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ORIGINAL PAPER

Protein and Amino Acid Solubilization using *Bacillus cereus*, *Bacillus velesensis*, and *Chryseobacterium* sp. from Chemical Extraction Protein Residue

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Abstract The exploitation of natural resources and increased environmental pollution have stressed the need for more valued use of residues generated by the fish processing plants, and species with low commercial value. Protein hydrolysis processes—whether chemical or enzymatic—generate insoluble proteins from bones, scales, and skin, which are not recovered and are often used as animal feed or disposed off into the environment. As an alternative, insoluble proteins could be converted in useful biomass protein concentrates or amino acids, by using microbial proteases. This work examines the solubilization of insoluble proteins discarded in the process of pH change in fish residues from Whitemouth croaker (*Micropogonias furnieri*), through the use of bacterial proteases. Temperature and pH conditions in the fermentations were adjusted for each microorganism and time was set at 96 h. Two substrates (acid and alkaline), three microorganism strains, and the substrate concentration used were examined. Among the three strains, *Bacillus velesensis* reached the higher proteolytic activity (47.56 U mL^{-1}), followed by *Chryseobacterium* sp. with 23.46 U mL^{-1}. *Bacillus cereus* (3.13 U mL^{-1}) showed low proteolytic activity. *B. velesensis* was the bacterium that presented better results with the analyzed substrates, achieving larger amount of soluble protein and free amino acids. The findings showed that these bacteria could be used to solubilize proteins from fish byproducts, which may be particularly useful to increase the yield of hydrolysis process or food formulations.

Keywords Bacterial proteases · Fish · Insoluble proteins · Solubilization

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Introduction

The use of a pH-change process to obtain fish muscle proteins may result in improved yields and increased added value to sea-based products (Gildberg et al. 2002; Perez-Mateos et al. 2004; Costa et al. 2005; Kristinsson and Liang 2006). During this process, the production of highly functional and stable protein out of byproducts from fish industrialization has received special attention. However, a considerable amount of insoluble proteins from bones, skin, connective tissue, cell membranes, and scales is generated. These insoluble materials could be hydrolyzed by specific proteases, resulting in peptides and amino acids that can be used for several purposes (Kristinsson and Liang 2006; Lempek et al. 2007). The use of protein isolates could be

increased with an assessment of all fractions after the solubilization of muscle proteins by finding new uses for the insoluble fraction (Kristinsson and Ingadottir 2006). Few studies were conducted on the evaluation of the insoluble portion. The process might have its value significantly enhanced if it is continued (Slizyte et al. 2005).

Fibrous proteins are abundantly available as byproducts of agroindustrial processing. Horns, hooves, feathers, nails, scales, thorns, and hair may be converted in useful biomass, such as protein and amino acid concentrates, through the use of microbial enzymes (Anwar and Saleemuddin 1998; Aurrekoetxea and Perera 2001; Hulse 2004). However, the current processes to obtain feather meal are expensive and also destroy certain amino acids, yielding a product with poor digestibility and variable nutrient quality (Wang and Parsons 1997). Indeed, it is recognized that digestive enzymes, such as trypsin and pepsin, digest feather protein poorly. The rupture of insoluble macromolecules, such as collagen, elastin, and keratin, depends on the microbial secretion of extracellular enzymes that have activity on the compact surface of those molecules (Aspmo et al. 2005; Riffel et al. 2007; Brandelli 2008).

Keratin is a structural protein found in feathers, scales, wool, and hair, which is abundantly distributed in nature. It is resistant to the degradation by common proteases, such as trypsin, pepsin, and papain (Papadopoulos et al. 1986; Shih 1993). This property is mainly associated to the molecular composition and conformation of the amino acids found in keratin, which are tightly packed in the α-helix or β-sheet into a supercoiled polypeptidic chain, because of the high degree of cross-linking by disulfide bonds, hydrogen bonding and hydrophobic interactions, keratin is insoluble and shows high mechanical stability and resistance to proteolysis (Parry and North 1998). However, keratin will not be present in nature, since it may be degraded by microorganisms (Onifade et al. 1998; Gupta and Ramnani 2006).

