

Evaluation of biobutanol production by *Clostridium beijerinckii* NRRL B-592 using sweet sorghum as carbon source

Avaliação da produção de biobutanol por *Clostridium beijerinckii* NRRL B-592 usando sorgo sacarino como fonte de carbono

Luiz Jardel Visioli¹ Eliana Albornoz Alves¹ Aline Trindade¹ Raquel Cristine Kuhn¹
Marcio Schwaab¹ Marcio Antonio Mazutti^{1*}

ABSTRACT

*In this research it was evaluated the production of biobutanol by *Clostridium beijerinckii* NRRL B-592 using sweet sorghum juice as carbon source. Operational variables, like pH and initial inoculum size, as well as supplementation of industrial media with yeast extract and tryptone, were evaluated. The maximum butanol obtained was 2.12g kg⁻¹ using 12.5% of inoculum size, 0.05g 100mL⁻¹ of tryptone and 0.1g 100mL⁻¹ of yeast extract and initial pH of 5.5. The main contribution of this research was to show a systematic procedure for development of a low cost industrial media for biobutanol production from sweet sorghum.*

Key words: biobutanol production, industrial media, biofuel, sweet sorghum.

RESUMO

*Neste trabalho, foi avaliada a produção de biobutanol por *Clostridium beijerinckii* NRRL B-592 usando o caldo de sorgo sacarino como fonte de carbono. Foram avaliadas as variáveis operacionais como pH e densidade de inóculo, bem como a suplementação de meios industriais com extrato de levedura e tripton. A máxima concentração de butanol obtida foi de 2,12g kg⁻¹, utilizando 12,5% de inóculo, 0,05g 100mL⁻¹ de tripton e 0,1g 100mL⁻¹ de extrato de levedura e pH inicial de 5,5. A principal contribuição deste trabalho foi o de mostrar um procedimento sistemático para o desenvolvimento de um meio industrial de baixo custo para a produção de biobutanol a partir de sorgo sacarino.*

Palavras-chave: biobutanol, meio industrial, biocombustíveis, sorgo sacarino.

INTRODUCTION

The increase at oil prices and the necessity to diversify the energetic chain have generated the

interest in the production of renewable biofuels worldwide (QURESHI & EZEJI, 2008). Ethanol and butanol production from fermentation are largely reported as good alternatives to replace the fossil fuels consumption. Although the industrial production of ethanol by fermentation is consolidated, mainly in Brazil, biobutanol industry is slowly reappearing after ceased operation by the end of 1960s (QURESHI et al., 2007). Butanol presents better proprieties than ethanol, making it a candidate to replace gasoline and it can be considered as a bulk chemical precursor for the production of chemicals (LU et al., 2012). In this scenario the researches on biobutanol production are very important to optimize, reduce costs and becomes this production easier (MARIANO et al., 2010).

The anaerobic fermentation of a sugar source by strains of *Clostridium* spp. is the main used biotechnological route for butanol production. However, this fermentation produces significant amounts of ethanol and acetone becoming known as ABE (Acetone–Butanol–Ethanol) fermentation (JONES & WOODS, 1986). The most reported clostridium strains to butanol production are: *acetobutylicum*, *saccharobutylicum*, *beijerinckii*, *butylicum*, *aurantibutyricum* and *tetanomorphum* (QURESHI & EZEJI, 2008). The anaerobic condition maintenance during the fermentation is indispensable, increasing the operation cost of the process (EZEJI et al., 2013).

