Cloning of A Gene Encoding Protease from *Bacillus halodurans* CM1 into *Escherichia coli* DH5*α*

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Abstract

Bacillus halodurans strain CM1 was an Indonesia alkalothermophilic bacteria isolated from Cimanggu Hot Spring, Bandung, West Java. The activity of alkalo thermophilic protease enzyme from *B. halodurans* CM1 was detected. Nowadays, alkalothermophilic protease enzyme was applied for the eco-friendly industrial purpose, for example, as additive substance in detergent product. For the production and application of protease in the future, the cloning of protease gene from *B. halodurans* CM1 into *E. coli* was conducted. The protease gene was isolated from *B. halodurans* by PCR approach using primers designed based on the GenBank database. The PCR product then ligated into pGEM-T Easy vector, transformed into *Escherichia coli* DH5α, verified, and analyzed using DNA sequencing and bioinformatic tools BLAST. The results showed that 1086 bp protease gene was obtained and had 99% similarity with that of alkalostable protease from *B. halodurans* C-125. When the culture of this positive recombinant *E. coli* DH5α containing the protease gene spotted onto calcium caseinate agar, the clear zone appeared after incubation at 50 °C. It showed that the protease gene was expressed in this recombinant *E.coli* DH5α.

Keywords: alkalothermophilic protease; *Bacillus halodurans* CM1; gene cloning

INTRODUCTION

Microbial enzymes have significant roles in application in ecofriendly industrial purposes. Especially, according to Tambekar et al., alkalothermophilic enzymes that have high stability in extreme temperature and alkaline condition promise wide application [1]. Along with Takami et al Akita et al., and Mamo et al. report, these alkalothermophilic enzymes are pectinase, amylase, and cellulose, glucanase, xylanase, and protease in [2,3,4].

As an additive in detergent, enzymatic catalysis reaction from alkalothermophilic protease was effective to eradicate the blood stain without damaging clothes. The protease enzyme as additive substance in laundering was an ideal alternative to the current harmful chemical cpmpound for health and environment, for example, chlorine [5]. However, due to the main advantage of heat stable, the alkalothermophilic protrase also dealt in another industrial processes such as dehairing, degumming, medical, environmental waste, even in the food and beverage industrial preparation. Protease enzyme that shows high kerqatinolytic activities was used in the destruction of the hair process belongs to the leather industry. Protease as an alternative substance to replace hydrogen sulfide and sodium sulfide, which are toxic and creating safety hazard [6,7]. In the silk industry, protease enzyme has an important role to remove raw fibers at the degumming process. Protease enzyme referred to the surgical importance was used as a good cleaner for instruments fouled by blood. Conventionally, in the food and beverage industry, protease enzyme was exploited in protein hydrolysates of high nutritional value (example food formulation to soy and cheese, the therapeutic effect of dietary products, fortification fruit juice, etc) [7,8]. Biodegradation of waste which is rich in protein compounds such as fowl feathers and tomato processing waste using protease improved the velocity of recycling pollutant [9].

The source of the alkalothermophilic enzymes could be a microbe that living in high temperature and

high pH such as *Bacillus halodurans* [1]. One of the *B. halodurans* strain isolated from Indonesia habitat recently was *B.halodurans* CM1. It potentially produced amylase, lipase, protease, and gelatinase [10]. This bacterium produced alkalothermophilic xylanase at a high level [11].

In this study, the presence of alkalothermophilic protease gene was confirmed by PCR isolation. Cloning of the target protease gene, analyses of DNA sequence, and qualitative protease activity assay have been conducted.

MATERIAL AND METHODS

Bacterial strain and plasmid

Bacillus halodurans CM1 isolated from Cimanggu Hot Springs, Bandung, West Java was used as a resource for protease gene. *B.halodurans* CM1 was inoculated in medium containing xylan absed on Horikoshi et al. at pH 9 at 50ºC [12]. *E.coli* DH5α (BPPT culture collection) was used as a host cell for gene cloning. These bacteria grow in LB agar medium at pH 7 at 37ºC. pGEM T-Easy (Promega, USA) was used as a cloning plasmid vector.

Confirmation of Protease Activity

The proteolysis activity of *B.halodurans* CM1 was confirmed in skim milk agar medium pH 7 and incubated at 50ºC. the medium was based on Harrigan and McCance work [13] with the following components: (A) tryptone 2g, yeast extract 1g, and NaCl 2g in 140 mL deionized water (w/v), pH was adjusted until 7. Solid medium was prepared by the addition 2% agar (w/v). (B) skim milk in 4 g in 60mL deionized water (w/v) was pasteurized at 110ºC in 20 minutes. Medium (A) and (B) was mixed instantly after sterilized.

