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Recombinant Expression and Characterization of Endoglucanase Isolated from Iranian *Bacillus subtilis*

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Abstract

Introduction: Endo-β-1,4-glucanase is the first enzyme in conversion of cellulose to fermentable sugars. The objectives of this study were to clone and characterize a thermostable endo-β-1,4-glucanase enzyme of *Bacillus subtilis* DR-8806 obtained from water samples from Dig Rostam, a hot mineral spring in Kerman, Iran.

Materials and Methods: Endo-β-1,4-glucanase gene from a thermostable *B. subtilis* bacterium was cloned and expressed in *Escherichia coli*. The recombinant proteins of the expression cell were tested by western blotting analysis. The enzymatic activity of the recombinant endoglucanase was measured using dinitrosalicylic acid method and carboxymethyl cellulose as substrate. Bioinformatics analysis was done to characterize domain organization and protein family through Pfam search server and PROSITE.

Results: Based on 16S ribosomal RNA sequence analysis, *Bacillus* is characterized and named as *B. subtilis* DR-8806. Western blot analysis verified the recombinant endoglucanase by detecting a specific band of ~55 kDa. Amino acid homology analysis of the protein showed 99% homology with that of endoglucanase from *B. subtilis*. The optimum temperature for enzyme reaction was attained at a temperature of 55°C. The cellulolytic activity of endo-β-1,4-glucanase protein determined 8.5 IU mL^{-1}. It showed that endoglucanase amino acid sequence contains a glycosyl hydrolase family 5, linker domain and a cellulose binding type 3 domain. The GH5 domain also contained a glycosyl hydrolase catalytic core.

Conclusions: It is possible to consider the purified endo-β-1,4-glucanase of *B. subtilis* DR-8806 as an efficient cellulose producer. Further research is required to examine the industrial applications of this study.

Keywords: Endoglucanase Enzyme, Cloning, Expression, *Bacillus subtilis*

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Introduction

Cellulose is one of the most abundant organic polymers on earth, and the largest form of fixed carbon in nature.[1] According to enzymatic mode of action and substrate specificity, cellulases are divided into three different types including: Endo-β-1,4-glucanase (endoglucanase, EC 3.2.1.4), exo-β-1,4-glucanase (exoglucanase, EC 3.2.1.91), and β-1,4-glucosidas (β-glucosidase, EC 3.2.1.21).[2]

Endoglucanase is the most significant among major cellulase groups because endoglucanase action causes a non-reducing end that is essential for the action of exoglucanase and cellobiase.[3] The synergistic action of some cellulolytic enzymes, known as cellulases, is required to convert cellulose into fermentable sugar, such as glucose.[2] Several studies have focused on the enzymatic degradation of cellulose by cellulases due to their numerous roles in bioconversions of agricultural wastes. Today, cellulose and related enzymes are widely used in agriculture and animal husbandry and other fields including detergents, nutrition, winemaking, animal nutrition, pulp and paper industry, etc.[4,5] Furthermore, recent research have focused on the industrial conversion of biomass containing cellulose into fermentable sugars in order to produce ethanol as an alternative fuel.[6,7] Enzymatic stability is essential for all these applications.

In order to industrially use cellulose enzyme, high-scale production with the highest level of activity and stability, but with the lowest cost, is very important. The use of biotechnology and production by recombinant microorganism is one of the best and most effective methods for the industrial production of this enzyme.

Selecting the right type of expression system for protein production is one of the most important factors in producing enzymes at high rates.[8] Bacteria such as *Escherichia coli* and lactic acid bacteria, yeasts and fungi are the best hosts in expression of recombinant enzymes.[9] The desirable and advantageous characteristics of these species have resulted in an increase in the number of biotechnological applications.[10] Bacterial expression systems have attracted special attention

