

## A psychrotrophic *Burkholderia cepacia* strain isolated from refrigerated raw milk showing proteolytic activity and adhesion to stainless steel

Maria de Fátima Barros Leal Nörnberg, Marilene Lenz Mentges, Silvana Terra Silveira, Eduardo César Tondo and Adriano Brandelli\*

Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul, 91501-970 Porto Alegre, Brasil

Received 5 August 2010; accepted for publication 1 March 2011; first published online 1 April 2011

The proteolytic activity of a psychrotrophic strain of *Burkholderia cepacia* isolated from refrigerated raw milk was characterized. *Bur. cepacia* produced proteolytic activity during growth at refrigeration temperature, with maximum activity at pH 6–7. The enzyme showed relative thermal stability in the range 40–50 °C during 25 min, and maintained 80% its initial activity at 76 °C/30 s. Milk coagulation assay showed that the crude protease from *Bur. cepacia* caused coagulation from day 2 for skimmed milk, whereas coagulation was observed from day 5 for whole milk. The adherence of this strain to stainless steel was evaluated, and the substrata had around  $10^7$  CFU/cm<sup>2</sup> after 15 to 60 min incubation. Results on biofilm development suggest that this bacterium could adhere and to form biofilms even at refrigeration temperatures. These results indicate that *Bur. cepacia* may represent a potential hazardous to milk and dairy products.

**Keywords:** adherence, *Burkholderia cepacia*, milk spoilage, proteolysis.

Refrigeration has an important impact on the microbiological quality of raw milk, by decreasing the presence of mesophilic bacteria, the main cause of acidification. However, the refrigeration temperature at the milk farms often ranges between 5 and 10 °C, which allow the development of psychrotrophic microorganisms (Cousin, 1982; Eneroth et al. 1998). Although most psychrotrophic bacteria are destroyed by the conventional thermal treatments employed in the dairy industry, such treatments have minor effects on their enzymes (Tondo et al. 2004). The intracellular enzymes and those associated with the cell wall can be released to the milk when bacterial lysis takes place after the thermal treatment and, in this way, together with the extracellular enzymes, hydrolyze milk components (Chen et al. 2003). In this context, the multiplication of psychrotrophic microbiota producing thermostable enzymes is a major factor influencing the quality of dairy products produced with raw milk stored at 7 °C or less for prolonged periods (Søraugh & Stepaniak, 1997). Milk gelation and deterioration of sensory characteristics are harmful effects caused by psychrotrophic bacteria (Shah, 1994; Datta & Deeth, 2003).

In addition, the elevated growth of psychrotrophic microorganisms may generate an adequate support for adherence of bacteria resulting in biofilms (Costerton et al. 1999; Kusumaningrum et al. 2003). Gram-negative bacteria like *Pseudomonas* are largely recognized by their capability to produce large amounts of exopolysaccharides, which contribute to adhesion and biofilm growth (Drenkard & Ausubel, 2002). The cell physical interaction with a surface through extracellular materials like polysaccharides or proteins, produced by the bacterium, withstands biofilm formation (Carpentier, 1997). Thus, microorganisms continue to grow, generating a highly appropriate substratum for the adherence of other bacteria, including pathogens (Costerton et al. 1999). This explains why surfaces of equipment and tools used in food production are focuses of bacterial contamination (Holah & Thorpe, 1999). Besides, organisms in biofilms are more resistant to the action of chemical and physical agents (Costerton et al. 1999; Sinde & Carballo, 2000).

Psychrotrophic gram-negative bacteria have been related to deleterious effects on milk, but research has been mostly focused on *Pseudomonas* spp. (Eneroth et al. 1998; Martins et al. 2006). There is growing evidence that other genera may have similar relevance by producing strong proteolytic activity (Nörnberg et al. 2010). The aim of this work was to evaluate the proteolytic activity of a *Burkholderia cepacia*

\*For correspondence; e-mail: abrand@ufrgs.br

strain isolated from refrigerated raw milk, and its capability of milk-coagulation and adhesion to stainless steel surfaces.

## Materials and Methods

### Microorganism

A psychrotrophic and proteolytic *Bur. cepacia* strain isolated from refrigerated raw milk was used (Nörnberg et al. 2010). The strain was maintained in BHI broth containing 20% (v/v) glycerol at  $-20^{\circ}\text{C}$ . The bacterium was cultivated twice in TSB at  $37^{\circ}\text{C}$  and then plated in milk agar plates before experiments.

