

ARTICLE

# Purification and characterization of a *Bacillus subtilis* keratinase and its prospective application in feed industry

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**ABSTRACT** We have isolated a *Bacillus subtilis* strain (RSE163) from soil and explored for keratinase production. Keratinase was purified using chromatographic methods (Sephadex G-75 and Q Sepharose) resulting in 8.42-fold purification with 3303 U/mg specific activity. The purified enzyme displayed 3 bands in close proximity between 20 to 22 kDa in SDS-PAGE which were apparent to the zone of hydrolysis in gelatin zymogram. Enzyme was stable over a wide pH (7.0-10.0) and temperature (30 °C to 70 °C) range with optimum activity at pH 9.0 and 60 °C. Keratinase activity was stimulated in presence of Mn<sup>2+</sup>, β-mercaptoethanol and surfactants (Triton-X and Tween-80) and inhibited by Fe<sup>3+</sup>, Cd<sup>2+</sup>, K<sup>+</sup>, PMSF (phenyl methane sulfonyl fluoride) and other chelating and reducing agents. The enzyme efficiently hydrolyzed a variety of complex protein substrates (chicken feather, keratin hydrolyzate and casein) and enzyme kinetics parameters were determined using Lineweaver Burk plot (Km = 6.6 mg/ml, Vmax = 5 U/ml/min). Hydrolyzed feather keratin obtained through fermentation with *B. subtilis* RSE163 has been explored for its cytotoxicity using liver cell line (HepG2). No cytotoxicity has been determined up to 0.015% concentration of hydrolyzed product indicating its potential applicability as feed supplement.

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**KEY WORDS**

amino acids  
animal feed  
cytotoxicity  
keratinase

## Introduction

World-wide poultry processing plants produce 3000 tons of feathers weekly as troublesome waste (Peddu et al. 2009). The accretions of feathers in large quantity lead to environmental problems with transmission of neurodegenerative diseases (Sahni et al. 2015). The feather keratin characteristically attributed for the presence of amino acids like glutamic acid, serine, histidine, valine, arginine, tyrosine, methionine, phenylalanine isoleucine, leucine, lysine, cysteine and aspartic acid (Gupta and Singh 2014; Kumar et al. 2011). Conventional methods (physical and chemical) used for the processing of feather waste destruct the quality of amino acids and leads to environmental problem (Tiwarly and Gupta 2012). Degradation of chicken feather using microbial enzymes establishes a safe economic and eco-friendly method for the utilization of feather waste (Gupta et al. 2015). Keratinases are one of them with a knack to hydrolyze insoluble keratin into valuable amino acids more efficiently than other proteases

pepsin, trypsin and papain (Gradisar et al. 2005). Production of keratinase has been reported from various fungal and bacterial sources including many species from the *Bacillus* genus (Ramnani et al. 2005; More et al. 2013). Keratinases also expressed great diversity with respect to their different biochemical and biophysical properties. Most of the microbial keratinases are alkaline or neutral proteases showing optimum pH and temperature ranging 7.5-9.0 and 40-85 °C, respectively (Selvam and Vishnupriya 2012).

Keratinases belong to the subtilisin family of serine protease identified by specific substrates and inhibitors (Selvam and Vishnupriya 2012). Keratinase act as potential catalyst in degradation of keratinaceous waste without affecting the other structural protein collagen (Macedo et al. 2005). Undeniably, keratin hydrolyzates obtained from keratin wastes are an inexpensive and alternative protein sources for animal feed. Feed quality is very crucial for the health of animals. Widespread acquaintance has been built up for the safety and nutritional testing of novel food supplements for animals (Maurici et al. 2007). The toxicity of substances can be evaluated by *in vitro* studies using different cell lines which prevent the direct testing on animal models (Parasuraman 2011).

Our previous study documented a new feather-degrading *Bacillus subtilis* RSE163 strain, which could completely

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degrade feather substrates within 72 h in submerged cultivation (Gupta and Singh 2014). The aim of this study was to purify and characterize the keratinase produced by *B. subtilis* RSE163, and to evaluate the cytotoxicity of keratin hydrolysates obtained after *B. subtilis* RSE163 fermentation on feather substrates.

