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Research article

Heterologous expression and enhanced production of β-1,4-glucanase of *Bacillus halodurans* C-125 in *Escherichia coli*

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ABSTRACT

Background: Recombinant DNA technology enables us to produce proteins with desired properties and insubstantial amount for industrial applications. Endo-1, 4-β-glucanases (Egl) is one of the major enzyme involved in degradation of cellulose, an important component of plant cell wall. The present study was aimed at enhancing the production of endo-1, 4-β-glucanases (Egl) of *Bacillus halodurans* in *Escherichia coli.*
Results: A putative Egl gene of *Bacillus Halodurans* was expressed in *E. coli* by cloning in pET 22b (+). On induction with isopropyl-b-D-1-thiogalactopyranoside, the enzyme expression reached upto ~20% of the cell protein producing 29.2 mg/liter culture. An increase in cell density to 12 in auto-inducing LB medium (absorbance at 600 nm) enhanced β-glucanase production up to 5.4 fold. The molecular mass of the enzyme was determined to be 39 KDa, which is nearly the same as the calculated value. Protein sequence was analyzed by CDD, Pfam, I TASSER, COACH, PROCHECK Servers and putative amino acids involved in the formation of catalytic, substrate and metal binding domains were identified. Phylogenetic analysis of the β-glucanases of *B. halodurans* was performed and position of Egl among other members of the genus *Bacillus* producing endo-glucanases was determined. Temperature and pH optima of the enzyme were found to be 60°C and 8.0, respectively, under the assay conditions.
Conclusion: Production of endo-1, 4 β-glucanase enzymes from *B. halodurans* increased several folds when cloned in pET vector and expressed in *E. coli*. To our knowledge, this is the first report of high-level expression and characterization of an endo-1, 4 β-glucanases from *B. halodurans*.
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1. Introduction

Endo-1,4-β-glucanases (EC 3.2.1.4) play a significant role for hydrolyzing large and complex cellulose chains into oligosaccharides [1]. These enzymes act randomly on the interior β 1,4 linkages of the less crystalline region, resulting in a rapid change in the degree of polymerization, which is favorable for further processing of cellulose to glucose [2].

Cellulases are produced by several types of fungi, actinomycetes, and bacteria including actinomycetes [3,4]. For commercial scale production of cellulases (particularly endoglucanases), cost effectiveness is desirable. Recombinant expression of endo-1,4-β-glucanases in *E. coli* enables robust and cost effective production and ultimately better saccharification of cellulosic substrates.

Several researchers previously reported the production of endo-1,4-β-glucanases in bacteria [5,6,7]. Genes encoding the enzymes from different sources, including *cel C* of *Clostridium thermocellulum* [8], *Cel5A* of *Eubacterium cellulosolvens* [9] and *celA* of *Thermobifida fusca* [10], *Cel9A* of *Cytophaga hutchinsonii* [11] have been cloned and expressed in *E. coli*. Facultative alkalophilic bacterium *Bacillus halodurans* C-125 grows well at pH 7 to 10.5. *Bacillus halodurans* C-125 genome (NCBI accession no. NC_002570.2) contains 4076 genes (85), of which several are putative endo-1, 4 β-glucanase genes (*Egl*). One of the genes, designated as BH_RS15755, found in position 324, 8335 to 324, 9420 in the genome sequence, encodes a putative endo-1,4 β-glucanase (*Egl*) of 361 amino acids and belongs to peptidase M42 family (UniProt ID: wp_010899276) [12]. The function of the protein of this gene is predicted *in silico* using gene prediction method by homology modeling. Here we reported for the first time

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the functional analysis of this gene product by cloning, expression, and characterization of this endo-1,4 β-glucanase (*Egl*) in *E. coli*.

2. Materials and methods

2.1. PCR Amplification and Cloning of Egl

B. halodurans C-125 was grown aerobically at 37°C in 50 ml LB broth (Sigma-Aldrich, USA). Genomic DNA was extracted as reported elsewhere [13] with some modifications. Endo-1,4 β-glucanase gene was amplified by PCR using genomic DNA as a template with gene specific forward FEgl (5'-*CATATG*GCACAATTAGACGAACGATTG-3′) and reverse primer REgl (5'-*GGATCC*TTAATCAAATGTAATGCGGTTCACG-3′) such that *Nde*I and *Bam*HI recognition sequences (shown respectively as underline and italicized) were placed at 5′ and 3′ ends of the coding region. The PCR reactions were set in 50 μl reaction volume and conditions were as follows; one cycle of initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 30 s, extension at 72°C for 60 s and a cycle of the final extension at 72°C for 20 min [14].

The amplified product was purified using Invitrogen gel extraction kit, T/A cloned in pTZ57R/T vector, and then subcloned between *Nde*I and *Bam*HI sites of the pET-22b (+) vector to generate chimeric pET-Egl expression plasmid, which was maintained in *E. coli* DH5α, selected on LB medium containing 100 μg/ml ampicillin. The junction sequences of the recombinant plasmid were confirmed by Sanger's sequencing.

2.2. Expression analysis of Egl

Competent *E. coli* BL21(DE3) CodonPlus were transformed with the recombinant plasmid pET-Egl for expression analysis. Transformants (containing recombinant vector) were confirmed by restriction digestion and colony PCR. For expression analysis, one of the positive colony was grown overnight in 25 ml LB-ampicillin medium at 37°C. One percent of this overnight culture was used to inoculate 200-ml fresh medium followed by incubation at 37°C in an orbital incubator shaker at 150 rpm. Protein expression was induced with either 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at culture density A_{600} of 0.5–0.6. Cells were allowed to grow till the maximum growth was attained (~12 h) and then harvested by centrifugation at 4500 rpm for 15 min. During cultivation, 1-ml aliquots were collected after every 2 h to monitor the growth (A_{600}) and analyze protein expression by 12% SDS-PAGE [15]. Percentage of Egl was determined by densitometric scanning of the Coomassie stained gel.

