DOI:10.1021/jf100803g

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Extracellular Expression of Keratinase from *Bacillus licheniformis* ER-15 in *Escherichia coli*

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Keratinase (ker BL) from *Bacillus licheniformis* ER-15 was cloned into vector pEZZ18 for extracellular expression in *Escherichia coli* HB101. Recombinant keratinase was secreted with high specific activity (75 units/mg) under non-inducible conditions after 36 h at 37 °C and 300 rpm in a shake flask. Protein was concentrated and, subsequently, purified by ion-exchange chromatography using Q-sepharose with 95.8% yield. The recombinant keratinase was a serine protease and most active in the pH range of 8−12 and at 60 °C. The enzyme was stable over a wide pH range of 4−12 for 3 h. ker BL degraded bovine serum albumin, casein, azocasein, gelatin, and feather. *E. coli* HB101 harboring pEZZ18 ker BL2 degraded chicken feather completely within 24 h at 37 °C.

KEYWORDS: Keratinase; *Escherichia coli* HB101; pEZZ18; non-inducible expression; purification

INTRODUCTION

Keratinases are specialty proteases that degrade hard-to-degrade proteins, such as keratins (*1*). Keratin is a structural protein of the epidermis and its variety of appendages such as hair, nail, wool, horn, feather, etc. (*2*). By virtue of their action on insoluble tough proteins, keratinases have been lately recognized as prospective proteases for the degradation of β-amyloid plaques and prion protein (*3*). Besides this, keratinases find application in a variety of industrial sectors, such as feed, fertilizers, detergents, and pharmaceuticals (*1*−*3*).

To date, a large number of keratinases have been isolated from bacteria, actinomycetes, and fungi (*1*−*3*). However, the major limitation for widespread application of keratinases is their economical production in heterologous hosts. In this respect, several keratinases have been cloned and expressed. However, functional expression of only a few keratinases from *Pseudomonas*, *Streptomyces*, *Bacillus*, and *Nocordiopsis* has been achieved in heterologous hosts, such as *Bacillus*, *Streptomyces*, and *Pichia* strains (*4*−*7*).

Of all keratinases, ker A from different strains of *Bacillus licheniformis* has been cloned and expressed several times in different hosts, such as *Bacillus subtilis*, *Bacillus megaterium*, and *Pichia pastoris* (*7*−*9*). Efforts have been made to improve its extracellular production in *Bacillus* using integrated plasmids and also strong promoters of the amylase and xylanase genes (*7*, *10*). To date, functional expression of keratinase has not been achieved in *Escherichia coli*, where it has been reported to accumulate largely in intracellular space in the form of inclusion bodies, which requires refolding for activation of the enzyme (*2*). Hence, extracellular functional expression in *E. coli* would serve as an easy way of expressing proteins for their production and biochemical characterization. Here, we report the extracellular functional expression of keratinase (ker BL) from *B. licheniformis* ER-15 using the *spa* promoter and signal in *E. coli* host and its biochemical characterization.

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MATERIALS AND METHODS

For the present study, the cloning vector pGEMT Easy vector was purchased from Promega, India, and pEZZ18 was purchased from GE Healthcare Life Sciences, India. All restriction enzymes and T4 DNA ligases were procured from Banglore GeneI, Bengaluru, India.

Bacterial Strains and Growth Conditions. The feather degrading strain of *B. licheniformis* ER-15 isolated from soil was used as a source of genomic DNA for ker BL gene cloning. The strain was identified by sequence analysis of 1.4 kb amplicon of 16S rRNA gene and submitted to Gene Bank (accession number EU293872). *B. licheniformis* ER-15 was grown in Luria−Bertani (LB) medium at 37 °C and 200 rpm for 18 h, while *E. coli* was grown in LB medium at 37 °C and 300 rpm for 24 h for uninduced gene expression. Antibiotic ampicillin was used at 100 μg/mL in the LB medium.