## Materials and Methods

### **Bacterium strain**

The previously isolated and identified *Bacillus subtilis* RSE163 (NCBI Accession No. JQ887983) was used in this study (Gupta and Singh 2014).

### **Keratinase production and enzyme assay**

Keratinase production and enzyme assay were performed according to the protocol of Gupta and Singh (2014). An increase of the absorbance with 0.01 was considered as 1 Unit of keratinase per ml in 1 hour under the assay conditions (Rajput et al. 2010).

### **Purification of keratinase**

The crude production medium (50 ml) was centrifuged at 6720 g for 15 min to remove cells, debris and other solid particles. Then, ammonium sulfate was slowly added to the crude filtrate up to 80% (w/v) saturation. The solution was left overnight at 4 °C and the protein precipitate was separated by centrifugation at 6720 g for 10 min. The pellet was dissolved in the smallest possible volume of phosphate buffer (0.1 M, pH 7.5), and was dialyzed (cellophane membrane, Sigma) against double distilled water at 4 °C for 48 h. This concentrated sample was loaded onto a Sephadex G-75 gel filtration column (50 mm x 15 mm) equilibrated and eluted with Tris-HCl buffer (pH 7.5) at a flow rate of 1 ml/min. The keratinase active fractions were pooled and assayed for keratinase activity according to Gupta and Singh (2014). This active solution was further purified with a Q Sepharose Fast Flow column (65 mm x 10 mm; Sigma-Aldrich) pre-equilibrated with 50 mM glycine NaOH buffer (pH 10), and then eluted with a linear gradient of 0.6 N NaCl at flow rate of 1 ml/min. The keratinase active fractions were pooled and concentrated by freeze-drying, and stored at -10 °C. The enzyme recovery and fold purification were calculated in term of specific activity. Concentration of protein was determined by Bradford assay. Five hundred microliters of protein sample was added with 4.5 ml of Bradford's reagent, mixed well and absorbance was read at 595 nm. Protein was quantified in comparison with a BSA (Bovine serum albumin) standard curve and was

expressed as mg/ml (Bradford 1976).

### **Molecular weight determination**

SDS PAGE technique was applied for the determination of the molecular weight of purified keratinase, which was performed on 12% resolving gel and 4% stacking gel with the help of Mini-protean II 2-D cell apparatus (Bio-Rad Laboratories, Hercules, CA). Purified protein samples were mixed with sample buffer at a ratio of 1:1, and heated at boiling water bath for 5 min and loaded to polymerized gel with unstained molecular weight marker (Broad Range Protein Marker, Merck-Genei). The electrophoresis unit was run at 120 V (constant voltage) for 60 min to separate the protein sample. The gel was carefully removed and silver stained as per the protocol given by Sorensen et al. (2002) for the visualization of protein band.

### **Zymogram studies**

Zymogram studies were performed using 0.2% (w/v) gelatin as substrate co-polymerized with 12% resolving gel and 4% stacking gel. Purified protein was mixed with sample buffer and loaded to polymerized gel without any heating. After electrophoresis, gel were washed with solution of Tris/HCl buffer (50 mM, pH 7.5) and 2.5% Triton X-100 on orbital shaker for 45 min. The gel was washed with glycine/NaOH buffer (0.5 M, pH 9.0) and kept for incubation at 37 °C for overnight. Next day gel was stained with Coomassie Brilliant Blue Dye R-250 at room temperature for 30 min. Area of clear bands appears against a dark blue stained background where the substrate has been degraded by the enzyme.

### **Characterization of purified keratinase**

#### *Effect of pH and temperature on keratinase activity and stability*

Optimum temperature for the enzyme activity was determined by varying the incubation at 30 to 80 °C at pH 9 (0.5 M glycine/NaOH buffer) for 1 hour. In order to investigate the thermo stability, enzyme was pre-incubated at temperatures 30 to 70 °C for 2 h without the substrate. Samples were taken after every 30 min and assayed for activity under standard conditions (Gupta and Singh 2014).

The optimum pH was determined using the following buffers (50 mM): sodium citrate buffer (pH 3-5), sodium phosphate buffer (pH 6-8) and glycine/NaOH buffer (pH 9-11). For stability studies, enzyme was pre-incubated in different buffers without substrate for 2 hours. Samples were taken after every 30 min and assayed for activity under standard conditions.

