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Optimization of Production and Partial Characterization of Keratinase Produced by *Bacillus thuringiensis* strain Bt407 Isolated from Poultry Soil

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A B S T R A C T

Microbial keratinases have become biotechnologically important since they target the hydrolysis of highly rigid, strongly cross-linked structural polypeptide "keratin" recalcitrant to the commonly known proteolytic enzymes. Soil samples collected from different poultry shops were enriched for keratinase producers on Whole feather agar containing whole feathers as a sole Carbon and Nitrogen source. Among 11 bacterial isolates, 6 isolates showed protease activity. The best keratinase producing bacterium K10 was selected and identified as *Bacillus thuringiensis* strain Bt407, based on morphological, cultural, biochemical characteristics and 16S rRNA sequence analysis. The isolate exhibited maximum keratinase production (94.52U/ml) in a optimized feather meal medium containing Feather meal (2%), Yeast extract (1%), Starch (1%), $MgSO_4$ $6H_2O$ (0.003%), $CaCl_2$ (0.5mM), KH_2PO_4 (0.5%), K_2HPO_4 (0.3%), NaCl (0.5%), pH 7, inoculated with 1% v/v pre-grown cell mass and incubated at 37°C on rotary shaker (120 rpm) for 48 hours. The optimum enzyme activity was observed at 55°C and pH 8. Metal ions like Ca^{+2}, Mg^{+2} and Ba^{+2} were seen to enhance enzyme activity whereas Cd^{+2}, Cu^{+2}, Fe^{+3}, Hg^{+2} and Zn^{+2} were observed to inhibit enzyme activity. Inhibitors such as SDS helped to retain the activity of the enzyme while 2- mercaptoethanol, DMSO and EDTA were seen to inhibit the enzyme activity. The molecular weight of the keratinase was found to be 33kDa by SDS-PAGE method. Zymography was carried out to show protease activity of the keratinase. Depilatory action of keratinase on goat skin was also demonstrated. The applications of the enzyme as a detergent additive and enzyme hydrolyzed feather meal in bacteriological medium as nitrogen source were also studied. Of the tested keratinous materials used as substrates, the production of enzyme was seen to be more in the presence of human nails than human hairs.

Introduction

Keratin forms a major structural component of vertebrate skin (epidermis) and its appendages viz. hair, feathers, nails, horns, hoofs, scales and wool (Feughelman, 1985). It is used in hair care products, animal feed and fibres for textiles (Mitsuiki *et al.,* 2004). Keratin is an insoluble fibrous protein macromolecule with very high stability and low degradation rate. On the basis of secondary structural conformation, keratins have been grouped into α (α-helix of hair and wool) and β (β-sheets of feather) (Voet and Voet, 1995; Akhtar and Edwards, 1997). Keratins are also classified as

Type I (acidic) keratin and Type II (basic) keratin and have molecular weights ranging from 30 KDa to 70 KDa (Steinert, 1993; Selvam and Vishnupriya, 2012). There are approximately 30 different types of keratins which are generally grouped into epithelial keratins (in epithelia cells) and trichocytic keratins which make up hair, nails, horns and reptilian scale.

Keratins are also classified as soft keratins and hard keratins (Zhou *et al.,* 1988; Chou *et al.,* 2015). Soft keratins (those containing up to 1 % sulphur) which constitute less amount of disulfide bonds are commonly present in skin and callus structure (epithelial keratin) and are more pliable. Hard keratins which are tough (those containing up to 5 % sulphur), constitute large amount of disulfide bond and exist in form of α keratin and β keratin (trichocytic keratin) (Voet and Voet, 1995; Schrooyen *et al.,* 2001).

The most distinctive feature of keratin is, they are mechanically robust and chemically unreactive (high resistance to proteolytic degradation) due to presence of higher degree of cross-linking by disulphide bridges, salt bridges, hydrogen bonds and hydrophobic interactions. Keratin is poorly susceptible to degradation and digestion by most common peptidases such as trypsin, pepsin and papin (Bockel *et al.,* 1995; Balaji *et al.,* 2008; Jones *et al.,* 1997).

Keratinase [E.C.3.4.21/24/99.11] are proteases able to degrade the scleroprotein keratin. Keratinases belong to group of proteolytic enzymes which have ability to hydrolyze insoluble protein keratin more efficiently than other proteases (Onifade *et al.,* 1998). Due to the strength and stability of keratin, very few microorganisms are able to degrade keratin and utilize it as carbon, nitrogen and sulphur source (Marchisio, 2000). Total degradation of keratinous material by specialized microorganisms can generate a chain of events involving breaking of disulfide bridges (sulfitolysis) and proteolysis (Błyskal, 2009; Ramnani *et al.,* 2005). It is due to the cooperative action of keratinolytic protease and disulfide reductase enzyme which may be produced by same or different microorganisms (Gupta *et al.,* 2015).

Microbial keratinases are mostly extracellular enzymes which are inducible in nature but some are membrane linked (cell bound) and intracellular. Keratinases are by and large serine or metalloproteases which are capable of degrading the structural keratinous protein (Gupta and Ramnani, 2006). Keratinase are also classified into two major groups "endopeptidases and exoproteases" based on their site of cleavage action. These enzymes are produced by bacteria, actinomycetes and fungi which can hydrolyze a large number of keratin substrates (Saibabu *et al.,* 2013*;* Tork *et al.,* 2010). Bacterial keratinases are also capable of degrading other proteins like casein, collagen and gelatin (Balakumar *et al.,* 2013).

There are reports of isolating keratinase producing microorganisms from poultry soil, poultry wastes, poultry farm, poultry processing industry, feather and hair dumping sites and barbers' landfill (Deivasigamani and Alagappan, 2008; Gioppo *et al.,* 2009; Sahoo *et al.,* 2015; Syed *et al.,* 2009; Kaul and Sumbali, 1997; Moallaei *et al.,* 2006; Xu *et al.,* 2009; Manoj Kumar *et al.,* 2016; Shah, 2015).

A major proportion of feather degrading bacteria belong to Gram positive category of *Bacillus spp.* which includes *Bacillus licheniformis, Bacillus amyloliquefaceins, Brevibacillus brevis* US575, *Bacillus tequilensis* strain Q7, *Bacillus pumilus, Bacillus cereus, Bacillus subtilis* and *Bacillus halodurans* (Jaouadi *et al.,* 2013*;* Jaouadi *et*

al., 2015; Lin *et al.,* 1992; Cortezi *et al.,* 2008; Kumar *et al.,* 2010; Adiguzel *et al.,* 2009; Mazotto *et al.,* 2010; Prakash *et al.,* 2010). Other Gram positive feather degrading bacteria include *Micrococcus spp.*, *Nesterenkonia* sp. AL20, *Arthrobacter creatinolyticus* KP015744, *Caldicoprobacter algeriensis, Kytococcus sedentarius, Micrococcus luteus,* and *Clostridium spp,* (Ionata *et al.,* 2008; Kate and Pethe, 2014; Bakhtiar *et al.,* 2005; Longshaw *et al.,* 2002; Laba *et al.,* 2015). Keratinolytic gram negative bacteria belong to *Pseudomonas spp.*, *Vibrio spp., Chryseobacterium spp., Xanthomonas spp.*, Lysobacter NCIMB 9497, *Acinetobacter* sp. PD 12, *Stenotrophomonas* and *Fervidobacterium spp.* (Allpress *et al.,* 2002; Sangali and Brandelli, 2000, De Toni *et al.,* 2002, Yamamura *et al.,* 2002, Lucas *et al.,* 2003; Shah, 2015; Cao *et al.,* 2009). Actinomyces species such as *Streptomyces flavis* 2BG (mesophilic) and *Microbispora aerata* IMBAS-11A (thermophilic), *Nocardiopsis dassonvillei* NRC2aza (Gushterova *et al.,* 2005; Azza, 2013) can also degrade keratin. Keratinases are also produced by fungi including *Paecilomyces marquandii, Myrothecium verrucaria, Aspergillus flavus* Strain K-03, Cladosporium and Trichoderma, *Cyberlindnera fabianii* Nrc3, *Purpureocillium lilacinum* LPS # 876, *Chrysosporium georgiae, Doratomyces microspores, Onygena corvine and Candida parapsilosis* (Veselá and Friedrich, 2009; Gioppo *et al.,* 2009; Kim, 2007; Patience *et al.,* 2015; Naghy *et al.,* 1998; Friedrich and Kern, 2003; Huang *et al.,* 2015; Duarte *et al.,* 2011).

The current studies focuses on isolation and identification of keratinase producing isolate obtained from soil around the poultry shops. It further discusses the optimization of keratinase production. It also reports partial purification, characterization and applications of keratinase enzyme.

Materials and Methods

Collection of samples

The soil samples were collected from the vicinity of the locations of four poultry shops in Mumbai city in the clean dry plastic containers

Enrichment

One gm of soil sample was mixed with 10 ml of Distilled water. The mixture was shaken well and allowed to settle for 30 min. Five ml of soil supernatant was inoculated in 95ml of Whole feather basal medium in flask. This medium was prepared using Whole feathers (20g/L) that were incorporated in Feather Basal broth Medium which is composed of NaCl (0.5g/L,) K_2HPO_4 (0.3g/L), KH_2PO_4 (0.4g/L), pH 7.4 (Tork *et al.,* 2010). The flasks were incubated at 37°C in shaker condition (120 rpm) till visible degradation or disappearance of the feathers (Agrahari and Wadhwa, 2010).

Isolation

Feather-meal agar plates were prepared by adding NaCl (0.5g/L), K_2HPO_4 (0.3g/L), KH_2PO_4 (0.4g/L), Agar (20g/L) and Feather meal (20g/L), which was used as a sole source of carbon and nitrogen. Its pH was adjusted to 7.4 (Selvam and Vishnupriya, 2012).

The feather meal was prepared from native chicken feathers which were cut with scissors into small pieces of 3 to 4 cm long and washed several times with tap water. Defatting of feather pieces was done by soaking them in a mixture of chloroform: methanol (1:1) for 2 days followed by chloroform: acetone: methanol (4:1:3) for 2 days. The solvent was replaced every day. The feathers were finally washed several times with tap water to eliminate the solvent residual, dried for 24 hrs in an oven at 50°C and grinded using electrical

mixer blender (Kenstar Senator PCMG 0120) and used as feather meal (Saibabu *et al.,* 2013; Riffel and Brandelli, 2006). The enriched suspension was streaked on feather meal agar plates and plates were incubated at 37°C for 72hrs. Single colonies were isolated and screened for their ability to produce protease by using Skimmed Milk Agar plates (Riffel *et al.,* 2003; Tork *et al.,* 2010).The cultures were spot-inoculated on Skimmed Milk Agar plates and incubated at 37°C for 24 - 48 hrs. The colonies showing a zone of clearance of >11mm around the colony was considered to have a proteolytic activity and hence Keratinolytic activity (Riffel and Brandelli, 2006).

