*Madras Agric.J.,* 2018; doi:xxxxxxxxx

RESEARCH ARTICLE

# Endo-glucanase producing thermophillic *Bacillus subtilis*: gene isolation and structure function prediction

*J.Beslin Joshi, Priyadharshini R and Sivakumar Uthandi**

Biocatalysis Lab., Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore- 641 003

ABSTRACT

Cellulase producing thermophillic bacteria, *Bacillus subtilis* VSDB5 was isolated from biotrap based enrichment at hot springs in Himachal Pradesh. Molecular confirmation for the presence of cellulase in B. licheniformis using spe- cific primers amplified partial gene fragment around 1300 bp. Cellulase gene specific PCR followed by sequencing and BLAST analysis revealed the fragment had 98% homology *to Endoglucanase gene* of *Bacillus subtilis* 168. Mul- tiple alignment and homology modelling revealed it belongs to GH5 endoglucanase with its structure containing a classical $(\beta/\alpha)_8$-barrel fold with conserved active site residues, Glu257 and Glu165.

Keywords: Cellulase, hydrolysis, Endoglucanase, Bacillus subtilis, cloning

DRAFT

## *Introduction*

Cellulose is a linear polysaccharide of glucose residues with β-1, 4-glycosidic linkages. Abundant availability of cellulose makes it an attractive raw ma- terial for producing many industrially important com- modity products. With the help of cellulolytic system, cellulose can be converted to glucose which is a multiutility product, in a much cheaper and biologi- cally favourable process. Cellulolysis is basically the biological process controlled and processed by the enzymes of cellulase system. Cellulase enzyme sys- tem comprises three classes of soluble extracellular enzymes: 1,4-β-endoglucanase, 1,4-β-exoglucanase and β-glucosidase (β-D-glucoside glucohydrolase or cellobiase). Endoglucanase is responsible for 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 wa- ter-soluble cellodextrin to glucose (Shewale, 1982).

Cellulases have been reported from several liv- ing organisms and among them fungal cellulase are mainly used in industrial application. The tolerance of high temperatures improves the enzyme robust- ness and increases the enzyme reaction rates need- ed for industrial-scale processes thereby decreasing the amount of enzyme needed (Kumar and Wyman, 2008). Hence search for thermostable enzymes is still ongoing. With advancement in molecular techniques,

the cellulase gene can be modified with desirable character or over-expressed in non-host organism for mass production. With this background the present investigation aims at search of cellulase producing thermophillic bacteria, isolation of cellulase gene and predict its protein structure functions.

*Methods*

Isolation and screening of thermophillic biocatalysts

Thermophillic bacterial isolate, VSDB5 was made from the saw dust biomass enriched at hot springs of Vasist in Himachal Pradesh. The purified isolate was screened for the production of biomass hydrolysing enzymes, cellulase and xylanase. The mi- crobial cultures were spot inoculated in CMC contain- ing minimal media and were incubated at 50°C for 48
h. The CMC cantaining plates were stained with 1% congo red followed by destaining with 1M NaCl for 20 minutes each (Salem *et al*., 2008). Positive isolates showed a zone of clearance around the cell growth. The hydrolytic capacity is calculated by the ratio be- tween the diameters of the clear zone by diameter of the colony.

*Genomic DNA extraction and PCR amplification*

The genomic DNA from VSDB5 was extracted using CTAB method (Melody, 1997). 16S rRNA se- quence was amplified using universal primers: 27 F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5'-ACGGCTACCTTGTTACGACTT-3') (Weisberg *et al*.,
1991), cellulase gene using gene-specific primers: Ba_EN1F (5' CCAGTAGCCAAGAATGGCCAGC 3') and

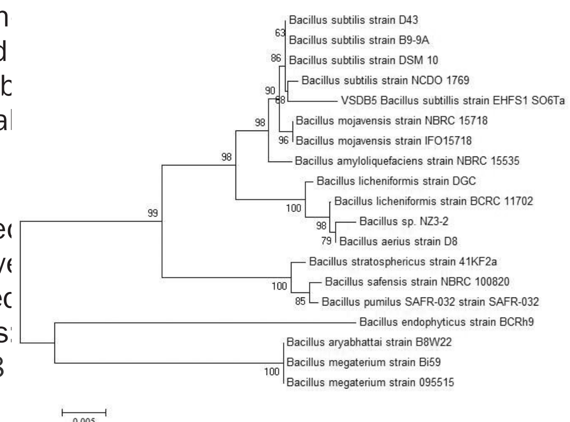
Ba_EN1R (5' GGAATAATCGCCGCTTTGTGC 3')(Ashe *et al*., 2014). The PCR product was resolved by electro- phoresis in 1.2% agarose gel in 1 X TAE buffer. Gels were stained with ethidium bromide (10 mg. $ml^{-1}$) and visualized on a gel documentation system and gel images were digitalized. The amplified PCR products were visualized by electrophoresis in 1.5% agarose gels and documented using a Bio-rad Gel DocXR+ sys- tem (Hercules,CA,USA).For bacterial identification, the 16S rRNA amplified PCR products were purified using GeneJET PCR Purification Kit (Thermoscientific, USA) and were sequenced commercially at Eurofins, India.

