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Improvement in extracellular protease production by the marine antarctic yeast *Rhodotorula mucilaginosa* L7

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ABSTRACT

Microorganisms from extreme and restrictive eco systems, such as the Antarctic continent, are of great interest due to their ability to synthesize products of commercial value. Among these, enzymes from psychrotolerant and psychrophilic microorganisms offer potential economical benefits due to their high activity at low and moderate temperatures. The cold adapted yeast *Rhodotorula mucilaginosa* L7 was selected out of 97 yeasts isolated from Antarctica as having the highest extracellular proteolytic activity in preliminary tests. The present study was aimed at evaluating the effects of nutrient composition (peptone, rice bran extract, ammonium sulfate, sodium chloride) and physicochemical parameters (temperature and pH) on its proteolytic activity. A 2^{6-2} fractional factorial design experiment followed by a central composite design (CCD 2^3) was performed to optimize the culture conditions and improve the extracellular proteolytic activity. The results indicated that the presence of peptone in the medium was the most influential factor in protease production. Enzymatic activity was enhanced by the interaction between low glucose and peptone concentrations. The optimization of culture conditions with the aid of mathematical modeling enabled a c. 45% increase in proteolytic activity and at the same time reduced the amount of glucose and peptone required for the culture. Thus culture conditions established in this work may be employed in the biotechnological production of this protease.

Introduction

The biodiversity of restrictive ecosystems can be explored to obtain different products for use in a wide variety of processes. Due to the extreme climatic conditions in Antarctica, the development of any living form is highly limited [1–3]. Nevertheless, it is inhabited by a variety of microorganisms, which have created special strategies to survive [4]. Some of them are psychrotrophs, representatives of mesophilic species that grew during the Antarctic summer and were adapted to the cold weather, whereas obligatory psychrophilics require low temperatures for their growth [5,6]. In both cases, adaptation to low temperatures is achieved mainly by changes in enzyme activities. The enzymes from psychrophilic microorganisms possess higher catalytic activities at lower temperatures and have lower thermostability than their mesophilic counterparts, due to their higher molecular flexibility [4,7]. Proteases, lipases, xylanases, and amylases from microorganisms, such as filamentous fungi and yeasts, have shown considerable commercial potential as they are secreted to the extracellular environment and are easy to recover [6,8]. The use of enzymes from psychrophilic and psychrotrophic microorganisms in industrial processes can reduce electricity consumption and make the processes more cost-effective [9].

The worldwide sale of industrial enzymes was $4.5 billion in 2012 and may exceed $7.1 billion by 2018 [10]. Proteolytic enzymes are among the most important for industrial biotechnological applications, sharing approximately 60% of the global industrial enzyme market [11]. Proteases are mainly used in the detergent (primarily from the genus *Bacillus*), food, and pharmaceutical industries, fine chemicals, leather processing, and in industrial waste treatment [12–19]. They also carry potential for use in ethanol production and other industrial purposes [20]. The

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majority of yeasts produce acidic aspartyl proteases while a few produce serine peptidases or neutral and alkaline proteases [19]. Fungal proteases are mostly acidic in nature and hence preferred in the food and pharmaceutical industries [21]. The industrial production of yeast proteases has not been exploited to a large extent [22,23].

Many studies have evaluated the influence of nutritional conditions, such as the effect of glucose as carbon source supplemented by meat peptone and casein peptone as nitrogen source, on fungal protease production [24,25]. However, there are several studies that have tried diverse sources of carbon and nitrogen (sucrose, starch, glucose, fructose, peptone, yeast extract, sodium nitrate, and ammonium sulfate) as well as agro-industrial by-products (soybean meal, rice bran, and wheat bran) [21,22,26–30]. In addition, physicochemical parameters, such as temperature and pH, have also shown to enhance protease production by microorganisms [22,27,31,32].

The current study aimed to establish the optimal culture conditions for protease production by the marine Antarctic yeast *Rhodotorula mucilaginosa* L7. In addition, we evaluated the possibility of reducing the cost of the culture medium by using byproducts of food industry like rice bran and also the effect of different nitrogen sources (peptone, rice bran extract, and ammonium sulfate) and physicochemical parameters (temperature and pH) on the protease production.

Material and methods

Microorganism, inoculum, and culture conditions

The yeast strain, previously isolated from an Antarctic marine alga and taxonomically identified as *Rhodotorula mucilaginosa* on the basis of 26S rDNA gene, was used in this study [6]. The strain was deposited in the Brazilian Collection of Environmental and Industrial Microorganisms under the access number CBMAI 1528. The yeast was maintained on Sabouraud dextrose agar at 15 °C in the laboratory.

