Molecular and biochemical characterization of a thermostable keratinase from *Bacillus altitudinis* RBDV1

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Received: 4 August 2017 / Accepted: 20 January 2018 / Published online: 2 February 2018
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Abstract

A thermostable keratinase designated as KBALT was purified from *Bacillus altitudinis* RBDV1 from a poultry farm in Gujarat, India. The molecular weight of the native KBALT (nKBALT) purified using ammonium sulfate and ion exchange and gel permeation chromatography with a 40% yield and 80-fold purification was estimated to be ~ 43 kDa. The gene for KBALT was successfully cloned, sequenced and expressed in *Escherichia coli*. Recombinant KBALT (rKBALT) when purified using a single step Ni-NTA His affinity chromatography achieved a yield of 38.20% and a 76.4-fold purification. Comparison of the deduced amino acid sequence of rKBALT with known proteases of *Bacillus* species and inhibitory effect of PMSF suggest that rKBALT was a subtilisin-like serine protease. Both native and rKBALT exhibited higher activity at 85 °C and pH 8.0 in the presence of Mg²⁺, Mn²⁺, Zn²⁺, Ba²⁺ and Fe³⁺ metal ions. Interestingly, 70% of their activity was retained at temperatures ranging from 35 to > 95 °C. The keratinolytic activity of both nKBALT and rKBALT was enhanced in the presence of reducing agents. They exhibited broad substrate specificity towards various protein substrates. KBALT was determined for its kinetic properties by calculating its K_m (0.61 mg/ml) and V_{max} (1673 U/mg/min) values. These results suggest KBALT as a robust and promising contender for enzymatic processing of keratinous wastes in waste processing plants.

Keywords *Bacillus altitudinis* · Keratinase · Protease · Heterologous expression

Introduction

Keratinases are proteases that are capable of degrading extremely rigid and insoluble keratinous substrates (Lin et al. 1992) into valuable products such as rare amino acids, feedstuffs, fertilizers and compounds of medical importance (Radha and Gunasekaran 2009). So far, a large number of keratinases have been isolated from fungi, bacteria, and actinomycetes (Gopinath et al. 2015). Keratinases from members of the genera *Microsporum* and *Trichophyton* (dermatophytes) (Kumar et al. 2016; Lange et al. 2016; Gradisar et al. 2005) and *Paecilomyces marquandii* and *Doratomyces microspores* which are non-dermatophytes have been studied

in great detail (Gradisar et al. 2005). Another limitations in application of keratinases is in large scale production, which is usually achieved by cloning the keratinase gene in heterologous hosts (Onifade 1998; Anitha and Palanivelu 2012; Selvam and Vishnupriya 2012). In this respect, cloning and expression of several keratinases has been carried out inside potential hosts but only a few keratinases have been successfully expressed in heterologous hosts such as *Bacillus* spp. and *Pichia strains* (Felix 2002; Lin et al. 1997; Sharma and Gupta 2010). The versatility of *Escherichia coli* has made it to the list of the most preferable hosts in expression studies but, it has hardly achieved any functional expression in case of proteases (Zhang and Li 2013). Commercially, these proteases account for sixty percent of the total enzyme market sale. Therefore, identifying new microbial sources of Keratinase (EC 3.4.99) holds great promise.

In this report, we describe a keratinase from *Bacillus altitudinis* strain RBDV1 which was overexpressed in *E. coli*, purified and characterized.

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Materials and methods

Identification of microorganism

The keratinolytic bacterial culture was provided by Protein Engineering Laboratory, P. D. Patel Institute of Applied Sciences, Charotar University of Science and Technology, Changa, Anand, India. The culture was maintained at 4 °C on Brain Heart Infusion agar medium. The pure culture of the bacterium was studied morphologically. Cells were gram stained and observed microscopically. The molecular profile of bacterium was carried out by 16S rRNA isolation and sequencing. The 16S r-RNA sequence was subjected for Nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/>) to identify the strain. Sequences were aligned to the representative *Bacillus* 16S r-RNA and neighbor joining tree was constructed using Molecular Evolution Genetic Analysis (MEGA, version 6.0). This sequence was deposited in the NCBI Gene Bankit nucleotide Sequence databases as *B. altitudinis* RBDV1, under accession number KF982823.1

