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Lipase Production by *Aspergillus niger* 11T53A14 in Wheat Bran Using Experimental Design Methodology

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Abstract Lipases have various industrial applications and their production by filamentous fungi in solid state fermentation (SSF) process is of interest. In this work the lipase enzyme production by the mutant *Aspergillus niger* 11T51A14, which is a well-known lipase producer was studied using wheat bran as substrate. Microorganism used in fermentation process was inoculated at a concentration of 10^7 spores/g_m into the medium containing nitrogen (ammonium sulfate), sunflower soapstock and wheat bran. The SSF process for enzyme production was carried out in aerated columns immersed in a water bath at 32ºC for 72 h. The process was studied using a central composite rotatable design 2^2, where variables tested were the nitrogen concentration (0.32-0.88 % w/w) and the volume of liquid (60.9-89.1 mL). The lipase activity was analyzed in enzymatic extracts. The wheat bran, in the fermentation medium, acted as a carbohydrate source and as an inducer for the lipase production. The maximum enzyme activity found was 153.4 U/g_{dm}, with a nitrogen concentration of 0.6 % and 89.1 mL of volume of liquid. The response surface model demonstrated that higher volume of liquid had a positive effect in lipase activity, while nitrogen concentration showed an optimal range (0.4-0.6%).

Keywords: *soapstock, solid state fermentation, fungi, agroindustrial waste*

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1. Introduction

Lipases, triacylglycerol hydrolases, are an important group of biotechnologically relevant enzymes [1], that catalyze the hydrolysis of triglycerides to free fatty acids and glycerol. These enzymes can also catalyze esterification, interesterification, acidolysis, alcoholysis and aminolysis in addition to the hydrolytic activity on triglycerides [2]. Due to its variety of applications, the biotechnological uses for lipases are steadily increasing [3], including uses in production of high value products in pharmaceuticals, agrochemicals and in aroma compounds [4].

Lipases are produced widely in nature, but fungic lipases have received increased attention due to the high production titers obtained in these microorganisms. Among fungi, well-known producers are *Penicillium restrictum* [5], *Candida rugosa* [6], *Colletotrichum gloeosporioides* [7], *Aspergillus niger* [8] and *Sporobolomyces ruberrimus* [9]. However, lipase production levels (or titers) and activity changes according to each microorganism and especially with the conditions employed in the cultivation. Thus, it is important to analyze and evaluate the variables and conditions that may influence lipase production and activity.

Lipase production is affected by several variables, such as, nitrogen source, carbon sources and inducers, initial pH, culture temperature, metal ions, microorganisms and fermentation process [10].

Filamentous fungi, *Aspergillus* spp. is a great producer of lipase [11], [12] and [13]. Solid state fermentation (SSF) with the mutant *A. Niger* 11T51A14 has proven to be an efficient way to produce enzymes [14]. SSF microbial cultures are closer to their natural habitat and hence their activity is increased [15].

SSF is defined as the growth of microorganisms on solid materials in the absence of or near absence of free water. However, substrate must contain enough moisture, in the absorbed form within the solid matrix [16]. There are several advantages of SSF, such as high productivity, extended stability of products, low production costs [17] and the use of numerous agricultural waste products as substrates [18]. Despite of these advantages, parameters for high enzyme yield and productivities need to be carefully optimized.

Agro-industrial residues are generally considered the best substrates for SSF processes and enzyme production [19] since they are an abundant, renewable, cheap [20] and contain carbon, nitrogen and minerals sources [21]. Several renewable residues can be used to grow microorganism in the SSF process for enzyme production, such as, babassu cake [22], soybean, rice husk [23], wheat bran [11] and palm oil mill effluents [24]. Wheat bran has been used in SSF processes [19], since it is supported, and which is also a substrate rich in carbon source for microorganism growth [25] and [14].

Present work studied some variables aiming optimal lipase production by mutant *Aspergillus niger* 11T53A14 with the use of wheat bran as substrate.

2. Material and Methods

2.1. Wheat Bran and Sunflower Soapstock

Lipid content of wheat bran grain (size ≤ 5 mm), supplied by Bunge Alimentos S.A. (Rio de Janeiro, Brazil), was analyzed following the methodology of direct extraction in Soxhlet [26].

