Carvalho dos Santos, Tamires; Souza Cavalcanti, Ingrid; Ferreira Bonomo, Renata Cristina; Batista Santana, Nivio; Franco, Marcelo

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Universidade Federal de Santa Maria
Santa Maria, Brasil

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Otimização da produção de enzimas celulolíticas obtidas de *Aspergillus niger* utilizando o resíduo da manga como substrato

Tamires Carvalho dos Santos1 Ingrid Souza Cavalcanti1 Renata Cristina Ferreira Bonomo1
Nivio Batista Santana1 Marcelo Franco*

ABSTRACT

The present paper analyses the effects of water activity (0.88, 0.94 and 0.97) and of fermentation time (24, 48, 72, 96 and 120 hours) on the kinetic activity of enzymes cellulolytic, produced during the solid state fermentation of waste from the improvement of mango, with the aid of fungus species *Aspergillus niger*. Solid state fermentation was carried out at 35°C inside a bacteriological incubator. The statistical results indicated that the best activity for enzyme CMCase was 7.26U g\(^{-1}\) after 74.51 hours of fermentation, whereas for enzyme FPase was 2.55U g\(^{-1}\) after 98.52 hours, both presenting best results in approximately 0.928 of water activity. Pareto charts have showed that fermentation time has greater effect over the activity of enzyme CMCase, while the water activity variable has greater effect over enzyme FPase activity. During fermentation the fungus synthesized the enzymes without the need of inductors other than mango residue and water.

Key words: *Aspergillus niger*, Pareto charts, solid state fermentation,

RESUMO

Neste trabalho, foram analisados o efeito da atividade de água (0.88, 0.94 e 0.97) e do tempo de fermentação (24, 48, 72, 96 e 120 horas) sobre a atividade cinética das enzimas celulolíticas, produzidas durante a fermentação em estado sólido do resíduo do beneficiamento de manga com a utilização da espécie fúngica *Aspergillus niger*. A fermentação em estado sólido foi realizada a 35°C em estufa bacteriológica. Os resultados estatísticos indicaram que a melhor atividade para a enzima CMCase foi de 7,26U g\(^{-1}\) após 74,51 horas de fermentação, enquanto que para a enzima FPase esse valor foi de 2,55U g\(^{-1}\). Após 98,52 horas, através dos resultados obtidos pela aplicação da metodologia de superfície de resposta, ambas as enzimas apresentaram melhores resultados em aproximadamente 0,928 de atividade de água. Nos gráficos de Pareto, observamos que o tempo de fermentação tem maior efeito sobre a atividade da enzima CMCase, enquanto que a variável atividade de água exerce maior efeito sobre a atividade da enzima FPase. Durante a fermentação, o fungo secretou as enzimas sem a necessidade de qualquer inductor além do resíduo de manga e água.


INTRODUCTION

Waste output and byproducts are inherent to all productive sectors. With the improvement of ecological awareness by the end of the 20th century, it became clear that humankind’s major challenge for the coming decades is to balance the production of goods and services with economic growth, social equality and environmental sustainability (GALEMBECK et al., 2009).

Environmental concern leads to the feasibility of projects that promote the sustainability of production systems. Contrary to what happened in the past when waste was improperly disposed of, today’s concepts of minimization, recovery and reuse of byproducts are being increasingly disseminated (LAUFENBERG et al., 2003; BALAT et al., 2008).

In Brazil, the quantity of agro-industrial byproducts such as bagasse, bran, peel and seeds in general is expressive, and nowadays, concepts...
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The solid fermentation process becomes more efficient with the use of filamentous fungi (HOLKER et al., 2004). Fungi of the *Aspergillus* genus are economically important, and are used in numerous fermentations, including the production of organic acids and biosurfactants, and is also the most used microorganism in the production of enzymes (HAQA et al., 2003).

The ability of decomposing cellulosic biomass into glucose, which may be converted into added value products and energy, has turned cellulase into one of the most investigated multicomponent enzyme systems (TENGERDY & SZAKACS, 2003). The objective of this study is to optimize the production of exoglucanases and endoglucanases by applying solid state fermentation to the waste of mango improvement.

**MATERIAL AND METHODS**

The microorganism studied was a strain of *Aspergillus niger* of the Agro-Industrial Residue Laboratory - LABRA of the Bahia Southeast State University – UESB, Itapetinga campus. The waste was provided by a fruit pulp improvement agro-industry located in the southeast region of Bahia, dried in a SOLAB drying and sterilization incubator at 70°C for 24 hours and then grinded to an approximate grain size of 0.2mm grinding of the biomass is necessary to reduce cellulose crystallinity. The process increases the material's contact surface, which is important for the fermentation processes.

The sporulated culture (inclined, acidified PDA HIMEDIA) incubated at 35°C for 7 days in a bacteriological incubator (model SL 101 SOLAB) was suspended in Tween 80 (VETEC) solution. The number of spores in suspension was counted using a double mirror Neubauer chamber and a binocular BIOVAL L1000 microscope.

