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PRODUCTION OF ALKALINE PROTEASE FROM *Cellulosimicrobium cellulans*

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ABSTRACT

Cellulosimicrobium cellulans is one of the microorganisms that produces a wide variety of yeast cell wall-degrading enzymes, β-1,3-glucanase, protease and chitinase. Dried cells of *Saccharomyces cerevisiae* were used as carbon and nitrogen source for cell growth and protease production. The medium components KH_2PO_4, KOH and dried yeast cells showed a significant effect ($p<0.05$) on the factorial fractional design. A second design was prepared using two factors: pH and percentage of dried yeast cells. The results showed that the culture medium for the maximum production of protease was 0.2 g/l of $MgSO_4.7H_2O$, 2.0 g/l of $(NH_4)_2SO_4$ and 8% of dried yeast cells in 0.15M phosphate buffer at pH 8.0. The maximum alkaline protease production was 7.0 ± 0.27 U/ml over the center point. Crude protease showed best activity at 50ºC and pH 7.0-8.0, and was stable at 50ºC.

Keywords: response surface, medium optimization, alkaline protease, *Cellulosimicrobium cellulans*, actinomycete.

INTRODUCTION

Actinomycetes, a Gram-positive mycelium forming bacterial group, are able degrade macromolecules, being efficient in the breakdown of proteins (25). The microbiology of Brazilian soil, which has special environmental characteristics and is rich in actinomycete populations, has not been very well explored and constituting an excellent source for the search for new enzymes (5). *C. cellulans* is an actinomycete that was isolated from residues of an alcohol fermentation industry. This microorganism produces an extracellular enzyme complex during growth, which consists mainly of β-1,3-glucanase, chitinase and alkaline protease. This enzyme complex is capable of lysing yeast cell walls.

Proteases are probably the most important class of enzymes, which constitute about 65% of the total industrial enzyme market (13). Proteases have applications in various industries such as the detergent, food, pharmaceutical, leather and silk industries (22). In recent years the use of alkaline protease as an industrial catalyst has increased. These enzymes exhibit high catalytic activity and are economically feasible. Various physiological activities have been detected in the hydrolysates derived from the proteolytic hydrolysis of many food proteins. For example, antioxidative peptides were isolated from a hydrolysate prepared with microbial protease (4).

The optimization of culture medium components for alkaline protease production by microorganisms, mainly *Bacillus*, was presented in several recent articles (3,13,15,17,23). Some of these components were glucose, corn starch, yeast extract, corn steep liquor, sucrose, casein, malt extract, polypeptone, fructose corn syrup, maltose, potato starch, molasses, whey, soybean meal and several salts. The aim of these papers was increase the protease production and the cellular growth.

The cost of enzyme production is a major obstacle in its successful industrial application. Statistical approaches offer ideal ways for process optimization studies in biotechnology, and have advantages because they use the fundamental principles of statistics, randomization and replication (13). In view of the promising applicability of the alkaline protease, it should be produced in high yields in a low-cost medium. Within this context, the purpose of the present study was to determine the optimum culture medium and the best fermentation conditions for the maximum protease production from *Cellulosimicrobium*

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cellulans, as well as to partially characterize the crude enzyme.

MATERIAL AND METHODS

Microorganism

The culture of *C. cellulans* was isolated from alcohol fermentation industrial waste in the Food Biochemistry Laboratory, UNICAMP, and identified by the Korean Institute of Bioscience & Biotechnology.

Protease Production

Protease production from *C. cellulans* was carried out in 50 ml of sterile medium containing 13.6 g/l of KH_2PO_4, 2.0 g/l of $(NH_4)_2SO_4$, 4.2 g/l of KOH, 0.2 g/l of $MgSO_4.7H_2O$, 0.001 g/l of $Fe_2(SO_4)_3.6H_2O$, 1 mg/l of thiamine, 1 mg/l of biotin and 1% of dried *S. cerevisiae* cells (20, with modification). Initial pH of the culture medium was 7.5. The culture medium was inoculated with a 10% inoculum of a 15 hour culture of *C. cellulans* and incubated at 30ºC with shaking (150 rpm) for 24 hours. The culture was centrifuged at 10000g for 10 minutes at 5ºC. Protease activity of the supernatant was determined.

