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Hydrolysis of Insoluble Fish Protein Residue from Whitemouth croaker (*Micropogonias furnieri*) by Fungi

Vilásia Guimarães Martins[1], Simone Canabarro Palezi[1], Jorge Alberto Vieira Costa[2] and Carlos Prentice[1*]

[1]Laboratório de Tecnologia de Alimentos; Escola de Química e Alimentos; Universidade Federal do Rio Grande; Rio Grande - RS - Brasil. [2]Laboratório de Engenharia Bioquímica; Escola de Química e Alimentos; Universidade Federal do Rio Grande; Rio Grande - RS - Brasil

ABSTRACT

A significant amount of insoluble fibrous protein, in the form of feather, hair, scales, skin and others are available as co-products of agro industrial processing. These wastes are rich in keratin and collagen. This study evaluated different fungi for the hydrolysis of insoluble fish protein residues. Proteins resulting from Micropogonias furnieri *wastes through pH-shifting process were dried and milled for fermentation for 96 h. This resulted the production of keratinolytic enzymes in the medium.* Trichoderma *sp. on alkaline substrate (28.99 U mL^{-1}) and* Penicillium *sp. on acidic substrate (31.20 U mL^{-1}) showed the highest proteolytic activities.* Penicillium *sp. showed the largest free amino acid solubilization (0.146 mg mL^{-1}) and* Fusarium *sp. the highest protein solubilization (6.17 mg mL^{-1}).*

Key words: fish waste, fungi, fibrous proteins, hydrolysis, *Whitemouth croaker*

INTRODUCTION

The exploitation of natural resources and increasing environmental pollution requires the need of a most valuable use of wastes generated from the processing plants and fish species of low commercial value. Generally, this material is converted into animal feed (Ferreira and Hultin 1994). The protein hydrolysis processes, either chemical, or enzymatic, generate insoluble proteins from the bones, skin and scales, which are not recovered and are often discharged to the environment (Guerard et al. 2002). However, the insoluble protein can be converted into useful biomass as proteins, or amino acids concentrated by microbial protease (Anwar and Saleemuddin 1998).

The fishing industry of Rio Grande (Brazil) processes a wide variety of species: however, only a portion is used as food for direct human consumption, the remaining constitutes a by-product rich in proteins and lipids that can be transformed into various useful products, such as surimi, or protein hydrolysates (Lempek et al. 2007). Whitemouth croaker (*Micropogonias furnieri*) is a fish of low commercial value, highly caught by fishing ships in southern Brazil, and has been industrialized by the fishing industries of Rio Grande, creating a large amount of residue (Martins et al. 2011).

The use of agro industrial wastes provides value to the industry and increases energy conservation and recycling conscious (Aguiar et al. 2010). These facts have stimulated the studies to convert the waste keratin products to value-added products. Although the keratin hydrolysis by microbial

*Author for correspondence: dqmprent@furg.br

enzymes has been described previously (Noval and Nickersen 1959), studies on keratinase have been increasing. However, the mechanism of keratin biodegradation by microorganisms remains unclear (Kunert and Stransky 1988; Kunert 1992; Böckle and Müller, 1997; Brandelli 2008). These studies have been focused on proteolytic microorganisms.

Collagen is an insoluble fibrous protein that makes up the majority of bone tissue, skin, and connective tissues. It has a spatial structure, and high molecular weight and insoluble in water. Keratins are insoluble structural proteins of scales, feathers and are known for their high stability. The keratin chain is well packed into α-helical, or β-helix in a polypeptide chain supercoiled (Kreplak et al. 2004), resulting in high mechanical stability and resistance to common proteolytic enzymes such as pepsin, trypsin, and papain (Jones et al. 1999).

Filamentous fungi synthesize a variety of hydrolytic enzymes. Many species are used in industrial process for the production of enzymes such as proteases, carbohydrases, and lipases. Friedrich et al. (1999) evaluated several fungal strains to identify active producers of keratinase for use in medical products and cosmestics, which included *Penicillium*, *Aspergillus*, *Fusarium* and *Trichoderma*. Keratinolytic enzymes are produced by fungi, bacteria and actinomycetes, which have been frequently isolated from the soils with keratin deposited materials (Santos et al. 1996; Kaul and Sumbali 1997). The strains of *A. fumigatus* and *A. flavus* producing keratinases have been described by Santos et al. (1996). Keratinases are included in a particular class of proteolytic enzymes that are able to degrade the keratinolytic substrates. The modification of the structure of keratin may be carried out by thermal, or enzymatic hydrolysis by reducing disulfide and peptide bonds (Williams et al. 1990, Kim et al. 2005). However, current processes for obtaining these residues are expensive and can destroy certain amino acids, yielding a product with a low digestibility and variable nutritional quality (Wang and Parsons 1997). Thus, the present study aimed to evaluate the proteolytic activity of ten fungal strains on insoluble substrates, resulting from a pH-shifting process carried out from the wastes of Whitemouth croaker (*Micropogonias furnieri*).

