Production, characterization, and immobilization of protease from the yeast *Rhodotorula oryzipicola*

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Abstract

The protease was produced extracellularly in submerged fermentation by the yeast *Rhodotorula oryzipicola* using different sources of nitrogen and maximum activity (6.54×10^{-3} U/mg) was obtained in medium containing 2% casein (w/v). Purification of the protease by gel filtration chromatography resulted in a 3.07-fold increase of specific protease activity. The optimal pH and temperature for enzyme activity were 6.51 and 63.04 °C, respectively. Incubation in the presence of some salts enhanced enzyme activity, which peaked under 0.01 M BaCl₂. The enzyme retained about 90% of enzymatic activity at temperatures 50–60 °C. The commercially

available enzyme carriers evaluated, silica gel, Celite 545, and chitosan effectively immobilized the protease. The enzyme immobilized in Celite 545 retained 73.53% of the initial activity after 15 reuse cycles. These results are quite promising for large-scale production and immobilization of protease from *R. oryzipicola*, as the high operational stability of the immobilized enzyme lowers production costs in biotechnological applications that require high enzymatic activity and stability under high temperatures. © 2020 International Union of Biochemistry and Molecular Biology, Inc. Volume 0, Number 0, Pages 1–11, 2020

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1. Introduction

Enzymes are used as catalysts to induce chemical reactions and boost production processes. They are used in several chemical and industrial processes, such as tanning, brewing, baking, animal feed, and biofuel production [1]. The enzyme market was responsible for generating about US\$ 4.61 billion in 2016, and this value is expected to reach US\$ 6.30 billion in

2022. This growing demand is related to the expansion of food-processing facilities and the increasing number of tanneries and textile-manufacturing facilities [2].

Brazil is an enzyme-importing country, and enzymes are still scarcely used in industrial processes when compared to other countries. Thus, there is an urge to close this gap and establish Brazil as a major player in enzyme technology [3].

Enzymes can have a key role in many industries that strive to implement processes that meet the demands of a rapidly growing population, while dealing with the depletion of natural resources [4]. The use of enzymes allows industries to address both regulations and customer demands for clean technologies and clean, sustainable production methods, altogether resulting in low environmental impacts. Such strategy provides a viable solution for the next generation of production and manufacturing due to reduced costs, energy expenditures, use of water and chemicals, and production time [5–7]. The growing worldwide awareness towards the need

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Highlights

- *Rhodotorula oryzzicola* was used to obtain protease.
- Protease was purified by gel filtration chromatography.
- Silica gel, Celite, and chitosan effectively immobilized the protease.
- Protease immobilized in Celite retained 73.53% of the initial activity after 15 reuse cycles.

for less pollution and cleaning the environment has favored the gradual replacement of chemical processes by enzymatic processes [3,8].

Enzymes of microbial origin have significant potential in waste management and consequently in the development of the environment, unlike enzymes of plant origin that need large planting areas and have greater requirements as to climatic factors and seasons for their development [4,8].

Microorganisms are the main source of these enzymes, as they are able to use a vast array of low-cost, widely available substrates and are easily grown on a large scale, allowing for high-yield enzyme production through faster, easier, and lower cost processes, and releasing an impressive amount of biocatalysts for different culture media [9–12].

Among the various types of enzymes, proteases have stood out in the industrial environment, leading the market in 2016 [1]. There are already studies indicating that the addition of protease in fermentations, such as corn fermented to ethanol, can generate potential savings due to lower costs involved [13].

Proteases are responsible for catalyzing the hydrolysis of protein molecules into large fragments, and peptidases hydrolyze these polypeptide fragments into amino acids. They can be classified as acidic, neutral, or alkaline proteases according to the pH at which they become active [14, 15]. Due to the industrial demand for proteolytic enzymes with high specificity and stability, the search for new sources of these enzymes continues to be encouraged [16].

The use of yeasts as a source of biocatalysts has some advantages, such as production of high concentrations of enzymes, shorter fermentation cycles, use of low-cost fermentation media, and high diversity of enzymes that catalyze the same reaction, which allows flexibility in conditions of use and low pathogenicity [17].

