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Production and Stability of Protease from *Candida buinensis*

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Abstract Cow raw milk from dairy cooperatives was examined for its microbial composition. Among the isolates identified, 17.6% were yeasts. The most frequent genus was *Candida*, although members belonging to the genera *Brettanomyces*, *Dekkera*, and *Geotricum* were also identified. Although qualitative and quantitative tests for extracellular proteolytic activity were positive for all the species isolated, *Candida buinensis* showed the highest response (23.5 U/mg); therefore, it was selected for subsequent investigation. The results of fermentations carried out at variable temperature, pH, and soybean flour concentration, according to a 2^3 full factorial design, demonstrated that this yeast ensured the highest production of extracellular proteases (573 U/mL) when cultivated at 35 °C, pH 6.5, and using soybean flour concentrations in the range 0.1–0.5% (*w*/*v*). The cell-free supernatants showed the highest activity at 25 °C and pH 7.0, and satisfactory stability in the ranges 25–30 °C and pH 7–9. The first-order rate constants of protease inactivation in the cell-free supernatants were calculated at different temperatures from semi-log plots of the residual activity versus time and then used in Arrhenius and Eyring plots to estimate the main thermodynamic parameters of thermoinactivation (E^*=40.0 kJ/mol; ΔH^*=37.3 kJ/mol; ΔS^*=−197.5 J/mol K; ΔG^*=101 kJ/mol).

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Introduction

Proteases have been investigated for practical purposes, as cleaning and hair removing agents, in detergents, leather processing, silver recovery, medical applications, food processing, chemical industry, and waste treatment [1, 2]. Moreover, their wide diversity and action specificity range have attracted the attention of biotechnologists worldwide [3]. Although protease production is an inherent property of all organisms, only those microorganisms producing a substantial amount of extracellular protease can be commercially exploited [4].

It is well known that extracellular protease production by microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, as well as physical factors such as pH, temperature, inoculum size, dissolved oxygen level, and incubation time [5]. However, there is not an optimal medium for the production of protease by different microbial species because each of them requires specific cultivation conditions [6].

Soybean flour (SF) is an inexpensive component of media for protease production by bacteria, being used as nitrogen, carbon, and vitamins source; therefore, its use as alternative component for proteases production by yeasts would be of a certain industrial concern [7–12].

The use of microorganisms as biotechnological sources of industrially relevant enzymes has stimulated a renewed interest in the identification of news producers such as yeasts [13, 14]. Several researchers [13, 15–17] have demonstrated that yeasts are able to produce enzymes with biotechnological potential, but only a few yeast proteases have been studied for alternative potential applications [18–20].

Terrestrial and marine yeasts have been described as protease producers such as *Candida lipolytica* [3], *Yarrowia lipolytica* [3], and *Aureobasidium pullulans* [2]. Other important sources of yeasts are many cheese varieties and raw milks, where they live in association with lactic bacteria [15, 16]. Dairy products offer a special niche for the development of yeasts with specific activity [21]. Different caseinolytic yeasts have been identified, including some species of *Kluyveromyces* and *Candida* such as *Candida punicea*, *Candida lipolytica*, *Candida aquatica* and *Candida curiosa* [14].

The aims of this work were (a) to select yeasts species from raw milk able to release extracellular proteases, (b) to identify the variables most influencing protease production by *Candida buinensis* by means of a 2^3 factorial design, (c) to determine the best conditions for its activity and stability, and (d) to estimate the thermodynamic parameters of its thermal inactivation.

Materials and Methods

Milk Samples and Yeast Isolation

The milk samples were collected in a dairy products cooperative at the Cachoeirinha district of Pernambuco, Brazil. The milk samples were filtered through quantitative filter and utilized for microorganism isolation.

Samples were serially diluted tenfold in sterile distilled water (10^2, 10^4, 10^6, and 10^8); 0.001 mL of the dilutions were inoculated in Man Rogosa Sharpe agar medium (Difco, Detroit, MI) and incubated for 3 days at 30 °C. The yeast colonies were randomly selected, through the macroscopic and microscopic characteristics of the colonies, from each plate and purified by subsequent streaking on Sabourand agar (Difco, Detroit, MI). The slants thus obtained were maintained at 4 °C.