The inoculum was prepared from cells cultured for 24 h on Sabouraud glucose agar containing ($g\,L^{-1}$): agar 17, glucose 20, and peptone 10 at 25 °C (previously reported to be the optimum growth temperature for *R. mucilaginosa L7*). The freshly grown cells were transferred to a 1000 mL Erlenmeyer flask containing 400 mL Sabouraud glucose broth ($g\,L^{-1}$): glucose 20, peptone 10 at pH 5.5, and incubated at 25 °C in a rotatory shaker at 150 rpm. After 24 h culture, yeast cells were harvested by centrifugation at $2000 \times g$ for 15m. The cells were washed twice and resuspended in sterile water. The standardized inoculum of 1×10^8 cells mL^{-1} was transferred to 125 mL Erlenmeyer flasks containing 50 mL culture medium and incubated for 120 h under 150 rpm agitation. Aliquots were taken every 24 h, and the broth was centrifuged at $2000 \times g$ for 10 min to remove the cells. The supernatants were used to determine protein concentration, protease activity, pH and sugar content as functions of time

Experimental design

A 2^{6-2} fractional factorial design (Table S1) was used to assess the impact of the six independent factors (nutrient variation and physicochemical parameters) on the extracellular proteolytic activity of *R. mucilaginosa* L7. The initial values for each factor were taken from the literature. The 2^{6-2} fractional factorial design is a type of a two-level factorial design (+1 or −1) and requires fewer runs than the comparable fractional design. It also allows investigation of n^{-1} factors in at least n experiments [28,31–35]. The design matrix included data from triplicates to determine standard error. The design matrix was created by STATISTICA 7 from Statsoft Inc. USA and the standardized effect was calculated using following first-order polynomial model:

$$y = \beta 0 + \sum \beta i x i \quad (1)$$

where y is the predicted response, β0 is the model intercept, βi is the linear coefficient and xi is the independent variable level. The evaluated variables included pH and temperature of the medium, sodium chloride (NaCl) concentration and nitrogen source, i.e., two organic or complex sources (peptone and rice bran extract) and one inorganic source (ammonium sulfate) at different concentrations. The standard glucose concentration ($20\,g\,L^{-1}$) was used as carbon source in all assays.

Results from the above experimental design were used to establish the 2^3 statistical experimental design and the central composite design (CCD) with a star configuration. A total of six axial and three central points, making 17 experiments to improve the protease production by *R. mucilaginosa* L7, were used [36]. From these experiments a second-order model was derived to predict proteolytic activity based on concentrations of peptone or rice bran extract and glucose sources. The quadratic model used to predict the optimal point was as follows:

$$y = \beta 0 + \sum \beta i x i + \sum \beta i i x 2\ i + \sum \beta i j x i x j \quad (2)$$

where y is the predicted response, β0 is the model intercept, xi and xj are the independent variable levels, βi, βii, and βij are the linear, quadratic and interaction coefficients, respectively. The physicochemical parameters (pH 4.0 and temperature 10 °C for 120 h at 150 rpm) favoring the proteolytic activity during the 2^{6-2} fractional factorial design were used. Samples were collected at every 24 h.

Data were analyzed using STATISTICA software version 7.0. The feasibility of the second-order model equation was expressed by R^2 (coefficient of determination). The data between groups were analyzed using ANOVA followed by *F*-test. For the screened variables (2^{6-2} fractional factorial design) the level of significance was $P < 0.1$, and for the central composite design was $P < 0.05$. The response surface plot was generated to analyze the effect of independent variables on the proteolytic activity.

All experiments were performed in triplicate under optimized conditions and the adequacy of the model equation was confirmed. The values predicted were considered as controls.

Proteolytic activity

The proteolytic activity of yeast cell extracts was determined using azocasein as a proteinase substrate as described by Charney and Tomarelli [37]. 500 μL of culture supernatant were mixed with 500 μL of 1% (w/v) azocasein in 50 mM sodium acetate buffer (pH 5.0) and incubated at 37 °C for 40 min. The reaction was stopped by adding 500 μL trichloroacetic acid (10% w/v) and centrifuged at 4025xg for 15 min. The supernatant was collected and 500 μL was added to 500 μL 0.5 M KOH, mixed well and absorbance measured by spectrophotometer at 430 nm. The samples were assayed in duplicate and the activity was expressed in units of enzyme activity (U), where one unit indicates the amount of enzyme required to increase absorbance by 0.001 under conditions used [24,37]. The proteolytic activity of different samples was compared with the activity of purified protease ($1\,mg\,mL^{-1}$) determined by the same method.

Glucose concentration

Glucose concentrations were determined by HPLC using “Bio-Rad Aminex” HPX–87 H column kept at 45 °C; 0.01 N sulfuric acid

