Characterization of Bacillus cereus isolated from fermented cabbage and conventional optimization of extracellular protease production

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Citation


Abstract

A strain of Bacillus cereus, isolated from fermented cabbage, was tested a laboratory scale in order to evaluate the production of extracellular protease on batch cultivation in Modified Basal Medium without agar (MB). Furthermore optimization of culture medium and cultivation conditions were carried out too. B. cereus spp. presented a typical growth kinetic with a short lag phase. The best conditions for B. cereus spp. growth were found at pH 7, 32ºC and in MB with glucose. Respecting to protease production, the best expression were at pH 8 and 32ºC. At this temperature the addition of modifiers in the fermentation media did not produce a significant improvement in the protease production. The novel properties of the protease liberated by this strain could have important benefic for the biotechnology industry.

INTRODUCTION

The biosynthesis of proteolytic enzymes by microorganisms is not only of scientific but also of great practical importance. Bacteria, moulds and yeast are some of the microorganisms that are able to produce proteases. Proteases are the most important kind of enzymes from an industrial point of view; they execute a large variety of functions and have important biotechnological applications. They could be useful in leather processing, laundry detergents, producing of protein hydrolysates and food processing (Salem S.R. et al., 2009). Proteases are commonly classified according to their optimum pH: acidic protease, neutral protease and alkaline protease. There have been extensive researches on the properties and functionalities of acidic or alkaline proteases (Dunn-Coleman N. et al.).

Among bacteria, Bacillus strains are the most important producers of commercial proteases (Ghorbel-Frikha B. et al., 2005), being specific producers of extracellular protease. Strains of Bacillus cereus possess high proteolytic potential, expressed by their ability to synthesize and excrete protease into the medium. This spore forming, mesophilic and relatively anaerobic rod is potent in terms of biosynthesis of various hydrolytic enzymes, including proteases, alpha- and beta-glucanases or lipases. Proteolytic enzymes containing serine proteinases (Shaheen M. et al., 2008), Ca²⁺- and Zn²⁺- dependent metaloproteinases (Karbalaei-Heidari H.R. et al., 2007), keratinases (Ghosh A. et al., 2008; Brandelli A. et al., 2005) and collagenases (Nip W.K. et al., 2006) are known to be produced by B. cereus. Microbial proteases account for approximately 60% of the total enzyme sales in the world (Banik R.M. et al., 2004). One of the several challenges faced by industrial application of microbial proteases is to obtain its optimal activity and stability. Each organism or strain has its own special conditions for maximum enzyme production. Extracellular protease production by microorganisms is highly influenced by media components, variation in carbon/nitrogen ratio, presence of some easily metabolically sugars, such as glucose (Beg Q.A.K., 2003) and presence of metal ions (Secades P. et al., 1999). Several others factors, such as aeration, inoculums density, pH, temperature and incubation time also affect the amount of protease production and their interaction plays an important role in the synthesis of these enzymes (Puri S. et al., 2002). Zambare et al. (2007) described an extracellular protease produced by Bacillus cereus that grow on a medium containing starch, wheat bran and soya flour (SWS).

Taken into account the potential uses of the highly demanded Bacillus proteases, there is a need for the research of new strains of bacteria that produce enzymes with novel properties and the development of low cost industrial medium formulations. In commercial practice, the optimization of medium composition is done to maintain a
balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. Recently, in a previous work (Pérez Borla et al., 2009) through the utilization of a simple and reliable method, three proteolytic bacteria were isolated from fermented cabbage: Lactococcus lactis subsp. lactis, Enterococcus hirae and Bacillus cereus using an economic and easily available source. In the light of the above facts, the objective of this study was to optimize the fermentation medium in order to enhance the protease production from Bacillus cereus previously isolated from fermented cabbage with possible novel characteristics. Moreover the morphological and physiological characterization of this proteolytic bacterium was performed.

