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Production of keratinase from a new strain of *Pseudomonas aeruginosa* gmp and its application for the removal of dyed keratin waste

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ABSTRACT

Keratins are insoluble structural proteins of skin, hair, horn, wool and claws. It is resistant to the action of common proteolytic enzymes however keratin can be hydrolyzed by microorganisms that can produce keratinolytic enzyme or keratinase. The objective of this work was the production of keratinase and degradation of keratin from microorganism isolated from poultry soil in Trichy. The biochemical and molecular characterization study reveals that the bacterial strain is *Pseudomonas aeruginosa* gmp with accession number JX027506. The optimum temperature and pH for keratinase production was found to be 70^0C and 7.0. The time course for keratinase production was found to be around 6 days. *Pseudomonas aueruginosa* gmp could degrade waste dyed keratin in 12 days.

KEYWORDS

Keratinase;
Pseudomonas aueruginosa gmp;
16s rRNA.

INTRODUCTION

Environmental pollution due to the generation of solid keratinous waste remains a concern till date. This includes waste generated as feather keratin, waste from human foot care centers and tanneries. The mostly problematic are feathers which are produced in large quantities and are the most abundant keratinous material. Annually the generation of feather waste crosses several million tons in the world[1]. Keratin is a tough insoluble protein and a major constituent of feather, hair, horn, hoof and claws[2,3]. As much as 90% of feathers are made up of keratin, the insoluble structural protein.

Kertinases are a class of serine or metalloprotease that are capable of degrading the insoluble keratinous substrate. Keratinolytic enzyme has been isolated from bacteria, fungi and acetinomycetes. The production and purification of keratinase from non-pathogenic microbial sources are important inorder to completely degrade feather keratin to produce an useful products[4,5].

The specific mechanism of keratin degradation by keratinase is still not well elucidation but appears to occur in two main steps: deamination and cleavage of disulphide bond. Deamination creates an alkaline environment needed for substrate swelling, sulphitolysis and proteolytic attack[6,7]. However, Willam *et al* (1990)[8] reported that keratinase degradation of keratins occurred without an accompanying release of soluble sulfhydryl,

which suggest that the degradation possibly follow different pathways, depending on the type and the origin of the keratinase and the prevailing degradation conditions. Therefore, keratinases may show variable activity towards different keratin substrates as well.

The objective of this paper mainly focuses on to isolate a novel keratinolytic organism from poultry soil in Trichy, Tamil Nadu that could effectively degrade keratin and also for production of keratinase. Also screening for the ability of the keratinolytic microorganism for degradation of dyed keratin was done.

MATERIALS AND METHODS

Isolation and screening of keratinase producing bacteria

Soil sample was collected from poultry soil in Trichy, Tamil Nadu, India. 1 g soil sample was serially diluted and a dilution of 10^{-5} was suspended in 100 ml peptone broth and kept for growth for 3 days. This suspension was then re inoculated into Feather meal media of following composition in g/l: KH_2PO_4 0.4, K_2HPO_4 0.3, Yeast extract 0.1, NaCl 0.5, Feather 10 (pH 7.0). The culture broth in which feather degradation was confirmed visually was used to further streak the feather meal agar plates of composition in g/l: Feather meal 10, NaCl 0.5, KH_2PO_4 0.4, K_2HPO_4 0.3, Yeast extract 0.1, Agar 15. Organism was selected based on ability to hydrolyze keratin in feather meal media.

Feather substrate

Chicken feather were collected from local poultry plant. It was washed and cleaned twice with detergent and rinsed with sterile distilled water and dried in hot air oven and stored till further use.

