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Cloning, expression and structure simulation of keratinase from *Bacillus licheniformis* strain MZK05

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ABSTRACT

Aims: *Bacillus licheniformis* MZK-05 is a keratinolytic bacterium having potential in dehairing of leather and feather hydrolysis. The present study aimed at to improving the production level of keratinase through gene cloning and expression of recombinant keratinase.
Methodology and results: *Bacillus licheniformis* MZK-05 produced an amplicon of 1,156 bp in a polymerase chain reaction while targeting the gene, *kerA,* responsible for the enzyme keratinase. The amplicon was subsequently cloned into the plasmid vector pGEX-6p-2 for expression in *Escherichia coli* BL21. A 58 kD GST-KerA fusion protein was expressed upon IPTG induction which was eventually cleaved by PreScission protease that produced a 39 kD protein. A corresponding increase in proteolytic (312 U/mL) and keratinolytic (196 U/mL) activity were observed with the expressed keratinase. Specific enzyme activities for protease and keratinase, an indication of efficiency of the enzyme, were 2621.84 U/mg and 1647 U/mg, respectively and the specific keratinase activity was the highest activity ever reported by any recombinant bacterial strain.
Conclusion, significance and impact study: Since the production of keratinase by wild type strain is limited to a certain level, the industrial need could be met by improving the production level through gene cloning and expression of recombinant keratinase. In this connection, the cloning of *kerA* gene from *B. licheniformis* MZK-05 into pGEX-6p-2 vector, its expression in *Escherichia coli* BL21 host and prediction of 3-D model of the expressed protein were performed which will be the basis for industrial production of keratinase in Bangladesh.

Keywords: 3-D model simulation, *Bacillus licheniformis* MZK-05, cloning, expression, keratinases

INTRODUCTION

Keratinases are a group of proteases that exert keratinolytic activity on insoluble keratin which are fibrous and insoluble structural proteins, extensively cross-linked with disulphide, hydrogen and hydrophobic bonds that result in mechanical stability and resistance to common proteolytic enzymes such as pepsin, trypsin and papain. Keratinase belongs to the subtilisin family of serine proteases and has a high homology with subtilisin Carlsberg from *Bacillus subtilis* (Evans *et al.*, 2000).

Keratinases have, therefore, great importance in eco-friendly dehairing processing of leathers in leather industry, feed and bio fertilizers production from feather and treatment of keratin-rich wastes from poultry etc. This enzyme has long been studied for dehairing processes in the leather industry and it was demonstrated that keratinase from *Bacillus* spp. was able to perform the dehairing by removing various proteins of hide especially the keratin, elastin and matrix protein (Takami *et al.*, 1992). Dehairing of goat skin was performed by using keratinase from *Streptomycetes* sp. (Mukhopadhyay and Chandra,1993).

Its application reduced the use of lime and sulphide up to 50% required in non-enzymatic process (Kalisz *et al.*, 1988). The chemicals causing pollution in pre-tanning processes are lime, sodium sulphide and caustic soda. In fact, one third of the pollution caused by the leather industries results from the wastes generated during dehairing operations.

Processed leather is one of the major export items in Bangladesh and in a survey it was found that about 200 tanneries in Bangladesh mostly employ conventional method of lime sulphide process in bating step and other harsh chemicals in soaking and dehairing steps of hide processing, which is hazardous to both environment and economy.

Bacillus licheniformis MZK-03 and MZK-05 strains, previously isolated, exhibited promising keratinase activity in dehairing of hide and feather hydrolysis (Hoq *et al.*,

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2005). Crude protease and keratinase enzymes alone - (without chemicals) were able to dehair the goat skin completely with increased area yield (98%), less wrinkled grain and smooth surface but no swelling of the skin (Hossain *et al.*, 2008). Combined treatment of the enzymes with 50% chemicals was much better as it de-haired the skin from the hair root, retained desired fibrous structure, and rendered moderate area yield (94%). Hence, the use of keratinase from indigenous *B. licheniformis* strains can be a useful alternative in eco-friendly de-hairing step of hide processing over conventional method.

MATERIALS AND METHODS

Bacterial strains, vector and growth conditions

Bacillus licheniformis strain MZK-05, previously isolated from feather-decomposed soil and identified by 16S rRNA typing (Hoq *et al.*, 2005) was used as the source of genomic DNA for *ker*A gene. Commercial vector pGEX-6p-2 (*tac*, *lacI*^{q}) (Amersham Bioscience, Pharmacia, USA) was used, expression of which is under the control of *tac* promoter that is induced by the lactose analog isopropyl β-D thiogalactoside (IPTG) and expression is prevented by the repressor protein encoded by *lacI*^{q} gene without IPTG. *Escherichia coli* DH5α was used as cloning host and *Escherichia coli* BL21 (*ompT*$^-$ *lon*$^-$) was used as expression host for high level expression of GST fusion protein. All strains were maintained at −70 °C by 15% Glycerol stock. LB broth and LB agar were used for subculture, cell propagation, culture preservation and maintenance in all necessary cases. The medium was supplemented with ampicillin (100 µg/mL) whenever needed. Both LB agar and broth were incubated at 37 °C providing aeration for liquid culture by shaking at 150 rpm in an orbital shaker (New Brunswick, USA).