in the production of recombinant enzymes due to their quick growth and high density on inexpensive substrates and the availability of an increasingly large number of cloning vectors and the mutant host strains. Different genes of bacterial cellulose have been cloned and expressed in different hosts.[11] Endoglucanase can be produced by different organisms such as microbes, fungi, plants and animals.[12,13] One of the most excellent sources of cellulases is bacteria.[14] Reports have shown that the main bacterial sources of endoglucanase are: *Bacillus*, *Chlostridium*, *Cellulomonas*, and *Erwinia* species.[15] Some of the members of *Bacillaceae* produce exocellular endoglucanase; for example, an exocellular endoglucanase was produced by *Bacillus subtilis* DLG.[15] *B. subtilis* which is known as a gram-positive bacterium, produces several enzymes in order to decompose a variety of substrates. Some extremophiles (alkaliphiles and thermophiles) and moderate microorganisms were used to clone endoglucanasegenes.[16,17] In an investigation on endoglucanase bacterial sources, researchers have discovered that *B. subtilis* species can produce thermostable enzyme with tolerance temperature of 55°C and optimal pH of 5-8.[18] In another study, the optimal temperature and pH of endoglucanase enzyme of *B. subtilis* IARI-SP-1 was 50–60°C and 8, respectively. Endoglucanases are active in different pH and temperatures. There are particular trends in which endoglucanases can be used in industrial processes without incurring any additional costs to adjust pH and temperature. Thermostable endoglucanase is a suitable candidate for practical applications.[19] In this study, we have shown the successful characterization and production of recombinant endo-β-1,4-glucanases from a *B. subtilis* DR-8806 isolated from a hot mineral spring.

Materials and Methods

Bacterial Strains, Plasmids and Culture Conditions

Two strains of *E. coli* bacteria including *E. coli* DH5α and BL21 (DE3) were used as hosts for both plasmid preparation and expression, respectively. A new *B. subtilis* strain collected from "Dig Rostam", a hot mineral spring in Kerman-Iran, was used as thermostable recombinant endoglucanase. InsTAclone™ kit (Thermo Scientific, USA) with pTZ57R/T vector was used for endoglucanase gene sub-cloning, and pET-21a(+) (Novagen, USA) was used as vector for gene expression. The DNA, plasmid, gel extraction kit and polymerase chain reaction (PCR) amplification and purification kit were purchased from Bioneer (Bioneer, South Korea). The DNA restriction enzymes were obtained from Thermo Scientific (Thermo Scientific, USA). All other reagents such as IPTG, X-gal and ampicillin were purchased from Sigma-Aldrich (USA).

Isolation and Characterization of Bacillus subtilis Strain

Water samples were collected from "Dig Rostam", a hot mineral spring in Kerman-Iran, to search for a specific microbe that could produce thermostable endoglucanase. Then, we managed to isolate a strong positive clone by applying both Congo red staining and screening the size of opaque circular around the colonies on avicel selective plates which demonstrated high Cellulases activity. In order to recognize bacteria, 16S ribosomal RNA gene was amplified using PCR. The primers had the following sequences: 16sFw: 5′-AGTTTGATCCTGGCTCAG-3′ and 16sRv: 5′-GGCTTACCTTGTTACGACTT-3′. The PCR reaction was carried out with the following cycling parameter: 94°C for 10 minutes, followed by 32 cycles of 30 seconds at 94°C, 30 seconds at 50°C and 60 seconds at 72°C, with a final extension for 10 minutes at 72°C. PCR purification kit (Bioneer, South Korea) was used to purify the amplified product. The mixture of reaction included 50 ng template DNA, 2.5 µL of each primer stock (0.5 µM), 2.5 µL of dNTPs mixture (0.2 mM), 2.5 µL of 10X *Taq* buffer, 1 U of Taq polymerase and the volume was made up to 25 µL. Nucleotide sequencing of the purified PCR product was carried out by Macrogen Company (Macrogen, South Korea).

DNA Amplification

The new isolated *B. subtilis* was used as a template for PCR. A truncated version of endoglucanase gene, without its native signal sequences and stop codon, was amplified with the following primers; CGGGATCCCGGCATCAGCAGCAGGGACA and CCGCTCGAGATTTGGTTCTGTTCCCCAAATCAGTTT. *BamHI and Xho*I restriction enzyme sites were introduced by the primer to facilitate subsequent cloning into the expression vector pET-21a (+). Oligonucleotides have been designed according to published endoglucanase sequences of *B. subtilis* and were synthesized from the Macrogen Company (South Korea). PCR was carried out with the following cycling temperature: 94°C for 10 minutes, followed by 34 cycles of 30 seconds at 94°C, 30 seconds at 54°C and 45 seconds at 72°C, with a final extension for 10 minutes at 72°C. The reaction mixture was as described above. The amplified product was purified by PCR purification kit (Bioneer, South Korea).

