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Research Paper

Milk-deteriorating exoenzymes from *Pseudomonas fluorescens* 041 isolated from refrigerated raw milk

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Abstract

The practice of refrigerating raw milk at the farm has provided a selective advantage for psychrotrophic bacteria that produce heat-stable proteases and lipases causing severe quality problems to the dairy industry. In this work, a protease (AprX) and a lipase (LipM) produced by *Pseudomonas fluorescens* 041, a highly proteolytic and lipolytic strain isolated from raw milk obtained from a Brazilian farm, have been purified and characterized. Both enzymes were purified as recombinant proteins from *Escherichia coli*. The AprX metalloprotease exhibited activity in a broad temperature range, including refrigeration, with a maximum activity at 37 °C. It was active in a pH range of 4.0 to 9.0. This protease had maximum activity with the substrates casein and gelatin in the presence of Ca^{+2}. The LipM lipase had a maximum activity at 25 °C and a broad pH optimum ranging from 7.0 to 10. It exhibited the highest activity, in the presence of Ca^{+2}, on substrates with long-chain fatty acid residues. These results confirm the spoilage potential of strain 041 in milk due to, at least in part, these two enzymes. The work highlights the importance of studies of this kind with strains isolated in Brazil, which has a recent history on the implementation of the cold chain at the dairy farm.

Keywords: raw milk, food deterioration, *Pseudomonas fluorescens*, extracellular protease, extracellular lipase.

Introduction

In Brazil, the practice of refrigerating raw milk at the dairy farm started in the 90s, was officially instituted by the government in 2002 and it is still being implemented in some areas of the country [Brasil, 2002; Brasil, 2011]. The refrigeration of raw milk in the farm and dairy industries has improved the quality and shelf life of milk and dairy products. However, it does not prevent the growth of psychrotrophic microorganisms that produce heat-stable enzymes such as proteases and lipases (Cousin 1982; Sorhaug and Stepaniak 1997; Decherni *et al.*, 2005; De Jonghe *et al.*, 2010; Corrêa *et al.*, 2011; Baglinière *et al.*, 2013; Quigley *et al.*, 2013).

Many of these enzymes are produced by *Pseudomonas fluorescens*, a frequent psychrotrophic spoilage bacterium found in milk (Wiedmann *et al.*, 2000; Dogan and Boor, 2003; Pinto *et al*,. 2006; Dufour *et al*.; 2008; Marchand *et al.*, 2009; Baglinière *et al.*, 2013). As hydrolytic enzymes from this bacterium are generally not inactivated by pasteurization or even by Ultra-High Temperature (UHT) treatment (Griffiths *et al.*, 1981; Chen *et al.*, 2003; De Jonghe *et al.*, 2010; Baglinière *et al.*, 2013), they can cause severe problems in the dairy industry such as milk protein hydrolysis, development of off-flavors, shelf-life reduction, decrease of yield during cheese production, milk heat-stability loss, and gelation of UHT milk (Fairbairn and Law, 1986; Datta and Deeth, 2001; Chen *et al.*, 2003; Dufour *et al.*, 2008; Baglinière *et al.*, 2013).

A commom type of protease produced by *P. fluorescens* is metalloprotease. This class of enzyme contains one zinc atom and up to eight calcium atoms, conferring thermostability to the protein (Sorhaug and Stepaniak, 1997). These authors listed some important

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characteristics of the metalloproteases secreted by strains of *P. fluorescens* including temperature optimum between 30 and 45 °C, a significant residual activity at 4 °C, and a pH optimum in the neutral range. These authors pointed out that a heat treatment of milk sufficient to fully inactivate these enzymes would also create unacceptable changes in the product and it is therefore unpractical for the dairy industry.

Microorganisms that produce lipolytic enzymes, such as *P. fluorescens*, are important in the dairy industry because they can produce rancid flavors and odors in milk and dairy products that make these foods unacceptable to consumers (Cousin 1982). Bacterial lipases generally have molecular masses between 30 to 50 kDa, and the pH optimum is slightly alkaline (in the range of 7 to 9) (Chen *et al.*, 2003; Chakraborty and Paulraj, 2009; Boran and Ugur 2010; Anbu, 2014). Lipase production by *P. fluorescens* is influenced by the type and concentration of carbon and nitrogen sources, iron, pH, dissolved oxygen concentration, and growth temperature (Cousin 1982; Burger *et al.*, 2000; Woods *et al.*, 2001; Rajmohan *et al.*, 2001).

The present work aimed at the molecular characterization of a protease and a lipase produced by *P. fluorescens* 041, a highly milk deteriorating strain isolated from refrigerated raw milk obtained from a Brazilian farm. Both enzymes were overexpressed in *Escherichia coli*, purified to homogeneity by affinity chromatography and biochemically characterized in order to evaluate their role in the spoilage of milk components.

Material and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *P. fluorescens* 041 and 07A strains were isolated from refrigerated raw milk as highly proteolytic and lipolytic psychrotrophic bacteria (Martins *et al.*, 2005; Pinto *et al.*, 2006; Pinto *et al.*, 2010).

Growth conditions

P. fluorescens was cultured in TYEP (tryptone 1%, yeast extract 0.25%, KH_2PO_4 0.1%, K_2HPO_4 0.1%, and $CaCl_2$ 0.25%) broth at 25 °C with aeration or in 12% (w/v) reconstituted skim milk powder. *E. coli* XL1-Blue was cultured in Luria-Bertani (LB) broth or on LB agar plates at 37 °C, as required.

DNA manipulations, PCR reactions and sequencing

DNA manipulations

Cloning, restriction enzyme analysis, and transformation of *E. coli* were performed using established procedures (Sambrook *et al.*, 1989). PCR was performed with TaKaRa Ex Taq polymerase (TaKaRa Shuzo, Shiga, Japan). Plasmid DNA was isolated using the QIAprep Spin Miniprep kit, and chromosomal DNA was purified with the DNeasy tissue kit. DNA fragments were purified from agarose gels by using the QIAquick gel extraction kit (all kits from Qiagen, Hilden, Germany).

Amplification and sequencing of the protease and lipase genes by PCR

The reaction consisted of 2.0 mM $MgCl_2$, 5.0 μL of 10X buffer Ex *Taq*, 2.5 mM deoxynucleotide triphosphates (dNTPs), 0.5 μM of each primer, 1 U of Ex *Taq* DNA polymerase, and 40 ng of DNA in a final volume of 50 μL. Primers based on the sequences of the *aprX* (GenBank accession numbers DQ146945, AY298902, AF216700, AY973251) and *lip* gene (GenBank accession numbers AF216702, AY694785, M86350, S77830, D11455, AB063391, AY304500, AY673674, M74125, AY700013) of other *P. fluorescens* strains were designed (Table 2), and synthesized by Microsynth (Zürich, Switzerland). The reactions were carried out in a T3 thermocycler (Biometra®, Biolabo Scientific Instruments, Zürich, Switzerland).

The M13 Forward and Reverse Primers were used to sequence the *aprX* and *lipM* genes of *P. fluorescens* 041

Table 1 - Bacterial strains and plasmids.