Primer designing and *ker*A gene amplification

The primers were designed from the alignment of the sequences obtained for *ker*A genes from different *Bacillus licheniformis* strains. Six sequences that exhibited maximum 100% and minimum 95% similarity were aligned and 18 bp consensus sequences at 5'- 3' initiated with start codon (Figure 1A) and at 3'- 5' including stop codon (Figure 1B) were chosen for forward and reverse primers respectively. Restriction sites for *Bam*HI and *Xho*I were then patched before the 5'-end with two additional nucleotides (Table 1) so that the amplicon could be ligated at right reading frame of the cloning vector after restriction digestion. Chromosomal DNA of *B. licheniformis* MZK05 was extracted and purified. PCR was then performed with the designed primers (Table 1) in 30 µL reaction mixture [containing 10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 1.0 µM of each primer, 50-100 ng of chromosomal DNA and 0.5 U of *Taq* DNA polymerase (Promega, USA)] in a thermal cycler (MJ Mini™ BIO RAD, USA) by 35 cycles (95 °C for 50 sec, 56 °C for 45 sec, 72 °C for 60 sec) with an initial denaturation step at 95 °C for 4 min and a final extension step at 72 °C for 10 min. PCR products were analysed by electrophoresis in 1.5% (w/v) agarose gel submerged in 1× TBE buffer.

Figure 1: Primer designing to amplify *ker*A gene from *Bacillus licheniformis* MZK05 A) 18 bases started from start codon-ATG were chosen as part of the forward primer, B) 18 bases ended with stop codon-TAA was selected as part of the reverse primer.

Table 1: Properties of the primers designed to amplify *kerA* gene (IDT, USA).

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Primer	Sequence (5'-3')	Restriction site	Length	GC%	T_m °C
Fwd	AC**GGATCC**ATGATGAGGAAAAAGAGT	*Bam* HI	26 bp	42.3	58
Rev	AT**CTCGAG**TTATTGAGCGGCAGCTTC	*Xho* I	26 bp	50	60.7

Construction of recombinant strain

The obtained PCR product and the vector were digested with *Bam*HI and *Xho*I (TaKaRa, Japan) and inserted into the corresponding sites of vector pGEX-6P-2 by ligation with T_4-DNA ligase to obtain the recombinant expression vector pGEX-6P-2-kerA. The vector: insert ratio was maintained 1:0, 1:1 and 1:3 in the ligation reaction.

Generation of recombinant expression vector was assessed by transforming the ligation mixture into chemically competent *Escherichia coli* DH5α cells and spreading them over LB agar supplemented with ampicillin (100 µg/mL). Putative *kerA* clones obtained for 1:1 and 1:3, were transferred into LB broth supplemented with ampicillin (100 µg/mL) and following overnight incubation, were checked by colony PCR for the presence of *ker*A gene. The PCR positive clones were preserved and further propagated to extract pGEX-6p-2-kerA recombinant vector. The recombinant vector was then digested with *Bam*HI and *Xho*I for further assessment of vector-insert ligation following agarose gel electrophoresis.

Expression of recombinant pGEX-6P-2-kerA

The recombinant vector, pGEX-6P-2-kerA, was transformed into the chemically developed competent *Escherichia coli* BL21 cells for the expression of GST-kerA fusion protein. *Escherichia coli* BL21 carrying pGEX-6P-2-KerA was cultured overnight at 37 °C in LB medium containing ampicillin (100 µg/mL) and was used as inoculum to start IPTG induced protein expression. Fresh LB ampicillin medium was inoculated with the overnight culture to start with an OD_{600nm}= 0.1 and incubated at 37 °C with shaking at 200 rpm. IPTG was added aseptically in the culture when the OD_{600nm} reached 0.5 and three different concentrations were applied (i.e. 0.1, 0.3 and 0.5 mM IPTG in 3 separate flasks) for optimization. Simultaneously to optimize the induction time, 1 mL of culture from each flask was collected aseptically at 1 h. interval up to 5 h. Then the cells were harvested by centrifugation at 10,000× *g* for 10 min at 4 °C (Tomy, MX-305, high speed Refrigerated micro centrifuge, Japan) and resuspended in 100 µL of 1× Laemmli buffer to denature the protein incubating at 95 °C for 5 min. Equal amount of protein from each IPTG concentration and each time interval was then analyzed by SDS-PAGE in a 10% separating gel.

