Ethanol Production using *Zymomonas mobilis* cells immobilized on Cane Bagasse

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Abstract. With rising oil prices and decreasing availability, as well as damage to the environment, it is necessary to look alternatives for the production of liquid fuels such as ethanol. For its production several supports have been used, in this work was carried out a study of the ethanol production using cells of *Zymomonas mobilis* immobilized on bagasse of cane as support which showed a good ability to adsorb bacterial cells, so the system was operated at continuous culture at different dilution rates D (from 0.05 to 0.33 h^{-1}) and different concentrations of sucrose (from 50 to 300 g L^{-1}). The best results were achieved at a sucrose concentration of 200 g L^{-1} operating at a dilution rate of 0.262 h^{-1}, reaching an ethanol production of 78.2 g L^{-1}, having a sucrose consumption of 97 % and a yield of bioethanol from sucrose of 0.40 g EtOH /g sucrose, representing 78.4% with respect to the maximum theoretical yield.

Keywords: Zymomonas mobilis, bioethanol, sugar cane bagasse, immobilized cell, continuous culture.

1. Introduction

Bioethanol is a biofuel obtained through the anaerobic fermentation of a variety of substrates: 1). Carbohydrates such as sucrose, glucose and fructose, (Lee & Huang, 2000) cane molasses (Behera et al, 2012); 2). Starches (Altuntas and Ozcelik, 2013) and/or 3). Lignocellulosic substrates with previous hydrolyze (Wirawan et al., 2012). *Saccharomyces cerevisiae* and *Z. mobilis* are the main microorganisms used for ethanol production (Tian et al., 2005), the last one has several advantages since it is less sensitive to inhibition by product, moreover less biomass is generated due to the metabolic pathway it employs, the Entner Duodoroff Patway (Valverde and Olalde, 2009); *Z. mobilis* transports the sucrose through facilitated diffusion, therefore tolerates high concentrations of sugar hence for ethanol production the inhibition process begins above 160 g of glucose L^{-1} (Khoja et al, 2015).

Different reaction systems have been used to carry out the fermentation process: with free cells culture and immobilized culture; an advantage of the use of immobilized cells is that it can operate continuously (Altuntas and Ozcelik, 2013). The immobilized systems use supports, one of the most used are the calcium alginate beads (Behera et al., 2010).

Z. mobilis showed lower conversion of substrate to ethanol from sucrose compared to glucose or fructose, even the formation of by-products (sorbitol and levan) was greatly reduced when an equimolar mixture of glucose and fructose was used as the source of carbon in continuous culture (Favela and Baratti, 1987).

Z. mobilis and *S. cerevisiae* have been used for the production of ethanol from organic waste, in a system co-immobilized in calcium alginate achieving higher productivities with *S. cerevisiae* (Ruiz et al, 2016).

This study proposes the use of sugarcane bagasse as a support to implement a continuous system for ethanol production using *Z. mobilis* immobilized in cane bagasse, hence the objective of this study is the process kinetic evaluation for determinate the best operating conditions of the reactor and to offer an efficient, productive, economic and easy to scale technological alternative.

2. Matherial and methods

2.1. Microorganism and grown medium

For this study the bacterium *Z. mobilis* CDBB-B-603 obtained from the national collection of microbial strains and cell cultures of the Research and Advanced Studies Center was used (CINVESTAV, IPN, Mexico). The strain was preserved in tubes with inclined culture medium of: glucose, peptone, yeast extract (YPG) implemented by the national selection of microbial strains and cell cultures. The corresponding resiembras were done every 6 months.

For the preservation of the bacteria, a solid culture medium was used with the following formulation in g L^{-1}: yeast extract, 3.0; Glucose, 20; Peptone, 10; Agar 20.

The composition of the fermentation medium is as follows in (g L^{-1}): $CaCl_2$, 0.01; $MgSO_4$, 0.007; $(NH_4)_2SO_4$, 0.04; yeast extract 0.02.

The culture medium was autoclaved at 121 ° C for 20 minutes, the concentration of the compounds of the production medium were adjusted proportionally to the final sucrose concentration required for each experiment, without presenting caramelization.

2.2. Operation of the bioreactor packed with bagasse

The bioreactor is a cylindrical glass column, with base and metal lid supported by metal rods that gives it stability. It has an internal diameter of 3.7 cm and 31 cm of height (glass column) with a volume of operation of 0.305 L, it has no pH, temperature and dissolved oxygen controls. The system was packed using cane bagasse as a support, a pre-treatment was applied to the support, consisting of a series of washes with cold water and hot water and then dried in the oven at 90° C for two hours.

