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CHARACTERIZATION OF A MICROBIAL KERATINASE WITH LEATHER DEHAIRING AND FEATHER WASTE MANAGEMENT POTENTIAL

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ABSTRACT

The aim of the study was purification and biochemical characterization of a keratinase enzyme from indigenous microorganism and its promising potential biotechnological applications. The increasing demand of keratinases in different industrial sectors calls for the need of more robust and stable keratinases with potential industrial applications. Keratinase produced by *Bacillus* D2 strain was purified by gel filtration and Q-Sepharose chromatograph. The enzyme showed a specific activity of 525 U/mg with overall recovery of 23.8%. Molecular size of purified enzyme after SDS-PAGE and zymogram was found 40 kDa. Purified keratinase had an optimal pH 8.5 and optimal temperature 50°C. Enzyme displayed pH stability in pH range of 7.5–9.0 and thermal stability up to 60°C. Amongst reducing agents, sodium sulfite and dithiothreitol (DTT) reduced relative activity however; SDS increased activity at 5 mM concentration. Keratinase enzyme was found solvent stable at 0.5% and 1% concentration. $MgCl_2$ enhanced activity up to 108% and 104% at 5 and 10 mM concentrations, respectively. Enzymatic treatment of goat skin and cow hide resulted in dehairing and removal of scud and keratin skin layer resultantly smooth intact skin surface was observed. However, conventionally treated hides produced dark and hard skin surface due to partial elimination of keratin layer. Potential of *Bacillus* strain for conversion of feather keratin waste in to valuable feather meal suggests its usefulness in poultry industry and eventually reducing environmental pollution hazards.

Key words: Characterization, Feather waste, Keratinase, Leather dehairing, Protein purification

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INTRODUCTION

Proteases are group of enzymes that break proteins by hydrolyzing their peptide bonds and refer as proteinases, proteases, or peptidases. Proteases are classified under class of hydrolases (E.C.3), and grouped as peptidases or peptide hydrolases subclass (E.C.3.4) (Govarthanan *et al.*, 2015; Motyan *et al.*, 2013). Keratinase (E.C.3.4.99.11) is one of these protease enzymes which has ability to disrupt highly stable keratin proteins having hydrogen, hydrophobic and disulfide bonds (Khardenavis *et al.*, 2009). Keratin proteins have compact structure of helices making 40–60 kDa keratins and β–sheets in about 10 kDa keratins making a compact structure. The secondary structures give them higher stability and resilience against most of the proteolytic enzymes including pepsin, trypsin and papain (Gegeckasa *et al.*, 2015). Keratinases are enzymes that target these insoluble proteins and their hydrophobic amino acids thus differentiated from rest of the protease group members that work on soluble proteins (Yong, *et al*., 2020).

Limited numbers of known microorganisms are reported that produce keratinases to degrade keratin polypeptide chains. Different microorganism groups including fungi, actinomycetes, and bacteria have shown ability to degrade insoluble and soluble proteins (Li, 2019). These microbes have been investigated for optimum keratinases production to meet the needs of industries such as cosmetic, medicine, prion degradation and leather industry. In detergent industry, keratinases could replace traditional protease additives as they have strong characteristics including alkaline stability, oxidation stability, temperature tolerance, and detergent compatibility (Tork *et al.*, 2013). One of the most promising applications of keratinases is the industrial production of cost effective, nutria–rich, environment friendly feather meal for poultry and animal feed. Keratinases have been successfully explored and used in enzymatic leather dehairing in leather processing, fertilizers production with slow release nitrogen ability, and in biomedical industry as in production of biodegradable coatings and films (Brandelli, 2008; Elhoul *et al.*, 2016).

The increase use of poultry worldwide has resulted in millions of tons of feather waste. Keratinous feather waste is made up of 90% of keratin which is difficult to hydrolyze due to stable secondary structures (Said *et al.,* 2019). This keratinous waste due to inefficient utilization creates serious environmental pollution hazards due to incineration of this waste (Brandelli and Riffel, 2005). Keratinase producing microbes are increasingly used for degradation of poultry feather waste and its

recycling. Lakshmi *et al.* (2013) characterize two strains, *B. cereus* and *B. subtilis* which completely degrade keratin. Wu *et al.* (2017) report a thermophilic feather degrading bacterium *Meiothermus taiwanensis* WR-220 which completely degrades feather in 2 days at 65°C and pH 10. This study elaborates thermomophile keratinases for keratin waste recycle and for industrial applications. Kumar *et al*. (2017) isolate *Chrysosporium tropicum* and *Malbranchea* sp. from soil to produce nitrogen rich organic fertilizer from degradation of feathers. Proteins and amino acids are released in submerged state fermentation after degradation of feather waste. The low cost organic manure produced is used for restoration of soil nutrition and for better growth of rice germination and peas. Kalaikumaria1 *et al.* (2019) have reported the complete dehairing of sheep skin by the keratinase and improved quality of the leather crust produced.

Keeping in consideration the increase in demand for keratinases in leather, poultry, animal feed and other industries, there is a calamitous need of more robust and stable keratinases which can be employed for lowering the burden of environmental pollution. The present study describes production and characterization of an efficient keratinase from local isolate as well as its potential role in hide dehairing.

MATERIALS AND METHODS

Keratinolytic bacterium was isolated from dumping soils of poultry sites located in suburbs of the city. Bacterial strains producing high levels of keratinase activity were selected on casein agar plates.