The ability of the genus *Rhodotorula* to produce different enzymes of industrial interest is known within the scientific community. These enzymes include phenylalanine ammonium lyase [18], fructosyltransferase [19], β -fructofuranosidase [20], superoxide dismutase, catalase [21], and laccase [22], with main applicability to the food industry. Moreover, production of proteolytic enzymes has been reported for these species *R. mucilaginosa* [23–26], *R. glutinis* [24, 27], and *R. minuta* [24].

The species *R. oryzzicola* was isolated in the semiarid region of Bahia, with few records available so far. There are reports in the literature on its ability to produce cellulase, an

enzyme characterized by its stability at high temperatures and activity at neutral pH [28]. Thus, this study aims to test the ability of *R. oryzzicola* to produce proteases in different conditions, purify, characterize, and immobilize them in order to improve their stability at different pH and temperature values and, by enabling biocatalyst reuse, to develop more effective protease-based catalyzed processes.

2. Materials and Methods

2.1. Microorganism used

Samples of *R. oryzzicola* for fermentation runs were obtained from the Culture Collection of Microorganisms, from Bahia, of the State University of Feira de Santana (UEFS). The yeast was grown in YM agar medium (0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 1% glucose, 2% agar, and chloramphenicol) at 28 °C for 72 H. All experiments were performed in triplicate.

2.2. Selection of medium for the fermentation process

The yeast *R. oryzzicola* was fermented in different media containing gelatin and casein, according to the following formulations: (i) casein medium containing glucose, peptone, yeast extract, and casein; (ii) malt extract medium with 2% malt extract and 1% gelatin; (iii) Sabouraud medium with peptone and glucose. The formulation that promoted the best result was used as basis to implement other statistical tests.

2.3. Fermentation

For inoculum standardization, the colonies were inoculated in a Petri dish containing YM agar medium and incubated at 28 °C for 72 H. After this time interval, the colonies were diluted in 0.45% saline solution in order to reach a standardized inoculum of 10^7 microorganisms upon spectrophotometric readings at 600 nm. Next, 10 mL of inoculum was inoculated in 100 mL of medium. Submerged fermentation was carried out at 28 °C under 100 rpm orbital shaking (TECNAL 720), and after 120 H the biomass was separated from the enzyme-rich supernatant by centrifugation (10,000g/10 Min/4 °C).

2.4. Determination of proteolytic activity

The casein digestion method was used to determine proteolytic activity [29]. The reaction mixture containing 100 μ L of enzymatic extract and 1 mL of 0.5% casein, prepared in sodium phosphate buffer (0.2 M, pH 6.0), was incubated for 20 Min at 35 °C. At the end of the reaction, an aliquot of 100 μ L of the mixture was collected and added to 5 mL of the Bradford reagent [30], followed by a spectrophotometer (Varian-Cary 50) reading at 595 nm.

One enzyme unit (U) was defined as the amount of enzyme as necessary to consume 1 μ g of casein per minute (expressed as U/mL). The specific activity was determined by the ratio between enzyme activity (U/mL) and protein concentration (mg/mL) and was expressed as U/mg. All measurements were performed in triplicate.

2.5. Purification

The enzyme was purified from the culture filtrate using 70% ethanol solution and Sephacryl-S200 column chromatography.

Seventy percent of ethanol solution was added to 3 mL of the crude enzyme extract in a 2:1 (v/v) ratio. The tubes containing the solution were left to stand for 15 Min, after which they were centrifuged at 10,000g, at 4 °C for 15 Min. The supernatant was discarded, and 50 mM phosphate buffer was added in the same amount as the initial extract to resuspend the precipitate.

After ethanol precipitation, 1 mL of the solution containing the enzyme sample was applied to the Sephacryl – S200 column (1.1 × 20 cm) using the Akta Prime system (Amersham Bioscience®) equilibrated with 50 mM sodium phosphate buffer, pH 7.0.

2.5.1. Determination of total proteins

The total protein concentration throughout the different steps in protease purification was determined using the Bradford method [30], with bovine serum albumin as reference protein for the standard curve.

