

# ALKALINE PROTEASE PRODUCTION BY *Bacillus licheniformis* LBA 46 IN A BENCH REACTOR: EFFECT OF TEMPERATURE AND AGITATION

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**Abstract** - The production of protease from *Bacillus licheniformis* LBA 46 was studied in a 6 L reactor using the experimental design tool. The higher protease production was obtained in the exponential phase of growth reaching maximum activity (~3,000 U/mL) after 48 h of fermentation at 30 °C and 300 rpm in a culture medium made of agroindustrial by-products. In the thermostability study, the semi-purified enzyme retained about 78% of the initial activity after 120 min at 50 °C. The protease was purified 3.33 times by ammonium sulfate precipitation and DEAE-Sepharose column chromatography and had a molecular mass estimated at 40 kDa by SDS-PAGE. The purified protease showed optimum activity at 50 and 60 °C, optimal activity in pH 8.5 and stability in the range between pH 5-10 after 24 h of incubation at 4 °C, presenting more than 86% of the initial activity.

**Keywords:** *Bacillus licheniformis*; Fermentation; Optimization; Protease; Purification.

## INTRODUCTION

Industries of food, pharmaceutical, agricultural and medical have been taking advantage of using *Bacillus* sp. because of their wide range of physiological characteristics and ability to produce enzymes and other metabolites (Gupta et al. 2002; Schallmeyer et al. 2004; Voigt et al., 2004). *Bacillus subtilis* and *Bacillus licheniformis* species are attractive industrial microorganisms recognized as GRAS (generally recognized as safe), which have high growth rates leading to shorter fermentation times and possess the ability to secrete extracellular proteins (Ward et al. 2009; Ward 2011; Parrado et al. 2014).

Since the advent of enzymology, one of the most important classes of hydrolytic enzymes, which have been extensively studied, is the microbial proteases (Furhan and Sharma 2014; Hadjidj et al. 2018). Each enzyme has a peculiar characteristic of performance, which makes it suitable for several applications. The

alkaline proteases were initially marketed for use in detergents and the market for these industrial enzymes expanded substantially during the 1960s. Until nowadays they are one of the most widely studied groups of enzymes because of their extensive types of application in several sectors such as detergent, textile, leather and food industries (Benmradi et al. 2016; Bouacem et al. 2016; Ward et al. 2009). The main microbial strains used in enzyme production are still *Bacillus* species, used principally to produce alkaline serine proteases and neutral proteases (Schallmeyer et al. 2004; Ward 2011; Pant et al. 2015).

On an industrial scale, microorganisms are cultivated in reactors under the best conditions of production (Moo-Young and Chisti 1994; Gupta et al. 2002). According to Potumarthi et al. (2007), mixing in the reactor is important during the production of proteases, which is transmitted by aeration and agitation. The temperature is another important function to control in a fermentative process, so

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there is a necessity of defining a better combination of these factors within the fermentation process for maximum efficiency and productivity. Based on this, the aim of this study was to verify the effects of modifying temperature and agitation conditions during the submerged fermentation of *B. licheniformis* LBA 46 in a reactor on protease production, using the experimental design process.

## MATERIALS AND METHODS

### Fermentation

#### *Microorganism and culture medium*

The microorganism used was a strain of *B. licheniformis* LBA 46 from the culture collection of the Laboratory of Food Biochemistry, School of Food Engineering, UNICAMP, Brazil. The culture medium used was proposed by Contesini (2014), with modifications, containing agroindustrial by-products as carbon and nitrogen sources (32 g/L of sugar cane molasses (Fio de Ouro<sup>®</sup>); 6 g/L of corn steep liquor (Corn Products<sup>®</sup>); 2 g/L of yeast extract (Prodex-Lac SD<sup>®</sup>) and 20 g/L dried whey (Alibra<sup>®</sup>), adjusted to pH 7).

#### *Inoculum preparation*

The microorganism was grown in nutrient agar (1 g/L of meat extract; 2 g/L of yeast extract; 5 g/L of peptone; 5 g/L of sodium chloride and 15 g/L of agar, pH 7) slants and incubated at 30 °C for 18-24 h. After growth, a bacterial cell suspension was prepared by adjusting the absorbance at 620 nm to 0.49-0.51. Erlenmeyer flasks containing the cell suspension and culture medium were incubated at 30 °C and 200 rpm for 36-40 h.

#### *Submerged fermentation in a reactor*

The fermentation of *B. licheniformis* LBA 46 was performed in a New Brunswick Bioflo II reactor with a capacity for 6 L and a working volume of 3 L. The inoculum represented 10% of the culture medium and was prepared as described above. Foaming was controlled during fermentation using the anti-foam DC\*FG-10 (Dow Corning<sup>®</sup>), which dripped automatically when the foam level reached the sensor. The air flow rate was maintained at 0.8 vvm. The pH value was monitored using a calibrated potentiometer. The total fermentation time was 72 h, and samples (15 mL) were collected every 12 h and centrifuged at 11,000 x g for 15 min at 5 °C. The cell-free supernatant was used as the enzyme extract for determination of protease activity.

#### *Optimization of temperature and agitation*

A factorial design with 4 possible combinations and 3 central points was used to optimize and evaluate

the effects of temperature and agitation for the reactor fermentation, resulting in a total of 7 tests, which were carried out in random order. Table 1 shows the coded and real values of the variables studied.