Keratinase assay

Preparation of crude enzyme extract

Cultures were grown on sterile feather meal broth medium on shaker incubator (120 rpm) at 37°C for 24hrs.The broth was centrifuged at 3000 rpm for 20 mins. The cell pellet was washed and resuspended in phosphate buffer saline pH 7.2. Washed cells (5ml) are then inoculated in 100ml of Feather meal basal medium and incubated for 24hrs, 48 hrs and 72 hrs at 37°C on shaker incubator (120rpm). After incubation an aliquot of the broth is centrifuged and the supernatant containing the enzyme extract was assayed by Keratin azure assay. The protein content was estimated by Folin- Lowry method (Lowry *et al.,* 1951).

Keratinase azure assay

This assay was carried out by using the modified method of. Bressollier *et al.,* (1999). Keratin azure (Sigma Aldrich, Germany Sigma) was used as a substrate. One ml of crude enzyme extract was incubated with 5mg of keratin azure in 1 ml of 50 mM Tris HCl buffer (pH 8.5) at 50°C for 1 hr with constant agitation at 100 rpm. The mixture was centrifuged at 3000rpm for 20 mins to remove the insoluble substrate. The supernatant was measured spectrophotometrically (UV-Visible Spectrophotometer- Agilent Technologies Cary 60 UV- vis) at 595 nm for the release of the azo dye. All assays were done in triplicate. One unit (U) of keratinase was defined as the amount of enzyme causing 0.01 increase in absorbance between sample and control at 595 nm after one hour under the conditions given. The culture showing good proteolytic activity was selected and studied for their best keratinase producing ability in the shortest period of time (Letourneau *et al.,* 1998; Sangali and Brandelli., 2000; Cortezi *et al.,* 2008; Anbu *et al.,* 2005; Suntornsuk *et al.,* 2005).

Protein estimation

Protein concentration of the supernatant was determined by using the Folin–Lowry Ciocalteau method. Bovine serum albumin was used as a standard and the colour developed was read at 660nm (Lowry *et al.,* 1951)

Identification

Identification was carried out on the basis of morphological, cultural and biochemical properties using Bergey's Manual of Bacteriology 8th Edition (1974). Further confirmation of the strain was done by 16s rRNA sequencing analysis (Codon Biosciences Pvt. Limited, Goa).

Media optimization for keratinase production

Sterile feather meal basal medium100ml was inoculated with 5ml culture suspension of the isolate (0.1 O.D. at 530nm) and were incubated in shaker incubator at 37°C for 48hrs. Static condition was maintained as control. The 100 ml of sterile feather meal

basal production medium was prepared in different flasks and inoculated with 5% inoculum. To check effect of temperature each flask was incubated at different temperature such as 28°C, 37°C, 45°C and 55°C for 48 hours. To study the effect of pH each flask of 100ml medium adjusted to different pH such as 5, 6, 7, 8, 9 and 10 using 1 N NaOH and 1 N HCl. To demonstrate the effect of carbon source each flask was added with 1% of different carbon sources such as Glucose, Lactose, Maltose, Xylose, Mannitol, Glycerol and Starch.

To investigate the effect of nitrogen source each flask was added with 1% different sources of nitrogen such as Urea, Yeast extract, Peptone, and Tryptone. To observe the effect of salt concentration each flask was added with different concentrations of $MgSO_4$ $6H_2O$ (0.5mg, 1mg, 3mg, 5mg) and different concentrations of $CaCl_2$ (0.5mM, 1mM, 1.5mM and 2mM).

A final enzyme assay was done after the isolate was grown in the optimized medium with components such as NaCl 0.05%, KH_2PO_4 0.07%, K_2HPO_4 0.14%, starch 1%, yeast extract 1%, $CaCl_2$ 0.5mM, $MgSO_4$ $6H_2O$ 0.003%, feather meal 1%, pH 7 at 37°C on shaker conditions(120 rpm) for 48 hrs.

Partial purification of Keratinase enzyme

The crude extract fluids (200 ml) were precipitated by 80% saturation using ammonium sulphate. The addition of salt was done with constant stirring on a magnetic stirrer in an ice bath. The protein precipitate obtained was separated by centrifugation at 10,000 rpm for 10 min and the pellet was dissolved with minimum volume of Tris-HCl buffer pH 8. The dissolved sample was dialyzed (Cellophane membrane, Sigma) against Tris-HCl buffer pH 8 for 8h. The dialyzed enzyme was collected and assayed for its enzyme and protein activity. The specific activity and % yield was calculated (Lakshmi, 2013; Mukhopadhyay and Chandra, 1990; Mehta *et al.*, 2014).

Effect of different parameters on the activity of keratinase

Effect of pH on enzyme activity

To determine the effect of pH, enzyme assay (Keratin azure assay) was performed by preincubating 0.2ml of partially purified enzyme with 0.8ml of different pH buffers (Phosphate Buffer pH 7, Tris-HCl pH 8, Glycine- NaOH pH 9 and 10) at 30°C (Tatineni *et al.,* 2008).

Effect of temperature on enzyme activity

To determine the effect of temperatures on keratinase activity 1ml of enzyme with 1ml of Tris-HCl buffer pH8 was incubated for 1hr at different temperatures such as 28°C, 37°C, 55°C, 65°C, 75°C, 80°C and 85°C. Later Keratin azure assay was carried out (Tatineni *et al.,* 2008).

Effect of metal ions on enzyme activity

To determine the effect of divalent cations on keratinase activity the purified fraction of enzyme was preincubated in the presence of Zinc, magnesium, copper, calcium, mercury, cadmium and barium (Zn^{+2}, Mg^{+2}, Cu^{+2}, Ca^{+2}, Cd^{+2}, Fe^{+3} and Ba^{+2}) chloride form at 2mM concentration for 15mins at 30°C. Later Keratin azure assay was carried out (Kainoor and Naik, 2010; Suntornsuk *et al.*, 2005).

Effect of inhibitors on enzyme activity

To determine the effect of different inhibitors on enzyme acitivity 1ml of enzyme was preincubated with 1ml of 5 mM inhibitor solutions (EDTA, SDS, 2-Mercaptoethanol, DMSO)) for 15 mins at 30°C. Later Keratin azure assay was carried out (Kainoor and Naik, 2010; Suntornsuk, 2005).

Determination of molecular weight

In order to determine the molecular weight of this partially purified enzyme Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out. SDS-PAGE was performed with 12% polyacrylamide gels as described by (Laemmli, 1970). Molecular weight markers (molecular weights, 14,000 to 170,000; Boehringer, Mannheim, Germany) were included and stained with 1% Coomasie brilliant blue R-250 and then destained.

Zymography

To prepare a zymogram, proteinase samples were mixed with electrophoresis sample buffer without heat denaturation prior to electrophoresis. SDS-PAGE was carried out at 4°C by using a 12% polyacrylamide gel. After electrophoresis, the gel was washed with 2.5% (vol/vol) Triton X-100 for 30 min and then with 50 mM Tris-HCl (pH 8) for 30 min. Casein (2%, wt/vol) in 50 mM Tris-HCl buffer (pH 8.5) was then poured onto the gel slab containing proteases. After 1hr of incubation at 50°C, the gel was stained with Coomassie brilliant blue R-250 prepared in solvent containing methanol: acetic acid: water (40:10:50) and then destained. Protease bands appeared as clear zones on a blue background (Bressollier *et al.,* 1999).

Similarly after electrophoresis, the gel was soaked in 1% (w/v) Triton-X-100 for 15 min and then in glycine-NaOH buffer (pH 10) for 30 min at 50°C. The gel was now over layered on 1.2% agarose plate containing 0.5% casein in 50 mM glycine-NaOH buffer (pH-8.5) for 1hr at 50°C and stained to visualize hydrolysed clear band. Hydrolysis of casein was observed on plate surrounding the gel slab (Kainoor and Naik, 2010; Jaouadi *et al.,* 2013).

Keratinase production using hair and nail as substrates

Five ml culture suspension of 20hrs old log phase washed culture (0.1O.D. at 530nm) was inoculated in sterile 100ml basal mineral medium containing 1% human hairs / nails and were incubated at 37°C for 7 days. Keratin azure assay was carried out at 24 hrs, 48hrs, 96hrs, 120 hrs and 144 hrs, 168 hrs (Suntornsuk *et al.,* 2005).

Application of keratinase

Keratinase treated feather meal as bacteriological media additive

Keratinase enzyme is a feather degrading enzyme which produces peptides and soluble proteins (amino acids) as the degradation products. These degradation products can be used as source of carbon and nitrogen in bacteriological media for growth of laboratory cultures (Ramakrishnan *et al.,* 2011).

Media were used as follows

Sterile Nutrient Agar

Sterile medium with 1% degraded feather meal+ 0.5% NaCl+ 2.5%Agar, pH 7.2

Sterile plates with medium containing 0.5%NaCl+ 2.5%Agar and pH 7.2.

The isolate was grown in sterile whole Feather basal medium and incubated at 37°C for 48 hrs. The grown medium was centrifuged at 5000 rpm for 20mins and the precipitate was obtained. The precipitate was washed with sterile distilled water and it was autoclaved and incorporated in the medium as stated above. The laboratory cultures (24 hrs old log phase culture of *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Salmonella paratyphi B, Proteus vulgaris and*

Pseudomonas areuginosa were spot inoculated on them and sterile media controls were also maintained. All these plates were incubated at 37°C for 48 hrs. Growth was checked after incubation.

Keratinase enzyme as detergent additive (Wash performance)

Wash performance analysis of cotton cloth with blood and egg yolk stains was conducted and efficacy of the Keratinase for use as detergent additive was assessed (Pathak and Deshmukh, 2012; Adinarayana *et al.,* 2003). Application of keratinase enzyme produced by the isolate as a detergent additive was studied on white cotton cloth pieces (4×4 cm) stained with human blood and egg yolk. 7mg/ml of Ariel detergent powder solutions were prepared. These were heated in boiling water bath for 20 min to inactivate the inherent enzymes. The following sets were prepared and studied.

Flask with distilled water (100 ml) + stained cloth (cloth stained with blood / egg yolk).

Flask with distilled water (100 ml) + stained cloth (cloth stained with blood / egg yolk) + 1 ml heat inactivated ariel detergent (7mg/ml).

Flask with distilled water (100 ml) + stained cloth (cloth stained with blood / egg yolk) + 1 ml heat inactivated ariel detergent (7mg/ml) + 2 ml of enzyme solution.

The piece of cloth was rinsed in above flasks for 30 minutes.

After incubation, cloth pieces were taken out, rinsed with water and dried.

Visual examination of various pieces exhibited the effect of enzyme in removal of stains. Untreated cloth pieces stained with blood / egg yolk were taken as control.

Dehairing capacity of keratinase on goat skin

Freshly salted goat hide was cut into pieces of approximately 2×2 cm and washed with distilled water repeatedly to remove salt and extraneous matter. After brief air drying, the hide was weighed (weight of the pieces was maintained 3 g and transferred to a flask containing 10-ml sterile distilled water (Macedo *et al.,* 2005). The soaked hide pieces were incubated with the enzyme (1 % w/v) at 37°C along with control which was goat hide of the same size with saline instead of enzyme (Pillai and Archana, 2008; Gurav *et al.,* 2016). Incubations were carried out for 18 hrs after which hair was scraped off gently from the hides.

