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RESEARCH ARTICLE

Endo-glucanase Producing Thermophilic *Bacillus subtilis*: Gene Isolation and Structure-Function Prediction

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ABSTRACT

Thermophilic bacteria, *Bacillus subtilis* VSDB5 isolated from the hot springs of Vashist had cellulase activity. Molecular confirmation for the presence of cellulase gene in the B. *subtilis* genome amplified partial gene fragment around 1300 bp. The full-length endo-glucanase gene isolated using specific primers was 1500 bp. Sequencing and BLAST analysis revealed the fragment had 98% homology *to endo-glucanase* gene of *Bacillus subtilis* 168. Multiple alignments and homology modelling revealed that it belongs to GH5 endo-glucanase with its structure containing a classical $(\beta/\alpha)_8$, TIM-barrel fold with conserved active site residues, Glu257 and Glu165. The isolated *endo-glucanase* gene sequence was submitted in NCBI, and the accession was MK424591.

Keywords: *Cellulase; Hydrolysis; Endo-glucanase; Bacillus subtilis; Cloning.*

INTRODUCTION

Cellulose is a linear polysaccharide of glucose residues with β-1, 4-glycosidic linkages. The abundant availability of cellulose makes it an attractive raw material for producing many industrially important commodity products. Cellulose can be converted to glucose, a multi-utility product, in a much cheaper and biologically favorable process. Cellulolysis is basically the biological process controlled and processed by the enzymes of cellulase system. Cellulase enzyme system comprises three classes of soluble extracellular enzymes: 1,4-β-endoglucanase, 1,4-β-exoglucanase and β-glucosidase (β-D-glucoside glucohydrolase or cellobiase). Endo-glucanase is responsible for the random cleavage of β-1,4-glycosidic bonds along a cellulose chain. Exoglucanase is necessary for cleavage of the non-reducing end of a cellulose chain and splitting of the elementary fibrils from the crystalline cellulose, and β-1,4-glucosidase hydrolyses cellobiose and water-soluble cellodextrin to glucose (Shewale, 1982).

Cellulases have been reported from several living organisms, and among them, fungal cellulases have major industrial applications. High-temperature tolerance improves the enzyme robustness and increases the enzyme reaction rates needed for industrial-scale processes, thereby decreasing the amount of enzyme needed (Kumar and Wyman, 2008). Hence the search for thermostable enzymes is still ongoing. With the advancement in molecular techniques, the cellulase gene can be modified with a desirable character or over-expressed in a non-host organism for mass production. With this background, the present investigation aims at search of the cellulase gene from thermophilic bacteria and to predict the protein structure functions.

MATERIALS AND METHODS

Screening for cellulase producing thermophilic biocatalysts

Thermophilic bacterial isolate, VSDB5, was screened for the production of biomass hydrolyzing enzyme, cellulase. The microbial culture was spot inoculated in CMC containing minimal media and were incubated at 50 °C for 48 h. Later the plates were stained with 1% congo red, followed by destaining with 1M NaCl for 20 min each (Salem *et al*., 2008). Positive isolates showed a zone of clearance around the cell growth. The hydrolytic capacity is calculated by the ratio between the diameters of the clear zone by the diameter of the colony.

Genomic DNA extraction and PCR amplification

The genomic DNA from VSDB5 was extracted using CTAB method (Wilkie, 1997). Cellulase gene was amplified using the gene-specific primers: Ba_EN1F (5' CCAGTAGCCAAGAATGGCCAGC 3') and Ba_EN1R (5' GGAATAATCGCCGCTTTGTGC 3') (Ashe et al., 2014). The PCR product was resolved by electrophoresis in 1.2% agarose gel in 1 X TAE buffer. Gels were stained with ethidium bromide (10 mg. ml^{-1}) and documented using a Bio-rad Gel DocXR+ system (Hercules, CA, USA). The amplified PCR products were purified using GeneJET PCR

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Purification Kit (Thermo Scientific, USA) and were sequenced at Eurofins, India.

Primer designing and isolation of endo-glucanase gene

The *endo-glucanase* gene-specific primer for *Bacillus* was designed using Oligo perfect designer based on the *endo-glucanase* gene sequence (EF070195) available in the NCBI database. The full-length *endoglucanase* gene was amplified using the specific primer set with the PCR conditions as follows: 95 °C for 5 min; 35 cycles of 94 °C for 1min, 54 °C for 1 min, and 72 °C for 1.5 min; and 72 °C for 10 min. The amplified PCR fragment was gel eluted (PrepEase Gel Extraction Kit, Canada) and cloned into a pGEM-T easy vector (Promega, USA). The positive colonies were selected based on the blue-white selection and PCR. The positive clones were sent for sequencing (Eurofins, Bangalore).

