Finding stable cellulase and xylanase: evaluation of the synergistic effect of pH and temperature

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Ethanol from lignocellulosic biomass has been recognized as one of the most promising alternatives for the production of renewable and sustainable energy. However, one of the major bottlenecks holding back its commercialization is the high costs of the enzymes needed for biomass conversion. In this work, we studied the enzymes produced from a selected strain of *Aspergillus niger* under solid state fermentation. The cellulase and xylanase enzymatic cocktail was characterized in terms of pH and temperature by using response surface methodology. Thermostability and kinetic parameters were also determined. The statistical analysis of pH and temperature effects on enzymatic activity showed a synergistic interaction of these two variables, thus enabling to find a pH and temperature range in which the enzymes have a higher activity. The results obtained allowed the construction of mathematical models used to predict endoglucanase, β-glucosidase and xylanase activities under different pH and temperature conditions. Optimum temperature values for all three enzymes were found to be in the range between 35°C and 60°C, and the optimum pH range was found between 4 and 5.5. The methodology employed here was very effective in estimating enzyme behavior under different process conditions.

Introduction

The biochemical route applying enzymatic hydrolysis for the conversion of lignocellulosic biomass into biofuels is considered advantageous when compared to the chemical or thermo-chemical routes, since it can reach higher conversion yields with lower environmental impacts and milder process operation conditions. However, this route still requires developing technologies to reduce the costs of the enzymes that degrade biomass [1–4]. Despite the cost of these enzymes, developing the know-how of their production process is a strategic issue. In addition, the pool of enzymes that will be more effective is strongly dependent on the type of biomass and on the pre-treatment technology chosen. Hence, it is a logical choice trying to produce tailor-made pool of enzymes for the specific feedstock to be processed. Another important issue is that these enzymes need to fulfill special requirements in terms of optimum pH, temperature and heat stability, since process configurations can vary from separate hydrolysis and fermentation to simultaneous saccharification and fermentation process. The optimal temperature for enzymatic hydrolysis is usually higher (around 55°C when using fungal enzymes) than that of fermentation by yeasts (around 30°C), and a compromise has to be found when using combined process configurations [5].

Microbial enzyme production through a solid state fermentation (SSF) process has been described as a better option in terms of product stability when compared to submerged fermentation [6]. Different microorganisms can produce cellulolytic enzymes, with the filamentous fungus *Trichoderma reesei* being the most used industrially. For an efficient biomass hydrolysis, a pool of enzymes that include cellulase, xylanase, and other accessory enzymes is required. In this sense, *Aspergillus niger* has been widely investi-
gated given the complete enzymatic complex produced by them [7]. Other features of Aspergillus species regard the production of thermostable enzymes [8] and higher production levels of β-glucosidase when compared to T. reesei [9].

Several studies focusing on the characterization of cellulase and xylanase in terms of the pH and temperature parameters have been made because of a large interest in understanding the action of these enzymes under different conditions. However, most of these studies describe the evaluation of one parameter at a time [10–13]. Finding the optimal pH and temperature includes a study of both variables at the same time. In this context, the use of statistical experimental design followed by response surface methodology (RSM) to identify the optimal values of pH and temperature can be very effective to analyze the relationships among these parameters. The conventional method for optimizing a multi-variable system analyzing one factor at a time can be very time consuming and besides, such methodology does not access the interaction effects between the variables. This interaction can result in a synergistic effect, that is, a pH and a temperature condition in which the enzymes have a higher activity value.

In the work herein, we studied the production and characterization of cel lulolytic enzymes produced by a selected strain of the filamentous fungi Aspergillus niger under solid state fermentation. The cellulase and xylanase enzymatic cocktail was thoroughly characterized in terms of the variables pH and temperature by using response surface methodology to evaluate the interaction of these effects on the enzyme activity and find a pH and temperature range in which the enzymes have a higher activity. The cellulase system was evaluated in terms of endoglucanase and β-glucosidase activities. The enzyme thermostability at 37 and 50°C and endoglucanase kinetics parameters were also evaluated.

