



International Journal of **Biological Chemistry**

ISSN 1819-155X



Academic
Journals Inc.

www.academicjournals.com

Gene Fragments that Encodes Inulin Hydrolysis Enzyme from Genomic *Bacillus licheniformis*: Isolation by PCR Technique Using New Primers

¹Minda Azhar, ²Dessy Natalia, ³Sumaryati Syukur, ⁴Vovien and ⁴Jamsari

¹Laboratory of Biochemistry, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang, Jl. Prof. Dr. Hamka, Air Tawar, Padang, 25131, Indonesia

²Biochemistry Research Division, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Jl. Ganesha 10, Bandung, 40132, Indonesia

³Laboratory of Biochemistry, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Andalas, Padang, 25163, Indonesia

⁴Laboratory of Biotechnology, Faculty of Agriculture, Universitas Andalas, Padang, 25163, Indonesia

Corresponding Author: Minda Azhar, Laboratory of Biochemistry, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang, Jl. Prof. Dr. Hamka, Air Tawar, Padang, 25131, Indonesia
Tel: +62 57420106 Fax: +62 7058772

ABSTRACT

Fructose and Fructo-Oligosaccharides (FOS) are derived more practical from the enzymatic hydrolysis reaction of inulin. In this study, the gene fragments that encodes inulin hydrolysis enzyme were isolated from genomic *B. licheniformis* by Polymerase Chain Reaction (PCR) technique using new primers designed on conserved domain in family GH32 enzymes. Gene fragments were cloned into pGEM-T vector with *E. coli* as host cells and determined the sequence of nucleotide bases. Size of the gene fragment have been found 539 bp using the DPE.sIF and DPE.eR primer pair. The gene fragment encodes 179 amino acid residues of protein fragment. The protein fragment has high homology with levanase of *Bacillus subtilis* BSn5, levanase of *Bacillus subtilis* subsp. subtilis str 168, exoinulinase of *Pseudomonas mucidolents*, exoinulinase of *Paenibacillus polymyxa*, exoinulinase of *Geobacillus stearothermophilus*, exoinulinase of *Paenibacillus Aloe* sp-11 and exoinulinase of *Paenibacillus polymyxa* SC2. The homology were 97, 97, 62, 62, 61, 62 and 62%, respectively. The primers can use to isolate gene fragment that encodes inulin hydrolysis enzyme from the *Bacillus* genus.

Key words: *Bacillus*, inulin hydrolysis enzyme, levanase gene, inulinase gene, family GH32

INTRODUCTION

Fructose and Fructo-Oligosaccharides (FOS) are very important compound in the food, beverage and pharmaceutical industry (Tohamy, 2006; Sirisansaneeyakul *et al.*, 2007; Singh and Gill, 2006). Fructose is a natural sweetener. Fructose is safer than sucrose because it has beneficial effects on diabetes patients, increase iron absorption in children and have higher sweet capacity (Singh and Gill, 2006). Otherwise, sucrose is known to cause diseases associated with corpulence, cariogenicity, arteriosclerosis and diabetes (Ricca *et al.*, 2007). The FOS is known as a functional food because it has a positive influence on the composition of the human gut microflora (Roberfroid *et al.*, 1998; Kaplan and Hutkins, 2003).

Fructose and FOS are obtained more practical from enzymatic hydrolysis reaction of inulin. Inulinase or levanase can be used as biocatalyst for enzymatic hydrolysis reaction of inulin. They are belonging to Glycoside Hydrolase family (GH)32. Inulinase and levanase can catalyze the hydrolysis of inulin or levan substrate. Inulinase of *Bacillus polymyxa* can hydrolyze sucrose, levan, raffinosa and inulin substrates (Kwon *et al.*, 2003). Exoinulinase from *Aspergillus awamori* can hydrolyze inulin and levan substrates through exo-action mode released fructose (Arand *et al.*, 2002). Levanase from *Bacillus subtilis* was expressed in *Escherichia coli* active on levan, inulin and sucrose substrates (Wanker *et al.*, 1995) while exolevanase from *Gluconacetobacter diazotrophicus* SRT4 can hydrolyze levan, inulin and sucrose substrates (Menendez *et al.*, 2002).

Action mode of endo-or exo-levanase and inulinase on inulin generate different products. Fructose production from inulin can be used exoinulinase or exolevanase while FOS production from inulin can be used endoinulinase or endolevanase. The combination of endo-and exo-action mode have strong synergistic effect to produce fructose from inulin (Sirisansaneeyakul *et al.*, 2007). Thus, bacteria that expresses levanase and inulinase have potential applications for inulin transformation to fructose or FOS. Therefore, the search for the gene encoding of inulinase and levanase carried out until now.

Inulinase gene has been isolated from genomic *Pichia guilliermondii* yeast by using the PCR technique. The gene have the Open Reading Frame (ORF) 1542 bp which encodes 514 amino acid residues of the inulinase protein (Zhang *et al.*, 2009). Levanase gene was isolated from genomic libraries of *Gluconacetobacter diazotrophicus* SRT4 and expressed in *Escherichia coli* (Menendez *et al.*, 2002) while levanase gene from *Bacillus subtilis* has been expressed in *Saccharomyces cerevisiae* (Martel *et al.*, 2011).

The information of inulin degrading bacteria from hot springs Bukik Kili Solok and Padang Balimbiang in West Sumatra began to be studied in 2009. Isolate bacteria from the hot springs have been identified in genotype and phenotype (Azhar, 2013). In this study, the isolation of a gene fragment encoding of inulin hydrolysis enzyme from genomic DNA *Bacillus licheniformis* by PCR technique using new primers designed on conserved domain in family GH32 enzymes was presented. The gene fragments were cloned and determined the nucleotide base sequences.

MATERIALS AND METHODS

Bacteria: *Bacillus licheniformis* UBCT-007 was used as source of genomic DNA. The bacteria have been identified in phenotype and genotype (Azhar, 2013). The bacteria was cultured in the medium inulin as the sole carbon source (g L^{-1}), 2 g $(\text{NH}_4)_2\text{SO}_4$, 14 g KH_2PO_4 , 6 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g trisodium citrate, 10 g inulin or inulin-RBB and 20 g agar (Castro *et al.*, 1995).

