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Produção e caracterização da amilase obtida de *Rhizopus microsporus* var. *oligosporus*

Production and characterization of amylase obtained from *Rhizopus microsporus* var. *oligosporus*

Producción y caracterización de la amilasa obtenida de *Rhizopus microsporus* var. *oligosporus*

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Resumo

As amilases possuem a capacidade de catalisar a ligação α-1,4 do amido liberando glicose e dextrina, com destaque em diversos campos industriais. A pesquisa buscou caracterizar a enzima amilase obtida do fungo *Rhizopus microsporus* var. *oligosporus*, através da metodologia de Doehlert, avaliando o comportamento da enzima frente as variações de pH e temperatura. A produção da amilase ocorreu com a utilização do amido como indutor, e a caracterização foi realizada através da metodologia de superfície de resposta, com a análise do pH em 5 níveis (3,0, 4,0, 5,0, 6,0 e 7,0) e temperatura em 3 níveis (30, 50 e 70 °C). A avaliação da termoestabilidade da amilase ocorreu à 60, 70 e 80ºC. A aplicação do modelo experimental indicou que a amilase obtida de *R. microsporus* var. *oligosporus* apresenta melhor desenvolvimento catalítico em temperaturas entre 40ºC e 55ºC e pH entre 2,5 e 3,2. A avaliação da termoestabilidade indicou que o aumento da temperatura influencia negativamente na atividade catalítica da amilase. O modelo experimental conduziu à compreensão das condições favoráveis à produção de amilases de *R. microsporus* var. *oligosporus*.

Palavras-chave: Biocatálise; Termoestabilidade; Metodologia de Superfície de Resposta.

Abstract

Amylases have the ability to catalyze the α-1,4 binding of starch by releasing glucose and dextrin, with prominence in several industrial fields. The research sought to characterize the enzyme amylase

obtained from the fungus *Rhizopus microsporus* var. *oligosporus*, through the Doehlert methodology, evaluating the behavior of the enzyme in the face of variations in pH and temperature. The production of amylase occurred with the use of starch as an inducer, and the characterization was carried out using the response surface methodology, with pH analysis at 5 levels (3.0, 4.0, 5.0, 6.0 and 7.0) and temperature in 3 levels (30, 50 and 70°C). The evaluation of the amylase thermostability occurred at 60, 70 and 80ºC. The application of the experimental model indicated that the amylase obtained from *R. microsporus* var. *oligosporus* shows better catalytic development at temperatures between 40ºC and 55ºC and pH between 2.5 and 3.2. The evaluation of thermostability indicated that the increase in temperature negatively influences the catalytic activity of amylase. The experimental model led to the understanding of the conditions favorable to the production of amylases from *R. microsporus* var. *oligosporus*.

Keywords: Biocatalysis; Thermostability; Response Surface Methodology.

Resumen

Las amilasas tienen la capacidad de catalizar la unión α-1,4 del almidón mediante la liberación de glucosa y dextrina, con prominencia en varios campos industriales. La investigación buscó caracterizar la enzima amilasa obtenida del hongo *Rhizopus microsporus* var. *oligosporus*, utilizando la metodología de Doehlert, evaluando el comportamiento de la enzima ante variaciones de pH y temperatura. La producción de amilasa se produjo con el uso de almidón como inductor, y la caracterización se realizó utilizando la metodología de superficie de respuesta, con análisis de pH a 5 niveles (3.0, 4.0, 5.0, 6.0 y 7.0) y temperatura en 3 niveles (30, 50 y 70 ° C). La evaluación de la termoestabilidad de la amilasa se realizó a 60, 70 y 80ºC. La aplicación del modelo experimental indicó que la amilasa obtenida de *R. microsporus* var. *oligosporus* muestra un mejor desarrollo catalítico a temperaturas entre 40ºC y 55ºC y pH entre 2.5 y 3.2. La evaluación de la termoestabilidad indicó que el aumento de la temperatura influye negativamente en la actividad catalítica de la amilasa. El modelo experimental condujo a la comprensión de las condiciones favorables para la producción de amilasas de *R. microsporus* var. *Oligosporus*.