Strains, vectors and media

Escherichia coli DH5 α cells and *E. coli* BL21 cells were grown aerobically on Luria–Bertani (LB) medium at 37 °C for recombinant plasmid amplification and gene expression, respectively. The pCold-TF plasmid (TaKaRa, Japan) was used for gene cloning. The restriction enzymes were purchased from New England Biology Labs (USA). The emerald master mix for PCR was purchased from Takara. Plasmids were extracted from *E. coli* using Qiagen's plasmid isolation kit (Omega, USA). All other reagents were of analytical grade.

Cloning and expression of KBALT in *Escherichia coli*

Bacillus altitudinis RBDV1 was grown on brain heart infusion agar at 37 °C overnight. DNA was isolated using lysozyme and proteinase K. For the expression of KBALT from strain *B. altitudinis* RBDV1, 1130 bp of KBALT gene was amplified using manually designed primers of ketker FP 5'ACTAGATAT KpnI ATGAGGAAAAAGAGTTTT TGGCTT 3' and ketker RP 5'AAGCTTCGAXBa ITTG AGCGGCAGCTTCGACATT 3'. The excised product was cloned into the pCold-TF vector using standard procedures and sequenced by SciGenom. This sequence was deposited in the NCBI Gene Bankit nucleotide Sequence databases, under accession number KX953193. The identity of the sequence was surveyed at the GenBank (National Centre

for Biotechnology Information, NIH, USA) using BLAST and FASTA programs (Altschul et al. 1997).

The nucleotide sequence was analyzed using the Open Reading frame Finder (ORF) tool (<https://www.ncbi.nlm.nih.gov/orffinder/>). The signal peptide was predicted by the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>). DNA and protein sequence alignments were carried out using the blastn and blastp programs (<http://www.ncbi.nlm.nih.gov/BLAST/>), respectively. Multiple sequence alignments were carried out with Clustal W. Various physico-chemical properties were computed with the help of ExPASy ProtParam tool (<http://web.expasy.org/protparam/>).

The *E. coli* host strain BL21 was transformed with the sequence confirmed plasmid for expression. The transformant was inoculated in the medium containing 100 μ g/ml of ampicillin, and cultured at 55 °C with shaking conditions. At OD₆₀₀ = 0.4–0.5, the culture solution was refrigerated at 15 °C and was left to stand for 30 min. IPTG at a final concentration of 0.1 mM was added and the culture incubated at 15 °C for 24 h with shaking. The cell pellets were suspended in 5 ml 50 mM tris buffer (pH 8.0) and homogenized by sonication, and then centrifuged at 16,000g for 10 min at 4 °C. The supernatant containing the crude keratinase was purified using 1 ml Ni–NTA His-Tag affinity chromatography according to the manufacturers' instructions. Aliquots of the purified rKBALT were visualized by 12% SDS-PAGE gels and then stored at – 70 °C. Protein was quantified using Bradford reagent against BSA as a standard.

Level of Protein expression and purification were examined by enzyme activity. It was estimated using modified Sivakumar et al. (2012a, b) method. Initially 5 mg of keratin azure was added to 1.5 ml centrifuge tube along with 50 mM phosphate buffer (pH 8.0) at 85 °C for 1 h. A 0.2 ml aliquot of supernatant of enzyme was added to keratin azure, mixed and incubated for 15 min. The reaction was terminated by adding 0.5 ml 10% TCA. The reaction mixture was filtered and analyzed for the activity. The absorbance of the filtrate was measured at 595 nm. The control was prepared by adding the TCA before the addition of the enzyme solution. The unit activity was defined as 0.01 unit increase in the absorbance at 595 nm as compared to the control after 15 min of incubation.

Enzyme characteristics

The molecular weight of nKBALT was estimated by Sephadex G-75 column chromatography. To examine the purity and determine the subunit molecular weight, discontinuous SDS-PAGE was performed and visualized using Coomassie blue R-250.

