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GENETIC DIVESITY AND EXTRACELULLAR ENZYMATIC ACTIVITY OF *BACILLUS LICHENIFORMIS* STRAINS FROM MILK POWDER

Marcela J. González, Jorge A. Olivera, Jorge Bermúdez, Stella M. Reginensi*

Address(es): MSc. Marcela J. González,
Unidad de Tecnología de los Alimentos, Facultad de Agronomía, Garzón 780, CP 12900, Montevideo, Uruguay, + 598 23547991.

*Corresponding author: marcejoan@gmail.com

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ABSTRACT

A total of 379 *B. licheniformis* strains isolated from commercial milk powder were characterized genotypically and phenotypically. RAPD analysis yielded three different profiles, which include all isolates in this study, which could be assigned to strain F (n=375) or strain G (n=4), strain F also could be divided into two groups (group 1, n = 117; group 2, n = 258). Clustering by pairwise sequence similarity and phylogenetic relationships between isolates based on comparisons of the 16S rRNA gene sequence, showed two well defined groups. Group I contains all isolates tested belonging to genotype F, and Group II consists of three G genotype isolates. A total of 32 isolates, respecting the representation of each genotype, were randomly selected for extracellular enzymatic activity plate assays. Most isolates (25 out of 32) showed extracellular proteinase, lipase and amylase activity. Hydrolytic activities tested in this study are strain-dependent and none enzymatic activity could be linked to a defined group at genetic level. Preliminar characterization of proteolytic crude enzyme extract suggests the presence of a metal-activated serine protease active at an optimun temperature of 60 ºC. The exoenzymes production and its variation against different factors such as temperature, is isolate dependent so these results indicate that not all *B. licheniformis* strains may mean the same risk to process or product quality**.**

Keywords: *Bacillus licheniformis*, milk powder, RAPD, protease activity

INTRODUCTION

Spore-forming bacilli are common contaminants in dairy products and have been detected throughout the dairy processing, including dairy farm environments, storage and transportation tanks, and dairy processing plants (**Crielly *et al*., 1994; Postollec *et al*., 2012**). Spore-forming microorganisms enter processing plants from farm environments via milk, and in some cases multiplying within processing stages where conditions are suitable for bacterial growth. Thermal processes based on high temperatures are used in food industries to guarantee stability and safety of the products. However, these thermal treatments are not always sufficient to inactivate all spore-forming bacteria, especially those that are highly heat-resistant. In most cases spore-forming bacteria do not present safety concerns, however they can impact on spoilage and product specification requirements, as in the case of thermophilic bacilli contaminants in milk powders (**Reginensi *et al*., 2011; Ronimus *et al*., 2003; Rueckert *et al*., 2004; Scott *et al*., 2007**). The presence of the thermophilic bacilli in dairy products is indicator of poor hygiene and high counts are unacceptable, since they can lead to product defects caused by the production of heat-stable enzymes, such as proteinases and lipases, and acids capable to spoil the final product (**Chopra and Mathur, 1984; Cosentino *et al*., 1997; Chen *et al*., 2004**).

In a survey of milk powders from numerous countries, *Bacillus licheniformis*, *Anoxybacillus flavithermus* and *Geobacillus* spp. constituted 92% of spore-forming species isolated (**Ronimus *et al*., 2003**). *B. licheniformis* is a bacterium commonly found in nature, is the thermophilic bacilli most commonly isolated from raw milk (**Crielly *et al*., 1994**) and has been described as one of the two predominant species in Uruguayan dairy powders (**Reginensi *et al*., 2011**). It is a Gram positive rod-shaped spore-forming bacteria, mobile and facultative anaerobic. Belonging to *B. subtilis* group of the genus *Bacillus*, further comprising *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus*, *B. atrophaeus*, *B. mojavensis*, *B. sonorensis*, *B. vallismortis*, *B. firmus*, *B. lentus* and *B. sporothermodurans* (**Fritze, 2004**). This organism is of great interest not only because of its predominance in the production line of milk powder but also for the broad technological potential of the bacterium itself and its extracellular products (**Schallmey *et al*., 2004**). Proteases are used in detergent and leather industry, while amylases are used in textile and paper manufacture (**Priest, 1977**). However, these enzymes can be a primary source of spoilage of milk and the manufactured dairy products. Intensive heat treatments (i.e. UHT) at dairy industry are able to lower the load of these microorganism, but exoenzymes may be thermostable (i.e. proteases and lipases) and only partially inactivated with further action on the product (**Chen *et al*., 2003**). Several authors confirmed that bacterial proteinases and lipases found in milk survived all the heat treatments applied during the manufacture of milk powder (e.g., pasteurisation and spray-drying) (**Celestino *et al*. 1997a,b; Chen *et al*., 2004**), and these enzymes remained active in reconstituted milk even after a further 6 months storage at 25 ºC (**Celestino *et al*., 1997b**).