Yeast Identification

The identification was realized by morphological characterization [18], reproduction type [22], physiological characterization based on urea hydrolysis [23], sugar and salt assimilation tests, and fermentations tests [22].

Screening of Proteolytic Yeasts

Strains isolated and identified were inoculated in the Manachini solution [16] containing gelatin (10% *w*/*v*) for 72 h at 30 °C and 150 rpm in orbital shaker. The cell-free supernatants were clarified by centrifugation (5,500×*g*) for 15 min, and 120 μL were placed into cup plates containing milk–gelatin–agar medium. Each plate was incubated for 3 days at 30 °C. The ability to digest casein was demonstrated by the formation of transparent halos on the plates and expressed in millimeter. Positive strains were submitted to the quantitative test described by Moreira et al. [8]. The proteolytic activity was determined in the cell-free supernatants obtained by centrifugation (5,500×*g*) for 5 min at 25 °C. A 1.0% (*w*/*v*) Azocasein (Sigma Chemical, St. Louis, MO) in 0.1 M Tris–HCl buffer solution (pH 7.6) containing 1 mM $CaCl_2$ was used as substrate [24]. One activity unit was defined as the amount of enzyme that produced a 1.0 increase in the optical density within 1 h at 440 nm. The residual protein concentration after the proteolytic activity assay was determined according to Bradford [25], using bovine serum albumin as a standard.

Fermentation Conditions

The fermentations were carried out in Erlenmeyer flasks (250 mL) containing 50 mL of the culture medium described by Porto et al. [7], consisting in 0.1% (*w*/*v*) NH_4Cl, 0.06% (*w*/*v*) $MgSO_4{\cdot}7H_2O$, 0.453% (*w*/*v*) K_2HPO_4, and 0.1 mL mineral solution (1 mg/mL $FeSO_4{\cdot}7H_2O$, 1 mg/mL $MnCl_2{\cdot}4H_2O$, 1 mg/mL $ZnSO_4{\cdot}H_2O$ and 1 mg/mL $CaCl_2$) and SF at concentration according to the selected full factorial design described later. After inoculating cells up to a concentration of 10^6 cells/mL in Erlenmeyer flasks containing the production medium, fermentations were performed in an orbital shaker at 200 rpm for 72 h. Samples were collected every 24 h and assayed for pH, total protein, proteolytic activity, and biomass concentration by dry weight.

Experimental Design and Modeling of Enzymatic Activity

Runs were carried out according to a 2^3 full factorial design in order to identify and quantify the effects and interactions of SF concentration, initial pH of the medium, and temperature (independent variables) on the enzyme activity (response), taken as an indirect measure of protease production. To this purpose, a set of ten experiments with two replicates at the central points was performed. The range and levels of the components (factors or independent variables) under study are given in Table 1.

Table 1 Factor levels employed in the 2^3 design used for the study on protease production by *Candida buinensis*.

Factors	Level		
	Low (−1)	Center (0)	High (+1)
Soybean flour concentration (% *w*/*w*)	0.1	0.3	0.5
Initial pH of the medium	6.5	7.5	8.5
Temperature (ºC)	25	30	35

The following linear regression model was employed to predict the response:

$$Y = b_0 + \sum b_i x_i + \sum b_i x_j + \sum b_{ij} x_i x_j \quad (1)$$

where b_0 is the interception coefficient, b_i are the linear coefficients, b_{ij} are the interaction coefficients and x_i and x_j are the coded values of the independent variables.

The goodness of fit of the model was evaluated by the determination coefficient (R^2) and the analysis of variance (ANOVA) at a confidence level of $p \leq 0.05$. All statistical and graphical analyses were carried out using the "Statistic 8.0" software (StatSoft, Tulsa, OK).

Effects of the pH and Temperature on the Enzymatic Activity and Stability

For the determination of the optimum pH of the enzyme, the pH of the reaction mixture containing buffer and 1% (*w*/*v*) azocasein was varied over the range 5.2–9.0. The buffers used were 0.1 M citrate–phosphate (pH 5.0–6.2), 0.1 M sodium phosphate (pH 5.8–8.0), and 0.1 M Tris–HCl (pH 7.2–9.0). The pH stability was determined by pre-incubating the cell-free supernatants into the selected buffer in the same pH range. For the determination of optimum temperature, the reaction mixture containing 1% (*w*/*v*) azocasein in 0.1 M Tris–HCl buffer (pH 7.6) and the cell-free supernatants were incubated over a temperature range of 25–80 °C. Tests of activity and stability at variable pH and temperature were performed, varying the incubation time from 30 to 120 min. After incubation, the samples were submitted to determination of residual proteolytic activity.