Gel was then stained in staining solution [0.02% CBB-G250 in 2% (w/v)] phosphoric acid, 5% aluminum sulfate and 10% ethanol for 2 h.

Purification

The GST-kerA fusion protein was expressed as described after optimizing the IPTG concentration and time. The liquid culture (50 mL) was then transferred into falcon tube and the cells were harvested by centrifugation at 7700× *g* for 10 min at 4 °C (Tomy, MX-305, Japan) and resuspended in 2.5 mL of 1× PBS **(**140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) from which 10 µL was preserved for SDS-PAGE. The suspended cells were then lysed using microtip sonicator in short bursts keeping in ice. Triton X-100 was then added for a final concentration of 1% and mixed gently for 30 min for the solubilization of the fusion protein. Then, 1.0 mL of 50% slurry of Glutathione Sepharose 4B was added into the bacterial sonicate and kept at room temperature for 30 min with gentle mixing. The supernatant was then separated carefully by decanting following centrifugation at 500× *g* for 5 min.

The sediment was then washed thrice with 5 mL of 1× PBS and once with 5 mL PreScission cleavage buffer (50 mM Tris-HCl; pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). The pellet was then resuspended by gentle mixing in 500 µL of PreScission protease mixture [40 µL (80 units) of PreScission Protease added into 960 µL of PreScission Cleavage Buffer] and incubated at 5 °C for 4 h. The supernatant containing KerA protein was finally collected very carefully following centrifugation at 500× *g* for 5 min and was analyzed by SDS-PAGE.

Protease and keratinase assay

Protease activity of the purified KerA was determined by a modified method described by Kreger and Lockwood (1981) using azo-casein (Sigma, USA) as the substrate. Briefly, 400 µL of appropriate dilution of KerA was added to 400 µL of 1% azo-casein solution (suspended in 0.05 M Tris- HCl; pH 8.5) and the mixture was incubated at 37 °C for 1 h. The reaction was terminated by adding 135 µL of 35% trichloroacetic acid (TCA) and left for 15 min on ice followed by centrifugation at 13000 rpm, at 4 °C for 10 min. Then, 750 µL of the supernatant was neutralized with equal volume of freshly prepared 1.0 N NaOH by gentle mixing. Absorbance (OD_{440nm}) of the mixture was then measured in a UV spectrophotometer (Thermo spectronic Genesys, USA) keeping the solution from a parallel reaction as blank where TCA was added before the enzyme. One unit of protease activity was defined as the amount of enzyme required to yield an increase in absorbance (OD_{440nm}) of 0.01 in 1 h at 37 °C.

Keratinolytic activity was determined by the method of (Bressollier *et al.*, 1999). The enzyme was incubated with 5 mg of keratin-azure (Sigma, USA) in 1 mL of 50 mM/l Na- phosphate (pH 7.5) at 37 °C for 1 h with constant agitation (900 rpm). One unit of keratinase activity was

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defined as the amount of enzyme required to result in an increase in absorbance (OD_{595}) of 0.01 in 1 h.

Nucleotide sequence and deduced amino acid sequence

The sequence of the *kerA* amplicon was determined by the dideoxy cycle sequencing method (Sanger *et al.*, 1992) on a model ABI 373 automated DNA sequencer (DNA Sequencing Laboratory, CARS, University of Dhaka, Bangladesh) and deposited in GenBank under the accession no. KJ661542. The similarity for the sequence was searched at the GenBank (National Centre for Biotechnology Information, NIH, USA) using BLAST and FASTA programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The amino acid sequence of the *kerA* ORF was deduced with the aid of the software MEGA 5.2.

3-D model building for KerA protein

Three dimensional model of KerA protein was built with ProMod Version 3.70 from a fully automated server, SWISS-MODEL (http://swissmodel.expasy.org/). Homology modeling typically comprises the following steps: (i) template identification, (ii) template selection, (iii) model building and (iv) model quality estimation (Sali and Blundell, 1993; Schwede *et al.*, 2008). The deduced amino acid sequences were used in this process to build the models based on the target-template alignment. Coordinates which are conserved between the target and the template were copied from the template to the model. Insertions and deletions were remodeled using a fragment library. Side chains were then rebuilt. Finally, the geometry of the resulting model was regularized by using a force field. In case loop modeling with ProMod (Guex and Peitsch, 1997) does not give satisfactory results, an alternative model was built with MODELLER (Sali and Blundell, 1993). The global and per-residue model quality has been assessed using the QMEAN scoring function (Benkert *et al.*, 2011).