Protease Gene Amplification

The alkalotermophilic protease gene was amplified from the genome DNA of *B.halodurans* CM1 as the result of previous extraction using phenol-chloroform method [14]. The protease gene locus based on complete genome mapping of *B.halodurans* C-125 was chosen for primer design [2].

The amplification of alkalotermophilic protease gene was conducted using PCR Hot Start DNA Taq Polymerase (KAPPA, USA). Primers for protease gene ORF were designed based on the GenBank database included, Protease-ORF-fwd (5'–ATGAGACAAAGTCTAAAAGTTATGG–3') and Protease-ORF-rev (5'–CTATTGTGTTGCACGTCCAGCATG–3'). The termal cycler PCR (Eppendorf, Germany) running under the following conditions: denaturation at 9 5ºC for 30 sec, annealing 56ºC 30 sec, extension 56ºC 2 min for 30 cycles, then followed by elongation at 72ºC for 10 min.

Cloning of Gene Encoding Protease

The obtained fragment DNA then ligated inside of the pGEM-T Easy plasmid using T4 DNA Ligase. The pGEM T-Easy recombinant plasmid was transformed into *E.coli* DH5α. The foreign DNA could be transferred ito *E.coli* DH5α competent cell through the heat shock method [15].

The transformants *E.coli* DH5α were inoculated in LB-ampicillin agar medium. X-Gal and IPTG were spread on the medium surface for implemented white blue screening. Some selected white colonies bacteria that were predicted containing protease gene were re-cultured in LB ampicillin medium. Extraction of plasmid inside the white colony bacteria was carried out with alkaline lysis method [16]. pGEM T-Easy plasmid recombinant was restricted by endonuclease restriction enzyme *Eco*RI to verify the existence of inserted gene. Sequencing the DNA fragment encoding protease with universal primer forward and primer reverse M13 was performed by First Base.

Partial Proteolysis Activity Assay

The positive single colony of recombinant *E.coli* DH5α was cultured in Luria Bertani (LB) pH 7 as a starter medium. Incubated overnight at 37ºC for cultivation. Afterwards, to produce high protei recombinant, transformants *E.coli* DH5α was re-cultured in LB skim milk medium for 2-3 h at 37ºC. As ab

inducer to transcript protease gene, IPTG was added into production medium.

The culture of this recombinant *E.coli* DH5α from LB skim milk medium was spotted in calcium caseinate agar medium. The culture was incubated at 50^{C} overnight. The expression of protease gene was tested through the qualitative assay on the calcium caseinate agar medium according to Cheeseman study [17]. Calcium caseinate 6,04 g in 120 mL deionized water (w/v). Solid medium was prepared by the addition of 1% agar (w/v).

RESULT AND DISCUSSION

Refreshment and Confirmation of *Bacillus halodurans* Stock

The proteolytic activity of *B.halodurans* CM1 was confirmed in LB skim milk agar medium (**Fig.1**). A clear zone appeared after 24-48 h since the culture spotted onto agar and incubated at 50°C.

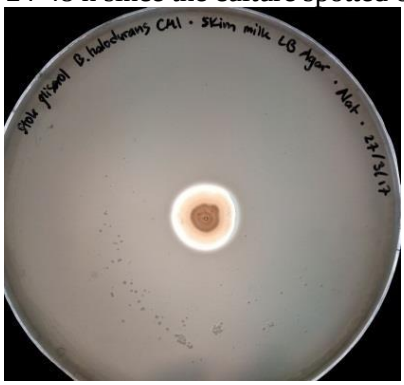

Figure 1. *Bacillus halodurans* CM1 Culture Spotted on LB Skim Milk Agar Medium Showed Protease Activity

A clear zone around of *B.halodurans* CM1 spot indicated protease enzyme activity. The raw milk's protein (casein), in the formed of white colloid within substrate had been degradated into amino acids. The bacteria used these amino acids as their nitrogen source [18].

Amplification of Protease Gene from *B.halodurans* CM1

The PCR product size was about 1100 bp as expected. The primers amplified gene encoding protease, based on the GenBank database have exact size of 1086 bp.

Transformation and White-Blue Screening

To select a positive transformants colony, culture of transformants *E.coli* DH5α was grown in a selective antibiotic medium agar. The LB agar medium was spread with ampicillin. An *amp*^{r} gene that encoded in the pGEM T-Easy plasmid elicited resistance to ampicillin antibiotic. Only *E.coli* DH5α with pGEM T-Easy recombinant inside be able to live in that medium [19].