The economics of the biobutanol production are largely dependent on the cost of the

¹Departamento de Engenharia Química, Universidade Federal de Santa Maria (UFSM), Av. Roraima, 1000, 97105-900, Santa Maria, RS, Brasil. E-mail: mazutti@ufsm.br. *Corresponding author.

fermentation substrate (GARCÍA et al., 2011). In this sense, it is very important to find a cheap and available raw material to be used in this fermentation. The residues are an alternative defended for many authors (QURESHI & EZEJI, 2008). However this kind of substrate has limited availability and presents great variability on the characteristics. The sweet sorghum can be an attractive sugar source to biobutanol production. Sweet sorghum (*Sorghum bicolor* (L.) Moench) is a high biomass and sugar-yielding crop. Its juice contains similar quantities of glucose and sucrose and the productivity is high (YU et al., 2012). Furthermore the sweet sorghum has better adaptation to climates colder than tropical. In the Brazilian case its use could be questioned because of the sugar cane cultivation, however the adaptation to the climate makes the sweet sorghum interesting to production in southern regions of the country. Another important advantage is the sowing as the sorghum is planted by seeds, this process becomes easier than the sugar cane case which is planted by the stalk cuts (WHITFIELD et al., 2012).

However, one of the difficulties in the use of industrial media is the fact that bacteria are much sensitivity to any variability of culture media. Based on this aspect, this research is focused on the production of biobutanol by *Clostridium beijerinckii* NRRL B-592 using sweet sorghum juice as raw material. Operational variables as pH and initial inoculum size as well as supplementation of industrial media with yeast extract, and tryptone were evaluated by means of experimental methodology.

MATERIAL AND METHODS

Microorganism, culture maintenance, inoculum preparation and microorganism manipulation

The microorganism used in this study was *Clostridium beijerinckii* NRRL B-592 kindly provided by the United State Department of Agriculture (USDA) - Agricultural Research Service (ARS). The microorganism was shipped lyophilized in the vacuum medium. In the reactivation of the strain it was used the Reinforced Clostridium Medium (RCM) (KHAMAISEH et al., 2012). This procedure was done during 48-62 hours at 37°C. The temperature used is the one reported as the optimum to cellular growth by *Clostridium beijerinckii* NRRL B-592 by ARS in its home page. The microorganism growth was carried out in a sealed test-tube to prevent the oxygen entering.

The microorganism was storage in RCM medium at temperature of 4-7°C. After the period

of storage the microorganism was grown again in the RCM without heat shock. The inoculums were prepared using 10% (v/v) of seed solution of microorganism from storage. This growth occurred in a sealed test-tube during 24 hours at 37°C and then was replicated again to test-tube using 10% of growth solution, and this last grown 24 hours, being ready to be a fermentation inoculum.

The RCM medium used in the procedures was made using reagents from Sigma-Aldrich®. This medium contains (g L⁻¹) yeast extract 3.0, meat extract 10.0, peptone 10.0, soluble starch 1.0, L-cysteine hydrochloride 0.5, sodium acetate 3.0, agar 0.5, NaCl 5.0 and pH 6.8. After the preparation the medium used in the inoculation was autoclaved at 121°C for 20 minutes.

The manipulation necessary during inoculation, to inoculum preparation to fermentation starts was done into an anaerobic chamber (glove box), hand-made in the laboratory, using a polypropylene stuff box, a neoprene glove, Polyvinyl chloride connections and glue enabling the necessary manipulation to implement the fermentation. Before the inoculation start a flux of nitrogen (99.9% of purity provided from Air liquid®) was turned on and maintained during 20 minutes to ensure oxygen-free in the box. The medium was sparged to remove oxygen traces before inoculation.

Fermentations

The fermentation process was carried out using sweet sorghum as sugar source. Sorghum juice was provided by micro distillery of Universidade Federal de Santa Maria - Brazil, and was farmed in the geographic coordinates 29° 41' 29" south, 53° 48' 3" west. The sugar concentration of in nature sorghum juice was determined using a DNS methodology described by MILLER (1959), which was maintained constant at 60g L⁻¹ for all fermentations. The biobutanol production medium containing sweet sorghum juice as carbon source was supplemented with (g L⁻¹): KH₂PO₄ 0.5, K₂HPO₄·3H₂O 0.65, MgSO₄·7H₂O 0.2, FeSO₄·7H₂O 0.01, MnSO₄·H₂O 0.01, NaCl 0.01, and sodium thioglycolate 1.0 (LIU et al.; 2010). All the reagents used were from Sigma-Aldrich®. The medium also was supplemented with tryptone and yeast extract which were used as independent variables in the experimental design and will be better presented below. The use of yeast extract in ABE fermentation was reported by LIU et al. (2010) and the use of tryptone and yeast extract were reported by AL-SHORDANI (2012) as required supplements to the medium. Oxygen-free nitrogen

was sparged in the medium during 15 minutes. Afterwards, the fermentation media was autoclaved at 121°C for 20 minutes.