RESULTS

Cloning of *kerA* gene from *Bacillus licheniformis* MZK-05

PCR amplification with the designed primers revealed a distinctive 1156 bp fragment from *B. licheniformis* MZK-05 template DNA (Figure 2A). Upon ligation of vector (pGEX-6p-2) and insert (*kerA* amplicon), both of which were double digested (Figure 2B), and transformation into the *E. coli* DH5α, ampicillin resistant clones (at 1:1 and 1:3 insert-vector ratio) were obtained on LB-ampicillin agar medium. Colony PCR performed with the clones produced the desired amplicon of 1156 bp (Figure 2C) and digestion of the pGEX-6p-2-kerA recombinant vector with *Bam*H1 and *Xho*1 restriction enzyme revealed the bands of vector and insert upon agarose gel electrophoresis which indicated the successful cloning of *kerA* gene (Figure 2D).

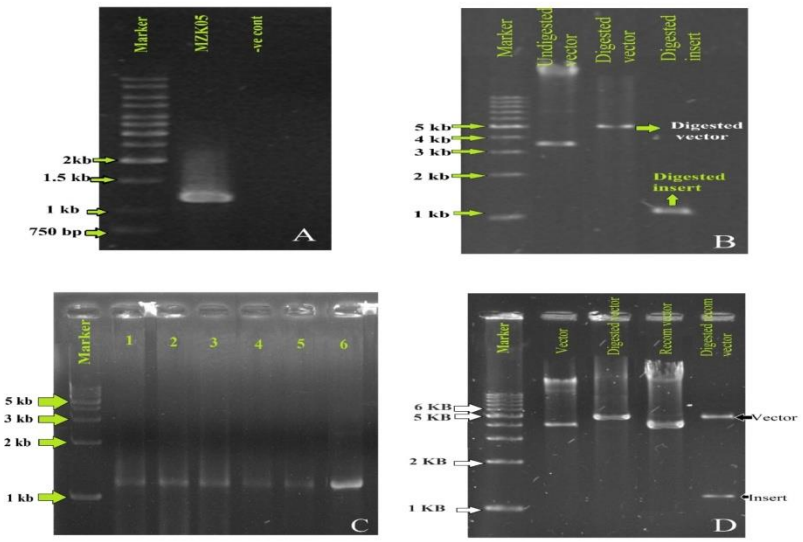

Figure 2: Agarose gel electrophoresis of A, PCR product (amplified *kerA* gene) of expected 1156 bp. (Marker: 1 kb DNA ladder, Fermentas, USA); B, Undigested vector in supercoiled form, moves faster than the digested vector that is in linear form. So, undigested vector appeared to be about 3.5 kb which upon digestion appeared to be about 5 kb (real size of the vector). C, Confirmation of the clones harboring pGEX-6p-2-kerA recombinant vector by PCR amplification for *kerA* gene. Lanes other than DNA marker are labeled as per the clone number (1-6). D, Double digestion of both pGEX-6p-2 and pGEX-6p-2-kerA vector produced vector in linear form and vector as well as insert respectively. (Marker in A, B, C and D: 1 Kb DNA ladder, TaKaRa, Japan)

Expression of *kerA* gene in *Escherichia coli* BL21

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The pGEX-6p-2-kerA was transformed into *E. coli* BL21 and expression of GST-kerA fusion protein was observed within 1 h of induction at 0.1 mM IPTG concentration (Figure 3A). The optimum time and concentration for maximum expression of the desired protein were determined to be 3 h with 0.3 mM IPTG (Figure 3B). From SDS-PAGE analysis, the molecular weight of the GST-kerA fusion protein was determined to be 58 kD (Figure 3C). Glutathione Sepharose 4B batch purification followed by PreScission protease cleavage produced the KerA protein of about 39 kD (Figure 3C).

The yield of purified protein was approximately 119 mg/L and the protease as well as keratinase activity of the purified keratinase was found to be 312 U/mL and 196 U/mL respectively. The specific protease and keratinase activity was also determined and those were found to be 2621.84 U/mg and 1647 U/mg of protein respectively (Figure 4).