Results and Discussion

Sample collection, enrichment, isolation and identification

A screening program was employed to obtain bacterial isolates capable of producing feather degrading extracellular keratinase enzyme using feather (keratin) as sole carbon and nitrogen source. Four soil samples from different poultry shops from Mumbai were successfully enriched in Whole feather broth medium. The enriched broth culture from soil sample number 1 was used for all further experimentation as turbidity of the medium and degradation of the feathers appeared to be more. The enriched sample was streaked on feather meal basal medium agar and 11 isolates K1, K2, K3, K5, K6, K7, K8, K9, K10, K11 and K12 were obtained. All the 11 isolates were checked for their protease activity using Skimmed milk agar plates. Only 5 bacterial isolates number K1, K5, K6, K10 and K12 showed larger zones of clearance i.e. protease activity around the colony on Skimmed milk agar plates (results not shown). This suggested that these isolates might

possess keratinase activity as well. The above 5 isolates were grown on whole feather basal broth for 24 hrs, 48 hrs and 72 hrs. The capability of these isolates to produce maximum keratinase in the shortest period of time was studied. Out of 5 bacterial isolates, the isolate K10 was found to be the best producer of keratinase in 48 hrs as shown in the figure 1 and 2. A significant reduction in enzyme production was observed in 72 hrs, thereby proving that the isolates produced maximum enzyme in the log phase. The whole feather basal medium was completely turbid, suggesting disintegration and degradation of the feathers in the medium. Protein concentration was estimated by employing Folin-Lowry method. K10 showed specific activity of 11.96 after 48 hrs which was the highest among the specific activities of all the five isolates (Table 1). The isolate K10 which was showing maximum keratinase activity was identified as *Bacillus thuringiensis* strain Bt407 by morphological, cultural, biochemical and 16S rRNA sequence analysis. It is gram-positive, sporulating *Bacillus* which is soil-dwelling bacterium, commonly used as a biological pesticide. *B. thuringiensis* also occurs naturally in the gut of caterpillars of various types of moths and butterflies, as well on leaf surfaces, aquatic environments, animal faeces, insect rich environments, flour mills and grain storage facilities.

There are reports of isolating keratinase producing microorganisms from poultry soil, poultry wastes, poultry farm, poultry processing industry, feather and hair dumping sites and barbers' landfill (Deivasigamani and Alagappan, 2008; Gioppo *et al.,* 2009; Sahoo *et al.,* 2015; Syed *et al.,* 2009; Kaul and Sumbali, 1997; Moallaei *et al.,* 2006; Xu *et al.,* 2009; Manoj Kumar *et al.,* 2016; Shah, 2015). Similar Keratinase producing bacterial strains of *Bacillus thuringiensis* were also isolated from the chicken feather dumping site (Sivakumar *et al.,* 2012; Shanker *et al.,* 2014).

The presence of keratinous substrates usually induces keratinase production (Mazotto *et al.,* 2010). In our studies enrichment and isolation of keratinase producer was carried out by using medium containing Whole feather as a sole carbon and nitrogen source which had induced higher keratinase activity (Lin *et al.,* 1992; Szabo *et al.,* 2000; Gushterva *et al.,* 2005). Feather meal was used for isolation of keratinase producer by many scientists (Tapia and Simoes,2008; Ramya *et al.,* 2014; Mousavi *et al.,* 2013; Govarthanan *et al.,* 2011; Shah, 2015; Fakhfakh-Zouari *et al.,* 2010). The abilities of bacterial isolates to produce keratinase were also confirmed in earlier reports by cultivation on agar plates containing skimmed milk (Tork *et al.,* 2010; Allpress *et al.,* 2002; Preethi *et al.,* 2015; Ramya *et al.,* 2014; Kazi *et al.,* 2015). Several *Bacillus* sp. including *Bacillus licheniformis, Bacillus amyloliquefaceins, Brevibacillus brevis* US575, *Bacillus pumilus, Bacillus cereus, Bacillus subtilis, Bacillus halodurans* have been documented to be potential sources of keratinases (Jaouadi *et al.,* 2013; Lin *et al.,* 1992; Cortezi *et al.,* 2008; Kumar *et al.,* 2010; Adiguzel *et al.,* 2009; Mazotto *et al.,* 2010; Prakash *et al.,* 2010). There are other documentary evidences of keratinase production by other bacterial species like *Bacillus subtilis, Bacillus coagulans, Bacillus cereus and Bacillus pumilus* (Femi-Ola *et al.,* 2015), *Bacillus polymyxa* and *Bacillus cereus* (Laba and Rodziewicz, 2010), *Bacillus megaterium* (Saibabu *et al.,* 2013), *Bacillus megaterium* RS1(Shankar *et al.,* 2013), *Bacillus pseudofirmus* FA30-01(Kojima *et al.*,2006), *Brevibacillus thermoruber* T1E(Bihari *et al.*,2010), *Bacillus safensis* LAU 13(Lateefa *et al.,* 2015), *Bacillus mycoides, Bacillus cereus, Bacillus vallismortis, Bacillus mojavensis* (Preethi *et al.,* 2015), *Bacillus weihenstephanensis* PKD5 (Sahoo *et al.,* 2015), *Bacillus pumilus* AR57, *Kocuria rosea* (Bernal *et al.,* 2006). Besides these, vast diversity also exists among other

bacterial sources with keratinases being described from *Fervidobacterium, Lysobacter, Nocardiopsis, Microbacterium* (Thys *et al.,* 2004), *Clostridium sporogenes* (Ionata *et al.,* 2008), *Chryseobacterium* (Nam *et al.,* 2002; Allpress *et al.,* 2002; Mitsuiki *et al.,* 2004; Silveira *et al.,* 2010), *Nocardiopsis dassonvillei* NRC2aza (Azza, 2013), *Stenotrophomonas* (Cao *et al.,* 2009), *Chryseobacterium* sp. (Brandelli and Riffel, 2005), *Vibrio* sp. kr2. (Grazziotin *et al.,* 2006), *Paecilomyces marquandii* (Veselá and Friedrich, 2009), *Myrothecium verrucaria* (Gioppo *et al.,* 2009), *Aspergillus flavus* Strain K-03 (Kim, 2007), Cladosporium and Trichoderma (Patience *et al.,* 2015), Lysobacter NCIMB 9497 (Allpress *et al.,* 2002), *Vibrio* (Sangali and Brandelli, 2000*), Cyberlindnera fabianii* Nrc3 (Azza, 2015), *Nesterenkonia* sp. AL20 (Bakhtiar *et al.,* 2005), *Purpureocillium lilacinum* LPS # 876 (Ivana *et al.,*2013), *Penicillium* spp. Morsy1 (El-Gendy, 2009), *Onygena corvina* (Huang *et al.,* 2015), *Acinetobacter* sp. PD 12 (Shah, 2015), *Arthrobacter creatinolyticus* KP015744 (Kate and Pethe, 2014), *Chrysosporium georgiae* (El-Naghy *et al.,* 1998), *Doratomyces microspores* (Friedrich and Kern, 2003), *Streptomyces flavis* 2BG (mesophilic) and *Microbispora aerata* IMBAS-11A (thermophilic)(Gushterova *et al.,* 2005), *Serratia* sp. HPC 1383 (Khardenavis *et al.,* 2009), *Micrococcus luteus* (Laba *et al.,* 2015), *Kytococcus sedentarius* (Longshaw *et al.,* 2002), *Cunninghamella echinulata* (More, 2013), *Malbranchea gypsea* (Singh, 1999) and *Candida parapsilosis* (Vermelho *et al.,* 2009).

Effect of various co-carbon sources on keratinase production

Though all sugars were seen to support the production of Keratinase in case of *Bacillus thuringiensis* strain Bt407 grown in Feather Basal Medium with 1% Starch showed the highest production of enzyme (87.37 U/ml) and degradation of feathers, followed by media containing Xylose, Glycerol and Glucose (Fig. 3). Enzyme production and feather degradation in medium containing 1% Maltose, Mannitol or Lactose showed scanty feather degradation and consequently exhibited low levels of keratinase production. Our results are in accordance with the reports where *Bacillus* sp strains (MBF11 and MBF21), *Bacillus* sp. JB 99, *Bacillus megaterium* RS1, *Streptomyces gulbargensis* DAS 131 and *Streptomyces minutiscleroticus* DNA38, *B.cereus* TS1 showed maximum keratinase production in presence of starch (Laxmi *et al.,* 2013; Kainoor and Naik, 2010; Shankar *et al.,* 2013; Allure *et al.,* 2015; Sivakumar *et al.,* 2013; Syed *et al.,* 2009). However, in earlier studies *Bacillus thuringiensis* TS2 strain (Sivakumar *et al.,* 2012) showed maximum keratinase production in presence of mannitol and minimum in presence of lactose whereas in case of *B.cereus* KER17, *Chryseobacterium sp.* RBT and *Streptomyces exfoliatus CFS1068* starch has shown negative impact on feather degradation process (Gurav *et al.,* 2016; Rayudu *et al.,* 2011; Jain *et al.,* 2012).

Previous studies also showed that in case of *Bacillus licheniformis* RG1 (Ramnani and Gupta, 2004), *Bacillus subtilis* MTCC9102 (Kumar *et al.,* 2010), *B. megaterium* F7-1 (Park and Son, 2009), *Bacillus Subtilis* Subsp *Subtilis* (Manju and Shanmugam, 2013), *Bacillus halodurans* strain PPKS-2 (Pathange *et al.,*2009), *Bacillus subtilis* (MTCC9102) (Kumar *et al.,* 2010), Streptomyces sp., *Stenotrophomonas* sp., (Yamamura *et al.,*2002; Jeong *et al.,* 2010), *Microbacterium* and many fungal species (Kazi *et al.,* 2013) glucose does have positive effect on Keratinase production. There are previous reports where *Bacillus subtilis* strain RM-01, *Myrothecium verrucaria* and *B.subtilis S8* produced maximum keratinase in presence of maltose (10%, w/w), bagasse and Sorbitol as best co-carbon source respectively (Rai *et al.,* 2009; Gioppo *et al.,* 2009; Jeong *et al.,*

2010a). Nevertheless, carbohydrate inhibition of keratinase production was observed in certain strains of bacteria (Brandelli and Riffle, 2005; Givskov *et al.,* 1991; Wang and Shih, 1999; Thys *et al.,* 2004; Yamamura *et al.,* 2002a; Bernal *et al.,* 2003; Gassesse *et al.,* 2003; Suntornsuk and Suntornsuk, 2003; Kainoor and Naik, 2010; Daroit *et al.,* 2011; Lo *et al.,* 2012; Sahoo *et al.,* 2015; Mabrouk, 2008).