### Bacterial growth

*Bur. cepacia* was cultured in sterile milk at  $10^{\circ}\text{C}$  for up to 72 h. Bacterial growth was monitored by serial dilutions to  $10^{-8}$  in sterile NaCl solution, and plating in triplicate onto plate count agar (Difco, Detroit, USA). Plates were incubated for 24 h at  $37^{\circ}\text{C}$  and counts performed among 30–100 colonies. Samples were monitored for proteolytic activity as described elsewhere (Tondo et al. 2004).

### Production of protease

A colony from milk agar plate was inoculated in mineral medium (0.5 g NaCl/l, 0.4 g  $\text{K}_2\text{HPO}_4$ /l, 0.3 g  $\text{K}_2\text{HPO}_4$ /l), containing 10 g casein/l, pH adjusted to 7.0. The production of protease was in 250 ml flasks containing 50 ml of the medium by incubation for 72 h at  $37^{\circ}\text{C}$  in an orbital shaker. The culture was centrifuged at 10,000 g for 10 min and the supernatant was used as crude enzyme.

### Proteolytic activity

Proteolytic activity was assayed using azocasein (Sigma, St. Louis, MO, USA) as substrate (Thys et al. 2004). The enzyme solution (100  $\mu\text{l}$ ) was mixed with 100  $\mu\text{l}$  0.1 M-sodium phosphate buffer pH 7. Then, 100  $\mu\text{l}$  10 mg azocasein/ml was added. The mixture was incubated for 60 min at  $37^{\circ}\text{C}$  and the reaction was stopped by adding 500  $\mu\text{l}$  trichloroacetic acid (TCA) 30% (w/v). After centrifugation at 10,000 g for 5 min, 800  $\mu\text{l}$  supernatant were mixed with 200  $\mu\text{l}$  1.8 M-NaOH. The absorbance at 420 nm was measured in a Shimadzu UV1240 spectrophotometer (Shimadzu, Tokyo, Japan). One enzyme unit was defined as the amount that caused an increase of 0.01 in absorbance at 420 nm in the assay conditions (Thys et al. 2004).

### Characterization of proteolytic activity

The effects of temperature and pH on the proteolytic activity were determined. Thermal stability was evaluated by pre-incubation of the enzyme for up to 25 min at 40, 45 and  $50^{\circ}\text{C}$ . The enzyme was also incubated at either 37, 76,  $100^{\circ}\text{C}$  for 30 s or  $142^{\circ}\text{C}$  for 10 s, and then residual activity

was measured as described above. The assay for optimum pH was developed using 0.1 mol/l of either sodium citrate buffer (pH 4 and 5), sodium phosphate buffer (pH 6, 7, and 8), or sodium carbonate buffer (pH 9 and 10).

### Milk coagulation

*Bur. cepacia* was grown in TSB by 24 h at  $37^{\circ}\text{C}$ . The culture was centrifuged at 10,000 g for 5 min and the supernatant was filtered through a 0.22  $\mu\text{m}$  membrane. Different amounts of this crude enzyme preparation (80 U/ml protease) were added to either whole or skim UHT milk. These samples were incubated at room temperature for up to 7 days. Milk coagulation was visually inspected at regular intervals of 24 h. Aliquots of thermally inactivated enzyme ( $100^{\circ}\text{C}/10$  min) served as controls.

Aliquots of the samples were adjusted to pH 4.6 with 2 M-HCl, followed by centrifugation at 11,600 g for 20 min to precipitate caseins and denatured soluble whey proteins. The concentration of amino acids in the resulting supernatants was measured by the ninhydrin method (Moore & Stein, 1954).

### Adherence to stainless steel

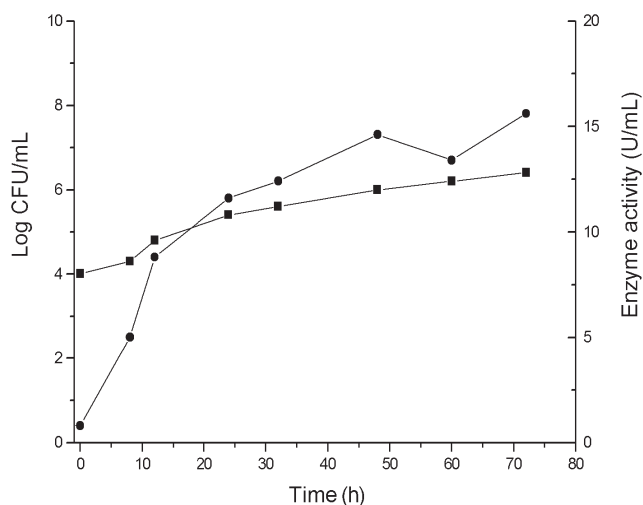
Stainless steel AISI 316 (Metalbras, Porto Alegre, Brazil) coupons of  $2 \times 2$  cm and 0.1 cm thick were used. Before the adhesion tests, coupons were degreased with a neutral detergent (3%, v/v) for 1 h, rinsed with 70% (v/v) ethanol, and then washed with MilliQ water. After that, the coupons were dried at  $60^{\circ}\text{C}$  for 2 h and autoclaved at  $121^{\circ}\text{C}$ , for 15 min in sealed tubes (Rossoni & Gaylarde, 2000).