2.6. Determination of optimum pH and temperature

Response surface methodology was used to predict the effect of operational pH and temperature in enzyme activity. The Doehlert experimental design had two variables, activity temperature and pH, and three replicates at the center of the domain, totaling nine experiments. Casein (0.5%) was prepared in sodium phosphate buffer for the pH values: 5.5, 6.0, 6.5, 7.0, and 7.5. The experiments were carried out at different temperatures for 20 Min, and the specific enzyme activity (U/mg) was quantified at each point.

The data obtained were transformed into relative proteolytic activity for plotting the graphs. Experimental errors were evaluated based on the replication of the central point. The experimental data were processed using the STATISTICA software version 7.0, where the lack of fit of the model to the experimental data was also tested through analysis of variance (ANOVA).

2.7. Effect of ions on enzyme activity

The following salts were added to the reaction medium, containing casein as substrate: magnesium chloride (MgCl₂), calcium chloride (CaCl₂), sodium chloride (NaCl), potassium chloride (KCl), ammonium chloride (NH₄Cl), barium chloride (BaCl₂), cobalt chloride (CoCl₂), aluminum sulfate (Al₂(SO₄)₃), magnesium sulfate (MgSO₄), ferrous sulfate (FeSO₄), manganese sulfate (MnSO₄), ammonium sulfate ((NH₄)₂SO₄), potassium sulfate (K₂SO₄), and sodium sulfate (Na₂SO₄) in concentrations of 0.1 M and 0.01 M. Activity without the addition of ions was set at 100%. The enzymatic activity was determined as described in Section 2.4.

2.8. Thermostability

Thermostability was assessed by collecting enzyme samples at different time intervals (0, 10, 20, 30, 40, 50, and 60 Min)

upon incubation at different temperatures (50, 60, 70, 80, and 90 °C). After this incubation period, the test tubes containing the enzymes were cooled in an ice bath and enzyme activity was evaluated as described in Section 2.4.

2.9. Enzyme immobilization

Three commercially available carriers were tested for enzyme immobilization: silica gel, Celite 545, and chitosan (Sigma Chemical Co., St Louis, MO, USA). 0.5 mL of 50 mM phosphate buffer pH 6.5 and 0.10 g of each support to be tested were added to 1 mL of crude enzyme extract of protease. The suspension was thoroughly mixed by stirring (100 rpm) at 25 °C for 120 Min. Carrier particles containing the immobilized enzyme were allowed to settle by gravity, the supernatant was removed with a micropipette, and the immobilized enzyme was washed twice with 2.5 mL of the same buffer. After sedimentation, the supernatant was removed with a micropipette. The amount of proteins in the supernatants and the enzyme activity of the immobilized crude extract (CE) were evaluated.

Enzyme immobilization was evaluated according to the following parameters:

- (1) Yield of the immobilization process (%):

$$YP = \frac{\text{Activity of the immobilized CE}}{\text{Activity of the CE}} \times 100$$

- (2) Protein retention (%) (amount of immobilized enzyme):

$$PR (\%) = \frac{\text{Protein in the supernatant}}{\text{Total protein in the CE}} \times 100$$

2.9.1. Determination of the optimum temperature and pH of the immobilized enzyme

The proteolytic activity of the immobilized enzyme was evaluated at different temperatures (50, 70, and 90 °C) and at different pH values (5.5, 6.0, 6.5, 7.0, 7.5), with casein solution (0.5%) in 0.2 M sodium phosphate buffer. The experiments were carried for 20 Min, and the specific enzyme activity (U/mg) was quantified at each point. The data obtained were transformed into relative proteolytic activity for plotting the graphs.

2.9.2. Reuse of the immobilized enzyme

The reuse of the immobilized enzyme was carried out in 20 Min batch runs. After each run (cycle), the immobilized enzyme was washed twice with 2.5 mL of 50 mM phosphate buffer, pH 6.5. After sedimentation, the supernatant was removed with a micropipette and the enzymatic activity of the immobilized crude extract was performed according to Section 2.4. The result was presented in relative activity (%), and the activity in the first cycle was set at 100%. All tests were performed in triplicate.