Effect of various co- nitrogen sources on the production of keratinase

Bacillus thuringiensis strain Bt407 was inoculated into Feather Basal medium containing 1% Yeast extract showed the highest production of enzyme and degradation of feathers, (79.26 U/ml) followed by media containing 1% Urea. Growth media containing 1% Tryptone or Peptone as the nitrogen source did not show feather degradation to the degree seen in media containing the other nitrogenous compounds (Fig. 4). Similar results were showed by *Bacillus subtilis* AMR (Mazotto *et al.,* 2010), *Bacillus* sp. JB 99 (Kainoor and Naik, 2010), *B.licheniformis* KMBVP (Vidhya and Palaniswamy, 2013), *Bacillus polymyxa* B20 and *Bacillus cereus* B5esz (Łaba and Rodziewicz, 2010) and *Streptomyces minutiscleroticus* DNA38 (Allure *et al.,* 2015). However, *Bacillus thuringiensis* TS2 strain reported to produce maximum keratinase in presence of peptone and minimum in presence of ammonium nitrate (Sivakumar *et al.,* 2012) whereas *Bacillus subtilis* (MTCC9102) demonstrated maximum keratinase production in presence of peptone (Kumar *et al.,* 2010). But earlier studies reported that *B.pumilus* FH9, *B. megaterium* F7 and *Stenotrophomonas maltophilia* R13 exhibited maximum keratinase production in presence of yeast extract + ammonium chloride, Tryptone and 0.12% polypeptone respectively (El-Refai *et al.,* 2005; Park and Son 2009; Jeong *et al.,* 2010). *Chryseobacterium sp. RBT* suppressed the keratinase activity in presence of inorganic and/or organic nitrogen sources (Gurav *et al.,* 2016).

Effect of incubation period on the production of keratinase

Maximum keratinase production was observed at 48 hours after inoculation of the isolate *Bacillus thuringiensis* strain Bt407 in Feather meal basal medium (Figure 2). Similar results were obtained for *Bacillus subtilis* NCIM 2724 (Harde *et al.,* 2011), *B.subtilis* MTCC 9102 (Kumar *et al.,* 2010), *Bacillus pumilus* FH9 (El-Refai *et al.,* 2005), *B. cereus* LAU08 (Lateef *et al.,* 2010) and *Stenotrophomonas maltophila* R-13 (Jeong *et al.,* 2010a). However, *Bacillus thuringiensis* TS2 was reported to produce optimum keratinase after 96 hrs of incubation (Sivakumar *et al.,* 2012) and *Bacillus thurengenesis* SN2 produced optimum keratinase in 5 days (Agrahari and Wadhwa, 2010). A decrease in the production of the enzyme after 48hrs of incubation can be due to the inactivation of the enzyme by metabolic end products accumulated in the medium or due to the depletion of nutrients in the medium (Anbu *et al.,* 2005) There were studies where optimum keratinase was produced by *Bacillus* sp. JB 99 and *Microbacterium* after 36 hrs (Kainoor and Naik, 2010; Thys *et al.,* 2004). Jeong *et al.,* (2010) reported *Bacillus* sp. SH-517 produce optimum keratinase after 40hrs whereas *Bacillus* sp. FK 28, *Bacillus pseudofirmus*, *Xanthomonas maltophila* POA-1 and *Arthrobacter creatinolyticus* KP015744 demonstrated maximum yield of enzyme in 72hrs (Pissuwan and Suntornsuk, 2001; Kate and Pethe, 2014; De Toni *et al.,* 2002; Kojima *et al.,* 2006). Optimum period of 5 days for keratinase production for *B.megaterium* F7-1 (Park and Son, 2007), *Bacillus* sp. FK46 (Suntornsuk and Suntornsuk, 2003), *Streptomyces gulbargensis* (Syed *et al.,* 2009)

and *Bacillus licheniformis* KMBVP (Vidhya and Palaniswamy, 2013) was also reported. *Bacillus subtilis* Subsp *Subtilis* and *Brevibacillus thermoruber* T1E with incubation of 7 days reported to give maximum enzyme yield (Manju and Shanmugam, 2013; Bihari *et al.,* 2010) whereas 8 days was reported for *Bacillus subtilis* AMR (Mazotto *et al.,* 2010).

Effect of various concentrations on Magnesium salt and calcium salt on the production of keratinase

Maximum keratinase production was observed in Feather Basal medium supplemented with 3mg of $MgSO_4$ $6H_2O$/100ml of medium by the isolate *Bacillus thuringiensis* strain Bt407. When inoculated in media containing $MgSO_4$ $6H_2O$ in concentrations greater than or lesser than 3mg/100ml, a reduction in synthesis of the keratinase by *Bacillus thuringiensis* strain Bt407 was observed (Fig. 5). *Bacillus thuringiensis* strain Bt407, when grown in Feather Basal medium containing 0.5 mM $CaCl_2$ 2 H_2O, was found to induce higher enzyme production. A higher concentration of $CaCl_2$ of 1mM or 2mM was seen to repress the production of keratinase (Fig. 6). In earlier studies the basic medium used for fermentation of the feather-degrading microorganisms such as *Bacillus subtilis* RSE163 (Gupta and Singh, 2013), *Bacillus* sp. JB 99 (Kainoor and Naik, 2008), *Bacillus halodurans* Strain PPKS-2 (Pathange *et al.,* 2009), *S. albidus* E4 and *S. griseoaurantiacus* E5 (Kansoh *et al.,* 2009) and Trichophyton sp. HA-2 (Anbu *et al.,* 2008) contained $MgSO_4$.The protease production is slightly stimulated by $CaCl_2$ and $MgCl_2$ and strongly inhibited by $CuSO_4$. It was reported that the maximum keratinase production by *Bacillus subtilis* KD-N2 was obtained when the initial concentrations of $MgSO_4$ was 0.91 g/L (Cai and Zheng, 2009) whereas *Streptomyces gulbargensis* DAS 131 produced keratinase in a medium containing $CaCl_2$-0.2% and $MgSO_4$ $7H_2O$-0.2% (Syed *et al.,* 2009). Previous studies showed that basal medium containing $MgSO_4$ $7H_2O$ and $CaCl_2$ were used for keratinase production in case of *Bacillus subtilis* (Pillai and Archana, 2008), *Bacillus* sp.SCB-3 (Lee *et al.,* 2002), *Bacillus megaterium* (Saibabu *et al.,* 2013), *Bacillus pumilus* FH9 keratinolytic (El-Refai *et al.*,2005), *Bacillus subtilis* MTCC 9102 (Balaji *et al.,* 2008), Streptomyces sp: (Ramakrishnan *et al.,* 2011), *Bacillus cereus* Wu2 (Lo *et al.,* 2012), *Pseudomonas aeruginosa* C11 (Han *et al.*,2012), *Aspergillus oryzae* NRRL-447 (Ali *et al.,* 2011), *Actinomyces* sp (Jayalakshmi *et al.*,2010) and *Scopulariopsis brevicaulis* (Anbu *et al.,* 2007).

Effect of pH and incubation temperature on the production of keratinase

The medium was adjusted to different pH values such as pH 5, 6, 7, 8, 9 and 10 and was assayed to obtain the optimum pH for the maximum production of keratinase by *Bacillus thuringiensis* strain Bt407. The maximum keratinase production was observed at pH 7 as shown in figure 7. Different temperatures such as 28°C, 37°C, 45°C and 55°C were tested to obtain the optimum temperature for the maximum production of keratinase by *Bacillus thuringiensis* strain Bt407.The temperature at which the production of keratinase was maximum was observed to be 37°C (Fig. 8). However, maximum keratinase for *Bacillus thuringiensis* TS2 was observed at pH 10.0 and 50°C (Sivakumar *et al.,* 2012) and *Bacillus thurengenesis* SN2 showed optimum enzyme production at 30°C and pH 7.5 (Agrahari and Wadhwa, 2010). Previous studies showed that maximum keratinase production by various bacterial species exhibited at different temperatures and pH values such as for *Bacillus cereus TS1* was at pH 9 and temperature 50°C (Sivakumar *et al.,* 2013),

Bacillus pumilus ZED17 at pH 10 and 37°C (Talebi *et al.,* 2013), *B. licheniformis* KMBVP strain at 40°C and pH 8 (Vidhya and Palaniswamy, 20130), *Bacillus amyloliquefaciens* at pH 8 and 50°C (Cortezi *et al.,* 2008), *Bacillus subtilis* strain RM-01 at 50°C and pH 8 (Rai *et al.,* 2009), *Bacillus* sp. JB 99 at 45°C and pH 10 (Kainoor and Naik, 2010), *Bacillus* sp. MBRL 575 at 30°C and pH 9-10 (Ningthoujam and Kshetri, 2016), *Brevibacillus thermoruber* T1E at 50°C and pH 6.5 (Bihari *et al.,* 2010), *Stenotrophomonas maltophilia* at 30°C and 7.0 (Jeong *et al.,* 2010), Lysobacter sp. A03 at temperature of 20°C and pH 7 (Pereira *et al.,* 2014), *Streptomyces minutiscleroticus* DNA38 at 45°C and pH 9 (Allure *et al.,* 2015) and *Bacillus subtilis* at pH 11 and 40ºC (Mousavi *et al.,* 2013). Proteolytic activities of most organisms are seen to be 30-37°C, whereas some Keratinolytic bacteria show feather degrading capacities at higher temperature (Riffel and Brandelli, 2006).

Effect of static and shaker conditions on the production of keratinase

Bacillus thuringiensis strain Bt407 showed a higher enzyme activity when incubated under agitation on a shaker at 120 rpm as compared to incubation in a static condition (Fig. 9). Similar results were obtained for *Bacillus thuringiensis TS2* (Sivakumar *et al.,* 2012*), Bacillus thurengenesis* SN2 (Agrahari and Wadhwa, 2010) and *Bacillus licheniformis* PWD1 (Lin *et al.,* 1996). Aeration is required for many bacterial species such as *Bacillus* sp. FK28 (150rpm), *Bacillus licheniformis* RG1 (250 rpm), *Bacillus halodurans* PPKS-2 (180rpm), *Bacillus* sp. P7 (130rpm) *Vibrio sp. Kr2* (180rpm), *Chryseobacterium sp. kr6* (180rpm), *B.pumilus* FH-9 (200 rpm), *B.megaterium* F7-1(200 rpm), *Streptomyces exfoliatus CFS1068* (150 rpm) and *Stenotrophomonas maltophila* R-13 (200 rpm) (Pissuwan and Suntornsuk, 2001; Ramnani and Gupta, 2004; Sangali and Brandelli, 2000; Riffel *et al.,* 2003; Jeong *et al.,* 2010b; Park and Son, 2007; El-Refai *et al.,* 2005; Jain *et al.,* 2012; Pathange *et al.,* 2009; Correa *et al.,* 2009) for maximum keratinase production. However, *Thermoanaerobacter keratinophilus* sp. nov. (Rissen and Antranikian, 2001), *Fervidobacterium islandicum* AW-1 (Nam *et al.,* 2002) and *Fervidobacterium pennavorans* (Friedrich and Antranikian, 1996) reported to require static condition for maximum yield of keratinase.

