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Submerged Culture Screening of Two Strains of *Streptomyces* sp. with High Keratinolytic Activity

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ABSTRACT

Keratinases can be used for the production of potentially important hydrolyzed proteins and chemicals. This study investigated the keratinolytic activity of *Streptomyces* sp on keratinaceous materials like wool. High levels of proteolytic and keratinolytic activity were obtained after 96 h of culture when two *Streptomyces* sp strains were grown on basal medium containing mineral salts and 3% (w/v) of defatted wool as a source of energy, carbon, and nitrogen. The cell-free culture filtrates exhibited rapid proteolytic digestion of keratin powder. Currently, the authors are testing whether the enzymatic activity obtained is in fact keratinolytic, and not only an alkaline protease activity.

Index Entries: Keratinases; enzymatic hydrolysis; *Streptomyces* sp.; M-Zyme; alkaline proteases.

INTRODUCTION

Keratin forms mammalian outer tissues such as hair, nails, feathers, and horn. Keratinous proteins are insoluble and resistant to degradation by common proteolytic enzymes, because of their extensive crosslinking by disulfide bonds, hydrogen bonds, and hydrophobic interactions. This insolubility and resistance to proteolytic enzymes makes them inaccessible to most living organisms.

However, keratin does not accumulate in nature. Despite the unusual stability of keratinous proteins, several microbial keratinolytic proteases have been reported, such as the keratinase of *Trichophyton mentagrophytes*

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(1). Keratinolytic activity has also been reported for some species of *Aspergillus (2)*, proteinase K of *Tritirachium album (3)*, the alkaline protease of *Streptomyces* sp *(4)*, the thermostabile alkaline protease of *Bacillus* sp *(5)*, and a purified keratinase from *Streptomyces fradie*, which solubilizes wool keratin *(6)*.

Laboratory research efforts are focusing on the processing of waste animal protein into either value-added byproducts or derivatives that are more quickly recycled in the environment. Biodegradation by microorganisms possessing keratinolytic activity is a possible method for improving the nutritional value of keratinaceous waste. Microbial keratinases can be used as food additives to improve the digestibility and nutritious value of proteins, since keratin contains all common amino acids, and mainly differs from other structural fibrous proteins in its high cysteine content. Enhanced production and processing of keratinase enzymes would benefit applications such as converting keratineous materials and waste proteins into usable amino acids.

In this article the authors report the production of alkaline protease activity and keratinolytic activity by submerged culture of two different strains of *Streptomyces* sp using defatted wool as an inducer in a simple mineral salts medium.

MATERIALS AND METHODS

Microorganisms

Streptomyces sp KER p-08 and *Streptomyces* sp KER p-17 were isolated in our laboratory from samples of soil from slaughterhouses that were enriched with strands of wool and pieces of cattle hoof. These strains have been shown to possess the ability to digest native keratin rapidly, as previously reported *(7,8)*.

The actinomycete strains were grown at 29 ± 1°C for 5 d on potato dextrose slants, and maintained on the same medium at 4°C. Subcultures were prepared at monthly intervals.

Alkaline Protease and Keratinase Production

Six grams of defatted wool were placed in 500-mL Erlenmeyer flasks containing 180 mL of fermentation medium. All chemicals used in this culture medium were industrial grade, and tap water was used according to the basal salt formula, indicated in Table 1. In our experiments, keratin was neither autoclaved nor sterilized. It was washed with water, extracted with chloroform–methanol *(9)*, and soaked several times in 0.05 *M* phosphate buffer, 1m*M* Mg^{2+}, pH 7.8, then rinsed with water and methanol, and air-dried at room temperature. The wool was either cut with scissors to a length of 3–5 mm, or with a knife to pieces of 1–3 mm length.

Table 1
Culture Medium Composition Utilized for the Production of Keratinolytic Activity by Submerged Fermentation

Industrial-grade medium	(G/L)
Defatted wool[a] (*size: 3 mm*)	30.0
K_2HPO_4	1.2
KH_2PO_4	1.0
KNO_3	3.0
$MgSO_4 \cdot 7H_2O$	0.6
$MnCl_2$	0.3
$ZnSO_4 \cdot 7H_2O$	0.6
$FeSO_4 \cdot 2H_2O$	0.6
$CaCl_2$	0.5
pH	8.0
Tap water	1.0 L

All the constituents are industrial grade chemicals.

