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ORIGINAL ARTICLE

Keratinolytic enzyme-mediated biodegradation of recalcitrant poultry feathers waste by newly isolated *Bacillus* sp. NKSP-7 under submerged fermentation

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Abstract

Microbial and enzymatic degradation of keratin waste is more preferred over various conventional approaches which are costly and not environmentally suitable. Diverse niches are auspicious for the discovery of new microorganisms. To discover novel keratinolytic bacteria, 60 isolates from different poultry dumping sites were initially screened, and among these found a potent keratinolytic isolate (NKSP-7) that displayed higher feather-degrading ability. The selected isolate was identified as *Bacillus* sp. NKSP-7 based on 16S rDNA sequencing as well as physiochemical and morphological characteristics. The strain NKSP-7 showed complete hydrolysis of native chicken feathers (10 g/L) in nutrient medium after 24 h of incubation at 37 °C under agitation (150 rev/min) and produced thermostable extracellular keratinase. The crude enzyme displayed maximal keratinolytic activity (34.7 U/mL) in phosphate buffer of pH 7.0, and at 60 °C using keratin azure as a substrate. Keratinolytic enzyme showed stability at 20–65 °C for 4 h over the pH range of 5.5–8.0. No obvious inhibitory influence was perceived by cations, organic solvents, EDTA, and detergents. Whereas, enzyme activity was enhanced by adding β-mercaptoethanol, Na^{+}, Cd^{2+}, and Mn^{2+}. All these notable features of keratinase make it a promising candidate for various industrial applications especially for dehairing process in leather industry, bioconversion of poultry waste, and in detergents formulations.

Introduction

Severe deficiency of protein resources and increase in demand for protein emphasize to rummage new-fangled and sustainable protein sources from different waste materials (keratinous wastes), and produce innovative functional materials in an eco-friendly way (Peng et al. 2019; Sharma and Devi 2018). Globally, millions of tons of keratinous wastes are generated from various processing industries (husbandry, herding, and poultry), which are the prevalent and abundant reservoir of pure keratin (Bhari et al. 2018). Management and recycling of these wastes have become one of the major environmental concerns due to the recalcitrant and complex structure of keratin (Tamreihao et al. 2019).

Keratin, a stubborn protein is pervasive and one of the most abundant biopolymers in nature, which is present in hair, scales, nail, feather, hooves, wool, and horns. These proteins are fibrous, insoluble, and structurally complex with excessive weak interactions like hydrogen bonds, hydrophobic interactions, and disulfide bridges, which make their molecular structure strong and resistant to the mechanical stress and the action of common proteolytic enzymes (pepsin, papain, or trypsin) (Gopinath et al. 2015; Ningthoujam et al. 2016). The high cysteine content and consequent disulfide bridges contribute to the stability and recalcitrant nature of keratin making it fairly resistant to degradation (Kshetri and Ningthoujam 2016).

Feathers are mainly constituted of keratin and account for about 5–7% of the total weight of a mature bird thereby producing considerable quantity of keratin-based poultry wastes that decomposed very slowly and thus, become a massive environmental concern (Ningthoujam et al. 2016). Feathers are the considerable source of amino acids, and minerals such as calcium (Ca), copper (Cu), iron (Fe), nitrogen (N), magnesium (Mg), manganese (Mn), potassium (K), phosphorus (P),

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and zinc (Zn) (Tamreihao et al. 2019). Several conventional and non-conventional approaches such as steam pressure, burning, landfilling, and acid/alkali hydrolysis have been utilized, either alone or in combination for recycling of keratinous wastes to produce fertilizers, glues, and foodstuffs. These strategies not only require enormous energy for processing but also cause obvious pollution and resultantly some essential amino acids such as methionine, tryptophan, and lysine are destroyed (Cascarosa et al. 2012).

Keratinase (E.C.3.4.21/24/99.11) form a group of proteolytic enzyme able to hydrolyze insoluble keratins (feathers, wool, nail, and hair) more efficiently than other proteases (Kshetri and Ningthoujam 2016; Kurane and Attar 2017). Keratinases are predominately produced and active in the presence of keratinous substrate, and act on peptide bonds in keratin and convert it into simplified forms (Gopinath et al., 2015). These enzymes are omnipresent in nature and widely found in bacteria, actinomyces, and fungi especially dermatophytic species. Potent keratinolytic bacteria, mostly belonging to genus *Bacillus*, have been reported (Kshetri and Ningthoujam 2016; Sekar et al. 2016; Abdel-Fattah et al. 2018; Bhari et al. 2018). Other bacterial genera *Chryseobacterium* sp. (Brandelli 2005); *Pseudomonas* sp. (Tork et al. 2010); *Staphylococcus aureus* (Raju and Divakar 2013); and *Amycolatopsis* sp. Strain MBRL 40 (Ningthoujam et al. 2016) have also been reported as keratin decomposers.

Keratinolytic organisms and their specific proteolytic enzymes (keratinases) offer an alternatively conspicuous, eco-friendly, and economical approach for the proper degradation and recycling of keratinous waste materials into worthy byproducts (Sharma and Devi 2018). Owing to their diverse biotechnological implications and multifarious properties, keratinase can be considered as promising enzyme for the manufacturing of protein supplements, biomedical products, leather processing, cosmetic, fiber modification, animal nutrients, processing of feather-meal for bio-fertilizer and feed, detergent formulation, pharmaceutical industries, waste management, and bioremediation (Kshetri and Ningthoujam 2016; Bhari et al. 2018; Sharma and Devi 2018). In the recent time, the quest of efficient keratinolytic microorganism with high potential to hydrolyze keratin, has become the goal of research worldwide.

The present research work describes the isolation and screening of efficient keratinolytic bacteria from local poultry dump sites, and investigation of their ability to degrade biowaste (feathers) generated by poultry-processing industries. Hence, the best keratinolytic bacterium with highest chicken feather degradation ability in short period of time and optimal enzyme activity was identified by using various biochemical tests and 16S rRNA gene sequencing after which, preliminary characterization of crude keratinolytic enzyme from potent bacterial isolate (*Bacillus* sp. NKSP-7) was studied.

Materials and methods

Chemicals and reagents

All chemicals, media components, substrates, salts, and reagents were purchased from Sigma-Aldrich Co. (St Louis, USA) and Merck (Darmstadt, Germany), unless otherwise mentioned. However, PCR components, gel extraction kit, DNA, and protein marker were obtained from Thermo Fisher Scientific Inc. (USA).