The hydrolysis of insoluble proteins is known among *Bacillus* spp. Many strains of *Bacillus licheniformis* and *Bacillus subtilis* are described as keratinolytic and/or collagenolytic (Lin et al. 1999; Suh and Lee 2001). Other species, like *Bacillus pumilus* and *Bacillus cereus* also produce keratinases (Kim et al. 2001; Werlang and Brandelli 2005). *Chryseobacterium* strains have been isolated from several ecosystems, such as water, soil, fish, marine environments, and clinical samples. Many strains of *Chryseobacterium* show high proteolytic activity (Jooste and Britz 1986; Yamaguchi and Yokoe 2000).

Collagen is the main organic constituent in bones. It is the major component of the extracellular matrix, a biomaterial that provides structural integrity for multicellular animals, through the formation of a supporting structure for their cells or organs. The molecular structure of collagen is recognized by its typical amino acid composition (Miller and Parker 1984; Luiten et al. 2003). The polypeptidic chains are composed of numerous, repeated tripeptide units of the Gly-Pro-X type, where X is often proline, modified after the translation to hydroxyproline (Vieille and Zeikus 2001; Watanabe 2004).

Considering the biotechnological potential of those microorganisms, the present work has the aim of evaluating the potential of *B. cereus*, *B. velesensis*, and *Chryseobacterium* sp. to hydrolyze insoluble proteins, byproducts from the production of Whitemouth croaker (*Micropogonias furnieri*) protein isolates.

Material and Methods

Raw Material

A fish specie was used, the Whitemouth croaker (*M. furnieri*), obtained at fish processing companies from the city of Rio Grande, in the state of Rio Grande do Sul. The fish was transported in ice-filled containers to the Food Technology Laboratory at Federal University of Rio Grande, where processing took place. Then, the fish was immediately washed with chlorinated water, beheaded, eviscerated, and filleted. The waste, such as visceras, bones, scales, skin, and head, were placed in plastic containers and stored frozen at −18 °C, pending use.

Insoluble Fish Substrate

Two kinds of insoluble residues were used as substrate, both resulting from the production of isolated fish protein through pH change. First, two processes of chemical solubilization (acid and alkaline) were conducted to obtain the protein isolate from wastes of fish. Samples were homogenized with distilled water (proportion 5:1, water–substrate). The reaction was conducted in a closed reactor, under stirring and controlled temperature. The alkalinizing agent was 1 mol l^{-1} NaOH and the acidifying agent was 1 mol l^{-1} HCl. Acid solubilization was performed under pH 2.5 for 20 min at 30 °C and alkaline solubilization was carried out at pH 12 for 20 min at 20 °C (Santos 2006). After solubilization, the substrate was centrifuged at 9,000×*g* for 15 min. During centrifugation, the samples were separated in three phases. The upper phase (neutral lipids) was discarded, the middle phase (soluble proteins) was subjected to isoeletric protein precipitation, and the bottom phase (insoluble proteins) was reserved for further processing. The insoluble proteins were dehydrated in a tray-dryer for 13 h at 50 °C. After that, it was ground in a knife-mill, in order to standardize the particle size as 1 mm, and then it is used in the fermentation.

Microorganisms

Bacterial strains designated as *Chryseobacterium* sp. strain kr6 (Riffel et al. 2003) and *B. cereus* strain kr16 (Werlang and Brandelli 2005) were isolated from feather waste and *B. velesensis* strain P11 (Giongo et al. 2007) was isolated from aquatic environment of the Brazilian Amazon basin (Manaus, Brazil). These microorganisms were chosen by their known keratinolytic activity that were tested before in feathers keratin. The bacteria were maintained at 4 °C in feather meal agar plates (Sangali and Brandelli 2000).

