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Isolation and Molecular Characterization of Extracellular Keratinase from Xanthomonas sp.: A Potential Approach in Feather Waste Management

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Abstract - **The poultry processing industry produces approx 8.5 billion tons of feathers as by product peryear worldwide. According to Asian Network for Scientific Information India's contributioalone is 350 million tons in 2010. This has become a part of solid waste management since it is tough to degrade feathers due to highly rigid structure rendered by extensive disulfide bonds andcross-linkages. In this present study, out of the isolated fifteen different keratinase producing bacterial stains, three isolates (belonged to the genera *Xanthomonas*) were selected to determine degradation of feathers. Complete degradation of 20g/L feather was achieved in just 5 days without any foul smell. Keratinase activity of 536.66 U/ml was noted after 48 hours incubation. Keratinase produced by *Xanthomonas* was isolated and purified using carboxymethyl cellulose ion–exchange and Sephadex G-75 gel chromatographies. The specific activity of the purified keratinase relative to that in original medium was approximately 86.9 fold. SDS-PAGE analysis and Sephadex G-75 chromatography indicated that the purified keratinase is monomeric and has a molecular mass of 31kDa. Under standard assay condition, the temperature and pH optimum of keratinase was 45°C and 9. Thus, this novel Xanthomonas strain can act as a potential tool to solve the feather waste pollution in short time span. The purified alkaline keratinase hasmultitude of applications in various industries.**

Keywords: Xanthomonas, **Keratinase, Degradation, Feather, Waste Management**

I. Introduction

Around 30 billion chickens, per year, are killed across the world which generates four billion pound (18,14,369 tons) of poultry feather. According to a recent report in leading newspaper India's contribution alone is 350 million tons (Asian Network for Scientific Information, 2010). The poultry feathers are dumped, used for land filling which involves problems in storage, handling, emissions control and ash disposal. Discarded feather also causes various human ailments including chlorosis, mycoplasmosis and fowl cholera (Williams *et al.,* 1990). These wastes largely comprise of the insoluble structural protein keratin. Keratins are structurally split up into α and β keratins. β keratins are mostly characterized in reptiles (i.e. snakes) and birds (Parry *et al.,*1998), whereas α keratins are predominantly found in mammals like humans (Fraser *et al.,* 1986).

In avian feathers, filaments contain pair of twisted β-sheet domains, each formed with a 32 residue domain (Parry *et al.,*1998). The most distinctive feature of keratin is the high concentration of half-cystine residues cross linked and packed as super coiled polypeptides, providing high mechanical stability. They are in soluble and hard to degrade due to highly rigid structure rendered by extensive disulphide bonds and cross linkages. The disulphide cross links produce a compact three dimensional network as a result of intermolecular disulphide bonds between the domains of constituent molecules. Due to this, their degradation by commonly known proteolytic enzymes like trypsin, pepsin and papain is hindered. Hence, there is a demand for developing biotechnological alternatives for recycling of such wastes. Depending upon the nature of keratin a variety of microorganisms exhibit different mechanisms to utilize them as sole source of carbon and nitrogen and in turn play an important role in the degradation of keratin rich wastes.

Despite the recalcitrance, keratin wastes can be efficiently degraded by a myriad of bacteria, actinomycetes and fungi. The most keratinolytic group among fungi belongs to the following genera: *Chrysosporium, Aspergillus, Alternaria, Trichurus, Curvularia, Cladosporium, Fusarium,*. However, they do not have much commercial value as they are categorized as dermatophytes. Among bacterial strains *Lysobacter, Nesternokia, Kocurica, Microbacterium, Vibrio, Xanthomonas, Stenotrophomonas, Chryseobacterium* (Riffel *et al.,* 2003; Yamamura *et al.,* 2002), *Fervidobacterium, Thermoanaerobacter, Bacillus* and *Nesternokia* (Friedrich *et al.,* 2001; Nam *et al.,* 2002) are known keratinolytic bacteria. Besides these, actinomycetes

from the *Streptomyces group,* and the *Thermoactinomyces group,* (Ignatova *et al.,* 1999) is commonly described as keratin degraders.

