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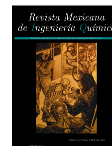

ETHANOL PRODUCTION BY *Zymomonas mobilis* NRRL B-806 FROM ENZYMATIC HYDROLYSATES OF *Eucalyptus globulus*

PRODUCCIÓN DE ETANOL A PARTIR DE HIDROLIZADOS ENZIMÁTICOS DE *Eucalyptus globulus* USANDO *Zymomonas mobilis* NRRL B-806

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Abstract

Ethanol production by *Zymomonas mobilis* NRRL-806 was assessed using different enzymatic hydrolysates from pretreated *Eucalyptus globulus* saw dust pulp. Results showed that when enzymatic hydrolysate containing 79.5 g L^{-1} of glucose and 8.81 g L^{-1} of acetic acid, a maximum ethanol yield of 92% and a productivity of 1.16 g L^{-1} h^{-1} were obtained, with a total of 37 g L^{-1} of ethanol after 27 h. Acetic acid concentration present in enzymatic hydrolysates was the main factor that contributed to decrease ethanol yield and productivity, in the case of hydrolysate *E*, where acetic acid concentration was higher (12.8 g L^{-1}), ethanol yield and productivity were 80% and 0.99 g L^{-1} h^{-1} respectively.

Keywords: *Eucalyptus globulus*, enzymatic hydrolysates, *Zymomonas mobilis* NRRL-806, ethanol yield, acetic acid.

Resumen

Se evaluó la producción de etanol por *Zymomonas mobilis* NRRL-806 a partir de diferentes hidrolizados enzimáticos de pulpa de aserrín de *Eucalyptus globulus* pretratada. Los resultados mostraron un máximo rendimiento de etanol de 92% y una productividad de 1.16 g L^{-1} h^{-1}, con una concentración total de etanol de 37 g L^{-1} después de 27 h, cuando el hidrolizado enzimático contenía 79.5 g L^{-1} de glucosa y 8.81 g L^{-1} de ácido acético. La concentración de ácido acético presente en los hidrolizados enzimáticos fue el principal factor que contribuyó a la disminución del rendimiento y productividad de etanol, en el caso del hidrolizado *E*, en donde la concentración de ácido acético fue mayor (12.8 g L^{-1}), el rendimiento y la productividad de etanol fueron de 80% y 0.99 g L^{-1} h^{-1} respectivamente.

Palabras clave: *Eucalyptus globulus*, hidrolizados enzimáticos, *Zymomonas mobilis* NRRL-806, rendimiento de etanol, ácido acético.

1 Introduction

The imminent ending of the non-renewable natural energetic resources brings the necessity to research for new renewable natural energetic resources, as well as the maximization of productivity and lowering the costs of already known processes. Biomass-based energy sources constitute an important alternative to help solving this problem (Letti and Karp, 2012; Domínguez-Maldonado *et al*., 2014). Lignocellulosic biomass is the most abundant renewable biological resource and, since is outside the human food chain is also an attractive and relatively inexpensive raw material (Pereira *et al*., 2012). Lignocellulose is an abundant natural carbohydrate formed by biopolymers such as cellulose, hemicellulose and lignin, which can be transformed into substitute renewable energy resource by microbial conversion

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(Roberto *et al.*, 2003). The advantages of using them as raw materials for ethanol production are their low cost, easy availability, avoidance of conflict with their use for food, and the potential production of fuel from lignin (Liu *et al.*, 2012). However, the heterogeneous and complex structure of lignocellulosic makes their fractionation and further benefit difficult (Romaní *et al.*, 2012). The main stages of the cellulosic ethanol production process are pre-treatment, hydrolysis, fermentation, distillation, and further fuel upgrading (Starfelt *et al.*, 2012). There are at least three methods of hydrolysis, including dilute acid hydrolysis, concentrated acid hydrolysis, and enzymatic hydrolysis. The enzyme-based process is more environmental-friendly and gives higher hydrolysis yield than acid hydrolysis (Zheng *et al.*, 2009). Therefore enzymatic hydrolysis is advantageous because of its low toxicity, low utility cost and low corrosion compared to acid or alkaline hydrolysis (Sun and Cheng, 2002; Taherzadeh and Karimi, 2007). In enzymatic hydrolysis the enzymes catalyze only specific reactions, and as result no other side reactions occur or byproducts are formed and hydrolysis has the potential to achieve higher yield of reducing sugars (Mukhopadhyay *et al.*, 2011). Here cellulase and hemicellulase enzymes cleave the bonds of cellulose and hemicellulose respectively. Cellulase enzymes involve endo and exoglucanase and β-glucosidases. Hemicellulolytic enzymes are more complex and are a mixture of at least eight enzymes. Cellulose is hydrolysed to glucose whereas hemicellulose gives rise to several pentoses and hexoses (Sarkar *et al.*, 2012).