Bacterial identification and phylogenetic analysis

The cultural, morphological and biochemical characteristics of the microorganisms were identified with Bergeys Manual of Determinative Bacteriology[9]. The molecular characterization was done by using 16S rRNA sequencing technique. The genomic DNA was extracted from the given organism using standard procedures[10]. The 16S rRNA gene amplification was performed using the universal primers. Forward primer: 5'AGAGTTTGATCCTGGCTCAG-3' and Reverse Primer: 5'-ACGGCTACCTTGTTACGACTT-3'. The amplified product was sequenced and the data was analyzed for its similarity by using BLAST[11] and by using MEGA 4.1 software[12] the phylogenetic analysis was done and the tree obtained by means of neighbor-joining method was analyzed for related organisms[13].

Keratinolytic and proteolytic activity assay

Kerationolytic activity and proteolytic activity was assessed spectrophotometrically by modified Folin-Ciocalteau method[3].

Protein determination

The protein content of the sample was determined using Lowry's method[14].

Fermentation of keratinase enzyme production and characterization

The media used for keratinase production was feather meal media of the following composition in g/L: KH_2PO_4 0.4, K_2HPO_4 0.3, Yeast extract 0.1, NaCl 0.5, Feather 10 (pH 7). Feathers in the media served as the carbon and nitrogen sources.

Effect of carbon and nitrogen sources

The effect of different carbon and nitrogen source on keratinase was studied with additional carbon and nitrogen sources like glucose, maltose, fructose, galactose, sucrose, lactose, soy meal media, yeast extract, gelatin and peptone. The concentration of these carbon and nitrogen sources was 0.5 g/L of media.

Effect of initial pH and temperature for keratinase production

Several factors such as pH, growth substrate, and effect of heat significantly influence the synthesis of keratinases in a fermentation culture[15]. A proper balance of these factors results in optimum synthesis of specific proteases of interest. To determine the optimization conditions for keratinase production, fermentation cultures were setup at temperature ranges from 20-90°C and pH ranges from 5-10.

Extraction and partial purification of keratinase enzyme

Ammonium sulphate precipitation of keratinase

Keratinase enzyme was partially purified with 30%

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ammonium sulphate precipitation. Centrifugation was carried out at 10,000 rpm, 20 min at 4°C and dialysis was carried out overnight against the phosphate buffer with alternating change of buffer.

Characterization of keratinase enzyme

1. Effect of pH and temperature on enzyme activity and stability

The effect of pH and temperature on the enzyme activity and stability was determined at 37°C using various pH buffers. Sodium citrate (pH 3.0–4.0), Sodium phosphate (5.0-6.0), KH_2PO_4-K_2HPO_4 (7.0-8.0), sodium carbonate (9.0-10.0), Glycine NaOH (11.0-12.0). The effect of temperature was tested by varying the temperature of the enzyme reaction (20 to 90°C). Enzymes were incubated for 30 minutes at varying temperature between 20-90°C. The residual activity was measured using keratinase assay.

2. Effect of metal ions and inhibitors

The chemicals like EDTA, SDS, Triton X-100 and metal ions (10mM) like Mg^{2+}, Ca^{2+}, Al^{3+}, Mn^{2+} were used to study the inhibitory effects and influencing activity of keratinase enzyme.

3. Substrate hydrolysis

The hydrolytic efficiency of keratinase was studied by incubating the enzymes with different substrates namely feather, hair, nails, human epidermis, wool by substituting these as a substitute of keratin. The keratinolytic activity was determined. The % relative activity was measured.

Degradation of dyed keratin waste

Malachite green solution was prepared at a concentration of 0.01g in 100 ml at a pH 5.0. The adsorption of dye onto chicken feathers was done by agitating 50 ml of dye solution in conical flask with chicken feathers which served as adsorbent. Experiments were conducted at room temperature and at a constant speed of 200 rpm, maintaining the pH at 5.0. These flasks were kept in shaker for 24 hours and then the solution was filtered using whattman filter paper. The dye adsorbed feathers obtained after adsorption experiments were used as the carbon and nitrogen sources to check the degradation efficiency by the isolated organism.