Palabras clave: Biocatálisis; Termoestabilidad; Metodología de la Superficie de Respuesta.

1. Introduction

Enzymes are products of high added value and, with the introduction of biocatalysis, numerous enzyme-based processes have been marketed in order to generate relevant products on the market (Choi, Han, & Kim, 2015).

The use of enzymes has gained space by presenting high specificity and not generating undesirable parallel products as occurs in chemical synthesis reactions, in addition to the easy

regulation of enzymatic activity through variables such as temperature and pH. As a result, enzymatic industrial processes become easy to control, relatively simple, energy efficient and low-cost (Porter, Rusli, & Ollis, 2016).

Enzymes are involved in various industrial processes such as food production, beverages, polymer synthesis, pulp and paper industry, textile, cosmetic, detergent, bioremediation, feed production. In the field of medicine, they are applied in diagnosis, as an indicator of diseases and in therapy (R. Singh, Kumar, Mittal, & Mehta, 2016)

Enzymes can be obtained from vegetables, animals and microorganisms. However, microbial enzymes are the preferred source because they are more stable, with simpler, more economical production and requiring less need for time and space. In addition, procurement processes are facilitated (Singh et al., 2016; Raveendran et al., 2018).

Amylases consist of a group of hydrolases that catalyze the breakdown of starch molecules into dextrin, maltose and glucose units (Simair et al., 2017). They are important in industrial processes and have the largest share in the world enzyme market (Abdulaal, 2018). The main applications are related to the production of sweeteners, syrups, papers, detergents, ethanol, acetone and lactic acid (Christopher & Kumbalwar, 2015; Wang et al., 2016).

The identification of potential microorganisms producing enzymes is essential to ensure supply to industrial processes. Moreover, research aimed not only at obtaining, but also on the optimization of catalytic activity needs to be stimulated, in order to enable processes and improve products from an industrial point of view. Thus, the aim of this study was to produce and characterize amylase secreted by *Rhizopus microsporus* var. *oligosporus*. The Doehlert planning was used to determine the optimal conditions of enzymatic activity and thermostability was investigated, with a view to application in the industrial sector.

2. Material and Methods

2.1 Microorganism

R. microsporus var. *oligosporus* was obtained from the Collection of Reference Microorganisms in Sanitary Surveillance (FIOCRUZ, Rio de Janeiro, Brazil). The fungus was kept in Potato Dextrose Agar (BDA) at 4ºC.

2.2 Amylase production

The fungus was previously cultivated in BDA medium for 7 days at 30ºC (BOD SL200/90 Incubator- SOLAB). The fungus was inoculated in liquid medium containing starch (10 g/L), yeast extract (3 g/L), malt extract (3 g/L) and magnesium sulfate (0.758 g/L) at pH 7.0, following the methodology of Chang et al. (2018) with modifications. Then, 1 x 10^7 spores/mL of the microorganism was inoculated in half. The fermentation process occurred in a rotary agitator (Incubator shaker SL 222, SOLAB) at 150 rpm for 120 h. After fermentation, the culture medium was vacuum filtered and the supernatant used as raw enzymatic extract.

2.3 Enzymatic assay

The activity of amylase was determined by the methodology proposed by Miller (1956) with the principle of quantification of reducing sugars released in the reaction medium from the reaction of 0.1 mL of gross enzymatic extract with 0.1 mL of starch solution 1% in phosphate buffer pH 7.0 for 10 min at 50°C. After incubation, 0.2 mL of 3.5-diitrosaliclic acid (DNS) was added and the mixture subjected to 100°C for 5 min and cooled with 2 mL of distilled water. The activity was measured by reading the absorbance at 540 nm in spectrophotometer (Mars Spectro 560), which was compared with a standard glucose curve obtained under the same conditions.

2.4 Protein determination

Total protein determination was conducted followed by the Bradford (1976) method. Bovine serum albumin was used as standard.