**Table 1.** Factorial design, coded and real values of the variables studied (temperature and agitation).

Assay	T (°C)	Agitation (rpm)
1	-1 (30.0)	-1 (200)
2	1 (37.0)	-1 (200)
3	-1 (30.0)	1 (300)
4	1 (37.0)	1 (300)
5	0 (33.5)	0 (250)
6	0 (33.5)	0 (250)
7	0 (33.5)	0 (250)

#### *Kinetics of microbial growth and protease production*

The microbial growth kinetics of *B. licheniformis* LBA 46 and the protease production were carried out in a 6 L reactor containing 3 L composed of 32 g/L of sugar cane molasses (Fios de Ouro<sup>®</sup>); 6 g/L of corn steep liquor (Corn Products<sup>®</sup>); 2 g/L of yeast extract (Prodex-Lac SD<sup>®</sup>) and 20 g/L dried whey (Alibra<sup>®</sup>), adjusted to pH 7, at 300 rpm, 30 °C and 0.8 vvm. Samples of the culture media were collected at different times and inoculated into Petri dishes containing nutrient agar using the pour plate technique. Petri dishes were incubated at 30 °C for 24 h. Microbial growth was expressed as colony forming units (CFU)/mL. Protease activity, protein and reducing sugar were determined as described below.

#### *Protease activity determination*

Protease activity was determined according to the method described by Charney and Tomarelli (1947) and modified by Castro and Sato (2014), using azocasein as the substrate. The reaction mixture contained 0.5 mL of 0.5% azocasein in 0.05 M sodium phosphate buffer, pH 7, and 0.5 mL of the enzymatic extract which were incubated for 40 min at 60 °C. The reaction was stopped by adding 0.5 mL of 10% trichloroacetic acid (TCA). The reaction mixture was centrifuged at 17,000 x g for 15 min at 15 °C. An aliquot of 1 mL of the supernatant obtained was neutralized with 1 mL of 5 M KOH. One protease activity unit was defined as the amount of enzyme which caused an increase of 0.01 in absorbance at 428 nm.

#### *Protein and reducing sugar determination*

Protein quantification was carried out by Lowry's method with some modifications (Hartree, 1972). The calculations were based on a standard curve of bovine serum albumin (BSA) and were expressed in mg/mL. The reducing sugars were quantified with dinitrosalicylic acid, DNS (Miller, 1959). The calculations were based on a standard glucose curve and were expressed in mg/mL.

### Determination of kinetic and thermodynamic parameters of semi-purified protease

#### Activation energy and temperature coefficient ( $Q_{10}$ )

To determine the activation energy ( $E_a$ ), measurements of protease activity were performed with incubation at different temperatures, 30-80 °C.  $E_a$  was calculated from the slope of the plot of  $1000/T$  vs.  $\ln$  (protease activity),  $E_a = -\text{slope} \times R$ .

The value of the temperature coefficient,  $Q_{10}$ , was determined according to Eq. 1 (Dixon and Webb, 1979). This measure is used to relate the reaction rate with a 10 °C increase in the reaction temperature.

$$Q_{10} = \text{antilog} \varepsilon \left( \frac{E_a \times 10}{RT^2} \right) \quad (1)$$

where  $R$  is the gas constant (8.314 J/Kmol) and  $T$  is the absolute temperature (K).

#### Determination of $K_m$ and $V_{max}$

Kinetic parameters (Michaelis Mentem constants,  $K_m$  and maximum velocity,  $V_{max}$ ) were determined at the optimal temperature and pH of protease activity using different concentrations of azocasein as substrate (1-10 mg/mL).

#### Determination of kinetic and thermodynamic parameters for thermal inactivation

*Kinetic parameters for thermal inactivation* - To determine the thermal inactivation of the protease, the enzyme was incubated in 0.05 M sodium phosphate buffer, pH 7, at temperatures of 50-70 °C for 120 min in the absence of substrate. Samples were collected periodically throughout the incubation period and residual activity was determined at the optimal temperature and pH of protease activity.

The value of the deactivation constant ( $k_d$ ) expressed as an exponential decay and found by plotting  $\ln(A/A_0)$  vs. time was measured according to Eq. 2.

$$A = A_0 e^{-k_d t} \quad (2)$$

where  $A$  and  $A_0$  is the protease activity at a determined time  $t$  and at an initial time, respectively.

The activation energies for denaturation ( $E_{ad}$ ) were calculated by plotting  $\ln(k_d)$  vs.  $1/RT$  as described in Eq. 3. The time when the residual activity reaches 50% (apparent half-life) was estimated by Eq. 4. The  $D$ -value, which is defined as the time required for a 90% reduction in the initial enzyme activity at a specific temperature, was calculated as shown in Eq. 5.

$$k_d = A e^{\left( \frac{-E_{ad}}{RT} \right)} \quad (3)$$

$$t_{1/2} = \frac{\ln(0.5)}{k_d} \quad (4)$$

$$D = \frac{2.303}{k_d} \quad (5)$$

*Thermodynamic parameters for thermal inactivation* - Thermodynamic parameters of the protease were projected using the Eyring absolute rate expression (Eq. 6).

$$k_d = \left( \frac{k_b T}{h} \right) e^{\left( \frac{-\Delta H}{RT} \right)} e^{\left( \frac{\Delta S}{R} \right)} \quad (6)$$

where  $k_b$  is the Boltzmann constant ( $1.38 \times 10^{-23}$  J/K);  $T$  is the absolute temperature (K);  $h$  is the Planck constant ( $6.63 \times 10^{-34}$  J.s);  $\Delta H$  is the enthalpy of activation (kJ/mol) and  $\Delta S$  is the entropy of activation (J/mol K).

