Optimization of protease production and sequence analysis of the purified enzyme from the cold adapted yeast *Rhodotorula mucilaginosa* CBMAI 1528

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A R T I C L E   I N F O

A B S T R A C T

Enzymes from cold-adapted microorganisms are of high interest to industries due to their high activity at low and mild temperatures, which makes them suitable for their use in several processes that either require a supply of exogenous energy or involve the use of heat labile products. In this work, the protease production by the strain *Rhodotorula mucilaginosa* CBMAI 1528, previously isolated from the Antarctic continent, was optimized, and the purified enzyme analyzed. It was found that protease production was dependent on culture medium composition and growth temperature, being 20 °C and a culture medium containing both glucose and casein peptone (20 and 10 g/L, respectively) the optimal growing conditions in batch as well as in bioreactor. Moreover, mass spectrometry analysis revealed that the enzyme under study has a 100 % sequence identity with the deduced amino acid sequence of a putative aspartic protease from *Rhodotorula sp.* JG-1b (protein ID: KWU42276.1). This result was confirmed by the decrease of 95 % proteolytic activity by pepstatin A, a specific inhibitor of aspartic proteases. We propose that the enzyme reported here could be Rodothorulapepsin, a protein characterized in 1972 that did not have an associated sequence to date and has been classified as an orphan enzyme.

1. Introduction

In spite of severe environmental conditions, the Antarctic continent is inhabited by a highly diverse microbial population [1,2]. One of the most important challenges faced by the biota present there is to adapt to low temperatures by maintaining the rates of chemical reactions involved in the main cellular processes [3]. Some adaptations to these conditions are achieved by synthesizing cold active enzymes with more mobile or flexible catalytic centers than their mesophilic homologues, which allows them to have a higher specific activity at low temperatures and lower thermal stability [4]. Due to the possibility of reducing energy consumption, these enzymes are attractive for their use in industrial processes that involve the use of heat labile products and for those that require a supply of exogenous energy [5].

Antarctica is inhabited by a diverse group of fungi, of which the most abundant members are those of the genera *Cryptococcus*, *Penicillium* and *Rhodotorula*. These cold adapted cosmopolitan species were found mainly in association with seaweeds, due to the availability of organic substrates [6] and were found to be adapted to unfavorable conditions such as low temperatures, osmotic stress and high UV radiation [7,8].

Among the different species of the genus *Rhodotorula*, *R. mucilaginosa* has been receiving increasing attention due to its capability to grow in extreme ecosystems, and to produce valuable biotechnological products such as biosurfactants [9], proteases [10], lipases [11], carotenoids [12] and high levels of unsaturated fatty acids [13]. Moreover, it was found that *R. mucilaginosa* is able to remediate some specific contaminants [14], and that it

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represents an emergent opportunistic pathogen for humans and animals [15]. Altogether, this information indicates the importance of studying the biochemical properties of this yeast in order to be implemented in biotechnological processes.

Previously, Duarte et al. [16] recovered 97 yeast strains from marine and terrestrial Antarctic environments and analyzed their ability to produce lipases, xylanases and proteases. From these screenings, *R. mucilaginosa* CBMAI 1528 was selected as the main producer of extracellular proteases. Moreover, in previous assays, the protease secreted proved to be active at temperatures between 15 °C and 60 °C, with an optimal catalytic activity at pH 5.0 and 50 °C, and highly stable in the presence of NaCl [10]. Here, we report the optimization of protease production both in batch and bioreactor. In order to get a deeper characterization of the enzyme, we purified and then analyzed its sequence by mass spectrometry, which allowed us to identify it as an aspartic protease belonging to the peptidase A1 subfamily. We propose that this extracellular protease could be the Rodothorulapepsin characterized in 1972, which, until now, it was classified as one orphan enzyme, as it had an EC number without a related amino acid sequence. [17,18]. This is the first report that identifies the family and amino acid sequence of an extracellular protease from the genus *Rhodotorula*. To date, a large part of well- defined enzyme activities are still lacking an associated protein sequence, despite the immense progress of genomics and bioinformatics methods for function prediction [19]. The identification of the protease described in this work contributes to narrow the gap between knowledge of biochemical function and sequence information.

2. Materials and methods

2.1. Microorganism and growth conditions

The yeast under study (CBMAI 1528) was obtained during an expedition to Antarctica in the austral summer (2009 and 2010) by the Brazilian Antarctic Program team. The strain was selected among 97 isolated yeasts based on the highest extracellular proteolytic activity in milk agar Sabouraud plates (5 g/L tryptone from casein, 5 g/L peptone from animal tissue, 20 g/L dextrose) and based on the similarity of partial 26S rDNA gene, it was identified as *R. mucilaginosa* [16]. The yeast strain was stored in Sabouraud dextrose medium with 20 % (v/v) glycerol at −70 °C.

2.2. Effect of growth temperature on biomass and protease production

To evaluate the effect of growth temperature on biomass and extracellular protease production, 250 mL flasks containing 50 mL of fresh Sabouraud dextrose medium were inoculated with a 10^{-2} dilution of an overnight culture of *R. mucilaginosa* CBMAI 1528 grown at 20 °C. Each broth was incubated at 250 rpm under different temperatures (10, 15, 20, 25, 30 and 35 °C), and samples were taken every 24 h.

Biomass concentration was determined gravimetrically. Culture samples (1–10 mL) were filtered through membranes with 0.45 μm pore size (Sartorius, Goettingen, Germany) and then cells were dried at 200 mm Hg and 60 °C for 48 h until constant weight. Biomass concentration was expressed in grams of dry yeast per L of cultivation medium (g/L). Protease activity was determined on the extracellular medium of each sample by the azocasein method, as detailed below, and expressed as U/mL. Extreme care was taken to prevent volume losses. Data represent the mean values ± standard deviation of three independent measurements.

2.3. Protein quantification and protease assay

Total protein concentration was determined by Bradford method [20] using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany) with Bovine Serum Albumin (BSA) as a standard. The crude extract and the purified enzyme were assayed for proteolytic activity using azocasein (Sigma, St. Louis, MO, USA) as a substrate [21], under previously selected optimal conditions [10].

2.4. Optimization of protease production

To assess the impact of the nitrogen source on the extracellular protease production by *R. mucilaginosa* CBMAI 1528, several combinations of different organic nitrogen sources (casein tryptone, peptone from animal tissue, yeast extract, casamino acids and peptone from gelatin) were tested at two different concentrations (0 or 2.5 g/L) according to the 2^{5-1} fractional factorial design depicted in Table 1. The above organic nitrogen sources were selected because they are common components of culture media. Moreover, 0–2.5 g/L of each component in the different culture media was used in order to obtain a final concentration of organic nitrogen sources comparable to that of the standard medium (Saboraud).

For all the assays, the growth temperature was 20 °C, and glucose (20 g/L) was used as the carbon source. Total protease activity (U/mL) and biomass concentration (g/L) were determined after 48 h of growth. The results were expressed as mean values ± standard deviation of three independent measurements and statistically analyzed using the software Statistica version 10 (StatSoft, Tulsa, OK, USA). Protease activity and biomass concentration at the end of each experiment were compared with the results obtained with the Sabouraud dextrose medium under the same conditions.