Influence of pH and temperature on keratinase activity

The keratinase activities were determined at temperatures ranging from 25 to 95 °C using 5 mg keratin azure which was suspended in 0.80 ml of buffer. 0.20 ml of purified enzyme (2.5 g of protein) was added at thermal equilibrium. At each temperature the enzymes were incubated for 15 min in same buffer. The amount of product released was estimated by keratinase enzyme assay.

To study the effect of pH on the activities of both nKBALT and rKBALT, assays were performed at pH values ranging from 4.0 to 12.0. 5 mg of keratin azure was added to 0.80 ml of a buffer containing sodium phosphate (pH 6.0–6.5), tris base (7–8.5), sodium carbonate (pH 9.0–10) and glycine–NaOH (pH 10.5–12.0).

Effect of inhibitors, organic solvents, detergents, metal ions and reducing agents

Both nKBALT and rKBALT were preincubated at 55 °C for 10 min with the following inhibitors and chemicals; each 5 mM concentration of ethylenediaminetetracetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), β -mercaptoethanol (β -ME) and various metal ions at 1% (v/v) concentration of dimethyl sulfoxide (DMSO) and isopropanol and sodium dodecyl sulfate (SDS) 0.5% (w/v). The keratinase activity was measured in the absence of additives, the residual activity of all the samples were performed in triplicates.

Catalytic efficiency and substrate specificities for various proteins

The analysis of catalytic efficiency of both nKBALT and rKBALT was carried out at 85 °C using a range of 5 to 30 mg of keratin azure. Each concentration was treated with 10 μ g enzyme in 50 mM Tris–HCl, pH 8.0. The reaction mixtures were analyzed for keratin azure according to methods described in above section. K_m and V_{max} values were calculated from Line-weaver Burk plot. Both nKBALT and rKBALT were analyzed for specificities towards various protein substrates. Various proteinaceous substrates including

casein, gelatin, BSA, human hair, nail and chicken feather were treated with KBALT under conditions optimized for a reaction time of 15 min.

Data were determined from a Lineweaver–Burk plot using the nonlinear regression computer program Graf pad prism 6.0. All assays were performed in triplicate.

Results and discussion

Cloning and sequence analysis of keratinase

The sequence showed 96% identity to the putative alkaline serine protease from *Bacillus* strain (gil748704686|WP_039962807.1). The protein sequence showed 63.9% identity to the characterized subtilisin E protein, encoding aprE gene of *Bacillus subtilis* strain168 (UniProt ID P04189). Comparison of the deduced amino acid sequence of rKBALT with known proteases of other *Bacillus* species showed that rKBALT is a subtilisin-like serine proteases belonging to the Protease S8 family (Table 1). The complete open reading frame (ORF) consisted of 1130 bp with 49.7% G + C content, starting with ATG and terminating with a TAA codon, and encoded a 374-residue polypeptide with calculated mass of 39,370.74 Da. SignalP analysis revealed the existence of an N-terminal signal peptide at amino acid residues 1–31 (Fig. 1).

Expression and purification of recombinant enzyme

After 20 h of incubation, the keratinolytic activity of nKBALT was recorded at 169 U/ml with 6.14 U/mg of specific activity. As presented in Table 2, the purification steps resulted in 40% yield of 80-fold purified nKBALT with specific activity of 2080 U/mg.

In case of rKBALT, a one step of affinity chromatographic separation yielded 76.41-fold purification with yield of 38.20% having 1986 U/mg of specific activity. Relatively better activity of keratinase was obtained than earlier reported from *Bacillus licheniformis* MKU3 keratinase expressed in *P. pastoris* (pPZK3) which produced Maximum keratinase activity of 135 U/ml with 5.44 U/mg of specific activity after 96 h of growth (Radha and Gunasekaran 2009).

