



CLONING OF PARTIAL β -MANNANASE GENE FROM INDONESIA MARINE BACTERIA *Bacillus subtilis* LBF-005

Yopi*, Nanik Rahmani, Maghfirotul Amaniyah and Apridah Cameliawati Djohan

Laboratory of Biocatalyst and Fermentation, Research Center for Biotechnology/Indonesian Institute of Sciences (LIPI),
Jalan Raya Bogor, KM. 46 Cibinong, Bogor, West Java, Indonesia

Article history:

Received: 5 December 2016; Revised: 8 April 2017; Accepted: 29 April 2017

Abstract

The strain LBF-005 from marine bacteria have been already isolated and screened for mannanase degrading enzyme in submerged fermentation process. This strain was further identified by using 16S rRNA identification and showed that bacterium is belong to *Bacillus subtilis* that could produce mannanase with activity around 9.5 U/mL. The optimum pH and temperature for the activity of crude enzyme for mannanase were 6.0 and 50 °C, respectively. Cloning of mannanase gene from *B. subtilis* was conducted using six primer / set designed based on the homology analysis conserved region of several mannanases from bacteria (*Bacillus* sp.) glycosyl hydrolase (GH) family 26. Optimization of PCR conditions was performed by gradient PCR to obtain PCR product of β -mannanase gene. The PCR product was obtained by the third primer combination and was estimated to be around 972-bp. Analysis of the nucleotide sequence indicated that the sequence has similarity with mannanase gene from other *Bacillus* sp., such as the *B. subtilis* strain WLY-12, *B. subtilis* strain WL-8, *B. subtilis* strain CICC 10260, *B. subtilis* strain CD-25, *B. subtilis* strain G1, and *Bacillus* sp. SWU60 about 98%, 98%, 98%, 98%, 97% and 95%, respectively.

Keywords: marine bacteria, *Bacillus subtilis*, β -mannanase, partial gene

1. Introduction

Indonesia has abundance renewable carbon source biomasses containing high mannan polymer such as plantation products of palm kernel cake, copra cake, porang potato, etc. Utilization of their hetero-mannan biomass by using the enzymatic conversion to get derivate of monosaccharide and oligosaccharide can be processed further into bio-ethanol and functional food.

Mannan, one of the major constituent groups of hemicellulose in the wall of higher plants, are classified into four subfamilies: linear mannan, glucomannan, galactomannan, and galactoglucomannan (Liepman et al., 2007; Moreira & Filho, 2008; Schroder et al., 2009). Each of these polysaccharides consists of β -1,4-linked backbone containing mannose or a combination of glucose and mannose residues (Liepman et al., 2007).

The mannan-degrading enzymes, mannanases, are composed of β -mannanase (1,4- β -D-mannanmannohydrolase, EC 3.2.1.78), β -

mannosidase (1,4- β -D-mannopyranoside hydrolase, EC 3.2.1.25), and β -glucosidase (1,4- β -D-glucosidegluco hydrolase, EC 3.2.1.21) (Moreira & Filho, 2008). The mannanases are known to be produced by plants, animals, and various microorganisms, including of bacteria, fungi, and actinomycetes (Chauhan et al., 2012). On the basis of their sequence similarity, microbial mannanases are classified as glycosyl hydrolase (GH) family 5 or 26 (Henrissat, 1991). The GH family 26 is composed of mannanases from *Bacillus subtilis*, *Cellulomonas fimi*, *Clostridium thermocellum*, *Pseudomonas fluorescens*, *Piromyces* sp., and *Humicolainsolens* (Moreira & Filho, 2008).

β -mannanase is an endo-type enzyme, which responsible to hydrolyze β -1,4-linked internal linkages of the mannan backbone randomly, producing manno oligosaccharides (Dhawan & Kaur, 2007). β -mannanases have been used to a great extent in various industrial processes including in bleaching of pulp wood (Benech et al., 2007), feed industry (Lee et al., 2003; Zou et al., 2006), pharmaceutical, food, and

*Corresponding author.

E-mail: yopisunarya@gmail.com

textile industries (Chauhan et al., 2012). Furthermore, this type of enzyme also play an important role in the production of manno oligosaccharides. Previous studies have confirmed the enzymatic hydrolysis of different substrates by β -mannanase to produce manno oligosaccharides (Blibech et al., 2011; Jian et al., 2013; Wang et al., 2013).

Several genes encoding β -mannanase from plants, fungi and bacteria have been cloned and their encoded enzymes have been purified and characterized (Moreira & Filho, 2008). Bacterial mannanases considerable interests over the past years, especially *Bacillus* sp. harboring mannanase, because of its safety, fast growth, moderate stability, and ability to secrete mannanase into the medium (Mendoza et al., 1994). Genes encoding β -mannanase from *Bacillus* sp. have been cloned and expressed in heterologous expression systems, and properties of the recombinant β -mannanase have been characterized (Yanhe et al., 2004; Sumppunn et al., 2011; Liu et al., 2015).

Many studies have been performed focusing on discovering new endo-1,4- β -mannanases from marine microorganisms. Marine microorganisms have been regarded as a reservoir, not only for novel natural products, but also for valuable genes and enzymes (Guo et al., 2009). Gene cloning and sequencing of β -mannanase from different marine organisms also have been reported (Tamaru et al., 1997; Politz et al., 2000).