2.5. Elemental analysis

Elemental analyses of *R. mucilaginosa* biomass, Sabouraud-Dextrose medium, casein tryptone and yeast extract were performed using a CHNS analyzer, model 2400 series ii (Perkin Elmer, Waltham, MA, USA). Each sample (about 0.1 g) was packed in a silver boat and placed in the analyzer where it was turned to elements by heating at 1,150 °C, further oxidized and analyzed.

2.6. Growth experiments and bioreactor operation

Batch cultures were carried out in a lab scale 3 L bioreactor (Bioflo115 New Brunswick Scientific, Edison, NJ, USA) with 1.5 L working volume. From previous experiments, a positive correlation between airflow rate, agitation and protease production was observed, with a maximum protease production at 2.0 vvm and 500 rpm (data not shown). A k_La of 92 h^{-1} was determined under these conditions according to Potumarthi and Jetty [22]. After inoculation with 1.0 g/L biomass coming from a 18 h pre-inoculum culture, the bioreactor was operated at 20 °C in medium containing

Table 1
Factor levels of the 2^{5-1} fractional experimental design used for the optimization of protease production by *R. mucilaginosa* CBMAI 1528 using different organic nitrogen sources.

Variable	Symbol parameter	Level	
		Low (-1)	High (+1)
Casein tryptone (g/L)	X_1	0	2.5
Peptone from animal tissue (g/L)	X_2	0	2.5
Yeast extract (g/L)	X_3	0	2.5
Casamino acids (g/L)	X_4	0	2.5
Peptone from gelatin (g/L)	X_5	0	2.5

20 g/L glucose and 10 g/L casein tryptone. Samples were collected periodically to monitor biomass and glucose concentrations as well as protease activity. Glucose concentration in the medium was determined colourimetrically by measuring the absorbance at 500 nm according to the glucose oxidase method (Laborclin, Pinhais, PR, Brazil).

2.7. Determination of maximum growth rate, yield coefficient and volumetric productivity

The maximum growth rate (μ_{max}) and generation time were determined after growth curves preparation. Biomass concentration and glucose consumption were used to calculate the biomass/glucose yield coefficient ($Y_{X/S}$) expressed as g/g. The volumetric productivities of biomass (Q_X) and protease (Q_P) were calculated by dividing their concentrations by the corresponding culture time.

2.8. Protease purification, in-gel digestion, mass spectrometry, and database search

To purify the protease, 1.5 L of culture were centrifuged at 8,000 x*g* for 10 min, and the supernatant was filtered through a membrane with 0.47 mm-pore size to completely remove cells. Ammonium sulphate was added to the supernatant to reach 25 % (v/w) saturation. After centrifugation at 10,000 x*g* for 15 min at 4 °C, the precipitate was removed, and ammonium sulphate was added to the supernatant to reach 70 % (v/w) saturation. After centrifugation under the same conditions, the precipitate was dissolved in 50 mL of 50 mM sodium acetate buffer (pH 5.0) and dialyzed thoroughly against this buffer overnight. After that, the solution was applied to a CM-Sepharose Fast Flow column (GE Healthcare, Uppsala, Sweden), and purified as previously described [10].

The purified enzyme suspended in 50 mM sodium acetate buffer containing 2 % (w/v) sodium dodecyl sulfate (SDS), 5 % (v/v) β-mercaptoethanol, and 0.002 % (w/v) bromophenol blue was boiled at 100 °C for 5 min and then subjected to SDS-PAGE according to Laemmly [23]. The band was excised from the gel, transferred to 1.5 mL microcentrifuge tube and sent to CEBIQUIEM facilities (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina) for analyses. The sample was reduced (20 mM DTT, 45 min, 56 °C), alkylated (50 mM iodoacetamide, 45 min, in the dark at room temperature) and digested overnight with trypsin (12 h, 37 °C). Peptides were extracted with acetonitrile and the digested mixture was lyophilized, resuspended in 10 μL formic acid 0.1 % (w/v), desalted and analyzed by nano-high performance liquid chromatography coupled with electrospray ionization mass spectrometry Q Exactive Plus Orbitrap LC-MS/MS System, Thermo Scientific technology (Waltham, MA, USA). The collected data were analyzed with Proteome Discoverer™ software (version 1.4).

2.9. Inhibition assay

Purified protease was preincubated for 15 min at 20 °C with different protease inhibitors, namely 1.0 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 5.0 mg/mL 6-aminohexanoic acid, 0.1 mM Antipain, 0.1 mM Aprotinin, 4.0 mM benzamidine HCl, 0.04 mM Bestatin, 0.1 mM Chymostatin, 10 μM E-64, 1.0 mM EDTA, 1.0 mM N-ethylmaleimide, 0.1 mM Leupeptin, 1.0 μg/mL Pepstatin A, 10 μM Phosphoramidon, and Trypsin Inhibitor in 1:1 stoichiometric binding. Purified protease without any inhibitor was included as control. After that, the proteolytic activity of the reaction mixture was measured using the azocasein assay as described above.

3. Results and discussion

Rhodotorula mucilaginosa CBMAI 1528 was previously reported to produce the highest amounts of extracellular proteases among 97 isolated yeasts from Antarctica [16]. The possibility of reducing the cost of the culture medium by using by-products of the food industry had previously been investigated [24]. In order to scale-up the microbial mass and protease production we optimized the temperature and media conditions. Then to obtain further characterization of the enzyme the sequence was analyzed by means of mass spectrometry.

