

## PURIFICACIÓN Y CARACTERIZACIÓN DE UNA PROTEASA TERMOESTABLE ALCALINA PRODUCIDA POR *Yarrowia lipolytica*

## PURIFICATION AND CHARACTERIZATION OF A THERMOSTABLE ALKALINE PROTEASE PRODUCED BY *Yarrowia lipolytica*

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### Abstract

Purification of the extracellular protease produced by *Yarrowia lipolytica* was realized in four steps: ammonium sulfate precipitation, anionic exchange (2X) and gel filtration. The enzyme showed molecular weight of 61.5 kDa (SDS-PAGE) and optimum activity at 52.4°C at pH 10-11. The thermal stability was modified in presence of Ca<sup>2+</sup> (10 mM) providing an increase of 73, 6 and 11% at 40, 50 and 60°C respectively. The thermodynamic parameters (enthalpy and entropy) indicate that the stability of the enzyme is not provided by non-covalent linkages. Furthermore the ion Ca<sup>2+</sup> is important for thermodynamic stabilization of the enzymatic structure. The proteolytic activity was inhibited by PMSF; suggesting that the enzyme can be classified in the serine protease family. The results of thermodynamic stability allow classifying the protease studied as thermostable. The importance of the Ca<sup>2+</sup> on the thermostability was corroborated; this is the first report on thermal stability and thermodynamic properties of proteases produced by *Y. lipolytica*.

**Keywords:** *Yarrowia lipolytica*, protease, thermostable, thermodynamic stability.

### Resumen

La purificación de la proteasa extracelular producida por *Yarrowia lipolytica* se realizó en cuatro etapas: precipitación con sulfato de amonio, intercambio aniónico (2X) y filtración en gel. La enzima presentó un peso molecular de 61.5 kDa (SDS-PAGE) y actividad óptima a 52.4°C en el rango de pH 10-11. La estabilidad térmica se ve modificada en presencia de Ca<sup>2+</sup> (10 mM) observándose un aumento de 73, 6 y 11% a 40, 50 y 60°C respectivamente. Los parámetros termodinámicos (entropía y entalpía) indican que la estabilidad de la enzima no es debida a enlaces no covalentes. El ion Ca<sup>2+</sup> estabiliza termodinámicamente la estructura enzimática. La actividad proteolítica fue inhibida por PMSF, sugiriendo que la enzima puede clasificarse como serín proteasa. Este es el primer trabajo que reporta estudios de estabilidad térmica y propiedades termodinámicas de proteasas producidas por *Y. lipolytica*.

**Palabras clave:** *Yarrowia lipolytica*, proteasa, termoestable, estabilidad termodinámica.

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## 1 Introduction

Proteases are enzymes widely distributed in nature; they can be extracted from plants and animals however microorganisms represent an interesting source of proteases with clear industrial projection due to the rapid and profitable growth and high enzyme titers produced. Furthermore, the relatively easy genetic modification allows building of high productive strains (Peñalva and Gerbert, 2002). Yeast extracellular proteases are of particular interest for their high commercial applications and potential use in expression systems of heterologous proteins (Glover *et al.*, 1997).

Among the yeast reported for protease production are included *Candida lipolytica*, *Aureobasidium pullulans* and *Yarrowia lipolytica*. In particular, higher titers of extracellular protease have been attributed to *Y. lipolytica* under optimized conditions (Mazdak *et al.*, 2004). *Y. lipolytica* is an obligate aerobic dimorphic ascomycete considered as non-pathogen strain and it's approved as GRAS (Generally Recognized as Safe). *Y. lipolytica* secretes a large variety of metabolites such organic acids, lipases, phosphatases and proteases (Aloulou *et al.*, 2007; González-López *et al.*, 2006). Mazdak *et al.* (2004) reported that the XPR2 gene from *Y. lipolytica* encodes the inducible alkaline extracellular protease (AEP), a major protein secreted by this strain. González-López *et al.* (2006) reported that the type of protease secreted depends on the culture initial pH; then at initial acid pH, an acidic protease is produced (AXP), whereas at neutral or alkaline initial pH, production of an AEP is induced. Poza *et al.* (2007) characterized one AEP serine proteases from the subtilisin subfamily with molecular weight of 30 kDa and optimum activity pH of 9-10 when they used a neutral initial pH.