In general, these enzymes produced by microorganisms derived from soil, compost, or animal rumen. At present, there is an increasing need in searching for new source of mannanase with especially properties. Indonesia is a country which has tropical marine conditions that has plenty of tropical marine indigenous microbe biodiversity. The utilization of marine bacteria to produce mannanase has not been used widely in Indonesia. We have already collected of marine microbes from Bali Island and these bacteria have been investigated as a potential strain that can produce mannanase. This study focused on isolation and characterization of mannanase degrading enzyme from marine bacteria and one potential candidate, the strain LBF-005 was used for the cloning of partial gene of β -mannanase.

2. Material and Methods

2.1. Isolation, Screening and Characterization of Mannanase Degrading Bacteria

2.1.1. Isolation of mannanase degrading bacteria

Sea water and sediments were collected from Bali Island using direct sampling method. The medium

for screening and bacteria purification contained 0,5% mannan (Locust Bean Gum), 0.075% peptone, 0.05% yeast extract and some minerals compound (artificial sea water) and pH was adjusted to pH 6.0 (Modification of Mandels & Sternberg, 1976). The purified colonies were preserved at 4 °C for 16SrDNA identification and screening for mannanase production.

2.1.2. Screening of mannanase degrading bacteria

Pure cultures of bacteria were individually transferred onto LBG agar plates. After one day incubation, agar plates were flooded with 0.2% Congo red and allowed to stand for 30 min at room temperature. One molar NaCl was throughly used for counterstaining. The colonies having the large clear zone were selected for enzyme production and characterization, identification and partial cloning of mannanase gene.

2.2. Production and Characterization Mannanase from the Strain LBF-005

2.2.1. Production of mannanase enzyme

The quantitative activity of mannanase was investigated by growing the selected bacteria in 100 mL flask containing 10 mL of ASW medium and incubating for one day at 30 °C. Then, X mL of the preculture medium was seeded into 300 mL Flask containing 30 mL of ASW medium and incubated at 30 °C for six days. The supernatant was assayed for mannanase activity.

2.2.2. Mannanase activity assay

Mannanase activity was assayed by measuring the reducing sugars using dinitrosalicylic acid (DNS) method (Miller, 1959). An appropriately diluted enzyme solution (250 μ L) was incubated with 250 μ L of the substrate solution (0.5% of LBG (Sigma) in 50 mM sodium phosphate buffer, pH 7 at 60 °C for 15 min. The reaction was mixed with 750 μ L DNS solution, heated at 100 °C, for 10 min and cooled on ice for 10 min. The absorbance was measured at 540 nm. D-Mannose was used as standar. One unit of mannanase activity is defined as the amount of enzyme that liberates 1 μ mol of D-mannose per minute under the experimental condition given.

2.2.3. Characterization of pH and temperature optimum

The optimum pH of enzyme activity was examined at pH 3.0-10.0 under standard assay condition. Fifty mM of various buffers were used: sodium citrate (pH

3.0-5.0), sodium phosphate (pH 6.0-8.0), and glycine NaOH (pH 9.0-10.0). The enzyme reaction was incubated at 50 °C for 15 min in the presence of 0.5% (w/v) LBG. The effect of temperature on enzyme activity was performed at temperature ranging from 30-90 °C in 50 mM acetate buffer at optimum pH for 15 min.

2.2.4. Mannanase hydrolysis analysis by using thin-layer chromatography

The main product analysis of mannan substrates (LBG, Konjac glucomannan, Ivory nut, porang) reaction was carried at 50 °C in 50 mM sodium phosphate buffer, pH 6, containing 0.5% for each mannan substrate. The enzyme-substrate ratio (v/v) 1:1 and the reaction time (hours from 0, 0.5, 1, 2, 4, 8, 12 and 24). Reactions were carried out in 1.5 mL eppendorf containing 100 µL of reaction mixtures in dry block. Thin Layer chromatography (TLC) of manno oligosaccharide products was carried out on silica gel 60F₂₅₄ plates (Merck Art 20-20 cm) and eluen with a solvent mixture of n-butanol/ acetic acid/ water (12:6:6, v/v/v). Spots were visualized by spraying the sugar color (0.5 g α-diphenylamine, 25 mL acetone, 2.5 mL phosphate acid, 0.5 ml aniline) and subsequent heating at 120 °C for 15 min. All samples were applied in equal quantities (4µl). Glucose, mannose (Sigma–Aldrich, U.S.A.), mannobiose (M2), mannotriose (M3), mannotetraose (M4) and mannopentaose (M5) and mannoheksaose (all from Megazyme, Ireland) were used as a standard.

2.3. Identification and Phylogenetic Analysis 16S rDNA Sequence

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) technique using a pair of primer

(9F: 5'-GAGTTTGATCCTGGCTCAG-3') 1510R; 5'GGC TACC TTGT TACGA-3') (Burggraft et al., 1992). The obtained bands were stained and visualized by UV transilluminator. The sequence was confirmed via 1st BASE Sequencing, Singapore and then compared with others available in the GenBank database using multiple sequence alignment (ClustalW). The 16S rRNA nucleotide sequences was deposited in GenBank with accession number KY630523.

