African Journal of Biotechnology Vol. 7 (3), pp. 296-300, 5 February, 2008
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB07.404
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Production and partial characterization of keratinase produced by a microorganism isolated from poultry processing plant wastewater

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Accepted 16 October, 2007

A *Streptomyces* was isolated from poultry plant wastewater, showed high keratinolytic activity when cultured on feather meal medium. Optimum keratinolytic activity was observed at 40°C and pH 8.0. The enzyme also showed to be stable between 40 and 60°C. The keratinolytic activity was not inhibited by EDTA, DMSO and Tween 80. On the other hand, $CaCl_2$, $ZnCl_2$, and $BaCl_2$ slightly inhibited the keratinolytic activity. The *Streptomyces* isolated might be useful in leather, keratin waste treatment, animal feeding industry, and also cosmetic industry.

Key words: Keratinase, streptomyces, wastewater.

INTRODUCTION

The leather and fur plants as well as slaughterhouses throw away considerable amounts of materials containing keratin; such as wool, bristle, horns, feathers, hoof, etc. Until recent years, these materials along with other animal wastes were treated at high temperatures and then milled in order to produce the so-called “animal flour” and used as “protein supplement” into the feed mixtures of domestic animals. However, it was established that this flour is the carrier of the enigmatic cause (called prion) of some related disease (mad cow, swine fever, bovine spongiform encephalopathy, Creutzfeldt-Jakob disease, etc.). Nevertheless, these problems can be overcome by using incineration which is a reliable way of breaking the spread of prions (Gousterova et al., 2005).

Considering that these keratin wastes have high protein content, they could be used as a source of protein and amino acids for animal feed and any other applications. Keratin in its native state is not easily degraded even by other common proteolytic enzymes like trypsin, papain and pepsin, due to the composition and molecular configuration of constituent amino acids in addition to the disulphide bonds and also its insolubility. Current process such hydrothermal process can destroy certain amino acids producing a product with poor digestibility and also with variable nutrient quality (Wang and Parsons, 1977).

Microorganisms which produce specific proteases (keratinase) may have important use in biotechnological processes involving keratin-containing wastes from leather and poultry industries by developing non polluting processes (Onifade et al., 1998; Wang and Shih, 1999; Gradisar et al., 2000; Sangali and Brandelli, 2000; Kim et al., 2001; Allpress et al., 2002; Longshaw et al., 2002; Yamamura et al., 2002; Gessesse et al., 2003; Singh, 2003). A number of keratinolytic microorganisms have been reported, including some species of *Bacillus* (Atalo and Gashe, 1993; Willimas et al., 1990), *Actinomycetes* (Böckle et al., 1995; Young and Smith, 1975) and fungi (Kushawa, 1983; Santos et al., 1996).

In this report, we describe the isolation and characterization of a microorganism from poultry processing plant wastewater showing keratynolitic activity in Rio Claro, São Paulo Brazil.

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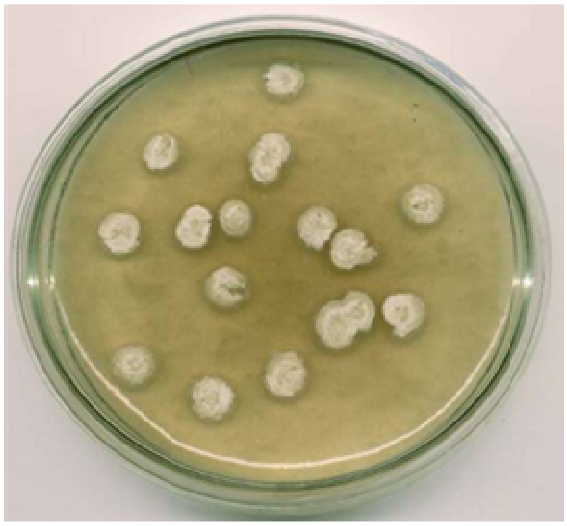

Figure 1. Colonies of the isolate on feather meal agar after incubation at 35°C and 48 h.

MATERIALS AND METHODS

Isolation of keratinolytic microorganism

Water samples were collected from different sites within a local poultry industry. The samples were flooded in saline solution 0.85%, suspension up to 10^{-5} were made and used to streak feather meal agar plates (10 gl^{-1} feather meal; 0.5 gl^{-1} NaCl; 0.3 gl^{-1} K_2HPO_4; 0.4 gl^{-1} KH_2PO_4; 0.1 gl^{-1} MgCl-$6H_2O$; 0.1 gl^{-1} yeast extract; 15 gl^{-1} agar, pH 7.5) which were incubated at 37°C for 1 ± 5 d.