Enzyme production: Keratinase enzyme was produced by inoculating 100 µL of bacterial glycerol stock in sterile 20 mL of LB broth for 24 hours in shaking incubator at 50°C. From this culture, 2 mL of bacterial culture was added to 50 mL of autoclaved feather meal media in shaking incubator for different time periods. The culture was centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was assayed for enzyme activity and protein concentration. The influences of initial pH, temperature, incubation time, different concentrations of carbon sources were studied to achieve maximal bacterial growth and enzyme production.

Keratinase and protein assay: Keratinase activity was determined according to method of Cai *et al.* (2008). Keratin azure was used as substrate in reaction mixture containing diluted enzyme with Tris buffer of pH 8.5 at 50°C for 30 min. Reaction was terminated by 20% trichloro-acetic acid (TCA) followed by centrifugation at 12,000 rpm and supernatant absorbance was taken at 575 nm against control (Shimadzu UV-250, Japan). One unit of keratinase activity is estimated as an increase of absorbance A_{575} by 0.01 under the assay conditions. Soluble proteins in supernatant were determined by the method described by Bradford (1976) using bovine serum albumin (BSA) as standard.

Purification of keratinase enzyme: The supernatant containing crude enzyme was precipitated at different ammonium sulphate concentrations. After dialysis, the lyophilized sample was loaded on Sephadex G–100 column (Pharmacia Chemical Co., Sweden) equilibrated with 50 mM Tris buffer of pH 8.5 for gel filtration chromatography. Fractions of 1 mL were collected and assayed for both enzyme and protein concentration. The active fractions after gel filtration were subjected to Q-Sepharose column (Sigma Chemical Co., USA), equilibrated with 50 mM Tris buffer of pH 8.5. Active protein fractions of 1 mL each was collected and analyzed for keratinase activity and protein contents.

Homogeneity and molecular weight of keratinase enzyme was determined by SDS–PAGE as described by Laemmli (1970). Protein denaturation was done before loading on gel by heating at 85°C for 2 min and mixed with loading dye containing DTT, SDS, bromophenol blue at 7.7%, 10.0%, 0.1% w/v and 50% glycerol (v/v) in 0.4 M Tris buffer (pH 6.8).

Biochemical characterization of keratinase: After purification, keratinase was characterized for optimum activity by assessing enzyme activity at different pH and temperature. Optimal pH was determined by establishing the pH of assay reaction with 50 mM Mcllvaine buffer ranging pH 5.0 to 7.0, 50 mM Tris buffer of pH 7.5 to 8.5 and 50 mM diethanolamine buffer of pH ranging 9.0 to 10.0. Stability of purified keratinize at different pH (5.0-10.0) was studied by pre incubating suitably diluted enzyme in buffers of various pH at room temperature for one hour and then determining the residual activity under the standard assay conditions.

Optimal temperature of purified keratinase enzyme was executed at different temperatures (30–90°C) by incubating purified enzyme activity with substrate in 0.05 M optimized buffer. Keratinase thermal stability was achieved by pre–incubating enzyme in optimized buffer at temperatures ranging 30–90°C for two hours before adding substrate and then determined remaining activity using standard conditions.

Keratinases belong to serine or metallo proteases class of enzyme so their activity is affected by different ions and inhibitors. The effect of inhibitors at 1 mM and 5 mM concentration, solvents at 0.5% and 1% concentrations and metal ions at 5 mM and 10 mM concentration were assayed for enzyme activity.

Caseinolytic, gelatinolytic and keratinolytic activities of purified keratinase were detected on different substrates by agar gel diffusion method. Casein, gelatin and keratin substrate prepared in buffer agar plates and enzyme (1 U) was loaded in the wells. The plates were incubated at 50°C for two hours and stained with Coomassie brilliant blue R-250 dye.

Hide dehairing: One ml of enzyme was added to cow hide and goat skin (16 cm^2 pieces, 100 g by weight) in 100 mL of 50 mM Tris buffer of pH 8.5 at 40°C under shaking conditions for 6–18 hours. The goat skin and cow hide were also subjected to conventional method by treating them with sodium sulfide and lime for dehairing and then comparing with control and biologically treated hides.

Chicken feather degradation: Feathers washed with detergent and tap water were dried at 37°C and stored at room temperature for further use. Feather substrate hydrolysis (10 g/L) by the microbial isolates was analyzed at 48, 72 and 96 hours by weight loss method as described by Tiwary and Gupta (2010). Protein concentration in culture supernatant was determined as described earlier. An increase in protein level in supernatant indicates the extent of keratin hydrolysis. The culture medium (100 mL) was filtered and undigested feathers were dried at 37°C. At each time interval, percentage weight loss of feathers was determined to study the bioremediation potential of the microorganism.

Statistical analysis: All the experiments were conducted in triplicates and the results were taken as means and standard deviation (mean± SD) of the three independently performed experiments. Results were analyzed statistically with the help of ANOVA test using SPSS software version 10.1. The means were compared for statistically significance at $p \leq 0.05$.

RESULTS

Screening of keratinase producing strain: Twenty-two keratinolytic strains based on casein hydrolysis clear zone were isolated from soil samples. The ratio of diameter of hydrolysis zone with that of colony was used as selection criteria. Highest ratio was shown by 6 isolates. Strain D2 showed highest extracellular keratinase activity after 36 hours of incubation therefore selected for further studies. As a result of initial morphological and physiochemical characteristics, the potent enzyme producing strain was identified to be a *Bacillus* species and named as *Bacillus* strain D2.

Fermentation conditions: Culture conditions of *Bacillus* strain were optimized by changing different growth parameters one by one i.e., pH, temperature, incubation time. After optimization, the microbe produced maximum keratinase enzyme activity at 50°C in the medium of pH 8.5 containing chicken feathers as a carbon source at the concentration of 10 g/L after 36 hours of fermentation period.