2.3. Purification of endo-1,4 β-glucanase

Culture was grown in 250 ml LB media was harvested by centrifugation (6500 rpm, 10 min), resuspended in 50 ml of 50 mM glycine-NaOH buffer (pH 8.0) and disrupted by sonication (10 × 30 s bursts with intervals of 1 min between successive pulses) in a UP 400s Ultrasonicator (Dr. Hielscher GmbH, Germany). Lysate thus obtained was again centrifuged as described previously, and soluble proteins in the supernatant were purified for the enzyme activity assay. For purification supernatant were precipitated by adding ammonium sulfate slowly to 75% saturation at 4°C and incubated for 6 h. The precipitates were collected by centrifugation at 6500 rpm for 30 min at 4°C. The precipitates were dissolved in 25 ml of 50 mM glycine-NaOH buffer (pH 8.0) and dialyzed overnight against three changes of the same buffer. The solution was filtered through 0.45 μm Millipore filter and loaded on the Sephadex G-75 column (1.6 × 40) for gel filtration chromatography. After equilibration with 50 mM glycine-NaOH buffer (pH 8.0), the sample was loaded and the proteins were eluted with the same buffer at a flow rate of 0.5 ml per min. Collected fractions were analyzed for Egl activity.

2.4. Enzyme (Endo-1,4 β-glucanase) assay

Endo-1, 4 β-glucanase activity of the supernatant after sonication and purified fractions was determined by dinitrosalicylic acid (DNS) method [16]. For the assay, 0.5 ml of the appropriately diluted enzyme in 0.05 M Tris-Cl buffer (pH 8.0) along with 1% Carboxymethylcellulose (Sigma Chemicals Co. USA) was incubated at 60°C for 20 min in a shaking water bath. After incubation, the reaction was stopped by adding 3 ml of DNS reagent and heating in boiling water for 10 min. The liberated reducing sugars in the supernatant were quantified at 590 nm and activity was calculated from glucose standard graph. One unit of enzyme activity is defined as the amount of enzyme which released 1 μmol of reducing sugars equivalent to glucose per minute under the standard assay conditions.

2.5. Endo-1,4 β-glucanase characterization

Effect of pH on enzyme activity at 60°C was determined by maintaining a different pH of assay mixtures with 0.05 M potassium phosphate buffer (pH 6.0–7.0), 0.05 M Tris-Cl buffer (pH 7.5–9.0) and glycine-NaOH buffer (pH 8.0–10.0).

Stability was expressed as a percentage of residual activity. Stability at different pH (6.0–10.0) was determined by incubating suitably diluted enzyme in buffers of various pH at room temperature for 1 h and determining the residual activity at 60°C under the standard assay conditions. Thermal stability was determined by pre-incubating enzyme in 0.05 M glycine-NaOH buffer pH 8.5 at various temperatures ranging from 30–70°C. The enzyme samples were drawn after 1 h, and residual enzyme activity was determined as below:

$$\text{Residual activity (percent)} = (\text{enzyme activity before incubation} - \text{enzyme activity after incubation}) \times 100$$

2.6. In Silico protein characterization and phylogenetic analysis

Protein sequences of endo-1,4 β-glucanase from 20 different species were retrieved using the BLAST program available at the NCBI server. Multiple sequence alignments were performed using the Clustal W program with default parameters using the alignment explorer option of the MEGA 5.2.1 software package [17]. The distance was calculated by the maximum parsimony model [18] and a phylogenetic tree was constructed [19]. The 3-D structure of the protein was predicted by I-TASSER [20], and different domains were predicted by PROCHECK [21], COACH [22] server, and family was checked by Pfam database [23].

3. Results and discussion

3.1. Construction of recombinant expression plasmid

About 1.086-nucleotide long fragment of Egl containing *Nde*I and *Bam*HI sites at the 5′ and 3′ termini, respectively, was PCR amplified with different $MgCl_2$ concentrations (1.5, 2.0, 2.5 mM was used) to optimize the PCR conditions (Fig. 1). Two millimolars of $MgCl_2$ was found to be the best concentration for amplification (Fig. 1). Variations of the Mg^{2+} concentration below 4 mM, can improve the performance of PCR by affecting the specificity (lower concentrations raise specificity, higher concentrations lower the specificity) [24]. The amplified PCR product was cloned in T7/*lac* promoter based pET-22b (+) vector at *Nde*I/*Bam*HI sites to generate pET-Egl expression plasmid (Fig. 2). The recombinant plasmid was first maintained in *E. coli* DH5α for vector propagation and then transformed into *E. coli* BL21 (DE3) CodonPlus for expression studies. The presence of the insert in positive transformants was confirmed by colony PCR (Fig. S1)

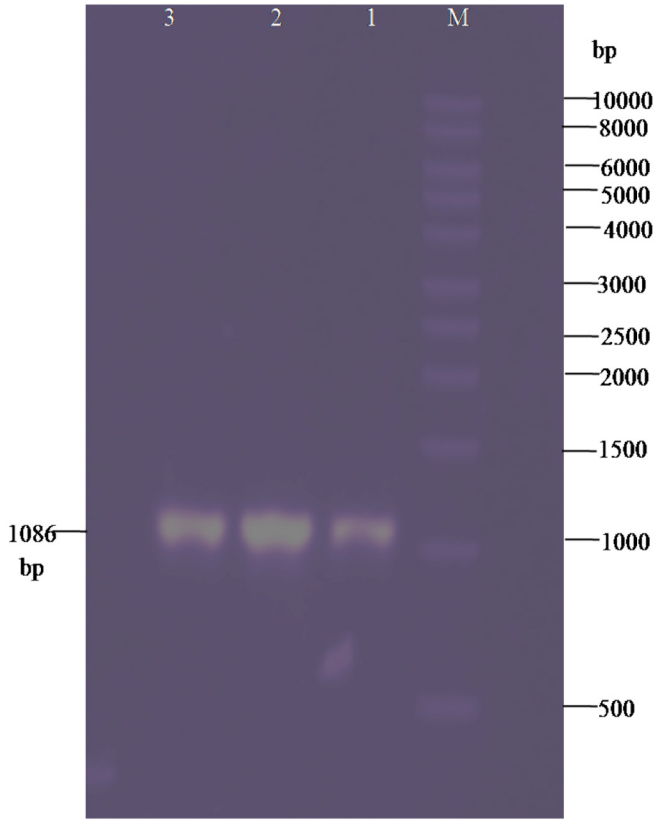

Fig. 1. 1% Agarose gel electrophoresis of the PCR product amplified with FEgl and REgl primers using different $MgCl_2$ concentrations. M, is the molecular weight marker (GeneRuler™ 1-kb DNA ladder by Sigma); Lane 1–3, PCR product amplified with 1.5, 2.0 and 2.5-mM $MgCl_2$ concentration.

and restriction digestion (Fig. 3), which showed the presence of a 1.086 kb fragment. Use of two different restriction sites at 5′ and 3′ of the coding region of the gene of interest resulted in a successful in-frame insertion of the gene for expression studies as reported previously [19,20].