Cloning of the ker BL Gene. Amplification of the ker BL gene from chromosomal DNA of *B. licheniformis* ER-15 was carried out by the polymerase chain reaction (PCR) using gene-specific primers. Primers were designed in accordance with the open reading frame (ORF) of the putative ker BL gene of *B. licheniformis* DSM 13/ATCC 14580. Signal peptide was identified using the Signal P 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), and pro-sequence was marked from the available database. Two primers ker BL F1, including complete pro-sequence, and ker BL F2, 57-nucleotide-truncated pro-sequence, respectively, were designed. Cloning sites of *Sac* I and *Kpn* I were introduced into the forward primers ker BL F1 *Sac* (5′-GAGCTCGCTCAACCGGCGAAAAAT-3′) and ker BL F2 *Sac* (5′-GAGCTCGCTCAACCGGCGAAAAAT-3′) and reverse primer ker BL R *Kpn* (5′-GGTACCTTATTGAGCGGCAGCTTCGACA-3′), respectively.

A ∼1 kb fragment was obtained through 35 cycle of PCR, cloned into pGEMT Easy vector, and transformed into *E. coli* DH5α cells. Transformants carrying recombinant plasmid were selected on LB agar medium supplemented with ampicillin and 0.01% (w/v) isopropyl-β-D-thiogalactopyranoside (IPTG) and X-gal by blue white selection. The insert was reconfirmed by colony PCR.

Construction of Recombinant ker BL in pEZZ18 Vector and Transformation in *E. coli* HB101. pGEMT plasmid DNA carrying ker BL1 and ker BL2 amplicon was digested with *Sac* I and *Kpn* I and inserted

 Published on Web 07/02/2010

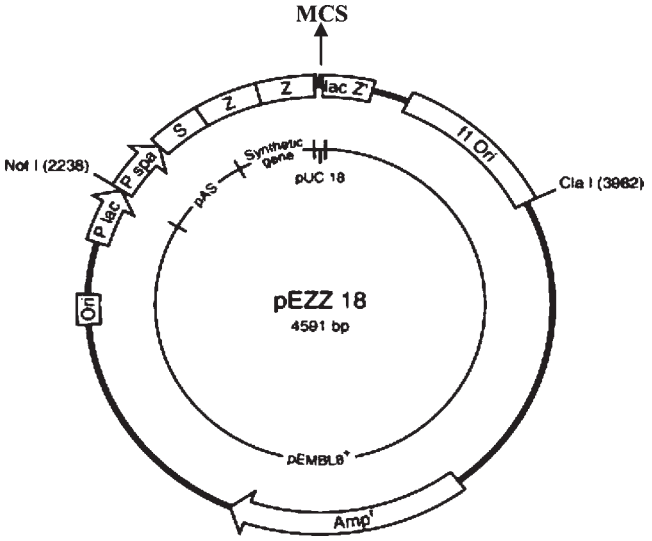

Figure 1. Vector map of expression vector pEZZ18. The expression is non-inducible, controlled by the *spa* promoter and protein A signal. The expressed protein becomes secreted with the "ZZ" peptide.

into the corresponding sites of pEZZ18 (**Figure 1**) to yield pEZZ18 ker BL1 and ker BL2, respectively, and transformed into *E. coli* HB101. Transformants were plated on a LB agar plate containing ampicillin, 0.032% (w/v) X-gal, and 0.01% (w/v) IPTG and incubated at 37 °C for 16 h. White transformants were selected and checked for expression. A negative control (vector without insert) was transformed into *E. coli* HB101.

Expression of Recombinant pEZZ18 ker BL1 and pEZZ18 ker BL2. For expression of recombinant enzyme (ker BL1 and BL2), 10 clones each of pEZZ18 ker BL1 and pEZZ18 ker BL2 were grown in LB medium containing ampicillin and incubated at 37 °C and 300 rpm. Extracellular expression was checked after 24 h by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis, and qualitative and quantitative estimation of keratinase activity was checked on azocasein and keratin azure, respectively. *E. coli* HB101 containing only pEZZ18 vector (without insert) was taken as the control.

Keratinase Production Using *E. coli* pEZZ18 ker BL2. Time kinetics for ker BL2 production was performed in LB medium at 37 °C and 300 rpm for a period of 3−48 h in the presence of ampicillin. Growth was monitored by A_{600}. The total protein was analyzed using the Bradford reagent, and keratinase and amidolytic activity were determined using keratin azure and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (*N*-succinyl-AAPF-pNA) as a substrate, respectively.