2.9.3. Thermostability of the immobilized enzyme

The thermostability of the immobilized enzyme was evaluated by collecting biocatalyst samples at different time intervals (0, 10, 20, 30, 40, 50, and 60 Min) upon incubation at different temperatures (50, 60, 70, 80, and 90 °C). After this incubation

TABLE 1
Comparison of *R. oryzzicola* enzymatic activity after two purification steps

	Total protein (mg)	Activity (U/mL)	Specific activity (U/mg)	Purification fold
Crude extract	1.97	0.1262	6.54×10^{-3}	1.00
Gel filtration	0.23	0.403	2.01×10^{-2}	3.07

period, biocatalyst activity was evaluated as described in Section 2.4.

3. Results and Discussion

3.1. Microorganism and selection of the culture medium for fermentation

The genus *Rhodotorula* has been shown to be a producer of proteolytic enzymes, hence strains from this genus are sound candidates to screen for protease activity [23,31–33]. The yeast *R. oryzzicola* was selected for the production of protease because it was previously isolated from an environment that experiences extreme climate conditions that change during the year. In order to thrive under such harsh conditions, the metabolic apparatus of this yeast is likely to be adapted to the production of biomolecules, such as enzymes, resistant to different temperatures, an attractive feature for industrial application. Accordingly, *R. oryzzicola* has been shown to produce a thermophilic cellulase but its potential as suitable source of enzymes for industrial application is vastly unexplored [34]. In this study, it was possible to evidence the ability of *R. oryzzicola* to produce proteases.

Among the three different media used for the production of protease, the casein-containing medium allowed the highest expression of proteases, with specific activity of 6.54×10^{-3} U/mg, followed by malt extract medium (specific activity of 5.7×10^{-3} U/mg) and by peptone-containing medium (4.6×10^{-3} U/mg). In the study by Chandrasekaran and Sathiyabama [35], casein at a concentration of 1% was selected as a suitable inducer for protease production, while other inducers tested led to low levels of protease production.

The production of proteases generally requires complex sources of nitrogen, such as casein, peptone, or yeast extract. The protease of *R. mucilaginosa* was significantly secreted in the presence of peptone, while enzyme activity increased by the interaction between low concentrations of glucose and peptone [23].

Previous studies showed that protease production by *R. mucilaginosa* was dependent on the composition of the medium, the optimal production conditions involving the use of peptone, casein, and glucose as nitrogen and carbon sources [33]. The availability of nutrients in the culture medium can also regulate the expression and secretion of different sets of proteases, as well as their biochemical properties, through the use of different types of substrate as source of carbon and nitrogen [36, 37].

Thus, it was shown that different compositions of the fermentation medium can directly influence enzyme production by the same microbial species. This still corroborates the work by Chaud et al. [23] who suggested that microorganisms have individual nutritional preference for maximum enzyme production.

3.2. Purification of the enzyme

The enzyme was purified in two steps with a final specific activity of 2.01×10^{-2} (U/mg). Purification with gel filtration proved to be efficient, as it increased the specific activity of the enzyme till 3.07-fold over the levels in the crude extract (Table 1).

After ethanol precipitation and Sephacryl-S200 column chromatography, spectrophotometric reading at 280 nm allowed to identify the significant absorbance peak in fractions 17–22. The same range of fractions depicted noticeable proteolytic activity, whereas other fractions did not depict proteolytic activity (Fig. 1).

Proteases of *Graphium putredinis* and *Trichoderma harzianum* were purified using ethanol precipitation and column chromatography, allowing for a recovery factor of up to 36.49% of the protein with purity increased up to 18.80-fold [38]. The alkaline protease produced by *Beauveria* sp. was purified by ammonium sulfate precipitation and ion exchange chromatography, allowing for a 10.2-fold increase in purity and 38.6% protein recovery [39].

3.3. Effect of pH and temperature on protease activity

Each enzyme has an optimum pH and temperature. An enzyme can undergo ionizations and acquire momentary charges, as well as suffer the rupture of bonds due to the temperature of the medium leading to loss of catalytic activity [3]. The characterization of enzyme activity regarding this operational variable is paramount to identify the conditions that maximize activity.

