J. Viet. Env. **2012**, Vol. 3, No. 2, pp. 80-86
DOI: 10.13141/jve.vol3.no2.pp80-86

JOURNAL of VIETNAMESE ENVIRONMENT

Molecular cloning gene and nucleotide sequence of the gene encoding an endo-1,4-β-glucanase from *Bacillus* sp VLSH08 strain applying to biomass hydrolysis

Tách dòng và xác định trình tự gen endo-1,4-β – glucanase từ chủng vi khuẩn Bacillus sp VLSH08 ứng dụng để thủy phân sinh khối

Research article

Phan, Minh Thi Tuyet[1]; Nguyen, Viet Quoc[1]; Le, Hy Gia[2]; Nguyen, Thoa Kim[1]; Tran, Man Dinh[1]*

[1]*Lab of Biomaterial Technology;* [2]*Lab of Fermentation Technology, Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau giay, Hanoi, Vietnam*

Bacillus sp VLSH08 screened from sea wetland in Nam Dinh province produces an extracellular endo-1,4-beta-glucanase. According to the results of the classified Kit API 50/CHB as well as sequence of 1500 bp fragment coding for 16S rRNA gene of the *Bacillus* sp VLSH 08 strain showed that the taxonomical characteristics between the strain VLSH 08 and *Bacillus amyloliquefaciene* JN999857 are similar of 98%. Culture supernatant of this strain showed optimal cellulase activity at pH 5.8 and 60 Celsius degree and that was enhanced 2.03 times in the presence of 5 mM Co^{2+}. Moreover, the gene encoding endo-1,4-beta-glucanase from this strain was cloned in *Escherichia coli* using pCR2.1 vector. The entire gene for the enzyme contained a 1500-bp single open reading frame encoding 500 amino acids, including a 29-amino acid signal peptide. The amino acid sequence of this enzyme is very close to that of an EG of *Bacillus subtilis* (EU022560.1) and an EG of *Bacillus amyloliquefaciene* (EU022559.1) which all belong to the cellulase family E2. A cocktail of enzyme containing this endo-1,4-beta-glucanase used for biomass hydrolysis indicated that the cellulose conversion attained to 72.76% cellulose after 48 hours.

Chủng vi khuẩn Bacillus sp VLSH08 được tuyển chọn từ tập hợp chủng vi khuẩn phân lập ở vùng ngập mặn tỉnh Nam Định có khả năng sinh tổng hợp enzyme endo-1,4-beta-glucanase ngoại bào. Kết quả phân loại chủng vi khuẩn Bacillus sp VLSH08 bằng Kit hóa sinh API 50/CHB cũng như trình tự gen mã hóa 16S rRNA cho thấy độ tương đồng của chủng Bacillus sp VLSH08 và chủng Bacillus amyloliquefaciene JN999857 đạt 98%. Dịch lên men của chủng được sử dụng làm nguồn enzyme thô để nghiên cứu hoạt độ tối ưu của enzyme ở pH 5,8 và nhiệt đô 60°C. Hoạt tính enzyme tăng 2,03 lần khi có mặt 5 mM ion Co^{2+}. Đồng thời, gen mã hóa cho enzyme endo-1,4-beta-glucanase cũng được tách dòng trong tế bào Escherichia coli sử dụng vector pCR 2.1. Gen mã hóa cho enzyme này có chiều dài 1500 bp, mã hóa cho 500 axit amin, bao gồm 29 axit amin của chuỗi peptid tín hiệu. So sánh cho thấy trình tự gen endo-1,4-beta-glucanase của chủng Bacillus sp VLSH08 có độ tương đồng cao với enzyme này của chủng Bacillus subtilis (EU022560.1) và của chủng Bacillus amyloliquefaciene (EU022559.1). Tất cả các enzyme nhóm này đều thuộc họ cellulase E2. Enzyme của chủng này cũng đã được phối trộn với các enzyme khác tạo thành cocktail để thủy phân sinh khối cho kết quả cellulose bị thủy phân 72,76% sau 48 giờ.

Keywords: cellulase, endo-1,4-beta-glucanase, *Bacillus*, biomass hydrolysis, molecular cloning

* Corresponding author
E-mail: tdman@ibt.ac.vn

http://dx.doi.org/10.13141/JVE
ISSN: 2193-6471

1. Introduction

Lignocelluloses are the most abundant plant cell wall components found in the biosphere and also the most voluminous waste produced by our society. The chemical properties of the components of lignocellulosics make them a substrate of enormous biotechnological value (Malherbe and Cloete, 2003). Large amounts of lignocellulosic "waste" are generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agroindustries and they pose an environmental pollution problem (Howard et al., 2003). Cellulases are a group of hydrolytic enzymes and capable of degrading lignocellulose materials which are mainly divided into three types: endo-1,4-*β*-D-glucanase (EC 3.2.1.4), exo-1,4-*β*-D-glucanase (EC 3.2.1.91), and *β*-glucosidases (EC 3.2.1.21) depended on the cleavage sites of polysaccharide molecules. Endo-1,4-β-glucanases are broadly spread group of cellulolytic enzymes that act on cellulose by randomly hydrolyzing internal β-1,4-D-glycosidic bonds resulting in decreased length of polymer and increased concentration of reducing sugar (Onsori et al., 2005).