Purification and Characterization of ker BL2. The selected clone of *E. coli* HB101 pEZZ18 ker BL2 was grown at 37 °C and 300 rpm in LB broth containing 100 μg/mL ampicillin for 36 h at 300 rpm. After 36 h, the broth was centrifuged at 6000*g*. ker BL2 was concentrated by ultrafiltration (Sartorious, Germany) using 10 kDa cutoff and purified using a Q-sepharose column at pH 10. Purity was checked on 10% SDS−PAGE and high-performance liquid chromatography (HPLC) using acetonitrile and water (90:10, v/v) as the mobile phase on a C-18 column (Shimadzu, Japan) and ultraviolet (UV) detector.

The effect of pH was studied at the pH range of 4−12 in various 50 mM buffers: pH 4−6, citrate buffer; pH 7−8, phosphate buffer; pH 9−10, glycine−NaOH buffer; pH 11, phosphate−hydroxide buffer; and pH 12, hydroxide−chloride buffer. For pH stability, the enzyme was incubated with respective buffer for 2 h and the activity was determined at pH 10 and 60 °C. The effect of the temperature was studied at temperature range from 30 to 80 °C. Different inhibitors, namely, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), bromoacetic acid, mercaptoethanol, iodoacetic acid, *p*-chloromercuric benzoic acid, trypsin inhibitor, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were mixed with the enzyme in 1 and 5 mM concentrations, and activity was checked on keratin azure. The effect of metals on enzyme activity was studied using 5 and 10 mM divalent cations viz. Mg^{2+}, Hg^{2+}, Ba^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, and Ca^{2+}. Different divalent metal ions were mixed with the enzyme and incubated at room temperature for 1 h. Enzyme activity in the absence of metal ions was considered as the control.

Kinetic constants were studied on *N*-succinyl-AAPF-pNA and azocasein in the range of 0.1−2 mM and 10−100 mg, respectively.

Keratinase Activity. Expression of keratinase was checked by the keratinase assay using keratin azure (Sigma-Aldrich, Banglore, India) as a substrate. A 5 mL reaction mixture contained 20 mg of keratin azure, 50 mM glycine−NaOH buffer (pH 10), and properly diluted enzyme. The reaction mixture was incubated at 60 °C for 1 h, and 4 mL of 5% trichloroacetic acid (TCA) was added to stop the reaction. The reaction mixture was then kept at room temperature for 30 min. Further, this mixture was centrifuged. Absorbance was measured at 595 nm, and 0.01 optical density (OD) was considered as 1 unit of enzyme (*9*).

Protein Estimation. Protein was estimated using the Bradford reagent with properly diluted protein in 2.25 mL of Bradford reagent. Absorbance was checked at 595 nm.

Feather Degradation by the *E. coli* HB101 pEZZ18 ker BL2. Feather degrading potential of ker BL2 was assessed by studying feather degradation by *E. coli* HB101 harboring ker BL2 plasmid in LB medium containing 250 mg and 100 μg/mL ampicillin (feather LB medium). A 2% overnight growth inoculum was added to this feather LB medium and incubated at 37 °C for 36 h at 300 rpm. Visual observation of feather degradation was performed after every 12 h. The experiment with *E. coli* HB101 containing pEZZ18 vector without insert was taken as the control.

RESULTS

Bacterial Strain. A feather degrading strain of *B. licheniformis* ER-15 was isolated from soil. The strain degraded chicken feather within 18 h at 37 °C. It was identified on the basis of a 16S rRNA gene sequence (accession number EU29372). It shared 99% homology with *B. licheniformis* DSM 13/ATCC 14580. The keratinase gene was amplified from the ORF of the ker BL gene in the genome of *B. licheniformis* DSM 13/ATCC 14580.

Cloning and Sequence Analysis of ker BL. The gene encoding the complete and truncated pro-sequence was amplified by PCR, cloned into pGEM-T easy vector, and subjected to sequence analysis. The nucleotide sequence shared 99% identity with ker A. The deduced amino acid sequence shared 99 and 96% identity with other keratinase from the *B. licheniformis* strain and Subtilisin Carlsberg, respectively. The amino acid sequence was different at only one position in comparison to ker A, where V 224 was replaced with A. These differences with respect to the same protein suggest that differences may arise because of strain diversity.