Biobutanol production was carried out in 250mL conical flasks containing 100mL of culture medium with concentrations of supplements, initial inoculum concentration and pH defined by the experimental design. Fermentation was started with defined inoculum concentration and pH and the rotation of 150rpm in the shaker (Tecnal® shaker model TE-420) at 37°C for 96 hours. The fermented broth was centrifuged at 4000rpm at 4°C by 15min (Eppendorf®, model 5804R). After this process the sample was stored at -15°C to further analysis.

To evaluate the effects of initial pH, inoculum size, concentrations of tryptone and yeast extract a Plackett-Burman design with eight fermentations plus three central points (PB8) was conceived. Table 1 presents the range investigated for each independent variable. Based on the analysis of PB8, a second Plackett-Burman design with eight fermentations plus three central points (PB8) was conceived to evaluate the effects of initial pH, inoculum size, concentrations of tryptone and yeast extract, where the evaluated range for each variable is presented in table 2. All the results were analyzed using the software Statistica® 7.0 (Statsoft Inc., Tulsa, OK, USA), considering a significance level of 90%.

Analytical procedures

Determination of solvents (acetone-butanol-ethanol) was carried out by gas chromatography (Shimadzu, GC – 17A) using a capillary column DB-Wax (30m x 0.25mm x 0.25µm) with polyethylene glycol as the stationary phase. The injection was done in a split injector (ratio 1:20)

at temperature of 220°C, where samples 0.2µL were injected. The detector of flame ionization (FID) was maintained at temperature of 250°C and its flame was operating with hydrogen and synthetic air. The column was operated according to the following column temperature gradient programming: 40°C (8min); 20°C min⁻¹ up to 180°C and maintained at this temperature for 10min. Nitrogen was the carrier gas with a flow rate of 0.8mL min⁻¹. Methyl ethyl ketone (Merk) was used as internal standard (QURESHI et al., 2007).

RESULTS AND DISCUSSION

Table 1 presents the results referring to butanol production obtained in the PB8. As can be seen, the production occurred only at some specific experimental conditions at low concentrations. However, the production occurred mainly in the runs carried out at initial pH of 6.0, indicating that the range evaluated for pH was not appropriated for this microorganism. This was confirmed by analysis of effects, expressed in the form of Pareto chart presented in the figure 1, where pH presented negative effect on butanol production at a significance of 89% (P<0.11).

Based on the results obtained in the first experimental design a second PB8 was conceived. The variables investigated were the same, being altered the range. The results obtained in the second PB8 design are presented in table 2. The highest production was 2.12±0.07g kg⁻¹ at central point of the PB8. In other runs, the production was low. Data of table 2 were used to compute the main effects of independent variables, which are presented in figure 2. As can be seen, all variables investigated were not significant in the range studied. However, the condition of central point is

Table 1 - Matrix of the first PB8 to evaluate the influence of process variables on biobutanol production.