In nature, cellulases have been detected in many microorganisms including in bacteria and filamentous fungi. Most cellulases are multienzyme systems and are active over alkaphilic, acidic or neutral pH ranges. In particular, considering the common use of endo-1,4-β-glucanases has been exploited in a vast range of biotechnological applications like brewing industry (Celestino et al., 2006), vinification (Blattel et al., 2011), animal feed production (Walsh et al., 1995), and waste management (Liu et al., 2004). Genes encoding these endo-1,4-β-glucanases have been cloned from different species of *Bacillus* such as *B. subtilis* (Cantwell and McConnell, 1983), *B. amyloliquefaciens* (Borriset et al., 1985), *B. circulans* (Bueno et al., 1990), *B. polymyxa* (Gosalbes et al., 1991), *B. licheniformis* (Lloberas et al., 1991), and *B. brevis* (Louw et al., 1993), and most of these genes have been cloned and expressed in heterologous hosts such as *Escherichia coli*, *B. subtilis* and yeast (Bueno et al., 1990; Cantwell et al., 1988; Gormley et al., 1988; Hecker et al., 1988). Here, a *Bacillus* sp. VLSH08, isolated from sea wetland in Nam Dinh province, was found to produce an endo-1,4-*β*-D-glucanase. Due to wide scope of this enzyme's applications, this study describes the molecular cloning of gene encoding for this enzyme and the determination of its nucleotide sequence. Moreover, a whole amino acid sequence of this enzyme is compared with those of other protein sequences published before. The potential of the recombinant enzyme is assessed for applications in biomass hydrolysis which is further utilised for different industrial productions.

2. Materials and Methods

2.1 Materials

2.1.2 Bacterial strain, plasmid, and cultural conditions

Bacillus sp VLSH08 strain isolated from sea wetland in Nam Dinh province, was used as the source of the gene for endo-1,4-*β*-D-glucanase. The *E. coli* TOP10F' (F'{*lac*I^q Tn*10* (Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G) was used as hosts for cloning and sequencing. Both *E. coli* and plasmid pCR2.1 were included in TOPO® TA Cloning® Kit which was purchased from Invitrogen. The *Bacillus* sp VLSH08 was cultivated in the mineral agar broth contained (w/v) 2% Carboxymethyl Cellulose (Sigma-Aldrich), 0.25% yeast extract (Difco), 0.5% K_2HPO_4, 0.05% NaCl, 0.02% $MgSO_4.7H_2O$ and 0.06% $(NH_4)_2SO_4$, and was used for the propagation of *Bacillus* sp. VLSH08. Solid media contained 1% (w/v) Agar-agar (Ha Long, Vietnam).

E. coli strains harbouring plasmids were grown on LB broth (BD Difco) supplemented with 100 µg/ml ampicillin.

2.2 Methods

2.2.1 Microbiological methods

Observation of cells and colony shape under microscope and SEM; determination of Gram property; CMC hydrolysis capacity were followed Nguyen LD et al (Nguyen et al., 1972 and 1976). Biochemical characteristics for classification of *Bacillus* sp VLSH08 was carried out according to the manual of Kit API 50/CHB (bioMérieux) and the results were analysed using APIWEB software.

2.2.2 Preparation of DNA

Bacillus sp VLSH08 was grown aerobically overnight at 37^{o}C in LB broth. Bacterial chromosomal DNA was purified as manufacture instruction of GeneJET™ Genomic DNA Purification Kit (Thermo Scientific).

2.2.3 DNA manipulation

The total DNA of *Bacillus* sp VLSH08 strain plays as a template to amplify the fragment coding for 16S rRNA gene. A pair of primers 16SF (5'-AGAGTTTGATCCTGGCTCAG-3') and 16SR (5-TACGGTTACCTTG TTACGACTT-3') was used in the following reaction: Taq polymerase buffer (10x) 5 µl; dNTPs (10 mM) 2 µl; Dream Taq polymerase (5000 U/ml) 0.3 µl (Thermo Scientific); 16SF primer (10 pmol) 1 µl; 16SR primer (10 pmol) 1 µl; DNA template (20 ng) 2 µl; water 38.7 µl. The reaction was carried out in the Thermal cycler PT-100 (MJ Reasearch Inc.) with the following program: 95 ^{o}C 3 min; 95 ^{o}C 1 min; 55 ^{o}C 1 min; 72 ^{o}C 1 min 15 sec; 30 cycles repeat from step 2 to step 4; 72 ^{o}C 7 min; 4 ^{o}C: endless. The PCR product was analyzed on 1% agarose gel and purified by using GeneJET™ Gel Extraction Kit (Thermo Scientific) before sending to sequencing.