MATERIALS AND METHODS

Raw material

Whitemouth croaker (*Micropogonias furnieri*) was obtained from fish processing companies at Rio Grande, Southern Brazil. The fish was transported in ice-filled containers to the laboratory washed immediately with chlorinated water, beheaded, eviscerated, and filleted. The waste such as viscera, bones, scales, skin and head, were placed in plastic containers and stored frozen at -18°C.

Microorganisms

Ten different fungal strains were used: four strains of *Aspergillus* sp. (E7, E17, E19 and O5), two of *Fusarium* sp. (E1 and E5), two of *Penicillium* sp. (E12 and E20) and two of *Trichoderma* sp. (E13 and E18) isolated by Laboratory of Fermentation of University of Passo Fundo, Brazil. The strains were maintained at 4^{o}C on slant of potato-dextrose agar (PDA) medium with 1% (v/v) additional agar-agar.

Insoluble fish substrates

Two kinds of insoluble residues were used as substrates, both resulting from the production of fish protein isolate through pH shifting process. First, two processes of chemical solubilization (acid and alkaline) were conducted to obtain the protein isolate from wastes such as viscera, bones, scales, skin and head. Samples were homogenized in a mechanical stirring at 150 rpm for 5 min with distilled water (water:substrate, 5:1). 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 at pH 2.5 and 30^{o}C for 20 min and alkaline solubilization was carried out at pH 12.0 and 20ºC for 20 min (Martins et al. 2009). After solubilization, the substrate was centrifuged at 9000 x *g* for 15 min. During centrifugation, the samples were separated in three phases. The upper phase (neutral lipids) and the middle phase (soluble proteins) were discarded and the bottom phase (insoluble proteins) was reserved for further processing. The insoluble proteins were dehydrated in a tray-dryer for 13 h at 50ºC. After that, it was ground in a knife-mill in order to standardize the particle size as 1 mm; then, it was used in the fermentation.

Fermentative Process
Spores were scraped from the slopes into 5.0 mL of 0.2% (v/v) aqueous Tween 80 and 0.5 mL of the suspension transferred to each of two Roux flasks containing PDA and incubated at 30°C for five days. Spores were scraped off the PDA, suspended in 0.2% (v/v) aqueous Tween 80 solution and enumerated using a Neubauer counting chamber (Martins et al. 2006). During the submerged 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} $CaCl_2.2H_2O$) and 2% substrate. The fermentation was carried out under aseptic conditions in 250 mL erlenmeyer flasks containing 100 mL of autoclaved medium and the initial pH was adjusted to 4.5 using 1 x 10^6 spores mL^{-1} as inoculum. The flasks were incubated at 30°C in an orbital shaker at 100 rpm for 96 h. Samples were withdrawn each 24 h for pH measurement.

Proteolytic Activity
The samples were centrifuged at 10,000 x *g* for 5 min and enzyme activity was measured in the supernatant 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}) with 25 mmol L^{-1} tris HCl buffer (pH 8.0). 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 (TCA) and keeping at 10 °C for 10 min. The mixture was then centrifuged at 10,000 x *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 Biospectro Sp-22 spectrophotometer. A control was simultaneously run using distilled water. 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 2.0 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 5.0 mL of 50% (v/v) ethanol was added to the tubes. The analysis was performed in triplicate and the absorbance at 560 nm was measured in a Biospectro SP-22 spectrophotometer. A control was simultaneously run using distilled water (100 µL). 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 (BSA) as a standard protein. The absorbance at 750 nm was measured in a Biospectro SP-22 spectrophotometer. A control was simultaneously run using distilled water (200 µL).

Statistical Analysis
All the analyses were conducted in triplicates and at each 24 h along the fermentative process. The data were evaluated by the analysis of variance (ANOVA) using Tukey´s test.