The enzymatic assay at different incubation temperatures and pH values allowed to establish that the activity of the protease produced by *R. oryzzicola* peaked at 60 °C and pH 6.5 (Table 2). Beyond these temperature and pH values, a decrease in proteolytic activity was observed, which is related to protein denaturation/unfolding. The pH of the reaction medium has been shown to modulate the activity of proteases [40].

This model was also used to generate response surface graphics. The statistical analysis of the effects of pH and temperature showed that temperature positively influenced

FIG. 1

Purification of *R. oryzae* protease on Sephacryl S-200 column.

TABLE 2

Doeblert experimental design used to optimize the production of R. oryzae protease

Experiment	Temperature (°C)	pH	Enzyme activity
1	80	6.0	0.007295238
2	80	7.0	0.007142857
3	60	5.5	0.00372619
4 (C)	60 (C)	6.5 (C)	0.011328571
5 (C)	60 (C)	6.5 (C)	0.011235714
6 (C)	60 (C)	6.5 (C)	0.010578571
7	40	7.5	0.005628571
8	40	6.0	0.006771429
9	40	7.0	0.00495

Abbreviation: (C): Central point,

enzyme activity at a significance level of 5% ($P < 0.5$), as can be seen in the Pareto diagram (Figs. 2A and 2B). The interaction of the two variables did not show a positive result for the proteolytic activity of the enzyme.

Using the STATISTICA 7.0, it was possible to establish that optimal proteolytic activity occurred at 63.04 °C and pH of 6.51. Bearing in mind that a major challenge for the industry is to find enzymes that can withstand harsh conditions such as temperature and pH shifts [3], the present work that seeks to identify different pH and temperatures where enzymes are

active is expected to contribute to bring the focus of research centers closer to industrial needs.

The model used is predictive because the regression coefficients were highly significant (21.36 and 9.48, respectively, for calculated F and tabulated F), and the percentage of variation computed by the model proved to be adequate ($R^2 = 97\%$). As for the lack-of-fit test, the calculated F (9.01) was lower than the tabulated F (18.81), indicating that the residues did not significantly interfere with the results obtained (Table 3).

3.4. Effect of salts on protease activity

Enzymatic activity can be altered in the presence of some salts; they can increase the hydrolytic power or reduce the activity of enzyme, a behavior also reported when proteases are involved [39,41,42].

Enzymatic activity was measured in the presence of each salt used at concentrations of 0.01 M and 0.1 M. There was an increase in enzyme activity at 0.01 M concentration, while at 0.1 M concentration of some salts inactivated the enzyme. Table 4 shows percentages with standard deviations of the residual activity of the enzyme resulting from the addition of different salts.

A positive effect on the proteolytic activity was noticeable for different salts at a concentration of 0.01 M. Thus, in the presence of barium chloride and of manganese sulfate, the proteolytic activity increased up to 50% and 14%, respectively, as compared to the activity without additives. When the concentration of salts was increased to 0.1 M, barium chloride continued to act as a cofactor, but with an increase in activity of only 6%. Enzyme activity was reduced in the presence of the remaining salts, up to 73.4% in the presence of calcium chloride. Magnesium chloride and potassium chloride inactivated the enzyme.

TABLE 3 ANOVA for optimization of protease activity

Variation source	SQ	DF	QM	F calculated	F tabulated (CI 95%)
Regression	0.10	5	0.019269	21.36466	9.01
Residual	0.00	3	0.000902		
Lack-of-fit	0.00	1	0.002234	9.482419	18.81
Pure error	0.00	2	0.000236		
SQ total	0.10				

Abbreviations: CI, confidence interval; DF, degrees of freedom; QM, quadratic mean; QS, quadratic sum.