Bacillus thuringiensis strain Bt407 exhibited a much higher enzyme activity (94.52Units/ml) when grown in optimized medium in comparison to the Whole feather basal medium (49.75Units/ml) which was used initially for the production of keratinase. In our studies optimized medium doubled the yield of enzyme.

Keratinase production using human hairs and nails

A steady increase in the keratinase enzyme production by *Bacillus thuringiensis* strain Bt407 in optimized medium containing human nails and human hairs was observed till 144hrs and after which there was decline in enzyme production (Fig. 10). Human nails were degraded efficiently than human hairs by the isolate. Similar results were obtained for *Bacillus pumilis* F3-4, *B. megaterium* F7-1, *B. cereus* TS1, *B. licheniformis* FK 14, *Bacillus* sp, *Bacilus licheniformis* PWD-1, *Microbacterium* sp., *Scopulariopsis brevicaulis*, *Streptomyces gulbargensis* DAS 131 and *Chryseobacterium* sp. RBT strain (Peddu *et al.,* 2009; Son *et al.,* 2008; Park and Son, 2007; Gurav *et al.,* 2016; Sivakumar *et al.,* 2013; Thys *et al.,* 2004; Cheng *et al.,* 1995; Sharaf and Khalil, 2011; Syed *et al.,* 2009).

Fig.1 Different isolates showing degradation of feathers

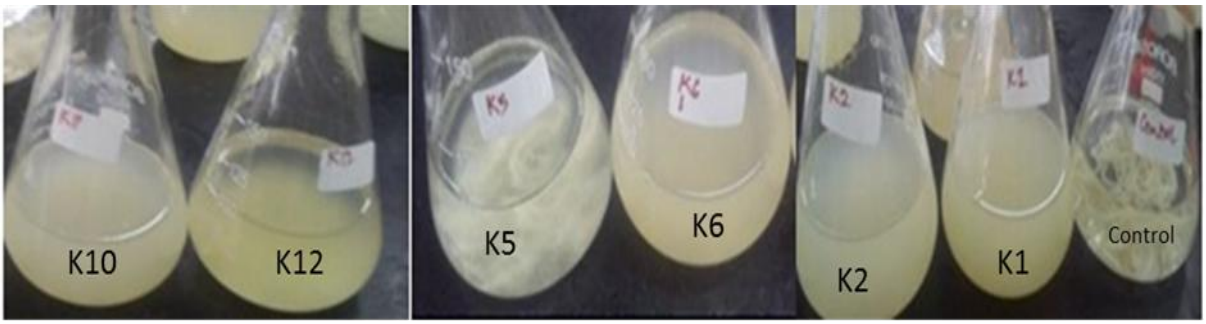


Fig.2 Keratinase assay of different isolates at various incubation periods

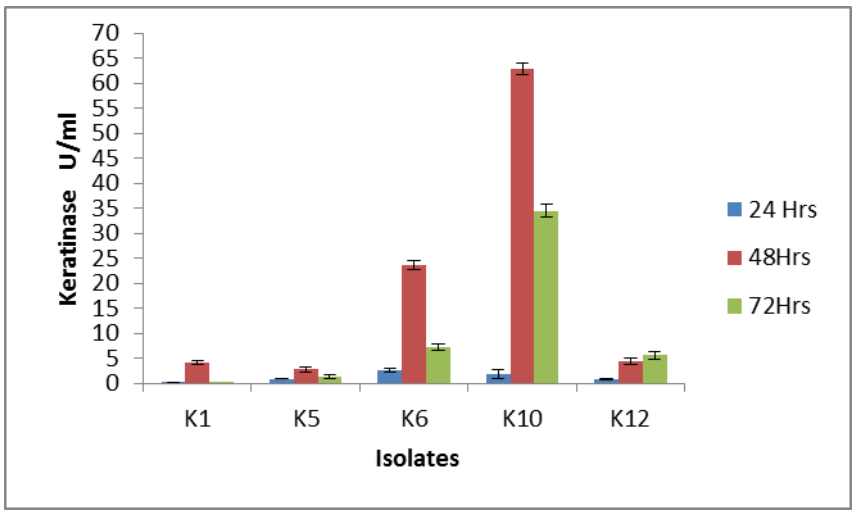


Fig.3 Effect of different co-carbon sources on keratinase production by *Bacillus thuringiensis* strain Bt407

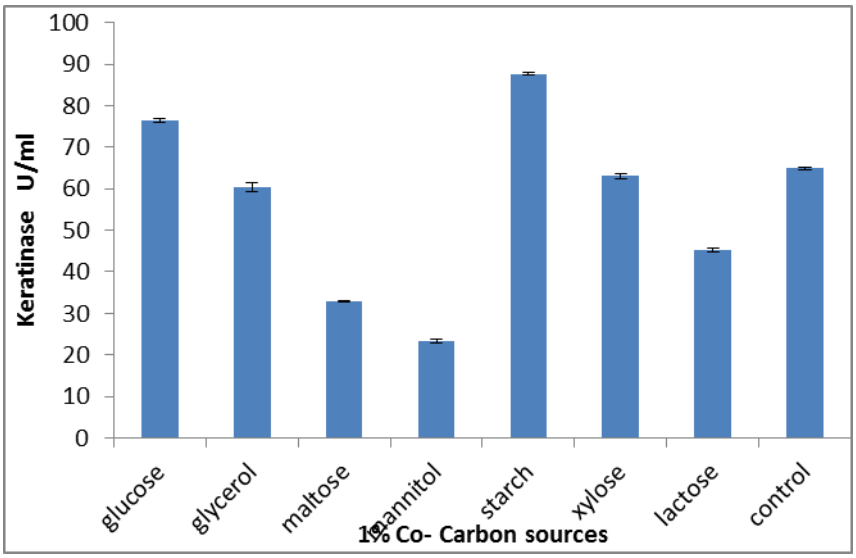


Fig.4 Effect of different co-nitrogen sources on keratinase production by *Bacillus thuringiensis* strain Bt407

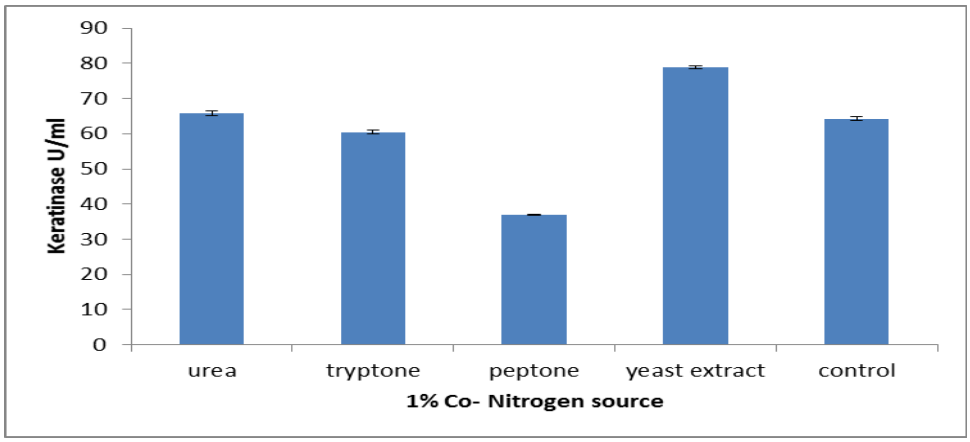


Fig.5 Effect of $MgSO_4$ concentration on keratinase production by *Bacillus thuringiensis* strain Bt407

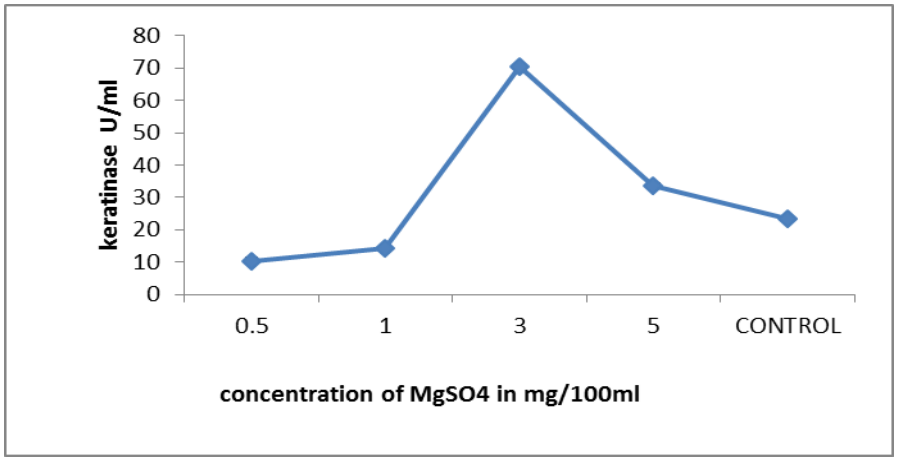


Fig.6 Effect of $CaCl_2$ concentration on keratinase production by *Bacillus thuringiensis* strain Bt407

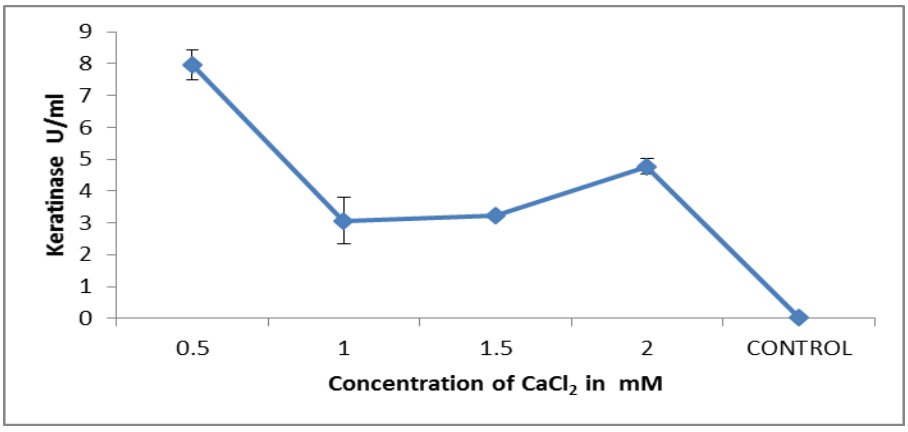


Fig.7 Effect of different pH values on keratinase production by *Bacillus thuringiensis* strain Bt407

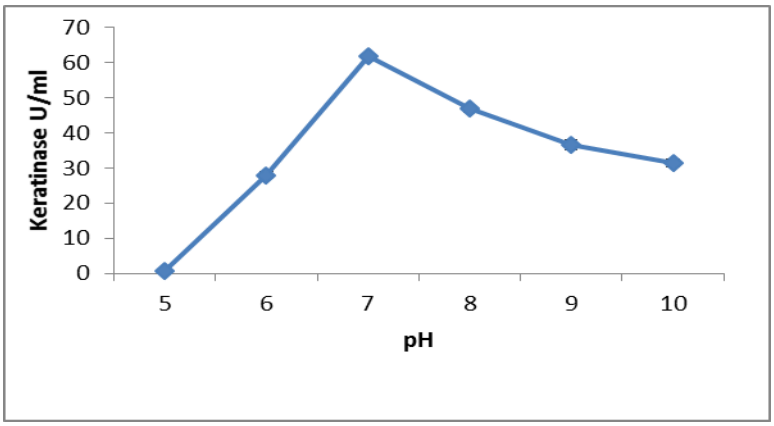


Fig.8 Effect of different temperatures on keratinase production by *Bacillus thuringiensis* strain Bt407