The enthalpy of activation,  $\Delta H$ , was calculated using Eq. 7. The activation free energy,  $\Delta G$  was calculated using Eq. 8 and the activation entropy,  $\Delta S$  was determined according to Eq. 9. All terms were previously described in the equations above.

$$\Delta H = E_{ad} - RT \quad (7)$$

$$\Delta G = -RT \ln \left( \frac{k_d h}{k_b T} \right) \quad (8)$$

$$\Delta S = \frac{(\Delta H - \Delta G)}{T} \quad (9)$$

### Purification and characterization of purified protease

The protease of *B. licheniformis* LBA 46 strain was produced using the optimized conditions of temperature and agitation. The supernatant was separated by centrifugation and fractionated with 80% ammonium sulfate. The precipitate was dissolved in 0.05 M phosphate buffer, pH 7, and dialyzed against distilled water at 5 °C and freeze-dried. The freeze-dried protease was applied to a 20 mL DEAE-Sepharose ion exchange column (HiPrep™ DEAE FF 16/10, GE, Little Chalfont, UK) equilibrated with 0.05 M sodium phosphate buffer, pH 7, and the proteins were eluted (5 mL/min) with a linear 0 to 1 M sodium chloride gradient (Äkta Purifier, GE, Little Chalfont, UK). Fractions containing protease activity were pooled and analyzed by SDS-PAGE (Vertical Slab Mini-Protean Electrophoresis System, Bio-Rad Laboratories, Hercules, CA, USA) as described by Laemmli (1970). The run was performed at 110 V for 30 min. The molecular weight of the enzyme was estimated using molecular mass markers (Thermo Fisher Scientific Ruler™ Unstained Protein Ladder) ranging from 10 to 200 kDa. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250.

### Determination of the optimum pH of activity and stability of purified protease

The effect of pH on protease activity was determined by univariate assay using 0.1 M acetate buffer (pH 4-5), 0.1 M sodium phosphate buffer (pH 6-8), 0.1 M Tris-HCl buffer (pH 9), 0.1 M carbonate-bicarbonate buffer (pH 10) and 0.1 M NaOH-bicarbonate buffer (pH 11).

The effect of pH on protease stability was determined using the same buffers and pH values already mentioned. The enzyme solutions were incubated at different pH values for 24 h at 4 °C in the absence of substrate. The residual enzyme activity was then determined. The results were expressed as a percentage and relative activity.

### Determination of the optimum temperature of activity and stability of purified protease

The protease activity was tested at different temperatures (between 30 °C and 80 °C, pH 7). Relative activities were determined by defining the maximum enzyme activity, at a specific temperature, as 100%.

The thermal stability of the enzyme was evaluated by preincubation at various temperatures (between 30 °C and 80 °C, pH 7) for 1 h with subsequent cooling, and the residual enzymatic activity was determined.

### Statistical analysis

The experimental design, matrix and statistical analysis were developed using the Statistica 7.0

program (Statsoft®/Dell, USA), Tukey's test and Pearson correlation were carried out in Minitab 16.1.1 (Minitab Inc., USA). All the analyses were carried out in triplicate and evaluated considering a *p*-value lower than 10% ( $p \leq 0.10$ ).