Strain or plasmid	Description	Reference or source
Strains		
E. coli XL1-Blue	Cloning and subcloning host *sup*E44, *hsd*R17, *end*A1, *rec*A1, g*yr*A96, *thi*1, *rel*A1, *lac*-F[*pro*AB+, *lac*Iq, *lac*Z-M15, Tn10 (*tet*F)]	Bullock et al., 1987
P. fluorescens 07A	Wild type	Martins *et al.*, 2005
P. fluorescens 041	Wild type	Martins *et al.*, 2005
Plasmids		
pCR2.1-TOPO	Cloning vector, *lacZ*α fragment containing MCS, f1 origin, ColE1, Km^r Ap^r	Invitrogen
pQE30-Xa	Vector for the insertion of a Factor Xa Protease recognition site C-terminal of the 6xHis tag, T5 promoter, *lac* operator, ribosome binding site, ATG start codon, His tag sequence, multiple cloning sites, stop codons in all three reading frames, Col E1 origin of replication, Ap^r	Qiagen
pQE30-Xa-aprX041	1.43 kb fragment containing *aprX* from *P. fluorescens* 041 in pQE30-Xa, Ap^r	This study
pQE30-Xa-lipM041	1.42 kb fragment containing *lipM* from *P. fluorescens* 041 in pQE30-Xa, Ap^r	This study

Table 2 - Primers used to amplify the *aprX* and *lipM* gene by PCR.

Primer	Sequence (5'-3')	Aplication
Apr-F	TTATGTCAAAAGTAAAAGAC	Amplification of *aprX* gene
Apr-R	TCAGGCTACGATGTCACTG	Amplification of *aprX* gene
APRX-F	ATTGGATCCAAAGCTATTGTATCTGCCGCG	Amplification of *aprX* gene and preparation for cloning in pQE-30Xa
APRX-R	ATTGAGCTCTCAGGCTACGATGTCACTGGC	Amplification of *aprX* gene and preparation for cloning in pQE-30Xa
Lip-F	ATGGGTRTSTTYGACTATAAAAACC	Amplification of *lipM* gene
Lip-R	TTAACCGATCACAATCCCCTCC	Amplification of *lipM* gene
LIPM-F	ATTGGATCCAACCTCGGTACCGAGGACTC	Amplification of *lipM* gene and preparation for cloning in pQE-30Xa
LIPM-R	ATTGAGCTCTTAACCGATCACAATCCCCTCCC	Amplification of *lipM* gene and preparation for cloning in pQE-30Xa

The introduced restriction sites BamHI and *Sac*I are underlined.

cloned into pCR2.1-TOPO according to description of Invitrogen.

Cloning, heterologous expression and purification of *P. fluorescens* 041 protease and lipase

Once the complete sequences of the *aprX* and *lipM* genes were obtained, primers were designed (Table 2) to amplify the open reading frames (ORF) by PCR using the bacterial genomic DNA as a template and TaKaRa Ex Taq as DNA-polymerase. The primers generated *Bam*HI and *Sac*I sites at the 5' and 3' ends of the amplified fragments, respectively.

The amplified DNA fragments of 1,434 bp and 1,422 bp, containing the *aprX* and *lipM* structural genes, respectively, were digested with *Bam*HI and *Sac*I and ligated into vector pQE-30Xa (Qiagen) previously cut with the same restriction enzymes. Plasmids harbouring the *aprX* or *lipM* ORFs inserted downstream of the T5 promoter were named pQE-30Xa-aprX041 or pQE-30Xa-lipM041. The plasmids were subsequently transformed into the expression strain *E. coli* XL1-Blue.

For overproduction of AprX and LipM, *E. coli* XL1-Blue cells carrying pQE-30Xa-aprX041 or pQE-30Xa-lipM041 were grown in dYT medium (tryptone 1.6%, yeast extract 1.0%, NaCl 0.5%, and glucose 0.2%) containing ampicillin (100 μg mL^{-1}) at 37 °C under shaking at 300 rpm. At an optical density of 0.5 at 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM, in order to induce the expression of *aprX* and *lipM*. After 5 h incubation at 37 °C, the cells were collected by centrifugation at 10,000 *g* for 30 min, resuspended in 50 mM Tris-HCl (pH 8.0) and centrifuged at 10,000 *g* for 30 min, followed by two washing steps with 50 mM Tris-HCl pH 8.0, NaCl 150 mM. The resulting cell pellets were finally resuspended in lysis buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0) and the recombinant histidine-tagged enzymes were purified under denaturing conditions using the Ni-NTA Spin Columns (Qiagen) according to the suppliers' instructions. After purification, the enzymes were subjected to overnight dialysis with Tris-HCl 20 mM, pH 8.0, $CaCl_2$ 5 mM at 4 °C to allow renaturation.

Protein quantification, SDS-PAGE and zymograms

Protein concentration of the purified AprX and LipM enzyme solutions was determined by using the method of Bradford (1976). Proteins were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [Laemmli, 1970]. After electrophoresis the gels were stained with Coomassie brilliant blue.

Exoprotease activities of *P. fluorescens* culture supernatants, resolved proteases after precipitation with ammonium sulfate, and recombinant expressed AprX protease were visualized in SDS-PAGE-gels supplemented with 0.2% (w/v) azocasein (Christensen *et al.*, 2003). After electrophoresis, proteins were renaturated by washing twice in 50 mM Tris-HCl, pH 7.5, 25% (v/v) isopropanol for 15 min at room temperature and once in 50 mM Tris-HCl, pH 7.5. After overnight renaturation at 4 °C, the zymogram was incubated for 4 h in 5 mM $CaCl_2$ and 50 mM Tris-HCl, pH 8.0 at 40 °C. Prior to detection, the gel was washed in 1 M NaOH for 5 min. Protease activity was detected as colourless zones in an orange background.

For the analysis of the lipase pattern after SDS-PAGE, proteins were renaturated as above described. The gels were overlaid with the fluorescent substrate methyl-umbelliferyl-butyrate (0.01 M in dimethylformamide) in order to detect lipolytic activity using UV-light (360 nm) to visualize blue fluorescent bands.

Identification of proteins by mass spectrometry

P. fluorescens 041 was grown in TYEP medium at 25 °C for 48 h. The cells were removed from the medium by centrifugation at 10,000 *g* for 30 min, the supernatant was sterilized by filtration, and the proteins were precipitated with ammonium sulfate. Samples were centrifuged 20 min at 10,000 *g* and the supernatant was discarded. Pellets were washed twice with an 85% ammonium sulfate solution (w/v), and again centrifuged. The pellets were dissolved in 50 Mm Tris-HCl, pH 8.0 and dialysed overnight at 4 °C against Tris-HCl 50 mM, pH 8.0, $CaCl_2$ 5 mM. Aliquots of

15 µL of the dialysed samples were separated on SDS-PAGE (12%) gels. Coomassie-stained protein bands were excised, digested with trypsin and analysed by mass spectrometry (Riedel *et al.*, 2006).

Enzyme assays

Proteolytic activity was investigated on azocasein, according to Christensen *et al.* (2003), by incubating 250 µL of 2% azocasein (w/v) with 150 µL sterile filtered culture supernatant or with 75 µL of the purified AprX. Lipolytic activity on *p*-nitrophenyl palmitate was investigated by incubating 1 mL of substrate with 100 µL supernatant from overnight cultures or with 50 µL of the purified lipase LipM.

Characterization of purified enzymes

The proteolytic and lipolytic activities of purified AprX and LipM were determined as described above at various incubation temperatures (4, 25, 30, 37, 40, 45, 50, and 60 °C) and at various pH values using the following buffer systems: sodium succinate (pH 4.0, 5.0, 6.0), Tris-HCl (pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0), and glycine-NaOH (pH 9.0,10.0,11.0,12.0,13.0).