**Table 1.** Percentage cytotoxicity of hydrolyzed product after 72 h of treatment using HepG2 cells.

Test item	Concentration	Cytotoxicity (%)
Doxorubicin ( $\mu\text{M}$ )	0.1	51
	1.0	77
	5.0	78
Hydrolyzed product (%)	0.015	2
	0.03	43
	0.06	53
	0.25	70
	1	79
	1.75	79
	2.5	82
	5	83
	10	85
	25	86
	50	87

#### Effect of metal ions and chemical reagents on keratinase activity

The effects of metal ions using different metal salts ( $\text{CaCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{MnSO}_4$ ,  $\text{ZnSO}_4$ ,  $\text{CoCl}_2$ ,  $\text{HgCl}_2$ ,  $\text{CdCl}_2$ ,  $\text{NaCl}$ ,  $\text{KCl}$ ,  $\text{FeCl}_3$ ) and reagents [phenyl methane sulfonyl fluoride (PMSF), ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), idoacetamide, aminocaproic acid, dithiothreitol (DTT) and  $\beta$ -mercaptoethanol] on enzyme activity was studied by assaying the activity at the final concentration of 1 mM and 5 mM in the reaction mixture. The effect of SDS (sodium dodecyl sulphate), Triton-X and Tween-80 was determined by the addition of these compounds at a final concentration of 1% in the reaction mixture.

#### Effect of incubation time on keratin hydrolysis

To study the effect of incubation time on keratin hydrolysis, the enzyme was incubated with 25 mg of chicken feather as substrate for 10 to 70 min under standard conditions (Gupta and Singh 2014).

#### Effect of enzyme concentration on keratin hydrolysis

The effect of enzyme concentration on keratinase activity

was assayed by varying the enzyme volumes in the range of 0.02 to 0.1 ml (60-350 U) under standard conditions (Gupta and Singh 2014).

#### Determination of $V_{max}$ and $K_m$

Kinetic parameters of the enzyme were determined by measuring the enzyme activity at different substrate concentrations (10-80 mg). The  $K_m$  and  $V_{max}$  values were determined by Lineweaver-Burk plot.

#### Substrate specificity

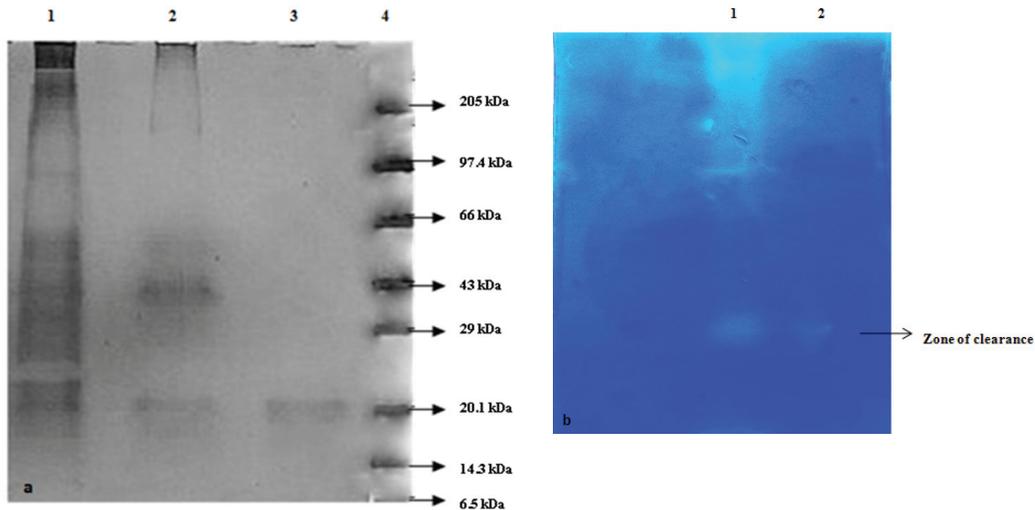
The substrate specificity of purified keratinase was determined using 25 mg of protein substrates (feather, hydrolyzed keratin, keratin azure, casein, collagen, BSA and gelatin) under the standard conditions (Gupta and Singh 2014). Hydrolysis was determined at 280 nm by measuring TCA-soluble peptides released from the substrates.