Materials and methods

Microorganism

The microorganism used in this study was a wild-type strain of A. niger from the Embrapa Food Technology collection, Rio de Janeiro, Brazil. The culture was kept in dry sand under freezing conditions (−18°C). Microorganism activation was carried out in basic medium agar slants incubated for 7 days at 32°C [14]. After this period, conidia were harvested by adding 10 mL of 0.1% Tween-80 to the slant.

Inoculum preparation

A volume of 1 mL of the spore suspension was inoculated into a 250 mL Erlenmeyer flask containing ground corn cob and a nutrient medium, according to [14]. The inoculum was incubated for 5 days at 32°C. The spore concentration in the suspension was determined by counting it in a Neubauer chamber. The inoculum volume used to inoculate the fermentation medium was calculated to obtain a final concentration of 10⁷ spores per gram of solid medium.

Fermentation conditions

Solid state fermentations were carried out in 500 mL Erlenmeyer flasks using wheat bran with a moisture level of 60% (adjusted with 0.9% (w/v) ammonium sulfate solution in 0.1 mol/L HCl) as a solid substrate. Fermentation was carried out at 32°C for 72 h. After this period, the enzymes were extracted by adding 0.2 mol/L acetate buffer at pH 4.5. The samples were stirred at 120 rpm for 1 h and the enzymatic solution was recovered by filtration. The recovered enzyme extracts were stored at –18°C for further analysis.

Experimental design

A full factorial design followed by response surface analysis was used to evaluate the effect of two variables – temperature and pH – and their possible interaction in the enzymatic activity. The experimental design selected was a central composite design comprising eleven runs, corresponding to four cube points, four axial points and three central points, with the experiments carried out in a random order [15]. The factors and levels investigated are shown in Table 1. The dependent variables (responses) were endoglucanase, β-glucosidase and xylanase activities. The Statistica software (Statsoft, version 7.0) was used to analyze the experimental data, the generation of the ANOVA (analysis of variance) data, and the plotting of response surfaces.

Enzyme assays

Standard endoglucanase activity was measured using an assay based on Ghose methodology [16]. Briefly, a volume of the appropriately diluted enzyme extract was incubated at 50°C for 10 min with a 1% CMC (Sigma, USA) solution prepared in 0.05 mol/L citrate buffer at pH 4.8 as a substrate. One unit of endoglucanase activity corresponds to 1 μmol of glucose released per minute at pH 4.8 and 50°C. Standard xylanase activity was measured according to Ref. [17], by

<table>
<thead>
<tr>
<th>Trial</th>
<th>T (°C)</th>
<th>pH</th>
<th>Endoglucanase (IU/mL)</th>
<th>β-Glucosidase (IU/mL)</th>
<th>Xylanase (IU/mL)</th>
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<tr>
<td>4</td>
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<td>3(−1)</td>
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<td>4,5(0)</td>
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<td>15.95</td>
<td>10.92</td>
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</table>

Table 1: Experimental conditions and results of the statistical experimental design for endoglucanase, β-glucosidase, and xylanase activities.
incubating a volume of the appropriately diluted enzyme extract at 50°C for 30 min with a 1% oat spelt xylan (Sigma, USA) solution prepared in 0.10 mol/L sodium acetate buffer pH 5.0 as a substrate. One unit of xylanase activity corresponds to 1 μmol of xylose released per minute at pH 5.0 and 50°C. The quantification of the reducing groups released from both the assays was performed according to the DNS method [18]. The enzyme extract dilution should release an amount of reducing sugar in the reaction that fits within the linear region of the standard curve used for quantification. β-Glucosidase activity was determined by incubating an appropriate enzyme volume in 1 mL of a 0.015 mol/L solution of cellobiose (Sigma, USA) prepared in 0.05 mol/L sodium citrate buffer pH 4.8 as a substrate for 30 min at 50°C. The reaction was stopped by submersion in boiling water for 5 min. The quantification of the glucose released was performed by using an enzymatic kit for glucose measurement (Laborlab, Brazil).

