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HYDROLYTIC POTENTIAL OF A PSYCHROTROPHIC *PSEUDOMONAS* ISOLATED FROM REFRIGERATED RAW MILK

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ABSTRACT

The production of extracellular hydrolases by a psychrotrophic bacterium isolated from refrigerated raw milk, and identified as a *Pseudomonas* sp. belonging to the *Pseudomonas jenssenii* group, was studied. This bacterium produced proteolytic and lipolytic enzymes in all media investigated (skim milk, cheese whey, casein broth, and tryptone soy broth). High levels of α-glucosidase were produced in skim milk broth. Hydrolytic enzymes detected in skim milk broth are of particular concern, indicating that these enzymes could be produced by *Pseudomonas* sp. during the cold storage of raw milk, contributing to the spoilage problem in milk and dairy products.

Key words: *Pseudomonas*; hydrolases; protease; lipase; glycosidase

Food spoilage results in high economical impacts to the food industry, and microbial activity is the most common cause of this. The microbiota colonizing a particular food depends on the product characteristics and how it is processed and stored. Spoilage is most rapid in proteinaceous foods such as milk and dairy products, since these are highly nutritious, possess near-neutrality pH values and high moisture content, thus allowing the growth of a wide range of microorganisms (9,12). Milk normally acquires contaminating bacteria during milking, handling and processing (3).

The cold storage of raw milk and dairy products is a prerequisite for the dairy industry. Although such practice on farms and in processing plants reduces the spoilage by mesophilic microorganisms, it will not prevent deterioration by psychrotrophic microorganisms (12,26). Therefore, psychrotrophic microorganisms are predominant in raw milk microbiota during the storage, producing hydrolytic enzymes such as proteases and lipases which are responsible for many quality issues, defects and deterioration, resulting in the limited shelf-life of dairy products (3,20). Although psychrotrophs are usually destroyed by heat treatments, the extracellular enzymes produced by these microorganisms are usually thermostable, keeping their activities even after pasteurization and even ultra-high temperature (UHT) treatments (8,25). Enzymatic spoilage without concomitant bacterial growth is of special concern in UHT milks (26).

Pseudomonas spp. are the predominant psychrotrophic bacteria isolated from refrigerated raw milk, being among the major spoilage agents in the dairy industry (8,20). The aim of this study was to investigate the profile of extracellular

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hydrolases produced by a psychrotrophic pseudomonad isolated from refrigerated raw milk.

The strain 1A4R was isolated from refrigerated raw milk in Plate Count Agar (PCA; Mast Diagnostics, Merseyside, UK) after 7 days at 7°C (21), and selected by producing large clearing zones on skim milk agar (SMA) plates (24). Cultures routinely maintained on SMA at 4°C were subcultured periodically. For inoculum preparation, the isolate was inoculated on SMA plates and incubated at 30°C for 24 h. The cultures were gently scraped from the agar surface, added to a sterile solution of 0.85% (w/v) NaCl and mixed until a homogeneous suspension with $O.D._{600}$ of 0.5 was obtained.

Bacterial identification was conducted based on morphological and biochemical tests (11,14), and sequence of the 16S rDNA. DNA was extracted from overnight cultures using the Promega Wizard SV Genomic DNA kit (Promega Corp., Madison, WI, USA). The amplification of 16S rDNA by PCR with the specific primers 27f (5'-GAGTTTGATCCTGG CTCAG-3') and 1525r (5'-AGAAAGGAGGTGATCCAGCC-3') was conducted using a Mastercycler® Personal Eppendorf (Eppendorf AG, Hamburg, Germany). PCR product was sequenced in the ACTGene Laboratory (Centro de Biotecnologia, UFRGS, Porto Alegre, Brazil) using the automatic sequencer ABI-PRISM 3100 Genetic Analyzer armed with 50 cm capillaries and POP6 polymer (Applied Biosystems, Foster City, CA, USA). Sequencing data were collected using the software Data Collection v1.0.1 (Applied Biosystems). The BLAST algorithm was used to retrieve for homologous sequences in GenBank (http://www.ncbi.nlm. nih.gov) using the software CLUSTAL W (27). The 16S rDNA sequence obtained for *Pseudomonas* sp. 1A4R has been submitted to GenBank under the accession number HM173665.