Endo-1,4-*β*-D-glucanase gene (1.5 kb) was amplified by polymerase chain reaction using the following set of forward and reverse primers: F8 (5'-CTAATTTGGTTCTGA TCC-3') and R8 (5'- ATGAAACGGTCAATCTCT-3'). The 50 µl reaction mixture contained 5 µl of 10 x Taq polymerase buffer; 2 µl of 10 mM dNTPs; 0.5 U of Dream Taq polymerase; 1 µl of each primer and 1 µl of DNA genomic. The reaction mixture was incubated in a

thermal cycler at 95 ^{o}C for 5 min and then underwent 30 amplification cycles of 1 min at 95 ^{o}C, 1 min at 45 ^{o}C; 2 min at 72 ^{o}C; followed by a final incubation of 7 min at 72 ^{o}C. The PCR product was checked on 1% agarose gel and purified by using GeneJET™ Gel Extraction Kit.

2.2.4 Cloning of endo-1,4-β-D-glucanase gene in pCR2.1

The purified *Bacillus* sp VLSH08 endo-1,4-β-D-glucanase gene was ligated into cloning vector pCR2.1 (3.9 kb) according to the instruction of the TOPO® TA Cloning® Kit (Invitrogen). The ligated mixture was transformed into *E. coli* TOP10F'competent cells included in the cloning Kit. Transformed cells were selected on LB agar plates containing isopropyl-β-D-thiogalactopyranoside (IPTG), X-Gal and ampicillin by blue/white screening technique. Plates were then incubated at 37^{o}C overnight. Positive clones were picked up, inoculated in LB medium plus ampicillin. The plasmids were prepared by using GeneJET™ Plasmid Miniprep Kit (Thermo Scientific) and then were further checked by digestion with *Eco*RI restriction enzyme. Finally, confirmation of positive clone was performed by sequencing using a pair of M13 primers. The alignments of sequences between endo-1,4-β-D-glucanase gene of *Bacillus* sp VLSH08 and those of reported genes in GenBank were performed using the program CLUSTALW (Thompson et al., 1994). Translations of amino acids were carried out by using the Translate EXPASY tool (Gasteiger et al., 2003).

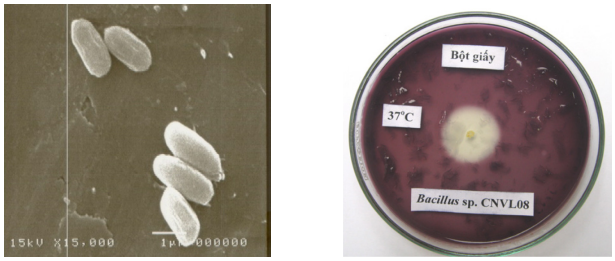
2.2.5 Enzyme assay

The fermented broth was centrifuged at 6000 rpm for 20 min. The supernatant was analysed for estimation of endo-1,4-*β*-D-glucanase at appropriate pH and temperature values by using dinitrosalicylic acid (DNS) method (Miller, 1959). CMC was used as substrate for enzyme assay. The reducing sugar liberated was measured at 550 nm in spectrophotometer using glucose as standard. One unit of enzyme activity was defined as the amount of enzyme that liberates one µmole of reducing sugar by glucose per ml per minute from appropriate substrate under assay conditions.

3. Results and discussion

3.1 Identification of *Bacillus* sp VLSH08

Strain VLSH08 was isolated from sea wetland in Nam Dinh province. Microscopy showed that this strain belongs to the *Bacillus* genus. This strain produced small, round, mucous, non-pigmented colonies with a diameter of about 1 mm. Morphological determination by SEM showed the cells were 0.5 - 0.7 µm wide and 1.4 - 1.6 µm long. The endospore was terminal and ellipse-shaped, and placed in the middle of the mother cell (Figure 1a). The Gram-positive staining of the cell wall was also demonstrated by light microscopy. We examined the cellulase activity of the VLSH08 strain cultured in the mineral agar broth containing 2% CMC or pulp at 37^{o}C, which resulted in a clearing zone after 24 - 48 hours (Figure 1b).



a b

Figure 1. Morphological cells of *Bacillus* sp VLSH08 strain under SEM (a) and its cellulase producing capacity (b).

In order to further classify strain VLSH08, a specific Kit for genus *Bacilli* identification, API 50/CHB (bioMérieux, France), was used in approximately 48 hours. The analysis results using APIWEB software indicated that the strain VLSH08 was similar of 98% as *B. amyloliquefaciene* (data not shown). Analysis of a 16S rRNA partial sequence showed 98% homology (Figure 2) with that of *B. amyloliquefaciens* subsp. *plantarum* strain NMCZ1 (Accession No: JN999857.1) and *B. subtilis* SNT3 (Accession No: JQ285941.1); 97% homology with that of *B. licheniformis* ATCC 14580 (Accession No: NC006270.3) and *B. licheniformis* ATCC 14580 (Accession No: NC006322.1). Based on these results, the bacterium was designated *Bacillus* sp VLSH08.

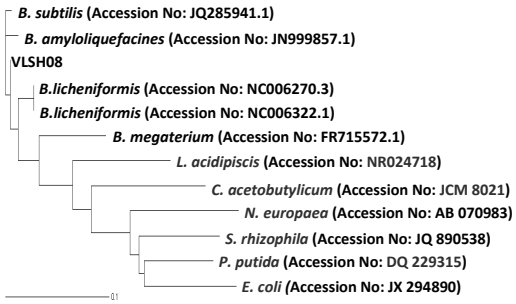


Figure 2. Phylogenetic tree showing the evolutionary position of strain VLSH08 among the genus *Bacillus* and six different genera. GenBank accession numbers are given in *parentheses*. The *bar* represents one substitution per 10 nucleotides.

