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Preliminary Assessment of the Production of Solvents from Different Carbon Sources Using *Clostridium beijerinckii* NCIMB 8052

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In Colombia, the government has implement policy instruments aimed at promoting the production of biofuels of second and third generation through incentives and the National Development Plan. Standard laboratory tests were preformed in which the carbon and nitrogen ratio was evaluated. As carbon sources were used: glucose, sucrose, xylose and glycerin. In test tubes, the maximum yield obtained was 0.736 g g^{-1} with 9.36 g L^{-1} of ABE using glucose. While at shake flask using glucose or sucrose as carbon source were produced 10.16 g L^{-1} of ABE and 8.46 g L^{-1}, respectively. This preliminary characterization allows to consider the evaluation of solvent production using Colombian agro-industrial by-products using the strain *C. beijerinckii* NCIMB 8052.

1. Introduction

Different Clostridium gender microorganisms have been used for solvents production. They were described by first time in 1880 by Prazmowski. Later on has been reported a great number of publications about the recognition, and the early history of the gender (Cato *et al.,* 1986; Dürre*,* 1998). One of the most important characteristics in the gender Clostridium is based on the use of organic compounds for obtaining energy. This gender has received a lot of attention in the last years due to its capacity to produce solvents of industrial interest as acetone and 1,3-propanodiol. The *Clostridium beijerinckii* (before clostridium *acetobutylicum*) is a spore forming bacterium, strictly anaerobia that produces acetone, butanol and small quantities of ethanol in sugary substrates (Jones and Woods, 1986). The fermentation acetone-butanol-ethanol (ABE) was considered an important industrial process during the first half of the XX century. This was based on the use of such substrates as corn. The butanol that can be used as fuel has other uses in the industry of foods, plastics and chemistry (Ezeji *et al*., 2005). In this work, the production of solvents was characterized in the fermentation ABE using different sources of carbon (glucose, xylose, sucrose and glycerine) and the relationship carbon: nitrogen by means of C. *beijerinckii* NCIMB 8052. For the best conditions it was carried out the process fermentative to elermeyers scale.

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2. Materials and methods

2.1 Microorganism activation

Spores of *Clostridium beijeriinckii* NCIMB 8052 was activated in 300 mL of activation medium (Yeast extract, 3g L^{-1}; Peptone, 10 g L^{-1}; Meat extract, 10 g L^{-1}; Soluble starch, 10 g L^{-1}; NaCl, 5g L^{-1}; L-Cysteine, 0.5 g L^{-1}; Glucose, 5 g L^{-1}, Sodium acetate 3 g L^{-1}) at 37°C, pH 6.8±0.2 and 250 rpm.

2.2 Evaluation of the source of carbon source and the relationship carbon:nitrogen in the production of solvents in the ABE fermentation

A preliminary evaluation of the effect of carbon source and carbon:nitrogen relation on the assessment of solvent production was carried out at test tube level. Fermentations were carried out in 9 mL of medium (Glucose, 20 g L^{-1}; KH_2PO_4, 0.5 g L^{-1}; $K_2HPO_4 \cdot 3H_2O$, 0.5 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.2 g L^{-1}; $MnSO_4 \cdot H_2O$, 0.01 g L^{-1}; $FeSO4 \cdot 7H_2O$, 0.01 g L^{-1}; NaCl, 0.01 g L^{-1}; Ammonium acetate, 2.2 g L^{-1}; p-Aminobenzoic acid, 0.001 g L^{-1}; biotin, 0.00001 g L^{-1}, and adjusted to pH 6.8, Monot *et al*., 1982) inoculated with 1 mL of activation medium with *C. beijerinckii* of an OD 1.3 (~4.2 mg mL^{-1}), and incubated under anaerobic conditions at 37 °C for 80 h. The used carbon sources were glucose, xylose, sucrose and glycerol in concentrations of the 2, 4 and 6 $\%^{w}/_{v}$. For each carbon concentration, independent assays with ammonium acetate at 1, 1.5 and 2 $\%^{w}/_{v}$ were done. Each trial was made in triplicate.

For all substrates, fermentations in 1000 mL shake flask with 180 mL of the described above fermentation medium were carried with 4 and 1.5 $\%^{w}/_{v}$ of carbon and nitrogen sources, respectively. The fermentation medium was inoculated with 20 mL of activation medium with C. *beijerinckii* of an OD 1.3 (~4.2 mg mL^{-1}), and incubated at 37 °C and 250 rpm during 90 or 115 h. Samples of 2 mL were removed during the fermentation for solvent analysis.