The phenotypical characteristics indicate the isolate belongs to the genus *Pseudomonas* (11), being mostly equivalent to *Pseudomonas asplenii* and *Pseudomonas jenssenii* (28). The 16S rDNA sequencing data indicated that the strain was taxonomically close to *Pseudomonas* species. The sequence shared 99% of similarity with *Pseudomonas koreensis* (13), which clusters with *P. jenssenii* (28). According to these characteristics the isolate was identified as a *Pseudomonas* sp. belonging to the *P. jenssenii* group. Strains of the *P. jenssenni* cluster have not been associated to milk spoilage, which is frequently related to *Pseudomonas fluorescens*, *Pseudomonas putida* and *Pseudomonas fragi* as prevalent species (17,19,29).

Production of extracellular enzymes (protease, lipase and glycosidases) was carried out by submerged (shaked-flask) cultivations using casein (C10B, 10 g/L; C50B, 50 g/L), cheese whey powder (CWPB; 10 g/L) or UHT skim milk (SMB; 10 mL/L) as substrates in mineral medium (2 g/L KH_2PO_4, 7 g/L K_2HPO_4, 0.2 g/L $CaCl_2$ and 0.2 g/L $MgSO_4$). Tryptone soy broth (TSB) was also evaluated. The initial pH of the media was adjusted to 7.0 before sterilization. Erlenmeyer flasks containing 50 mL of medium were inoculated with 500 µL (1%, v/v) of a bacterial suspension and incubated at 30°C in an orbital shaker (125 rpm) for 48 h. After this period the culture media were centrifuged (10,000 × *g* for 10 min), and supernatants were utilized as enzyme source.

Extracellular protease activity was estimated using azocasein (Sigma, St. Louis, MO, USA) as substrate (24). One unit (U) of protease was defined as the amount of enzyme that caused an increase of 0.1 absorbance unit at the defined assay conditions.

Lipase activity was evaluated using *p*-nitrophenyl-palmitate (*p*NPP; Sigma) as substrate (3). Substrate solution was prepared with 1 mL of *p*NPP (0.4 mg/mL) dissolved in isopropanol and 9 mL of Tris-HCl buffer (100 mM; pH 7.0) containing Triton X-100 (0.04%, v/v) and arabic gum (0.11% w/v). The reaction (1 mL) contained 800 µL of substrate solution and 200 µL of enzyme sample. After incubation at 37 °C for 60 min, the release of *p*NP was determined at 405 nm. One unit (U) of lipase was defined as the amount of enzyme that released 1 µmol of *p*NP at the above conditions.

Activities of α-glucosidase, β-glucosidase, α-

galactosidase, β-galactosidase, N-acetyl-β-glucosaminidase and N-acetyl-β-galactosaminidase were evaluated using the respective *p*NP-derivatives (Sigma) as substrates (4). Release of *p*NP was determined by measuring the absorbance at 405 nm, and one unit (U) of glycosidase was defined as the amount of enzyme that released 1 μmol of *p*NP at the assay conditions.

The profile of extracellular enzymes (protease, lipase, glycosidases) produced by *Pseudomonas* sp. 1A4R during growth on different media is presented in Table 1. Preliminary results showed the proteolytic potential of strain 1A4R during growth on skim milk agar plates, where prominent clearing zones were observed (data not shown). Subsequently, protease production was investigated in submerged cultivations. Extracellular proteolytic activities of *Pseudomonas* 1A4R were higher on TSB, followed by CWPB, C50B, SMB and C10B (Table 1). During growth on casein broths, which contained this substrate as the only carbon and nitrogen source, protease production showed to be positively correlated with the casein content, suggesting that casein could act as an inducer of protease production in *Pseudomonas* 1A4R.