Collection of samples

The soil samples were collected with the permission of responsible authorities from different chicken sale centers, poultry farms, and feather processing areas (dump sites) including Sajjad poultry farm (SPF) near Jallo Park, Jallo Park soil (JPS), local chicken sale centers (LCSC), Jallo Park hen cages (JPHC), compost sites of feathers waste at Jallo park (CSJP), poultry farm in Noor kot Shakkargarh (NKPF), poultry farm near Shahdara, Lahore (SHP), domestic waste of hens and feathers (DC) and chicken sale centers at Tollinton market (TWM), Lahore, Pakistan. All samples were collected in sterile plastic zip bags and then processed for the isolation of keratinolytic bacteria.

Preparation of feather meal powder

Feathers of chicken were procured from local poultry market and chicken sale centers. White chicken feathers were exhaustively washed with warm water followed by soaking them in a chloroform: methanol (1:1) mixture for 1 day and afterward in acetone: chloroform: methanol (1:4:3) mixture for 1 day. After that, feathers were washed many times with distilled water to remove all the residues of solvent, dried at 50 °C for overnight, and then milled using electrical blender, and stored at room temperature in a bottle until further used. Feather meal powder was used as a source of keratin substrate for the isolation of keratinase producing (feather degrading) bacteria (Tork et al. 2010). In contrast, for the production of keratinase enzyme only washed and sterilized whole feathers were used.

Isolation of keratinolytic bacteria

Keratinolytic bacteria were isolated from various poultry dumping sites of Lahore and its surrounding cities, Pakistan. Each soil sample (5 g) was mixed individually in 50 mL of sterile saline in Erlenmeyer's flasks, and the resultant suspension was serially diluted (up to 10^{-9}) followed by spreading on nutrient medium (Merck, Germany) agar plates and incubated at 37 °C for 24 h. Morphologically distinct bacterial colonies were streaked individually and maintained on nutrient medium agar slants until further use.

Screening of proteolytic bacteria

For screening of proteolytic bacterial strains, each isolate was inoculated on skimmed milk agar plates (2.8% v/v, skimmed milk in nutrient agar medium) and incubated at 37 °C for 1–2 days. The positive bacterial colonies with peak clear zones displayed hydrolytic ability were selected for further experiments.

Screening of keratinolytic bacteria

The keratinolytic ability of isolates was investigated by inoculating bacterial strains on feather-meal minimal salt medium (MSM) agar plates containing NaCl (0.05%, w/v), $MgCl_2.6H_2O$ (0.01%, w/v), NH_4Cl (0.05%, w/v), KH_2PO_4 (0.03%, w/v), K_2HPO_4 (0.04%, w/v), milled chicken feathers (1.0%, w/v), and bacteriological agar (1.5%, w/v), pH 7.5. After inoculation, the plates were incubated at 37 °C for 1–3 days. Bacterial colonies that developed clear zones by the hydrolysis of keratin were selected and purified by repeated streaking on same medium, and were finally maintained on nutrient agar slants.

Chicken feathers degradation by isolates

Isolated keratinolytic bacterial strains from agar slants were cultivated individually in 10 mL nutrient medium and kept at 37 °C for overnight in a rotary shaker. These cultures (1%, v/v) were used to inoculate 50 mL nutrient medium supplemented with 1% (w/v) of whole chicken feathers instead of feather powder (as a source of carbon and nitrogen) in Erlenmeyer's flasks and incubated up to 96 h (4 days) at 30–50 °C, under agitation (50–200 rev/min). Percentage of keratin feather degradation was determined after regular interval of 6 h (Kurane and Attar, 2017). All flasks were also monitored visually and compared to the control flask (without inoculum). After incubation, the remaining feathers were filtered, washed, dried using pre-weighed filter paper, and weighed to calculate percentage degradation using following equation.

$$\text{Percentage of degradation} = \text{TWF}-\text{RWF}/\text{TWF} \times 100$$

where, TWF is total weight of feathers and RWF is residual weight of feathers.

Keratinolytic bacterial isolates showing maximum feathers degradation were selected for further experimental studies.

Optimal production of keratinolytic enzyme

To evaluate the optimal conditions for keratinolytic enzyme production and feathers degradation, the selected keratinolytic isolates were cultivated (as described above) in nutrient medium containing 1% (w/v) chicken feathers and incubated at various temperatures ranging from 30 to 50 °C individually up to 96 h (4 days) in a rotary shaker (50–200 rev/min). The cultures were withdrawn after regular interval of 6 h to analyze the keratinase activity in extracellular, intracellular, and cell-bound fractions. After incubation and filtration of degrading content, the culture filtrate was harvested by centrifugation (10,000×*g* at 4 °C, 20 min), and the resultant culture supernatant was used as extracellular fraction. The pellet was washed with Tris-HCl buffer (50 mM, pH 8.0) and resuspended in the same buffer, sonicated (10 × 30 s bursts with 60 s intervals between successive pulses in a Ultra Sonicator, UP 400 s) to disrupt bacterial cells, and then centrifuged (10,000×*g* at 4 °C, 20 min). Supernatant was used as intracellular fraction, and the pellet (cell debris) was resuspended using same Tris-HCl buffer and used as cell-bound fraction. All cell fractions (soluble and insoluble) were assayed to determine the optimal keratinolytic activity.

Analytical approaches

The growth of keratinolytic bacterial culture was evaluated by the described method of Basar et al. (2010). To analyze the bacterial growth, regularly withdrawn samples (of 6 h) were subjected to centrifugation (10,000×*g* for 10 min, 4 °C), the resultant cell pellets were resuspended in sodium chloride (0.9%, w/v) and OD_{600nm} (optical density) was measured. To scrutinize dry cell weight (DCW), cell suspensions were filtered using membrane filter and the retentate were dried in a preheated oven for 24 h at 80 °C. The relationship of OD and DCW was assessed from many experiments, which demonstrated that one unit (U) of OD_{600nm} was approximately equivalent to 0.6 g DCW/L (Basar et al. 2010).