Protein instability at high temperature is one of the main setbacks in extending the enzymes industrial application, and then protein thermostability has been vigorously studied in the biophysical and biotechnological areas. With the understanding of the factors that regulate protein thermostability, the enzymes thermostability could be modeled by rational design (Pack and Yoo, 2003). The objective of this work was to characterize the thermostable extracellular protease produced by *Y. lipolytica* in solid state culture (SSC) in order to provide information on the responsible factors for enzyme thermostability.

## 2 Materials and methods

### 2.1 Microorganism and inoculum

*Y. lipolytica* from coconut copra was provided by the Laboratory of Bioprocess from the University of Guadalajara (UdeG) Mexico. The strain was maintained on potato dextrose agar (PDA) at 4°C. The yeast was propagated in conical flasks (125 mL) with 30 mL of PDA at 45°C for 7 days. The cells were harvested with 25 mL of tween-80 (0.01%) and used as inoculum.

### 2.2 Protease production by solid state culture

The protease production was performed in tubular columns (25 x 200 mm) as previously described by Saucedo *et al.* (1992). Polyurethane foam and fish flour were used as inert support and substrate respectively at 70/30 (w/w) ratio. The culture medium was (g/L): KH<sub>2</sub>PO<sub>4</sub>, 1; MgSO<sub>4</sub> 7H<sub>2</sub>O, 0.5; KCl, 0.5 in phosphate buffer 0.1 M, pH 7 (Sandhya *et al.*, 2005). The inoculum was adjusted at 2×10<sup>7</sup> cells per g of dry matter and moisture content at 50%. The culture was maintained at 45°C and aeration rate of 40 mL/min for 72 h.

#### 2.2.1 Enzyme extraction

The enzymatic extract was obtained by adding 10 mL of TRIS-HCl buffer (20 mM, pH 8) per gram of fermented matter; the slurry was gently stirred for 30 min at 4°C. The suspension was filtered and centrifuged at 15 000g for 15 min at 4 °C (Beckman Coulter 25R) to remove cells and other debris. The supernatant was stored at 4°C and used as protease extract.

### 2.3 Protein assay

Protein content was measured following the method reported by Bradford using bovine serum albumin (BSA) as standard (Bradford, 1976).

### 2.4 Assay of protease activity

Protease activity was determined by the modified method of Johnvesly and Naik (2001). The enzyme (50 μL) was added to 950 μL of 1% of casein Hammerstein (w/v) (dissolved in 50 mM phosphate buffer pH 7) pre-incubated at 50°C for 5 min.

The enzymatic reaction was performed during 15 min and stopped by addition of 1.5 ml of 5% (w/v) trichloroacetic acid (TCA). The mixture was centrifuged at 15,000g at 4°C for 15 min and filtered through Whatman No. 1. The soluble peptides in TCA were estimated spectrophotometrically at 280 nm. A standard curve of tyrosine was used as reference. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute under the assay conditions.

## 2.5 Enzyme purification

The enzyme extract was precipitated by ammonium sulphate at 80% saturation and the suspension was kept overnight with mild stirring at 4°C. The precipitated protein was separated by centrifugation at 15 000g, 30 min at 4°C and re-dissolved in 20 mM phosphate buffer pH 6 and dialyzed for 24 h against the same buffer with three changes of buffer. The dialyzed extract was loaded onto anion exchange chromatography column (AEC) (5 mL, HiTrap<sup>TM</sup> Q FF GE Healthcare®) previously equilibrated with the buffer above and eluted with a linear gradient from 0 to 2 M of NaCl in the same buffer. Fractions with protease activity were pulled and re-chromatographed in the same column but this time the enzymatic extract was loaded with Tris-HCl buffer 20 mM pH 9 and eluted with a linear gradient from 0 to 1 M NaCl in the same buffer. The flow rate was 1mL/min in both times of AEC. The major protease activity fractions from the second AEC were pulled and subjected to gel filtration (GF) in a 50 mL column packed with Ultrogel® AcA 34 previously equilibrated with Tris-HCl (20 mM ,pH 9) added with NaCl (200 mM). Load and elution were performed at 0.5 mL/min. In all cases, separation was performed in FPLC Biologic LP (Bio-Rad®).

## 2.6 Characterization of the pure enzyme

### 2.6.1 Molecular weight determination

The molecular weight and purification progress were followed by electrophoresis (silver stain, SDS-PAGE) (Laemmli, 1970).