Identification of the microorganisms that may be contributing to milk powder spoilage can help in implementing preventive and corrective actions, with an approach directed to find critical control points through the dairy chain. Molecular methods able to rapidly detect and identify thermophilic contaminants are essential to improve the industrial response. *B. licheniformis* can be isolated from commercial milk powder under 'mesophile' and 'thermophile' growth conditions (**Reginensi *et al*., 2011**). Various methods, including multi-locus enzyme electrophoresis and phenotypic analysis (**Duncan *et al*., 1994**), random amplification of polymorphic DNA (RAPD) (**Ronimus *et al*., 2003**), *rpoB* and *gyrA* sequencing (**De Clerck and De Vos, 2004**), and bacitracin synthetase gene sequences (**Ishihara *et al*., 2002**) are currently used for genotyping *B. licheniformis.* Two or three different subgroups have been discriminated by this methods. Recently, a multi-locus variable number tandem repeat analysis (MLVA) method and combined with high resolution melt analysis (MLV-HRMA) have been developed for genotyping *B. licheniformis* (**Dhakal *et al*., 2013**). Nineteen genotypes could be identified using this methodology, many of which are correlated with previously defined by RAPD-PCR (**Ronimus *et al*., 2003**).

In this study, we evaluated genotypic and phenotypic biodiversity of *B. licheniformis* strains isolated from Uruguayan commercial milk powder, with emphasis on the production of extracellular enzymes. A better undestanding of the diversity will allow the validation of industrial process optimization to monitor and track *B. licheniformis* wild strains as an aid to minimize quality and safety problems for food processors.

MATERIAL AND METHODS

Bacterial isolates and culture conditions

Three hundred and seventy nine bacteria identified as *B. licheniformis* isolated from commercial milk powder manufactured in Uruguay belonging to our laboratory collection were used in this study. Isolates were prepared from frozen stocks after growth and transfer twice in fresh Triptic Soy Broth (TSB, Oxoid Ltd., UK) at 37 °C for 24 h prior to growth at different temperatures on Plate Count Agar (PCA, Oxoid Ltd., UK). A 100-fold dilution of each overnight culture was plated in duplicate on PCA and incubated at 37 °C or 55 °C for 24-48 h.

Isolation of total DNA

Bacterial cell cultures were grown overnight in Tryptic Soy Broth (TSB, Oxoid Ltd., UK) and cells were harvested at 10,000 rpm for 5 min in a Spectrafuge 7M tabletop centrifuge (Labnet International Inc., USA). Cell pellets were suspended in 200 µL TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). DNA was purified using a Genomic DNA purification kit (Fermentas International Inc., USA) following the manufacturer's instructions. Purified DNA was suspended in 40 µL TE buffer and used as template in amplification reactions. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific Incorporation, Wilmington, DE, USA).

RAPD-PCR analysis

Random amplification of polymorphic DNA (RAPD) analysis was carried out in 25 µL reaction mixtures containing 1× Thermo buffer (Fermentas, USA), 2.5 mM $MgCl_2$, 200 µM of each dNTP (Fermentas, USA), 1U Taq polymerase (Fermentas, USA), 1 µM primer OPR13 (5′-GGACGACAAG-3′) and 20 ng template DNA. PCR amplifications were done in a Corbett CG1-96 thermal cycler with a palmtop computer interface (Corbett Research Ltd., Cambridge, UK). PCR cycling parameters included a denaturation step at 94 °C for 3 min and 45 s; 35 cycles each consisting of 94 °C for 15 s, 36 °C for 15 s and 72 °C for 2 min; and a final extension step at 72 °C for 4 min. (**Ronimus *et al.*, 1997**). Control reaction mixtures lacking template DNA were included with each analysis. RAPD-PCR reactions were electrophoresed on 1.8 % agarose-gels using 0.5× TBE buffer (45 mM Tris, 45 mM Boric acid, 1 mM EDTA, pH=8.0) as running buffer at 10 V/cm for 1 h, stained with GoodView Nucleic Acid Stain 5%(v/v) (SBS Genetech Co. Ltd, China) and visualized and photographed on a UV transilluminator.