These microorganisms are capable of producing extracellular keratinase enzyme that helps in the degradation of this recalcitrant wastes. Keratinase [EC 3.4.21/24/99.11] are large serine or metallo proteases. The enzymes have the capacity to act on compact substrates better than other comparable proteolytic enzymes; this distinguishes keratinase from other proteases and peptidases. The microbial degradation of the insoluble keratin depends on the secretion of enzymes with the ability to act on the surface of these substrates. In this sense, keratinase is reported to be mainly extracellular, and the production of such enzymes is generally induced by keratinous substrates such as feathers (Ramnani *et al.,* 2006).

Keratinase from microorganisms have attracted a great deal of attention in the recent decade, particularly due to their multitude of industrial applications such as in the feed, fertilizer, detergent, leather and pharmaceutical industries. Currently, the most promising application of keratinase / keratinolytic microorganisms is the production of nutritious, cost-effective, environmentally benign feather meal for poultry. This poultry feed can also be applied for organic farming. As keratinase have the ability to bind and hydrolyze solid substrates like feather, this important property can be successfully applied in detergent industry as they are required to act on protein substrates attached to solid surfaces, making them attractive additives for hard-surface cleaners (Ramnani *et al.,* 2006). An extended application of keratinase in detergents is their use as additives for cleaning up of drains clogged with keratinous wastes.

Till date most purified keratinase cannot completely solubilize native keratin (Ignatova *et al.,* 1999; Ramnani *et al.,* 2005), their exact nature and uniqueness for keratinolysis is still not clear. Therefore, there is always a need of enzyme isolation from new sources to meet industrial and environmental demand.

II. Materials And Methods

A. ***Processing of Feathers***

Feathers were collected from poultry farms and poultry dumping sites near Siliguri, India. They were washed thoroughly three times with warm water. The washed feathers were packed in autoclavable plastic bags and autoclaved at 121°C at 1 atm. pressure for 90 minutes. After sterilization feathers were dried at 30°C in hot air oven for 3 days. Dried feathers were then cut into fine pieces and stored in plastic bags for future use.

*B.****Isolation And Screening of Feather Degrading Bacteria***

Soil sample was collected from the feather waste dumping sites in Siliguri, India. 1gm of the collected poultry soil was serially diluted till 10^{-5} dilution using standard protocol in order to reduce the initial number of microorganisms. The last three dilutions (10^{-3}, 10^{-4} and 10^{-5}) were inoculated into feather meal broth (NaCl-0.5g/L, K_2HPO_4 - 0.3g/L, KH_2PO_4--0.4g/L, $MgCl_2.6H_2O$-0.1g/L, NH_4Cl-0.5g/L, Feather – 10g/L) and were incubated at 37°C for 48 hours. After 48 hours of incubation period, 1 ml of inoculam from each dilution was spread plated onto the feather meal agar and was incubated at 37ºC for 72 hours. The colonies obtained were purified by repeatedly streaking them on feather meal agar to obtain pure isolated colonies. All the experiments were performed in duplicate.

C. ***Selection and Characterization of Best Keratinolytic Strains***

In order to obtain the best keratinolytic bacterial strains all the colonies from the feather meal agar plates were further inoculated on keratin agar plates by single streak method. Among those colonies, only the colonies exhibiting the highest zone of hydrolysis were selected for further works**.** The morphological and biochemical characterization of the selected bacterial strains were determined by performing following biochemical tests IMViC Test, Hydrogen Sulfide Test, Urease Test, catalase test, Nitrate Reduction Test, Carbohydrate Fermentation, Oxidation, Starch hydrolysis, lipid hydrolysis, cellulose hydrolysis and Gelatin liquefaction (Cappuccino and Sherman, 2004). These tests were performed in duplicates.

D. Antibiotic Sensitivity Determination of Isolates

More than 10 different antibiotics (Clindamycin, Vancomycin, Ofloxacin, Ampicillin, Streptomycin, Erythromycin, Penicillin G, Tetracycline, Gentamicin, Amikacin, Tobramycin, & Co-Trimazine) were selected. The bacterial isolates were spread plated on Mueller Hinton agar plates along with the antibiotic discs and incubated at 37 °C for 24 hours. The area of the inhibition zones was measured.

E. Measurement of Bacterial Growth

Overnight grown culture of isolates was inoculated in 0.2% feather meal broth. Growth of the isolate was determined by recording the absorbance at 620 nm at different time intervals for three days. The same procedure was repeated with increased concentration (0.5%, 1%, 2% and 5%) of the feathers in the media.