**Determination of optimum enzyme pH and temperature**

Optimum pH and temperature for endoglucanase, β-glucosidase and xylanase were determined by assaying the corresponding activity at different temperatures (from 23 to 87°C) and pH values (from 2.4 to 6.6), selected according to the experimental design conditions.

**Enzyme thermostability**

Endoglucanase, β-glucosidase and xylanase thermostabilities at temperatures of 37 and 50°C were evaluated by measuring the residual enzyme activity after every 24 h during a 96 h incubation period in the absence of substrate. The enzyme’s half-lives were calculated according to [19] using the single-step non-first-order model proposed by Sadana and Henley [20] to fit the experimental data. The activity time expression used (Eq. (1)) relates the activity (a) to the parameter $k_1$, the first-order deactivation rate constant, and the parameter $\alpha_1$, the long-term level of activity:

$$a = (1 - \alpha) \exp(-k_1 \cdot t) + \alpha_1 \quad (1)$$

The two-parameter model was fitted to the residual activity data using the Levenberg–Marquardt method of iterative convergence, at 0.95 confidence level.

**Determination of endoglucanase kinetic parameters**

Different concentrations of CMC prepared in 0.05 mol/L citrate buffer at pH 4.8 (from 1 to 50 mg/mL) were used to determine the endoglucanase reaction rates. The affinity constant, $K_m$, and the maximal rate of reaction, $V_{max}$, were calculated from double-reciprocal plots according to the method of Lineweaver and Burk [21].

**Results and discussion**

**pH and temperature effect on enzymatic activity**

The pH and temperature effects on endoglucanase, β-glucosidase and xylanase activities present in the cellulolytic enzyme complex produced by the filamentous fungus A. niger grown on wheat bran under SSF were evaluated by using the statistical design of experiments and response surface methodology analysis. Table 1 presents the results of the complete factorial design for endoglucanase, β-glucosidase and xylanase activities under the different conditions evaluated. Table 2 exhibits the coefficients of the mathematical model and statistical parameters.

The data analysis allowed defining an optimum range of temperature and pH for higher enzyme activity, as well the degree of significance of each variable and their interaction. All the main effects were statistically significant ($p$-value < 0.1) for endoglucanase and xylanase, except for the synergistic effect between temperature and pH for xylanase. For β-glucosidase the linear and quadratic pH effects, as well as the quadratic effect of temperature, were significant at 95% confidence limit ($p$-value < 0.05). The pH (quadratic effect) and temperature (both linear and quadratic effects) variables showed a negative effect on all three enzymatic activities, within the range evaluated. The negative effect of the factors means that an increase in one of them will reduce the enzymatic activity. As for endoglucanase activity, the significance of the interaction effect between pH and temperature revealed the synergistic effect of these variables. The pH effect was higher than the temperature effect, as can be verified for the coefficient values listed in Table 2. A similar result was found by Singh et al. [22] on the optimization of parameters for thermostable cellulase activity from Aspergillus heteromorphus. The authors found that the change in temperature was less important than the change in pH, within the range evaluated. As the pH varies, the charge of the substrate

### Table 2

<table>
<thead>
<tr>
<th>Coefficient values and statistical analysis for endoglucanase, β-glucosidase, and xylanase activities</th>
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</thead>
<tbody>
<tr>
<td><strong>Endoglucanase</strong></td>
</tr>
<tr>
<td><strong>Coefficients</strong></td>
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<tr>
<td>T</td>
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<td>pH</td>
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<tr>
<td>pH²</td>
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<tr>
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<tr>
<td>F value</td>
</tr>
<tr>
<td>$F_{cal}/F_{listed}$</td>
</tr>
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</table>

* $R_i$ coefficient of determination.
** Significant at 0.1 level.
*** Significant at 0.05 level.
FIGURE 1
Surface response showing the pH and temperature effect on (a) endoglucanase, (b) β-glucosidase and (c) xylanase activities present in the cellulolytic enzyme complex produced by the filamentous fungus A. niger grown on wheat bran under SSF.