Table 1 Amino acid contents of rKBALT, P04189, and P29142

AA	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
rKBALT	12.4	3	6.9	3.3	0.8	4.4	1.9	8.8	2.5	4.9	6.3	6.9	1.9	1.6	4.7	11	5.2	0.8	3.8	8.8
P04189	12.9	1.3	4.7	3.4	0	3.7	3.1	9.7	2.1	5.5	5.8	6.6	2.4	2.1	3.7	13.1	6.3	1.0	4.2	8.4
P29142	12.3	1.3	4.7	3.4	0	3.7	3.1	9.7	2.1	5.5	5.8	6.6	2.4	2.1	3.7	13.9	5.8	1.0	4.2	8.7

Calculations were performed using all of the open reading frames described in the genomic sequences present in GenBank and UniProt

Fig. 1 Sequence alignment of rKBALT and P04189. Arrow marks indicate the active site residues. Letters in white represent the identical positions found in the two sequences. The numbers represent the positions of the amino acid residues present in the sequence. *Bacillus altitudinis*; P04189, keratinase from *Bacillus subtilis*

SDS–PAGE analysis revealed the nKBALT and rKBALT as MONOMER with molecular masses of ~ 43 and ~ 51.0 kDa, respectively (Fig. 2). This difference in the molecular weight between rKBALT from nKBALT could have been due to the process of glycosylation and the presence of N-terminal 6xHis-tag on the recombinant protein. Similarly, keratinases of 45 and 55 kDa size encoding *Ker A* from *Bacillus licheniformis* was reported by Hu et al. (2013). Baweja and Singh (2017) reported ~ 53.0 kDa alkaline protease from *Bacillus pumilus* MP27. Likewise, keratinase of *B. pumilus* KS12 of 45 kDa was reported by Rajput et al. (2010), whereas, alkaline protease of 33 kDa was found in *Cunninghamella echinulata* (More et al. 2013).

Physiochemical characteristics of KBALT

The pH profile of rKBALT was almost similar to nKBALT (Fig. 3). Both enzymes showed optimal activity at pH 8.0

and retained 84% activity at pH 9.0. pH values below 6 and above 11 showed < 15 of its original activity. Both rKBALT and nKBALT showed stability at pH range of 7–10 with > 70% activity. It was < 40% active for pH < 6 and > 11 (Fig. 4). Subtilisins need deprotonated histidine, therefore, they exhibit pH optima at values ranging from 7.0 to 10.0 (Gödde et al. 2005). Keratinase of several bacillus and fungal species show similar pH profile (Bressollier et al. 1999; Saibabu et al. 2013; Sivakumar et al. 2012a, b; Gradisar et al. 2005).

Both rKBALT and nKBALT exhibited highest activity at 85 °C. rKBALT showed 79% relative activity at 65 °C, 83% at 75 °C and 89% at 95 °C. The activity got decreased to 12.49 and 29.17% at incubation temperatures 35 and 45 °C, respectively. Whereas, nKBALT exhibited 82% at 65 °C, 89% at 75 °C and 96% at 95 °C (Fig. 5). Thermostability of nKBALT was found out to be slightly better than rKBALT (Fig. 6). Guo and Ma (2008) and Sareen et al. (2005) also show the similar pattern where keratinase of parent micro-organism has exhibited better thermostability. In our work, thermal inactivation of rKBALT followed half-lives of 96, 44, 16, 6, and 3 h at 25, 55, 75, 85, and 100 °C, respectively. The enzyme from *Streptomyces albidoflavus* had a half-life of 72 min at 60 °C (Bressollier et al. 1999). Whereas *Fervidobacterium islandicum* AW-1 and *Chrysosporium keratinophilum* showed maximum activity at 100 and 90 °C with a half-life of 90 and 30 min (Gupta and Ramnani 2006).

Metal ions and inhibitors showed similar effects on nKBALT and rKBALT (Table 3). Ca^{2+} , Zn^{2+} , Ba^{2+} , Fe^{3+} , Mn^{2+} and Mg^{2+} enhanced enzyme activity (Guo and Ma 2008; Kumar et al. 2011), while Pb^{3+} , Ag^{2+} and Hg^{2+} repressed the activity. Similar effects were reported by Radha and Gunasekaran (2007) where heavy metals had inhibited the activity of enzyme. It is a well known fact that PMSF inhibits serine proteases (Pushpam et al. 2011; Nayaka 2013). In present study, activity of enzyme was completely inhibited by PMSF indicating that it is a serine protease. DMSO and chelating agent EDTA had no effect on keratinase activity. Similar kind of stability was reported by Peña-montes et al. (2008) in presence of EDTA. However, many studies have also shown the slight inhibitory effect of EDTA on activity of keratinases, which implies the need for