The catalytic activity of the protease produced by *Cryptococcus* sp. was increased by about 22%, upon addition of $MgCl_2$ to the reaction medium, compared to the control without additives, whereas Ca^{2+} had negligible influence on the enzyme activity [16]. The proteolytic activity of the enzyme produced by *R. mucilaginosa* decreased by 20% in the presence of NaCl at the concentration between 0.5 and 1.0 M. However, it remained stable without losing activity at higher concentrations of NaCl [33]. Stability and increased activity of the enzyme in the presence of $BaCl_2$ and $MnSO_4$ at low concentrations makes the use of these salts suitable as additives for future formulations for the production of protease by *R. orydicola*.

3.5. Thermostability

As for the thermostability of the protease, long-term residual activity tests were carried out in the temperature range from 50 to 90 °C. The protease of *R. orydicola* presented a good thermostability profile, retaining about 90% of activity at temperatures between 50 and 60 °C, while activity was reduced to 75% at 70, 80, and 90 °C (Fig. 3).

Thermophilic microorganisms are obvious sources of thermostable enzymes. However, these enzymes can also be obtained from mesophilic and even psychrophilic microorganisms. This was corroborated by Lario et al. [33] who investigated the features of the protease of *R. mucilaginosa*, a yeast isolated from an Antarctica seaweed from, and observed that the enzyme had optimal activity at 50 °C.

The protease of *R. orydicola* showed to be suitable for industrial applications, which generally require proteases that are both active and stable under relatively hostile conditions, often comprising extremes of temperature and pH [43].

4. Enzyme Immobilization

Enzyme activity was evaluated on all carriers used for immobilization. Irrespectively of the carrier used, immobilized enzyme activity exceeded that of the free enzyme (Table 5). This result is promising because immobilization by adsorption can be easily performed. As the immobilized enzyme can be

TABLE 4 Evaluation of enzymatic activity under the effect of different salts at a concentration of 0.1 M and 0.01 M. The control activity was taken as 100%

Salt	Residual activity % (average + DP)	
	0.01 M	0.1 M
Control	100	100
Na ₂ SO ₄	90.71 ± 0.0002	93.48 ± 0.0008
K ₂ SO ₄	79.86 ± 0.0001	94.91 ± 0.0001
(NH ₄) ₂ SO ₄	90.54 ± 0.0009	63.7 ± 0.0001
MnSO ₄	114.28 ± 0.0001	44.59 ± 0.0006
FeSO ₄	83.57 ± 0.0007	83.07 ± 0.0001
MgSO ₄	84.28 ± 0.0001	59.24 ± 0.0002
Al ₂ (SO ₄) ₃	106.96 ± 0.0002	73.11 ± 0.0007
CaCl ₂	135 ± 0.0001	26.6 ± 0.0007
NaCl	21.42 ± 0.0001	33.33 ± 0.0002
MgCl ₂	69 ± 0.0004	–
KCl	107 ± 0.0001	–
NH ₄ Cl	104 ± 0.0008	46.66 ± 0.0004
BaCl ₂	150 ± 0.0002	106.66 ± 0.0004

–: absence of enzymatic activity.

TABLE 5 Total protein concentration in the supernatant after immobilization and enzyme activity of free and immobilized protease by *R. orizycola* adsorption. The activity of the free enzyme was set at 100%

Sample	Total protein	Enzyme activity (U/mg)
Free enzyme	61.85 ± 0.322	3.6 × 10 ⁻³ ± 0.008
Silica gel	39.37 ± 0.380	1.07 × 10 ⁻² ± 0.008
Celite 545	46.30 ± 0.972	1.11 × 10 ⁻² ± 0.018
Chitosan	32.78 ± 0.0503	5.44 × 10 ⁻² ± 0.089

TABLE 6 Yield of immobilization of *R. orizycola* protease by adsorption on different inorganic supports

Support	Process yield (%)	Protein retention (%)
Silica gel	122.53	36.79
Celite 545	123.85	25.15
Chitosan	262.32	47

by roughly 1.3-fold and 1.9-fold in silica gel and Celite 545, respectively (Table 6).

