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Research Article

Isolation and screening of keratinase enzyme producing *Bacillus mojavensis* par01 from poultry farm

Pardeep Kumar[1*], Yasmeen Faiz Kazi[1] and Muhammad Kamran Azim[2]
1. Institute of Microbiology, Shah Abdul Latif University, Khairpur, 66020, Sindh, Pakistan
2. Department of Biosciences, Mohammad Ali Jinnah University, Karachi,75400, Sindh, Pakistan
**Corresponding author's email:pardeepjanbhani@gmail.com*

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Abstract

Feathers are the most prevalent waste produced by poultry industry. In addition to seriously polluting the environment, an excessive amount of feathers also wastes protein resources. Currently, one method under consideration is the degradation of poultry waste by bacteria that produce keratinase. The keratinase plays a significant role in the biodegradation of chicken waste into animal feed and fertilizers. In this study, keratinolytic bacterium was isolated, screened, identified and cultural conditions were optimized for the maximum production of enzyme. Keratinase producing bacterium was isolated from soil of poultry farm and screened on skim milk agar followed by enzyme production using submerged fermentation. The isolate showing maximum zone of hydrolysis on skim milk agar was identified as *Bacillus mojavensis* on the basis of morphological, biochemical and contigs sequences of the strain Bacillus par01 and related bacteria using blast search and named as *Bacillus mojavensis* par01. Maximum keratinase production (160 U/mL) was achieved in 72 h using a minimal growth medium containing 1% (w/v) feather meal at 37 °C, pH 7.5 at 150 rpm. The crude keratinase was purified using ammonium sulfate precipitation, DEAE Sephadex A-50 and Q-Sepharose chromatographic techniques. The molecular mass of purified keratinase was 30 kDa. The overall purification factor was 13.5 fold**,** final yield was 40% and the specific activity of final product was 560 U/mg. This study shows that the good feather-degrading capacity of the selected *B. mojavensis* par01 strain may be used for its prospective biotechnological applications in the processing of poultry waste.

Keywords: Feather meal; Fermentation; Keratin; Precipitation; Purification

Introduction

The keratinases are a class of proteases that exhibits keratinolytic activity on fibrous, insoluble structural keratin proteins that have been extensively cross-linked with hydrogen, disulfide, and hydrophobic bonds that result in mechanical stability and resistance to common protease enzymes like trypsin, papain and pepsin **[1]**. Keratinases are endo-acting proteases that belong to protease family M36 (MEROPS database). Family M36 contains 687 proteases in all **[2]**.

Keratins, which occur mainly in epithelial cells, are known as fibrous proteins. Keratin proteins are not soluble in weak acid, alkali, water and organic solvents **[3]**. Keratin is found in the wool, horns, hooves, nails, hair and stratum corneum (outer layer of

skin).There are two forms of keratin, alpha and beta. Alpha-keratin is found in all vertebrates. It forms the outer layer of skin, wool, horns, nails and claws. The beta keratin is found in reptiles and birds. It is found in claws, nails and scales of reptiles and in claws, feathers and beaks of birds [4]. The amount of sulfur varies between the two kinds of keratin structures, giving the keratin a softer or tougher structure and having a greater impact on the rate of degradation of the keratinous material [5]. A crucial characteristic of these proteins is their high cystine concentration, which distinguishes keratin from other structural proteins like collagen and elastin [6]. In chicken feathers, you will find 85% of the crude protein, 70% of the amino acids, vitamins, high-value minerals, and other growth-promoting substances [7].

The main compound in most biological materials is the recalcitrant keratin. It is also the waste product in the manufacturing industries for poultry, slaughterhouse, leather and fur [3]. The handling of keratinous wastes produced by many industries is increasingly of significance on a global scale. Every year, the environment receives more than a billion tons of keratin waste. Despite several studies that transform this waste into valuable products, keratin waste from many different sources continues to be a less-explored biomaterial for making useful commodities. This shows that a significant keratin waste quantity is not properly disposed of or turned into valuable goods, but is instead tossed into the environment, contributing to environmental contamination [8]. The keratin waste from the polluted area can be removed with keratinolytic protease enzymes that are spread and secreted by numerous groups of microbes in nature. In addition to a few strains of Gram negative bacteria like *Stenotrophomonas maltophilia*, *Chryseobacterium indologenes* TKU014 and *Xanthomonas maltophilia*, a large variety of Gram positive bacteria including Bacillus species, *Lysobacter* sp. NCIMB 9497, *Nesternkonia* sp. AL-20, *Kocuria rosea* *Clostridium sporogenes* and *Microbacterium* sp. kr10 are confined as keratin degraders [7]. The Bacillus genus contains the vast majority of keratin-degrading bacteria [9]. The production of keratinase or other proteins by microbes is influenced by a number of variables, including temperature, pH, the quality of the carbon and nitrogen sources, the presence of aeration in the medium and various techniques such as optimizing the cultivation conditions and the medium composition to increase the yield of the enzyme [10].