Protease assay

The protease was assayed by Obata *et al.* (12) using casein as substrate. One unit of enzyme activity was defined as the amount of protease required to produce an increase in absorbance of 1.0 in 30 minutes (18) under the above assay conditions.

Identification of important nutrient components of the culture medium (fractional design)

A factorial design was used to estimate the effects of each medium component on enzyme production. Initially, eight components of the culture medium were studied. To screen the relative influence of these factors and their possible interactions in the experimental domain, a 2^{8-3} fractional factorial design was chosen. The assays were performed at 30ºC with agitation of 150 rpm. Table 1 presents the 35 assays performed and the respective concentrations of the culture medium components.

Central composite experimental design to optimize the medium components

A new design was prepared using two factors: pH and concentration of dried yeast cells. We used a central composite design to find the optimal concentration of two factors. For this purpose, a set of 11 experiments including 2^2 factorial experiments, three center point and four axial points ($\alpha = 1.41$) were conducted. The assays were performed at 30ºC with agitation of 150 rpm. The setting range for factors was as follows: pH, 6.6-9.4 and percentage of dried yeast cells, 4.0-11.0%.

A multiple regression analysis of the data was carried out with the statistical package (Statistica 5.0) and the second-order polynomial equation was obtained: $y = a_0 + a_1.x_1 + a_2.x_2 + a_3.x_1^2 + a_4.x_2^2 + a_5.x_1.x_2$; where a_0 is the intercept term; a_1, a_2 are linear coefficients; a_3, a_4 are squared coefficients and a_5 is the interaction coefficient.

Central composite design to optimize the fermentation conditions

An experimental design was prepared to evaluate the optimal temperature and agitation conditions for maximum protease production. The range studied in this experimental design was from 18 to 32ºC for the temperature and from 80 to 220 rpm for the agitation speed. Once the response was obtained, the data were correlated as second-order polynomial models.

The relationships between responses and variables were determined using the software Statistica® 5.0 from Statsoft Inc. (2325 East 13 th Street, Tulsa, OK, 74104, USA). The response surface graph indicates the effect of variables and determines their optimum levels for maximal protease production.

Protease production and kinetic of growth in the optimized culture medium

One loopfull of a 24 hour culture of *C. cellulans* was inoculated in 10ml of the optimized culture medium and incubated at 27ºC for 16 hours at 150 rpm. Five ml of this culture were transferred to 250 ml Erlenmeyer flasks containing 45 ml of the same culture medium, and incubated at 27ºC at 150 rpm. One ml samples were collected after 5, 10, 24, 48 and 72 hours of growth. The samples were transferred to tubes containing 9 ml of a 0.2% Tween 80 solution and mixed vigorously in a vortex for 5 minutes. The viable cell count was performed using serial dilutions on TYM agar plates, incubated at 30ºC. The number of colony forming units was determined by the drop method (9).

Crude enzyme characterization

To investigate the effect of temperature on protease activity, the protease assay was performed in the temperature range from 20 to 96ºC at pH 7.5. The influence of pH was investigated using 50 mM buffer solutions ranging from 2.6 to 10.7, at the optimum temperature previously determined. To determine the enzyme stability, the crude enzyme was incubated at temperatures ranging from 5 to 80ºC in a 50 mM phosphate buffer pH 7.5. After 15 minutes incubation, the reaction mixture was assayed, and the residual enzymatic activity was measured. The crude enzyme was incubated at pH ranging from 2.6 to 10.7 at 30ºC during 1 hour, and then the residual enzymatic activity was determined.

RESULTS AND DISCUSSION

Factorial fractional design ($2^{8-3)}$ to estimate the effects of the culture medium components on protease production.

Table 1 show the factorial fractional design matrix and Table 2 shows the main effects in the fractional experimental design. The culture medium components that showed significant effects

($p<0.05$) on protease production were KH_2PO_4, KOH and dried yeast cells. The effect of KH_2PO_4 was -0.077. The negative effect indicates that an increase in the concentration of this compound (level -1 to level +1) in the culture medium resulted in 0.077 U/ml less protease activity; this represents a reduction of 35.6% (0.216 to 0.139 U/ml) in the protease activity. The effects of KOH and dried yeast cells were 0.164 and 0.128. Thus, the increase in these variables from level -1 to level +1 resulted in an increase of 170.8% (0.096 to 0.260 U/ml) and 112.3% (0.114 to 0.242) in the protease activity, respectively.