Purification of keratinase: The purification results of keratinase enzyme of *Bacillus* strain were summarized in Table 1. The 36 hours bacterial culture was centrifuged at 8,000 rpm for 10 min and supernatant was used as enzyme source. Enzyme was precipitated with ammonium sulphate at 60% saturation. Precipitates, obtained after centrifugation at 10,000 rpm for 20 min, were suspended in minimal volume of buffer and dialyzed overnight with 50 mM Tris buffer of pH 8.5. One ml of sample was loaded on Sephadex G-100 column pre–equilibrated with Tris buffer and eluted with the same buffer with 40 ml/h flow rate (Fig. 1).

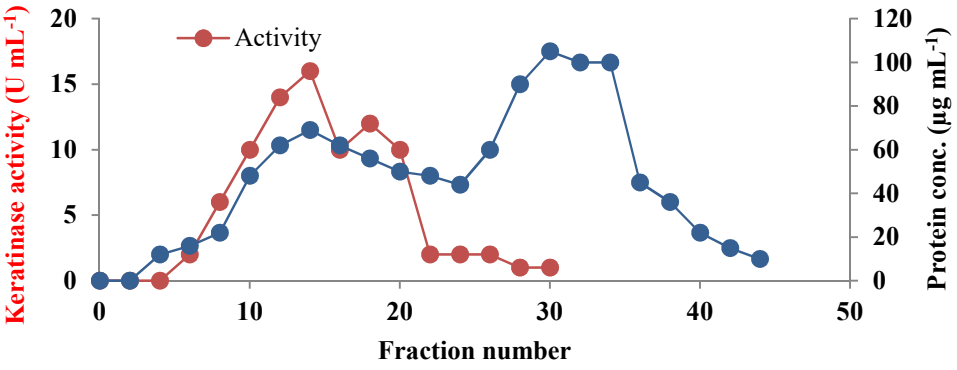

Fig. 1: Gel filtration chromatography of *Bacillus* strain D2 culture supernatant after ammonium sulfate precipitation.

Enzyme activity and protein concentration were detected in all fractions while active fractions were collected and then subjected to Q–Sepharose ion exchange chromatography. Bound proteins were eluted with NaCl gradient 0–1.0 M NaCl in 50 mM Tris buffer of 8.5 pH as shown in Fig. 2. The overall recovery was 23.8% while purified enzyme showed specific activity of 525 U/mg of protein.

Table 1: Purification scheme of keratinase from *Bacillus* strain D2.

Purification steps	Keratinase activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)	Recovery (%)
Crude extract	1475±19	10.2±0.5	114.6	100
Ammonium sulfate precipitation	1125±11	6.5±0.2	173.0	76.27
Gel filtration chromatography	515±16	2.2±0.26	234.0	34.91
Ion exchange chromatography	210±9	0.4±.01	525.0	23.8

The data is representative of the mean ± Standard Deviation.

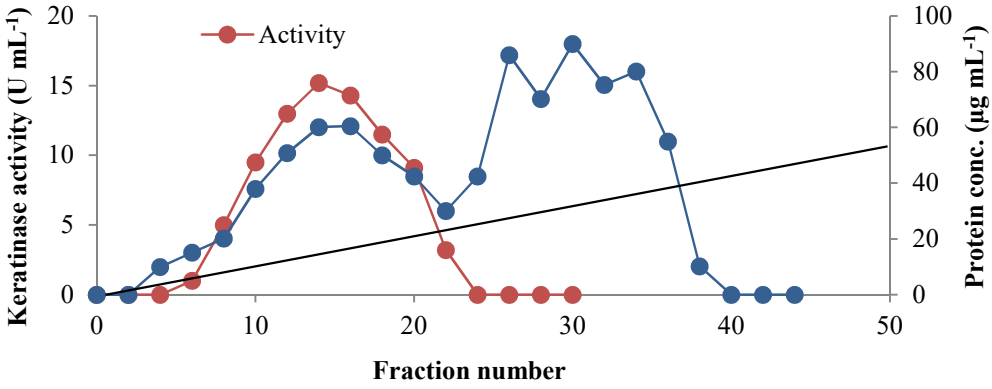

Fig. 2. Q-Sepharose chromatography of *Bacillus* strain D2 culture supernatant after Gel filtration chromatography. _____ NaCl Conc.

Molecular mass determination and zymogram analysis: The protein samples obtained after different steps of purification were analyzed on 12% SDS-PAGE. Crude extract obtained after dialysis showed multiple bands corresponding to cocktail of proteases produced by the bacteria along with the keratin breakdown products which are also protein in nature. The purified keratinase showed a single band on SDS-PAGE with molecular weight of 40 kDa as estimated from relative mobility of reference proteins as shown in Fig. 3(a).

The purified enzyme was also subjected to native polyacrylamide gel electrophoresis and single activity band was observed when analyzed by zymography technique. The casein in the gel was hydrolyzed by the enzyme leaving behind clear zone where the Coomassie brilliant blue dye could not bind. The rest of the gel gave blue color due to the presence of casein copolymerized with acrylamide (Fig. 3b).

Influence of pH on activity and stability: The influence of pH on purified keratinase is shown in Figure 4. The substrate was dissolved in buffers of different pH 5.0–10.0. Considerable variation in the activity of purified enzyme was not observed in pH range 8.0–9.0 when examined under standard conditions though optimum pH of 8.5 was established for enzyme activity.

Stability of purified enzyme was investigated in buffer solutions of various pH (5.0–10.0) and protein samples were placed at room temperature for one hour. The enzyme was found stable over pH of 7.5–9.0. After pH 9.5, there was a rapid decrease in stability and about half of activity was lost at pH 10.0 when incubated for one hour at room temperature (Fig. 4).