2.5 Optimum pH determination and amylase temperature

The optimum pH and temperature studies of amylase were performed by applying the response surface methodology using doehlert planning. The pH variable was studied at 5 levels (3.0, 4.0, 5.0, 6.0 and 7.0) and temperature at 3 levels (30ºC, 50ºC and 70°C), with the central point performed in triplicate to evaluate experimental errors.

2.6 Evaluation of thermostability

The thermomostability was studied at different temperatures (60ºC, 70ºC and 80ºC) and for increasing periods of time (10, 30 and 50 min) at constant pH equal to 5.0.

2.7 Statistical analyses

The experiments were carried out in triplicate and Statistic software® version 7.0 and variance analysis (ANOVA) were used for data analysis.

3. Results and discussion

The nature by which enzymes have attracted attention in catalysis on an industrial scale, is primarily due to their functional characteristics, selectivity, activity and stability at a wide range of pH and temperature, lower generation of by-products, because they are industrially sustainable and thus less harmful to the environment (Singh & Bajaj, 2017; Chapman, Ismail, & Dinu, 2018).

Temperature directly influences enzymatic reactions, increasing or decreasing the catalytic speed of reactions. Enzymes that operate below the temperatures considered optimal for their performance can have their activity decreased with consequent lower generation of the desired product. In addition, above-optimal temperatures can lead to enzyme denaturation and inactivation, except in cases where the enzyme has thermoloterance. With this, knowing the optimal ranges of enzymatic activity is of paramount importance for the optimization of the processes that use them.

Table 1 presents the results of enzymatic activity (UA) found after applying the Doehlert planning to determine optimum pH and temperature for the production of amylase by *R. microsporus* var. *oligosporus*.

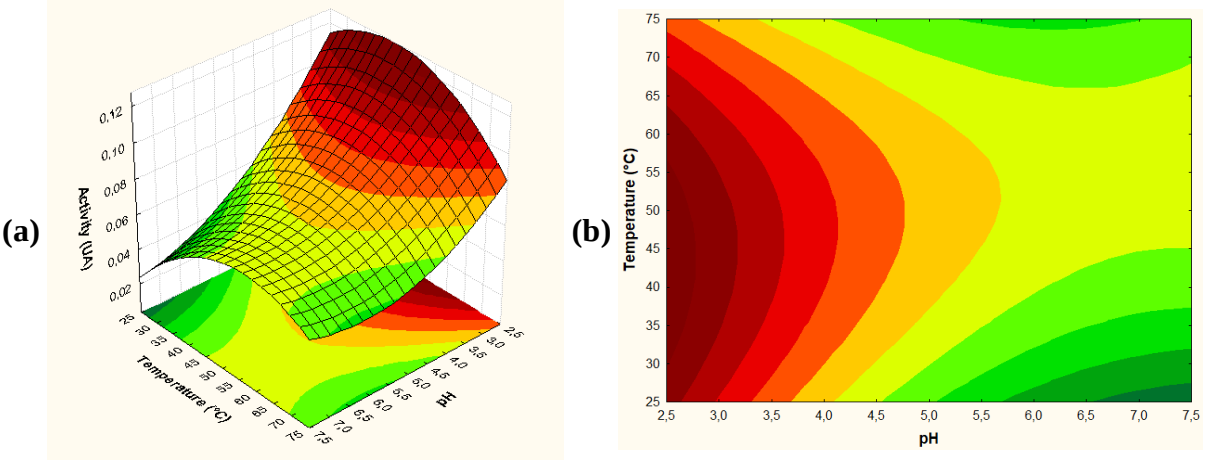
Table 1. Doehlert planning for optimum pH and temperature analysis for amylase production by *R. microsporus* var. *oligosoporus*.

Nº	pH	Temperature	UA*
1	4 (-0,5)	70 (+0,866)	0,07227
2	6 (+0,5)	70 (+0,866)	0,05474
3	3 (-1)	50 (0)	0,11209
4C	5 (0)	50 (0)	0,08433
4C	5 (0)	50 (0)	0,07099
4C	5 (0)	50 (0)	0,07556
5	7 (+1)	50 (0)	0,06478
6	4 (-0,5)	30 (-0,866)	0,08122
7	6 (+0,5)	30 (-0,866)	0,04524

*UA: µmol/min. Source: Authors.