Table 2 Purification of nKBALT and rKBALT from *B. altitudinis* RBDV1

Purification step	Protein (mg)	Enzyme (units)	Specific activity (U/mg)	Fold purification	% yield
Culture filtrate	30	780	26	1	100
Ammonium sulfate fraction	10.58	495	46	1.79	63.46
IEC	5	396	79.2	3.04	50.76
GPC	0.15	312	2080	80	40
Affinity chromatography (rKBALT)	0.15	278	1986	76.41	38.20

Fig. 2 SDS-PAGE 12% showing rKBALT protein purified by affinity chromatography. Lane 1—rKBALT (~ 51 KDa), lane 2—Prestained protein ladder, lane 3—DE3 lysate and lane 4—whole cell expression (~ 51 KDa)

Fig. 3 Effect of pH on keratinase activity. The activity assay was performed at 55 °C in buffers with pH ranging from 4.0 to 12.0

metal ion to act on substrate (Gupta et al. 2015). Reducing agents such as SDS, DTT and b-ME enhanced keratinolytic activity. However, Bressollier et al. (1999) reported no effect of above mentioned agents on Serine Proteinase obtained from *Streptomyces albidoflavus*.

Substrate specificity and kinetic parameters

Both nKBALT and rKBALT exhibited substrate preference towards chicken feather, followed by keratin azure,

Fig. 4 Effect of pH on stability of keratinase activity. After incubating purified rKBALT at 55 °C for up to 250 min in buffers ranging from 4.0 to 12.0, activity was measured in 50 mM Tris–Cl buffer (pH 8.0) at 85 °C

Fig. 5 Effect of temperature on keratinase activity. The activity measured in 50 mM Tris–Cl buffer (pH 8.0)

human hair and human nail. They showed comparatively less substrate preference towards BSA and casein. Both nKBALT and rKBALT did not show collagenase activity. Gupta et al. (2015) reported analogous specificity of keratinase from *B. subtilis* RSE163. These results vary distinctly from substrate specificity of keratinase from *Aspergillus oryzae* (Farag and Hassan 2004) and *B. pumilus* FH9 (Abdel-Naby 2017) which showed comparatively less affinity towards β -keratinous substrates. KBALT, therefore, has the potential to be developed as a prospective catalyst for hydrolysis of both soluble and insoluble, rigid proteinaceous substrates. Its highest affinity towards chicken feathers can be beneficial in recycling the feathers in poultry processing plants.

The K_m and V_{max} values of KBALT calculated from Lineweaver–Burk plot were 0.61 mg/ml and 1673 U/mg/min, respectively. These values suggest that KBALT is more efficient than keratinase of *Bacillus subtilis* having K_m and V_{max} 6.6 mg/ml and 5 U/ml/min, respectively (Gupta et al. 2015). Values of K_m and V_{max} of Purified keratinase from *Bacillus thuringiensis* TS2 were 5.97 mg/ml and 265.3 μ mol/min/mg, respectively (Sivakumar et al. 2012a, b).

Fig. 6 Thermostability of purified nKBALT and rKBALT. The enzyme was pre incubated at 55, 65, 75 and 85 °C in 50 mM Tris–Cl buffer (pH 8.0) and aliquots were removed at specific time interval for the measurement of residual activity at 85 °C. Each value represents the mean of triplicates with standard deviation

Table 3 Effect of inhibitors and metal effectors on enzyme activity of nKBALT and rKBALT