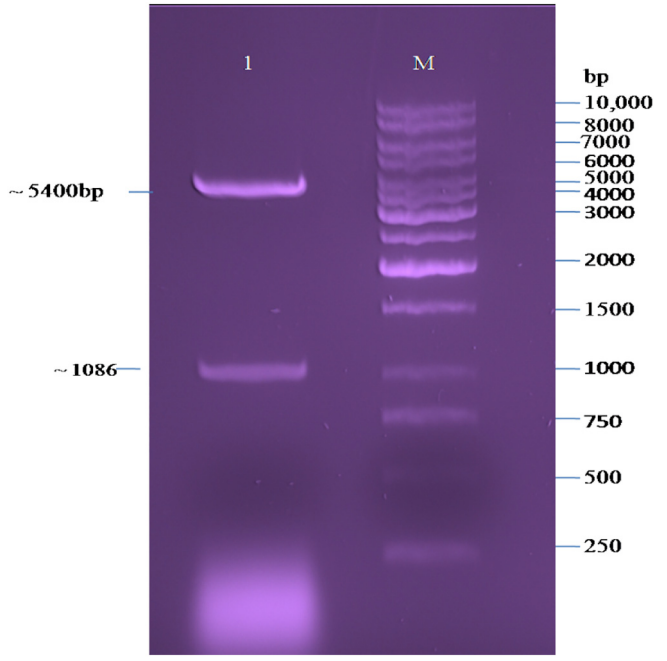

Fig. 3. Restriction analysis of recombinant plasmids pET-Egl on 1% agarose gel. Lane 1, Double cut plasmid (pET-Egl) with *Nde*I and *Bam*HI, M on the right panel base pairs of 1 kb DNA ladder are shown to relate the size of the insert.

3.2. Heterologus expression of Egl

E. coli cells harboring pET-Egl were grown in LB-ampicillin and induced initially with IPTG. Protein analysis of the induced *E. coli* cells by SDS-PAGE showed a band at a position corresponding to ~39 kDa, which matches the calculated molecular mass of Egl (Fig. 4). As

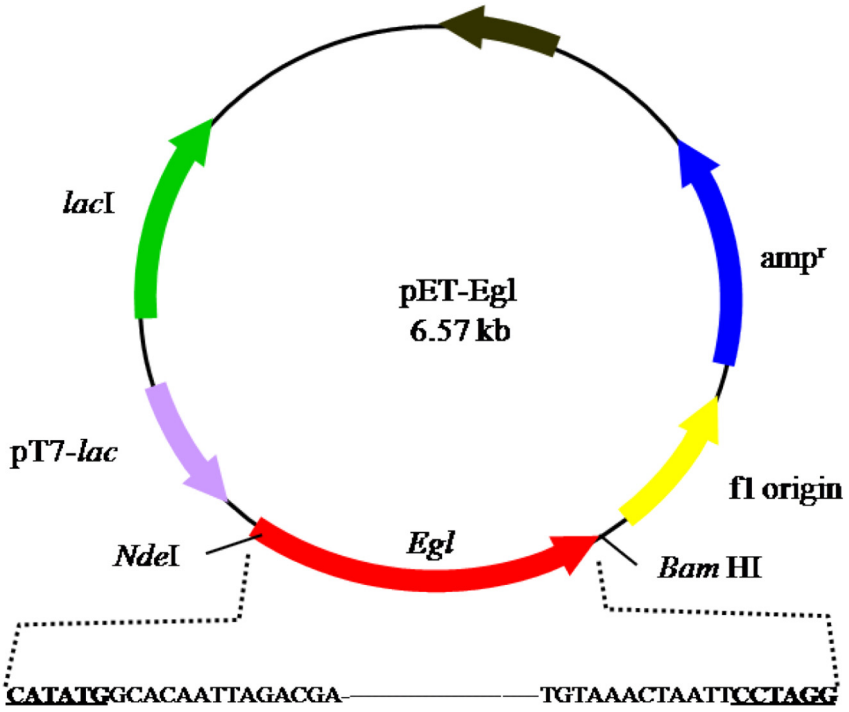

Fig. 2. Construction of recombinant plasmids pET-Egl. A 1.08 kb long Egl gene was cloned in pET-22b (+) expression vector downstream of the T7-*lac* promoter (pT7-*lac*) to generate pET-Egl. fi ori, F1 origin of replication; amp^r, gene for ampicillin resistance; *lac*I, Lac repressor gene.