### 2.6.2 Effect of pH on activity and stability

The effect of pH on the activity of the pure protease was evaluated at 50°C at different pH values. The pH of the substrate (casein 1 %) was adjusted by the following buffers: 0.1 M sodium phosphate (pH 6 and 7); 0.1 M Tris-HCl (pH 8 and 9); 0.1 M glycine-NaOH

(pH 10 and 11); sodium carbonate (pH 12). For pH stability the enzyme was diluted in the pH buffer to be tested and incubated at 25°C for 2 h. The residual protease activity (%) was subsequently determined as described above.

### 2.6.3 Effect of temperature on activity and stability

The effect of temperature on enzyme activity was evaluated from 40-80°C in presence and absence of Ca<sup>2+</sup> (10 mM). Optima temperature activity was pointed out by means of Arrhenius correlation (Iqbal *et al.*, 2003; Siddiqui *et al.*, 1997).

Thermal stability (thermal inactivation) was studied from 40 to 70°C for 60 min both, in presence and absence of calcium (10 mM) (Adinarayana *et al.*, 2003). Aliquots were withdrawn at different intervals and residual activity was measured as described above.

### 2.6.4 Thermodynamic studies

Thermal stability data were fitted and first order rate constant ( $k_d$ ) was determined by linear regression of  $\ln V$  versus time ( $t$ ). The thermodynamic data were calculated by rearranging the Eyring's absolute rate equation:

$$\ln\left(\frac{k_d}{T}\right) = \ln\left(\frac{k_B}{h}\right) + \frac{\Delta S^*}{R} - \frac{\Delta H^*}{RT} \quad (1)$$

where  $k_d$ ,  $T$ ,  $k_B$ ,  $h$ ,  $\Delta S^*$ ,  $\Delta H^*$  and  $R$  stand for specific reaction velocity, absolute temperature, Boltzmann's constant, Planck's constant, activation entropy, activation enthalpy and gas constant respectively (Iqbal *et al.*, 2003).

### 2.6.5 Inhibition studies

The effect of protease inhibitors (5mM): phenylmethylsulphonyl fluoride (PMSF),  $\beta$ -mercaptoethanol and ethylene diamine tetra acetic acid (EDTA) on the activity of the pure enzyme was individually determinate by pre-incubation of the enzyme on the inhibitor solution at 25°C for 30 min and residual activity was measured. Residual activities in presence of the enzyme inhibitors were compared to the activity obtained without inhibitors (Charles *et al.*, 2008).

### 3 Results and discussion

#### 3.1 Protease production

Production of bioactive compounds by SSC is taking a renewed importance due to its high productivity in short time fermentations (Ruíz-Leza *et al.*, 2007). Figure 1 shows that protease production by *Y. lipolytica* in SSC increases with time and the maximum protease activity (252 U/mL) is observed at 36 h culture, after this time the enzyme production decreases. The reduction in enzyme production through time might be due to nutrients limitation and hydrolysis of the protease itself (Sandhya *et al.*, 2005). The time of highest protease production observed in this work (36 h) is shorter than protease production by *Candida humicola* (96 h) and *Candida buinensis* (48 h) regardless of the previously optimized processes in both cases (Ray *et al.*, 1992; De Araújo *et al.*, 2010). The time production reported in this work represents a time reduction of 62 and 25% respectively. Nevertheless, *Aerobasidium pullulans* and *Kluyveromyces marxianus* presented maximum protease production at 30 h (Chi *et al.*, 2007; Ramírez-Zavala *et al.*, 2004), however, the authors do not mention the activity levels.

#### 3.2 Enzyme purification

Purification of extracellular protease from *Y. lipolytica* is summarized in Table 1. The enzyme was purified 9.3- fold with 13.5% final yield. SDS-PAGE (silver stained) analysis of the purification progress shows a single band at 61.5 kDa after GF (Fig. 2). The molecular weight of proteases reported for *Y. lipolytica*, *Debaryomyces hansenii* and *Candida humicola* are from 30 to 36 kDa (Poza *et al.*, 2007; Bolumar *et al.*, 2005; Ray *et al.*, 1992). Hernández-Montañez *et al.* (2007) reported a neutral intracellular protease (aminopeptidase) produced by *Y. lipolytica* with molecular weight of 97 kDa, highest that the extracellular protease studied in this work but similar to proteases from *Debaryomyces hansenii* (101 and 370 kDa) (Bolumar *et al.*, 2003a; Bolumar *et al.*, 2003b). The molecular weight of the pure protease is comparable with proteases produced from *Debaryomyces hansenii* (42 and 68 kDa), *kluyveromyces marxianus* (46 kDa) and *Saccharomyces cerevisiae* (62 kDa) (Bolumar *et al.*, 2008; Ramírez-Zavala *et al.*, 2004; Kuhn *et al.*, 1974). The molecular weight estimated for the protease studied is higher than the characteristic molecular weight presented by