MATERIALS AND METHODS

STRAIN AND CULTURE CONDITIONS

Bacillus cereus spp. bacteria were previously isolated from fermented cabbage by Pérez Borla et al (2009) and identified in CERELA center (CONICET, Tucumán, Argentina; 2008). The cells were maintained at -18°C in BHI (Breath and Heart Infusion, Britania, Buenos Aires, Argentina) soft agar (0.75% agar) supplemented with glycerol 20% (V/V) as a crioprotector (Waldir E. et al., 2007). The strain was activated twice by subculturing in a BHI, incubated at 32°C during 24h. Firstly, a culture loop was inoculated in 8 ml BHI. After 24 h, 2 ml of the culture was centrifuged at 100 rpm for 3 min at 4°C. The precipitated was added to 25 ml fresh BHI and statically incubated at 32°C during 20-24 h. This culture (0.5 % v/v) was allow to grow in Minimal Broth (MB), a agarless Modified Basal Medium (MM) (Pérez Borla et al., 2009) containing 0.1% bacteriological glucose (w/v) (Britania, Lot.095, Buenos Aires, Argentina) and 0.25% yeast extract (w/v) (Acumedia, Lot. 66-22, Maryland). The bacterial cells were grown in MB in a 250 ml Erlenmeyer flask on an orbital shaker at 80 rpm. The pH of aliquots taken in sterile conditions from inoculated MB was measured periodically during fermentation by a pH-meter (Hanna Instruments Model 201, Portugal).

EFFECT OF PHYSICOCHEMICAL CONDITIONS ON MICROORGANISM GROWTH

TEMPERATURE EFFECT ON MICROORGANISM GROWTH

To investigate growth at different temperatures, Bacillus cereus spp. was grown in MB (pH 7) at four temperatures: 10, 20, 32 and 40°C. The bacterial growth was developed in 250 ml Erlenmeyer flasks with MB and 0.5% v/v of Bacillus cereus spp. on an orbital shaker at 80 rpm. The aliquots were always taken in sterile conditions and the bacterial growth was quantified by reading the optical density (the absorbance of the culture) at 600 nm (Spectrum SP-2000 UV, Zhejiang, China). Growth was monitored each hour from 0 till 12 hours and, a last lecture, at 24 incubation hours. At 10°C the time intervals were extended to 30, 36, 48 and 72 hours. All experiments were carried out in triplicate.

PH EFFECT ON MICROORGANISM GROWTH

Bacillus cereus strain (0.5% v/v) was inoculated in MB, buffered at different pH (3.0, 4.0, 5.7, 7.0 and 8.0) using 0.1M citric acid (Biopack, Buenos Aires, Argentina), 0.2 M KH₂PO₄ anhydride (Biopack, Buenos Aires, Argentina) and 0.2 M K₂HPO₄ anhydride (Biopack, Buenos Aires, Argentina) instead of distilled water to prepare the solution. The 250 ml Erlenmeyer flasks were incubated at 32°C on an orbital shaker at 80 rpm, during the assay period (24 hours). For each assayed pH value, bacterial growth was evaluated as the optical density at 600 nm in one hour intervals from 0 till 12 hours and, a last lecture, at 24 incubation hours. All experiments were carried out in triplicate.

INCUBATION MEDIA MODIFIERS EFFECT ON MICROORGANISM GROWTH

The effect of different modifiers on Bacillus cereus spp. growth was examined. Cultures were complemented with different modifiers. The microorganism was grown in MB (pH 7.0) supplemented with CaCl₂ (1 mM) (Anedro, Buenos Aires, Argentina), KCl (1 mM) (Alun, Metroquímica, Buenos Aires, Argentina) bacteriological glucose (2 g/l) and meat peptone (2.5 g/l) (Britania, Lot. 2342, Buenos Aires, Argentina) to replace yeast extract. These complemented media were denominated MB + Ca²⁺, MB + K⁺, MB + Glu and MB + Peptone, respectively. The 250 ml Erlenmeyer flasks containing the modified MBs and of Bacillus cereus spp. (0.5% v/v) were incubated at the assayed temperatures (10, 20, 32 and 40°C) on an orbital shaker at 80 rpm. Growth was monitored each hour from 0 till 12 hours and, a last lecture, at 24 incubation hours. At 10°C the time intervals were extended to 30, 36, 48 and 72 hours. All experiments were carried out in triplicate.

