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Influence of Clostridium butyricum inoculum age on glycerol fermentation

Influência da idade do inóculo de Clostridium butyricum na fermentação de glicerol

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Mariana F. D. Corrêa
Graduate Student. Institute of Chemistry, Universidade Federal do Rio de Janeiro; Av. Athos da Silveira Ramos, 149 – Cidade Universitária CEP: 21941-909
anamari_200@yahoo.com.br

Tatiana F. Ferreira
Professor, Department of Organic Processes, Universidade Federal do Rio de Janeiro; Av. Athos da Silveira Ramos, 149 – Cidade Universitário CEP: 21941-909
tatiana@eq.ufrj.br

Felipe S. dos Santos
Professor, Department of Biochemical Engineering, Universidade Federal do Rio de Janeiro; Av. Athos da Silveira Ramos, 149 – Cidade Universitária CEP: 21941-909
fsombra@eq.ufrj.br

Fernanda F. Martins
Professor, Campus Duque de Caxias, Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro; Av. República do Paraguai, 120 - Vila Sarapui, Duque de Caxias - RJ, CEP: 25050-100;
fernanda.martins@ifrj.edu.br

ABSTRACT
The high production of biodiesel in recent years has generated a large amount of crude glycerol that cannot be absorbed and totally process in the industry. In this way, new studies focused on using this residue have become important to promote the development of biodiesel industry. The 1,3-propanediol (1,3-PDO) is an organic compound with bifunctional character that has great potential to be used in several reactions of polymer synthesis. It is usually used in polycondensation to produce polyesters, polyurethanes and polyethers. 1,3-propanediol (1,3-PDO) and organic acids are simultaneously produced by *Clostridium butyricum* growing on glycerol. The purpose of this study was to evaluate the growth kinetics of *Clostridium butyricum* on glycerol-based medium and to observe the influence of inoculum age on the product formed, such as 1,3-propanediol and butyric acid. The results showed the kinetic stages of the *Clostridium butyricum* growing on glycerol, however no significant difference between the products formed was observed in different conditions studied.

Keywords: Glycerol fermentation, 1,3-propanediol, *Clostridium butyricum*, inoculum age.

RESUMO
A considerável produção de biodiesel recentemente vem gerando uma grande quantidade de glicerina que não pode ser absorvida e totalmente processada pela indústria. Dessa forma, novos estudos

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focados no uso desse resíduo tornaram-se importante para a expansão da indústria de biodiesel. O 1,3-propanodiol (1,3-PDO) é um composto orgânico com caráter bifuncional que possui grande potencial para ser utilizado em diversas reações de síntese de polímeros. Geralmente é usado em reação de policondensação para produzir poliésteres, poliuretanos e poliéteres. O 1,3-propanodiol (1,3-PDO) e ácidos orgânicos são simultaneamente produzidos durante crescimento de *Clostridium butyricum* em glicerol. O objetivo deste estudo foi avaliar a cinética de crescimento de *Clostridium butyricum* em meio de cultura contendo glicerol e observar influência da idade do inóculo nos produtos formados, como 1,3-propanodiol e ácido butírico. Foi possível identificar os estágios cinéticos de crescimento de *Clostridium butyricum* em glicerol, contudo nenhuma diferença significativa entre os produtos formados foi observada nas diferentes condições estudadas.

Palavras-chave: Fermentação de glicerol, 1,3-propanodiol, *Clostridium butyricum*, idade do inóculo.

1 INTRODUCTION

In biodiesel industry, a large amount of crude glycerol is produced, and it is a low-cost sub-product with no final disposal. Several studies have tested bioorganic routes to find a sustainable and economic process for using the crude glycerol (Ribeiro et al., 2020).

The *Clostridium butyricum* was proposed by Prazmowski in 1880. The genus *Clostridium* presents some properties, such as spore forming, anaerobic and a Gram-positive microorganism and can be found in the intestinal tract of humans and animals. It is also frequently found in soil converting organic matter and helping soil mineralization. *Clostridium* is able to metabolize several substrates (monoglycerides, diglycerides, glycerol, carbon monoxide, cellulose, and more) into other products. Biofuels, such as ethanol and butanol, and several chemicals, such as acetone, 1,3-propanediol, and butyric acid, can be produced by those microorganisms through fermentative processes with high conversion efficiency. *Clostridium* spp. are known to produce mainly butanol, ethanol, lactic acid and acetic acid from glycerol, a by-product of biodiesel industry, as unique carbon source (Chatzifragkou et al., 2011; Ferreira et al., 2014; Pachapur et al., 2015; Liberato et al., 2019)

The microbial fermentation of glycerol to produce 1,3-propanediol (1,3-PDO) has attracted much attention owing to its renewability, environmental friendliness, ease of operation and the increasing demand for this glycol (Guo et al., 2017; Wojtusik et al., 2015). 1,3-PDO is a specialty chemical that presents a wide range of industrial applications such as adhesives, antifreeze, laminates, solvents and polymers (Ferreira et al. 2014; Kivistö et al., 2012).