The assays were carried out in Erlenmeyers flasks containing 10g of mango waste, to which were added 5ml, 10ml and 15ml of sterile water the water activity values were 0.88, 0.94 and 0.97 respectively, and were determined in a BASEQ aqualab. The flasks containing waste and water medium were sterilized in autoclaving at 121°C for 1h. A quantity of 10⁶ spores per gram of dry basis substratum was added to the suspension. The incubations were conducted at 35°C in a SOLAB model SL 101 bacteriological incubator. Following the fermentation process, the enzyme extract was mechanically extracted with a 50mL sodium citrate tampon solution (VETEC) with a pH of 4.8 at 50mM. The enzyme extract that resulted from the fermentation was centrifuged at 900 G-force for 10 minutes in a CETRIBIO model 80-2B centrifuge.
The method chosen to determine the activity of CMCase is based on the dose of reducing sugars produced by the degradation of carboxymethylcellulose (CROMOLINE) at 2% per volume previously diluted in a sodium citrate solution with a pH of 4.8 at 50mM. The dinitrosalicylic acid method was used for quantification (DNS) (MILLER, 1959). Reaction assays were conducted by adding to an assay tube 0.5mL of sodium citrate tampon solution with a pH of 4.8 at 50mM, 0.5mL of enzyme extract and 0.5mL of CMC (2% per volume). The reaction control was carried out in another tube, to which were added 0.5mL of the same tampon solution and 0.5mL of enzyme extract. The samples were incubated in a bacteriological incubator, subjected to QUIMIS orbital shaking at 50°C and 150rpm for 10 minutes. The reaction was interrupted with the addition of 0.5mL of DNS. The tubes were submerged in boiling water for 5 minutes and soon after, 6.5mL of distilled water were added for a subsequent measurement of absorbance in the 540nm range, carried out in a BEL PHOTONICS 2000UV spectrophotometer.

The FPase activity, that is, filter paper activity, results from the degradation of a strip of Whatman filter paper measuring 1.0cm x 6.0cm (SANTOS et al., 2011). The tube containing the reaction assay received the addition of 1.0mL of sodium citrate tampon solution with a pH of 4.8 at 50mM, 0.5mL of enzyme extract and a strip of filter paper. Another tube received the addition of 1mL of the same tampon solution and 0.5mL of enzyme extract. The third tube, which was the substrate control, received the addition of 1.5mL of tampon solution and a strip of filter paper. The samples were left in an incubator at 50°C for 1 hour. The reaction was interrupted with the addition of 3mL of DNS. The tubes were placed in boiling water for 5 minutes and soon after, 20mL of distilled water were added for the subsequent measurement of absorbance in the 540nm range, carried out in a spectrophotometer.

The standard curve was plotted with the determination of glucose in concentrations of 0.2 to 1.0g L⁻¹ using the DNS method described by MILLER (1959). The enzyme activity unit (U) was defined as the quantity of enzyme capable of releasing 1 μmol of reducing sugars per minute at 50°C, the enzyme activity being expressed in U g⁻¹. Absorbance was measured in a model SP 2000UV spectrophotometer.

The experiments were carried out in an entirely randomized design (ERD) with treatments organized in a 3x5 factor scheme with 2 repetitions, totaling 30 experimental points. Three levels of water activity were used (0.88, 0.94, 0.97) as well as five levels of time factor (24h, 48h, 72h, 96h, 120h). The results obtained were analyzed using Analysis Variance (ANOVA) with a 5% probability for the F test (Fisher) and Regression Analysis for the significance of parameters (Student Test, P<0.10), analysis of residues and coefficient of determination (R²). All statistical analyses were conducted using the statistics program package SAEG v.8.1 (RIBEIRO, 2001).

RESULTS AND DISCUSSION

The experimental results for enzyme activity under the water activity and time conditions studied were submitted to ANOVA and regression analysis. The regression analysis was carried out in order to adjust the mathematical models to the experiment data with the objective of identifying an optimal region for the variable activity studied (enzyme activity) in the light of the established independent variables (fermentation time and water activity). The two independent variables had a significant effect on both enzymes. The behavior of the systems studied can be described by a second-order polynomial model (Equations 1 and 2), where the term of interaction among the independent variables was non-significant (P>0.10). The quadratic model for both variables adjusted for each of the enzymes studied showed the results R²=0.81 and R²=0.73 respectively, for CMCase and FPase. From figure 1, Pareto charts, it is possible to verify that for CMCase, the fermentation time has a more significant effect over enzyme activity, whereas for enzyme FPase the water activity variable is the one having more significant effect.

The equations were generated to fit the experimental data using response surface methodology for the optimization of experimental parameters, as shown below. The reduced models concerning each enzyme and microorganism are described by the equations 1 and 2:

\[ \text{CM}\text{Case} = - 679,697 + 1468,76a - 792,633a^2 + 0,233250t - 0,00156527t^2 \]  

\[ \text{FP}\text{ase} = - 679,697 + 1468,76a - 792,633a^2 + 0,233250t - 0,00156527t^2 \]

These regression models were used to determine the response surface concerning the response variable by relating two independent variables, as shown in figure 2.

