

Keratinase Production From Feathers Wastes Using Some Local *Streptomyces* Isolates

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Abstract: Various soil samples collected from different localities in Egypt were enriched to isolate different *Streptomyces* spp. Only, 21 of these isolates showed quantitative protease activity. Under restricted medium conditions that contain feather as a sole carbon and nitrogen source, eight isolates showed keratinolytic activities. Isolates no. 4 and 5 that showed high keratinase activities were identified on the bases of the International *Streptomyces* Project (ISP) and were designated as *S. albidus* E4 and *S. griseoaurantiacus* E5. Some cultural conditions were tested to attain maximum keratinase production. Galactose supported the highest level of production for both strains. Ammonium nitrate was a good nitrogen source for the production of keratinase by *S. albidus* E4. Maximum enzyme production was reached on the 5th day of incubation of the shaking culture at 30°C and pH 8.0 by *Streptomyces* spp. E4 and E5. Of the tested keratinous materials used as substrates for keratinase production, feathers, wool, nails and hair were well utilized by both strains.

Key words: *Streptomyces* species, morphological & physiological characteristics, Cell wall analysis, Keratinase production and keratinous wastes.

INTRODUCTION

The Keratin - degrading microorganism thrives under different ecological and environmental conditions, and they demonstrate a wide ranging capacity to solubilize keratinous substrates as well as other compact proteinous substrates. Several species of bacteria, actinomycetes and fungi produce keratinase enzyme to degrade keratinous material like, feather so known as keratin degraders.

The actinomycetes are generally recognized to represent a large and heterogeneous group of microorganisms comprising several families, genera and numerous species (Waksman, 1959 and Krassilnikov, 1970). They play an important ecological role in biodegradation, many are commercially important, either in the production of bioactive compounds such as antibiotics and enzymes or in useful biological processes such as biodegradation and waste treatment (Chitte *et al.*, 1999; Szabo *et al.*, 2000; Kansoh and Nagieb, 2004 and Gousterova *et al.*, 2005).

Keratinaceous materials such as feather, wool and hair are insoluble and resistant of degradation by common proteolytic enzymes. Feathers represent over 90% protein, the major component being β -keratin, a fibrous and insoluble structural protein extensively cross-linked by disulfide, hydrogen and hydrophobic bonds. Owing to their insoluble nature, feathers are resistant to degradation by common microbial proteases. Thus, the several million tons of feathers generated annually by the livestock industry leads to troublesome environmental pollution and wastage of protein-rich reserve (Onifade *et al.*, 1998; Gousterova *et al.*, 2005 and Grazziotin *et al.* 2006).

Keratinolytic enzymes are widespread in nature and are elaborated by a compendium of microorganisms largely isolated from poultry wastes. (Nitisinprasert *et al.* 1999; Gousterova *et al.*, 2005 and Cai *et al.*, 2008). Keratinases [EC 3.4.21/24/99.11] are by and large serine or metallo proteases capable of degrading the structure forming keratinous proteins. Since most of the purified keratinases known to date cannot completely solubilize native keratin. Nonetheless, keratinases in nature have been continuously contributing to voluminous keratin containing wastes in the form of hair, feathers, dead birds and animals (Onifade *et al.*, 1998 and Gupta & Ramnani, 2006). These enzymes are also used or could be applied in the food industry, textile, medicine, cosmetics, leather and poultry processing industry (Gupta & Ramnani, 2006).

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MATERIALS AND METHODS

Sampling and Isolation of streptomycetes: Feather degrading isolates were randomly collected from different sources as feather, agricultural soil and poultry grange soil. Isolation was carried out according to the dilution technique (Johnson *et al.*, 1959). *Streptomyces* colonies were selected according to their morphological characteristics that have different colored aerial mycelium with deep sitting colonies.

Preparation of Keratin:

The collected native feathers were prepared according to the method of Friedrich *et al.* (1999). Keratin was obtained from the prepared feathers by the modified method of Wawrzkievicz *et al.* (1987) and used as a keratin powder.

Detection of Proteolytic and Keratinolytic Activities:

The chosen isolates were screened qualitatively on casein-agar medium and quantitatively in submerged culture of the same medium. It contained (g/l): Casein, 10.0; Glucose, 2.0; K₂HPO₄, 0.9; MgSO₄ · 7 H₂O, 0.2; NaCl, 0.2; FeSO₄ · 7H₂O, 0.003; (NH₄)₂NO₃, 1.0; MgCl₂, 0.02; ZnSO₄, 0.002 and agar-agar, 20. The pH of the medium was adjusted to pH 7.0 before autoclaving. The positive isolates were selected and screened quantitatively for their keratinolytic activity in submerged culture of the feather basal salt medium (F.B.M). It contained (g/l): Feather, 20.0; K₂HPO₄, 1.0; MgSO₄ · 7 H₂O, 0.5; NaCl, 0.5; CaCO₃, 3.0; FeSO₄ · 7H₂O, 0.01 and trace elements solution, 1.0 ml. The pH of the medium was adjusted to pH 7.2 ± 0.2 before autoclaving.