2.4. Cloning of Partial β-mannanase Gene from Marine Bacteria B. subtilis LBF-005

2.4.1. Isolation of DNA genomic B. subtilis LBF-005

B. Subtilis LBF-005 was cultivated in medium containing YE (1g/L), pepton (5g/L), and ASW (38 g/ L) at 28 °C 180 rpm for overnight. Washed cells from 5 ml overnight culture were isolated by Wizard®genomic DNA purification kit (Promega, USA) according to the manufacturer’s instructions. Quantification of the DNA genome was estimated with nanophotometer by lid factor 50.

2.4.2. Design of mannanase primers

Six sets of forward-reverse primer set were designed based on the homology analysis of several conserve mannanase region from bacteria (*Bacillus* sp.) glycosyl hydrolase (GH) family 26. Each pair of primer were marked as: PC1, PC2, PC3, PC4, PC5, PC6, and PC7 (Table 1).

2.4.3. Cloning of partial β-mannanase

The DNA genome of *B. Subtilis* LBF-005 was used as template for PCR amplification. PCR conditions of

Table 1. Design of six forward-reverse primers set, primer combinations and target of amplification of partial mannanase gene

No	Primers	Sequences	Primer combinations	Target (bp)
1	31-F	ccY gTV AAY ccW AAT gcM cAg cAg AcR AcA AA	PC1 = 31F-169R	438
2	96-F	AgA ggA Tgg cTK gAA AcA gcR	PC2 = 31F- 186R	495
3	169-R	AAg Kcc gTc Rgc AAT TTT gcT gAg cA	PC3 = 31F-343R	972
4	186-F	TTc Agg ccg cTg cAT gAA ATg AAC	PC4 = 96F-169R	242
5	186-R	gTT cAT TTc ATg cAg cgg ccT gAA	PC5 = 96F-186R	299
6	343-R	TT ccA TAT TTc Kcc cTT RTT VAg YgT ccA gcT	PC6 = 96F-343R PC7 = 186F-343R	776 408

seven primers combination were optimized by gradient PCR with conditions as follows: 94 °C for 2 min; 30 cycles of 98 °C for 10sec, 45-57 °C for 30sec, 68 °C for 1,5 min; and 68 °C for 5 min. The PCR mixture contained 2.5 µl 10X buffer KOD plus-neo (Toyobo, Japan), 2.5 µl 2 mM dNTP, 1.5 µL 25 mM MgSO₄, 0.75 µl of each primer (10 pmol), 1 µL of genomic DNA (15 ng), 0.5 µL KOD plus-neo and supplemented with 15.5 µL of ddH₂O to a final volume of 25 µl. The PCR products was analyzed by electrophoresis on 1% agarose gel. The target PRC product was then purified by wizard SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's instructions.

2.4.4. Analysis sequence

The amplicon was sequenced by 1st BASE Sequencing, Singapore. Partial mannanase gene sequence was analyzed with nucleotide BLAST search in Gen Bank National Center for Biotechnology Information (NCBI), either BLASTX for amino acid analysis (Altschul et al., 1997). Phylogenetic relationship of nucleotide sequence gene was analyzed with other closely related mannanase from GenBank and aligned by MEGA5 software using ClustalW as multiple alignment parameters; and *Neighbour joining algorithm* to estimate phylogenetic tree (Hall, 2013). For amino acid analysis, amino acid sequences of *Bacillus* mannanases were collected

from GenBank and aligned by Custal Omega software (version 1.2.1) as multiple alignment parameters (Sievers et al., 2011).

3. Results and Discussion

3.1. Isolation, Screening and Characterization of Mannanase Degrading Enzyme from Marine Bacteria

In this study, bacteria were isolated from marine source (Bali Island) and screened for mannanase production using the locust bean galactomannan (LBG) substrate assay. The isolation succeeded to collect 11 isolate that produce mannanase but only the strain of LBF-005 which produced the highest mannanase activity using congo red analysis. The result showed the clear zone on agar plates using Congo red staining indicating that these isolates secrete considerable amount of mannanase enzymes (Figure 1A). The quantitative enzyme test have been conducted to determine the ability for each isolates to produce enzyme by using commercial substrates as a carbon source. The strain of LBF-005 produce enzyme using commercial substrate LBG as a carbon source with optimum activity at 72 hours culture (9.5 U/mL) (Figure 1B).

The effect of pH on activity of mannanase from the strain of LBF-005 were carried out in range 3-10, shown in Figure 1C. The crude mannanase from the