EXTRACELLULAR PROTEASE ACTIVITY: QUALITATIVE AND QUANTITATIVE ASSAY

For enzymatic extract preparation, Bacillus cereus spp. was grown in MB for 24 h at the assay temperatures (10, 20, 32
and 40°C) and pH (5.7, 7.0 and 8.0). Cell-free supernatants were obtained by centrifugation at 10,000 rpm for 10 min at 4 ºC. The cell free culture supernatants were used as protease source.

The protease activity was assayed through quantitative and qualitative enzymatic assays at regular intervals of 0, 1, 2, 5, 6, 7, 8 and 24 hours. Protease activity was measured using azocasein as the substrate. The assay mixture contained 100 µl of protease source and 500 µl of 2% (w/v) azocasein (Sigma-A-2765 Lot.043K7021) in 10 mM Tris-HCl, pH 7.5. After incubation in a thermal bath (Lauda E-300) at 37ºC for 90 min, the reaction was stopped by adding 600 µl of 20% (w/v) trichloroacetic acid (TCA) and keeping the mixture at room temperature. A vortex mixture was used to insure complete mixing at various stages of these assay procedures. The mixture was centrifuged at 10,000 rpm for 5 min. 1 ml aliquot of the cell free supernatant was taken. The absorbance of the supernatant was determined at 335 nm by using of precision quartz cells (Hellma- Type Nº 10-QS-light path 10 mm). Reaction blank was prepared by adding the TCA to the substrate solution immediately before the enzyme preparation was added. Proteolytic activity was defined as the difference between the sample and blank absorbance at 335 nm. One unit of absolute proteolytic activity (UA) was the amount of enzyme that caused a change of absorbance of 0.01 at 335 nm in 90 min at 37°C. One unit of relative proteolytic activity unit (U) was defined as an increase of one absorbance unit at 335 nm in 90 min at 37°C per growth (absorbance of culture measured at 600 nm).

Qualitative proteolytic activity assay was evaluated in Modified Basal Medium (MM) supplemented with 6.2 g/l protein of skim milk plates (5 g/l of casein) (Pérez Borla et al., 2009). Wells 5 mm in diameter were cut under sterile conditions into these agar plates. A 20 µl aliquot of the growth culture was placed into the well. These plates were then incubated aerobically at 32°C during 48 hours and bacterial proteolytic activity was observed daily. Proteolytic activity of Bacillus cereus spp. was detected on the basis of appearance of clear zones around the bacterial colonies (hydrolyzed zones). The clear zone (6 mm or more, measured from the well center) in the medium surrounding the bacterial growth indicated positive proteolytic activity (Pérez Borla et al., 2009). Diameters of the clear circular area resulting from proteolysis around the wells (haloes) were measured and the results expressed as the square of the diameter, according to Cooper (1963). Proteolytic activity was negative when no visible clearing halo was present.

The protease substrate specificity of Bacillus cereus spp. was evaluated in MM supplemented with 5 g/L protein of: gelatin, bovine serum albumin, ovalbumin, gluten, milk whey or hemoglobin, respectively. Plates were incubated at 32°C for 7 days and bacterial proteolytic activity was observed daily. The enzymatic hydrolysis of the protein was studied by qualitative assay as described above. A 300 g/l trichloroacetic acid (TCA) solution was flooded on the agar plates only when it was necessary to observe protein hydrolysis.

MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE CULTURE:

Bacillus cereus spp. was grown in MB buffer pH 7 during 24 h at 32 ºC. General morphologic characteristics of cells and spores development were monitored by analyzing fresh cultures. Broth preparations were observed in a phase contrast microscopy (Phase contrast Microscopy Leica DC 100 – x 1000). Gram stain and Schaeffer-Fulton spore stain were applied to the characterization of strain with the microscopy.

To analyze motility, B. cereus cells were collected with a sterile toothpick from a fresh culture in BHI and used to inoculate by introducing vertically in a sterile tubes containing 0.3% brain and heart agar. The tubes were incubated at 32°C and evaluated over time during 48 hours to observe the swim possibility of the strain.