RESULTS AND DISCUSSION

Changes in pH medium during the fermentation
The initial pH of the fermentative medium was adjusted to 4.5. In some runs the pH remained constant around pH 4.0 for 72 h and increased in the last 24 h: in some other cases a gradual increase occurred from the initial pH 4.5 up to the final range between 7.0 and 8.0. Sangali and Brandelli (2000) also observed an increase in the pH values during the cultivation on raw feather degradation, indicating its strong keratinolytic character. Organisms with higher keratinolytic activity alkalinize the medium to a greater extent than those exhibiting lower keratinolytic activity (Kaul and Sumbali 1997). This tendency to alkalinize the medium results from the production of ammonia due to deamination of peptides and amino acids originating from keratin degradation. The resulting increase of pH is typical of microorganisms growing on protein substrates (De Toni et al. 2002; Riffel et al. 2003; Gradisar et al. 2005). The pH increase during the fermentative process is an important indiciation of the keratinolytic potential of microorganisms (Kim et al. 2001). During the keratin degradation, the thiol groups are also released. The thiol formation is accomplished by the reduction of disulfide bonds by both enzymatic (disulfide redutase) and

chemical (sulfite and tiosulfate) mechanisms (Yamamura et al. 2002; Ramnani et al. 2005).

Evaluation of soluble protein during fermentative process

During the fermentative process the maximum substrates resulting from acid and alkaline hydrolysis was among 24, 48 and 72 h. After reaching their maximum, both substrates indicated a decrease in the concentration. This behavior suggested that the microorganisms consumed part of the soluble protein that was available in the medium. In some cases, the concentration of soluble protein increased again after the reduction. The same behavior was observed by Riffel et al. (2003), who evaluated the soluble protein of feather keratin, when the maximum was reached at 24 h and a reduction occurred afterwards. In the alkaline substrate *Fusarium* E5 (6.17 mg mL^{-1}) at 72 h and *Aspergillus* E7 (5.72 mg mL^{-1}) at 96 h resulted highest protein. In the acidic substrate, the maximum attained was 0.987 mg mL^{-1} for the strain O5 at 96 h. This was the only microorganism that obtained the maximum at this time; all the other strains reached the maximum in shorter times. Santos et al. (1996), working with *A. fumigatus* in a medium containing 1% of chicken feathers showed small amounts of soluble proteins with the maximum of 0.31 mg mL^{-1} at pH 7.2.

Martins et al. (2011) evaluated the capacity of some bacteria with potential keratinolytic for the solubilization of the protein present in the residues of acidic and alkaline hydrolysis of fish wastes, which were studied in similar conditions to the present study. *Bacillus velesensis* gave the maximum yield with the alkaline substrate (3.58 mg mL^{-1}) at 24 h with 3% substrate concentration. In the acidic substrate, the maximum yield was 3.29 mg mL^{-1} at 48 h with the same bacterium and substrate concentration. The results showed the good efficiency of these strains when compared to other bacteria on the same substrates. The soluble protein concentration was higher than the free amino acids concentration in the majority of the steps of cultivation process. *Aspergillus* O5 attained the maximum protein solubilization in the acidic substrate and *Fusarium* E5 in the alkaline substrate.

Solubilization of free amino acids

Figure 1 shows the solubilization of amino acids during the fermentative process for each evaluated fungus in both the substrates. In the alkaline substrate, the amino acids release started from the beginning, but in the acidic substrate, an effective release started just from 24 h into fermentation.

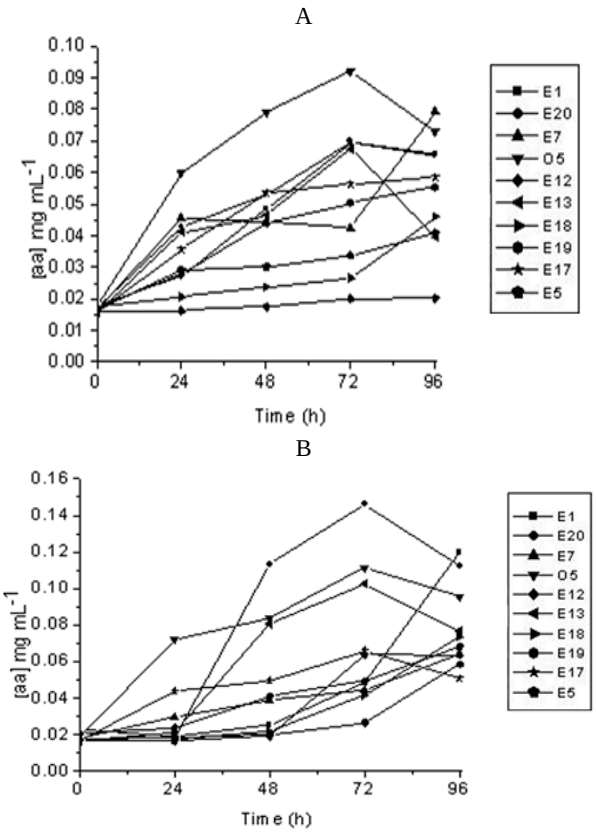

Figure 1 - Free amino acids concentration during the fermentative process (A) Alkaline substrate and (B) Acid substrate. E1 (*Fusarium* sp.), E20 (*Penicillium* sp.), E7 (*Aspergillus* sp.), O5 (*Aspergillus* sp.), E12 (*Penicillium* sp.), E13 (*Trichoderma* sp.), E18 (*Trichoderma* sp.), E19 (*Aspergillus* sp.), E17 (*Aspergillus* sp.) and E5 (*Fusarium* sp.).