Reagents	Residual activity (%)	
	nKBALT	rKBALT
Control	97 ± 2.3	98 ± 1.3
Ca ²⁺	127 ± 0.5	125.9 ± 1.8
Zn ²⁺	129.4 ± 0.4	125.3 ± 0.2
Ba ²⁺	115.4 ± 0.4	113.6 ± 0.4
Fe ³⁺	107.1 ± 0.3	108.2 ± 0.1
Mn ²⁺	104.5 ± 2.7	101.9 ± 0.9
Mg ²⁺	134.7 ± 0.6	132.2 ± 4.5
Pb ³⁺	20.0 ± 0.3	14.0 ± 1.4
Ag ²⁺	18.7 ± 0.7	21.5 ± 0.5
Hg ²⁺	2.7 ± 0.1	3.5 ± 0.1
PMSF	0.0 ± 0.0	0.0 ± 0.0
DMSO	101.4 ± 0.4	103.9 ± 1.3
EDTA	102.3 ± 1.4	102 ± 0.3
SDS	128.2 ± 0.3	134.8 ± 0.6
DTT	98.7 ± 0.6	96.8 ± 2
β-ME	102.5 ± 0.8	100.8 ± 0.7

Conclusion

The nKBALT enzyme obtained from *B. altitudinis* RBDV1 is a potential biocatalyst. The rKBALT also showed enzyme properties identical to the nKBALT. Thermostability of both enzymes could allow the degradation of feedstock even at high temperatures. Their highest affinity towards chicken feathers could be beneficial in recycling the recalcitrant feather waste. The broad substrate specificity along with the absence of collagenase activity indicate towards their possible application in detergent as well as leather industries.

Acknowledgements Authors are thankful to P.G. Department of Bio-sciences, Sardar Patel University for providing facilities to conduct research.

Compliance with ethical standards

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

References

- Altschul S, Madden TL (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25(17):3389–3402. <https://doi.org/10.1093/nar/25.17.3389>
- Anitha TS, Palanivelu P (2012) Research article production and characterization of keratinolytic protease(s) from the fungus, *Aspergillus parasiticus*. *Fungal Genom Biol* 2(2):87–93
- Bressollier P, Letourneau F et al (1999) Purification and characterization of a keratinolytic serine proteinase from *Streptomyces albidoflavus*. *Appl Environ Microbiol* 65(6):2570–2576
- Farag AM, Hassan MA (2004) Purification, characterization and immobilization of a keratinase from *Aspergillus oryzae*. *Enzyme Microb Technol* 34:85–93
- Felix CR (2002) Heterologous production of *Aspergillus fumigatus* keratinase in *Pichia pastoris*. *World J Microbiol Biotechnol* 18:563–568
- Gödde C et al (2005) Cloning and expression of islandisin, a new thermostable subtilisin from *Fervidobacterium islandicum*, in *Escherichia coli*. *Appl Environ Microbiol* 71(7):3951–3958. <https://doi.org/10.1128/AEM.71.7.3951-3958.2005>
- Gopinath SCB et al (2015) Biotechnological aspects and perspective of microbial keratinase production. *Biomed Res Int* 2015(2015):140726. <https://doi.org/10.1155/2015/140726>
- Gradisar H et al (2005) Similarities and specificities of fungal keratinolytic proteases: comparison of keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* to some known proteases. *Appl Environ Microbiol* 71(7):3420–3426
- Guo J, Ma Y (2008) High-level expression, purification and characterization of recombinant *Aspergillus oryzae* alkaline protease in *Pichia pastoris*. *Protein Expr Purif* 58:301–308
- Gupta MR, Ramnani P (2006) Microbial keratinases and their prospective applications: an overview. *Appl Microbiol Biotechnol* 70:21–33
- Gupta S, Nigam A, Singh R (2015) Purification and characterization of a *Bacillus subtilis* keratinase and its prospective application in feed industry. *Acta Biol Szeged* 59(2):197–204

