Full Length Research Paper

Response surface optimization of D(-)-lactic acid production by Lactobacillus SMI8 using corn steep liquor and yeast autolysate as an alternative nitrogen source

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The production of D(-) lactic acid from Lactobacillus LMI8 sp. was studied, using 2 low-cost nitrogen sources: corn steep liquor (CSL) and yeast autolysate (YA). A central composite design was used to determine maximal lactic acid production. The surface response method was used to evaluate the results of the experiments. The assays were performed in 250 mL Erlenmeyer flasks containing 100 mL of production medium maintained under refrigerated incubation at 200 rpm and a temperature of 37 ± 1°C for 48 h of fermentation. Lactic acid production was significantly affected by the isolated CSL as well as the interaction between CSL and YA. Maximal production of D(+) lactic acid was 41.42 g/L – a value located at the central point, which corresponded to 15 g/L of CSL and 5 g/L of YA.

Key words: D(-)-Lactic acid, Lactobacillus, corn steep liquor, yeast autolysate, response surface methodology, medium optimization.

INTRODUCTION

Lactic acid is the common name given to 2-hydroxypropanoic acid (Datta et al., 1995), which is a versatile product that has found applications in diverse fields as an acidulant in the food and pharmaceutical industries (Schepers et al., 2002). With the development and commercialization of biopolymers, lactic acid use has increased considerably and 20 to 30% of the production was estimated to be in these new applications (Datta and Henry, 2006).

Lactic acid can be obtained either through chemical synthesis or fermentative processes (Chotani et al., 2000). The chemical pathway always leads to the formation of a racemic mixture, in which concentrations of the D(-) and L(+) forms are equal. In fermentative processes, only one of these forms is obtained [D(+) or L(-)], depending on the bacterium chosen, or a mixture of the isomers is obtained in varied compositions (Hofvendahl and Hahn-Hagerdal, 2000).

It has recently been discovered that the mixture of the polymers D (-) and L (+) furnishes a recemic crystal known as complex-ester. This type of complex is characterized by a greater resistance to high temperatures, approximately 50ºC higher than polymerization in the L(+) form (Roy et al., 2002; Ikada et al., 1987; Tsuji et al., 1991; Takaaki et al., 2006). This has generated an increased important of the production of the D(-) isomer.

Lactic acid bacteria make up a group of diverse microorganisms that are widely distributed in nature and associated to plants (kale, corn, barley), meat, milk products, porridge and silage. These lactobacilli are very demanding microorganisms that require fermentable carbohydrates, amino acids, B complex vitamins, nucleic acids and minerals for growth as well as nutrients that are specific to each strain (Gomes and Malcata, 1999).

A number of byproducts and raw materials from the food and/or agriculture industries have been employed for microorganism growth due to their considerable availability and low cost. Examples include cheese whey, corn steep liquor, corn syrup, distillery yeast and molasses (Moraes et al., 1991). In Brazil, lactic acid production...
MATERIALS AND METHODS

Microorganisms

The *Lactobacillus* sp. LMI8 used in the present study was isolated from a decantation tank of residuals from the flour industry (Plaza Starch Mill-Santa Maria-SP, Brazil). The culture was stored in Man, Rogosa and Sharpe (MRS) medium with 20% (v/v) glycerol at -80°C.

Isolation of LMI8 culture

The successive dilution method was employed and plating was performed using the “pour plate” method. Bacterial strain selection was based on the greatest production of the D(-) lactic acid isomer from the culture medium used in the fermentation process.

Sugarcane molasses

Sugarcane molasses was obtained from the santa lucia sugar processing plant located in the region of Araras-SP, Brazil. The molasses was diluted and hydrolyzed at a ratio of 1 mL of H$_2$SO$_4$ (20%) in 100 mL of molasses solution and heated for 20 min. After hydrolysis, the pH of the molasses was adjusted to 6.5 with 4.0 M of KOH.

