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# Evaluation of temperature and moisture response surface on the lipase from pumpkin seeds fermentation using *Aspergillus niger*

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**ABSTRACT.** Agro-industrial waste has shown great potential for use in solid-state fermentation due to its low cost and promoting the growth of microorganisms. This study evaluated the interaction between temperature and moisture response surface on lipase from the fermentation of pumpkin seed flour, using *Aspergillus niger*. Fermentations were performed in Petri dishes in an experimental design using a $2^2$ matrix with four replications at the center point and four axial points, varying substrate moisture between 30 and 60% and temperature between 30 and 40ºC. The maximum hydrolytic activity (71.88 U $g^{-1}$ dry weight) was obtained using flour with 30% moisture and 30°C temperature in 120 hours of fermentation. The interaction between fermentation temperature and initial moisture content of the residue was the parameter that most influenced the process at 95% significance level. The response surface analysis showed that the maximum lipase production can be obtained under temperature and moisture conditions of the flour ranging from 28 to 34.5°C and 25 to 32.5%; or between 32 and 40°C and 58 and 65%, respectively. Pumpkin seed flour showed potential for obtaining microbial lipase.

**Keywords:** agro-industrial waste, fermentation, solid state, microbial enzyme.

## Avaliação entre temperatura e umidade por superfície de resposta sobre lipase da fermentação de sementes de abóbora utilizando *Aspergillus niger*

**RESUMO.** Os resíduos agroindustriais têm apresentado grande potencial para utilização em fermentação em estado sólido por possuírem baixo custo e favorecerem o crescimento de microrganismos. Foi avaliada a interação entre temperatura e umidade por superfície de resposta sobre lipase obtida da fermentação da farinha de sementes de abóbora utilizando *Aspergillus niger*. As fermentações foram realizadas em placas de Petri, segundo planejamento experimental empregando a matriz $2^2$ com quatro repetições no ponto central e quatro pontos axiais, variando-se a umidade do substrato entre 30 e 60% e a temperatura entre 30 e 40ºC. A máxima atividade hidrolítica (71,88 U $g^{-1}$ seca) foi obtida quando utilizado farinha contendo 30% de umidade e temperatura de 30°C em 120h de fermentação. A interação entre a temperatura de fermentação e a umidade inicial do resíduo foi o parâmetro que mais influenciou o processo em nível de 95% de significância. A análise de superfície de resposta demonstrou que a produção máxima de lipase pode ser obtida em condições de temperatura e umidade da farinha entre 28 e 34,5°C e 25 e 32,5% ou entre 32 e 40°C e 58 e 65%, respectivamente. A farinha de sementes de abóbora demonstrou potencial para obtenção de lipase microbiana.

**Palavras-chave:** resíduo agroindustrial, processo fermentativo, estado sólido, enzima microbiana.

## Introduction

Among the enzymes of interest stand out industrial lipases, which are hydrolases acting on ester bonds of triacylglycerols, having the lipid compounds as natural substrate, and can be produced by plants, animals, bacteria and fungi (POLAINA; MACCABE, 2007). Lipases can be used in a wide range of industrial processes, such as: reduced fermentation time in the brewing industry, softening bread, improves crumb structure and controls the non-enzymatic browning, enhancing the aroma and hydrolysis of fat milk in the dairy industry, synthesis of short chain fatty acids esters and alcohols (HASSAN et al., 2006).

The enzymes produced by fungi are those that currently have received greater attention due to ease of production by fermentation processes, the production speed, ease of recovery from the culture medium and the fact that, mostly are not harmful to human health. Among the genera of fungi used for lipase production stand out: *Aspergillus*, *Rhizopus*, *Mucor*, *Penicillium, Geotrichum* and *Rhizomucor* (BURKERT et al., 2004; CARVALHO et al., 2005;

DELLA et al., 2006; GUTARRA et al., 2009; MENONCIN et al., 2009; MHETRAS et al., 2009; MOHAMED et al., 2010; PASTORE et al., 2003; RAJENDRAN; THANGAVELU, 2009; RODRIGUEZ et al., 2006).