The biotechnological application of keratinases requires the production of these enzymes in sufficient amounts for commercial purposes. The production of keratinase is usually induced by keratin and, thus, a keratinous substrate (hair, chicken feathers, feather meal) is often added to the cultivation medium [7, 11].

These microbial keratinases are considered as promising biocatalysts for several purposes including application in the field of agroindustry for the production of feed hydrolysates, feed supplements and nitrogen fertilizers. These keratinases also used in pharmaceutical/biomedical field like hydrolysis of prion, enhanced drug delivery and for dermatological treatments. Keratinases also have a potential role in industries like cosmetics, leather processing (tannery), fiber modification (textiles), detergents, modification of protein functionality (foods), biopolymers (films, coatings, glues), wastewater treatment/waste management and bioconversion of keratinous wastes for subsequent production of methane or biohydrogen [7]. The aim of the current study was to isolate, screen, and identify the most promising keratinolytic bacterial strain from poultry farm soil and optimization of

various cultural conditions for the production of maximum enzyme yield.

Materials and Methods

Collection of soil samples

Soil samples were collected from the poultry farms in Khairpur City in sterile plastic bags. Afterward soil samples were examined and processed in the Microbiology research lab, Shah Abdul Latif University Khairpur for the isolation of keratin degrading microorganisms.

Preparation of feather meal broth

Chicken feathers were thoroughly washed with distilled water, allowed to air dry, then cut into little pieces with the help of scissor and then autoclaved. The processed feathers are referred as feather meal [12]. The following ingredients were included in the keratinase production medium (g/100mL) feather meal 1gm, NaCl 0.05, K_2HPO_4 0.14, KH_2PO_4 0.07, $MgCl_2.6H_2O$ 0.02, $CaCl_2$ 0.025, yeast extract 0.3 and pH 7.5.

Isolation and screening of keratin degrading bacteria from soil

Soil dilutions were prepared with 0.9% saline, inoculated with feather meal broth, and incubated at 37°C in incubator shaker (Innova 4900) at 150rpm. Once there was noticeable feather hydrolysis observed, and then serial dilutions of the culture suspensions were inoculated on skim milk agar plates for the screening of bacteria that secrete proteases [12,13]. The plates were then incubated for 24 hours at 37°C. On skim milk agar, isolated bacterial colonies that showed a clear zone were selected for further research. The isolates were distinguished by colonial, morphological, biochemical features as described by Bergey's manual® of systematic bacteriology [14].

Molecular identification of bacterial isolate

For this molecular identification, genomic DNA of isolate was sent to Macrogen Company (Korea) for commercial analysis. After obtaining results, an assembled genome of Bacillus par01 was sent to PATRIC's full genome analysis service, then genome was annotated using RAST tool kit (RASTtk).

Culture conditions for keratinase production

A 250 ml Erlenmeyer flask containing 90 ml of feather meal broth was used to cultivate the selected keratinolytic bacterial strain. 10 ml of overnight-grown bacterial culture was used as the inoculum, which was then incubated for 6 days at 150 rpm. For the control, the feather meal broth without inoculum was used. The growth of bacteria was observed through visible turbidity. The culture medium was centrifuged at 10,000 rpm for 10 minutes, after being filtered to eliminate any leftover feather meal. The keratinase activity was determined in supernatant by keratin azure assay.

Keratin azure assay

Keratinase activity was measured by using keratin azure (Sigma USA) as a substrate. The keratin azure was frozen at -20°C and then crushed into a fine powder for the keratinase assay. The 5mg powder of keratin azure was added in 1ml 50mmol/L Tris HCl buffer pH 8.0. The reaction mixture of keratin azure (1mL) and culture filtrate (1mL) was incubated in incubator shaker at 37°C, 200 rpm for 30 minutes. Keratin azure solution (1mL) was rotated in the same buffer at 37°C for 30 minutes as a control. After incubation period, 2mL of 0.4mol/L trichloroacetic acid (TCA) was added in both the test and control tubes to stop the reaction. Then 1mL of culture filtrate was added into the control tube. The reaction mixtures were then filtered, and absorbance (optical density) was taken at 595 nm. The amount of enzyme responsible for a 0.01 absorbance increase between the control and sample at the given conditions at 595 nm was used to compute one unit of kertatinolytic activity [15].