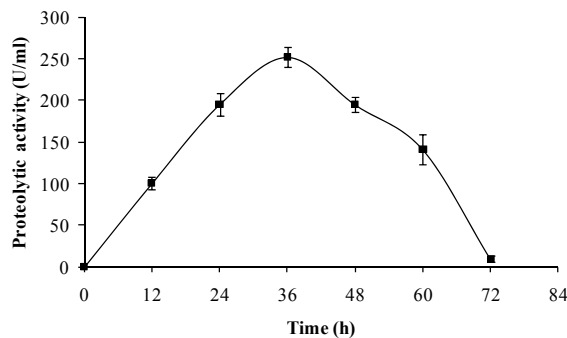


Fig. 1. Kinetic of protease production by *Y. lipolytica* in SSC at 45°C

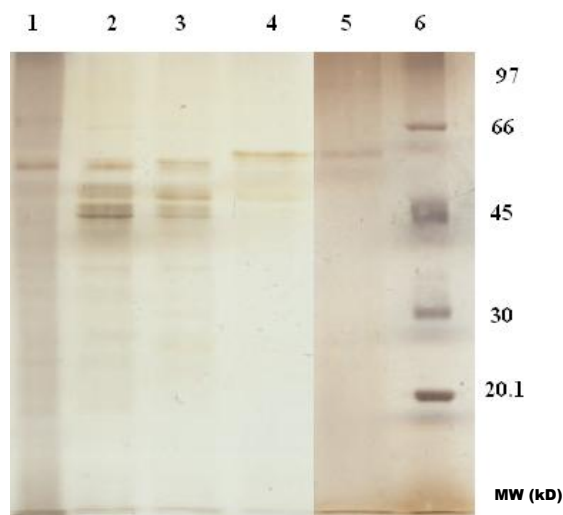


Fig. 2. SDS-PAGE gel electrophoresis throughout purification. Lane: 1) enzymatic extract, 2) ammonium sulphate precipitation, 3) anionic exchange 1, 4) anionic exchange 2, 5) gel filtration, 6) molecular weight markers (MWkDa)

monomeric proteases produced by other yeast strains, probably due to some post-transcriptional modification (Spiro, 2002).

#### 3.3 Effect of pH on activity and stability

The protease activity at different pH values is shown in Fig. 3. The protease is most active in a pH range from 6 to 12 showing at least 62% of maximum activity. However, optimal activity was observed at pH from 10-11. The pH values for optimal activity are in accordance with the extracellular protease produced by *Yarrowia lipolytica* CX161-1B and *Aerobasidium pullulans* that showed optimum activity at pH values

Table 1. Purification of extracellular protease from *Yarrowia lipolytica*

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (Fold)
Enzymatic extract	563	38.23	15	100	1
Amonium sulfate precipitation	304	14.17	21	53.9	1.5
Anion exchange 1	191	6.81	28	33.9	1.9
Anion exchange 2	146	2.96	49	25.9	3.3
Gel filtration	76	0.55	137	13.5	9.3

of 9-10 and 9.5-10.5 respectively (Ogrydziak and Scharf, 1982; Donaghy and McKay, 1993). A large number of alkaline proteases produced by *Aerobasidium pullulans*, *Candida humicola* and *Debaryomyces hansenii* showed highest activity at alkaline pH from 8 to 9 (Chi et al., 2007; Ray et al., 1992; Bolumar et al., 2005; Bolumar et al., 2008).

The enzyme was stable over a broad range of pH from 7 to 12 retaining more than 92% of residual activity. However at pH 5 and 6 retained 74 and 65% of activity respectively, after incubation for 2 h (Fig. 4). These results differ the ones reported by Ramírez-Zavala et al. (2004) whom reported that a protease from *Y. lipolytica* is stable at pH from 4 to 8, then the strain studied provide a wide pH range of high activity and gives a major advantage in industrial processes.