Expression of Recombinant ker BL. The constructed vector denoted as pEZZ18 ker BL1 and pEZZ18 ker BL2 (a truncated pro-sequence) was transformed into *E. coli* HB101. Positive clones were monitored for extracellular expression at 37 °C and 300 rpm for 24 h. ker BL1 and ker BL2 showed 0.75 and 9.75 units/mL after 24 h at 37 °C and 300 rpm. ker BL1 showed ∼43 kDa protein, while ker BL2 was ∼28 kDa protein (panels **A** and **B** of **Figure 2**). Later, ker BL1 was identified as unprocessed keratinase with 14 kDa IgG domain, and ker BL2 was an active auto-processed protein of 28 kDa mass.

Time Kinetics for ker BL2 Production. Keratinase production by *E. coli* pEZZ18 ker BL2 was studied in LB medium every 6 h until 48 h at 37 °C and 300 rpm. Keratinase activity in extracellular broth was observed after 18 h on keratin azure with specific activity of 25 units/mg protein, which linearly increased up to 75 units/mg (11.25 units/mL activity and 150 μg/mL protein) in 36 h (**Figure 3**). A decrease in keratinase activity was observed after 48 h. Production was compared to the data available on keratinase from other strains of *B. licheniformis* in different heterologous hosts (**Table 1**).

Purification of ker BL2. The culture supernatant (1 L) was concentrated 10 times using 10 kDa molecular cutoff through ultrafiltartion, purified on anion-exchange Q-sepharose matrix,

Table 1. Comparison of the Expression of Keratinases in Various Expression Systems

source	expression host	vector/promoter	induction type and localization	production time (h)	production (units/mL, units/mg)	reference
B. licheniformis ER-15	*E. coli*	pEZZ18/spa	non-inducible/extracellular	36	11.25, 75	present work
B. licheniformis MKU3	*E. coli*	pET 30b/lac Z	IPTG/periplasmic/intracellular	4	74.32, ND[a]	*7*
B. licheniformis MKU3	*B. megaterium*	pWHK3/xylanase	xylose/extracellular	24	186.33, 17.25	*7*
B. licheniformis MKU3	*B. megaterium*	pWAK3/amylase	extracellular	36	168.6, 14.59	*9*
B. licheniformis MKU3	*P. pastoris*	pPICZαA	methanol/extracellular	96	135, 5.44	*9*
B. licheniformis PWD-1	*B. subtilis*	pLAT10	ND/extracellular	72	140, ND	*10*
B. licheniformis PWD-1	*B. licheniformis*	pE194	ND/extrcellular	36	35, ND	*10*
B. licheniformis PWD-1	*Pichia* X33	pPICZαA	methanol/extracellular	144	285, ND	*12*

[a] ND = not described.

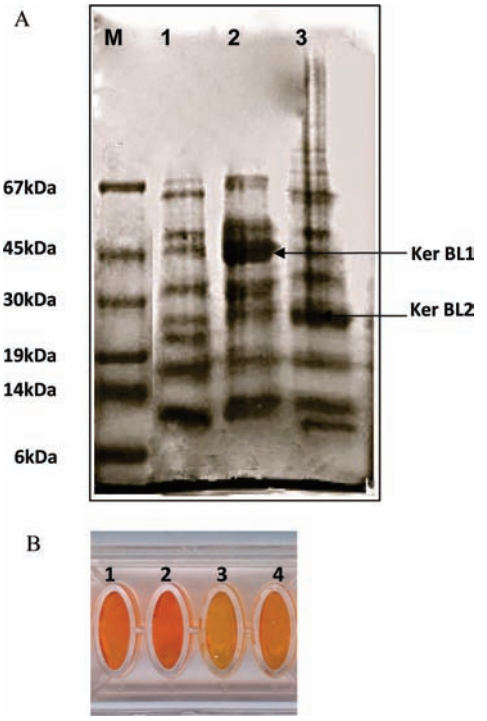

Figure 2. Expression of recombinant ker BL1 and ker BL2 in pEZZ18 *E. coli* HB101. (A) Expression of ker BL1 and ker BL2 on SDS−PAGE: lane M, protein marker; lane 1, extracellular protein of *E. coli* HB101 containing only pEZZ18 vector; lane 2, extracellular protein of *E. coli* pEZZ18 ker BL1; lane 3, extracellular protein of *E. coli* pEZZ18 ker BL2. (B) Hydrolysis of azocasein by extracellular broth of recombinant *E. coli* HB10, 1 ker BL1, and ker BL2 by the plate assay: lane 1, control 1 (*E. coli* HB101 without vector); lane 2, control 2 (*E. coli* HB101 with pEZZ18 vector only); lane 3, *E. coli* HB101 pEZZ18 ker BL2; lane 4, *E. coli* HB101 pEZZ18 ker BL1.