Run	Initial pH	Inoculum (%)	Tryptone (g 100mL ⁻¹)	Yeast extract (g 100mL ⁻¹)	Butanol production (g kg ⁻¹)
1	8 (1)	5 (-1)	0 (-1)	0.4 (1)	0.00
2	8 (1)	20 (1)	0 (-1)	0 (-1)	0.00
3	8 (1)	20 (1)	0.2 (1)	0 (-1)	0.00
4	6 (-1)	20 (1)	0.2 (1)	0.4 (1)	0.79
5	8 (1)	5 (-1)	0.2 (1)	0.4 (1)	0.06
6	6 (-1)	20 (1)	0 (-1)	0.4 (1)	0.00
7	6 (-1)	5 (-1)	0.2 (1)	0 (-1)	0.17
8	6 (-1)	5 (-1)	0 (-1)	0 (-1)	0.68
9	7 (0)	12.5 (0)	0.1 (0)	0.2 (0)	0.00
10	7 (0)	12.5 (0)	0.1 (0)	0.2 (0)	0.00
11	7 (0)	12.5 (0)	0.1 (0)	0.2 (0)	0.05

Table 2 - Matrix of the second PB8 to evaluate the influence of process variables on biobutanol production.

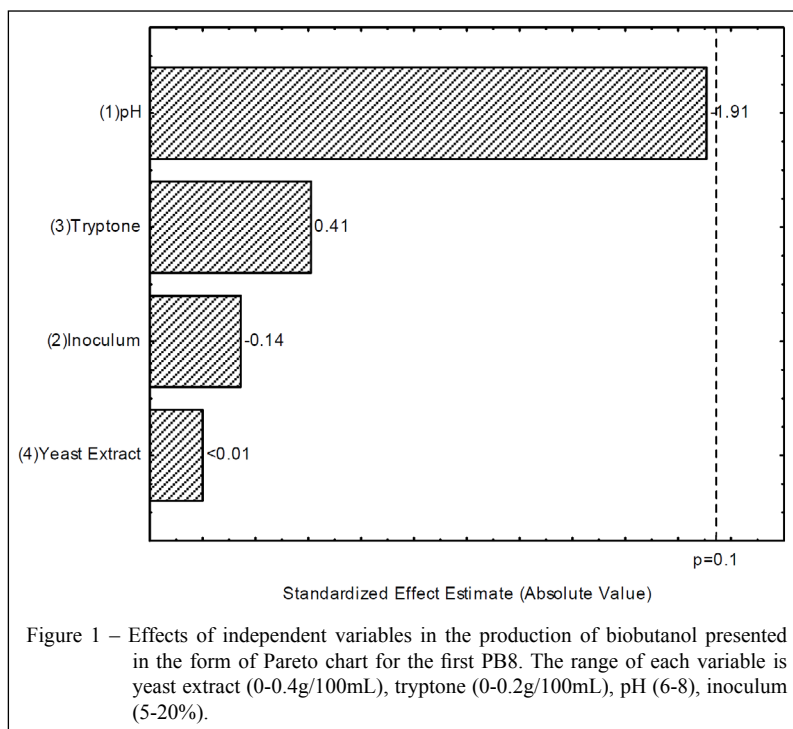
Run	Initial pH	Inoculum (%)	Tryptone (g 100mL ⁻¹)	Yeast extract (g 100mL ⁻¹)	Butanol production (g kg ⁻¹)
1	6 (1)	5 (-1)	0 (-1)	0.2 (1)	1.64
2	6 (1)	20 (1)	0 (-1)	0 (-1)	0.10
3	6 (1)	20 (1)	0.1 (1)	0 (-1)	0.28
4	5 (-1)	20 (1)	0.1 (1)	0.2 (1)	0.00
5	6 (1)	5 (-1)	0.1 (1)	0.2 (1)	0.67
6	5 (-1)	20 (1)	0 (-1)	0.2 (1)	0.00
7	5 (-1)	5 (-1)	0.1 (1)	0 (-1)	0.00
8	5 (-1)	5 (-1)	0 (-1)	0 (-1)	0.00
9	5.5 (0)	12.5 (0)	0.05 (0)	0.1 (0)	2.13
10	5.5 (0)	12.5 (0)	0.05 (0)	0.1 (0)	2.18
11	5.5 (0)	12.5 (0)	0.05 (0)	0.1 (0)	2.05