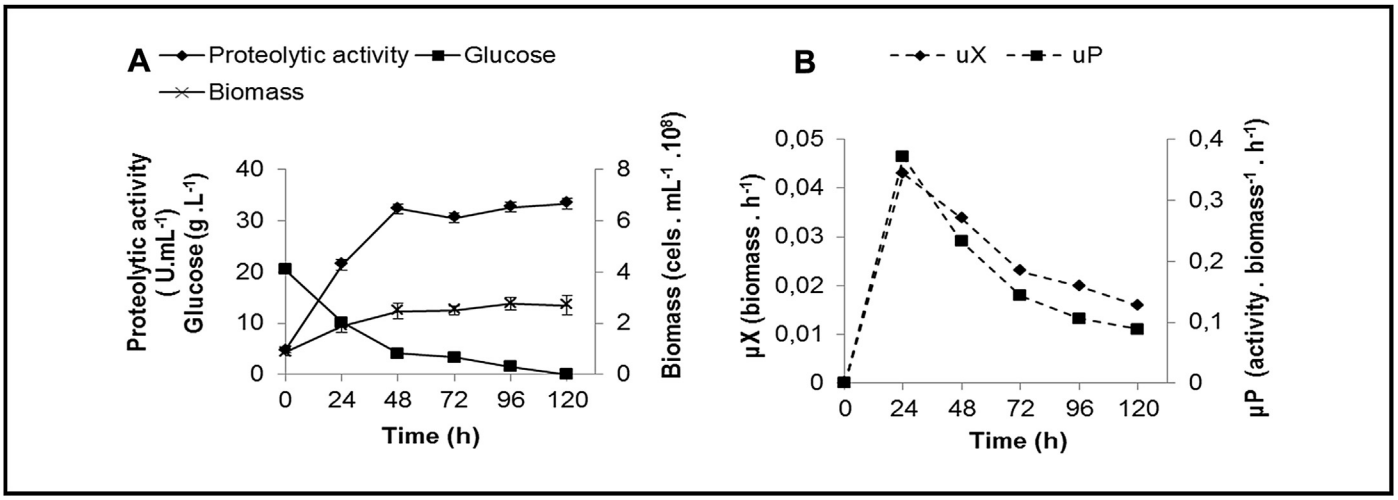

Fig. 1. (A) Profile of extracellular proteolytic activity ($U\,mL^{-1}$), glucose consumption ($g\,L^{-1}$) and growth ($cells\,mL^{-1} \times 10^8$) of *R. mucilaginosa* L7; (B) Kinetic profile of the biomass (μ_X) and protease production (μ_P). Both analyses were performed in Sabouraud glucose broth ($g\,L^{-1}$): glucose 20, peptone 10; at pH 5.5, 25 °C, in a rotatory shaker at 150 rpm, for 120 h with an inoculum of 1×10^8 cells mL^{-1}.

eluent, and 0.6 mL min^{-1} flow rate. Previously the samples were diluted and filtered on "Sep-Pack" C18 (Millipore).

Cell concentration

The cells were stained with methylene blue (0.01% w/v in sodium citrate) and the cell concentration (number of cells per mL) was measured using the Neubauer counting chamber [38]. Methylene blue was used to clarify yeast cells and to differentiate dead and living cells.

Results and discussion

R. mucilaginosa L7 was selected as having the highest extracellular proteolytic activity among 97 fungal strains isolated from the Antarctic [6]. In this study, the impact of variation in nutrient and physicochemical parameters on extracellular protease production was evaluated.

Maximum extracellular proteolytic activity recorded in basal medium (Sabouraud glucose broth) was 33.36 U mL^{-1}, achieved after 120 h of cultivation. This coincided with the stationary cell growth phase and the carbon source depletion (Fig. 1A). However, the maximum enzyme production rate was observed at 24 h of culture during the exponential microbial growth phase (Fig. 1B). These results showed that *in vitro* the proteolytic activity is directly correlated with maximum cell growth and glucose consumption (Fig. 1). Similar protease synthesis dynamics correlated with cell growth has been observed for *Leucosporidium antarcticum* psychrophilic yeast [22]. The results support the earlier findings that revealed the correlation between the stationary cell growth phase and the maximum proteolytic activity. This was also observed with different microorganisms, under different growth conditions [12,23,24,28,39]. According to Gupta et al., protease production is regulated by nutrient amount in the culture medium as well as biomass production [40]. Although peak protease production was observed at 48 h, in the following experiments proteolytic activity was determined during the stationary phase of growth, after 120 h of culture, due to the modifications performed in the culture conditions (pH value, temperature and medium composition) which could modify the growth rate of the yeast.

First, a 2^{6-2} fractional factorial design was used to evaluate protease production at different temperatures, pH values and

Table 1
2^{6-2} Fractional Factorial design to evaluate the influence of six variables on the extracellular proteolytic activity of proteases from *R. mucilaginosa* L7 after 120 h cultivation at 150 rpm. Glucose (20 g L^{-1}) was used as carbon source in all assays.

Run	pH	Temperature (°C)	NaCl ($g\,L^{-1}$)	Peptone ($g\,L^{-1}$)	Ammonium sulfate ($g\,L^{-1}$)	Rice bran extract ($g\,L^{-1}$)	Proteolyticactivity ($U\,mL^{-1}$)
1	4	10	0	0	0	0	0,88
2	8	10	0	0	2	0	0,00
3	4	25	0	0	2	20	0,15
4	8	25	0	0	0	20	0,00
5	4	10	5	0	2	20	1,29
6	8	10	5	0	0	20	0,85
7	4	25	5	0	0	0	0,24
8	8	25	5	0	2	0	3,63
9	4	10	0	10	0	20	23,98
10	8	10	0	10	2	20	8,84
11	4	25	0	10	2	0	3,49
12	8	25	0	10	0	0	5,19
13	4	10	5	10	2	0	13,09
14	8	10	5	10	0	0	3,00
15	4	25	5	10	0	20	7,43
16	8	25	5	10	2	20	8,78
17	6	17,5	2,5	5	1	10	3,40
18	6	17,5	2,5	5	1	10	2,88
19	6	17,5	2,5	5	1	10	2,97