STATISTICAL ANALYSIS

The growth and the proteolytic activity of B. cereus spp. were performed in three independents experiments by triplicate. For the 3-point assay, the means for each point were plotted against the culture time and the standard deviation of triplicate determinations was represented (error bars). Data were analyzed by analysis of variance (one way ANOVA) using a statistical package MATLAB 7.2. Probability level was fixed to p< 0.1.

RESULTS

EFFECT OF PHYSICOCHEMICAL CONDITIONS ON MICROORGANISM GROWTH:

TEMPERATURE EFFECT ON MICROORGANISM GROWTH:

Figure 1 shows the time course of patterns of cell growth for Bacillus cereus spp. grown at different temperatures: 10, 20,
Characterization of Bacillus cereus isolated from fermented cabbage and conventional optimization of extracellular protease production

32 and 40°C. B. cereus spp. was able to grow at 20, 32 and 40°C showing typical growth profiles with an exponential phase that began in a range of third and forth hour and ended between sixth and ninth hours. For these temperatures the exponential phase lasted about tree hours with a maximum specific growth rates. During this period, the cell growth curve presented a linear profile. The time course of Bacillus cereus spp. at 10°C did not show significant cells growth.

The maximum optical density (0.9) was observed at 32°C so this temperature was considered optimal for the cell growth.

**Figure 1**
Figure 1. Kinetic growth profiles of . at different temperatures: 10°C (□), 20°C (○), 32°C (◆) and 40°C (▲) in MB buffered at pH 7.0 on a rotary shaker. Each point is the mean of three determinations; standard errors were less than 10% of the means.

**INCUBATION MEDIA MODIFIERS EFFECT ON MICROORGANISM GROWTH**

Figure 3 shows the effect of different modifiers in MB at optimal conditions of temperature and pH; 32°C and 7.0 respectively. The use of glucose as modifier showed an increase in the optical cells density, reaching the maximum in the seventh hour of growth. This result indicated that the exponential phase was prolonged compared to control sample (only MB). The other modifiers (Ca²⁺, K⁺ and Peptone) presented an inhibitor effect in the cell growth compared to control sample.

**Figure 3**
Figure 3. Kinetics growth profiles of . grown in MB buffered at 32°C and pH 7.0 on a rotary shaker enriched with different modifiers: MB (□), MB + Ca (◆), MB + K (○), MB + Glu (x) and MB + Peptone (?). Each point is the mean of three determinations; standard errors were less than 10% of the means.

At the latency phase similar cell growth profiles were obtained for pH 7.0 and 5.7.

**Figure 2**
Figure 2. Kinetic growth profiles of . grown at 32°C in MB at buffer pH 5.7 (□); pH 7.0 (○) and pH 8.0 (▲) on a rotary shaker. Each point is the mean of three determinations; standard errors were less than 10% of the means.

**PH EFFECT ON MICROORGANISM GROWTH**

To establish the optimum pH growth, Bacillus cereus spp. was incubated at 32°C (the optimal temperature) in MB buffered at five different pHs. Figure 2 shows the kinetic growth profiles in MB at pH 5.7, 7.0 and 8.0. Cells growth at pH 7.0 showed the maximum absorbance values and the lag phase was extended till the third hour. The lowest absorbance values were observed at pH 8.0 starting the accelerated phase at the fourth hour. The bacteria growth at pH 5.7 was intermediate between both pH 7.0 and 8.0.

The Bacillus cereus spp. growth in MB enriched with these modifiers at others temperatures (10°C, 20°C and 40°C) was also investigated. These results are shown in Figure 4A, B
and C. At 10°C none of the modifiers could shorten the lag phase. At 20°C, the MB enrichment with glucose did not affect Bacillus cereus spp. growth compared with the control without modifiers. The addition of peptone, K⁺ and Ca²⁺ reduced significantly the cell growth. At 40°C the addition of glucose increased Bacillus cereus spp. growth significantly with the maximum optical density. The others modifiers did not influence Bacillus cereus spp. growth at this temperature compared with the control sample. As figure 3 and 4C showed, glucose was found to be a good carbon source for the growth of the strain at 32 and 40°C.