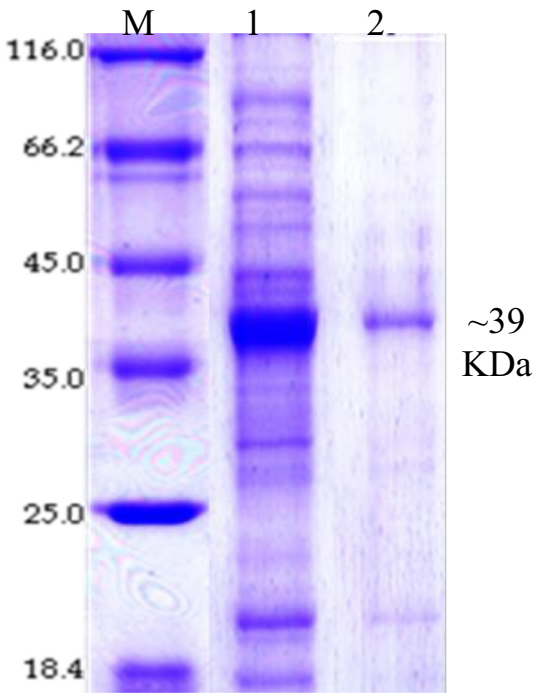

Fig. 4. SDS-PAGE of the total cell proteins of *E. coli* BL21 (DE3) harboring pET-Egl at optimized growth conditions (10-mM lactose, after 12-h of post-induction). Lane M, protein molecular weight marker; lane 1, Total cell protein and lane 2, purified Egl.

expected, no band of similar size and intensity was present in the uninduced cells. The expression levels increased rapidly reaching a maximum of ~20% of the total *E. coli* cellular proteins within ~12 h of induction and remained constant thereafter. Optimal IPTG concentration for induction was found to be 1 mM (Fig. S2).

The alternative inducer of the *lac* operon is lactose, and 10 mM lactose (Fig. S3) was used as an inducer for the expression of recombinant pET-Egl (Fig. 4). The protein expression Egl obtained with lactose is comparable to expression with IPTG (Table 1). The main drawbacks of IPTG are its expense, toxicity to humans and inhibit the growth of microorganisms [21,22]. Therefore employed lactose (10 mM) instead of IPTG (1 mM), especially in the case of large-scale preparations reduces the cost of expression up to 0.3% [23,24]. Lactose supported the exponential growth of *E. coli* to relatively higher cell densities (Table 1), thereby, increasing the production of Egl by 5.44 fold as compared to IPTG (Table 1). Use of lactose in replacement of IPTG as an inducer for expression of enzymes and other recombinant proteins has already been suggested by Khan et al. [25] and Naz et al. [26].

3.3. Purification of Egl

Two-step procedure was adopted for purification of endoglucanase and enzyme from 250 ml of the sonicated cell lysate supernatant was precipitated with 75% saturated ammonium sulfate. After dialysis protein showed 1.88 fold purification with a recovery of ~64.4%. The pooled fractions containing Egl activity from the gel filtration column showed specific activity 4.77 U/mg, with 2.62-fold purification and a recovery of ~56% (Table 2). Previously specific activities of β-glucanase from various microorganisms vary in the range of 5–150 U/mg [27,28,29].

3.4. Thermal and pH stability of Egl

Egl showed the highest activity at pH 8.0 in Tris-Cl buffer (Fig. 5A). The enzyme retained 95% residual activity at pH 8.5–9.0 when incubated at 60°C for 1 h. The enzyme, however, was found to lose activity at pH >9.0, the rate of inactivation increasing with increasing pH. At pH 9.5, the residual activity was only about 20% after incubation at 60°C for 1 h (Fig. 5B). The optimal temperature under the assay conditions was 60°C. The enzyme activity was 4.2 U/ml when assayed against CMC as substrate (Fig. 5C). Egl retained 85% of its activity on incubation at temperatures up to 60°C for 1 h, losing activity with longer incubation periods. At higher temperatures, a sharper decline in the activity was observed (Fig. 5D). The optimal temperature for CMCase activities of *Bacillus* strains was in the range of 40–60°C [30,31]. The characteristics of recombinant Egl of the present study are also similar to a previous report [32] where the isolated enzymes were active at pH 10.0 and temperature 50°C.

3.5. Molecular characterization and phylogeny of Egl

The enzyme was characterized by its sequence similarities with endo-1,4 β-glucanase genes from related *Bacillus* species and a phylogenetic tree was constructed using the maximum parsimony [33]. The Egl sequence tree separated *Bacillus halodurans* C-125 as a distinct clade from other *Bacilli (B. subtilis, B. methanolicus, B. licheniformis, B. megaterium and B. cereus)* as shown in Fig. 6. The evolutionary history was inferred using the Maximum Parsimony method and Bootstrap scores (500 replicates) are shown next to the branch. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the

Table 1
Comparison of Egl production when induced with 1-mM IPTG and 10-mM lactose at 37°C.

Time (Hours)	IPTG				Lactose			
	Optical Density OD $_{600nm}$	TC[a] mg/l	% age of expression	Egl mg/l	Optical Density OD $_{600nm}$	TC[a] mg/l	% age of expression	Egl mg/l
4	1.1	152	6	12.0	2.1	590	6	35
8	2.1	230	8	18.4	4.5	955	11	105
12	3.5	290	10	29.2	7.9	1450	11	159.5

[a] Total cell proteins after sonication.

Table 2
Summary of purification of Egl expressed in *E. coli* after induced with lactose at 37°C.

Purification Steps	Total protein (mg)	Total activity (Ul)	Specific activity (U/mg)	Recovery (%)	Purification yield (fold)
Sonicated cell lysate supernatant	620	1125	1.82	100	1
Ammonium sulfate precipitation	320	725	2.26	64.4	1.88
Sephadex G-75 Gel filtration	132	630	4.77	56	2.62