Primer designing and isolation of *endoglucanase*Gene

*Identification of cellulase producing thermophillic bacteria*

The PCR amplification of 16S rRNA sequence resulted in the amplification of 1.5 kb fragment. PCR product was sequenced and has been analysed by NCBI BLAST and the nearest match from GenBank data was reported. DNA sequencing and phylogenetic analysis revealed that the thermophillic bacteria was gram positive and belong to the phylum Firmicutes. The isolate VSDB5 was closely related to *Bacillus sub- tilis* strain *B9-9A* with a 99% of identity. The phyloge- netic tree was constructed on the aligned datasets using neighbor joining (NJ) method using the program MEGA 6.0 (Figure 1).

The *endoglucanase* gene specific primer for *Ba- cillus* was designed using Oligo perfect designer based on the *endoglucanase* gene sequence (EF070195) available in the NCBI database. The *endoglucanase* gene was amplified using BI_EN 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 respectively. Th
(PrepEase Gel Extraction Kit, Canada) and cloned
The positive colonies were selected based on the b
clones were sent for sequencing (Eurofins, Bangal




*Sequence-structure analysis:*

The Endo-glucanase of VSDB5 was analyzed
sequence and structure homologues were retrieve
The MSA was prepared using Bioedit sequence ec
made using swiss-model automated server (https:
were predicted based on the *Bacillus subtilis* 168

DRAFT

was drawn using PyMol software (Ver 0.97).