Sunflower soapstock was provided by Indústrias Granfino S/A (Nova Iguaçu, RJ, Brazil).

The official AOCS [27] method was used to analyze neutral oil determination, pH, soap content, ash and minerals.

2.2. Microorganism

Aspergillus niger mutant strain 11T53A14 was previously selected as good lipase producer [14]. This strain, a mutant from the Embrapa Food Technology Collection, was maintained with 2.0 % (w/v) of olive oil as the carbon source [28]. The activation was done in the same basic agar medium.

2.2.1. Inoculum Preparation

Spores from five to seven-days -old agar slant cultures were used to inoculate corn cob medium for fungi propagation. After five days at 32°C, a spore suspension was prepared by adding 20 mL of 0.1 % (v/v) of Tween 80 per flask and then filtered using gauze to remove corn cob [28]. The number of conidia/mL in the suspension was quantified in a Neubauer chamber.

2.3. Fermentation Medium

The fermentation medium contained 100 g of wheat bran grain (size ≤ 5 mm), humidified with a solution containing nitrogen source (ammonium sulfate) and inducer (sunflower soapstock) in a concentration of 3 % (w/w). Concentration of ammonium sulfate used is related to the concentration of nitrogen required. For that reason, this nutrient was dissolved in the amount of water as established in the experimental design. Nitrogen concentration and the volume of liquid (moisture-related) are indicated in the experimental design (Table 1). The fermentation medium was autoclaved at 121ºC at 1 atm for 15 min.

Variables nitrogen concentration and volume of liquid, and their values were selected based on preliminary studies, as the variables that influenced the process and they were identified through experimental design.

Table 1. Experimental and calculated values of lipase activity for the different experimental conditions (real and coded variable values) of the Central Composite Rotatable Design (CCRD) 2^2

Run	Nitrogen concentration (% w/w)	Volume of liquid (mL)	Lipase activity (U/g_{dm})
1	0.4(-1)	65(-1)	95.1
2	0.8(+1)	65(-1)	72.4
3	0.4 (-1)	85(+1)	146.6
4	0.8(+1)	85(+1)	114.3
5	0.32(-1.41)	75(0)	131.9
6	0.88(+1.41)	75(0)	115.3
7	0.6(0)	60.9(-1.41)	124.2
8	0.6(0)	89.1(+1.41)	153.4
9	0.6(0)	75(0)	142.3
10	0.6(0)	75(0)	151.2
11	0.6(0)	75(0)	129.8

2.4. Solid State Fermentation

Assays were carried out using a column reactor. Glass columns were filled with 20 g of the fermentation medium inoculated with a spore suspension equivalent to 10^7 spores/g_m (g_m – gram of medium). Columns were immersed in a thermostatic bath at 32 °C and aerated with dried air at a 1 vvm (volume of air per volume of medium per minute) for 72 h.

2.5. Experimental Design

A central composite rotatable design (CCRD) 2^2 with three central points and two star points for each independent variable was used in this work. Coded and real values are shown in Table 1. The independent variables studied for the production of lipase were nitrogen concentration (0.32-0.88 % w/w) and volume of liquid (60.9-89.1 mL). Dependent variable was enzymatic activity (U/g_{dm}). Real values of these variables were selected based on preliminary studies.

Results were analyzed using the software program STATISTICA 6.0 (Statsoft Inc. 2325, Tusla, OK, USA).

2.6. Enzyme Extraction

Enzyme extraction was carried out using 2.5 mL of 100 mM sodium phosphate buffer (pH 7.0) per gram of fermented medium [29]. Enzymatic extract was filtered using filter paper (J. Prolab 42) and a microfiltration membrane (0.45 µm) and then enzyme activity was determined.

2.7. Enzyme Assay

Lipase activity was analyzed in crude enzyme extracts, using a titrimetric method which is based on the quantification of fatty acids released after the reaction between olive oil emulsion and enzyme extract. Reaction mixture containing 5 mL of the emulsion (50 mL of water, 50 mL of olive oil and 7 g of gum arabic prepared by mixing for 3 min at room temperature), 4 mL of 200 mM sodium phosphate buffer (pH 7.0) and 1 mL enzyme extract was incubated for 15 min at 35ºC. Reaction was stopped by adding 10 mL of 1:1:1 acetone/ethanol/water solution and the amount of the fatty acids was quantified

through the titration reaction with 0.05 M NaOH in a titrator (Metrohm Titrino 794, Herisau, Switzerland) until pH end-point of 11 [30]. Blank assays were conducted adding the enzyme just before titration. One unit (U) of lipase activity was defined as amount of enzyme which produces 1 µmol of fatty acids per minute under assay conditions. Lipase activity is represented by U per gram of dry medium (U/g_{dm}) (g_{dm} - gram of dry medium). All analyses were carried out in triplicates.