The coupons were immersed in 10 ml TSB containing 8 log CFU/ml *Bur. cepacia*. Three coupons of stainless steel were immersed in the cultures for 15, 30 and 60 min, without shaking, at room temperature (Kusumaningrum et al. 2003). After that, the coupons were washed with PBS (phosphate buffer saline; pH 7.2) to remove the poorly adhered cells. The stainless steel coupons were immersed in 10 ml PBS before sonication process using an ultrasound bath (Unique USC 700) with a frequency of 40 kHz. Each coupon was sonicated for 2 periods of 10 min, aiming the release of adhered cells from coupons surfaces.

Decimal dilutions of PBS containing each sonicated coupon were made, and 20  $\mu\text{l}$  of each dilution were plated in TSA. The plates were incubated for 24 h at  $37^{\circ}\text{C}$  and CFU/cm<sup>2</sup> were determined (Hood & Zottola, 1997). All the counts were made in triplicate and each experiment was repeated twice.

### Biofilm growth assay

The capability of *Bur. cepacia* to form biofilms was determined using the microplate assay involving crystal violet staining (Burton et al. 2007). A 20  $\mu\text{l}$  inoculum of *Bur. cepacia* ( $10^5$  CFU/ml) was added to 280  $\mu\text{l}$  TSB in 96-well polystyrene microplates (Nunc, Rockville, USA).



**Fig. 1.** Time course of protease production during growth of *Bur. cepacia* in UHT milk. The cultures were developed in 100 ml flasks containing 20 ml sterile milk at 10 °C. Bacterial counts (CFU/ml; squares) and protease activity (circles) were determined at the indicated times.

Microplates were incubated at 4, 10, or 25 °C for up to 96 h. After incubation, the medium was gently removed and microplates were washed three times with distilled water. Microplates were stained for 15 min with a 4 mg/ml crystal violet solution, washed three times with distilled water, and air-dried for 60 min. The stain was then dissolved in 400  $\mu$ l 95% (v/v) ethanol and the absorbance at 570 nm was measured (O'Toole & Kolter, 1998).

## Results and Discussion

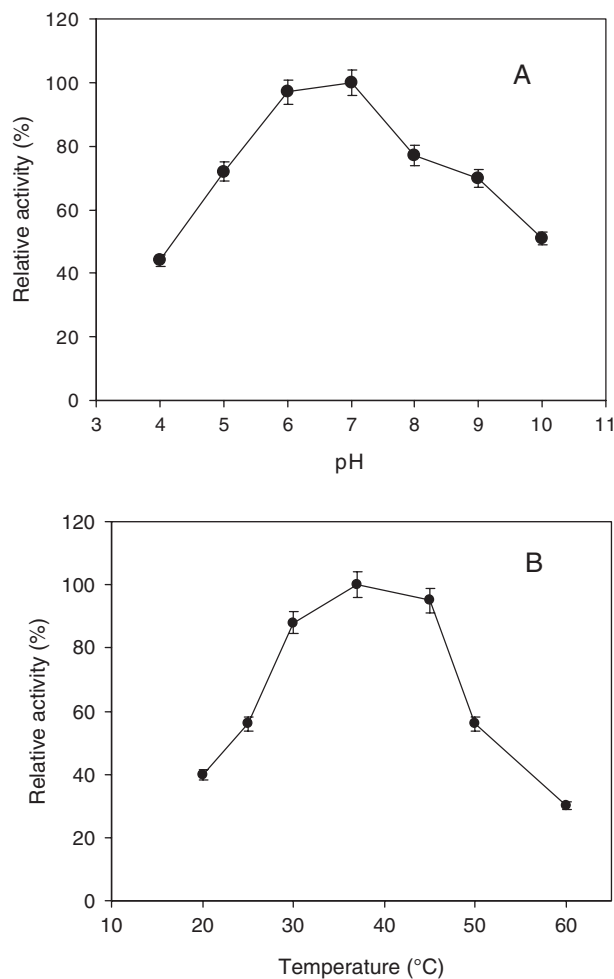
### Proteolytic activity of *Bur. cepacia*