The agro-industrial residues have shown great potential for use as substrate in solid state fermentation, due to the low cost, the possibility of manufacturing high added value products, and because they present in their composition organic matter liable to be consumed by microorganisms (PELIZER et al., 2007). Several agro-industrial residues have shown potential for the production of lipases such as sugarcane bagasse (ELLAIAH et al., 2004; PELIZER et al., 2007), soybean meal (MENONCIN et al., 2009), wheat bran (MARTINS et al., 2002; SILVA et al., 2005), apple pomace (PAGANINI et al., 2005) and castor bean cake (GODOY et al., 2009). The success of solid-state fermentation depends on several factors such as temperature, pH, substrate moisture, aeration, inoculum concentration, type of substrate and microorganism species. The study on the influence of these parameters is very important to maximize the production of compounds of biotechnological interest (BELLON-MAUREL et al., 2003).

Among the filamentous fungi that can be used in solid state fermentation, *Aspergillus niger* has been highlighted in relation to others in the production of microbial lipase, in addition, there are several reports in the literature about the use of this microorganism to obtain lipase by fermentation in solid state (DUTRA et al., 2008; EDWINOLIVER et al., 2010; KAMINI et al., 1998; MAHADIK et al., 2002; MENEZES et al., 2006). In this context, the objective of this study was to evaluate, by means of response surface methodology, the influence of temperature and moisture of the substrate on the obtaining a lipase from the solid-state fermentation of pumpkin seed flour, using the microorganism *Aspergillus niger*.

## Material and methods

The survey was conducted at the Laboratory of Food Microbiology from the Department of Food Technology, Federal University of Sergipe and in the Food Research Laboratory from the Institute of Technology and Research (ITP, Aracaju, Sergipe State).

### Materials

Pumpkin seeds of *Curcubita moschata* were purchased at street markets of the city of Aracaju, State of Sergipe. The microorganism *Aspergillus niger* IOC 3677 was purchased from the collection of cultures from the Oswaldo Cruz Institute (Rio de Janeiro State, Brazil) preserved in tubes with slanted nutrient agar and stored at 4°C. All reagents used were of analytical grade.

### Preparation of the pumpkin seed meal

Pumpkins seeds were subjected to drying (Pardal- EP 100) at 60°C for 8h and then crushed until getting an average diameter of 1.06 mm, sterilized by autoclaving at 121°C for 15 min. and stored in sterile glass vials until use. The flour was analyzed for lipid content through hot extraction in a Soxhlet extraction apparatus (Nova Ética, Brazil) using ethyl ether as solvent (IAL, 2005), obtaining 43% lipids.

### Microbial lipase production from pumpkin seed waste

The fermentations were conducted in Petri dishes containing 10 g of pumpkin seed flour and a spore suspension of *Aspergillus niger* ($10^6$ cells $mL^{-1}$). The kinetics of lipase production was monitored throughout the fermentation by removing a Petri dish from the oven to perform the analysis every 24h. The enzyme extraction was performed by adding to the fermented, sodium phosphate buffer 0.1 M, pH 7.0, in the proportion 1:5 (mass: volume), keeping in stirring at 30°C for 15 min. Then the material was centrifuged (Eppendorf Centrifuge 5804R) at 120 x g for 10 min., yielding the crude enzyme extract. The influence of fermentation temperature (30-40°C) and initial moisture content of the flour (30-60%) on the lipase production was evaluated by the response surface methodology, using the central composite rotational design (CCRD) $2^2$ with four replicates at the central point and four axial points (RODRIGUES; IEMMA, 2009). The statistical analyses were performed using the program STATISTIC 6.0.

