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Cloning of a Gene Encoding Protease from *Bacillus halodurans* CM1 into *Escherichia coli* DH5α and Expression Analyses of the Gene Product

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Abstract

Bacillus halodurans strain CM1 is an Indonesia alkalothermophilic bacterium isolated from Cimanggu Hot Spring, Bandung, West Java. This bacterial strain produces high levels of thermoalkalophilic xylanase. It has also been predicted to produce other potential industrial enzymes, including protease. For production and application of protease in the future, the protease gene from *B. halodurans* CM1 was cloned into *Escherichia coli*. The protease gene was isolated from *B. halodurans* CM1 by the PCR approach using primers designed based on the GenBank. The PCR product was then ligated into pGEM-T Easy vector, transformed into *E. coli* DH5α, verified, and analyzed based on DNA sequencing data using the BLAST search tool. A 1086-bp protease gene was obtained that exhibited a very high sequence similarity (99%) with that of alkaline protease gene from *B. halodurans* C-125. When the culture of this positive recombinant *E. coli* DH5α containing the protease gene was spotted onto calcium caseinate agar, a clear zone appeared after incubation at 50°C. This result demonstrated that the protease gene was expressed in this recombinant *E.coli* DH5α.

Abstrak

Kloning Gen Protease dari *Bacillus halodurans* CM1 ke *Escherichia coli* DH5α dan Analisis Ekspresi Produk Gen. *Bacillus halodurans* galur CM1 adalah bakteri alkalotermofilik yang diisolasi dari sumber air panas Cimanggu, Bandung, Jawa Barat, Indonesia. Galur CM1 diketahui memproduksi xilanase termoalkalofilik dengan level yang tinggi. Bakteri tersebut diprediksi menghasilkan enzim industri potensial lainnya, termasuk protease. Dalam rangka pemanfaatan galur CM1 khususnya dalam produksi dan aplikasi protease di masa depan, gen protease dari *B. halodurans* CM1 dikloning ke *Escherichia coli*. Gen protease diisolasi dari *B. halodurans* CM1 dengan teknik PCR menggunakan primer yang dirancang berdasarkan data di GenBank. Produk PCR diligasi ke dalam vektor pGEM-T Easy, ditransformasi ke dalam *E. coli* DH5α, diverifikasi, dan dianalisis berdasarkan data sequence DNA menggunakan pencarian homologi BLAST. Hasil gen protease yang diperoleh sepanjang 1086-*bp*, menunjukkan kesamaan urutan *sequence* yang sangat tinggi (99%) dengan gen alkaline protease dari *B. halodurans* C-125. Biakan *E. coli* DH5α rekombinan yang positif mengandung gen protease tersebut menunjukkan zona bening pada agar yang mengandung kalsium caseinat, yang diinkubasi pada 50°C. Hasil studi ini menunjukkan bahwa gen protease berhasil diekspresikan dalam *E.coli* DH5α rekombinan tersebut.

Keywords: protease; Bacillus halodurans CM1; gene cloning

Introduction

Microbial enzymes play significant roles in ecofriendly industrial applications. Tambekar et al. reported that alkalothermophilic enzymes that are highly stable under extreme temperature and alkaline conditions have the potential to be used in a variety of applications [1]. Some of the examples of these industrially applicable alkalothermophilic enzymes are pectinase, amylase, cellulase, glucanase, xylanase, and protease [2,3,4].
Protease is one of the most potential enzymes used in several fields. In addition to having a broad range of

applications in industrial processes and products, proteases are representative of the most worldwide enzyme sales. As an additive in detergent, alkalothermophilic protease is effective in removing blood stains without damaging the clothes. Protease used as an additive substance in laundering is an ideal alternative to replace the currently used harmful chemical compound for health and environment, for example, chlorine [5]. It is also used in other industrial applications such as degumming, medicines, and even in the preparation of foods and beverages. Furthermore, proteases possessing high keratinolytic activities are used in the dehairing process in leather industry. These enzymes could minimize the use of toxic compounds such as hydrogen sulfide and sodium sulfide, thereby resulting in limited hazards [6,7]. In the silk industry, protease plays an important role in removing raw fibers during the process of degumming. Conventionally, in the food and beverage industry, protease is used in protein hydrolysis of the product containing high protein content to increase its nutritional value (for example, food formulation of soy and cheese, the therapeutic effect of dietary products, fortification fruit juice, etc) [7,8]. The use of protease also accelerates the biodegradation of waste that is rich in protein compounds, such as fowl feathers [9].