Isolation of genomic DNA bacteria: Culture in exponential phase was put in 1.5 mL micro tube, then centrifuged at 13,000-16,000x g for 5 min. Isolation and purification of genomic DNA bacteria was carried out according to the procedure in the Wizard Genomic DNA Purification Kit (Promega, 2010).

Isolation of gene fragment that encodes inulin hydrolysis enzyme: The gene fragment that encodes inulin hydrolysis enzyme was isolated from the genomic DNA of *B. licheniformis* by PCR technique using degenerative primers. The primers were designed based on the alignment conserved domain of amino acid residues of exolevanase and exoinulinase from several *Bacillus* genus. The degenerative primers have been synthesized by Macrogen in Korea.

The gene fragment that encodes inulin hydrolysis enzyme was amplified by using PCR technique in a final concentration of MgCl₂ and annealing temperature variation (adjusted with forward and reverse primer T_m). The composition of the master mix as follows: 2.5 µL 10x Dream Taq buffer, 0.5 µL 10 mM dNTP mix, concentration variations of MgCl₂ (2, 3 and 4 mM), 0.5 µL forward primer 20 µM, 0.5 µL reverse primer 20 µM, 0.5 µL samples (75 ng µL⁻¹), 0.125 µL Dream Taq DNA polymerase. ddH₂O was added to a volume of 25 µL. The PCR process was done at initial denaturation 94°C for 2 min, denaturation 94°C for 60 sec, annealing temperature variation for 60 sec, elongation 72°C for 60 sec, final elongation 72°C for 5 min. The PCR cycles were done 29 times. Amplicon was amplified on optimum condition of PCR, purified and cloned in pGEM-T Easy vector. Amplicons were electrophoresed using agarose gel.

Agarose electrophoresis: Amplicons were electrophoresed by using 0.8% agarose gel. Agarose was dissolved in 1x TAE buffer and heated to boil. After temperature of agarose solution was about 40°C, agarose solution was added EtBr, poured in a tray and mounted comb. The tray was placed on a vessel containing 1x TAE buffer, then comb removed gently. The DNA samples were mixed with bromphenol blue loading buffer (Fermentas) and put in the gel wells. Determination of size and approximate DNA concentration were used 1 kb DNA ladder marker (Fermentas). Electrophoresis was performed at a voltage of 80 V for 35 min. Electrophoresis results were observed under UV light.

Cloning of gene fragments that encodes inulin hydrolysis enzyme and sequencing: Gene fragment that encodes inulin hydrolysis enzyme was ligated to pGEM-T Easy vector with a mole ratio of DNA fragments to vector of 3:1 (Promega, 2010). The mixture of ligation reaction were 5 µL of ligation buffer 2 x rapid T4 DNA ligase, 1 µL of pGEM-T Easy 50 ng, 1 µL of T4 DNA ligase (3 Weiss units µL⁻¹), 1 µL of insert 20 ng µL⁻¹. ddH₂O was added to final volume of 10 µL. The reaction mixture was incubated at room temperature for 1 h or at 4°C for overnight. Ligation results (5 µL) were transformed in *E. coli* TOP10F⁺ according to the procedure of Sambrook *et al.* (1989). Recombinant DNA that carried the DNA fragment (insert DNA) was isolated from white transformant colonies according to the procedure in the High-Speed Plasmid Mini Kit (Geneaid). Recombinant DNA (1000 ng) were sent to Macrogen in Korea for sequencing by using T7 universal primer.

Bioinformatics: Nucleotide sequences in a file format AB1 was analyzed by using the SeqMen and EditSeq program of DNA STAR program. Sequence similarity with another amino acid residues of protein in the GenBank database was determined by using the BLASTx program (<http://www.ncbi.nlm.nih.gov>). Amino acid residues of proteins and nucleotides of genes were aligned by using the ClustalW2 program (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

The interaction of intra-and inter-molecular primer and estimation of primer annealing temperature (T_a) were determined by using oligos program. The estimated T_m primer was determined by using the OligoAnalyzer 3.1 program (<http://www.idtdna.com/analyzer/applications/oligoanalyzer/>). The nucleotide sequences of the gene were translated into amino acid residue sequences by using the standard genetic code at <http://web.expasy.org/translate/>.

RESULTS AND DISCUSSION

Design of new degenerative primer for gene fragment isolation that encodes inulin hydrolysis enzyme: Gene fragments that encodes inulin hydrolysis enzyme from genomic DNA

Bacillus licheniformis were isolated by PCR techniques by using the degenerative primers. Degenerative primers were designed based on conserved region in alignment of amino acids residues of several exoinulinase and exolevanase from *Bacillus* genus in the GenBank database. Exoinulinase alignment were exoinulinase from *Geobacillus stearothermophilus* (BAC45010.1), *Paenibacillus polymyxa* (AAL82575.1), *Bacillus subtilis* (AAK00768.2), *Bacillus* sp. snu-7 (AAK54126.1) and a hypothetical protein from *Bacillus licheniformis* Bli04178 ATCC 14580 while exolevanase from *Bacillus subtilis* (ADV93476.1). Figure 1 shows the amino acid residues alignment of the enzymes.

The size of the gene fragment that encodes inulin hydrolysis enzyme as PCR product using DPE.slF and DPE.dR primer pair were estimated about 400 bp while the DPE.slF and DPE.eR primer pair were estimated about 550 bp. The amplicon nucleotide sequences of 400 bp contained in 550 bp. The position of these primers were in the conserved domains as shown clearly in Fig. 1.

Amplification of gene fragment that encodes inulin hydrolysis enzyme: Amplification of the gene fragment that encodes inulin hydrolysis enzyme from genomic *B. licheniformis* UBCT-007 was performed at PCR optimum conditions. Determination of amplification optimum condition was performed at final MgCl₂ concentration variation and annealing temperature variations. Mg²⁺ ions and annealing temperature are important factors that affected the PCR process (Kramer and Coen, 2003). In the PCR process by using the Dream Tag DNA Polymerase, MgCl₂ has been added to the Dream Tag Buffer 10x with a final 2 mM MgCl₂ concentration. Final MgCl₂ concentration can be increased to 4 mM (Thermo Scientific, 2011). Therefore, variations of the final MgCl₂ concentration were chosen 2, 3 and 4 mM.