Identification of Streptomycetes:

The active isolates were chosen, tested and identified on the bases of the observations made on International Streptomyces Project (ISP) media. Morphological, Cultural, Physiological and biochemical characterizations, Antimicrobial activities, and Cell hydrolysate analysis were examined as recommended by Shirling and Gottlieb (1966).

Cultivation of the Selected Strains for Keratinase Production:

Experiments were carried out in shaken cultures to determine the physiological and physical conditions that would affect the keratinase production of the selected strains. Preincubation in shaking flasks was routinely used to prepare the inoculum of the organism. In all experiments Erlenmeyer flasks (250 ml) containing 50 ml of the feather basal salt medium (F.B.M.) were inoculated with 2 % (v/v) of the pre-incubated broth culture. The flasks were incubated shaken at 120 rpm at 30°C for 5 days. Prior to assay, the fermentation broth was centrifuged to separate the filtrate.

Enzyme Assay:

Protease Activity:

A modified version of Hindazoltilk *et al.* (1983) was used and the solubilized proteins were measured using the method of Lowry *et al.* (1951). One unit of the enzyme activity (U) was defined as the amount of the enzyme that liberates 1 µg of amino acids equivalent to tyrosine per min. under the experimental conditions.

Keratinase Activity:

Keratinase activity was measured using modified method of Friedrich *et al.* (1999). One ml of the enzyme solution was incubated with keratin powder (1% w/v) in 2 ml 0.1 M phosphate buffer pH 8.0 for 1h at 40°C in a shaking water bath. The reaction was stopped by adding 2ml of 20 % TCA and the clear supernatant was estimated according to Lowry *et al.*, (1951). One unit of keratinase activity was defined as the amount of enzyme that liberates one µg of amino acids equivalent to tyrosine per 1h under the experimental conditions.

Effect of Different Factors:

The feather basal salt medium (F.B.M) was supplemented with 1% (w/v) starch, lactose, galactose, glucose, fructose, mannose, sucrose or glycerol to study the effects of addition different carbon sources on keratinase production. Addition of equimolar amounts of different inorganic and organic nitrogen sources on keratinase production were also studied. Similarly, various phosphate salts were substituted for the phosphate salt in the F.B.M to study their effect on keratinase production. The optimized F.B.Ms were adjusted to the appropriate pH value of 5.0 to 10.0, inoculated with different inoculum sizes and study their effects on keratinase production at different sample sizes for various aeration.

RESULTS AND DISCUSSION

Selection and Characterization of Feather Degrading *Streptomyces*:

From various soil samples collected from different habitats, the active isolates having proteolytic activity were screened quantitatively for their keratinolytic activities in a basal salt medium containing feather as a sole carbon and nitrogen source (F.B.M). Under these restricted conditions only 8 isolates of 27 ones showed keratinolytic activities in shaken culture medium (F.B.M) at different incubation periods (4, 5, 6, 7&10 days). The isolates no. 4 and 5 showed high keratinase activities after 5 days of incubation (Table 1). So, those isolates were used in the succeeding work.

Table 1: Keratinolytic activity of some *Streptomyces* isolates in submerged cultures at different incubation period.

<i>Streptomyces</i> Isolates no.	Keratinolytic activity (U/ml) (days)			
	4	5	7	10
3	0.28	0.77	0.88	0.88
4	0.83	1.43	1.43	1.27
5	0.66	1.16	1.16	0.80
6	0.55	0.88	1.10	0.61
11	-	0.28	0.23	0.28
12	0.50	0.93	0.88	1.10
13	-	0.33	0.50	0.44
18	0.44	0.94	0.94	0.18

Most of the reports available on keratinases (keratin-degraders) grouped them as inducible enzymes requiring keratin as exogenous inducer and the nature of the enzyme is based on casinolytic rather than keratinolytic activity (Manczinger *et al.*, 2003).

Identification of the Selected Isolates No. 4 & 5:

Morphological Characteristics:

The microscopic examination of 14 days old culture of the selected isolates no.4 and no.5 revealed that aerial mycelia were morphologically related to the section Rectiflexibiles RF and to the section spirals, respectively (Fig.1 & 3). Both isolates have smooth spore mass surfaces (Fig. 2 & 4).