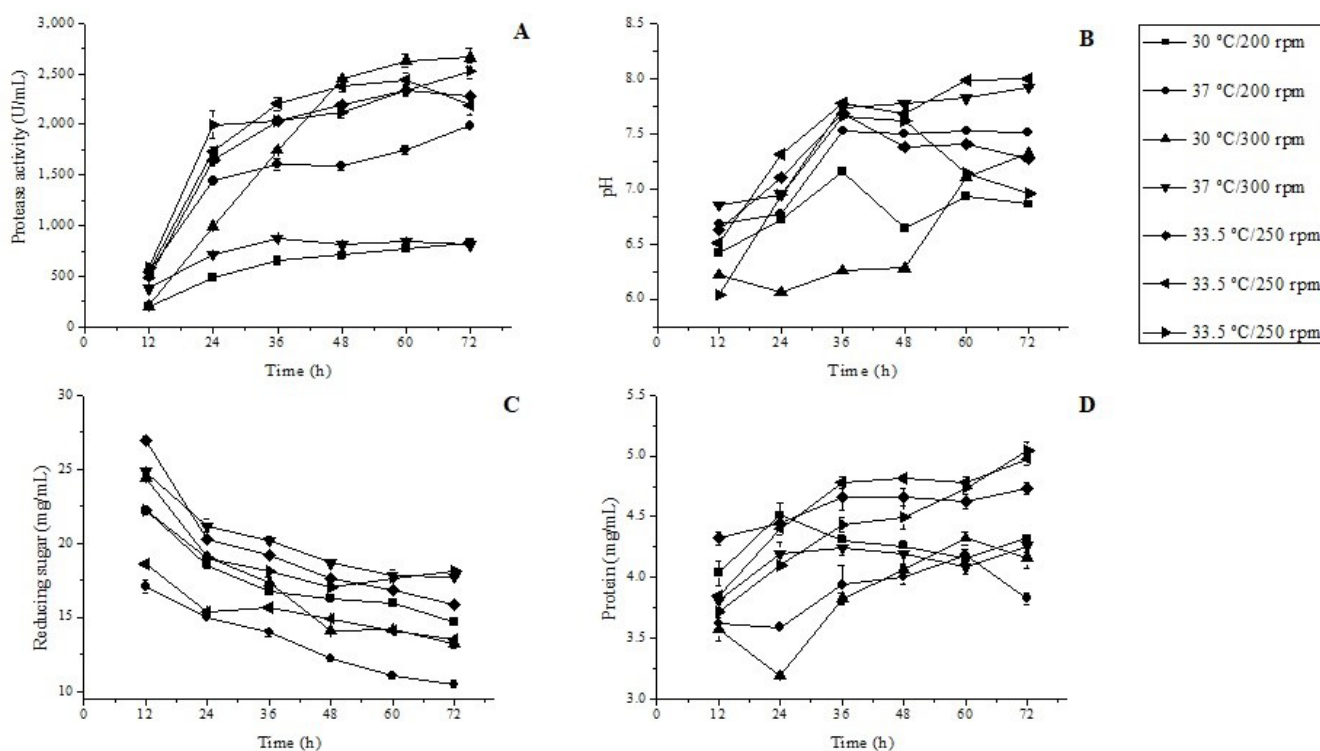
## RESULTS AND DISCUSSION

### Experimental design for the kinetics of protease production in a reactor

Preliminary tests were performed to verify the effects of temperature (in the range of 30-37 °C) on extracellular protease production by *B. licheniformis* LBA 46. Using 100 mL of culture medium in Erlenmeyer flasks, it was found that temperature variations caused variations in the protease production. Therefore, the reactor studies were carried out using the reactor in this temperature range. Figure 1 presents the responses obtained in the factorial design for the effect of temperature and agitation during the kinetics of protease production by *B. licheniformis* LBA 46 in a reactor for 72 h of fermentation.

The estimated enzymatic activity for the kinetics at 72 h (Figure 1A) showed that the values remained high (> 1000 U/mL) in 71.43% of the assays analyzed. Under the conditions evaluated, the microorganism produced protease in the range from 30-37 °C and 200-300 rpm.

It can be observed that, for the majority of the analyzed times, the central points (assays 5-7) and



**Figure 1.** Kinetics of the protease production (A), pH values (B), reducing sugars (C) and protein (D) measured during fermentation of *B. licheniformis* LBA 46 in a bench reactor during 72 h of fermentation.

assay 3 presented values of activity greater than the other tests. This information indicates that there is no adequate fit for a 1st order model, so there is a necessity of evaluating the curvature. According to the  $p$ -value obtained ( $p \leq 0.10$ ), the analysis of curvature was significant. Table 2 presents the estimated regression coefficients for each variable, their interaction and the statistical analysis of each effect for significance assessment.

For protease activity, the temperature, the agitation and the interaction between them showed effects on the factorial design, and the calculated  $p$ -value confirmed the presence of all significant effects after 48 h of fermentation with 90% of confidence (Table 2). The maximum protease activity was reached under the conditions of assay 3 (30 °C and 300 rpm), which presented activities of 2,448.83, 2,627.33 and

**Table 2.** Regression coefficients, standard error,  $t_{calc}$  and  $p$ -value during the protease production by *B. licheniformis* LBA 46 in a bench reactor during 72 h of fermentation.

Variables	Coefficient	Standard error	$t_{calc}^*$	$p$ -value
12 h of fermentation				
Mean	333.96	22.97	14.54	0.005
Curvature	204.17	35.09	5.82	0.028
Temperature	130.96	22.97	5.70	0.029
Agitation	-40.54	22.97	-1.76	0.219
Interaction	-42.87	22.97	-1.87	0.203
24 h of fermentation				
Mean	909.13	92.10	9.87	0.0101
Curvature	884.43	140.69	6.29	0.0244
Temperature	169.46	92.10	1.84	0.2072
Agitation	-53.38	92.10	-0.58	0.6208
Interaction	-308.88	92.10	-3.35	0.0786
36 h of fermentation				
Mean	1,221.50	49.44	24.71	0.0016
Curvature	868.19	75.52	11.50	0.0075
Temperature	19.83	49.44	0.40	0.7271
Agitation	88.67	49.44	1.79	0.2148
Interaction	-453.83	49.44	-9.18	0.0117
48 h of fermentation				
Mean	1,301.13	66.60	19.54	0.0026
Curvature	926.53	101.73	9.11	0.0118
Temperature	-277.38	66.60	-4.17	0.0531
Agitation	329.29	66.60	4.94	0.0386
Interaction	-541.04	66.60	-8.12	0.0148
60 h of fermentation				
Mean	1,499.17	30.91	48.50	0.0004
Curvature	871.31	47.21	18.45	0.0029
Temperature	-202.42	30.91	-6.55	0.0225
Agitation	235.67	30.91	7.62	0.0168
Interaction	-690.08	30.91	-22.33	0.0020
72 h of fermentation				
Mean	1,571.21	88.34	17.79	0.0031
Curvature	761.35	134.95	5.64	0.0300
Temperature	-173.54	88.34	-1.96	0.1884
Agitation	163.63	88.34	1.85	0.2052
Interaction	-752.79	88.34	-8.52	0.0135

\* $t_{calc}$  calculated with 3 degrees of freedom.  
 $R^2 > 0.96$  for all responses.