3.2 Cloning of the endo-1,4-β-glucanase of *Bacillus* sp VLSH08 and its sequence analysis

Bacillus sp VLSH08 was grown in LB medium and genomic DNA was isolated to use as template for endoglucanase amplification. Most of endoglucanase genes were obtained by PCR or by constructing DNA libraries. Here, according to the reported cellulase gene of *B. amyloliquefaciens* (gi: 154183738) and the same gene of *B. subtilis* (gi: 599673), the PCR product from *Bacillus* sp VLSH08 was amplified about 1500 bp and other non-specific bands (Figure 3). A 1500 bp band of the PCR product was recovered from the agarose gel and ligated to pCR2.1 vector using T4 ligase and then recombinant plasmids were transformed into *E. coli* TOP10F'.

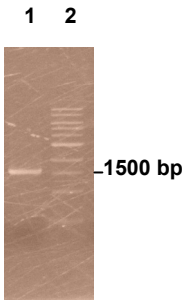


Figure 3. PCR amplification of endo-1,4-β-glucanase gene. Lane 1: endo-1,4-β-glucanase (1.5 kb). Lane 2: 1 kb ladder (New England Biolabs).

Rabbani et al. (2009) and Aftab et al. (2012) also reported to use TA vectors such as pCR2.1 or pTZ57R/T for cloning procedures (Rabbani et al., 2009; Aftab et al., 2012). Positive clones were identified using blue/white screening. Furthermore, the recombinant plasmids from them was isolated and restricted with EcoRI to separate the ligated product from the plasmid. The restricted plasmid was run on the gel and appearance of 1.5 kb band confirmed the ligation of endoglucanase gene with plasmid. The plasmid was then sent to National Key Laboratory of Gene Technology (Institute of Biotechnology, Vietnam) for sequencing. The nucleotide sequence of the 1.5 kb inserted in pCR2.1 was determined using a pair of M13 primers. Only one large open reading frame (ORF) beginning with an ATG codon and ending with a TAG codon (Figure 4). BLAST(P) result revealed a modular enzyme composed of a signal peptide (1-29), a catalytic domain (50-296) of Glycosyl Hydrolase family 5 (GH5) and a substrate binding domain (356-437) of Cellulose Binding Module 3 (CBM 3). The modular organization (GH5-CBM 3) of endoglucanase are also found in many *Bacillus* genus such as in *B. licheniformis* strain B-41362 (Bischoff et al., 2007), *B. amyloliquefaciens* DL-3 (Lee et al., 2008), *B. amyloliquefaciens* PMS3.1 (Nurachman et al., 2010)and *B. subtilis* (Li et al., 2009) The ORF in the nucleotide sequence encoded 500 amino acid residues, as indicated under the nucleotide sequence. The deduced sequence from amino acid 24 to 27 (Ala-Ser-Pro-Ala) resembles the recognition site of signal peptidase (Perlman and Halvorson, 1983). The endo-1,4-β-glucanase in *Bacillus* sp VLSH08 may possibly be cleaved by a signal peptidase at the bond between Ala 24 and Ser 25 during secretion across the cytoplasmic membrane. The sequence of *endo-1,4-β-glucanase* gene reported in this paper has been submitted to the NCBI database under accession no AEA30146.

```
ATG AAA CGG TCA ATC TCT ATT TTT ATT CCG TGT TTA TTG ATT ACA GTA TTG ACA ATG GGC GGC TTG CAG   69
 M   K   R   S   I   S   I   F   I   P   C   L   L   I   T   V   L   T   M   G   G   L   Q   23

GCT TCG CCG GCA TCA GAA GCA GGG ACA AAA ACC CCA GTA GCC AAG AAT GGG CAG CTT AGC ATA AAA GGA   138
 A   S   P   A   S   E   A   G   T   K   T   P   V   A   K   N   G   Q   L   S   I   K   G   46

ACA CAG CTC GTA AAC CGG GAC GGC AAA GCG GTA CAA TTG AAA GGG ATC AGT TCA CAT GGA TTG CAA TGG   207
 T   Q   L   V   N   R   D   G   K   A   V   Q   L   K   G   I   S   S   H   G   L   Q   W   69

TAT GGC GAT TTT GTC AAT AAA GAC AGC TTA AAA TGG CTG AGA GAC GAT TGG GGC ATA ACC GTT TTC CGC   276
 Y   G   D   F   V   N   K   D   S   L   K   W   L   R   D   D   W   G   I   T   V   F   R   92

GCG GCG ATG TAT ACG GCA GAT GGC GGT TAT ATT GAT AAT CCG TCC GTG AAA AAT AAA GTA AAA GAA GCG   345