Kinetic and Thermodynamic Parameters of Thermal Inactivation

The progressive inactivation of proteases in cell-free supernatants was kinetically described as a first-order irreversible reaction, whose temperature-dependent rate constant, k (min^{-1}), was estimated at each temperature from the semi-log plot of the residual activity with respect to the initial one (ψ) versus time (t):

$$\ln \psi = -k\,t \quad (2)$$

On the other hand, all the thermodynamic parameters of thermal inactivation of proteases were estimated by a combination of the well-known Arrhenius and Eyring equations.

In particular, the activation energy, E^*(kJ/mol), has been estimated by regression from the slope of the straight line obtained plotting ln$k$ versus $1/T$, according to Arrhenius:

$$\ln k = \ln A - \frac{E^*}{RT} \quad (3)$$

where A is the frequency factor or Arrhenius constant (min^{-1}), R the ideal gas constant (8.3145 J/mol K), and T the absolute temperature (K).

The related enthalpy (ΔH^*) and entropy (ΔS^*) of activation were estimated by regression analysis of $\ln(k/T)$ versus $1/T$ [26], according to the equation of Eyring [27] derived from the transition state theory:

$$\ln\frac{k}{T} = \ln\frac{k_B}{h} + \frac{\Delta S^*}{R} - \frac{\Delta H^*}{RT} \tag{4}$$

where k_B is the Boltzmann constant ($1.381 \cdot 10^{-23}$ J/K) and h the Plank's constant ($1.104 \cdot 10^{-35}$ J min).

Finally, the free energy of activation (ΔG^*) was estimated from the corresponding values of enthalpy and entropy by the equation:

$$\Delta G^* = \Delta H^* - T\Delta S^* \tag{5}$$

Results and Discussion

Yeast Isolation and Identification

A total of 51 microorganisms were isolated in the milk samples, and 17.6% of these were identified summarily as yeasts. The low yeast number in raw milk may be due to competitive utilization for the substrates especially by the quickly growing psychotropic bacteria or to inhibition by metabolites excreted by bacteria. The species isolated and identified were deposited in the URM collection of Federal University of Pernambuco, Brazil (Table 2).

In this study, the *Candida* genus was the most frequently encountered one in the cow milk samples. These results are in accordance with those reported for fermented milks or handicraft cheese [15], milks of Apulia region, Italy [16], and African fermented milks [28, 29].

Table 2 Identification and proteolytic activity of yeast species isolated from raw cow milk produced in Cachoeirinha-PE, Brazil.

Specie [18]	Mycotec URM number UFPE-Br	Halo diameter (mm)[a]	Specific proteolytic activity (U/mg)[b]
Candida glabrata	4682	8	nt[c]
C. buinensis	4674	20	23.5
C. diversa	4680	18	15
C. butyri	4679	22	15.6
C. peltata (Yarrow)	4681	5	nt
C. norvegica (Reiersol)	4677	18	4.8
Bretanomyces clausenii (*Dekkera anomala* asexual form)	4675	15	22.7
Dekkera bruxellensis	4676	7	nt
Geotrichum candidum (Link)	4673	10	nt

[a] Qualitative test after 3 days in Gelatin-milk-agar at 30°C

[b] Quantitative test

[c] Not tested because the halo diameter was shorter than 10 mm

Selection of the Proteolytic Yeast

As shown by the transparent halos for casein degradation ability (Table 2), all the species isolated and identified were positive for qualitative proteolytic activity, even if the subsequent quantitative proteolytic tests were carried out only for the species showing positive halos with diameter larger than 10 mm. The *Candida* species tested showed satisfactory proteolytic activity (between 4.8 and 23.5 U/mg), a result that agrees with the ones obtained by Corbo et al. [16], who observed the ability of several *Candida* strains present in dairy products to hydrolyze the milk protein. Since the species that showed the highest proteolytic activity was *C. buinensis* (23.5 U/mg), it was selected for subsequent protease production in SF medium.