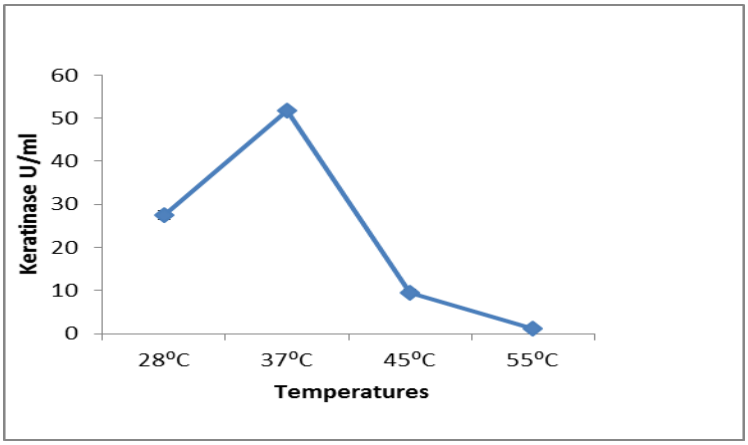


Fig.9 Effect of aeration on keratinase production by *Bacillus thuringiensis* strain Bt407

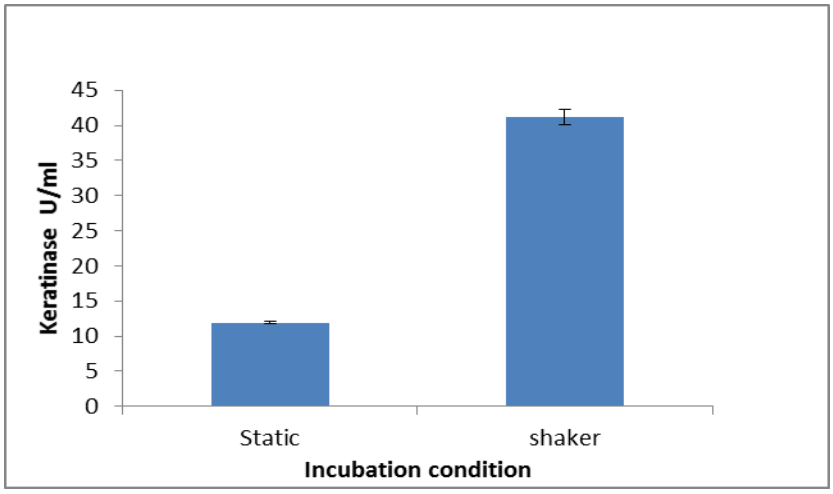


Fig.10 Utilization of Human hairs and human nails as substrates for keratinase production by *Bacillus thuringiensis* strain Bt407 at different incubation period

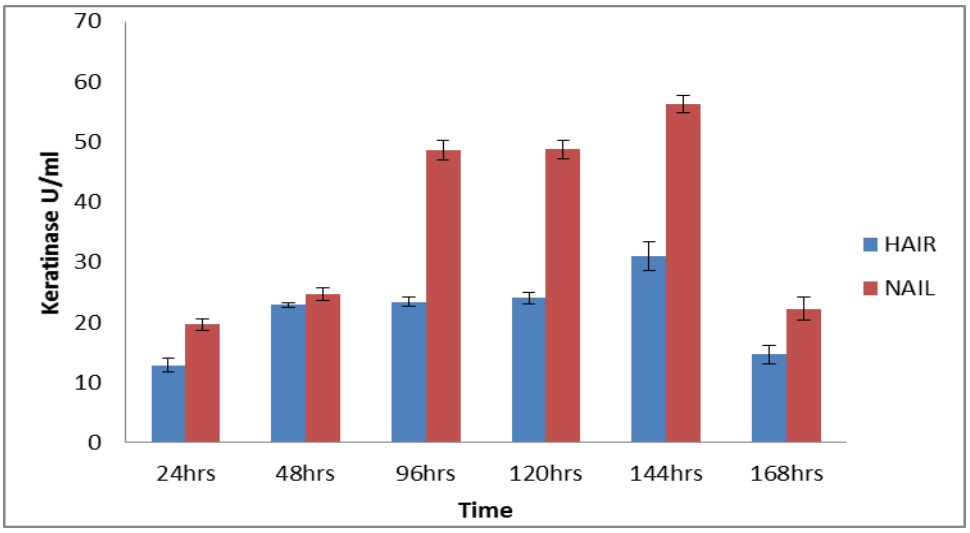


Fig.11 [A] SDS -PAGE of keratinase enzyme, [B] Zymogram, [C] Proteolytic activity of keratinase on casein agar plate