The effects of thiamine, biotin and $Fe_2(SO_4)_3.6H_2O$ were not significant ($p>0.05$), meaning that they were not necessary for protease production since that the lowest studied level for they was concentration zero.

The effect of $MgSO_4.7H_2O$ and $(NH_4)_2SO_4$ were also not significant in the studied levels, however, they were kept at the

Table 1. Design matrix for the 2^{8-3} fractional factorial design and the response after analysis.

Run	KH_2PO_4	$(NH_4)_2SO_4$	KOH	$MgSO_4.7H_2O$	$Fe(SO_4)_3.6H_2O$	Thiamine	Biotin	yeast cells	Protease Activity
	g/l				10^{-3} g/l			%	U/ml
1	19.6	3.0	6.4	0.3	2.0	2.0	2.0	1.5	0.27
2	19.6	3.0	6.4	0.3	0.0	2.0	2.0	0.5	0.10
3	19.6	3.0	6.4	0.1	2.0	2.0	0.0	0.5	0.10
4	19.6	3.0	6.4	0.1	0.0	2.0	0.0	1.5	0.23
5	19.6	3.0	2.0	0.3	2.0	0.0	2.0	0.5	0.06
6	19.6	3.0	2.0	0.3	0.0	0.0	2.0	1.5	0.15
7	19.6	3.0	2.0	0.1	2.0	0.0	0.0	1.5	0.21
8	19.6	3.0	2.0	0.1	0.0	0.0	0.0	0.5	0.07
9	19.6	1.0	6.4	0.3	2.0	0.0	0.0	0.5	0.07
10	19.6	1.0	6.4	0.3	0.0	0.0	0.0	1.5	0.24
11	19.6	1.0	6.4	0.1	2.0	0.0	2.0	1.5	0.20
12	19.6	1.0	6.4	0.1	0.0	0.0	2.0	0.5	0.09
13	19.6	1.0	2.0	0.3	2.0	2.0	0.0	1.5	0.12
14	19.6	1.0	2.0	0.3	0.0	2.0	0.0	0.5	0.09
15	19.6	1.0	2.0	0.1	2.0	2.0	2.0	0.5	0.08
16	19.6	1.0	2.0	0.1	0.0	2.0	2.0	1.5	0.15
17	7.6	3.0	6.4	0.3	2.0	0.0	0.0	1.5	0.54
18	7.6	3.0	6.4	0.3	0.0	0.0	0.0	0.5	0.19
19	7.6	3.0	6.4	0.1	2.0	0.0	2.0	0.5	0.28
20	7.6	3.0	6.4	0.1	0.0	0.0	2.0	1.5	0.55
21	7.6	3.0	2.0	0.3	2.0	2.0	0.0	0.5	.0.08
22	7.6	3.0	2.0	0.3	0.0	2.0	0.0	1.5	0.09
23	7.6	3.0	2.0	0.1	2.0	2.0	2.0	1.5	0.09
24	7.6	3.0	2.0	0.1	0.0	2.0	2.0	0.5	0.03
25	7.6	1.0	6.4	0.3	2.0	2.0	2.0	0.5	0.24
26	7.6	1.0	6.4	0.3	0.0	2.0	2.0	1.5	0.23
27	7.6	1.0	6.4	0.1	2.0	2.0	0.0	1.5	0.65
28	7.6	1.0	6.4	0.1	0.0	2.0	0.0	0.5	0.18
29	7.6	1.0	2.0	0.3	2.0	0.0	2.0	1.5	0.03
30	7.6	1.0	2.0	0.3	0.0	0.0	2.0	0.5	0.09
31	7.6	1.0	2.0	0.1	2.0	0.0	0.0	0.5	0.07
32	7.6	1.0	2.0	0.1	0.0	0.0	0.0	1.5	0.12
33*	13.6	2.0	4.2	0.2	1.0	1.0	1.0	1.0	0.19
34*	13.6	2.0	4.2	0.2	1.0	1.0	1.0	1.0	0.18
35*	13.6	2.0	4.2	0.2	1.0	1.0	1.0	1.0	0.23

* center point

Table 2. Main effects of the variables on protease production (2^{8-3} fractional design).