Chitosan is a natural polymer that has been widely used as a carrier for enzyme immobilization [44]. Such wide use of chitosan can be ascribed to the ability of the polymer to retain twice as many enzymes due to the existence of some hydroxyl and amino groups that bind to the enzyme more easily, and also to the high porosity of chitosan [45–47]. These characteristics may have influenced the activity of the protease produced by *R. orizycola*, increasing it by up to 262% compared to the control.

The silica used in the immobilization of enzymes is an attractive support to the industrial sector due to its toxicological safety and its ability to promote the stability of the enzyme and its reuse [48] besides long-term durability and efficiency [49]. Moreover, silica-immobilized enzymes have been shown to outperform the free form, as reported by Soleimani et al. [50].

The use of Celite 545 as protease carrier proved more promising than other types of inorganic supports [51]. Celite 545 is a highly porous material, with low cost and large adhesion area, also showing chemical inertia and has been used to immobilize different enzymes with excellent results [52–54].

To expand the large-scale use of immobilized enzymes requires the resolution of technological challenges, such as the constant search for new supports and immobilization protocols that maintain the catalytic activity of the enzyme after the immobilization process [55].

4.1. Reuse of the immobilized enzyme

Immobilized enzyme formulations were evaluated throughout for 15 reuse cycles (Fig. 4). Although the protease immobilized in chitosan showed excellent results in the hydrolysis of the

FIG. 3 Thermostability of *R. orizycola* protease.

reused for several catalytic cycles, there is also a reduction in the cost of producing and obtaining the enzyme.

The highest immobilization yield (262.32%) and protein retention (47%) were observed when protease was immobilized in chitosan, besting the immobilization yield in Celite 545 and silica gel by a little over twofold and protein retention,

FIG. 4

Operational stability of *R. oryzzicola* protease immobilized on different supports. Activity obtained in the first cycle was taken as 100% activity.

protein, it falls short in operational stability, since the residual activity of the immobilized enzyme decreased to 67% after the first cycle. After the second cycle, the chitosan-immobilized protease lost all catalytic activity, so the use of this enzyme formulation was discontinued.

The residual activity of the silica-gel based biocatalyst decreased to 22.9% in the 11th reuse cycle, and, like the chitosan support, its use was discontinued. The loss of activity of the immobilized enzyme can probably be due to conformational changes, instability enzyme, and leakage [56, 57].

The protease immobilized in Celite 545, on the other hand, showed good stability throughout the 15 reuse cycles, with a final residual activity of 73.53%. According to reports in the literature, immobilized enzymes that retain up to 50% of their initial activity after 10 successive reuse cycles are considered to have excellent operational stability [58, 59].

With the technique of enzymatic immobilization on solid supports and subsequent reuse of the enzyme, it is possible to reduce the costs of enzyme production, since new processes of enzyme production and purification become unnecessary [60].

The results confirmed that the protease produced by *R. oryzzicola* immobilized by adsorption on Celite 545 had increased stability and reuse. This support should therefore be used, as it is an excellent carrier for enzyme immobilization. Moreover, it has been suggested that carriers allowing for activity retention above 60% of the initial activity of the enzyme after 15 reuse cycles are recommended for further focused studies with enzyme immobilization [61].

4.2. Optimization of the pH and temperature of the protease produced by *R. oryzzicola* immobilized on Celite 545

The enzymatic assay under different temperatures and pH of incubation showed that the highest activity of the protease produced by *R. oryzzicola* immobilized on the inorganic support Celite 545 occurred at 60 °C and pH 6.5 (Table 7). At other

TABLE 7

Doehlert experimental design for optimization of the enzymatic activity of R. oryzzicola protease immobilized on Celite 545

Experiment	Temperature (°C)	pH	Enzyme activity
1	80	6.0	0.0428
2	80	7.0	0.0954
3	60	5.5	0.1166
4 (C)	60 (C)	6.5 (C)	0.2739
5 (C)	60 (C)	6.5 (C)	0.2368
6 (C)	60 (C)	6.5 (C)	0.2739
7	40	7.5	0.09980
8	40	6.0	0.1220
9	40	7.0	0.1146

temperatures, it was possible to observe a decrease in residual activity. Studies that seek to characterize the effect of temperature as well as the effect of pH on enzyme activity are important to determine the ideal conditions of the enzyme [62].