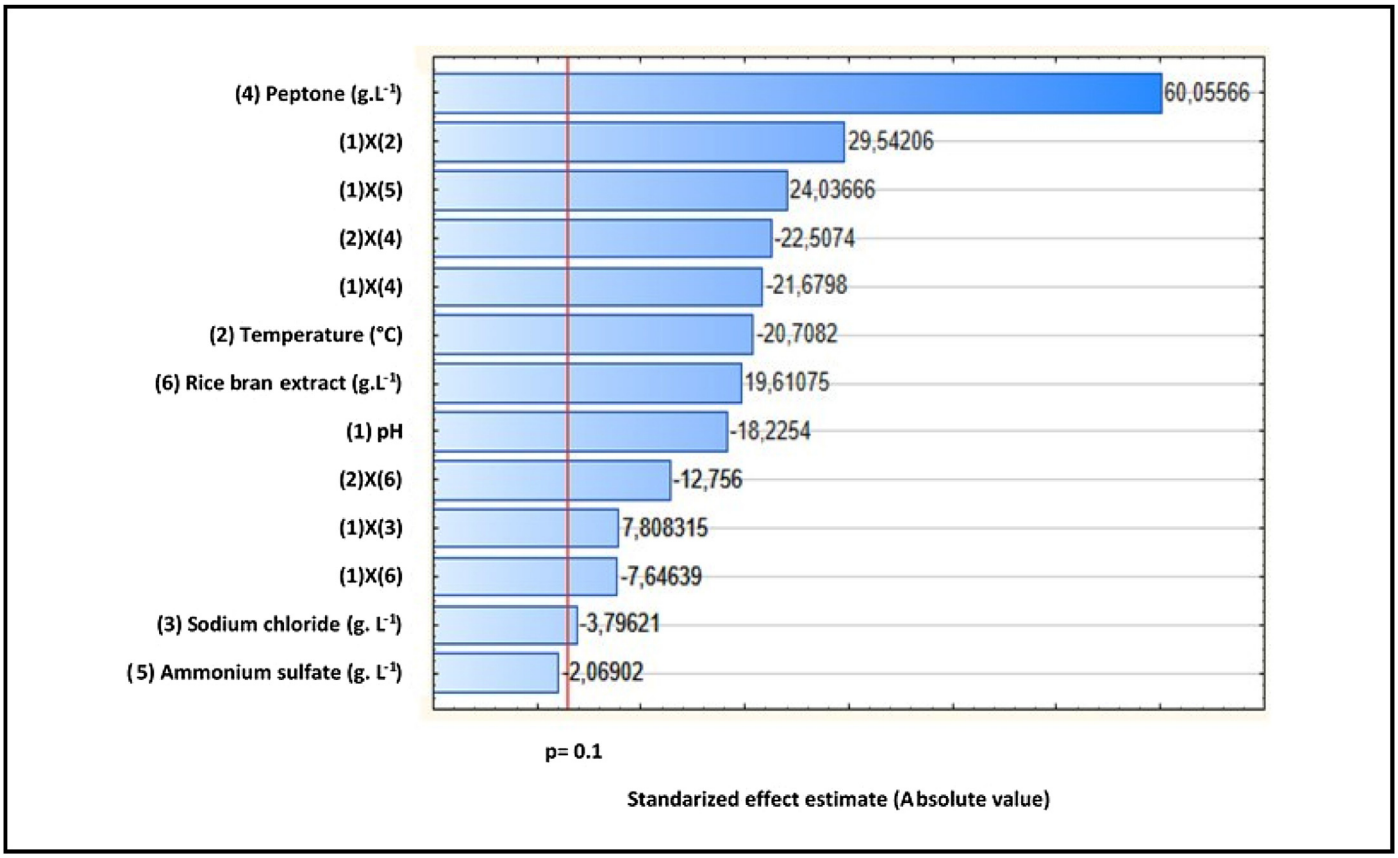

Fig. 2. Pareto diagram showing effects of six independent variables (in descending order of occurrence) on extracellular protease production by *R. mucilaginosa* L7. Standard glucose concentration (20 g L^{-1}) was used as carbon source in all assays in a rotary shaker at 150 rpm for 120 h.

concentrations of NaCl, peptone, ammonium sulfate and rice bran extract, as shown in Table 1. All assays were performed after 120 h of culture, in which glucose (20 g L^{-1}) was used as a carbon source. The maximum proteolytic activity (23.98 U mL^{-1} in run 9) was observed at pH4.0,10 °C, and at the maximal concentrations of peptone (10 g L^{-1}) and rice bran extract (20 g L^{-1}). Table 1 shows that the proteolytic activity was low when the organic nitrogen source (peptone) was absent from the medium. Interestingly, the addition of an inorganic nitrogen source such as ammonium sulfate did not enhance the protease activity. Similar results were also noted in an earlier study [34]. The production of proteases often requires complex nitrogen sources such as casein, peptone, yeast extract, bovine hemoglobin, and albumin [24,28,31,41]. However, protease production by the marine yeast *Aureobasidium pullulans* is better with an inorganic nitrogen source (sodium nitrate), whereas the organic sources including the complex ones impaired the enzyme production [33]. This suggests that each microorganism has its own nutrient preferences for maximum enzyme production.