In order to determine heat stability of purified AprX and LipM, they were incubated for 5, 10, 15, 20, 30 and 60 min at 50, 60, 70, 80, 90, and 100 °C. They were also incubated at 65 °C for 30 min and 72 °C for 20 s to simulate the milk pasteurization treatments.

To investigate the effect of metal ions on purified AprX and LipM, the reaction mixture was supplemented with 1 mM of each of the following compounds: $MnS0_4$, $CoCl_2$, $ZnSO_4$, $FeSO_4$, $MgSO_4$, or $FeCl_3$. The effect of protease inhibitors on the proteolytic activity of purified AprX was determined by supplementing the reaction mixture with 1 mM PMSF, 1 mM EDTA, 1 mM Pefabloc SC, 2% (w/v) SDS, 4 M urea, 0.1% (w/v) DTT, and 0.1% (v/v) β-mercaptoethanol and subsequent measuring the residual activities on azocasein substrate.

The substrate specificity of purified AprX was determined on casein, elastin, collagen, bovine serum albumin, and gelatin. The reaction mixture consisted of 0.4% (w/v) of each protein in 400 µL of 50 mM Tris-HCl, pH 6.5 and 150 µL of enzyme solution. After incubation at 37 °C for 1 h, the mixture was withdrawn and the increase in the amount of free amino groups was determined by the ninhydrin method according to Setyorini *et al.* (2006).

Activities of purified LipM on different *p*-nitrophenyl fatty acid esters (*p*- nitrophenyl acetate, *p*-nitrophenyl butyrate, *p*-nitrophenyl palmitate, and *p*-nitrophenyl phosphorylcholine) were also measured according to the assay for lipolytic activity as described above.

Results

Milk-deteriorating hydrolytic activities of *P. fluorescens*

P. fluorescens 041 showed higher proteolytic (Figure 1A) and lipolytic (Figure 1B) activities than the strain 07A. Moreover, strain 041 exhibited a higher capacity to hydrolyze milk than *P. fluorescens* 07A when both strains were inoculated into 12% (w/v) reconstituted skim milk (Figure 1C). Therefore, *P. fluorescens* 041 was selected for further analysis of its hydrolytic extracellular enzymes.

SDS-PAGE analysis of ammonium sulfate precipitated protein from supernatants of TYEP cultures of *P. fluorescens* 041 demonstrated the presence of multiple protein bands (Figure 1D). Proteolytic activity of the dominant 50 kDa band was demonstrated by a zymogram incorporating azocasein (Figure 2). Mass spectrometry analysis of the major proteolytic protein band identified this protein as a metalloprotease, which is commonly referred to as AprX. Analysis of the bands with lower molecular weight that showed proteolytic activity (Figure 1D) revealed that these bands were actually degradation products of AprX.

Cloning and sequencing of protease and lipase genes

Primers based on sequences of homologous proteases and lipases from other *P. fluorescens* strains were synthesized and used to PCR amplify a segment encoding these enzymes from *P. fluorescens* 041 genome. Electrophoresis of the PCR products revealed a single product of about 1,500 bp for both genes. These PCR products were sequenced to reveal their identity as *aprX* and *lipM* genes. The *aprX* and the *lipM* genes of *P. fluorescens* 041 comprised open reading frames of 1,434 bp and 1,425 bp and coded for proteins with 477 and 474 amino acids, respectively. Based on the amino acid sequences, the molecular mass of both enzymes was predicted to be 49.365 kDa and 49.811 kDa, which was confirmed by SDS-PAGE analysis of the purified enzymes (Figure 2, line 3 and 5). The isoeletric point for AprX was 4.46 and 4.36 for LipM, as determined by using Protean (DNA Star Lasergene 7). They were active on zymograms after renaturation in buffer containing 1 mM of $CaCl_2$ (Figure 2, line 4 and 6).

The *aprX* gene of *P. fluorescens* 041 showed 97% identity with the extracellular alkaline metalloprotease (*aprX*) gene of *P. fluorescens* strain A506 and with the protease (*aprX*) gene of *P. fluorescens* strain F. The *lipM* gene of *P. fluorescens* 041 showed 93% identity with polyurethanase lipase A (*pulA*) gene and 86% with the lipase (*lipA*) gene of *P. fluorescens* strain A506.

Biochemical characterization of AprX and LipM

The temperature optimum of activity of the purified protease of *P. fluorescens* 041 was 37 °C (Figure 3A). Moreover it showed activity under conditions of refrigera-