FIGURE 2
Thermostability at 37 °C and 50 °C of (a) endoglucanase, (b) β-glucosidase and (c) xylanase present in the cellulolytic complex produced by the filamentous fungus A. niger grown on wheat bran under SSF.
and ionic components of the substrate changes, affecting the activity of the enzymes [23].

The ANOVA analysis for endoglucanase, β-glucosidase, and xylanase activities showed that the coefficient of correlation (0.95, 0.94, and 0.89, respectively) and the F test (5.77, 7.91, and 4.10 times higher than the listed F value at 90% level of confidence, respectively) were satisfactory for the prediction of the models used to describe the response surface plots of the enzyme activities as a function of pH and temperature (Fig. 1). The statistical analysis revealed that the optimum values of temperature for all three enzymes were found to be in the range between 35°C and 60°C, and the optimum pH range was found between 4 and 5.5. Higher experimental values of enzymatic activity were found at the condition of the central point, which is at pH 4.5 and 55°C.

Other studies on the characterization of endoglucanase, β-glucosidase, and xylanase report optimum pH and temperatures at specific values. Saquib et al. [13] found an optimum temperature of 64.3°C for endoglucanase produced by A. fumigatus under SSF in wheat straw. Regarding the use of thermophilic fungi, the optimal temperature of endoglucanase activity produced by Thermaosacus aurantiacus was of 75.8°C [10]. Watanabe et al. [24] report an optimum temperature of 55°C and pH of 4.5 for β-glucosidase from A. niger. Xiong et al. [11] found a maximum activity at 70°C and optimum pH of 6.5 for the xylanase produced by Thermomycetes lanuginosus. The advantage of using the statistical methodology was the definition of an optimum temperature and pH range, rather than a specific value, allowing more flexibility during process development.

**Thermostability**

Thermostability can be defined as the retention of activity after heating an enzyme extract at a selected temperature for a prolonged period of time. A way to express thermostability is to measure the half-life of enzyme activity at elevated temperatures [25]. The half-life of an enzyme is the time after which the enzyme activity reduces to 50% of the original activity at a given temperature [13]. Here, we used a model proposed by Sadana and Henley [20] to fit the experimental data and the enzyme’s half-lives were then calculated using the fitted model according to Tardioli et al. [19]. Table 3 shows the results of the model parameters \( k_1 \) and \( \alpha_1 \) and the half-life for endoglucanase, β-glucosidase, and xylanase activities. As for endoglucanase stability, after 96 h at 37°C, endoglucanase retained virtually 100% of its activity (Fig. 2a) and the model proposed did not fit the experimental data, whereas at 50°C its half-life was 43.3 h. As for β-glucosidase, a high stability at 37°C and 50°C was also observed (Fig. 2b), being that at 50°C a first order model (\( \alpha_1 \) equal to zero) fitted better the experimental data resulting in a calculated half-life of 148.1 h. In terms of endoglucanase and β-glucosidase thermostabilities, our results were similar to the ones of Soni et al. [26], who found that these enzymes produced by Aspergillus sp. retained 66% and 84% of the activity, respectively, after 72 h of incubation at 50°C, when compared to our results of 45% and 80% residual activity, respectively, after incubation under the same conditions.

Regarding xylanase, its half-life was 243.2 h at 37°C (value obtained by using a first order model) and this value dropped to 90.1 h when incubation was at 50°C. The residual activity of xylanase for these two temperatures is shown in Fig. 2c. These results show that the xylanase enzymes produced here present a higher thermostability than many of the other reported xylanase from Aspergillus strains. Shah and Madamwar [27] found that xylanase produced by a newly isolated Aspergillus foetidus was highly stable at 40°C but retained only 36% of its activity at 50°C after 3 h. Castro et al. [8] evaluated the thermostability of xylanases produced by a thermotolerant Aspergillus strain and found that in the absence of substrate, the systems retained 50% and 30% of their activity after 60 min of incubation at 50°C.