All the employed variables except ammonium sulfate significantly influenced the protease production ($p < 0.1$) (Fig. 2). So far, no published study has employed the experimental design used here to optimize protease production by yeast. The majority have evaluated alkaline protease production by bacteria, mainly from the genus *Bacillus*. A recent study has applied this methodology to a new *Bacillus* strain and revealed that peptone (1.4 g L^{-1}) has significant effects on proteases production [42]. Thus, results of the present study corroborate these earlier findings.

The presence of rice bran extract in the culture medium was significantly correlated with the growth and protease production in *R. mucilaginosa* L7, which can be attributed to its high nutritional value [28]. The physicochemical parameters (pH and temperature) also showed significant effects (Fig. 2). The maximum protease production at low temperature (10 °C) in psychrotolerant yeasts suggests that enzymatic adaptation strategies such as structural changes might provide high enzyme activity [7,43]. On the other hand, the greater protease production at low pH during runs 9 and 13 suggests that the protease produced by *R. mucilaginosa* L7 could be acidic in nature. Protease production was not favored by NaCl (Table 1 and Fig. 2). However, it is worth mentioning that seawater contains many other salts besides NaCl. Studies in our laboratory on marine-derived filamentous fungi under salinity conditions showed that artificial seawater (ASW) was much better than NaCl-supplemented medium for marine-derived fungal cultivation (unpublished data).

From the results of the 2^{6-2} fractional factorial design, maximum protease production was obtained at pH value 4, temperature of 10 °C, 10 g L^{-1} peptone, 20 g L^{-1} rice bran extract, 20 g L^{-1} glucose and in the absence of NaCl and ammonium sulfate. Since a positive correlation was observed between peptone, rice bran and the protease activity, a new experimental design was devised to improve the production of the enzyme. For this, a 2^3 central composite design (CCD) with a total of 17 runs at the optimal conditions obtained in the previous experiments (pH value 4, 10 °C, during 120 h) was employed, with varying concentrations of glucose, peptone and rice bran extract. The results are presented in Table 2. The highest extracellular protease production, i.e., 39.18 U mL^{-1} was achieved during run 1, when concentrations of peptone, rice bran extract and glucose were 11, 14 and 14 g L^{-1}, respectively.

R. mucilaginosa L7 showed some statistically significant results ($p < 0.05$) in the regression coefficient for the model that predict

Table 2
2^3 Central composite design (CCD), with star configuration (six axial points) and three central points to increase culture conditions for *R. mucilaginosa* L7, after 120 h cultivation at pH 4.0 and 10 °C, at 150 rpm.

Run	Peptone ($g\,L^{-1}$)	Rice bran extract ($g\,L^{-1}$)	Glucose ($g\,L^{-1}$)	Proteoliticactivity ($U\,mL^{-1}$)	y predict	Absolut error	Relative Error
1	11	14	14	39,18	37,8	1,38	0,37765
2	14	14	14	36,98	34,82	2,155	0,34762
3	11	26	14	35,96	37,80	−1,845	0,37851
4	14	26	14	33,78	34,82	−1,04	0,34851
5	11	14	26	34,09	35,70	−1,605	0,35747
6	14	14	26	24,63	23,64	0,99	0,23599
7	11	26	26	35,69	35,70	−0,015	0,35700
8	14	26	26	22,56	23,64	−1,08	0,23688
9	10	20	20	38,54	36,83	1,7082	0,36782
10	15	20	20	24,02	24,10	−0,1782	0,24201
11	12,5	10	20	35,57	34,29	1,272984	0,34256
12	12,5	30	20	33,42	34,29	−0,872016	0,34318
13	12,5	20	10	38,82	39,31	−0,485136	0,39318
14	12,5	20	30	29,06	28,15	0,905064	0,28118
15	12,5	20	20	32,15	30,51	1,635	0,30459
16	12,5	20	20	26,90	30,51	−3,615	0,30644
17	12,5	20	20	31,29	30,51	0,78	0,30485

proteolytic activity such as glucose linear and quadratic terms, linear peptone, rice bran extract quadratic terms and peptone x glucose interaction (Table 3). The multiple regression analysis resulted in a second-order model; Eq. (3) evaluated protease activities according to the nitrogen sources (peptone and rice bran extract) and glucose as follows:

$$\text{Protease activity} = 30.51 - 3.76\ \text{peptone} + 1.34\ (\text{rice bran extract})^2 - 3.32\ \text{glucose} + 1.14\ (\text{glucose})^2 - 2.27\ (\text{peptone} \times \text{glucose}) \quad (3)$$