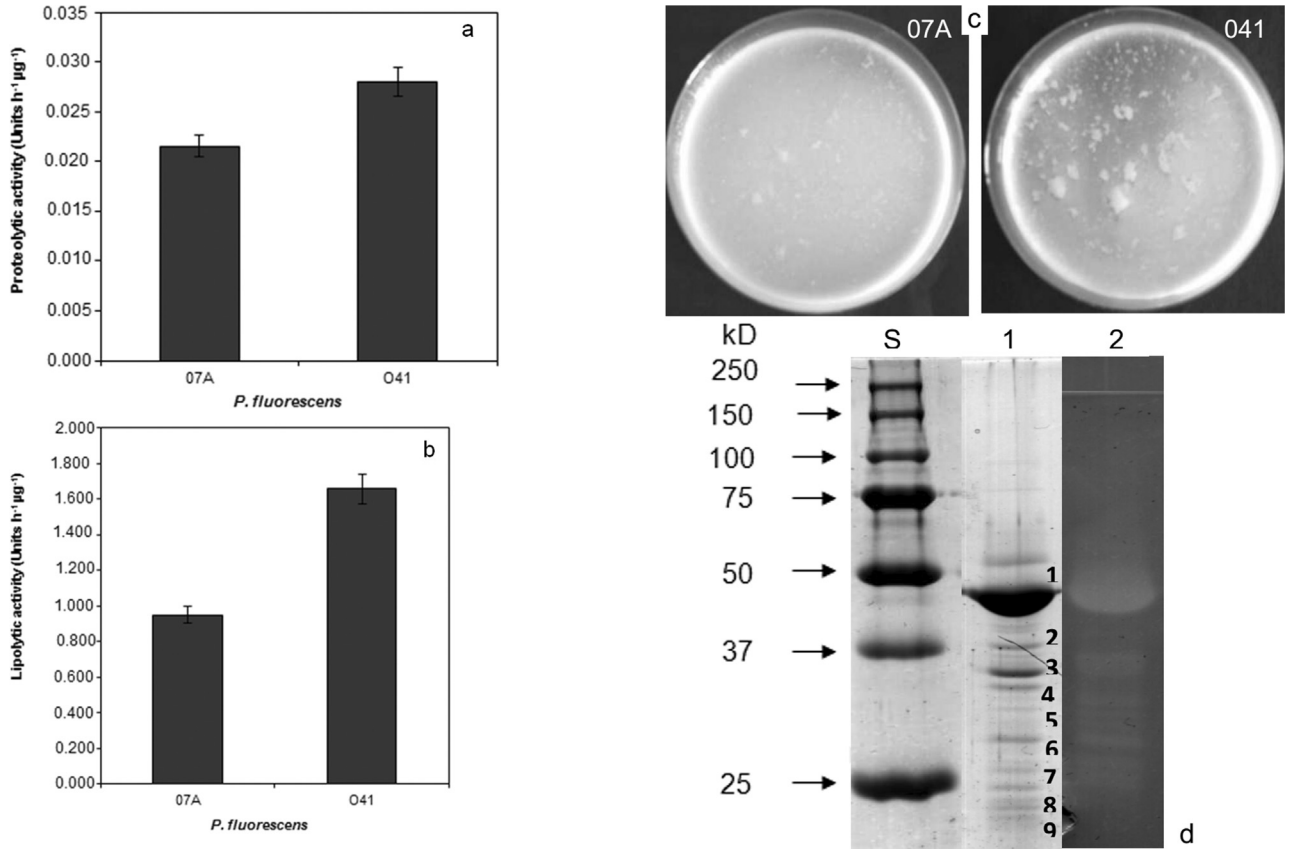

Figure 1 - Production of extracellular hydrolytic enzymes by *P. fluorescens*. A: Proteolytic activity in the supernatant of TYEP medium; B: Lipolytic activity in the supernatant of TYEP medium; C: Samples of reconstituted skin milk powder (12%) inoculated with *P. fluorescens* 07A and 041 after 18 h of incubation at 25 °C. Data represent the average of duplicate experiments; D: Coomassie-stained SDS-PAGE and azocasein zymogram on 12% PAA-gels visualizing protease production by *P. fluorescens* grown in TYEP medium supplemented with 0.25% $CaCl_2$. Lanes S: molar mass standard (Biorad); lane 1: SDS-PAGE of ammonium sulfate precipitated proteins of *P. fluorescens* 041 supernatant; lane 2: azocasein zymogram of ammonium sulfate precipitated proteins of *P. fluorescens* 041 after proteins precipitation with ammonium sulfate.

tion from 4 °C to 7 °C, and low activity in temperatures higher than 45 °C (Figure 3A).

The pH optimum of AprX is between 6.0 and 6.5 (Figure 3B). The protease still exhibits 36% residual activity at pH 4.0 and 62% at pH 9.0.

The protease activity was strongly decreased by pre-incubating the enzyme at different temperatures (Figure 3C); the residual activity of the protease after 60 min at 50, 60, 70, 80, 90 and 100 °C was between 2 and 4%. Inactivation of the metalloprotease at temperature and time conditions used during the pasteurization process were also evaluated: AprX showed 70% residual activity when it was treated at 75 °C for 20 s (HTST treatment: high temperature and short time) and 4% residual activity when it was incubated at 65 °C for 30 min (LTLT: low temperature and long time).

Proteolytic activity of AprX was strongly dependent on the presence of Ca^{2+}. However, other metal ions reduced the proteolytic activity (Table 3). The activity of AprX was decreased when 1 mM EDTA, an inhibitor that specifically acts on metalloproteases, was added to the reaction mixture, confirming the type of enzyme. In addition, AprX was strongly inhibited by denaturing and reducing agents such as SDS, dithiothreitol (DTT), β-mercaptoethanol, and urea (Table 4).

The alkaline metalloprotease was further tested for its capability to hydrolyze different substrates such as casein, bovine serum albumin, collagen, elastin, and gelatin. The highest activities were found on gelatin (100%) and casein (87.6%), followed by collagen (57%), elastin (41.2%), and bovine serum albumin (39.8%).

The temperature optimum of the purified lipase was 25 °C (Figure 4A). LipM showed a residual activity of 3.7% at 4 °C and exhibited low activities at temperatures higher than 37 °C (Figure 4A). Besides, LipM showed the highest lipase activity at pH 7.5 (Figure 4B). At pH values lower than 6.0 and higher than 11.0 only residual lipase activities could be detected (Figure 4B).

The lipase activity after 60 min of pre-incubation at 50, 60, 70, 80, 90 and 100 °C was nearly undetected (Figure 4C). The treatment of 65 °C for 30 min (LTLT), and 75 °C for 20 s (HTST), reduced the lipolytic activity to 13.2% and 25.4%, respectively.

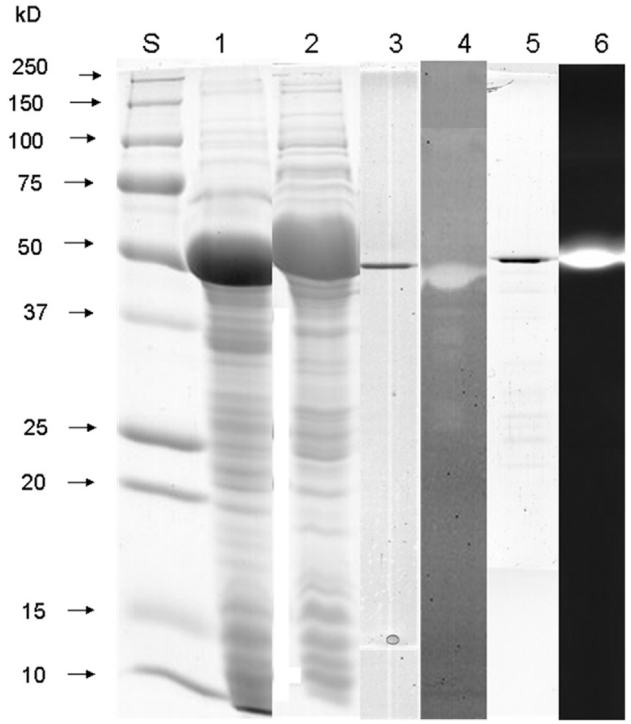

Figure 2 - Coomassie-stained SDS-PAGE and zymogram gel on 12% PAA-gels visualizing recombinant AprX and LipM. Lane S: molecular mass standard (Biorad); lane 1: SDS-PAGE of crude extract of *E. coli* XL1-Blue carrying pQE30-Xa-aprX-041; lane 2: SDS-PAGE of crude extract of *E. coli* XL1-Blue carrying pQE30-Xa-lipM-041; lane 3: SDS-PAGE of purified AprX; lane 4: azocasein zymogram of purified AprX; lane 5: SDS-PAGE of purified LipM; lane 6: MU-butyrate zymogram of purified LipM.

Lipase activity was dependent on Ca^{+2} ions in the renaturation buffer. The same was observed for the protease activity. The presence of ions other than Ca^{+2} reduced the lipolytic activity (Table 3). Among the tested substrates, LipM exhibited the highest activity for *p*-nitrophenyl palmitate (100%), followed by *p*-nitrophenyl butyrate (73%), *p*-nitrophenyl acetate (20%), and *p*-nitrophenyl phosphorylcholine (11%). The highest activity on substrates with long-chain fatty acid residues such as *p*-nitrophenyl palmitate indicates that the enzyme has esterolytic and lipolytic activities.