3. Results and Discussion

3.1. Characterization of Wheat Bran and Sunflower Soapstock

Wheat bran used in this work was analyzed and contained 3.55±0.92 % of lipids. In relation to physicochemical analysis of sunflower soapstock, it contained water 29.72±0.94 % (w/w), soap (expressed as sodium oleate) 47.50±1.20 % (w/w), neutral oil 18.11±3.21 % (w/w), ash 7.15±0.01 % (w/w) and had pH 8.60.

3.2. Lipase Production in Solid State Fermentation

Variables studied in experimental design and lipase activity results after SSF are listed in Table 1, where are presents real and coded values for independent variables.

The lowest activity was obtained from run 2 (nitrogen concentration of 0.8 % and volume of liquid of 65 mL). On the other hand maximum enzyme activity (153.4 U/g_{dm}) was observed in run 8 of experimental design, with a nitrogen concentration of 0.6 % and 89.1 mL of volume of liquid. Similar work done by Damaso and coworkers [14] using corn soapstock as an inducer for 48 h of SSF yielded a lipase activity of 62.7 U/g_{dm}. In other study, the combination of three substrates (wheat bran, wheat rawa and coconut oil cake) in SSF with *Aspergillus niger* for 96 h the lipase activity was 628.7 U/g_{dm} [31]. In the work done by Ferraz et al. [9], lipase production with *Sporobolomyces ruberrimus* using rice meal as the substrate, in SSF for 48 h, at 30 ºC and 70% moisture, gave a lipase activity of about 91.9 U/g.

In this work *Aspergillus niger* 11T53A14 was grown on wheat bran as substrate for enzyme production in SSF. Some authors have described wheat bran as the best carbon source for enzyme production, due to the high amount of carbohydrate present in the substrate/or fibers [11,25,32,33]. This agricultural residue was used as carbon source and support for microbial growth, and contained 3.55% of lipids. Similar values were found in the work done by Sharma et al [34] in relation to lipid content (4.1%).

Presence of lipids as constituent of medium can induce lipase production and may stimulate increasing of productivity [14]. In the work carried out by Santos et al [35], the presence of soapstock increased lipase activity using wheat bran as the substrate added of sunflower soapstock as inducer. This cultivation medium resulted in increasing in the lipase activity, i. e. from 183.3 to 201.8 U/g_{dm}. In the other work, which was also used *A. niger* strain, the need for lipid material was estimated comparing a medium lacking oil with a medium containing olive oil. The addition of oil to the medium increased lipase production significantly, but lipase biosynthesis occurred in medium without lipids [36].

3.3. Statistical Analysis of the Influence of Variables on the Lipase Activity

Statistical analysis was performed for the CCRD (2^2) to find optimization values of independent variables for lipase production by *A. niger* 11T53A14. Due to the great heterogeneity of the SSF and the inherent variations of enzyme production, variables were considered significant with p-values smaller than 10% ($p < 0.1$). Table 2 shows the regression coefficient, the standard deviation and values of t and p-value.

Despite the linear term of nitrogen concentration, which showed $p = 0.12$, was higher than the region of acceptance, this was included in the model, because the *p*- value of this variable was close to the significance level (90%) considered. Parameters that were not included in the model were volume of liquid quadratic and the interaction, because had no significant effect on lipase activity.

Table 2. Regression coefficient for the lipase activity

Variables	Regression coefficient	Standard error	*t* (2)	*p*-value
Mean*	141.20	6.21	22.75	0.00
(1) Nitrogen concentration (L)*	-9.83	3.81	-2.58	0.12
Nitrogen concentration (Q)*	-14.90	4.54	-3.28	0.08
(2) Volume of liquid (L)*	16.87	3.81	4.43	0.05
Volume of liquid(Q)	-7.25	4.54	-1.60	0.25
1 L x 2 L	-2.40	5.38	-0.45	0.70

* Variables statistically significant ($p<0.1$)

Analysis of variance (ANOVA) of model is summarized in Table 3, along with coefficient of determination (R^2) and F_{cal} and F_{tab} for the model.