Protease Production

The results of protease production, in terms of protease activity in the cell-free supernatants obtained by *C. buinensis* fermentations performed according to the 2^3 full factorial design, are summarized in Table 3. The numbers from one to eight refer to the experimental conditions corresponding to combinations of the variables tested, while nine and ten to the central point.

The highest protease production by *C. buinensis* (573 U/mL) took place after 48 h in the fermentations performed at 35 °C, pH 6.5, and 0.1–0.5% (*w*/*v*) SF; therefore, such a fermentation time was always adopted for the statistical analysis. This activity was one order of magnitude higher than that reported for chymotrypsin-like protease by a mutant of *Tricoderma harzianum* (80 U/mL) after 2–3 days [30].

Some significant findings of the statistical analyses are evident in Fig. 1, where the Pareto chart shows the estimated effects of the independent variables (pH, temperature, and SF concentration) and of their interactions on the proteolytic activity, according to a decreasing order of magnitude. Whereas the length of each bar is proportional to the standardized effect, bars extending beyond the vertical line correspond to the effects statistically significant at a confidence level of 95%. It should be noted that only the pH main effect (3) and its interaction with temperature (1*3) were significant.

Table 3 Results of protease production, in terms of protease activity in the cell-free supernatants, by *Candida buinensis* fermentations performed according to the 2^3 full factorial design.

Run[a]	SF[b] (%)	Temperature (ºC)	pH	Protease activity (U/mL)
1	0.1	25	6.5	547
2	0.1	35	6.5	573
3	0.5	25	6.5	560
4	0.5	35	6.5	573
5	0.1	25	8.5	547
6	0.1	35	8.5	527
7	0.5	25	8.5	553
8	0.5	35	8.5	487
9(C)	0.3	30	7.5	520
10(C)	0.3	30	7.5	517

[a] Numbers 1 to 8 refer to runs at variable levels of the three independent variables, while 9 and 10 to runs performed under the experimental conditions of the central point

[b] Soybean flour concentration

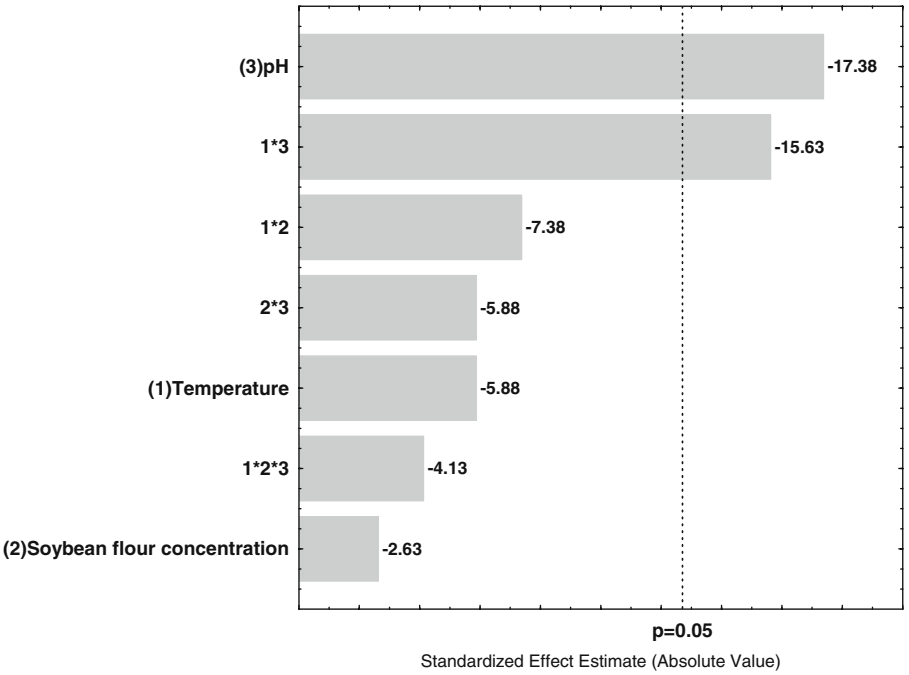

Fig. 1 Pareto chart showing the individual effects of variables (*1* temperature, *2* soybean flour concentration, *3* pH) and their interactions (1*2, 1*3, 2*3, 1*2*3) on the extracellular proteolytic activity of *C. buinensis* cell-free supernatants

In particular, the negative sign of the pH effect means that protease production was favored by a pH decrease and achieved maximum values (547–573 U/mL) at pH 6.5. Moreover, the negative effect also of the interaction between pH and temperature means that the simultaneous decrease in the levels of both independent variables led to an improvement in protease production.