[a] The wool was neither autoclaved nor sterilized. It was either cut with scissors or knife to pieces of 1–3 and 3–5-mm long, washed with water, extracted with chloroform–methanol and soaked several times in 0.05 *M* phosphate buffer, 1 m*M* $MgCl_2$, pH 7.0, then rinsed with water and methanol, and air-dried at room temperature. The wool was treated prior to being added to the medium previously sterilized by autoclaving.

Inocula were prepared by harvesting spores from 1-wk-old PDA slants of the two strains in the proportion 3:1 in sterile distilled water containing 0.1–0.3 mL of Tween-80.

Fermentation flasks were inoculated to give 10^6–10^7 spores/mL. Incubation was at 29 ± 1°C. The pH value was initially adjusted to 8.0 before sterilization.

During the culturing, samples were removed aseptically and centrifuged at 3200 g, to obtain cell-free filtrates.

Enzyme Activity Assays and Soluble Protein Determination

These assays were performed with the supernatants after removing the cell growth and residual wool. Removal of the filamentous growth from cultures of *Streptomyces* grown in shake flasks on wool–salts medium was readily accomplished by vacuum filtration through Whatman (Maidstone, UK) No. 1 paper.

Protein content was determined according to Lowry *(10)* in aliquots of cell-free culture filtrates after ±4°C overnight dialysis, with bovine serum albumin as a standard. Culture filtrates of both strains of *Streptomyces* sp.

were concentrated 5× by ultrafiltration (mol wt cutoff >10,000), with respect to the initial soluble protein content, or until both proteins reached approximately the same concentration.

Alkaline protease activity was measured by the Anson's method modified by Keay et al. *(11,12)*. The concentrated culture filtrates were incubated with 5% casein in 1 mL buffer 50 m*M* Tris-HCl (pH 7.8), containing 1 m*M* $CaCl_2$ for 30 min at 37°C. The reaction was stopped by adding 2 mL of TCA solution (0.11 *M* trichloroacetic acid, 0.22 *M* CH_3COONa, 0.33 *M* CH_3COOH). After 30 min of incubation at 30°C, the precipitate was removed by centrifugation and the absorption of the supernatant was measured at 280 nm. One unit of protease activity is defined as the amount of enzyme required to release 1 μmol of tyrosine in 30 min at 37°C, using casein as substrate.

The determination of keratinolytic activity was estimated by incubating concentrated culture filtrates with 10 mg of keratin powder in 1 mL of buffer A (50 m*M* Tris-HCl buffer, pH 7.8, containing 1 m*M* $CaCl_2$) with vigorous shaking for 1 h at 37°C. The reaction was stopped by adding 2 mL of TCA solution (0.11 *M* trichloroacetic acid, 0.22 *M* CH_3COONa, 0.33 *M* CH_3COOH). After 30 min of incubation at 30°C, the substrate was removed by centrifugation and the absorption of the supernatant was measured at 280 nm, as described by Shu-Wen et al. *(13)*. One unit of keratinase activity is defined as the amount of enzyme required to release 1 μmol of tyrosine in 60 min at 37°C, using keratin powder as substrate.

Enzymatic Hydrolysis

A keratin-digestion assay was devised, in which the amount of protein solubilized during the incubation period was estimated spectrophotometrically at 280 nm. Keratin powder was hydrolyzed by a commercial purified keratinase, the M-Zyme-(Merck) *(14)*, by the cell-free culture filtrates obtained from *Streptomyces* sp strains, and by other microbial alkaline proteases. The enzymes were incubated with 20 mg substrate (keratin powder, Merck, Rahway, NJ) in 1 mL buffer (0.05 *M* Tris-HCl containing 1m*M* $MgSO_4$, pH 7.8) with shaking during 3 h at 37°C, and filtered through a Whatman No. 2 paper. Absorption of the supernatant at 280 nm was measured after (TCA) precipitation.

RESULTS AND DISCUSSION

As previously reported *(8)*, some preliminary tests were carried out at the authors' laboratory using dyed keratin powder (keratin-azure, Merck). After a final screening in Petri dishes using casein medium, many strains of filamentous fungi and some actinomycetes were obtained. In the present work, the production of alkaline protease and keratinolytic activities by two different strains of *Streptomyces*, chosen for the previously indicated reasons was determined by submerged culture in a cheap and simplified