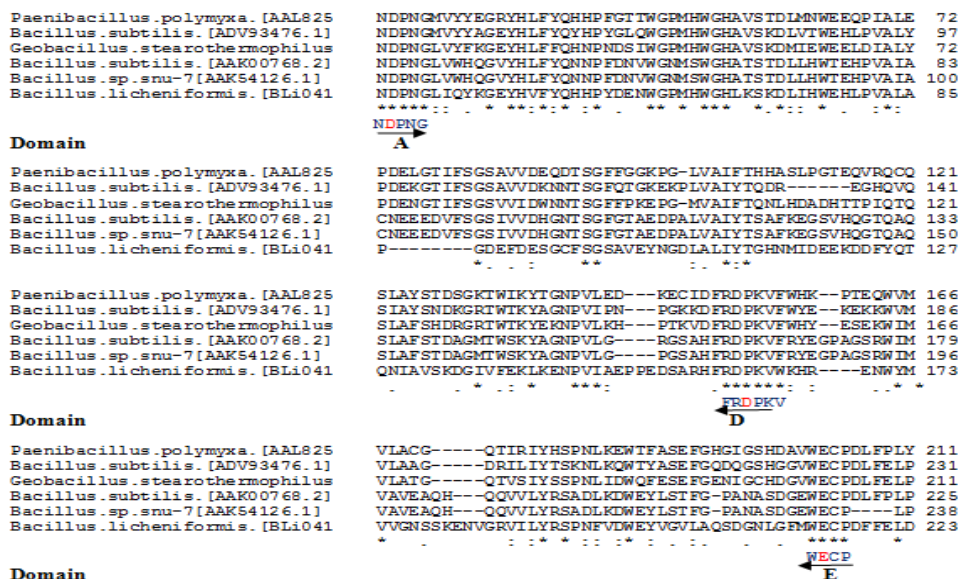


Fig. 1: Alignment of amino acid residues of exoinulinase and exolevanase from *Bacillus* genus. *Geobacillus stearothermophilus* (BAC45010.1), *Paenibacillus polymyxa* (AAL82575.1), *Bacillus subtilis* (AAK00768.2), *Bacillus* sp. snu-7 (AAK54126.1), *Bacillus licheniformis* (Bli04178), *Bacillus subtilis* (ADV93476.1). A, D and E domains are conserved domain of GH32 family

The selection of annealing temperature variation in the PCR process based on the T_m value of the primer pairs. The variation was 10 points below the temperature T_m . Degenerative primer that used to amplify the gene fragment that encodes inulin hydrolysis enzyme from genomic DNA *Bacillus licheniformis* UBCT-007 were listed in Table 1. Other factors affected PCR process are the sequence and length of primers, template concentration, dNTP concentration, denaturation, annealing and elongation time (Ahmed, 2006). Elongation time was used in the PCR process depending on the size of the amplified DNA fragment. An approximate speed of elongation is 1 kb every minute for Dream Tag DNA polymerase (Thermo Scientific, 2011).

Amplification of the gene fragment that encodes inulin hydrolysis enzyme from *B. licheniformis* UBCT-007 using DPE.slF and DPE.dR primer pair performed at annealing temperatures 44.0, 47.1, 50.7 and 52.0°C with the final concentration of 2, 3 and 4 mM $MgCl_2$. The results of the gene fragment electrophoresis on agarose gel is shown in Fig. 2. At the annealing temperature of 50.7°C and final concentration of 4 mM $MgCl_2$ was produced one thick band amplicon about 400 bp (line 10). Bright band below 250 bp DNA marker was the primer residues. This condition was defined as the amplified optimum conditions of the gene fragment that encodes inulin hydrolysis enzyme using DPE.slF and DPE.dR primer pair. Initial denaturation for 2 min at 94°C and final elongation for 7 min at 72°C. Conditions of each cycle PCR were denaturation for 60 sec, annealing time for 60 sec and elongation time for 60 sec. The amplicon was produced by using the PCR technique under the optimum PCR condition. Amplicons were purified and cloned in pGEM-T Easy vector.

Amplification of the gene fragment that encodes inulin hydrolysis enzyme from genomic *B. licheniformis* UBCT-007 using the PCR technique with DPE.slF and DPE.eR primer pairs performed at annealing temperatures of 48.0, 49.6, 52.1 and 56.0°C and $MgCl_2$ concentration of 2, 3 and 4 mM. Amplicons on agarose gel electrophoresis are shown in Fig. 3. Close observation of Fig. 3, it is revealed that at 56°C annealing temperature and the 3 mM $MgCl_2$ final concentration were produced one band amplicons around 550 bp (line 5). Condition of each PCR cycle was denaturation for 45 sec, annealing time for 30 sec and elongation time for 45 sec. The conditions

Table 1: Degenerative primer to amplify of gene fragment that encodes inulin hydrolysis enzyme

Primer	Conserved domain	Direction	Sequences of nucleotides	Size (bp)	$T_m(^{\circ}C)$	Reference
DPE.slF	A	Forward	TGG MTR AAY GAY CCM AAC GGA C	22	58,4	This study
DPE.dR	D	Reverse	TRS CRR AAC ACY TTV GGR TC	20	52,3	This study
DPE.eR	E	Reverse	GGR CAT TCC CAY WCV CCR TC	20	58,4	This study
-	A	Forward	TGG ATG AAY GAY CCN AAY GG	20	-	Zhang <i>et al.</i> (2009)
-	D	Reverse	TGR AAR AAN ACY TTN GGT	19	-	Zhang <i>et al.</i> (2009)
-	A	Forward	TGG ATG AAY GAY CCI AAY GGI CTI G	25	-	Basran <i>et al.</i> (2010)
-	D	Reverse	R AAY ACY TTI GGR TCY CTR AAR TC	24	-	Basran <i>et al.</i> (2010)