Enzyme activity assay

Keratinase enzyme activity was determined using keratin azure as substrate according to modified protocol of Cai et al. (2008). Keratin azure was frozen at − 40 °C for 4 h and subsequently ground into a fine powder. Powder keratin azure (5 mg) was suspended in 1 mL phosphate buffer (0.05 M, pH 7.0). Assay was conducted in triplicate by incubating reaction mixture that contained 1 mL crude enzyme and 1 mL keratin suspension at 60 °C for 2 h with constant agitation (150 rev/min). The reaction was stopped after incubation with the addition of 2 mL trichloroacetic acid (TCA, 10% *w/v*) and subsequently centrifugated (6000×*g* at 4 °C, 15 min) to remove substrate. The resultant supernatant was used to measure the release of azo dye at OD_{595nm} against a control. A control test was also run along experimental reactions that contained only 1 mL keratin azure suspension. After incubation, 1 mL enzyme and 2 mL TCA (10%, w/v) were added in the control. One unit of keratinolytic enzyme activity

was defined as an increase in absorbance of 0.01 per minute as compared to control under conditions described above.

Morphological and physiological characterization

Bacteria that displayed high production of keratinolytic enzymes were identified and characterized by different morphological and biochemical tests including colony morphology (color, shape, and size), Gram staining, endospore staining, motility test, methyl red test, indole test, oxidase test, nitrate reduction test, ureases test, gelatin hydrolysis test, catalase test, casein hydrolysis test, starch hydrolysis tests, carbohydrate fermentation (lactose, glucose, and sucrose), and hydrogen sulphide (H_2S) production test according to *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994).

Molecular identification of potent isolates

The 16S rRNA gene sequencing was performed for the identification and phylogenetic analysis of best keratinolytic isolates. Genomic DNA was isolated by phenol-chloroform method from the potent keratinolytic strains, and employed to amplify respective gene by PCR using a pair of primers, F-5′- AGAGTTTGATCMTGGCTCAG-3′ and R-5'-TACGGYTACCTTGTTACGACTT-3′. The amplification reaction mixture contained $MgCl_2$ (25 mM), dNTP (2.5 mM), primers (10 pM each), reaction buffer (1X), genomic DNA (30–40 ng), *Taq* polymerase (1.5 U), and made the final volume with nuclease-free water. Optimal amplification conditions consisted of 1 cycle for 3 min at 95 °C (initial DNA denaturation); 30 cycles of 95 °C (1 min), 54 °C (1 min) and 72 °C (1 min), and final extension for 10 min at 72 °C. The amplified product was analyzed on agarose gel (0.8%, w/v) with ethidium bromide (0.5 μg/mL) and purified using gene clean kit before being sent for sequencing. The sequence was compared and aligned using BLAST server to search homologous sequences available in GenBank database of NCBI and Clustal Omega.

Preliminary characterization of crude keratinase

Preliminary characterization of crude keratinolytic enzyme from the best bacterial isolate was studied using keratin azure as substrate.

Determination of molecular mass

The molecular mass of crude extracellular keratinase enzyme from potent isolate was determined by SDS-PAGE analysis. The concentration of protein was measured according to dye binding method using bovine serum albumin (BSA) as standard (Bradford 1976).

Effect of pH and various buffer system

To determine the best pH and buffer system for optimal activity of enzyme, various buffer systems of different pH were used including sodium acetate (pH 4.0–5.5), McIlvaine (pH 3.0–7.5), Tris-Cl (pH 7.0–9.0), HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (pH 7.0–8.5), phosphate (pH 5.5–7.5), and CAPS [3-(cyclohexylamino)-1-propanasulfonic acid] (pH 9.0–11.0) buffers. The enzyme was diluted suitably in buffers from pH 3.0–11.0, with increment of 0.5 unit of pH, and incubated for 2 h at 60 °C with substrate. In order to study pH stability, crude keratinolytic enzyme was pre-incubated in different buffers (pH 3.0–11.0) for 4 h at 40 °C in McIlvaine (pH 3.0–5.0), phosphate (pH 5.5–7.5), HEPES (pH 7.0–8.5), and CAPS (pH 9.0–11.0), and then enzyme residual activity was assayed under described assay conditions.

Effect of temperature

The effect of temperature on keratinase catalytic efficiency was determined by incubating crude enzyme at temperature ranging from 20 to 100 °C (with increment of 5 °C) using phosphate buffer (pH 7.0). Thermal stability of crude keratinase was scrutinized by pre-incubating enzyme in the absence of substrate at temperature ranging from 20 to 85 °C for 4 h at pH 7.0 buffer, and residual activity was measured under optimal assay conditions.

Effect of various chemicals

The influence of various chemicals (metallic cations, surfactant, organic solvents and chemical reagents) on the catalytic efficiency of crude keratinase were studied by adding 10 mM cations (Ba^{2+}, K^{1+}, Na^{1+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, and Zn^{2+}), chelating agent (EDTA), β-mercaptoethanol, Tris, Urea, and 10 mg/mL surfactant (SDS, Triton X-100, and Tween-80) in the reaction mixture. Similarly, the effect of 50% (*v*/v) different organic solvents (acetone, isopropanol, acetonitrile, chloroform, ethanol, glycerol, ethyl acetate, n-butanol, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), and methanol) on enzyme were also investigated. All inhibitors were added individually in the reaction mixture and the enzyme activity was measured under described assay conditions (pH 7.0 and 60 °C). The activity without adding any additive was considered as 100%. All results were compiled as the percentage of relative activity.

Statistical analysis

All experimental tests (triplicates) were performed in CRD (completely randomized design); student's *t* test and one-

way ANOVA (analysis of variance) was used to statistically analyze all data. To study the comparison of means, Duncan's multiple range test was applied using the SPSS (statistical package for the social sciences) software, after finding the significance results ($p \leq 0.05$) of ANOVA.

Results

Isolation and screening of keratinolytic isolates

Initially, 135 bacterial strains were isolated but only 60 isolates exhibited proteolytic activity on skimmed-milk agar plate by producing clear zones, two best isolates showed maximum zones (Fig. S1a in supplementary material). These isolates were selected for secondary screening on feather-meal agar plates. Among 60 isolates, only 5 colonies (SPF-9, NKSP-7, NKSP-9, JPS-9, and SHP-5) showed highest clear zones (maximum keratinolytic activity) on feather-meal plates (Fig. S1b in supplementary material) as compared to others (Table 1).

Chicken feathers degradation by isolates

All bacterial isolates (60 strains) were evaluated individually for their capability to degrade white chicken feathers (keratin wastes). Some isolates exhibited noteworthy ability to degrade feathers, among which, 5 isolates (SPF-9, NKSP-7, NKSP-9, JPS-9, and SHP-5) showed more than 80% feather's degradation after 24 h and 20 isolates revealed more than 60% of degradation whereas, some did not possess keratinolytic activity (Table 1). Among the most prominent 5 keratinolytic bacterial isolates, NKSP-7 and NKSP-9 (Fig. S2a and S2b in supplementary material) displayed $88.02 \pm 0.021\%$ ($p \leq 0.05$) and $86.11 \pm 0.401\%$ ($p \leq 0.05$) feathers' degradation, respectively. However, almost all feather's barbules and raches were degraded by inoculating NKSP-7 and NKSP-9 isolates (Fig. S2c in supplementary material) after 24 h of incubation at 37 °C under 150 rev/min.