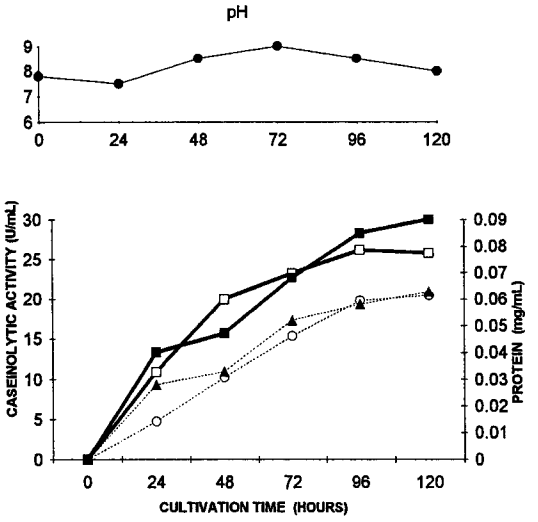

Fig. 1. Production of caseinolytic activity and soluble protein in cell-free culture filtrates of KER p-08 (-■-), (-▲-) and KER p-17 (-□-), (-○-) *Streptomyces* sp. strains, respectively, as well as pH variation (-●-) on liquid fermentation using defatted wool.

medium. Figure 1 shows caseinolytic activity, protein content in cell-free culture filtrates, and pH variation over 120 h of growth of *Streptomyces* sp under liquid fermentation on defatted wool.

Considerable keratinolytic activity was produced by the *Streptomyces* sp strains (KER p-08 and KER p-17) after 72 h.

It is important to notice in Figure 2 that maximal enzyme production was achieved in a medium containing 3.0% (w/v) defatted wool, as a sole carbon and organic nitrogen source, and mineral salts at pH 7.7 and 30°C for 96 h.

Table 1 shows the gross composition of the culture medium used for all the fermentations. The use of tap water did not show negative effects on the production of alkaline protease and keratinolytic activities. Table 2 compares enzymatic activities evaluated at maximal production time with reference to the soluble protein content present in culture filtrates obtained during fermentation.

Keratin that has been denatured and degraded by treatments such as sterilization and ball milling can be decomposed by organisms that are incapable of attacking native keratin *(15)*. The pretreatments mentioned drastically decrease the cysteine content of wool *(16)*. Upon destruction of the disulfide bonds, keratin becomes easily denatured, and then loses its natural insolubility and resistance to proteases.

As indicated by Noval and Nickerson *(17)*, a basic question involved in studies on microbial decomposition of keratin is this: Do the microorganisms decompose native keratin during their growth on keratinaceous substrates, or do they grow at the expense of nonkeratinaceous nutrients (fats,

Table 2
Caseinolytic and Keratinolytic Activities Obtained in Submerged Fermentation for 96 h by the Strains of *Streptomyces* sp

Enzymatic activities (U/mL)	*KER p-08*	*KER p-17*
CASEINOLYTIC	26.2	15.2
KERATINOLYTIC	19.8	9.4

The proteolytic activity was measured by Anson's method modified by Keay et al. *(11,12)*. One unit of protease activity is defined as the amount of enzyme required to release 1 μmol of tyrosine in 30 min at 37°C, using casein as substrate.

The keratinolytic activity was estimated according Shu-Wen *(13)*. One unit of keratinase activity is defined as the amount of enzyme required to release 1 μmol of tyrosine in 60 min. at 37°C, using keratin powder as substrate.

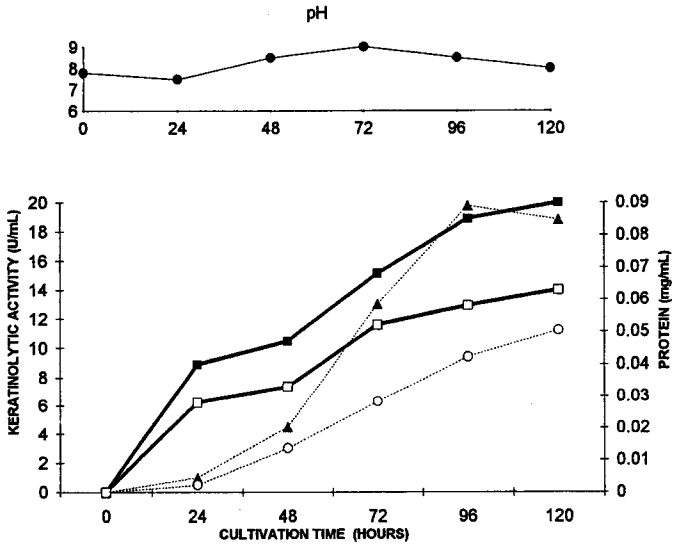

Fig. 2. Production of keratinolytic activity and soluble protein in cell-free culture filtrates of KER p-08 (-■-), (-▲-) and KER p-17 (-□-), (-○-) *Streptomyces* sp. strains, respectively, as well as pH variation (-●-) on liquid fermentation using defatted wool.

carbohydrates, and proteins) that are usually present as minor constituents of such substrates? In seeking an answer to this question, the following experimental requirements must be met: Nondenatured keratin must be used as the substrate, and the evidence that keratin has been decomposed must be unequivocal.