M: A/C, R: A/G, Y: C/T, S: G/C, V: A/G/C, W: A/T, N: A/G/C/T

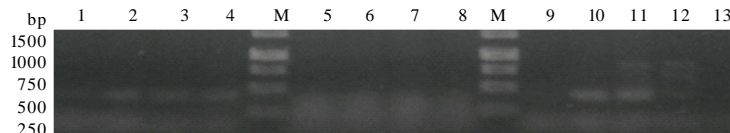


Fig. 2: PCR amplification of gene fragment encoding inulin hydrolyzing enzyme using DPE.slF dan DPE.dR. primer. Line 1-4, 5-8 and 9-12: Amplicon at annealing temperature 52.0, 50.7, 47.1 and 44.0°C with final concentration of $MgCl_2$ 2, 3, 4 mM, respectively. Line 13: Negative control. Line 10: One band amplicon, about 400 bp. M (marker): 1 kb DNA ladder. Smear bands under marker 250 bp were unused primers from the PCR reaction

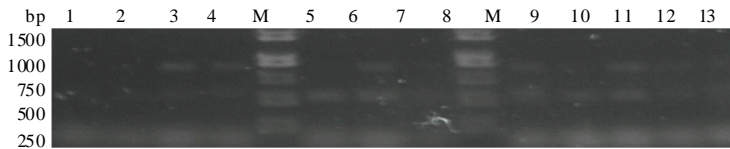


Fig. 3: PCR amplification of gene fragment that encodes inulin hydrolysis enzyme using DPE.slF and DPE.eR primers. Line 1-4, 5-8, 9-12: Amplicon at annealing temperature 56.0, 52.1, 49.6 and 48.0°C with final concentration of MgCl₂ 2, 3 and 4 mM, respectively. Line 5: One band amplicon about 550 bp. Smear bands under marker 250 bp were unused primers from the PCR reaction. M (marker): 1 kb DNA ladder

were optimum conditions to amplify the gene fragment that encodes inulin hydrolysis enzyme by using DPE.slF and DPE.eR primers. In the conditions, amplicons were produced using the PCR technique, purified and cloned in pGEM-T Easy vector.

Cloning of the gene fragment that encodes inulin hydrolysis enzyme in pGEM-T

Easy: Each pure amplicon of approximately 400 and 550 bp was ligated to pGEM-T Easy vector. The DNA ligation was transformed in *E. coli* TOP10F⁺ cells by heat shock method, grown in LB medium containing ampicillin, IPTG (isopropyl-thiogalactoside) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). There were white and blue transformant colonies on the media surface at 16 h later. White transformant colonies contained the pGEM-T Easy with insert DNA. Blue transformant colonies carried pGEM-T Easy without insert DNA. X-gal is a β-galactosidase substrate that is analogous to lactose. The IPTG is an inducer of the lacZ gene. LacZ gene express β-galactosidase which would catalyze break the bond on X-gal produces galactose and 5-bromo-4-chloro-3-Hydroxyindole. 5-bromo-4-chloro-3-Hydroxyindole are oxidized further to 5,5'-dibromo-4,4'-dichloro-indigo. The compound gives blue color in the transformant cells. Expression of β-galactosidase will be disrupted if the pGEM-T Easy contains the insert DNA, because the insert DNA is ligated to the lacZ gene in the vector. The position of cutting side of the EcoRV restriction enzyme was added thymidine at both ends of the vector (Promega, 2010). The pGEM-T Easy also contains genes that express enzymes to degradate ampicillin. Therefore, the transformants can live in LB medium containing ampicillin.

White transformants colony that carried pGEM-T Easy with insert DNA were screened by using colony PCR method. Colony PCR method is very quick and easy to screen bacterial colonies containing specific DNA sequences by using PCR technique without DNA purification from the bacteria (Woodman, 2005). In this method, the time in initial denaturation process of PCR was increased to 5 min to allow bacterial lysis and DNA denaturation. Colony PCR products that align with the insert DNA fragment was bacteria colony contains recombinant DNA with desired insert DNA (Fig. 4). The pGEM-T Easy containing desired insert DNA was isolated and purified to be used as a template in sequencing of the inserts DNA using T7 primer.

Nucleotide sequences of the gene fragment that encodes inulin hydrolysis enzyme and deduced amino acid sequences:

The size of the gene fragments that encodes inulin hydrolysis enzyme were found 398 and 539 bp by using DPE.slF and DPE.dR, DPE.slF and DPE.eR primer pairs, respectively. The nucleotide sequences of the gene fragment is shown in Fig. 5. In the nucleotide sequences of 539 bp DNA fragment was found the nucleotide sequences of 398 bp DNA fragment. This is suitable with the draft position of degenerative primer of inulin

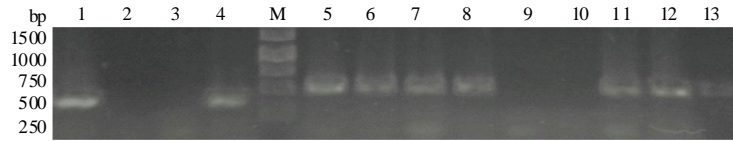


Fig. 4: PCR amplification of colony PCR from white transformant colony. Line 1 and 4 amplicon colony PCR using DPE.slF and DPE.dR primers. Line 5-8, 11 and 12: Amplicon colony PCR using DPE.slF and DPE.eR primers with, Line 13: Positip control. M (marker): 1 kb DNA ladder. Smear bands under marker 250 bp were unused primers from the PCR reaction