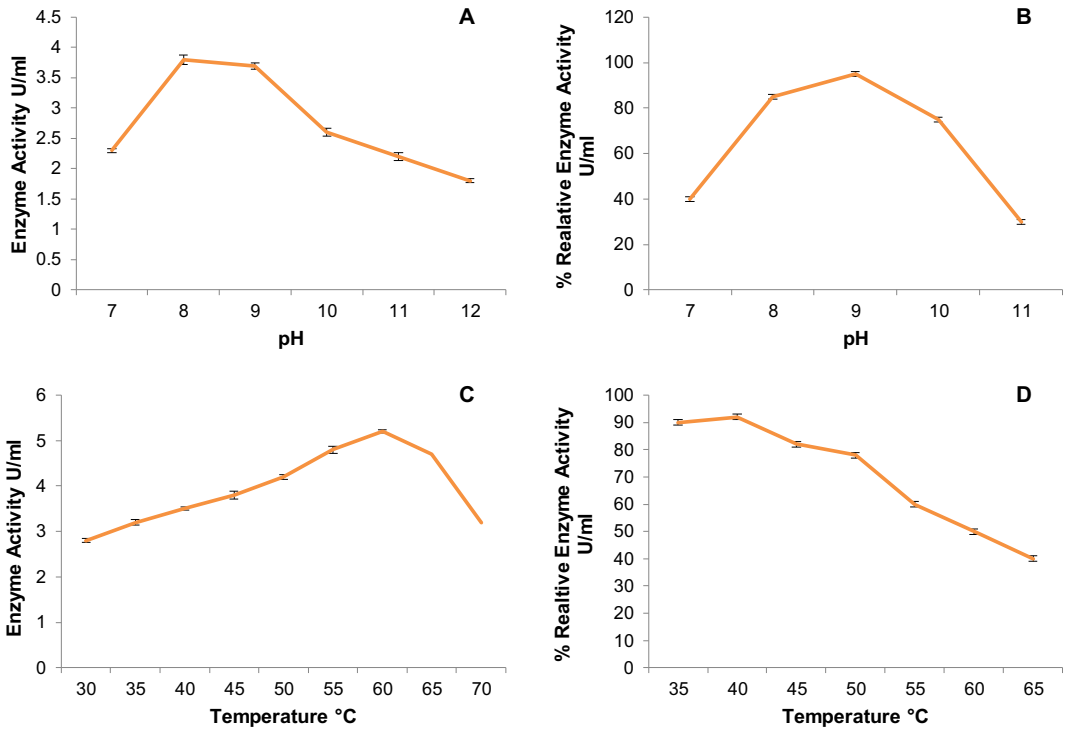

Fig. 5. Effect of pH on activity (A) and stability of Egl (B) and Effect of temperature on activity (C) and stability (D) of recombinant Egl.

random addition of sequences (100 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. The relative positions of these genes are well supported by bootstrapping.

3.6. 3-D structure and active site prediction

Since no single structure of Engl is available in Protein Data Bank (PDB), the 3-D homology model was built by using an online web server I-TASSER (Fig. 7). It selects templates by multiple threading

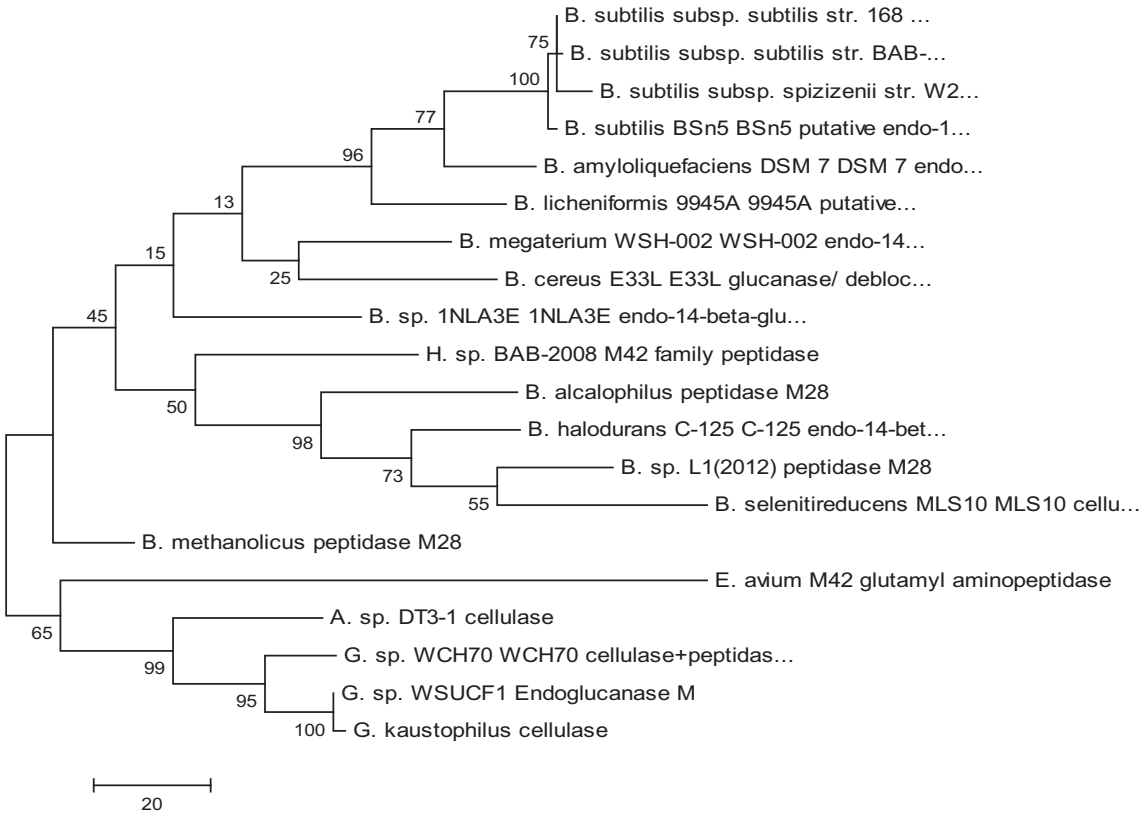

Fig. 6. Phylogenetic dendrogram of the complete nucleotide sequences of the endo-1, 4 β-glucanase genes from 20 different species available in the databases.

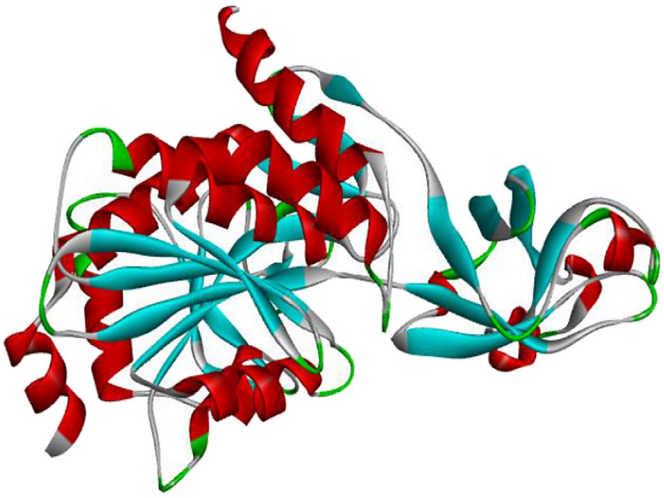

Fig. 7. 3-D structure of Engl predicted by I-TASSER.