and eluted in unbound fraction with 95.8% recovery (**Table 2**). SDS−PAGE analysis revealed that it was a monomeric 28 kDa protein, and purity was confirmed by HPLC (panels **A** and **B** of **Figure 4**).

Characterization of Recombinant ker BL2. ker BL2 exhibited activity in the pH range of 8−12 with optima at pH 10, and more than 80% activity was observed at pH 11 and 12 (**Figure 5A**). The enzyme exhibited stablility over a wide range of pH 4−12 for 3 h. The enzyme was most active between 50 and 60 °C, with optima at 60 °C. The enzyme was thermostable with $t_{1/2}$ of 2 h at 50 °C and 10 min at 60 °C (data not shown).

The enzyme was completely inhibited by 5 mM PMSF. Nearly 65 and 78% residual activity was observed in the presence of 5 mM reducing agents, DTT and β-mercaptoethanol (β-ME), respectively.

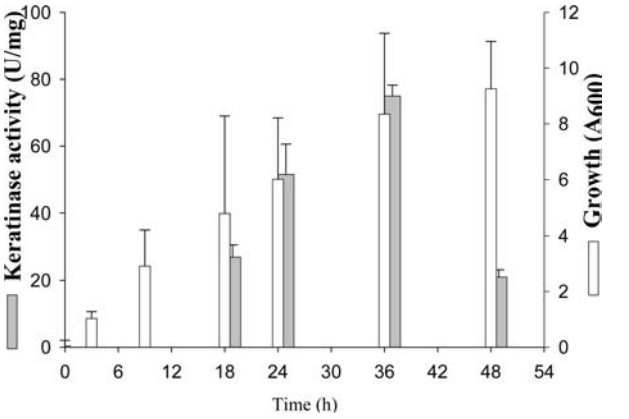

Figure 3. Time kinetics of ker BL2 production by recombinant *E. coli* HB101 ker BL2 at 37 °C and 300 rpm.

Table 2. Purification Summary of ker BL2 Using Anion-Exchange Chromatography

purification steps	total protein (mg)	total activity (units)	specific activity (units/mg)	purification fold	yield (%)
culture supernatant	150	11250.0	75	1	100
10 kDa cutoff	100	10991.0	109.9	1.46	97.7
Q-sepharose	80	10777.5	134.7	1.79	95.8

Among various substrates, keratinase hydrolyzed bovine serum albumin (BSA), casein, and azocasein more efficiently in comparison to insoluble substrates (**Table 3**). Amidolytic activity of ker BL2 showed that it hydrolyzed AAPF, Ala-Ala-Ala (AAA), and Gly-Leu only (**Table 4**).

Kinetic constants of ker BL2 were determined on azocasein and *N*-succinyl-AAPF-pNA. K_m and V_{max} on azocasein were 263.1 μg/mL and 3.33 μg mL^{-1} min^{-1}, respectively. Kinetic parameters K_m and V_{max} on *N*-succinyl-AAPF-pNA were observed to be 0.575 mM and 250 mM/min.

Feather Degradation by *E. coli* HB101 pEZZ18 ker BL2. *E. coli* HB101 harboring pEZZ18 ker BL2 plasmid could degrade chicken feather within 24 h, and shafts were also dissolved within 36 h of incubation. The feather was not degraded in control *E. coli* HB101 with pEZZ18 vector without insert even after 48 h of incubation (data not shown).