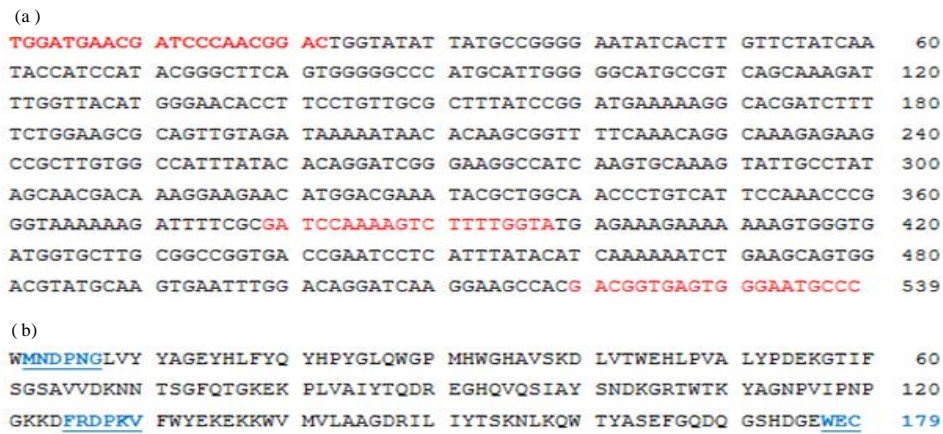


Fig. 5(a-b): (a) Nucleotide sequences of gene fragment. DPE.slF, DPE.dR and DPE.eR (red letter) and (b) Amino acid residues of protein fragment. Blue letter are conserved domain A, D and E, respectively

hydrolysis enzymes from *Bacillus* genus bacteria as can be seen in Fig. 1. Deduction of 539 bp DNA gene fragment that encodes inulin hydrolysis enzyme from *Bacillus licheniformis* UBCT-007 generates protein fragment along the 179 amino acid residues (Fig. 5).

Conserved domain of family GH32 enzyme groups were found no less eight domains: A, B, B1, C, D, E, F and G (Goosen, 2007). Position of PCR primers were designed in conserved domain A, D and E. Three amino acid residues, namely amino acid residue D (Asp) in the conserved domain A and D, amino acid residue E (Glu) in the conserved domain E. The residues act directly on the glycoside bond hydrolysis reaction of inulin (Nagem *et al.*, 2004). The residues are catalytic residues in the enzyme of inulin hydrolysis reaction. Therefore, conserved domains A, D and E are used as primers for amplifying the gene fragment that encodes inulin hydrolysis enzyme in genomic DNA *B. licheniformis*. Conserved domain A was used as forward primer while the conserved domain D and E were used reverse primers (Table 1). Zhang *et al.* (2009) made forward primer in conserved domain A and reverse primer in conserved domain D to amplify the gene fragment of exoinulinase from marine yeast *Pichia guilliermondii* (Zhang *et al.*, 2009) while Basran amplified exoinulinase gene fragment from endophytic bacteria of *Black solenostemon scutellarioides* plant (Basran *et al.*, 2010).

The size of the gene fragment that encodes inulin hydrolysis enzyme was found 398 bp using DPE.slF and DPE primer pair. The BLASTx results indicated that the fragment of this protein has specific hit GH32 β -fructosidase with two residues of active site and four residues of the substrate

binding. The protein fragment has high homology with levanase from *Bacillus subtilis*, levanase from *Bacillus majovensis*, exoinulinase from *Geobacillus stearothermophilus*, exoinulinase *Pseudomonas mucidolents*, exoinulinase from *Paenibacillus polymyxa*, exoinulinase from *Paenibacillus Aloe* sp-11, exoinulinase from *Paenibacillus polymyxa* SC2 and exoinulinase of chain A from *Aspergillus awamori*. The homology were 98, 90, 64, 63, 63, 62, 62 and 51%, respectively (Table 2).

The size of gene fragment that encodes inulin hydrolysis enzyme was found 539 bp by using DPE.slF and DPE.eR primer pairs (Fig. 5). At the nucleotide sequences of 539 bp DNA fragment was found the nucleotide sequences of 398 bp DNA fragment. It is appropriate with the draft position of degenerative primer in inulin hydrolyzing enzymes from bacteria *Bacillus* genus (Fig. 1). BLASTx results indicated that the protein fragment has a specific hit GH32 β -fructosidase with three and five residues of the active site residues of the substrate binding. Thus, in the 539 bp fragment was found one more residue of the active site and the substrate-binding residues.

Gene fragment deduction (539 bp) that that encodes inulin hydrolysis enzyme from *Bacillus licheniformis* UBCT-007 generates protein fragment along 179 amino acid residues (Fig. 5). At the protein fragment was found conserved domains A, B, B1, C, D and E. The domains are six domains of eight domains (A, B, B1, C, D, E, F and G) group of family GH32 enzymes (Goosen, 2007). Domain A, D and E are position of the DPE.slF, DPE.dR and DPE.eR primer while domains F and G located after the domain E. Thus, the gene fragment has been found in *Bacillus licheniformis* UBCT-007 which showed the family GH32 enzyme group.

Protein fragments along the 179 amino acid residues have high homology with levanase from *Bacillus subtilis* BSn5, levanase *Bacillus subtilis* subsp. *subtilis* str 168, exoinulinase *Pseudomonas mucidolents*, exoinulinase *Paenibacillus polymyxa*, exoinulinase *Geobacillus stearothermophilus*, exoinulinase *Paenibacillus Aloe* sp-11 and exoinulinase *Paenibacillus polymyxa* SC2. The homology were 97, 97, 62, 62, 61, 62 and 62%, respectively (Table 3). Thus, gene fragments that encodes inulin hydrolysis enzyme have been found in *Bacillus licheniformis* UBCT-007. The sequences of protein fragment are estimated belong to a levanase group. Levanase and inulinase are family GH32 enzyme group.