3.1. Cell growth and protease production at different temperatures

To assess the effect of temperature (*T*) on cell growth and extracellular protease production by *R. mucilaginosa* CBMAI 1528, cells were batch-wise cultured at temperatures from 10 to 35 °C in 250 mL flaks with Sabouraud dextrose medium. Samples were collected every 24 h up to 96 h. As shown in Fig. 1A, this cold adapted strain, which had previously been isolated from an Antarctic marine algae, was able to grow slowly even at temperatures as low as 10 °C or as high as 35 °C. However, it reached a mean maximum biomass concentration of 7.5 g/L at between 20
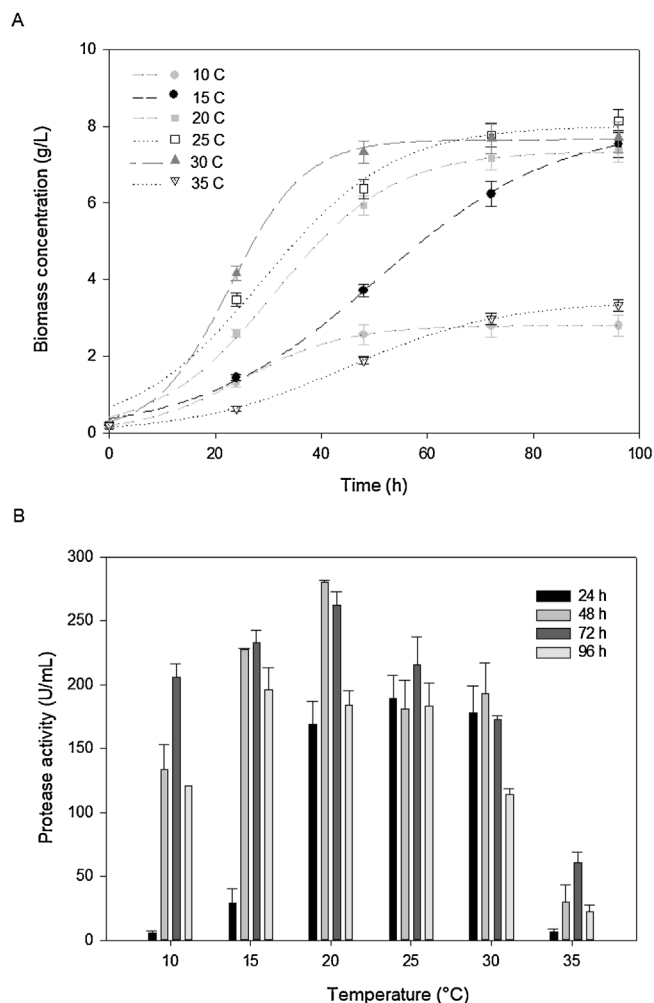
Fig. 1. Effect of temperature on A) *R. mucilaginosa* CBMAI 1528 dry biomass concentration (g/L) and B) extracellular protease activity (U/mL). The strain was cultured at 10, 15, 20, 25, 30 and 35 °C in 250 mL Erlenmeyer flasks with Sabouraud dextrose medium, and samples were collected every 24 h to perform both determinations. The data are expressed as mean values ± standard deviations of three independent assays.

and 30 °C after 72 h, which corresponds to a volumetric biomass productivity (Q_X) of about 0.1 g/L.h.Furthermore, protease production was also dependent on growth temperature. In fact, the extracellular protease activity reached a maximum value of 280 ± 1.7 U/mL at 20 °C after 48 h of growth (Fig. 1B). Based on these results, 20 °C was selected as the optimal temperature for yeast growth in subsequent experiments.

According to Morita [25], psychrophilic taxa show an optimum growth at 15 °C or lower and cannot grow above 20 °C, while psychrotolerant taxa typically have maximum growth temperature higher than 20 °C, and still keep the ability to grow at low temperatures. Therefore, *R. mucilaginosa* CBMAI can be classified as a psychrotolerant organism.

3.2. Optimization of the culture media for protease production

Fungi have the ability to use many compounds as sole nitrogen sources. In the absence of the preferred nitrogen sources (ammonium and glutamine) they can also use less assimilated compounds such as nitrate, urea, purines, pyrimidines, uric acid and proteins [26], through the transcriptional activation of genes encoding enzymes and permeases required for the generation of ammonia performed by their metabolism. These responses to the quality of nitrogen source in the growth medium are termed nitrogen catabolite repression [27]. So, to evaluate the influence of the nitrogen source on the extracellular protease production by *R. mucilaginosa* CBMAI 1528, batch cultures were grown in 250 mL flasks for 48 h at 20 °C and 250 rpm. Glucose was used as the sole carbon source with 16 different combinations of 5 selected organic nitrogen sources, through a 2^{5-1} fractional factorial design depicted in the Materials and Methods section 2.4 (Table 1).

Results in terms of protease activity and biomass concentrations are summarized in Table 2. The highest values of extracellular protease activity occurred in runs 4 (441.5 ± 35.5 U/mL), 6 (420.5 ± 47.6 U/mL) and 8 (422.8 ± 47.8 U/mL). As stressed in legend of Table 2, protease activity of *R. mucilaginosa* CBMAI 1528 on Sabouraud dextrose medium was 279.4 ± 12.4 U/mL; approximately 1.5 fold lower than in runs 4, 6 and 8, which contain casein tryptone as a component of the culture media. These results demonstrate the importance of the nitrogen source on the culture medium to stimulate protease production and/or enzyme release by the yeast.

On the other hand, the highest biomass production occurred in run 16 (15.30 ± 0.52 g/L) carried out in the simultaneous presence of all the nitrogen sources. These results suggest that a higher biomass production may be not associated to a higher protease production.

To identify the preferred nitrogen source for protease production, all these results were subjected to a Statistical analysis using a Pareto chart (Fig. 2). In this graph, the length of each bar is proportional to the standardized effect of the related variable or interaction, and the bars extending beyond the vertical line correspond to the statistically significant effects at a confidence level of 95 %. Thus, according to Fig. 2, protease production was stimulated in media containing casein tryptone (X_1, positive sign),
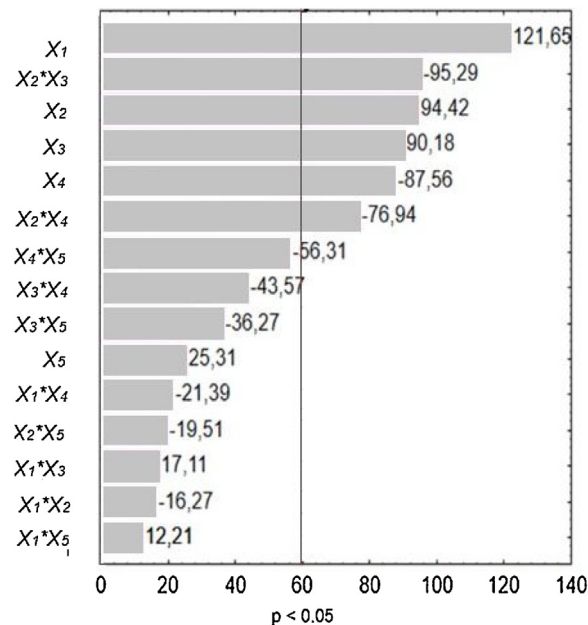
Fig. 2. Pareto diagram showing the effects of five different organic nitrogen sources on extracellular protease production by *R. mucilaginosa* CBMAI 1528. Glucose at concentration of 20 g/L was used as a carbon source in runs carried out at 20 °C in flasks shaken at 250 rpm for 48 h. X_1: Casein tryptone (g/L), X_2: Peptone from animal tissue (g/L), X_3: Yeast extract (g/L), X_4: Casamino acids (g/L), X_5: Peptone from gelatin (g/L).

Table 2
Experimental values of extracellular protease activity (U/mL) and biomass concentration (g/L) at the end of batch runs carried out in flasks using different organic nitrogen sources, according to the 2^{5-1} fractional factorial design depicted in Table 1.