To evaluate if the enzymatic activity obtained in our experiments really represents keratinolytic activity, several microbial enzymes, includ-

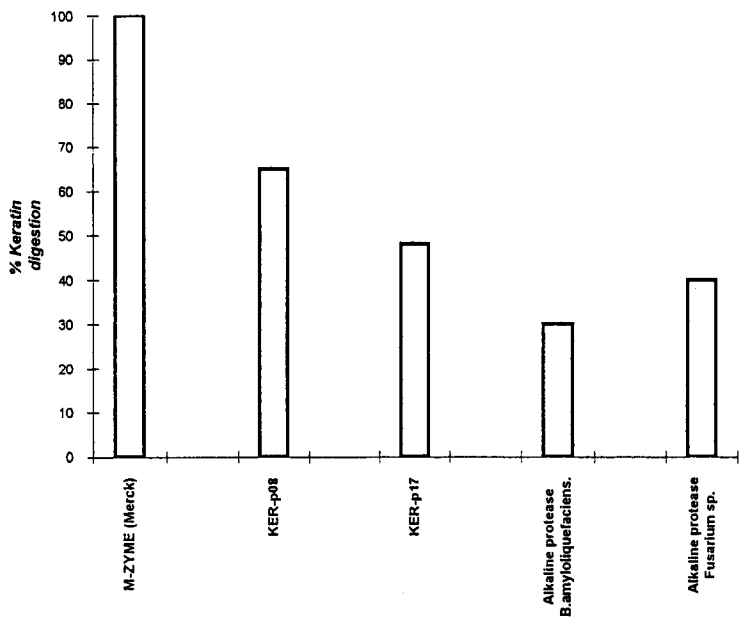

Fig. 3. Hydrolysis of keratin powder with a purified keratinase, two alkaline proteases and the cell-free culture filtrates obtained from *Streptomyces* sp. The enzymes were incubated with 20 mg substrate (keratin powder, Merck) in 1 mL of buffer, with shaking for 3 h at 37°C. Absorption at 280 nm of supernatant was measured after TCA precipitation.

ing a commercial preparation, were tested. Figure 3 shows the effects of the cell-free culture filtrates on keratin powder.

The percentage of keratin digestion of the filtrates with respect to purified keratinase was about 65% for the KER p-08 enzyme and 48% for the KER p-17 enzyme. This result partially supports hypothesis that the obtained enzymatic activity is keratinolytic, because the alkaline protease activity obtained was much lower.

In conclusion, a good level of keratinolytic activity was obtained with the KER p-08 strain of *Streptomyces* sp after 96 h by submerged culture at 37°C in the evaluated culture medium. The keratinolytic activity observed with this strain was compared with that of some microbial proteases. Keratinolytic activity in cell-free culture filtrates from *Streptomyces* sp hydrolyzes several types of substrates, and exhibits high proteolytic activity, compared to other alkaline proteases. The highest hydrolysis level was obtained with keratin powder, but it is necessary to test other keratinaceous materials like feathers, wool, hide, and so on, in order to demonstrate real keratinolytic activity, and not the presence of an alkaline protease in the culture filtrates.

Not much research is presently done in Mexico on the use of native keratinaceous materials that are considered agroindustrial or cattle wastes. Enhanced production of *Streptomyces* sp KER p-08 and processing of kera-

tinase will benefit applications such as recycling hides, feathers, wool, and waste proteins as usable peptides. These molecules have nutritional value and can be used as animal food supplements, or in other industrial applications. Microbial proteases have extensive commercial applications, and their use in agricultural applications could be increased.

There is considerable interest in producing chemicals from renewable resources. This increasing interest is justified by recent technological advances that can create economically competitive processes. These technologies must be environmentally acceptable and produce acceptable marketable materials. For these reasons, the authors are working to turn keratinaceous materials (an abundant renewable feedstock) into a wide variety of industrial chemicals. However, numerous technical improvements must be achieved before the potential global benefits of these biological processes can be fully realized.

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