RESULT AND DISCUSSION

Isolation and characterization of feather degrading bacterium

The results of morphological, biochemical tests and molecular characterization of the isolated strain using 16srRNA sequencing showed high similarities of *Pseudomonas aeruginosa* and it was deposited in GenBank with the accession number JX 027506 and named as *Pseudomonas aeruginosa* gmp. Gram positive bacteria's like Bacillus sp., and Streptomyces sp., were widely used to degrade feather keratin. However only few reports are available for the degradation efficiency for gram negative bacteria's like *Vibrio* sp, *Chryseobacterium* sp, *Pseudomonas aeruginosa* sp[16-18]. TABLE 1 explain the biochemical characteristics of isolated bacteria show the rod shaped, green pigment producing, gram negative, non motile, catalase

TABLE 1 : Morphological and biochemical characteristics of *Pseudomonas aeruginosa* gmp

TEST	OBSERVATION
Shape	Rod
Gram reaction	Gram negative
Motility	Non motile
Catalase	+
Oxidase	+
Indole	-
MR	-
VP	-
Simmons citrate	+
Triple iron sugar	+
Amylase	+
Pectinase	+
Lipase	+
Arginine	+
Lysine	-
Dextrose	+
Fructose	+
Lactose	+
Sorbitol	-
Sucrose	+
Xylose	-
Gelatinase	+
Cellulose	+
Starch	+

positive, gelatinase positive bacteria. The phylogenetic tree of 16s rRNA gene sequence which shows the position of *Pseudomonas aeruginosa* gmp (MNF7) (Data not shown). Thus it confirms the isolated new organism is *Pseudomonas aeruginosa*.

Effect of carbon and nitrogen sources

The addition of carbon and nitrogen sources caused an increase in production of keratinase. Figure1 shows the effect of different carbon sources on keratinase production. However media supplemented with maltose shows highest enzyme production (133.9 U/ml). Whereas addition of other carbon sources like glucose, fructose and lactose shows minimum keratinase production. Figure 2 depicted the effect of organic and inorganic nitrogen source on keratinase enzyme production. Among all organic nitrogen sources yeast extract exhibited maximum keratinase production (85.1 U/ml) followed by soy meal (77.6 U/ml) and the effect of inorganic nitrogen sources ammonium sulphate induce enzyme production (63.9 U/ml) followed by potassium nitrate (58.6 U/ml). The carbon source espe-