Run	X_1 (g/L)	X_2 (g/L)	X_3 (g/L)	X_4 (g/L)	X_5 (g/L)	Protease activity (U/mL)	Biomass concentration (g/L)
1	0	0	0	0	2.5	56.3 ± 10.2	0.78 ± 0.03
2	2.5	0	0	0	0	61.1 ± 6.8	0.53 ± 0.02
3	0	2.5	0	0	0	233.5 ± 2.9	0.63 ± 0.10
4	**2.5**	**2.5**	**0**	**0**	**2.5**	**441.5 ± 35.5**	**8.05 ± 0.2**
5	0	0	2.5	0	0	179.3 ± 32.0	0.60 ± 0.10
6	**2.5**	**0**	**2.5**	**0**	**2.5**	**420.5 ± 47.6**	**11.73 ± 0.38**
7	0	2.5	2.5	0	2.5	304.7 ± 21.3	12.29 ± 0.47
8	**2.5**	**2.5**	**2.5**	**0**	**0**	**422.8 ± 47.8**	**12.79 ± 0.50**
9	0	0	0	2.5	0	41.7 ± 15.7	0.60 ± 0.03
10	2.5	0	0	2.5	2.5	165.9 ± 0.2	5.54 ± 0.09
11	0	2.5	0	2.5	2.5	163.8 ± 5.0	6.05 ± 0.16
12	2.5	2.5	0	2.5	0	244.9 ± 1.4	11.61 ± 0.34
13	0	0	2.5	2.5	2.5	142.8 ± 3.4	6.21 ± 0.23
14	2.5	0	2.5	2.5	0	324.2 ± 36.9	12.76 ± 0.52
15	0	2.5	2.5	2.5	0	160.7 ± 30.2	12.22 ± 0.51
16	**2.5**	**2.5**	**2.5**	**2.5**	**2.5**	**175.1 ± 3.6**	**15.30 ± 0.52**

Under the same conditions (48 h of growth at 250 rpm, 20 °C and 20 g/L of dextrose), the protease activity of *R. mucilaginosa* CBMAI 1528 on Sabouraud dextrose medium was 279.4 ± 12.4 U/mL, and the biomass concentration 6.8 ± 0.3 g/L. X_1: casein tryptone (g/L), X_2: peptone from animal tissue (g/L), X_3: yeast extract (g/L), X_4: casamino acids (g/L), X_5: peptone from gelatin (g/L).

Table 3
Composition of the different culture media tested to optimize protease production by *R. mucilaginosa* CBMAI 1528.

Medium	Glucose (g/L)	Yeast extract (g/L)	Casein tryptone (g/L)	C:N ratio
1	20	0	10	9.72
2	20	5	5	10.25
3	20	10	0	10.87
4	30	0	15	9.72
5	30	7.5	7.5	10.25
6	30	15	0	10.87
7	40	0	20	9.72
8	40	10	10	10.25
9	40	20	0	10.87

The strain was grown on different media at 20 °C, pH 5.6, in flasks shaken at 250 rpm for 48 h. Aliquots from these cultures were taken to determine protease activity and biomass concentration.

peptone from animal tissue (X_2, positive sign), and yeast extract (X_3, positive sign). However, it was negatively affected in media containing casamino acids (X_4, negative sign). Moreover, consistent with the above simplified evaluation, casein tryptone (X_1) was the culture component that most promoted protease production. The negative interaction between peptone from animal tissue (X_2) and yeast extract (X_3) indicates that these two medium components were mutually exclusive for protease production. Based on these results, we infer that the optimized medium for protease production should be composed of casein tryptone with yeast extract or peptone from animal tissue as organic nitrogen sources.

These data demonstrate that protease production by *R. mucilaginosa* CBMAI 1528 greatly depends on medium composition. Similar results were previously reported for other microorganisms. For example, protease secretion by *Candida humicola* and *Candida albicans* was promoted when the culture media was composed by proteins and repressed when composed by amino acids and ammonium salts or by nitrogen starvation [28–30]. Moreover, casein and BSA are commonly used to induce the production and secretion of proteases [31–34]. A possible explanation for this behavior is that proteins can be used as nitrogen sources by yeasts only when preferred nitrogen sources, such as amino acids, ammonium and urea, are unavailable [31]. In addition, protease-encoding genes are usually repressed at high concentrations of nitrogen sources other than proteins [35].

Based on these results and considering the elemental composition of both the yeast and the culture medium, a final optimization was attempted to improve extracellular protease production by *R. mucilaginosa* CBMAI 1528. To optimally cultivate any microorganism, not only must the medium contain all the chemical elements required for its growth, but also the elemental composition of such medium must reflect the elemental composition of the organism [36]. For this reason, the C:N ratio of a cell extract of *R. mucilaginosa* CBMAI 1528 was determined and then considered to optimize protease production. The C:N ratio of *R. mucilaginosa* cell extract was 9.86 (Table S1), a value close to that of the Sabouraud dextrose medium, in which the yeast was grown (C:N 9.47). Considering the results of the fractional factorial design, and the C:N ratio of the yeast under study, protease production was then evaluated on different culture media with a similar C:N ratio (9.72–10.87) and composed of glucose and casein tryptone and/or yeast extract as nitrogen source (Table S1 and Table 3).

