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# Identification of proteolytic genes from Bacillus lehensis G1

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**Abstract:** Protease is an enzyme that catalyses the hydrolysis of peptide bond in polypeptide chain and hold a wide range of applications in industry. The aim of this study is to clone and to express several genes encoding proteases from alkalitolerant bacteria Bacillus lehensis strain G1. A total of 13 genes encoding proteases have been selected using bioinformatics approach. These genes were then amplified using polymerase chain reaction (PCR) method. Subsequently, the PCR product was cloned into cloning vector pGEM®T easy and transformed into competent cell E. coli DH5α. The transformants were further verified by sequencing. The positive cloned were subcloned into the expression vector and were then expressed in Luria Bertani medium in the present of IPTG using E. coli BL21. The expressions of recombinant proteases were optimized for several hours at different temperatures, 16-37°C. Furthermore, structural prediction was performed using Modeller v9.18 for BleG1_1940. Each generated model was verified for overall completeness and bias, using PROCHECK, ERRAT, and Verify 3D. The overall quality of the model was relatively good with percentage of Ramachandran plot is 96.3%, PROCHECK is 86.2% and ERRAT score is 95%,.

**Key words:** *Recombinant enzyme, Bacillus lehensis, protease, homology modelling*

## INTRODUCTION

Protease is an enzyme that could cleave other proteins or itself that carried many functions from protein recycling, pathogenicity, nutrient digestion, and immune system. Understanding their biological roles; could allow proteases to be manipulated in many industries, in therapeutics and also academia [1]. Many studies have been carried out on the application of microbial proteases in detergents, foods, pharmaceuticals, leather dehairing, bio-waste disposal, silk degumming, and silver recovery [2,3,4,5,6,7,8]. There are several methods available to improve the yield and activity of proteases such as optimization in fermentation, molecular recombinant and site directed mutagenesis [9].

Proteolytic enzyme from alkaliphile bacteria are generally used in many industries, thus finding a new bacteria strain with high catalytic activity and thermostability is always essential, valuable, useful and yet challenging. Proteases could be produced from several sources, such as animals, bacteria, fungi, and plant although, in general, microbial proteases are widely used. Among the bacteria, *Bacillus* species are often used as source of proteases due to high amount of active enzyme with pH, temperature and solvent stabilities such as *B. korieensis*, *B. circulans*, *B. mojavensis* and *B. pumilus* [2,10,11,12].

In this study, *B. lehensis* is a gram-positive, endospore-forming that able to grow under extreme pH conditions as it was isolated from natural alkaline environments [13]. the genome of newly isolated *B. lehensis* G1 from rubber plantation in Johor, Malaysia [14] has been sequenced, and this could offer a platform for their protein to be extensively study through bioinformatics' approach. *B. lehensis* G1 genome contain 4017 protein-coding sequences with approximately 70% assigned biological functions [15]. The availability of this bacterial genome may facilitate researcher to find new potential area to explore, as there are already few articles reported on *B. lehensis* G1 such as cyclodextrin glucanotransferase [16,17,18], membrane protein [19], and signal peptide [20,21]. In this paper, ongoing works on recombinant proteases isolated from *B. lehensis* G1 together with the molecular structure prediction will be discussed. This

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research could provide new opportunities for the discovery, production and modification of novel protease to be used in highly alkaline conditions.

## MATERIALS AND METHODS

### *Bioinformatics analysis*

Multiple sequence alignment of amino acid from *B. lehensis* G1 genome (http://www.mgi-nibm.my/bacillus_lehensis_g1/) were carried out by overlapping the sequences with the Uniprot database (http://www.uniprot.org/) and MEROPS database (https://www.ebi.ac.uk/merops/) in order to find potential proteases. The presence of a putative secretion signal peptide at N-terminus were predicted using SignalP 4.0 server [22], whilst transmembrane were calculated using TMHMM server v 2.0 3].

### Strains, media and vectors

DNA genome was extracted from *B. lehensis* G1 using Wizard® Genomic DNA Purification Kit (Promega, USA. The genome was used as a template for amplification of alkaline protease genes. *Escherichia coli* DH5α was used for the construction and propagation of vector molecules, whilst *E. coli* BL21 (DE3), Origami 2 (DE3) and Rosetta-gami™ 2 (DE3) (Novagen, USA) was used as expression host, respectively. The expression was carried out in Luria Bertani (LB) medium (10 g bacto-tryptone, 5 g bacto-yeast extract, and 10 g sodium chloride in 1L) supplemented with Ampicillin (100 µg/ml) and Kanamycin (100 µg/ml). Vector pGEM®-T Easy (Promega, USA) was used for cloning whilst pET28b (+), pET32 Ek/LIC (Novagen, USA) and pET200/ D-TOPO® (Invitrogen, USA) was used as an expression vector.