 A   A   M   Y   T   A   D   G   G   Y   I   D   N   P   S   V   K   N   K   V   K   E   A   115

GTT GAA GCG GCA AAA GAA CTT GGG ATA TAT GTC ATC ATT GAC TGG CAT ATC TTA AAT GAC GGC AAC CCA   414
 V   E   A   A   K   E   L   G   I   Y   V   I   I   D   W   H   I   L   N   D   G   N   P   138

AAC CAA CAT AAA GAG AAG GCA AAA GAT TTT TTT AAG GAA ATG TCA AGT CTT TAC GGA AAC ACG CCA AAC   483
 N   Q   H   K   E   K   A   K   D   F   F   K   E   M   S   S   L   Y   G   N   T   P   N   161

GTC ATT TAT GAA ATT GCA AAC GAA CCA AAC GGT GAC GTG AAC TGG AAG CGT GAT ATT AAA CCG TAT GCG   552
 V   I   Y   E   I   A   N   E   P   N   G   D   V   N   W   K   R   D   I   K   P   Y   A   184

GAA GAA GCG ATT TCC GTT ATC CGC AAA AAT GAT CCA GAC AAC ATC ATC ATT GTC GGA ACC GGT ACA TGG   621
 E   E   A   I   S   V   I   R   K   N   D   P   D   N   I   I   I   V   G   T   G   T   W   207

AGC CAA GAT GTG AAT GAT GCA GCC GAT GAT CAG CTA AAA GAT GCA AAC GTC ATG TAC GCG CTT CAT TTT   690
 S   Q   D   V   N   D   A   A   D   D   Q   L   K   D   A   N   V   M   Y   A   L   H   F   230

TAT GCC GGC ACA CAC GGC CAA TCT TTA CGG GAT AAA GCA AAC TAT GCA CTC AGT AAA GGA GCG CCT ATT   759
 Y   A   G   T   H   G   Q   S   L   R   D   K   A   N   Y   A   L   S   K   G   A   P   I   253

TTC GTG ACG GAA TGG GGA ACA AGC GAC GCG TCT GGA AAT GGC GGT GTA TTC CTT GAC CAG TCG CGG GAA   828
 F   V   T   E   W   G   T   S   D   A   S   G   N   G   G   V   F   L   D   Q   S   R   E   276

TGG CTG AAT TAT CTC GAC AGC AAG AAC ATC AGC TGG GTG AAC TGG AAT CTT TCT GAT AAG CAG GAA TCA   897
 W   L   N   Y   L   D   S   K   N   I   S   W   V   N   W   N   L   S   D   K   Q   E   S   299

TCC TCA GCG TTA AAG CCG GGA GCA TCT AAA ACA GGC GGC TGG CCG CTT ACA GAT TTA ACT GCT TCA GGA   966
 S   S   A   L   K   P   G   A   S   K   T   G   G   W   P   L   T   D   L   T   A   S   G   322

ACA TTC GTA AGA GAA AAC ATT CGC GGC AAC AAA GAT TCA ACG AAG GAT GCC CCT GAA ACG CCA GCA CAA  1035
 T   F   V   R   E   N   I   R   G   N   K   D   S   T   K   D   A   P   E   T   P   A   Q   345

GAT AAT CCC GCA CAG GAA AAA GGC ATT TCC GTA CAA TAC AAA GCA GGG GAT GGG GGT GTG AAC AGC AAC  1104
 D   N   P   A   Q   E   K   G   I   S   V   Q   Y   K   A   G   D   G   G   V   N   S   N   368
```

```
CAA ATC CGC CCG CAG CTT CAC ATA AAA AAT AAC GGC AAT GCG ACG GTT GAT TTA AAA GAT GTC ACT GCC  1173
 Q   I   R   P   Q   L   H   I   K   N   N   G   N   A   T   V   D   L   K   D   V   T   A   391

CGT TAC TGG TAT AAC GCG AAA AAC AAA GGC CAA AAC TAT GAC TGT GAC TAC GCG CAG ATT GGA TGC CGC  1242
 R   Y   W   Y   N   A   K   N   K   G   Q   N   Y   D   C   D   Y   A   Q   I   G   C   R   414

AAT CTG ATC TAC AAA TTT GTG ACG CTG CAT AAA CCT AAG CAA GGT GCA GAT ACC TAT TTG GAA CTG GGG  1311
 N   L   I   Y   K   F   V   T   L   H   K   P   K   Q   G   A   D   T   Y   L   E   L   G   437

TTT AAA ACA GGA ACG CTG TCA CCG GGA GCA AGC ACA GGG AAT TTT CCG CTT CGT CTT CAC AAT GAT GAC  1380
 F   K   T   G   T   L   S   P   G   A   S   T   G   N   F   P   L   R   L   H   N   D   D   460

TGG AGT AAT TAT GCA CAA AGC GGC GAT TAT TCC TTT TTT CAA TCA AAT ACG TTT AAA ACA ACG AAA AAA  1449
 W   S   N   Y   A   Q   S   G   D   Y   S   F   F   Q   S   N   T   F   K   T   T   K   K   483

ATT ACA TTA TAT CAT CAA GGA AAC CAG ATT GGG GGA TCA GAA CCA AAT TAG   1500
 I   T   L   Y   H   Q   G   N   Q   I   G   G   S   E   P   N   *    500
```

Figure 4. The nucleotide and amino acid sequences of endo-1,4-β-glucanase from Bacillus sp VLSH08. The nucleotide sequence of the coding strand is given from 5' to 3', and the deduced amino acid sequence is shown under the nucleotide sequence. Numbering of both nucleotides and amino acid starts with the beginning of the coding sequence. A putative signal sequence is indicated by bold letters.