Factor	Effect	p
Mean	0.180*	0.001*
KH_2PO_4	-0.077*	0.014*
$(NH_4)_2SO_4$	0.024	0.121
KOH	0.164*	0.003*
$MgSO_4.7H_2O$	-0.032	0.076
$Fe(SO_4)_3.6H_2O$	0.031	0.0820
Thiamine	-0.014	0.264
Biotin	-0.026	0.111
Dried yeast cells	0.128*	0.005*

* Statistically significant values ($p<0.05$).

concentration used in the initial medium, 0.2 and 2.0 g/l respectively, because they were not studied at concentration zero.

KH_2PO_4 and KOH showed a significant effect. The significant role of phosphate ions in protease production is in agreement with others reports (10). These compounds are also related to the pH of the culture medium, thus the influence of pH on protease production was evaluated in a new experimental design.

Central composite design to optimize the medium components

More two experimental designs were performed after the first design. They showed that the protease production increased when pH and the percentage of dried yeast cells increased. Based in these results, the levels studied in the design presented in Table 3 were defined.

C. cellulans produced 0.20 ± 0.03 U/ml protease with the initial medium. Table 3 shows the central composite design matrix.

Protease activity around the center points was 7.16 ± 0.27 U/ml. The Analysis of Variance of the optimization study showed that the model-F value was 15.04. This value is more than three times the value of $F_{3,7}$ value (4.35), indicating that the quadratic model has a good fit .

Fig. 1 shows the estimated response surface for protease production based on two statistically significant factors. The highest protease production was observed when the value of the culture medium pH was between 7.5 and 8.5, and the dried yeast cell concentration between 7.0 and 9.0%.

The regression equation obtained from the analysis of variance (ANOVA) indicated that the R^2 value (correlation coefficient) is 0.86 (a value > 0.75 indicates aptness of the model). This value ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that 86% of the variability in the response could be explained by the model.

Table 3. Results obtained in the central composite design for culture medium optimization.

Experiment	pH	% Dried yeast cells	Protease activity (U/ml)
1	9.0	10.0	4.94
2	9.0	5.0	2.42
3	7.0	10.0	4.30
4	7.0	5.0	3.88
5	9.4	7.5	3.98
6	6.6	7.5	5.42
7	8.0	11.0	5.82
8	8.0	4.0	3.36
9*	8.0	7.5	7.18
10*	8.0	7.5	6.88
11*	8.0	7.5	7.42

* center point.

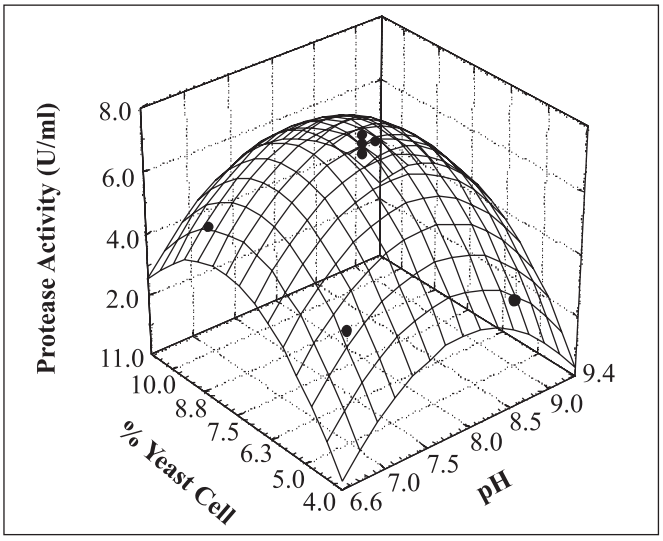

Figure 1. Response surface curve of protease production (U/ml) by *C. cellulans* as a function of dried yeast cell percentage and pH. Protease activity (U/ml) = 7.16 - 1.42 pH^2 + 0.80 DYC - 1.48 DYC^2, where pH is the value of the coded pH and DYC, the value of the coded percentage of dried yeast cell.

The culture medium for maximum protease production was 0.2 g/l of $MgSO_4.7H_2O$, 2.0 g/l of $(NH_4)_2SO_4$ and 8.0% of dried yeast cells in 0.15M phosphate buffer pH 8.0.