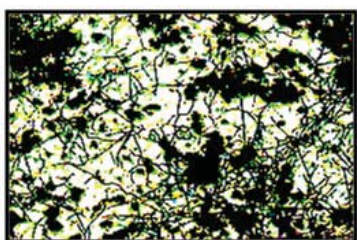


Fig. 1: Photograph of spore chains of isolate 4 (X3000).

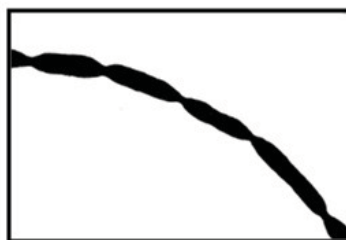


Fig. 2: Electron micrograph of spore of isolate 4 (X16000).

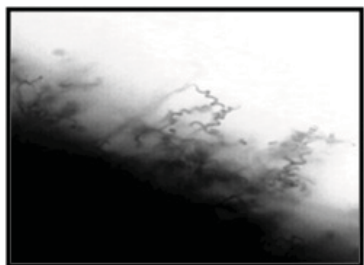


Fig. 3: Photograph of spore chains of isolate 5 (X16000).

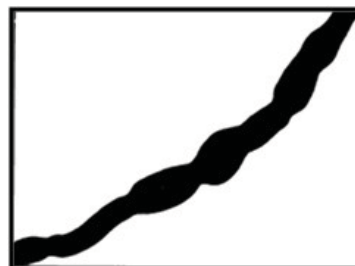


Fig. 4: Electron micrograph of spore of isolate 5 (X3000).

Cultural Characteristics:

The Cultural characteristics studies revealed that the isolates 4 and 5 grew well on the tested media either organic or synthetic media. The aerial mass color was in the yellow-color series and gray-color series for the isolates 4 and 5, respectively. The color of the substrate mycelium showed no distinctive pigment. Diffused pigments were sometimes observed (data not shown).

Physiological and Biochemical Characteristics:

The physiological and biochemical characteristics studies of the isolates 4 and 5 (Table 2) revealed that both isolates did not produce melanoid pigments on peptone-yeast extract-iron agar or tyrosine agar.

Table 2: Physiological and biochemical characteristics of isolates no. 4 and 5.

Character state	Results Isolate 4	Results Isolate 5
Melanin pigment production:		
- Iron agar	-	-
- Tyrosine agar	-	-
Enzyme activities:		
- Proteolysis	++	+
- Lipolysis	+	+
- Lecithinase	±	±
Hydrolysis activities:		
- Chitin	-	-
- Pectin	-	-
- gelatin	+	+
Nitrate reduction:	+	+
H ₂ S Production:	-	±
Utilization of different carbon sources:		
- Control: Negative control	-	±
Positive control (glucose)	+	+
- L-arabinose	±	+
- D-xylose	+	+
- I-Inositol	±	+
- D-mannitol	+	+
- D-fructose	+	+
- Rhamnose	+	+
- Sucrose	±	±
- Raffinose	-	±
- D- galactose	+	+
Tolerance to NaCl:	Up to 6%	7%
Growth temperature:	20 - 37°C	15-35°C
pH range of growth:	6.0 - 9.0	7.0-8.0

Negative control: No sugars; Positive control: Glucose sugars - : Negative result +: Positive result ±: Doubtful (weak, variable or trace of growth).

Enzyme activities of the isolate 4 and 5 showed high and moderate proteolytic activities, respectively. Both isolates showed moderate lipolysis and weak licithinase activities. The utilization of various carbohydrates by the selected isolates suggests a moderate pattern of carbon assimilation by isolate 4 and the tested sugars were well utilized by isolate 5. Isolates 4 and 5 tolerated NaCl up to 6.0 % and 7%, respectively. Well growth was recorded at a temperature range of 20 to 37c and at pH range of 6.0 to 9.0 for isolate 4. A temperature range of 15 to 35°C and pH range of 7.0 to 8.0 was optimum for well growth of isolate 5.

Isolates 4 and 5 reduced nitrate and couldn't hydrolyze chitin or pectin. H₂S production was not detected by isolate 4 but was relatively produced by isolate 5.

Antimicrobial Activities:

Table (3) showed the antimicrobial activities of the isolates 4 and 5 against Gram +ve and Gram -ve bacteria, yeasts and fungi. Strain no. 4 was active against Gram positive bacteria with relative or weak effect on fungi and yeast. No response was detected on Gram negative bacteria. Strain no.5 showed a broad spectrum antimicrobial activities but weak activity was detected on Gram negative bacteria.