Table 4 Analysis of variance for protease production.

Variable	Sum of squares	Degrees of freedom	Means of squares	*F* ratio	*p* value[a]
(1) Temperature[b]	276.12	1	276.12	61.36	0.08
(2) Soybean flour concentration[b]	55.12	1	55.12	12.25	0.17
(3) pH	2,415.12	1	2,415.12	536.69	0.02
1*2[b]	435.12	1	435.12	96.69	0.06
1*3	1,953.12	1	1,953.12	434.03	0.03
2*3[b]	276.12	1	276.12	61.36	0.08
Lack of fit	1,335.15	2	667.57	148.35	0.06
Pure error	4.50	1	4.50		
Total SS	6750.40	9			

$R^2 = 0.80$

[a] Confidence level of 95% (>0.05)

[b] Not statistically significant values

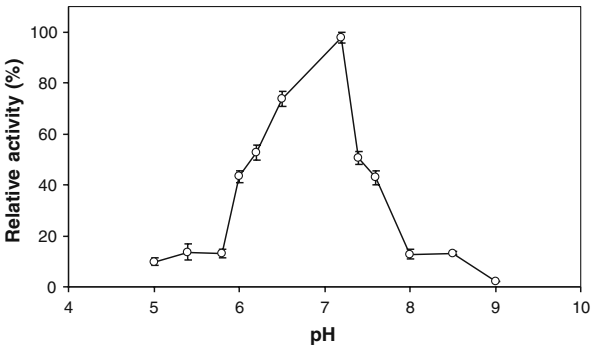

Fig. 2 Effect of pH on the proteolytic activity of *C. buinensis* cell-free supernatants. Proteolytic activity is expressed as percentage with respect to its maximum value at pH 7.2 (531 U/mL)

This behavior is consistent with the well-known strong dependence of cell growth and enzyme production on the extracellular pH [20], although optimum pH is well known to differ from one organism to the other. In particular, Chi et al. [2] observed that a decrease in the initial pH of culture medium improved the release of extracellular alkaline protease by *A. pullulans*, with the highest yield occurring at pH 6.0.

Contrarily to the generally accepted opinion that extracellular protease secretion by microorganisms is substantially influenced by medium components, including carbon source, nitrogen source, and trace elements [31], the level of SF, utilized in the present study as nitrogen, vitamin, and carbon source, did not significantly influence protease production according to the full factorial design. The protease activity by *C. buinensis* was in fact the same (573 U/mL) at both tested SF levels (0.1% and 0.5%) at pH 6.5 and 35 °C. This result, which suggests that the highest protease production could be ensured by a minimum amount of this component, is of a certain industrial concern, taking into account that around 30–40% of the production cost of industrial enzymes can be accounted for the cost of the growth medium [32].

The value of the determination coefficient (R^2=0.80) indicates that the model explained 80% of the variability data. The statistical significance of the model (Eq. 1) was also confirmed by the low significant lack of fit (p=0.06) and the very low pure error (4.5) (Table 4). Moreover, it was found to adequately fit the data at a confidence level of 95% ($p \leq 0.05$). These results demonstrate that both temperature and pH were important independent variables governing the protease production by *C. buinensis*.

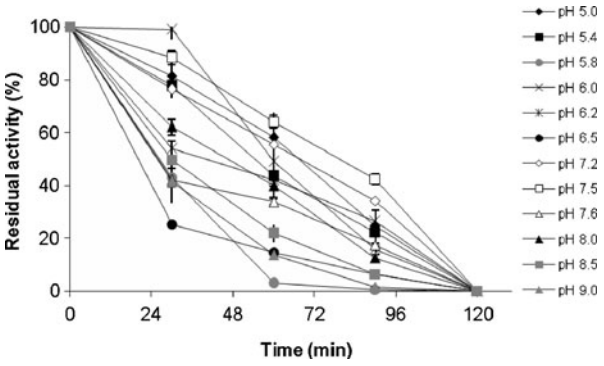

Fig. 3 pH stability profile of *C. buinensis* protease in cell-free supernatants after 30, 60, and 90 min of incubation. Values are expressed as percentage residual activity with respect to the initial one