Table 3. Analysis of variance for lipase activity

Source of Variation	Sum of squares	Degree of freedom	Mean square	$F_{calculated}$
Regression	4041.55	3	1347.18	4.37
Residual	2157.72	7	308.24	
Total	6199.28	10		

$R^2 = 70.32$

According to the ANOVA (Table 3), Fisher's *F*-value in the present study (4.37) was higher than *F* tabulated value ($F_{3;\,7;\,0,1} = 3.07$) and the coefficient of correlation obtained (0.70) made it possible to validate the codified model obtained. Model of experimental results was adjusted according to following Equation 1, where Y is lipase activity (U/g_{dm}) x_1 is variable nitrogen concentration, and x_2 is variable volume of liquid.

$$Y = 141.20 - 9.83\,x_1 - 14.87\,x_1^2 + 16.87\,x_2 \qquad (1)$$

The model expressed by Equation (1) was used to generate the response surfaces, presented in Figure 1. This response surface demonstrated that higher volume of liquid (more than 85 mL) had a positive effect in lipase activity, while nitrogen concentration showed an optimal range (0.5-0.7%).

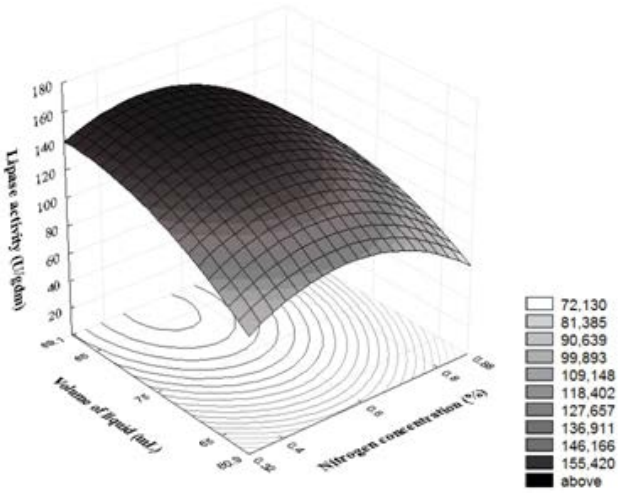

Figure 1. Surface response for lipase activity obtained by mutant *Aspergillus niger* as a function of the variables nitrogen concentration and volume of ammonium sulfate solution

Moisture content is a critical factor in SSF, an increase or decrease in the moisture content affected the enzyme production significantly [37]. In this work it was provided by addition of liquid containing nitrogen source. According to Figure 1, lipase activity was greater in the run with the most volume of liquid, i.e. a high moisture fermentation medium. However, according by Mahadik et al. [11], higher moisture levels cause a decreased porosity of substrate, lower oxygen transfer and alteration in structure of the wheat bran particles. This could occur because of the air entering in the column is not humidified and can dry the environment over time, thus the best medium was the one that began with more water. On the other hand the lower activity was obtained with lower moisture content (run 2).

Generally, high concentrations of the nitrogen source in the fermentation medium are effective to enhance lipase production by microorganisms [38]. Lipase activity increased with increasing nitrogen concentration up to a point (about 0.7%), where it began to decrease (Figure 1). This result may be due to the limited availability of these nitrogen sources to the fungus in the media supplemented with ammonium sulphate.

4. Conclusions

Wheat bran is a good substrate for lipase production in SSF because besides being a source of carbon and supporting the fungus has lipids that assist in the production of this enzyme. The addition of lipid substrate as oils is essential to obtain highest lipase production. The response surface methodology was used to enhance the production of lipase. The highest lipase production was achieved with the highest values of volume of liquid and nitrogen concentration between 0.4-0.6 % (w/w). Volume of liquid and nitrogen concentration presented significance at p–value $\leq$ 0.1, showing that lipase activity can be obtained in volumes of liquid greater than 89 mL.

A large number of factors determines the increase of lipase activity and can be tested using sunflower soapstock and wheat bran as inducer and substrate respectively.

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