The addition of IPTG and X-Gal substance were to find out the result of transformation. IPTG as a lactose analog compound served to induce the activity of *lacZ* gene that also encoded on pGEM T-Easy plasmid vector. An enzyme that initiates the activation of *lacZ* gene identified as β-galactosidase. The activation of β-galactosidase mean activation of operon Lac system. Thus, only transformants *E.coli* DH5α which carrying pGEM T-Easy plasmid be able to metabolism any lactose on the susbtrate through operon Lac system [20].

X-Gal was a chromogenic substance. The way of X-Gal expression was to change the colour of cell into blue. The living cell absorbed X-Gal in formed a 5'-dibromo-4,4'-dichloro-indigo as their pigment. First, X-Gal needed to be hydrolyzed before could be absorbed. While the activity of β-galactosidase was detected through operon Lac mechanism, X-Gal was self-hydrolyzed into a 5'-dibromo-4,4'-dichloro-indigo [20].

On pGEM T-Easy plasmid, protease gene in this study was inserted precisely in *lacZ* gene locus.

Insertion of protease gene ensured inhibited operon Lac system. So, transformants *E.coli* DH5α with recombinant pGEM T-Easy plasmid have an inability to hydrolyzed X-Gal. Therefore, the allegedly positive transformants *E.coli* DH5α colonies carrying recombinant pGEM T-Easy plasmid without X-Gal activity should be white. The allegedly negative colonies should be blue.

Recombinant Plasmid Verification using Restriction Enzyme

The successful cloning was confirmed by the analyses using restriction enzymes of extracted plasmids. *Eco*RI restriction endonuclease enzyme used to cut the recombinant plasmid and the result showed DNA fragmentwithht 3000 bp and around 1100 bp.

The approximately 3000 bp DNA fragment was confirmed as pGEM T-Easy plasmid. The true size of pGEM T-Easy plasmid was 3015 bp length. Meanwhile, an approximately 1100 bp DNA fragment was thought to be a gene encoding protease. The protease gene had isolated by *Eco*RI enzyme. This enzyme cutting off the 52nd and the 1144th base sequence of pGEM T-Easy.

Qualitative Assay

The qualitative assay was done to ensure proteolysis activity. When protease gene inside transformants *E.coli* DH5α was expressed, a clear zone appeared around the culture. A clear zone was formed from peptide bond termination reaction of casein (**Fig.2**). The thermophilic profile of protease enzyme was characterized after incubated culture at 50^{o}C.

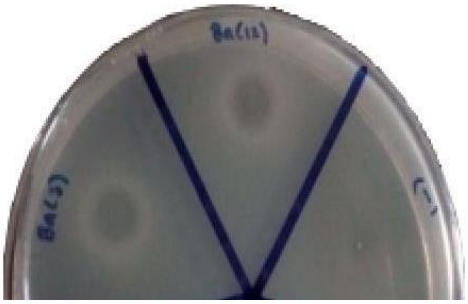

Figure 2. Culture recombinant *E.coli* DH5α (8a) Compared to Negative Control (-) in Calcium Caseinate Agar Medium

The protease enzyme that ligated in restriction site was expressed as functional enzymes resulting from utilized T7 and SP6 promoters. Both of these promoters regulated the translation of the *lacZ* gene in pGEM T-Easy plasmid.

Analysis of DNA Sequencing

For futher bioinformatic characterization of the protease recombinant gene, sequencing was conducted. DNA fragment that ligated in pGEM T-Easy size was verified 1086 bp length. Analysis using BLAST (**Fig.3**) showed 99% similarity with *B.okuhidensis* peptides and the gene encoding thermostable alkaline protease frim *B.halodurans*. This high percentage explained that protease gene from tge present study have closed consecutive compared to the nucleotides encoding alkalotermohilic protease belong to *B.okuhidensis* and to other *B.halodurans* strain [21,22].

Alignments Download GenPept Graphics

Description	Max score	Total score	Query cover	E value	Ident	Accession
peptidase S8 [Bacillus okuhidensis]	699	699	99%	0.0	99%	WP_053432556.1
thermostable alkaline protease [Bacillus halodurans]	698	698	99%	0.0	99%	WP_010897028.1
extracellular alkaline serine protease [Bacillus halodurans]	698	698	99%	0.0	99%	SBU87532.1
thermostable alkaline protease [Bacillus clausii]	667	667	99%	0.0	99%	ABI26631.1
prepro-thermostable alkaline protease [Bacillus halodurans]	664	664	99%	0.0	97%	BAA02443.2
high-alkaline serine proteinase (EC 3.4.21.-) precursor - Bacillus sp. (strain AH-101)	662	662	99%	0.0	97%	A48373
thermostable alkaline protease precursor [Bacillus sp. JB99]	659	659	99%	0.0	96%	ADD64465.1
peptidase S8 [Bacillus pseudofirmus]	417	417	99%	2e-142	61%	WP_012957236.1
prepro-subtilisin ALP I [Bacillus sp.]	416	416	99%	8e-142	60%	BAA06158.1
peptidase S8 [Bacillus marmarensis]	415	415	99%	2e-141	60%	WP_022628745.1
peptidase S8 [Bacillus sp. LL01]	409	409	93%	3e-139	62%	WP_047973137.1