2.3 Analytical methods

Solvents (ethanol, butanol and acetone) and acids (acetic and butyric) obtained during the fermentations were analyzed by headsapace-gas chromatography (GC-HS, GC-2010, Shimadzu, USA) equipped with a Heliflex®ATTMWax (25 m, 1.20 µm, i.d 0.53mm, model 13141 Alltech) capillary column and flame ionization detector (FID). The inlet temperature was set at 150°C, helium served as the carrier gas with a flow of 3.0 mL min^{-1} in splitless mode. The oven temperature program ramped from 60°C (held the initial temperature for 2 minutes) to 200°C at 20°C min^{-1}. the temperature was held at 240 °C for 4 min. The detector temperature was heat at 250 °C with a hydrogen flow of 40 mL min^{-1} and air flow of 400 mL min^{-1} with constant makeup flow at 40 mL min^{-1}. Samples were preheated during 10 min at 80 °C and 500 rpm in the HS, the injection temperature was 80°C.

Solvent production yields were determined by the relation of concentration of total solvents and the carbon source consumed. For glycerol this yield was based on initial glycerol concentration. Productivity was calculated as the relation of total concentration of the fermentation ABE (gL^{-1}) and fermentation time (Qureshi *et al*., 2007). Sucrose and xylose were determined by using 3,5-(dinitrosalicylic) (DNS), and glucoses by a Biosystem Glucose Kit.

3. Results and Discussion

Table 1 shows the production of acetone, butanol, ethanol, and acetic and butyric acids by *C. beijerinckii* NCIMB 8052 using different carbon sources and carbon:nitrogen relations. These variables have demonstrated to be an important factor in ABE fermentation using *C. beijerinckii* NCIMB 8052. It is observed that a concentration increase of the carbon source favor the production of solvents. However, xylose and glycerol concentration increase did not present this performance. For glycerol, it could be due to its metabolic route that favors the formation of other solvents, like 1-3 propanodiol, diminishing the production of ABE (Cárdenas *et al.*, 2006). While for xylose, it is observed that nitrogen concentration affects the production of solvents since for the selected xylose concentration there was not a clear tendency. Also, it is notice that solvent yields decrease or keep constant at 2% ammonium acetate regarding to the yields obtained at 1.5 % of salt, obtaining at this concentration the major maximum yields. The biggest obtained productivities for each carbon source were 0.13, 0.21, 0.11 and 0.071 g L^{-1} h^{-1}, for glucose, xylose, sucrose and glycerol, respectively. This shows that monosacarides favored solvent production more than sucrose and glycerol. Meanwhile, a nitrogen concentration increase further than 1.5% did not present a notable advantaje in the production of solvents but favor acid production. Monot *et al*., (1982) reported for *C. acetobutylicum* that high nitrogen (> 2.2 g L^{-1}) concentrations favor the production of acids decreasing the solvent production. Obtained and reported results allowed to select concentrations of carbon and nitrogen of 4 and 1.5 %, respectively, for shake flaks rehearsals.

Table 1. *Solvents and acids concentrations (g L^{-1}) for different carbon sources and carbon:nitrogen ratios.*

	GLUCOSE					SUCROSE				
CS : NS[a]	Acetone	Butanol	Ethanol	Acetic	Butyric	Acetone	Butanol	Ethanol	Acetic	Butyric
2 : 1	1.06 ± 0.01	2.98 ± 0.03	0.68 ± 0.01	2.53 ± 0.02	0.43 ± 0.05	1.03 ± 0.01	2.69 ± 0.11	2.72 ± 0.59	3.88 ± 0.75	8.82 ± 0.19
2 : 1.5	0.96 ± 0.05	2.95 ± 0.82	0.70 ± 0.02	4.69 ± 0.42	0.94 ± 0.14	0.97 ± 0.63	2.67 ± 0.08	2.86 ± 0.65	3.84 ± 0.87	8.76 ± 0.91
2 : 2	1.00 ± 0.06	3.23 ± 0.06	0.69 ± 0.02	5.12 ± 0.38	1.12 ± 0.12	1.02 ± 0.07	2.73 ± 0.01	2.84 ± 0.83	3.64 ± 0.36	9.11 ± 0.08
4 : 1	1.59 ± 0.00	2.74 ± 0.01	1.00 ± 0.00	2.49 ± 0.03	0.27 ± 0.01	1.50 ± 0.08	2.68 ± 0.09	3.23 ± 0.45	5.60 ± 0.46	4.19 ± 0.73
4 : 1.5	1.57 ± 0.02	3.62 ± 0.08	1.06 ± 0.03	2.35 ± 0.03	0.37 ± 0.04	1.47 ± 0.01	2.81 ± 0.00	3.18 ± 0.07	5.96 ± 0.59	4.55 ± 0.57
4 : 2	1.58 ± 0.01	3.37 ± 0.02	1.07 ± 0.02	3.36 ± 0.09	0.86 ± 0.19	1.49 ± 0.03	3.72 ± 0.15	3.18 ± 0.09	6.31 ± 0.40	4.79 ± 0.81
6 : 1	3.02 ± 0.03	4.15 ± 0.22	2.20 ± 0.07	3.05 ± 0.02	0.75 ± 0.47	2.94 ± 0.00	2.99 ± 0.06	2.27 ± 0.08	12.01 ±0.93	2.93 ± 0.58
6 : 1.5	3.16 ± 0.01	3.46 ± 0.08	2.10 ± 0.03	2.48 ± 0.01	0.25 ± 0.07	2.92 ± 0.04	3.08 ± 0.05	2.27 ± 0.08	11.29± 0.10	2.90 ± 0.80
6 : 2	3.16 ± 0.01	3.98 ± 0.13	2.22 ± 0.03	2.61 ± 0.03	0.63 ± 0.18	2.96 ± 0.07	3.17 ± 0.04	2.35 ± 0.90	11.24 ±0.24	2.89 ± 0.64