F. Keratinase Activity Assay

Keratinase activity was assayed with azo-proteins as a substrate by the following method. The reaction mixture contained 120 µl of enzyme preparation and 480 µl of (10 g/L) azocasein. The mixture was incubated for 30 min at 45˚C in water bath, and the reaction was stopped by the addition of TCA (100g/l). After centrifugation at 10,000g for 5 min, 800 µl of the supernatant were added to 200 µl of 1.8 N NaOH and the absorbance were determined at 420 nm. One unit (U/ml) of keratinolytic activity was defined as an increase of corrected absorbance of 420nm (A420) (Gradisar *et al.,* 2005) with the control for 0.01 per min under the conditions described above and calculated by the following equation: U= volume of final reaction * n * O.D / 0.01* time, where n= dilution.

G. Purification and Molecular Weight of Crude Enzymes

Bacterial isolate was cultivated in feather meal broth for 72 h at 37°C. Culture medium was pre filterated through glass wool to remove un-degraded feathers. Bacterial cells were removed by using centrifuge under cooling (4°C) at 5000 rpm for 10 min. Protein was precipitated by adding solid ammonium sulphate (40%) at 4°C for 24 h. The precipitate was collected by centrifugation at 5000x g for 40 min and was dissolved in a minimal volume of 25 mM potassium phosphate buffer, pH 5.8, the enzyme solution was desalted by dialysis with a cellulose dialysis bag (3500 Da pore-size) in 25 mM potassium phosphate buffer at pH 5.8. After dialysis, the enzyme was dissolved in 2 mL dist. water and applied on carboxymethyl cellulose. The active fractions were collected, lyophilized and applied on sephadex G75. The active fractions were used to determine the molecular weight and enzyme assays. Protein content (mg/mlG1) was determined by Bradford assay (Bradford, 1976).

H. Molecular Weight Determination

Molecular weight of keratinase was detected according to Laemmli (1970) on a 0.75 mm thick polyacrylamide slab gel (7-8 cm) using mini gel system Bio-Rad. Low molecular weight protein standard ranged from 14-97 KD was used to determine the molecular weight of the purified keriatinase. The protein markers were run along side of the sample (25 µg protein sample was used) and protein bands were visualized by Coomassie blue R350 staining method.

I. Characterization of Crude Enzyme

1. Effect of pH

The optimum pH of the crude enzyme was found by dissolving the azocasein in 50mM Tris-HCl buffer at various pH 5, 6, 7, 8 & 9. The experiment on the effect of pH on enzyme stability was carried out by incubating the enzyme solution at pH 5, 6, 7, 8 & 9. Then this enzyme activity was determined by the standard enzyme assay.

2. Effect of Temperature

Thermo stability of the crude enzyme was assessed by incubating the enzyme with the substrate (azocasein solution prepared according to optimum pH i.e. pH 7) at varying temperature range from 30 to 60 °C. Then the enzyme activity was determined by the standard enzyme assay.

3. Effect of Substrate Concentration

With the optimum pH and temperature as constant, optimum substrate concentration was determined by extracting enzyme from broth containing different concentration (0.2%, 0.5%, 1%, 2% and 5%) of. The enzyme activity was then determined using the standard enzyme assay.

4. Effect of Incubation Time

With the optimum pH of the crude enzyme as constant the optimum incubation time was found by incubating the enzyme with the substrate at varying incubation times ranging from 15 to 40 min. Then the enzyme activity was determined using the standard enzyme assay.

J. Degradation of Feather by Isolates

The degradation capabilities of the selected strains were determined by growing the isolates in feather meal broth containing different concentrations (0.2%, 0.5%, 1%, 2% & 5%) of feather for 5 days. The feather degrading activity was visually inspected with the solubilization of feather after every 24 hours intervals (Riffel and Brandelli 2002).

III. Results

1. Isolation of Feather-Degrading Bacteria

Fifteen keratinase producing bacterial stains were isolated from the mentioned site and their morphological and gram character were summarized in Table I. In order to

segregate the best keratinolytic bacterial strain among these fifteen strains, the colonies were checked for the highest zone of hydrolysis along the line of streaking on the keratin agar plates. The highest zone of hydrolysis was found in case of three isolates (KE1, KA, KX). These isolates were thus selected to carry out the further studies.

2. Biochemical Characterization of Isolated Strains

The biochemical characterization of the strains (KE1, KA, KX) were investigated according to the methods described in Bergey's Manual of Determinative Bacteriology. Results were summarized in Table II. The morphological examinations and physiological tests suggest that all the three strains (KE1, KA, KX) belongs to the genera *Xanthomonas*.