Cloning and Expression of Recombinant Endoglucanase

In accordance with the manufacturer's instructions, pTZ57R/T vector from InsTAclone™ kit (Thermo Scientific, USA) was used to sub-clone the amplified PCR product with a flanking restriction site. After transformation of *E. coli* DH5α supplemented with 100 µg/mL ampicillin for white-blue screening. White colonies on the plate were cultured and the plasmids were extracted and purified. This recombinant plasmid was named pTZ57R/T-endoglucanase. The pTZ57R/T-endoglucanase was digested by appropriate restriction endonucleases. After that, electrophoresis on agarose gel was performed and the fragment (~1438 bp) was recovered and purified using AccuPrep Gel Purification Kit (Bioneer, South Korea). The fragment was cloned into expression plasmid pET21a(+) and transformed into competent *E. coli* BL21 (DE3) for the protein expression and further sequence analysis. The construct was a fusion protein with a C-terminal 6x histidine tag and N-terminal T7-tag. After optimization of IPTG concentration, time of induction and some other factors, it was shown that a final concentration of 0.5 mM IPTG is appropriate to express recombinant endoglucanase protein in *E. coli* BL21 (DE3). Therefore, the culture was

induced by a final concentration of 0.5 mM IPTG for 2-4 hours and cultured again for 4 hours. Cells were harvested at 0, 1, 2, 3 and 4 hours by centrifugation (6000 rpm for 10 minutes at 4°C), and were then suspended in PBS (pH 7.3). The cells were wrapped by sonication at 20 MHz for 3 minutes at 0°C and the supernatant was collected by centrifugation (13 000 rpm for 30 minutes at 4°C) and stored at -20°C.

SDS–PAGE

For SDS-PAGE analysis, 15 µL of the supernatant samples were mixed with 5 µL of sample buffer containing 1mM Tris (pH= 6.8), 2-mercaptoethanol (0.5%), glycerol (50%), SDS (10%) and bromophenol blue (1%). Then samples were loaded into appropriate wells for electrophoresis (12% SDS-PAGE) after being heated for 3 minutes at 100°C. The gel was stained with a mixture of Coomassie Brilliant Blue R-250 (Sigma, USA) in 30% methanol and 10% acetic acid. The electrophoretic was carried out at 200V for 80 minutes.

Western Blotting

To examine specific reactions of the recombinant protein, western-blot analysis was performed. Following SDS–PAGE, electro-transference of endoglucanase recombinant protein in the gels, into nitrocellulose paper was carried out at 100V for 120 minutes with transfer buffer (29 g/L of Tris, 14.5 g/L of glycine, 1.85 g/L of SDS, and 10% of methanol). The unreacted sites of the membranes were blocked by incubating in blocking solution (5% (w/v) of nonfat milk powder in TBST (150 mM NaCl, 20 mM Tris-HCl, 0.05% Tween 20 (v/v), (pH 8.0)) for 2 hours at room temperature. The incubation of the membrane was carried out with 6×His antibody (Sigma, USA) for 1 hour at room temperature, followed by washing with PBS-Tween 20. Afterwards, the membrane was subjected to horseradish peroxidase (HRP)-conjugated second antibody (Sigma, USA) incubation at 37°C for 1 hour. To exposure of bands, after washing, a developing solution (DAB, 30% H_2O_2, 1% $CoCl_2$ in PBS) was used.

Endoglucanase Activity Assay

The measurement of the quantity of reducing sugars following reaction with DNS method[20] revealed the endoglucanase activity. The reducing sugar release was assessed at 550 nm in spectrophotometer using glucose as the standard. One unit of cellulase activity was defined as the amount of enzyme necessary to release 1 µmol of reducing sugar (glucose equivalents) per minute. The reaction mixture which contained1 ml of 2% carboxy methylcellulose (*CMC*) (Sigma, USA) in 0.05 M acetate buffer (pH 4.8) and 1 mL of cell lysate incubated at 50°C for 60 minutes and the reducing sugar produced was distinguished through dinitrosalicylic acid–DNS procedure[16] by glucose as a sugar standard. Blanks were prepared with inactivated enzymes. Different ranges of temperature (25–80°C) were applied to measure the effects of temperature on the enzyme activity. Thermal stability studies were carried out by incubating the enzyme at different temperatures (30–65°C) for 1 hour.