Table 1. Extracellular enzyme activities produced by *Pseudomonas* sp. during growth on different media†

Medium‡	Protease (U/mL)	Lipase (U/mL)	α-glucosidase§ (U/mL)	*N*-acetyl-β-glucosaminidase (U/mL)
C10B	66.3 ± 3.0 [a]	0.08 ± 0.01 [a]	0.06 ± 0.002 [a]	0.02 ± 0.01 [a]
C50B	94.5 ± 4.5 [b]	0.03 ± 0.003 [b]	0.03 ± 0.002 [b]	-
CWPB	161.8 ± 6.1 [c]	0.37 ± 0.02 [c]	-	-
TSB	178.5 ± 7.6 [d]	0.06 ± 0.01 [a]	-	-
SMB	67.9 ± 3.4 [a]	0.31 ± 0.02 [d]	0.67 ± 0.03 [c]	0.01 ± 0.01 [a]

† Values are expressed as mean ± standard deviation of three independent experiments.
‡ C10B: casein 10 g/L broth; C50B: casein 50 g/L broth; CWPB: cheese whey powder broth; TSB: tryptone soy broth; SMB: skim milk broth.
§ β-glucosidase, α-galactosidase, β-galactosidase and *N*-acetyl-β-galactosaminidase activities were not detected.
[a] Data were subjected to analysis of variance and, when appropriate, to Tukey's test to examine where the differences existed. Values were considered significantly different from each other when $P<0.05$.

Extracellular protease production was also evaluated by gelatin zymograms. Culture supernatants were electrophoresed on 16% polyacrylamide gels containing 1 mg/mL gelatin. After electrophoresis, gels were washed twice with 20 mM Tris-HCl buffer (pH 8.0) containing 2.5% (v/v) Triton X-100 for 30 min, and then with the same buffer without detergent for 60 min. After 12 h of incubation at 30°C in the last buffer, gels were stained with Coomassie Brillant Blue R-250 (Sigma) and then destained. Protease bands appear as clear zones on a blue background due to gelatin hydrolysis.

On zymogram analysis, *Pseudomonas* 1A4R showed to produce at least one proteolytic enzyme during growth on SMB, and at least two proteases on CWPB, TSB and casein broths (Fig. 1). Large activity zones on the zymogram were observed for proteases produced on CWPB and TSB, which may be a function of enzymes migrating in the native conformation, and/or the result of a larger amount of protease (Table 1). However, it is not clear if these proteolysis bands corresponded to different monomeric proteases or to aggregates or multimers (19). Rajmohan *et al*. (22) reported the production of five proteases by three different *Pseudomonas* strains, whereas Nicodème *et al*. (19) showed that *Pseudomonas* sp. LBSA1 isolated from raw milk produced only one extracellular protease. The majority of *Pseudomonas* species, and other psychrotrophs, produce a single type of monomeric (40-55 kDa) neutral metalloprotease, with optimum pH of 6.5-8.0 (3,20,26).

Proteolytic activities detected on skim milk broth are of particular concern, since these enzymes could be produced during the cold storage of raw milk and contribute to the

spoilage of milk after heat treatment. The casein content and pH of normal milk were shown to be suitable for the action of proteases produced by psychrotrophs isolated from raw milk (20,25). Actually, casein hydrolysis results in the destabilization of the casein micelle and the production of small peptides that contribute to bitter flavors in milk and milk products (6,12). Proteolysis is a main factor limiting the shelf-life of UHT milk due to flavor and texture changes, with the eventual formation of a gel. This process is of special economical importance since only trace levels of protease are required to cause gelation of UHT milk during storage (5).