Sequence-structure analysis:

The endo-glucanase of VSDB5 was analyzed in the RCSB server (www.rcsb.org). The sequence and structure homologs were retrieved for multiple sequence alignment (MSA). The MSA was prepared using Bioedit sequence editor (Version 7.2.5). The structure was made using swiss-model automated server (https://swissmodel.expasy.org). The active sites were predicted based on the *Bacillus subtilis* 168 (3pzt) sequence alignment. The structure was drawn using PyMol software (Ver 0.97).

RESULTS AND DISCUSSION

Qualitative screening for cellulase production by thermophilic bacteria

The complex cellulose polymer is broken down into simpler sugars by the cellulase enzyme complex. Cellulase production by several living organisms viz., plant, microbes, insects have been reported. Cellulolytic bacterial species include *Bacillus, Trichonympha, Clostridium, Actinomycetes, Bacteroides succinogenes, Butyrivibrio fibrisolvens, Ruminococcus albus,* and *Methanobrevibacter ruminantium* (Schwarz, 2001 and Ekperigin, 2007). In the present study, thermophilic bacteria, VSDB5 isolated from the hot springs of Vashist (~65 °C), Himachal Pradesh, produced cellulase enzyme with a hydrolytic capacity of 7.33. The substrate hydrolysis was visualized as a yellow color clearing zone around the positive colonies.

Table 1. Analysis of Bacillus cellulase gene sequence

Primer used	Sample	Sequence homology			
		Organism	Gene	Accession No.	Per cent homology
Cellulase	VSDB5	*Bacillus subtilis* subsp. *subtilis* strain 168G	*Endo-glucanase gene*	CP016852.1	98

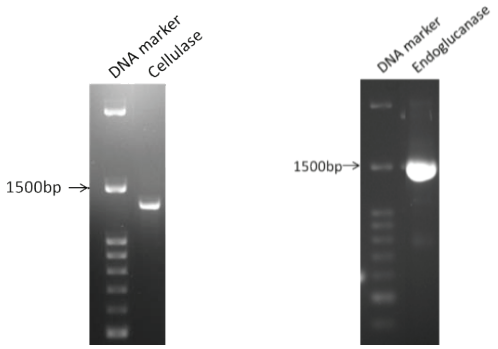
Figure 1. Qualitative assay for cellulase production by thermophilic bacteria

The qualitative assay revealed massive cellulose utilization around the thermophilic bacteria, *Bacillus* bacteria, VSDB5 (Figure 1).

Bacillus sp. is gram-positive bacteria with a high level of extracellular enzyme production capacity, which attracted its application in many industries. Cellulase enzyme production by several *Bacillus* species has been reported (Bhalla *et al*., 2012; Pandey *et al*., 2014; Sharma *et al*., 2015 and Vyas *et al*., 2016). Optimal cellulase activities of different isolates like *Bacillus circulans* (4.80 IU/ml) and *Bacillus subtilis* (4.64 IU/ml) were well reported at pH 6 after incubation at 40 ^{0}C (Otajevwo *et al*., 2011).

Molecular screening for cellulase in VSDB5 genome

The thermophilic bacteria VSDB5 was screened for the presence of cellulase gene at a molecular level. PCR analysis amplified a product of around 1300 bp for cellulase primer (Figure 2A). The PCR product was purified and sequenced. The sequence was analyzed by NCBI BLAST, and the nearest match from GenBank data was reported (Table 1).



A.Partial gene B. Full length gene

Figure 2. PCR amplification of cellulase gene in thermophilic bacteria

Isolation of full-length endo-glucanase gene from thermophilic Bacillus subtilis

The endo-glucanase gene was amplified from thermophilic bacteria VSDB5 using an endo-glucanase gene-specific primer. The amplification resulted in 1500 bp gene fragment (Figure 2B). The gene fragment was eluted, cloned, and transformed into *E. coli* DH5α cells. From the PCR positive white colony JB-VSDB5-EN-3, the plasmid was isolated and sequenced. Blast analysis showed 99 % homology to other endo-glucanase genes from *Bacillus,* and the phylogenetic tree was constructed on the aligned datasets using the neighbor joining (NJ) method (Figure 3). The nearest match was to *Bacillus subtilis subsp. subtilis strain 168G* Endo-glucanase gene (CP016852.1). Several cellulase genes from *B. subtilis* have been isolated and are available in NCBI database eg. AAK39540.1, AAK94871.1, ABK63475.1, and CAA47429.1 (Li *et al*., 2008).

