

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

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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 Co²⁺. 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 độ 60°C. Hoạt tính enzyme tăng 2,03 lần khi có mặt 5 mM ion Co²⁺. 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

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 lignocelluloses 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' $\{lacI^q$ Tn10 (Tet^R) $\}$ *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX*74 *recA1* *araD*139 Δ (*ara-leu*)7697 *galU* *galK* *rpsL* *endA1* *nupG*) 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% K₂HPO₄, 0.05% NaCl, 0.02% MgSO₄.7H₂O and 0.06% (NH₄)₂SO₄, 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 μ g/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°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'-TACGGTTACCTG TTACGACTT-3') was used in the following reaction: Taq polymerase buffer (10x) 5 μ l; dNTPs (10 mM) 2 μ l; Dream Taq polymerase (5000 U/ml) 0.3 μ l (Thermo Scientific); 16SF primer (10 pmol) 1 μ l; 16SR primer (10 pmol) 1 μ l; DNA template (20 ng) 2 μ l; water 38.7 μ l. The reaction was carried out in the Thermal cycler PT-100 (MJ Research Inc.) with the following program: 95 °C 3 min; 95 °C 1 min; 55 °C 1 min; 72 °C 1 min 15 sec; 30 cycles repeat from step 2 to step 4; 72 °C 7 min; 4 °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'-CTAATTTGGTTCTGATCC-3') and R8 (5'-ATGAAACGGTCAATCTCT-3'). The 50 μ l reaction mixture contained 5 μ l of 10 x Taq polymerase buffer; 2 μ l of 10 mM dNTPs; 0.5 U of Dream Taq polymerase; 1 μ l of each primer and 1 μ l of DNA genomic. The reaction mixture was incubated in a

thermal cycler at 95 °C for 5 min and then underwent 30 amplification cycles of 1 min at 95 °C, 1 min at 45 °C; 2 min at 72 °C; followed by a final incubation of 7 min at 72 °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°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°C, which resulted in a clearing zone after 24 - 48 hours (Figure 1b).



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. amyloliquefaciens* (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.

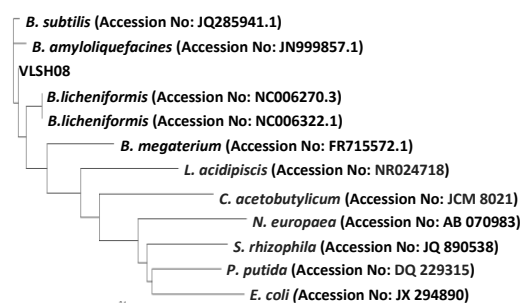


Figure 2. Phylogenetic tree showing the evolutionary position of strain VLSH08 among the genus *Bacillus* and six different genera. GenBank accession numbers are given in parentheses. The bar represents one substitution per 10 nucleotides.

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'.

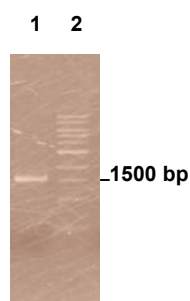


Figure 3. PCR amplification of endo-1,4-β-glucanase gene. Lane 1: endo-1,4-β-glucanase (1.5 kb). Lane 2: 1 kb ladder (New England Biolabs).

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

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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 ACG 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

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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°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°C for 24 hours. Furthermore, the enzyme was very stable at 60°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°C and 70°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 Ca²⁺, Mg²⁺, K⁺, Co²⁺ and Zn²⁺ 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 Ca²⁺ and Zn²⁺ 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 Co²⁺ 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, Co²⁺ 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°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-1,4- β -glucanase are needed.

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