As observed in Fig. 3, protease production in all the media tested was higher than the one obtained in the previous assay (1.4–2.4 folds). This demonstrates the importance of considering the elemental composition of the organism to design the culture medium. The lowest biomass concentrations were obtained when casein tryptone was used as the only organic nitrogen source (media 1, 4 and 7), while the highest values were obtained when both components were present in the medium (media 2, 5 and 8). The highest extracellular protease activities were obtained in presence of casein tryptone (media 1, 4 and 7), and the lowest ones when yeast extract was used as nitrogen source (media 3, 6 and 9). Moreover, for each media (only casein tryptone, only yeast extract or both organic nitrogen sources), protease activity was very similar almost irrespectively of both glucose (20−40 g/L) and total nitrogen source (10−20 g/L) concentrations. It is a fact that to reduce the cultivation costs it is necessary to reduce to a minimum the number of ingredients of the culture medium and their concentrations [36]. Therefore, medium 1, which contains both glucose and casein tryptone at the lowest concentrations (20 and 10 g/L, respectively), was selected as the best one to perform batch
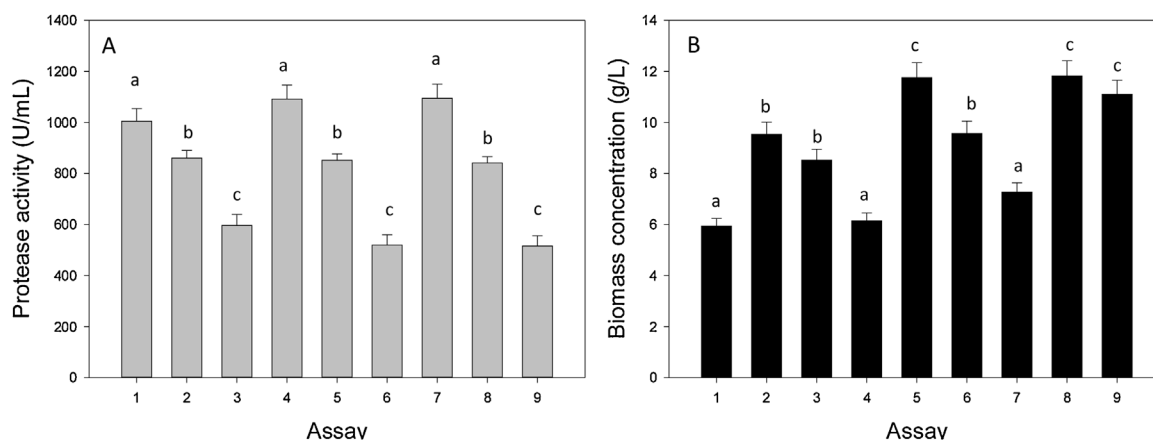
Fig. 3. (A) Protease production (U/mL) and (B) biomass concentration (g/L) by *R. mucilaginosa* CBMAI 1528. The strain was grown on different culture media at 20 °C, pH 5.6, in flasks shaken at 250 rpm for 48 h. Compositions of the different culture media (1-9) are listed in Table 3. Data are means ± s.d. n = 3, where samples labelled with identical letters are not significant at P < 0.05.
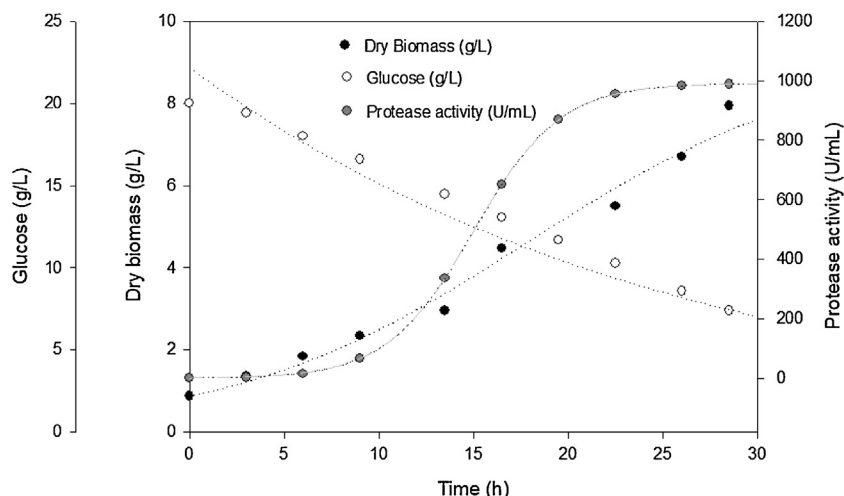
Fig. 4. Time course of a batch culture of *R. mucilaginosa* CBMAI 1528. The strain was cultured in medium containing 20 g/L glucose and 10 g/L of casein tryptone at 20 °C, pH 5.6, in shake flasks at 250 rpm for about 30 h, with an initial dry cell concentration of 1 g/L. Aliquots from this culture were taken every 3 h to determine protease activity, dry biomass and glucose concentration. (●) Dry biomass concentration (g/L); (○) glucose concentration (g/L); (●) protease activity (U/mL).
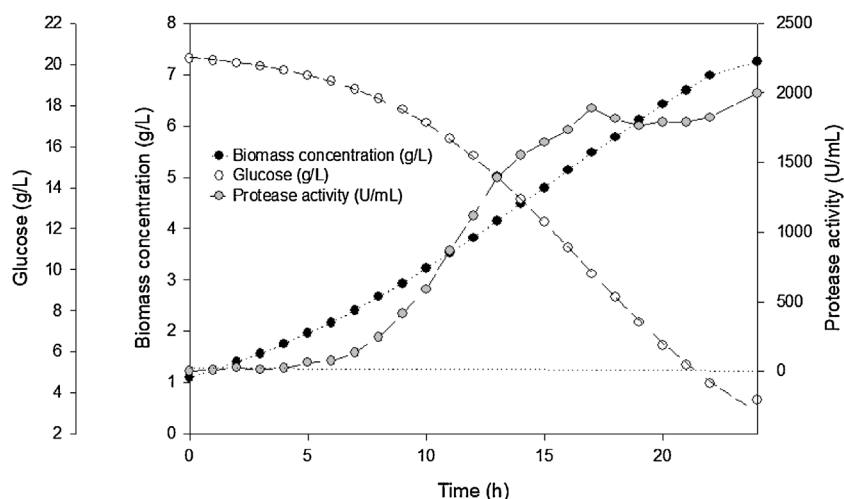
Fig. 5. Time course of a batch culture of *R. mucilaginosa* CBMAI 1528 in bioreactor. The strain was cultured on medium containing 20 g/L glucose and 10 g/L of casein tryptone for 24 h at 20 °C, pH 5.6, 200 vvm, 500 rpm and K_La =92 h^{-1}, with an initial dry biomass concentration of 1 g/L. Aliquots from this culture were taken every hour to determine protease activity, dry biomass and glucose concentration. (●) Dry biomass concentration (g/L); (○) glucose concentration (g/L); (●) protease activity (U/mL).

Table 4
Tryptic fragments from digestion of purified enzyme identified by LC–MS/MS against UNIPROT protein database.

Sequence	Master Protein Accessions	Theoretical MH+ [Da]	XCorr Sequest HT	Confidence Sequest HT
LATQNSAASELYLGGLNSAK	A0A125PHT0	2008.03456	5.46	High
VAANSFSFR	A0A125PHT0	998.50541	2.99	High
ASTGSDSLTSYQNDNLWAGK	A0A125PHT0	2114.96252	2.62	High

R. mucilaginosa CBMAI 1528 culture in shaken flasks for protease production. It is interesting to note that between the three tested media, the selected one has the most similar C:N ratio to the yeast under study (C:N 9.72 and 9.86 respectively). However, more studies are required to assess the impact of the C:N ratio on protease production.

3.3. Growth experiments and bioreactor operation

R. mucilaginosa CBMAI 1528 was cultured at 20 °C, pH 5.6 in shake flasks at 250 rpm for about 30 h, with an initial biomass concentration of 1 g/L in medium which contained 20 g/L glucose

and 10 g/L of casein tryptone. Aliquots were taken every 3 h to determine protease activity and dry biomass concentration (Fig. 4). Under these conditions, the maximum specific growth rate (μ_{max}) was 0.18 h^{-1}, and the generation time (T_g) 3.85 h. After 28 h of culture, 56 % of the carbon source was consumed, with a biomass productivity (Q_X) of 0.28 g/L.h. On the other hand, this parameter achieved a maximum value (0.45 g/L.h) after only 3 h, whereas the maximum protease productivity (Q_p) was 44.4 U/mL.h after 22.5 h.

Under similar conditions (20 °C, 20 g/L glucose, 10 g/L casein tryptone and 1 g/L of inoculum), the values obtained in bioreactor were very close to those obtained in shake flasks: μ_{max} (0.20 h^{-1}), T_g (3.46 h) and Q_X (0.35 g/L.h after 21 h). Nevertheless, the

percentage of carbon source consumption (86 % after 24 h), maximum Q_x (0.93 g/L.h after 2 h) and maximum Q_p (111.2 U/mL.h after 17 h) were remarkably higher (Fig. 5), likely due to the positive effect of a more efficient aeration in bioreactor.