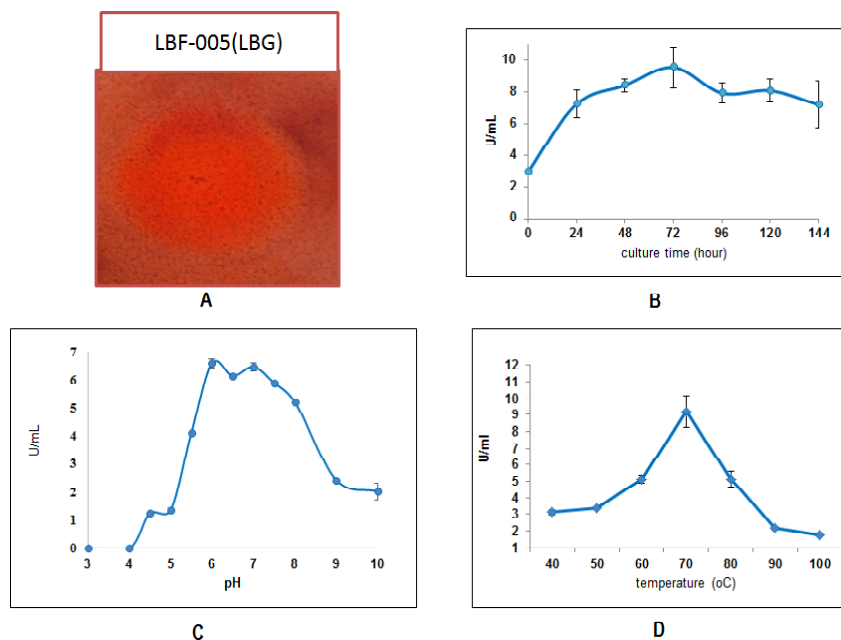


Figure 1. (A) Congo red analysis from the strain of LBF-005, (B) Mannanase production profile from the strain of LBF-005 grown in ASW medium with shaking (180 rpm) at 30 °C, activities (in U/mL) was monitored for 6 days cultures (C) and (D) characterization of pH and temperature from the strain of LBF-005.

strain of LBF-005 was active at a wide range of pH from 5.0-10.0. The highest mannanase activity was observed at pH 6.0. Increasing or decreasing pH beyond these resulted in declining in enzyme activity. Any change in pH cause changed in the enzyme active site (Irfan et al., 2012).

The effect of temperature was studied on each enzyme activity at 30 °C to 100 °C. Figure 1D shows the effect of temperature on the activity of mannanase from the strain of LBF-005 showing optimum temperature of 70 °C with activity of 9.5 U/mL. As the temperature increased from 40 °C enzyme activity increased but activity started to decline as temperature increased above 80 °C and become completely denatured at 100 °C. Similar findings were also reported by some species of *Bacillus* sp., *B.subtilis* YJ1 which have optimum temperature of 50 °C (Kim et al., 2009).

Endo- β -mannanase is an important hydrolytic enzyme responsible for degradation of internal β -1.4-

mannosidic linkages of mannan polysaccharides at random. The patterns of the strain of LBF-005 hydrolysis using some mannan polysaccharides were analyzed by TLC. Hydrolysis of ivory nut and galactomannan and LBG, porang potato, konjac glucomannan produced mannobiose throughout incubation as can be shown at Figure 2A and 2B, respectively.

2.3. Identification and Phylogenetic Analysis rDNA Sequence

Identification of a nearly full length sequence of 16S rRNA (1500 bp) gene for the strain of LBF-005 was determined, and based on the sequence identity of 16S rRNA gene (Figure 3A) against the GenBank database, indicates that the strain of LBF-005 was closely related to the members of the genus *Bacillus*. The phylogenetic analysis showed about 99% similarity between the strain of LBF-005 and *Bacillus subtilis* (Figure 3B).