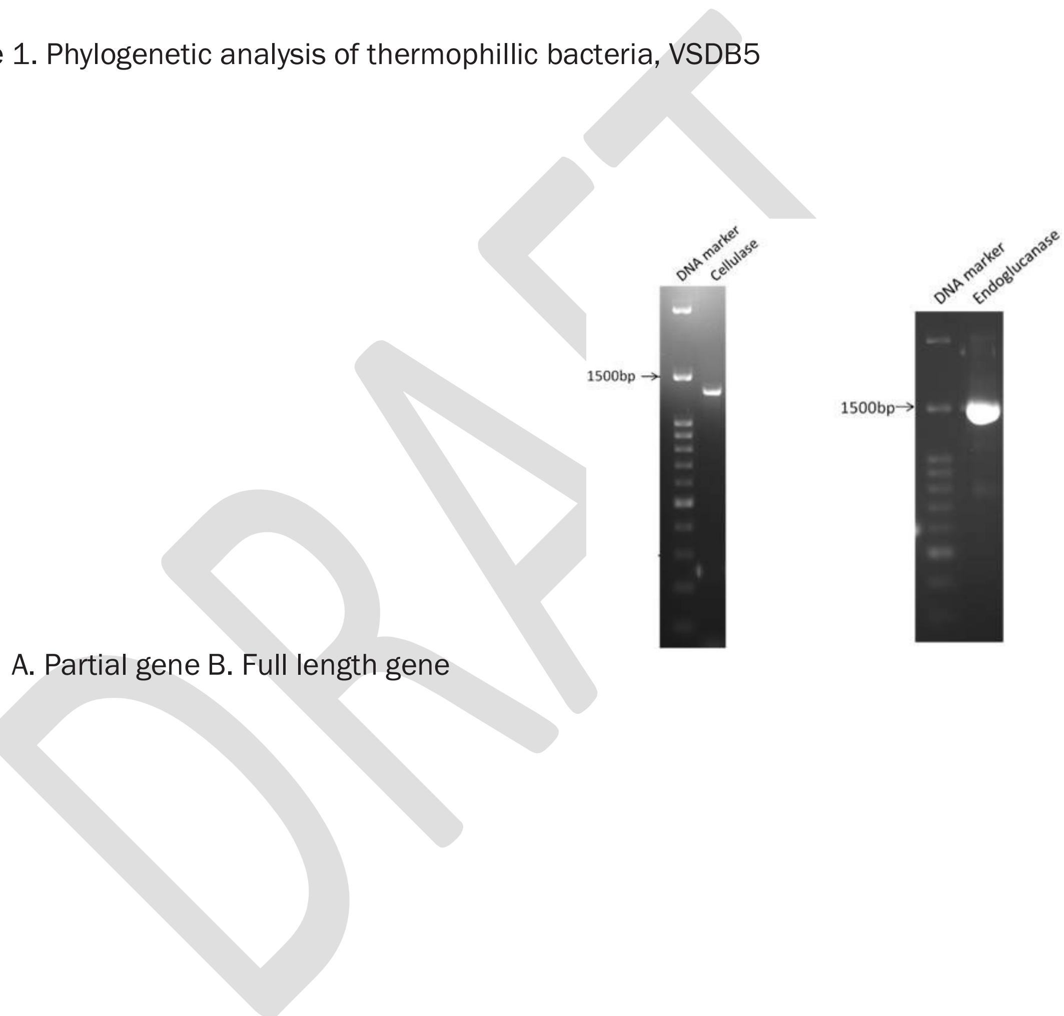
## Results

*Qualitative screening for cellulase production by*

### thermophillic bacteria

Thermophillic bacteria, VSDB5 isolated from saw dust biomass enriched at hot springs of Vasist (~65 °C), HP produced cellulase enzyme with hydro- lytic capacity of 7.33. The substrate hydrolysis was visualised as yellow colour clearing zone around the positive colonies.

Figure 1. Phylogenetic analysis of thermophillic bacteria, VSDB5




A. Partial gene B. Full length gene

| 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 | Endoglucanase gene | CP016852.1 | 98 |

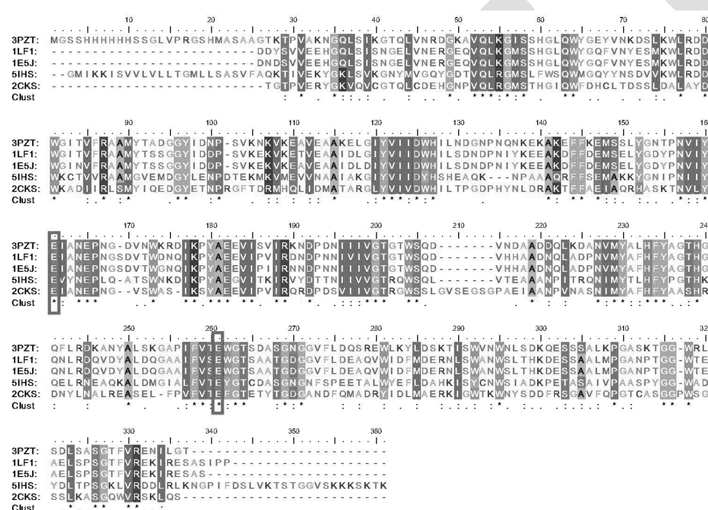


Figure 3. Multiple sequence alignment (MSA) of endo-glucanase from close homologues of VSDB5. The active site residue is boxed.

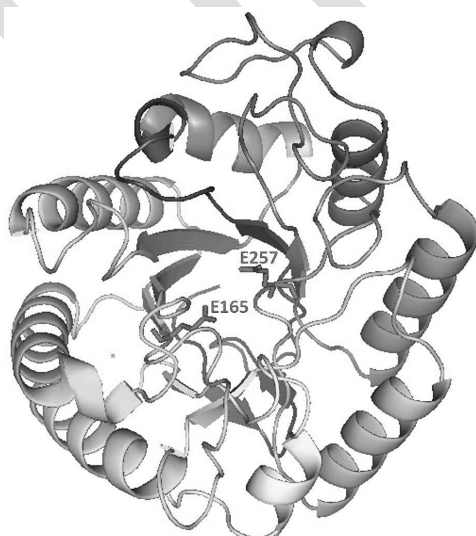



Figure 4. Swiss-model predicted structure of VSDB5. The template was *Bacillus subtilis* 168 (3pzt) which shared 100% identity with VSDB5. The active site residues Glu165 and Glu257 are shown in sticks.

*PCR based screening for cellulase*

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

*Isolation of endoglucanase gene from thermophillic*

*Bacillus subtilis*

The endoglucanase gene was amplified from thermo- phillic bacteria VSDB5 using endoglucanase gene specific primer. The amplification resulted in 1500bp 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, plasmid was isolated and se- quencing is under process.

Sequence and structure analysis of *Bacillus subtilis*

*VSDB5 endo-glucanase*

*Bacillus subtilis* VSDB5 endo-glucanase (EG VSDB5) showed 100 percent identity to endo-1,4-be- ta-glucanase from *Bacillus subtilis* 168 (3pzt). Other organism which 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 re- spectively (Fig 3). The homology model of VSDB5 was made using swiss-homology modelling server using *Bacillus subtilis* 168 (3pzt) as template (Fig 4).