Zymomonas mobilis has been intensively studied in ethanol fermentation. *Zymomonas mobilis* metabolizes sugar via the Entner-Doudoroff (ED) pathway, which produces less ATP and less biomass. More carbon sources are thus channeled to ethanol, resulting in an even higher ethanol yield than that found with the native ethanol fermenting yeast *Saccharomyces cerevisiae*. In addition, because of producing less ATP during ethanol fermentation, *Z. mobilis* maintains a higher glucose metabolic flux, normally three to five fold that of *S. cerevisiae* (Wirawan *et al.*, 2012). The aim of this work was to evaluate the production of ethanol from different enzymatic hydrolysates from pretreated *Eucalyptus globulus* saw dust pulp.

2 Materials and methods

2.1 *Microorganism and media*

Zymomonas mobilis NRRL B-806 strain was provided by the Environmental Biotechnology Lab. of Biochemistry Engineering Faculty, Pontificia Universidad Catolica de Valparaiso, Chile (PUCV). *Zymomonas mobilis* NRRL B-806 was cultivated on M2 solid medium containing per liter: 100 g glucose, 5 g yeast extract, 2 g KH_2PO_4, 1 g $MgSO_4$ and 1.5 g $(NH_4)_2SO_4$ at 30 ºC. Inoculum was prepared from the stock culture, using the same culture medium. The growth was carried out at 30 ºC for 48 hours.

2.2 *Eucalyptus globulus pretreatment*

Eucalyptus globulus saw dust pulp pretreatment was carried out in a stainless steel reactor under the following conditions: H_2SO_4 1% at 176 °C for 10 minutes followed by a quick decompression to atmospheric pressure. The solids were separated by filtering and washed with tap water to neutrality. The solid residue was dried in a forced-air oven at 105 °C.

2.3 *Enzymatic hydrolysates*

The study was carried out using three different enzymatic hydrolysates from pretreated *Eucalyptus globulus* saw dust pulp under the following conditions: (FPU:UI pNPG ratio and enzyme/sustrate relation). Enzymes used were of the series NS500 Novozymes (Bagsvaerd Denmark), cellulase (NS50013) and β-glucosidase (NS50010). Enzymatic hydrolysates compositions are shown in Table 1 obtained under the following conditions; 50 °C, with a stirring rate of 200 rpm during 72 h.

2.4 *Hydrolysates fermentation process*

Enzymatic hydrolysates were supplemented with nutrients of M2 medium at a pH 5.5 (without glucose). Experiments were carried out in 250 mL Erlenmeyer flasks, each containing 100 ml de hydrolysates. In the case of the hydrolysate *E*, hydrolysates C and D were mixed in a ratio 1:1 to obtain 75 ml of final volume. A control was studied containing M2 medium, using glucose with an initial concentration of 100 g L^{-1}. All the flasks were inoculated with 5 mL of cellular suspension (0.15 g L^{-1}). Incubation time was 51 h, at 30 °C with a stirring rate of 150 rpm.

Table 1. Enzymatic hydrolysates contents from pretreated *Eucalyptus globulus* saw dust pulp used for ethanol production by *Zymomonas mobilis* NRRL B-806.

Parameter	Hydrolysate *A*	Hydrolysate *B*	Hydrolysate *C*	Hydrolysate *D*
FPU:UI pNPG ratio	1:3	1:2	1:3	1:2
Enzyme/sustrate (%)	0.45	0.2	0.2	0.45
Glucose g L^{-1}	87.27	79.55	76.91*	
Xylose g L^{-1}	7.85	6.82	6.99*	
Arabinose g L^{-1}	10.36	5.77	9.15*	
Cellobiose g L^{-1}	11.55	7.06	7.75*	
Other carbohydrates g L^{-1}	6.35	6.5	6.15*	
Acetic acid g L^{-1}	8.87	8.81	12.8*	
Formic acid g L^{-1}	0.08	0.05	0.1*	
Glycerol g L^{-1}	0	0	0*	

* Hydrolysate E: C and D enzymatic hydrolysates mixture.