Central composite design to optimize the fermentation conditions

Table 4 shows the central composite design matrix. Optimization of the fermentation conditions presented an

increase of about 16% in protease activity (8.28 ± 0.29 U/ml). The analysis of variance showed that the variation in agitation speed during microbial growth did not significantly affect protease production. Enzyme production was only influenced by fermentation temperature, showing a significant effect of -1.13 on protease production.

The analysis of variance showed that the statistical coded model was significant and predictive. The model-F value (21.87) was 7 times higher than the $F_{2,8}$ value (3.11) and the R^2 value was 0.84 ($p<0.10$).

Table 4. Results of the runs of the central composite design for optimization of the fermentation conditions.

Run	Temperature (ºC)	Agitation (rpm)	Protease Activity (U/ml)
1	30	200	7.14
2	30	100	7.52
3	20	200	8.42
4	20	100	8.50
5	18	150	7.80
6	32	150	6.90
7	25	80	8.59
8	25	220	8.89
9*	25	150	8.55
10*	25	150	7.98
11*	25	150	8.31

* center point.

The response surface presented in Fig. 2 indicates that the highest protease activity was observed when the fermentation was performed at temperatures between 20 and 27ºC.

Culture medium optimization produced an increase of about 36 times in protease activity when compared with the activity in the initial culture medium (0.20 ± 0.03 U/ml, 7.16 ± 0.27 U/ml), optimization of the fermentation conditions presented an increase of about 16% (7.16 ± 0.27 U/ml, 8.28 ± 0.29 U/ml).

There is a growing acceptance of use of statistical experimental designs in biotechnology. Many scientists have reported satisfactory optimization of protease production from microbial sources using the statistical approach (7,16,24). In this study, response surface methodology was shown to be efficient for the optimization of the enzyme production.

In view of the commercial utility of the enzyme, a cost-effective media formulation becomes a primary concern (3). Protease production by *C. cellulans* in the initial medium was very low. Optimization of this culture medium showed that is possible to increase protease production and reduce the cost of the culture medium with the use of yeast cells. Yeast cells are

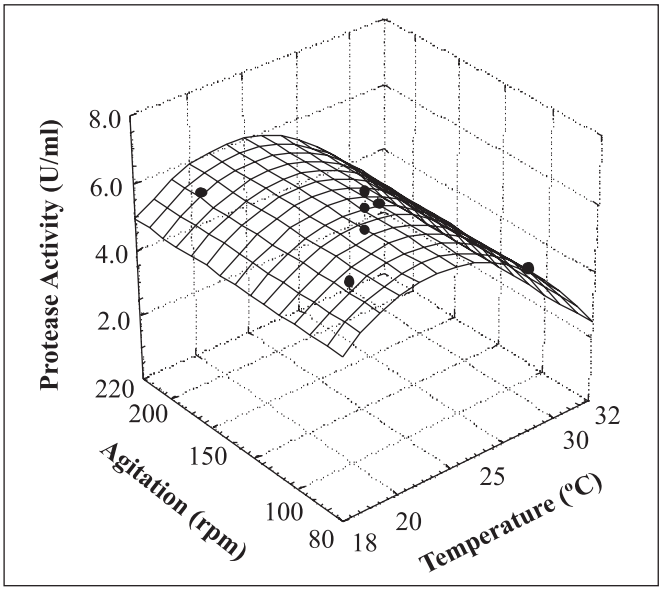

Figure 2. Response surface curve of protease production as a function of temperature and agitation speed. Protease activity (U/ml) = 8.46 - 0.44 T - 0.56 T^2, where T is the coded value for the temperature.

a residue of the alcohol fermentation industry and are a rich source of carbon and nitrogen for the cellular growth and alkaline protease production. The use of the yeast cell wall was only possible because *C. cellulans* is a yeast lysing microorganism (YLM) and it is capable of using the cell walls of yeasts as source of nutrients. Moreover, this culture medium is hardly contaminated by nonlytic microorganisms.

Protease production and kinetic of growth in the optimized culture medium

Protease was produced during the exponential growth phase (Fig. 3). Protease activity was detected from the early stages in the culture medium, showing that the yeast cell was an excellent nutrients source. The activity increased greatly during the exponential growth phase, reaching a plateau during the stationary phase. Maximum alkaline protease production was observed after 24 hours of incubation.

