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Screening of Keratinolytic Bacteria and Keratinase Production by using Pseudomonas aeruginosa isolated from Poultry

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ABSTRACT Enzymes keratinase used for degradation of keratins and it is found in animal hair, nails, hoofs and feathers. Feather waste is generated in large amounts as a by-product of commercial poultry processing and cause serious environmental problems. In the present study a total of 25 strains were isolated and identified based on morphological and biochemical Characterization and analysis of 16S rDNA sequences. Among them a potential feather degrading organism Pseudomonas aeruginosa K13 was shown highest Keratinolytic activity and its protein concentration was 280 U/ml. The intense feather degradation was achieved at 400C and at pH to 7 and 8. The metal ions including Zn2+ and Cd2+ enhance the keratinolytic activity and Hg2+ and Mg2+ inhibits the keratinolytic activity. The activity of 60% precipitate enzyme was found to be 1056 U/ml. This precipitate was subjected to Ion exchange chromatography for further purification process. The fraction number 17 shows 11.74 mg/ml concentration of protein and enzymatic activity was found to be 11.44 U/ml. From the gel, it was observed that molecular mass of keratinase of K13 isolate was 38 kDa. The potential bacterial isolates can be used for degradation of feathers waste of commercial poultry processing.

KEYWORDS : Keratinase, Poultry farm, Protease, feather, protein, bacteria

INTRODUCTION

Poultry processing units as well as poultry farming has become one of the major units for producing certain poultry products nowadays. Indian Poultry Industry is emerging as the world's second largest market and growing at a phenomenal rate of 12 to 15% every year (Agrahari et al., 2010; The Ministry of Food Processing Industries, India). Feather waste of poultry farms comprises high percentage of keratins (Poole et al., 2009). Feather keratin is not easily degradable protein. Disposal of poultry waste constitute a problem of environmental pollution. Different approaches like land filling, burning, acid or alkali hydrolysis of feathers are the methods for the poultry waste treatment. Acid or alkali treatment of feathers may lead to destruction of amino acids and thus decreasing the nutritive value of the protein.

Keratinase producing microorganisms are being increasingly utilized for degradation and recycling of poultry feather waste. Certain microorganisms produce proteases or keratinase which hydrolyze keratin without loss of amino acids. Hydrolysis of poultry waste by microbial keratinase and improving the nutritional value represent the innovative and ecofriendly approach for treatment of keratinous waste. This enzyme was isolated from bacterial spp. like Bacillus licheniformis, Pseudomonas, Chryseobacterium, Burkholderia. Bernall et al., (2006) also studied different parameters of keratinase. This is a potential enzyme applied for removing hair and feather in the poultry industry (Takami et al., 1992), and for nutritional upgrading of feather meal and conversion of feathers into a feed protein in feed industry. Keratin hydrolysate can be used commercially as organic fertilizers and production of rare amino acids.

They are widely used in commercial products, for example in laundry and dishwashing detergents, cosmetics, food processing, skincare ointments, and contact lens cleaners and for research purposes in synthetic organic chemistry (Gupta et al., 2002; Krishna, 2002).

The aim of this study was to obtain a keratinolytic enzyme with high ability to degrade the keratinous wastes from poultry industry. Use of keratinase or keratinolytic microorganisms is the alternative for recycling of keratinous waste as well as degradation of feathers waste of commercial poultry processing.

MATERIALS AND METHODS

Soil sample collection: Collection of soil sample was done from different poultry processing units and feather dumping sites at Vita region of Ahmednagar District, Maharashtra State India. These poultry farms were selected as these regions are having highest number of poultry units and are convenient for isolation of feather degrading microbes. The samples were collected from about 10-12 cm depth of the soil at poultry farms. All the samples were collected in polythene bags and processed on the same day for the isolation of keratinolytic bacteria.

Isolation and screening of keratinolytic bacteria: Soil samples were suspended in Basal salt medium (composition) and kept for growth at 300C for four days for enrichment. This suspension was further inoculated in Nutrient agar medium and Hichrome Bacillus agar medium for isolation of Keratinase producers. All the isolates were reinnoculated in sterile milk agar plates for primary screening of keratinase producers. The isolates with clear zone of hydrolysis on milk agar were selected for further study.

Screening for keratinase production: Isolates showing largest clear zone of hydrolysis were tested for feather degradation. The flasks containing basal salt medium and feathers were inoculated with the isolates and were incubated at temperature 300C on a rotary shaker at 140 rpm for 4 days. Feather degradation in culture broth was confirmed visually. After 24 hours of incubation at regular interval the activity of keratinase was measured and the isolates showing maximum activity were selected for further analysis.

