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Butanol production by *Clostridium pasteurianum* NRRL-598 using corn steep liquor as nutrient source

Produção de butanol por Clostridium pasteurianum NRRL-598 usando licor de maceração de milho como fonte de nutrientes

DOI:10.34117/bjdv6n7-238

Recebimento dos originais: 03/06/2020
Aceitação para publicação: 10/07/2020

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ABSTRACT
Butanol has become an interesting substitute for ethanol in additives and fuel applications due to its physical chemical properties. Several Clostridium strains are able to produce ABE (acetone, butanol and ethanol) solvents by fermentation. Efforts have been made to reduce cost of butanol biotechnological production in order to become this route competitive when compared to petrochemical technology. This study aimed to evaluate the use of corn steep liquor, a residue of corn industry, as unique nutrient source in culture medium for butanol production by Clostridium pasteurianum NRRL B-598. The strain is capable of producing butanol using only corn steep liquor as nutrient source. The yield was 0.30 gButanol/gglucose and the butanol productivity was 0.11 $g.L^{-1}.h^{-1}$ using 20 $g.L^{-1}$ of corn steep liquor.

Keywords: Butanol, corn steep liquor, Clostridium pasteurianum, ABE fermentation.

RESUMO
O butanol tornou-se um substituto interessante para o etanol em aplicações de aditivos e combustíveis devido às suas propriedades físico-químicas. Várias linhagens de Clostridium são capazes de produzir solventes ABE (acetona, butanol e etanol) por fermentação. Esforços foram feitos para reduzir o custo da produção biotecnológica de butanol, a fim de tornar essa rota competitiva quando comparada à tecnologia petroquímica. Este estudo teve como objetivo avaliar o uso do licor de maceração de milho, um resíduo da indústria do milho, como fonte única de nutrientes no meio de cultura para a produção de butanol por Clostridium pasteurianum NRRL B-598. A cepa é capaz de produzir butanol usando apenas licor de maceração de milho como fonte de nutrientes. O rendimento foi de 0,30

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gButanol / gglucose e a produtividade de butanol foi de 0,11 g.L-1.h-1 usando 20 g.L-1 de licor de maceração.

Palavras-chave: Butanol, licor de maceração de milho, Clostridium pasteurianum, fermentação ABE.

1 INTRODUCTION

Solventogenic Clostridia are capable of producing butanol, acetone and other metabolites from different substrates by ABE (acetone, butanol and ethanol) fermentation. ABE fermentation is composed of two phases: acidogenic and solventogenic. The first is characterized by organic acid production (butyric and acetic), and the second by assimilation of these acids as a carbon source for subsequent transformation into acetone, butanol, and ethanol (Jones and Woods, 1986; Liberato et al., 2019). The most used solventogenic clostridial strains for butanol production are *C. acetobutylicum*, *C. beijerinckii*, *C. pasteurianum*, and *C. saccharoperbutylacetonicum* (Sun et al. Liberato et al., 2019).

Lately, the interest in using butanol as biofuel has increased due to its advantages over ethanol, such as its higher energy content and lower volatility. It can be used as a fuel additive or a fuel (replacing the gasoline). Butanol is also an important building block for the chemical industry, being used as an intermediate to the production of methacrylate esters, butyl acrylate, butyl glycol ether, butyl acetate, and plasticizers (Lee et al., 2008; Jin et al. 2011; Liberato et al., 2019). For many years, butanol production by ABE fermentation was commercially applied. After the establishment of a cost effective petrochemical technology, the biotechnological route suffered a decrease (Patakova et al. 2011).

Aiming to reduce the cost of this bioprocess, the use of agro-industrial residues has been considered. The corn wet-milling process generates a residue rich in nitrogen, known as corn steep liquor (CSL). CSL is composed of several amino acids and some minerals usually employed as enzymes co-factors (Maddipati et al., 2011; Liberato et al., 2019; Afonso et al., 2020). Besides that, Ahn et al. (2011) observed an increase in the butanol production by C. *pasteurianum* DSM 525 when lactic acid was used in the culture medium. The corn steep liquor used in this study contains around 4.5 $g.L^{-1}$ of lactic acid.

The present study aimed to evaluate the use of CSL as sole nutrient source in the production of butanol by *Clostridium pasteurianum* NRRL B-598, using glucose as substrate.

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2. MATERIALS AND METHODS

2.1 MICROBIAL CULTURE

Clostridium pasteurianum NRRL B-598 obtained from the *Agriculture Research Service (ARS) Culture Collection* was used in this study. The microbial culture was stored in serum glass bottles containing 50 mL of *Reinforced Clostridial Medium* (RCM) at 4°C.

2.2 EXPERIMENTAL METHODOLOGY

The experiments were performed in serum flasks containing 50 mL of culture medium and 5 mL of grown cells undergone prior reactivation during 30 minutes in orbital shaker. 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. Bottles were incubated at 37°C and 150 rpm in orbital shaker for 72 hours. Samples were collected every 24 hours to analyze cell density, substrate consumption, products formation, and pH.