Due to their versatile applications, several research groups have isolated, produced, and characterized proteases [10,11]. The genus *Bacillus* is probably the most important bacterial source of native proteases and is capable of producing high yields of neutral and alkaline proteolytic enzymes [11]. In addition to the production of native proteases from wild-type microorganisms, processes such as manipulating their production and genetic engineering approaches have also been carried out. Several research groups have conducted cloning and expression analysis of the protease gene from bacteria, especially from the genus *Bacillus*, for example, the alkaline protease gene from *B. subtilis* [9], the intracellular serine protease from *B. megaterium* [12], or alkaline protease from *B. circulans* [13]. However, based on our literature search, we found only one study describing the metallopeptidase gene cloned from *B. halodurans* that was isolated in Kenya [14]. On the other hand, several enzyme genes have been cloned from *B. halodurans* species, such as α-carbonic anhydrase [15], rhamnose isomerase [16], purine nucleoside phosphorylase [17], and xylosidase [18]. However, as mentioned above, the cloning of protease gene from *B. halodurans* has been less studied. Therefore, the cloning of protease gene from the Indonesian *B. halodurans* CM1 strain could be a novel study and could contribute to the advancement of science.

Being one of the alkalophilic bacterial strains recently isolated from the Indonesian habitat, *B. halodurans* CM1 could survive at high temperature and high pH and is a source of alkalothermophilic enzymes. It potentially produces amylase, lipase, protease, and gelatinase [19]. This bacterium also produces high levels of alkalothermophilic xylanase [20,21].

In this study, we isolated the protease gene by PCR cloning from this *B. halodurans* CM1. We also conducted cloning of the target protease gene, analyzes of DNA sequence, and analyzes of expression based on qualitative and quantitative protease activity assays.

Material and Methods

Bacterial strains and plasmid. *Bacillus halodurans* CM1, a collection of Agency for the Assessment and Application of Technology (BPPT)-Culture Collection (BPPT-CC), LAPTIAB-BPPT, isolated from Cimanggu Hot Spring, Bandung, West Java, was used as a source for the protease gene [19]. The 16S rDNA sequence of this strain has already been submitted to GenBank with the accession number JN903769. CM1 was inoculated into medium containing xylan, according to Horikoshi *et al*. [22], at pH 9 and 50 °C. *E. coli* DH5α, which was also deposited in BPPT-CC, LAPTIAB-BPPT, Puspiptek-Serpong, was used as a host cell for gene cloning. This bacterium was grown in Luria Bertani (LB) agar medium at pH 7 and at 37 °C. pGEM-TEasy (Promega, USA) was used as a cloning vector plasmid.

Confirmation of protease activity. The proteolytic activity of *B. halodurans* CM1 was confirmed in skim milk agar medium at pH 7 and incubated at 50 °C. The medium was prepared according to Harrigan and McCance [23], with addition of the following components: (A) tryptone (Himedia, India) 2 g, yeast extract 1 g (Himedia, India), and NaCl (Himedia, India) 2 g in 140 mL of deionized water (w/v); pH was adjusted to 7. The solid medium was prepared by the addition of 2% agar (w/v). (B) Skim milk 4 g added to 60 mL of deionized water (w/v) was pasteurized at 110 °C for 20 min. Medium (A) and medium (B) were mixed immediately after sterilization.

Protease gene amplification. The protease gene was amplified using genomic DNA of *B. halodurans* CM1 as a template. The DNA was previously extracted using the phenol–chloroform method [24]. The protease gene locus based on complete genome mapping of *B. halodurans* C-125 (GCA_000011145.1) was chosen for designing the primers [2].