Identification of the Keratinolytic bacterial strain

The morphological and biochemical characteristics of the isolated bacterium were studied according to Bergey's Manual of Systematic Bacteriology (Brenner et al., 2004). Further identification of the microorganism was performed through 16S rDNA sequencing. Chromosomal DNA was extracted from 18 h culture cells using a CTAB method (Xu et al., 2007). The 16S rDNA was amplified by PCR using a DNA thermal cycler (Prism, MWG-Biotech). The bacterial 16S rDNA primers used for gene amplification and sequencing were 8f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACCTTGTTACGACTT-3'). After initial denaturation at 95°C for 10 min, the PCR reaction was carried out using Pyrobest Taq polymerase. The conditions for each cycle were as follows: denaturation at 94°C for 1min, annealing at 57°C for 1min, and extension at 72°C for 90 s. A final extension step for 10min at 72°C was performed at the end of the 35 cycles. The amplified PCR products were sequenced by geneOmbio technologies, Pune; India. Nucleotide sequence of the amplified PCR products was compared with other deposited sequences in the Genbank via the online programme BLAST (basic local alignment search tool).

Protein estimation and keratinolytic activity determination: The isolates were cultured at 30°C for 4 days on a rotary shaker at 180 rpm for the production of keratinolytic protease. The culture medium (g/L, pH 7.5) was supplemented with feather powder, 30; trypton, 5; MgSO4, 0.1; yeast extract, 5; K2HPO4, 1 and distilled water. Protein concentration was determined at 750 nm according to Lowry method (Scopes 1982) using bovine serum albumin (BSA) as the standard. The keratinolytic activity was determined spectrophotometrically using a modified Folin-Ciocalteu method (Margesin et al., 1991). Keratin meal was used as the substrate. The 1 g keratin azure was suspended in 100ml 50 mmol/L Tris-HCl buffer (pH 7.0). The reaction mixture contained 1 mL keratin meal suspension and 1 mL crude enzyme. The reactions were carried out at 500C within a boiling water bath for 10 min. After incubation, the reactions were stopped by adding 2mL 0.4 mol/L trichloracetic acid (TCA) and followed by filtration to remove the substrate. The filtrate was spectrophotometrically measured at 660 nm. One unit (U) of keratinase activity was defined as the amount of enzyme causing 0.01 increases in absorbance between sample and control at 660nm after one hour under the conditions given.

Formula for the Enzyme activity Units / ml enzyme = 4 x O.D. at 660nm x Dilution Factor / 0.01 x Time of incubation

Purification of the keratinolytic protease

The culture supernatant was obtained by centrifugation at 10,000×g for 5 min, then solid ammonium sulfate was gradually added to 30% saturation. The precipitate formed was removed by centrifugation at 10,000×g for 5 min. After further addition of solid ammonium sulfate to supernatant until 50% saturation, the resulting precipitate was collected by centrifugation at 10,000×g for 5 min. The collected precipitate was dissolved in Tris–HCl buffer (25 mM, pH 7.8), dialyzed against 10 mM of the same buffer for 24 h, and concentrated. Next purification step of the concentrated sample was carried out through a sephadex G-75 gel filtration column equilibrated with 10 mM Tris–HCl buffer (pH 7.8). The keratinolytic active fractions were eluted with the same buffer, pooled and concentrated. Insoluble substances were removed by centrifugation at 10,000×g for 5 min, and the sample was applied to a DEAE sepharose fast-flow column equilibrated with 10 mM Tris–HCl buffer (pH 7.8). Elution was performed with a linear gradient of Tris–HCl buffer (10 mM, pH 7.8) containing NaCl from 0 to 0.5 M. The fractions possessing keratinolytic activity were pooled and concentrated. The active fractions eluted from DEAE sepharose fast-flow column were used for keratinolytic activity analysis. All purification steps were performed at 4°C. To examine the purity and determine the molecular weight of the enzyme, discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed with 12% polyacrylamide gel as described (Laemmli et al., 1970). The protein bands were stained with a solution of Coomassie blue R-250.