Taxonomical studies and identification of the microorganism

The morphological, cultural and physiological characteristics of the isolated bacterium were compared with data from Bergey's Manual of Systematic Bacteriology (Brenner, 1984). The identification of the microorganism was performed through rRNA 16S sequence and compared to the sequences deposited at the GENBANK.

Growth determination

The optimal growth conditions of the isolate were determined using feather meal, crude feather meal, and crude feathers, incubated in an orbital shaker at 160 rev min^{-1}, in flasks containing 100 ml of the medium preconized by Williams et al. (1990) using the following temperatures; 30, 40, 50°C and pHs; 4.0, 5.0, 6.0, 7.0, 8.0, 9.0. Bacterial growth was monitored by measuring the colony forming units (cfu). Samples of 9 mL were withdrawn at each 24 h and diluted to 10^{-8} in saline solution (0.85%, m/v) and loaded (15 ml) in triplicate onto nutrient agar plates. Plates were incubated at 35°C for 24 h and counts done among 30 ± 100 colonies.

Enzyme assay

Keratynolitic activity was measured using a modified protocol by Bressollier et al. (1999), Lin et al. (1992) and Kim et al. (2001) using Keratin Azure (Sigma Aldrich, St. Quentin Fallavier, France) as substrate. The reaction mixture consisted of 800 µL of the enzyme solution with 3.2 mL of TRIS buffer (50 mmol), pH 8.5 and 0.4% Keratin Azure (Sigma Aldrich, St. Quentin Fallavier, France) were incubated at 50°C for 1 h, the reaction was stopped by adding 1.2 mL of TCA at a final concentration of 100 g l^{-1}, the samples were filtered and the absorbance of the supernatant was determined at 595 nm. One unit of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01 at 595 nm for 1 h at 50°C.

Effect of the pH and temperature on enzyme activity

The effect of the pH was studied by assaying the enzyme using McIlvaine buffers pH 4.0, 5.0 and 6.0; KH_2PO_4 / NaOH, pH 7.0; Tris–HCl, pH 8.0 and 9.0; $NaHCO_3$ / NaOH, pH 10.0. The effect of the temperature was measured by incubating the enzyme at temperatures ranging from 30 to 60°C. Thermostability was assessed by incubating the enzyme in the above temperature range in buffer pH 8.0 for 15 to 240 min at each temperature. The residual activity was compared to the enzyme activity determined at 40°C pH 8.0.

Effect of metal ions and inhibitors on enzyme activity

The enzyme activity was measured in the presence of 2, 5 and 10 mM of $MnCl_2.4H_2O$, ZnCl, HgCl, $BaCl.2H_2O$, $CuSO_4.5H_2O$, $MgCl_2$ and $CaCl_2$. The effect of inhibitors was measured by assaying the enzyme in the presence of 0.1 – 0.5% Triton X –100, 2, 5 and 10 mM EDTA, 0.1 – 0.5% Tween 80, 0.1 – 0.5% DMSO, 0.1 – 0.5% isopropanol, 0.1 – 0.5% mercaptoethanol at 50°C for 1 h.

RESULTS

Isolation of keratinolytic microorganism

After being incubated for 48 h, a plate containing feather meal and agar showed the growth of a single colony, which was re-isolated using the same culture medium, subsequently tested for keratinolytic activity, using the medium preconized by Williams et al. (1990) using commercial feather meal. Feather meal was necessary to induce keratinase production. Figure 1 shows colonies of the isolate on feather meal agar.

Taxonomical studies and identification of the microorganism

Table 1 showed morphological and biochemical characteristics of the isolate, the identification of the microorganism was performed through rRNA 16S sequence and compared to the sequences deposited at the GENBANK, which indicated that the isolate is a *Streptomyces*.

Growth determination

Maximum growth was observed at pH 8.0 and 40°C. These results are in good agreement to other growth determination. Figures 2 and 3 showed the effect of pH

Table 1. Morphological, cultural and physiological characteristics of keratinase-producing bacterial.

Morphological Characteristics	Inference
Form	Coccus
Gram Stain	Positive
Spore	Non Forming
Cultural Characteristics	
Feather Meal Agar	Undulate, moist, circular, yellowish
Physiological Characteristics	
Catalase	Positive
Oxidase	Negative
O-F	Negative
VP Test	Positive
TSI 24h	k/k red
O-F	Negative
Citrate	Negative
Indol	Negative
Nitrate Reduction	Positive
Gelatina Liquefaction	Positive
Lipase	Negative
Lisine Descarboxilase	Negative
Ornithine Descarboxilase	Negative
Arginine Dehidrolase	Positive
Motility	Negative
DNAse	Negative

and temperature on the growth of the isolate.

Enzyme assay

The enzyme assay carried out using Keratin Azure (Sigma Aldrich, St. Quentin Fallavier, France) as substrate is shown in Figures 4 and 5.