### DNA manipulation and protein expression

Standard recombinant DNA routines were carried out according to the methods described in [24]. Polymerase Chain Reaction (PCR) amplification genes were carried out using gene-specific primers and *pfu* DNA polymerase (Promega, USA) according to the manufacturer's specifications. Restriction sites *Nde1* and *Xho1* were added at the N-terminus and at the C-terminus, respectively, to ensure correct orientation of the gene in the expression vector. The cloning vectors were then digested, purified (QIAquick gel purification kit, Qiagen) and ligated into the multiple cloning site of pET28b (+). *B. lehensis* G1 recombinant proteins were expressed in LB medium at 16-37°C, 150-250 rpm rotary shaking and 0.01-1mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) induction for four hours, six hours and overnight. Extracted protein was obtained using BugBuster Protein Extraction Reagent (Novagen CA, USA) and sonication.

### Polyacrylamide gel electrophoresis and Western Blot

Protein concentration was measured using the Quick Start™ Bradford Protein Assay kit according to the manufacturer's specifications (BioRad, USA). The molecular mass of the recombinant proteases was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli method [25]. Protein bands were visualized via staining with Coomassie brilliant blue R-250 and destained in destained solution ( 20% methanol in 10% acetic acid solution). Western blot was carried out to ensure correct protein band were expressed together with His tag protein [26].

### Enzyme assay

Proteases activities were assayed according to [7]. The activity was measured by adding substrate casein (1%) prepared in the glycine-NaOH buffer (pH 10) and was incubated at 37°C for 30 min. The reaction was terminated by adding 5.0% trichloroacetic acid (TCA) followed by incubation for 30 min in ice. The OD (optical density) of the supernatants was measured at 280 nm, with one unit of alkaline protease activity was defined as the amount of enzyme liberating 1 µg of tyrosine/min under assay conditions. Enzyme units were calculated using tyrosine (0–100 µg) as standard.

### Homology modelling

Homology modelling was used to construct the tertiary structure of one target protease; BleG1_1940. The template was selected from Protein Data Bank (PDB) based on sequence similarity using SWISS-MODEL workspace server. The three-dimensional structures was then built using MODELLER (version 9.18) with Chimera v1.11.2 software was used to view the generated model. Quality of the predicted model was assessed using Verify 3D [27], PROCHECK [28], and ERRAT [29].

## RESULTS AND DISCUSSION

There are 2818 proteins with assigned biological roles in *B. lehensis* G1 [15], however, after alignment with proteases from Uniprot and MEROPS database, there were only 300 protein candidates available. Following that, 13 candidate genes were chosen after the removal of predicted transmembrane proteins. 12 proteases gene were successfully cloned and one protease (BleG1_3423) was unsuccessful to amplify. All proteases were cloned in pGEM®-T Easy with size ranging between 525-1137 bp with the start and a stop codon at the N and C termini, respectively. Whilst, prediction of signal sequence using SignalP 4.0 server showed that two genes; BleG1_3207 and BleG1_1058 contained signal peptide. BLAST analysis of the

pGEM®-T Easy vector containing proteases sequences revealed that all proteases has 100% match with the genome database *B. lehensis* G1. The nucleotide sequences were translated into amino acid using ExPASy web-based tools to estimate the molecular mass and pI value (Table 1). The clones were later digested with *Nde1* F and *Xho1* R, and subsequently ligated into pET28b (+). Proteases genes in pET28b (+) vector was initially transformed into *E. coli* DH5α to select the positive clones and then was transform into *E. coli* BL21 (DE3) for recombinant expression. Expression was carried out in LB medium containing kanamycin (100 µg/mL) under various conditions.

Table 1 Summary of thirteen proteases gene with assigned NCBI accession number, calculated protein size and predicted isoelectric point,

| Name | Size (bp) | kDa | pI | NCBI Accession No. |
|---|---|---|---|---|
| BleG1_3041 | 663 | 19.1 | 5.35 | AIC95658 |
| BleG1_1242 | 783 | 29.0 | 5.86 | AIC93863 |
| BleG1_3207 | 1137 | 38.8 | 4.72 | AIC95824 |
| BleG1_3006 | 618 | 24.3 | 4.58 | WP0384B2701 |
| BleG1_2536 | 693 | 22.8 | 5.66 | WP038481565 |
| BleG1_3196 | 819 | 29.9 | 5.36 | WP038483149 |
| BleG1_3228 | 768 | 27.8 | 6.58 | WP038483224 |
| BleG1_3423 | 594 | 20.0 | 4.36 | WP051667653 |
| BleG1_0055 | 747 | 27.1 | 5.61 | WP038475814 |
| BleG1_0791 | 864 | 33.0 | 5.30 | WP051667334 |
| BleG1_1047 | 741 | 26.9 | 5.64 | WP038478032 |
| BleG1_1940 | 525 | 19.1 | 5.35 | WP038480120 |
| BleG1_1058 | 918 | 33.9 | 4.33 | WP051667390 |