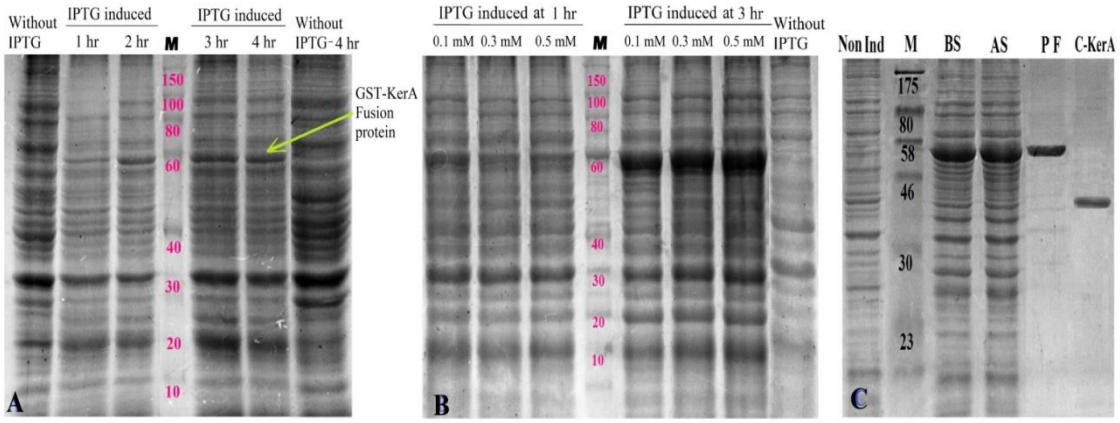

Figure 3: Expression of GST-KerA fusion protein. A, Time required for maximum protein expression at 0.1 mM IPTG; B, IPTG concentration required for maximum protein expression; C, Purification of recombinant protein. Non Ind, Non induced; BS, Before sonication; AS, After sonication; PF, Purified fusion protein; C- KerA: KerA protein after cleavage by PreScission protease free from GST tag. (M, Pre-stained protein Marker, NEB, USA).

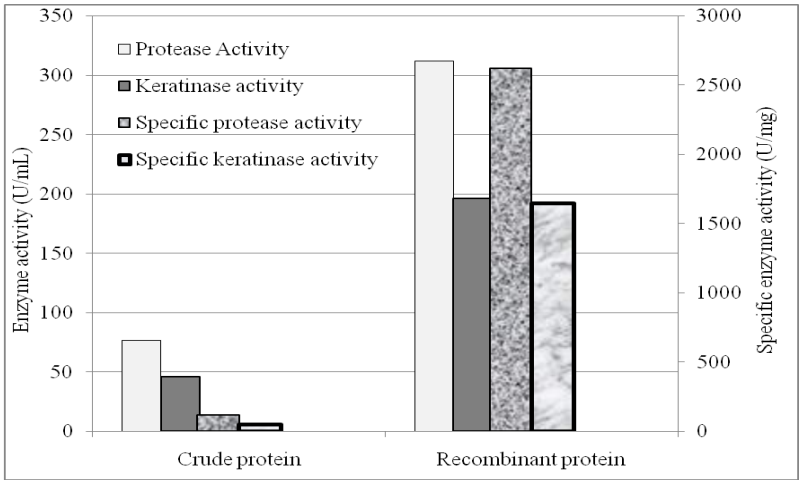

Figure 4: Comparison of yield in enzyme activity between crude and protein recombinant. A 4-fold increase both for enzyme and specific enzyme activity was observed.

Sequence analysis

Sequence analysis of *kerA* indicated that the insert was in proper orientation and correct junction to maintain the reading frame. The deduced amino acid sequences of *kerA* gene of *B. licheniformis* MZK-05 showed 100% identity with thermostable keratinase from *Bacillus licheniformis* DS23, KerA of *Bacillus licheniformis* OWU 1411T and 98.4% identity with kerA of *B. licheniformis* PWD-1 (*B. licheniformis* ATCC 53757). The insert contained an ORF of 1137 nucleotides from which amino acid sequences were deduced and 379 amino acids were revealed with an N-terminal signal peptide consisting of 29 residues (Figure 5), a pro-peptide of 76 residues followed by the matured protein comprising of 274 residues and thus the prokeratinase of 350 residues. The

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estimated molecular mass of matured keratinase deduced from the amino acid sequence was 27330 Da. Sequence alignment of the amino acids from matured KerA protein revealed that Tyr26 instead of Phe26, Asn86 for Ser86 and Ser211 for Asn211 are present (Figure 6).

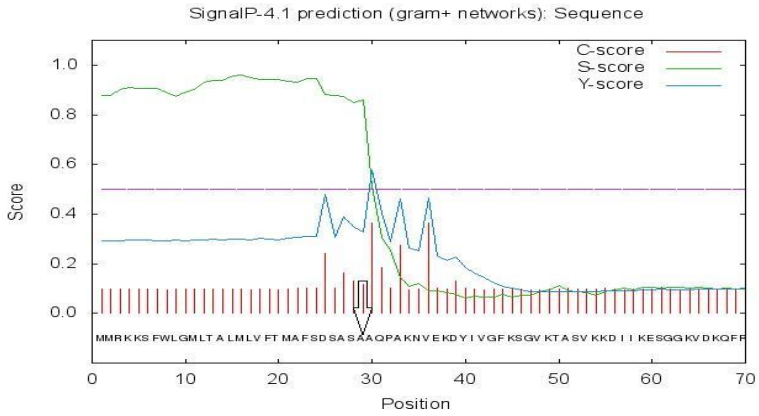

Figure 5: Signal peptide was predicted by SignalP 4.1 from the deduced amino acid sequences which started from Met and finished at Ala, a total of 29 amino acids.

