of the 1,3-PD seemed growth-associated, with a propanediol global yield $Y_{P/S}$ of 0.54 – 0.66 $mol.mol^{-1}$. In general, the conversion of glycerol into 1,3-PD can be considered as relatively "specialized", since by using natural strains, 1,3-PD is produced only when glycerol if found as sole substrate in the medium (Biebl *et al*., 1999; Papanikolau *et al*., 2004; Zeng and Biebl, 2002). Overall, the present work exhibits promising results regarding the completion of 1,3-PD fermentation and as a next step, a series of batch experiments can be carried out in a bioreactor, using crude glycerol.

ACKNOWLEDGEMENTS

We would like to thank Dr. Eulalia C. P. A. Ximenes of Department of Antibiotics (DANTI/UFPE– Brazil) for supplying the *Clostridium acetobutylicum* ATCC 4259 and Dr. Andrelina M. P. Santos and Msc. Solange M. Vasconcelos for chromatography analysis. We also gratefully acknowledge the financial support from CNPq, Facepe and BNB.

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Received: April 25, 2012; Accepted: June 18, 2012