3. Determination of Antibiotic Sensitivity

The antibiotic sensitivity of the isolates was determined using a wide range of antibiotics. As shown in figure 1, among the 12 different antibiotics highest zone of inhibition was found against clindamycin in all three isolated stains (KE1, KA, KX). Though all the strain belong to the genera *Xanthomonas* but strain KE1 showed multiple resistances against penicillin, ampicillin and co-trimazine where as strain KA was highly susceptible to all the antibiotics.

4. Measurement of Bacterial Growth

Growth of the isolates was determined by inoculating them in feather meal broth containing 0.2% of feather and recording the absorbance at 620 nm for three days. Each strain showed different growth patterns. The bacterial strain KE1 had an initial lag phase of 6 hours after which the growth curve gradually increased, thus confirming the log phase from 7 hours till 18 hours. Log phase continued till 30 hours after which the isolate gradually reached its stationary phase. Strain KX showed steady increase in growth from the 6 hours and the log phrase continued till 72 hours. Strain KA showed sigmoid growth pattern. It had a prolonged lag phrase till 12 hours. Growth curve gradually took a steep peak and the strain attained log phrase after 12 hours incubation and continued till 42 hours after which it entered the stationery phrase as shown in figure 2. As *Xanthomonas* strain KX shows prolonged log phrase in presence of feather, it was further used to degrade feather.

5 Keratinase Activity Assay

The production of keratinase by *Xanthomonas* strain KX was determined during feather biodegradation, based on the hydrolysis of azoprotein. The keratinase activity was determined using different concentrations of feather at an interval of 24 hours. Initially with lower concentration of feathers, keratinolytic activity was obtained after 24 hours but as incubation time increased all the strains showed increased keratinase production. Maximum keratin production was observed after 48 hours incubation. Keratinase activity was noted 536.66 U/ml after 48 hours incubation (Figure 3). Gradually, keratinase production decreased after 72 hours incubation.

6. Purification and Molecular Weight of Keratinase enzyme:

Purification of microbial enzyme was carried out using different column chromatography and the purified enzyme had more specific activity after the final step. Purification was carried out using two types of column chromatography. Total and specific activities were summarized in Table III. Importantly, the second column sephadex G75 column chromatography used for enzyme purification provided the best yield with an increased purity. This step eliminated proteins that might inhibit keratinase activity. A single protein band was shown on SDS-PAGE gel electrophoresis at 31 kDa (Figure 4).

7. Characterization of Crude Enzyme

The optimum pH of the crude enzyme was found by dissolving the azoprotein in 50mM Tris-HCl buffer at various pH. The keratinase was active in neutral and alkaline condition and showed activity in the range of pH 7 to 10 with an optimum activity at pH 9 as shown in Figure 5 (a). The enzyme activity was studied over a broad range of temperature (30-60°C). The influence of temperature on the keratinase activity was examined at pH 9.0 with azoprotein as substrate. The results showed that the enzyme was active between 30°C and 50 °C with an optimum temperature of 45 °C (Figure 5 b). Further increase in the temperature from 50°C to 60°C resulted in loss of keratinase activity. It has been found that with the increase in substrate concentration the amount of keratinase produced was also increased accordingly as the strain was successfully acclimatized shown in the figure 5 (d). The influence of incubation time on activity of keratinase was determined by extracting enzyme and incubating this

enzyme with the substrate at varying incubation times ranging from 15 to 40 min with the optimum pH and temperature as constant.Maximum activity was observed in the range of 20 to 30 minutes. The optimum incubation time was found to be 20 minutes as shown in the Figure 5 (c).

8 Degradation of Feather by Isolate

The feather degrading capacities of *Xanthomonas* strain KX were inspected visually. The isolates were grown in feather meal broth containing different concentrations of feathers (0.2%, 0.5%, 1%, 2% and 5% w/v). It was observed that all the isolates were able to grow and degrade feathers within 5 days but the rachises were not completely degraded (Figure 6). 70% degradation was observed in presence of 5% (w/v) feather in 2 days. No foul smell was produced during the degradation process.

IV. Discussion

Keratinaceous materials such as feather, wool and hair are insoluble and resistant of degradation by common proteolytic enzymes. 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 poultry industry leads to troublesome environmental pollution and wastage of protein-rich reserve (Grazziotin *et al.,* 2006). Keratinase is an effective enzyme useful for degradation of feather, hair and chicken wastes. It can act converting keratin (insoluble materials) into soluble amino. The keratin degrading microorganism thrives under different ecological and environmental conditions including soil, air, fodder, sand and different environmental wastes (Kansoh *et al.,* 2009).