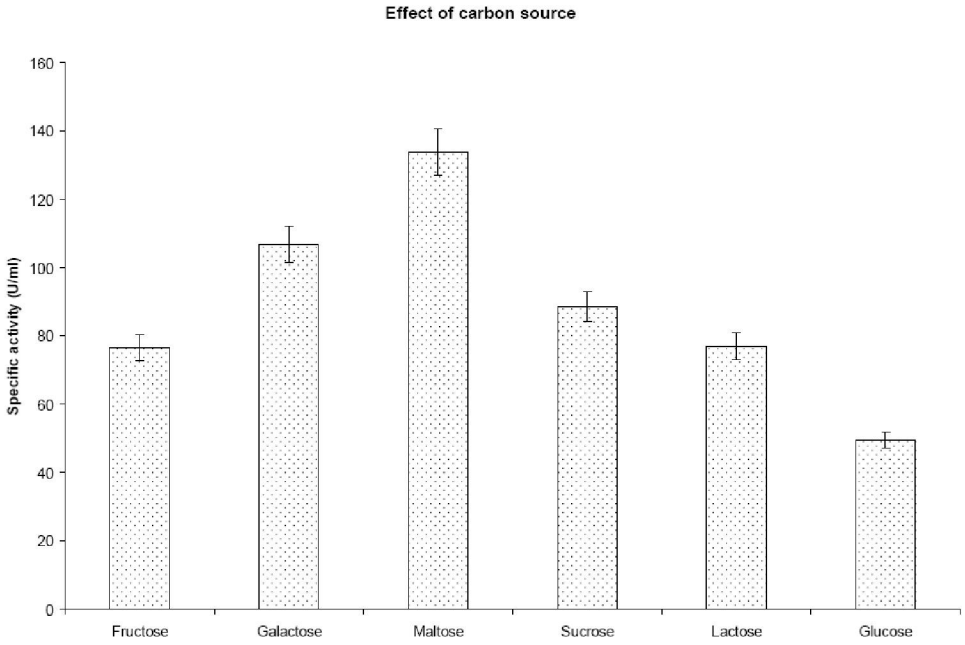

Figure 1 : Effect of carbon source on keratinase production

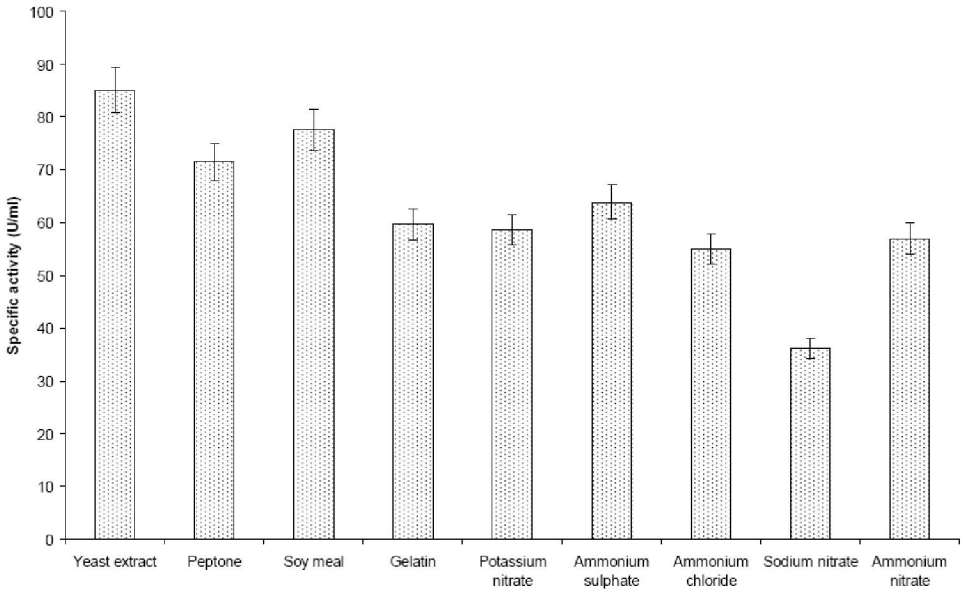

Figure 2 : Effect of organic and inorganic nitrogen source on keratinase production

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cially glucose inhibited the production of keratinase because it has tendency to act as a catabolite repressor[4,15]. In this case yeast extract served as inducer, soya meal also induced the production to some extent.

Effect of initial pH and temperature on keratinase production

The optimum pH for the production of keratinase was found to be 7.0 (Figure 3). The production of keratinase was low in the media with acidic pH 5.0 and in alkaline pH of 10.0. This shows that the optimum pH for the production of keratinase is 7.0. The effect of initial pH is very important parameter for the growth and morphology of the bacteria. If the pH of the medium is increased it affects the metabolic activity and morphology of the bacteria[18,19]. Production of keratinase enzyme was maximum at 40 ^{0}C (Figure 4). At this temperature the enzyme production was 89.9U/