2.5 Analytical methods

Carbohydrates concentrations, inhibitor concentrations and ethanol production were determined by HPLC (Agilent 1260 HPLC, IR Detector,). A Biorad HPX-87-H column was used, enabling quantification of glucose, xylose, celobiose, arabinose, formic acid, acetic acid, ethanol. Operational conditions were H_2SO_4 5mM as mobile phase, at a flow rate of 0.6 mL/min, and 45 °C. All samples were centrifuged at 10,000 rpm for 5 min, and filtered and dilutes in HPLC water before being analyzed. Cellular growth was determined by measuring optical density of cells using a UV/Vis spectrometer (Cary 50Bio) at 660 nm and correlated with dry weight. All the analyses were performed in triplicate.

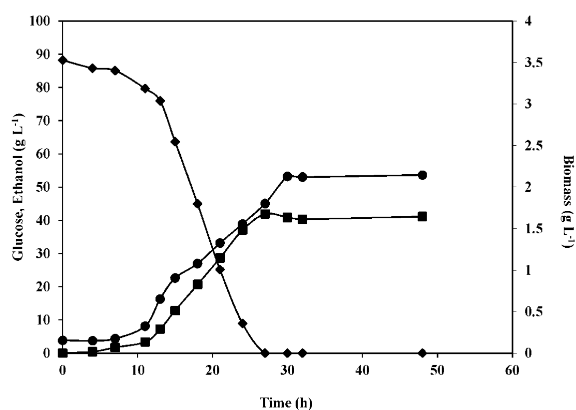

Fig. 1. Glucose consumption (♦), ethanol production (■) and biomass growth (•) in control reactor with *Zymomonas mobilis* NRRL B-806 using M2 medium at 30 °C.

3 Results and discussion

Figure 1 shows glucose consumption, ethanol production and biomass growth in control reactor with *Zymomonas mobilis* NRRL B-806 using M2 medium at 30 °C. Glucose was totally consumed after 30 h and at this point maximum ethanol production and biomass growth was achieved with a 41.1 g L^{-1} and 2.1 g L^{-1} respectively, with an ethanol yield ($Y_{E/S}$) of 0.46 $g_{ethanol}\ g^{-1}_{glucose}$ (90% in relation to theoretical value) and a productivity of 1.37 g L^{-1} h^{-1} (see Table 2). Growth curve had a correlation with glucose consumption, showing that after 30 h a stationary phase was reached, achieving a yield of 2.43 $\times 10^{-2}$ $g_{biomass}\ g^{-1}_{glucose}$ (2.43 %). Low biomass production is normally observed in *Zymomonas mobilis*, and cell growth and fermentation are not linked (Parker *et al*., 1997). According to Rogers *et al*. (1982) approximately 2% of the carbon source is converted into biomass. This occurs due to Entner-Doudoroff pathway used by this microorganism. This pathway yields only a single mole of ATP per mole of carbohydrate fermented, giving Zymomonas the lowest molar growth yield reported for bacteria (Swings and De Ley, 1977).

Figure 2 shows glucose consumption and ethanol production with *Zymomonas mobilis* NRRL B-806 from different enzymatic hydrolysates of pretreated *Eucalyptus globulus* saw dust pulp (A, B and E) with medium M2 (without glucose) at 30 °C. Ethanol production with *Zymomonas mobilis* NRRL B-806

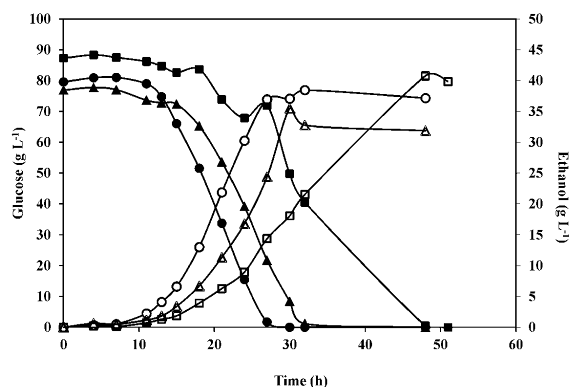

Fig. 2. Glucose consumption (solid markers) and ethanol production (unfilled markers) by *Zymomonas mobilis* NRRL B-806 with different enzymatic hydrolysates from pretreated *Eucalyptus globulus* saw dust pulp at 30 °C, hydrolysate *A* (■ □); hydrolysate B: (● o) ; hydrolysate E: (▲ △).