Once the reactor was inoculated, it was kept in batch culture for 72 hours to allow the immobilization of the bacterium on the bagasse. Cell sedimentation was prevented by providing sterile air which would keep the cells dispersed throughout the reaction volume and allow their adhesion to the carrier. Subsequently the free cells were drained and the fresh medium was fed to packed bioreactor to initiate ethanol production in continuous culture by Z. *mobilis* cells immobilized on cane bagasse.

For ethanol production sucrose concentration of 50, 100, 150, 200, 250 and 300 g L was varied, and the system was operated continuously at seven different dilution rates (D): 0052, 0098, 0157, 0262, 0281 and 0.327 h^{-1}.

The dynamic equilibrium state for each tested dilution rate was reached at the five replacement volumes of the bioreactor and the samples were taken from the top of the column using a sterile syringe. Each sample was determined the concentration of free cells, residual sucrose and ethanol produced.

2.3. Determination of ethanol concentration

The ethanol concentration was determined with a VARIAN CP-3800 gas chromatograph with flame ionization detector (FID) and WCOT fused silica CP7860 (30 m x 0.25 mm) capillary column. Helium was used as a carrier gas at a flow of 60 mL min^{-1}. The operating temperature conditions were: oven 160 ºC, injector 220 ºC and detector 250 ºC, without temperature ramp and 10 Split.

The sucrose concentration was determined indirectly by quantifying the residual glucose by the glucose oxidase-peroxidase method (Gochman and Schmitz, 1972) and by stoichiometry the residual sucrose was obtained.

3. Results and discusion

3.1 Kinetics of Ethanol production

The concentration of ethanol was increased by increasing the concentration of sucrose in the range of 50 to 200 g L^{-1}, at higher concentrations ethanol production was inhibited (Figure 1).

When the sucrose concentration in the feed was kept constant, the ethanol produced was constant in the range of dilution rates between 0.05 to 0.262 h-1.

The highest productivities were obtained when the sucrose supplied was 200 g/L, so the highest productivity was obtained at D = 0.262 h^{-1}, reaching a productivity of 52.4 g ethanol L^{-1} h^{-1}.

3.2 Kinetics of sucrose consumption

When the concentration of the sucrose fed varied between 50 and 200 g L^{-1}, it was completely consumed in the range of dilution rates between 0.05 and 0.2 h^{-1} (Figure 2), residual sucrose accumulated, with increasing dilution rate (D). At higher concentrations of 250 g L^{-1}, the substrate consumption was deficient and accumulated, when the sucrose concentration was 300 g L^{-1} there was no consumption so the residual sucrose reached the feed value.

*3.3 Yield ethanol:sucrose (*Yp/s*)*

The experimental yields were calculated considering the ethanol produced and the sucrose consumed, then compared with the theoretical yield of 0.538 g Ethanol / g sucrose obtained considering the stoichiometric reaction:

$$C_{12}H_{22}O_{11} \longrightarrow 4\,C_2H_6O + 4\,CO_2$$

For sucrose feed concentrations between 50 and 200 g L^{-1}, yields of ethanol Yp/s were increased with increasing substrate concentration and did not depend on the rate of dilution (Figure 3).

The best experimental yield of 0.401 g of ethanol g^{-1} sucrose was obtained at 200 g L^{-1} of sucrose, it was kept constant in the range of the dilution rates between 0.05 to 0.327 h^{-1}, representing 78.4% with respect to the theoretical maximum yield of 0.538 g g^{-1}.

At higher concentrations yield decreased by the inhibitory effect of the substrate on *Z. mobilis*.