Optimization of fermentation conditions

The fermentation conditions were optimized for the production of enzyme. Initial pH (6-

9), fermentation temperature (30-50 ^{o}C), feather meal (%w/v) and fermentation time (24-144 hours) were studied to observe the effect of these parameters on the quantity of enzyme production.

Keratinase purification

The crude enzyme was centrifuged (8,000 rpm), at 4^{o}C to remove bacterial cells and other particles. The enzyme was precipitated from cell free supernatant till70% saturation with solid ammonium sulfate and then allowed to precipitate for 12 hours. The resultant precipitate was obtained by centrifugation (10,000 rpm, 4^{o}C for 30 min), dissolved in a small volume of 50 mM Tris-HCl buffer (pH 8.0), and dialyzed overnight against the same buffer. A DEAE Sephadex A-50 matrix chromatography column (1.0 x 35 cm) was loaded with the dialyzed enzyme and equilibrated with 50 mM Tris HCl buffer (pH 8.0). Keratinase-positive fractions were combined and put on a Q-Sepharose column (2.0 x 5.0 cm) that had been pre-equilibrated with 50mM Tris-HCl buffer (pH 8.0). A gradient of 0-1 mol l^{-1} NaCl in a 50 mM Tris HCl buffer at pH 8.0 was used to elute the column. The active fractions were combined and used for additional research. The keratinase activity from the purification steps was confirmed by keratin azure assay as described above [16].

Protein estimation

The Bradford method [17] was used to determine the protein concentration, using bovine serum albumin (BSA) as the standard. The protein concentration was measured spectrophotometrically at 595 nm.

Molecular weight determination

The molecular weight of keratinase was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was carried out on 12.5% resolving gel and 5% stacking gel using BioRad mini gel electrophoresis instrument [18]. Gel was stained with Coomassie brilliant blue R-250. The electrophoretic migration of the keratinase was compared with a BlueStep^{TM} broad range protein marker (Amresco).

Statistical analysis

All the experiments were performed in triplicates. Data was analyzed by using MS Office 2007. Results were expressed as means±standard error. Differences were considered significant at $p<0.05$.

Results

Isolation and screening of keratinase producing strain

A bacterial isolate that degrades feathers was isolated from feather meal broth inoculated with soil samples. Proteolytic bacteria were screened on skim milk agar, inoculated with feather meal culture. The colonies that displayed a clear zone were further selected and the colony exhibiting the highest zone of hydrolysis on skim milk agar (Fig. 1) was selected for enzyme production. After incubation, degradation of feather was observed in flask. Extracellular keratinase was secreted by a bacterial isolate using feather keratin as the only carbon source.

Identification of keratinase producing isolate

The bacterial isolate was identified up to the genus level and designated as Bacillus par01 based on the colonial, morphological, biochemical properties and with reference to Bergey's manual of systematic bacteriology (Table 1). Additional confirmation came from the contig sequences of the strain Bacillus par01 and associated bacteria through blast search. A unique genome identifier of 1386.1398 was given to the Bacillus par01 genome after it was annotated using the RAST tool kit (RASTtk).

Figure 1. Zone of hydrolysis on skimmed milk agar

Table 1. Cultural and Biochemical Characteristics of Bacillus par01

Test	Result	Test	Result
Gram stain	Gram +ve	Spore Formation	+ve
Colony shape	Circular	Catalase	+ve
Shape	Rods	Casein hydrolysis	+ve
Colony color	Opaque	Oxidase	+ve
6.5% NaCl growth	+ve	Lactose fermentation	-ve
Motility	motile	Nitrate reduction	+ve
Lecithin degradation	-ve	Mannitol fermentation	+ve
β Haemolysis Test	+ve	Starch hydrolysis	+ve
Penicillin sensitivity test	+ve	Gelatin hydrolysis	+ve
Anaerobic growth	-ve	Urea degradation	-ve
Growth at 55^0C	+ve	Indole test	-ve

