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.

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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
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.

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 (NH4)2SO4, 2 g KH2PO4, 0.5 g MgSO4·7H2O and 0.5 g FeSO4 (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 (10,000 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):

\[ E_{fi} = (\bar{y}_+) - (\bar{y}_-), \]

where \(E_{fi}\) is the effect of the \(i\)th factor on the ethanol production, and \((\bar{y}_+)\) and \((\bar{y}_-)\) 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
selecting the statistically significant factors, TRS concentration in sugar cane molasses \((X_1)\), temperature \((X_2)\) and growth time \((X_3)\), therefore, two new levels for each factor were chosen according to the experimental design, shown in Table 2. The results of this factorial design evidenced that TRS concentration in sugar cane molasses \((X_1)\) and temperature \((X_2)\) are significant factors for ethanol production. In this case, a new full factorial design was employed to investigate the simultaneous effect of these two factors on the response. The experiments were carried out with a central point and star design, which consist in an identical planning, turning from 45° regarding to the original orientation, where the variables \(X_1\) and \(X_2\) were at a distance of \(\sqrt{2} (1.414)\) from the central point, adding up to 11 experiments, being 3 in the central point and 4 at the star design (Table 3). All the experiments were carried out in duplicate.

The RMS used in the present study is a central composite involving two different factors. Once the experiments are performed, the coefficients of linear and polynomial models are calculated using the Eqs. (2) and (3):

\[
Y = b_0 + \sum_{i=1}^{k} b_{i}X_i + e \quad \text{linear model (2)}
\]

\[
Y = b_0 + \sum_{i=1}^{k} b_{i}X_i + \sum_{i<j}^{k} b_{ij}X_iX_j + e \quad \text{quadratic model (3)}
\]

where, \(ij\) are linear and quadratic coefficients, respectively, while \(b\) is the regression coefficients, \(k\) the number of factors studied and optimized in the experiment and \(e\) is random error. The significance of each coefficient was determined using a student’s test.

### 3. Results and discussion

The first step of the statistical approach to the analysis optimization was to establish the criteria that will define the experimental factors that have a significant effect on the response variables. Therefore, to optimize the ethanol production it was first performed as a \(2^4\) factorial design. Four relevant factors for the fermentative process were selected in a factorial design \(2^4\). The variables, studied simultaneously were: TRS concentration in sugar cane molasses \((150\) and \(250\) g L\(^{-1}\)), temperature \((25^\circ\) and \(35^\circ\) C), agitation \((180\) oscillations per minute and static culture) and culture time \((12\) and \(24\) h), as shown in Table 1.

The main effects of the three factors concentration in sugar cane molasses \((2.72)\), temperature \((3.27)\), and \((8.47)\) are all positives, and agitation rate is negative \((-6.66)\). The growth time \((X_4)\) main effect is the most significant factorial design effect value for the production of ethanol. The inclusion of agitation rate reduces the average ethanol production. Therefore, the subsequent runs were performed in a static format. Higher ethanol productions, \(22.69\) g L\(^{-1}\) and \(30.08\) g L\(^{-1}\) were obtained in static culture \((run 11\) and \(12\) in Table 1). According to Lee and Huang (2000) \(Z.\ mobilis\) is able to obtain an ethanol production close to the theoretical one from glucose through Entner–Dudoroff pathway under aerobic conditions.

Based upon the results obtained in the \(2^4\) factorial design a \(2^3\) factorial design was developed using new variation levels in order to move sequentially in the direction of maximizing the ethanol production. To define the best culture conditions it was necessary to test new sugar concentrations, temperature and culture time in a \(2^3\) factorial

### Table 2

\(2^3\) Central composite design for investigating the effects of TRS in molasses, temperature and culture time on the ethanol production by \(Z.\ mobilis\) ATCC 29191

<table>
<thead>
<tr>
<th>Factors</th>
<th>Real levels</th>
<th>Coded levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>(X_1) Molasses (g L(^{-1}))</td>
<td>200</td>
<td>250</td>
</tr>
<tr>
<td>(X_2) Temperature (°C)</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>(X_3) Time of growth (h)</td>
<td>24</td>
<td>36</td>
</tr>
</tbody>
</table>

### Table 3

\(2^2\) Central composite design and star design investigating the effects of TRS in molasses and temperature on the ethanol production by \(Z.\ mobilis\) ATCC 29191

\(Y_{stab}\) (%) = substrate conversion.

