

RESEARCH ARTICLE

## Optimization and Purification of Keratinase from *Bacillus anthracis* with Dehairing Application

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### Abstract

The feather waste is treated by land filling or burning which requires a lot of expenditure and also responsible for air, soil and water contamination. The common methods to degrade feathers consume a huge amount of energy and are not good for environment. The biological degradation of feather waste is an environmental friendly method. In this study, bacterial strain MKR-9 was used and identified as *Bacillus anthracis* by using 16S rRNA sequencing. The keratinase production was carried in keratin medium by using strain MKR-9. During optimization, the maximum keratinase production was observed with 4% inoculum, 1% (w/v) substrate concentration, 40°C temperature, 7.0 pH, 3 days of incubation and presence of methionine. The keratinase production was suppressed by the presence of all used carbon and nitrogen source. The crude keratinase was stable at 40°C and 7.0 pH after pre-incubation of enzyme for 6 h. The crude keratinase can completely remove the goat skin hair after 24 hr of treatment. On the basis of this finding, crude keratinase mediated process can be employed in lather industry for the removal of hair as it is environmental friendly method. After ammonium sulphate precipitation (80% saturation) and dialysis, the partially purified keratinase was further purified by Sephadex G-75 column chromatography. The protein content, enzyme activity, specific activity, yield and overall purification were measured as 16.38 mg, 9652 U, 589.25 U/mg, 44.89% and 7.93 respectively. The purity of keratinase was confirmed by SDS- PAGE with a single band of 34 kDa.

**Keywords:** rRNA sequencing, keratinase, dehairing, purification, Sephadex G-75.

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## INTRODUCTION

A huge amount of keratinaceous waste is accumulated in our environment in the shape of chicken feathers, skin, hair, nails, hoofs and horns. This matter can be transformed into valuable products of great business value by using biodegradation. It has been estimated that 400 million tons chicks are processed each week globally; the daily build up of feather waste accumulates five million tons (Hans *et al.*, 2012). Feathers represent 5-7 % of whole weight of fully grown chicken (Joshi *et al.*, 2007). Keratinases have application in pharmaceutical, feed, fertilizer, detergent and leather industry. The enzyme is known as "green chemicals" due to their eco-friendly nature. The keratinolytic bacteria were isolated from soil and poultry compost (Manczinger *et al.*, 2003; Zerdani *et al.*, 2004). The majority of purified keratinases known till now cannot totally degrade native keratin. The study of keratinolysis is not fully elucidated till now (Ramnani *et al.*, 2005). In addition, they are helpful for removing the hair from hides during leather processing and solubilization of prions-the contributory agent of prion diseases (Macedo *et al.*, 2005; Tiwary and Gupta, 2010; Langeveld *et al.*, 2003). It is significant to biologically degrade the accumulated feather waste which is ecofriendly. Keeping in view of feather waste accumulation, optimization, purification of keratinase was studied by using bacterial strain MKR-9 with dehairing ability.

## MATERIALS AND METHODS

### Enzyme production and keratinase assay

In this study, bacterial strain MKR-9 was used and identified by 16S rRNA sequencing from Institute of Microbial Technology Chandigarh, India. The taxonomic relationship was studied by using EZ-TAXON tool. The 16S rRNA sequence was submitted to Gen Bank. The phylogenetic tree was generated by using neighbor joining algorithm. The enzyme production was carried out in keratin medium by inoculating MKR-9 strain for 3 days. The composition of keratin medium was in  $g\ l^{-1}$  as (10 feather powder; 0.5 NaCl; 0.3  $K_2HPO_4$ ; 0.4  $KH_2PO_4$ ; 0.1  $MgSO_4 \cdot 6H_2O$ ; pH 7.0). The cell free extract was used to evaluate the keratinase activity. The keratinolytic activity was measured with feather as a substrate with some