The statistical difference was analyzed by ANOVA (Table S2). The model is predictive, since the regression coefficient values were highly significant (23. 82 and 3. 2, respectively for *F* test and *F* tabulated values), and the percentage of variation computed by the model was suitable ($R^2 = 91\%$). This model was further used to generate response surface plots (Fig. 3). According to the response surface plots, proteolytic activity was high when low amounts of peptone and glucose were present in the culture medium (Fig. 3A). However, for the rice bran extract and peptone interaction, it remained high at peptone concentrations lower than half, i.e., $12.5\,g\,L^{-1}$ (Fig. 3B). This suggests that the rice bran extract and glucose may have different influence on the C:N (carbon:nitrogen) ratio. Several studies have shown that the presence of complex nitrogen source and glucose are key factors in protease production [40,42,44]. In addition, a few have demonstrated that higher concentrations of these nutrients reduce proteolytic activity in various microorganisms, which is mainly attributed to catabolic repression by glucose [28,33,34,39,45].

Finally, protease production was tested under the optimized cultivation conditions predicted by the second-order equation, to verify the adequacy of the model. *R. mucilaginosa* L7 was cultured in a medium composed of peptone, glucose, and rice bran extract (at concentrations of 10, 10, and $20\,g\,L^{-1}$, respectively) at pH 4.0 and temperature 10 °C, and aliquots were taken every 24 h. The highest proteolytic activity of $48.55\,U\,mL^{-1}$ was recorded after 96 h cultivation (Fig. 4). These data confirmed the adequacy of the model, since it produced a 45.53% (from 33.36 to $48.55\,U\,mL^{-1}$) increase in protease production from the initial production level in Sabouraud dextrose broth (at pH 5.5 and 25 °C after 120 h). Moreover, the optimal conditions predicted by this model increased proteolytic activity by 102.46% (i.e., from 23.98 to $48.55\,U\,mL^{-1}$) and 23.91% (i.e., from 39.18 to $48.55\,U\,mL^{-1}$) with respect to production under the optimal conditions during the first and second experimental designs, respectively, and at the same pH, temperature and culture duration. There was little difference in proteolytic activity obtained during culture in Sabouraud-dextrose medium at 25 °C for 48 h and 120 h culture ($32.28\,U\,mL^{-1}$ vs. $33.36\,U\,mL^{-1}$, respectively). However, when *R. mucilaginosa* L7 was cultured in the optimized medium composed of $10\,g\,L^{-1}$ of peptone, $10\,g\,L^{-1}$ of glucose and $20\,g\,L^{-1}$ of rice bran extract at 10 °C, the activity after 120 h of culture was higher than that recorded after 48 h of culture (16.75 and $47.92\,U\,mL^{-1}$ at 48 and 120 h, respectively). These results suggest that at 10 °C the growth of yeast was slower but the protease production was actually higher which could be attributed to adaptive strategies employed by this yeast to survive under extreme conditions in Antarctica.

The first experimental design demonstrated that rice bran had a positive effect on enzyme production. On the other hand, the second experimental design allowed us to reduce the glucose

Table 3
Regression coefficient for *R. mucilaginosa* L7 extracellular protease activity (nutritional conditions were set out in the matrix of the experimental design 2^3 Central composite design (CCD) (Table 2), after 120 h cultivation at pH 4.0 and 10 °C, at 150 rpm).

Factor	Regression coefficient	Standard error	*t*-value	*p*- value
Mean	30,14828	1,054198	28,59831	0,000000
Peptone (L)	−3,76434	0,495350	−7,59935	0,000126*
Peptone (Q)	0,28467	0,545716	0,52164	0,618015
Rice branextract (L)	−0,76979	0,495350	−1,55403	0,164125
Rice branextract (Q)	1,42465	0,545716	2,61061	0,034882*
Glucose(L)	−3,32179	0,495350	−6,70595	0,000276*
Glucose(Q)	1,22801	0,545716	2,25028	0,059174*
Peptone X Rice bran extract	−0,45375	0,646920	−0,70140	0,505695
Peptone X Glucose	−2,27625	0,646920	−3,51860	0,009746*
Rice bran extract X Glucose	0,74250	0,646920	1,14775	0,288781