2,661.17 U/mL after 48, 60 and 72 h of fermentation, corresponding to protease productivity values equals to 51, 46.8 and 36.7 U/mL.h, respectively. The values for the coefficients of temperature and agitation from 48 h of fermentation were high, negative for temperature and positive for agitation, which means that, when the temperature decreased and the agitation increased, the protease activity was at its highest. The assay 3 fitted perfectly with these conditions, and it was chosen for the protease production.

Figure 1 (B, C, D) shows the pH values, reducing sugars and protein measured during fermentation by *B. licheniformis* LBA 46 in a reactor. The pH of the culture medium provides some important information. The initial pH (7) of the culture medium decreased to 6-6.8 after 12 h and then increased to reach 6.8-8 after 72 h in the most assays. The pH initially dropped, probably due to acid production from glucose utilization during the growth phase with the increase in the number of microbial cells, but when the enzymatic production was initiated, the pH started to increase (Singh et al., 2004). The culture medium used is a complex medium, which presents a variety of proteins and peptides from the yeast extract, dried whey protein and corn steep liquor. According to Chu et al. (1992), the acidification or alkalization of the medium during the microbial growth reflects the substrate consumption. When microbial cells use organic nitrogen (amino acids and proteins), the medium becomes more alkaline, resulting in a pH increase, and when ammonium ion is used, the medium turns more acidic, resulting in a pH decrease.

Consumption of sugars and protein synthesis were consistent with cell growth. The sugars were consumed and decreased with the advance of the fermentation, the expected behavior, because the sugars are fermented by the microorganisms during their growth to supply their metabolic needs. According to Figure 1C, the consumption of sugars had a similar profile for the 7 assays. Protein content of the culture medium increased in all assays, reaching about 3.5-5 mg/mL after 72 h of fermentation (Figure 1D), representing the increase in the protease production.

Dey et al. (2016) evaluated the improvement of protease production by *B. licheniformis* NCIM-2042 in a 2.2 L bioreactor containing 30.8 g/L starch, 78.89 g/L soybean meal, 0.5 g/L  $MgSO_4$ , and 5.3 g/L NaCl, pH 7.4. The effect of aeration (1, 2 and 3 vvm) and agitation (150-210 rpm) were tested and the maximum protease production, 382.46 U/mL, was achieved using 180 rpm and 2 vvm, after 84 h of incubation at 37 °C. On the contrary, in this study a higher protease production (>2400 U/mL) was obtained after 48 h of fermentation using lower temperature and lower agitation than those used by Dey et al. (2016).

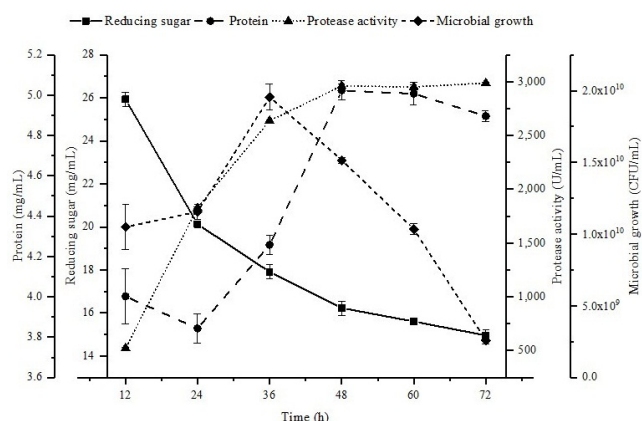
Chuprom et al. (2016) studied the enhancement of halophilic protease production by *Halobacterium* sp. strain LBU50301 using statistical design response to optimize the medium composition. Using 18.62 g/L gelatin, 9.13 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 27.95% (w/v) NaCl, pH 7.88 as culture medium the protease production increased 13-fold from 17.80 U/mL in Erlenmeyer flasks to 231.33 U/mL in a laboratory fermenter. According to the authors, the production of proteases obtained in the reactor was higher than that obtained in the fermentation of the Erlenmeyer flasks, since the reactor systems provide more precise control of parameters such as pH, aeration and stirring speed. As in this work, Chuprom et al. (2016) also observed that the optimization tool is useful for increasing the enzymatic production, as well as the use of a reactor. However, the values of enzyme activity found in this study were higher than those mentioned above. The protease of *B. licheniformis* LBA 46 was produced in greater quantity (>2400 U/mL) when produced in a reactor with optimized conditions of temperature and agitation (30 °C and 300 rpm). The strain of *B. licheniformis* LBA 46, in the conditions studied, was a better protease producer than the strain investigated by Chuprom et al. (2016).

Besides the activity values reported by Dey et al. (2016) and Chuprom et al. (2016) being lower than those found in this study, the culture medium used by them was synthetic, unlike the culture medium used in this work, which was composed of agro-industrial low-cost by-products.