Fermentative Process

The strains were cultivated in a mineral salt medium (0.5 g L^{-1} NaCl, 0.4 g L^{-1} KH_2PO_4, 0.015 g L^{-1} $CaCl_2$ $2H_2O$), containing different concentrations of acid and alkaline substrate, according to the experimental design (see below). The process was carried out under aseptic conditions in 250 mL erlenmeyer flasks containing 100 mL of autoclaved medium and initial pH adjusted to 8.0 for kr6 and 7.0 for kr16 and P11. Subsequently, 1 mL of the starter culture was aseptically transferred to 100 mL of medium, which supply 10^6 ufc mL^{-1}. The flasks were incubated at 30 °C (kr6) and 37 °C (kr16 and P11) in an orbital shaker at 150 rpm for 96 h.

Cellular Concentration

The cellular concentration was monitored by measuring the colony forming units (cfu) mL^{-1}, as described elsewhere (Sangali and Brandelli 2000). The bacterial suspension was diluted to 10^{-5} in phosphate-buffered saline, 100 μL of sample in 900 μL of NaCl 0.85%. The samples were then homogenized and loaded (20 μl) in triplicate onto nutrient tryptic soy agar plates, which were incubated for 48 h at 37 °C.

Enzyme Activity

Enzyme activity was measured as described elsewhere (Thys et al. 2004), using azocasein as substrate. The enzyme solution (120 μL) was added to 480 μL of azocasein solution (10 mg mL^{-1}) in reaction 25 mmol L^{-1} tris HCl buffer pH 8. The mixture was incubated at 45 °C for 40 min and the reaction was stopped by adding 600 μL of 10% (*w*/*v*) trichloroacetic acid and leaving the preparation on 10 °C for 10 min. The mixture was then centrifuged at 10,000×*g* for 5 min and 800 μL of the supernatant was added to 200 μL of 1.8 mol L^{-1} NaOH. Absorbance at 420 nm was measured with a Hitachi U-1100 spectrophotometer (Hitachi, Tokyo, Japan). One unit of enzyme activity was defined as the amount of protein that resulted in an increase of absorbance at 420 nm of 0.01, under the assay conditions used.

Free Amino Acid Concentration

The free amino acid concentration was measured as described elsewhere (Moore 1968). Samples (100 μL) were added to 2 mL of 0.1 mol L^{-1} phosphate buffer pH 7.2, then 500 μL of these samples were mixed with 500 μL of 50 mg mL^{-1} ninhydrin. The mixture was incubated at 100 °C for 15 min followed by an ice bath to reach room temperature. Then, 5 mL of 50% (*v*/*v*) ethanol were added to the tubes. The analysis was performed in triplicate and the absorbance at 560 nm was measured in a Shimadzu UV/VIS mini-1240 spectrophotometer. A control was simultaneously prepared using distilled water (100 μL) instead of the sample. The standard curve was developed with glycine.

Soluble Protein

The concentration of soluble protein was determined by the Folin phenol reagent method (Lowry et al. 1951) with bovine serum albumin as a standard protein. Briefly, 200 μL of the sample were added to 2.5 mL of combined reagent (5 g L^{-1} $CuSO_4$ $5H_2O$ and 10 g L^{-1} $KNaC_4H_4O_6$ $4H_2O$ in 20 g L^{-1} $NaCO_3$) and incubated for 10 min at room temperature. Then, 300 μL of Folin-Ciocalteau's reagent were added with dilution ratio of 1:1 (Folin–water) and incubated for additional 30 min. The absorbance at 750 nm was measured in a Hitachi U-110 spectrophotometer. A control was simultaneously prepared using distilled water (200 μL) instead the sample.

Experimental Design

A mixed factorial experimental design ($3^2 \times 2^1$) was applied. The variables studied were substrate type, microorganism, and substrate concentration in the cultivation medium. Table 1 shows the factors assessed and their respective variations. All analyses were conducted as triplicates and at each 24 h along the fermentative process. For each analysis, the time with the most expressive response was distinguished and then the statistical analysis was performed. The software used was Statistic 6.0.