Bioinformatics Analysis

As this enzyme generated from a new isolated *B. subtilis*, thus we decided to exert a bioinformatics analysis. The BLAST servers applied for the homology analysis. The alignment of the DNA and protein sequences was performed by applying BLASTN and BLASTP, respectively. The resulted amino acid sequence by those bioinformatics programs was analyzed for theoretical isoelectric point (pI) and Molecular weight (Mw) measured using ExPASy tools.[21] The SWISS-MODEL method[22] was applied to predict the tertiary structure. Multiple sequence alignments were conducted by Clustal W. Each ORF (open reading frame) was analyzed to characterize the protein family and domain organization through the Pfam search server[21] and PROSITE.[23]

Results

To confirmed thermostable cellulase in the bacterium, water samples were collected from the hot spring. A clone was isolated and showed that was gram positive, rod-shaped and aerobic. To identify the strain of bacteria, the microscope assay was performed and it was proven that the bacterium belonged to *Bacillus* strain. According to the prevalent method, 16S ribosomal RNA analysis was amplified and 16S ribosomal RNA of the thermostable *Bacillus* was sequenced (Accession number: JF309277.1). Partial sequence of 16S ribosomal RNA gene showed 99% homology with some other *B. subtilis* strains. The phylogenic tree between 16S ribosomal RNA sequence obtained in this study and the sequences derived from NCBI is shown in Figure 1. The phylogenic tree is drawn using neighbor-joining method by Mega 5 software. According to the above-mentioned results, it can be proved that investigated *Bacillus* was *B. subtilis,* which entitled *B. subtilis* DR-8806.

Endoglucanase gene of *B. subtilis* of DR-8806 strain was amplified (1438 bp). The electrophoresis of PCR product derived from gene encoding endoglucanase is shown in Figure 2a. Then it was cloned in pET21a (+). In order to observe endoglucanase expression, the pET21a (+) vector was transformed into *E. coli* BL21 (DE3). The endoglucanase gene was expressed in *E. coli* with T7-tag and His-tag fusion protein under the control of T7-phage promoter. The inserted endoglucanase of *B. subtilis* DR-8806 was sequenced and deposited in the gene bank database (Accession number: KF361708.1). The ORF of the endoglucanase gene consists of 1491 nucleotides, which encodes a protein of 497 amino acids with a predicted molecular weight of 55 kDa. To further verify the endoglucanase gene product in *E. coli*, silver staining showed a distinct band with a molecular mass of approximately 55 kDa (Figure 2b), which accorded to that deduced from gene sequence of the mature protein lacking the signal peptide and fusion tags. The synthesized protein contains 470 amino acids in length with a recorded molecular mass of 52 kDa.

The effect of temperature on the activity of the non-purified enzyme was considered in various thermal statuses. The optimal activity of the enzyme was specified to be 55°C. Moreover, in temperatures of 40-60°C, more than 60% of