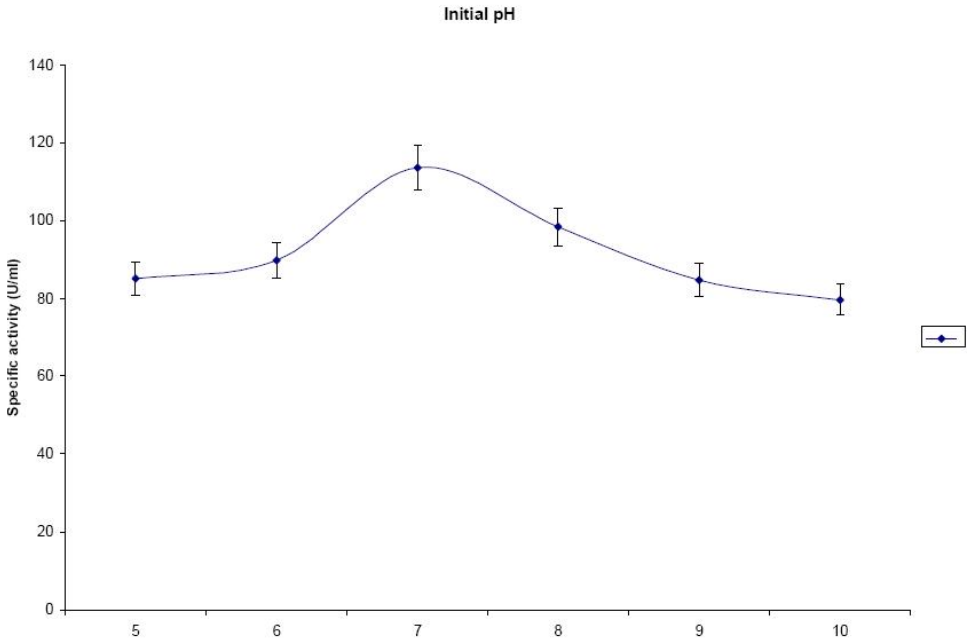

Figure 3 : Effect of initial pH of keratinase production

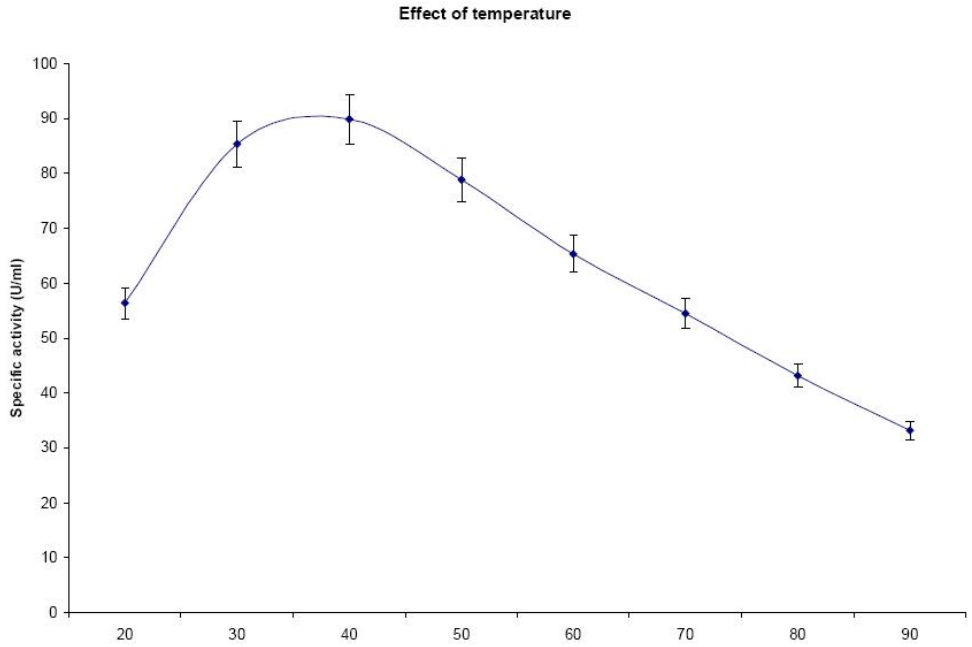

Figure 4 : Effect of temperature on keratinase production from *Pseudomonas aeruginosa* gmp.

ml followed by 30 ^{0}C (85.4 U/ml). Similar observation was also found keratinase production from *pseudomonas* sp.,[19].