Phylogenetic analysis

The reference and representative genomes are provided by PATRIC, and they are used in the phylogenetic analysis. Mash/MinHash was used to find the representative and nearest reference genomes. From these genomes, PATRIC global protein families (PGFams) were selected to infer the evolutionary position of this genome. MUSCLE was used to align the protein sequences from these families, and the nucleotides for each of those sequences were then mapped to the protein alignment. A data matrix was created by concatenating the joint set of amino acid and nucleotide alignments. RaxML was then used to analyze this matrix, and fast bootstrapping was employed to create the support values in the tree. Phylogenetic analysis revealed a 100% similarity between the strain Bacillus par01 and *B. mojavensis* (RO-H-1 1051501.3). The phylogenetic tree that was created (Fig. 2) revealed that the strain belonged to the clade of *B. mojavensis*, a species that produces keratinase. The isolate is referred as *B. mojavensis* par01.

Keratinase production and optimization of fermentation conditions

Bacillus mojavensis par01 produced extracellular keratinase in the culture medium containing feather meal (1%) as the sole carbon and nitrogen sources. In the first 24 hours, keratinase activity was low, but as the bacteria expanded after 72 hours, the enzyme activity peaked at 80 U/mL. Enzyme activity decreased steadily as fermentation

duration increased and reached at 20 U/mL after 144 hours (Fig. 3a). The maximum keratinase activities were 155 and 160 U/mL at the initial pH of 7.5 and fermentation temperature of 37°C respectively. However, subsequent increases in pH and temperature suppressed the enzyme activity (Fig. 3b, 3c).

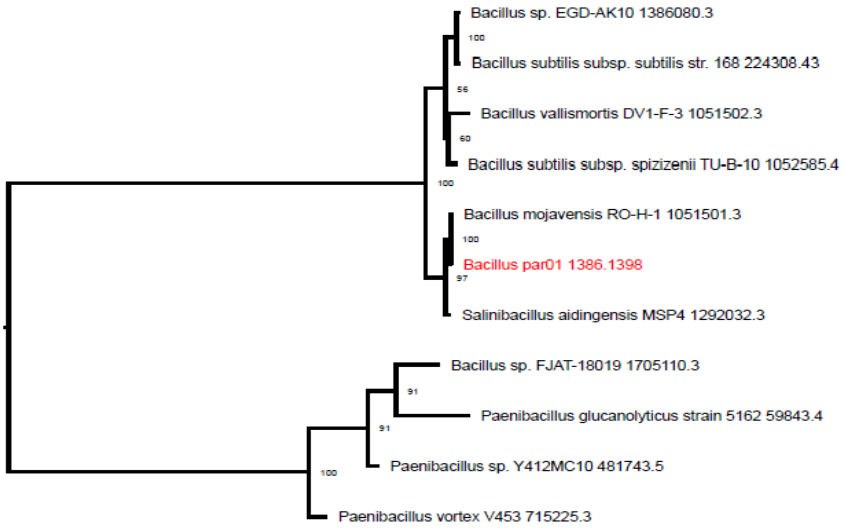

Figure 2. Phylogenetic tree constructed from contigs sequences of the strain Bacillus par01 and related bacteria using blast search.