\(Y_{eth}\) (g g\(^{-1}\)) = yield ethanol for substrate.

\(Q_{eth}\) (g L\(^{-1}\) h\(^{-1}\)) = ethanol productivity.
analysis, with a central point. The values of the new variables are listed in Table 2.

The results of the 2^3 design showed that the condition of 200 g L\(^{-1}\) of TRS and temperature 30 °C was the most favorable, achieving 54.83 g L\(^{-1}\) after a 48-hour-culture time. The time was a decisive factor, once the ethanol production increased to more than 60% from 24 to 48 h. By comparison Bandaru et al. (2006) reported a maximum ethanol concentration (55.3 g L\(^{-1}\)) at 32.4 °C, pH of 4.93 after 17.24 h from sago starch using Z. mobilis MTCC 92. Davis et al. (article in press) reported similar values (54 g L\(^{-1}\)) for Z. mobilis ZM4 from hydrolysed waste starch stream.

In the central point, 250 g L\(^{-1}\) and 35 °C, ethanol production was an average of 31.15 g L\(^{-1}\). The decrease in ethanol production at high sugar concentration occurred due to an increase in the osmotic pressure that is one of the essential factors for by-products synthesis such as sorbitol and levan. The molasses was an industrial sucrose-containing substrates that has been reported to contain substantial salt content (Bekers et al., 2000). At 35 °C and 300 g L\(^{-1}\) sugars concentration on molasses Cazetta et al. (2005) obtained maximum sorbitol production by Z. mobilis ATCC 29191.

The temperature of 40 °C was negative for fermentative process, resulting in lower productions, 4.6 g L\(^{-1}\). Numerous studies have shown that temperatures above 37 °C are detrimental for ethanol production (Lee et al., 1981; Skotinicki et al., 1981; Lyness and Doelle, 1981; Diez and Yokoya, 1996a). Based on the results of 2^3 factorial design, it was performed as a 2^2 factorial design, with central composite design, resulting in 11 experiments (Table 3). In this stage the time was fixed in 48 h.

With the central composite design it was possible to confirm that maximum ethanol concentration occurred at the central point, 55.8 g L\(^{-1}\) on average (Fig. 1 and Table 3). These values are similar to the ones described for ethanol production from sucrose (Skotinicki et al., 1981; Lyness and Doelle, 1981; Sreekumar et al., 1999) and sago starch (Bandaru et al., 2006), which confirmed that the microorganism showed an optimal adaptation to the non-treated molasses. The ethanol productivity was a mean of 1.1 g L\(^{-1}\) h\(^{-1}\).

A multiple regression analysis of the data was used to describe the variables under study taking into account linear, quadratic and cross product terms for each factor. The significance of the equation parameters on ethanol production was assessed by the F test.

According to the RSM methodology, it was not possible to fit the data obtained to either the linear or quadratic mathematical model, however, there was evidence of a slight curvature in the response surface. Since the average response at the center point was larger than the average response at the vertices, the surface was slightly convex.

The uncoupling between the biomass and ethanol production can be observed clearly in these experiments (Tables 1–3). Low biomass production is normally observed in Z. 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 sugar fermented, giving Zymomonas the lowest molar growth yield reported for a bacterium (Swings and DeLey, 1977).

The pH of the medium remained constant during the experiments, varying from 6.0 at the beginning to 5.6, on average \(n\) (Fig. 2). The pH has also been described as a factor that strongly interferes in the fermentative processes. However, according to Diez and Yokoya (1996b) molasses exhibits a buffering effect. This regulatory action depends of molasses chemical composition. The main stabilizer compounds of the pH are weak acids and amino acids that act in the acid range, mainly between pH 3.0 and 5.0, or phosphates, whose buffering effects occur in the range of 6.0 and 7.0. Falcão de Moraes et al. (1981) noted that Z. mobilis possesses hugh tolerance at pH variations from 3.5 to 7.5, and its optimum at a range of 5.0–7.0. Buzato (1984), observed no substantial oscillations on the alco-
holic yield at a pH range of 5.0–6.0, showing that there is no major influence of this factor when Z. mobilis is cultivated on molasses.

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References


Further reading