Keratinolytic enzyme production and activity analysis

All 60 isolates were cultivated individually in nutrient broth medium supplement with 1% (w/v) feathers and incubated up to 96 h (4 days) at 30–50 °C temperature with shaking (50–200 rev/min). Some bacterial isolates have the ability to utilize chicken feathers as a nitrogen and carbon source, and keratinase enzyme was produced using an inexpensive substrate under submerged fermentation. Regularly withdrawn culture samples (6 h) were subjected to evaluate the activity of keratinase enzyme. Keratinolytic enzyme activity from isolates was observed in cell fractions such as extracellular (crude supernatant), cell-bound (cell pellet), and intracellular (lysis cells), but maximum enzyme activity was found in crude supernatant followed by intracellular and cell-bound fractions. Intracellular and cell-bound fractions exhibited 30–35% and 10–15% relative activity respectively as compared to the extracellular fraction that was considered as 100%.

Maximum feather degradation and extracellular keratinase enzyme production was observed when submerged fermentation was conducted using a potent keratinolytic isolate NKSP-7 that exhibited high extracellular keratinolytic activity (34.7 U/mL) and dry cell weight (8.76 g DCW/L), after 24 h of incubation with agitation (150 rev/min) at 37 °C which was considerably great than all other isolates (Table 1); therefore, NKSP-7 was selected for further experimental study.

Morphological and physiological characterization

Five SPF-9, NKSP-7, NKSP-9, JPS-9, and SHP-5 keratinolytic isolates were identified and characterized by different morphological, cultural, and biochemical tests. These isolates proved to be aerobic, motile, gram-positive, rod-shaped, and spore-forming bacilli (with central or subterminal endospore per cell). They formed creamy white circular colonies on feather-meal agar plate and were able to degrade chicken feather. While irregular, convex and mucoid colonies appeared on nutrient agar plate. They were also strong catalase and oxidase positive. The results demonstrated that these isolates were identified as the members of genus *Bacillus.* All cultural, morphological, biochemical, and physiological characteristics of these strains are summarized in Table 2.

Molecular identification of potent isolates

Among 5 isolates, the 2 best NKSP-7 and NKSP-9 strains were selected for molecular identification. The length of the obtained 16S rRNA gene amplicons was ~ 1.5 kb (Fig. S3 in supplementary material). The 16S rRNA sequencing and gene-based phylogenetic analysis suggested that NKSP-7 and NKSP-9 isolates showed high sequence homology to other species of *Bacillus*, which confirmed that both strains belong to genus *Bacillus*. The sequence of NKSP-7 exhibited 95% similarity to *Bacillus thuringiensis* (GenBank Ac. No. MK875170.1), whereas NKSP-9 sequence showed 96% homology to *Bacillus cereus* (GenBank Ac. No. MK641669.1). On the basis of sequence analysis, NKSP-7 and NKSP-9 keratinolytic bacteria were identified as *Bacillus* sp. NKSP-7 and *Bacillus* sp. NKSP-9, respectively.

Preliminary characterization of crude keratinase

The selected potent keratinolytic isolate *Bacillus* sp. NKSP-7 was grown in nutrient medium containing chicken feathers (1% w/v). The intense poultry feather degradation was

Table 1 Screening of isolates with the formation of zone of clearance on feather agar meal and skimmed milk agar medium, degree of degradation, and enzyme activity of the isolates after 24 h incubation at 37 °C under agitation (150 rev/min) in nutrient broth containing whole feathers (10 g/L)

Sr. #	Isolates designation	DZC on FAM medium (cm)	DZC on SMA medium (cm)	DD (%)	Enzyme activity (U mL^{-1})
1.	LCSC-1	1.0	0.2	10.10 ± 0.711	2.33 ± 0.680
2.	LCSC-3	1.3	0.5	8.03 ± 0.233	0.92 ± 0.132
3.	LCSC-5	0.3	0.1	16.05 ± 0.410	1.20 ± 0.401
4.	LCSC-8	0.3	0.1	2.01 ± 0.08	0.62 ± 0.102
5.	LCSC-9	0.6	0.3	8.02 ± 0.211	0.15 ± 0.025
6.	SPF-1	2.7	0.9	64.04 ± 0.303	10.61 ± 0.141
7.	SPF-5	1.0	0.4	26.13 ± 0.381	2.51 ± 0.183
8.	*SPF-9*	*3.6*	*1.1*	*88.01* ± 0.062	*26.21* ± 0.465
9.	SPF-7	1.3	0.7	74.06 ± 0.033	19.42 ± 0.872
10.	SPF-5	2.4	0.9	60.05 ± 0.091	14.21 ± 0.813
11.	SPF-4	1.4	0.3	42.11 ± 0.113	8.10 ± 1.712
12.	TWM-3	1.0	0.6	20.03 ± 0.472	1.92 ± 0.085
13.	TWM-5	1.0	0.3	24.60 ± 0.501	2.43 ± 0.276
14.	TWM-7	1.0	0.2	22.33 ± 0.044	2.21 ± 0.321
15.	TWM-8	2.0	0.9	57.82 ± 0.075	5.23 ± 0.133
16.	TWM-9	0.2	0.3	2.04 ± 0.054	–
17.	NKSP-1	1.8	0.5	72.04 ± 0.141	11.80 ± 1.461
18.	NKSP-4	2.7	1.1	70.11 ± 0.094	18.61 ± 0.753
19.	NKSP-5	2.2	1.2	63.26 ± 0.171	10.20 ± 0.041
20.	*NKSP-7*	*3.8*	*1.3*	*88.02* ± 0.021	*34.70* ± 0.572
21.	NKSP-8	0.6	0.2	6.07 ± 0.391	–
22.	*NKSP-9*	*3.7*	*1.2*	*86.11* ± 0.401	*32.50* ± 0.931
23.	JPS-1	1.2	0.2	16.04 ± 0.073	3.51 ± 0.045
24.	JPS-3	1.0	0.2	10.11 ± 0.662	1.21 ± 0.027
25.	JPS-5	2.3	0.4	61.63 ± 0.106	12.24 ± 0.044
26.	JPS-7	1.0	0.5	30.02 ± 0.681	3.87 ± 0.067
27.	JPS-8	1.0	0.2	7.01 ± 0.106	0.59 ± 0.077
28.	JPS-10	1.8	0.3	37.41 ± 0.592	5.61 ± 0.133
29.	*JPS-9*	*3.0*	*1.0*	*84.11 ± 0.0453*	*20.21 ± 0.283*
30.	JPS-2	2.7	0.9	64.63 ± 0.0431	6.54 ± 0.377
31.	JPS-4	2.4	0.8	70.61 ± 0.088	10.02 ± 0.221
32.	JPS-6	1.3	0.3	46.60 ± 0.714	8.14 ± 0.137
33.	SHP-9	2.0	0.4	50.40 ± 0.517	9.76 ± 0.044
34.	SHP-3	0.8	0.2	8.81 ± 0.077	1.22 ± 0.081
35.	*SHP-5*	*2.9*	*1.0*	*80.14 ± 0.021*	*24.50 ± 0.166*
36.	SHP-3	2.1	1.0	32.21 ± 0.045	6.24 ± 0.216
37.	SHP-7	2.5	0.8	76.04 ± 0.133	20.60 ± 0.392
38.	SHP-6	0.4	0.3	4.42 ± 0.082	–
39.	SHP-8	2.3	0.5	67.60 ± 0.131	15.21 ± 0.084
40.	SHP-1	0.6	0.2	16.44 ± 0.075	2.11 ± 0.455
41.	SHP-2	1.0	0.6	21.25 ± 0.105	4.67 ± 0.076
42.	CSJP-1	2.2	0.5	62.64 ± 0.941	8.98 ± 0.088
43.	CSJP-5	2.7	0.8	73.82 ± 0.681	13.73 ± 0.174
44.	CSJP-3	1.1	0.5	58.83 ± 0.662	10.33 ± 1.062
45.	CSJP-7	2.7	1.0	78.41 ± 0.377	14.21 ± 0.773
46.	CSJP-8	2.0	0.7	51.04 ± 0.041	5.93 ± 0.083
47.	CSJP-10	1.3	0.2	34.22 ± 1.051	4.28 ± 0.0361
48.	CSJP-9	2.7	0.9	79.84 ± 0.901	16.53 ± 0.125