DISCUSSION

In the present report, keratinase from *B. licheniformis* was expressed extracellularly in *E. coli*. This is the first report of the functional extracellular expression of keratinase from *B. licheniformis* in an *E. coli* system. By and large, it is well-documented that

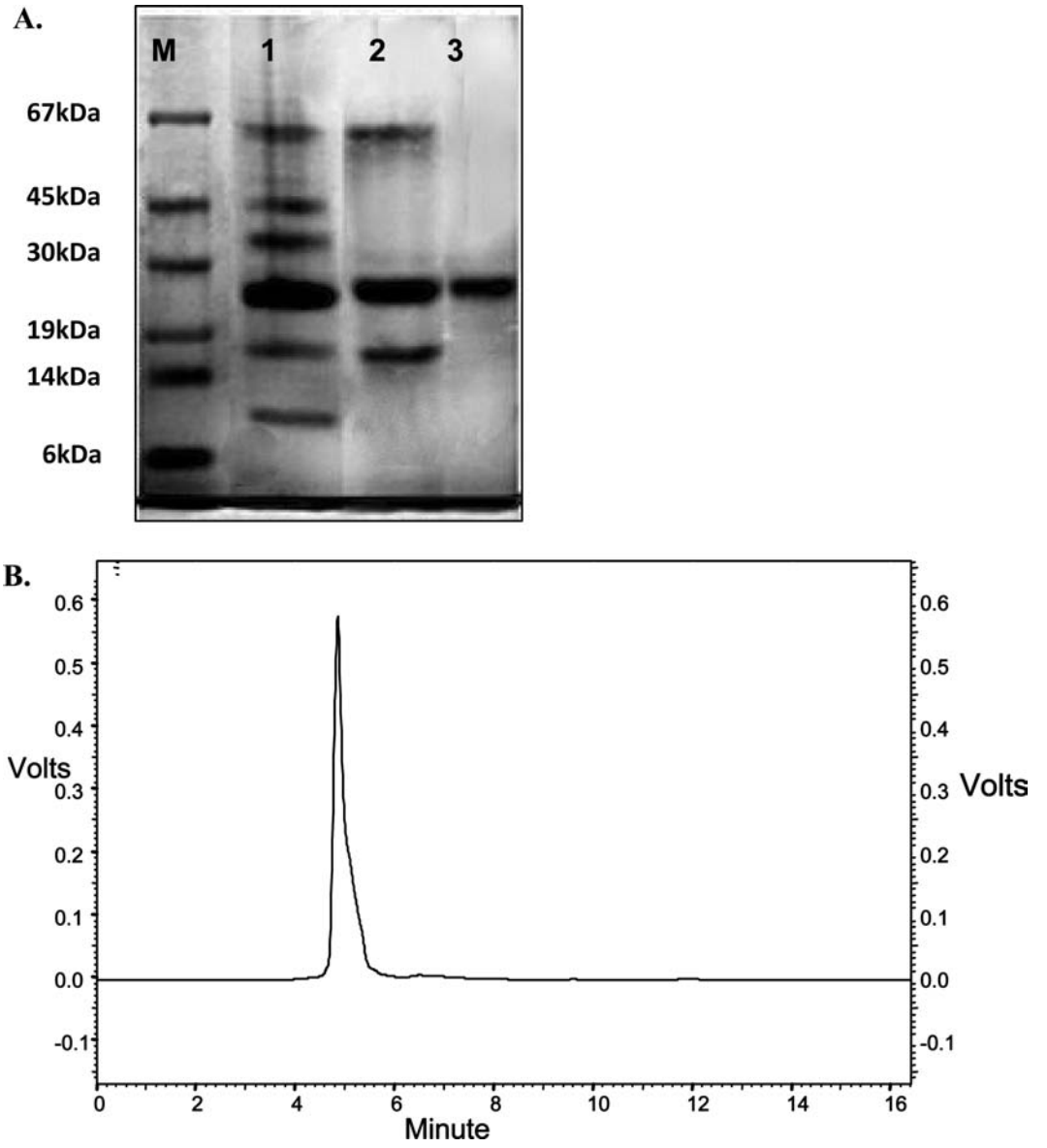

Figure 4. SDS−PAGE and HPLC analysis of purified ker BL2. (A) Lane M, protein marker; lane 1, crude broth of ker BL2 after 36 h of production; lane 2, concentrated broth after ultrafiltration; lane 3, purified ker BL2 after Q-sepharose anion exchange. (B) Protein was eluted from the C18 column using acetonitrile/water (9:1) as the mobile phase and detected at 280 nm after 5.3 min.