Maximum protease production was observed after 24 hours of fermentation, when the stationary phase of microbial growth begins. Protease production was shown to be directly related to biomass production as reported by Puri *et al.* (16) and Chauhan and Gupta (3). Azeredo *et al.* (2) and Chauhan *et al.* (3) reported maximum protease production by Bacillus sp. after 96 hours of incubation. Thys *et al.* (24) reported maximum enzyme production by *Microbacterium* sp upon 48-96 h of incubation. The decline in protease production in prolonged incubation may be due to autolysis or the proteolytic activity of other proteases (3).

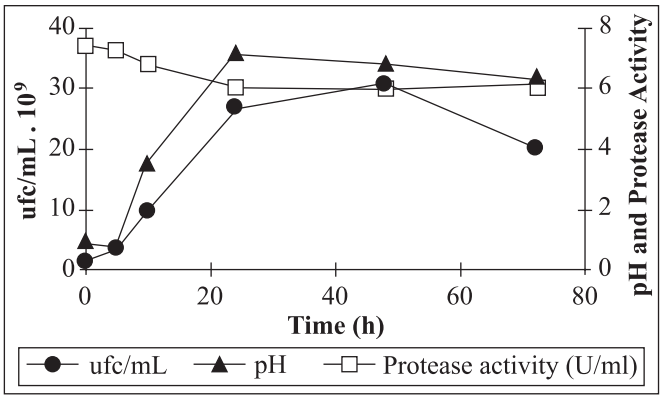

Figure 3. Kinetics of growth and protease production by *C. cellulans* in the optimized culture medium.

Protease properties in crude extract

The maximum activity was observed at 50ºC. Thermophilic proteases from Actinomycetes, with high activity at 70ºC, have been reported for *Thermoactinomyces vulgaris* (7) and *Streptomyces thermovulgaris* (26). The strain of actinomycete *Arthrobacter* sp presented high activity at 55ºC (1). Alkaline proteases from *Bacillus* sp. presented optimum temperature about 55 to 70ºC (11,14,21).

The optimum pH of proteolytic activity was found to be 7.0-8.0, but significant levels of activity (80%) were still detected between pH 8.0 and 10.7. There are reports of alkaline proteases from *Bacillus* sp. that presents the maximum activity at pH 8.0 to 9.0 (8,11,21). Proteases from Actinomycetes *Arthrobacter luteus, Arthrobacter* sp and *Oerskovia xanthineolytica* presented high activity at pH 10.5, 11.0 and 9.5-11.0, respectively (1,6,19).

C. cellulans protease was stable (around 90% of activity) in a range of temperature from 5 to 55ºC after 15 minutes incubation. At low temperatures (-5ºC), the crude enzyme preparation retained 96.8% of its activity after 2 months. The enzyme was stable between pH 7.0 and 9.0 after 1 hour of incubation.

C. cellulans is a very promising strain for biotechnological application. The alkaline protease of this microorganism was produced in a low cost medium, providing a novel and effective alternative for the production of a higher value product.

RESUMO

Produção de protease alcalina por *Cellulosimicrobium cellulans*

Cellulosimicrobium cellulans é um microrganismo que produz uma variedade de enzimas que hidrolisam a parede celular de leveduras: β-1,3-glucanase, protease e quitinase. Células desidratadas de *Saccharomyces cerevisiae* foram usadas como fonte de carbono e nitrogênio para o crescimento celular e produção de protease. Os componentes do meio de cultura: KH_2PO_4, KOH e células de levedura desidratadas mostraram efeitos significativos ($p<0,05$) no planejamento experimental fracionário. Um segundo planejamento foi preparado usando dois fatores: pH e porcentagem de células de levedura desidratadas. Os resultados mostraram que o meio de cultura para a produção máxima de protease foi 0,2 g/L de $MgSO_4.7H_2O$; 2,0 g/L de $(NH_4)_2SO_4$ e 8% de células de levedura desidratadas em tampão fosfato 0,15M e pH 8,0. A produção máxima de protease alcalina foi 7,0 ± 0,27 U/mL no ponto central. A protease bruta apresentou atividade ótima a 50ºC e pH 7,0-8,0; e foi estável a 50ºC.

Palavras-chave: superfície de resposta, otimização de meio, protease alcalina, *Cellulosimicrobium cellulans*, actinomiceto.

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