3.4. Protease characterization: mass spectrometry analysis and inhibition assay

In order to get a further characterization of the extracellular protease secreted by *R. mucilaginosa* CBMAI 1528, the enzyme was

purified and then its sequence analyzed by mass spectrometry. To do that, 1.5 L of fermented broth obtained in bioreactor was centrifuged, and the enzymes present in the supernatant were precipitated with ammonium sulfate. The precipitate was then dissolved in sodium acetate buffer, dialyzed, and finally loaded on a CM-Sepharose Fast Flow column. Only one protein peak appeared in the elution profile, which coincided with the one displaying proteolytic activity (data not shown). A Coomassie stained SDS-PAGE showed a unique band of 34.5 kDa (Fig. S1). In order to ascertain the *Rhodotorula* extracellular protease identity, the
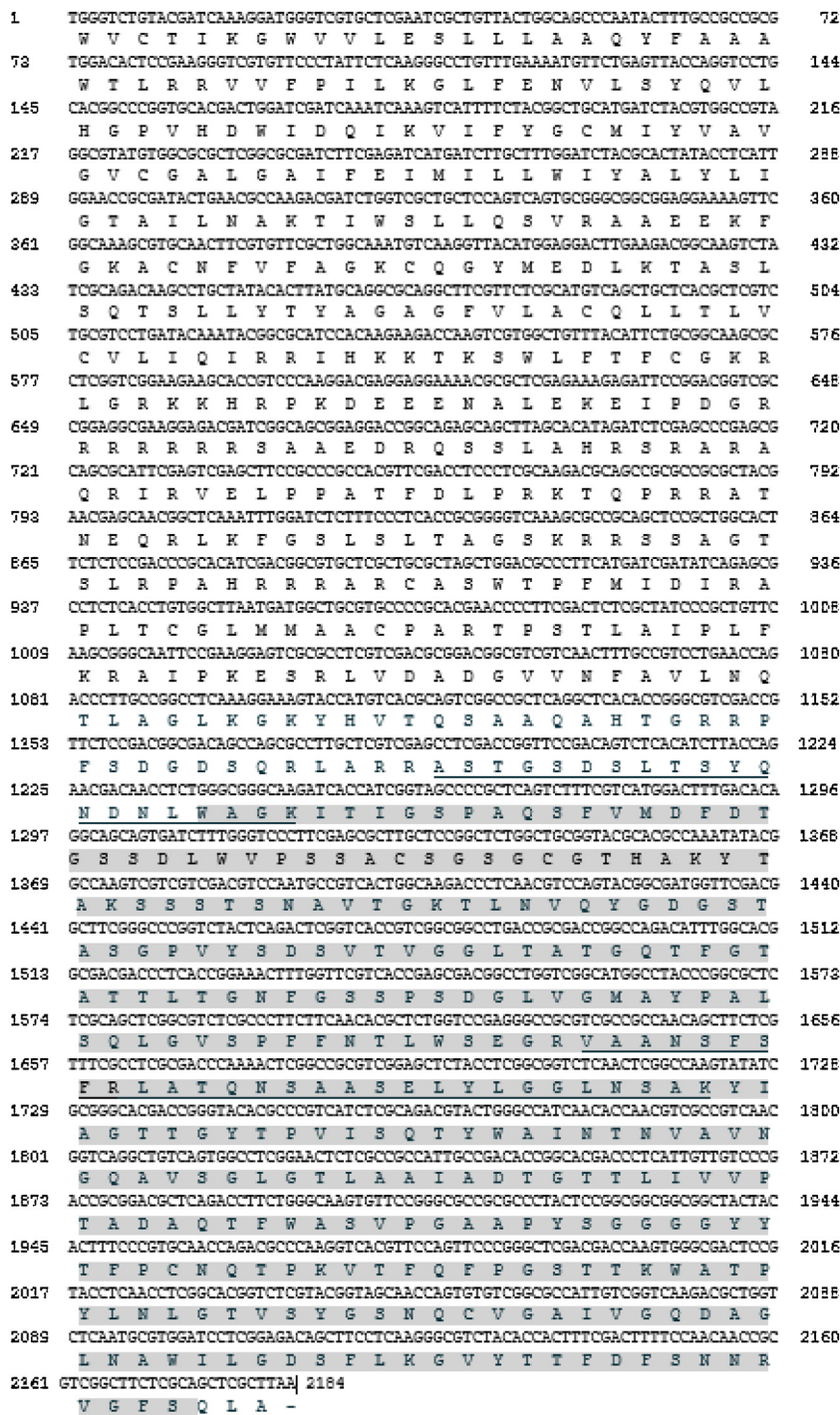