### 3.4 Effect of temperature on activity and stability

Enzyme activity in the temperature range from 35 to 70°C was evaluated in absence and presence of 10 mM Ca<sup>2+</sup> (Figs. 5 and 6). The enzyme showed an optimal activity at 52.7 (5 U/mL) and 52.4°C (5.6 U/mL) in presence and absence of Ca<sup>2+</sup> respectively. The results shown that the optimal temperature in presence or absence of Ca<sup>2+</sup> are equal; nevertheless the enzyme activity was 12% increased in presence of Ca<sup>2+</sup> suggesting that Ca<sup>2+</sup> had an assisting effect on enzyme activity by providing stability. The optima temperature showed for the protease studied in this work is higher than the proteases produced by *Y. lipolytica* reported by Hernández-Montañez et al. (2007), Ogrydziak and Scharf (1982) and Jing et al. (2009) which display their optimum temperature at 37, 40, 45°C. Furthermore, the protease studied showed optimum temperature value higher than proteases produced by *Candida humicola* (37°C), *Debaryomyces hansenii* (37°C),

*Kluyveromyces marxianus* (45 °C), *Aerobasillum pullulans* (45°C) and *Debaryomyces hansenii* (Ray et al., 1992; Bolumar et al., 2003b; Ramírez-Zavala et al., 2004; Chi et al., 2007; Bolumar et al., 2003a).

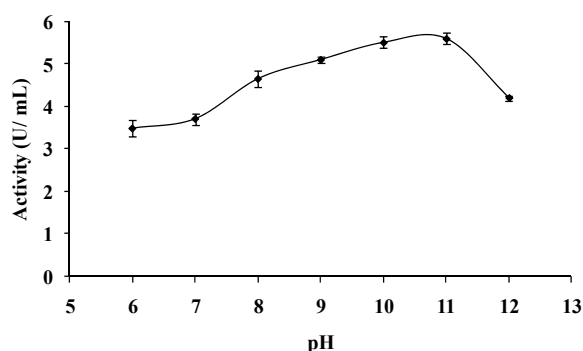


Fig. 3. Effect of pH on the activity of protease produced by *Y. lipolytica*.

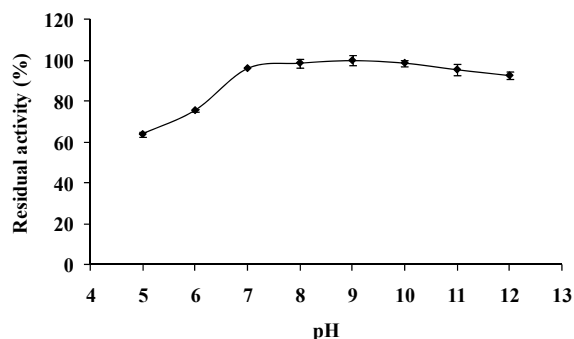


Fig. 4. Effect of pH on the stability of alkaline protease produced by *Y. lipolytica*.

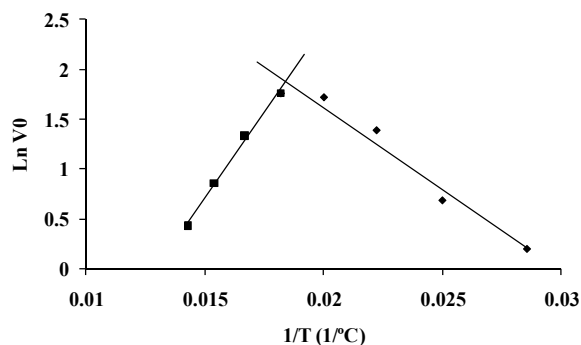


Fig. 5. Arrhenius plot of the alkaline protease produced by *Y. lipolytica*.

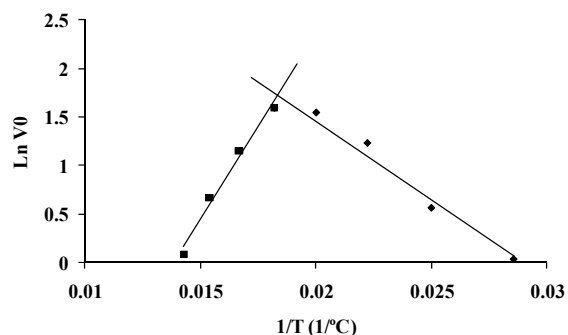


Fig. 6. Arrhenius plot of the alkaline protease produced by *Y. lipolytica*.