Characterization of keratinase from *Pseudomonas aeruginosa* gmp

Effect of temperature on keratinolytic activity and stability

The maximum activity and stability of the purified enzyme was found to be at 70°C and 50-80°C respectively. It retains more than 80% residual activity at these temperatures and maximum stability was attain at 70°C (Figure 5). This shows the thermostable nature of the keratinase produced from newly isolated *Pseudomonas aeruginosa gmp*. There are also reports suggest the keratinase activity and stability was at around 50°C for *Pseudomonas aeruginosa* KS-1[20] which is contrasting to our findings. Because of the thermostability nature of keratinolytic enzyme it has been used for various industrial processes.

Effect of pH on keratinolytic activity and stability

The pH activity and stability of keratinase enzyme purified from the bacteria was shown in Figure 6. The keratinase activity was found to be in the range of 6.0-9.0 which is slightly acidic to alkaline condition and optimum activity was at 7.0. The stability of the enzyme was assessed at p H ranges from 3.0-12.0 and the optimum was found to be from 5.0-9.0 and shows optimum stability at pH 7.0. Previous literature suggest that there keratinase from bacteria are found to be in between 7.0-12.0 [21].

Hydrolysis of various substrates by keratinase

This strain could effectively hydrolyse several soluble and insoluble proteins (TABLE 2). The keratinase was able to hydrolyse various substrates in order Gelatin> Human epidermis> Nail> Wool> Hair> Feather. This widens the use of keratinase from *Pseudomonas aeruginosa* because of its substrate specificity. Keratinases are reported to have broad substrate specificity.

Effect of metal ions and inhibitors on activity of keratinase

TABLE 3 shows the effect of metal ions and inhibitors on activity of keratinase enzyme produced from *Pseudomonas aeruginosa* gmp. Metal ions were found to inhibit activity of keratinase in contradictory to some reports but it should be taken note that keratinase from gram negative bacteria are different from gram positive. EDTA was found to strongly inhibit the activity of keratinase in accordance with previous works done by Sanaa *et al*. 2008[22]. The order was Al^{3+}> Mn^{2+}> Triton X-100> SDS> Ca^{2+}EDTA> Mg^{2+}.

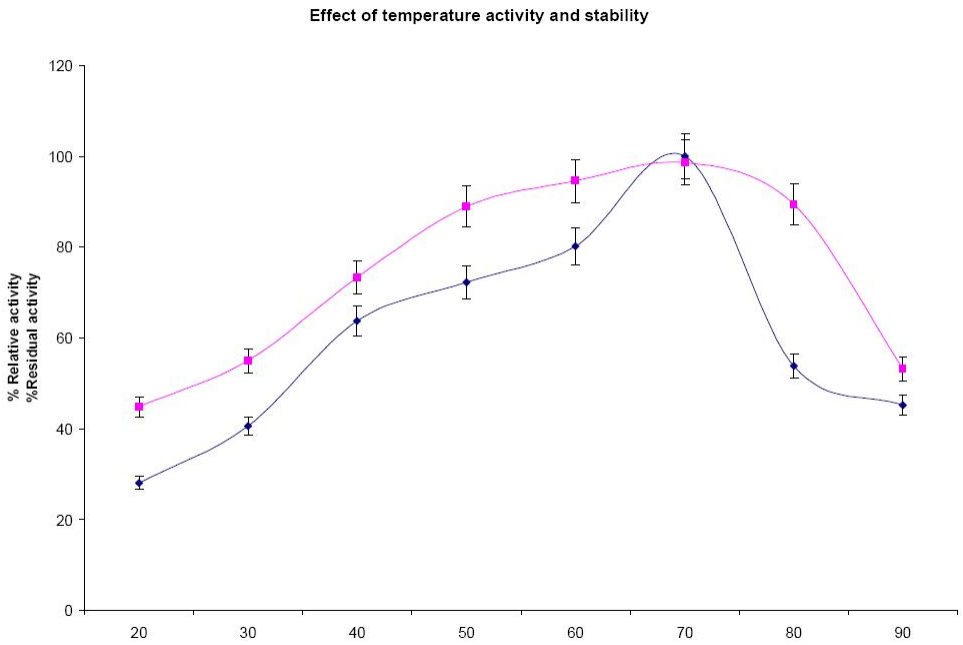

Figure 5 : Effect of temperature activity and stability of keratinase.