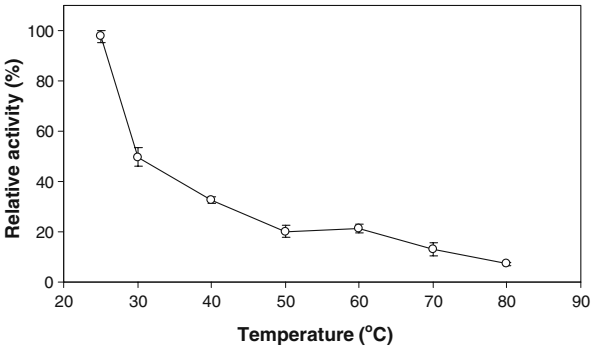

Fig. 4 Effect of temperature on the proteolytic activity of *C. buinensis* cell-free supernatants. Proteolytic activity is expressed as percentage with respect to its maximum value at 25 °C (534 U/mL)

Effect of pH on Activity and Stability

Proteases produced by *C. buinensis* were then submitted to tests of relative activity versus time performed in the pH range 5.2–9.0 in the presence of three different buffers.

As shown in Fig. 2, the proteolytic activity progressively increased with the pH, reached a maximum value (531 U/mL) at pH 7.2 in a Tris–HCl buffer, and then drastically fell under alkaline conditions to a minimum value at pH 9.0. Similar results were found by Bolumar et al. [33] for protease produced by *Debaryomyces hansenii* that showed maximum activity at pH 7.5, although this enzyme was active in a broad range (pH 5.0–9.5). Contrarily to these results, optimum proteolytic activities under alkaline conditions were observed for *A. pullulans* (pH 9) [2], *Candida albicans* URM3622 (pH 8.2) [20], and *Bacillus* sp. strain MO-1 (pH 8.0) [34].

In the present work, we also observed retention of more than 98% of the maximum activity at pH 6.0 after 30 min and 42% at pH 7.5 after 90 min, and negligible activity at pH 5.8 and 9.0 (Fig. 3). These optima are quite different from the acidic and alkaline proteases reported by Nakayama et al. [35] for *Bacillus* sp. strain NTAP-1 and by Lima et al. [20] for *C. albicans*, respectively, but in reasonable agreement with those of common bacterial collagenases, which are known to have optimum pH in the range 7–8 [36].

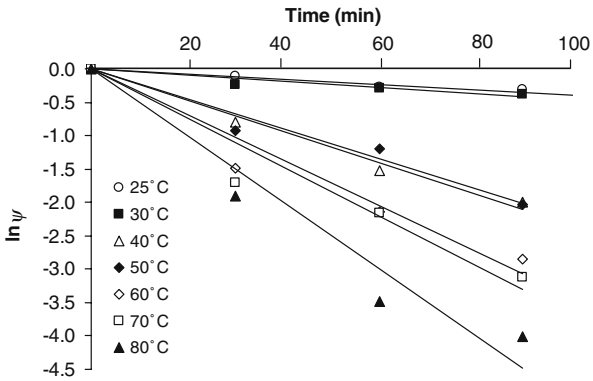

Fig. 5 Semi-log plot of the residual activity of *C. buinensis* proteases versus time in cell-free supernatants at different temperatures

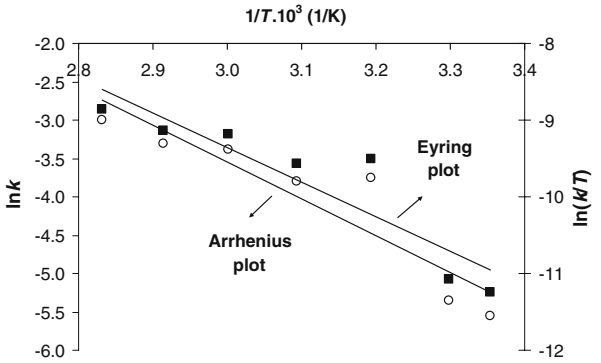

Fig. 6 Arrhenius (*white circle*) and Erying (*filled square*) plots of thermal stability of *C. buinensis* proteases in cell-free supernatants