In this study, effort was made to isolate and characterize a novel bacterial strain that can completely degrade keratin rich wastes into soluble and useful materials. Out of fifteen bacterial isolates recovered from the poultry wastes, three isolates (KE1, KX, KA) has been found to be the best producer of keratinase. This may be due to their high metabolic rate and adaptation to use keratin as carbon and nitrogen source. They degraded feather keratin by using it as a primary source of energy, carbon, nitrogen and sulfur. It was clear that presence of feather in the growth medium act as an enzyme inducer (Ramnani *et al.,* 2005).

The identification of these bacterial isolates was based on several physiological and biochemical tests. Microscopic observation of the isolates showed gram negative motile rod shaped cells. Preliminary biochemical and physiological tests indicate that all three strains (KE1, KA, KX) belonged to the genera *Xanthomonas*.

The degradation of high feather (5% w/v) content by *Xanthomonas* strain KX was a unique characteristic. Complete degradation of 2% (w/v) feather and 70% degradation of 5% (w/v) feather were achieved in just 5 days. Previous reports on degradation on feather wastes were limited till 2% (w/v) feather. This makes the isolated *Xanthomonas* KX strain a potential tool for feather wastes management.

Degradation of feather keratin is hard not only to the supercoiled helical structure of polypeptide chains but also to the strength of intermolecular disulfide bonds and other molecular interactions (Middlebrook *et al.,* 1941). One of the possible mechanisms in breaking down keratin is the reduction of disulfide bonds. For instance, pretreatment of keratin with a reducing agent, such as thioglycolate, disrupts the disulfide bonds and increases digestibility by trypsin (Goddard *et al.,* 1934). However, such a mechanism is not likely in the case of the keratinase. The molecular mechanism of the enzymatic action remains to be determined.

The purification of keratinase was effective and efficient. Protein was purified about 86.9 fold while 37% of the total activity was retained. However, decrease in the total enzyme unit concentration was observed after ion exchange chromatography. This phenomenon was repeatedly observed. The concentration of the proteins may be such that the keratinase is inhibited by an unknown but co-concentrated factor. The keratinase molecule is monomeric. Molecular weights were found to be identical when determined by two different methods, SDS-PAGE and Sephadex G-75 gel chromatography.

The keratinolytic activities were detected between 35 and 45°C with an optimum at 45 °C. An optimum keratin-degrading activity at mesophilic temperatures is a desirable characteristic because these microorganisms may achieve hydrolysis with reduced energy input. The enzyme was active in neutral and alkaline condition and showed activity in the range of pH 6 to 10 with an optimum activity at pH 9. It has been found that initially with lower concentration of feathers (till 1% w/v) highest activity was obtained after 24 hours; however as the substrate concentration was gradually increased to 2% and 5%, maximum production obtained after 72 hours, which indicated successful acclimatization of the strain.

V. Conclusion

To conclude, though much work has been done on feather degrading microorganisms, to the best of our knowledge very few reports are available on feather degrading *Xanthomonas* strain. This newly discovered *Xanthomonas* sp has the capability to degrade feather wastes in short time span without producing any foul smell. Purified keratinase from *Xanthomonas* sp. was characterized in terms of its biochemical properties and found to have a broad temperature and pH specificity. The purification and characterization studies of the keratinase have provided the basis to develop further the production and uses of this enzyme.

Table I Morphological Characterization and Zone of Keratin Hydrolysis by Isolated Strains

