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Fermentation of molasses by *Zymomonas mobilis*: Effects of temperature and sugar concentration on ethanol production

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Abstract

Fermentations utilizing strains of *Zymomonas mobilis*, in place of the traditional yeasts, have been proposed due their ethanol yields being close to theoretical. Ethanol production from sugar cane molasses was analyzed under different culture conditions using *Z. mobilis* in batch fermentation. The total reducing sugars (TRS) concentrations in the molasses, temperature, agitation and culture time effects were studied simultaneously through factorial design. The best conditions for ethanol production were 200 g L^{-1} of total reducing sugars in the molasses, temperature of 30 °C and static culture and time of fermentation of 48 h, achieving 55.8 g L^{-1}. The pH of the medium was kept constant during the experiments, showing that molasses presents a buffering effect.

Keywords: Ethanol; *Zymomonas mobilis*; Sugar cane molasses; Factorial design

1. Introduction

The depletion of fossil fuel reserves, the unstable panorama of the petrol prices and more recently, increasing environmental and political pressures (Davis et al., 2005) has increased industrial focus toward alternative fuel sources, and encouraged the search of products originated from biomass, as renewable sources of energy.

In this context, fermentative processes stand out, where microbial metabolism is used for the transformation of simple raw materials in products with high aggregate value. Among these, ethanol is one of the best examples of how fermentation can match market needs satisfactorily. Even though the fermentative process for ethanol production is well known, the production costs are still the key impediment wide use of ethanol as fuel. Therefore, the development of a fermentation process using economical carbon sources is important for the biofuel ethanol production on a commercial scale (Tanaka et al., 1999; Tao et al., 2005). Many studies have been done that focus on production improvement and decreasing its costs (Sreenath and Jeffries, 2000; Davis et al., 2005; Ruanglek et al., 2006; Mohagheghi et al., 2006).

Zymomonas mobilis, a Gram-negative bacterium, have been attracting increasing attention for fuel ethanol. It is an osmo- and ethanol-tolerant bacterium and it has shown higher specific rates of glucose uptake and ethanol production (Rogers et al., 1982, 1997) via the Entner-Doudoroff pathway under anaerobic conditions. *Z. mobilis* may have a greater potential for industrial ethanol production from raw sugar, sugarcane juice and sugarcane syrup (Lee and Huang, 2000).

Molasses is an agro-industrial by-product often used in alcohol distilleries (Jiménez et al., 2004) due to the presence of fermentative sugars, being an optimal carbon source for the microorganism metabolism. Sugar cane molasses is an abundant agro-industrial material produced in Brazil and other tropical countries and its low cost is an important

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factor for the economical viability of substances production by fermentation.

The traditional one-at-a-time optimization strategy is relatively simple, and the individual effects of medium factors can be graphically depicted without the need of the statistical analysis. Unfortunately, it frequently fails to locate the region of optimum response in such procedures. In this case, fractional and/or full factorial design provides an efficient approach to optimization. A combination of factors generating a certain optimum response can be identified though factorial design and the use of response surface methodology (RSM) (Box et al., 1978).

The response-surface methodology is an empirical modeling system that assesses the relationship between a group of variables that can be controlled experimentally and the observed response (Sreekumar et al., 1999; Hamsaveni et al., 2001). Response surface methodology (RSM) is a useful model to study the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments (Hamsaveni et al., 2001). The aim of this work was to study the influence between four factors and their interaction to optimize the ethanol production by *Z. mobilis* ATCC 29191 in sugar cane molasses using factorial design and analysis by RSM. The selected factors were sugar concentration on molasses, temperature, agitation rate and culture time. The measured responses were ethanol and biomass.

Table 1
2^4 Factorial experimental design investigating the effect of TRS concentration in molasses, temperature, agitation rate and culture time to ethanol production by *Z. mobilis* ATCC 29191

Run	Variables in coded levels				Measured responses	
	X_1	X_2	X_3	X_4	Ethanol (g L^{-1})	Biomass (g L^{-1})
1	−	−	−	−	5.74	0.89
2	+	−	−	−	5.38	0.81
3	−	+	−	−	7.95	0.75
4	+	+	−	−	6.98	0.65
5	−	−	+	−	2.97	0.56
6	+	−	+	−	9.89	0.31
7	−	+	+	−	4.09	0.53
8	+	+	+	−	1.98	0.41
9	−	−	−	+	19.33	1.07
10	+	−	−	+	7.31	1.22
11	−	+	−	+	22.69	1.29
12	+	+	−	+	30.08	1.41
13	−	−	+	+	2.94	0.81
14	+	−	+	+	12.20	1.07
15	−	+	+	+	2.23	0.65
16	+	+	+	+	15.91	0.43

	Factors	Real levels	
		−1	+1
X_1	Molasses (g L^{-1})	150	250
X_2	Temperature (°C)	25	35
X_3	Agitation rate (rpm)	0	180
X_4	Time of cultivation (h)	12	24

2. Methods

2.1. Microorganism and culture conditions

The strain used was *Z. mobilis* ATCC 29191. The strain was maintained on agar plates containing (per liter): 200 g glucose, 10 g yeast extract, 5 g peptone, 1 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and 0.5 g $FeSO_4$ (Merck). The culture medium was sterilized at 121 °C for 15 min. The cultures were maintained at 4 °C and renewed every five weeks.

The inoculum culture was grown composed with sucrose at 200 g L^{-1} and the components mentioned previously. The cell concentration was standardized to 0.2 g L^{-1}, determined by turbidimetry at $\lambda = 605$ nm. The batch fermentations were carried out in duplicate in the sugar cane molasses culture medium, in the different culture conditions, according to the experimental design (Table 1).

2.2. Analytical methods

After each fermentation, the culture was centrifuged (10,0000 rpm for 15 min) and the biomass concentration was determined by measuring the turbidity of diluted sample at 605 nm using a standard curve of absorbance against dry cell mass. The total reducing sugars (TRS) were quantified according to Somogy (1945) and Nelson (1944). Ethanol was determined by Gas Chromatography (GC) Shimadzu, using a DBWAX column (30.0 × 0.25 cm) with a flux of 40 ml min^{-1} and isopropanol as an internal standard.

2.3. Experimental design

The conditions to optimize *Z. mobilis* ethanol production by controlling fermentation variables were performed using a factorial design and analysis of the results by response surface methodology (Box et al., 1978; Barros et al., 1995). As a preliminary step for optimization, the most important factors were screened by applying the full 2^4 factorial design. The main effects for each of the factors studied were defined by the Eq. (1):

$$Ef_i = (\bar{y}_+)_i - (\bar{y}_-)_i \tag{1}$$

where Ef_i is the effect of the *i*th factor on the ethanol production, and $(\bar{y}_+)_i$ and $(\bar{y}_-)_i$ are the average ethanol productions values at the high (+) and low (−) levels of the factor. Interaction effects of two or more factors are also calculated using this equation. In these calculations, the ethanol production values attributed to the (+) and (−) levels were determined by multiplying the sign in the columns of design matrix for the factors involved in the interaction. The following independent variables were included X_1 = total reducing sugars (TRS), X_2 = temperature (°C), X_3 = agitation (rpm) and X_4 = culture time (h) shown in Table 1. The dependent variables were ethanol and biomass production. This preliminary analysis facilitated