Effect of Temperature on Activity and Stability

Figure 4 shows that the cell-free supernatants activity progressively decreased with increasing temperature within the selected range; the maximum proteolytic activity (534 U/mL) did in fact occur at the lowest temperature (25 °C). This temperature is quite different from the optimum values reported for different proteases from *Candida caseinolytica* (37 °C) [3], *D. hansenii* (37 °C) [33], *A. pullulans* (45 °C) [2], and *C. albicans* (45 °C) [20].

Figure 5 shows the semi-log plots of extracellular protease thermal stability expressed as residual activities at different temperatures with respect to the initial ones. Up to 30 °C, the residual activity after 90 min kept higher than 68%, whereas it fell to less than 6% for $T \geq$ 60 °C. Beyond 60 °C, a drastic reduction was observed, with the residual activity falling to zero at 80 °C, thereby highlighting complete thermal inactivation. Ramírez-Zavala et al. [14] reported that the activity of a serine carboxypeptidase from *Kluyveromyces marxianus* decreased significantly over 60 °C, with almost complete inactivation (>99%) after 30 min at 55 °C. Ma et al. [37] observed that an alkaline protease from *A. pullulans* was stable up to 20 °C, whereas inactivated rapidly over 45 °C and totally at 50 °C for 30 min.

From the slopes of these straight lines, we estimated with fair correlation ($0.86 \leq R^2 \leq 0.96$) the values of the first-order kinetic constant (k), which varied from 0.039 min^{-1} at 25 °C to 0.0498 min^{-1} at 80 °C, corresponding to half-lives of 178 and 13.9 min, respectively. These values confirm the satisfactory thermostability of proteases in the cell-free supernatants at temperatures not exceeding 30 °C.

As explained in "Materials and Methods", the estimation of the thermodynamic parameters of the system required the elaboration of the Arrhenius and Eyring type plots illustrated in Fig. 6. In particular, according to Eq. 3, the Arrhenius plot of ln$k$ versus $1/T$ allowed estimating the activation energy of protease thermal inactivation in the cell-free supernatants.

Table 5 lists the activation parameters referred to the above-supposed irreversible first-order inactivation. The activation enthalpy exhibited a positive value ($\Delta H^* = 37.3$ kJ/mol),

Table 5 Thermodynamic parameters of thermal inactivation of protease from *Candida buinensis* in cell-free supernatants.

E^* (kJ/mol)	ΔH^* (kJ/mol)	ΔG^* (kJ/mol)	ΔS^* (J/mol·K)[a]
40.0	37.3	101	−197.5

[a] Estimated at a reference temperature of 50 °C

consistently with the fact that the enzyme inactivation is an endothermic process. Moreover, the activation entropy was negative (ΔS^*=−197.5 J/mol K) as expected by the formation of a transition state with more rigid structure compared to the ternary one of the stable proteases. As previously suggested, to explain similar thermodynamic profile of clavulanic acid degradation (ΔH^*=36.5 kJ/mol; ΔS^*=−219.7 J/mol K; ΔG^*=103.5 kJ/mol), protease inactivation could have been influenced by some interaction with other substances either initially present in the medium or produced by the microorganism [38]. Furthermore, the activation energy (40 kJ/mol) well compares with that reported for clavulanic acid degradation (E^*=39 kJ/mol), which proves the general validity of the proposed inactivation model.

Conclusions

The isolation and identification of proteolytic yeast strains from raw cow milk allowed selecting a strain of *C. buinensis* as the best protease producer. The influence of three independent variables (temperature, pH and SF concentration) and interactions among them on the extracellular protease production by this yeast was investigated through a 2^3 full factorial design. The thermodynamic parameters of the enzyme thermoinactivation were also estimated by a combination of the Arrhenius and Eyring models. The protease exhibited important properties such as broad stability ranges of pH and temperature. Due to these properties, they could be profitably exploited in industrial applications as an environmentally friendly hydrolysis agent alternative to the chemical ones. Then, the use of an inexpensive medium as cheap nitrogen source, as the SF proposed in this study, could be of great significance to scale up this process to the industrial scale.

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