	XYLOSE					GLYCEROL				
CS : NS[a]	Acetone	Butanol	Ethanol	Acetic	Butyric	Acetone	Butanol	Ethanol	Acetic	Butyric
2 : 1	1.00 ± 0.09	2.83 ± 0.20	2.69 ± 0.21	2.77 ± 0.27	2.54 ± 0.32	0.96 ± 0.16	0.67 ± 0.05	1.37 ± 0.38	1.14 ± 0.06	1.96 ± 0.12
2 : 1.5	1.00 ± 0.04	2.77 ± 0.10	2.64 ± 0.02	3.57 ± 0.70	1.89 ± 0.11	0.97 ± 0.16	0.67 ± 0.01	1.32 ± 0.11	1.11 ± 0.14	1.12 ± 0.34
2 : 2	1.01 ± 0.01	2.75 ± 0.19	2.70 ± 0.27	2.91 ± 0.59	1.73 ± 0.07	0.98 ± 0.03	0.65 ± 0.00	1.34 ± 0.21	1.09 ± 0.09	2.01 ± 0.53
4 : 1	1.49 ± 0.06	2.90 ± 0.01	4.01 ± 0.15	2.47 ± 0.26	0.81 ± 0.01	1.47 ± 0.05	0.88 ± 0.05	1.44 ± 0.24	1.13 ± 0.02	1.34 ± 0.03
4 : 1.5	1.53 ± 0.04	3.53 ± 0.36	4.18 ± 0.33	2.61 ± 0.24	1.01 ± 0.08	1.45 ± 0.07	0.88 ± 0.02	1.45 ± 0.07	1.12 ± 0.21	2.13 ± 0.41
4 : 2	1.50 ± 0.08	3.21 ± 0.28	4.08 ± 0.37	2.54 ± 0.33	0.95 ± 0.02	1.45 ± 0.05	0.88 ± 0.01	1.45 ± 0.01	1.63 ± 0.16	1.81 ± 0.88
6 : 1	3.02 ± 0.04	3.56 ± 0.44	8.30 ± 0.16	2.38 ± 0.07	0.63 ± 0.05	2.91 ± 0.04	0.66 ± 0.01	1.59 ± 0.04	1.14 ± 0.09	1.68 ± 0.45
6 : 1.5	3.11 ± 0.05	2.88 ± 0.19	7.94 ± 0.05	2.36 ± 0.19	0.57 ± 0.02	2.90 ± 0.05	0.64 ± 0.05	1.60 ± 0.08	1.13 ± 0.29	1.76 ± 0.37
6 : 2	2.91 ± 0.02	2.85 ± 0.07	7.93 ± 0.09	2.30 ± 0.13	0.86 ± 0.09	2.90 ± 0.02	0.68 ± 0.11	1.60 ± 0.05	1.14 ± 0.40	1.92 ± 0.38
2 : 1	1.00 ± 0.09	2.83 ± 0.20	2.69 ± 0.21	2.77 ± 0.27	2.54 ± 0.32	0.96 ± 0.16	0.67 ± 0.05	1.37 ± 0.38	1.14 ± 0.06	1.96 ± 0.12

a CS : NS Carbon and nitrogen source respectively.