The endo-1,4-β-glucanase protein sequence of *Bacillus* sp VLSH08 had 95% identity with the reported that of *B. subtilis* DLG (Accession No. P07983.2) (Robson and Chambliss, 1987), 91% identity with Cel5 *Bacillus subtilis* (Accession No. P10475.1) (MacKay et al., 1986)and with Cel5 *Pectobacterium carotovorum* subsp. *carotovorum* (Accession No. Q.59395.1) (Mäe et al., 1995)

3.3 Endo-1,4-β-glucanase activity of *Bacillus* sp VLSH08 and its applications

Bacillus sp VLSH08 was inoculated in the mineral medium broth in 48 hours. The supernatant obtained after centrifugation was used as crude enzyme for determination of enzyme properties. When using CMC-Na as the substrate, native endo-1,4-β-glucanase of this strain had maximal activity of 13.42 ± 0.71 IU/ml at pH 5.8 and 60^{o}C. This activity was higher than those of *Bacillus* sp CH43 and *Bacillus* sp HR68 (9 IU/ml) (Mawadza et al., 2000), however, it was much lower than other enzymes of *B. amyloliquefaciens* DL-3 (153 IU/ml), *B. subtilis* subsp. *subtilis* A-53 (109 IU/ml) (**Lee** et al., 2008; Kim et al., 2009). The enzyme showed more than 50% of the maximum activity at pH 4-8, therefore this enzyme was very stable over a wide pH range, and retained nearly 100% of the original activity after incubation at pH 5.4-6.2, 60^{o}C for 24 hours. Furthermore, the enzyme was very stable at 60^{o}C which was still retained 97.36 ± 3.94 % relative activity at pH 5.8 after 24 hours of incubation. However, when increasing the incubated temperature up to 65^{o}C and 70^{o}C, the relative activities were decreased to 45.03 ± 4.84 % and 13.11 ± 5.12 %, respectively, in the same condition (data not shown). The biochemical properties of endo-1,4-β-glucanase showed the optimal temperature of this enzyme. Endo-1,4-β-glucanase with good thermal stability and substantial biological activity at pH 5.0-6.0 are well suited for application in various industrial processes, especially in the brewing process (McCarthy et al., 2005).

On the other hand, we examined the effects of some metal ions on enzyme activity at 1 mM and 5 mM concentrations. Most of additives used in this study, except for EDTA, enhanced enzyme activity as shown in Table 1. The EDTA inhibited endo-1,4-β-glucanase activity shown in decreasing the relative activity to 86.53% and 73.06% corresponding with 1 mM and 5 mM additive concentration, respectively. Relative activities of endoglucanase in the presence of 1 mM Ca^{2+}, Mg^{2+}, K^{+}, Co^{2+} and Zn^{2+} were 114.5%, 102.6%, 110.4%, 183.4%, and 111.9%, respectively. When increasing the concentration of metal ions up to 5 mM, most of the cases also enhanced the relative activities, except for Ca^{2+} and Zn^{2+} cases which showed the activities were reduced 7% and 26.9% compared with that at low concentration, respectively. Especially, the endo-1,4-β-glucanase activity was strongly enhanced by Co^{2+} which was raised up to 1.83 and 2.03 times compared with control case. Unlike previous report on inhibition of CMCase produced by a bacterial strain isolated from soil, Co^{2+} inhibited the enzyme activity whereas EDTA enhanced the activity of the endo-1,4-β-glucanase activity produced by *B. subtilis* subsp. *subtilis* A-53 (Kim et al., 2009).

Table 1. Effect of metal ions and chemical reagents on endo-1,4-β-glucanase activity

Additives	Relative activity (%)	
	1 mM	**5 mM**
Control	100.0	100.0
EDTA	86.5	73.1
Ca^{2+}	114.5	107.5
Mg^{2+}	102.6	111.4
K^{+}	110.4	116.6
Co^{2+}	183.4	203.6
Zn^{2+}	111.9	85.0

Member of the genus *Bacillus* was known to produce and secrete into culture media a broad variety of hydrolytic enzymes such as α-amylases, proteases, glucanases, and restriction endonucleases, etc. They represent one of the most important groups of bacteria not only for the production of commercially valuable enzymes but also for study of the secretion mechanism of extracellular enzymes (Khan and Husaini, 2006). Molecular cloning with targeted proteins is well documented in some *Bacillus* species. Moreover, some *Bacillus* strains which bear the generally regarded as safe (GRAS) such as *B. subtilis*, *B. megaterium* and *B. licheniformis* are now played as the most widely used hosts for the production of recombinant proteins.