Discussion

Numerous *Pseudomonas* spp. have been shown to produce and secrete hydrolytic enzymes (McCarthy *et al.*, 2004; Burger *et al.*, 2000; Woods *et al.*, 2001; Pinto *et al.*, 2006; Pinto *et al.*, 2010; Liao and McCallus 1998; Koka and Weimer, 2000; Maunsell *et al.*, 2006; Mu *et al.*, 2009; Jankiewicz *et al.*, 2010; De Jonghe *et al.*, 2011). Interestingly, in this study it was verified that *aprX* encodes for the major, if not the only extracellular protease produced by *P. fluorescens* 041. Mass spectrometry analysis of low molecular weight bands that showed proteolytic activity on the azocasein zymogram were identified as degradation products of the metalloprotease AprX. These results are in agreement with the findings of Liao and McCallus (1998)

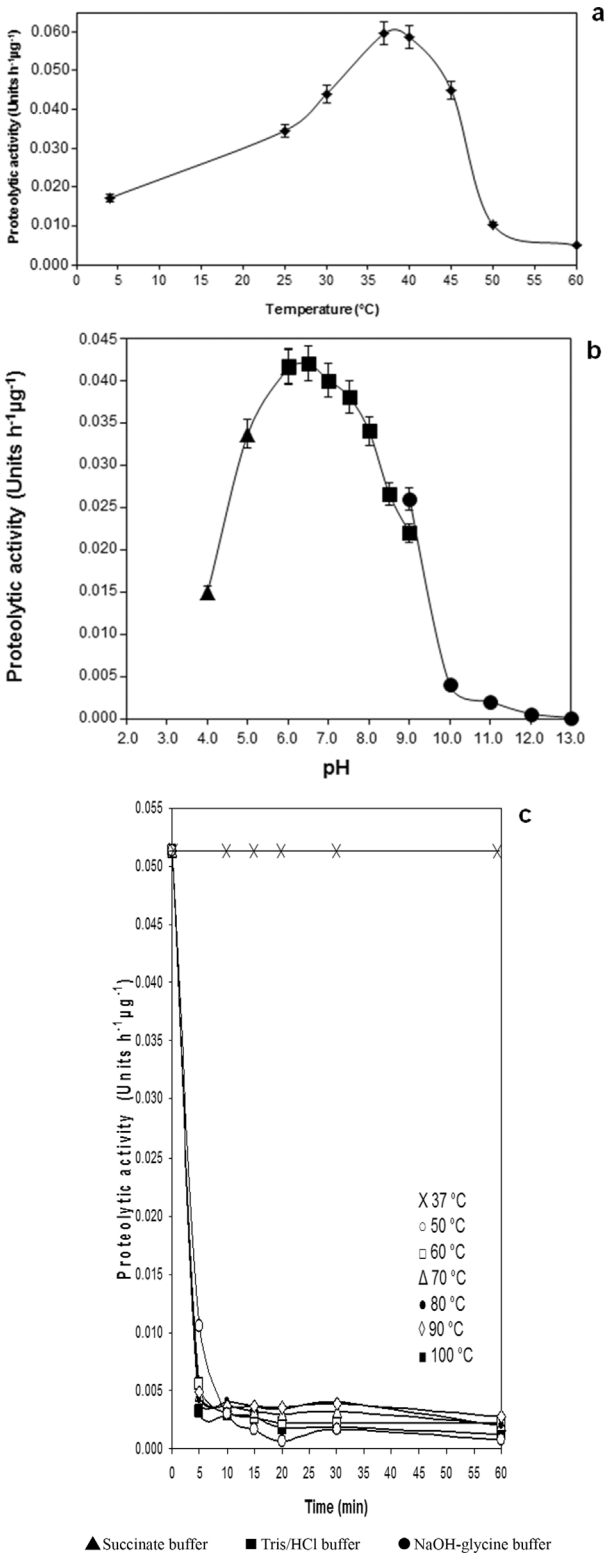

Figure 3 - Biochemical characteristics of AprX. A: Temperature optimum of purified AprX on azocasein; B: pH-optimum of purified AprX on azocasein. C: Effect of heat treatment on proteolytic activity of purified AprX. Data represent the average of duplicate experiments.

Table 3 - Effect of metal ions on the activities of alkaline metalloprotease and lipase.

Metal ion	Relative activity (%)	
	Alkaline metalloprotease[a]	Lipase[b]
None	100 ± 2	100 ± 2
Mn^{2+}	73 ± 1	61 ± 1
Co^{2+}	48 ± 3	59 ± 8
Zn^{2+}	86 ± 3	49 ± 0
Fe^{2+}	90 ± 5	48 ± 3
Fe^{3+}	102 ± 1	65 ± 2
Mg^{2+}	100 ± 1	50 ± 3

[a]A reaction mixture containing 250 µL of 2% (w/v) azocasein in 50 mM Tris/HCl (pH 8.0), 75 µL of AprX, and 1 mM of each metal ion was incubated at 37 °C for 12 h. The remaining activity was then measured, as described in the text. Results show the mean value (n = 3) plus or minus the standard deviation.
[b]A reaction mixture containing 1 mL of substrate (one volume of 0.3% (w/v) *p*-nitrophenyl palmitate in isopropanol and nine volumes 0.2% (w/v) sodium desoxycholate and 0.1% (w/v) gummi arabicum in 50 mM sodium phosphate buffer, pH 8.0), 50 µL of LipM, and 1 mM of each metal ions was incubated at 25 °C for 20 min. The remaining activity was then measured, as described in the text. Results show the mean value (n = 3) plus or minus the standard deviation.

Table 4 - Effect of inhibitors, denaturing and reducing agents on the activity of alkaline metalloprotease.

Compound	Relative activity (%)[c]
Inhibitor[a]	
None	100 ± 1
PMSF	95 ± 2
EDTA	51 ± 3
Pefabloc SC	89 ± 1
Denaturing and reducing agent[b]	
None	100 ± 2
SDS	6 ± 1
Urea	38 ± 5
DTT	24 ± 3
β-mercaptoethanol	44 ± 2

[a]A reaction mixture containing 250 µL of 2% (w/v) azocasein in 50 mM Tris/HCl (pH 8.0), 75 µL of AprX, and 1 mM of each inhibitor was incubated at 37 °C for 12 h. The remaining activity was then measured, as described in the text.
[b]A reaction mixture containing 250 µL of 2% (w/v) azocasein in 50 mM Tris/HCl (pH 8.0), 75 µL of AprX, and 2% (w/v) SDS, 4 M urea, 0.1% (w/v) DTT, or 0.1% (v/v) β-mercaptoethanol in 50 mM Tris/HCl (pH 8.0) was incubated at 37 °C for 12 h. The remaining activity was then measured, as described in the text.
[c]Results show the mean value (n = 3) plus or minus the standard deviation.