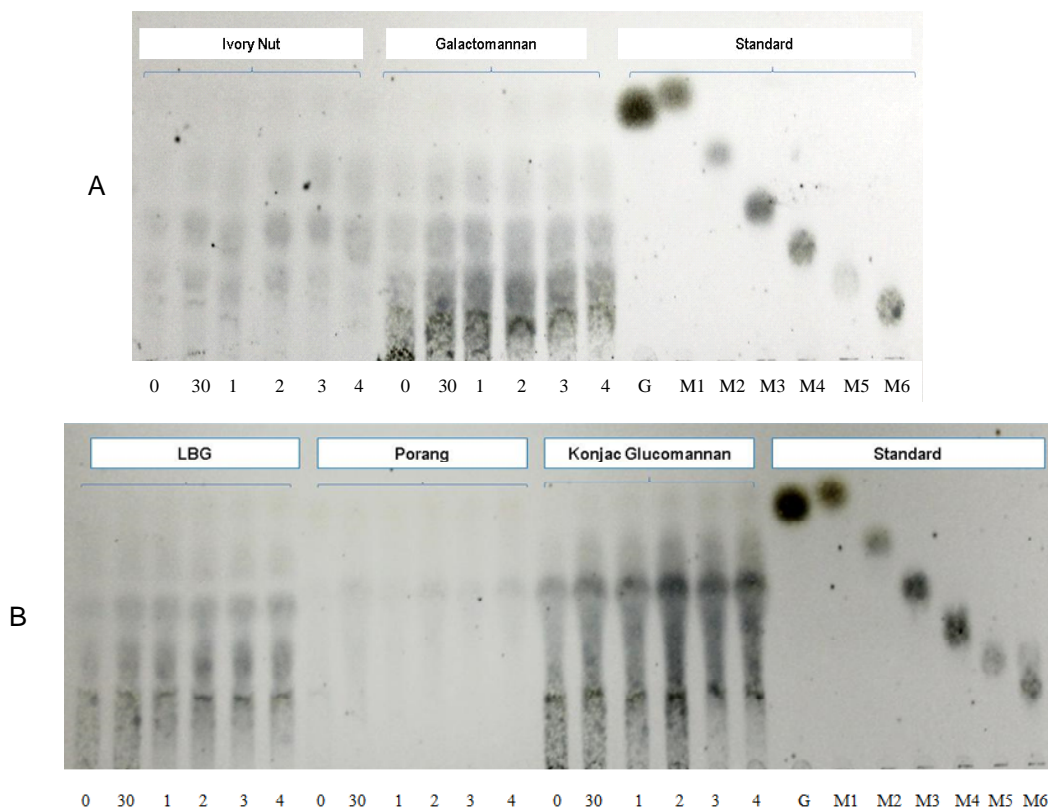


Figure 2. Thin layer chromatography analysis of hydrolysis products LBF-005 of mannanase using various mannan as substrat (galactomannan, LBG, porang, konjac glucomannan and ivory nut). Standards (STD): glucose (G), mannobiose (M2), mannotriose (M3), mannotetraose (M4), mannopentaoase (M5) and mannohexaoase (M6). Substrate : enzyme (v/v) = 1:10 and 1:5, Substrate concentration 0.5% in 50 mM sodium phosphate buffer (pH 6.0), reaction time (hours) : 0, 0.30, 1, 2, 3 and 4 at 30 °C.

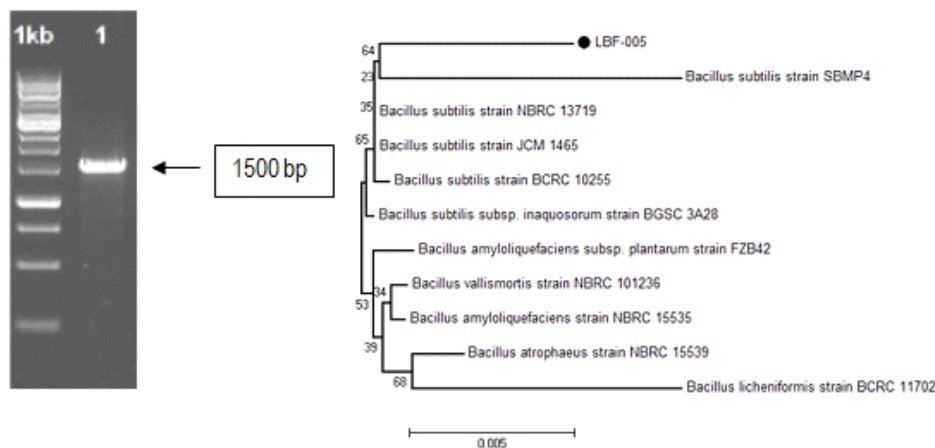


Figure 3. (A) Analysis of 16s rDNA from the strain of LBF-005. Lane 1 1Kb DNA Ladder, lane 2 the strain of LBF-005 (B) Phylogenetic tree analysis from the strain of LBF-005 and 16s rDNA sequence from other *B. subtilis*. The phylogenetic tree was constructed using neighbour joining algorithm in MEGA 6.06 software. Bar, 5% estimated sequence divergence.

3. Cloning of Partial β -mannanase Gene from Marine Bacteria *B. subtilis* LBF-005

different length of DNA target amplification as follow: PC1, PC2, PC3, PC4, PC5, PC6, and PC7 (Table 1).

3.1. Design Primers of Partial Mannanase Gene

Eleven conserve regions were identified by six amino acid sequences alignment of mannanase gene GH 26 from *Bacillus*, including *B. subtilis* WL-7 (AAT27435), *B. subtilis* BCC41051 (GU982918), *B. subtilis* WL-3 (AAZ95239), *B. subtilis* Z-2 (AAV84100), *Bacillus* SP. 5H (BAA31711), and *B. subtilis* NM-39 (BAA07178). Based on this information, six conserve regions were chosen as forward-reverse primers set and were combined as seven primer combinations with

3.2. Partial Gene Cloning and Analysis

The DNA target encoding partial mannanase gene from *B. subtilis* LBF-005 was obtained by third primer combination. Single fragment of PCR product of DNA target was showed by lane 10, at annealing temperature of 56.16 °C (Figure 4). The band was estimated to be around 972 bp as DNA target amplification of third primer combination. Purified amplicon of targeted DNA showed the same length as amplified DNA (Figure 5).

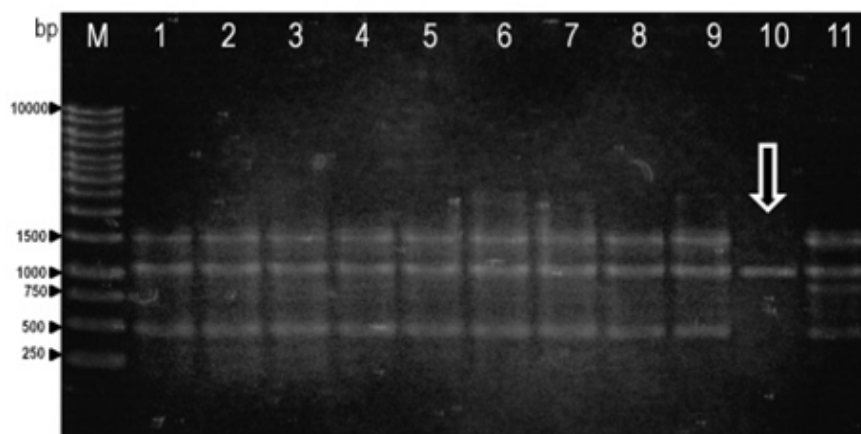


Figure 4. Optimization of PCR condition by gradient PCR using third primer combination. (Lane M: 1 kb DNA Ladder Fermentas; Lane 1-11: PCR product by annealing temperature 45-57 °C).