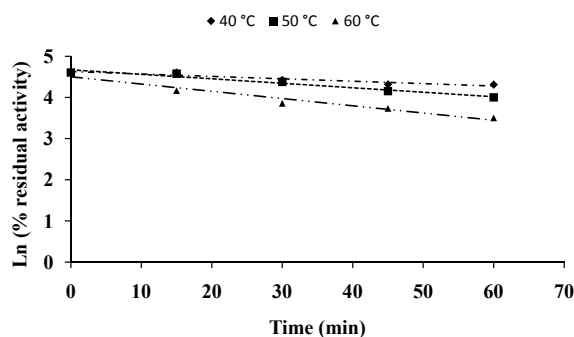


Fig. 7. Thermal stability of the alkaline protease produced by *Y. lipolytica*.

The thermal stability of the pure protease in presence and absence of  $\text{Ca}^{2+}$  (10 mM) is shown in Figs. 7 and 8. With the experimental data at different temperature values and the use of reverse lineal model (Azeredo *et al.*, 2004), the half live ( $t_{1/2}$ ) was calculated (Table 2 and 3). The thermal stability profile of the protease showed that  $\text{Ca}^{2+}$  is required for enzyme stability because  $t_{1/2}$  is increased by 61, 6 and 11% at 40, 50 and 60°C respectively in presence

of  $\text{Ca}^{2+}$ ; whereas at 70°C no activity was detected in absence of  $\text{Ca}^{2+}$  and a  $t_{1/2}$  value of 6 min was observed when  $\text{Ca}^{2+}$  was added. The improvement in protease thermostability in presence of  $\text{Ca}^{2+}$  may be explained by the interactions inside the protein's molecules and by binding the ion to the autolysis sites avoiding the self-protolysis. Ramírez-Zavala *et al.* (2004) reported that the activity of serine carboxypeptidase from *Kluyveromyces marxianus* decreased significantly over 60°C; with almost complete inactivation after 30 min at 55°C; and Ma *et al.* (2007) observed that a protease produced by *Aerobasillum pullulans* was stable up to 20°C and totally inactivated at 50°C. The enzyme characterized in here, provides higher thermostability at higher temperatures. This is the first report of thermal stability of proteases produced by *Y. lipolytica*.

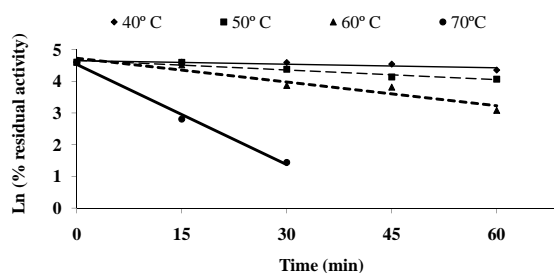


Fig. 8. Thermal stability of the alkaline protease produced by *Y. lipolytica* in presence of 10 mM of  $\text{Ca}^{2+}$ .

### 3.5 Thermodynamic studies

Tables 2 and 3 list the inactivation parameters of thermal inactivation at temperatures from 40 to 70°C. The results shows that in both cases (absence and presence of  $\text{Ca}^{2+}$ ) the enthalpy ( $\Delta H^*$ ) is independent of temperature, thus there is no change in enzyme heat capacity, indicating that the enzyme stability is not provided by non-covalent linkages, since  $\Delta H^*$  value can be correlated with the number of non-covalent broken bonds during the process of protein denaturation (Ortega *et al.*, 2004). The results showed that  $\Delta G^*$  is slightly higher in presence of  $\text{Ca}^{2+}$  indicating additional stability and in consequence the importance of  $\text{Ca}^{2+}$  to increase stability on the protease structure (Volquen *et al.*, 2009). All entropy values ( $\Delta S^*$ ) for thermal inactivation of the protease produced by *Y. lipolytica* from 40 to 60 °C were negative, however when  $\text{Ca}^{2+}$  was added, a slightly increase of  $\Delta S^*$  indicate that the structure is better

organized than in absence of  $\text{Ca}^{2+}$  (Table 2 and 3). The most common cause of the heat inactivation on most enzymes is the loss of the native conformation (unfolding of the active tertiary protein structure to a disordered polypeptide), a process defined as thermodenaturation, which takes place as result of the increased molecular mobility at elevated temperature (Volquen *et al.*, 2009; Iqbal *et al.*, 2003).