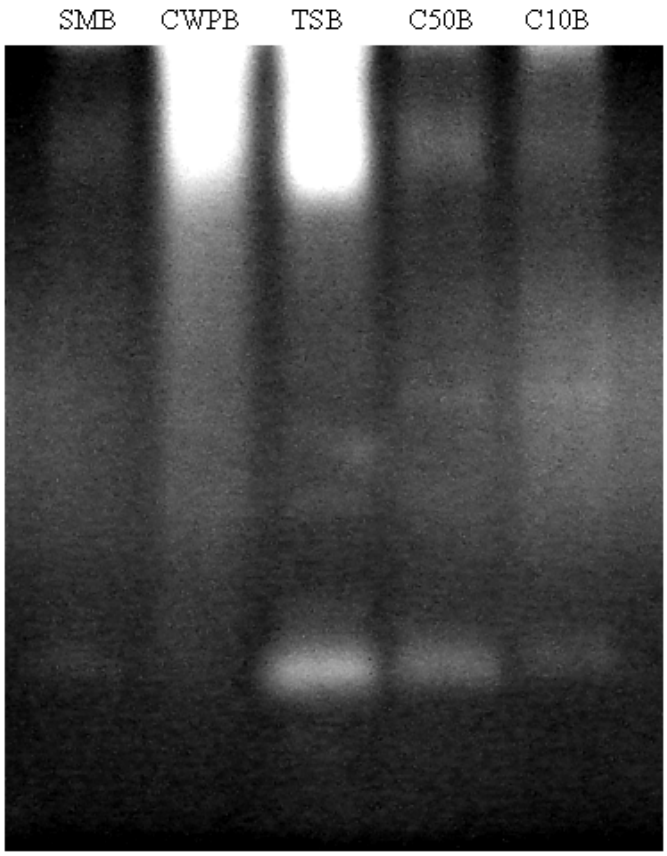

Figure 1. Gelatin zymogram of *Pseudomonas* sp. culture supernatants. This strain was grown on skim milk broth (SMB), cheese whey powder broth (CWPB), tryptone soy broth (TSB), casein 50 g/L broth (C50B) and casein 10 g/L broth (C10B).

Exploitation of *Pseudomonas* spp. as promising enzyme producers is a reality (10,15). As *Pseudomonas* 1A4R produced high protease levels in TSB and CWPB, investigations on the applications of such proteolytic enzymes are pertinent. In addition, the genus *Pseudomonas* is considered as an important lipase producer, being biotechnologically exploited (10). *Pseudomonas* 1A4R showed higher lipolytic activity on CWPB, followed by SMB. Milk is a good medium for lipase production, since the synthesis of such enzymes can be stimulated by lipids, such as milkfat (3). In this sense, lipase activity is generally higher in whole milk than in skimmed milk (6). Contrarily, low levels of lipase were produced on TSB and casein media (Table 1), indicating that the production of lipase is induced by lipids present in culture media.

In milk, lipase activity leads to spoilage through the hydrolysis of triglycerides, with the preferential release of medium and short-chain fatty acids. The hydrolysis of as little as l% of the milk triglycerides could lead to rancid off-flavors (3,12). Specifically, Dogan and Boor (7) reported that of 338 *Pseudomonas* spp. isolated from raw and pasteurized milk, 67% were lipase positive, and rancid flavors were associated with the presence of lipase-producing strains. As lipases can cause spoilage of milk and dairy products even at low concentrations (1), the production of such enzymes by *Pseudomonas* 1A4R might negatively affect the quality of milk and dairy products.

Among the extracellular glycosidases investigated, *Pseudomonas* 1A4R produced only high α-glucosidase activity in SMB medium. In the remaining culture media, glycosidases were produced only at low levels or were not produced at all (Table 1). Contrarily, the hydrolysis of *β*-anomers of glucose and galactose by psychrotrophic bacteria was reported to occur more rapidly than their respective α-anomers (15). Deeth *et al*. (6) observed the production of β-galactosidase by *Pseudomonas* spp. in both skim and whole milk. However, production of α-glycosidases was not studied by those authors. Marin *et al*. (16) reported that *P. fluorescens* was able to

release monosaccharides (such as glucose, galactose and *N*-acetylglucosamine) from glycoproteins of skim milk, washed-cream buttermilk and milk fat globule membrane (MFGM). The release of monosaccharides, observed during the storage of UHT milk, might indicate the presence of glycosidase activities produced by psychrotrophics (2,23). These extracellular enzymes might play an important role in the shelf-life of milk and dairy products, since the hydrolysis of glycoconjugates may render the non-carbohydrate portion of a molecule accessible to specific hydrolases (6,16).

Microbial contamination of raw milk is a crucial event determining the quality of the fluid milk and milk products. The main sources of bacterial contamination of raw milk are the interior of the udder, cows' teats, and the milking, storage and transporting equipments (3,18). As different bacterial strains yield different levels of enzymes with different stabilities, the identification of spoilage bacteria is important (3). The results obtained in SMB are of particular interest, since such enzymes might be produced by *Pseudomonas* 1A4R during the cold storage of raw milk, and subsequently act on the three major components of milk (proteins, lipids and carbohydrates), leading to a potential spoilage of this product and its derivatives.

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