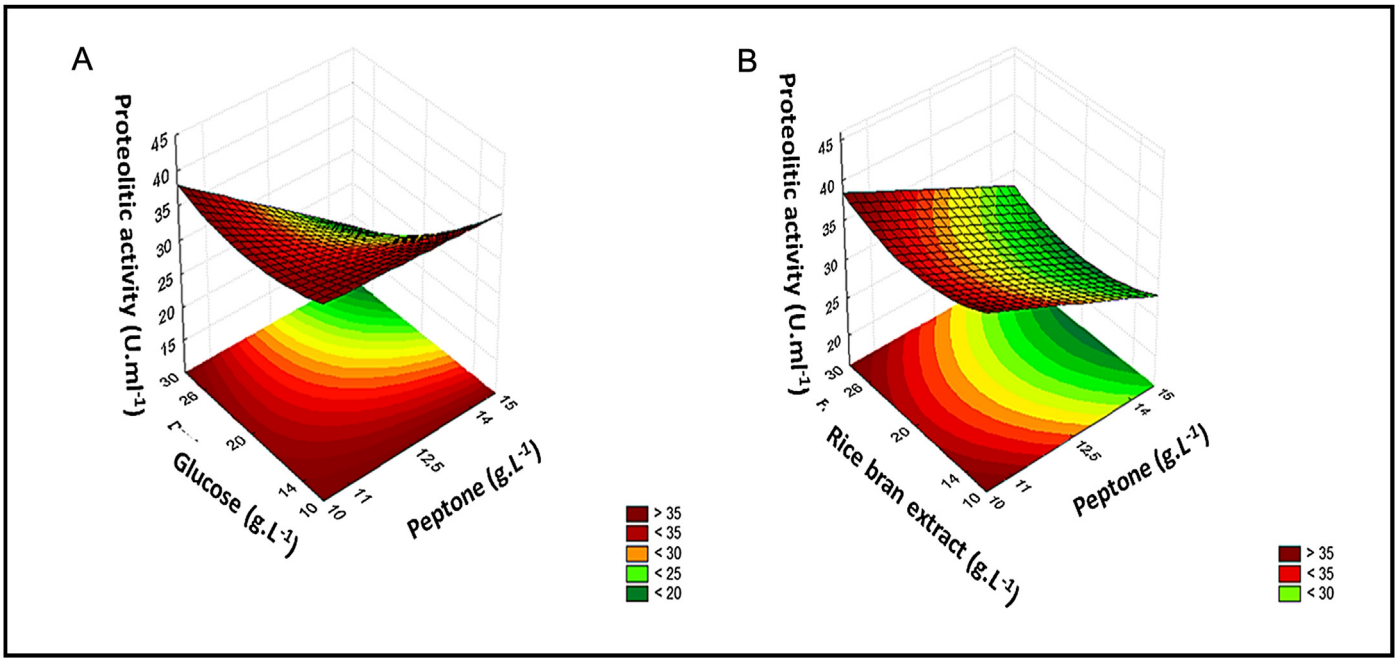

Fig. 3. Surface response plots and protease activity by *R. mucilaginosa* L7 in terms of interaction of glucose and peptone levels (A), and rice bran extract and peptone levels (B). Nutritional conditions set out in the matrix of the experimental design 2^3 CCD (Table 2).

concentration in the culture medium (from 20 g L^{-1} to 14 g L^{-1}) and further increased protease production by 63.18% compared to the first experimental design (23.98–39.18 U mL^{-1}). Similarly, in the optimized medium obtained with the statistical model (Fig. 4) protease production was increased when glucose and peptone concentrations in culture medium were reduced from 14 g L^{-1} to 10 g L^{-1} for glucose and from 11 g L^{-1} to 10 g L^{-1} for peptone. However, the concentration of the rice bran was increased from 14 to 20 g L^{-1}. From the economical and sustainable point of view, all these changes in the composition of culture medium are beneficial. Finally, the proteolytic activity after culture under optimal conditions suggested by the model (10 g L^{-1} of glucose, 10 g L^{-1} of peptone and 20 g L^{-1} of rice bran, pH 4 and 10 °C) were comparable at 96 and 120 h (see Fig. 4). Thus, it could be more convenient and economic to cultivate the yeast for 96 h rather than 120 h. Furthermore, the current study detected changes in the metabolic pathways of the yeast during the time it was kept on Sabouraud dextrose agar. There was decreased pigmentation over time as the characteristic salmon color of yeast at the beginning of the experiments appeared to be less intense or absent (Fig. 5). This might possibly be due to the lack of exposure to the Antarctic climatic conditions such as UV radiation and temperature, which resulted in adaptation of this yeast.

Conclusion

The current study has optimized culture conditions, including composition of nutrient medium (peptone, glucose, rice bran extract, NaCl, and ammonium sulfate) and physicochemical parameters (temperature and pH) to enhance protease production by *R. mucilaginosa* L7. The results indicated that protease production was influenced mainly by the presence of peptone in