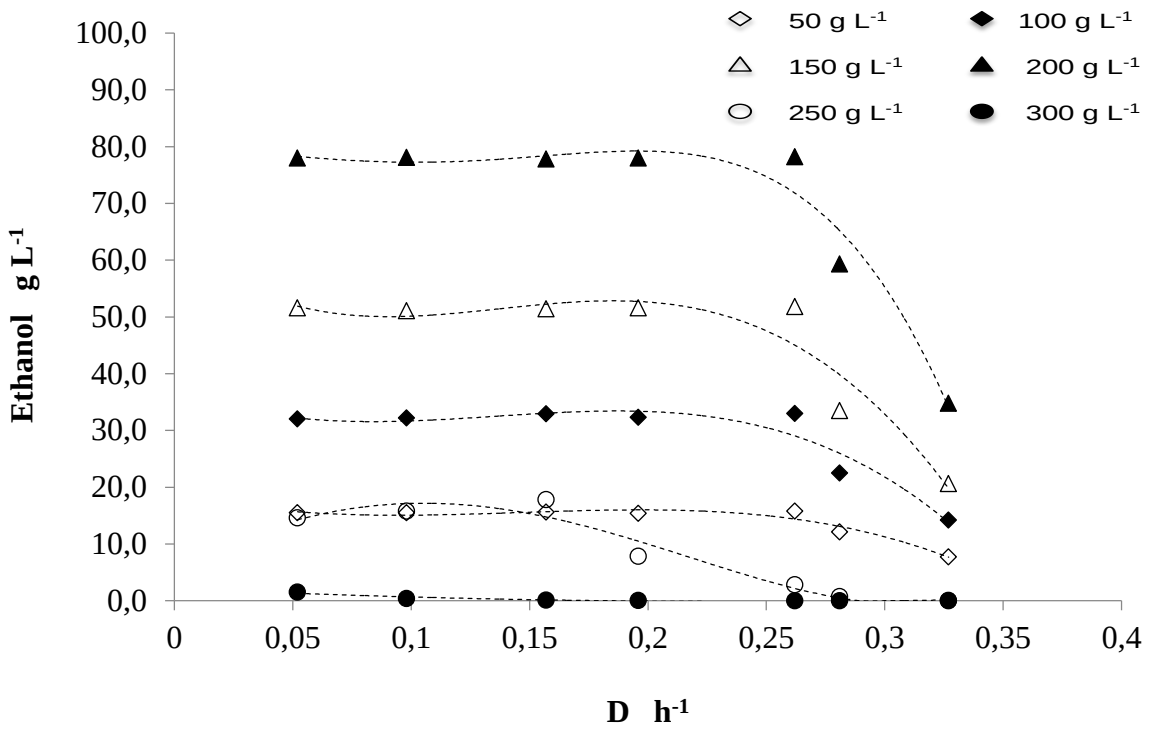

Figure 1. Kinetics of ethanol production in continuous culture of *Zymomonas mobilis* immobilized on cane bagasse at different concentrations of sucrose in feed.