*Bur. cepacia* was able to grow and produce proteolytic activity during cultivation at 10 °C in milk (Fig. 1). Maximum proteolytic activity was observed after 24 h, but it was also detected from 8 h. This indicates that this bacterium can secrete proteolytic enzymes during incubation at refrigeration temperatures. After 24 h growth, protein precipitation was observed indicating that coagulation occurred during growth of *Bur. cepacia* in milk.

Further studies on the proteolytic system of *Bur. cepacia* were developed after growth on casein broth. The proteolytic activity increased for up to 72 h, when a maximum activity of 85.9 U/ml was reached. Notable activity was also detected at 24 h (48 U/ml) and 48 h (72.4 U/ml).

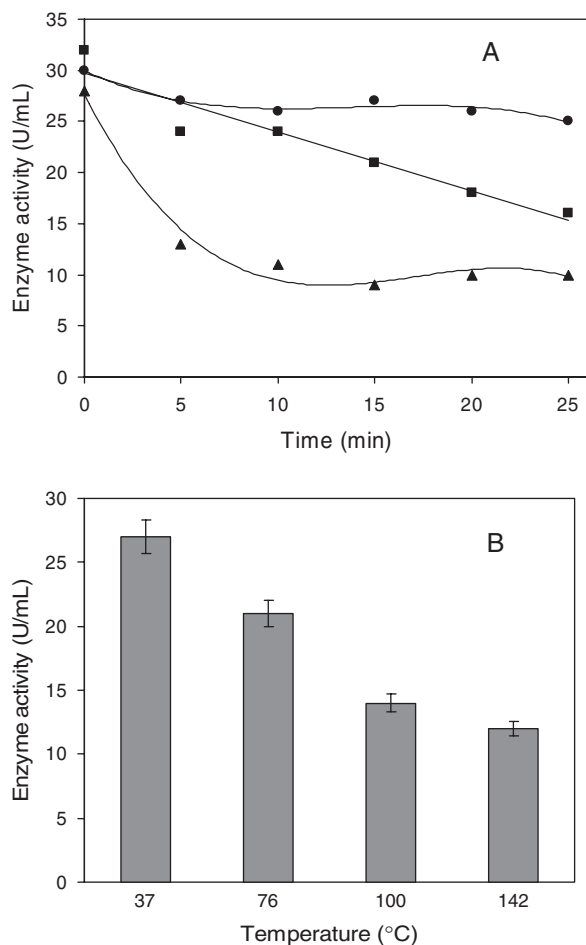
The optimum pH for proteolytic activity of *Bur. cepacia* was investigated in the range of pH 4 to 10. Maximum activity was observed between pH 6 and 7, and a reduced level of activity was observed under more acidic and alkaline pH values (Fig. 2A).

The effect of temperature on the activity of *Bur. cepacia* protease was evaluated. The maximum proteolytic activity



**Fig. 2.** Effect of pH (A) and temperature (B) on proteolytic activity of *Bur. cepacia*.

was observed within the range 35–45 °C, with accentuated decrease outside this range (Fig. 2B). The thermal stability was evaluated within 25 min at the temperatures of 40, 45, and 50 °C. When the enzyme was incubated at 40 °C, a high level of activity was observed within the established time maintaining at least 80% of the initial activity (Fig. 3A). By regression analysis, the residual activity correlates with time through a polynomial equation ( $R^2=0.994$ ). At the temperature of 45 °C, the enzyme showed a linear reduction ( $R^2=0.913$ ) of its activity during the incubation time (Fig. 3A). At 50 °C, there was a rapid reduction of the activity within 5 min, with about 52% of the initial activity remaining (Fig. 3A). Continued incubation at 50 °C caused little additional inactivation and at least 40% residual activity was observed. A polynomial relationship was observed with a  $R^2=0.981$ . In addition, the enzyme maintained about 80% its initial activity after 30 s incubation at 76 °C, and 40% its initial activity at 142 °C for 10 s (Fig. 3B). These results suggest that the enzyme is relatively heat resistant and is not completely inactivated by heat treatments conventionally applied to milk, in particular to pasteurization conditions.