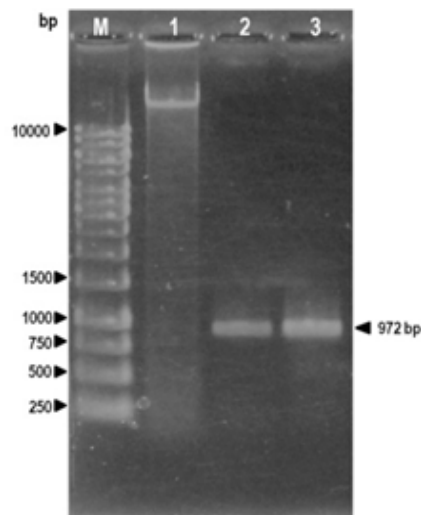


Figure 5. Analysis of mannanase gene. Lane M: 1 kb DNA ladder, Lane 1: DNA genome of *B. subtilis* LBF-005, Lane 2: Amplified DNA by third primer combination, Lane 3: purification product of amplified DNA.

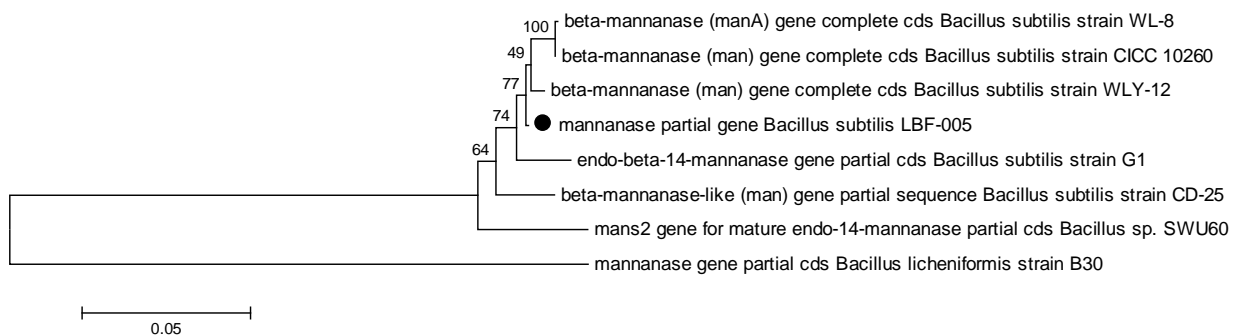


Figure 6. Phylogenetic tree of *Bacillus subtilis* LBF-005 β -mannanase gene sequence and mannanase gene sequence from other *Bacillus subtilis*. The phylogenetic tree was constructed using neighbour joining algorithm in MEGA 6.06 software. Mannanase gene from *Bacillus licheniformis* strain B30 has been taken as an out-group. Bar, 5% estimated sequence divergence.

Cloning full length the sequence of mannanase from *B. subtilis* LBF-005 will continue by in vitro cloning kit. Primer for in vitro cloning was design based on the partial gene of this mannanase.

3.4. Analysis Sequence

Amplified gene of partial β -mannanase gene was sequenced. A BLAST search of the nucleotide sequence of partial β -mannanase gene *B. subtilis* LBF-005 in the NCBI database showed an identity of $\leq 98\%$ with corresponding nucleotide sequences of mananase from *B. subtilis* strain WLY-12 (98%) (KC979152), *B. subtilis* strain WL-8 (98%) (HM149533), *B. subtilis* strain CICC 10260 (98%) (GQ859468), *B. subtilis* strain CD-25 (98%) (EU755327), *B. subtilis* strain G1 (97%) (DQ309335),

and *Bacillus sp.* SWU 60 (95%) (LC074726). The phylogenetic tree showed that *B. subtilis* LBF-005 β -mannanase gene sequence was under the group of mannanase from *Bacillus sp.* (Figure 6).

Sequence analysis of the of the partial β -mannanase gene from *B. subtilis* LBF-005 and subsequent homology search analysis using BLASTX program showed that the amino acid had an identity of $\leq 98\%$ with related amino acid mannanase gene from other *Bacillus*, include beta-mannosidase of *Bacillus sp.* LM 4-2 (98%) (WP_046380815), endo-beta-1,4-mannanase of *Bacillus subtilis* (97%) (ACY00389), beta-mannanase of *Bacillus subtilis* (97%) (ACX94026), mannan endo-1,4-beta-mannosidase *Bacillus subtilis* (97%) (WP_015715339), partial endo-beta-1,4-mannanase, *Bacillus subtilis* (97%) (ABC61374), and beta-

