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# Thermostable keratinase from *Bacillus pumilus* KS12: Production, chitin crosslinking and degradation of Sup35NM aggregates

Rinky Rajput, Rani Gupta *

*Department of Microbiology, University of Delhi, South Campus, New Delhi 110021, India*

## HIGHLIGHTS

- A 7-fold increase in keratinase production by *Bacillus pumilus* was achieved by RSM.
- Keratinase was purified by thermal precipitation with purity of 3.2-fold.
- Enzyme was immobilized on chitin by covalent crosslinking.
- Immobilized keratinase showed better catalytic rate and improved pH and thermostability.
- Immobilized keratinase degraded Sup35NM at pH 7.0 and 37 °C.

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## ABSTRACT

Production of thermostable keratinase from *Bacillus pumilus* KS12 was enhanced up to seven fold by statistical methods. The enzyme was partially purified by ultrafiltration followed by thermal precipitation with purity of 3.2-fold and recovery of 89%. Keratinase was immobilized using covalent method by crosslinking 2 mg protein (688 U/mg) onto 1 g chitin activated with 2.5% (v/v) glutaraldehyde for 60 min. Its comparative biochemical studies with that of free keratinase revealed the shift in optimum pH with increased stability towards pH from 9.0 to 10.0 and temperature. Also, it showed statistically significant improved hydrolysis of a number of soluble and insoluble substrates in comparison to free keratinase. Owing to improved catalytic efficiency of immobilized keratinase, its potential for degradation of Sup35NM was evaluated, where 100 μg of enzyme could degrade 60 μg Sup35NM after 60 min at pH 7.0 and 37 °C.



## 1. Introduction

Keratins are hard-to-degrade structural proteins that form structures like feather, hair, horn, hooves, nail, scales, among other epidermal appendages (Onifade et al., 1998). The intensive crosslinkage in keratin hinders their degradation by commonly known proteolytic enzymes like trypsin, pepsin and papain but are efficiently degraded by a selective class of proteases, i.e. keratinases (Papadopoulos, 1986). Keratinases are enzymes which target mostly hydrophobic amino acid-rich, insoluble proteins. Therefore, they are easily demarcated from proteases as they have >50% activity on insoluble keratin when compared with soluble proteins (Gupta and Ramnani, 2006; Brandelli et al., 2010). Beside their applications in various conventional biotechnological sectors of detergent, feed and fertilizer industries (Gupta and Ramnani, 2006; Brandelli, 2008), they have gained importance for degradation of protease resistant proteins (Gupta et al., 2012). Prion protein is one such recalcitrant protein and its decontamination is a challenging area. Several physio-chemical methods are known for prion decontamination (Chen et al., 2005; Wang et al., 2007) but not all address the issue of infectivity of degraded protein. Therefore, enzymatic degradation is specific and can target the infective domain leading to non-infectivity of degraded products. Various microbial keratinases have been documented to have potential to degrade prion protein (Gupta et al., 2012) and thus, added momentum to keratinase research in this sector. In this reference, protein Sup35NM, a translation termination factor derived from *Saccharomyces cerevisiae* (Zhouravleva et al., 1995; Stansfield et al., 1995) is being used in laboratory as a non-pathogenic model prion like protein which has physical and chemical properties almost similar to that of infective mammalian prion $PrP^{SC}$ protein (Paushkin et al., 1996). In our previous report, the present 27 kDa thermostable keratinase from *Bacillus pumilus* KS12 was cloned and produced in heterologous host *Escherichia coli*, and was reported to degrade Sup35NM aggregates (Rajput and Gupta, 2011). However, an extracellular protein yield from recombinant *E. coli* was low as it does

* Corresponding author. Tel.: +91 11 24111933; fax: +91 11 24115270.
*E-mail addresses:* ranigupta15@rediffmail.com, ranigupta15@yahoo.com (R. Gupta).

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not secrete high amount of protein and thus, is difficult to exploit on large scale. In this respect, the wild strain of *B. pumilus* KS12 was explored for optimizing enzyme yield using statistical approaches, followed by standardization of downstream processing using membrane filtration and thermal precipitation. This work also includes immobilization of keratinase using chitin crosslinking and its comparative biochemical characterization with that of free keratinase. Its usage in degradation of Sup35NM has also been studied.

## 2. Methods

### 2.1. Bacterial strain

A potential feather-degrading strain of *B. pumilus* KS12 previously isolated from garden soil of University of Delhi South Campus, New Delhi, India was used for the present study (Rajput et al., 2010).

### 2.2. Keratinase production

Feather peptone medium (FPM) containing 0.5% (w/v) whole chicken feather, 0.5% (w/v) glucose, 0.5% (w/v) peptone, 0.1% (w/v) $KH_2PO_4$, 0.3% (w/v) $K_2HPO_4$, pH 7.0 was used for keratinase production (Ramnani and Gupta, 2004). Fifty micro liters medium was dispensed in 250 mL Erlenmeyer flask and sterilized at 110 °C for 20 min. Each flask was inoculated with 1 mL of 14 h old seed culture ($23 \times 10^8$ cfu/mL) prepared in nutrient broth, and incubated at 37 °C, 200 rpm (New Brunswick Scientific Shaker, Edison NJ, USA) for 24 h. Culture broth was centrifuged at $7441 \times g$ for 10 min (Sigma Laboratory Centrifuge 3K15) and the supernatant was used for keratinase assay.