Sample	Gram	Morphology	Arrangement	Shape	Surface	Colour	Margin	Elevation	Opacity	Keratinase
KA	-	Small rod	Chains	round	Smooth	Yellow	Entire	Raised	translucent	+++
KA2	+	Long rod	Chains	Circular	Rough	White	Entire	Raised	Opaque	+
KA3	+	Small rod	Chains	Circular	Rough	White	Lobate	Raised	Opaque	+
KX	-	rod	Chains	Circular	Rough	yellow	Entire	Raised	Opaque	+++
KA5	-	Cocci	Chains	Circular	Smooth	Glistening	Entire	Flat	Translucent	++
KA6	+	Cocci	Clusters	Irregular	Rough	White	Entire	Raised	Opaque	+
KE1	-	Small rod	Short chains	round	Smooth	yellow	Entire	Raised	Translucent	+++
KA8	-	Cocci	Pairs	Circular	Smooth	Glistening	Undulate	Raised	Translucent	++
KA9	+	Small rod	Chains	Circular	Rough	White	Entire	Raised	Opaque	+
KA10	+	Long rod	Single	Circular	Rough	Yellow	Entire	Flat	Opaque	+
KA11	+	Long rod	Single	Circular	Rough	White	Entire	Raised	Opaque	+
KA12	-	Cocci	Clusters	Circular	Rough	White	Lobate	Raised	Opaque	++
KA13	+	Cocci	Chains	Circular	Rough	Yellow	Undulat	Raised	Opaque	+
KA14	+	Small rod	Single	Circular	Rough	Orange	Entire	Raised	Opaque	+
KA15	+	Long rod	Chains	Circular	Rough	White	Entire	Raised	Opaque	+

Table II Biochemical Characterization of The Isolated Strain Showing the Highest Keratinase Activity

Biochemical Characteristics	KE1	KX	KA
Indole production	-	-	-
Methyl Red Reaction	+	+	+
Voges-Proskauer Reaction	-	-	-
Citrate utilization	-	-	-
Catalase	++	++	+++
Gelatin liquefaction	+++	++	+++
Starch Hydrolysis	++	+++	++
Lipid Hydrolysis	+	+	+
Casein Hydrolysis	+++	++	+++
Cellulose Hydrolysis	-	-	-
Hydrogen sulphide Production	+	-	-
Urease Activity	-	-	-
Oxidation/Fermentation	+	+	+
Nitrate Reduction	-	-	-

(Symbol: '+': positive, '++': moderately positive '+++': highly positive and '-': negative).