Table 1 (continued)

Sr. #	Isolates designation	DZC on FAM medium (cm)	DZC on SMA medium (cm)	DD (%)	Enzyme activity (U mL^{-1})
49.	CSJP-2	1.5	0.4	49.62 ± 0.022	4.10 ± 0.043
50.	JPHC-1	0.3	0.1	5.61 ± 0.113	–
51.	JPHC-2	0.9	0.4	19.03 ± 0.411	2.14 ± 1.022
52.	JPHC-5	1.2	0.5	23.81 ± 0.571	3.94 ± 0.331
53.	JPHC-3	0.8	0.3	18.62 ± 0.254	2.73 ± 0.041
54.	JPHC-6	0.3	0.3	4.04 ± 1.062	–
55.	JPHC-7	0.6	0.1	10.41 ± 0.201	2.21 ± 0.571
56.	JPHC-8	0.4	0.2	3.05 ± 0.920	0.91 ± 0.077
57.	DC-5	0.2	0.2	2.61 ± 0.304	0.76 ± 0.112
58.	DC-7	0.4	0.1	5.62 ± 0.080	1.79 ± 0.071
59.	DC-3	0.5	0.2	9.02 ± 0.662	1.85 ± 0.033
60.	DC-9	0.1	0.1	2.04 ± 0.255	–

Five isolates i.e. SPF-9, NKSP-7, NKSP-9, JPS-9, and SHP-5 are in italic that showed more than 80% feather's degradation

The crude enzyme activity of isolates was assessed using keratin azure (5 mg) as substrate. Results are the mean of triplicates and ± expressed the standard deviations (SD) that differ significantly at $p \leq 0.05$

SPF Sajjad poultry farm; *NKSP* Noorkot shakargarh poultry farm; *SHP* Shahdra poultry farm, *CSJP* compost sites Jallo Park; *JPHC* Jallo Park hen cages; *DC* domestic cages; *TWM* Tollinton Market; *JPS* Jallo Park soil; *LCSC* local chicken sale centers. Degree of Degradation (%), DD (%); diameter of zone of clearance on feather agar meal (FAM) medium (cm), DZC on FAM medium; diameter of zone of clearance on skim milk agar (SMA) medium (cm), DZC on SMA medium; (−), Denotes minimal or no activity

Table 2 Morphological, cultural, and physiological characteristics of SPF-9, NKSP-7, NKSP-9, JPS-9, and SHP-5 keratinolytic bacterial isolates

Morphological characteristics	Inference
Shape	Rod
Size	1 × 2 μm
Gram staining	Positive
Spore	Spore forming
Endospore staining	Positive
Cultural characteristics	**Inference**
Feather agar	Creamy white color colonies, circular, rough
Skimmed milk agar	Creamy white color colonies, round
Nutrient agar	Creamy yellowish color colonies, mucoid, irregular, convex
Biochemical tests	**Inference**
Catalase	Positive
Oxidase	Positive
Indole production	Positive
Nitrate reduction	Positive
Motility	Positive
Starch hydrolysis	Positive
Casein hydrolysis	Positive
Urease hydrolysis	Positive
Gelatin hydrolysis	Positive
Methyl red test	Negative
H_2S production	Negative
Carbohydrate fermentation with lactose	Positive
Sucrose	Positive
Glucose	Positive

attained when cultivated at 37 °C for 24 h with initial pH of medium adjusted to 7.0, under agitation (150 rev/min). After incubation, the crude supernatant was used for the preliminary characterization of keratinase using keratin azure as substrate at described assay conditions.

Determination of molecular mass

The molecular mass of crude extracellular fraction of keratinase enzyme from *Bacillus* sp. NKSP-7 was determined using SDS-PAGE analysis. The prominent band of protein at position of 25 kDa was observed (Fig. 1).

Effect of pH and various buffer system

Six different buffer systems were used to evaluate the optimal buffer and pH for extracellular keratinase from *Bacillus* sp. NKSP-7, and it displayed optimal activity with phosphate buffer (pH 7.0). Enzyme revealed more than 75% of peak activity at pH 5.5–7.5 and 60% activity at pH 9.0 (Fig. 2a). The crude keratinase was quite stable over a wide range of pH from 5.5–8.0 and retained more than 85% of initial activity at pH 5.0 and 8.5 (Fig. 2b), when tested in respective buffers at 40 °C for 4 h in the absence of substrate.

