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Effect of temperature on the lipolytic and proteolytic activity of *Bacillus cereus* isolated from dairy products

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Abstract

Bacillus cereus is a bacterium with deteriorating potential for dairy products, by being a psychrotrophic organism producer of lipases and proteases. This study evaluated the psychrotrophic behavior, lipolytic and proteolytic activity at 30ºC, 10ºC and 7ºC of 86 strains of *B. cereus* lato sensu isolated from dairy products, marketed in Southern Brazil. It was also evaluated the optimal temperature for protease production. No strain grew at 7ºC; but at 10ºC, 84.9% of strains have grown. Only one strain had lipolytic activity at 30ºC, and none at 7ºC. At 10ºC, 16.3% of strains produced lipases. All the strains presented proteolytic activity at 30ºC; and at 10ºC, 72.1% had this activity, and at 7ºC, only 4.6%, an amount significantly lower ($p < 0.05$). The temperature of 20ºC promoted the highest proteolytic activity, and at 10ºC, the lowest activity. *B. cereus* can produce lipases and proteases at room and marginal chilling temperatures, causing technological defects in dairy products stored under these conditions.

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Introduction

Bacillus cereus has raised concern in dairy industry by having deteriorating potential. This because *B. cereus* is a psychrotrophic microorganism able to grow under chilling temperature and produce lipases and proteases, enzymes that change the components of dairy products (García-Armesto and Sutherland, 1997; Giffel *et al.*, 1997; Svensson *et al.*, 2004; Chen *et al.*, 2004; Zhou *et al.*, 2010).

Changes associated with contamination of dairy products by *B. cereus* are important from the technological point of view, since they reduce the shelf life and bring up undesirable sensory attributes (Almeida *et al.*, 2000; Chen *et al.*, 2004; Froom and Boor, 2004; Rezende-Lago *et al.*, 2007; De Jonghe *et al.*, 2010).

Proteases may cause unpleasant tastes and gelation in UHT milk (Silva, 2004; Vidal-Martins, 2005; Janstová *et al.*, 2006; Murugan and Villi, 2009), produce bitter taste and decrease in solubility of milk powder (Celestino *et al.*, 1997; Chen *et al.*, 2003), beyond the decrease in cheese yield (Furtado, 2005). Lipases act on milk fatty acids, causing the off-flavor in pasteurized and UHT milk, milk powder and cheese (Celestino *et al.*, 1997; Chen *et al.*, 2003; Fromm and Boor, 2004; Furtado, 2005).

Few are the information about the hydrolases production by *B. cereus* in dairy products marketed in Brazil. Regarding the technological problems that *B. cereus* may cause in dairy products, this study aimed to evaluate the ability of *B. cereus* strains in producing lipases and proteases at different temperatures, determining the optimal temperature of proteases, and assess if the strains have psychrotrophic behavior.

Material and Methods

It was evaluated 86 strains *B. cereus* lato sensu isolated from samples of UHT milk, milk powder and pasteurized milk marketed in Southern Brazil, confirmed from biochemical tests. Analyses were performed in the period from March 2010 to November 2011, in the laboratory of food microbiology of the Graduate Program in Food Engineering of the Federal University of Paraná.

The strains were resuspended in TSB broth at 30ºC for 24 hours and then replated in specific culture media to evaluate the psychrotrophic behavior, lipolytic and proteolytic activities.

To evaluate the proteolytic activity, the strains were plated on milk agar. The milk agar was prepared with plate count agar (Himedia Laboratories Ltd.) supplemented with 1% skimmed milk powder (Beerens and Luquet, 1990). The plates were incubated at 30ºC for 48 hours, 10ºC for 7 days, and at 7ºC for 10 days, and considered positive the colonies with transparent halo.

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For the evaluation of lipolytic activity, the strains were plated on tributyrin agar, prepared with plate count agar (Himedia Laboratories Ltd.) supplemented with 1% tributyrin (Sigma-Aldrich Co.) (Beerens and Luquet, 1990). The plates were incubated at 30ºC for 48 hours, at 10ºC for 7 days, and at 7ºC for 10 days, and considered positive the colonies with transparent halo.

The frequencies of positive evaluations for enzymatic activities among the different incubation temperatures were compared by testing differences between proportions, with application of Chi-Square Fisher's exact test (Kaps and Lamberson, 2009).