Isolate identification by 16S rDNA sequence analysis

Several isolates having distinct OPR13 RAPD profiles were identified by 16S rDNA sequence analysis. Twenty-five microliter reaction mixtures contained 1× Thermo buffer (Fermentas, USA), 2.5 mM $MgCl_2$, 200 µM of each dNTP (Fermentas, USA), 1U Taq polymerase (Fermentas, USA), 0.2 mM of each PCR primer (fD1 and rD1) and 20 ng template DNA. Primers fD1 5′-AGAGTTTGATCCTGGCTCAG-3′ and rD1 5′-AAGGAGGTGATCCAGCC-3′ were used to amplify a 1540 bp genome fragment with 16S rRNA gene sequences (**Weisburg *et al.*, 1991**). PCRs were performed using an initial denaturation step at 94 °C for 7 min, then 35 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 10 min, and experiments included negative controls with no added DNA template. Amplified fragments were purified and sequenced by Macrogen Sequencing Service, Korea, using an ABI PRISM 3730XL capillary sequencer (Applied Biosystems, CA, USA). DNA sequences were compared with those of the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify type strains with highest similarity, and were aligned against each other using Clustal W package (**Thompson *et al.*, 1994**). Phylogenetic trees were constructed using neighbourjoining method (**Saitou and Nei, 1987)** by MEGA4 software (**Tamura et al., 2007)**.

Enzymatic activity

From a total of 379 isolates of *B. licheniformis* genotypically characterized, a total of 32 isolates, respecting the representation of each genotype, were randomly selected for extracellular enzymatic activity. The proteolytic activity was tested by culturing the strains on 50% skim milk agar and incubating the plates at 37°C and 55ºC for 24 – 48 h. Presence of clear zone of hydrolysis around the colonies was taken as positive for proteolysis. The lipolytic activity was tested by culturing the strains in Spirit Blue agar supplemented with 30% of lipase reagent (Difco, Becton Dickinson and Company, Nevada, USA) and incubating the plates at 37°C for 3 days and screening the plates for the presence of clear zone of hydrolysis as described by **Starr and Burkholder (1942)**. The amylolytic activity was tested by culturing the strains on PCA plates supplemented with 1% soluble starch (w/v, Difco) and incubating the plates at 37°C for 24 – 48 h. Amylase activity was detected by flooding the plates with Gram's iodine solution (0.203 g of I_2 and 5.2g of KI in 100 ml of aqueous solution), active isolates were detected as bright clear haloes around the colonies.

Preparation of crude enzyme

All proteolytic isolates were cultivated in 250 ml nutrient broth with vigorous shaking (150 rpm) at 37 °C for 30 h. The culture was then centrifuged at 15,000 rpm for 30 min at 4 ºC. The cell free culture supernatant was filtered using 0.22 µm membranes (Millipore, Bedford, MA, USA) and maintained at -40 ºC for subsequent experiments.

Quantification of proteolytic activity

Determination of the enzyme activity was performed using the azocasein method (**Andrews and Asenjo, 1986**). 250 µl of the crude enzymes were incubated at 37 °C in 500 µl mixture containing 1% azocasein and 0.5 M tris-(hydroxymethyl)-aminomethane (TRIS) buffer pH 7.5 for 1 h. To terminate the reaction, 0.5 ml of 1.5M trichloroacetic acid (TCA) was added. All samples were allowed to stand for 15 min and the supernatant was collected after centrifugation (10,000 rpm; 15min). Protelytic activity was determined by measuring the absorbance at 340 nm. One unit of enzyme activity (U) was defined as the amount of cell-free supernatant required to increase one unit of absorbance at 340 nm in the assay conditions.

Effect of temperature on proteolytic activity and influence of protease inhibitors

The effect of temperature on enzyme activity was determined by carrying out the enzyme assay in a temperature range between 30-70 °C at pH 7.5. Enzyme activity at each temperature was measured as described above. The effects of active site inhibitors on protease activity were studied using phenylmethylsulfonylfluoride (PMSF), iodoacetimidate and ethylene diamine tetra acetic acid (EDTA). To determine whether the activity of proteases could be affected, each inhibitor was added to the crude enzymes and incubated at 35 ºC for 20 min. Protease activity was then measured using the azocasein method.

RESULTS AND DISCUSSION

RAPD genotyping of *Bacillus licheniformis* strains.