### Determination of hydrolytic activity

The enzyme activity of crude enzyme extract was determined by the titration method, modified from Soares et al. (2006). The substrate was prepared using 25 mL of olive oil and 25 mL of gum arabic at 7% in distilled water. In Erlenmeyer flasks were added 5 mL of the substrate, 2 mL of sodium phosphate buffer at 0.1 M, pH 7.0, and 1 mL of crude enzyme extract; the mixture was incubated at 37°C for 5 min. The reaction was stopped with acetone and ethanol (1:1, v:v). The fatty acids released were titrated using a solution of 0.04 M KOH, using phenolphthalein as indicator. One activity unit was defined in terms of amount of enzyme to release 1µmol of fatty acid per min. of reaction, under the experimental conditions.

### Determination of microbial growth

Indirect quantification of cell growth was performed by measuring glucosamine, using methods described by Aidoo et al. (1981). The procedure consisted of adding 0.5 g of fermented to 5 mL of 6 M hydrochloric acid, keeping the mixture in a boiling water bath for 2h. Then the sample was cooled and filtered under vacuum. It was added a drop of alcoholic solution of phenolphthalein (0.5% w $v^{-1}$) to the supernatant (1 mL), neutralizing the solution with 3 N sodium hydroxide (until getting pink color). Then, it was performed the reverse titration by adding a solution of 1% $KHSO_4$ until the pink color disappears, completing the volume with distilled water. After the extraction, it was mixed 1 mL of the solution to 1 mL of acetyl acetone in 0.5 N $Na_2CO_3$, keeping in boiling water bath for 20 min. After cooling the samples, were added 6 mL of ethanol and 1 mL of Ehrlich's reagent (2.67 g p-dimethylaminobenzaldehyde in 30 mL of ethanol/hydrochloric acid 1:1). The tubes were then incubated at 65°C for 10 min. and the absorbance was read in a spectrophotometer at 530 nm. To determine the glucosamine concentration it was used a standard curve ranging from 0 to 0.2 g $mL^{-1}$.

## Results and discussion

### Kinetics of lipase production

The kinetics of lipase production was monitored throughout the fermentation process (Figures 1 and 2) in order to determine, for each experiment, at which time was obtained the maximum hydrolytic activity. The results are presented in Table 1.

**Table 1.** Coded levels and real values of the independent variables of the factorial design and maximum hydrolytic activity obtained in each fermentation.

| Exp | $X_1$ | $X_2$ | T (°C) | U (%) | Hydrolytic Activity (U $g^{-1}$) |
|---|---|---|---|---|---|
| 1 | -1 | -1 | 30 | 30 | 71.88 ± 0.0 |
| 2 | +1 | -1 | 40 | 30 | 26.07 ± 2.82 |
| 3 | -1 | +1 | 30 | 60 | 53.39 ± 5.44 |
| 4 | +1 | +1 | 40 | 60 | 54.37 ± 0.0 |
| 5 | -1.41 | 0 | 28 | 45 | 46.29 ± 4.22 |
| 6 | +1.41 | 0 | 42 | 45 | 25.54 ± 0.0 |
| 7 | 0 | -1.41 | 35 | 24 | 58.67 ± 4.83 |
| 8 | 0 | +1.41 | 35 | 66 | 70.56 ± 6.11 |
| 9 | 0 | 0 | 35 | 45 | 60.00 ± 0.0 |
| 10 | 0 | 0 | 35 | 45 | 56.27 ± 0.0 |
| 11 | 0 | 0 | 35 | 45 | 57.25 ± 3.40 |
| 12 | 0 | 0 | 35 | 45 | 64.44 ± 3.85 |

$X_1$: Coded values for temperature (T). $X_2$: Coded values for moisture (U).

It was observed in all fermentations that the lipase production increased over the fermentation time, reaching a maximum value followed by a stability or drop (Figures 1 and 2). In experiments 1; 2; 3; 4; 5; 6; 7; 8; 9; 10; 11 and 12, it was obtained maximum hydrolytic activity of 71.88, 26.07, 53.39, 54.37, 46.00, 29.00, 25.54, 58.67, 70.56, 60.00, 56.27, 57.25 and 64.64 U $g^{-1}$ dry, at 120, 24, 264, 216, 96, 96, 240, 264, 96, 120, 144 and 96h of fermentation, respectively.