Cell Hydrolysate Analysis:

The Cell hydrolysate analysis of the isolates 4 and 5 showed that the cell wall was characterized by the presence of LL-DAP amino acid. No diagnostic sugars in the whole cell hydrolysis were detected.

Table 3: Antibiosis of *Streptomyces* strains 4 and 5 towards various microbial members.

Microorganisms	Type of organism	Response Isolate 4	Response Isolate
<i>Bacillus cereus</i>	Gram +ve bacteria	+	+
<i>Bacillus subtilis</i>	Gram +ve bacteria	+	+
<i>Staphylococcus aureus</i>	Gram +ve bacteria	+	+
<i>Escherichia coli</i>	Gram -ve bacteria	-	±
<i>Aspergillus flavus</i>	Fungi	-	+
<i>Aspergillus niger</i>	Fungi	±	+
<i>Aspergillus terreus</i>	Fungi	+	+
<i>Botrytis allii</i>	Fungi	-	+
<i>Diplodia oryzae</i>	Fungi	-	+
<i>Fusarium oxysporum</i>	Fungi	-	+
<i>Macrophomina phaseoli</i>	Fungi	-	+
<i>Candida albicans</i>	Yeast	+	+
<i>Candida pseudotropicalis</i>	Yeast	±	+
<i>Rhodotorula minuta</i>	Yeast	±	±3

+: Positive - : Negative ±: Weak

Taxonomic Identification:

Taxonomical identification of isolates 4 and 5 showed that they were belonging to the genus *Streptomyces*. They were closely resembles in morphology and cultural characteristics to *Streptomyces albidus* and *Streptomyces griseoaurantiacus*, respectively. So, they were designated as *Streptomyces albidus* E4. and *Streptomyces griseoaurantiacus* E5.

Induction of Keratinase in *Streptomyces* spp. E4 and E5:

Of all the tested carbon sources (1% w/v) that added to F.B.M medium (Fig. 5), galactose supported the highest level of production for both *S. spp. E4* and *E5*. Other, sugars though they supported the growth of the organisms, they repressed the enzyme production than the galactose level. The activities of respiratory enzymes in cells grown on different carbon sources explained the differences of the oxidative ability of cells grown aerobically on glucose and galactose. Glucose has been shown to be a repressor of the respiratory and citric acid-cycle enzymes under aerobic and anaerobic conditions unlike the metabolism of galactose (Polakis and Bartley, 1965).

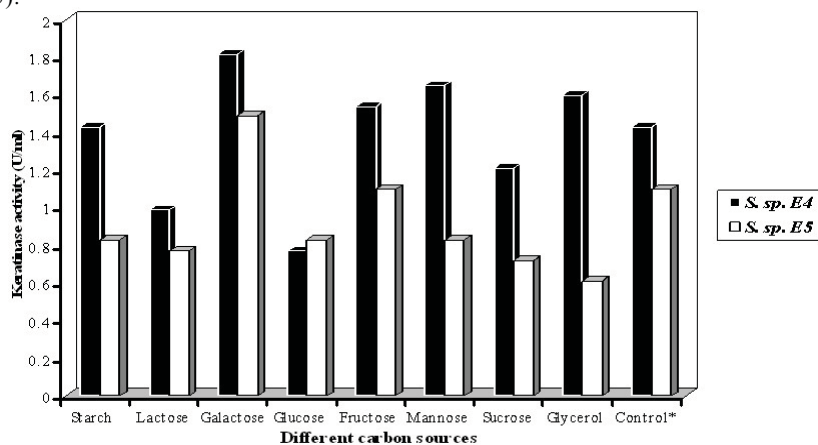


Fig. 5: Effect of various carbon sources on keratinase production by *Streptomyces albidus* E4 and *Streptomyces griseoaurantiacus* E5.

Fig. (6) shows that the concentration of 0.2% and 0.4% (w/v) galactose supported the highest production of keratinase by *Streptomyces* sp. E4 and *Streptomyces* sp. E5, respectively. Further, increase of the concentration cause gradual decrease in the keratinase production.

Ammonium nitrate was a good nitrogen source for the production of keratinase by *S.sp.E4*, in the F.B.M supplemented with 0.2 % galactose (Fig. 7). None of the tested inorganic nitrogen salts supported the production of keratinase by *Streptomyces* sp. E5. Deletion of the organic nitrogen source was more preferable as the medium protein concentration was increased. In most microorganisms, both inorganic and organic forms of nitrogen are metabolized to produce amino acids, nucleic acids, proteins and cell wall components.