using enzymatic hydrolysate A reached a maximum ethanol production of 39.8 g L^{-1} at 48 h, observing that glucose consumption rate increased after 20 h, contributing this to the long time required for total glucose consumption and to reach ethanol production stationary phase (48 h). Ethanol yield was 0.45 $g_{ethanol}\ g^{-1}_{glucose}$ (88% in relation to theoretical value) and a productivity of 0.83 g L^{-1} h^{-1} (see Table 1). In the case of enzymatic hydrolysate B it was observed that glucose was consumed in less time and achieving a higher ethanol yield, with a maximum ethanol concentration of 37 g L^{-1} at 32 h, with no significant increase after 27 h, at this point glucose was almost totally consumed. Ethanol yield was 0.47 $g_{ethanol}\ g^{-1}_{glucose}$ (92% in relation to theoretical value) with a productivity of 1.16 g L^{-1} h^{-1} (see Table 2),

In the case of enzymatic hydrolysate *E*, it can be observed that glucose was totally consumed after 32 h, obtaining a maximum ethanol concentration of 31.8 g L^{-1}. Ethanol yield was 0.41 $g_{ethanol}\ g^{-1}_{glucose}$ (80% in relation to theoretical value) and a productivity of 0.99 g L^{-1} h^{-1} (see Table 2). Low ethanol yield may be attributed to the high concentration of acetic acid present in the hydrolysate E (12.8 g L^{-1}), causing a partial inhibition of *Zymomonas mobilis* NRRL B-806 (Figure 2).

Kim *et al*. (2000) mentioned that acetic acid has a potential inhibitory effect over *Z. mobilis* (10.9 g L^{-1} at pH = 6.0), followed by vanillin (0.04 g L^{-1}), syringaldehyde (0.13 g L^{-1}) hydroxymethylfurfural (0.9 g L^{-1}) and furfural (0.3 g L^{-1}) (Rogers *et al*., 2007) Previous reports by our research group (Rios-Gonzalez *et al*., 2012), in relation to inhibitory effects of acetic acid in ethanol production by *Zymomonas mobilis* NRRL B-806 from pretreated *Eucalyptus globulus* saw dust pulp (enriched with glucose), showed that ethanol yield was 86 % and 4.7 % when acetic acid concentration was 6.8 g L^{-1} and 17.9 g L^{-1} respectively.

Table 3 shows xylose, arabinose and cellobiose concentration during fermentation process by *Zymomonas mobilis* NRRL B-806 from pretreated *Eucalyptus globulus* saw dust pulp. No significant changes in concentration for the three carbohydrates during fermentation process were observed. This is attributed to the limited range of carbohydrates that *Zymomonas mobilis* can use as carbon source, which convert only hexoses such as glucose, fructose and sucrose (Rogers *et al*., 2007).

Table 2. Fermentation kinetic parameters using different enzymatic hydrolysates from pretreated *Eucalyptus globulus* saw dust pulp for ethanol production by *Zymomonas mobilis* NRRL B-806.

Kinetic Parameters	M2 Control	Hydrolysate *A*	Hydrolysate *B*	Hydrolysate *E*
$Y_{X/S}$	0.0243	-	-	-
$Y_{E/S}$	0.46 (90)	0.45 (88)	0.47 (92)	0.41 (80)
EP	1.37	0.83	1.16	0.99

$Y_{X/S}$ Biomass yield ($g_{biomass}\ g^{-1}_{glucose}$).
$Y_{E/S}$ Ethanol yield ($g_{ethanol}\ g^{-1}_{glucose}$).
EP Ethanol productivity ($g_{ethanol}$ L^{-1} h^{-1})

Table 3. Xylose, arabinose and cellobiose concentrations during fermentation process by *Zymomonas mobilis* NRRL B-806 from pretreated *Eucalyptus globulus* saw dust pulp.