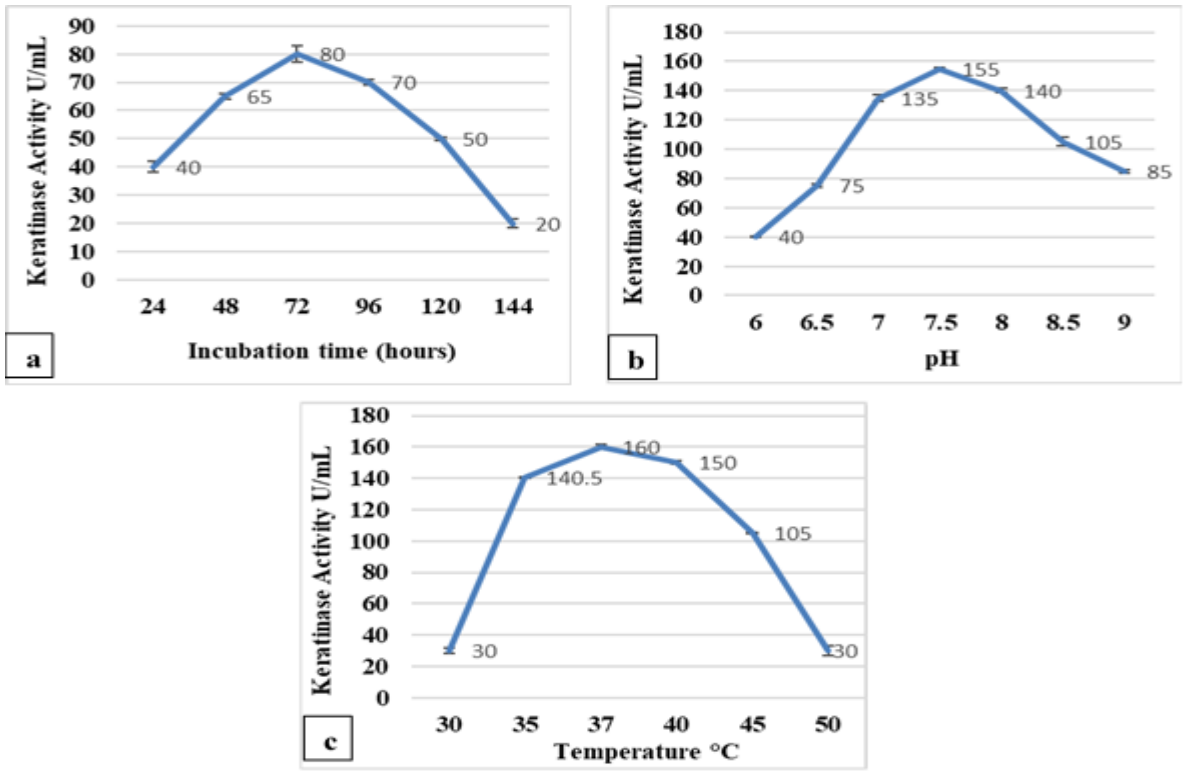

Figure 3. Effect of different parameters on the production of keratinase by *B. mojavensis* par01: (a) incubation time; (b) pH; (c) temperature

Keratinase purification

The crude enzyme produced by *B mojavensis* par01 was concentrated by centrifugation and precipitation with 70% ammonium sulfate. Enzyme was then dialyzed and subjected to gel filtration on a sephadex G-50 column. Q-Sepharose ion exchange chromatography was used to further purify the most active fractions from the sephadex G-50 column. The final yield was 40%, while the overall purification factor was 13.5 fold. The specific activity of final product was 560 U/mg (Table 2).

Determination of molecular weight

The molecular weight of the purified keratinase was calculated by comparing the electrophoretic mobility of the keratinase with the electrophoretic mobilities of marker proteins. SDS PAGE results revealed a single protein band, demonstrating the homogeneity of the enzyme's purification. The apparent molecular weight was 30 kDa (Fig.4).

Table 2. Purification profile of keratinase enzyme from *Bacillus mojavensis* par01

Purification Stage	Total Protein (mg)	Total Enzyme Activity (*U)	Specific Enzyme Activity (U/mg)	Purification (Fold)	Yield (%)
Crude culture supernatant	315	16080	41.3	1.0-fold	100
Ammonium sulfate saturation	135	8290	61	1.4-fold	51.5
DEAE-Sephadex A-50	14	6720	480	11.6-fold	41.79
Q-Sepharose	11.5	6440	560	13.5-fold	40

*Unit (U): The increase of 0.1 absorption is equal to one unit of keratinase activity

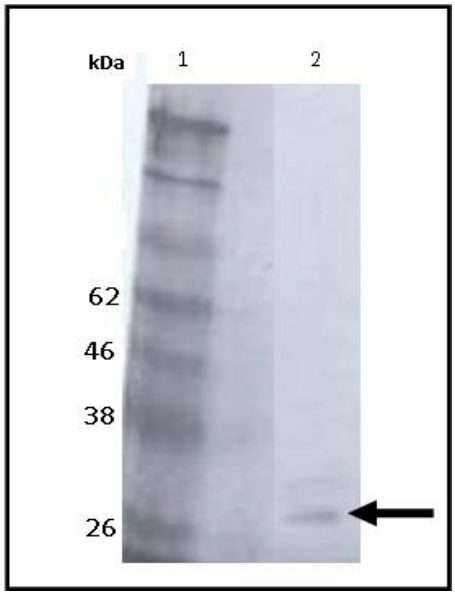

Figure 4. SDS-PAGE of the purified Keratinase. Lane 1: Protein marker, BlueStep™ broad range (Amresco), Lane 2 (Arrow): purified keratinase.