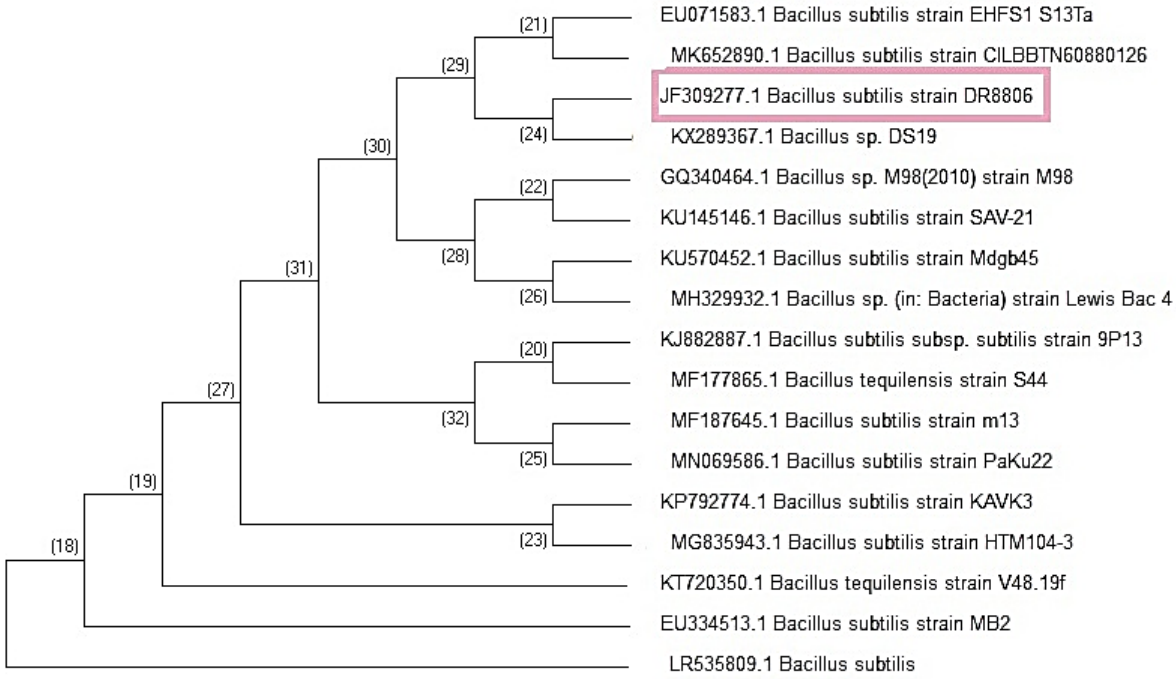

Figure 1. The Phylogenic Tree Between the 16S Ribosomal RNA Sequence of *Bacillus subtilis* DR-8806 (Accession number: JF309277.1) With 16S Ribosomal RNA Sequences of Some Other *Bacillus*.

the maximal activity was observed. However, endoglucanase exhibited approximately 40% activity at 25°C and 65°C (Figure 3). Based on the results shown by thermo stability, it was determined that the enzyme was thermostable as it conserved more than 97% and about 80% activity when incubated at 50°C and 60°C, respectively for 1 hour (Figure 3).

The structure analysis through ExPASy proteomics tools revealed that pI and Mw of the mature peptide were about 7.63 and 52.26 kDa, respectively. When it fused into vector pET21a (+), its pI decreased to 7.27 and Mw increased to 55.13 kDa, for the fusion of T7-tag, His-tag and peripheral amino acids. Based on NCBI-CDD, the whole amino acid sequence showed a high resemblance to the protein of BglC (*E*<7e-53) and BLASTP results of adjusted amino acid sequence of endoglucanase revealed the highest similarity with proteins coding for endo-1,4-beta-glucanases in *B. subtilis*. The amino acid sequences had more than 99% congruity with the reported endo-1, 4-beta-glucanases (CAA97610.1, AGN52749.1, WP 003231540.1, etc) of *B. subtilis*. The identification and structure of protein domain was performed by ExPASy proteomics server. When the sequence was fully aligned against the profiles, only one hit was found including a CBM3 (carbohydrate binding module type-3) domain profile. The sequence was scanned against the patterns, and showed that it had one distinctive pattern; glycosyl hydrolases family 5 domain signatures. Further sequence analysis revealed that endoglucanase amino acid sequence comprised of three main areas including glycosyl hydrolase family 5 domain (*E*< 7.2e-61), linker domain and a cellulose binding type 3 domain (*E*< 1.7e-27) (Figure 4). The GH5 domain also contained a glycosyl hydrolase catalytic core (Figure 4). We point out this endoglucanase from *B. subtilis* DR-8806as BsCelDR.

Like another endoglucanase, CBM3 domain of BsCelDR was found at C-terminal of catalytic domain and GH5 was at its N-terminus. To be more specific, GH5 domain at N-terminal had a catalytic module property, containing a total of 247 residues from amino acids 21 to 267. The C-terminal domain involved 150 residues from amino acid 321 to 470, which was associated to the function of binding to the cellulose surface. The total number of amino acids in both GH5 and CBM-3 domains was 331.