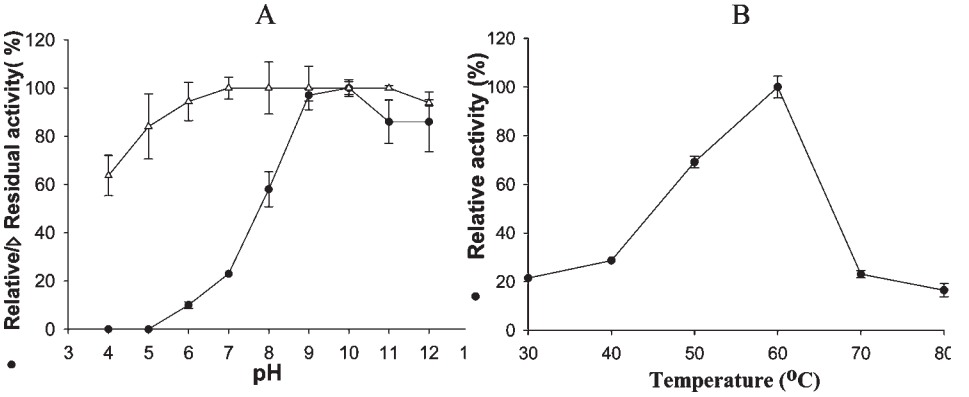

Figure 5. Keratinase activity of ker BL2 as a function of (A) pH and (B) temperature. (A) Keratinase activity of ker BL2 was studied using 50 mM buffers: pH 7−8, phosphate buffer; pH 9−10, glycine−NaOH buffer; pH 11, phosphate−hydroxide buffer; and pH 12, hydroxide−chloride buffer, at 60 °C on keratin azure. The pH stability was studied by incubating ker BL2 in various buffers in the pH range of 4−12 for 1 h at 30 °C, and residual activity was determined on keratin azure. The maximum activity of 104 units/mg protein was considered as 100% activity. (B) Keratinase activity of ker BL2 was studied at pH 10 buffer by incubating the reaction mixture at different temperatures for 1 h. The maximum activity of 104 units/mg protein was considered as 100% activity.

Table 3. Activity of ker BL2 on Different Soluble and Insoluble Protein Substrates[a]

substrate	keratinase activity (units/mg)
casein	2200 ± 3.25
BSA	2750 ± 9.45
gelatin	882 ± 6.34
azocasein	2311 ± 10.22
meat	174 ± 1.23
hemoglobin	151 ± 2.24
feather	248 ± 3.45
keratin azure	109 ± 2.67

[a] To study substrate specificity of ker BL2, 20 mg of insoluble substrates and enzyme was incubated at 60 °C for 1 h and the reaction was stopped by 4 mL of 5% TCA. The mixture was centrifuged at 5700*g*, and absorbance was measured at 280 nm. In the case of azocasein and keratin azure, absorbance was measured at 440 and 595 nm, respectively. An increase in 0.01 OD was considered as 1 unit of enzyme.

Table 4. Amidolytic Activity of ker BL2 on Different Synthetic Substrates[a]

substrate	amidolytic activity (units/mg)
N-CBZ-1-Phe-pNA	0
N-succinyl-Ala-Ala-Ala-pNA	99.8 ± 1.45
N-succinyl-Ala-Ala-Pro-Phe-pNA	2299 ± 12.67
N-succinyl-Arg-pNA	0
N-succinyl-Gly-Leu-pNA	134 ± 2.67
N-CBZ-Tyr-pNA	0
N-succinyl-Phe-pNA	0
N-succinyl-Pro-Phe-Arg-pNA	0

[a] Different *p*-nitroanilides (10 mM) were used as the substrate. The reaction was carried out at pH 8 and 60 °C, and released *p*-nitroaniline was measured at 410 nm. One unit was defined as the enzyme required to release 1 μmol of *p*-nitroaniline in 1 min.

proteases are difficult to express in heterologous hosts because they become lethal to the cell and hamper cell growth or they become accumulated as inactive inclusion bodies that require refolding of the protein (*2*). In this respect, extracellular expression is always desired for industrially useful products for the ease of downstream processing.

In the present investigation, extracellular expression was obtained using a *spa* promoter and protein A signal along with "ZZ" domain based on IgG-binding sites. Under the direction of the protein A signal, expressed protein becomes secreted as a fusion protein with "ZZ" peptides under a non-inducible system. This vector has been used for expression of several other proteins (*11*).