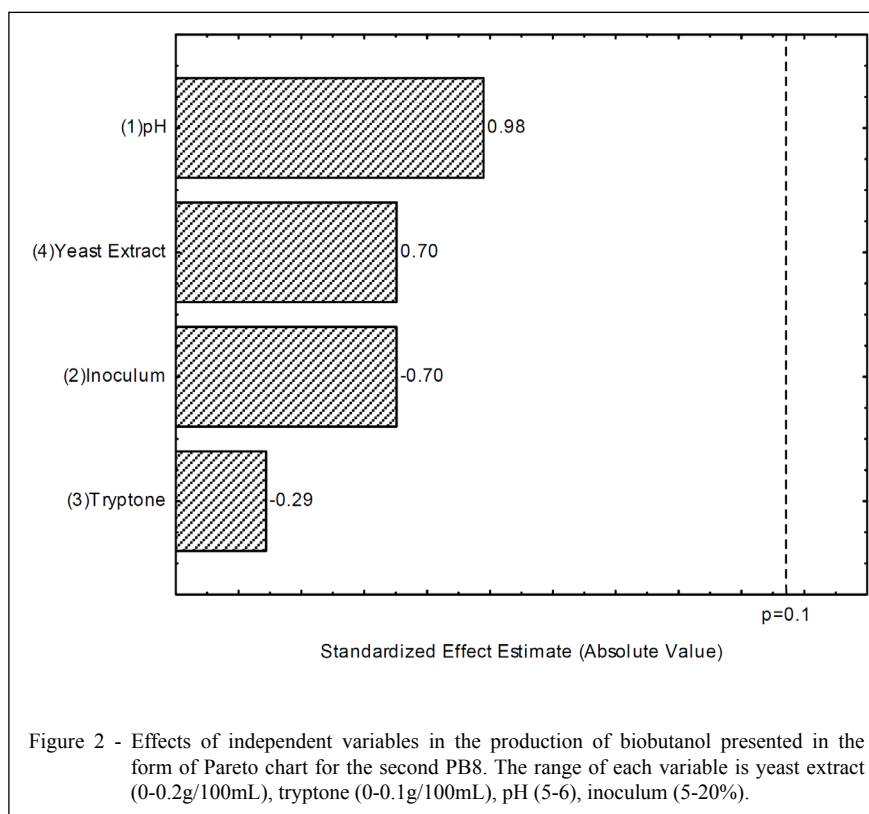
not considered in the calculation of the effects. In this sense, there is the indicative that central the condition of central point is the best for biobutanol production.

The impossibility of determination of which variables are really significant in the both experimental design occurs because in the both cases the numbers of helpful run was halved with the not butanol production on pH 8, 7 and 5. This undertakes the ability of PLACKETT & BURMAN design to determinate the real significance of each variable. However the results obtained in these experiments could be important to encourage new researches in order to better understanding the true necessity of

the addition of supplementation on fermentation medium. The increase on the concentration of yeast extract and tryptone added enlarged substantially the total cost associated to raw material in the biofuel production. Thus, the results detect in this research could be important to the reduction of the process cost and to be helpful to enable the biofuel production viability.

One important aspect of this result is that the greatest butanol production using *Clostridium beijerinckii* NRRL B-592 and sweet sorghum (60g L⁻¹ of total sugars) requires low concentration of yeast extract and tryptone, which are expensive





nutrient for industrial fermentation. This result demonstrates that the sweet sorghum can be used for clostridium culture development not requiring a lot of expensive additional compounds, making this biofuel more attractive.

CONCLUSION

In this research it was evaluated the production of biobutanol by *Clostridium beijerinckii* NRRL B-592 using sweet sorghum juice as carbon source. Maximum butanol obtained was 2.12g kg⁻¹ using 12.5% of inoculum size, 0.05g 100mL⁻¹ of tryptone and 0.1g 100mL⁻¹ of yeast extract and initial pH of 5.5. The research shows that it is possible to reduce the medium supplementation to biobutanol production, and so decrease the total cost associated to it. The main contribution of this research was to show a systematic procedure to develop a low cost industrial media for biobutanol production from sweet sorghum.

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