Medium and growth conditions

The inoculum was prepared through the transference of 2 mL of stock culture to Erlenmeyer flasks containing 100 mL of growth medium (MRS). The MRS growth medium was made up of peptone (10 g/L), yeast extract (5 g/L), meat extract (10 g/L), glucose (20 g/L), sodium acetate (5 g/L), ammonium citrate (2 g/L), K$_2$HPO$_4$ (5 g/L), Na$_2$HPO$_4$.2H$_2$O (2 g/L), MgSO$_4$.7H$_2$O (0.1 g/L) and MnSO$_4$.4H$_2$O (0.05 g/L). The inoculated medium was incubated at 37 ± 1°C for 18 h in a refrigerated incubator (New Brunswick, USA) at 200 rpm. Initial pH of the medium was adjusted to 6.7. A total of 10% (v/v) of the inoculum was transferred to 250 mL Erlenmeyer flasks containing 100 mL of production medium (modified MRS medium) agitated at 200 rpm in a refrigerated incubator at 37 ± 1°C for 48 h.
Table 1. Results of lactic acid production obtained in experiments employing the LMI8 isolate.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>CSL (g/L)</th>
<th>YA (g/L)</th>
<th>Lactic acid (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.3</td>
<td>2.5</td>
<td>15.32</td>
</tr>
<tr>
<td>2</td>
<td>4.3</td>
<td>7.5</td>
<td>20.43</td>
</tr>
<tr>
<td>3</td>
<td>25.7</td>
<td>2.5</td>
<td>35.23</td>
</tr>
<tr>
<td>4</td>
<td>25.7</td>
<td>7.5</td>
<td>22.7</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>5</td>
<td>12.11</td>
</tr>
<tr>
<td>6</td>
<td>30.1</td>
<td>5</td>
<td>25.39</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>1.47</td>
<td>24.16</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>8.52</td>
<td>31.27</td>
</tr>
<tr>
<td>9</td>
<td>15</td>
<td>5</td>
<td>41.42</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>5</td>
<td>41.23</td>
</tr>
<tr>
<td>11</td>
<td>15</td>
<td>5</td>
<td>39.98</td>
</tr>
</tbody>
</table>

Concentration of corn steep liquor (CSL); Concentration of yeast autolysate (YA).

led to a reduction in fermentation time, reaching maximal lactic acid production. Wee et al. (2006) studied the effect of CSL on lactic acid production using different concentrations (15 to 60 g/L) and obtained the same final concentration of lactic acid in all experiments, but the time needed to achieve this concentration decreased significantly when using greater concentrations of CSL. Yu et al. (2007) used sugarcane molasses ranging from 12 to 60 g/L and CSL ranging from 24 to 56 g/L and report that the increase in molasses and CSL provided greater lactic acid production in a shorter fermentation time. Altaf et al. (2006) report that peptone and yeast extract are the main nitrogen sources used for the production of lactic acid and if alternative sources were used, the final product would be smaller and the fermentation time would increase. This likely occurs because yeast extract is an excellent source of B complex vitamins and is often used to provide these factors to the bacteriological culture media, which are often considered indispensable to obtaining faster growth and production rates of lactic acid by lactic bacteria (Selmer-Olsen and Sorhaug, 1998). The high cost of yeast extract, however, has a negative impact when used in industrial processes (Hurok et al., 2005).

In Experiment 5 (Table 1), there was lactic acid production in the absence of CSL. Despite being less significant within the fermentation process (Equation 1), YA provided a good interaction with CSL, generating greater lactic acid production. It should be stressed that calcium carbonate plays a crucial role in lactic acid fermentation, as it buffers the medium. During the production of lactic acid, calcium carbonate is converted into calcium lactate and maintains the pH. The presence of 10% (w/v) calcium carbonate in the production medium was found to be good for lactic acid production, which adversely affected bacterial growth.

The precision of the results of the experiments can be seen in the assessment of the results obtained at the central point of the design, where the difference between responses was close to 1%.

The identification of significant parameters was performed through testing the hypotheses using the student’s t-test. The maximal likelihood of error on the test was established as 5%. Thus, parameters with a level of significance greater than 5% were discounted.