Based on the Doehlert matrix and using the STATISTICA 7.0, it was possible to establish that catalytic activity peaked at of 59.54 °C and pH 6.82. The optimum temperature of the immobilized enzyme was slightly lower than the optimum temperature of the free enzyme.

This matrix model was used to generate response surface graphics and a Pareto diagram, indicating that the optimal activity of the enzyme is located at the central point of the matrix and that both temperature and pH influence the activity of this enzyme at the level of significance of 5% ($P < 0.5$) (Figs. 5A and 5B).

The model used is predictive, since the values of the regression coefficients were highly significant (28.39 and 0.76, respectively, for calculated F and tabulated F), and the percentage of variation computed by the model proved to be adequate ($R^2 = 97\%$). As for the lack-of-fit test, the calculated $F = 9.01$ was smaller than the tabulated $F = 18.81$, indicating that the residues did not significantly interfere with the results obtained (Table 8).

4.3. Thermostability of the enzyme immobilized on Celite 545

The immobilized protease from *R. oryzzicola* displayed thermostability profile that was less favorable than that of the free enzyme. The protease of *R. oryzzicola* presented a good thermostability profile, retaining more than 90% of activity at 50 °C for up to 60 Min, at 60 °C, the residual activity was above 90% only for 20 Min, falling to 40% after 60 Min of incubation. At 70 °C, there was a 20% loss of residual activity in the first 10 Min, and the enzyme showed about 30% of residual

FIG. 5

(A) Response surface for optimization of protease characterization showing the effects of pH and temperature (°C) for immobilized enzyme. (B) Pareto chart of standardized effects of the variables studied in the planning—influence of pH and temperature on the proteolytic activity of the *R. oryzzicola* enzyme immobilized in Celite 545.

TABLE 8

ANOVA for optimization of protease activity immobilized on Celite 545 support

Variation source	SQ	DF	QM	F calculated	F tabulated (CI 95%)	R ²
Regression	0.13	5	0.026	28.39631	9.01	0.972599
Residual	0.00	4	0.000916			
Lack-of-fit	0.00	1	0.000744	0.764515	18.81	
Pure error	0.00	3	0.000973			
SQ total	0.13					

Abbreviations: CI, confidence interval; DF, degrees of freedom; QM, quadratic mean; QS, quadratic sum;

FIG. 6

Thermostability of immobilized *R. oryzzicola* protease in Celite 545.

activity after 60 Min at that temperature. At 80 and 90 °C, the enzyme showed activity only in the first 20 Min of incubation (Fig. 6).

This difference in stability may be related to the fact that the immobilized enzyme has a more rigid conformation, since it is linked to the support, and when denaturation occurs, the enzyme remains in this state, losing part or all of its catalytic activity. The free enzyme goes through a cooling process right after incubation at different temperatures, which can influence the native conformation of the enzyme, allowing it to exert its total or partial catalytic activity.

Crescimbeni et al. [63] suggested that high temperatures affect the dissociation state of the ionizable functional groups involved in enzyme activity and in the interaction with the substrate, leading to the denaturation and ultimate inactivation of the protease. Celite 545 likely lacks a good mechanism of heat absorption from the external environment, making it impossible to minimize the temperature increase inside the enzyme microenvironment, leading to its inactivation.

5. Conclusion

The present work focused on the production of proteases from the yeast *R. oryzzicola* isolated in the semiarid region of Bahia,

an unprecedented finding for the species, which proved to be a good producer of the enzyme of interest. The data found in this study indicate that the enzyme, in either free or immobilized form, can be used in biotechnological processes that require adverse operational conditions, since both had their optimum point of operation above 50 °C. The enzyme also showed good thermal stability, with a half-life of 60 Min.

The carrier selected for immobilization proved to be efficient, retaining up to 73% of catalytic activity, after 15 reuse cycles, without the need for new production, thus lowering the costs of enzyme production. Therefore, Celite 545 is suggested as a sound carrier for enzyme immobilization.

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7. Conflict of Interests

The authors declare that there are no conflicts of interest.

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