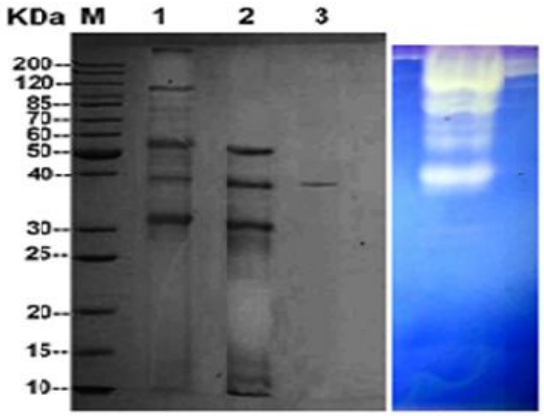

Fig. 3. (a) SDS-PAGE analysis of keratinase. Lane M: protein marker; Lane 1: After ammonium sulphate precipitation; Lane 2: After gel filtration chromatography; Lane 3: After Ion exchange chromatography (b) Zymogram of keratinase.

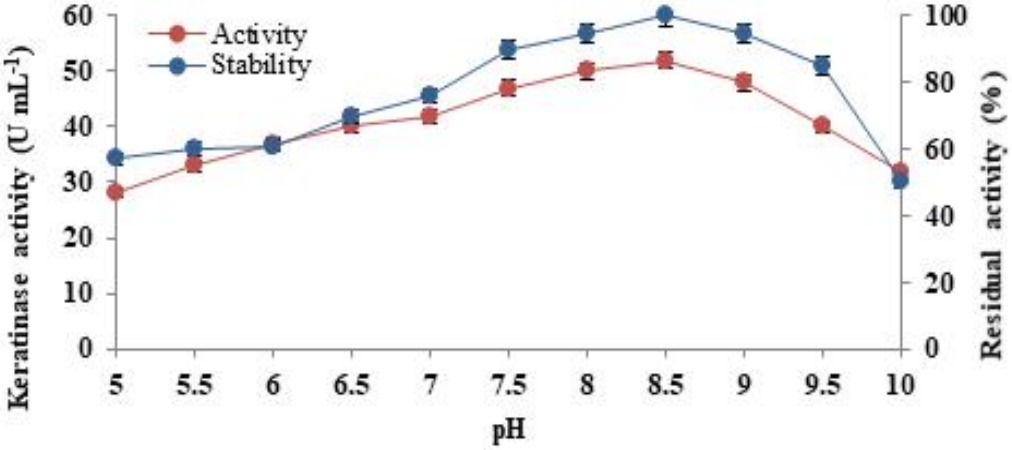

Fig. 4. Effect of pH on activity and stability of purified keratinase. The data is representative of the mean of three experiments.

Influence of temperature on activity and stability: The optimum assay temperature for keratinase activity was examined after incubating the enzyme with substrate at temperatures 30–90ºC at optimized pH using standard conditions. Enzyme activity was enhanced with temperature up to 50ºC and then there was a gradual decline in activity (Fig. 5).

Temperature stability of purified keratinase was explored after incubating enzyme at different temperatures range (30–90°C) and residual activity was then measured after two hours. The temperature contour indicated that up to 60°C purified enzyme preserved maximum activity while about 80% activity was retained at 70°C (Fig. 5).

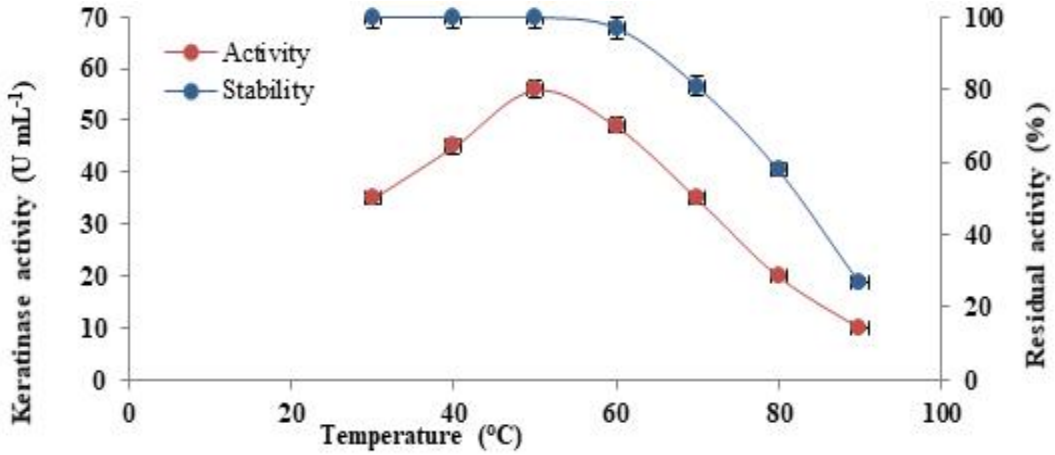

Fig. 5. Effect of temperature on the activity and stability of purified keratinase. The data is representative of the mean of three experiments.