**Figure 4**

Figure 4. Kinetics growth profiles of Bacillus cereus grown in MB (pH 7) enriched with different modifiers and incubated at different temperatures: MB (?), MB + Ca (?), MB + K (?), MB + Glu (x) and MB + Peptone (?). Cells were grown at 10Â°C (A), 20Â°C (B) and 40Â°C (C) on a rotary shaker. Each point is the mean of three determinations; standard errors were less than 10% of the means.

**Figure 5**

**EXTRACELLULAR PROTEASE ACTIVITY: QUALITATIVE AND QUANTITATIVE ASSAY**

Extracellular proteolytic activity of the Bacillus cereus strain was measured (at different growth times) as was described in Materials and Methods (2.3) The incubation time was defined analyzing the difference in the absorbance lectures reading at 335 nm at regular intervals of time (0, 30, 60, 90, 120 and 150 minutes) and the optimal time (90 minutes) was selected (data not shown).

Maximum relative activity of Bacillus cereus protease was obtained in MB the first 3-4 hour of growth corresponding to the acceleration phase (Figure 5A). Non proteolytic activity, expressed as relative activity, was observed during the stationary phase.

Figure 5B shows the absolute proteolytic activity during Bacillus growth. The protease activity began at the lag phase extending its protease action during the exponential phase. The highest absolute proteolytic activity was found at the exponential phase. At it was expected, non absolute proteolytic activity was observed during the stationary phase. The maximum cell concentration was at 6 h of incubation (Figure 1) and the highest enzyme activity was at 5 h with values of absorbance (600 nm) of 0.879 and 11.84 UA, respectively.

Parallel to these measures, we determined the proteolytic activity halos in MM plates supplemented with milk (Figure 5C). Aliquots of cell culture incubated in MB were placed in MM plates wells. Under these conditions, the maximum proteolytic halos observed took place during the lag and exponential phases. Notorious small halos were detected in the stationary phase, inducing on that a lower protease activity was present at this phase, indicating a poor protease activity at this phase.

When spores were inoculated again in MM agar plates supplemented with milk, they found adequate conditions to begin their vegetative growth producing proteolytic enzymes, breaking proteins and obtaining energy. To support this hypothesis, the halo diameter square was quantified but using only cell free culture supernatants (protease source) obtained at the same Bacillus growth times as Figure 5C. The values in terms of the diameter square of the clear zone surrounding the protease source were lower that those which appeared by inoculation of the active culture (Figure 5D). As a constant, the highest activity of protease was found, from the lag phase to the end of the exponential phase. In the stationary growth period a minimal halo was detected and it was smaller compared to the halo found when culture was inoculated. The halo which appeared in the stationary phase could be present by the enzymatic hydrolysis of the nitrogen source present in the
MB (yeast extract).

**Figure 6**

Figure 5. Time course of protease production by *B. cereus* isolated from culture grown in MB at pH 7 and 32°C. Relative proteolytic activity (A); absolute proteolytic activity (B); diameter square of proteolytic halos from culture grown in MM (C) and diameter square of proteolytic halos from crude enzyme (D). Standard deviations are represented by error bars.

The results on the pH effect on protease activity were shown in Figure 6 A and B. As was mentioned, 7.0 was the optimum pH for *Bacillus cereus* spp. growth (Figure 2). However for the proteolytic activity expression the optimum pH was 8.0 despite of a reduced microbial growth at this pH value (Figure 2). For the three pH analyzed the higher extracellular proteolytic activity occurred during the lag and exponential phases (Figure 6 A and B). If activity was expressed as absolute values it is noticeable that during the stationary phase (pH 8.0) a residual extracellular proteolytic activity was detected. As at pH 8.0 *B. cereus* spp. cells grew slowly could depleted the substrate gradually expressing longer the extracellular protease activity (Figure 6B).
The effect of growth temperature on specific extracellular proteolytic activity is shown in Figure 7 A and B. Different information was obtained if the activity was expressed in a relative or absolute way. At 10°C Bacillus grew significantly slower showing the maximum relative extracellular proteolytic activity compared with Bacillus growing at 32°C, (Figure 7 A). Once again, at 10°C the microorganisms could deplete the substrate nutrients gradually showing a remaining proteolytic activity during the stationary phase.