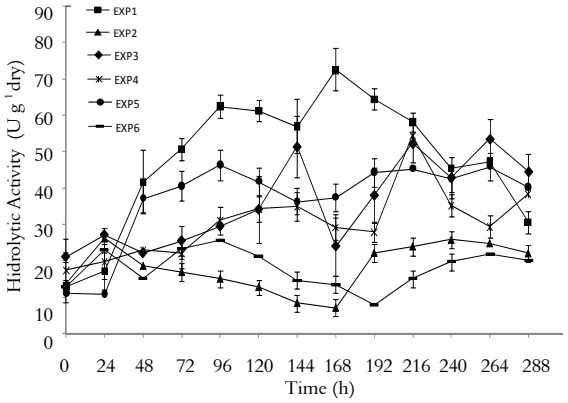


**Figure 1.** Kinetics of solid-state fermentation for lipase production (Experiments 1-6).

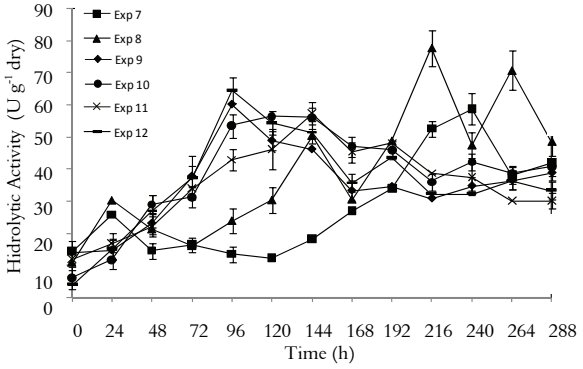


**Figure 2**. Kinetics of solid-state fermentation for lipase production (Experiments 7-12).

The maximum hydrolytic activity (71.88 U $g^{-1}$ dry) was obtained in experiment 1, when using waste containing 30% moisture and 30°C in 120h of fermentation (repeated in triplicate). The results of this study were higher than those found by Kamini et al. (1998), which fermented peanut cake, rice bran and sugarcane bagasse with the fungus *Aspergillus niger* ($10^8$ spores $mL^{-1}$) and has obtained in 120 fermentation hours, hydrolytic activities of 10; 35 and 5 U $g^{-1}$ dry, respectively, and Colla et al. (2010) which fermented a mixture of soybean meal and rice husk with *Aspergillus* sp. ($10^6$ spores $g^{-1}$) and achieved a maximum hydrolytic activity of 25 U $g^{-1}$ dry in 96 fermentation hours.

However, some researchers have obtained higher hydrolytic activities in solid state fermentation of waste, such as Edwinoliver et al. (2010), Mahadik et al. (2002), Martins et al. (2008) and Kamini et al. (1998), which obtained maximum hydrolytic activity of 521.6, 340, 120, 169.0 and 198.3 U $g^{-1}$ dry after the fermentation of a mixture of soybean meal, cake of coconut and wheat, wheat bran, rice husk meal, cake of sesame and wheat bran with *Aspergillus niger*, respectively.

### Kinetics of microbial growth

The kinetics of microbial growth was determined (in triplicate) during fermentation, in which it was used 30% of initial moisture of the substrate, and temperature of 30°C (which had the highest production of lipase). The results are shown in Figure 3.

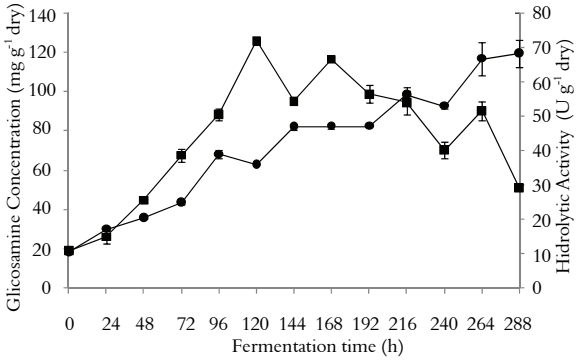


**Figure 3.** Fermentation of pumpkin seed flour, using 30% moisture of the substrate and temperature of 30°C. Hydrolytic activity (■) and glucosamine concentration (•).