After cloning, endo-1,4-β-glucanase of *Bacillus* sp VLSH08 was fused with promoter of α-amylase gene of *B. subtilis* 168M which then that cassette was transferred in the shuttle vector pHV33 and expressed in *B. subtilis* 168M (unpublished). To et al (2011) used this recombinant enzyme to combine with other enzymes such as exo-1,4-β-glucanase, β-glucosidase and lactase in order to create a cocktail "*Helper enzymes*" for biomass hydrolysis. A low "*Helper enzymes"* cocktail dose was used to hydrolyse lignocellulose from thermo-alkaline pretreated sugarcane bagasse which showed the biomass conversion was gained 72.76% cellulose after 48 hours at 50^{o}C and pH 4.8. Phenolic inhibitors of the fermentation in the hydrolysate issued from lignin during pretreatment was removed at 57.17%, resulting in an ethanol recovery of 77% (To et al., 2011). However, in order to increase the catalytic efficiency of the cocktail for the bioethanol development, evolution approaches of each enzyme including endo-1,4β-glucanase are needed.

4. Acknowledgement

We would like to thank the Laboratory of Gene Technology (Institute of Biotechnology, Vietnam) for kindly supporting us determination of endo-1,4-β-glucanase sequence.

5. References

[1] Aftab, S., Aftab, M.N., Haq I.U., Javed, M.M., Zafar, A. and Iqbal, I. 2012. Cloning and expression of endo-1,4–β-glucanase gene from Bacillus licheniformis ATCC 14580 into *Escherichia coli* BL21 (DE 3). Afr. J. Biotech. 11(12): 2846-2854.

[2] Bischoff, K., Liu, S and Hughes, S.R. 2007. Cloning and characterization of a recombinant family 5 endoglucanase from *Bacillus lichenisformis* strain B-41361. Process Biochem. 42: 1150-1154.

[3] Blattel, V., Larisika, M., Pfeiffer, P., Nowak, C., Eich, A., Eckelt, J., Konig, H. 2011. β-1,3-Glucanase from *Delftia tsuruhatensis* strain MV01 and its potential application in vinification. Appl. Environ. Microbiol. 77(3): 983-990.

[4] Borris, R., Baumlein, H., and Hofemeister, J. 1985. Expression in *Escherichia coli* of a cloned β-glucanase gene from *Bacillus amyloliquefaciens*. Appl. Microbiol. Biotechnol. 22: 63-71.

[5] Bueno, A., Vazquez de Aldana, C.R., Correa, J., Villa, T.G., del Rey, F. 1990. Synthesis and secretion of a Bacillus circulans WL-12 1,3-1,4-β-D-glucanase in *Escherichia coli*. J. Bacteriol. 172: 2160-2167.

[6] Cantwell, B.A., and McConnell, D.J. 1983. Molecular cloning and expression of a *Bacillus subtilis* β-glucanase gene in *Escherichia coli*. Gene 23: 211-219.

[7] Cantwell, B.A., Sharp, P.M., Gormley E., and McConnell, D.J. (Ed) (1988). Molecular cloning of *Bacillus* β-glucanases, p. 181-201. In J.-P. Aubert, P. Beguin, and J. Millet (ed.), Biochemistry and genetics of cellulose degradation. Academic Press, London.

[8] Celestino, K.R.S., Cunha, R.B., Felix, C.R. 2006. Characterization of a β-glucanase produced by *Rhizopus microsporus* var. *microsporus*, and its potential for application in the brewing industry. BMC Biochem. 7: 23-31.

[9] Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R.D., Bairoch, A. 2003. Expaxy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 31, 3784-3788.

[10] Gormley, E.P., Cantwell, B.A., Barker, P.J., Gilmour, R.S., and McConnell, D.J. 1988. Secretion and processing of the *Bacillus subtilis* endo-β-1,3-1,4-glucanase in *Escherichia coli*. Mol. Microbiol. 2: 813-819.

[11] Gosalbes, M., Perez-Gonzalez, J.A., Gonzalez, R., and Navarro, A. 1991. Two β-glycanase genes are clustered in *Bacillus polymyxa*: molecular cloning, expression, and sequence analysis of genes encoding a xylanase and an endo-β-(1,3)-(1,4)-glucanase. J. Bacteriol. 173 : 7705-7710.

[12] Hecker, M., Riethdorf, S., Bauer, C., Schroeter, A., and Rainer. B. 1988. Expression of a cloned β-glucanase gene from *Bacillus amyloliquefaciens* in an *Escherichia coli* relA strain after plasmid amplification. Mol. Gen. Genet. 215: 181-183.

[13] Howard, R.L., Abotsi, E., Jansen van Rensburg, E.L. and Howard, S. 2003. Lignocellulose biotechnology: issues of bioconversion and enzyme production. Afr. J. Biotech. 2(12): 602-619.

[14] Khan, F.A.B., Husaini, A.A.S. 2006. Enhancing α-amylase and cellulase in vivo enzyme expressions on sago pith residue using *Bacillus amyloliquefaciens* UMAS 1002. Biotechnol. (Pakistan) 5: 391-403.

[15] Kim, B.K., Lee, B.H., Lee, Y.J., Jin, I.H., Chung, C,H., Lee, J.W. 2009. Purification and characterization of carboxymethylcellulase isolated from a marine bacterium *Bacillus subtilis* subsp. *subtilis* A-53. Enzyme Mircrob. Technol. 44: 411-416.