Table 2: Homology of DNA fragment 398 bp with inulin hydrolysis enzyme from bacteria

Accession number of GenBank	Description	Homology (%)
YP004204503.1	Levanase from <i>Bacillus subtilis</i>	98
ZP10515038.1	Levanase from <i>Bacillus majovensis</i>	90
BAC45010.1	Exoinulinase from <i>Geobacillus stearothermophilus</i>	64
AAF44125.1	Exoinulinase from <i>Pseudomonas mucidolents</i>	63
AAL82575.1	Exoinulinase from <i>Paenibacillus polymyxa</i>	63
FHS58800.1	Exoinulinase from <i>Paenibacillus</i> sp. Aloe-11	62
08202772.1	Exoinulinase from <i>Paenibacillus</i> polymyxa SC2	62
IY9MA	Chain A exoinulinase from <i>Aspergillus awamori</i>	51

Table 3: Homology of DNA fragment 539 bp with levanase and exoinulinase bacteria

Accession number of GenBank	Description	Homology (%)
YP004204503.1	Levanase <i>Bacillus subtilis</i> BSn5	97
NP390581.1	Levanase <i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168	97
AAF44125.1	Exoinulinase <i>Pseudomonas mucidolents</i>	62
AAL82575.1	Exoinulinase <i>Paenibacillus polymyxa</i>	62
BAC45010.1	Exoinulinase <i>Geobacillus stearothermophilus</i>	61
ZP09772525.1	Exoinulinase <i>Paenibacillus</i> sp. Aloe-11	62
YP003945276.1	Exoinulinase <i>Paenibacillus polymyxa</i> SC2	62

Alignment of the protein fragments 179 amino acids residues from *B. licheniformis* UBCT-007 with levanase from *Bacillus subtilis* BSn5, levanase from *Bacillus subtilis* subsp. subtilis str.168, exoinulinase from *Paenibacillus polymyxa*, exoinulinase from *Geobacillus stearothermophilus* and exoinulinase from *Bacillus* sp. snu-7 shows that the protein fragment has the highest homology (closest resemblance) with levanase from *Bacillus subtilis* (Table 3 and Fig. 6). Homology between the protein fragment with levanase from a variety of different *Bacillus subtilis* strains: *B. subtilis* (CAA29137.1), *B. subtilis* BSn5 (ADV93476.1), *B. subtilis* subsp. str168 (NP390581.1), *B. subtilis* subsp. str SC-8 (EHA31108.1) and *B. subtilis* subsp. natto.BEST 195 (BAI86177.1) is 97%. Homology between the compared levanase is 98 and 100%. Alignment of levanase protein fragments from various *Bacillus subtilis* strains is shown in Fig. 7. Thus, gene fragment of levanase have been found in *Bacillus licheniformis* UBCT-007.

```

lev. B. subtilis subsp str168 [NP390581.1] EANWMNDPDMGMVYYAGEYHLFYQYHPYGLQWGMHWGHAUSKDLVTWEHL 92
lev. B. subtilis subsp strSC-8 [EHA31108.1] EANWMNDPDMGMVYYAGEYHLFYQYHPYGLQWGMHWGHAUSKDLVTWEHL 100
lev. B. subtilis [CAA29137.1] EANWMNDPDMGMVYYAGEYHLFYQYHPYGLQWGMHWGHAUSKDLVTWEHL 68
lev. B. subtilis BSn5 [ADV93476.1] EANWMNDPDMGMVYYAGEYHLFYQYHPYGLQWGMHWGHAUSKDLVTWEHL 92
lev. B. subtilis subsp.natto.BEST195[BAI86177.1] EANWMNDPDMGMVYYAGEYHLFYQYHPYGLQWGMHWGHAUSKDLVTWEHL 100
Fragmen protein-179 --WLNDPGLVVYAGEYHLFYQYHPYGLQWGMHWGHAUSKDLVTWEHL 47
*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*

lev. B. subtilis subsp str168 [NP390581.1] PVALYPDEKGTIFSGSAVWDKNNITSGFQTGKEKPLVAIYITQDREGHQVQS 142
lev. B. subtilis subsp strSC-8 [EHA31108.1] PVALYPDEKGTIFSGSAVWDKNNITSGFQTGKEKPLVAIYITQDREGHQVQS 150
lev. B. subtilis [CAA29137.1] PVALYPDEKGTIFSGSAVWDKNNITSGFQTGKEKPLVAIYITQDREGHQVQS 118
lev. B. subtilis BSn5 [ADV93476.1] PVALYPDEKGTIFSGSAVWDKNNITSGFQTGKEKPLVAIYITQDREGHQVQS 142
lev. B. subtilis subsp.natto.BEST195[BAI86177.1] PVALYPDEKGTIFSGSAVWDKNNITSGFQTGKEKPLVAIYITQDREGHQVQS 150
Fragmen protein-179 PVALYPDEKGTIFSGSAVWDKNNITSGFQTGKEKPLVAIYITQDREGHQVQS 97
*****

lev. B. subtilis subsp str168 [NP390581.1] IAYSNDKGRITWTKYAGNFVIPNPGKKDFRDPKVFVWYEEKKHWVTVLAAGD 192
lev. B. subtilis subsp strSC-8 [EHA31108.1] IAYSNDKGRITWTKYAGNFVIPNPGKKDFRDPKVFVWYEEKKHWVTVLAAGD 200
lev. B. subtilis [CAA29137.1] IAYSNDKGRITWTKYAGNFVIPNPGKKDFRDPKVFVWYEEKKHWVTVLAAGD 168
lev. B. subtilis BSn5 [ADV93476.1] IAYSNDKGRITWTKYAGNFVIPNPGKKDFRDPKVFVWYEEKKHWVTVLAAGD 192
lev. B. subtilis subsp.natto.BEST195[BAI86177.1] IAYSNDKGRITWTKYAGNFVIPNPGKKDFRDPKVFVWYEEKKHWVTVLAAGD 200
Fragmen protein-179 IAYSNDKGRITWTKYAGNFVIPNPGKKDFRDPKVFVWYEEKKHWVTVLAAGD 147
*****

lev. B. subtilis subsp str168 [NP390581.1] RILIYTSKNLKQWTYASEFGDQSSHGGSUWECFDLFLFVVDGNGNPKQKWV 242
lev. B. subtilis subsp strSC-8 [EHA31108.1] RILIYTSKNLKQWTYASEFGDQSSHGGSUWECFDLFLFVVDGNGNPKQKWV 250
lev. B. subtilis [CAA29137.1] RILIYTSKNLKQWTYASEFGDQSSHGGSUWECFDLFLFVVDGNGNPKQKWV 218
lev. B. subtilis BSn5 [ADV93476.1] RILIYTSKNLKQWTYASEFGDQSSHGGSUWECFDLFLFVVDGNGNPKQKWV 242
lev. B. subtilis subsp.natto.BEST195[BAI86177.1] RILIYTSKNLKQWTYASEFGDQSSHGGSUWECFDLFLFVVDGNGNPKQKWV 250
Fragmen protein-179 RILIYTSKNLKQWTYASEFGDQSSHGGSUWEC----- 179
*****