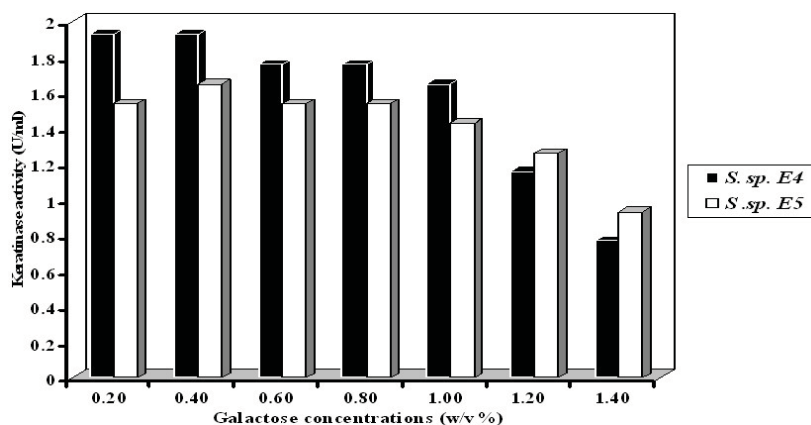


Fig. 6: Effect of different concentrations of galactose on keratinase production by *Streptomyces albidus* E4 and *Streptomyces griseoaurantiacus* E5.

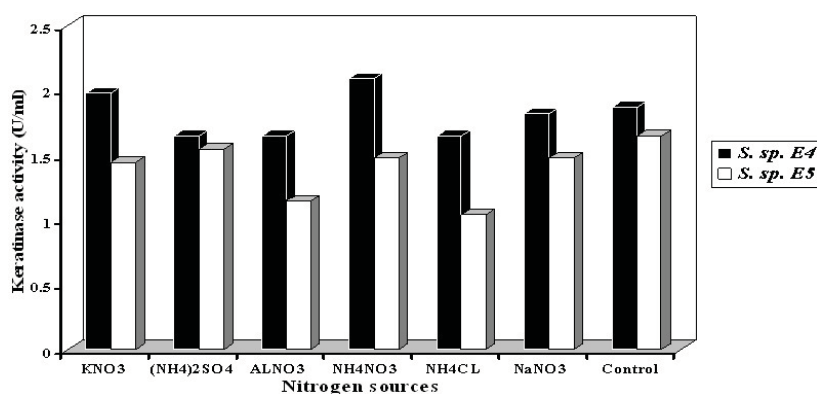


Fig. 7: Effect of inorganic nitrogen sources on keratinase production by *Streptomyces albidus* E4 and *Streptomyces griseoaurantiacus* E5.

Fig. (8) shows that 0.06 % (w/v) of ammonium nitrate was the best concentration for optimum keratinase production by *Streptomyces* sp. E4. Further increase of ammonium nitrate concentration resulted in a proportional decrease of keratinase production.

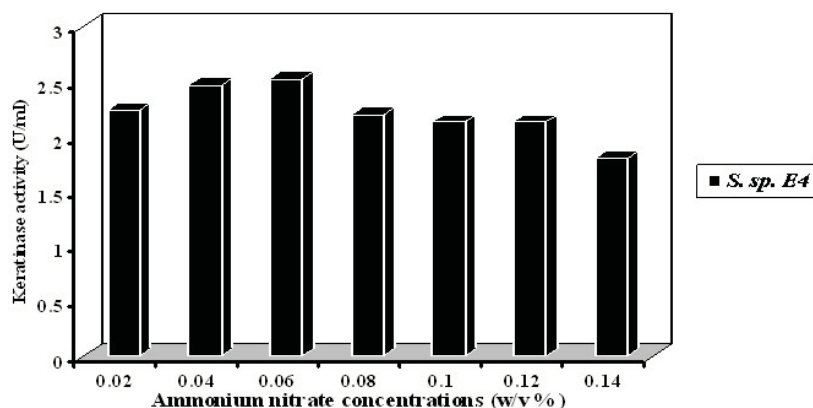


Fig. 8: Effect of different concentrations of ammonium nitrate on keratinase production by *Streptomyces albidus* E4.