*Discussion*

The complex cellulose polymer is broken down to simpler sugars by the enzyme cellulase complex. Cellulase production by several living organisms' viz., plant, microbes, insects have been reported. Cellulo- lytic bacterial species include *Bacillus, Trichonympha, Clostridium, Actinomycetes, Bacteroides succino- genes, Butyrivibrio fibrisolvens, Ruminococcus albus and Methanobrevibacter ruminantium* (Schwarz, 2001 and Ekperigin, 2007). In present study, a ther- mophillic bacteria producing cellulase was isolat- ed from the enriched saw dust substrate at the hot springs of of HP. The organism was identified based on the 16S rRNA sequence as Bacillus subtilis with 99% identity to sequence in the NCBI database. Ba- cillus sp. is gram positive bacteria with high level of extra cellular enzyme production capacity which at- tracted its application in many industries. Cellulase

DRAFT

enzyme production by several Bacillus species has been reported (Bhalla *et al.*, 2012 and Sharma *et al*., 2015). 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 incuba- tion at 40ºC (Otajevwo *et al*., 2011).

Molecular confirmation of the cellulase gene from thermophillic *B. subtilis* (VSDB5) was carried out using Ba_EN F and R primers. The amplified 1300bp cellulase xylanase gene fragments were sequenced and the BLAST analysis of the gene sequence showed 98% homology 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). The *endoglucanase* gene of about 1500 bp was isolated from *B. subtilis* (VSDB5) and sequencing is under progress. This gene can be over-expressed in non-host organism for mass production and used in industry. Endoglucanase along with BGL brings bet- ter biomass hydrolysis in bioethanol production. Co- expression of *endoglucanase A* from an endophytic *Bacillus pumilus* and the hyperthermophilic β-glucosi- dase A (*BglA*) from *Fervidobacterium* sp. in *Escherich- ia coli* presented 30-fold increase in reducing sugar content from CMC compared to unmodified strain (Rodrigues *et al*., 2010).

## Conclusion

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 thermophillic biocatalysts with better enzyme activity. Understanding the cellulase gene will help to improve its stability and activityusing modern biotech- nological tools. In this study we have identified cellu- lase producing thermophillic *Bacillus subtilis* (VSDB5) and the presence of *cellulase* gene was confirmed. The 1500bp endoglucanase gene fragment was isolat- ed. Through over-expression of this gene in suitable expression host characterisation and mass production of the endoglucanase enzyme can be achieved which will find a major application in various industries for production of biomass derived products.

## References


Ashe S, Maji U.J, Sen, R, Mohanty S, Maiti N.K (2014) Spe- cific oligonucleotide primers for detection of endog- lucanase positive *Bacillus subtilis* by PCR. 3 Biotech 4(5): 461-465


DRAFT

Bhalla A, Bansal N, Kumar S, Bischoff K.M, Sani R.K (2013) Improved lignocellulose conversion to biofu- els with thermophilic bacteria and thermostable en- zymes. Bioresour. Technol. 128: 751–759.

Ekperigin M. M (2007) Preliminary studies of cellulase pro- duction by *Acinetobacter anitratus* and *Branhamella sp*. African Journal of Biotechnology 6(1): 28–33.

Li W, Zhang W, Yang M, Chen Y (2008) Cloning of the Ther- mostable Cellulase Gene from Newly Isolated *Bacil- lus subtilis* and its Expression in *Escherichia coli.* Mol Biotechnol 40:195–201.

Otajevwo F.D, Aluyi H.S.A (2011) Cultural conditions nec- essary for optimal cellulase yield by cellulolytic bac- terial organisms as they relate to residual sugars re- leased in broth medium," *Modern App. Sci.,* vol*5(3):* 141-151.

Rodrigues A, Cavalett A, Lima A.O.S (2010) Enhancement of *Escherichia coli* cellulolytic activity by coproduc- tion of betaglucosidase and endoglucanase enzymes 13(5):1-6.

Salem F, Ahmed S, Jamil A (2008) Isolation of a xylan de- grading gene from genomic DNA library of a thermo- philic fungus *Chaetomium thetmophile* ATCC 28076. Pak. J. Bot., 40: 1225-1230.

Schwarz W. H (2001) The cellulosome and cellulose degra- dation by anaerobic bacteria. Applied Microbiology and Biotechnology 56(5-6): 634–649.

Sharma A, Tewari R, Soni S.K (2015) Application of Statis- tical Approach for Optimizing CMCase Production by *Bacillus tequilensis* S28 Strain via Submerged Fermentation Using Wheat Bran as Carbon Source. International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering 9(1): 76-86.

Shewale J. G (1982) Glucosidase: its role in cellulase synthe- sis and hydrolysis of cellulose. International Journal of Biochemistry 14(6): 435–443.

Weisberg W.G, Barns S.M, Pelletier B.A, Lane D.J (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173: 697–703.

DRAFT