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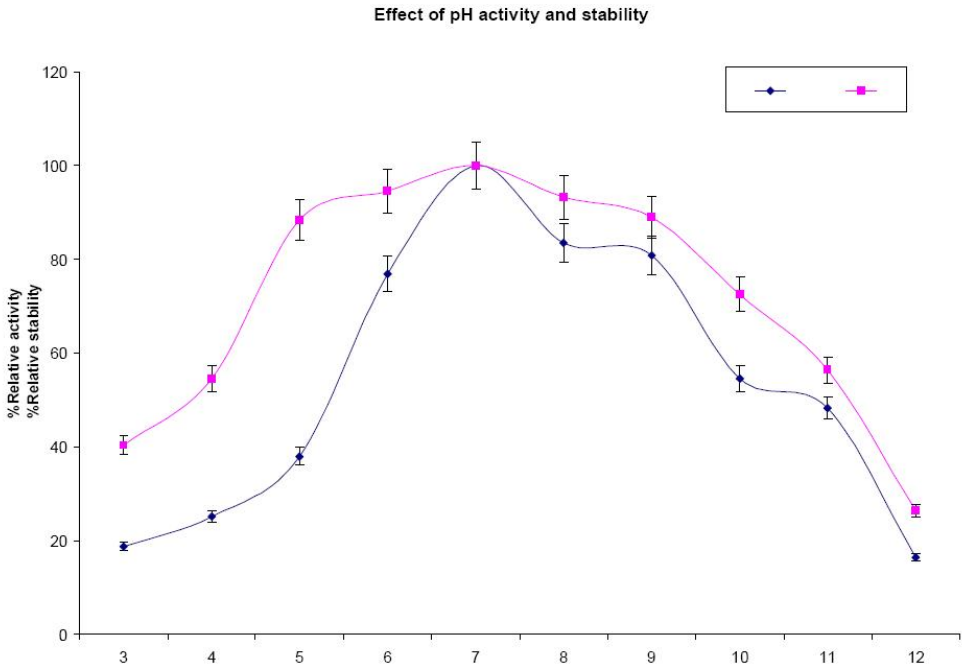

Figure 6 : Effect of pH activity and stability on keratinase enzyme.

TABLE 2 : Substrate hydrolysis by keratinase

Substrates	% Relative activity
Feather	100
Hair	110
Nail	124
Gelatin	142
Wool	85
Human epidermis	108

TABLE 3 : Effect of metal ions and inhibitors on keratatinase activity

Compound	% Residual Activity
Control	100
SDS	78
EDTA	52
Triton X-100	80
Mg^{2+}	48
Ca^{2+}	74
Al^{3+}	92
Mn^{2+}	83

Application for the degradation of dyed waste keratin

Adsorption kinetics of dye using chicken feathers has already been studied[23]. 3 hours is the required saturation time for the adsorption of dye using chicken feathers as adsorbent. *Pseudomonas aeruginosa* gmp could successfully degrade the dyed keratin in 12 days (Data not shown).

CONCLUSION

Pseudomonas aeruginosa gmp (JX027506) was isolated from poultry soil and its efficiency of keratinase enzyme production was studied. The characteristics of keratinase enzyme were found to be at 70ÚC and 7.0. The time course for keratinase production was found to be around 6 days and it was capable to degrade waste dyed keratin in 12 days.

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