Influence of inhibitors, solvents and metals on keratinase activity: Impact of different inhibitors including ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), β–mercaptoethanol, urea, sodium sulfite, DTT and phenylmethylsulfonyl fluoride (PMSF) at 1 mM and 5 mM concentration was explored (Fig. 6). The relative enzyme activity was determined with respect to control taken as 100%. The enzyme retained 89 and 91% activity at 1 mM while 82% and 88% relative activity at 5 mM concentration with thiol reagents such as sodium sulfite and DTT, respectively. In the presence of urea, the activity was considerably reduced to 55% and 51% at 1 mM and 5 mM concentration while β–mercaptoethanol gave 72% and 71% relative keratinase activity at these two concentrations. On the other side, the addition of SDS in the assay mixtures increased the activity by 9% and 3% at 1 mM and 5 mM concentration, respectively while EDTA in either case did not produce substantial influence on enzyme activity. PMSF was found to be a strong inhibitor for keratinase enzyme at both concentrations; resultantly 75% inhibition occurred with 1 mM concentration and 90% inhibition with 5 mM PMSF.

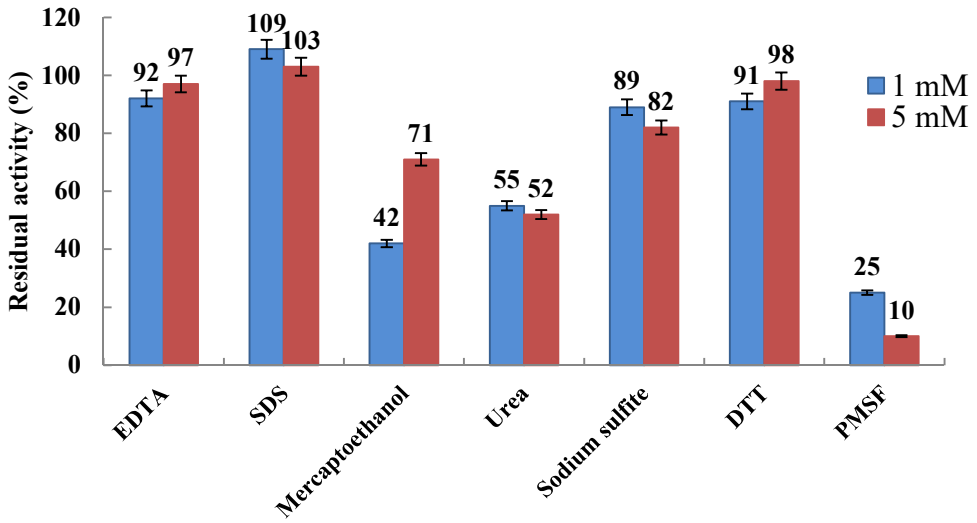

Fig. 6. Effect of various inhibitors on purified keratinase activity. The data is representative of the mean of three experiments.

The keratinase enzyme was found to be solvent stable with all solvents when used their 0.5% and 1% concentration; methanol, ethanol, isopropanol, DMSO, Triton X-100 and Tween 80 (Fig. 7). No significant decrease in enzyme activity was observed with reference to control by methanol, ethanol and isopropanol at both concentrations retaining 91–98% enzyme activity. While DMSO and Tween 80 at 0.5% concentration increased the relative activity by 103% and 118%, respectively.

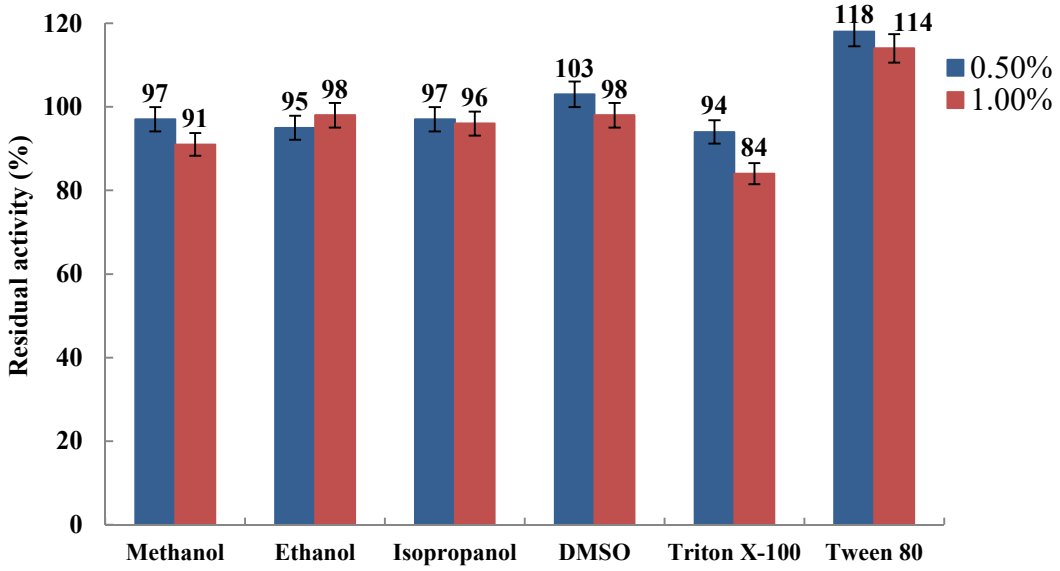

Fig. 7. Effect of various solvents on purified keratinase activity. The data is representative of the mean of three experiments.

Influence of various metal ions on keratinase was analyzed at two concentrations i.e. 5 and 10 mM. $MnCl_2$ significantly reduced the enzyme activity to 65% at 5 mM and 53% at 10 mM concentration while comparatively; less reduction in enzyme activity was detected in case of $ZnCl_2$ retaining 96% activity at 5 mM and 74% activity at 10 mM concentration. $CuSO_4$ at both these concentrations did not produce considerable impact on purified enzyme retaining 93–82% activity while purified enzyme was found stable with $CaCl_2$ preserving 98–96% enzyme activity. However, $MgCl_2$ in the assay reaction mixtures enhanced the activity up to 108% and 104% at 5 mM and 10 mM concentrations, respectively (Fig. 8).