Results and Discussion

The highest cell concentrations were found for *Chryseobacterium* sp. in both substrates, acid (4×10^9 cfu mL^{-1}) and alkaline (3×10^9 cfu mL^{-1}). The acid substrate provided the highest concentrations, despite of the substrate concentration

Table 1 Factors evaluated and respectively levels of performed experiments

Variable	−1	Level 0	+1
Substrate	Acid	Not applied	Alkaline
Microorganism	*Chryseobacterium* sp. kr6	*Bacillus cereus* kr16	*Bacillus velesensis* P11
Substrate concentration (g L^{-1})	10	20	30

used, the *Chryseobacterium* sp. reached the concentration 10 g L^{-1} (2×10^9 ufc mL^{-1}), 20 g L^{-1} (4×10^9 ufc mL^{-1}) and 30 g L^{-1} (2.5×10^9 ufc mL^{-1}). *B. cereus* reached maximum values around 1×10^8 ufc mL^{-1}, after 96 h of cultivation, regardless of substrate concentration and substrate type. *B. velesensis* reached the best values at the concentration of substrate 20 g L^{-1}, alkaline (3.6×10^8 cfu mL^{-1}) and acid (1.3×10^8 cfu mL^{-1}) substrate, in the other concentrations the maximum values were around 4×10^7 cfu mL^{-1}.

Table 2 shows the description of factorial design experiments ($3^2\times2^1$) for soluble proteins, free amino acids, and proteolytic activity at time that they were studied.

Soluble Proteins

The soluble protein concentration was determined along the fermentation process (Fig. 1). It was verified that for the substrate resulting from alkaline hydrolysis, the highest concentration was reached at 24 h for most samples. For the substrate resulting from acid hydrolysis, the highest levels were observed at 48 h. This fact shows that the microorganisms were more able to solubilize proteins in alkaline substrate. After reaching the highest level of soluble protein, a reduction in its concentration was observed for both substrates. This reduction probably was caused by enzymes released in the medium that achieve protein consumption. However, in the acid substrate, it started to increase again at 96 h, whereas in the alkaline substrate, the amount of soluble protein tends to stabilize. These results show that probably the acid substrate still have protein insoluble, then hydrolysis keep going, and in the alkaline substrate all the protein insoluble was already hydrolyzed. The same occurred in a study with *B. cereus* cultivated at 30 °C in medium with feather meal at pH 7. The study showed that the production of soluble protein had an increase up to 48 h, and then became stable. The amount of soluble protein was around 0.6 mg mL^{-1} (Kim et al. 2001). The cultivation of *Chryseobacterium* sp. kr6 in a medium containing chicken feathers at pH 8 and 30 °C resulted in the highest concentration of soluble protein at 48 h (2.5 mg mL^{-1}), decreasing to 1.6 mg mL^{-1} at 192 h (Riffel et al. 2003).

The concentration of soluble protein was higher than the concentration of free amino acids in all fermentation steps. The bacterium P11 was the microorganism that reached the highest degree of protein solubilization, in comparison with the other two.

The factorial design analysis had three steps, using a comparison between two bacteria. Considering that the highest solubilization occurred in two distinct times, the analysis was set for 24 and 48 h. When comparing the behavior for kr6

Table 2 Results of factorial design experiments for soluble proteins and amino acids (mg mL^{-1}) and proteolytic activity (U mL^{-1})