Microbial growth increased with fermentation time reaching its maximum value (123.46 mg $g^{-1}$ dry) in 288h of fermentation. The increase in microbial growth was not proportional to the increase in hydrolytic activity, since the maximum value of lipase production was achieved in 120h of fermentation. One hypothesis for this is that the high microbial growth promoted an higher production of proteases than lipases, resulting in a decrease of hydrolytic activity, pattern also observed by Dutra et al. (2008).

The values obtained in this study were higher than those found by Chiou and Wu (2004), Menezes et al. (2006), Edwinoliver et al. (2010), Kamini et al. (1998), Dutra et al. (2008) and Hamidi-Esfahani et al. (2004), which obtained maximum growth of 21.6 mg $g^{-1}$ dry of *Aspergillus oryzae* in Koji prepared with extruded rice; 21.54 mg $g^{-1}$ dry of *Aspergillus niger* in fermented waste of passion fruit and wheat bran; 20 mg $g^{-1}$ dry of *Aspergillus niger* in a mixture of soybean meal, cake of coconut and wheat; 5.9 mg $g^{-1}$ dry of *Aspergillus niger* in sesame cake; 15 mg $g^{-1}$ dry of *Aspergillus niger* in wheat bran; and 50 mg $g^{-1}$ of dry *Aspergillus niger* in wheat bran, respectively. This result demonstrated the potential of pumpkin seed meal as a natural substrate for the growth of *Aspergillus niger*, regarding that no nutrient was added to the flour.

### Influence of parameters on lipase production by solid state fermentation - assessment of response surface

It was evaluated the influence of temperature and initial moisture of substrate on the lipase production through the fermentation of pumpkin seeds. The Table 2 shows the estimative of the effects and which parameters were statistically significant in the production of lipase.

**Table 2.** Estimative of the effects for the hydrolytic activity and statistical analysis of the fermentation process.

| Parameters | Effect | Standard-error | t | *p* | Confidence limit – 95% | Confidence limit + 95% |
|---|---|---|---|---|---|---|
| Principal | 59.4900 | 1.829 | 32.524 | 0.000064* | 53.669 | 65.311 |
| U(L) | 6.6562 | 2.587 | 2.573 | 0.082266 | -1.576 | 14.888 |
| U(Q) | 5.7062 | 2.892 | 1.973 | 0.143023 | -3.498 | 14.910 |
| T(L) | -18.5437 | 2.587 | -7.169 | 0.005592* | -26.776 | -10.311 |
| T(Q) | -22.9938 | 2.892 | -7.950 | 0.004150* | -32.198 | -13.781 |
| U(L).T(L) | 23.3950 | 3.658 | 6.395 | 0.007744* | 11.753 | 35.037 |

*Significant factors ($p < 0.05$).

The linear and quadratic moisture was not statistically significant for the determination of the hydrolytic activity at 95% level ($p < 0.05$), i.e., it had no effect on the lipase production. The significant parameters were the temperature (linear and quadratic) and the interaction between temperature and moisture. The Pareto chart (Figure 4) shows that the effect on the hydrolytic activity will be more significant the more it is to the right of the red line. The length of each bar is proportional to the variable effect. According to the graph, the temperature (quadratic) was the variable that most interfered with the hydrolytic activity (lipase production), but negatively (negative coefficient), i.e., the higher the temperature the lower the hydrolytic activity. The interaction between moisture and temperature had a positive effect on the hydrolytic activity. The moisture was the parameter that least affected the hydrolytic activity.