Three hundred and seventy nine *B. licheniformis* isolates were characterized genotypically. RAPD analysis of isolates yielded three different RAPD profiles, which include all isolates in this study (Fig. 1). All three profiles matched those described previously (**Ronimus *et al.*, 2003; Rüeckert *et al.*, 2004; Reginensi *et al.*, 2011**), corresponding to strain F (n = 375), and strain G (n = 4), although strain F could be divided into two groups (group 1, n =117 ; group 2, n = 258). A more detailed analysis of the RAPD patterns showed that almost all isolates identified as *B. licheniformis* belonged to these two groups. Both profiles corresponded to strain F, but differed from each other by the presence of an additional band of approximately 650 bp in group 2 (Fig. 1, Lanes 3, 6, 7, 9,10). A third group within this species had a different RAPD profile corresponding to strain G (Fig. 1, Lanes 12 to 14), but this group had a small contribution to *B. licheniformis* contamination in the powdered milk samples (4 out of 379 isolates). Distribution of *B. licheniformis* isolates agreed with other reports in which isolates of strain F (groups 1 and 2) were the dominant isolates in milk powder from New Zealand and Australia (**Ronimus *et al*., 2003, Dhakal *et al*., 2013**) as well as in milk powders manufactured in other countries (**Rüeckert *et al*., 2004**). The regular occurrence of *B. licheniformis* in powdered milk samples is likely due to the widespread distribution as a frequent contaminant in the dairy environment, like feed concentrate (**Scheldeman *et al*., 2005; Vaerewijck *et al*., 2001**), feces (**Scheldeman *et al*., 2005**), soiling of the udder and teats (**Waes, 1976**) and raw milk (**Scheldeman *et al*., 2005**).

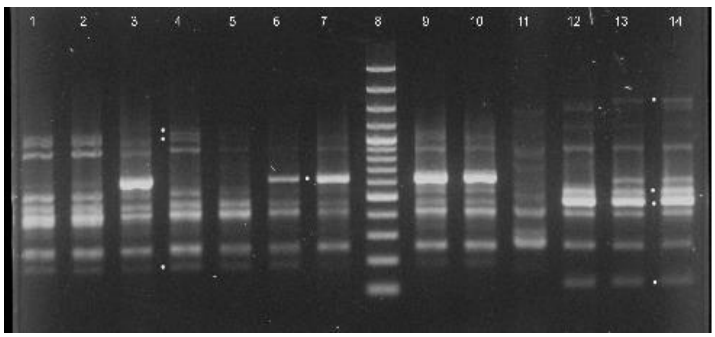

Figure 1 RAPD fingerprint profile of *B. licheniformis* isolates . Lanes 1, 2, 4, 5 and 11 *B. licheniformis* F isolates subgroup 1; lanes 3, 6, 7, 9,10, *B. licheniformis* F isolates subgroup 2 with distinctive band at 650 bp; lanes 12–14 *B. lichenifomis* G isolates; lane 8 DNA molecular mass standard ((Generuler 100bp

DNA Ladder Plus, Fermentas, bandas: 3000pb, 2000pb, 1500pb, 1200pb, 1000pb, 900pb, 800pb, 700pb, 600pb, 500pb, 400pb, 300pb, 200pb, 100pb). * Characteristics bands defined for each genotype are indicated.

Pairwise sequence similarity and phylogenetic relationships between isolates were inferred from comparisons of the 16S rRNA gene sequence. Clustering into two groups well defined was observed (Fig. 2). Group I contains all isolates tested belonging to genotype F, and Group II consists of three G genotype isolates. **Ronimus *et al.* (2003)** reference sequences of F (AY751766.1) and G (AY672764.1) isolates were also included and they appear clustered in group I and II, respectively. Our results corresponded well with previous findings of two different lineages within *B. licheniformis* that seem to have evolved differently (**De Clerck and De Vos, 2004, Madslien *et al*., 2012**). Several techniques were used to study the genotypic diversity among isolates of *B. licheniformis* from different sources. On the basis of different DNA fingerprinting methods two major groups were determined (**De Clerck and De Vos, 2004, De Jonghe *et al*., 2008, Banyko and Vyletelova, 2009**), as well as by *gyrA*, *rpoB* and *bac* sequence analysis (**De Clerck and De Vos, 2004, Ishihara *et al*., 2002**). A recent analysis of diversity within the species *B. licheniformis* typing was performed by multilocus isolates sequences food contaminants. The evolutionary relationship inferred by the analysis of six "house keeping " genes, showed the presence of two groups or lineages (major one from the other) that appear to have evolved separately (**Madslien *et al*., 2012**).