Run	Substrate	Microorganism	Substrate concentration	Soluble proteins (24 h)	Soluble proteins (48 h)	Amino acids (48 h)	Proteolytic activity (72 h)
1	−1	−1	−1	0.293	0.695	0.046	12.883
2	−1	−1	0	0.504	1.123	0.082	6.383
3	−1	−1	+1	0.848	1.863	0.114	5.841
4	−1	0	−1	0.204	0.900	0.047	1.512
5	−1	0	0	0.874	1.378	0.092	1.991
6	−1	0	+1	1.576	1.851	0.114	2.416
7	−1	+1	−1	1.544	2.265	0.058	38.633
8	−1	+1	0	2.361	3.293	0.104	35.933
9	−1	+1	+1	3.000	3.631	0.131	27.316
10	+1	−1	−1	0.791	0.900	0.049	20.433
11	+1	−1	0	2.374	1.576	0.100	15.883
12	+1	−1	+1	1.665	2.859	0.122	18.750
13	+1	0	−1	1.334	1.270	0.045	0.695
14	+1	0	0	1.774	2.565	0.070	2.229
15	+1	0	+1	3.331	2.565	0.091	1.629
16	+1	+1	−1	1.065	0.925	0.048	1.858
17	+1	+1	0	2.406	2.208	0.081	1.441
18	+1	+1	+1	3.587	3.338	0.087	2.066

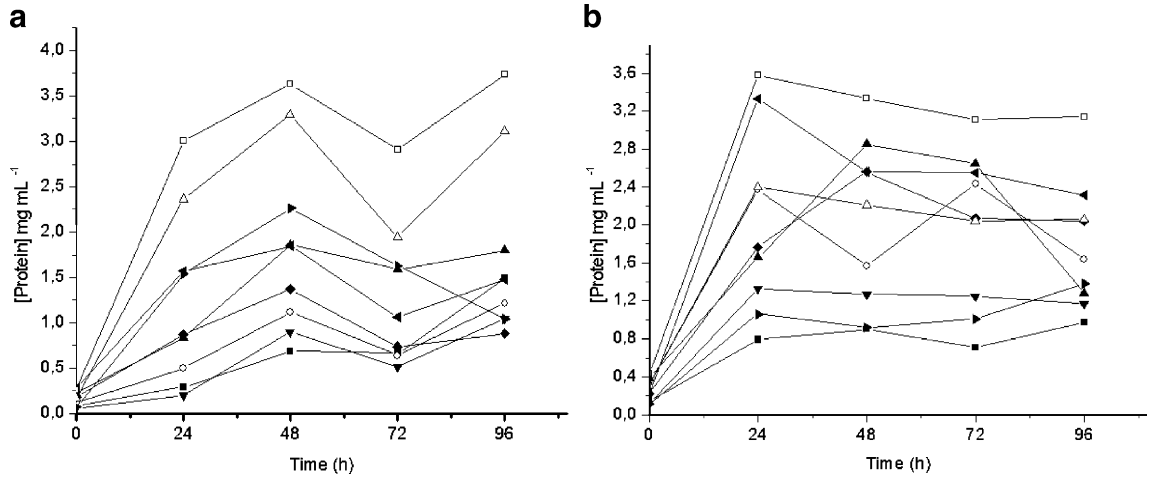

Fig. 1 Soluble protein concentration during the fermentation in both substrates **a** acid and **b** alkaline. *Filled square* kr6 (10 g L^{-1}); *unfilled circle* kr6 (20 g L^{-1}); *filled triangle* kr6 (30 g L^{-1}); *filled inverted triangle* kr16 (10 g L^{-1}); *filled diamond* kr16 (20 g L^{-1}); ◀ kr16 (30 g L^{-1}); ▶ P11 (10 g L^{-1}); *unfilled triangle* P11 (20 g L^{-1}); *unfilled square* P11 (30 g L^{-1})

and P11, the significant variables ($p<0.05$) were microorganisms and the substrate concentration used, for both time periods examined, showing the best results for P11 in the concentration of 30 g L^{-1}. When assessing kr6 and kr16, the significant variables ($p<0.05$) were substrate type and substrate concentration for the 48-h period. These bacteria presented very similar values of soluble protein. When analyzing kr16 and P11, it was found that for the 24-h period all variables were significant; for the 48-h period, only the microorganism and the substrate concentration were significant. After that analysis, it was found that the most important variables for protein solubilization were the microorganism used and the substrate concentration, showing that P11 was the strain with the best results, and the substrate concentration of 30 g L^{-1} was the most efficient.