SDS-PAGE (Figure 1), western blot and protease assay have showed that seven out of twelve recombinant proteases were insoluble form, whilst another five enzymes were not expressed. The major problem in protein expression would be the formation of "inclusion bodies" that occur when the heterologous protein overexpressed [30]. The insoluble protein could be formed by the association of partially folded protein or misfolded protein [31]. Although *B. lehensis* G1's proteases are difficult to express recombinantly, it does not seem unusual, as for subtilisin from *Bacillus amyloliquefaciens* DC-4 and *Bacillus* sp. DJ-4 [32,33] and nattokinase from *B. subtilis* [34] exhibit similar pattern of expression.

There are several ways to improve protein solubility such as (i) reducing the rate of protein synthesis, (ii) denaturing and refolding, (iii) changing growth condition and (iv) adding chaperones or fusion partner. *B. lehensis* G1 proteases were undergo several modifications and optimizations such as changing the expression vector, host expression strain and expression condition; e.g. reducing temperature and IPTG concentration. All 12 genes were again expressed in two different vectors, pET32 Ek/LIC and pET200/D-TOPO® with several *E. coli* strains. Recombinant plasmid pET32 Ek/LIC and pET200/D-TOPO® from the selected clones were isolated and transformed into *E. coli* BL21 (DE3), BL21 star™ (DE3), Origami 2 (DE3) and Rosetta-gami™ 2 (DE3),respectively. The recombinants were expressed in the *E. coli* strains to obtain soluble proteases. pET32 Ek/LIC was used as Trx•Tag™ (thioredoxin protein) allows the formation of disulfide bonds in the *E. coli* cytoplasm which enhances the solubility of target

proteins [35]. Whilst pET200/D-TOPO® was expressed in BL21 Star™ as of lack *rne131* gene might improve mRNA stability and thus enhance the protein expression for non-expressed proteases such as BleG1_ 1242, BleG1_ 3207, BleG1_ 3006, BleG1_ 1047 and BleG1_ 1058[36].

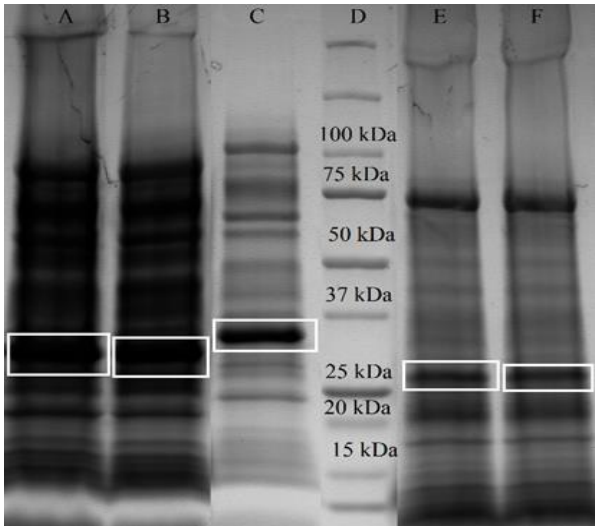


Figure 1 SDS PAGE of insoluble proteases. (A) BleG1_3228-27.8 kDa, (B) BleG1_0055-27.1 kDa, (C) BleG1_0791-33 kDa, (D)Biorad Precicion Plus Protein™ Unstained Standard, (E) BleG1_3041-24.3 kDa and (F) BleG1_2536-25.6 kDa. Proteases size might be bigger than expected size due to vector expression proteins.

*E. coli* heterologous expression of *B. lehensis* G1 proteases seems to be difficult with all protein remains insoluble after countless screening of expression conditions. This suggests that proteases are difficult target for soluble expression in *E. coli* as changing the expression vector, host strains and expression condition did not alter the formation of protein aggregates. The formation of inclusion body are unlikely to be caused by the presence of rare codon, since expression in *E. coli* Rosetta-gami 2 (DE3) did not improve solubility. *E. coli* Origami 2 (DE3) and Rosetta-gami 2 (DE3) which carried two mutations in thioredoxin reductase (*trx-B*) and glutathione reductase (*gor*) for efficient folding of proteins was failed to aid in *B. lehensis* G1 protease formation. Furthermore, beside conventional IPTG induction, expression using auto-induction was also carried out and still no changes observed on *B. lehensis* G1 protease activity nor solubility. Initial attempt of denaturing and refolding for BleG1_2536 and BleG1_0055 did not show a promising result. However, refolding of subtilisin from *Bacillus* sp DJ-4 (rf-subDJ-4) and *B. amyloliquefaciens* DC-4 (subtilisin DFE) showed that their enzymatic activity were retained after denaturing-refolding step using urea and was dialyzed overnight [32,33]. This result suggest that *B. lehensis* G1 proteases behave differently from other recombinant proteins that were previously reported to be effortlessly refolded preceding to *E. coli* expression.