The effect of different parameters on enzyme activity: Enzyme characterization was done by Allpress et al., (2002) method. The optimum pH of the crude enzyme was determined by using the keration at various buffers using 50 mM acetate buffer (pH 4-5.5), 50mM phosphate buffer (6-7.5), and 50 mM Tris-HCl buffer (8-9) Based on the above mentioned pH the keratin solution is prepared for various buffers. The experiment on the effect of pH on enzyme stability was carried out by incubating the enzyme solution at pH ranges of 4-9. Then the activity determined by standard enzyme assay. The optimum pH of the crude enzyme as constant the optimum temperature was determined by incubating the 1ml of crude enzyme with 1 ml of substrate (keratin solution was prepared according to optimum pH) at varying temperature range from 300C to 800C. The experiment on the effect of temperature on enzyme stability was carried out by incubating the enzyme solution at temperature ranges of 30- 800C. Then the activity determined by standard enzyme assay. The Km and Vmax value for the crude keratinase is determined by using different concentration of keratin. For this stock solution of keratin is prepared by dissolving 0.5 g in 50 ml phosphate buffer of pH 7. The stock solution is diluted for different concentration (0.1 – 1.0 g) in the series of test tubes using phosphate buffer. Then the activity determined by standard enzyme assay.

Effect of various metal ions on enzyme activity: To study the influence of metal ion, crude enzyme was incubated in the presence of Zinc, magnesium, mercury and cadmium (Zn2+, Mg2+, Hg2+ and Cd2+) at 1-3 mM concentration for 1hr at 370C. Then the activity was determined by standard enzyme assay. The purified protease were preincubated in barbital buffer (pH 7.5) at 30°C for 10 min with Ca2+, Mg2+, Mn2+, Co2+, Cu2+, Zn2+, Hg2+, Pb2+, Fe2+, Li+, ethylenediaminetetracetic acid (EDTA), 2-mercaptoethanol, dithiothreitol (DTT) and phenylmethylsulfonyl fuoride (PMSF) at 2.0 and 10 mM, and with gycerol and dimethyl sulfoxide (DMSO) at 1% and 5% (v/v). The activity of the control without addition of any additive was taken as 100%.

RESULTS AND DISCUSSIONS

Screening of keratinolytic bacteria and Keratinase Production: In the present study a total of 25 strains were isolated from ten poultry processing units. Primary isolation was done on Nutrient agar medium and for rapid identification all the isolates were transferred on Hichrome Bacillus Agar Medium.

All the 25 isolates allowed growing on milk agar and on feather meal agar for screening keratinolytic activity. Out of 25 isolates, the isolates belonging to genera, Bacillus, and Pseudomonos were effective protease producers. Among the 25 isolates, strain K13 was found to have good keratinolytic activity when inoculated on solid agar plate at 30°C for 2 days by measuring the diameter of hydrolysis zone (Table 1). Bacillus spp (K1) isolated showed clear hydrolytic zone of 23 mm, whereas as Pseudomonas spp. (K13) showed the hydrolytic zone of 34mm (Figure 2). Saha and Dhanasekaran, (2010), also carried out primary screening of keratinolytic actinobacteria from keratin waste dumped soil in Thiruchirapalli and Namakkal, Tamilnadu, India using milk agar medium. Feathers could be completely degraded by strain K13 in liquid broth at 30°C for 4 days (Figure 1) and raw feathers be deeply degraded by the purified keratinolytic protease at 37°C for 24-48 h .

Table 1: Screening and keratinolytic activity of the isolates, Zone diameter of the isolates (zone of hydrolysis) on milk agar

Culture number	Name of the isolates	Zone diameter in mm
K1	Bacillus pumilis	23mm
K2	Pseudomonos fluroscence	26 mm
K3	Pseudomonos aeruginosa	26 mm
K4	Pseudomonos hibiscola	28 mm
K5	Pseudomonos hibiscola	29mm
K6	Bacillus subtilis	17 mm
K7	Bacillus megaterium	14 mm
K8	Bacillus cereus	16 mm
K9	Bacillus brevis	14 mm
K10	Bacillus subtilis	21 mm
K11	Bacillus megaterium	16 mm
K12	Bacillus cereus	17mm
K13	Pseudomonos aeruginosa	34mm
K14	Bacillus subtilis	20 mm
K15	Bacillus cereus	20 mm
K16	Bacillus thuringenesis	16 mm

K17	Bacillus subtilis	20 mm
K18	Bacillus cereus	20 mm
K19	Bacillus megaterium	17mm
K20	Bacillus pumilis	14 mm
K21	Bacillus subtilis	24 mm
K22	Pseudomonos fluroscence	23 mm
K23	Pseudomonos aeruginosa	30 mm
K24	Pseudomonos hibiscola	29 mm
K25	Pseudomonos hibiscola	28 mm