modifications in methodology (Wawrzekiewicz *et al.*, 1987). The 1.0 ml enzyme was incubated with 0.1 g of feather powder and suspended in 5 ml of phosphate buffer (pH 7.0). The buffer and feather powder was used as control. The reaction mixtures were incubated at 37°C for 2 h at 120 rpm. After incubation, the reaction was quenched by adding 2 ml of 10% trichloro acetic acid. The control sample was also prepared in the same way and enzyme solution was added after quenching of reaction. The mixture was filtered by using Whatman No.1 filter paper. The absorbance of filtrate was measured at 280 nm. One unit (U) of keratinolytic activity was defined as the amount of enzyme that resulted in an increase in absorbance of 0.01 at 280 nm under above said conditions. The protein concentration ( $\mu g/ml$ ) was determined by using Lowry method (Lowry *et al.*, 1951).

### Optimization of keratinase, stability and dehairing application

The one variable at-a-time approach was used for optimization of keratinase production. The culture supernatant was used to measure the enzymatic activity. All the optimization experiments were performed in triplicate. The different conditions like (inoculum size, substrate concentration, temperature and pH) were optimized. The various carbon sources (1% w/v), nitrogen sources (0.5% w/v) and different amino acid at 5 mM concentration were optimized by measuring the enzymatic activity after three days of incubation. The effect of pH on the stability of crude keratinase was examined by pre-incubating 1.0 ml of enzyme and 1.0 ml of buffers at different pH in the absence of substrate for 6 h at room temperature. The thermal stability was examined by pre-incubating the enzyme at different temperatures ranging from 40-70°C for 6 h in the absence of substrate. The samples were analysed at regular interval of 2 h to measure the keratinase activity. The dehairing assay was performed by using a simple method (Alexandre *et al.*, 2005). The goat skin was treated with crude keratinase for 24 h at 37°C and 120 rpm. The samples of goat skin were fixed and examined at regular time interval (6 h, 12 h, 18 h and 24 h) by scraping of hair with the help of fingers.

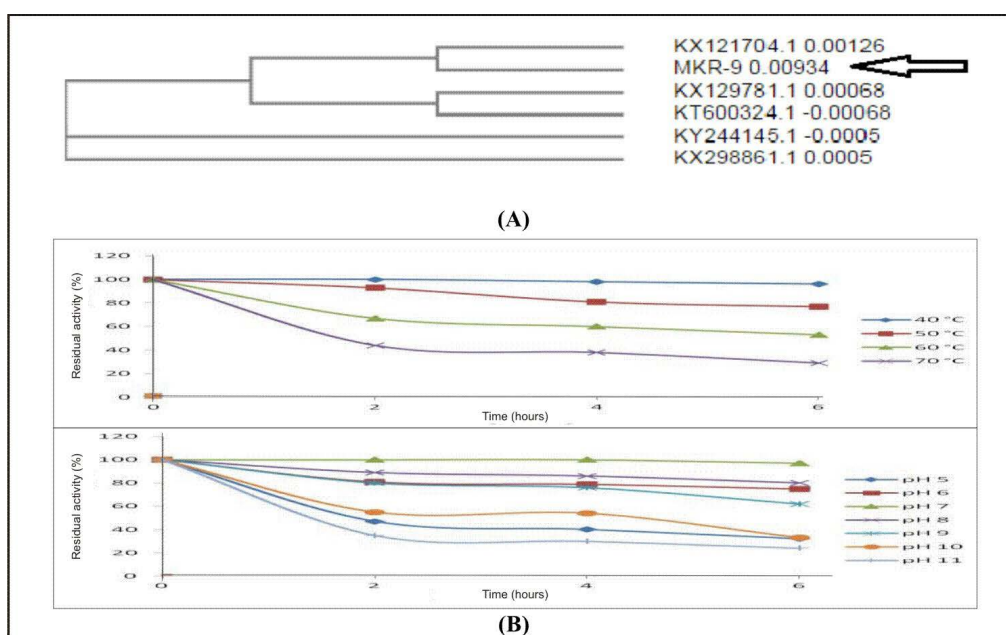
### Purification and characterization of keratinase