Effect of temperature

Keratinase from *Bacillus* sp. NKSP-7 showed optimal activity at 60 °C and maintained above 70% of optimal activity over a range of temperature at 45–75 °C, whereas was rapidly inactivated at higher temperatures (Fig. 3). Keratinolytic enzyme exhibited worthy thermostability at 20–65 °C temperature in pH 7.0 buffer for 4 h, whereas above 75 °C enzyme was less stable (Fig. 3).

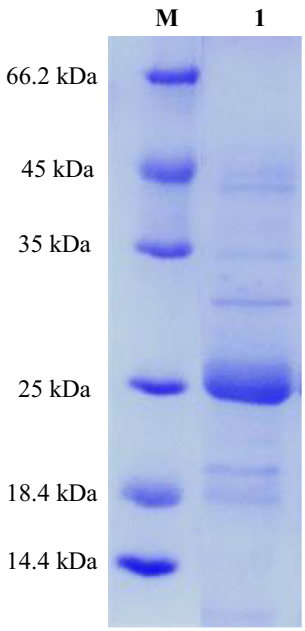

Fig. 1 SDS-PAGE analysis of extracellular keratinase from *Bacillus* sp. NKSP-7. Lane M, Thermo Scientific Protein Marker (Catalog # 26610); lane 1: crude keratinase from *Bacillus* sp. NKSP-7

The storage stability of crude keratinase was scrutinized occasionally, and it was observed that enzyme has 100% stability at 4 °C (cool lab) and at room temperature for 20 days and 120 days, respectively without any significant decline in activity.

Effect of various chemicals on crude keratinase

Enzyme did not show any obvious constrain in the presence of Ba^{2+}, Ca^{2+}, K^{+}, Ni^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Co^{2+}, Tween-80, EDTA, Tris, SDS, Urea, and Triton X-100. Only in the presence of Hg^{2+} enzyme activity was repressed up to certain limit. Whereas activity was enhanced by adding β-mercaptoethanol, Na^{+}, Cd^{2+} and Mn^{2+} up to 132.8%, 112.5%, 115.8%, and 110.5%, respectively. Similarly, no inhibitory effect was observed with the addition of 50% (v/v) organic solvents (Table 3).

Discussion

Globally, poultry farms and poultry-processing industries produce an immense amount of keratin-feather waste that create a serious problem of solid waste. Chicken feathers contain about 90% keratin protein that cannot be degraded easily in natural environmental conditions. Their appropriate and sustainable disposal method is a foremost constraint for waste management processing plants. However, keratinolytic microorganisms and enzymes might be used to degrade this abundant insoluble keratin waste into nutritionally rich meal (peptides and amino acids), with prospective applications as organic fertilizer, in foodstuff and animal feed as a supplement (Bhari et al. 2018). Microbial conversion of keratinolytic wastes into valuable byproducts is an impending ecofriendly and alternative approach for the removal of poultry feathers (Abdel-Fattah et al. 2018). Therefore, the present study was focused to isolate bacterial strains from poultry dumping site that possess the ability to hydrolyze feather. The newly isolate *Bacillus* sp. NKSP-7 was easily cultivated in simple medium with chicken feathers as carbon and nitrogen sources, and recycled recalcitrant keratin-rich feather waste using extracellular keratinase enzyme. *Bacillus* sp. NKSP-7 has promising potential of bioconversion of poultry-feather into protein-rich feedstuff.

Considering the incessant voluminous keratin wastes in the environment, 45 soil samples from 9 different poultry waste and feather dumping sites of Lahore and its surrounding cities were selected to isolate potential keratinolytic bacterial strains. Among 60 isolates, only 5 isolates SPF-9, NKSP-7, NKSP-9, JPS-9, and SHP-5 showed prominent feather degradation

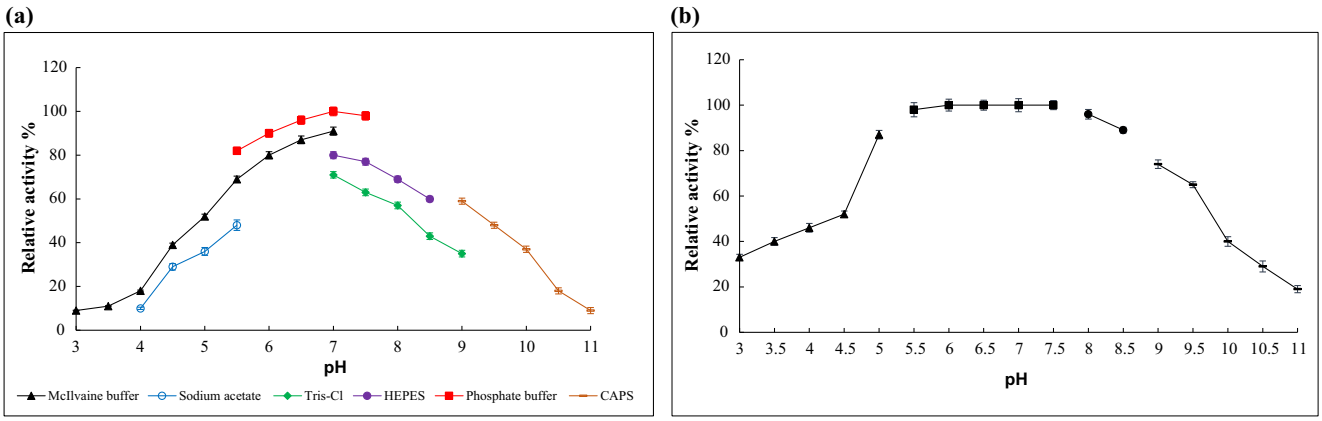

Fig. 2 **a** Effect of pH and various buffer system on crude keratinolytic enzyme from *Bacillus* sp. NKSP-7. Crude enzyme was incubated in sodium acetate (*open circle,* pH 4.0–5.5), McIlvaine (*closed triangle,* pH 3.0–7.0), Tris-Cl (*closed diamond,* pH 7.0–9.0), HEPES (*closed circle*, pH 7.0–8.5), phosphate (*closed square,* pH 5.5–7.5), and CAPS (*dash*, pH 9.0–11.0), under standard assay conditions. The maximum activity was observed at pH 7.0 with phosphate buffer, defined as 100% activity to calculate the relative activity. **b** pH stability was determined by pre-incubating enzyme in different pH buffers (3.0–11.0) at 40 °C for 4 h, using McIlvaine (*closed triangle,* pH 3.0–5.0), phosphate (*closed square,* pH 5.5–7.5), HEPES (*closed circle,* pH 8.0–8.5), and CAPS (*dash*, pH 9.0–11.0) buffers. Enzyme activity without pre-incubation is described as 100%. Reported data is the average of at least three independent experiments with the standard deviation (± SD) presented as error bars, which differ significantly at $p \leq 0.05$

ability and keratinolytic activity as compared to others (Table 1). The dumping sites contain large amount of feathers and native microbes may have adapted to utilize feather-keratin as substrate. Various previous studies have reported the isolation of keratinolytic bacteria from dumping sites of feathers that exhibited the ability to degrade keratin (Sekar et al. 2016; Kurane and Attar 2017; Bhari et al. 2018).