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                        80        90        100       110       120       130       140
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MZK05 KerA      IINAAKAKLDKEALEEVKNDPDVAYVEEDHVAHALAQTVPYGIPLIKADKVQAQGYKGANVKVAVLDTGI
4gi3.1.A KerA   ------------------------------------.....................F..............
1c3l.1.A SUBTIL ------------------------------------.....................F..............
1yu6.1.A Subtil ----------------------------------M.....................F..............

                       150       160       170       180       190       200       210
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MZK05 KerA      QASHPDLNVVGGASFVAGEAYNTDGNGHGTHVAGTVAALDNTTGVLGVAPNVSLYAVKVLNSSGSGSYSG
4gi3.1.A KerA   ..................................................S...................
1c3l.1.A SUBTIL ..................................................S...................
1yu6.1.A Subtil ..................................................S...................

                       220       230       240       250       260       270       280
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MZK05 KerA      IVSGIEWATTNGMDVINMSLGGPSGSTAMKQAVDNAYARGVVVVAAAGNSGSSGNTNTIGYPAKYDSVIA
4gi3.1.A KerA   ......................A.................................................
1c3l.1.A SUBTIL ......................A.............................S.................
1yu6.1.A Subtil ......................A............................N..S.................

                       290       300       310       320       330       340       350
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MZK05 KerA      VGAVDSNSNRASFSSVGAELEVMAPGAGVYSTYPTSTYATLNGTSMASPHVAGAAALILSKHPNLSASQV
4gi3.1.A KerA   ..................................N.................................
1c3l.1.A SUBTIL ..................................N.................................
1yu6.1.A Subtil ..................................N.................................

                       360       370
                ....|....|....|....|....|....
MZK05 KerA      RNRLSSTATYLGSSFYYGKGLINVEAAAQ
4gi3.1.A KerA   ..............................
1c3l.1.A SUBTIL ..............................
1yu6.1.A Subtil ..............................
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Figure 6: Alignment of mature protein sequence (274 aa) of *B. licheniformis* strains MZK-05 with the highly similar KerA and Subtilisin Carlsberg proteins of other strains. SC, Subtilisin Carlsberg. In the KerA from *B. licheniformis* MZK-05, discrepancies with other sequences, i.e. Tyr26 instead of Phe26, Asn86 and Ser211 instead of Ser86 and Asn211 respectively were observed.

Protein structure simulation

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From the homology modeling results, a total of 317 templates were found to match the target sequence at different similarity index. This list was filtered by a heuristic down to 52 from which 3 models were built based on the maximum sequence coverage, similarity and identity.

As to determine the 3D structure from the above mentioned 3 models, the correct one was considered to be similar to Subtilisin Carlsberg (PDB- 1c3l.1.A). The structure model and the hydrogen added surface of the protein were shown in Figure 7A and Figure 7B, respectively. This structure was chosen for its maximum sequence identity and Ca^{2+} binding site (Figure 7C) with Subtilisin Carlsberg. Like all subtilisins, three residues at the active site (Figure 7D) forming the catalytic triad (Asp-32, His-63 and Ser-220), are also conserved in the keratinase of *B. licheniformis* MZK-05. There is no disulfide bond in the protein, as presence of Cysteine was not observed in the sequence (Ceroni *et al.*, 2006). There were 6 N-Glycosylation sites in the matured protein sequence which are 76NTTG, 86NVSL, 96NSSG, 122NMSL, 217NGTS and 249NLSA.

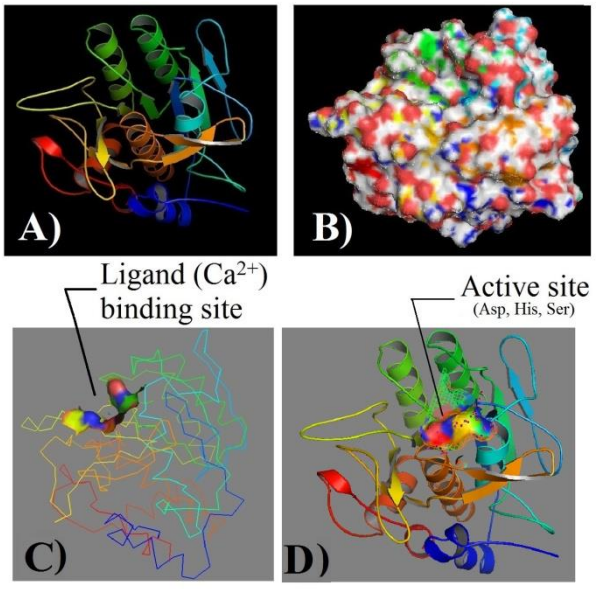

Figure 7: 3-D modeling of KerA protein from *Bacillus licheniformis* MZK-05. A) Overall view of the mature Keratinase enzyme where N-terminal to C-terminal is indicated by rainbow colour (Blue to red). B) Surface of the modeled protein when H- ions are present. C) Ligand (Ca^{2+}) binding site of the protein. D) Active site of the protein (Asp, His, Ser). All these analysis was performed using PyMOL$^{(TM)}$ Educational Product – Copyright (C) 2010 Schrodinger, LLC (http://www.pymol.org/).