The bioconversion of glycerol into 1,3-PDO is generally performed under anaerobic, micro anaerobic or facultative anaerobic conditions, by different microorganisms, such as *Klebsiella pneumoniae, Klebsiella oxytoca, Clostridium butyricum, Clostridium carboxidivorans, Clostridium diolis, C. ragsdalei,, C. ljungdahlii, Enterobacter agglomerans, Citrobacter freundii* and *Lactobacillus brevis* (Guo et al., 2017; Kivistö et al., 2012; Wojtusik et al., 2015; Liberato et al., 2019). A halophilic

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fermentative bacterium, *Halanaerobium saccharolyticum* subsp. *saccharolyticum* can also produce 1,3-PDO from glycerol (Kivistö et al., 2010).

Gallardo et al. (2017) observed that the inoculum age is related to the products formed in the *Clostridium pasteurianum* fermentation. Therefore, a study of growth kinetics and inoculum age is important to optimize the 1,3-PDO production by *Clostridium butyricum* (Yun et al., 2018; Zhu et al., 2012). So, this study aimed to evaluate the influence of *C. butyricum* inoculum age on the products formed during the glycerol fermentation. *C. butyricum* is a promising biocatalyst to produce 1,3-PDO and butyric acid from crude glycerol.

2 MATERIALS AND METHODS

2.1 MICROBIAL CULTURE

Clostridium butyricum NCIMB 8082 was obtained from the *National Collection of Industrial and Marine Bacteria* (NCIMB) and stored in 50 mL serum glass bottles containing Reinforced Clostridial Medium (RCM) at 4ºC (inoculum).

2.2 EXPERIMENTAL METHODOLOGY

2.2.1 Growth kinetics

The *Clostridium butyricum* NCIMB 8082 growth kinetics was performed in serum glass bottles containing 50 mL of RCM and 3 mL of inoculum. The bottles were kept at 37ºC and 150 rpm in orbital shaker for 32 hours. Samples were collected at different time intervals to cell growth and pH measurement.

2.2.2 Inoculum Age

In order to study the influence of inoculum age, 5 mL of grown cells were transferred to serum glass bottles containing 50 mL of the culture medium and samples were taken at the beginning and after 24 h of fermentation to quantify the substrate consumption and the products formed. Two different inoculum ages were evaluated: 16-hour inoculum and 24-hour inoculum. These tests were carried out at 37ºC and 150 rpm in orbital shaker. The culture medium used was composed of 1.0g KH_2PO_4, 1.0g NH_4Cl, 0.3g $MgSO_4.7H_2O$, 0.02g $CaSO_4.2H_2O$, 20mg $FeSO_4.7H_2O$, 1.0g yeast extract, 12g of crude glycerol (solubilized in 1L of distilled water) and pH 8.5. After medium preparation, nitrogen gas was flushed in the liquid phase for 5 min. The glass bottles were sealed with gas impermeable butyl rubber septum stopper and aluminum seal and sterilized in autoclave at 0.5 atm for 20 min.

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2.3 ANALYTICAL METHODS

2.3.1 Cell Growth and pH Determination

The cell growth was determined by optical density (D.O.) in a spectrometer (Bel Photonics S2000) at 600 nm. Absorbance values were converted to dry weight ($g.L^{-1}$) by a factor obtained from standard curve previously obtained. The pH was analyzed in pH-meter (D22 Digimed).