```

Fig. 6: Protein fragment alignment of 179 amino acid residues from *B. licheniformis* with levanase and exoinulinase from *Bacillus* genus

```

lev. B. subtilis subsp str168 [NP390581.1] EANWMNDPDMGMVYYAGEYHLFYQYHPYGLQWGMHWGHAUSKDLVTWEHL 92
lev. B. subtilis subsp strSC-8 [EHA31108.1] EANWMNDPDMGMVYYAGEYHLFYQYHPYGLQWGMHWGHAUSKDLVTWEHL 100
lev. B. subtilis [CAA29137.1] EANWMNDPDMGMVYYAGEYHLFYQYHPYGLQWGMHWGHAUSKDLVTWEHL 68
lev. B. subtilis BSn5 [ADV93476.1] EANWMNDPDMGMVYYAGEYHLFYQYHPYGLQWGMHWGHAUSKDLVTWEHL 92
lev. B. subtilis subsp.natto.BEST195[BAI86177.1] EANWMNDPDMGMVYYAGEYHLFYQYHPYGLQWGMHWGHAUSKDLVTWEHL 100
Fragmen protein-179 --WLNDPGLVVYAGEYHLFYQYHPYGLQWGMHWGHAUSKDLVTWEHL 47
*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*

lev. B. subtilis subsp str168 [NP390581.1] PVALYPDEKGTIFSGSAVWDKNNITSGFQTGKEKPLVAIYITQDREGHQVQS 142
lev. B. subtilis subsp strSC-8 [EHA31108.1] PVALYPDEKGTIFSGSAVWDKNNITSGFQTGKEKPLVAIYITQDREGHQVQS 150
lev. B. subtilis [CAA29137.1] PVALYPDEKGTIFSGSAVWDKNNITSGFQTGKEKPLVAIYITQDREGHQVQS 118
lev. B. subtilis BSn5 [ADV93476.1] PVALYPDEKGTIFSGSAVWDKNNITSGFQTGKEKPLVAIYITQDREGHQVQS 142
lev. B. subtilis subsp.natto.BEST195[BAI86177.1] PVALYPDEKGTIFSGSAVWDKNNITSGFQTGKEKPLVAIYITQDREGHQVQS 150
Fragmen protein-179 PVALYPDEKGTIFSGSAVWDKNNITSGFQTGKEKPLVAIYITQDREGHQVQS 97
*****

lev. B. subtilis subsp str168 [NP390581.1] IAYSNDKGRITWTKYAGNFVIPNPGKKDFRDPKVFVWYEEKKHWVTVLAAGD 192
lev. B. subtilis subsp strSC-8 [EHA31108.1] IAYSNDKGRITWTKYAGNFVIPNPGKKDFRDPKVFVWYEEKKHWVTVLAAGD 200
lev. B. subtilis [CAA29137.1] IAYSNDKGRITWTKYAGNFVIPNPGKKDFRDPKVFVWYEEKKHWVTVLAAGD 168
lev. B. subtilis BSn5 [ADV93476.1] IAYSNDKGRITWTKYAGNFVIPNPGKKDFRDPKVFVWYEEKKHWVTVLAAGD 192
lev. B. subtilis subsp.natto.BEST195[BAI86177.1] IAYSNDKGRITWTKYAGNFVIPNPGKKDFRDPKVFVWYEEKKHWVTVLAAGD 200
Fragmen protein-179 IAYSNDKGRITWTKYAGNFVIPNPGKKDFRDPKVFVWYEEKKHWVTVLAAGD 147
*****

lev. B. subtilis subsp str168 [NP390581.1] RILIYTSKNLKQWTYASEFGDQSSHGGSUWECFDLFLFVVDGNGNPKQKWV 242
lev. B. subtilis subsp strSC-8 [EHA31108.1] RILIYTSKNLKQWTYASEFGDQSSHGGSUWECFDLFLFVVDGNGNPKQKWV 250
lev. B. subtilis [CAA29137.1] RILIYTSKNLKQWTYASEFGDQSSHGGSUWECFDLFLFVVDGNGNPKQKWV 218
lev. B. subtilis BSn5 [ADV93476.1] RILIYTSKNLKQWTYASEFGDQSSHGGSUWECFDLFLFVVDGNGNPKQKWV 242
lev. B. subtilis subsp.natto.BEST195[BAI86177.1] RILIYTSKNLKQWTYASEFGDQSSHGGSUWECFDLFLFVVDGNGNPKQKWV 250
Fragmen protein-179 RILIYTSKNLKQWTYASEFGDQSSHGGSUWEC----- 179
*****

```

Fig. 7: Protein fragment ailment of 179 amino acid residues from *B. licheniformis* with levanase from different *Bacillus subtilis* strain

Three dimensional model of protein fragment structure that consist of 179 amino acid residues shows β -propeller folding. Closest three dimensional model homology of the protein (50%) was exoinulinase from *Aspergillus awamori* (PDB code 1Y9G) (Kelley and Sternberg, 2009). Nevertheless, this research has found 539 bp gene fragment that encodes inulin hydrolysis enzyme from genomic *Bacillus licheniformis* UBCT-007 that belong to levanase. The gene fragment was isolated by PCR technique using degenerative primers that designed on conserved domain of exoinulinase and levanase in family GH32 enzyme from *Bacillus* genus.

CONCLUSION

In conclusion, the new primers, DPE.slF and DPE, DPE.slF and DPE.eR have been used to isolate gene fragment that encodes inulin hydrolysis enzyme from genomic DNA *Bacillus licheniformis* UBCT-007. Sizes of the gene fragments that encodes inulin hydrolysis enzyme were found 398 and 539 bp. The primers can be used to isolate gene fragment that encodes inulin hydrolysis enzyme from *Bacillus* genus.

ACKNOWLEDGMENT

This study was funded by Directorate General for Higher Education, Ministry of National Education, Indonesia under contract Number of 486/SP2H/PP/DP2M/VI/2010.