Free Amino Acids

Figure 2 shows amino acid concentration along 96 h of fermentation for each bacterium studied. The highest concentration of free amino acids was observed with the substrate concentration of 30 g L^{-1} for the three bacteria, which was expected, as it has the highest amount of protein. Strains kr16 and P11 had the best results for the acid substrate (0.115 and 0.178 mg mL^{-1}, respectively). The strain kr6 reached 0.126 mg mL^{-1} in alkaline substrate. In most assays, a decrease in the amino acid concentration was found after reaching the highest level, probably due to the consumption of those amino acids as nutrients by the microorganisms. A study by Riffel et al. (2003) with kr6, using chicken feathers as substrate, found that the free amino acid concentration increased up to 48 h, and then stabilized, remaining at about 0.5 mg mL^{-1}. Acting upon the insoluble proteins of the fish protein isolate studied in the present work, kr6 showed a similar behavior to that previously observed (Riffel et al. 2003) although with a lower amount of free amino acids—0.12 mg mL^{-1}.

Since the highest concentration of free amino acids was reached at 48 h for most experiments, the factorial design statistical analysis was performed for this incubation time. The statistical analysis was performed by dividing the design in three parts, assessing two bacteria each time. All

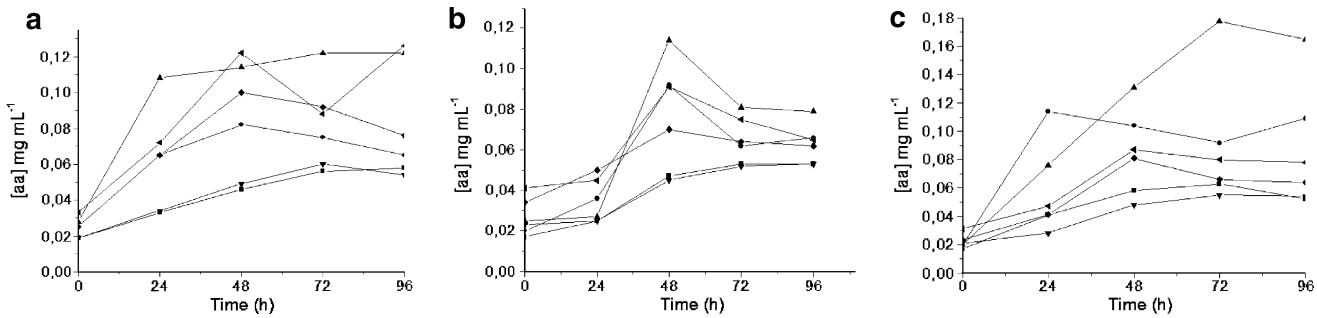

Fig. 2 Free amino acids concentration during the fermentative process for the bacteria, kr6 (**a**), kr16 (**b**), and P11 (**c**). *Filled square* acid (10 g L^{-1}); *filled circle* acid (20 g L^{-1}); *filled triangle* acid (30 g L^{-1}); *filled inverted triangle* alkaline (10 g L^{-1}); *filled diamond* alkaline (20 g L^{-1}); ◀ alkaline (30 g L^{-1})