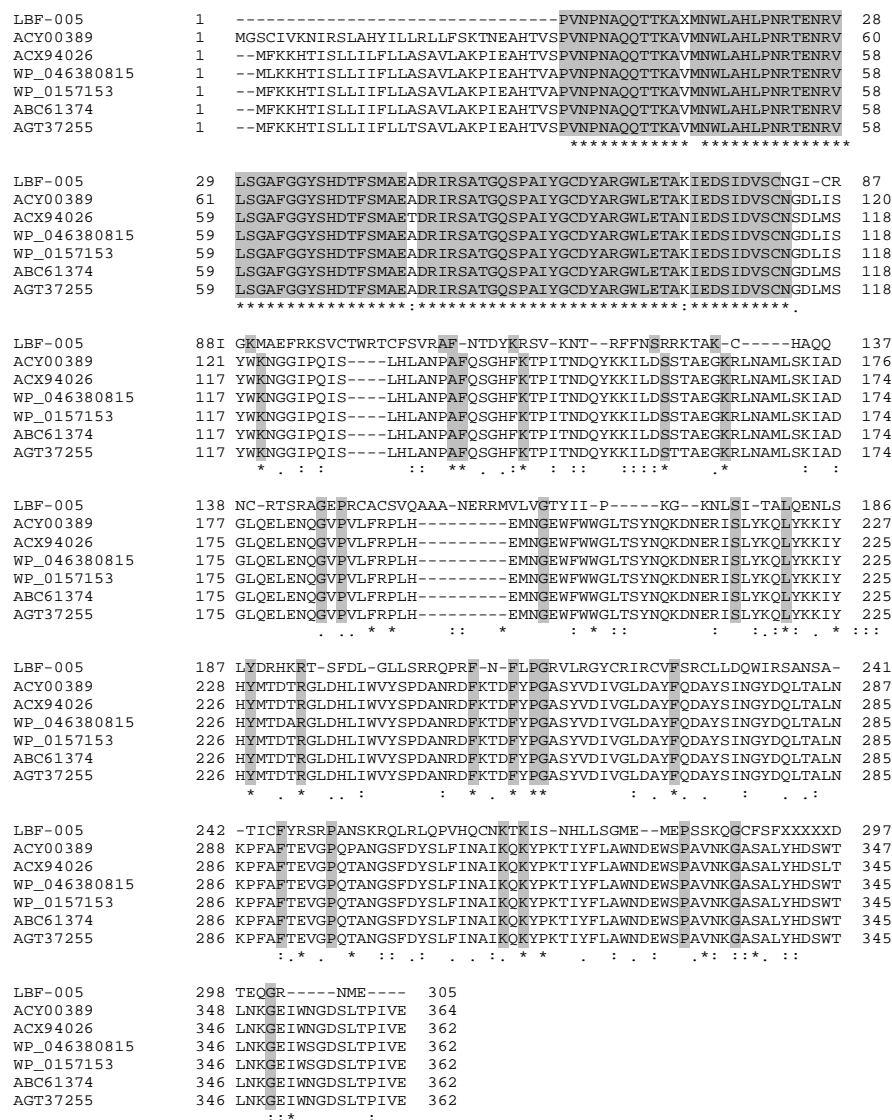


Figure 7. Multiple sequence alignment of deduced amino acid mannanase genes from partial mannanase *Bacillus subtilis* LBF-005 and mannanase gene sequence from other *Bacillus*. Representative mannanase genes are from: *endo*-beta-1,4-mannanase *Bacillus subtilis* (ACY00389); beta-mannanase *Bacillus subtilis* (ACX94026); beta-mannosidase *Bacillus* sp. LM 4-2(WP_046380815); mannan *endo*-1,4-beta-mannosidase *Bacillus subtilis* (WP_015715339); partial *endo*-beta-1,4-mannanase *Bacillus subtilis* (ABC61374); and beta-mannanase *Bacillus subtilis* (AGT37255). The multiple sequence alignment was calculated with ClustalO (1.2.1) program. The conservative area are grey.

mannanase of *Bacillus subtilis* (97%) (AGT37255). The putative conserved domains have been detected with glycosyl hydrolase family 26 (pfam02156) and ManB2 (COG4124) (Figure 7).

4. Conclusion

Isolation of mannolytic marine bacteria have been conducted and there is one potential isolate, i.e. the strain of LBF-005 which belongs to *B. subtilis* based

on 16S rRN Analysis. This strain could produce mannanase with activity around 9.5 U/mL. The optimum pH and temperature for mannanase was 6.0 and 50 °C respectively with activity of 9.5 U/mL. The partial β -mannanase gene from *B. subtilis* LBF-005 have cloned, wich estimated to be 972-bp and the analysis showed identity of $\leq 98\%$ in nucleotide sequences and $\leq 98\%$ in amino acid sequence with the known mannanase from GenBank. The next research were to obtain a whole gene encoding β -mannanase by

using in vitro cloning method, characterization of recombinant mannanase and application this enzyme for manno oligosaccharides production.

5. Acknowledgement

This research was supported by Toray Research Grant 2012 and research grant from Research Center for Biotechnology LIPI (Unggulan LIPI Pangan & Obat).