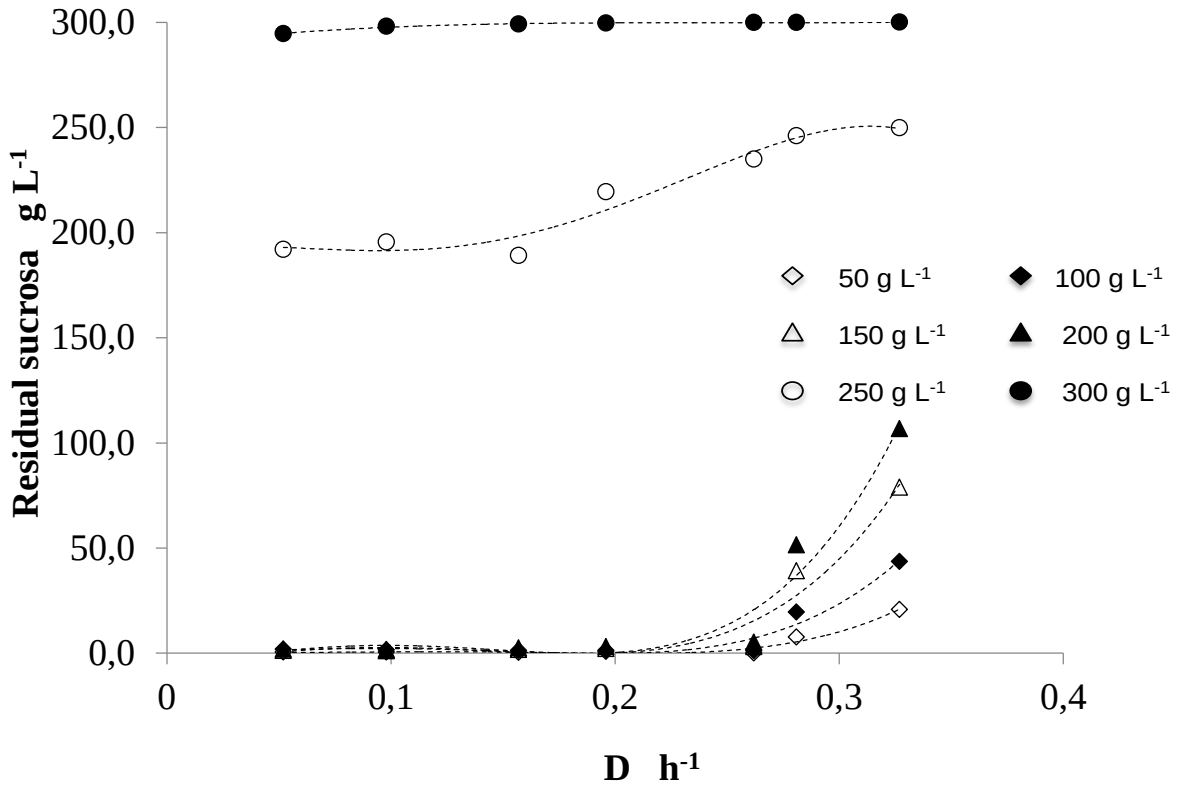

Figure 2. Residual sucrose in continuous culture of *Zymomonas mobilis* immobilized on cane bagasse at different concentrations of sucrose in feed

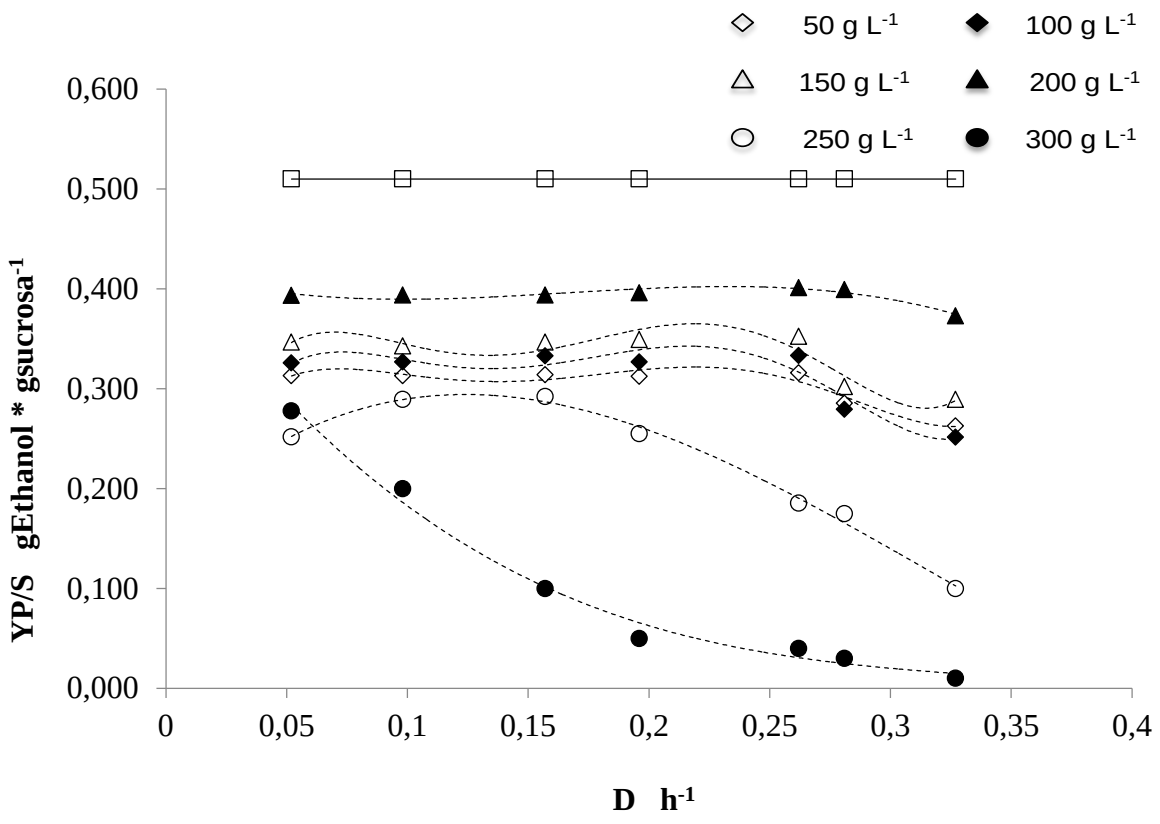

Figure 3. Substrate yields obtained in continuous culture of *Zymomonas mobilis* immobilized on cane bagasse at different concentrations of sucrose in feed.

One of the advantages of *Z. mobilis* is the ability to direct the consumption of the carbon source more toward ethanol production than towards biomass formation, under specific conditions such as high sugar concentration, anaerobic conditions and low pH (Leveau and Bouix , 2000), thus promoting the consumption of sucrose to produce ethanol.