Figure 3. Analysis Protease DNA Sequence using BLAST NCBI

Among *Bacillus* genus, *B.pseudofirmus* and *B.marmarenss* were also involved in bioindustry to produce protease enzyme [23,24]. Massive production of protease enzyme for commercial biocatalyst product most efficient to express in a new host. Cloning technology used *E.coli* or *B.subtilis* bacteria as a host cell were not charged a specifi environment to live, so that would compressed production cost [8].

Cloning in genetic engineering technology based on previous studies has been multiplied various microbial enzymes. Currently, bioindustries are more demanding for microbial extremophiles enzyme. The bacteria of *Bacillus* group which most widely used for amylase production from α and β-amylase genes were the *subtilis* and *licheniformis*. The starch thermo-bioconversion in industry was done by *Thermococcus* spp. and *Staphilothermus* spp. the bacteria from *Thermotoga* spp. were used as a source of xylanase and cellulose thermostable enzyme, whereas *Pyrociccus* spp. as a source of lipase enzyme [25].

Protein Translated

Base sequences from analyzed DNA sequencing were translated into protein. The uninterrupted amino acids translated into a protease enzyme from start codon until termination (**Fig.4**). the deduced amino acids showed that the ORF predicted functional enzyme.

Met R Q S L K V **Met** V L S T V A L L F **Met** A N P A A A S E E K K E Y L I V V E P E E
V S A Q S V E E S Y D V D V I H E F E E I P V I H A E L T K K E L K K L K K D P N V K
A I E E N A E V T I S Q T V P W G I S F I N T Q Q A H N R G I F G N G A R V A V L D T
G I A S H P D L R I A G G A S F I S S E P S Y H D N N G H G T H V A G T I A A L N N
S I G V L G V A P S A D L Y A V K V L D R N G S G S L A S V A Q G I E W A I N N
N **Met** H I I N **Met** S L G S A S G S S T L E L A V N R A N N A G I L L V G A A G N T G
R Q G V N Y P A R Y S G V **Met** A V A A V D Q N G Q R A S F S T Y G P E I E I S A P
G V N V N S T Y T G N R Y V S L S G T S **Met** A T P H V A G V A A L V K S R Y P S Y

Figure 4. The Deduced Amino Acids

CONCLUSSION

As a conclusion, the ORF encoding alkalotermophilic protease was cloned and the DNA sequence was analyzed. The ORF encoding protease from *B.halodurans* CM1 with 1086 bp length has been obtained. The protease gene had been proven have an ability to express as a functional thermostable protease enzyme. This gene has a potential to produce and apply in the industries. However, measuring the protease activity quantitatively is also needed to be done to determine the value of proteolysis activity. Further genetic

engineering for better expression must be done for application of protease in detergent industrial.

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REFERENCES

[1] Tambekar, D. H., Tambekar, S. D., Jadhac, A. S., & Babhulkar, B. V., *Isolation and Partial Characterization of Protease from Bacillus halodurans (AJ302709) from Alkaline Lonar Lake*, International Journal of Pharmaceutical Sciences and Research, **7**(11), pp. 4546–4549, Nov. 2016.

[2] Takami, H., Nakasone, K., Takaki, Y., Maeno, G., Sasaki, R., Masui, N., Fuji, F., Hirama, C.,Nakamura, Y., Ogasawara, N., Kuhara, S., & Horikoshi, K., *Complete Genome Sequence of the Alkaliphilic Bacterium Bacillus halodurans and Genomic Sequence Comparison with Bacillus subtilis*, Nucleic Acids Rcs, **28**(21), pp. 4317–4331, Aug. 2000.

[3] Akita, M., Kayatama, K., Hatada, Y., Ito, S., & Hirokoshi, K., *A Novel β-glucanase Gene from Bacillus halodurans C-125*, FEMS Microbiology, **248**, pp. 9–15, July 2005.