### 2.3. Source of keratin substrate

Chicken feather available from local poultry plants were used to prepare feather powder according to Rajput et al. (2011). It was used as a keratin substrate to perform keratinase assay.

### 2.4. Keratinase assay and protein estimation

Keratinase assay was performed according to Rajput et al. (2011). The assay was setup with 1 mL of enzyme, 4 mL of glycine–NaOH buffer (50 mM, pH 10.0) and 10 mg of feather powder at 60 °C for 1 h. The reaction was terminated with 4 mL of 5% (w/v) trichloroacetic acid (TCA). The feather and insoluble residues were separated by glass wool and the filtrate was centrifuged at $7441 \times g$ for 10 min. Proteolytic products in the supernatant were monitored by reading the absorbance at 280 nm against appropriate controls. An increase in absorbance of 0.01 at 280 nm was considered as equivalent to one unit of enzyme activity/mL/h under standard assay conditions.

The total protein was estimated by the Bradford method using bovine serum albumin (BSA) as a standard protein (Bradford, 1976).

### 2.5. Cell growth determination

The growth of the bacterium was estimated by dry weight method. Fermentation broth was treated with 0.1% (v/v) triton X-100 in order to disperse biomass adhered to chicken feather and then passed through glass wool to retain undegraded feather as described by Ramnani et al. (2005). Filtrate was pelleted by centrifugation and washed three times with saline and dried at 60 °C until constant weight.

### 2.6. Experimental design for optimization of medium components

Medium optimization was carried out by one-variable-at-a-time and statistical methods. Various physiological and nutritional parameters of production medium were standardized first by one-variable-at-a-time approach. Following this, signal parameters affecting keratinase production were identified by Plackett-Burman (PB) design and interactions between signal parameters were studied by response surface methodology (RSM).

#### 2.6.1. One-variable-at-a-time approach

*2.6.1.1. Selection of physiological parameters.* The effect of pH in the range of 7.0–11.0 using 50 mM phosphate/Tris–HCl (pH 7.0–8.0) and glycine–NaOH/carbonate (pH 9.0–11.0) buffer was studied in FM1. Also, the effects of temperature (30–40 °C) and agitation (150–250 rpm) on keratinase production were investigated.

*2.6.1.2. Selection of nutritional parameters.* Glucose as carbon and peptone as nitrogen source in FM1 medium were replaced with equimolar amount of different carbon source like fructose, galactose, lactose, maltose, mannitol, starch and sucrose, and different organic nitrogen source like beef extract, casein, malt extract, soy flour, soybean meal and urea, and inorganic nitrogen source like ammonium sulfate, ammonium nitrate. Glucose (0.5%, w/v) as carbon and soy flour (0.5%, w/v) as nitrogen source which resulted in higher keratinase activity were maintained as medium components, originating the FM2 medium.

#### 2.6.2. Plackett-Burman (PB) design

After selecting the favorable physiological and nutritional factors for keratinase production by *B. pumilus*, eight variables, namely chicken feather, glucose, soy flour, phosphate ($KH_2PO_4$ and $K_2HPO_4$), calcium chloride, magnesium chloride and incubation time were evaluated for their effect on enzyme production by PB design in FM2 medium (Table 1). The maximum and minimum range of each factor was selected to generate a set of 12 experiments using Design expert® 6.0. (Stat Ease, Inc., Minneapolis, USA) and their effect on keratinase production was determined by calculating *p*-value.

#### 2.6.3. Response surface methodology (RSM)

The optimum concentration and interaction of the signal parameters selected on the basis of *p*-values, were studied by RSM design using Central Composite Design (CCD) model in FM3 medium (modified FM2 medium with 5 mM calcium chloride). Each factor was studied at five different levels ($-\alpha$, $-1$, 0, $+1$, $+\alpha$) as presented in Table 2, where $\alpha$ values were calculated beyond the design space from the equidistance of the extremes. The data at $\alpha$ level was analyzed by point predication of the design. A set of thirty experimental runs with four factors and six repetitions at the center were generated using Design expert® 6.0. (Stat Ease). Biomass production was also considered as one of the response. The model was analyzed using ANOVA, 3D curves, contour and one-factor plots in order to study the interactions among various factors and to determine the optimum concentration of each for maximum keratinase production.

In order to determine the accuracy of the model, a combination of ten random experiments were setup within and beyond the design according to the conditions predicted by the model. Experiments were conducted with varying concentrations of glucose, soy flour, phosphate ($KH_2PO_4$ and $K_2HPO_4$) and incubation time in FM3 medium (Table 4). Finally, FM4 medium supporting maximum keratinase production was originated with following components, 0.5% (w/v) whole chicken feather, 1.25% (w/v) glucose, 2.0% (w/v) soy flour, 0.15% (w/v) $KH_2PO_4$, 0.5% (w/v) $K_2HPO_4$ and 5 mM (w/v) calcium chloride, pH 7.0.