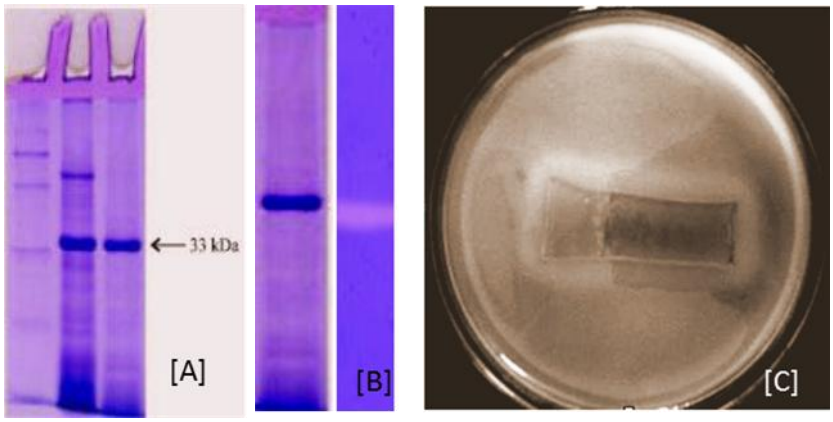


Fig.12 Effect of different pH values on enzyme activity

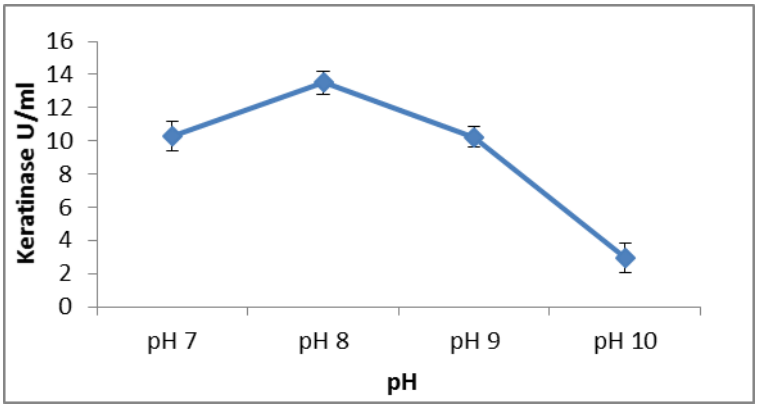


Fig.13 Effect of different temperatures on keratinase activity

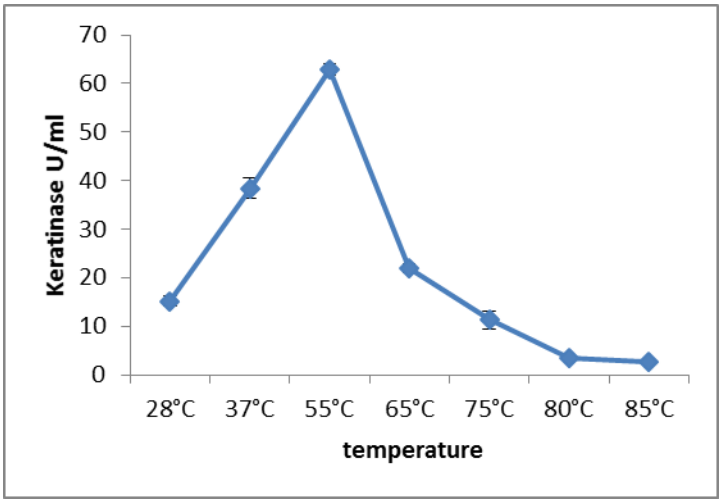


Fig.14 Effect of different metal ions on keratinase activity

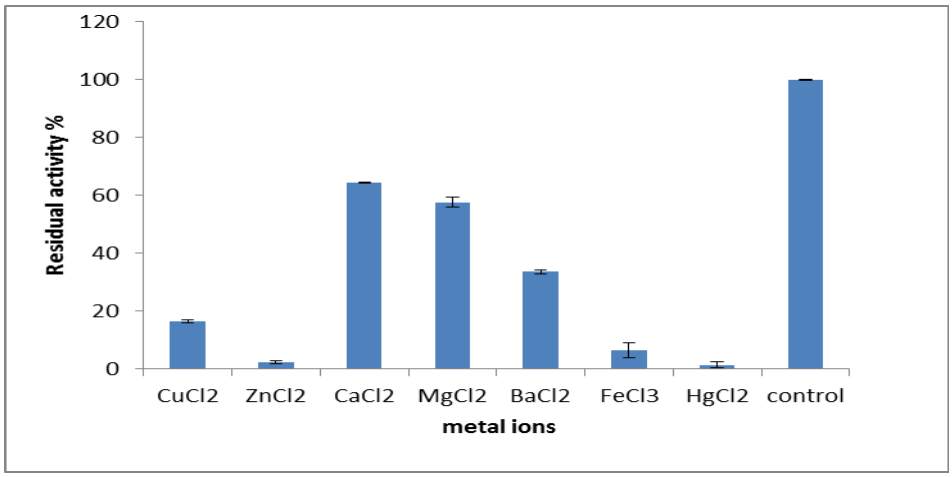


Fig.15 Effect of different inhibitors on keratinase activity

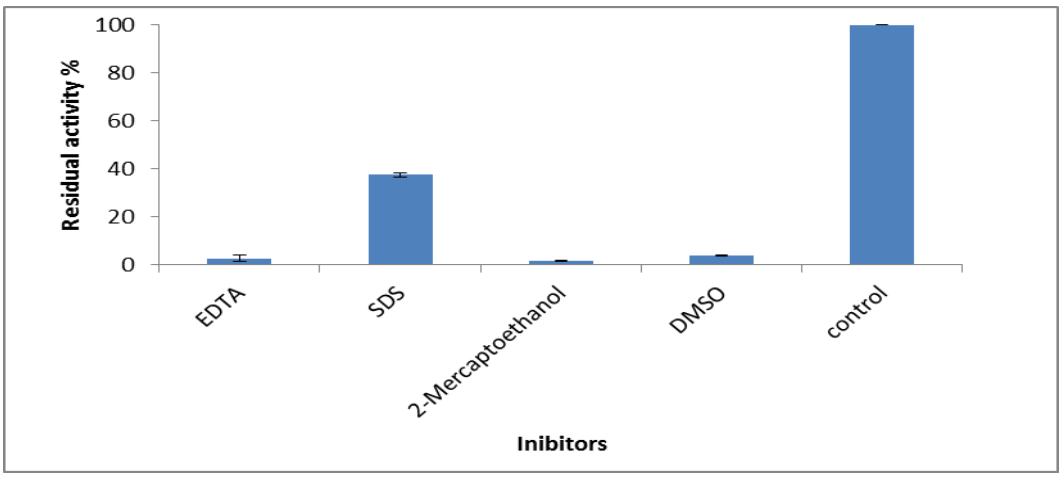
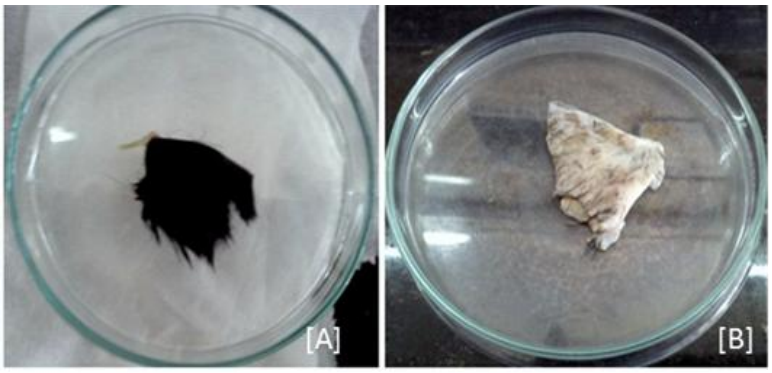


Fig.16 Effect of Keratinase on goat skin hair removal



[A] Before addition of enzyme. [B] After addition of enzyme

Fig.17a [A] Control: Blood stain rinsed by Distilled Water. [B] Blood + Ariel: Blood stain rinsed in Ariel detergent solution. [C] Blood + Ariel + Enzyme: Blood stain rinsed by detergent followed by enzyme produced by *Bacillus thuringiensis* strain Bt407

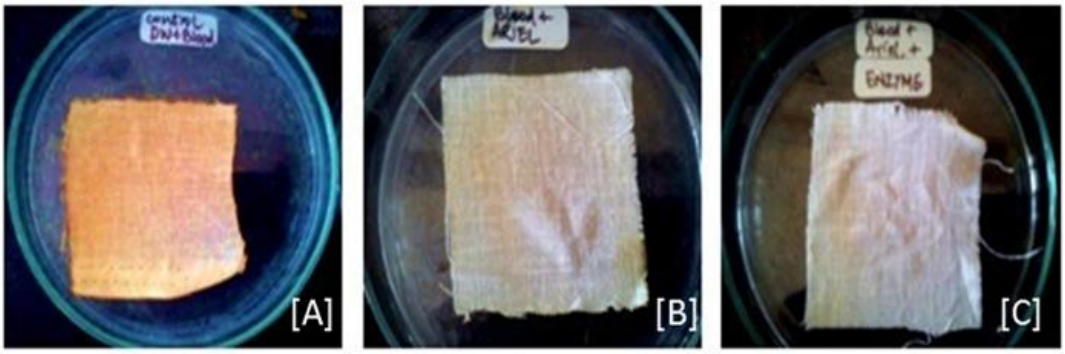


Fig.17b [A] Control: Egg yolk stain rinsed by Distilled Water. [B] Egg yolk stain + Ariel: Egg yolk stain rinsed in Ariel detergent solution. [C] Egg yolk stain + Ariel + Enzyme: Egg yolk stain rinsed by detergent followed by enzyme produced by the *Bacillus thuringiensis* strain Bt407

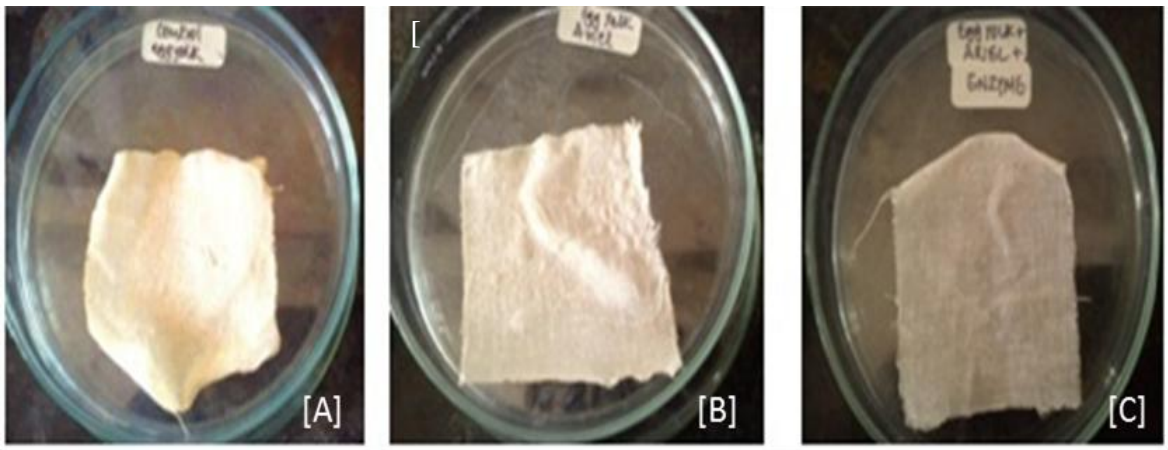


Table.1 Quantification of keratinase enzyme of different isolates at different Incubation time period

Isolate	24Hrs			48Hrs			72Hrs		
	U/ml	Mg protein /100ml	Specific activity U/mg	U/ml	Mg protein /100ml	Specific activity U/mg	U/ml	Mg protein /100ml	Specific activity U/mg
K1	0.22	12	0.001	4	9.62	0.41	0.023	80	0.0002
K5	0.89	30	0.029	2.5	16.5	0.151	1.34	67	0.002
K6	2.65	15	0.1766	23.68	14	1.68	7.24	34	0.212
K10	1.85	10	5.40	62.6	5.23	11.96	34.6	18.4	1.880
K12	0.72	16	0.045	4.5	28.2	0.160	5.6	12	0.466

Table.2 Purification studies of keratinase enzyme produced by *Bacillus thuringiensis* strain Bt407

Enzyme	Total protein content (mg/ml)	Enzyme U/ml	Specific activity U/mg
Crude extract	1.85	10	5.40
Ammonium sulphate precipitation	3.26	23	8.67
After Dialysis	5.45	62.62	11.96

Table.3 Use of feather hydrolysate in microbiological medium

Media	*E.coli*	*B.subtilis*	*P.vulgaris*	*S.aureus*	*P.aeruginosa*	*S. para B*
NaCl+ Agar	-	-	-	-	-	-
NaCl + Feather hydrolysate + Agar	+	+	+	+	+	+
Nutrient agar	+	+	+	+	+	+

Key += Growth, - = no growth

However, *B. subtilis* AMR produced higher yield of enzyme in medium containing hairs (Mazotto *et al.,* 2010), *Bacillus subtilis* KD-N2 could not hydrolyze human hair (Cai and Zheng, 2009) and *Bacillus licheniformis* FK-14 could neither hydrolyze human hairs nor human nails (Suntornsuk *et al.,* 2005). *Doratomyces microsporus* was able to produce enzyme to hydrolyze native keratin such as stratum corneum, porcine nails and human nails. The human stratum corneum was seen to have a much higher degree of enzyme production than the rest of the substrates (Gradisar *et al.,* 2000). A new *Bacillus subtilis* strain was able to hydrolyze sheep wool and human hair (Cai *et al.,* 2008). *Tichophyton mentagrophytes var. erinacei* was able to degrade human hair and guinea pig hair (Muhsin and Aubaid, 2000).

Purification Studies: Partial purification of Keratinase

A cell-free extract of the isolate which was grown in optimized medium was precipitated using the Ammonium sulfate method.

Following dialysis, an increase in the specific activity of the enzyme was observed (Table 2) Similar purification and dialysis technique was used to purify enzyme from *Stenotrophomonas* (Cao *et al.,* 2009), *Bacillus subtilis* KD-N2 (Cai *et al.,* 2008), *Streptomyces minutiscleroticus* DNA38 (Allure *et al.,* 2015), *Bacillus subtilis* (Manju and Shanmugam, 2013), *Streptomyces albus* (Nayaka *et al.,* 2013), *Bacillus tequilensis* strain Q7 (Jaouadi *et al.,* 2015), *Bacillus licheniformis* FK-14 (Suntornsuk *et al.,* 2005), *B. thuringenesis* SN2 (Agrahari and Wadhwa, 2010), *Bacillus cereus* MCM B-326 (Nilegaonkar *et al.,* 2007) and *Streptomyces minutiscleroticus* DNA38 (Allure *et al.,* 2015).

SDS- PAGE electrophoresis and Zymography

SDS-PAGE electrophoresis was carried out for the purified enzyme produced by *Bacillus thuringiensis* strain Bt407 and the molecular weight of the keratinase was found to be 33 kDa. However, molecular weight of keratinase in *B. thuringenesis* SN2 is 120 kDa, 80 kDa, 60 kDa and 40 kDa (Agrahari and Wadhwa, 2010). The molecular weight of major keratinases varies from 20 to 50 KDa (Bockle *et al.,* 1995). Likewise, it is similar to the keratinase MW of *Bacillus pseudofirmus* FA30-01 at 27 KDa (Kojima *et al.,* 2007). Similar results were obtained for *Bacillus licheniformis* keratinase (31.4 kDa) (Cheng *et al.,* 1995), *Bacillus licheniformis* FK-14 (35 kDa) (Suntornsuk *et al.,* 2005), *Bacillus subtilis* (30kDa) (Kazi *et al.,* 2015), *Bacillus pumilus* (32 kDa) (Huang *et al.,* 2003), *Bacillus subtilis* KD-N2 (30.5 kDa) (Cai *et al.,* 2008), *Bacillus* (38kDa) (Peddu *et al.,*2009), *Stenotrophomonas maltophilia* (35.2 kDa) (Cao *et al.,* 2009) and *Streptomyces minutiscleroticus* DNA 38 (29kDa) (Allure *et al.,* 2015). However, there were studies which were showing molecular weight of keratinase lower or higher than molecular weight shown in our studies viz. *Bacillus* sp. JB 99 (66kDa) (Kainoor and Naik, 2010), *Streptomyces albidoflavus* (18 kDa) (Bressollier *et al.,* 1999), *Fervidobacterum pennavorans* (130 kDa), (Friedrich *et al.,* 1995), *Bacillus* sp. SCB-3(134kDa) (Lee *et al.,* 2002), *B. cereus* DCUW (80kDa) (Ghosh *et al.,* 2008), *Bacillus tequilensis* strain Q7 (28.3 kDa) (Jaouadi *et al.,* 2015), *Bacillus subtilis* (40kDa) (Manju and Shanmugam, 2013), *Bacillus licheniformis* strain HK-1 (46kDa) (Korkmaz and Dincer, 2004), *Chrysosporium keratinophilum* (69 kDa) (Dozie *et al.*,1994), *Kocuria rosea* (240kDa) (Bernal *et al.,* 2006a) and *Bacillus megaterium* (41kDa) (Saibabu *et al.,* 2013). Zymography analysis in SDS – PAGE showed a single band which corresponded keratinase activity was observed by clear zone of proteolytic activity in the zymogram (Fig. 11 A and B). Similarly the gel was placed on a Sterile Casein agar plates. A zone of clearence was observed around the gel on the plate indicating the proteolytic activity of the keratinase (Fig. 11C). Similar studies about zymography of keratinase is also done in case of *Bacillus* sp (Pandian *et al.,* 2012), *Bacillus* sp. 50-3 (Zhang *et al.,* 2009), *Bacillus* sp. P45 (Daroit *et al.,* 2009), *Bacillus cereus* (Sousa *et al.,* 2007), *Bacillus subtilis* AMR (Mazotto *et al.,* 2010), Bacillius sp (Pandian *et al.,* 2012), *Bacillus tequilensis* strain Q7 (Jaouadi *et al.,* 2015), *Acinetobacter* sp. PD 12 (Shah, 2015), Streptomyces sp. strain 16(Xie *et al.,* 2010), Lysobacter sp. A03 (Pereira *et al.,* 2014), *Brevibacillus brevis* US575 (Jaouadi *et al.,* 2013) and *Onygena corvina* (Huang *et al.,* 2014).