Parameter (g L^{-1})	Initial concentration			Final concentration		
	A	B	E	A	B	E
Xylose	7.85	6.82	6.99	7.10	6.19	6.59
Arabinose	10.36	5.77	9.15	10.11	5.53	9.01
Cellobiose	11.55	7.06	7.75	11.21	7.01	7.49

Table 4. Main results reported in literature for ethanol production from lignocelullosic hydrolysates by strains of *Zymomonas mobilis*.

Feedstock	Conditions	Ethanol concentration	Reference
Sugarcane molasses	*Zymomonas mobilis* MCC 2427 Substrate 216 g/L of total reducing sugar Temperature of 31 °C pH 5.13 Time 44 h	58.4 g/L	(Maiti *et al*., 2011)
Mahuala flowers	*Zymomonas mobilis* MTCC 92 Substrate 100 g/L of mahuala flowers slurry Temperature of 30 °C pH 6.5 Time 96 h Cell concentration 10% (v/v)	38 g/L	(Behera *et al*., 2011)
Kitchen garbage	*Zymomonas mobilis* 10225 Substrate 70 g/L of reducing sugar Enzyme load 100 U/g wet mass Temperature 30°C pH 6 Time 40 h Cell concentration 10% (v/v)	52 g/L	(Ma *et al*., 2009)
Sugarcane bagasse	*Zymomonas mobilis* CP4 Substrate 80 g/L of glucose Enzyme load 25 FPU/g Temperature 30 °C pH 5 Time 36 h Stirring 150 rpm Cell concentration 4 g/L	60 g/L	(da Silveira dos Santos *et al*., 2010)
Carob pod extract	*Zymomonas mobilis* PTCC 1718 Substrate 113.82 g/L of glucose Temperature 30 °C pH 5.2 Time 36 h Stirring 80 rpm Cell concentration 0.017 g/ 50 mL	40 g/L	(Vaheed *et al*., 2011)
Eucalyptus globulus	*Zymomonas mobilis* NRRL-806 Substrate 79.5 g/L of glucose Temperature 30 °C pH 5.5 Time 32 h Stirring 150 rpm Cell concentration 0.15 g/L	38 g/L	(this work)

In table 4 are shown the most prominent results reported for ethanol production from lignocellulosic feedstocks by *Zymomonas mobilis* strains. As shown in this work and based on several reports (Maiti *et al*., 2011; Behera *et al*., 2011; Ma *et al*., 2009; da Silveira dos Santos *et al*., 2010; Vaheed *et al*., 2011) *Zymomonas mobilis* has the potential to revolutionize the fuel ethanol industry commercially; laboratory and pilot-scale operations indicate that it can generate nearly theoretical maximum yields from several feedstocks, including enzymatic hydrolyzate of wood-derived cellulose, such as *Eucalyptus globulus* saw dust pulp.

The main limitation of *Zymomonas mobilis* for a widespread industrial usage for ethanol production is its capacity to utilize only hexoses. Pentoses such as xylose and arabinose cannot be metabolized by *Zymomonas mobilis*, unless it is genetically manipulated (Yanese *et al*., 2005; Lawford *et al*., 1997). However as mentioned by da Silveira dos Santos *et al*. (2010) if only the cellulose fraction is used in a two-stream model process, non-modified *Zymomonas mobilis* strains can be a promising alternative for ethanol production on industrial scale.

Conclusions

Although in this study only acetic acid, formic acid were quantified, other inhibitory compounds

commonly found in lignocellulosic hydrolysates, may also be present in the medium, such as furfural, HMF, vanillin, syringaldehyde, and hydroxybenzoic acid, depending to the acid concentration, temperature and other conditions used for hydrolysis, and such compounds may also affect the metabolism of the microorganism used for fermentation. However, acetic acid is the most common fermentation inhibitor, causing a decrease in ethanol rate and yield. Its toxic effect is basically due to its undissociated form. Acetic acid present in the different enzymatic hydrolysates from pretreated *Eucalyptus globulus* saw dust pulp caused a delay in ethanol production. High acetic acid concentration had adverse effect causing a delay in ethanol production efficiency, maximum ethanol production and glucose consumption.

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Nomenclature

$Y_{X/S}$	biomass yield ($g_{biomass}$ $g^{-1}_{glucose}$).
$Y_{E/S}$	ethanol yield ($g_{ethanol}$ $g^{-1}_{glucose}$).
EP	ethanol productivity ($g_{ethanol}$ L^{-1} h^{-1})
t	time (h)

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