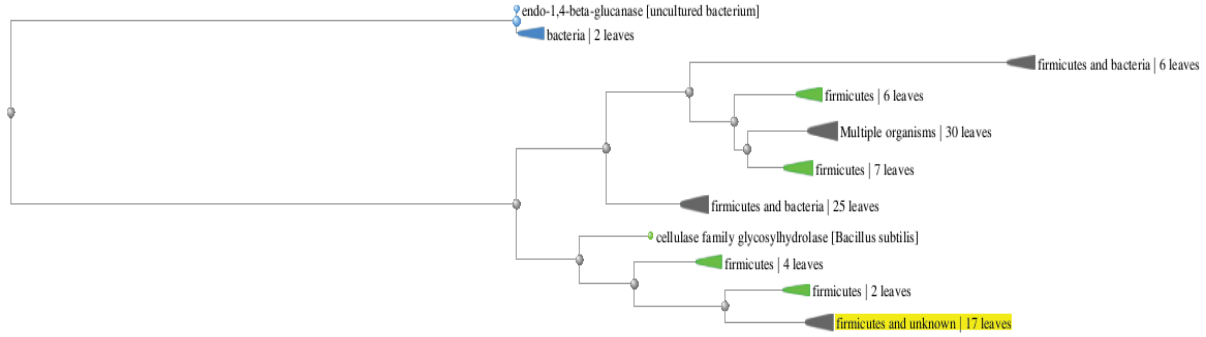


Figure 3. Phylogenetic tree constructed based on endo-glucanase gene sequence of *Bacillus subtilis* isolate with reference isolates

Protein Sequence and structure analysis of endo-glucanase of Bacillus subtilis VSDB5

Endo-glucanase from *Bacillus subtilis* VSDB5 (EG VSDB5) showed 100 percent identity to endo-1,4 beta-glucanase from *Bacillus subtilis* 168 (3pzt). Other organisms that showed sequence and structural match with VSDB5 were from alkalophilic *Bacillus* sp. (1lf1), *Bacillus agaradhaerens* (1e5j), *Cytophaga hutchinsonii* (5ihs), and *Thermobifida fusca* (2cks) with a percent identity of 67.9, 68.3, 50.3, and 42.8 respectively (Figure 4). The homology model of VSDB5 was made using a swiss-homology modelling server using *Bacillus subtilis* 168 (3pzt) as a template (Figure 5).

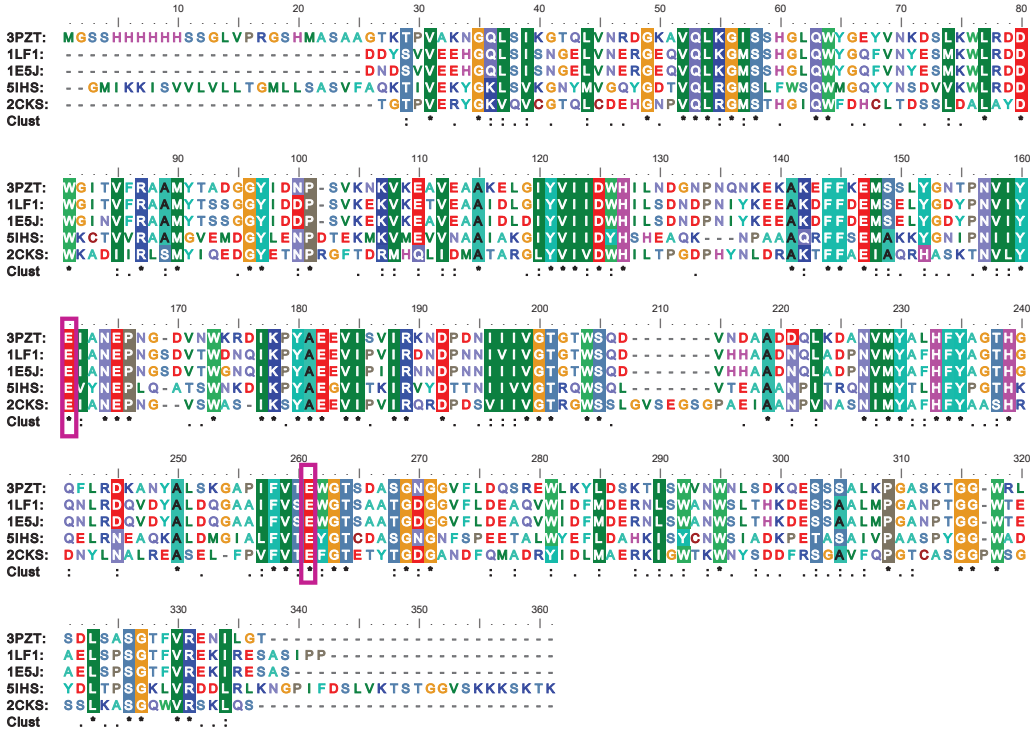


Figure 4. Multiple sequence alignment (MSA) of endo-glucanase from close homologues of VSDB5. The active site residue is boxed.