The protease gene was amplified using the Hot Start DNA Taq Polymerase (KAPPA, USA). The following primers for the protease gene ORF were designed based on the GenBank database: protease ORF forward (5′-ATGAGACAAAGTCTAAAAGTTATGG-3′) and protease ORF reverse (5′-CTATTGTGTTGCACGTCCAGC ATG-3 ′). The thermal cycler PCR (Eppendorf, Germany) was run under the following conditions: denaturation at

95 °C for 30 s, annealing at 56 °C for 30 s, extension at 56 °C for 2 min for 30 cycles, and then followed by elongation at 72 °C for 10 min.

Cloning of gene encoding protease, verification, and DNA sequencing. The obtained DNA fragment was then ligated into the pGEM-T Easy plasmid using T4 DNA Ligase between *EcoR* I site. The recombinant pGEM-T Easy plasmid was transformed into *E. coli* DH5α competent cells by the heat shock method [25]. The correctly constructed recombinant plasmid was designated as the pGEM-bhprot plasmid.

The *E. coli* DH5α transformants were inoculated into LB–ampicillin agar medium. X-Gal and IPTG were spread on the medium surface for white-blue screening. Some selected white colonies of bacteria that were predicted to contain the protease gene were re-cultured in LB–ampicillin medium. The plasmid from the white colonies of bacteria was extracted using the alkaline lysis method [26]. Recombinant pGEM-T Easy plasmid was cut by the endonuclease restriction enzyme *Eco*RI to verify the existence of the inserted fragment. Sequencing of the pGEM-bhprot plasmid containing the DNA fragment encoding the protease with the universal forward primer and the reverse primer M13 was performed by First Base (Singapore). The correct DNA sequence was then deposited in GenBank.

Partial proteolytic activity assay. The positive single colony of recombinant *E. coli* DH5α was cultured in LB medium at pH 7 as the starter medium. The culture was incubated overnight at 37 °C for the bacterial growth. Then, for producing the protease recombinant, the recombinant *E. coli* DH5α was re-inoculated into LB skim milk medium and incubated for 2–3 h at 37 °C. As an inducer to the transcript protease gene, IPTG was added into the production medium.

The culture of this recombinant *E. coli* DH5α from the LB skim milk medium was spotted on calcium caseinate agar medium. The culture was incubated overnight at 50 °C. The expression of protease gene was assessed through a qualitative assay of this calcium caseinate agar medium as described by Cheeseman [27]. The agar was prepared as follows: calcium caseinate 6.04 g was added to 120 mL of deionized water (w/v), and then the solid medium was prepared by the addition of 1% agar (w/v).

For quantitative assay, the enzyme extract was obtained from the cytoplasmic fraction from 200 mL of broth culture. The cell pellet was obtained by centrifugation and resuspended in 20 mL of phosphate buffer. The suspension was sonicated for 30 s with on/off for 5 min, and then the enzyme fraction was obtained after removing the cell debris by centrifugation. The activity assay was conducted based on the assay of the universal protease [28,29] that has been already modified by Amano K protease assay (http://cy-bio.com/Administrator/Order/20091222125934b.pdf). A 0.65% casein solution (625 µL) was placed in a vial and incubated at 37 °C for about 10 min. Next, the enzyme (125 µL) was added and mixed and then incubated at 37 °C for exactly 10 min. This was followed by addition of trichloroacetic acid (TCA, 625 µL) to stop the reaction. Then, the solutions were incubated at 37 °C for 15 min. after which the test solutions and the blank were filtered using a 0.45-µm polyethersulfone syringe filter. Filtration is required to remove any insoluble material from the samples. Then, 300 µL of the filtrate was added to sodium carbonate (750 µL), followed by addition of Folin's reagent (150 µL) immediately. Subsequently, the mixture was incubated at 37°C for 15 min. Then, the absorbance was measured at 660 nm. Tyrosine at different concentrations was used as a standard. One unit activity was defined as the amount of enzyme that produces 1 µmol of tyrosine per minute. To observe the protein band, SDS-PAGE analysis was also conducted according to Laemmli [30].