approaches and predicts full-length model by iterative structure assembly. I-TASSER predicts 3D models that can be evaluated based on C-score, TM-score, and RMSD. As the only single structure was predicted by I-TASSER for Egl and no other model was available for comparative study, this single structure based on confidence score (C-score) was used for the further computational study. The c-score value in I-TASSER ranges between −5 to 2, higher the C-score value better will be the predicted structure. Egl 3-D model predicted by I-TASSER showed the C-score value of 1.71. The structure predicted by I-TASSER was further validated based on Ramachandran Plot generated through PROCHECK (Fig. 8). The Ramachandran plot generated by PROCHECK showed 82.5% amino acids in red (favored), 15.6% in brown (allowed), 1% in yellow (generously allowed) and 1% in the disallowed region based on Phi and Psi angles Fig. 8. The model gave 97.45% ERRAT quality factor and 96.95% VERIFY3D 3D-1D score.

The protein sequence analysis of Egl of *Bacillus halodurans* C-125 in Conserved Domain Database (CDD) showed that the protein comprises of three sites for the function of the enzyme, oligomer interference, active site, and metal binding site. All three sites are

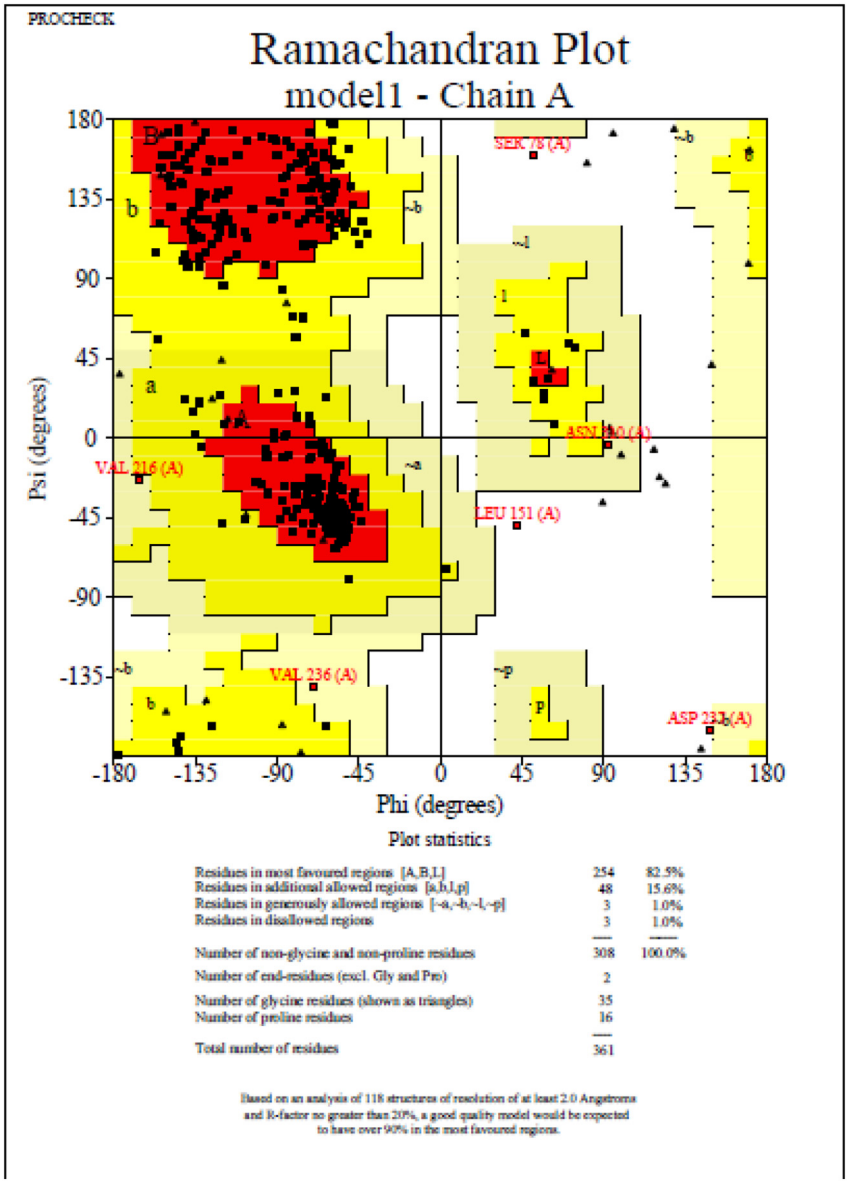

Fig. 8. Validation of 3-D model by Ramachandran Plot predicted by PROCHECK web server.

Table 3
Table showed the possible ligand binding sites of Egl predicted by by I-TASSER COACH Server.

Rank	C-score	Cluster size	PDB[a] hit	Ligand name[b]	Consensus binding residues
1	0.74	93	2cf4A	CO	68, 182, 183, 214, 215, 237
2	0.64	76	514mA	ZN	182, 215, 325
3	0.31	23	1v0yA	PEPTIDE	68,182, 214, 215, 237, 238, 239, 240, 295, 298, 299, 324, 325
4	0.21	3	2pokA	BGC	62, 232, 274, 278,349, 350
5	0.00	1	3GB0A	3GB0A00	18, 19, 106, 160, 161, 162
6	0.00	1	1R43A	1R43A00	214, 217, 218
7	0.00	1	1R43A	1R43A01	12, 16, 166
8	0.00	1	2QYVA	2QYVA02	10,13, 33, 34
9	0.00	1	2RB7B	2RB7B02	195, 196, 337, 341
10	0.00	1	1lokA	NA	280, 284, 290

[a] Protein Data Bank.
[b] Ligand Name with details is available in PDB.

representative of proteins belong to the family M42_Frv (conserved protein domain family database available at NCBI).