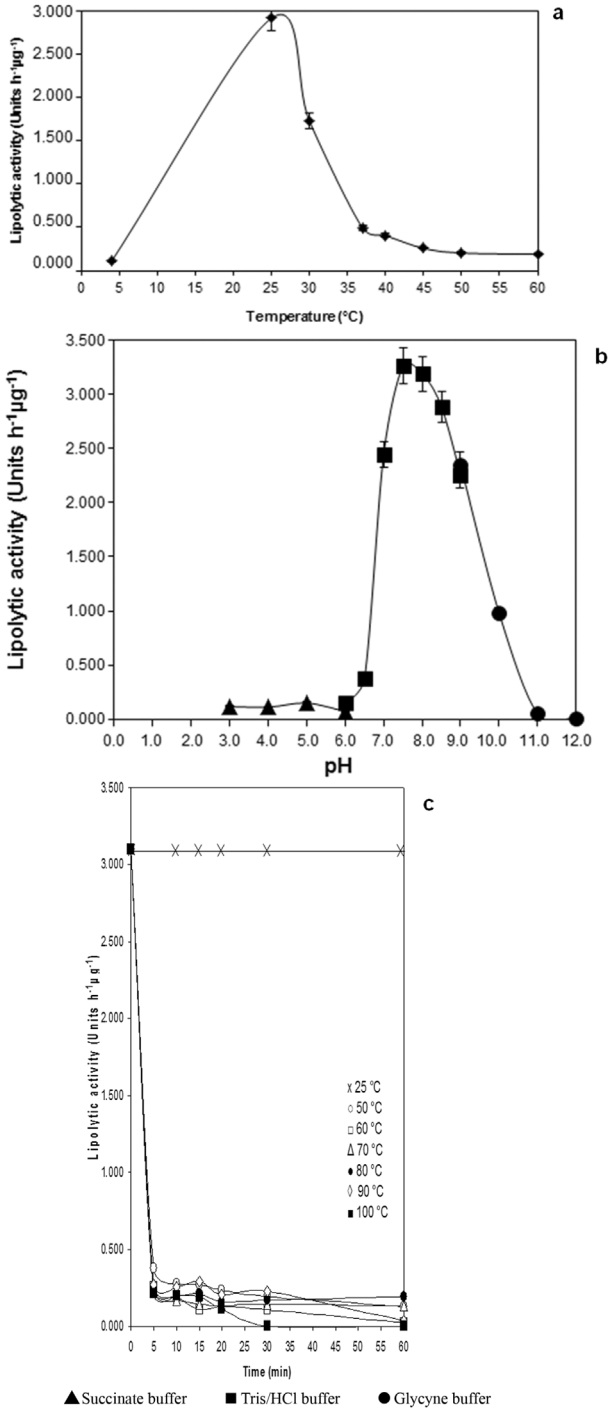

Figure 4 - Biochemical characteristics of LipM. A: Temperature optimum of purified LipM; B: pH-optimum of purified LipM. C: Effect of heat treatment for 60 min on lipolytic activity of purified LipM. Data represent the average of duplicate experiments.

who observed that *P. fluorescens* CY091 produces a unique extracellular 50 kDa protease, AprX. Our results are also in agreement with several other studies (Koka and Weimer, 2000; Mu *et al.*, 2009; Jankiewicz *et al.*, 2010). In contrast, Rajmohan *et al.* (2002) reported that another *P. fluorescens* isolated from milk produces five distinct proteases when they used the ultrafiltration technique to purify these enzymes. Nicodeme *et al.* (2005) observed the presence of more than one protease band for some strains of *Pseudomo-*

nas, while others produced just one protease, as revealed by a zymogram analyzes. According to Sørhaug and Stepaniak (1997) the number of secreted proteases depends strongly on the *P. fluorescens* strain. These findings highlight the great diversity of *P. fluorescens* isolates and reiterate the importance of studies aiming to elucidate the molecular mechanisms of the hydrolytic enzymes produced by these strains.

Once the AprX protein produced by strains of *P. fluorescens* isolated from raw milk showed high similarity with sequences from homologous enzymes in the database (Figure 5), it further confirms the possibility of using the *aprX* gene as a marker to detect *P. fluorescens* in milk by using PCR as described by Martins *et al.* (2005) and Machado *et al.* (2013). This approach would reduce the time for detecting these bacteria in raw milk giving flexibility for the dairy manager to choose the best use for a particular milk batch during processing.

Unlike many proteolytic and lipolytic enzymes described in the literature (Makhzoum *et al.*, 1996; Liao and McCallus, 1998; Rajmohan *et al.*, 2002; Chen *et al.*, 2003; Kojima and Shimizu, 2003; Nornberg *et al.*, 2009; Baglinière *et al.*, 2013; Anbu 2014), the protease and lipase evaluated in this study were relatively more sensitive to heat treatment. This could be attributed due to differences in the enzymes structures or to differences in experimental procedures, as the above mentioned studies have used purified enzymes from culture supernatants and we have purified those from overexpressing *E. coli* strains. However, some authors (Teo *et al.*, 2003; Jing *et al.*, 2010) verified that His-tag did not affect the metalloprotease activities of some strains, indicating that the recombinant metalloprotease was in an active form. Affinity tags have become essential tools for the production of recombinant proteins in a wide variety of settings (Waugh, 2011).

As the heat treatment and refrigeration processes adopted by the dairy industry during milk processing and storage do not fully inhibit enzymatic activity nor the growth of psychrotrophic bacteria, it is important to produce milk under stringent good manufacturing practices to limit contamination and bacterial spoilage.

Although LipM exhibits the conserved serine lipase catalytic domain, it presented somewhat lower similarity, as compared to AprX alignment, to the sequences described in the data base (Figure 6). LipM was also less heat stable than some lipases described by other authors (Knaut, 1978; Cousin, 1982; Makhzoum *et al.*, 1996; Boran and Ugur, 2013; Anbu, 2014), although low heat stability has also been observed (Chakraborty and Paulraj, 2009; Dahiya *et al.*, 2010). According to Cousin (1982), complete inactiva-

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AprX041    MSKVKDKAIV SAAQASTAYS QIDSFSHLYD RGGNLTVNGK PSYTVDQAAT QLLRDGAAYR   60
AY298902   MSKVKDKAIV SAAQASTAYS QIDSFSHLYD RGGNLTVNGK PSYTVDQAAT QLLRDGAAYR
DQ146945   MSKVKDKAIV SAAQASTAYS QIDSFSHLYD RGGNLTVNGK PSYTVDQAAT QLLRDGAAYR

AprX041    DFDGNGKIDL TYTFLTSATQ STMNKHGISG FSQFNTQQKA QAALAMQSWA DVANVTFTEK   120
AY298902   DFDGNGKIDL TYTFLTSATQ STMNKHGISG FSQFNTQQKA QAALAMQSWA DVANVTFTEK
DQ146945   DFDGNGKIDL TYTFLTSATQ STMNKHGISG FSQFNTQQKA QAALAMQSWA DVANVTFTEK

AprX041    ASGGDGHMTF GNYSGGQDGA AAFAYLPGTG AGYDGTSWYL TNNSYTPNKT PDLNNYGRQT   180
AY298902   ASGGDGHMTF GNYSSGQDGA AAFAYLPGTG AGYDGTSWYL TNNSYTPNKT PDLNNYGRQT
DQ146945   ASGGDGHMTF GNYSSGQDGA AAFAYLPGTG AGYDGTSWYL TNNSYTPNKT PDLNNYGRQT

AprX041    LTHEIGHTLG LAHPGDYNAG NGNPTYNDAT YGQDTRGYSL MSYWSESNTN QNFSKGGVEA   240
AY298902   LTHEIGHTLG LAHPGDYNAG NGNPTYNDAT YGQDTRGYSL MSYWSESNTN QNFSKGGVEA
DQ146945   LTHEIGHTLG LAHPGDYNAG NGNPTYNDAT YGQDTRGYSL MSYWSESNTN QNFSKGGVEA

AprX041    YASGPLIDDI AAIQKLYGAN LSTRATDTTY GFNSNTGRDF LSATSNADKL VFSVWDGGGN   300
AY298902   YASGPLIDDI AAIQKLYGAN LSTRATDTTY GFNSNTGRDF LSASSNADKL VFSVWDGGGN
DQ146945   YASGPLIDDI AAIQKLYGAN LSTRATDTTY GFNSNTGRDF LSATSNADKL VFSVWDGGGN