Effect of the pH and temperature on enzyme activity

The stability of the enzyme was observed between pH 8.0 and 9.0 (Figures 6 and 7), and temperature of 40 to 60°C.

Effect of metal ions and inhibitors on enzyme activity

The effects of metal ions on enzyme activity are shown on Table 2 and 3. The results showed that the enzyme was slightly reduced by the following metal ions; $CaCl_2$, $ZnCl_2$ EDTA and Triton X-100 mainly at 10 mM concentration. No significant changes were observed with the detergents and reducer agents.

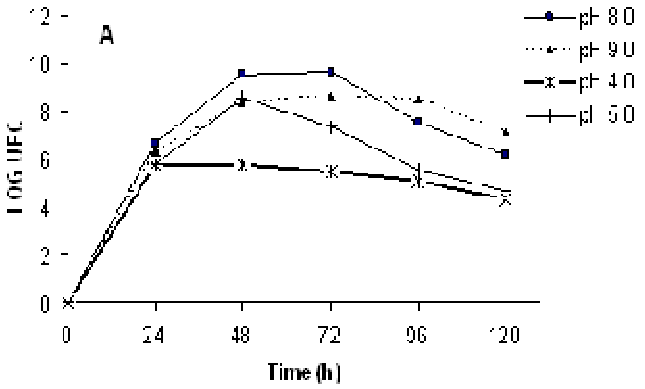

Figure 2. Growth curve *Streptomyces* at 30°C ad different pH.

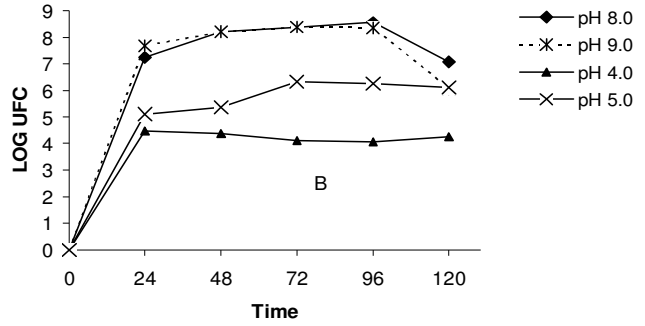

Figure 3. Growth curve of *Streptomyces* at 40°C and different pH.

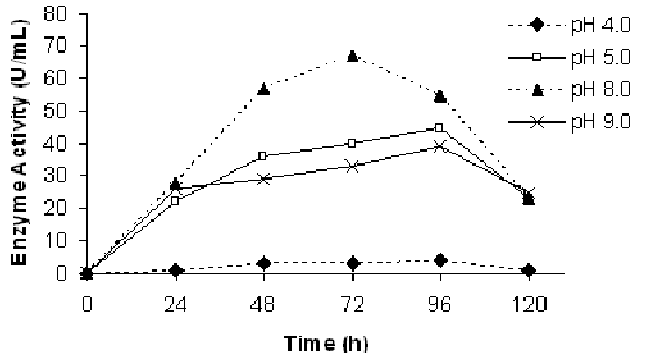

Figure 4. Enzyme activity of *Streptomyces* at 30°C different pH using Keratin Azure as substrate.

DISCUSSION

A bacterium was isolated from wastewater from poultry-processing plant, presenting feather-degrading properties. The identification of the microorganism, which was performed using biochemical tests and rRNA 16S sequence comparison, indicated that the isolate is a *Strep-*

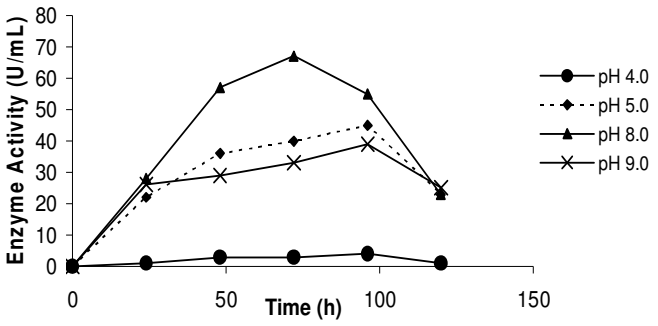

Figure 5. Enzyme activity of *Streptomyces* at 30°C different pH using Keratin Azure as substrate.