Results and Discussion

Refreshment and confirmation of protease activity of *B. halodurans* stock culture. The proteolytic activity of *B. halodurans* CM1 was confirmed in LB skim milk agar medium (Figure 1). A clear zone appeared after 24–48 h of spotting the bacterial culture onto the agar–skim milk medium and incubated at 50 °C. A clear zone

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

around the *B. halodurans* CM1 spot indicated protease enzyme activity. The raw milk protein (casein), in the form of white colloid within agar, was degraded into amino acids. The bacteria used these amino acids as their nitrogen source [31].

Amplification of protease gene from *B. halodurans* CM1. The PCR product size was about 1100 bp as expected (Figure 2A). The primers amplified the gene encoding protease, and based on the sequencing result, the DNA fragment had an exact size of 1086 bp.

Transformation and plasmid verification. To select a positive transformant colony with the correct target insert DNA, *E. coli* DH5α transformants were grown in a selective antibiotic (ampicillin) medium agar, supplemented with IPTG and X-Gal. IPTG as a lactose analog compound served to induce the activity of the *lacZ* (β-galactosidase) gene that was also encoded on the pGEM-T Easy plasmid vector. The activation of β-galactosidase implied the activation of the *lac* operon system. X-Gal is a chromogenic substance, and when it is degraded by β-galactosidase, it turns into blue color within the cell. The living cells absorb X-Gal in the form of 5′-dibromo-4,4′-dichloro-indigo as their pigment. X-Gal that must be hydrolyzed before could be absorbed. The activity of β-galactosidase was detected through the *lac* operon mechanism, while X-Gal was hydrolyzed into 5′-dibromo-4,4′-dichloro-indigo [32]. Therefore, white colonies that are resistant to ampicillin were predicted to contain the target insert DNA, which disrupted the *lacZ* gene. However, the exact DNA sequence must be verified further by restriction enzyme and DNA sequencing analyses.

Successful cloning was confirmed by analyzes using the restriction enzymes of the extracted plasmids. The *Eco*RI restriction endonuclease enzyme was used to cut the recombinant plasmid in two of *EcoR* I sites, and resulting 2 DNA fragments of 3000 bp and about 1100 bp (Figure 2B). The approximately 3000 bp DNA fragment was confirmed as pGEM-T Easy plasmid, as the exact size of the plasmid was 3015 bp (Figure 2B, 2C). Meanwhile, an approximately 1100-bp DNA fragment was predicted to be a gene encoding protease, which was obtained by the cut by the *Eco*RI enzyme (Fig. 2B, 2C). The correct inserted DNA based on the DNA sequencing result was then submitted to GenBank with the accession number MH674193.

At both the nucleotide and the amino acid level, the ORF of the protease gene isolated from *B. halodurans* CM1 showed 99% identity to that of the extracellular alkaline serine protease isolated from *B. halodurans* C-125 and a high homology with those from other *Bacillus*