Figure 2 illustrates combinations of the effects of independent variables on enzyme activity, and through the derivatives of equations 1 and 2 we can observe that the optimal activity point for enzyme CMCase was at time 74.51h and water activity 0.929, whereas for enzyme FPase time was 98.52h and water activity 0.927 (Table 1). We highlight that the fungus synthesized the enzyme (7.26U g⁻¹) for CMCase and
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2.55U g⁻¹ for FPase) without the need of any inductors or supplies other than the mango waste and water at various concentrations, thereby demonstrating that it is a constitutive enzyme.

Enzymes usually have expression control mechanism that can be stimulated or inhibited by products of the medium. The end products of a particular metabolic pathway are often inhibitors of enzymes that catalyze the first steps of the pathway. This mechanism is known as negative feedback. BIAZUS et al. (2006), working with corn malt, noted that in the production of enzymes the beginning is slow, then accelerates until it reaches its maximum value, thereafter, the concentration of products generated are inhibited and its activity is reduced, which was also observed in this study. OMEMU, et al. (2005) obtained higher yields of cassava starch hydrolysis by *A. niger* after 72 hours of fermentation, which agrees with ALVA et al. (2007), who also reported a higher enzymatic activity by Aspergillus. The decrease in activity with increasing incubation time may be due to the production of by-products resulting from microbial metabolism, besides of nutrient depletion, inhibiting the growth of the fungus and formation of the enzyme (SHAFIQUE et al., 2009).

The literature shows the production of endoglucanases by actinomycetes, particularly *Streptomycyes*, on different substrates. The strain of *Streptomycyes* T3-1, produced 40.3U mL⁻¹ in 1.5% CMC and ammonium sulfate, urea and peptone (JANG & CHEN 2003), but these nutrients were not used with low cost substrates. *Streptomycyes* sp. isolated from Canadian soil was cultivated in a solution containing Mandel peptone, 1.0% Tween 80 in crystalline cellulose and produced 11.8U mL⁻¹ of CMCase (ALANI et al., 2008) however, *Thermomonospora* sp. (GEORGE et al., 2001) when grown
on medium containing cellulose paper powder, yeast extract and Tween 80, showed a peak of 23U mL\(^{-1}\), whereas when grown on wheat bran activity was 8.5U mL\(^{-1}\). JORGENSEN & OLSSON, (2006) working with Penicillium brasiliunum IBT in a bioreactor in medium containing yeast extract and a type of wood from pine subjected to steam explosion, values of 0.59U mL\(^{-1}\) FPase. Trichoderma viride NCIM 1051 in 1.0% of sugarcane bagasse treated with NaOH resulted in an FPase activity of 0.4U mL\(^{-1}\) (ADSUL et al., 2004). Aspergillus niger IZ9 in medium containing sugarcane bagasse treated with sodium hydroxide (NaOH) showed peak activity of 0.2U mL\(^{-1}\) (AGUIAR & MENEZES 2000).

The water activity interval in which microbial development takes place in general ranges from 0.60 to 0.99, and the optimal value for growth oscilates between 0.90 and 0.99 (GERVAIS & MOLIN 2003). The water content is a factor that interferes with the excretion of enzymes by microorganisms. In solid-state fermentation the moisture promotes the growth of fungi through the transfer of O\(_2\), diffusion of nutrients in the solid substrate and temperature control. As the water content is limited, its control is essential to optimize the solid-state fermentation. The ideal water content forms an aqueous film on the surface, which facilitates the dissolution and transfer of nutrients and oxygen (GERVAIS & MOLIN 2003). In the present study, it has been observed that starting from water activity between 0.95 and 0.98, there was a drop in production for both studied enzymes. That could be related with fungal inhibition, marked by the extrapolation of the ideal water level for the development of the selected lineage, which could be influencing the metabolic route responsible for enzyme production.

The fungus used was shown to be effective enzyme production by fermentation, as mentioned by PANDEY et al. (2000) which points out that the microorganism Aspergillus is the most widely used enzyme production. The mathematical modeling of fermentation processes can be defined as the attempt to represent, mathematical equations, the mass balances, associated with biochemical changes that occur in the process and the speed with which these transformations take place.

**CONCLUSION**

The waste mango can be used as feedstock for the production of cellulolytic enzymes by cultivating the filamentous fungus Aspergillus niger by solid state fermentation. The differential performance in the production of enzymes demonstrated decreased the importance of mathematical modeling. Pareto charts, it is possible to verify that for CMCase, the fermentation

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**Table 1 - Optimal values for the effects time and water activity and temperature to enzymatic.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Time (h)</th>
<th>Water activity</th>
<th>Temperature (°C)</th>
<th>enzymatic activity (U g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMCase</td>
<td>71.54</td>
<td>0.929</td>
<td>35</td>
<td>7.26</td>
</tr>
<tr>
<td>FPase</td>
<td>98.52</td>
<td>0.927</td>
<td>35</td>
<td>2.55</td>
</tr>
</tbody>
</table>
time has a more significant effect over enzyme activity, whereas for enzyme FPase the water activity variable is the one having more significant effect.

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