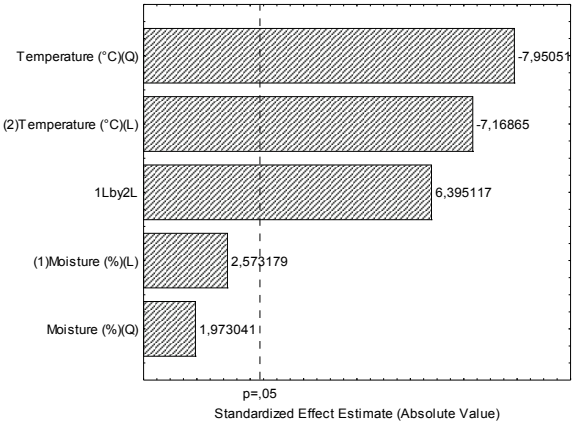


**Figure 4.** Pareto chart for the lipase production through the fermentation of pumpkin seed flour.

In Equation 1 is presented the second order encoded model for the lipase production as a function of the fermentation temperature and substrate moisture, where T is the temperature and U is the moisture.

$$\text{Hydrolytic activity (U g}^{-1}\text{ dry)} = 59.49 - 9.27\ T - 11.50\ T^2 + 11.70\ T\ U \quad (1)$$

The empirical model obtained was validated by the analysis of variance (ANOVA) presented in Table 3. The correlation coefficient $R^2 = 0.96745$ and the calculated F (about 8 times higher than the tabulated F = 4.39) have validated statistically the model and allowed obtaining the response surface.

**Table 3.** Analysis of variance (ANOVA) for the hydrolytic activity of lipase produced by FES.

| Source of variation | Sum Squared | Degree of freedom | Mean squared | Calculated F |
|---|---|---|---|---|
| Regression | 2346.605 | 5 | 469.321 | 35.66 |
| Residue | 78.959 | 6 | 13.16 | - |
| Lack of fit | 38.810 | 3 | - | - |
| Pure error | 40.149 | 3 | - | - |
| Total | 2425.564 | 11 | - | - |

Correlation coeficient $R^2 = 0.96745$; $F_{0.95;5;6} = 4.39$.

According to the response surface (Figure 5), the best conditions for the lipase production through solid state fermentation of pumpkin seed flour are: fermentation temperature between 28 and 34.5°C, and initial moisture of the flour from 25 to 32.5%; and fermentation temperature between 32 and 40°C and initial moisture of the flour from 58 to 65%.

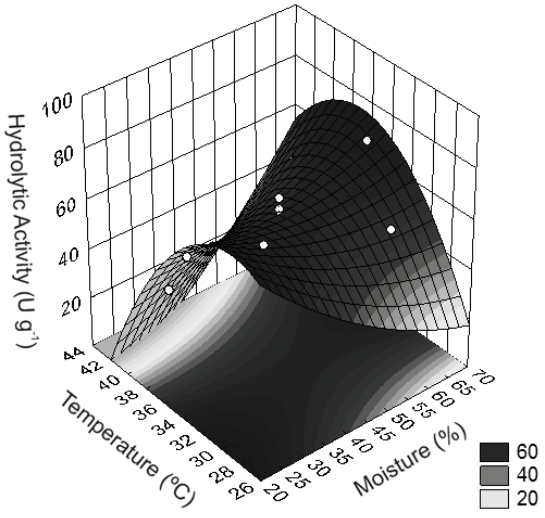


**Figure 5.** Response surface for the lipase production as a function of initial moisture of pumpkin seed meal and fermentation temperature.

## Conclusion

The interaction between temperature and moisture positively influences the lipase production through the fermentation of pumpkin seed meal, with maximum values between 28.0 and 34.5°C, and between 25 and 32.5%, or between 32 and 40°C, and between 58 and 65%, respectively. Given these findings, pumpkin seeds, commonly discarded in household waste, demonstrated to be a potential source of natural substrates (without adding nutrients) in solid state fermentation processes, aiming to obtain fungal lipase from *Aspergillus niger*.

## Acknowledgements

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