#### Determination of *in vitro* cytotoxicity of hydrolyzed keratin

The *in vitro* cytotoxic effect of hydrolyzed keratin obtained after complete degradation of chicken feather in cell free supernatant was evaluated using HepG2 cell line at Dabur Research Foundation Ghaziabad (Uttar Pradesh, India). The cells ( $8 \times 10^3$  cells/well) were treated with different concentrations of cell free supernatant ranging from 0.015-50% (v/v). Doxorubicin hydrochloride at concentrations ranging from 0.1-5  $\mu\text{M}$  was used for positive control cells. Untreated cells were considered as negative control. After addition of hydrolyzed product or doxorubicin hydrochloride, cells were incubated in a  $\text{CO}_2$  incubator at 37 °C, 5%  $\text{CO}_2$  for 72 h. After incubation, the cytotoxicity was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. 20  $\mu\text{l}$  of 5 mg/ml of MTT was added to each well and the microtitre plates were incubated at 37 °C for 3 h. The supernatant was aspirated and 150  $\mu\text{l}$  of DMSO (dimethyl sulfoxide) was added to each well to dissolve formazan crystals (Raghavan et al. 2015). The OD was measured at 540 nm using Biotek Reader. The percentage cytotoxicity was calculated.

**Table 2.** Purification steps for *B. subtilis* RSE163 keratinase.

	Activity (U)	Protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude	22750	58	392.24	100	1
Ammonium sulphate precipitation (80%)	19000	33.5	567.16	83.51%	1.44
Sephadex G-75	16800	7.55	2225	73.84%	5.67
Q Sepharose	14700	4.45	3303	64.61%	8.42



**Figure 1.** SDS-PAGE (a) and gelatin-gel zymogram (b) of the purified *B. subtilis* RSE163 keratinase. (a) Lane 1: crude enzyme; Lane 2: partially purified enzyme; Lane 3: purified enzyme; Lane 4: protein marker; (b) Lane 1: crude enzyme; Lane 2: purified enzyme showing zone of clearance.

The percentage cytotoxicity was calculated using the formula as mentioned in equation

$$\% \text{ Cytotoxicity} = (1 - X/C) \times 100$$

Where, X = absorbance of treated cells, C = absorbance of control cells

## Results

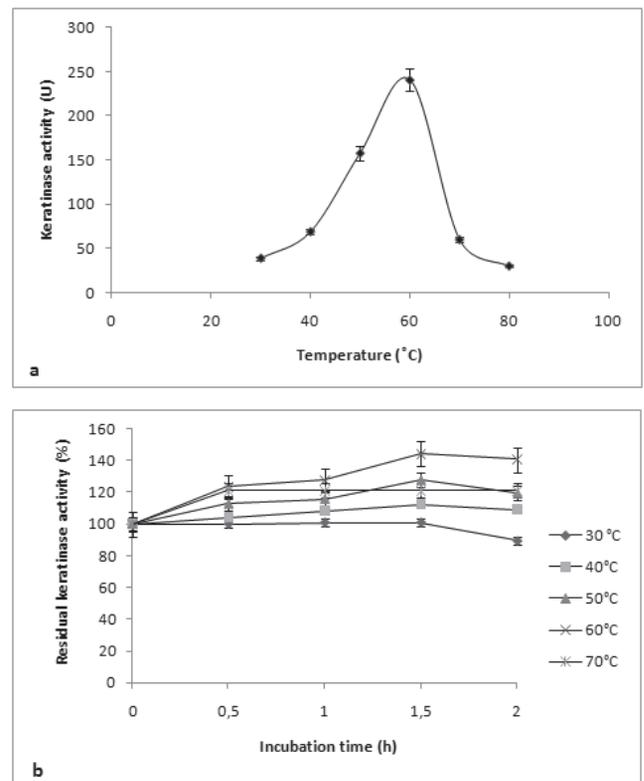
### Production of keratinase and in vitro cytotoxicity of hydrolyzed product