[4] Mamo, G., Hatti-kaul, R., & Mattiasson, B., *A Thermostable Alkaline Active Endo-β-1-4-Xylanase from Bacillus halodurans S7 : Purification and Characterization*, Biochimie, **39**, pp. 1492–1498, Mar. 2006.

[5] Han, X., Shiwa, Y., Itoh, M., Suzuki, T., Yoshikawa, H., Nakagawa, T., & Nagano, H., *Molecular Cloning and Sequence Analysis of an Extracellular Protease from Four Bacillus subtilis Strains*, Biosci. Biotechnol. Biochem, **77**(4), pp. 870–873, Apr. 2013.

[6] Dettmer, A., Cavalli, É.., Ayub, M. A. Z.., & Gutterres, M., *Environmentally Friendly Hide Unhairing: Enzymatic Hide Processing for the Replacement of Sodium Sulfide*, Journal of Cleaner Production, **47**, pp. 11-18, May 2013.

[7] Niehaus, F., Bertoldo, C., Kähler, M., & Antranikian, G., *Extremophiles as a Source of Novel Enzymes for Industrial Application*, Appl. Microbiol Biotechnol, **51**, pp. 711-729, Feb. 1999.

[8] Gupta, R., Beg, Q. K.., & Lorenz, P., *Bacterial Alkaline Proteases: Molecular Approaches and Industrial Applications*, Appl. Microbiol. Biotechnol, **59**, pp. 15-32, Apr. 2002.

[9] Zaghloul, T. I., *Cloned Bacillus subtilis Alkaline Protease (aprA) Gene Showing High Level of Kerationlytic Activity*, Applied Biochemistry and Biotechnology, **70-72**, pp. 199-205. 1998.

[10] Ulfah, M., Helianti, I., Wahyuntari, B., & Nurhayati, N., *Characterization of a New Thermoalkalophilic Xylanase-Producing Bacterial Strain Isolated from Cimanggu Hot Spring, West Java, Indonesia*, Microbiology Indonesia, **5**(3), pp. 139–143, Sept. 2011.

[11] Wibowo, S. G., Helianti, I., Suryani, A., & Wahyuntari, B, *Application of Response Surface Method in Optimization of Medium Composition for Xylanase Production by Bacillus halodurans CM1 in Submerged Fermentation*, Microbiology Indonesia, **10**(3), pp. 112-117, Sept. 2016.

[12] Horikoshi, K., *Alkaliphiles: Some Application of Their Products for Biotechnology*, Microbiology and Molecular Biology Reviews, **63**(4), pp. 735-750, Jan. 1999

[13] Harrigan, W. F. & McCance, M. E., *Laboratory Methods in Microbiology*, Academic Press, 1966. [14] Sambrook, J. & Russel, D. W., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory. 2001.

[15] Hanahan, D., *Studies on Transformation of Escherichia coli with Plasmids*. Journal of Molecular Biology, **166**(4), pp. 557-580, Jan. 1983.

[16] Bimboim, H.C. & Doly, J., *A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA*, Nucleic Acids Res., **7**(6), pp. 1513–1523, Aug. 1979.

[17] Cheeseman, G. C., *Action of Rennet and Other Proteolytic Enzymes on Casein-Agar Gels*, J. Dairy Rcs, **30**(1), pp. 17-23, Aug. 1963.

[18] Terplan, G., Rundfeldt, H. U., & Zaadhof, K. J., *Zur Eignung Verschiedener Nährböden För Die Bestimmung Der Gesamtkeimzahl Der*, Milch. Arch. Lebensmittelhyg, **18**, pp. 9–11. 1967.

[19] Lodish, H., A. B., & Zirpursky, S. L., *Molecular Cell Biology*, ed. 4, W. H. Freeman and Company. 2000.
[20] Brown, G. G., *Molecular Cloning: Selected Applications in Medicine and Biology*. 2011.
[21] Li, Z., Kawamura, Y., Shida, O., Yamagata, S., Deguchi, T., & Ezaki, T., *Bacillus okuhidensis* sp. Nov., *Isolated from the Okuhida Spa Area of Japan*. International Journal of Systematic and Evolutionary Microbiology, **52**(4), pp. 1205-1209. 2002.
[22] Porwal, S., Lal, S., Cheema, S., & Kalia, V. C., *Phylogeny in Aid of the Present and Novel Microbial lineages: Diversity in Bacillus*, PLoS ONE, **4**(2), pp. 1-27, Feb. 2009.
[23] Abdel-Fatah, Y. R., El-Enshashy, H. A., Soliman, N. A., & El-Gendi, H., *Bioprocess Development for Production of Alkaline Protease by Bacillus pseudofirmus Mn6 Through Statistical Experimental*