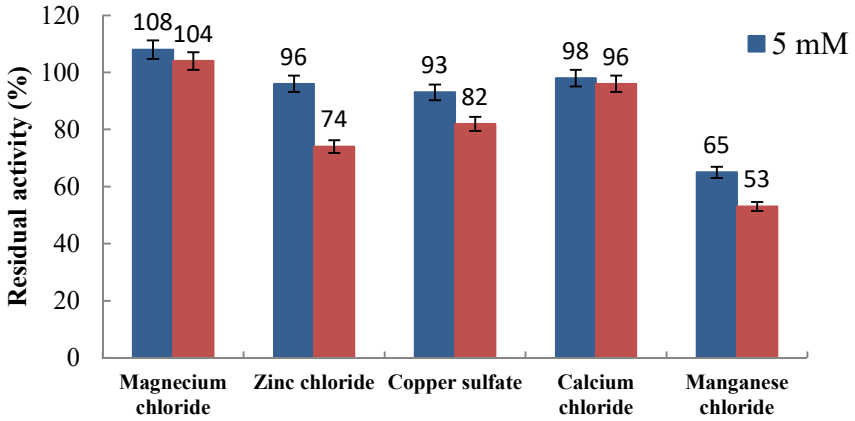

Fig. 8. Effect of various chemicals on purified keratinase activity. The data is representative of three experiments.

Caseinolytic, gelatinolytic and keratinolytic activity of keratinase: The potential of keratinase to hydrolyze substrates such as casein and gelatin along with keratin was explored in agar gel medium plates. 0.1% casein, gelatin and keratin substrates were prepared in agar plate and 0.1 mL (I U) enzyme was loaded in the wells. Coomassie brilliant blue staining of plates after incubation at 50°C for two hours showed clear zones of substrate hydrolysis displaying the caseinolytic and gelatinolytic activity of keratinase enzyme.

Hide dehairing potential of keratinase: The soaked cow hide and goat skin were treated with keratinase enzyme at 40°C in 50 mM Tris buffer of pH 8.5 for various time intervals and hairs were removed by manual scrapping. After 6 hours of enzymatic treatment, hairs were loosened and could be pulled out from the goat skin. Complete dehairing of goat skin was achieved within 18 hours along with the removal of scud and upper keratin skin layer (Fig. 9).

Fig. 9. Enzymatic dehairing of goat skin. (A) control goat skin, (B) keratinase treated dehaired pelt after 6 h, (C) after 12 h treatment, (D) after 18 h treatment, (E) hair removed from goat skin. (F) traditional dehairing of goat skin using sodium sulfide and lime

Cow hide, on the other hand, had thick hair and therefore, started to be removed after 12 hours of incubation. After 18 hours, the hide was left with partial scud and small hair and onward after 24 hours the hide was completely dehaired when compared to its controls as shown in Figure 10. The hide did not show any signs of collagen damage and smooth grain surface was observed. The dehaired hide had white appearance due to removal of epidermis and hair (Fig. 10E).

The goat skin and cow hide were also dehaired by conventional dehairing process using sodium sulfide and lime. The paste was applied on goat skin and cow hide and

manually dehaired by scrapping. The hides obtained by conventional method had more hard grain surface and epidermis giving it a dark surface due to partial removal of epidermis and keratin layer (Fig. 9F & 10F).

Fig. 10. Enzymatic dehairing of cow hide. (A) control cow hide, (B) keratinase treated dehaired pelt after 6 h, (C) after 12 h treatment, (D) after 18 h treatment, (E) after 24 h treatment, (F) traditional dehairing cow hide using sodium sulfide and lime

Feather keratin hydrolysis: Potential of *Bacillus* strain D2 for breakdown of feather keratin was analyzed at different incubation period *i.e.,* 48, 72 and 96 hours by weight loss method and protein content in the culture supernatant was determined (Fig. 11). An increase in protein concentration in the culture supernatant with the passage of time indicated the corresponding increase in hydrolysis of keratin. The culture medium was filtered and undigested feathers were dried at 37°C. Percentage weight loss of feathers was determined for each time interval.

After 24 hours incubation of feather keratin with bacteria, no significant change in feather structure was observed, however, after 48 hours, feathers appeared to be degraded and dense culture medium obtained. These changes were verified by observing the decrease in feather weight loss (39%) and increase in soluble protein content in the medium (Table 2). Further 72 hours of incubation showed increased feather hydrolysis and protein contents in the medium up to 52% and 1.6 mg/mL, respectively.

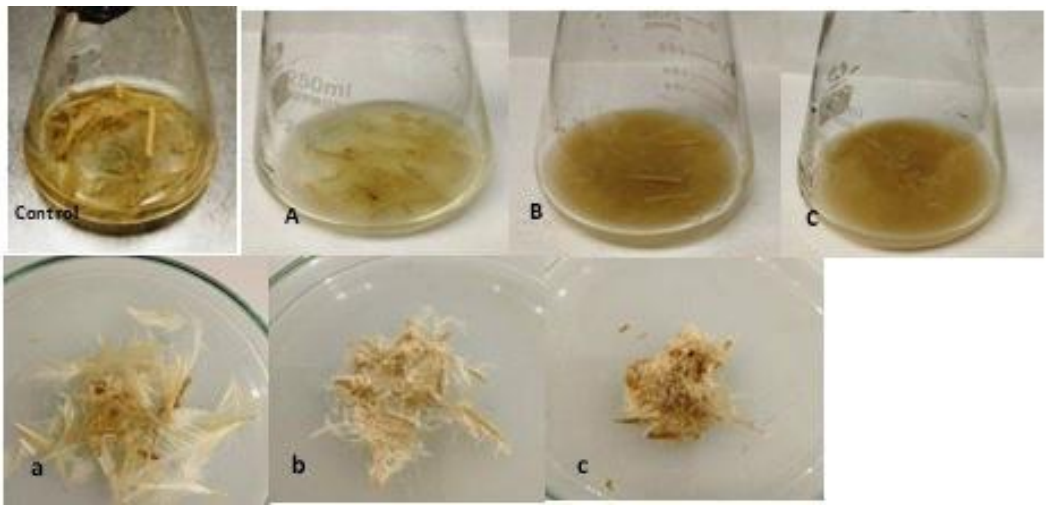

Fig. 11. Control: autoclaved feather media, A: feather degradation after 24 h, B: after 48 h, C: 72 h, a: undigested feathers left after 24 h, b: after 48 h, c: after 72 h