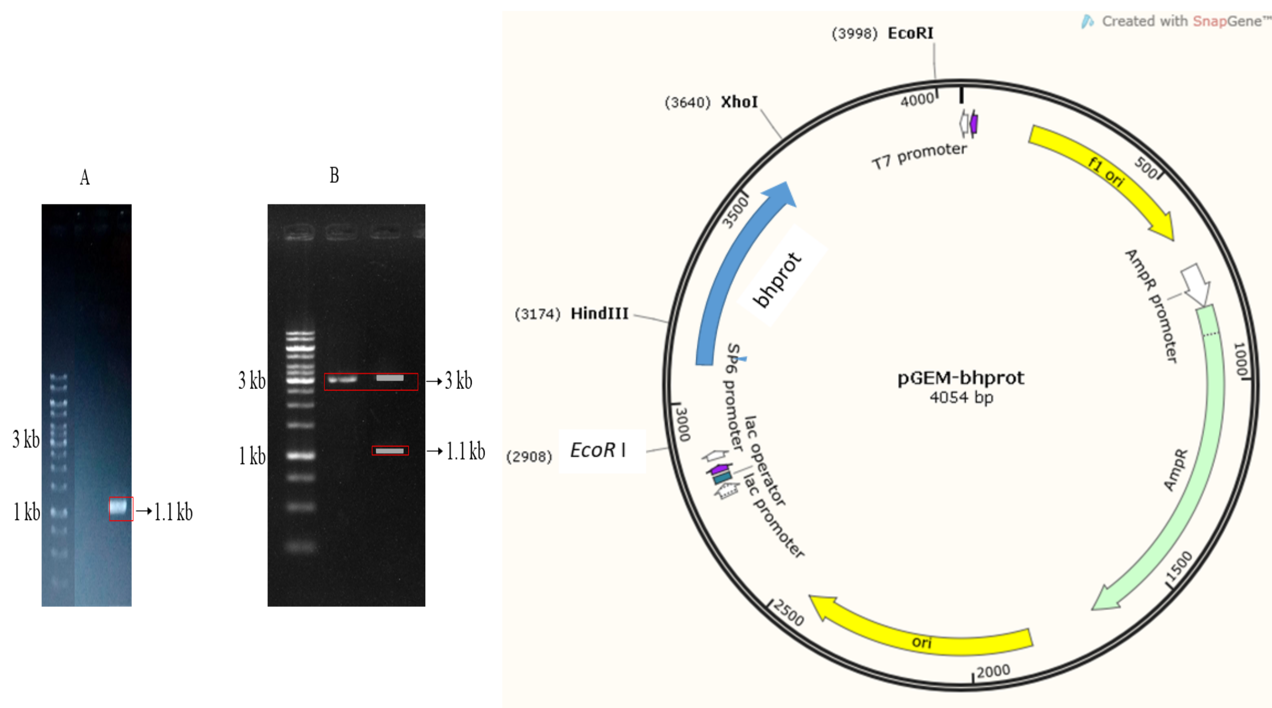

Figure 2. The Chronology of PCR Cloning of Protease Gene from *Bacillus halodurans* CM1 Into *Escherichia coli*. (A) The PCR Product with *B. halodurans* Genomic DNA as a Template DNA. (B) Plasmid Verification of Positive *E. coli* Transformant Using *Eco*RI as the Digesting Enzyme. Lane 1: DNA Marker; Lane 2: Vector pGEM-T Easy; Lane 3: Recombinant Plasmid After Cut by *Eco*RI. (C) The Map of Recombinant pGEM-T Plasmid Harboring the Open Reading Frame of Protease (pGEM-bhprot Plasmid)

spp. [33,34]. Since the extracellular alkaline serine protease gene of the C-125 strain has not yet been cloned, the analyses were conducted based on GenBank data only. The deduced amino acid of the target gene had peptidase S8 subtilase family motif and had a homology of 75% with the peptidase isolated from *B. pseudalcaliphilus* and 66% with that isolated from *B. pseudofirmus*.

According to the signal peptide prediction [35], this gene also has a consensus signal peptide that cleaves between the 24th and the 25th amino acid, thereby suggesting that the gene product should be extracellular in *B. halodurans* CM1 (Fig. 3). The recombinant *E. coli* containing the correct DNA sequence was then chosen for further qualitative and quantitative assays.

Qualitative and quantitative protease assays. The qualitative assay was performed to ensure the proteolytic activity. The protease gene that ligated in the restriction site was expressed as the functional gene that might be affected by the *lac* Z promoter (Fig. 2C). When the protease gene in the recombinant *E. coli* DH5α was expressed, a clear zone appeared around the spot. This clear zone was formed from the cleavage of the peptide bond of casein and degrading into small peptides (Fig. 4). In fact, when we assayed the protease activity, we did not detect any significant protease