The Doehlert design defines combinations for the levels of variables that must be applied experimentally to obtain the desired responses. The surface and area graphs (Figure 1) show the response obtained after applying the model.

Figure 1. Influence of pH and temperature on enzymatic activity of amylase produced by *R. microsporus* var. *oligosporus*. (a) Response surface graph. (b) Area chart.



Source: Authors.

The regression model provided for enzymatic activity in relation to pH and temperature in the experimental model is expressed by equation:

Activity (UA) = 0,1648 ± 0,0665 - (0,0525 ± 0,0177)pH + (0,0028 ± 0,0015)pH^2 + (0,0029 ± 0,0014)T + (0,0002 ± 0,0001)pH x T.

Where: T: Temperature in °C.

Amylases are enzymes responsible for the hydrolysis of α-1, 4-glycosidic bond in starch, releasing maltose and oligosaccharides. Microbial source enzymes are useful because they are economical and easy to obtain and manipulate, which allow their wide use in the industrial sector. The optimal conditions of action of enzymes may vary according to the microorganism that produces. Amylases of fungal origin are required because they act mainly at lower temperatures and more acidic pH, when compared to the conditions of bacterial amylases that act at high temperatures, around 95 to 105ºC and pH close to neutral, between 5.8 and 6.8 (Abdulaal, 2018; Yan, Xiangsong, & Xiang, 2019). From the inspection of the response surface (Figure 1) it is possible to consider that the amylase produced by *R. microsporus* var. *oligosporus* showed better activity in more acidic pH conditions and in a wide temperature range. The increase in the pH of the medium to values above 5 contribute to the decline of enzyme activity, as well as the increase in temperature at values above 70ºC approximately. Yan et al. (2019) in their studies reported similar results for the performance of amylase produced by the fungus *Fomitopsis palustres*, which presented higher activity in a pH range of 2 to 5 and ideal temperature at 60ºC.

The analysis of variance (ANOVA) was performed to estimate the lack of adjustment of the model against the results obtained experimentally (Table 2).

Table 2. Variance Analysis (ANOVA).

Variation Source	SQ	df	MQ	$F_{calculated}$	F_{tabled}
Regression	0,002806	5	0,000561	17,13	9,01
Residue	0,000098	3	3,28E-05		
Lack-of-fit	0,000006	1	0,000006	0,13996	18,01
Pure error	0,000092	2	0,000046		
Total SQ	0,002905	8			

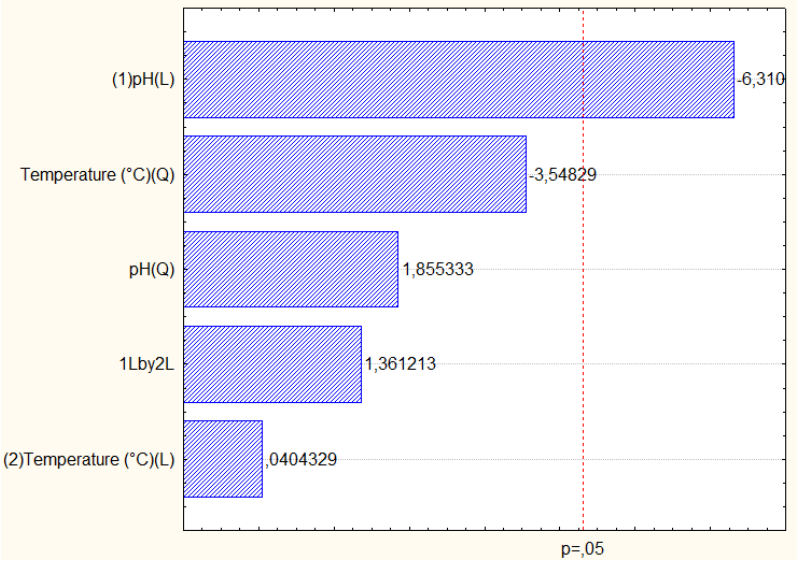
SQ: Quadratic sum; df: Degrees of freedom; MQ: Quadratic mean. Source: Authors.