REFERENCES

- Ahmed, Z., 2006. Optimization of PCR conditions *in vitro* for maximum amplification of DNA from *Xanthomonas campestris* 13551. *J. Applied Sci. Res.*, 2: 112-122.
- Arand, M., A.M. Golubev, J.R.B. Neto, I. Polikarpov and R. Wattiez *et al.*, 2002. Purification, characterization, gene cloning and preliminary X-ray data of the exo-inulinase from *Aspergillus awamori*. *Biochem. J.*, 362: 131-135.
- Azhar, M., 2013. Characterization of extracellular enzymes on inulin substrate and molecular levanase gene from thermophilic bacterium *Bacillus licheniformis* UBCT-007, local isolate of West Sumatra. Ph.D. Thesis, University of Andalas, Indonesia.
- Basran, N.F., S. Mustafa, R.A. Shamsuddin, A.M. Ali, R. Noormi and S. Subramaniam, 2010. Isolation and cloning of an inulinase gene from an endophytic bacteria. *Adv. Environ. Biol.*, 4: 392-396.
- Castro, G.R., M.D. Baigori and F.Sineriz, 1995. A plate technique for screening of inulin degrading microorganisms. *J. Microbiol. Meth.*, 22: 51-56.
- Goosen, C., 2007. Identification and characterization of glycoside hydrolase family 32 enzymes from *Aspergillus niger*. Ph.D. Thesis, University of Groningen, Netherlands.
- Kaplan, H. and R.W. Hutkins, 2003. Metabolism of fructooligosaccharides by *Lactobacillus paracasei* 1195. *Applied Environ. Microbiol.*, 69: 2217-2222.
- Kelley, L.A. and M.J.E. Sternberg, 2009. Protein structure prediction on the web: A case study using the Phyre server. *Nat. Protocols*, 4: 363-371.
- Kramer, M.F. and D.M. Coen, 2003. Enzymatic Amplification of DNA by PCR: Standard Procedure and Optimization. In: *Current Protocols in Immunology*, Coligan, J.E., A.D.A.M. Kruisbeek and D.H. Margulies (Eds.). John Wiley and Sons, Inc., New York, USA., ISBN-13: 978-0471142737, pp: 1-6.
- Kwon, H.J., S.J. Jeon, D.J. You, K.H. Kim and Y.K. Jeong *et al.*, 2003. Cloning and characterization of exoinulinase from *Bacillus polymyxa*. *Biotechnol. Lett.*, 25: 155-159.

- Martel, C.M., J.E. Parker, C.J. Jackson, A.G.S. Warrilow and N. Rolley *et al.*, 2011. Expression of bacterial levanase in yeast enables simultaneous saccharification and fermentation of grass juice to bioethanol. *Bioresour. Technol.*, 102: 1503-1508.
- Menendez, C., L. Hernandez, G. Selman, M.F. Mendoza, P. Hevia, M. Sotolongo and J.G. Arrieta, 2002. Molecular cloning and expression in *Escherichia coli* of an exo-lavanase gene from the endophytic bacterium *Gluconacetobacter diazotrophicus* SRT4. *Curr. Microbiol.*, 45: 5-12.
- Nagem, R.A.P., A.L. Rojas, A.M. Golubev, O.S. Korneeva and E.V. Eneyskaya *et al.*, 2004. Crystal structure of exo-inulinase from *Aspergillus awamori*: The enzyme fold and structural determinants of substrate recognition. *J. Mol. Biol.*, 344: 471-480.
- Promega, 2010. pGEM[®]-T and pGEM[®]-T easy vector systems technical manual. Instructions for Use of Products, Promega Corporation, Madison, WI., USA.
- Ricca, E., V. Calabro, S. Curcio and G. Iorio, 2007. The state of the art in the production of fructose from inulin enzymatic hydrolysis. *Crit. Rev. Biotechnol.*, 27: 129-145.
- Roberfroid, M.B., J.A.E. van Loo and G.R. Gibson, 1998. The bifidogenic nature of chicory inulin and its hydrolysis products. *J. Nutr.*, 128: 11-19.
- Sambrook, J., E.F. Fritish and T. Maniatis, 1989. *Molecular Cloning: A Laboratory Manual*. 2nd Edn., Cold Spring Harbor Laboratory Press, New York, USA., ISBN-13: 978-0879693091, Pages: 397.
- Singh, P. and P.K. Gill, 2006. Production of inulinases: Recent advances. *Food Technol. Biotechnol.*, 44: 151-162.
- Sirisansaneeyakul, S., N. Worawuthiyanan, W. Vanichsriratana, P. Srinophakun and Y. Chisti, 2007. Production of fructose from inulin using mixed inulinases from *Aspergillus niger* and *Candida guilliermondii*. *World J. Microbiol. Biotechnol.*, 23: 543-552.
- Thermo Scientific, 2011. DreamTaq DNA polymerase. Thermo Fisher Scientific Inc., Waltham, MA., USA. <http://www.thermoscientificbio.com/pcr-enzymes-master-mixes-and-reagents/dreamtaq-dna-polymerase/>
- Tohamy, E.Y., 2006. Purification and characterization of exoinulinase enzyme from *Sterptomyces grisenus*. *Pak. J. Biol. Sci.*, 9: 911-916.
- Wanker, E., A. Huber and H. Schwab, 1995. Purification and characterization of the *Bacillus subtilis* levanase produced in *Escherichia coli*. *Applied Environ. Microbiol.*, 5: 1953-1958.
- Woodman, M.E., 2005. Direct PCR of Intact Bacteria (Colony PCR). In: *Current Protocols in Microbiology*, Coico, R., T. Kowalik, J. Quarles, B. Stevenson and R. Taylor (Eds.). John Wiley and Sons, Inc., New York, USA., pp: A.3D.1-A.3D.6.
- Zhang, T., F. Gong, Z. Chi, G. Liu and Z. Chi *et al.*, 2009. Cloning and characterization of the inulinase gene from a marine yeast *Pichia guilliermondii* and its expression in *Pichia pastoris*. *Antonie van Leeuwenhoek*, 95: 13-22.