A three-dimensional structure was predicted by SWISS-MODEL and visualized in PyMOL. Hypothetical conformation of the enzymes was predicted and identified for two completely distinct structures at the tertiary level. The

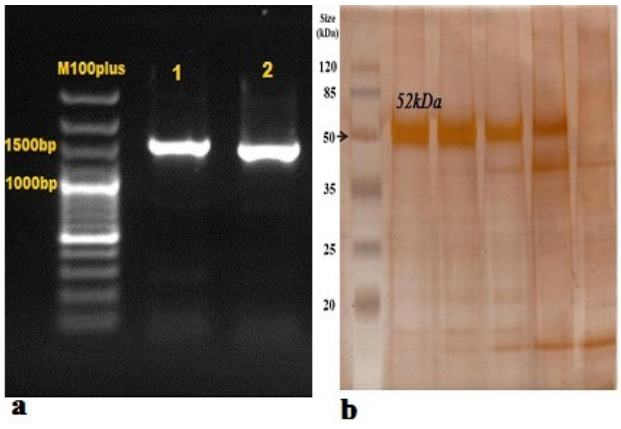

Figure 2. (a) Electrophoresis of the PCR Product Derived From the Gene Endoglucanase of *Bacillus subtilis* Dr-8806. **(b)** Western Blotting Analysis of Recombinant Protein, Confirmed That Endoglucanase Protein was Produced With ~52 Kda Molecular Weight.

3D structure of GH5 domain was predicted using 3pzt.1 as a specific template and the C-terminal domain was predicted using 2l8a.1 as the template. Sequence congruency of the template with GH5 and CBM3 domains were 99.67% and 98.63%, respectively.

B. subtilis BsCelDR belongs to the glycoside hydrolase family 5 and studies strongly confirmed the fact that β-glycanases of the same family share a similar architecture including conserved catalytic residues and a similar catalytic mechanism.[20] This suggested that the BsCelDR catalytic core structure surrounds a classical $(\beta/\alpha)_8$-barrel fold, with the active site located at the crevice formed by the C-terminal ends of β-strands. Such structure was the typical module for clan GH-A glycoside hydrolase families. The CBM3 folded into a β-sandwich fashion (Figure 5).

The catalytic machinery involved three types of amino acid residues, including Glu 140 and Glu 228, the aromatic gate keepers Trp 178 and Trp 262, and the polarizers His 200, Arg 63 and Thr 227 (Figure 6). From homology with other GH5 cellulases, Glu 140 is anticipated to be the proton donor, protonates the glycosidic bond and catalyzes the bond fission, and the Glu 228 is known as the nucleophile amino acid, which facilitates the reaction by stabilizing the resulting carbonium ion intermediate. Divalent ions are well-known cofactors of enzymes, either activating their catalytic machinery or having a structural role. Some evidences proved specific interactions of the Mn^{2+} ion with the catalytic core of BsCelDR. Manganese ion devotes as ligand in which placed in

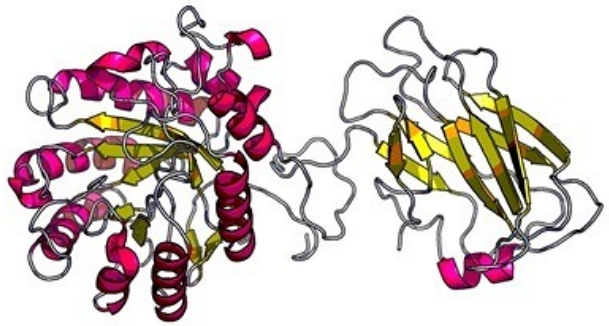

Figure 5. The Three-Dimensional Structure of Bsceldr Showed Two Completely Distinct Structures, one corresponding to the catalytic module (folds $(\alpha/\beta)_8$) and the other to the CBM3, which folds into a β–sandwich.