The MKR9 culture was grown in 800 ml nutrient broth, harvested at log phase and

washed twice with distilled water. The washed cells were transferred to 1 liter production medium under optimized conditions. The crude extract was obtained by using a cold centrifuge. The precipitation of proteins from crude extract was carried out by ammonium sulphate (80% saturation). The precipitated proteins were harvested by cold centrifugation and suspended in 20 ml, 50 mM phosphate buffer (pH 7.0) and then dialyzed. The dialyzed protein was assessed for its keratinase activity and protein concentration. The further purification of crude keratinase was carried out by using Sephadex G-75 column (90 x 1.5 cm) chromatography at 4 °C. The column was equilibrated with 50 mM potassium phosphate buffer (pH 7.0). The 3 ml concentrated protein was loaded. The elutions of protein were carried out in cold condition with same buffer at flow rate of 30 ml/h. The eighty fractions (5.0 ml each) were collected and analyzed individually for protein concentration by measuring absorbance at 280 nm. The fractions with higher absorbance were analysed for keratinase activity and pooled together. The purity of protein was further confirmed by SDS-PAGE. During characterization, keratinase activity of purified enzyme was analysed at various pH, temperatures, different metal ions (5 mM) and different chemicals (5 mM).

## RESULTS AND DISCUSSION

On the basis of 16S rRNA sequencing analysis, the culture was identified as *Bacillus anthracis* MKR-9 (KY798438). A distance based phylogenetic tree was constructed as shown in fig. 1(A). The optimization of keratinase production in the form of enzyme activity is shown in table 1. The maximum enzymatic activity ( $19.10 \pm 0.87$  U/ml) was observed after 3 days of incubation. At 3 days of incubation, the maximum enzymatic activity was observed with 4 % inoculum size, 1% (w/v) substrate, 40°C, 7.0 pH and methionine, measured as 18.86 U/ml, 23.7 U/ml, 18.96 U/ml 30.33 U/ml and 33.8 U/ml respectively. The keratinase production was suppressed in the presence of all used carbon and nitrogen sources. Other study shows similar results as keratin degradation varies with incubation time and maximum production was found with 4% inoculum size and neutral pH (Ramni and Gupta, 2004; Ramya et al., 2014; Brandelli et al., 2005). However, addition of carbon source decreases the keratinase production (Matikeviciene et al., 2011 and Kainoor et al., 2010). The stability of crude keratinase is shown in fig.1 (B). The crude keratinase was stable at 40°C and residual enzyme activity at 40, 50, 60 and 70°C recorded as 96%, 77%, 55% and 29%



**Fig. 1.** (A) Phylogenetic tree of MKR-9 strain and (B) stability of crude keratinase

respectively after pre-incubation of enzyme for 6 h. The keratinase was stable in wide range of pH, at pH 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0, the enzyme express 32 %, 75 %, 96 %, 80 %, 62 %, 34 % and 24 % residual activity respectively after pre-incubation for 6 h.