AprX041    DTLDFSGFTQ NQKINLTATS FSDVGGLVGN VSIAKGVTIE NAFGGAGNDL IIGNQVANTI   360
AY298902   DTLDFSGFTQ NQKINLTATS FSDVGGLVGN VSIAKGVTIE NAFGGSGNDL IIGNQVANTI
DQ146945   DTLDFSGFTQ NQKINLTATS FSDVGGLVGN VSIAKGVTIE NAFGGSGNDL IIGNQVANTI

AprX041    KGGAGNDLIY GGGGADQLWG GAGSDTFVYG ASSDSKPGAA DKIFDFTSGS DKIDLSGITK   420
AY298902   KGGAGNDLIY GGGGADQLWG GTGSDTFVYG ASSDSRPGAA DKIFDFTSGS DKIDLSGITK
DQ146945   KGGAGNDLIY GGGGADQLWG GTGSDTFVYG ASSDSRPGAA DKIFDFTSGS DKIDLSGITK

AprX041    GAGVTFVNAF TGHAGDAVLS YASGTNLGTL AVDFSGHGVA DFLVTTVGQA AASDIVA      477
AY298902   GAGVTFVNAF TGHAGDAVLT YASGTNLGTL AVDFSGHGVA DFLVTTVGQA AASDIVA
DQ146945   GAGVTFVNAF TGHAGDAVLT YASGTNLGTL AVDFSGHGVA DFLVTTVGQA AASDIVA
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Figure 5 - Multiple sequence alignment of deduced protease AprX from *P. fluorescens* 041 (this study), *P. fluorescens* A506 (Genbank accession number AY298902), and *P. fluorescens* strain F (Genbank accession number DQ146945). The differences in amino acid residues are indicated by gray shading, and the catalytic domain of neutral zinc metalloprotease is underlined. Boxed residues are thought to participate in Calcium binding.

Figure 6 - Multiple sequence alignment of deduced lipase LipM from *P. fluorescens* 041 (this study), Lip (Genbank accession number DQ305493), Lip68 (Genbank accession number AY694785), and LipA (Genbank accession number AF216702) from *P. fluorescens*. The differences in amino acid residues are indicated by gray shading and the catalytic domain of serine lipase is underlined.

tion of lipases was only obtained by autoclaving milk at 121 °C for 15 min. Knaut (1978) observed that lipases from *P. fluorescens* species were stable even above 100 °C. A heat-treatment of 98 °C for 14 to 25 min was necessary to inactivate lipases from some *Pseudomonas* species, including *P. fluorescens* and *P. fragi* (Cousin, 1982).

Overall, the biochemical properties of the purified protease and lipase from this work were similar to those found for proteases and lipases of other *P. fluorescens* strains isolated from raw milk (Makhzoum *et al.*, 1996; Kim *et al.*, 1997; Schokker and van Boekel, 1997; Liao and McCallus, 1998; Rajmohan *et al.*, 2002; Chen *et al.*, 2003; Kojima and Shimizu, 2003; Dufour *et al.*, 2008; Correa *et al.*, 2011; Baglinière *et al.*, 2013). It is worth to mention that AprX still exhibited 36% residual activity at pH 4.0, so if present in milk, this enzyme would not only affect the quality of pasteurized milk products but also of fermented products such as yogurt and cheese.

Surprisingly, no lipolytic activity could be detected when the renaturated SDS-PAGE was overlaid with the lipase substrate methylumbeliferyl-butyrate (results not shown). Probably this occurred due to the degradation of the enzyme by proteases or because Ca^{+2} was not added into the renaturation buffer, and the lipase may need this ion for correct folding.

The purification of AprX and LipM was important for the characterization of these spoilage enzymes and it would be interesting to use them to develop tools for improving their detection in milk. Nowadays, there is a great need for developing fast and reliable methods to detect spoilage enzymes directly from samples in order to determine the quality of milk that arrives at the dairy industry platform (Datta and Deeth, 2001). Most approaches currently available are time consuming, do not have good sensitivity or have detection limits that are too high. Besides characterizing these spoilage enzymes, it is important to estimate the extent of degradation of milk components, and thus further improve enzymatic methods to access milk quality.

In this work a protease and a lipase produced by *P. fluorescens* 041, a highly milk spoilage strain, isolated

from cooled raw milk were purified and characterized. The study showed that both enzymes presented similar biochemical properties to other enzymes from *P. fluorescens* strains isolated from raw milk. The differences that were observed could be accounted for the experimental procedures, especially the use of overexpressed recombinant proteins. The study confirms the spoilage potential of strain 041 in milk due to, at least in part, these two enzymes. The work highlights the importance of studies of this kind with *P. fluorescens* strains, the major spoilage bacteria contaminating milk produced in Brazil (Martins *et al.*, 2005) which has a recent history on the implementation of the cold chain at the dairy farm.

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References

Anbu P (2014) Characterization of an extracellular lipase by *Pseudomonas koreensis* BK-l07 isolated from soil. Prep Biochem Biotechnol 44:266-280.

Baglinière *et al.* (2013) Proteolysis of ultra high temperature-treated casein micelles by AprX enzyme from *Pseudomonas fluorescens* F induces their destabilization. Int Dairy J 31:55-61.

Boran R, Ugur A (2010) Partial purification and characterization of the organic solvent-tolerant lipase produced by *Pseudomonas fluorescens* RB02-3 isolated from milk. Prep Biochem Biotechnol 40:229-241.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein dye binding. Anal Biochem 72:248-274.

BRASIL (2002) Ministério da Agricultura, Pecuária e Abastecimento. Departamento de Inspeção de Produtos de origem Animal. Instrução Normativa n° 51, de 18 de setembro de 2002. Aprova os Regulamentos Técnicos de Produção, Identidade e Qualidade do Leite tipo A, do Leite tipo B, do Leite tipo C, do Leite Pasteurizado e do Leite Cru Refrigerado e o Regulamento Técnico da Coleta de Leite Cru Refrigerado e seu Transporte a Granel. Diário Oficial da República Federativa do Brasil. Brasília, 20 set. 2002, Seção I, p.13.

BRASIL (2011) Ministério da Agricultura, Pecuária e Abastecimento. Departamento de Inspeção de Produtos de origem Animal. Instrução Normativa n°62, de 29 de dezembro de 2011. Aprova o Regulamento Técnico de Produção, Identidade e Qualidade do Leite tipo A, o Regulamento Técnico de Identidade e Qualidade de Leite Cru Refrigerado, Leite Pasteurizado e o Regulamento Técnico da Coleta de Leite Cru Refrigerado e seu Transporte a Granel. Diário Oficial da República Federativa do Brasil. Brasília, 30 dez. 2011, Seção I.

Bullock WO, Fernandez JM, Short, JM (1987) XL-1 Blue: a high efficiency plasmid transforming recA *Escherichia coli* strain with β-galactosidase selection. Bio Techniques 5:376-377.

Burger M *et al.* (2000) Temperature regulation of protease in *Pseudomonas fluorescens* LD107d2 by an ECF sigma factor and a transmembrane activator. Microbiology 146:3149-3155.

Chakraborty K, Paulraj R (2009) Purification and Biochemical Characterization of an Extracellular Lipase from *Pseudomonas fluorescens* MTCC 2421. J Agri Food Chem 57:3859-3866.

Chen L, Daniel RM, Coolbear, T (2003) Detection and impact of protease and lipase activities in milk and milk powders. Int Dairy J 13:255-275.