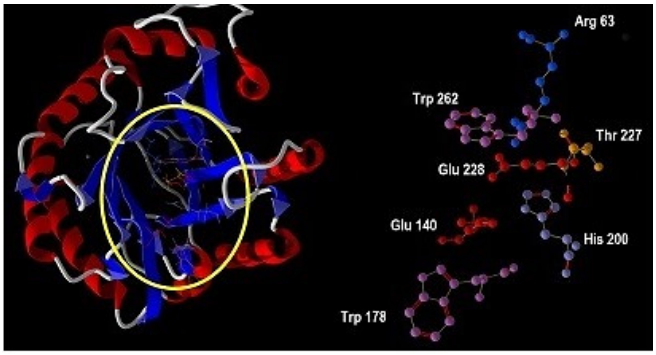

Figure 6. Active Site of the BsCelDR in Crystal Structure of GH5 Domain. Right: Zoom of the active site, showing the glutamate residues and other catalytically relevant residues.

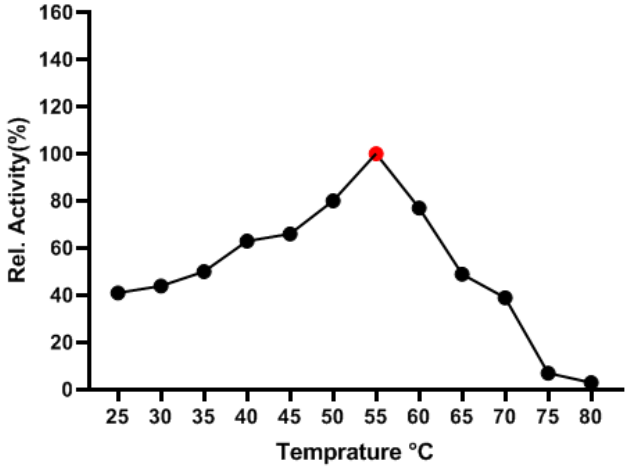

Figure 3. Relative Enzymatic Activity in Different Temperatures. The maximum activity was determined in 55°C.

contact with G128, D166, D168, N169, in crystal structures of BsCelDR (Figure 7).

Mn^{2+} significantly elevates the thermal stability of Cel5As by promoting T_m. Finally, the known motifs that shaped and was recognized in the sequence were analyzed using online motif scanning. Four potential Sequon or N-glycosylation sites were found (Asn-X-Ser or Asn-X-Thr) in the deduced amino acid sequence namely Asn263, Asn352, Asn386 and Asn411 (according to mature peptide nucleotide positions).

Discussion

In the current study, we successfully constructed expression vectors of endoglucanase gene and expressed the target protein through induction with IPTG under the control of a T7-phage promoter. The recombinant proteins were fused with His-tag and T7-tag.

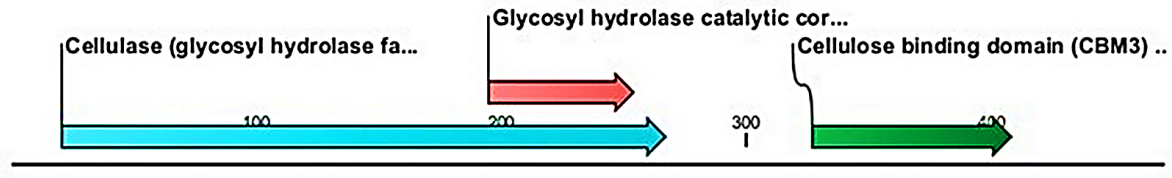

Figure 4. The Conserved Domains of BsCelDR.

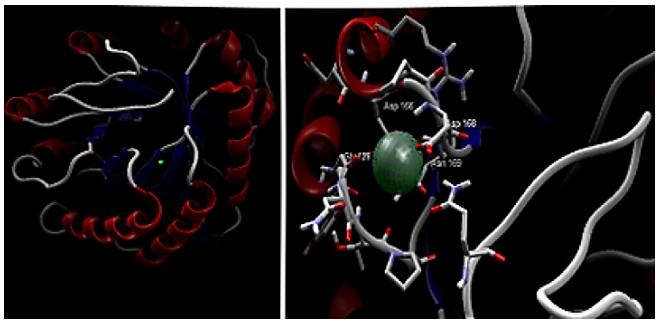

Figure 7. Left: Cartoon Representation of the Protein Structure, With the Manganese Ion Shown as a Green Sphere. Right: Schematic Representation for the Interactions of Protein Residues (Grey) and Manganese Ion (Green Sphere). Manganese ion devotes as ligand in which placed in contact with G128, D166, D168, N169, in crystal structure of the BsCelDR. Four potential Sequon or N-glycosylation sites were found (Asn-X-Ser or Asn-X-Thr) in the deduced amino acid sequence namely Asn263, Asn352, Asn386 and Asn411