In the alkaline substrate, the assay containing *Aspergillus* sp. O5 showed the highest soluble amino acids concentration (0.092 mg mL^{-1}) among the evaluated fungi at 72 h of fermentation. The minimal concentration (0.018 mg mL^{-1}) was obtained using *Penicillium* sp. E12 which remained the same during the whole process. Santos et al. (1996) evaluated *A. fumigatus* in a medium containing 1% of feather chicken at different pH, which reached maximum soluble amino acids of 0.55 mg mL^{-1}, which was higher than that obtained in this study. In the acidic substrate, a higher free amino acids concentration was reached when compared with the alkaline substrate. The maximum concentration was

obtained using *Fusarium* sp. E20 (0.146 mg mL^{-1}), followed by *Aspergillus* sp. O5 (0.111 mg mL^{-1}), both at 72 h. In the studies performed with bacteria on the same substrates as used in this study, similar results were obtained. The highest free amino acid concentration was obtained in the acidic substrate by *B. velesensis* (0.178 mg mL^{-1}), followed by the *B. cereus* (0.1146 mg mL^{-1}). In the alkaline substrate, the maximum was reached by *Chryseobacterium* sp. (0.1263 mg mL^{-1}) (Martins et al. 2011). The statistical analysis carried out at 72 h for both the substrates showed that the solubilization of free amino acids was significant (p<0.05) for all the strains with the exception of E1 and E5 for the acidic substrate and E1 and E20 for the alkaline substrate.

Proteolytic activity determination

Table 1 and 2 show the behavior of the microorganisms on both the substrates, all analyzes were performed in triplicate. It was observed that the highest proteolytic activity (31.20 U mL^{-1}) was reached using the acidic substrate by *Penicilium* sp. E20 at 72 h of the fermentative process. However, in general, the maximum activities were found in the alkaline substrate by *Trichoderma* sp. E13 (28.98 U mL^{-1}), *Aspergillus* sp. O5 (19.30 U mL^{-1}), *Fusarium* sp. E1 (16.34 U mL^{-1}) and *Penicilium* sp. E20 (14.73 U mL^{-1}). The other strains presented low proteolytic activity. In the acidic substrate, except strain E20, which reached the maximum activity (31.20 U mL^{-1}) at 72 h, the other two strains that presented highest activities were *Fusarium* sp. E1 and *Aspergillus* sp. O5, (5.36 and 5.90 U mL^{-1} respectively), although these activities were very low when compared with the E20. Notably, the strains that attained the maximum proteolytic activity in the acidic substrate were also the same that obtained the best results of enzyme production in the alkaline substrate.

Table 1 - Proteolytic activity in U mL^{-1} during the fermentative process on alkaline substrate.

Alkaline Substrate					
Fungi	**0**	**24 h**	**48 h**	**72 h**	**96 h**
E1	1.91±0.015	2.06±0.024	6.41±0.027	7.50±0.024	16.34±0.188
E20	1.19±0.012	7.30±0.040	5.24±0.186	5.77±0.210	14.73±0.204
E7	0.90±0.020	2.34±0.079	2.43±0.054	2.03±0.071	3.17±0.053
O5	1.13±0.072	6.06±0.095	6.07±0.030	5.31±0.016	19.30±0.027
E12	0.22±0.016	0.22±0.019	1.70±0.048	2.07±0.016	2.26±0.022
E13	1.40±0.025	4.68±0.160	14.07±0.030	28.99±0.041	19.18±0.082
E18	0.27±0.006	0.97±0.011	1.67±0.024	1.28±0.022	1.68±0.015
E19	0.62±0.019	1.80±0.029	1.84±0.029	2.61±0.029	3.16±0.079
E17	0.52±0.020	1.14±0.040	1.41±0.108	2.03±0.020	2.40±0.050
E5	1.12±0.015	1.29±0.034	3.16±0.054	3.84±0.016	6.14±0.002

Table 2 - Proteolytic activity in U mL^{-1} during the fermentative process on acid substrate.