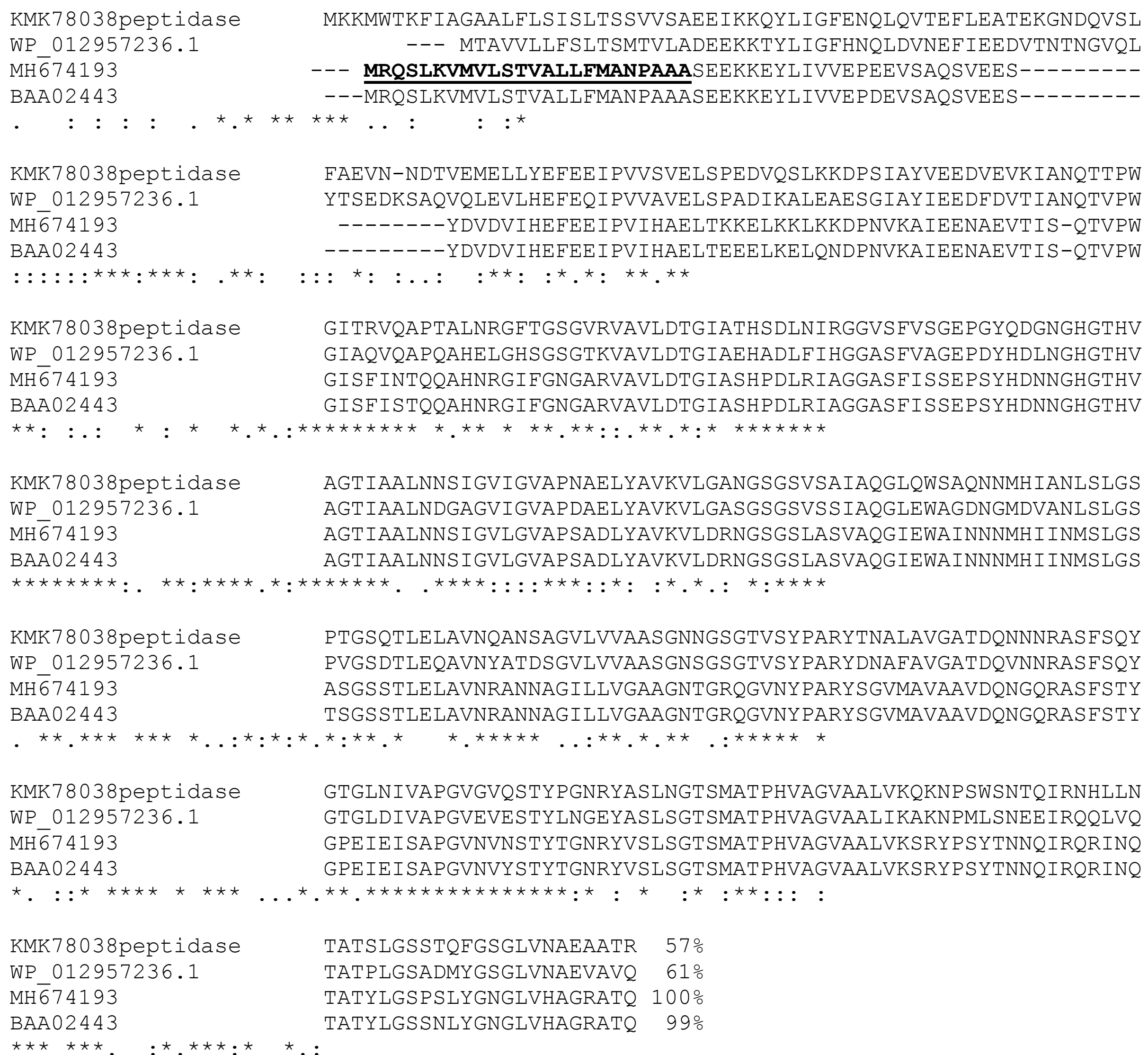

Figure 3. The Alignment of the Deduced Amino Acid of *Bacillus halodurans* Protease Gene with Other Proteases from Genbank. BAA02443: Protease Gene from *Bacillus halodurans* C-125; KMK78038: Peptidase S8 from *Bacillus pseudalcaliphilus*; WP_012957236.1: Peptidase S8 from *Bacillus pseudofirmus*; MH674193: this Study. The Underlined Deduced Amino Acid is the Predicted Signal Peptide

activity in the supernatant. However, there was a higher protease activity than that in the negative control in the cytoplasmic fraction. The activity of the gene product obtained from the recombinant *E.coli* at pH 7 and 37 °C was 24.83 ± 2.48 U/mL, which was higher than that of the negative control (*E. coli* without the plasmid), which was 1.88 ± 0.27 U/mL. This low expression might be because of the inclusion body or cytotoxicity. It has