Keratinase production produced by *S. sp.E4* and *S.sp.E5* was optimum with dipotassium hydrogen phosphate salt (Fig. 9). In general, the basic phosphates were more favourable for keratinase production. The best concentration of dipotassium hydrogen phosphate salt for keratinase production by *S. sp.E4* and *S.sp.E5* was 0.125 % w/v (Fig. 10). It is known that the absence of mineral sources of phosphorous in the medium causes a substantial drop in the activity and a decrease in the intensity of growth of the culture, which is due not only to the significance of phosphorous as an element of nutrition, but also to the buffering of the solutions of its salts. These results for keratinase induction are in agreement with those reported by Ignatova *et al.* (1999); Chitte *et al.* (1999) and Suntornsuk & Suntornsuk (2003).

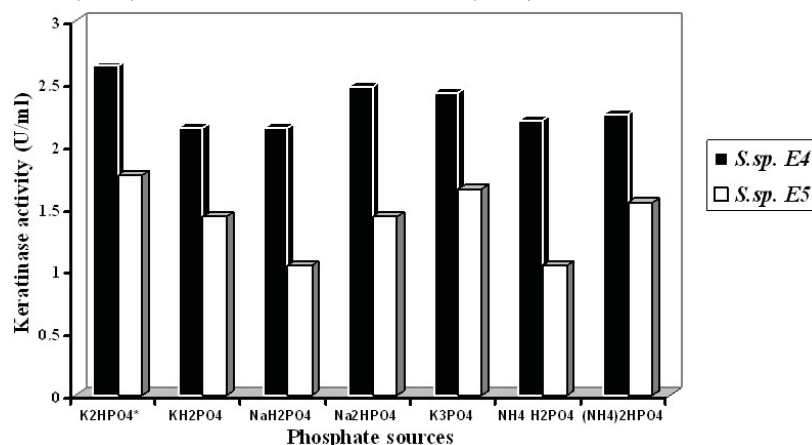


Fig. 9: Effect of different phosphate salts on keratinase production by *Streptomyces albidus E4* and *Streptomyces griseoaurantiacus E5*.

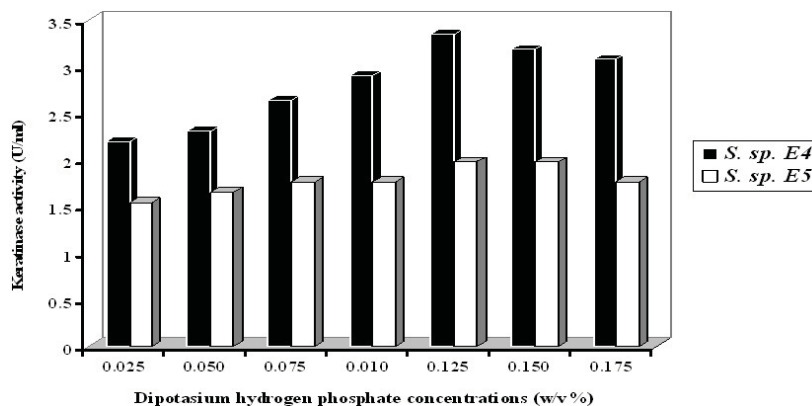


Fig. 10: Effect of different concentrations of dipotassium hydrogen phosphate salt on keratinase production by *Streptomyces albidus E4* and *Streptomyces griseoaurantiacus E5*.

The optimum activity was detected at a concentration of 4% (v/v) inoculum sizes for *S.sp.E4* and *S.sp.E5* (Fig.11). The optimum keratinase production by *S.sp.E4* and *S.sp.E5* was detected on the 5th day incubation period at initial pH 8.0 for both *S. spp.* with a slight increase of the final pH (Table 4). . During fermentation, the culture pH strongly affected many enzymatic processes and the transport of various components across the cell membrane. These results are in agreement with those reported by Williams *et al.* (1990); Friedrich and Antranikian (1996) and Chitte *et al.* (1999).

The keratinase production by *Streptomyces sp. E4* and *Streptomyces sp. E5* was measured at various volumes of the optimized media for each strain (Fig. 12). Optimum keratinase production was observed at 30 % (v/v) of the sample for both strains, but the viscosity of the medium was increased due to increase of the feather weight in the flask. So, we prefer to use 20% v/v as the difference degree of activity was less than 10%. This viscosity may reduce the oxygen supply and/or cause substrate inhibition or repression of keratinase production (Ignatova *et al.*, 1999 and Suntornsuk & Suntornsuk, 2003)