### Kinetics of microbial growth and protease production

In the fermentation of *B. licheniformis* LBA 46 in a 6 L reactor in the best conditions of temperature (30 °C) and agitation (300 rpm), according to assay 3, the protease was produced in the exponential phase of growth reaching maximum activity (~3,000 U/mL) after 48 h of fermentation. The reducing sugar content in the culture medium decreased to 17.9 mg/mL and 16.2 mg/mL after 36 and 48 h of fermentation, respectively. Cell growth decreased after 48 h of fermentation (Figure 2).

The production of proteases by *Bacillus* species is controlled by a number of complex mechanisms that occur during the transition between exponential and stationary phases. The production of enzymes is related to the growth phase of the microorganism (Strauch and Hock, 1993). According to Strauch and Hock (1993), Jisha et al. (2013) and Contesini (2014), proteases from *Bacillus* sp. are mainly produced during the stationary phase of microbial growth. The extracellular enzyme production pattern depends on the *Bacillus* strains (Jisha et al., 2013).



**Figure 2.** Fermentation of *B. licheniformis* LBA 46 in a bench reactor at temperature of 30 °C and agitation of 300 rpm: microbial growth, protease production, reducing sugar and protein content.

The highest activity of protease from *B. subtilis* UO-01 was reached after 15 h of fermentation (10 U/mL), when the microbial cells entered the post-exponential phase. This may occur due to the need for nutrients for microbial survival or due to the need for renewal of cell proteins at a lower growth rate. The enzyme production was associated with microbial growth (Blanco et al., 2016). Dias et al. (2008) observed maximum proteolytic activity of the enzyme produced by *B. subtilis* ATCC 6633 (839.8 U/mg) and *Bacillus* sp. UFLA 817 (975.9 U/mg) after 24 h of fermentation, coinciding with the end of the exponential phase of microbial growth. The protease production by *Bacillus cereus* VITSN04 also had its maximum activity (200 U/mL) associated with the exponential growth phase (Sundararajan et al., 2011).

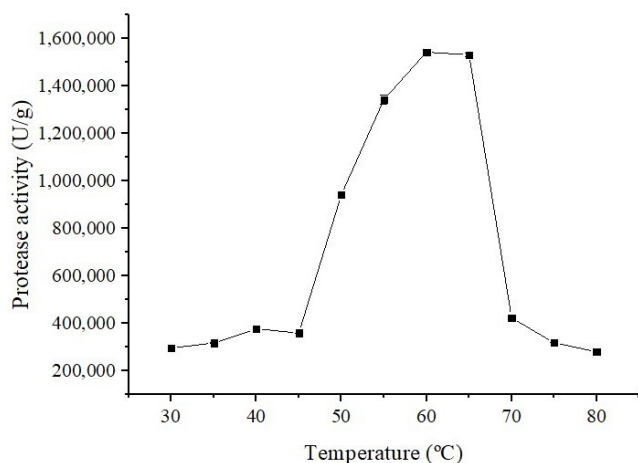
The protease from *Bacillus megaterium* was produced in accordance with the bacterial growth. The maximum protease activity (7 U/mL) was achieved during the stationary phase after 15 h of fermentation (Uttatree et al., 2017). Rao e Narasu (2007) observed that the maximum activity (215 U/mL) of the protease produced by *Bacillus firmus* 7728 was reached in the stationary phase after 48 h of growth.

The results presented in this study showed a high production of protease and high productivity, with values higher than those found in the mentioned literature, regardless of the microbial growth phase or the strain used in the production.

### Determination of the kinetic and thermodynamic parameters of semi-purified protease

#### Activation energy and $Q_{10}$ value

Figure 3 presents the protease activity at different temperatures (30-80 °C). It can be observed that the semi-purified protease from *B. licheniformis* LBA 46 showed high activity between 55-65 °C, with the optimum value at 60 and 65 °C.



**Figure 3.** Effect of temperature on the activity of semi-purified protease from *B. licheniformis* LBA 46.

For the range of temperature analyzed (30-80 °C) a linear variation could be observed with the increase in temperature, which suggests that the protease has a single conformation at the temperature of transition (Castro et al. 2014).

The  $E_a$  value (47.96 kJ/mol) was high, positive and showed good correlation ( $R^2 = 0.95$ ), within the temperature range studied. Abdel-Naby (2017) described a lower  $E_a$  of 17.31 kJ/mol for alkaline protease from *Bacillus stearothermophilus*. Souza et al. (2015) also found  $E_a$  (19.03 kJ/mol) for acid protease from *Aspergillus foetidus* lower than that described in this study. It can be observed that the proteases produced by different microorganisms have different  $E_a$  values. As related in this study, an alkaline protease from *Nacordiopsis alba* showed a high  $E_a$  value of 36.80 kJ/mol (Gohel and Singh, 2012). The high reported values mean that to make the activated enzyme-substrate complex, more energy is required.

The  $Q_{10}$  value is a kinetic parameter used to determine whether the catalytic reactions are controlled by temperature or other factors. According to Elias et al. (2014), the enzymatic reactions have values of  $Q_{10}$  ranging from 1 to 2. Values outside this range can be interpreted as indicative of the involvement of factors other than temperature in the control of the reaction rate. The  $Q_{10}$  value was determined to be in the range of 1.59-1.87, representing the rate of reaction which was affected only by a temperature increase.

#### Kinetic parameters, $K_m$ and $V_{max}$

The kinetic parameters were calculated according to a double reciprocal Lineweaver-Burk plot. The  $K_m$  value indicates the protease-substrate affinity and a low  $K_m$  value indicates higher affinity of the enzyme for the substrate. The  $V_{max}$  value could be defined as the maximum value of initial velocity when all active sites are occupied by the substrate.