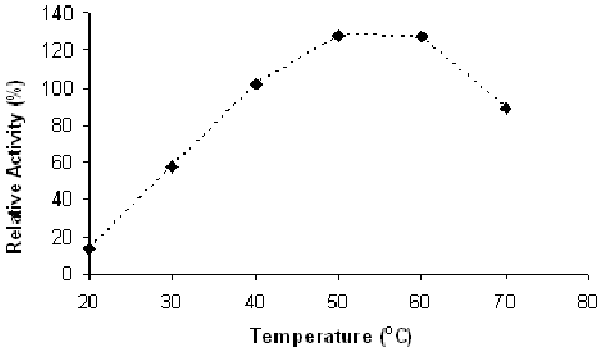

Figure 6. Effect of pH on keratinolytic using keratin azure as substrate.

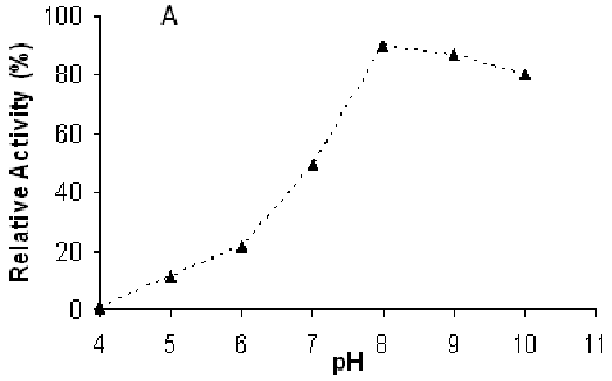

Figure 7. Thermal stability of keratinase of *Streptomyces* at 40°C, incubation time ranging from 15 to 240 min.

Table 2. Effect of some chemicals on keratinolytic activity.

Substances	Concentration	Relative activity (%)
Triton X -100	0.1%	109
	0.5%	114
EDTA	2 mM	73,6
	5 mM	63,2
	10 mM	60,6
TWEEN 80	0.1%	98
	0.5%	109
DMSO	0.1%	102
	0.5%	107
Isopropanol	0.1%	103
	0.5%	100
Mercaptanol	0.1%	119
	0.5%	108

Table 3. Effect of metal ions on keratinolytic activity.

Metal Ion	Concentration (mM)	Relative activity (%)
$MnCl_2.4H_2O$	2	91.19
	5	88.9
	10	78.7
$ZnCl_2$	2	65
	5	35.3
	10	24.5
$HgCl_2$	2	78.5
	5	63.2
	10	59.8
$BaCl.2H_2O$	2	107
	5	68.9
	10	55.6
$CuSO_4.5H_2O$	2	112.6
	5	102.7
	10	88.9
$MgCl_2$	2	118.3
	5	102.35
	10	99.78
$CaCl_2$	2	98.6
	5	74.5
	10	38.9

tomyces. The isolate showed optimum growth at mesophilic temperatures, as expected for a microorganism of environmental origin. The isolate showed maximum growth at 40°C and pH 8.0. These results are very close to the ones obtained by Böeckle et al. (1995) using *Streptomyces pactum*, and Young and Smith (1975) with *Streptomyces fradiae* and *S. pactum*. Other authors have considered that keratinolytic bacteria generally have optimum growth and feather degradation activity at high temperatures (Williams et al., 1990; Atalo

and Gashe, 1993; Böckle et al., 1995).

The levels of keratinase produced by this *Streptomyces* were variable during cultivation in different feather meals. According to Sangali and Brandelli (2000), this pattern may suggest that the enzyme is inducible, that is, substrate levels in the extracellular mileu regulate its secretion. Similar results were obtained by Cheng et al. (1995) with *Bacillus licheniformes* PWD1, and also by Macfarlane and Macfarlane (1992) using *Clostridium bifermentans*.

Thermostable proteases have been used mainly in detergent industry. These enzyme are obtained principally from thermophilic bacteria such as *Bacillus* genus, *Bacillus licheniformis* PWD1 being the most known. On the other hand, the proteases obtained from *Streptomycetes* are less thermostables, but according to the results obtained in this work a good stability (40 to 60°C) was achieved using this *Streptomyces*, which is similar to the previously isolated *Streptomyces* $S.K_{1-02}$ (Letourneau et al., 1998), *Streptomyces* sp. 594 (De Azeredo et al., 2006) and *Streptomyces thermoviolaceus* strain SD8 (Chitte et al., 1999).

Keratinase activity was affected by some metal ions, especially $CaCl_2$ and $ZnCl_2$. On the contrary $MgCl_2$ did not show any relevant effect. Anionic detergent such as Triton X-100 increased the keratinolytic activity; results that are close to the obtained by Korkmaz et al. (2003) using *Streptomyces* strain BA7. When DMSO was used, an increase in the enzyme activity was also noticed (Böckle et al., 1995), but EDTA decreases the activity.

According to the results, it is possible to conclude that the keratinase produced by this strain of *Streptomyces* could be used in biotechnological processes such as in feathers' degradation, in leather industry, and in wastewater treatment.

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