Effect of different pH and temperature on keratinase enzyme activity

The maximum activity of keratinase of *Bacillus thuringiensis* strain Bt407 was

observed at pH 8 and 55°C indicating that the enzyme is alkaliphilic and active at high temperature (Fig. 12 and 13). These results are in accordance with *Bacillus cereus* DCUW and *Chryseobacterium sp*. kr6 (Ghosh *et al.,* 2008; Riffel *et al.,* 2007). But there is slight difference in optimum pH and temperature of keratinase activity of othere starins of *Bacillus thuringiensis* TS2 and *B. thuringenesis* SN2 where optimum pH was 10 and optimum temperature was 50ºC (Sivakumar *et al.,* 2012; Agrahari and Wadhwa, 2010). For many *Bacillus* species optimum pH and temperature for keratinase activity were studied and it was found that in case of *Bacillus subtilis* temperature 37^{o}C and pH 10.0 (Kazi *et al.,* 2015), *for Bacillus subtilis* AMR temperature 50°C and pH9 (Mazotto *et al.,* 2010), for *Bacillus* sp. keratinase activity range was between 28°C - 55°C and pH optima was 8.5 (Peddu *et al.,*2009), for *Bacillus* sp. JB 99 temperature 65^{o}C and pH 10 (Kainoor and Naik, 2010), for *B. licheniformis* strain HK-1 temperature 60ºC and pH11.0 (Korkmaz and Dinçer, 2004), *for Bacillus* sp. 50-3 temperature 60°C and pH 10.0 (Zhang *et al.,* 2009), for *Bacillus safensis* LAU 13 temperature 40ºC and pH 7.5 (Lateef *et al.,* 2015), for *Bacillus polymyxa* B20 temperature 50ºC and pH 10.2 and for *Bacillus cereus* B5esz temperature 45ºC and pH 7.4(Łaba and Rodziewicz, 2010) was observed. For *Streptomyces sclerotialus* optimum temperature 55°C and pH 9.0 was recorded (Yadav *et al.,* 2011) and for *Streptomyces albus* optimum temperature and pH observed 40^{0}C and 7.0 respectively (Nayaka *et al.,* 2013).

Effect of different metal ions on keratinase activity

The effect of different metal ions on keratinase activity was studied. Metal ions like Ca^{+2}, Mg^{+2}, and Ba^{+2} were seen to enhance enzyme activity whereas Cd^{+2}, Cu^{+2}, Fe^{+3}, Hg^{+2} and Zn^{+2} were observed to inhibit keratinase activity from *Bacillus thuringiensis* strain Bt407 (Fig. 14). Similar results were reported in case of *Bacillus thuringienesis* SN2 (Agrahari and Wadhwa, 2010), *Bacillus thuringiensis* TS2 (Sivakumar *et al.,* 2012), *Bacillus* sp. P7 (Correa *et al.,* 2009), *Bacillus megaterium* (Saibabu *et al.,* 2013), *Bacillus* sp. JB 99(Kainoor and Naik, 2010), *Bacillus licheniformis* strain HK-1 (Korkmaz and Dinçer, 2004), *B. licheniformis* YJ4 (Lin and Yin, 2010), *Bacillus* sp (Peddu *et al.,*2009), *Bacillus subtilis* MTCC 9102 (Balaji *et al.,* 2008), *B. pumilus* CBS *Bacillus* species (Giongo *et al.,* 2007), *Brevibacillus brevis* US575 (Jaouadi *et al.,* 2013), *Fervidobacterium islandicum AW-1* (Nam *et al.,* 2002), *Arthrobacter creatinolyticus* KP015744 (Kate and Pethe, 2014), *Bacillus* sp. KG5 (Ahmetoglu *et al.,* 2015).

However, in case of *B. licheniformis* FK 14, even Zn^{+2} in addition to Ca^{+2}, Mg^{+2} and Ba^{+2} were seen to enhance keratinase activity (Suntornsuk *et al.,* 2005) and keratinase from *Bacillus safensis* LAU 13 was activated by Na^{+}, Ca^{+2}, $Fe^{+2}$and Mg^{+2} but inhibited by Mn^{+2}. A metal ion plays an important role for protease thermal stability as well (Bajorath *et al.,* 1988).The effect of various metal ions on the production of a keratinase from *Bacillus* sp.SCB-3 was investigated and it was found out that the addition of Zn^{+2}, Cu^{+2}, Co^{+2} and Hg^{+2} resulted in decrease in the enzyme production of the enzyme. But the addition of 0.1mM Ca^{+2} and Mg^{+2} gave an excellent production of enzyme suggesting that Ca^{+2} and Mg^{+2} maybe active ions in the native enzyme (Lee *et al.,* 2002; Suntornsuk *et al.,* 2005).

These results also suggest that Ca^{+2} ion is required for the enzyme activity or stability. The effect of Ca^{+2} on the enzyme under investigation may involve stabilization of the enzyme structure.

Effect of different inhibitors on keratinase activity

Keratinase produced by *Bacillus thuringiensis* strain Bt407 was observed to retain its 37.67% residual activity in the presence of SDS while 2- mercaptoethanol, EDTA and DMSO were seen to inhibit the enzyme activity (Fig. 15). However, *B. licheniformis* strain HK-1 keratinolytic activity was partially inhibited by EDTA, 1, 10-phenanthroline and PMSF and it was on the other hand increased by DMSO, TritonX-100 and SDS (Korkmaz and Dinçer, 2004). Other studies suggest that for *Bacillus subtilis* the keratinase activity was not inactivated in presence of EDTA (Kazi *et al.,* 2015) but affected by $HgCl_2$.

Use of feather hydrolysate as microbial feed in bacteriological media

Keratinase hydrolysed products were incorporated in the medium containing only salt and agar. Various laboratory cultures were grown on it at 37°C for 24 hrs as shown in Table 3. It was seen that chicken feathers degraded by keratinase obtained from isolate can be used for preparation of economical microbial feed for growth of different microbiological cultures. Similar degraded chicken feathers obtained after treatment with keratinase from Streptomyces sp. IF 5 were used to prepare economical microbiological medium which supported growth of different microorganisms (Ramakrishnan *et al.,* 2011).

Dehairing capacity of keratinase on goat skin

Alkaline Keratinases enables swelling of hair roots and subsequent attack of protease on hair follicle causing easy removal of hair. *Bacillus thuringiensis* strain Bt407 produced enzyme did not cause any damage to the skin but caused sucessful removal of hair from the goat skin after 24 hrs (Fig. 16). This means that the enzyme from this organism can be used as depilatory agent in tanning industry. Similar results were obtained *Bacillus pumilus* CBS Alkaline Proteinase (Jaouadi *et al.,* 2009), *Bacillus cereus* MCM B-326 (Zambare *et al.,* 2007) and *Bacillus safensis* LAU 13(Lateef *et al.,* 2015). A few reports that indicate that keratinases could be useful depilating agents are available (Letourneau *et al.,* 1998; Bressollier *et al.,* 1999; Allpress *et al.,* 2002; Friedrich and Kern, 2003). In fact, a keratinase from *B. subtilis* S14 (Macedo *et al.,* 2005) was reported to completely eliminate the need for toxic sodium sulfide. Thus, sulfide-based "hair-destroying dehairing" processes that pose an environmental threat by increasing the BOD could be replaced by keratinase-based cleaner "hair-saving dehairing" technology. *Brevibacillus brevis* US575 (Jaouadi *et al.,* 2013)

Application of keratinase enzyme as detergent additive (Wash performance)

Keratinase being a very sturdy enzyme is checked for its ability to act as a potent detergent additive which was determined by using a cotton cloth stained by Blood and egg yolk. Visual observations were made after subjecting the stained cloth pieces to various control and test solutions (Fig. 17a and 17b). The results indicate that the enzyme Keratinase was able to destain both blood and egg yolk stains on the cloth as shown in Figure 17 a(C) and 17 b(C) compared to wash performance of the detergent Figure 17 a(B) and 17 b(B). Similar results were obtained for alkaline proteinase of *Bacillus pumilus* CBS (Jaouadi *et al.,* 2009), *Bacillus subtilis* RM-01(Rai *et al.,* 2009), *Bacillus megaterium* (Saibabu *et al.,* 2013), *PaeniBacillus woosongensis* TKB2 (Paul *et al.,* 2014), *Bacillus safensis* LAU 13(Lateef *et al.,* 2015) and *Brevibacillus* sp. strain AS-S10-II (Rai

and Mukherjee, 2011; Gupta and Ramnani, 2006).

This study concludes that the optimization of production of keratinase from *Bacillus thuringiensis* Bt407 which was isolated` from poultry site soil in a medium containing Feather meal (2%), Yeast extract (1%), Starch (1%), $MgSO_4$ $6H_2O$ (0.003%), $CaCl_2$ (0.5mM), KH_2PO_4 (0.5%), K_2HPO_4 (0.3%), NaCl (0.5%), pH 7, inoculated with 1% v/v pre-grown cell mass and incubated at 37°C on rotary shaker (120 rpm) for 48 hours. Keratinase enzyme has optimum activity at 55ºC and pH 8 suggesting its thermophilic and alkaliphilic nature. Application of keratinase enzyme for depilatory action on goat skin, as a detergent additive and enzyme hydrolyzed feather meal in bacteriological medium as nitrogen source were also studied.

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