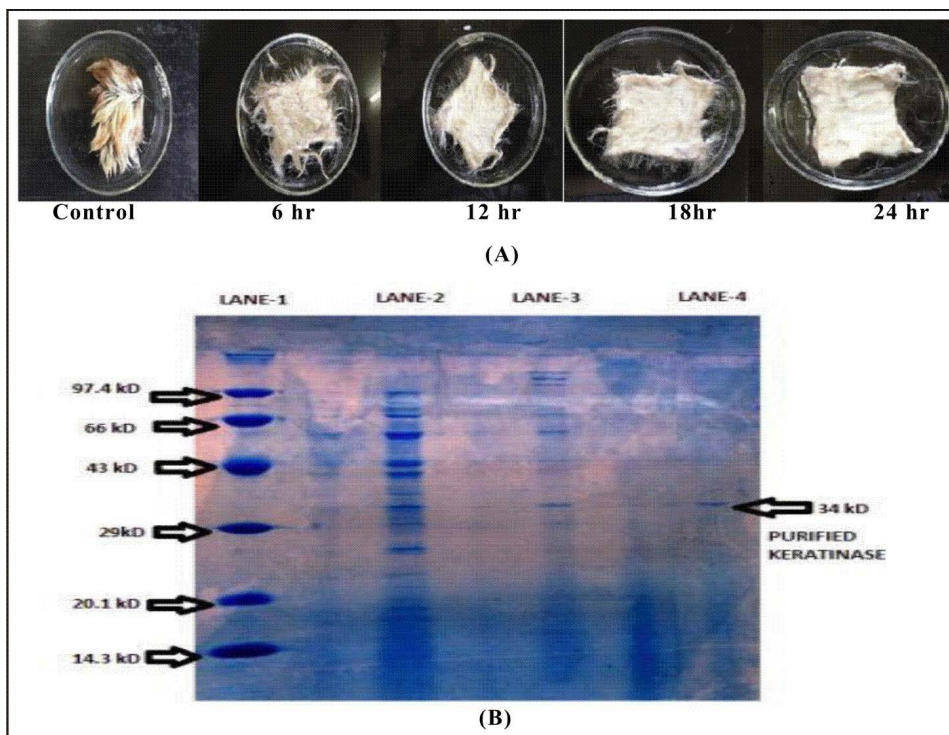
#### Keratinase in dehairing application

The action of crude keratinase in goat skin dehairing ability is shown in fig 2 (A). After beginning of dehairing at 6 h, 40 to 60% and 60 to 80% dehairing of goat skin was observed after 12 and 18 h of incubation respectively. The complete removal of hair from skin was observed after 24 h however, dehairing not occurred in case of control. In the present study, the dehairing ability was remarkable by using crude keratinase of *Bacillus anthracis* MKR-9. A *Bacillus subtilis* was reported with keratinase production and dehairing properties can be an alternative to sodium sulphide (Alexandre *et al.*, 2005). Keratinase opens the straight beginning of enzymatic dehairing devoid of changes in the time of the usual tanning process. The removal of skin hair by using crude

keatenase is a valuable process as it avoids high-pH effluents those occur in sulfide-using process, so it can be used at industrial level at 7.0 pH. The incubation time and 7 pH range is main feature of this enzyme which makes this enzyme an excellent applicant for dehairing, as it helps in industry tanning process.

#### Purification of keratinase

After ammonium sulphate precipitation and dialysis, the total protein, enzymatic activity, specific activity, purification fold and yield (in percentage) was measured as 65.21 mg, 18102 U, 277.59 U/mg, 3.75 fold and 84.19 % respectively. The partially purified keratinase was eluted through Sephadex G-75 column for further purification. A total of 80 eluted fractions were collected. The absorbance of 23<sup>rd</sup>, 24<sup>th</sup> and 25<sup>th</sup> eluted fractions was 2.641, 2.991 and 2.405 respectively at 280 nm. These fractions were mixed and analyzed. The protein content, enzyme activity, specific activity, yield and overall purification was measured as 16.38 mg, 9652 U, 589.25 U/mg, 44.89 % and 7.93 fold respectively



**Fig. 2.** (A) Goat skin treated with crude keratinase (B) electrophoresis of purified keratinase; protein marker-lane 1, crude protein - lane 2, protein after ammonium sulfate precipitation- lane 3, purified keratinase enzyme (lane 4)