Figure 1(A-D) presents the concentration profiles for solvents and acids produced by *C. beijeriincki* NCIMB 8052 at shake flask level for a carbon:nitrogen of 4:1.5%. The maximum quantity of solvents obtained was of 10.16 g L^{-1} after 34 h of fermentation

using glucose as substrate (Figure 1-A). Ezeji and Blaschek (2008) reported a production of 14.3 g L^{-1} of ABE in shake flasks containing 100 mL of fermentation medium at 35°C, pH 6.8, without agitation, and an initial glucose concentration of 55 g L^{-1}. Figure 1-B presents the concentration profiles for solvents produced from xylose fermentation. It is observed that the maximum quantity of ABE was 7.71 g L^{-1}. This production remained constant during approximately 20 h (from 76 to 96 h of fermentation). By the end of the fermentation butyric acid concentration was 2 g L^{-1}, higher than acetic acid concentration. On the contrary, Qureshi *et al*. (2008) obtained an ABE and butanol production of 12.80 and 8.60 g L^{-1}, respectively, using and initial xylose concentration of 15 g L^{-1}. This difference could be own to a possible microorganism sensibility to high xylose and butyric acid concentrations.

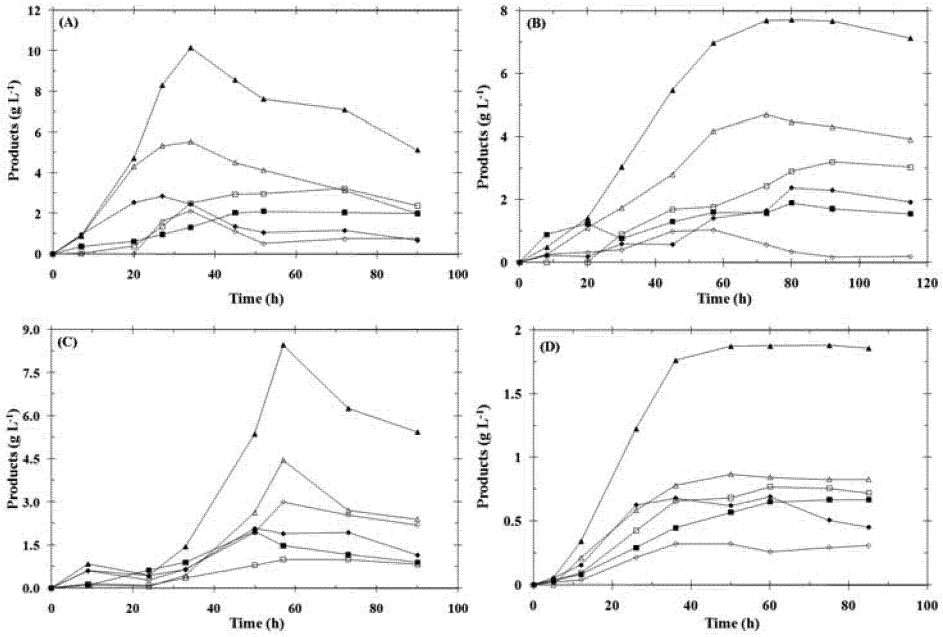

Figure 1. *Effect of carbon source on the production of acetone (□), ethanol (◇), butanol (⇔), acetic acid (■),butyric acid (♦) and total ABE (◢) for different carbon sources, glucose (A), xylose (B), sucrose (C) and glycerol (D).*

For the other carbon sources, sucrose and glycerol, solvents profiles are similar to obtain with glucose and xylose (Figure 1C-D). For sucrose, the maximum solvent production (8.46 g L^{-1}) was obtained at 57 h of fermentation (Figure C). This was lower (in ~17%) than obtained with glucose, but greater (in ~9%) than obtained with xylose. The highest quantity of butanol produced was 4.46 g L^{-1}. While for glycerol fermentations, no more than 1.881 g of ABE L^{-1} was obtained. Being this the lowest concentration obtained with regard to the other analyzed carbon sources. Nevertheless, butanol concentration profile remained constant during the last 30 h of fermentation.

The fermentations with glicerol presented no favorable results for ABE production. However, butanol production up to 17 g L^{-1} from glycerol fermentations using genetically modify *Clostridium* strains has been reported (Yazdani and Gonzalez, 2007). The obtained profiles of acids and solvents do not allow to determine clearly acidogenesis and solventogenesis stages.

4. Conclusions

The use of *Clostridium beijerinckii* NCIMB 8052 to produce solvents using different carbon is feasible process that depends on factors like carbon:nitrogen ratio, fermentation time, and chemical structure of the carbon source. It was noticed that solvent production was favored when sacharides, glucose, xylose and sucrose, were used as substrate. While fermentations carried out with glycerol did not favor the ABE production. The preliminar experimentation at tests tube scale confirms the use low nitrogen concentrations in the fermentation medium. Results present the strain *C. beijerinkii* NCIMB 8052 as an alternative to evaluate the production of solvents using hydrolysates of agro-industrial and industrial by-products. Nevertheless, assays with genetically modified Clostridium strains able to resist high butanol and butyric acid concentrations is desire to increase the productivity and solvent yields.

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