*B. subtilis* RSE163 could completely degrade chicken feather substrates in our previous study, and it showed maximum keratinase production of  $366 \pm 15.79$  U after 72 h (Gupta and Singh 2014). Mechanism of degradation and amino acid content of hydrolyzed product have also been reported which pointed out its possible application in feed industry (Sahni et al 2015; Gupta and Singh 2014). With this context, we carried out tests to evaluate the cytotoxicity of these keratin hydrolyzates. Data in Table 1 revealed that the hydrolyzed product was cytotoxic in 0.015-50% (v/v) concentrations after 72 h treatment. However, hydrolyzed product at 0.015% concentration exhibited negligible cytotoxic effect as compared to positive control.

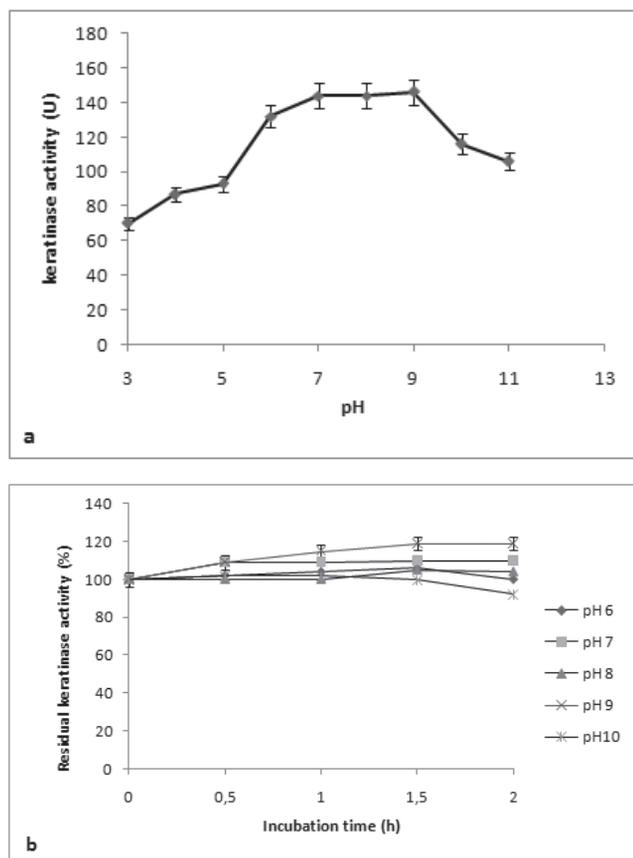
### Purification of the keratinase

Partial purification of keratinase was done by ammonium sulphate precipitation. Concentrated supernatant obtained from above were subjected to Sephadex gel filtration. Active

fractions obtained after gel filtration were pooled and further purified on a Q Sepharose ion exchange column. The overall



**Figure 2.** Effect of temperature on *B. subtilis* RSE163 keratinase activity (a) and stability (b).



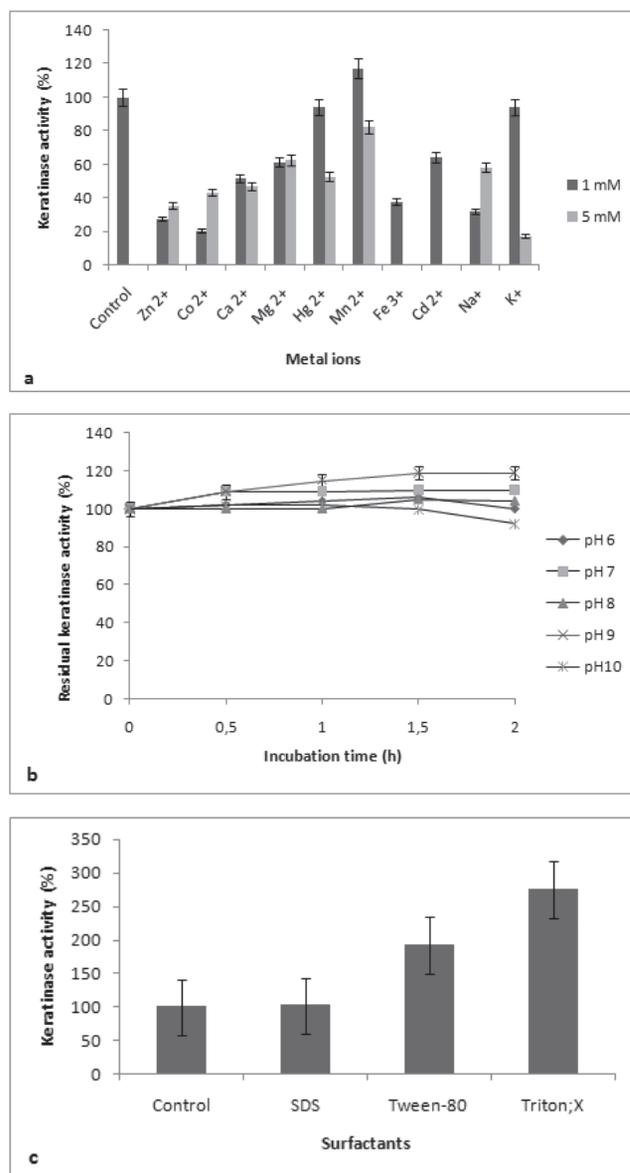
**Figure 3.** Effect of pH on *B. subtilis* RSE163 keratinase activity (a) and stability (b).