The enzyme showed, with good correlation ( $R^2 = 0.91$ ), Michaelis-Menten-type kinetics with  $K_m = 1.60$  mg/mL and a high  $V_{max} = 2 \times 10^6$  U/g. A similar  $K_m$  value (1.92 mg/mL) was reported by Souza et al. (2015) for acid protease from *A. foetidus* utilizing azocasein as substrate. A lower  $K_m$  value (0.44 mg/mL) was related for serine protease from *Aspergillus niger* (Castro et al., 2014) also using azocasein as substrate. Using casein as substrate, Abdel-Naby (2017) determined a higher  $K_m$  value (3.7 mg/mL) than the one found in this study for alkaline protease from *B. stearothermophilus*. It can be seen that the  $K_m$  value depends on the type of substrate evaluated and the enzyme-producing microorganism.

#### Thermal inactivation of semi-purified protease

The thermostability of semi-purified protease from *B. licheniformis* LBA 46 was studied in the range of 50-70 °C. The enzyme showed higher stability at a temperature of 50 °C, retaining above 80% of the initial activity after 120 min. The protease was rapidly inactivated at 70 °C in the absence of substrate, losing 84% of the initial activity after 30 min of incubation.

The half-life of an enzyme is defined as the amount of time required at a given temperature, capable of reducing its initial activity by half. According to Table 3, the semi-purified protease of *B. licheniformis* LBA 46 has high thermal resistance, requiring 693.15 min to reduce half of its activity at 50 °C and that value fell as the temperature increased, reaching 23.90 min at 70 °C. The  $D$ -value, which is the time required for a 90% reduction in the initial enzyme activity was also reduced with increasing temperature, ranging from 2,302.60 to 79.40 min between 50 and 70 °C. In relation to the inactivation rate constants ( $K_d$ ), the values increased with an increase in temperature, ranging from  $1.0 \times 10^{-3}$  to  $29 \times 10^{-3}$  min<sup>-1</sup>. The energy required for thermal inactivation (144.50 kJ/mol) was calculated using an Arrhenius plot. Abdel-Naby (2017) determined a similar value for  $E_a$  (105.5 kJ/mol) by studying an alkaline protease from *B. stearothermophilus*, which means that both enzymes require a similar amount of energy to be inactivated.

**Table 3.** Thermodynamic and kinetic parameters for thermal inactivation of semi-purified protease from *B. licheniformis* LBA 46.

T (°C)	$k_d$ (min <sup>-1</sup> )	$t_{1/2}$ (min)	D (min)	$R^2$	$E_{ad}$ (kJ/mol)
50	0.0010	693.15	2,302.60	0.87	144.50
55	0.0030	231.05	767.53	0.93	
60	0.0070	99.02	328.94	0.82	
65	0.0090	77.02	255.84	0.90	
70	0.0290	23.90	79.40	0.96	

Thermal inactivation of enzymes is accompanied by the breakdown of many non-covalent bonds, which represents an increase in the value of  $\Delta H$ . According to Batista et al. (2014), high  $\Delta H$  values are linked to high thermal stability of the enzyme. The opening or unfolding of the enzyme caused by heating increases its disordered state, which can be measured by the value of  $\Delta S$ . An enzymatic reaction can also be evaluated by measuring the change in  $\Delta G$  value during the conversion of an enzyme-substrate complex into a product (Riaz et al., 2007). A low  $\Delta G$  value suggests that this conversion is more spontaneous; however, high  $\Delta G$  values indicate high enzyme stability (Batista et al., 2014).

The values of  $\Delta H$ ,  $\Delta S$  and  $\Delta G$  practically did not vary within the temperatures analyzed (Table 4), in this case, these temperatures were not capable of causing visible changes in the enzymatic behavior, which remained constant within the range of temperature evaluated. The parameters of kinetic inactivation are important since they serve to define and model the use of enzymes in certain industrial applications.

**Table 4.** Thermodynamic parameters for thermal inactivation of semi-purified protease from *B. licheniformis* LBA 46.

T (°C)	$\Delta H$ (kJ/mol)	$\Delta S$ (J/mol.K)	$\Delta G$ (kJ/mol)
50	141.81	101.79	108.92
55	141.77	103.98	107.65
60	141.73	104.28	106.99
65	141.69	99.83	107.93
70	141.65	103.21	106.23

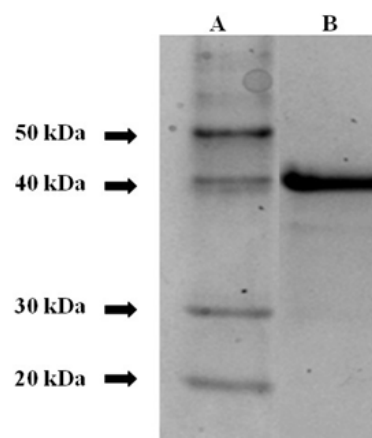
#### Purification of protease

The protease from *B. licheniformis* LBA 46 was purified 3.33 fold using 80% ammonium sulfate precipitation and using DEAE-Sepharose column chromatography. The purified protease showed a specific activity of 628.96 U/mg (Table 5).