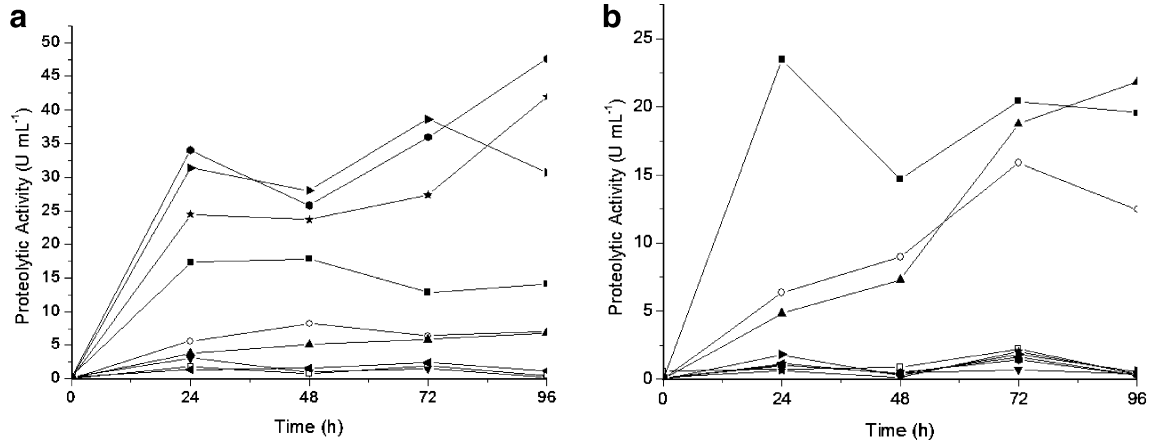

Fig. 3 Proteolytic activity in the acid (**a**) and alkaline (**b**) substrates, during the fermentative process with the different bacteria and concentrations. *Filled square* kr6 (10 g L^{-1}); *unfilled circle* kr6 (20 g L^{-1}); *filled triangle* kr6 (30 g L^{-1}); *filled inverted triangle* kr16 (10 g L^{-1}); *unfilled square* kr16 (20 g L^{-1}); ◀ kr16 (30 g L^{-1}); ▶ P11 (10 g L^{-1}); *filled circle* P11 (20 g L^{-1}); *filled star* P11 (30 g L^{-1})

analyses showed a significant difference ($p<0.05$) for the variable substrate concentration, despite the substrate type and microorganism used. The substrate type also presented a significant difference ($p<0.05$) between kr16 and P11, thus showing that the acid substrate provided a higher concentration of free amino acids. No significant differences ($p<0.05$) were found among the bacteria, as expected, since they had very close values (Fig. 2).

Proteolytic Activity

B. cereus (kr16) presented very low proteolytic activity when compared with the two other bacteria tested. Strains kr6 and P11 reached maximum values of 23 and 47 U mL^{-1}, respectively, whereas kr16 reached a maximum activity of 3 U mL^{-1}. Therefore, it was not assessed in the factorial design along with kr6 and P11. Study accomplished by Casarin et al. (2008) using fish meal and the bacteria *Chryseobacterium* sp. showed a very low proteolytic activity (1 U mL^{-1}).

Figure 3 shows the behavior of microorganisms for both acid and alkaline substrates. It can be seen that the highest proteolytic activity was reached when the acid substrate was used. Most experiments presented a reduction in proteolytic activity at 48 h, increasing again after this period. This pattern may suggest that the enzyme is inducible, i.e. substrate levels in the extracellular milieu regulate its secretion (Sangali and Brandelli 2000). These results resemble those observed for cultures of *Chryseobacterium* sp. (Riffel et al. 2003) and *Streptomyces* spp. (Sharma and Berwick 1991; Böckle et al. 1995) growing on keratin-rich substrates. The proteolytic activity reached the highest activity at 48 h, coinciding with the end of the exponential phase, then reducing and increasing again at 100 h. Protease production by *B. cereus* growing on chicken feathers showed a comparable trend as the production of soluble protein increased up to 48 h and then stabilized (Kim et al. 2001). The same behavior was found for *B. cereus* in our work. Esakkiraj et al. (2008) performed experiments using acid and alkaline hydrolysates of tuna waste for protease production,