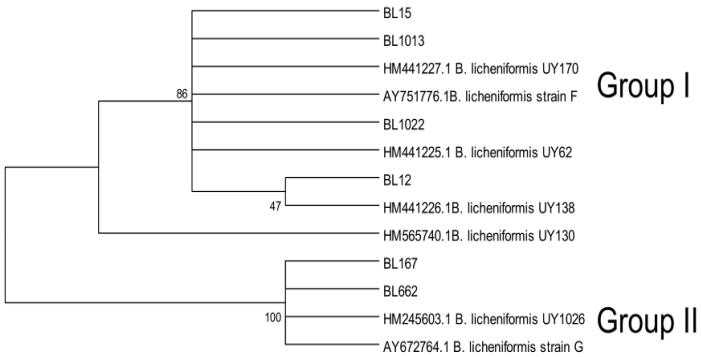

Figure 2 Phylogenetic tree obtained by Neighbor-joining analysis based on 16S rRNA gene sequences showing the phylogenetic position of isolates and type strains. Bootstrap values expressed as percentages of 1000 replications are shown at the branch points.

Extracellular enzymatic activity

A total of 32 isolates, respecting the representation of each genotype, were randomly selected for extracellular enzymatic activity plate assays. *Bacillus* species generally synthesise several extracellular enzymes, the maximum synthesis of which normally occurs in the late exponential and early stationary phases of growth, before sporulation (**Priest, 1977**). The results showed the ability of *B. licheniformis* isolates to produce exoenzymes with different hydrolytic activities (Table 1). Most isolates (25 out of 32) were screened as capable of degrading casein, starch and lipids. All the 32 tested isolates showed lipolytic activity, 26 isolates were able to hydrolyze starch, while the ability to hydrolyze casein was found in 30 and 31 isolates when incubation temperature were 37 and 55 °C, respectively (Table 1). None hydrolytic activity could be linked to a defined genetic group. Hydrolytic activities tested in this study were strain-dependent and proteolytic activity could eventualy (1 out of 31 isolates) be temperature dependent. This is an important fact to consider in the context of milk processing, and not all *B. licheniformis* strains represent the same risk to process or product quality.

Table 1 Qualitative assessment of extracellular enzymatic activity of *B. licheniformis* isolates.

Isolates	Proteolytic isolates		Amylolytic isolates	Lipolytic isolates
	37 ºC	55 ºC	37 ºC	37 ºC
Strain F group 1	14/14	14/14	9/14	14/14
Strain F group 2	13/14	14/14	14/14	14/14
Strain G	3/4	3/4	3/4	4/4

All *B. licheniformis* strains that exhibited a clear zone around their colonies on skim milk agar were grown on nutrient broth for 30 hours and tested for proteolytic activity by the azocasein assay. As well as other *Bacillus* species, production of protease by this *B. licheniformis* is dependent on cell growth, reaching the maximum yield at early stationary phase (**Olajuyigbe and Ajele, 2008)**. In the crude enzyme caseinolytic protease activity assay, no significant difference (P>0.05) among genotypic groups F and G was observed, but different levels of proteolytic activity were expressed by particular bacterial isolates (Fig. 3). BL1026 and BL27 showed highest proteolytic activity amongst all *B. licheniformis* isolates tested (Fig. 3). Consequently, these isolates were selected for further studies.

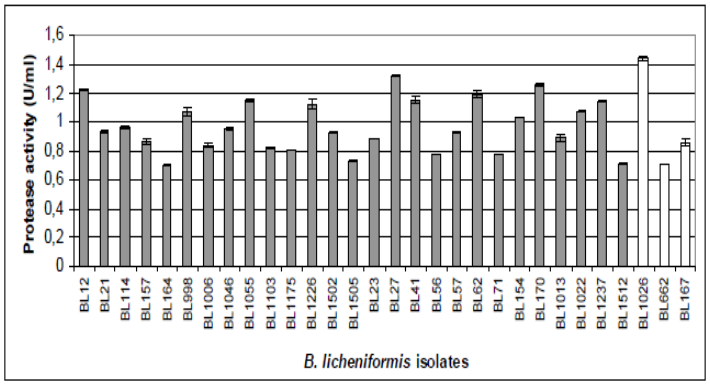

Figure 3 Proteolytic activity of *B. licheniformis* strain F (■) and strain G (□) isolates performed using azocasein method (**Andrews and Asenjo**, 1986). Experiments were performed in triplicate, and error bars indicate ±SD.