Thermostable enzymes in the hydrolysis of lignocellulosic materials have several potential advantages: higher specific activity (decreasing the amount of enzyme needed), higher stability (allowing elongated hydrolysis times) and increased flexibility for the process configurations [25]. Considering that in a simultaneous saccharification and fermentation process configuration a compromise between optimum conditions for enzymatic hydrolysis and fermentation needs to be achieved, the use of 37°C is a favorable condition, since the yeast usually has an optimal temperature around 30°C and enzymes around 50°C. Our results showed a highly stable enzymatic extract at this condition.

**Kinetic parameters**

The enzymatic extracts produced by SSF were used to estimate the endoglucanase kinetic parameters \( K_m \) and \( V_{max} \). These values are apparent values, since the extracts used were not purified. The Michaelis constant, \( K_m \), is a parameter related to the affinity of an enzyme to the substrate, with the value of the substrate concentration at which the enzyme acts at a rate corresponding to half the maximum reaction rate, \( V_{max} \). Thus, the higher the value of the constant \( K_m \), the lower the affinity of the enzyme to the substrate. The kinetic parameters \( K_m \) and \( V_{max} \) of endoglucanases were determined by typical Michaelis–Menten hyperbolic (Fig. 3) and Lineweaver–Burk double reciprocal plots. For endoglucanase at pH 4.8 and 50°C, CMC hydrolysis resulted in a \( K_m \) value of 40 mg/mL and a \( V_{max} \) value of 4.22 μmol/min mL. Aboul-Enein et al. [28] found a \( K_m \) of 5 mg/mL and \( V_{max} \) of 62.5 μmol/min mL for CMC.

### Table 3

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Endoglucanase</th>
<th>β-Glucosidase</th>
<th>Xylanase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37</td>
<td>50</td>
<td>37</td>
</tr>
<tr>
<td>( \alpha_1 ) (h⁻¹)</td>
<td>Nd</td>
<td>0.4191</td>
<td>0.8408</td>
</tr>
<tr>
<td>( K_1 )</td>
<td>Nd</td>
<td>0.0455</td>
<td>0.0327</td>
</tr>
<tr>
<td>Half-life (h)</td>
<td>Nd</td>
<td>43.3</td>
<td>341.5</td>
</tr>
</tbody>
</table>

*Half-lives calculated from the model equation. Nd, not determined.*
hydrolysis using a purified enzyme produced by a thermophilic actinomycete. Javed et al. [29] found a $K_m$ of 20 mg/mL for the hydrolysis of CMC at 55°C also using a purified endogluccanase produced by $A. oryzae$. Even though, the $K_m$ value obtained here was higher than those described in the literature, it was expected that the $K_m$ value for the purified enzyme would be lower than the one using a crude enzyme extract. Another point is that in the Michaelis–Menten curve, the maximal reaction rate $V_{max}$ is represented by a plateau, or region of zero-order kinetics. Fig. 3 shows that the region of zero-order kinetics was not achieved. This may be associated with the existence of other enzymes capable of acting on this substrate (CMC), since crude enzyme extract was used. Evaluation of higher substrate concentration was not possible because of the high viscosity of CMC solution.

**Conclusion**

The methodology employed here was very effective in estimating enzyme behavior under different pH and temperature conditions. Higher enzyme activities were found to be in the range between 35 and 60°C, and between pH 4 and 5.5. By using the statistical methodology it was possible to find an optimal temperature and pH range rather than a specific value, allowing more flexibility during process development. In terms of thermal stability, the enzymatic extract was found to be highly stable at both temperatures tested, 37 and 50°C. These results showed the potential of using in situ enzyme production as an alternative to contribute toward the economics of converting biomass into ethanol. Nevertheless, more studies are needed to improve enzyme activity as well as to carry out a full characterization of the other enzymes present in the cellulosytic complex.

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**References**