Christensen *et al.* (2003) Quorum-sensing-directed protein expression in *Serratia proteamaculans* B5a. Microbiology 149:471-483.

Correa APF, Daroit DJ, Velho RV, Brandelli A (2011) Hydrolytic potential of a psychrotrophic *Pseudomonas* isolated from refrigerated raw milk. Braz J Microbiol 42:1479-1484.

Cousin MA (1982) Presence and activity of psychrotrophic microorganisms in milk and dairy products. J Food Protect 45:172-207.

Datta N, Deeth HC (2001) Age gelation of UHT milk. Trans Inst Chem Eng 79:197-210.

Dahiya P, Arora P, Chaudhury A, Chand S, Dilbaghi D (2010) Characterization of an extracellular alkaline lipase from *Pseudomonas mendocina* M-37. J Basic Microbiol 50:420-426.

De Jonghe V *et al.* (2011) Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. Appl Environ Microbiol 77:460-70.

Decherni S, Benjelloun H, Lebeault JM (2005) Effect of modified atmospheres on the growth and extracellular enzyme activities of psychrotrophs in raw milk. Eng Life Sci 5:350-356.

Dogan B, Boor KJ (2003) Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. Appl Environ Microbiol 69:130-138.

Dufour *et al.* (2008) Molecular typing of industrial strains of *Pseudomonas* spp. isolated from milk and genetical and biochemical characterization of an extracellular protease produced by one of them. Int J Food Microbiol 125:188-196.

Fairbairn DJ, Law BA (1986) Proteinases of psychrotrophic bacteria: their production, properties, effects and control. J Dairy Res 53:139-77.

Griffiths MW, Philips JD, Muir DD (1981) Thermostability of proteases and lipases from a number of species of psychotrophic bacteria of dairy origin. J Appl Bacteriol 50:289-303.

Jankiewicz U, Szawlowska U, Sobanska (2010) Biochemical characterization of an alkaline metallopeptidase secreted by a *Pseudomonas fluorescens* isolated from soil. J Basic Microbiol 50:125-34.

Jing Y, Toubarro D, Hao Y, Simões N (2010) Cloning, characterisation and heterologous expression of an astacin metalloprotease, Sc-AST, from the entomoparasitic nematode *Steinernema carpocapsae*. Mol Biochem Parasit 174:101-108.

Jonghe *et al.* (2010) Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. App Env Microbiol 77:460-470.

Kim HJ *et al.* (1997) Purification and characterization of an extracellular metalloprotease from *Pseudomonas fluorescens*. J Biochem 121:82-88.

Knaut T (1978) Heat resistance of Pseudomonas lipases in milk. XX International Dairy Congress.

Kojima Y, Shimizu S (2003) Purification and characterization of the lipase from *Pseudomonas fluorescens* HU380. J Biosci Bioeng 96:219-26.

Koka R, Weimer BC (2000) Isolation and characterization of a protease from *Pseudomonas fluorescens* RO98. J Appl Microbiol 89:280-8.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Liao CH. McCallus, DE (1998) Biochemical and genetic characterization of an extracellular protease from *Pseudomonas fluorescens CY091.* Appl Environ Microbiol, 64:914-921.

Machado SG, Bazzolli, DMS, Vanetti MCD (2013) Development of a PCR method for detecting proteolytic psychrotrophic bacteria in raw milk. Int Dairy J 29:8-14.

Makhzoum A, Owusu-Apenten RK, Knapp JS (1996) Purification and properties of lipase from *Pseudomonas fluorescens* strain 2D. Int Dairy J 6:459-472.

Marchand *et al.* (2009) Heterogeneity of heat-resistant proteases from milk *Pseudomonas* species. Int J Food Microbiol 133:68-77.

Martins ML *et al.* (2005) Detection of the apr gene in proteolytic psychrotrophic bacteria isolated from refrigerated raw milk. Int J Food Microbiol 102:203-211.

Maunsell B, Adams C, O'Gara F (2006) Complex regulation of AprA metalloprotease in *Pseudomonas fluorescens* M114: evidence for the involvement of iron, the ECF sigma factor, PbrA and pseudobactin M114 siderophore. Microbiology 152:29-42.

McCarthy CN, Woods RG, Beacham IR (2004) Regulation of the aprX-lipA operon of *Pseudomonas fluorescens* B52: differential regulation of the proximal and distal genes, encoding protease and lipase, by *ompR-envZ.* FEMS Microbiol Lett 241:243-248.

Mu Z, Du M, Bai Y (2009) Purification and properties of a heat-stable enzyme of *Pseudomonas fluorescens* Rm12 from raw milk. Eur Food Res Technol 228:725-734.

Nicodeme *et al.* (2005) Extracellular protease activity of different *Pseudomonas* strains: dependence of proteolytic activity on culture conditions. J Appl Microbiol 99:641-8.

Nornber MFBL, Friedrich RSC, Weiss, RDN, Tondo EC, Brandelli, A (2009) Proteolytic activity among psychrotrophic bacteria isolated from refrigerated raw milk. Int J Dairy Technol 63:41-46.

Pinto CLO, Martins ML, Vanetti MCD (2006) Qualidade microbiológica de leite refrigerado e isolamento de bactérias psicrotróficas proteolíticas. Cienc Tecnol Aliment 26:1-7.

Pinto UM, Costa ED, Mantovani HC, Vanetti MCD (2010) The proteolytic activity of *Pseudomonas fluorescens* 07A isolated from milk is not regulated by quorum sensing signals. Braz J Microbiol 41:91-96.

Quigley *et al.* (2013) The complex microbiota of raw milk. FEMS Microbiol Rev 37:664-698.

Rajmohan S, Dodd CE, Waites, WM (2002) Enzymes from isolates of *Pseudomonas fluorescens* involved in food spoilage. J Appl Microbiol 93:205-13.

Riedel K, Carranza P, Gehrig P, Potthast F, Eberl L (2006) Towards the proteome of *Burkholderia cenocepacia* H111: setting up a 2-DE reference map. Proteomics 6:207-216.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor.

Schokker EP, van Boekel MAJS (1997) Production, purification and partial characterization of the extracellular proteinase from *Pseudomonas fluorescens* 22F. Int Dairy J 7:265-271.

Setyorini E, Takenaka S, Murakami S, Aoki K (2006) Purification and characterization of two novel halotolerant extracellular proteases from *Bacillus subtilis* strain FP-133. Biosci Biotechnol Biochem 70:433-40.

Sorhaug T, Stepaniak L (1997) Psychrotrophs and their enzymes in milk and dairy products: quality aspects. Trends Food Sci Tech 8:35-37.

Teo JWP, Zhang LH, Poh CL (2003) Cloning and characterization of a metalloprotease from *Vibrio harveyi* strain AP6. Gene 303:147-56.

Waugh DS (2011) An overview of enzymatic reagents for the removal of affinity tags. Protein Expres Purif 80:283-93.

Wiedmann M, Weilmeier D, Dineen SS, Ralyea R, Boor KJ (2000) Molecular and phenotypic characterization of *Pseudomonas* spp. isolated from milk. Appl Environ Microbiol 66:2085-95.

Woods RG, Burger M, Beven CA, Beacham IR (2001) The aprX-lipA operon of *Pseudomonas fluorescens* B52: a molecular analysis of metalloprotease and lipase production. Microbiology 147:345-54.

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