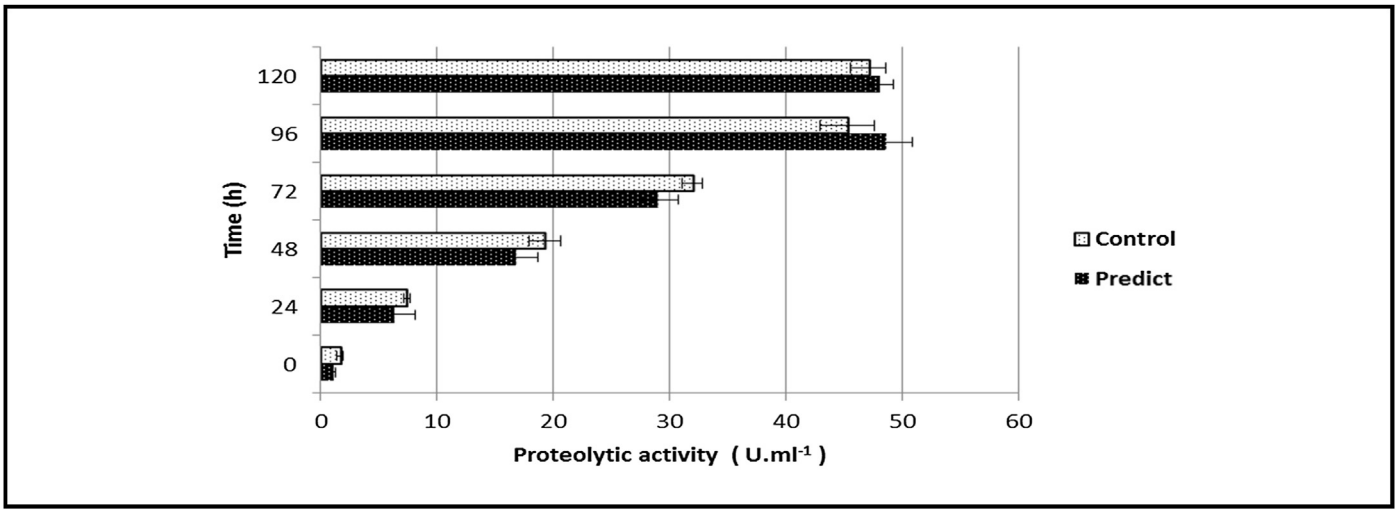

Fig. 4. Time-dependent extracellular proteolytic activity of *R. mucilaginosa* L7 under optimal conditions predicted by the model (composed of (g L^{-1}): peptone 10, glucose 10 and rice bran extract 20) (black bars) and the control experiment (CCD 2^3, run 1) composed of (g L^{-1}): peptone 11, glucose 14 and rice bran extract 14 (gray bars). Both experiments were performed at 10 °C, pH 4 and 150 rpm.

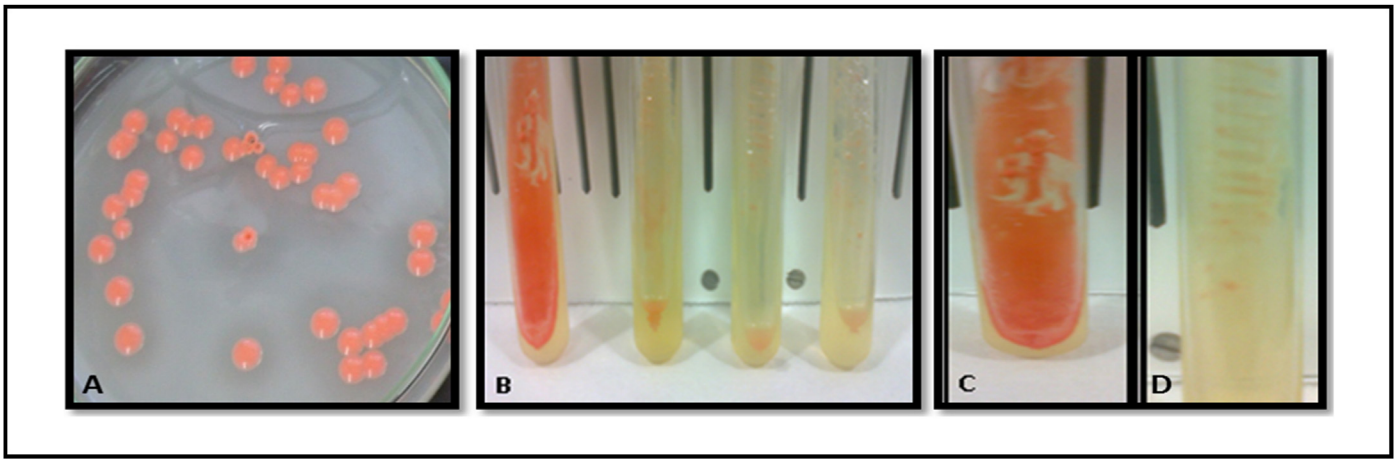

Fig. 5. Depigmentation of *R. mucilaginosa* L7 marine Antarctic yeast: (A) Colonies on Sabouraud agar with milk (10%) in Feb 2012; (B) Subcultures on Sabouraud agar over time (Feb 2012; Feb 2013; Mar 2013 and May 2013; (C) High magnification image of culture taken in Feb 2012; and (D) High magnification image of culture taken in May 2013.

the medium followed by temperature, rice bran extract, and pH. The protease produced after culture of yeast in nutrient medium, optimized with the help of mathematical model, showed a 45.53% increased proteolytic activity. The *R. mucilaginosa* L7 cultured in the laboratory showed adaptive properties, indicated by depigmentation over time possibly due to the absence of UV radiation. Although cultivation of Antarctic yeast is challenging, this work optimized culture conditions for *R. mucilaginous* L7 and its protease production. Further studies are required to establish a method of purification of the proteases and to evaluate their potential for industrial use.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nbt.2016.07.016.

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