purification factor was about 8.42-fold, with 64.61% recovery. The final product had a specific activity of about 3303 U/mg (Table 2). SDS-PAGE analysis showed that the purified sample displayed 3 bands in close proximity between 20 to 22 kDa (Fig. 1a). Zone of hydrolysis observed in the gelatin zymogram gel, indicated keratinase activity of the purified enzyme (Fig. 1b).

### Biochemical characterization of purified keratinase

#### Effect of temperature and pH on enzyme activity and stability

Keratinolytic activity of the purified protein was maximal at 60 °C (Fig. 2a). The enzyme was stable up to 90 min at 30 °C, and lost only 11% of its activity after 120 min. The enzyme was also stable at 40 °C to 60 °C retained its 100% activity up to 90 min and lost only 5-9% of its activity after 120 min. Moreover, incubation at 70 °C for 30 min enhanced the activity with 21% and the enzyme was stable up to 2 h at this

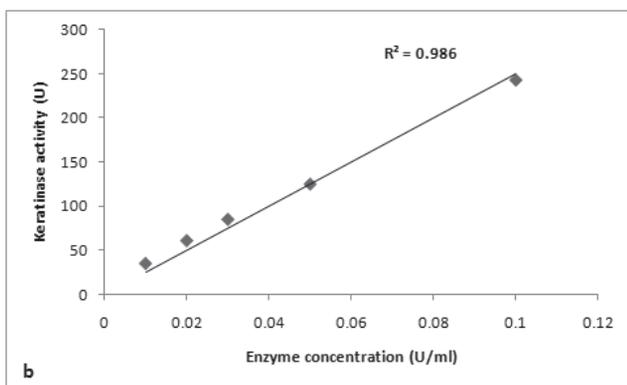
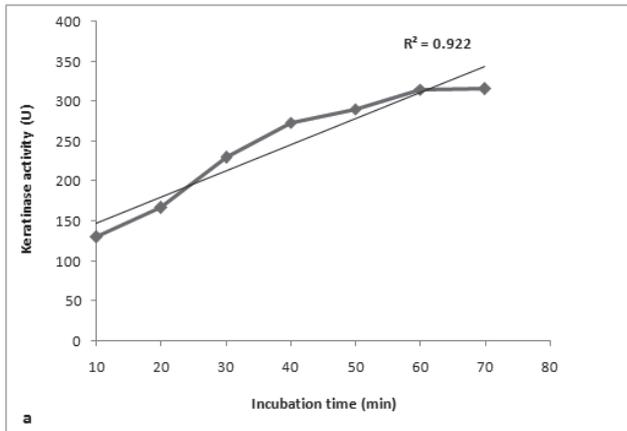


**Figure 4.** Effect of metal ions (a), inhibitors (b), reducing agents and surfactants (c) on *B. subtilis* RSE163 keratinase activity.

temperature (Fig. 2b). Keratinase was completely stable in pH range from pH 7.0-10.0 up to 120 min, and it had maximal activity at pH 9.0 (Fig. 3a and 3b).