Regression analysis presents a calculated value for fisher distribution greater than the tabulated value (17.13 > 9.01), which allows us to infer that the experimental model is well

adjusted, proven by the value obtained in the lack of adjustment with $F_{calculated}$ equal to 0.14, being lower than the F_{tabled} 18.01, which indicates that the results found are not random. The coefficient of determination R^2 was 0.96, which indicates that 96% of the results found are explained by the experimental model. The experimental results of enzymatic activity of this study were adjusted by multiple regression using Statistic software 7.0.

According to the Pareto diagram (Figure 2) the linear pH has a significant influence on the activity of amylase produced by *R. microsporus* var. *oligosporus*, while temperature has no significant relationship for enzymatic activity, as well as the temperature and pH ratio when analyzed together.

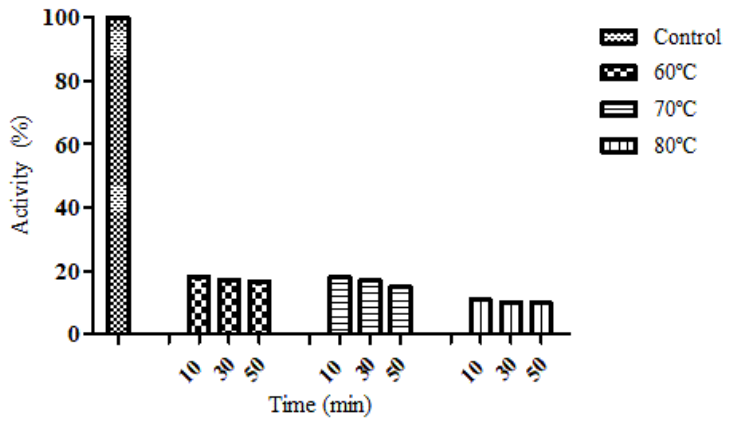
Figure 2. Pareto diagram for the production of amylase by *R. microsporus* var. *oligosporus*.



(Q) = Quadratic, (L) = Linear and 1Lby2L = pH + temperature. Source: Authors.

The amylase produced by *R. microsporus* var. *oligosporus* had its activity reduced when previously exposed to temperatures of 60º, 70 and 80ºC in the periods of 10, 30 and 50 minutes. With greater reduction when exposed to the temperature of 80ºC. The reduction of enzymatic activity of amylases obtained from fungi, when exposed to temperatures above 60ºC, are reported by other authors (Kelleci & Comlekcioglu, 2016; Xian, Wang, Luo, Feng, & Feng, 2015; Bussa, Moges, Muthuswamy, & Abdisa, 2019). The activity and stability of enzymes are factors sensitive to the action of temperature. As a result, the increase in temperature for a certain period of time may imply enzymatic denaturation.

Figure 3. Evaluation of the thermostability of amylase produced by *R. microsporus* var. *oligosporus*.



Source: Authors

Enzymatic characterization studies are useful in view of its wide industrial application. The enzyme amylase has been used in important processes industries, such as the production of glucose syrups, in the bakery industry, beverage production, among others. The vast biodiversity and the possibility of using microorganisms as potential producers of enzymes applicable to industrial processes justify the stimulation of studies that seek to optimize their production conditions and act in order to improve the performance of biocatalysis.

4. Conclusions

The experimental model adopted in the study allowed us to understand the favorable conditions for the action of the enzyme amylase secreted by the fungus *Rhizopus microsporus* var. *oligosporus*. The results showed that the enzyme could act in a wide temperature range, which involves milder temperatures and more acidic pH. Such conditions can make the process more economically viable when compared to other sources of procurement. There was no lack of adjustment in the applied model, which indicates that the results presented are consistent with the experimental reality.

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