If absolute activity was recorder, the maximum extracellular proteolytic activity was observed at 32 and 40°C, while at 32°C the activity was expressed early in the lag phase, at 40°C the maximum activity expression was during the exponential phase.

The effect of different modifiers on the extracellular protease production from Bacillus cereus spp. expressed as absolute extracellular proteolytic activity was determined for the different assayed temperature (10, 20, 32 and 40°C) (Figure 8 A, B, C and D). As can be seen each modifier affected extracellular protease expression differently related with incubation temperature. At 10°C all modifiers affected the extracellular protease expression increasing its activity between three and six times compared with the control sample (MB). However with the addition of peptone and glucose the activity was expressed in the lag phase, but the addition of K⁺ and Ca²⁺ delayed the protease expression to the late exponential phase.

At 20°C, and similar to the modifier effects observed at 10°C, a positive effect in the protease expression was observed. The activity increase ranged between seven and ten times compared with the control sample (MB). All modifiers expressed the protease activity during the lag and exponential phase. With glucose addition the maximum activity was present in the late exponential phase.
maintaining a high protease activity (14-15 UA) during the stationary phase. It was probably that microorganisms use the glucose as fast carbon source of MB. When an important biomass was reached, the microorganisms use its enzyme battery to hydrolyzed the protein substrate of MB toward obtains energy.

At 32°C only the MB enrichment with peptone appears to cause an accelerated protease expression reaching at 2 h its maximum activity (19 UA), after that the activity gradually decrease during time. K⁺ improved also the proteolytic expression compared with MB reaching the highest value in the late exponential phase. For all modifiers added a significant protease activity was observed during the stationary phase.

At 40°C any of the modifiers improved the proteolytic expression. For the control culture (MB) and with modifiers, the activity expression occurred during the late exponential phase.

In general, Ca²⁺ ions did not exert an enzyme stabilization role without effects in the protease activity at any of the assayed temperatures.

**Figure 10**

Figure 8. Effect of different modifiers on the protease activity from incubated in MB buffered at pH 7: MB (?), MB + Ca (?), MB + K (?), MB + Glu (x) and MB + Peptone (?), at 10°C (A), 20°C (B), 32°C (C) and 40°C (D).

Results were expressed as absolute proteolytic activity. Each point is the mean of three determinations; standard errors were less than 10% of the means.

The hydrolysis of tested protein substrates by B. cereus spp. protease and the presence of hydrolysis are shown in Table 1. Among the substrates tested, this protease hydrolyzed all the protein sources.

**Figure 11**

Table 1. Substrate specificity of protease in MM medium supplemented with different proteins.

<table>
<thead>
<tr>
<th>Protein source</th>
<th>Concentration (g/L)</th>
<th>Proteolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein from bovine milk</td>
<td>5.0</td>
<td>+</td>
</tr>
<tr>
<td>(6.2 g/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>5.0</td>
<td>+</td>
</tr>
<tr>
<td>Gluten</td>
<td>5.0</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>5.0</td>
<td>+</td>
</tr>
<tr>
<td>Milk whey</td>
<td>5.0</td>
<td>+</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>5.0</td>
<td>+</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>5.0</td>
<td>+</td>
</tr>
</tbody>
</table>

* The presence of clear halos in the culture medium indicated the presence of hydrolysis. The values shown are means of three independent determinations.

**MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE CULTURE**

The fermentation process developed in MB at 20, 32 and 40°C (pH 7.0) could be divided in four distinct phases, each of them with well-defined particularities: Phase I, vegetative growth; Phase II, transition to sporulation; Phase III, sporulation; Phase IV, spore maturation with cell lysis. Phase I corresponds to the beginning of the fermentation process and was characterized by a rapid period of adaptation of the cells to the culture medium, resulting in a short lag phase, due to the use of active inoculums, with reactivated vegetative cells only (Table 2, Picture 1). Phase II occurred between the 5 and the 8 hours of process and showed an abrupt morphology change forced by the beginning of the bacterium sporulation. Cell size presented a marked reduction without arrangements in pairs and chains. After that, the cells started to group and form small flacks (Picture 2). Phase III presented an intense sporulation of the culture. Although most of the cells were within large clumps (Picture 3). Phase IV was the last phase of the cellular cycle and was defined as the period of complete maturation of spores and partial lysis of sporulated cells population. The clumps size diminished while cell lysis increased continuously (Picture 4). The main characteristics of each
phase are summarized in Table 2.

The pH behavior at each phase during Bacillus cereus spp. growth in MB incubation medium was also included in Table 2.

Table 2. Main morphological and physiological characteristics of Bacillus cereus culture.

<table>
<thead>
<tr>
<th>Phase of cellular growth</th>
<th>Main characteristics</th>
<th>Phase contrast micrographs (x1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetative growth</td>
<td>Vegetative cells, isolate and chains, uniform. Fast decrease of the pH values and reached values of around 6.8 in the second hour, probably due to the use of glucose as fast carbon source and the formation of organic acids that reduce the pH of the medium.</td>
<td>Picture 1. At 2 h of fermentation.</td>
</tr>
<tr>
<td>Transition to sporulation</td>
<td>Rods with uniform dimensions. Rise of pH, reaching values near those established in the beginning, probably due to the use of proteins as energy source and the production of metabolites that raise pH values.</td>
<td>Picture 2. At 5 h of fermentation.</td>
</tr>
<tr>
<td>Sporulation (Phase III)</td>
<td>Endospores, shorter and isolated cells. No pH changes.</td>
<td>Picture 3. At 8 h of fermentation.</td>
</tr>
</tbody>
</table>
Swim motility of Bacillus cereus spp., assessed by cell movement through the water filed channels of 0.3% brain and heart agar plates, was observed.

**DISCUSSION**

Bacillus cereus strain, used in this study, is a mesophilic, gram positive, spore forming and motile exhibiting an optimum growth at 32ºC.

The strain of B. cereus presented a typical kinetic growth profile, showing a short lag phase, a marked exponential and stationary phases. The decline phase was not observed probably due to the cell sportulation that produced a significant decrease in the absorbance values because of the propriety of the spores to absorb light at the selected wavelength (600 nm).

The optimal pH for the microorganism growth 7, while the optimal pH for the enzyme production was found at pH 8. This result is accordance with several earlier reports showing pH optima for Bacillus cereus spp. protease expression being alkaline (Bani R.M. et al., 2004).

Despite of the strain was isolated from fermented cabbage, which juice had an acid pH between 3 and 4 (Pérez Borla et al., 2009), these pH ranges were not the optimal for B. cereus sp. growth and its extracellular protease production (data not shown). This behavior could indicate that B. cereus strain survives in this acid juice, acquiring a sporulated form. Then the spores were well reactivated in BHI leading to the spores germination and therefore dominate the vegetative cells into the medium.

Generally food preservation processes exposes bacteria to both lethal and sub-lethal stresses. Bacteria may have different mechanisms for surviving these extreme environmental stresses (Davidson P.M. et al., 2002).

In this context, the formation of endospores in response to acid stress during cabbage fermentation is a survival strategy for Bacillus cereus spp.

Our experimental results showed that the maximum amount of enzyme was produced by the bacterium in its exponential growth phase and a reduction in enzyme expression was noted beyond this period, precisely in the stationary phase. Similar results reported previously other authors (Gopal Joshu et al., 2007). Although the rapid proteolytic activity expression constituted a technologic advantage, in Fig. 5A when proteolytic activity was expressed relative to the bacterial growth, the reduced number of microorganisms could be the responsible for the enhanced proteolytic activity as this was not a benefit. For a technological point of view, an elevated enzyme concentration was desirable. The enzyme presents a significant absolute extracellular proteolytic activity at the beginning of the fermentation during the lag and exponential phases; following occurs a diminution of the absolute extracellular protease activity. Two independent facts could explain this behavior: the extracellular protease production is controlled by the level of a repressor which is directly or indirectly an intermediate in many pathways related to a feedback inhibition; or the low level of nutrients in the medium provoke a depletion in the enzyme activity, due to the morphological changes from vegetative to sporulated cell forms that not produce extracellular proteases. It must be emphasize that only vegetative cells are capable to liberate protease into the medium. The proteolytic activity of culture assayed in MM enrichment with milk indicates that the enzyme presents activity in the stationary phase inclusively; indicating that if the MM is complement with related substrate, then there is no drain of protease production during the stationary phase. This fact could indicate that limited substrate conditions observed during the stationary phase (Figure 5A and B) could be responsible for the absence of proteolytic activity in this period.