Comparative modelling is an alternative method to obtain 3D structure from amino acid sequence while experimental techniques unsuccessful. The aim is to generate 3D model that is comparable to the results achieved through X-ray crystallography or NMR. The multistep process of homology modelling consists of sequence alignment, template recognition, backbone generation, loop and side chain modelling followed by model optimization and validation [37]. BleG1_1940 was selected for homology modelling with 19.1 kDa calculated molecular mass. In BLAST analysis, the amino acid sequences of BleG1_1940 exhibited high similarity with *B. plakortidis* glutamine amidotransferase (NCBI Acession no. WP_055737683). Whilst in Conserved Domain Database (CCD), BleG1_1940 is matched with type 1 glutamine amidotransferase (GATase1)-like superfamily (ThiJ/DJ-1/PfpI domain) found in *Pyrococcus furiosus* and *Pyrococcus horikoshii*. The catalytic triad consisted of cysteine, histidine, and glutamic acid were also found in *B. amyloliquefaciens* [38]. Sequence alignment of BleG1_1940 with known 3D model in PDB database displayed 40% similarity with intracellular protease from *T. onnurineus* (PDB:3l18), and thus it was selected as template. The predicted structures were generated using Modeller v9.18 in PDB format and was sorted according to DOPE (discrete optimized protein energy) and GA341.

Figure 2 showed a representation of the BleG1_1940 predicted 3D model. The proposed model was represented as (A) ribbon and (B) superimposed with *T. onnurineus* showed the RMSD of 0.285 Å indicating a good overall structure alignment. BleG1_1940 model showed eight β strands and seven helices with the center was consist of six β strands (S1, S2, S5, S6, S7, and S8) and two strands (S3 and S4) on one side. Furthermore, the conserved catalytic residue; Cys106 was located in the sharp turn between β strand and α helix with S-I-C-H-G-P motif around the Cys106. These character are distinctive motif of strand-nucleophile-helix and catalytic triad in the α/β hydrolases. The S-I-C-H-G-P motif was also consistent with the consensus sequence motif of 'small-x-Nu-x-small-small' for α/β hydrolases which is involved in catalysis in the DJ-1 superfamily. All these features are similar to other members of the class DJ-1 superfamily (DJ-1/ThiJ/PfpI domain) of which crystal structures has been identified. DJ-1 superfamily possess highly diverse range of cellular functions with protease activity [39,40]. The location of the catalytic residues, consensus motif and the atom in the backbone is similar with the *T. onnurineus*, suggest that BleG1_1940 model was an accurate representation of the actual protein's structure.

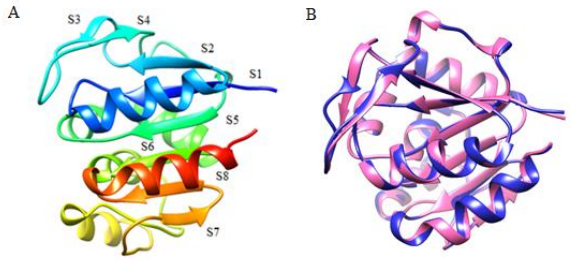


Figure 2 (A) General overview of BleG1_1940 in ribbon diagram (B) The superimposition of the BleG1_1940 of *B. lehensis* G1 (pink) with intracellular protease from *T. onnurineus* (blue).

The quality of the generated BleG1_1940 model was further verified using PROCHECK, ERRAT, and Verify 3D. The backbone conformation was verified by examining the Psi/Phi degrees in Ramachandran plot obtained from the PROCHECK analysis. Results showed that 86.2 % of the residues were located in the most favored region, whilst the remaining residues were found located in the additional and generously allowed regions. Model assessment with ERRAT has demonstrated that the BleG1_1940 3D model was 95% which indicate a high quality as this is the accepted range for ERRAT score [29]. Furthermore, Verify 3D analysis showed that 92.9 % of the BleG1_1940 residues had an average 3D–1D score greater than 0.2, suggesting that the predicted model is in satisfying quality with score greater than 80% [27]. Overall, the values obtained from allanalysis indicate that BleG1_1940 has a good overall structure.

## CONCLUSION

In conclusion, cloning and expression of *B. lehensis* G1's proteases genes were successfully carried out for the first time in *E. coli*. However, the recombinant enzymes require further optimization and investigation to obtain soluble and functional protein for crystallization and thus, application for industrially used. Currently, the expression of *B. lehensis* G1 protease genes using yeast expression system, *Pichia pastoris* is actively pursued.

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