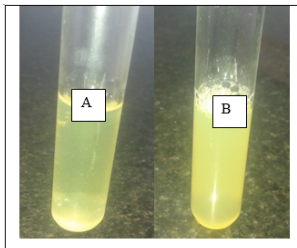
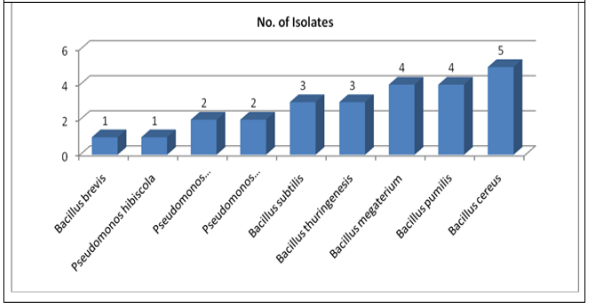


Fig.1 Feathers degradation by *Pseudomonas aeruginosa*

Fig.2 Keratinolytic activity after two days incubation

Identification of the Keratinolytic bacterial strain: The strains of keratinase producing bacteria were isolated and identified on the basis of morphological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (Brenner et al., 2004). The potential keratinase producer was confirmed by 16S rDNA sequencing. 16S rRNA gene sequence of strain K13 was submitted to GenBank and given accession number KJ754135. The 16S rRNA sequence of strain K13 showed highest similarity to that of P. aeruginosa NCBI 487

Fig.:4.1 Number of Bacterial Strains Isolated from Poultry Farms

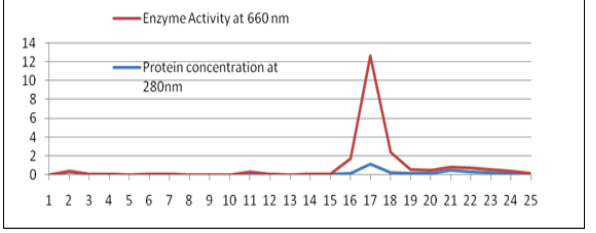


Purification of the keratinolytic protease: The protein concentration and Keratinolytic activity was checked to select the strain having high Keratinolytic activity. After 4 days the protein concentration and Keratinolytic activity was checked for the K13. It was found that the protein concentration for K13 it was 280 U/ml (mean Value). So for the further characterization of enzyme, the bacterial stain K13 was selected.

After checking the activity of the entire precipitate samples, the precipitate obtained after 30% saturation does not show activity hence the excess protein was removed and the precipitate obtained after 80% saturation shows activity hence the enzyme was precipitated at 60% saturation. The activity of 60% precipitate enzyme was found to be 1056 U/ml. This precipitate was subjected to Ion exchange chromatography for further purification process. The enzyme keratinase precipitated by 80% salt precipitation was further attempted to purifying by passing through DEAE cellulose column. Unbound enzymes were collected by passing buffer. The enzyme which was bound to the matrix was eluted with gradient buffer NaCl. The concentration of each fraction further analyzed by taking absorbency at 280 nm and enzymatic activity of each fraction is estimated by taking absorbency at 660 nm. Fraction number 2 of 0.1 M elution buffer, fraction number 17 of 0.4 M elution buffer and fraction number 21 and 22 from 0.5 M elution buffer show maximum concentration of protein. The Fraction number 2 shows 322 mg/ml concentration of protein and enzymatic activity was found to be 12U/ml, fraction number 17 shows 1174 mg/ml concentration of protein and enzymatic activity was found to be 11.44 U/ml and fraction number 21 and 22 shows 0.480 mg/ml and 0.366 mg/ml concentration of protein and enzymatic activity was found to be 32U/ml and 36 U/ml respectively. The fraction number 17 was further utilized for SDS-PAGE.

The elution profile of enzyme solution of DEAE cellulose column showed enzyme extract was separated in 25 fraction of different concentration of elution buffer i.e. NaCl. It was found that the fraction number 2, 17, 21 and 22 contained high activity of keratinase. The comparisons were made between the control set of protein bands with the bands obtained from the sample. From the gel, it was observed that K13 isolates of keratinase has a molecular mass of 38 kDa.