In this study, the result revealed that all isolates produced keratinase enzyme in all cell fractions but maximum activity was found in extracellular fraction than others. Some earlier studies have also been reported that *Bacillus* spp. predominantly produce extracellular keratinases (Abdel-Fattah et al. 2018; Bhari et al. 2018). Optimal enzyme activity from all experimental isolates weas perceived during the exponential phase of growth (after 24–48 h of fermentation) and reduced gradually with the longer time of incubation, suggesting that strains produced keratinase as primary metabolite. SPF-9, NKSP-7, NKSP-9, JPS-9, and SHP-5 isolates showed peak extracellular activity after 24 h (at 37 °C) (Table 1). Similarly, Kazi et al. (2015) demonstrated an extracellular keratinase from *Bacillus subtilis* showed optimal activity at the exponential phase with 32.5 U/mL at 37 °C after 72 h. While, *Bacillus subtilis* FDS15 showed high keratinolytic enzyme activity in supernatant with 16.5 U/mL (Sekar et al. 2016).

Initial morphological study of these 5 isolates revealed that they were gram-positive, aerobic, spore-forming bacilli, and adroit to degrade feather's keratin (Table 2). These strains were isolated from anaerobic habitat but optimally grew under aerobic conditions, as would be expected from the members of bacillaceae family (Vigneshwaran et al. 2010). Biochemical tests and characteristics revealed that SPF-9, NKSP-7, NKSP-9, JPS-9, and SHP-5 isolates may belong to genus *Bacillus* (Table 2.). Similar strategy of identification was adopted by various studies (Raju and Divakar 2013; Agrawal and Dalal 2015; Sekar et al. 2016). However, 16S rDNA sequencing confirmed that two most prominent keratinolytic strains, NKSP-7 and NKSP-9, are the member of *Bacillus* genus. On the basis of sequence analysis, these isolates were identified as new strains, *Bacillus* sp. NKSP-7 and *Bacillus* sp. NKSP-9.

Bacillus sp. NKSP-7 and NKSP-9 displayed significant bioconversion of feathers at 37 °C after 24 h. Extracellular keratinase was produced using feather (cheap substrate), by which cost of enzyme production can be reduced. Bhari et al. (2018) reported that *Pseudomonas aeruginosa* BSP10, *Bacillus licheniformis* LRP1, and *Bacillus aerius* NSMk2 degraded the chicken feathers in 120 h at 37 °C. Whereas *Bacillus thuringenesis*, *B. megaterium*, and *B. pumilus* (Agrahari and Wadhwa, 2010) showed degradation in 120 h at 30 °C, *Bacillus weihenstephanensis* PKD 5 (Sahoo et al. 2012) and another strain of *Bacillus licheniformis* (Vigneshwaran et al. 2010) displayed maximum degradation after 168 h of incubation at 37 °C and 40 °C, respectively. On the contrary, *Bacillus* spp. (Agrawal and Dalal 2015) and *Bacillus subtilis* FDS15 (Sekar et al. 2016) decomposed feathers within 30 days and 21 days, respectively at 37 °C.

Preliminary characterization of crude keratinase from *Bacillus* sp. NKSP-7 was performed. Enzyme showed best activity at pH 7.0 (Fig. 2a), and was stable over the pH range of 5.5–8.0 (Fig. 2b). Optimal pH of enzyme from NKSP-7 is quite similar to other *Bacillus* keratinases (Prasad et al. 2010; Vigneshwaran et al. 2010); whereas some displayed optimally activity at pH 8.0–9.0 (Ghasemi et al. 2012; Sahoo et al. 2012; Saibabu et al. 2013). Keratinase from NKSP-7 showed great

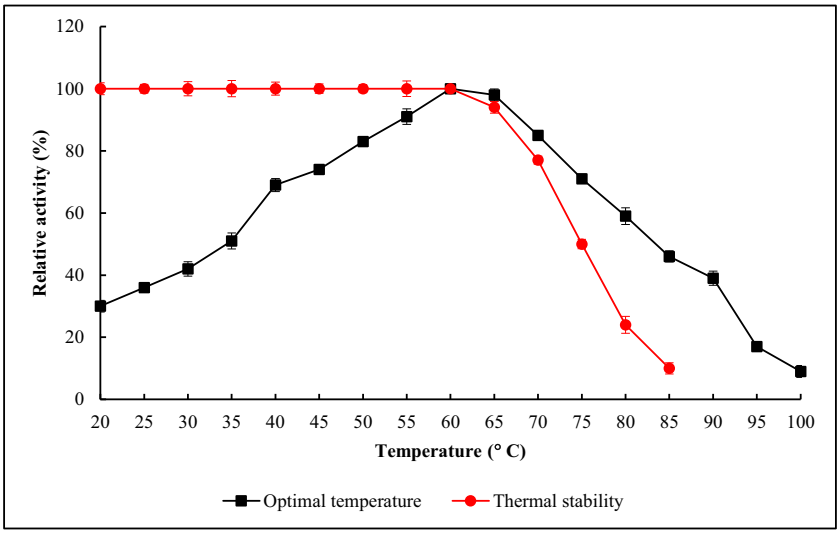

Fig. 3 Effect of temperature and stability of crude keratinase from *Bacillus* sp. NKSP-7. Temperature profile (*closed circle*) was determined by incubating enzyme at 20–100 °C in phosphate buffer (pH 7.0), under standard assay conditions. Enzyme was optimally activity at 60 °C, is considered as 100% activity to calculate the relative activity. Thermal stability (*closed square*) was determined by pre-incubating enzyme at 20–85 °C for 4 h at pH 7.0 buffer, activity without pre-incubation is taken as 100%. All values are the average of three individual experiments, Y-error bars show the standard deviation (±SD) among the triplicate reactions, which differ significantly at $p \leq 0.05$