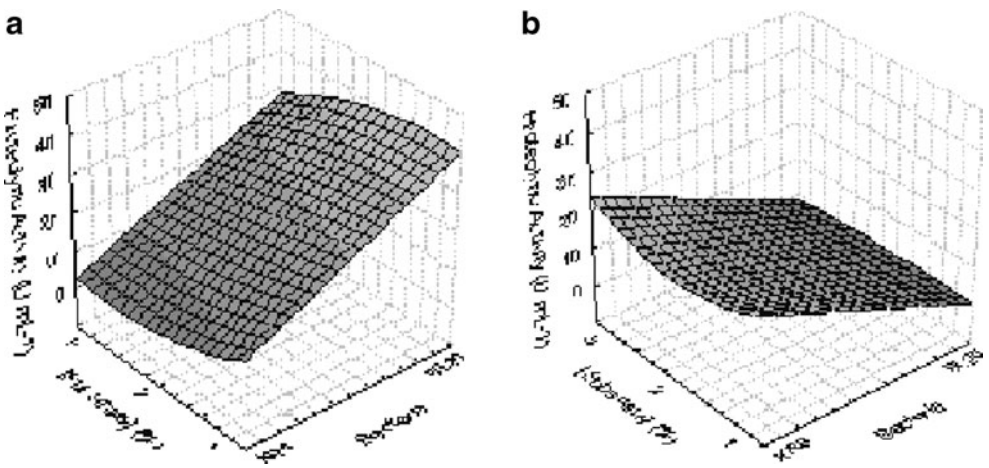

Fig. 4 Proteolytic activity on different substrate concentrations and bacteria in the acid (**a**) and alkaline (**b**) substrate at 72 h of fermentation

the results showed a protease activity of 60.37 and 65.96 U mL^{-1}, respectively. The maximum were attained at 48 h of fermentation and it decrease after. They obtained highest values, although we used just the insoluble protein resulting from the acid and alkaline hydrolysates and these authors performed the experiments with the entire protein hydrolysates.

Most proteolytic activity of kr6 and P11 was found to occur at 72 h; therefore, the mixed factorial experimental design $3^2 \times 2^1$ followed that time. The significant variables were the microorganism (p=0.045), substrate type (p= 0.008), and the interaction substrate/microorganism (p= 0.002). The microorganism behavior for the different substrate concentrations and acid and alkaline substrate types is shown in Fig. 4. Results show that in the studied conditions, most proteolytic activity for the acid substrate was reached by using *B. velesensis* (P11), in the substrate concentration of 10 g L^{-1}. On the other hand, for the alkaline substrate, the best results could be reached using *Chryseobacterium* sp. (kr6), also in the 10 g L^{-1} substrate concentration.

The substrate concentration not presented statistical significance for the proteolytic activity, this statement agree with Casarin et al. (2008) who studied different substrate concentrations, using the microorganism *Chryseobacterium* sp. on insoluble proteins. The variable less significant in the fermentative process was the substrate concentration.

Conclusion

The three bacteria used were capable of solubilize proteins and amino acids from scales, bones, skins, cell membranes, and cartilages, which are contained in the insoluble proteins discarded in the processes of acid and alkaline solubilization conducted with residues of Whitemouth croaker (*M. furnieri*) processing. The medium percentage of protein hydrolysated with these treatments regardless the bacteria used was between 50–60%.

B. velesensis (P11) was the bacterium that presented better results with the analyzed substrates, showing the largest proteolytic activities, and hence achieving larger amount of soluble protein and free amino acids.

Since no study has been found in the literature using residues from chemical hydrolysis of fish protein, three bacteria with keratinolytic activity were used to act upon the insoluble proteins. From the results found, additional investigation could be developed, such as the type of proteases produced during the fermentative process, considering that the type of material hydrolyzed provides evidence for keratinase and collagenase production. The amino acid composition of the resulting hydrolysates could also be studied.

Our laboratory is conducting similar research, examining ten different fungi in submerged fermentation, using the same fish residues as substrate.

The proteins, peptides, and amino acids recovered may be used to increase the yield in traditional processes of fish-based protein concentration. In addition, keratinolytic enzymes from bacteria may have important uses in biotechnological processes involving keratin-containing wastes from fish, poultry, and leather industries through the development of nonpolluting processes. Insoluble keratins can be converted after enzymatic hydrolysis to feedstuffs, fertilizers, glues, and films, used for the production of the rare amino acids serine, cysteine, and proline.

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