Cellulase and other cellulose-degrading enzymes are commonly produced in *B. subtilis* and may survive in isozymic forms in some other strains.[10,24,25] Because of its well-being ambiguity in the existing enzymes to cover all aspects of industrial needs, efforts are continuing to discover new genes of endoglucanase.[26] The estimation of the molecular weight resulted from SDS-PAGE was shown that the molecular weight of endoglucanase recombinant protein is almost 55kDa, which was similar to that deduced from gene sequence of β-1,4-endoglucanasemature protein. These results are closely similar to those obtained from other *Bacillus* sp.[17, 27] DNA sequencing studies have previously shown that *B. subtilis* endoglucanases are formed as precursor proteins with molecular mass of above 55 kDa; however, ultimately an extracellular protein with more than 35 kDa will be produced after processing which also involves the removal of a peptide segment from carboxy-terminus.[28]

Carboxymethyl cellulose substrates were used to measure endoglucanase activity. The media and cellular extract of *E. coli* harboring endoglucanase had a specific activity of 8.5 IU mL^{-1}. It is a fact that by enhancing the production and recovery of endoglucanase from the bacteria, it may become economically viable for industrial use. A four times increase in endoglucanase production by the recombinant cell rather than the wild type is suggested, since the application of *E. coli* system as a cell factory for endoglucanase over-production has a higher level of recombinant expression.[29]

The optimal temperature of recombinant cellulase obtained in our study was 55°C and the substrate was CMC. This optimal temperature was higher than those of *P. punctate*,[30] *Streptomyces lividans* 66,[31] *Bacillus* sp. KSM-S237,[32] *Streptomyces* sp. G12,[33] *B. subtilis* IARI-SP-1[29] and lower than the optimum temperature (65°C) reported by[32] for the purified endoglucanase from *Streptomyces rochei*. Some studies reported that the maximum endoglucanase activity from the *Bacillus* strain M-9 and *Bacillus halodurans* C-125 were obtained at 60°C.[33,34] Increased temperature might lead to an increase in kinetic energy of molecules and also in the enzyme activity. Further increase in temperature caused a decline in the enzyme activity due to denaturation of the enzyme. Moreover, more than 60% of the maximal activity of BsCelDR was observed at 40-60°C incubation for 60 minutes. Meanwhile, three strains of thermostable cellulase including cellulase of *Caldocellum saccharolyticum*,[35] cellulase of *Bacillus sp.* KSM-S237[31] and the cellulase from *B. subtilis* DR[36] were reported to preserve high enzyme activity at 70°C. Also, it was reported that cellulase of *Bacillus sp.* KSM-S237 can retain 30% of the original activity after being heated at 100°C for 10 minutes. [31] The cellulase from *B. subtilis* DR can retain 70% of its maximum activity at 75°C after incubation for 30 minutes.[36] Further sequence analysis confirmed that cellulose enzyme from *B. subtilis* strain DR-8806 belonged to glycosyl hydrolase 5 family, similar to other *Bacillus* cellulose.[1] Glycosyl hydrolases, containing cellulases, indicated a great multiplicity from the extensive variety of carbohydrate structures. In contrast to the conventional classification of enzymes based on the type of catalyzed reactions and substrate-specificity, a classification of glycosyl hydrolases into families based on amino acid sequence similarities was introduced to integrate both structural and mechanistic features of these enzymes which is regularly updated.[37]

Conclusions

In this study, B. subtilis was isolated from "Dig Rostam" a hot spring in Iran, which could be an effective producer of cellulose. Endo-β-1,4-glucanase obtained in our study was thermostable which one of the most important characteristics for the enzyme to maintain its activity during *pelleting* processes on feed materials in the animal feed industry. Therefore, this feature of the enzyme endoglucanase from bacillus subtilis makes it possible to use this enzyme in the *livestock feed* industry. Further researches are required to study its applications in other industries.

Authors' Contributions

All authors have contributed in the experimental design, data collection and writing of the manuscript.

Conflict of Interest Disclosures

The authors declare that they have no competing interests.

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