#### Effects of metal ions and reagents on enzyme activity

The purified *B. subtilis* RSE163 keratinolytic protein was also tested in the presence of various metal ions, potential inhibitor reagents and surfactants. After addition of Mn<sup>2+</sup>, a 1.7-fold increment in the enzyme activity could be observed, whereas keratinase activity was inhibited by Na<sup>+</sup>,

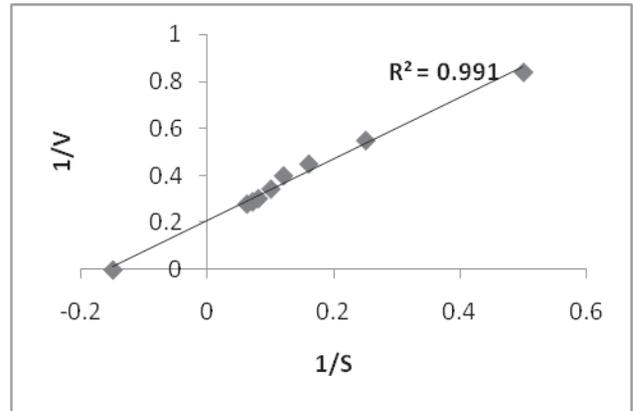


**Figure 5.** Effect of incubation time (a) and enzyme concentration (b) on keratin hydrolysis.

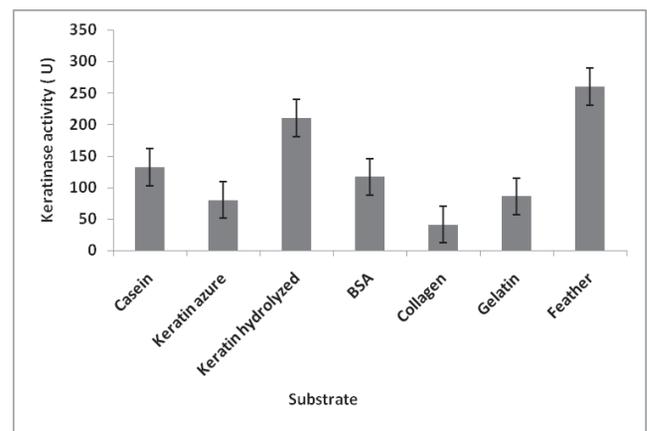
$\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{K}^{+}$ ,  $\text{Co}^{2+}$ ,  $\text{Hg}^{2+}$  and  $\text{Zn}^{2+}$  metal ions (Fig. 4a). Keratinase activity was totally inhibited by PMSF, partially inhibited by idoacetamide and the chelating agents EDTA and EGTA (Fig. 4b). The enzyme retained 76% of its activity in presence of aminocaproic acid, while 1 mM  $\beta$ -mercaptoethanol stimulated its activity up to 160%. DTT showed partial inhibition at 1 mM concentration and almost complete inhibition at 5 mM. Enzyme activity exhibited about two- or three-fold increment in the presence of Tween-80 or Triton X-100 surfactants, respectively, while SDS showed no effect on keratinase activity (Fig. 4c).

#### Kinetic parameters of purified keratinase

The rate of keratinase reaction reached its maximum in 60 min, and thereafter it remained constant (Fig. 5a); in addition, reaction velocity also increases with the increase of enzyme concentration as shown on Figure 5b.  $R^2$  value of both graphs (0.922 and 0.986) represents the fitness of data. The  $K_m$  and  $V_{max}$  of purified keratinase calculated from Lineweaver-Burk plot were 6.6 mg/ml and 5 U/ml/min, respectively (Fig. 6).



**Figure 6.** *B. subtilis* RSE163 keratinase: enzyme kinetics (Lineweaver-Burk plot).



**Figure 7.** Specificity of *B. subtilis* RSE163 keratinase in the presence of different substrates.

#### Substrate specificity

Keratinase represented broad substrate specificity since it could hydrolyze feather, keratin hydrolyzed, keratine azure, casein, collagen, BSA and gelatin substrates. The enzyme showed maximum affinity towards feather followed by keratin hydrolyzed, casein, BSA, keratin azure and gelatin (Fig. 7).