Determining the optimal temperature for protease production by *B. cereus* strains was done according to Nörnberg et al. (2010). The cultures stored in TSA were resuspended in TSB and incubated at 30ºC for 24 hours. The cultures were then inoculated into 100 mL of mineral medium (0.5 g/l NaCl; 0.4 g/l K_2HPO_4; 0.3 g/l KH_2PO_4), with 10 g/l of casein and pH adjusted to 7.0. The enzyme production was evaluated at different temperatures (10ºC, 20ºC and 30ºC), in orbital shaker for 72 hours. After this period, the medium was centrifuged at 10,000 g for five minutes to separate the biomass from the supernatant. The supernatant was used for the analysis of the proteolytic activity.

The proteolytic activity was determined using azocasein as substrate medium for 45 *B. cereus* inoculates. For this, 100 µL of the supernatant obtained from the cultures were added to 100 µL of 0.1M solution of sodium phosphate pH 7.0, and 100 µL azocasein (10 mg/ml) in eppendorf tube. The mixture was incubated at 37ºC for 60 min and the reaction was stopped by adding 500 µL of 30% of trichloroacetic acid. The solution was centrifuged at 10,000 g and 800 µL of the supernatant were mixed with 200 µL of 1.8M NaOH. The absorbance was measured with spectrophotometer at 420 nm. The increase in absorbance of 0.01 corresponded to one unit of proteolytic enzyme activity (PEA). Assays were done in triplicate (Nörnberg *et al*., 2010).

The comparison between the treatments in the different temperatures was performed by the non-parametric test for multiple comparisons between independent treatments, using the Kruskal-Wallis test, with 95% confidence, with the software Statistica version 8.0 (Hill and Lewicki, 2007).

Results and Discussion

The results of the Table 1 indicated that none of the 86 strains of *B. cereus* grew at 7ºC. But at 10ºC, 84.9% of the isolated *B. cereus* strains grew, and at 30ºC, all tested strains had grown.

Table 1. Evaluation of growth, lipolytic and proteolytic activity of *Bacillus cereus* strains isolated from dairy products at different temperatures (n=86)

Characteristics	30ºC / 48 h	10ºC / 7 d	7ºC / 10 d
Growth	86 (100.0%) ^{Aa}	73 (84.9%) ^{Aa}	0 (0.0%) ^{Bb}
Lipolytic activity	1 (1.2%) ^{Bb}	14 (16.3%) ^{Ab}	0 (0.0%) ^{Bb}
Proteolytic activity	86 (100.0%) ^{Aa}	62 (72.1%) ^{Aa}	4 (4.60%) ^{Ba}
Both enzymatic activities	1 (1.2%) ^{Bb}	13 (15.1%) ^{Ab}	0 (0.0%) ^{Bb}

Values with different upper cases in the same row or lower cases in the same column differ significantly by the Chi-Square Fisher's Exact test ($p < 0.05$).

In relation to the definition of psychrotrophics (microorganisms able to grow at 7ºC or below, regardless its optimal growth temperature) (McPhee and Griffiths, 2011), none of the *B. cereus* strains herein evaluated can be classified as psychrotrophic.

The strains that did not grow at 7ºC, but did at 10ºC, are classified as mesophilic (Lechner *et al.*, 1998). Thus, our data indicated that the strains of *B. cereus* isolated in the study region have predominantly mesophilic behavior, probably due to climatic conditions that favor this behavior.

These data differ from reported by authors in studies carried out in other countries. Giffel *et al*. (1997) found that all 106 strains of *B. cereus* isolated from dairy products in the Netherlands were able to grow at 10ºC for 7 days and 56 (53%) at 7ºC for the same period. García-Armesto and Sutherland (1997) examined 50 strains of *Bacillus* spp. isolated from milk and derivatives marketed in Spain and identified 26% (52%) as psychrotrophic *B. cereus*, able to grow at 6.5ºC for 10 days.

The occurrence of proteolytic and lipolytic strains of *B. cereus* under chilling temperatures was lower than observed in other studies (Svensson *et al*., 2004; Zhou *et al*., 2010). Considering the lipolytic activity, only one strain (1.2%) of *B. cereus* was positive at 30ºC and none at 7ºC (Table 1). However, at 10ºC, 16.3% of strains produced lipases. Based on this result, the lipase production by *B. cereus* seems to be higher in the range of 10ºC. But the occurrence of lipolytic strains was lower than the proteolytic strains.

All the strains of *B. cereus* isolated from dairy products had proteolytic activity at 30ºC. This a worrying statistic for samples stored at room temperature, such as the UHT milk and milk powder. At 10ºC for 7 days, 72.1% had proteolytic activity, and at 7ºC, only 4.6%, a significantly lower amount ($p < 0.05$).