DISCUSSION

The pGEX-6P-2 vector was chosen as the expression vector because of its tight and inducible high expression capacity, purification of the fusion protein by affinity chromatography in mild condition that minimizes the effects on the antigenicity and functional activity of the protein. The expression is under the control of *taq* promoter which is chemically inducible with lactose analog isopropyl β-D thiogalactoside (IPTG) and also engineered with an internal *lac*I^{q} gene, product of which acts as a repressor protein. It binds to the operator region of *tac* promoter that prevents expression until induction by IPTG. Thus a tight control over expression of insert is maintained. The vector has multiple cloning sites (mcs) and a PreScission Protease cleavage site is present between the GST tag and the mcs. Thus it offers the most efficient method for cleavage and purification of GST fusion proteins. Site-specific cleavage is performed with simultaneous immobilization of protease as well as the GST tag on the column and thereby removed from the purified protein in the elute fractions.

Restriction sites, *Bam*H I and *Xho* I were chosen from the mcs of pGEX-6p-2 as these two sites are normally absent in the restriction mapping of previously reported *kerA* ORFs. To keep the insert in proper orientation and correct junction for maintaining the reading frame, *Bam*H I was used in forward primer and *Xho* I was used in reverse primer. Thus the desired amplicon reached 1156 bp in length (1140+ 2×8).

After the accomplishment of PCR amplification, double digestion of both vector and insert and ligation, construction of recombinant DNA was checked by transforming the ligation mixture into the competent *Escherichia coli* DH5α cells. Transformation with undigested vector and digested vector alone were also performed as positive and negative control of the experiment. A good number of transformants were found for the ligation mixture (vector: insert-1:3) and 15 clones were selected randomly to check for insert by PCR. PCR positive clones were then used for the propagation and extraction of pGEX-6p-2-kerA. Double digestion also revealed two DNA bands upon agarose gel electrophoresis at around 1 kb and 5 kb.

The recombinant pGEX-6p-2-kerA vector was used for sequencing with primers designed from the upstream and downstream region of PreScission Protease and *Xho*1 restriction site respectively to see whether the insert was in proper orientation and correct junction to maintain the reading frame. It was confirmed from the sequence analysis that Gln-Gly-Pro-Leu-Gly-Ser-Met was maintained i.e. the insert was in proper orientation. Thus the cloning of *kerA* gene was confirmed and the attempt was taken to express the GST-KerA fusion protein.

The transformants of *E. coli* BL21 was prepared transforming the pGEX-6p-2-kerA vector and was induced with IPTG in LB- ampicillin broth. Expression of a 58 kD GST-KerA fusion protein took place when induced with IPTG at a concentration of 0.1 mM and maximum expression level was attained at 3 h (Figure 3A). Hence, expression up to 3 h was continued with different IPTG

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concentrations starting from 0.1 mM to 0.5 mM and it was observed that the optimum IPTG concentration was 0.3 mM as the expression level remained unchanged from 0.3 mM onward (Figure 3B).

Purification of fusion protein was performed by cleaving the KerA protein with PreScission Protease from its GST counterpart in Glutathione Sepharose 4B batch purification method. Thus, the protein band for KerA (39 kD) was observed after purification. As the heterologous expression of KerA was performed as GST-fusion protein, neither signal peptide nor pro-protein were processed after to generate mature protein. So, the molecular weight of the protein seemed to be unusual from other serine proteases which are around 30 kD. It was also clarified when the molecular weight of the protein in pro-protein and matured form was determined from the deduced amino acid sequences to be 35.65 and 27.3 kD respectively.

The concentration of purified protein was found to be 119 mg/L which is an appreciable yield. The protease and keratinase activity of the purified keratinase was found to be 312 U/mL and 196 U/mL respectively. A 4-fold increase both in protease and keratinase activity have been obtained by heterologous expression of keratinase than the wild-type organism. The specific protease and keratinase activity was also determined and those were found to be 2621.84 U/mg and 1647 U/mg of protein respectively.

The sequences of all reported mature subtilisins of *B. licheniformis* strains have strong identity of amino acid sequences (Radha and Gunasekaran, 2007). It was not different in our study too. The blast result for the deduced amino acid sequences of KerA from *B. licheniformis* MZK05 revealed that 30 hits were with more than or equal to 98% identity. With thermostable keratinase from *Bacillus licheniformis* DS23 and KerA of *Bacillus licheniformis* OWU 1411T, 100% identity was observed and 98.4% identity was observed with KerA of *B. licheniformis* PWD-1 (*B. licheniformis* ATCC 53757).