Table 2: Percentage feather degradation and protein concentration.

Incubation time (Hours)	Residual feather weight (%)	Protein concentration (mg/mL)	*P* value
24	80±1.2	0.5±0.05	<0.0005
48	61±0.44	1.2±0.03	<0.0005
72	48±0.81	1.6±0.06	<0.0005
P value	<0.0005	<0.0005	

The data is representative of the mean ± Standard Deviation. Values sharing same letters differ non significantly (P≤0.0005)

DISCUSSION

In this study, locally isolated keratinolytic bacteria *Bacillus* strain D2 showed fivefold increase in enzyme production after optimizing different growth conditions such as temperature, pH, incubation time and substrate concentration. Limited numbers of known microorganisms are reported that produce keratinases to degrade keratin polypeptide chains. Different microorganism groups have been investigated for keratinase enzyme to meet the needs of industries. *Bacillus* sp. is the most extensively studied keratinase producing bacteria having feather degrading potential (Kim *et al.*, 2001; Yousef *et al*., 2016). Different culture conditions significantly influenced the keratinase production by *Bacillus* strain D2. Maximal enzyme activity was produced in the medium of pH 8.5 containing chicken feathers as a sole carbon source at 10 g/L concentration after 36 hours of incubation at 50°C. *Bacillus licheniformis* is well characterized keratinolytic strain which has been extensively studied and enzyme product is also commercially available (Lin *et al.*, 1992). Other keratinase producing *Bacillus* species i.e. *B. cereus* (Akhter *et al.*, 2020), *B. licheniformis* (El-Komy 2019), *B. pumilus* (Kumar *et al.*, 2008), *B. subtilis* (Yong *et al*., 2020), *B. megaterium* (Radha and Gunasekaran, 2008), *B. thuringiensis* (Hassan *et al*., 2020) *B. halodurans* (Prakash *et al.*, 2010), *B. pseudofirmus* (Kojima *et al.*, 2006) and *Bacillus* sp. (Nnolim *et al.,* 2020) have been studied for their keratinolytic activity

The keratinase enzyme produced by *Bacillus* was purified using two purification procedures i.e. gel filtration and ion exchange chromatographic techniques. Molecular size of the purified enzyme after SDS-PAGE and zymogram was found to be 40 kDa that was analogous to the keratinase reported by Poopathi *et al.* (2014) and nearly similar to the keratinase BtKER which was purified as 39 kDa protein (Gegeckas *et al.*, 2014). Researchers have also reported low molecular weight keratinase enzymes contrary to our present study like 28 kDa keratinase from *Brevibacillus parabrevis* (Zhang *et al.*, 2016); 30 kDa keratinase of *B. megaterium* (Radha and Gunasekaran (2008). However, keratinases with high molecular mass as compared to keratinase purified by this study are reported in *B. cereus* (80 kDa) by Ghosh *et al.* (2008); *B. subtilis* (66 kDa) by Balaji *et al.* (2008) and *B. pumilus* (65 kDa) by Kumar *et al.* (2008).

Impact of pH on purified enzyme activity was examined in buffers of various pH by determining the amount of soluble proteins released after substrate hydrolysis. Purified enzyme showed activity in neutral as well as alkaline pH range however, exhibited maximum activity at 8.5. An optimum pH of 7.0 for the keratinase from *Caldicoprobacter algeriensis* and pH 8.0 for the keratinase from *Brevibacillus parabrevis* is reported by Bouacem *et al.* (2016) and Zhang *et al*. (2016), respectively. Contrarily, keratinases of *B. pumilus* FH9 (Abdel-Naby *et al*., 2017) and *Actinomadura keratinilytica* (Habbeche *et al*., 2014) exhibited pH 9.0 and 10.0 as optimum pH, respectively and even an optimum pH of 12.5 was reported by Benkiar *et al.* (2013) for keratinolytic protease isolated from *B. circulans*.

The influence of pH on the stability of the purified keratinase when incubated for one hour at room temperature revealed maximum retention of enzyme activity within pH range of 7.5 to 9.0 in comparison with pH ranging 7.0 to 11.0 reported by Briki *et al.* (2016) and more wide range of 5.0–10.0 by Tork *et al.* (2016).