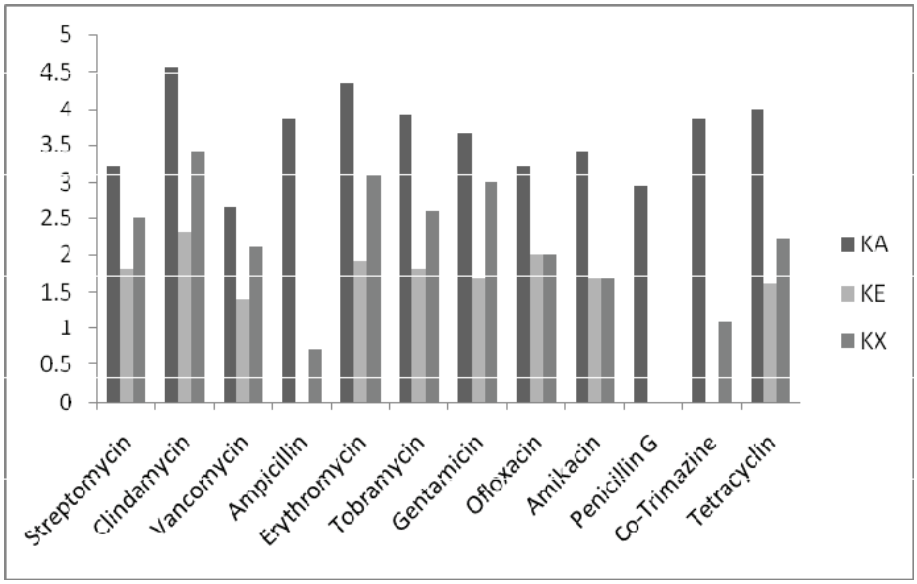


Fig.1 Antibiotic sensitivity determination of isolated strains

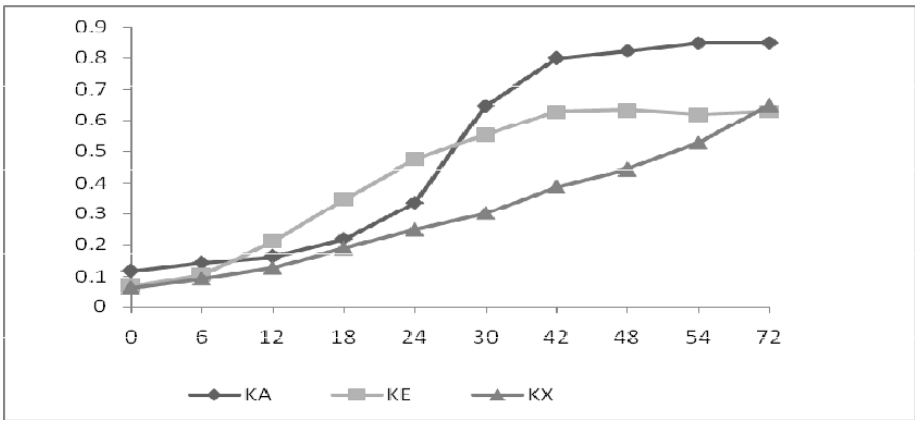


Fig. 2 Growth curve determination of the isolated strains

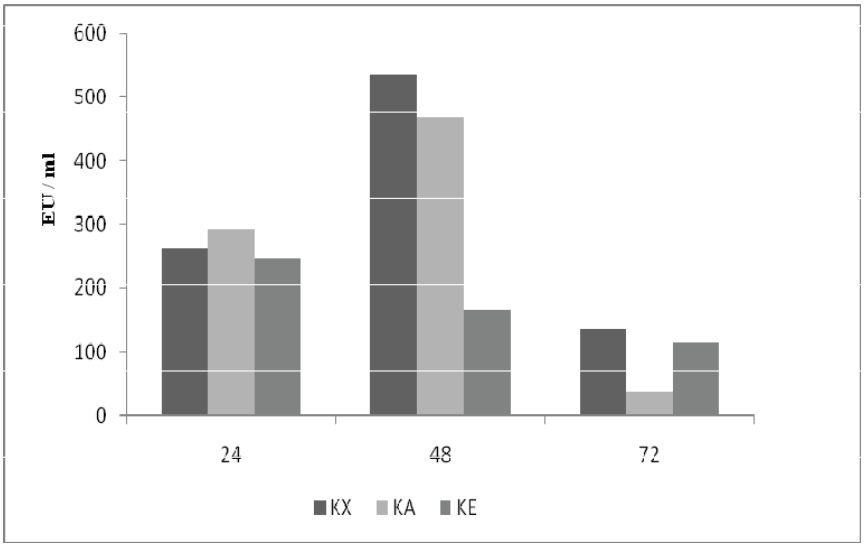


Fig. 3 Keratinase activity by the isolated strains

Table III Purification of Keratinase Enzyme

Step	Total protein (mg)	Total Activity (U)	Specific Activity (U /mg)	Purification Fold	Yield (%)
Ammonium sulphate	820	4071	4.96	1	100
CM-cellulose	18.1	1213	67	13.5	29.7
Sephadex G-75	3.49	1506	431.51	86.99	37

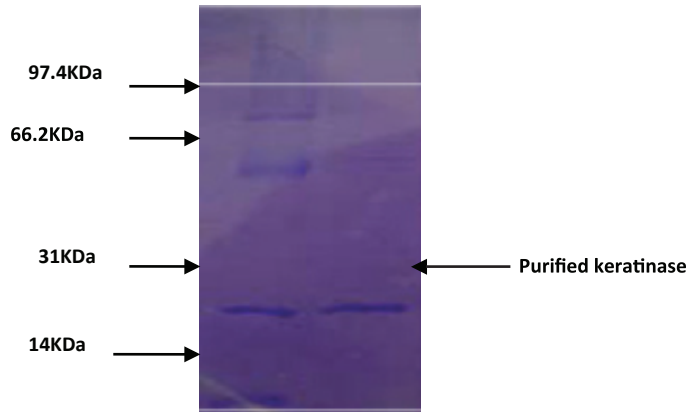
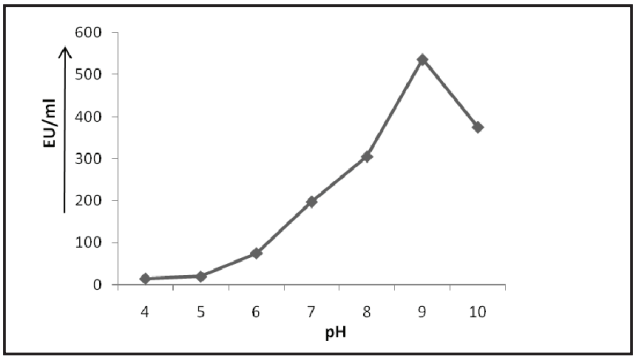
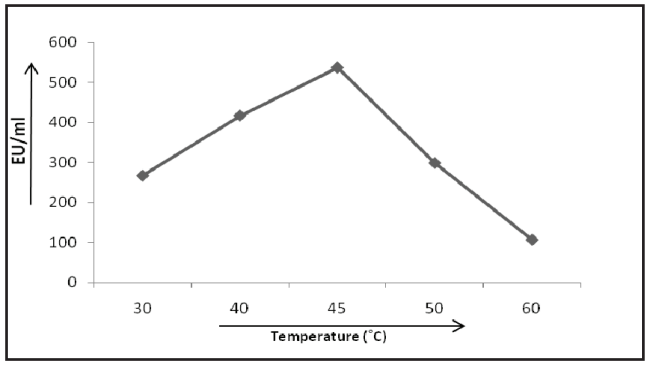