2.3.2 Analysis of metabolites

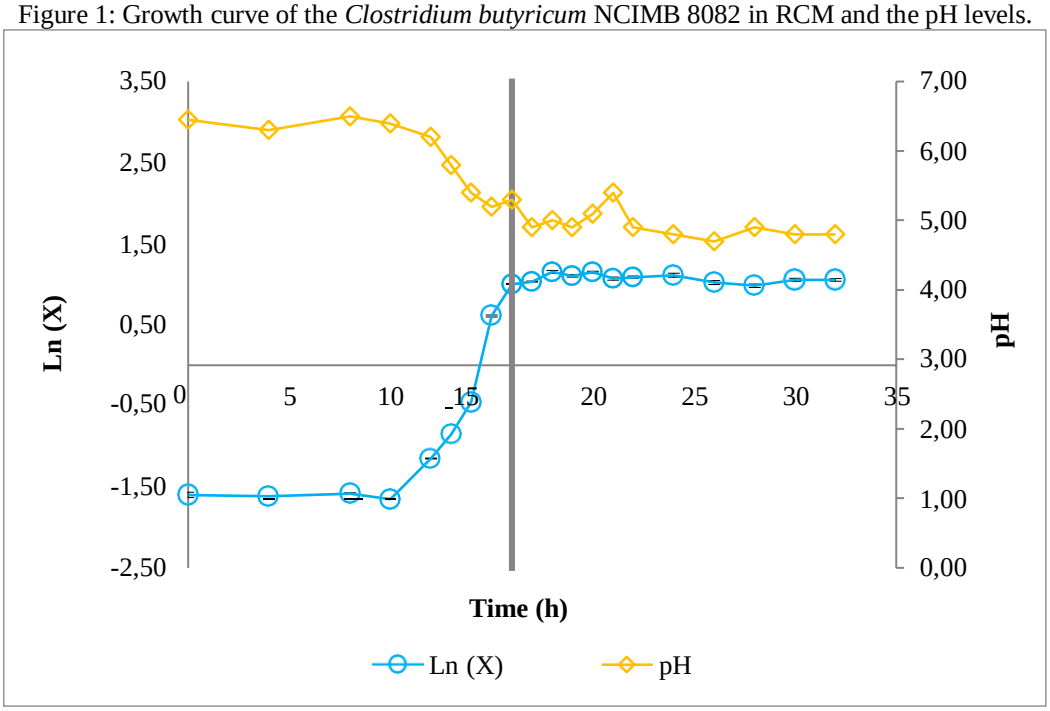
1,3-PDO, glycerol and by-products were analyzed by High Performance Liquid Chromatography (Shimadzu®) using the column Aminex® HPX-87H, 300 x 7.8 mm (Bio-Rad Laboratories Ltd) and IR detector (Shimadzu®). The mobile phase was 5 mM H_2SO_4 at flow rate 0,8 $mL.min^{-1}$; injection volume was 20 µL; and temperature analysis was 60°C (Martins et al., 2016).

3 RESULTS AND DISCUSSION

3.1 GROWTH KINETICS

The Figures 1 and 2 show the results obtained in the study of *Clostridium butyricum* NCIMB 8082 growth in RCM. Figure 1 presents three distinct phases of cell growth: the lag phase from 0 to 10 h; the exponential phase from 10 to 16 h; and the stationary one from 16 to 32 h. It is possible to observe a decrease in pH during the fermentation, being inversely proportional to biomass concentration. The pH reduction occurs due to glucose conversion into acetic and butyric acid which can be observed in Figure 2. The specific growth rate (µ) was 0.461 h^{-1}.

Figure 1: Growth curve of the *Clostridium butyricum* NCIMB 8082 in RCM and the pH levels.



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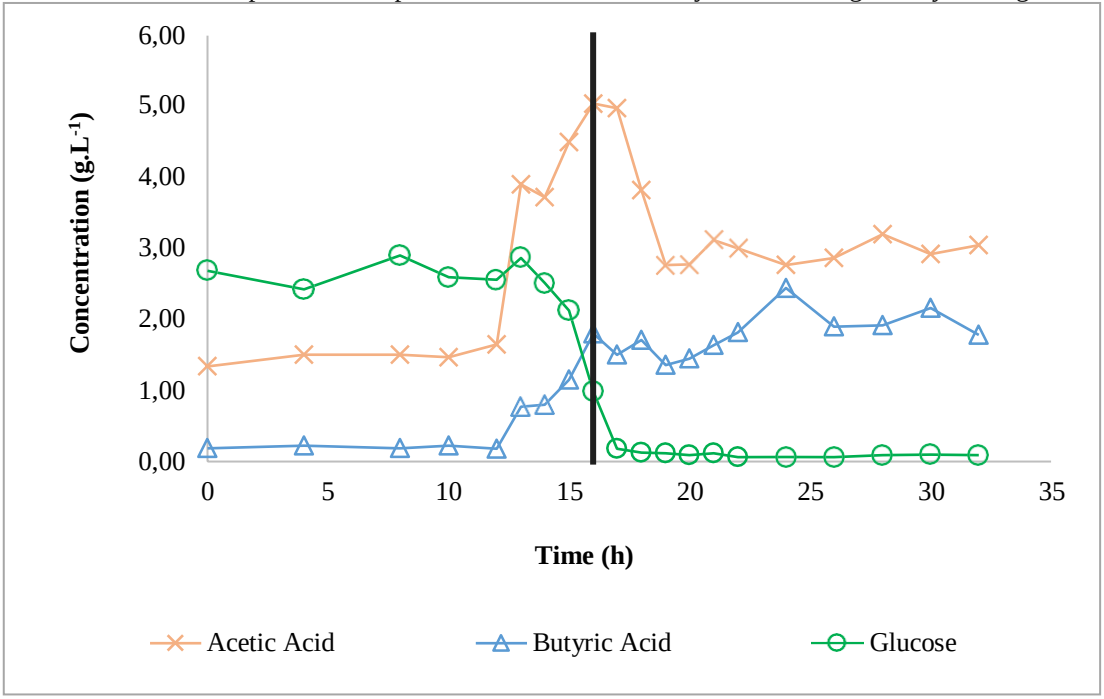
The highest acetic acid concentration was obtained after 16 hours of fermentation when glucose depletion is observed, that is, the end of exponential phase. Then, a decrease in acetic acid can be observed, followed by an increase in butyric acid concentration during stationary phase.