Also the influence of pH on B. cereus spp. growth support the hypothesis previously proposed about substrate exhaustion as mainly cause of the proteolytic activity absence in the stationary phase.

Based on the obtained results, the effect of the MB supplementation with different modifiers on the protease activity shows a variation with the incubation temperature. The results indicate that the production of protease required the presence of the assayed modifiers as a function of the temperature. At 10 and 20ºC all modifiers positively affected protease expression, but at 32 and 40ºC, the microorganism appeared not to require any modifiers to express the proteolytic activity. In contrast, other microorganisms show only very low level of specific-activity in no-supplemented synthetic medium (Nicodème M. et al., 2005). In a previous work, Ghorbel- Frinkha et al. (2005) reported that the production of protease by a B. cereus strain is absolutely calcium-dependent (Ghorbel-Frikha B. et al., 2005). So the fact that the Bacillus cereus strain isolated from fermented cabbage produced proteolytic alkaline enzymes in minimally
substrate conditions that no requires neither ions (Ca\textsuperscript{2+} or K\textsuperscript{+}) nor addition nutrients (peptone or glucose) could represent a novel type of enzyme at the optimal temperature (32°C) of enzyme production.

Growth temperature controlled the production of the protease by Bacillus cereus spp. In the range from 10 to 40°C, the proteolytic activity rapidly increased with temperature, and it drastically decreased at and below 20°C. On the basis of our results, we suggest that the optimal temperature of protease production was coincident with the optimal growth temperature. The effect of extreme temperature on the enzymatic activity has to be analyzed in further studies.

The B. cereus strains used for this study could have different applications in diverse industries (Horikoski K., 1999) due to their ability to hydrolyze many proteins sources such as casein, hemoglobin, gluten, gelatin, milk whey, ovalbumin and bovine serum albumin. This strain may be regarded as a potential starting culture due to its highly active proteolytic activity and hence rapid fermentation would be expected (Paniuwan Chantawannakul et al., 2002). A combination of different nitrogen source could be assay in a future study. Yeast extract and peptone was found to be the optimum nitrogen source for protease production for a B. cereus strain in previous study (Ghorbel-Frikha B. et al., 2005).

In future works we will emphasize the search of microorganisms that resist several technological stress condition during the different process and could produce desirable metabolic products. Proteases produced by Bacillisses species are the most important group of secondary metabolites that are widely exploited (Ferrari E. et al., 1993). Important advantages would obtain if undesirable microorganisms, like B. cereus spp., could act in favor of the biotechnological process mainly in the food industry where such microorganisms are frequently involve in contamination of equipments and products.

CONCLUSIONS

Many researchers are interested in the synthesis of peptides or aminocids using proteases, extracted from diverse sources. Microorganisms are a potentially important source of enzyme production. The relationship between the kinetic growth of the microorganisms and the culture conditions can not be extended proportionally to the proteolytic activity. Therefore, optimization of the protease production implicates the well knowledge of the physical and chemical factors that influence the growing of the microorganisms and the studied of the factor that affect directly or indirectly the protease activity.

In spite of the fact that several studies refer to Bacillus cereus as a food spoilage and food poisoning organism, really not desirable in the food industry (Durick K. et al., 2005), we recognize the use of B. cereus enzymes as an important resource for this industry. In future studies we will centre on the technological importance of the application of these enzymes for the obtaining of wished products.

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