Effect of temperature and inhibitors on protease activity

Proteases produced by mesophilic and thermophilic bacteria have been reported to be heat stable. The effect of temperature on protease activity was determined for isolates BL1026 and BL27 at temperatures between 30 and 70 °C. Temperature profiles on enzyme activity are shown in Figure 4. Optimum temperature for the enzyme was found to be 60 ºC for both isolates. Similar results were reported by other researchers where optimum temperature of 60 ºC was recorded for proteases from other *B. licheniformis* strains (**Öztürk *et al*., 2009; Bezawada *et al*., 2011; El Hadj-Ali *et al*., 2007**).

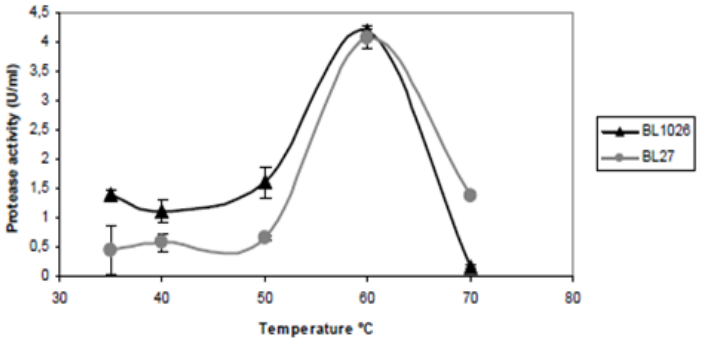

Figure 4 Effect of temperature on protease activity produced from *B. licheniformis* strains BL1026 and BL27. Experiments were performed in triplicate, and error bars indicate ±SD.

Inhibition studies primarily give an insight of the nature of an enzyme. The effect of a variety of enzyme inhibitors, such as chelating agent and group-specific reagent, on the activity was investigated (Table 2). The enzymes were strongly inhibited by the serine protease inhibitor (PMSF), indicating that both isolates produced serine proteases. Furthermore, the enzymes were equally affected when preincubated with EDTA, a metalloprotease inhibitor. Partial inhibition of many serine porteases by chelating agents has been reported (**El Hadj-Ali *et al*., 2007, Fakhfakh *et al*., 2009**) indicating the probable interaction of ions on the stabilization or activation of conformational structure of enzymes. These data indicate that the preparations contains a metal-activated enzyme with a serine residue at the active site.

Table 2 Effect of inhibitors on activity of protease crude enzyme extract obtained from *B. licheniformis* strains BL1026 and BL27.

Strain	Relative activity in the presence of [a]			
	No inhibitors	1 mM PMSF	1 mM EDTA	0,1 mM Iodoacetamide
BL1026	100	25 ± 3,4	24 ± 6,9	98 ± 0,4
BL27	100	19 ± 3,7	17 ± 1,7	99 ± 6,0

[a]The relative activity was calculated using the A340nm value of the control reaction (no inhibitors) as 100% enzyme activity. Experiments were performed in triplicate and SD is indicated.

Many commercial proteases (Protemex®, Neutrase®), mainly neutral or alkaline are produced by microorganisms belonging to the genus *Bacillus*. Most of these proteases have been cloned and characterized. These enzymes were characterized as serine proteases, cysteine proteases or metalloproteases. Only a protease of this genus was characterized as atypical aspartic protease described in *B. licheniformis* (**Carroll and Setlow, 2005**). Generally, *Bacillus* species have higher extracellular and intracellular proteolytic activity than other bacteria and they commonly produce subtilases, members of the superfamily of subtilisin-like serine proteinases (**Siezen and Leunissen, 1997**). These enzymes are active at neutral or alkaline pH. Many peptidases of this family are thermostable and the typical substrate for these enzymes is casein. Indications are that proteases produced by *B. licheniformis* isolates belong to this family but needed more comprehensive characterization.

CONCLUSION

These results confirm the diversity at genetic and phenotypic level of *B. licheniformis* isolated from milk powder. Up to now, it seems difficult to link a phenotypic characteristics to a defined group at genetic level. The study highligths the existence of groups with similar behaviors or characteritics but other strains with higher differences. The results of this work show that the exoenzymes production and its behaviour at different temperature tends to be isolate dependent. Further studies must be developed to determine the impact of enzyme activity and the number of microorganisms in the quality of dairy products.

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