Different culture mediums were studied in which corn steep liquor concentration varied from 10 to 50 $g.L^{-1}$, glucose concentration was 10 $g.L^{-1}$ and initial pH was set at 6.5.

2.3 ANALYTICAL METHODS

Cell growth 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. The pH was analyzed using pH-meter (D22 Digimed).

Glucose, butanol, and other by-products were quantified by High Performance Liquid Chromatography (Shimadzu®) equipped with column Aminex® HPX-87H, 300 x 7.8 mm (Bio-Rad Laboratories Ltd) and IR detector (Shimadzu®). Operating conditions were sample volume 20 µl; mobile phase 0.5 $mmol.L^{-1}$ H_2SO_4; flow rate 0.6 $ml.min^{-1}$; and column temperature 60°C (Ferreira et al., 2014).

3 RESULTS AND DISCUSSION

3.1 STUDY OF DIFFERENT CORN STEEP LIQUOR CONCENTRATIONS IN CULTURE MEDIUM

Figure 1A shows cell growth and glucose consumption in five different culture mediums: M10 (10$g.L^{-1}$ of CSL), M20 (20$g.L^{-1}$ of CSL), M30 (30$g.L^{-1}$ of CSL), M40 (40$g.L^{-1}$ of CSL), and M50 (50 $g.L^{-1}$ of CSL).

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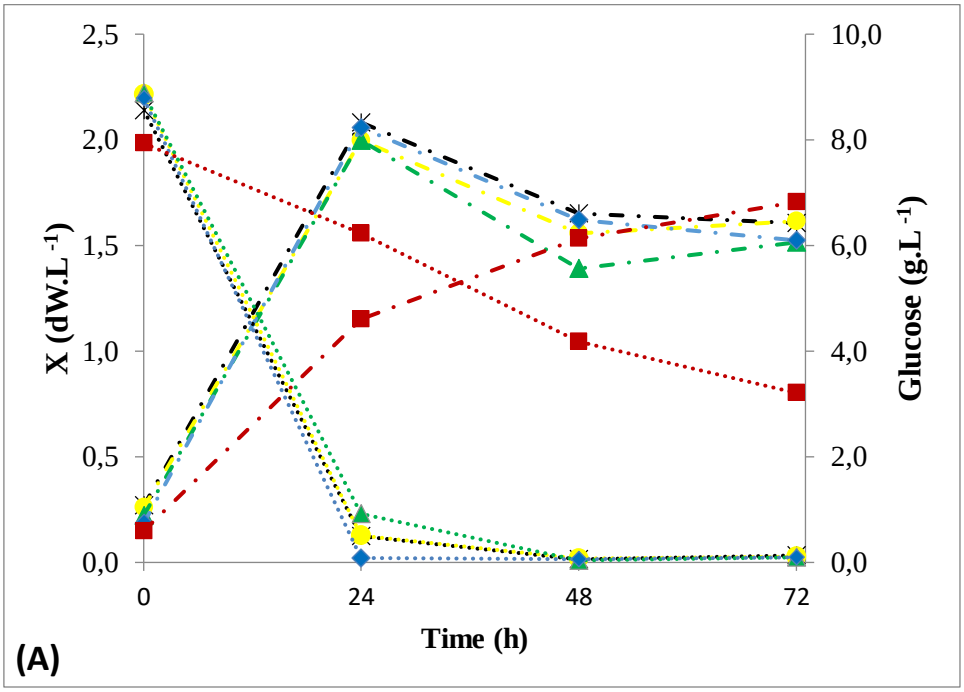
All studied conditions, except M10, showed maximum growth in 24 hours. M10 presented lower growth rate, probably due to lowest CSL concentration which might have been a limiting nutritional factor. Although M10 presents a lower growth rate, the final biomass concentration obtained (1.8 $dW.L^{-1}$) was slightly higher than other conditions, showing biomass might not have reached its maximum concentration in this condition. Also shown in Figure 1A, the consumption of glucose in M10 differs from others. In 72 hours of fermentation, the glucose was not depleted as observed after 24 hours of fermentation in other four conditions. Based on those results, it is assumed that CSL limits cell growth.

As shown in Figure 1B, M20 reached its maximum butanol production after 24 hours while others took longer. Presumably, due to substrate lower consumption rate, M10 final titer of butanol was 30% lower than the highest titer observed (M40), that were approximately 2.6 $g.L^{-1}$.