Murugan and Villi (2009) registered proteolytic activity in 64% of *B. cereus* strains incubated at 37ºC. The authors found a significant reduction ($p < 0.01$) in the casein nitrogen / total protein (NC/TP) in six hours of incubation, achieving a reduction of 4.15%

of NC/TP by the end of 30 hours.

One strain of *B. cereus* featured both lipolytic and proteolytic activity at 30ºC. This result was below that reported by Chen *et al*. (2004) who verified that all seven strains of *Bacillus* spp. isolated from milk power were able to synthetize both enzymes at 37ºC, a temperature not evaluated in the present study. At 10ºC, thirteen strains produced lipases and proteases simultaneously.

The temperature of 7ºC inhibited the production of lipases and reduced the protease production, indicating that the adequate chilling of dairy products is enough to ensure stability in relation to the enzymes produced by *B. cereus*. Nevertheless, we may frequently find perishables exposed to temperatures above recommended in shelves of supermarkets and bakeries. Thus, marginal chilling temperatures represent a deteriorating potential to dairy products, once *B. cereus* has high metabolic activity at 10ºC, growing and producing lipases and proteases, being thus a critical temperature for the storage of dairy products.

Janstová *et al*. (2006) registered that UHT milk samples stored for four months at 4ºC presented no alterations due to the action of lipases and proteases. Meantime, when stored at 24ºC, sensory changes were detected within three weeks, as well as reduced level of protein and free tyrosine and increased level of free fatty acids.

The attention of industry and researcher should be focused on dairy products stored at room temperature, since the occurrence of strains producers of proteases was highest at 30ºC. Taking into account that these products have a long shelf life, these enzymes may act on proteins, altering their sensory characteristics.

Determining the optimal temperature for protease production by *B. cereus* pointed that 20ºC promoted the highest proteolytic activity, and at 10ºC, the lowest activity (Figure 1). Results for the three tested temperatures were significantly different ($p < 0.05$) by the non-parametric Kruskal-Wallis test. No significant coefficient of determination was detected for linear and quadratic regression for the obtained data.

These results are similar to found by Wang and Jayarao (2001), which observed a higher production of proteases by *Pseudomonas* fluorescens at 22ºC than at 7ºC and at 32ºC. The highest production of proteases at 20ºC represents a deteriorating potential for samples stored at room temperature, considering that this is within the range of average annual temperature in Southern Brazil (SIMEPAR, 2011).

Comparing these results with those obtained in the evaluation of the occurrence of *B. cereus* in several dairy products and identification of psychrotrophic behavior, lipolytic and proteolytic activity of the isolated strains, there was a greater occurrence of proteolytic strains of *B. cereus* at 30ºC, but the protease production was higher in the range 20ºC. Moreover, despite the high incidence of proteolytic strains at 10ºC, the production of proteases at this temperature was lower than the others. This is because some strains of psychrotrophic bacteria have greater proteolytic capacity (Nörnberg *et al*., 2009).

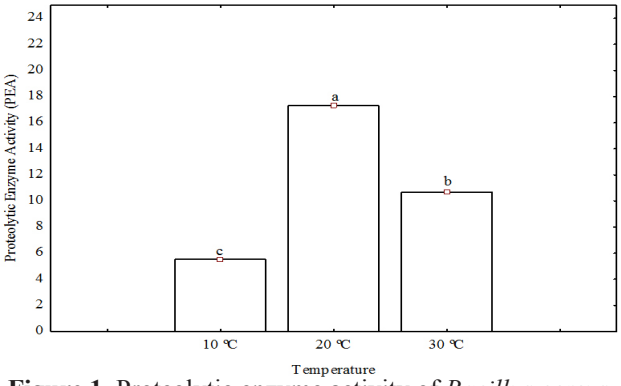

Figure 1. Proteolytic enzyme activity of *Bacillus cereus* isolated from dairy products under different temperatures (n=45).

Columns with different letters are significantly different ($p < 0.05$) by the non-parametric Kruskal-Wallis test.

Conclusions

Probably the control of the presence of *B. cereus* in dairy products is more critical in products stored at room or marginal chilling temperatures. *B. cereus* features low lipolytic activity, but its proteolytic potential is more pronounced, with a maximum production of proteases at room temperature. Under ideal chilling conditions, the production of these enzymes is inhibited. Dairy products stored at room temperature or under inadequate chilling conditions may bring about technological problems for dairy industry.

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