It was observed that ker BL with truncated pro-sequence (ker BL2) folded rapidly in comparison to complete pro-sequence (ker BL1), where it was recovered as inactive 43 kDa protein with an IgG domain. The pro-sequence has been known to play an important role in the folding of proteases, and possibly, the truncated pro-sequence folds faster (*13*). During auto-processing, the IgG domain is also cleaved along with the pro-sequence.

The maximum keratinase was obtained in 36 h with high specific activity of 75 units/mg, which is much higher than previously reported proteins from other *B. licheniformis* strains (*9*).

The high specific activity in the present investigation may be attributed to its extracellular nature of the protein, because the *E. coli* expression host has much less extracellular protein in comparison to the intracellular cytoplasmic protein (*9*).

The enzyme was purified with high yields of 96% and specific activity of 134.7 units/mg, which is significantly higher than the previously reported yields in *Bacillus* and *Pichia* systems, where only 9.3 and 1.4% enzyme could be recovered with specific activity of 1277.7 and 365.7 units/mg, respectively(*9*). This expression system was found to be better because much less extracellular protein was obtained in *E. coli*, while in the case of *Bacillus* and *Pichia*, more extracellular proteins are observed. ker BL2 was found to be a monomer of 28 kDa and was active, which is similar to the earlier reports (*9*, *12*). Keratinases from *Bacillus* sp. are reported to be 24−42 kDa (*3*).

ker BL2 exhibited activity and stability in the alkaline pH range of 8−12, with maximum activity at pH 10. The enzyme was stable over a wide pH range of 6−12 for a period of 3 h without the loss of much activity, and more than 50% activity was observed at pH 4. This property is different from the earlier reports from the same keratinase expressed in the *Bacillus* and *Pichia* systems, where activity range was neutral to alkaline and never beyond pH 10 (*9*, *12*). The highly alkaline nature of ker BL2 and stability in extreme acidic and alkaline conditions may be a result of conformational differences. However, this makes it a biotechnologically important enzyme for feed applications, where the enzyme requires stability in the acidic gastric environment and activity optima in the alkaline range (*2*). Further alkaline stability is advantageous in leather processing, where $Ca(OH)_2$ is used for the swelling of hides (*3*). A similar alkaline nature has been reported from *B. halodurans* AH101, with maximum activity at pH > 12.0 (*14*). Likewise, ker BL2 exhibited temperature optima at 60 °C, while previously expressed protein in *B. megaterium* was most active at 70 °C and ker A from *B. licheniformis* PWD-1 was at 50 °C (*9*, *15*). Kinetic constants have not been reported from all recombinant keratinases, except heterologous expression in *B. megaterium*. The present ker BL2 exhibited high K_m and low V_{max}, i.e., 0.575 mM and 250 mM/min, in comparison to an earlier report, where K_m and V_{max} were 0.201 mM and 61.09 units/s, respectively (*9*). These differences with respect to pH, temperature, and kinetic constant of the same expressed protein may be a result of different enzyme conformations obtained in various expression hosts.

ker BL2 was strongly inhibited by 5 mM PMSF being a serine protease, which is similar to earlier reports (*3*, *9*, *12*). In contrast to other reports, thiol and Ca^{2+} activation was not observed (*12*).

ker BL2 hydrolyzed soluble protein substrates, such as casein, BSA, and gelatin, more efficiently than insoluble substrates, such as feather, keratin azure, and hemoglobin, which is in confirmation with earlier reports for the same enzyme (*9*, *12*). Its keratinolytic potential was further established by the fact that *E. coli* HB101 harboring ker BL2 could degrade chicken feather. It is well-documented that keratin degradation is a redox-mediated process of keratinase, where redox is supplied by the live bacterial cell or reducing chemicals (*3*).

Thus, keratinase from *B. licheniformis* was expressed as a functional extracellular protein in an *E. coli* system using pEZZ18 vector with high specific activity. Hence, this system can be used for extracellular expression of proteases/keratinases from different organisms.

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Received for review March 4, 2010. Revised manuscript received June 9, 2010. Accepted June 9, 2010. The authors thank the University of Delhi for financial assistance through the DU-DST-PURSE program.