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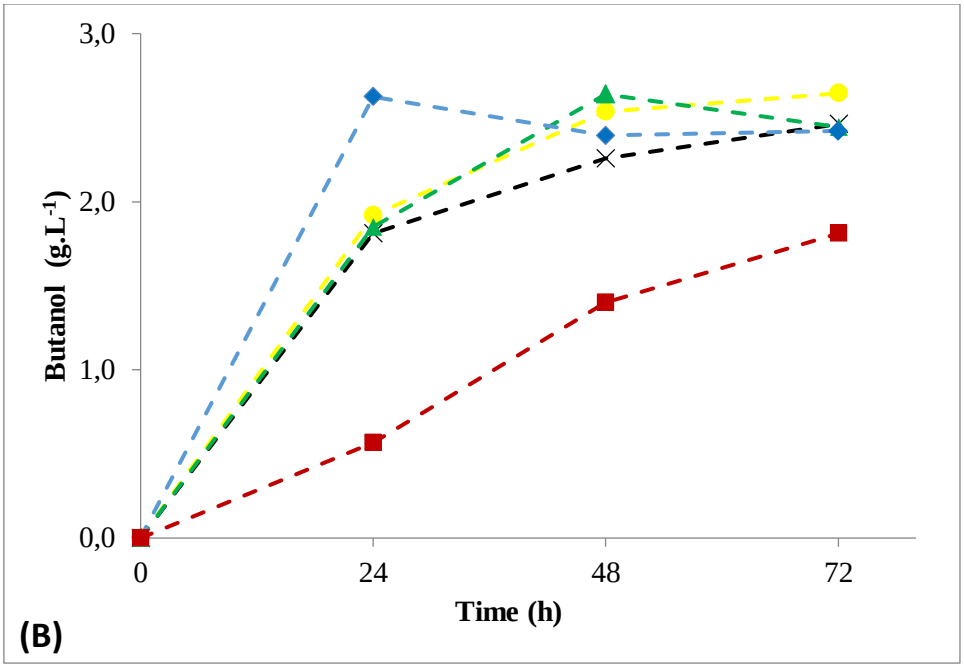

Figure 1. (A) Cell growth and glucose consumption, and (B) Butanol production in different conditions of glucose fermentation by Clostridium pasteurianum NRRL B-598 during 72 hours. Medium conditions are red square (M10), blue diamond (M20), green triangle (M30), yellow circle (M40), and black asterisk (M50). (– . –) represents biomass, (•••) glucose consumption, and
(– – –) butanol production Abbreviations: M10 represents culture medium containing 10 $g.L^{-1}$ of CSL, M20: 20 $g.L^{-1}$ of CSL, M30: 30 $g.L^{-1}$ of CSL, M40: 40 $g.L^{-1}$ of CSL and M50: 50 $g.L^{-1}$ of CSL, all of them contain 10 $g.L^{-1}$ of glucose.

3.2 BUTANOL PRODUCTIVITY AND YIELD

Given that in all studied conditions, the glucose was almost totally consumed after 24 hours and butanol production rate was higher in this period, except for M10, the parameters were calculated based on concentrations observed in 24 hours of fermentation.

Table 1 shows the parameters calculated. The lower yield ($Y_{B/S}$) of butanol was observed in conditions M30, M40 and M50 while the higher yield was observed in M10. Even though 10 $g.L^{-1}$ of CSL was a limiting cell growth factor, the selective use of glucose for butanol production was considerably high in this condition, especially knowing that theoretical yield is 0.41 $g_{butanol}/g_{glucose}$. In terms of productivity, M20 was the highest one. Although M10 presented an expressive yield, its productivity was quite low, which agrees to its low butanol production and glucose consumption rate.

Table 1. Yield ($Y_{B/S}$) and Productivity (Q_B) of butanol considering 24 hours of glucose fermentation by *Clostridium pasteurianum* NRRL B-598 in different CSL concentrations.

	$Y_{B/S}$ ($g_{Butanol}/g_{glucose}$)	Q_B ($g.L^{-1}.h^{-1}$)
M10	0.33	0.02
M20	0.30	0.11
M30	0.23	0.08
M40	0.23	0.08

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M50	0.23	0.08

Patakova et al. (2011) performed a batch fermentation with 40 $g.L^{-1}$ of initial glucose, also using *C. pasteurianum* NRRL B-598 and obtained a butanol yield of 0.18 $g_{butanol}/g_{glucose}$. Therefore, this work showed great results of yield considering only corn steep liquor and glucose were used as medium compounds.

4 CONCLUSIONS

Clostridium pasteurianum NRRL B-598 is able to produce butanol using corn steep liquor (CSL) as sole nutrient source in culture medium. Based on the results obtained herein, the medium composed of 20 $g.L^{-1}$ of CSL (M20) would be considered for further experiments since this condition showed the highest butanol concentration and productivity. In addition, the yield of M20 was 0.30 $g_{Butanol}/g_{glucose}$ which is similar to the highest yield obtained in this study (0.33 $g_{Butanol}/g_{glucose}$ for M10 condition); and the butanol productivity was 0.11 $g.L^{-1}.h^{-1}$.

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