[16] Lee, Y.J., Kim, B.K., Lee, B.H., Jo, K.I., and Lee, N.K. et al. 2008. Purification and characterization of cellulase produced by *Bacillus amyloliquefaciens* DL-3 utilizing rice hull. Bioresour. Technol. 99: 378-386.

[17] Li, W., Huan, X., Zhou, Y., Ma, Q., and Chen, Y. 2009. Simultaneous cloning and expression of two cellulase gene from *Bacillus subtilis* newly isolated from Golden Takin (Budorcas taxicolor Bedfordi). Biochem. Biophys. Res. Commun. 383: 397-400.

[18] Liu, B., Yang, Q., Zhou, Q., Song, J., Chen, D., Liu, H. 2004. Cloning and expression of the endo-β-glucanase III cDNA gene from *Trichoderma viride* AS3.3711. Acta Environ. Sinica 25 : 127-132.

[19] Lloberas, J., Perez-Pons, J.A., and Querol, E. 1991. Molecular cloning, expression and nucleotide sequence of the endo-β-1,3-1,4-glucanase from *Bacil-*

lus licheniformis. Predictive structural analyses of the encoded polypeptide. Eur. J. Biochem. 197: 337-343.

[20] Louw, M.E., Reid, S.J., and Watson, T.G. 1993. Characterization, cloning and sequencing of a thermostable endo-(1-3, 1-4)-beta-glucanase-encoding gene from an alkalophilic *Bacillus brevis*. Appl. Microbiol. Biotechnol. 38: 507-513.

[21] MacKay, R.M., Lo, A., Willick, G., Zuker, M., Baird, S., Dove, M., Moranelli, F., Seligy, V. 1986. Structure of a *Bacillus subtilis* endo-beta-1,4-glucanase gene. Nucleic Acids Res. 14(22): 9159-9170.

[22] Mäe, A., Heikinheimo, R., Palva, E.T. 1995. Structure and regulation of the *Erwinia carotovora* subspecies *carotovora* SCC3193 cellulase gene celV1 and the role of cellulase in phytopathogenicity. Mol Gen Genet. 247(1): 17-26.

[23] Malherbe, S., Cloete, T.E. 2003. Lignocellulose biodegradation: fundamentals and applications: A review. Environ. Sci. Biotechnol. 1: 105-114.

[24] Mawadza, C., Hatti-Kaul, R., Zvauya, R., and Mattiasson, B. 2000. Purification and characterization of cellulases produced by two *Bacillus* strains. J. Biotechnol. 83: 177–187.

[25] McCarthy, T.C., Lalor, E., Hanniffy, O., Savage, A.V., Tuohy, M.G. 2005. Comparison of wild-type and UV-mutant beta-glucanase-producing strains of *Talaromyces emersonii* with potential in brewing applications. J. Ind. Microbiol. Biotechnol. 32(4): 125-34.

[26] Miller, L.G. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. 31: 426-428.

[27] Nguyen, L.D., Nguyen, D.D., Dang, H.M., Nguyen, V.P., Nguyen, D.Q., Nguyen, P.T., Pham, V.T. (Ed) (1972) and (1976). Microbiological methods, Hanoi Science and Technology Publishing 1-2.

[28] Nurachman, Z., Kurniasih, S.D., Puspitawati, F., Hadi, S., Radjasa, O.K., and Natalia, D. 2010. Cloning of the endoglucanase gene from a *Bacillus amyloliquefaciens* PSM 3.1 in *Escherichia coli* revealed catalytic triad residues Thr-His-Glu. Am. J. Biochem. & Biotech. 6(4): 268-274.

[29] Onsori, H., Zamani, M.R., Motallebi, M., Zarghami, N. 2005. Identification of over producer strain of endo-1,4-glucanase in *Aspergillus* species: Characterization of crude carboxymethyl cellulase. Afr. J. Biotech. 4(1): 26-30.

[30] Perlman, D., and Halvorson, H.O. 1983. A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. J. Mol. Biol. 167: 391-409.

[31] Rabbani, M., Sadeghi, H.M.M., Ani, M., Chegini, K.G., Etemadifar, Z., Moazen, F. 2009. Cloning and nucleotide sequence of a lipase gene from a soil isolate. Res. Pharm. Sci. 4(1): 25-32.

[32] Robson, L.M., Chambliss, G.H. 1987. Endo-beta-1,4-glucanase gene of *Bacillus subtilis* DLG. J Bacteriol. 169(5): 2017-2025.

[33] Thompson, J.D., Higgins, D.G., Gibson, T.J. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-4680.

[34] To, K.A., Dang, T.T., Nguyen, X.S., Tran, D.M., Le, Q.H., Truong, Q.P., Do, T.H., Nguyen, Q.V., Le, D.K., Le, T., Phung, T.T. 2011. Saccharification and detoxication of sugar cane bagasse lignocelluloses for bioethanol fermentation by *in-house* enzyme mixture. Journal of Science and Technology. 49(1A), 439-445. ISSN 0866 708X.

[35] Walsh, G.A., Murphy, R.A., Killeen, G.F., Headon, D.R., Power, R.F. 1995. Technical note: detection and quantification of supplemental fungal β-glucanase activity in animal feed. J. Anim. Sci. 73: 1074-1076.