#### Biochemical characterization of purified protease

The molecular weight of purified protease from *B. licheniformis* LBA 46 was estimated as 40 kDa by SDS-PAGE (Figure 4).

Jalkute et al. (2017) purified the protease from *Bacillus safensis* CK about 7-fold by DEAE-cellulose column chromatography and estimated the



**Figure 4.** SDS-PAGE of purified protease from *B. licheniformis* LBA 46. (A) molecular mass markers and (B) purified protease.

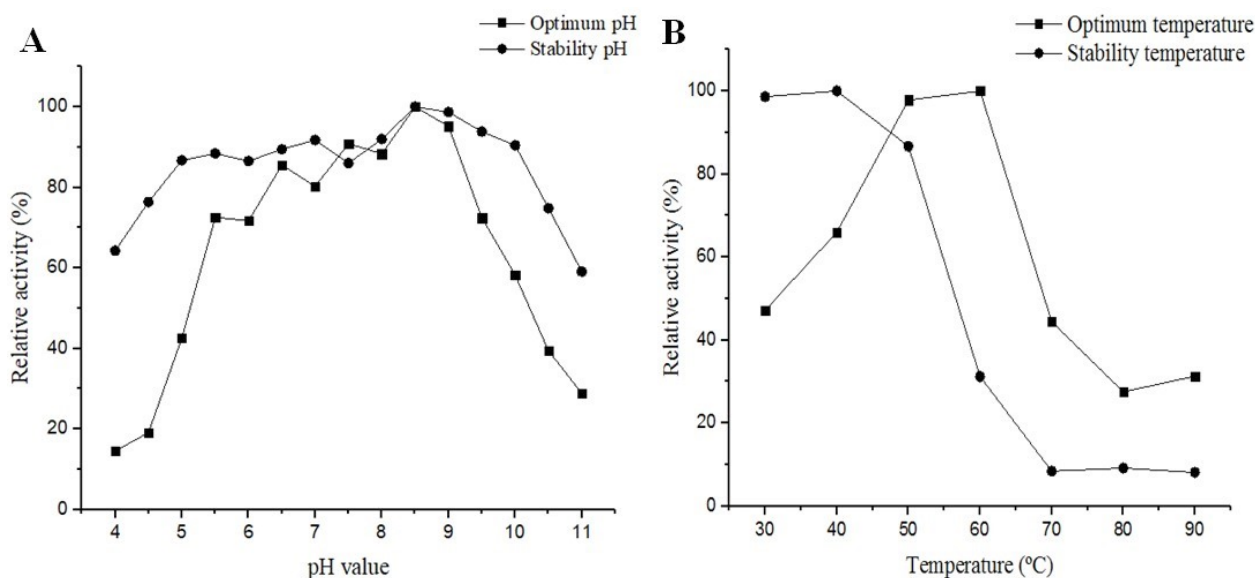
molecular weight (equal to that found in this study) of the protease at 40 kDa by SDS-PAGE. Other works have studied different types of proteases from *Bacillus* sp. purification using various methods with varied molecular weights. Annamalai et al. (2013) purified a 33 kDa protease from *Bacillus alveayuensis* CAS 5 using DEAE-cellulose and Sephadex G-50 columns. Jellouli et al. (2011) purified a 30 kDa protease from *B. licheniformis* MP1 using Sephadex G-100 and Mono Q-Sepharose columns. Lakshmi et al. (2018) purified a protease from *Bacillus cereus* strain S8 using ion exchange followed by gel filtration chromatography. The estimated molecular weight was 21.8 kDa. As in this work, several studies in the literature also described proteases with low molecular weight from *Bacillus* sp.: 15 kDa (Adinarayana et al. 2003), 17.10 kDa (Kim and Kim 2005), 20.10 kDa (Rai et al. 2009), 30 kDa (Hadjidj et al., 2018).

The purified protease of *B. licheniformis* LBA 46 presented high activity (> 80%) in the range of pH 6.5-9, optimal activity at pH 8.5 and low activity at pH 4.0 (15%). The purified protease was stable in the range of pH 5-10 after 24 h at 4 °C, retaining more than 86% of the initial activity (Figure 5A). The purified protease presented optimum activity at 50 and 60 °C at pH 7.0. The enzyme was stable at 40 °C for 1 h in pH 7 and retained 85% of the initial activity after 1 h of treatment at 50 °C, pH 7 (Figure 5B).

**Table 5.** Summary purification of protease from *B. licheniformis* LBA 46.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude extract	6,355,416.67	33,620.33	189.03	100	1
Ammonium sulfate precipitation	539,933.33	4,300.32	125.56	8.50	0.66
DEAE-Sepharose	327.90	0.52	628.96	0.005	3.33





**Figure 5.** Optimum and stability pH (A) and temperature (B) of purified protease from *B. licheniformis* LBA 46.

### CONCLUSIONS

A protease from *B. licheniformis* LBA 46 was produced using agroindustrial by-products as sources of carbon and nitrogen in a reactor. The highest protease activity was obtained after 48 h of fermentation at 30 °C and 300 rpm. The semi-purified protease showed high catalytic activity (~1,500,000 U/g) with an optimum at 60 and 65 °C. The purified protease presented optimum activity at 50 and 60 °C and was stable in the range of pH 5-10 after 24 h at 4 °C. This protease presented interesting characteristics for potential industrial application.

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