Acid Substrate					
Fungi	**0**	**24 h**	**48 h**	**72 h**	**96 h**
E1	0.80±0.036	1.45±0.073	2.40±0.014	2.83±0.119	5.64±0.087
E20	0.41±0.005	0.74±0.027	26.80±0.037	31.20±0.056	25.27±0.074
E7	0.72±0.041	0.61±0.004	0.74±0.025	0.47±0.016	1.24±0.016
O5	0.60±0.021	3.04±0.039	6.72±0.090	4.68±0.005	5.90±0.049
E12	0.51±0.047	0.31±0.015	0.41±0.016	0.51±0.001	0.55±0.006
E13	0.47±0.017	1.15±0.037	3.08±0.042	5.06±0.108	1.72±0.039
E18	0.36±0.012	0.28±0.010	0.91±0.068	2.04±0.012	3.76±0.046
E19	0.84±0.027	0.70±0.012	0.97±0.073	2.06±0.055	3.66±0.026
E17	0.74±0.025	1.08±0.005	1.72±0.015	1.83±0.010	2.05±0.008
E5	0.54±0.010	0.70±0.012	0.70±0.039	1.06±0.062	1.12±0.015

Most of the experiments showed the maximum proteolytic activity in 96 h. This might have occurred due to the long adaptation phase of the fungi in the cultivation medium, considering that the medium of growth was not the same as fermentative medium. The statistical analysis performed at 96 h for the acidic and alkaline substrates showed that the proteolytic activity of the majority of strains in both the substrates presented statistical significance (p<0.05). In acidic substrate, only between E5-E7 and E18-E19, these were not significant (p>0.05), and in alkaline substrate, between E7-E19, O5-E13 and E12-E17 also were not significant (p>0.05).

Martins et al. (2011) used the same substrates, but with bacteria and observed the maximum proteolytic activity in 48 h, coinciding with the end of the exponential phase; then a decrease occurred and it increased again in 96 h. Similar result was shown by Riffel et al. (2003) who worked with *Chryseobacterium* sp. kr6 in a medium containing 1% feather chicken at pH 8.0 and 30^{o}C. The maximum activity was obtained in 48 h. However, in both the cases, the microorganisms used were maintained in a medium similar to the fermentative medium. Martins et al. (2011) reported the maximum activity for the acid substrate with *B. velesensis* and for alkaline substrate by *Chryseobacterium* sp., which were 47.56 and 15.88 U mL^{-1}, respectively. Jonsson and Martin (1965) evaluated different strains of fungi for proteases production and mentioned that the amount of proteases produced varied highly depending on the strain and the medium used. In order to obtain a higher production of commercial protease, it is essential to optimize the fermentative medium for better growth of the fungi and protease production.

The fungi used were able to produce enzymes to solubilize the proteins and amino acids from fibrous protein such as scales, bones, skin and cartilage from the acidic and alkaline hydrolysis performed with Withemouth croaker (*Micropogonias furnieri*) processing wastes. The proteins, peptides and amino acids resulting from these processes could be used to improve the efficiency of the hydrolysis process, increasing the amount of protein recuperated. They can be also used to raise the nutritional value of food stuffs. *Penicillium* sp. E20, *Trichoderma* sp. E13, *Aspergillus* sp. O5 and *Fusarium* sp. O1 were shown best producers of enzymes, consequently they were able to solubilize the highest amounts of protein and amino acids. Thus, it would be interesting to study other several fibrous substrates and these microorganisms, considering their keratinolytic and collagenolytic potential.

Although there is a wide range of studies discussing a great variety of uses of fish wastes, the use of microorganisms to hydrolyze insoluble protein from fish waste are rare. There are some works in this area but hydrolyzing chicken feather, nails and horn.

The most common use of the fish waste is to recover the components for animal feed, as silage fermentation, fish meal, and fish oil, etc. (Arvanitoyannis and Kassaveti 2008) as well as for the production of biodiesel/biogas (Lanari and Franci 1998); carotenoids extraction (Sachinda et al. 2006); enzymes extraction (Tavares et al. 1997); source of nitrogen for seaweed cultivation (Anderson et al. 1999); collagen isolate (Nagai and Suzuki 2000); protein hydrolysate (Bhaskar et al. 2008; Martins et al. 2009); chrome immobilization (Ozawa et al. 2003); lactic acid production (Gao et al. 2006); carbon sources (Damasio et al. 2011) and hydrolysis of collagen and keratin from bacterium (Martins et al. 2011) and fungi which were the goals of this study. The proteins, peptides, and amino acids recovered could be used to increase the yield in the traditional processes of fish based protein concentration.

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