pH stability (5.5–8.0) in comparison with others keratinases (Vigneshwaran et al. 2010; Ghasemi et al. 2012; Abdel-Fattah et al. 2018). However, the result revealed that the structure of enzyme will be destabilized and altered at variant pH because the net charge can affect protein's free energy and hence deactivate it (Akram et al. 2018). Keratinase from NKSP-7 displayed peak activity at 60 °C (Fig. 3), which is analogous to other keratinolytic enzymes from *Bacillus* (Xu et al. 2009; Vigneshwaran et al. 2010); and higher than that of other (35–50 °C) (Prasad et al. 2010; Sahoo et al. 2012; Saibabu et al. 2013). Crude keratinase exhibited high thermal stability at 20–65 °C for 4 h as compared to other keratinases (Abdel-Fattah et al. 2018; Saibabu et al. 2013; Ghasemi et al. 2012; Sahoo et al. 2012). Keratinase from NKSP-7 showed a notable storage stability at 4 °C and room temperature (Ghasemi et al. 2012).

The metallic cations, surfactants, and organic solvents cause a conspicuous inhibitory influence on the activity of microbial enzymes. But keratinase from NKSP-7 did not show any obvious constrain with these additives (Table 3). However, the activity was slightly enhanced by adding β-mercaptoethanol, Na^{+}, Cd^{2+}, and Mn^{2+}; the similar behavior has been reported from *Bacillus* sp. MKR5 (Ghasemi et al. 2012). However, keratinase from NKSP-7 was strongly inhibited by Hg^{2+}, signifying that Hg^{2+} might affect catalysis by binding to –SH groups in the active site or/and Hg^{2+} bind to tryptophan residues and carboxyl groups of amino acids and decrease the enzyme activity due to denaturation. This observation is in line with some prior studies (Xu et al. 2009; Vigneshwaran et al. 2010; Saibabu et al. 2013). However, some cations stimulated the keratinase activity of NKSP-7, which might perform an essential function in the stability of enzyme's catalytic site and increase the affinity by stabilizing protein's structural conformation.

Generally, the activity of keratinases was inhibited with the addition of EDTA (Xu et al. 2009; Vigneshwaran et al. 2010). But keratinase from NKSP-7 did not show any inhibitory influence with EDTA, this behavior is in accord with keratinases from *Bacillus* sp. MKR5 (Ghasemi et al. 2012) and *Bacillus megaterium* (Saibabu et al. 2013). Hence, keratinase from NKSP-7 cannot be considered as metalloprotease and should possibly categorized as a serine keratinolytic protease. In the presence of β-mercaptoethanol (reducing agent), the activity of keratinase from NKSP-7 was enhanced, possibly through the reduction of disulfide bonds in the enzyme substrate. No noticeable effect was detected by adding isopropanol, methanol, n-butanol, ethanol, DMF, acetonitrile, acetone, and DMSO (Table 3); the comparable results has been reported by Xu et al. (2009) and Cai et al. (2008).

Conclusion

An efficient keratinolytic bacterial strain NKSP-7 was isolated from poultry dumping site, and molecular identification confirmed that it belongs to genus *Bacillus* (*Bacillus* sp. NKSP-7). The newly isolated strain *Bacillus* sp. NKSP-7 efficiently produced extracellular keratinase using recalcitrant keratin-rich feathers as substrate under submerged fermentation. Keratinase from NKSP-7 has promising potential of bioconversion (bioremediation) of feather waste into protein-rich feedstuff and also has impending applications for bio-waste management. The

Table 3 Effects of various chemical reagents on the activity of crude keratinase from *Bacillus* sp. NKSP-7

Effectors	Relative activity (%)
Control	100
Cations (10 mM)	
Ba^{2+}	101.8 ± 2.8431
Cu^{2+}	104.7 ± 1.5275
Ca^{2+}	102.6 ± 2.5166
Cd^{2+}	115.8 ± 1.914
Co^{2+}	99.8 ± 2.623
K^{1+}	100.4 ± 2.1633
Hg^{2+}	18.6 ± 1.2767
Fe^{2+}	106.4 ± 1.8877
Mn^{2+}	110.2 ± 2.0664
Mg^{2+}	102.3 ± 1.9502
Pb^{2+}	98.0 ± 2.0
Na^{2+}	112.5 ± 2.4515
Ni^{2+}	99.6 ± 1.5716
Zn^{2+}	101.2 ± 2.5632
Additives (10 mM)	
Chelating agent (EDTA)	109.4 ± 1.4526
β-Mercaptoethanol	132.8 ± 1.9035
Urea	100.4 ± 2.1633
Tris	108.2 ± 1.0583
Solvent (50% v/v)	
Isopropanol	92.6 ± 2.1548
Methanol	97.7 ± 1.4189
Ethanol	98.2 ± 2.0298
n-butanol	95.1 ± 1.5275
Chloroform	91.7 ± 1.9672
DMF	99.5 ± 0.8963
DMSO	97.3 ± 1.5308
Ethyl acetate	87.2 ± 1.3577
Acetonitrile	88.5 ± 1.0817
Glycerol	94.7 ± 1.7088
Acetone	91.5 ± 1.4731
Detergents (10 mg/mL)	
Triton-X-100	105.6 ± 2.1071
Tween-80	104.8 ± 1.5275
SDS	87.1 ± 1.1359

Relative activity was expressed as a percentage, and assessed using keratin azure (5 mg) under standard assay conditions. Results are the mean of three replicates and the ± represents the standard deviations (SD) of relative activity that differ significantly at $p \leq 0.05$

stability of keratinase at elevated temperature and variant pH, independency toward cations, detergents, and organic solvents make this biocatalyst a perceptible candidate for various industries such as agroindustry, cosmetic, animal feed, detergent, textile, pharmacy, waste water treatment, biofuels, and especially in leather industry for dehairing process.

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Author contribution IU Haq: designed the study and supervised all work.
F Akram wrote the manuscript, carried out experiments, analyzed the data and performed statistical analysis.
Z Jabbar: also carried out experiments work.

Compliance with ethical standards

The authors declare that they have no competing interests. We assure the integrity and quality of our research work. It is also stated that there is no plagiarism in this work and all points taken from other authors are well cited in the text. This study is completely independent and impartial.

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals N/A. This research did not involve human participants and/or animals.

Informed consent N/A. This research did not involve human participants.

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