**Table 1.** Optimization, characterization and purification scheme of keratinase

At different time interval (1, 2, 3, 4, 5, 6, 7 days)	4.96	7.8	19.1	17.73	16.86	16.1	14.7		
At 3 <sup>rd</sup> day with different concentration of feather (0.1%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3%)	12.67	15.48	23.7	22.43	20.38	18.46	16.08		
At 3 <sup>rd</sup> day with different inoculum size (2%, 4%, 6% and 8%)	16.74	18.86	15.47	10.85					
At 3 <sup>rd</sup> day with 25°C, 30°C, 35°C, 40°C, 50°C and 60°C	10.66	14.8	16.46	18.96	10.4	6.2			
At 3 <sup>rd</sup> day with 5, 6, 7, 8 and 9 pH	10.86	16.96	30.33	18.53	12.2				
At different carbon source (without carbon source, dextrose, maltose, sucrose, galactose and mannitol) under above optimized conditions	29.12	20.46	19.46	4.12	16	12.46			
At different nitrogen source (without nitrogen source, gellatin, peptone, casein, urea, yeast extract, ammonium chloride, beef extract and tryptone) under above optimized conditions	28.46	12.69	21.9	22.98	24.69	19.5	24.99	17.7	21.82
At different amino acid (without aa, alanine, methionine, lucine, asparagine, valine, cysteine and tryptophan) under above optimized conditions	21.6	18.5	33.8	27.96	1.63	31.7	6.6		
Characterization of keratinase at different parameter ((Enzymatic activity U/ml)									
At different temp (25°C, 30°C, 35°C, 40°C, 45°C and 50°C)	11.33	21.09	45.06	56.10	32.26	4.56			
At different pH (5, 6, 7, 8 and 9)	12.13	25.10	62.36	45.20	18.30				
At different carbon source (control, MgCl <sub>2</sub> , CaCl <sub>2</sub> , ZnCl <sub>2</sub> , CuSO <sub>4</sub> , CoCl <sub>2</sub> , HgCl <sub>2</sub> , DMSO, PMFS)	30.83	59.33	45.36	4.13	12.66	17.36	13.6	19.9	1.1
Purification scheme of keratinase									
Purification step	Total protein (mg)	Total enzyme activity	Specific activity (U/mg)	Yield (%)	Purification fold				
Crude enzyme	289.65	Units 21500	74.23	100%	1				
Ammonium sulphate precipitation	65.21	18102	277.59	84.19	3.75				
Sephadex G-75 chromatography	16.38	9652	589.25	44.89	7.93				

as shown in table 1. The purity of keratinase was confirmed by SDS- PAGE with a single band of approximately 34 kDa is shown in fig 2(B).

In characterization, maximum kera-tinase activity ( $56.1 \pm 1.3$  U/ml) and ( $62.36 \pm 1.66$  U/ml) and ( $59.33 \pm 0.8$  U/ml) was observed at 40 °C, and 7 pH as shown in table 1. Most of the metal ions had an inhibitory effect. In case of PMSF the enzymatic activity ( $1.13 \pm 0.73$  U/ml) reduced as compare to control. The reduction in enzyme activity was calculated as 96.47%. The enzyme was inhibited by EDTA, SDS and DMSO, although other protease inhibitors had minor effect. The enzyme activity in the presence of EDTA, SDS and DMSO was calculated as  $24.34 \pm 0.96$  U/ml,  $22.16 \pm 0.95$  U/ml and  $19.93 \pm 0.95$  U/ml respectively. The heavy metal ions such as  $\text{Cu}^{2+}$ ,  $\text{Hg}^{2+}$  and  $\text{Zn}^{2+}$  have inhibitory effects on keratinolytic activity (Thys et al., 2004).

## CONCLUSION

Keratinase is a valuable enzyme for degradation of keratinaceous waste. Keratin degradation by keratinase enzyme isolated from bacteria is an easy, economic and ecofriendly process. Purified enzyme was stable at wide range of pH and temperature and this can be considered as extra benefit. Thus Keartinase can be employed in various industries for dehairing application.

## ACKNOWLEDGEMENT

None

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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