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1      TGGGTCTGTACGATCAAAGGATGGGTCGTGCTCGAATCGCTGTTACTGGCAGCCCAATACTTTGCCGCCGCG    72
       W  V  C  T  I  K  G  W  V  V  L  E  S  L  L  L  A  A  Q  Y  F  A  A  A
73     TGGACACTCCGAAGGGTCGTGTTCCCTATTCTCAAGGGCCTGTTTGAAAATGTTCTGAGTTACCAGGTCCTG    144
       W  T  L  R  R  V  V  F  P  I  L  K  G  L  F  E  N  V  L  S  Y  Q  V  L
145    CACGGCCCGGTGCACGACTGGATCGATCAAATCAAAGTCATTTTCTACGGCTGCATGATCTACGTGGCCGTA    216
       H  G  P  V  H  D  W  I  D  Q  I  K  V  I  F  Y  G  C  M  I  Y  V  A  V
227    GGCGTATGTGGCGCGCTCGGCGCGATCTTCGAGATCATGATCTTGCTTTGGATCTACGCACTATACCTCATT    288
       G  V  C  G  A  L  G  A  I  F  E  I  M  I  L  L  W  I  Y  A  L  Y  L  I
289    GGAACCGCGATACTGAACGCCAAGACGATCTGGTCGCTGCTCCAGTCAGTGCGGGCGGCGGAGGAAAAGTTC    360
       G  T  A  I  L  N  A  K  T  I  W  S  L  L  Q  S  V  R  A  A  E  E  K  F
361    GGCAAAGCGTGCAACTTCGTGTTCGCTGGCAAATGTCAAGGTTACATGGAGGACTTGAAGACGGCAAGTCTA    432
       G  K  A  C  N  F  V  F  A  G  K  C  Q  G  Y  M  E  D  L  K  T  A  S  L
433    TCGCAGACAAGCCTGCTATACACTTATGCAGGCGCAGGCTTCGTTCTCGCATGTCAGCTGCTTCACGCTGTC    504
       S  Q  T  S  L  L  Y  T  Y  A  G  A  G  F  V  L  A  C  Q  L  L  T  L  V
505    TGCGTCCTGATACAAATACGGCGCATCCACAAGAAGACCAAGTCGTGGCTGTTTACATTCTGCGGCAAGCGC    576
       C  V  L  I  Q  I  R  R  I  H  K  K  T  K  S  W  L  F  T  F  C  G  K  R
577    CTCGGTCGGAAGAAGCACCGTCCCAAGGACGAGGAGGAAAACGCGCTCGAGAAAGAGATTCCGGACGGTCGC    648
       L  G  R  K  K  H  R  P  K  D  E  E  E  N  A  L  E  K  E  I  P  D  G  R
649    CGGAGGCGAAGGAGACGATCGGCAGCGGAGGACCGGCAGAGCAGCTTAGCACATAGATCTCGAGCCCGAGCG    720
       R  R  R  R  R  R  S  A  A  E  D  R  Q  S  S  L  A  H  R  S  R  A  R  A
721    CAGCGCATTCGAGTCGAGCTTCCGCCCGCCACGTTCGACCTCCCTCGCAAGACGCAGCCGGCCGCGCGCTACG    792
       Q  R  I  R  V  E  L  P  P  A  T  F  D  L  P  R  K  T  Q  P  R  R  A  T
793    AACGAGCAACGGCTCAAATTTGGATCTCTTTCCCTCACCGCGGGGTCAAAGCGCCGCAGCTCCGCTGGCACT    864
       N  E  Q  R  L  K  F  G  S  L  S  L  T  A  G  S  K  R  R  S  S  A  G  T
865    TCTCTCCGACCCGCACATCGACGGCGTGCTCGCTGCGCTAGCTGGACGCCCTTCATGATCGATATCAGAGCG    936
       S  L  R  P  A  H  R  R  R  A  R  C  A  S  W  T  P  F  M  I  D  I  R  A
937    CCTCTCACCTGTGGCTTAATGATGGCTGCGTGCCCCGCACGAACCCCTTCGACTCTCGCTATCCCGCTGTTC    1008
       P  L  T  C  G  L  M  M  A  A  C  P  A  R  T  P  S  T  L  A  I  P  L  F
1009   AAGCGGGCAATTCCGAAGGAGTCGCGCCTCGTCGACGCGGACGGTCGTCAACTTTGCCGTCCTGAACCAG    1080
       K  R  A  I  P  K  E  S  R  L  V  D  A  D  G  V  V  N  F  A  V  L  N  Q
1081   ACCCTTGCCGGCCTCAAAGGAAAGTACCATGTCACGCAGTCGGCCGCTCAGGCTCACACCGGGCGTCGACCG    1152
       T  L  A  G  L  K  G  K  Y  H  V  T  Q  S  A  A  Q  A  H  T  G  R  R  P
1153   TTCTCCGACGGCGACAGCCAGCGCCTTGCTCGTCGAGCCTCGACCGGTTCCGACAGTCTCACATCTTACCAG    1224
       F  S  D  G  D  S  Q  R  L  A  R  R  A̲  S̲  T̲  G̲  S̲  D̲  S̲  L̲  T̲  S̲  Y̲  Q̲
1225   AACGACAACCTCTGGGCGGGCAAGATCACCATCGGTAGCCCCGCTCAGTCTTTCGTCATGGACTTTGACACA    1296
       N̲  D̲  N̲  L̲  W̲  A̲  G̲  K̲  I̲  T̲  I̲  G̲  S̲  P̲  A̲  Q̲  S̲  F̲  V̲  M̲  D̲  F̲  D̲  T̲
1297   GGCAGCAGTGATCTTTGGGTCCCTTCGAGCGCTTGCTCCGGCTCTGGCTGCGGTACGCACGCCAAATATACG    1368
       G̲  S̲  S̲  D̲  L̲  W̲  V̲  P̲  S̲  S̲  A̲  C̲  S̲  G̲  S̲  G̲  C̲  G̲  T̲  H̲  A̲  K̲  Y̲  T̲
1369   GCCAAGTCGTCGTCGACGTCCAATGCCGTCACTGGCAAGACCCTCAACGTCCAGTACGGCGATGGTCGACG    1440
       A̲  K̲  S̲  S̲  S̲  T̲  S̲  N̲  A̲  V̲  T̲  G̲  K̲  T̲  L̲  N̲  V̲  Q̲  Y̲  G̲  D̲  G̲  S̲  T̲
1441   GCTTCGGGCCCGGTCTACTCAGACTCGGTCACCGTCGGCGGCCTGACCGCGACCGGCCAGACATTTGGCACG    1512
       A̲  S̲  G̲  P̲  V̲  Y̲  S̲  D̲  S̲  V̲  T̲  V̲  G̲  G̲  L̲  T̲  A̲  T̲  G̲  Q̲  T̲  F̲  G̲  T̲
1513   GCGACGACCCTCACCGGGAAACTTTGGTTCGTCACCGAGCGACGGCCTGGTCGGCATGGCCTACCCGGCCGTC    1573
       A̲  T̲  T̲  L̲  T̲  G̲  N̲  F̲  G̲  S̲  S̲  P̲  S̲  D̲  G̲  L̲  V̲  G̲  M̲  A̲  Y̲  P̲  A̲  L̲
1574   TCGCAGCTCGGCGTCTCGCCCTTCTTCAACACGCTCTGGTCCGAGGGCCGCGTCGCCGCCAACAGCTTCTCG    1656
       S̲  Q̲  L̲  G̲  V̲  S̲  P̲  F̲  F̲  N̲  T̲  L̲  W̲  S̲  E̲  G̲  R̲  V̲  A̲  A̲  N̲  S̲  F̲  S̲
1657   TTTCGCCTCGCGACCCAAAACTCGGCCGCGTCGGAGCTCTACCTCGGCGGTCTCAACTCGGCCAAGTATATC    1728
       F̲  R̲  L̲  A̲  T̲  Q̲  N̲  S̲  A̲  A̲  S̲  E̲  L̲  Y̲  L̲  G̲  G̲  L̲  N̲  S̲  A̲  K̲  Y̲  I̲
1729   GCGGGCACGACCGGGTACACGCCCGTCATCTCGCAGACGTACTGGGCCATCAACACCAACGTCGCCGTCAAC    1800
       A̲  G̲  T̲  T̲  G̲  Y̲  T̲  P̲  V̲  I̲  S̲  Q̲  T̲  Y̲  W̲  A̲  I̲  N̲  T̲  N̲  V̲  A̲  V̲  N̲
1801   GGTCAGGCTGTCAGTGGCCTCGGGAACTCTCGCCGCCATTGCCGACACCGGCACGACCCTCATTGTTGTCCCG    1872
       G̲  Q̲  A̲  V̲  S̲  G̲  L̲  G̲  T̲  L̲  A̲  A̲  I̲  A̲  D̲  T̲  G̲  T̲  T̲  L̲  I̲  V̲  V̲  P̲
1873   ACCGCGGACGCTCAGACCTTCTGGGCAAGTGTTCCGGGCGCCGCGCCCTACTCCGGCGGCGGCGGCTACTAC    1944
       T̲  A̲  D̲  A̲  Q̲  T̲  F̲  W̲  A̲  S̲  V̲  P̲  G̲  A̲  A̲  P̲  Y̲  S̲  G̲  G̲  G̲  G̲  Y̲  Y̲
1945   ACTTTCCCGTGCAACCAGACGCCCAAGGTCACGTTCCAGTTCCCGGGCTCGACGACCAAGTGGGCGACTCCG    2016
       T̲  F̲  P̲  C̲  N̲  Q̲  T̲  P̲  K̲  V̲  T̲  F̲  Q̲  F̲  P̲  G̲  S̲  T̲  T̲  K̲  W̲  A̲  T̲  P̲
2017   TACCTCAACCTCGGCACGGTCTCGTACGGTAGCAACCAGTGTGTCGGCGCCATTGTCGGTCAAGACGCTGGT    2088
       Y̲  L̲  N̲  L̲  G̲  T̲  V̲  S̲  Y̲  G̲  S̲  N̲  Q̲  C̲  V̲  G̲  A̲  I̲  V̲  G̲  Q̲  D̲  A̲  G̲
2089   CTCAATGCGTGGATCCTCGGAGACAGCTTCCTCAAGGGCGTCTACACCACTTTCGACTTTTCCAACAACCGC    2160
       L̲  N̲  A̲  W̲  I̲  L̲  G̲  D̲  S̲  F̲  L̲  K̲  G̲  V̲  Y̲  T̲  T̲  F̲  D̲  F̲  S̲  N̲  N̲  R̲
2161   GTCGGCTTCTCGCAGCTCGCTTAA|                                                  2184
       V̲  G̲  F̲  S̲  Q̲  L̲  A̲  -
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Fig. 6. *R. mucilaginosa* CBMAI 1528 protein with aspartic protease activity: partial nucleotide and amino acid sequences deducted from the homologous *Rhodotorula sp. JG-01b* gene RHOSPDRAFT_21543. Protease conserved Pfam domain PF00026 for members of the peptidase A1 family of proteases is highlighted (light grey) [39]. Tryptic peptides obtained by mass spectrometry are underlined.