Discussion

Keratinase enzyme has potential to degrade the tough and fibrous keratin protein present in feathers and hair therefore this enzyme has various industrial applications. The present study aimed to isolate, screen and identify novel keratinolytic bacteria and determine their optimum parameters for maximum

enzyme yield. In this study a bacterial strain that was obtained from a poultry farm exhibited the highest level of keratinolytic activity. This isolated strain of bacteria is a Gram-positive, rod-shaped, and spore-forming bacterium and this isolated strain was named as Bacillus par01. Previously keratinase secretion has been reported from Gram-positive bacteria including *Bacillus* and *Streptomyces* species and few Gram-negative bacterial species [19]. In some studies, two *Bacillus* species were discovered which secreted keratinase [20, 21].

In current study the Bacillus par01 was identified through morphological, biochemical and molecular analysis based on contigs sequences of the strain *Bacillus* par01 and related bacteria from blast search that has 100% sequence homology with *B. mojavensis* RO-H-1 (10515013) and now this bacterium *Bacillus* par01 is named as *B. mojavensis* par01.

Maximum extracellular keratinase was obtained after growth of the *B. mojavensis* par01 on the culture medium supplemented with feather meal (1%) as the sole carbon and nitrogen sources. Similar results were also obtained with *Bacillus* sp. JB99 that produced highest keratinase in presence of 1% feather meal [22]. High level of keratinase was also obtained with *Arthrobacter creatinolyticus* KP015744 in presence of 1% feather powder [23].

Depending on the optimum growing conditions, the keratinase production from *B. mojavensis* par01 was measured for 144 hours and the greatest keratinase yield (80 U/mL) was obtained after 72 hours of cultivation, then the keratinase secretion started to decline. Similarly, *B. cereus* LAU08 showed the highest enzyme production after 72 hours of incubation [24], while *B. cereus* YQ15 obtained the highest enzyme output at 36 hours of incubation [25]. *B. mojavensis* par01 isolate exhibited adequate keratinolytic activity in a wide range of acidic to slightly alkaline pH (6-9) with optimum pH 7.5 like keratinase from *Bacillus* sp. NKSP-7 at pH 7.5 [26] and from *B. subtilis* [27], where the maximum activity occurs in acidic to neutral pH range (pH 5–7). Additionally, a neutral to alkaline pH was also found to encourage the synthesis of keratinase in a variety of microorganisms [28, 29]. However, the majority of keratinase producing bacteria, actinomycetes, and fungi show that their optimum pH ranges from neutral to alkaline [30]. Temperature changes during fermentation frequently affect microorganism's metabolism and have a substantial impact on the production of target enzymes. According to this study, 37 °C is the optimum temperature for keratinase production, which is similar to the optimum growth temperatures for certain other bacterial keratinases from *B. mojavensis* FUM125 [21] and *B. pumilus* FH9 [1]. The *B. mojavensis* par01 isolate, however, was active in both acidic and alkaline environments, and it can be extensively used in a variety of industries. The keratinase was purified using gel filtration and ion exchange chromatography after being dialyzed and precipitated with 70% ammonium sulfate. Keratinase was homogeneously purified as shown by a single resolved protein band on SDS-PAGE. The keratinase has a molecular weight of 30 kDa, which was very close to *Bacillus subtilis* keratinase 30.5 kDa [15], *Bacillus mojavensis* FUM125 keratinase 33.5 kDa [21]. The molecular weight of distinct keratinases produced by various bacteria has been shown to range from 18 to 240 kDa [32]. The final yield was 40%, with a purification factor of 13.5 fold. The specific activity of final product was 560 U/mg.

Conclusion

Keratinases play a significant role in biotechnology as proteolytic enzymes due to their distinctive and varied physicochemical features. *Bacillus mojavensis* par01 was isolated, identified and screened for its ability

to produce the novel keratinase enzyme. This strain was capable for the complete feather degradation which indicates that this organism has potential to degrade keratin and thus can be used in various industries. The keratinase enzyme secreted in this study is capable of operating in a wide pH and temperature range, which is advantageous for its use in diverse industrial applications.

Authors' contributions

Conceived and designed the experiments: Pardeep Kumar, YF Kazi & M Kamran Azim, Performed the experiments: Pardeep Kumar, Analyzed the data: Pardeep Kumar & YF Kazi, Contributed materials/ analysis/ tools: Pardeep Kumar, YF Kazi & M Kamran Azim,Wrote the paper: Pardeep Kumar & YF Kazi.

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