Template based binding site was predicted using I-TASSER COACH server. It predicts ligand-binding site by combining five different programs namely TIM-SITE, S-SITE, COFACTOR, FINDSITE, and ConCavity. Ten possible ligands against their PDB hits and ranked based on their C-score value. Higher the C-score value better will be the ligand bound interactions (Table 3). Binding of ligands (as determined by C-score) showed the possible amino acid residues of Egl involved in the binding of enzyme with its substrate (Fig. 9). Possible binding sites for cellulose are at position 62, 232, 274, 278, 349 and 350 (as predicted by ranked 4th ligand BGC; beta- D-glucose in Table 3).

CDD search also showed the amino acids H68, D182, N214, V215, G218, D237, I240, M295, G298, G299, I324, H325 constituted the active site domain of Egl. Amino acids at position H68, D182, N214, V215, D237, H328 formed the metal binding sites of the enzyme as shown in Fig. 9 **(A and B).** The gene is found to be conserved in a number of thermophiles, archaea and pathogenic bacterial species; the closest structural homolog is *Thermotoga maritima* FrwX (34% identity), which is annotated as either a cellulase or an endoglucanase and is possibly involved in polysaccharide metabolism.

In this study, we reported *in silico* characterization of sequence features of the Egl of *Bacillus halodurans* C-125 as well as functional analysis of the gene by cloning and characterization of the Egl gene in *E. coli*. In this study by expressing the Egl in pET vector and using the alternate inducer, we demonstrated 5.44 fold increase in production of the enzyme. Enhancing the production of industrially important enzymes by heterologous expression showed a good alternative to conventional enzyme production.

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Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgment

We are also thankful to Prof. Waheed Akhtar Director, School of Biological Sciences, University of the Punjab, Lahore for generously providing vector system for expression studies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbt.2018.05.001.

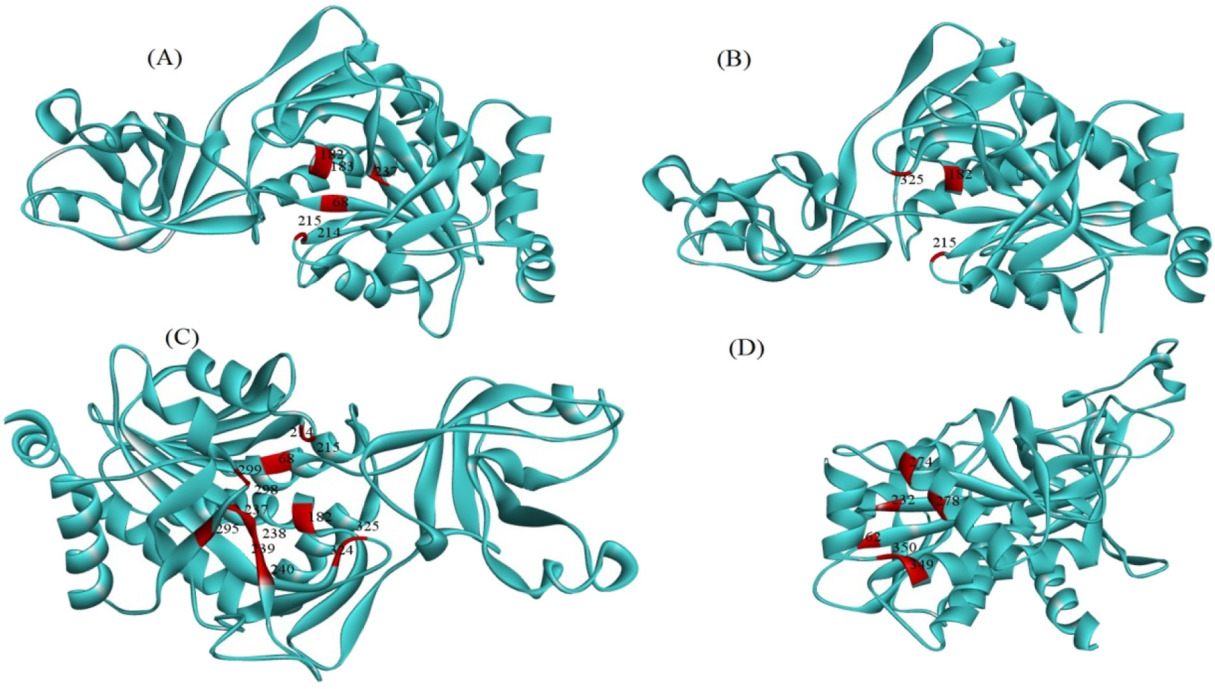

Fig. 9. Predictions of possible ligand banding sites by I-TASSER COACH server Engl enzyme shown in 3-D structures. (A) Binding with cobalt, (B) binding with Zn, (C) binding with peptide, (D) binding with Beta D glucose.

References

[1] Sturcova A, His I, Apperley DC, et al. Structural details of crystalline cellulose from higher plants. Biomacromolecules 2004;5(4):1333–9. https://doi.org/10.1021/bm034517p.

[2] Kadla Jf, Gilbert RD. Cellulose structure: a review. Cellul Chem Technol 2000;34(3–4):197–216.

[3] Sharada R, Venkateswarlu G, Venkateshwar S, et al. Production of cellulase-a review. Int J Pharm Chem Biol Sci 2013;3(4):1070–90.

[4] Rasul F, Afroz A, Rashid U, et al. Screening and characterization of cellulase producing bacteria from soil and waste (molasses) of sugar industry. Int J Biosci 2015;6(3):230–6. https://doi.org/10.12692/ijb/6.3.230-238.

[5] Kleman-Leyer, Gilkes NR, Miller RC, et al. Changes in the molecular size distribution of insoluble celluloses by the action of recombinant *Cellumonas fimi* cellulases. Biochem J 1994;302(2):463–9. https://doi.org/10.1042/bj3020463.

[6] Woodward J, Wiseman A. Fungal and other β-D-glucosidases, their properties and applications. Enzyme Microb Technol 1982;4(2):73–93. https://doi.org/10.1016/0141-0229(82)90084-9.

[7] Tomme P, Van Tilbeurgh H, Pettersson G, et al. Studies of the cellulolytic system of *Trichoderma reesei* QM9414. Eur. J. Biochem 1988;170(3):575–82. https://doi.org/10.1111/j.1432-1033.1988.tb13736.x.