The adjusted empirical equation that represents the synthesis of lactic acid is expressed in Equation 2:

\[
\text{Lactic acid} = 40.876 + 5.12X_1 - 4.41X_1X_2 - 11.01X_2^2 - 6.53X_2^2
\]  

(2)

The goodness of fit of the model was checked by the determination coefficient ($R^2$) and multiple correlation coefficient (R). In this case, the value of the $R^2$ (0.96) for Equation 2 indicates that the sample variation of 96.0% for lactic acid was attributed to the independent variables and only 4% of the total variation cannot be explained by the model. The value of the adjusted determination coefficient (adjusted $R^2 = 0.935$) is also high, which stresses the significance of the model. The high value of R (0.98) demonstrates a high degree of agreement between the experimental observations and predicted values. This correlation is also proven by the plot of predicted versus experimental values of lactic acid in Figure 1, as all the points cluster around the diagonal line, which means that no significant violations of the model were found.

The effects of the independent variables and their interactions on the formation of the product are illustrated...
Figure 2. Response surface and contour curve representing the effects of corn steep liquor and yeast autolysate concentrations on lactic acid production.

Table 2. Stationary point for lactic acid production and coded \( x_1 \) and \( x_2 \) values at the optimization point.

<table>
<thead>
<tr>
<th>( P_0 )</th>
<th>Lactic acid</th>
<th>Coordinates</th>
<th>Lactic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda_1 )</td>
<td>-12.233</td>
<td>( x_1 )</td>
<td>0.139</td>
</tr>
<tr>
<td>( \lambda_2 )</td>
<td>-5.833</td>
<td>( x_2 )</td>
<td>-0.029</td>
</tr>
</tbody>
</table>

in the analysis of the response surfaces (Figure 2) constructed from Equation 2.

Analyzing Figure 2, maximal lactic acid production occurred when nitrogen concentrations were near 15 g/L (CSL) and 5 g/L (YA). For greater and lower concentrations of CSL and YA, there was a reduction in response, likely due to either a lack or excess of nitrogen in the fermented medium. According to Wood and Holzapfel (1995) the genus *Lactobacillus* has complex nutritional requirements. As lactic acid synthesis through fermentation is associated to cell growth, there is no formation of product if the medium does not have an adequate concentration of nitrogen in order to promote this growth (Pritchard and Coolbear, 1993). On the other hand, high concentrations of nitrogen may lead to cell death and inhibition of the product.

An algorithm carried out on the Maple 9.5 program (Waterloo Maple, Inc., Canada) was used to calculate the stationary point \( (P_0) \) for synthesis of lactic acid. These values are displayed in Table 2. The \( \lambda \) values indicate that these responses have a maximal point, as they have equal and negative signs. Lactic acid production was 41.52 g/L at the optimization point from the coded \( x_1 \) and \( x_2 \) values (Table 2).

Comparing this value with lactic acid production values in experiments 9, 10 and 11 (Table 1), the results were practically the same, as the conditions of the maximal point were near to those of the central point. Thus, the corresponding values for the optimal concentrations of CSL and YA were 16.48 and 4.93 g/L, respectively. The model predicted a maximal response of 41.38 g/L of lactic acid at this point.

Wee et al. (2006) produced 48.6 g/L of lactic acid using wood hydrolysate (equivalent to 50 g/L of glucose) supplemented with 60 g/L of CSL in 36 h of fermentation. Bustos et al. (2004) obtained maximal lactic acid production (58.9 g/L) in 96 h of culturing using 5 g/L of CSL together with 3.6 g/L of yeast extract and 10 g/L of peptone. Using a mathematical model furnished the response surface method, Hauly et al. (2003) determined maximal concentrations values tested for sugarcane molasses (100 g/L), yeast extract (20 g/L) and peptone (4 g/L) as the best medium composition for the production of lactic acid. In practice, these conditions provided a production of 30.5 g/L of lactic acid.

**Conclusion**

The central composite design proved to be a very useful and applicable tool for determining the behavior of the variables studied in the production of lactic acid, thereby avoiding excessive analyses and offering generalized information on the influence of the independent parameters in the process. The *Lactobacillus* sp. LMI8 strain used in the present study achieved significant results regarding lactic acid production using corn steep liquor and yeast autolysate. Under optimal conditions (16.48 g/L of CSL and 4.93 g/L of YA) a lactic acid concentration of 41.52 g/L was obtained.
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REFERENCES