**Fig. 3.** (A) Thermal stability of *Bur. cepacia* protease at 40 °C (circles), 45 °C (squares) and 50 °C (triangles). (B) The enzyme was incubated at either 37, 76, 100 °C for 30 s or 142 °C for 10 s and then residual activity was measured.

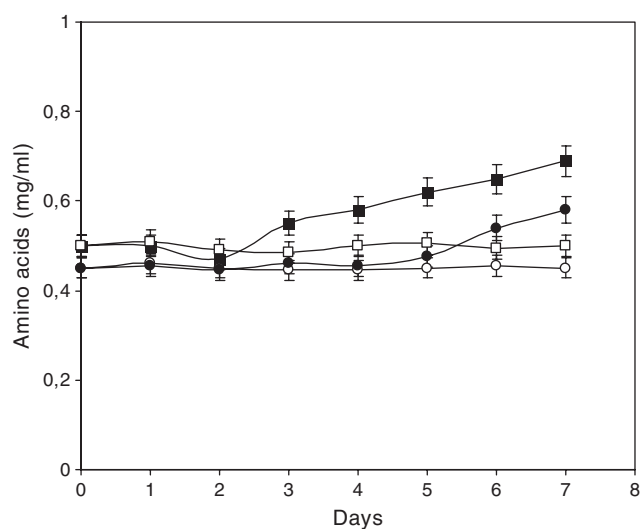
#### Milk coagulation

The effect of *Bur. cepacia* protease on milk coagulation was evaluated throughout 7 consecutive days, by visual inspection of skimmed and whole UHT milk samples artificially spoiled with the enzyme. The results are shown in Table 1. The enzyme added to the skimmed milk, independently of the amount (0.8, 1.6, or 4 U/ml), promoted milk coagulation from day 2, while the milk samples with inactivated enzyme (control group) did not coagulate. Concerning the whole milk samples, coagulation was also observed, but it started from day 5, while control samples did not coagulate until the day 7 of the evaluation. The fact that proteolysis occurs with higher velocity in skimmed milk samples than in whole UHT milk samples was also observed by Garcia-Risco et al. (1999), indicating that the rate of proteolysis is influenced by the fat level present in milk, in accordance to research by Lopez-Fandiño et al. (1993). The concentration of free amino acids increased during incubation time, whereas the values were nearly constant for control incubated samples (Fig. 4). These results indicate that the enzyme effectively

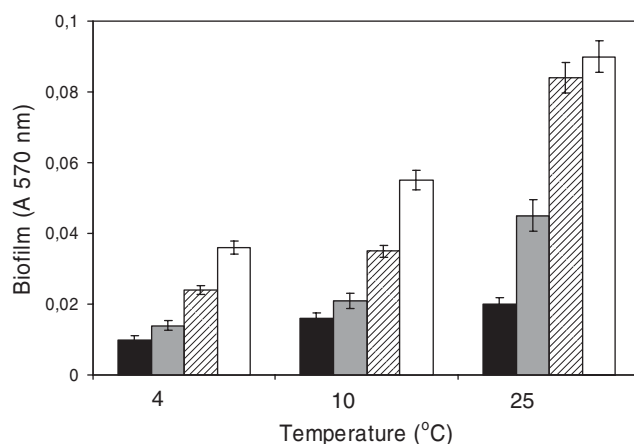
**Table 1.** Coagulation of skimmed and whole UHT milk samples by *Burkholderia cepacia* protease

Milk samples	Enzyme (U/ml)	Days						
		1	2	3	4	5	6	7
Skimmed	Control*	–	–	–	–	–	–	–
	4	–	+	+	+	+	+	+
	1.6	–	+	+	+	+	+	+
	0.8	–	+	+	+	+	+	+
Whole	Control*	–	–	–	–	–	–	–
	4	–	–	–	–	+	+	+
	1.6	–	–	–	–	+	+	+
	0.8	–	–	–	–	+	+	+

\*Control, 4 U/ml enzyme inactivated at 100 °C for 10 min



**Fig. 4.** Concentration of amino acids of whole (circles) and skimmed milk (squares) incubated with 4 U/ml protease (black symbols) or heat-inactivated protease (white symbols).



**Fig. 5.** Crystal violet staining of biofilms formed by *Bur. cepacia*. Values were determined after 24 (black bars), 48 (gray bars), 72 (dashed bars) and 96 h (white bars) incubation at the indicated temperatures.

hydrolyzed milk proteins and an increased amount of free amino acids and soluble peptides are present in the milk serum. This suggests that the enzyme continues to hydrolyze proteins after coagulation.