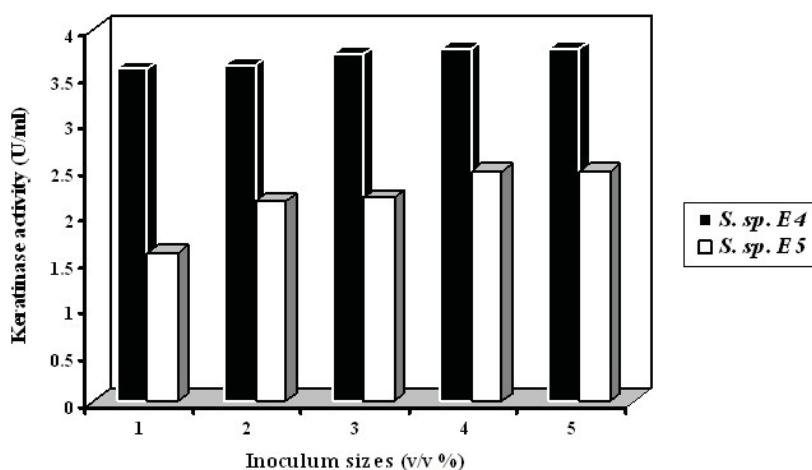


Fig. 11: Effect of inoculum sizes on keratinase production by *Streptomyces albidus* E4 and *Streptomyces griseoaur antiacus* E5.

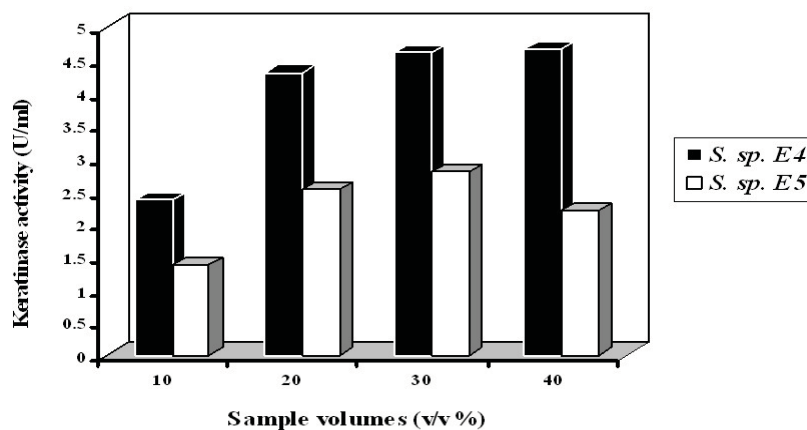


Fig. 12: Effect of aeration on keratinase production by *Streptomyces albidus* E4 and *Streptomyces griseoaurantiacus* E5.

Time Course of the Keratinase Production by *Streptomyces* spp. No. E4 and E5:

Time course from 2 to 10 days was followed in shaking and static incubated flasks containing the optimized culture conditions of *S.sp.E4* and *Streptomyces* sp. *E5* (Fig. 13). Maximum keratinase production was reached after 5 days shaking incubation period for *Streptomyces* sp. *E4* (4.7 U/ml) and *Streptomyces* sp. *E5* (3.0 U/ml). It seems to be stable on the 4th and 5th days of the static incubation period having maximum enzyme production on the 5th days for *Streptomyces* sp. *E4* (2.7 U/ml) and *Streptomyces* sp. *E5* (1.8 U/ml). A gradual decrease in activity was noticed up to the 10th day of either shaking or static incubation for the two strains.

The keratinase activities of the culture fluid of *Streptomyces albidus* E4 and *Streptomyces griseoaurantiacus* E5 were liberated during the exponential phase characteristic of extracellular enzymes. Maximum enzyme activity was reached on the 5th day at the end of the logarithmic phase and start of the stationary phase. Enzyme activity was relatively stable as it started to decline gradually during the stationary phase.

The time course for keratinase activity differs according to the strain and cultivation conditions. For instance, the maximum keratinase activity was achieved after 22h of incubation of *Streptomyces thermoviolaceus* SD8 (Chitte *et al.*, 1999), 60 h of *B. cereus* (Kim *et al.*, 2001), 72 h of *B. pumilus* and *Bacillus* sp. SCB-3 (Kim *et al.*, 2001 and Lee *et al.*, 2002) and it may take 5 day to have the maximum

keratinase activity of *B. pumilus* KUB-K0082 and *Streptomyces pactum* (Hanel *et al.*, 1991 and Buckle *et al.*, 1995) or 12-14 days of *Bacillus* sp. Fk46, *Dermatophilus congolensis* (Nitisinprasert *et al.* 1999, Suntornsuk & Suntornsuk, 2003).