In this study, substrate inhibition occurred when sucrose concentrations in feed were 250 and 300 g L^{-1}, inhibiting the consumption of sucrose and therefore the production of ethanol. At 250 g L^{-1} of sucrose fed a low sucrose consumption and low ethanol production was reached, but at 300 g L^{-1}, sucrose consumption was completely stopped and therefore there was no ethanol production.. The sucrose concentration in the medium was so high that it generated a high osmotic pressure on *Z. mobilis* diminishing its enzymatic and aqueous activity until the cellular lysis (Tortora et al, 2007).

In this process the maximum ethanol production was 78.2 g L^{-1} remaining below the reported of 86 g L^{-1} (Lee y Rogers, 1983). This result may be due to the fact that the system had no pH and temperature controls, but one advantage of this system is the high productivity achieved and the packed bed system with *Z. mobilis* immobilized on sugar cane bagasse remains stable for approximately 6 months of continuous operation.

4. Conclusions

An ethanol production system was implemented using *Zymomonas mobilis* CDBB-B-603 immobilized on cane bagasse, which was operated in continuous culture representing an efficient alternative, besides being economical and easy to operate.

The best ethanol production conditions were reached with an initial sucrose concentration of 200 g L^{-1} and a dilution rate D of 0.26 h^{-1}, whereby the maximum productivity was 52.4 g of ethanol L^{-1} h^{-1}, which can be maintained for long periods of time.

References

Lee W.C., Huang C.T., (2000), Modeling og Ethanol fermentation using Zymomonas mobilis ATCC 10988 grown on the media containing glucose and fructose, Biochemical Engineering Journal, 43(4) 1499-1507.

Behera S., Mohanty R. C., Ray R. C. (2012). Ethanol fermentation of sugarcane molasses by Zymomonas mobilis MTCC 92 immobilized in Luffa cylindrical L sponge discs and ca-alginate matrices. Brazilian Journal of Microbiology, 4(3) 217-227.

Altuntas E.G., Ozcelik (2013). Ethanol production from starch by co-immobilized amyloglucosidase – Zymomonas mobilis cells in a continuous-stirred bioreactor. Biotechnology and Biotechnological Equipment, 27(1), 3506-3512.

Wirawan F., Cheng C. L. Kao W. C., Lee D. J. Chang J. S. (2012), Cellulosic ethanol production performance with SSF and SHF processes using immobilized Zymomonas mobilis, Applied Energy, 100, 19-26.

Tian S., Wang J., Chen X., Yuan Z., Yang X. (2005) Ethanol production of immobilized Zymomonas, Acta Energiae Solaris Sinica. 26, 219-223.

Valverde, E. y Olalde V. (2009). Rutas, procesos y microorganismos implicados en la producción de biocombustibles, CONCYTEG, 54,1216-1233.

Khoja A. H., Ali E., Zafar K., Ansari A. A., Nawar A., Qayyum M. (2015). Comparative study of bioethanol production from sugarcane molasses by using Zymomonas mobilis and Saccharomyces cerevisiae, African Journal of Biotechnology, 14(31),2455-2462.

Behera S., Ray R. C., Mohanty R.C. (2010). Comparative study of bioethanol production from mahula (Madhuca latifolia L.) flowers by immobilized cells of Saccharomyces cerevisiae and Zymomonas mobilis in calcium alginate beads, Journal of Scientific and Industrial Research, 69(6),472-475.

Favela E. & Baratti J. (1987), Continuous ethanol production by Zymomonas mobilis from an equimolar mixture of glucose and fructose, Biomass, 13(2),75-85.

Ruiz_Marín A., Canedo-López Y., Narváez-García A., Robles-Heredia J.C. (2016), Production of etanol by Saccharomyces cerevisiae and Zymomonas mobilis coimmobilized: Proposal for the use of organic waste. Agrociencia, 50(5),551-563.

Gochman N, Schmitz JM. (1972), Application of a new peroxide indicator reaction to the specific, automated determination of glucose with glucose oxidase. Clin Chem, 18: 943-950.

Leveau, Y. J. y Bouix, M. (2000). Microbiología Industrial: Los microorganismos de interés industrial, Acribia Editorial, Zaragoza, España, pp 608.

Tortora G. J., Funke B. R. and Case, C. L. (2007). Crecimiento microbiano. Introducción a la microbiología. 9a. ed, editorial Médica Panamericana, Buenos Aires, Argentina, pp 159-186.

Lee K. J. and Rogers P. L. (1983). The fermentation kinetics of ethanol production by Zymomonas mobilis. The Chemical Engineering Journal, 27(2), B31-B38.