## Discussion

Keratinolytic microorganisms and their enzymes have significant scientific interest due to their capability to hydrolyze keratin into valuable peptides and amino acids (Saber et al. 2009; Gopinath et al. 2014). *B. subtilis* RSE163 was able to

grow and produce keratinase in presence of chicken feather as substrate, which stimulated the enzyme production to 366 U/ml after 3 days (Gupta and Singh 2014). Keratinase cleaved the hydrogen bonds of  $\beta$ -pleated sheets and released different amino acids from keratin. Here, this hydrolyzed product was further evaluated for its cytotoxicity using HepG2 cell line. Results showed negligible cytotoxic effect up to 0.015% of hydrolyzate concentration. These findings indicated that the hydrolyzed product in low concentrations may be suitable for animal nutrition as protein supplement.

The *B. subtilis* RSE163 extracellular keratinase has been purified with purity of 8.42-fold and specific activity of 3303 U/mg. Cai et al. (2008) also purified *B. subtilis* keratinase that showed specific activity of 63.3 U/mg with 12.7 purification folds. Purified keratinase had molecular weight of approximately 20 to 22 kDa which were apparent to the zone of hydrolysis in gelatin zymogram. Keratinases with similar molecular weight have been purified from *B. subtilis* KS 1 (25.4 kDa, Suh and Lee 2001) and *Bacillus pseudofirmus* (24 kDa, Gessesse et al. 2003).

The purified *B. subtilis* RSE163 keratinase was stable over a broad range of pH (7.0-10.0) and temperature (30 to 70 °C), and it has maximal keratinolytic activity at pH 9.0 and 60 °C. Thermostable keratinase were also reported from *Bacillus pumilus* KS12 (Rajput and Gupta 2013), *Meiothermus* sp. I40 (Kuo et al. 2012) and *Bacillus halodurans* PPKS-2 (Prakash et al. 2010), where the enzymes generally retained their activity at the temperature range of 60 to 70 °C.

Stimulation of keratinase in presence of metal ion like  $Mn^{2+}$  may due to the formation of salt or an ion bridge which maintain the confirmation of the enzyme-substrate complex (Balaji et al. 2008). Inhibition of keratinase activity by metal ions may be attributed to the formation of bridges between metal monohydroxide ( $MOH^+$ ) and catalytic ions at the active site (Sivakumar et al. 2013). The sensitivity of *B. subtilis* RSE163 keratinase towards PMSF suggests that the enzyme may be a serine protease. The PMSF can bind covalently to the active site (serine residue) of the enzyme and inactivates it (Suntornsuk et al. 2005). However, reducing agents able to break the disulphide bonds in the substrate keratin releasing different hydrolytic sites for the keratinolytic attack (Brandelli 2005; Cai et al. 2008; Gupta and Singh 2014).

The estimated  $K_m$  and  $V_{max}$  values for feather keratin were 6.6 mg/ml and 5 U/ml/min, respectively. Purified keratinase from *Bacillus thuringiensis* presented similar affinity for keratin ( $K_m$ : 5.97 mg/ml, Sivakumar et al. 2012). The  $K_m$  and  $V_{max}$  of purified keratinase of *B. pumilus* KS12 towards keratin were 0.25 mg/ml and 0.75  $\mu$ g/min/ml (Rajput et al. 2010), respectively; its  $K_m$  value was considerably lower than calculated for *B. subtilis* RSE163 keratinase.

The purified keratinase effectively hydrolyzed variety of complex protein substrates such as chicken feather, keratin hydrolyzed, keratine azure, casein, collagen, BSA and gelatin.

The enzyme showed maximum hydrolysis on feather wastes, which suggests a potential application in chicken feather-processing technologies. Rajput et al. (2010) also reported the maximum keratinolytic activity towards feather substrate.

It can be concluded that *B. subtilis* RSE163 is a source of a keratinolytic protease which may be a serine protease. The enzyme showed efficient hydrolysis towards feather keratin, and it has stability at broad pH and short-term tolerance to temperatures up to 70 °C. Cytotoxicity determination of hydrolyzed feather supposes the potential application of *B. subtilis* RSE163 for conversion of feather into digestible animal feed.

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