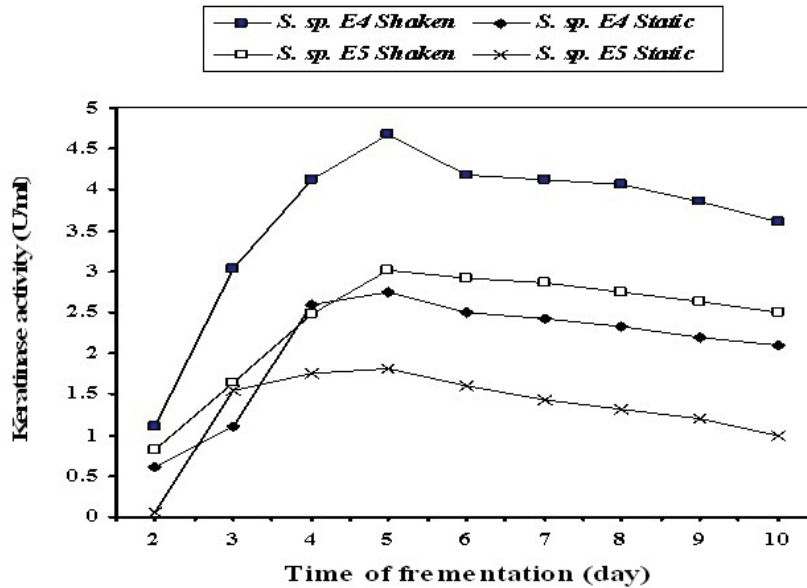


Fig. 13: Time course of the keratinase production by *Streptomyces albidus* E4 and *Streptomyces griseoaurantiacus* E5.

Effect of Various Keratinous Materials on Keratinase Production by *Streptomyces* spp. E4 and E5:

Various common keratinous materials were utilized as a sole carbon and nitrogen sources in the fermentation media at a concentration of 1% (w/v) for keratinase production by *Streptomyces* sp. E4 and *Streptomyces* sp. E5. Of the tested keratinous materials, feathers were well utilized by both strains (Fig. 14) followed by wool. *Streptomyces* sp. E4 and *Streptomyces* sp. E5 have similar activities on nails, while *Streptomyces* sp. E5 showed higher keratinase activity on hair. Keratins are grouped into hard (hair- feathers- nails- wool) and soft (skin- callus) keratin according to the sulfur content. On the basis of the secondary structural confirmation, keratins have been classified into α -keratin (α -helix of hair and wool) and β -keratin (β -sheets of feather) (Thys *et al.*, 2006 and Giongo *et al.*, 2007). Hydrolysis of feathers in the submerged culture by *Streptomyces albidus* E4 and *Streptomyces griseoaurantiacus* E5 compared with the control (without inoculums) was shown in Fig. (15).

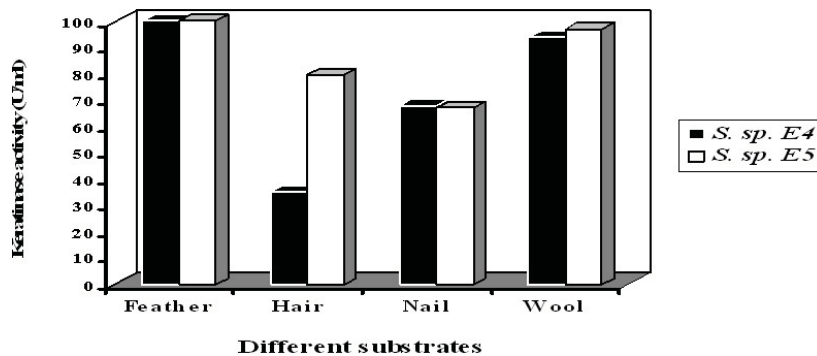


Fig. 14: Effect of different keratinous material on keratinase production by *Streptomyces albidus* E4 and *Streptomyces griseoaurantiacus* E5.

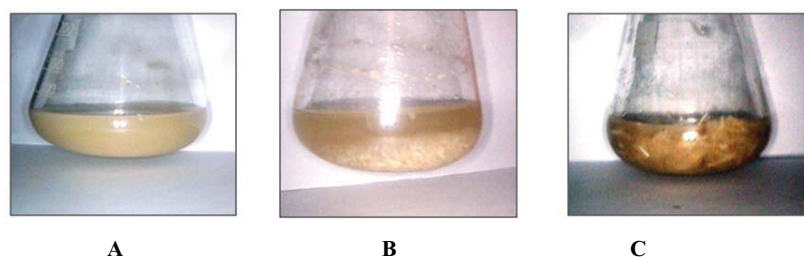


Fig. 15: Hydrolysis of feather in the submerged culture by *Streptomyces albidus* E4 (A) and *Streptomyces griseoaurantiacus* E5 (B) compared with the control (C) without inoculums.

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