The signal sequence obtained (Petersen *et al.*, 2011) has similar properties with 29 residues to other signal sequences from Gram positive bacteria. This includes a basic N-terminal segment followed by a stretch of uncharged residues. It was also reported that the Gram positive signal peptides are unusual both in length and charge compared with other prokaryotic and eukaryotic organisms and subtilisin Carlsberg signal peptide was typical for Gram positive bacteria with 29 residues (Jacobs *et al.*, 1985).

The N-terminal signal peptide is followed by a pro-peptide of 76 residues followed by the mature protein comprising of 274 residues and thus the prokeratinase of 350 residues. The amino acid residues at positions 150 and 153 are important for determining the specificity of Carlsberg protease (Takagi *et al.*, 1998) and for *B. licheniformis* MZK-05, amino acids at these positions are same as others, i.e. Ala-Ala-Ala-Gly.

Amino acids sequence alignment of the matured KerA from MZK-05 and PWD-1 revealed that differences are present in the following positions, i.e. Tyr26 instead of Phe26, Asn86/Ser86, Pro128/Ala128, Ser211/Asn211 and Ala222/Val222.

This divergence could be due to strain variation. The residue involved in catalysis is Ser220 and Val222 is one of the two residues near to the active site. It was reported that the larger side chain of Val222 in KerA in contrast to Ala222 may enhance keratin hydrolysis with the consequence of impaired kinetics on the charged amino acids at P1 (Evans *et al.*, 2000).

The three dimensional models of KerA_MZK05 were built with ProMod Version 3.70 simulating the 3-D models of PDB templates 4gi3.1.A, 1yu6.1.A, 1c3l.1.A whose sequence identities with our protein sequence were 98.54%, 97.81% and 98.18% respectively. Models simulating 4gi3.1.A, 1yu6.1.A and 1c3l.1.A were named as Model 01, Model 02 and Model 03 respectively. QMEAN scores estimated for Model 01, 02 and 03 were 0.60, 0.11 and -0.08 and were predicted with none, 1× Ca^{2+} and 2× Ca^{2+} ligand binding sites respectively.

Among these three models, Model 03 was considered to be the correct one because it was observed that the $CaCl_2$ has a positive influence over the keratinase activity of MZK-05. Addition of 0.5 mM $CaCl_2$ causes an increase of 20% in enzyme activity of the MZK-05 wild strain (Nizam *et al.*, 2006). Again, there are reports that Ca^{2+} plays an important role in stabilizing several members of the subtilisin protein family including subtilisin Carlsberg, subtilisin BPN or Proteinase K through two different Ca^{2+}-binding sites in these proteins (Pantoliano *et al.*, 1988). Thus, based on the ligand binding sites, Model 03 best fits the criteria.

In comparison to this, the absence of disulfide bonds in the enzyme was established in the present study which would facilitate easy and increased contact with the substrate resulting improved enzyme activity. The results were in conformance with the idea when the enzyme activity in shake flask and the specific activity were compared to the reports (Liu *et al.*, 2014). A 46% higher yield in keratinase activity (196 U/mL) was obtained in shake flask in this study than the reported activity (134 U/mL) by recombinant *Escherichia coli* BL21. On the other hand, they reported highest ever keratinase activity from recombinant *B. subtilis* (3,010 U/mL) and a moderate activity from *P. pastoris* (1,050 U/mL), when cultured in a 3.0 L bioreactor. Based on the specific enzyme activity, these results were compared with our study as it was done in shake flask. The reported specific activities of purified ker E (*E. coli* BL21), ker B (*B. subtilis*), and ker P (*P. pastoris*) were almost similar i.e. 1,206.8, 1,286.7, and 1,245.6 U/mg respectively, an indication of efficiency of the enzyme, whereas it was 1647 U/mg in our study which is 24% higher than their average activity. Actually, enzyme activity does not necessarily represent the efficiency of an enzyme unless the specific enzyme activity is determined. From this point of view, expression of a highly efficient keratinase enzyme was successful in our study which can be scaled up into a bioreactor for industrial level production.

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Regarding the protein expression, presence of six potential N-Glycosylation sites in the mature protein sequence also suggests expression at higher level in the eukaryotic expression systems such as Yeast and also enhanced expression by engineering additional glycosylation sites near the *N*-termini of the target protein.

CONCLUSION

The cloning of *kerA* gene from *B. licheniformis* MZK-05 into pGEX-6p-2 vector, its expression in *Escherichia coli* BL21 and prediction of 3-D model of the expressed protein as well as their active site and ligand binding site determination were performed which will be the basis for large scale production of keratinase for its technical applications. This has also provided the scope of protein engineering with the predicted model of keratinase.

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