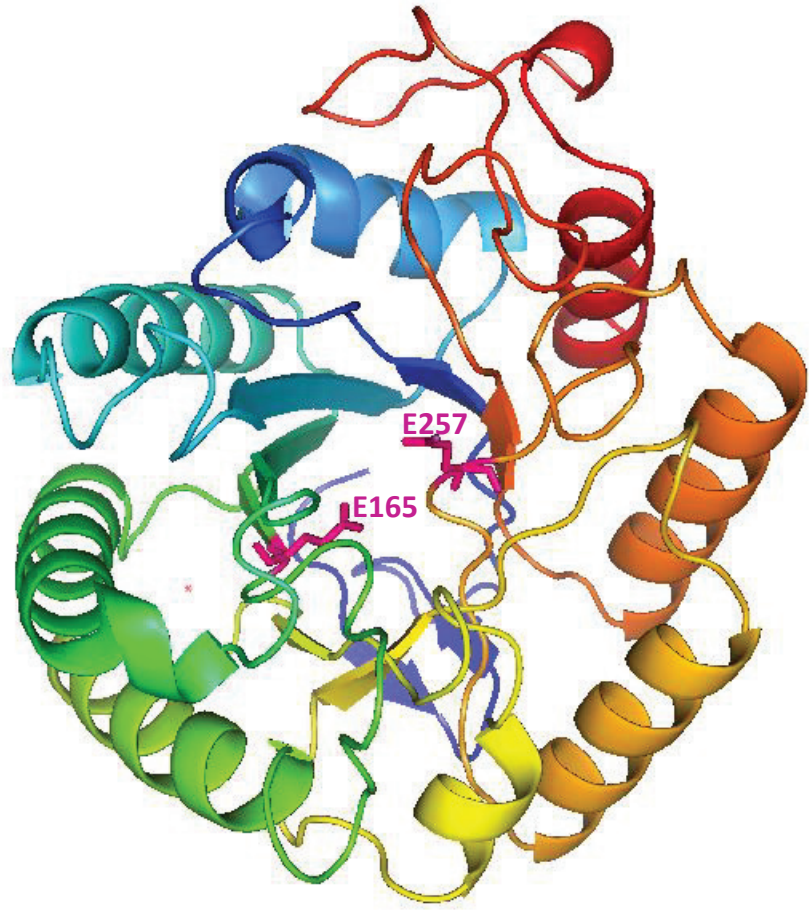


Figure 5. Swiss-model predicted structure of endo-glucanase from VSDB5. The template was *Bacillus subtilis* 168 (3pzt), which shared 100% identity with VSDB5. The active site residues Glu165 and Glu257 are shown in sticks.

We attempted to clone the full-length *endo-glucanase* gene from the thermophilic *B.subtilis* VSDB5. The full-length coding gene was about 1500 bp and the sequence was submitted in NCBI database under the accession MK424591. This gene can be over-expressed in non-host organisms for mass production and its utilization in the industry. Endo-glucanase, along with BGL brings better biomass hydrolysis in bioethanol production. Co-expression of *endo-glucanase A* from an endophytic *Bacillus pumilus* and the hyperthermophilic β-glucosidase A (*BglA*) from *Fervidobacterium* sp. in *Escherichia coli* presented a 30-fold increase in reducing sugar content from CMC compared to unmodified strain (Rodrigues *et al.*, 2010). Similarly, expression of *CelA*, *BglA* and *BglB* genes in *Bacillus subtilis* secreted endo-glucanase and β-Glucosidase into the media successfully (Yuan *et al*., 2013)

CONCLUSION

A cellulase is a wide group of enzyme with broad industrial applications. The search for novel enzymes with increased stability had led to the exploration of novel thermophilic biocatalysts with better enzyme activity. Understanding the cellulase gene will help to improve its stability and activity using modern biotechnological tools. In this study, we have identified cellulase producing thermophilic *Bacillus subtilis* VSDB5 and confirmed the presence of *cellulase* gene in its genome. The full length endo-glucanase coding gene of 1500 bp was cloned. Through over-expression of this gene in suitable expression host, characterization and mass production of the endo-glucanase enzyme can be achieved, which will find a major application in various industries for the production of biomass-derived products.

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