Protein concentration and enzyme activity after Ion-exchange chromatography



The effect of pH, temperature, substrate and metal ions on enzyme activity: The strain K13 grew well and completely degraded feather meal in the nutrient medium. The intense feather degrading was achieved in 40°C and initial pH adjusted to 7 and 8. Similar growth curve were observed with in this range of temperature and pH. The keratinase was active in neutral condition with an optimum activity at pH 7 with highest activity 400U/ml in phosphate buffer used. The enzyme was stable over a wide range of pH values, with the highest stability at pH 6-8 for 30 minutes. The enzyme had an optimum activity at the temperature of 60°C and was rapidly inactivated at higher temperature. Above 80°C, the keratinase was no longer active. The enzyme was unstable at high temperatures but was stable at moderate temperatures. The activity at 600C was 560 U/ml. The effect of substrate concentration on keratinase production was investigated. The affinity of the keratinase for keratin was determined at 60°C and pH 7.0 and it shows that the as the concentration of the substrate increases, activity of the enzyme increases. Ellaiah et al. (2002) stated that at high pH, the metabolic action of bacterium may be suppressed and thus it inhibits the enzyme production. Physical factors are important in any fermentation for optimization of biochemical production. The important physical factors that determine the bioprocess are pH, temperature, aeration and agitation. In the present study, the effect of temperature and pH on Keratinase enzyme activity revealed that 40°C at pH 7 was optimum and at the tested higher temperatures, the enzyme production decreased which might be due to growth reduction and enzyme inactivation or suppression of cell viability.

In the present study among the metal ions the Zn2+ and Cd2+ enhance the keratinolytic activity and Hg2+ and Mg2+ inhibits the keratinolytic activity. Vigneshwaran et al., (2010) observed that the crude keratinase belongs to the group of metalloproteases. Among the metal ions the Zn2+ and Mg2+ enhance the keratinolytic activity by 1.34 and 1.71 folds respectively and Cu2+, Hg2+ and Cd2+ inhibits the keratinolytic activity by 92,94 and 93% respectively. Keratinase inhibition by Hg2+ may suggest that a free cysteine is present at or near the active site. Lusterio et al., (1992) suggested that inhibition by Hg2+ is not just related to binding of the thiol groups but may be a result of an interaction with tryptophan residues or with the carbonyl group of amino acids in

the enzyme.

In the present study after 4 days incubation period the protein concentration and keratinolytic activity was checked for the K13. It was found that the protein concentration for K13 it was 280 U/ml (mean Value). Fraction number 17 shows 1174 mg/ml concentration of protein and enzymatic activity was found to be 11.44 U/ml. From the gel, it was observed that K13 isolates of keratinase has a molecular mass of 38 kDa. Venkata and Divakar, (2013) reported that enzyme production was assayed in submerged fermentation (SmF) condition. The maximum keratinase production was observed with maltose (120±2.6 U/ml), yeast extract (90±3.6 U/ml), ammonium sulphate (61±3.0 U/ml), pH 7.0 (114±4.1), temperature 40°C (110±1.9), Tween-80 (104±2.8U/ml), inoculum size level 5% (112±2.0U/ml) and incubation time 48 hours (101.01± 0.56U/ml) in the production medium.

Figure 4.8b : Effect of pH on enzyme activity

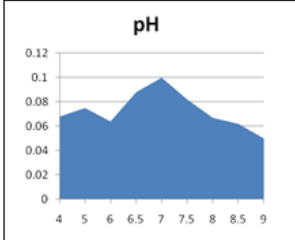


Fig-4.8c-Effect of temperature on enzyme activity.

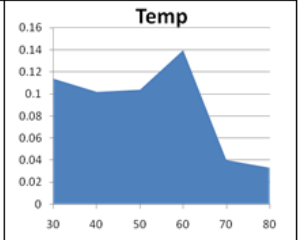


Figure 4.8d: Effect of Substrate Concentration

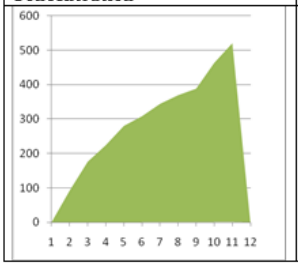
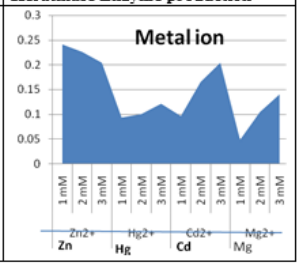


Figure 4.9: Effect of metal ions on Keratinase Enzyme production



CONCLUSION

The presence of such type of species in poultry waste is due to adaptation to utilize substrates like feathers. The most studied keratinolytic bacterium is Pseudomonas aeruginosa (K13) which possesses high keratinolytic activity. The bacterium and keratinase enzyme could be used for improving nutritive quality of animal feed containing feathers of poultry waste. The keratinolytic activity of keratin degrading isolate will have biotechnological application in various industrial processes involving keratin hydrolysis. Use of microbial keratinase is beneficial and economical approach for keratinous waste disposal and to prevent environmental pollution.

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