- Hu H et al (2013) Expression of a keratinase (kerA) gene from *Bacillus licheniformis* in *Escherichia coli* and characterization of the recombinant enzymes. *Biotechnol Lett* 35(2):239–244
- Abdel-Naby MA (2017) Structural characterization, catalytic, kinetic and thermodynamic properties of Keratinase from *Bacillus pumilus* FH9. *Int J Biol Macromol* 105(1):973–980. <https://doi.org/10.1016/j.ijbiomac.2017.07.118>
- Baweja M, Singh PK (2017) Cost effective characterization process and molecular dynamic simulation of detergent compatible alkaline protease from *Bacillus pumilus* strain MP27. *Process Biochem* 58:199–203
- Kumar EV et al (2011) A novel serine alkaline protease from *Bacillus altitudinis* GVC11 and its application as a dehairing agent. *Bio-process Biosyst Eng* 34(4):403–409
- Kumar V et al (2016) Recent developments in systems biology and metabolic engineering of plant–microbe interactions. *Front Plant Sci* 7:1–12
- Lange L, Huang Y, Busk PK (2016) Microbial decomposition of keratin in nature—a new hypothesis of industrial relevance. *Appl Microbiol Biotechnol* 100:2083–2096
- Lin X et al (1992) Purification and characterization of a keratinase from a feather-degrading *Bacillus licheniformis* strain *Bacillus licheniformis* strain. *Appl Environ Microbiol* 58(10):3271–3275.
- Lin X et al (1997) Expression of the *Bacillus licheniformis* PWD-1 keratinase gene in *B. subtilis*. *J Ind Biotechnol* 19(2):134–138
- More SS et al (2013) Purification and properties of a novel fungal alkaline keratinase from *Cunninghamella echinulata*. *Turk J Biochem* 38(1):68–74
- Nayaka S (2013) Purification and characterization of keratinase from hair-degrading *Streptomyces albus*. *Int J Bioassays* 2(3):599–604
- Onifade AA (1998) A review: potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. *Bioresour Technol* 66:1–11
- Peña-montes C et al (2008) Purification and biochemical characterization of a broad substrate specificity thermostable alkaline protease from *Aspergillus nidulans*. *Appl Environ Microbiol* 78:603–612
- Pushpam PL, Rajesh T, Gunasekaran P (2011) Identification and characterization of alkaline serine protease from goat skin surface metagenome. *AMB Express* 1(1):3. <https://doi.org/10.1186/2191-0855-1-3>
- Radha S, Gunasekaran P (2007) Cloning and expression of keratinase gene in *Bacillus megaterium* and optimization of fermentation conditions for the production of keratinase by recombinant strain. *J Appl Microbiol* 103:1301–1310
- Radha S, Gunasekaran P (2009) Purification and characterization of keratinase from recombinant *Pichia* and *Bacillus* strains. *Protein Expr Purif* 64(1):24–31. <https://doi.org/10.1016/j.pep.2008.10.008>
- Rajput R, Sharma R, Gupta R (2010) Biochemical characterization of a thiol-activated, oxidation stable keratinase from *Bacillus pumilus* KS12. *Enzyme Res* 2010:132148. <https://doi.org/10.4061/2010/132148>
- Saibabu V, Niyonzima FN, More SS (2013) Isolation, partial purification and characterization of keratinase from *Bacillus megaterium*. *Int Res J Biol Sci* 2(2):13–20
- Sareen R, Bornscheuer UT, Mishra P (2005) Cloning, functional expression and characterization of an alkaline protease from *Bacillus licheniformis*. *Biotechnol Lett* 9(5):1901–1907
- Selvam K, Vishnupriya B (2012) Biochemical and molecular characterization of microbial keratinase and its remarkable applications. *Int J Pharm Biol Arch* 3(2):267–275
- Sharma R, Gupta R (2010) Extracellular expression of keratinase Ker P from *Pseudomonas aeruginosa* in *E. coli*. *Biotechnol Lett* 32:1863–1868
- Sivakumar T, Shankar T, Vijayabaskar P et al (2012) Optimization for keratinase enzyme production using *Bacillus thuringiensis* TS2. *Acad J Plant Sci (AJPS)* 5(3):102–109
- Sivakumar T, Shankar T, Ramasubramanian V (2012b) Purification properties of *Bacillus thuringiensis* TS2 keratinase enzyme. *Am Eurasian J Agric Environ Sci* 12(12):1553–1557
- Zhang J, Li B (2013) Expression and characterization of extreme alkaline, oxidation-resistant keratinase from *Bacillus licheniformis* in recombinant *Bacillus subtilis* WB600 expression system and its application in wool fiber processing. *World J Microbiol Biotechnol* 29(5):825–832