References

- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25, 3389-3402. doi:10.1093/nar/25.17.3389.
- Benech, R.O., Xiaoming Li, Patton, D., Powlowski, J., Storms, R., Bourbonnais, R., Paice, M. & Tsang, A. (2007). Recombinant expression, characterization, and pulp prebleaching property of a *Phanerochaete chrysosporium* endo- β -1,4-mannanase. *Enzyme and Microbial Technology*, 41, 740-747. doi:10.1016/j.enzmictec.2007.06.012.
- Blibech, M., Chaari, F., Bhiri, F., Dammak, I., Ghorbel, R.E. & Chaabouni, S.E. (2011). Production of manno oligosaccharides from locust bean gum using immobilized *Penicillium occitanicum* mannanase. *Journal of Molecular Catalysis B: Enzymatic*, 73, 111-115. doi:10.1016/j.molcatb.2011.08.007.
- Chauhan, P.S., Puri, N., Sharma, P. & Gupta, N. (2012). Mannanases: microbial sources, production, properties and potential biotechnological applications. *Applied Microbiology and Biotechnology*, 93, 1817-1830. doi:10.1007/s00253-012-3887-5.
- Dhawan, S. & Kaur, J. (2007). Microbial mannanases: an overview of production and applications. *Critical Reviews in Biotechnology*, 27, 197-216. doi:10.1080/07388550701775919.
- Guo, B., Li, Y., Yue, Y.S., Zhao, H.L., Song, S.D., Sun, C.Y., Zhang, W.X., Chen, X.L., Zhang, X.Y., Zhou, B.C. & Zhang, Y.Z. (2013). Gene Cloning, Expression and Characterization of a Novel Xylanase from the Marine Bacterium, *Glaciecolomesophila* KMM241. *Marine Drugs*, 11, 1173-1187. doi:10.3390/md11041173.
- Hall, B.G. (2013). Building phylogenetic trees from molecular data with MEGA. *Molecular Biology and Evolution*, 1-7. doi:10.1093/molbev/mst012.
- Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal*, 280, 309-316. doi:10.1042/bj2800309.
- Jian, H.L., Zhu, L., Wang, W.M., Sun, D.F. & Jiang, J.X. (2013). Enzymatic production and characterization of manno-oligosaccharides from *Gleditsia sinensis* galactomannan gum. *International Journal of Biological Macromolecules*, 55, 282-288. <http://dx.doi.org/10.1016/j.ijbiomac.2013.01.025>.
- Lee, J.T., Bailey, C.A., & Cartwright, A. L. (2003). Betamannanase ameliorates viscosity-associated depression of growth in broiler chickens fed guar germ and hull fractions. *Poultry Science*, 82, 1925-1931. doi:10.1093/ps/82.12.1925
- Liepmann, A.H., Nairn, C.J., Willats, W.G.T., Sørensen, I., Roberts, A.W., and Keegstra, K. 2007. Functional genomic analysis supports conservation of function among cellulose synthase-like A gene family members and suggest diverse roles of mannans in plants. *Plant Physiology*, 143, 1881-1893. <http://dx.doi.org/10.1104/pp.106.093989>
- Liu, H.X., Gong, J.S., Li, H., Lu, Z.M., Li, H., Qian, J.Y., Xu, Z.H., & Shi, J.S. (2015). Biochemical characterization and cloning of an endo-1,4- β -mannanase from *Bacillus subtilis* YH12 with unusually broad substrate profile. *Process Biochemistry*, 50, 712-721. <http://dx.doi.org/10.1016/j.procbio.2015.02.011>.
- Ma, Y., Xue, Y., Dou, Y., Xu, Z., Tao, W., & Zhou, P. (2004). Characterization and gene cloning of a novel β -mannanase from alkaliphilic *Bacillus* sp. N16-5. *Extremophiles*, 8, 447-454. doi:10.1007/s00792-004-0405-4.
- Mendoza, N.S., Arai, M., Kawaguchi, T., Yoshida, T. & Joson, L.M. (1994). Purification and properties of mannanase from *Bacillus subtilis*. *World Journal of Microbiology and Biotechnology*, 10, 551-555. doi:10.1007/BF00367665.
- Moreira, L.R. & Filho, E.X. (2008). An overview of mannan structure and mannan degrading enzyme systems. *Applied Microbiology and Biotechnology*, 79, 165-178. doi:10.1007/s00253-008-1423-4.
- Politz, O., Krah, M.K., Thomsen, K., & Borriss, R. (2000). A highly thermostable endo-(1,4)- β -mannanase from the marine bacterium *Rhodothermus marinus*. *Applied Microbiology and Biotechnology*, 53, 715-721. doi:10.1007/s002530000351.
- Schroder, R., Atkinson, R.G., & Redgwell, R.J. (2009). Re-interpreting the role of endo- β -mannanases as mannan endo-transglycosylase/hydrolases in the plant cell wall. *Annals of Botany*, 104, 197-204. doi:10.1093/aob/mcp120.
- Sievers, F., Andreas, W., David, D., Toby, J.G., Kevin, K., Weizhong, L., Rodrigo, L., Hamish, M., Michael, R., Johannes, S., Julie, D.T. & Desmond, G.H. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, 7, 1-6. doi:10.1038/msb.2011.75.
- Sumppunn, P., Chaijan, S., Isarangkul, D., Wiyakrutta, S., & Meevootisom, V. (2011). Characterization, gene cloning, and heterologous expression of β -mannanase from a thermophilic *Bacillus subtilis*. *The Journal of Microbiology*, 49, 86-93. doi: 10.1007/s12275-011-0357-1.
- Tamaru, Y., Araki, T., Morishita, T., Kimura, T., Sakka, K. & Ohmiya, K. (1997). Cloning, DNA sequencing, and expression of the P-1,4-mannanase gene from a marine bacterium, *Vibrio* sp. strain MA-138. *Journal*