The activation parameters related to Eyring's model supposed as irreversible first order inactivation shows that activation enthalpy exhibited a positive values in absence of  $\text{Ca}^{2+}$  ( $\Delta H^* = 58.8$  kJ/mol) and in presence of  $\text{Ca}^{2+}$  ( $\Delta H^* = 85.3$  kJ/mol). The difference on the value of this parameter indicates major thermostability when  $\text{Ca}^{2+}$  is present, suggesting that the protein structure presents higher rigidity (De Araújo *et al.*, 2010). On the

other hand, the activation entropy was negative in both, absence ( $-102.2$  J/mol K) and presence of  $\text{Ca}^{2+}$  ( $-21.6$  J/mol K), when  $\text{Ca}^{2+}$  is present, the increase entropy value indicates reorganization of the molecular structure of the enzyme.

### 3.6 Inhibition studies

The protease activity was inhibited 88% in presence of PMSF (5 mM) suggesting that the extracellular protease can be classify in the serine protease family. PMSF is known to sulphonate the essential serine residue in the active site of the protease resulting in a loss of enzyme activity (Kumar, 2002). Minimal or no inhibitory effect on the protease activity was observed with the other protease inhibitors tested (data not show).

Table 2. Kinetic and thermodynamic parameters<sup>a</sup> for irreversible thermal inactivation of alkaline protease from *Y. lipolytica*.

T (°C)	$k_d$ (min <sup>-1</sup> )	$t_{1/2}$ (min)	$\Delta H^*$ (kJ/mol)	$\Delta G^*$ (kJ/mol)	$\Delta S^*$ (J/mol K)
40	0.0048	143	83.50	90.70	-22.99
50	0.0092	76	83.41	91.97	-26.47
60	0.0200	35	83.33	92.73	-28.21

<sup>a</sup> $k_d$  = First order rate constant for inactivation,  $t_{1/2}$  = Half live values from logarithmic plots of (relative residual activity) vs. incubation time and  $k_d = 1.6989/t_{1/2}$ ,  $\Delta H^* = E_a - RT$  where  $E_a$  is the activation energy,  $\Delta G = -RT \ln\{(kd.h)/k_B.T\}$ ,  $\Delta S^*$  is the entropy of irreversible inactivation and was calculated from  $\Delta S = (\Delta H^* - \Delta G^*)/T$ .

Table 3. Kinetic and thermodynamic parameters<sup>a</sup> for irreversible thermal inactivation of alkaline protease produced by *Y. lipolytica* in presence of 10 mM of calcium.

T (°C)	$k_d$ (min <sup>-1</sup> )	$t_{1/2}$ (min)	$\Delta H^*$ (kJ/mol)	$\Delta G^*$ (kJ/mol)	$\Delta S^*$ (J/mol K)
40	0.0028	247	84.85	92.12	-23.21
50	0.0092	75	84.77	91.95	-22.22
60	0.0213	33	84.69	92.56	-23.65

<sup>a</sup> $k_d$  = First order rate constant for inactivation,  $t_{1/2}$  = Half live values from logarithmic plots of (relative residual activity) vs. incubation time and  $k_d = 1.6989/t_{1/2}$ ,  $\Delta H^* = E_a - RT$  where  $E_a$  is activation energy,  $\Delta G = -RT \ln\{(kd.h)/k_B.T\}$ ,  $\Delta S^*$  is the entropy of irreversible inactivation and was calculated from  $\Delta S = (\Delta H^* - \Delta G^*)/T$ .

## Conclusions

Although many reports on production and purification of proteases by *Y. lipolytica* have been published, little information about thermal characteristics of this enzyme is available. In this study, we demonstrated that *Y. lipolytica* produce a thermostable serine protease in SSC at 45°C. The serine protease exhibited important properties such as broad range of pH stability and temperature. In presence of  $\text{Ca}^{2+}$  the

stability was improved up to 73% at 40°C. The results of thermodynamic stability allows to class the protease studied as thermostable, additionally the importance of the  $\text{Ca}^{2+}$  on the thermostability was corroborated; this is the first report of thermal stability and thermodynamic properties of proteases produced by *Y. lipolytica*.

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