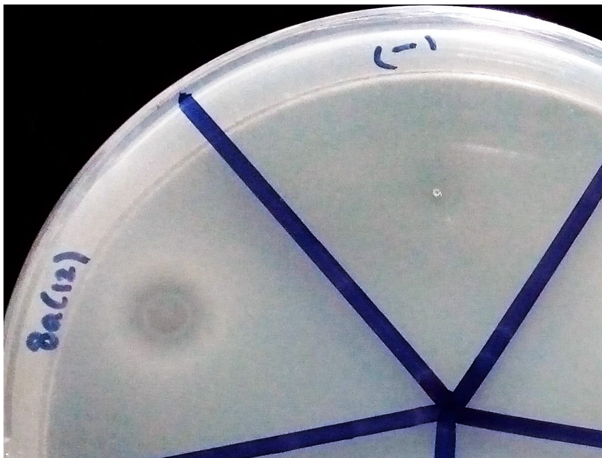

Figure 4. Culture Recombinant *E. coli* DH5α (8a) Compared with Negative control (−) in Calcium Caseinate Agar Medium

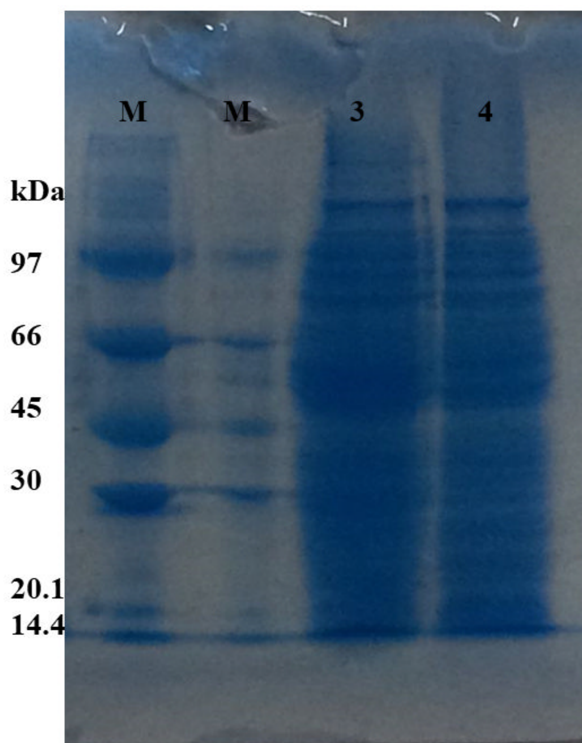

Figure 5. SDS-PAGE of Crude Extract of Recombinant *E. coli* DH5α (4) Compared with Negative Control (3) Fermented in LB Media Containing Skim Milk. M: Protein Marker

been observed that cloning and expression of proteases often fail due to their catalytic functions, which, in turn, cause toxicity in the *E. coli* heterologous host [36]. Due to this low activity, the SDS-PAGE analyzes did not show any difference between the crude extract of recombinant and non-recombinant *E. coli* (Fig. 5). Further optimization for this protease expression can be carried out by choosing tightly regulated expression using vector and a specific *E. coli* strain such as BL21(DE3) pLysS [36]. The cloning and expression of a bacterial strain from the genus *Bacillus* is also a considerable choice because *Bacillus* is a good enzyme secretor [8].

Conclusion

The ORF encoding the protease isolated from *B. halodurans* CM1 was cloned and the DNA sequence was analyzed. The ORF encoding the protease with 1086 bp length was also obtained. This protease showed high homology with that of the extracellular alkaline serine protease isolated from other *Bacillus* spp. and has a peptidase motif and also has a predicted signal peptide, suggesting that the gene product is extracellular in its native expression. The protease gene was functional, although the expression was found only in the *E.coli*'s intracellular form. Further genetic engineering to optimize the expression, such as choosing a more tightly regulated vector or a more suitable host such as *E. coli* BL21(DE3) pLysS or even *B. subtilis*, must be carried out in the future.

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