Table 5
Inhibitor reactivity.

Inhibitor	Specificity	Solvent	Inhibitor concentration	Residual activity (%)[a]
None				100.0
AEBSF[b]	Serine proteases	Water	1 mM	87.4
6- Aminohexanoic acid	Chymotrypsin, Factor VIIa, lysine carboxypeptidase, plasmin, and plasminogen activator	Water	40 mM	94.8
Antipain hydrochloride	Serine proteases	Water	0.1 mM	93.5
Aprotinin	Serine proteases	Water	0.1 mM	74.8
Benzamidine hydrochloride hydrate	Trypsin, trypsin-like enzymes, and serine proteases	Water	4 mM	91.6
Bestatin	Metalloproteases	Water	0.04 mM	100.0
Chymostatin	Chymotrypsin	DMSO[c]	0.1 mM	46.0
E-64	Cysteine proteases	Water	10 μM	94.2
EDTA	Metalloproteases	Water	1 mM	86.6
N-Ethylmaleimide	Cysteine proteases	Ethanol	1 mM	69.8
Leupeptin	Serine and Cysteine proteases	Water	0,1 mM	68.7
Pepstatin	**Aspartic proteases**	**Ethanol**	**1 μM**	**4.9**
Phosphoaramidon	Metalloproteases	Water	10 μM	86.4
Trypsin inhibitor	Trypsin	Water	1:1 stoichiometric binding	81.2

[a] Enzyme samples were incubated with the inhibitor for 15 min at 20 °C and then the remaining activity in the sample was determined. Each value represents the mean of three experiments. The activity in the absence of inhibitor was considered 100 %.
[b] AEBSF 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride.
[c] Dimethyl Sulfoxide (DMSO).

protein band obtained from the SDS-PAGE was in-gel digested with trypsin, and peptides were analyzed by MS/MS analysis. The obtained data were used to search for matching peptides from proteins in the UNIPROT database using the Mascot search program.

Table 4 shows the sequences of the three tryptic peptides obtained. BLASTp search against UniprotKB reference proteomes plus Swiss-Prot database revealed 100 % identity with an acid protease from Rhodotorula sp. JG-1b (Gene RHOSPDRAFT_21543; protein ID: KWU42276.1) with an E- value of $2.6e^{-8}$. Fig. 6 shows the analysis of the nucleotide and deduced protein sequences of the enzyme under study. BLASTp search allowed us to classify the enzyme as an aspartic protease belonging to the peptidase A1 subfamily, also known as the pepsin family. All these peptidases are endopeptidases, mostly secreted from cells as inactive proenzymes that are activated autocatalytically under acidic conditions. This probably explains the lower molecular weight of the purified protease in contrast to the higher molecular mass of the predicted protein. Finally, to provide further proof that the protease characterized in this study is an aspartic protease, the effect of different inhibitors on its activity was investigated. As shown in Table 5, only pepstatin, a specific inhibitor of aspartic proteases, almost completely inhibited the enzyme activity.

4. Conclusions

In this work, we describe the production, purification and identification of an enzyme with protease activity secreted by R. mucilaginosa CBMAI 1528, previously isolated from the Antarctic continent. Analysis of the effect of the temperature on yeast cell growth indicates that the strain was able to grow at temperatures as low as 10 °C and as high as 35 °C with a maximum between 20 and 30 °C, allowing us to classify this strain as psychrotolerant. The temperature also influenced protease production, reaching a maximum activity at 20 °C.

Analysis of medium composition on protease production by R. mucilaginosa CBMAI 1528 demonstrated that it was promoted by casein tryptone, peptone from animal tissue and yeast extract but repressed by casamino acids. Further optimization of media composition was performed taking into account the C:N ratio. Medium containing both glucose and casein tryptone was selected

as the best option to perform batch culture in shaken flasks. When the same culture medium was tested in the bioreactor, protease production and carbon source consumption were enhanced, thereby proving efficient scaling-up of the process.

The protein was purified by a two-step procedure, and the deduced amino acid sequence indicated that the protein is an aspartic protease belonging to the peptidase A1 subfamily, also known as the pepsin family. In accordance with this result, the activity of the enzyme was inhibited by pepstatin, a specific inhibitor of aspartic proteases. Interestingly, Murao et al. [37] reported during screening tests for yeast extracellular proteases, the isolation of a potent acid protease from *Rhodotorula glutinis*, which was subsequently purified and crystallized by Kamada et al. [18]. These authors found that the enzyme was stable in the pH range from 2.4 to 6.5 after 20 h of incubation at 37 °C. When this protease was compared with several other proteases obtained from various sources, it was found that the specific activity towards casein was potent and 1.7-fold higher than that of pepsin. Moreover, the enzyme was inactivated by a pepsin inhibitor produced from *Streptomyces naniwaensis*. Subsequently, Oda et al. [17] determined some physicochemical properties of the enzyme. The molecular weight was estimated to be approximately 30 kDa, and the isoelectric point 4.5. The enzyme was named as Rhodotorulapepsin (EC 3.4.23.26); however, until now, the enzyme has not had an associated sequence and has been classified as an orphan enzyme as it had a EC number without an associated sequence [38]. Based on the results collected here, we can reasonably propose that the aspartic protease described in the present study may be the Rodothorulapepsin described years ago.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00546.

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