Among the properties of enzymes, thermal stability marks them as a significant tool to be employed in different industrial areas. The purified keratinase was optimally active at 50°C similar to the optimum temperature of keratinase purified from *Caldicoprobacter algeriensis* (Bouacem *et al.*, 2016) and *B. pumilus* NRC21 (Tork *et al*., 2016). The optimal temperature of keratinase purified from various other microbes was considerably high such as 60°C for keratinase from *B. pumilus* (Abdel-Naby *et al.*, 2017), 70°C for keratinase from *Actinomadura keratinilytica* (Habbeche *et al*., 2013) and 85°C for keratinase from *B. circulans* (Benkiar *et al*., 2013) while temperature optima of keratinase isolated from *Bacillus* sp. SB12 (Briki *et al*., 2016) and *B. tequilensis* Q7 (Jaouadi *et al.*, 2015) were relatively low i.e. 37°C and 30°C, respectively. Purified enzyme of *Bacillus* strain D2 was found to be stable up to 60°C and could be well compared with keratinases of other *Bacillus* sp. such as keratinase of *B. pumilus* exhibiting stability up to 60°C (Tork *et al.*, 2016). Approximately 20% activity was lost at 70°C after incubation for two hours therefore, such properties made

the microbial strain a prospective and environment friendly tool for its industrial uses.

The stability of the enzyme in the presence of reducing agents like DTT indicates that reducing conditions are required for optimum keratinolytic process. Several reports have suggested that keratinases are mostly unable to hydrolyze keratin substrate in the absence of reducing agents (Fakhfakh-Zouari *et al.*, 2010). Reducing agents mostly reduce the disulfide bridges in the keratin substrate rather than directly affecting the enzyme (Gegeckas *et al.*, 2014) therefore, their addition enhances the breakdown of disulfide bonds of feather keratin. Our *Bacillus* keratinase showed 89 and 91% relative activity in the presence of sodium sulfite and DTT at 1 mM concentration, respectively. The enzyme activity was increased up to 109% and 103% in the respective presence of 1 mM and 5 mM SDS demonstrating its potential to be used as detergent additive.

Organic solvents may disrupt the hydrophobic core of the enzyme structure thereby deactivate the enzyme. Polar solvents can cause structural changes in the enzymes that affect interaction between the substrate and active site (Jaouadi *et al.*, 2013). Keeping in view these studies, various organic solvents were assayed for identifying their effects on the keratinase activity. Results obtained in our study showed that keratinase was solvent stable retaining 91–98% activity in the presence of ethanol, methanol, and isopropanol at 0.5% and 1% concentration whereas 3 and 18% rise in enzyme activity was observed with DMSO and Tween 80 at both concentrations suggesting it as a potential candidate for industrial application.

Metal ions form salt or ion bridges that stabilize the tertiary structure of the protein as well as the enzyme–substrate complex. Enzyme thermal stability is also maintained by metal ions. In our study, $MgCl_2$ improved the activity up to 108% while preserved its 98% activity in the presence of $CaCl_2$ supporting the results reported by Brandelli *et al*. (2010), but contrasting, $MnCl_2$ significantly reduced the enzyme activity at 10 mM concentration. Romero *et al*. (2001) have reported about 40% inhibition with Zn^{2+} and 35% decrease with Mg^{2+} while 12% increase in activity with Ca^{2+} ions. In contrast to these results, in our study, $ZnCl_2$ and $MgCl_2$ both did not show inhibitory effect on keratinase activity however interestingly retained and improved the enzyme activity in their presence. Abdel-Naby *et al.* (2017) have also reported Ca^{2+} and Mg^{2+} as activators of keratinase enzyme purified from *B. pumilus*. The purified enzyme along with keratinase activity also displayed caseinolytic and gelatinolytic activity showing its employment in various biotechnology fields.

Role of proteases and keratinase in skin dehairing has been reported previously as an alternative to chemically treated hides due to its promising role in reducing the pollution due to toxic chemicals along with improved quality of leather. (Hammami *et al.,* 2018; Ibrahim *et al*., 2019). Leather processing involves a series of processes including pre tanning techniques which causes about 70% of the pollution caused by leather processing. Lime, sodium sulfide and solid waste are responsible for higher TDS and BOD (Thanikaivelan *et al.*, 2004). Enzymatic dehairing of hides and skins resulted in reduction of effluent load thus protecting environment along with improved quality of the leather as compared to the harsh chemicals (Zhang *et al*., 2020). Ranjithkumar *et al*. (2017) elaborated that conventional leather dehairing process utilized large amounts of hazardous sodium sulfide and lime which under acidic conditions could liberate significant amount of hydrogen sulfide, generating detrimental effects in natural systems and serious waste disposal concerns. On the basis of such reports, the bioremediation potential of keratinase in leather dehairing was explored. The dehairing process in leather processing was mostly carried out at high pH range between 8.0 and 10.0; the keratinase enzyme in this study shows maximum activity in basic range (pH 8.5) thereby exhibited its prospective use in leather industry. Similar results are observed previously on goat skin by the *A. tamarri* alkaline protease at 30–37°C and pH 9.0–11.0 (Dayanandan *et al.*, 2003). The hide dehairing was achieved without any hazardous chemicals and the grain surface was smoother than that obtained by conventional method making the entire process eco–friendly. Hair pelt removed by enzymatic method were intact hair as compared to conventional method. This hair saving dehairing method with intact hair removal avoids the semi gelatinous and organic matter in waste water. This indicates that conventional lime sulfide method is harsher to the skin tissues as well as to the environment than enzymatic method. In this aspect, displaying feather degradation and hide dehairing abilities, *Bacillus* D2 keratinase may be considered a potential candidate for industrial application in biotechnological bioprocesses involving the hide dehairing, poultry feed production, and organic fertilizer.

Conclusion: Keratinases are considered as 'modern protease' because of its ever increasing demand in industry and their ability to digest extremely resilient hydrophobic proteins. Keratinases have been used in leather processing industry, as keratinases are economical and eco–friendly option than the conventional leather bating process. Undoubtedly, heat and pH constancy of enzymes are imperative criteria for their industrial uses, the keratinase isolated from *Bacillus* has heat and alkaline pH tolerance, its effectiveness in poultry and leather industrial area is promising.

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