Gelation is one of the main problems that affect UHT milk quality. The UHT process promotes the increase of milk viscosity, but not its coagulation, since heating time is short. During storage, milk viscosity increases until there is gel formation (gelatinization) showing that the product is no longer suitable for consumption. The mechanisms involved in the gelatinization phenomenon comprise basically alteration of milk proteins, association and dissociation of calcium ions, formation of polymers derived from Maillard reaction, formation and dissociation of  $\kappa$ -casein complex with whey proteins, and the participation of innate milk enzymes (plasmin) and proteases of psychrotrophic bacteria (Chen et al. 2003). The gelatinization phenomenon is induced, initially, by the action of thermal-resistant enzymes (proteases) present, naturally, in the milk or derived from bacteria, mainly those of the psychrotrophic group (Tondo et al. 2004). These enzymes present the ability of degrading caseins and promote the aggregation of micelles. When the psychrotrophic bacteria counts reaches a level about  $10^6$  CFU/l, protease production by such microorganisms is able to degrade considerable quantities of casein. According to Shah (1994), UHT milk samples, stored at 30 °C, showing protease levels higher than one ng/ml can present a bitter flavour and undergone gelatinization. Degrading action of proteases from psychrotrophic microorganisms is distinct among milk protein fractions.  $\kappa$ -Casein is the most susceptible to the action of these enzymes, while whey proteins are relatively protease-resistant. Koka & Weimer (2000), reported that the action of these psychrotrophic bacteria, mainly the proteases from the *Pseudomonas* spp., is similar to that presented by chymosin, an enzyme employed in enzymatic coagulation for cheese production. Faster appearance of the phenomenon of gelatinization in UHT milk is associated to the severity of heating process and to the levels of contamination due to the presence of psychrotrophic microorganisms.

#### Adherence capacity and biofilm formation

The capacity of *Bur. cepacia* 1A4 to adhere to stainless steel was investigated. The adherence levels on the stainless steel coupons were around  $10^7$  CFU/cm<sup>2</sup>, independent of the different immersion times evaluated ( $P < 0.05$ ). The values observed for 15, 30, and 60 min were  $2.56 \times 10^7$ ,  $4.0 \times 10^7$  and  $1.52 \times 10^7$  CFU/cm<sup>2</sup>, respectively.

Microbial adherence to surfaces can occur directly through contact with contaminated materials or indirectly through bacterial particles present in the atmosphere, when the process of bacterium adhesion to the substratum is initialized (Kusumaningrum et al. 2003). Some studies report that bacterial adhesion consists of cell attraction by the surface, followed by adsorption and later by adherence by the bacterium cell (Katsikogianni & Missirlis, 2004). Surfaces

with more free energy, such as steel and glass, are less hydrophobic. These surfaces generally allow less bacterial adherence than hydrophobic surfaces, such as teflon, nylon, and the vast variety of polymers, including polyethylene (Sinde & Carballo, 2000). However, elevated counts of *Bur. cepacia* were found on stainless steel substratum, indicating that this strain has a high capability to adhere and potentially form biofilms.

The biofilm formation by *Bur. cepacia* was evaluated by crystal violet staining of extracellular polysaccharides. This strain showed an increased biofilm formation on microplates, in a temperature-dependent manner (Fig. 5A). Although low absorbance values were observed at 4 °C, it suggests that this bacterium could adhere and form biofilms even at refrigeration temperatures. This strain of *Bur. cepacia* showed capability similar to other recognized food spoilage bacteria like *Pseudomonas putida*, *Citrobacter freundii* and *Proteus vulgaris*, to form biofilms at low temperatures (Michaels et al. 2003). Biofilms are formed in dairy processing lines despite cleaning-in-place procedures, representing a source of post-pasteurization contamination (Austin & Bergeron, 1995). The potential for biofilm formation increases the resistance to usual sanitization and cleaning procedures, which represent a risk to food industry.

#### Conclusions

*Burkholderia cepacia*, isolated from refrigerated raw milk, produced proteolytic activity during growth at low temperature. The enzyme was relatively heat resistant and caused coagulation of skimmed and whole milk samples within 2 and 5 days, respectively. *Bur. cepacia* showed a high level of adherence to stainless steel and capability to form biofilms even at refrigeration temperatures, which may represent a hazard to food industry. This is a concern for milk industry because the potential contamination of equipment and tools, since stainless steel is widely used.

This work was supported by CNPq and CAPES (Brazil).

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