Fig. 4 Molecular weight of keratinase isolated from Xanthomonas sp. by SDS-PAGE

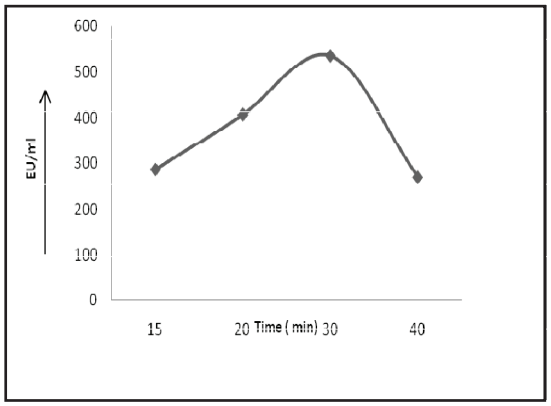
(First lane: molecular mass marker proteins. Rabbit phosphorylase B (97.4 KDa), bovine serum albumin (66.2 KDa), carbonic anhydrase (31 KDa) and lysozyme (14 KDa). Second lane: Purified keratinase)



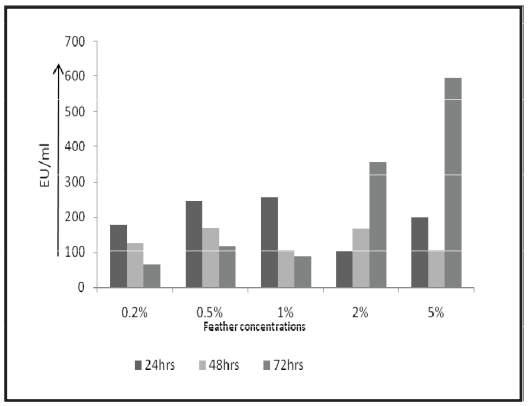
(a) Effect of pH



(b) Effect of temperature



(c) Effect of incubation time



(d) Effect of substrate concentration

Fig.5 Characterization of keratinase

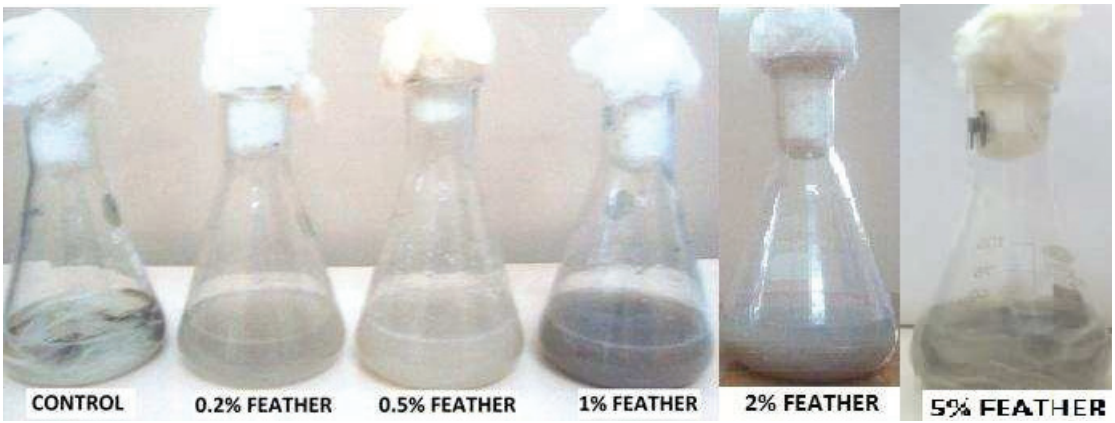


Fig. 6 Degradation of feather by Xanthomonas sp strain

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