Figure 2: The substrate consumption and the production of acetic and butyric acid during *C. butyricum* growth in RCM.



3.2 INOCULUM AGE

The results of glycerol fermentation using different *C. butyricum* inoculum ages are in Table 1. The final biomass concentration after 24 hours of fermentation was similar in both conditions, unlike the results obtained by Gallardo et al. (2017). The remaining glycerol after 24 hours of fermentation was higher in 24-hour inoculum essay.

Table 1: Concentration of biomass, glycerol, acetic acid, butyric acid, and 1,3-PDO using different inoculum ages.

Age	Time (h)	X (g.L^{-1})	Glycerol (g.L^{-1})	Acetic Acid (g.L^{-1})	1,3-PDO (g.L^{-1})	Butyric Acid (g.L^{-1})
16-hour	0	0.27 ± 0.01	9.14 ± 0.76	0.38 ± 0.02	0.00 ± 0.00	0.21 ± 0.04
	24	1.54 ± 0.03	0.56 ± 0.38	0.45 ± 0.20	1.67 ± 0.49	1.07 ± 0.14
24-hour	0	0.33 ± 0.01	9.27 ± 1.42	0.38 ± 0.06	0.00 ± 0.00	0.25 ± 0.08
	24	1.54 ± 0.02	1.40 ± 0.41	0.26 ± 0.07	1.54 ± 0.07	1.32 ± 0.01

X = biomass concentration

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Regarding the products, acetic acid production was not significant, butyric acid production was higher in 24-hour inoculum test, and 1,3-PDO production was similar in both conditions (Table 2).

Table 2: Yield of biomass, acetic acid, butyric acid, and 1,3-PDO using different inoculum ages.

	16-hour	**24-hour**
$Y_{X/S}$	0.148	0.154
$Y_{AA/S}$	0.008	-
$Y_{BA/S}$	0.100	0.136
$Y_{PDO/S}$	0.195	0.196

$Y_{X/S}$ = biomass yield (g biomass produced/g glycerol consumed); $Y_{AA/S}$ = (g acetic acid produced/g glycerol consumed); $Y_{BA/S}$ = (g butyric acid produced/g glycerol consumed); $Y_{PDO/S}$ = (g 1,3-PDO produced/g glycerol consumed).

4 CONCLUSIONS

Three different stages of cell growth can be observed in the growth kinetics: lag stage (0 - 10h), exponential stage (10 - 16h) and stationary stage (16 - 32h). Glucose was completely consumed during the fermentation and acetic and butyric acids were the major products formed. The specific growth rate obtained during the growth of *Clostridium butyricum* NCIMB 8082 in RCM was 0,461 h^{-1}.

Regarding the *Clostridium* inoculum age, no significant difference in 1,3-PDO production between 16-hour and 24-hour inoculums was observed. However, the butyric acid production was higher in 24-hour inoculum fermentation probably because this inoculum was obtained from stationary phase when *Clostridium* cells produce more butyric acid according to growth kinetics presented herein.

Although there is no difference in 1,3-PDO production during glycerol fermentation using different *Clostridium* inoculum ages, the 16-hour inoculum was obtained at the end of exponential phase when the cell metabolism is changing. A 14-hour inoculum may be a good option to produce 1,3-PDO by glycerol fermentation once it is in the middle of exponential phase.

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