[8] Tcika K, Zverlov VV, Velikodvorskaya GA. Synergism between *Clostridium thermocellum* cellulases cloned in *Escherichia coli*. Appl Biochem Biotechnol 1992;37(2):201–7. https://doi.org/10.1007/BF02921671.

[9] Yoda K, Toyoda A, Mukoyama Y, et al. Cloning, sequencing, and expression of a *Eubacterium cellulosolvens* 5 gene encoding an endoglucanase (Cel5A) with novel carbohydrate-binding modules, and properties of Cel5A. Appl Environ Microbiol 2005;71(10):5787–93. https://doi.org/10.1128/AEM.71.10.5787-5793.2005.

[10] Kostylev M, Alahuhta M, Chen M, et al. Cel48A from *Thermobifida fusca*: structure and site directed mutagenesis of key residues. Biotechnol Bioeng 2014;111(4):664–73. https://doi.org/10.1002/bit.25139.

[11] Fierobe HP, Bayer EA, Tardif C, et al. Degradation of cellulose substrates by cellulosome chimeras substrate targeting *versus* proximity of enzyme components. J Biol Chem 2002;277:49621–30. https://doi.org/10.1074/jbc.M207672200.

[12] Takami H, Nakasone K, Takaki Y, et al. Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. Nucleic Acids Res 2000;28(21):4317–31. https://doi.org/10.1093/nar/28.21.4317.

[13] Rodriguez RL, Tait RC. Recombinant DNA techniques, an introduction. 1st ed. Reading, MA, USA: Addison-Wesley Publishing Company; 1983 [236 pp.].

[14] Sambrook J, Russell DW. Molecular cloning. A laboratory manual. 3rd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 2001; 727–50.

[15] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;277:680–5. https://doi.org/10.1038/227680a0.

[16] Ghose TK. Measurement of cellulase activities. Pure Appl Chem 1987;59(2):257–68. https://doi.org/10.1351/pac198759020257.

[17] Kumar S, Nei M, Dudley J, et al. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 2008;9(4):299–306. https://doi.org/10.1093/bib/bbn017.

[18] De Laet J. Parsimony analysis of unaligned sequence data: maximization of homology and minimization of homoplasy, not minimization of operationally defined total cost or minimization of equally weighted transformations. Cladistics 2014;31(5):550–67. https://doi.org/10.1111/cla.12098.

[19] Kolaczkowski B, Thornton JW. Performance of maximum parsimony and likelihood phylogenetics when evolution is heterogeneous. Nature 2004;431:980–4. https://doi.org/10.1038/nature02917.

[20] Yang J, Yan R, Roy A, et al. The I-TASSER suite: protein structure and function prediction. Nat Methods 2015;12:7–8. https://doi.org/10.1038/nmeth.3213.

[21] Laskowski RA, MacArthur MW, Moss DS, et al. *PROCHECK*: a program to check the stereochemical quality of protein structures. J Appl Cryst 1993;26(2):283–91. https://doi.org/10.1107/S0021889892009944.

[22] Xue Z, Xu D, Wang Y, et al. ThreaDom: extracting protein domain boundary information from multiple threading alignments. Bioinformatics 2013;29(13):i247–56. https://doi.org/10.1093/bioinformatics/btt209.

[23] Finn RD, Tate J, Mistry J, et al. The Pfam protein families database. Nucleic Acids Res 2008;36(Suppl. 1):D281–8. https://doi.org/10.1093/nar/gkm960.

[24] Blanchard MM, Taillon-Miller P, Nowotny P, et al. PCR buffer optimization with a uniform temperature regimen to facilitate automation. Genome Res 1993;2:234–40. https://doi.org/10.1101/gr.2.3.234.

[25] Khan MA, Sadaf S, Sajjad M, et al. Production enhancement and refolding of caprine growth hormone expressed in *Escherichia coli*. Protein Expr Purif 2009;68(1):85–9. https://doi.org/10.1016/j.pep.2009.05.011.

[26] Naz S, Ikram N, Rajoka MI, et al. Enhanced production and characterization of a β-glucosidase from *Bacillus halodurans* expressed in *Escherichia coli*. Biochemistry (Mosc) 2010;75(4):513–8. https://doi.org/10.1134/S0006297910040164.

[27] Wood TK, Peretti SW. Effect of chemically induced, cloned gene expression on protein synthesis in *E. coli*. Biotechnol Bioeng 1991;38(4):397–412. https://doi.org/10.1002/bit.260380410.

[28] Bentley WE, Davis RH, Kompala DS. Dynamics of CAT expression in *E. coli*. Biotechnol Bioeng 1991;38(7):749–60. https://doi.org/10.1002/bit.260380709.

[29] Monteiro RA, Souza EM, Yates MG, et al. Use of lactose to induce expression of soluble NifA protein domains of *Herbaspirillum seropedicae* in *Escherichia coli*. Can J Microbiol 2000;46(11):1087–90. https://doi.org/10.1139/w00-094.

[30] Ou J, Wang L, Ding X, et al. Stationary phase protein overproduction is a fundamental capability of *Escherichia coli*. Biochem Biophys Res Commun 2004;314(1):174–80. https://doi.org/10.1016/j.bbrc.2003.12.077.

[31] Sadaf S, Khan MA, Akhtar MW. Production of bubaline somatotropin by auto-induction in *Escherichia coli*. Biotechnol Appl Biochem 2007;47(1):21–6. https://doi.org/10.1042/BA20060154.

[32] Fukumori F, Kudo T, Horikoshi K. Purification and properties of a cellulase from alkalophilic *Bacillus* sp. no. 1139. J Gen Microbiol 1985;131:3339–45. https://doi.org/10.1099/00221287-131-12-3339.

[33] Fukumori F, Sashihara N, Kudo T, et al. Nucleotide sequences of two cellulase genes from alkalophilic *Bacillus* sp. strain N-4 and their strong homology. J Bacteriol 1986;168(2):479–85. https://doi.org/10.1128/jb.168.2.479-485.1986.