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Biochemical Properties and Potential Application of Proteases from Alkalophilic *Bacillus lehensis* G1

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Running title: Characterization of Protease

Abstrak: Pencirian enzim ekstraselulur protease daripada bakteria Alkalophilic Malaysia *Bacillus lehensis* G1 telah dikaji. Enzim protease yang dirembeskan diuji pada agar susu skim 2%. Keputusan menunjukkan protease ekstraselular mampu mengekalkan aktiviti sehingga suhu 60°C di dalam julat pH yang luas iaitu 3 hingga 11 dengan suhu optimum pada 40°C dan pH optimum pada 7.0. Aktiviti enzim juga diperhatikan akan meningkat dengan penambahan beberapa ion iaitu Mn^{2+}, Fe^{2+}, Cu^{2+}, Mg^{2+} and Co^{2+}. Manakala, aktiviti protease didapati sedikit direncat dengan kehadiran ion Ca^{2+}, K^{+}, and Ni^{2+} dengan baki aktiviti sebanyak 85%, 81%, dan 75%. Protease ekstraselular juga didapati serasi dengan beberapa cecair detergen komersial dari Malaysia, yang menunjukkan protease ini boleh dimanfaatkan sebagai pembersih kotoran pada pakaian. Selain itu, potensi kegunaan protease yang dihasilkan oleh *B. lehensis* G1 ke atas penguraian gelatin dari filem X-ray yang telah digunakan juga telah dilakukan di dalam kajian ini.

Kata kunci: *Bacillus lehensis*, protease, alkalofilik, detergen, filem X-ray

Abstract: The biochemical properties of extracellular proteases enzymes from Malaysia Alkalophilic's bacteria, *Bacillus lehensis* G1, were investigated. The secreted enzymes were tested on 2% of skim milk agar. Results demonstrated that the enzyme could maintain the activity up to 60°C within an extensive range of pH from 3 to 11 with the optimal pH and temperature of 7.0 and 40°C, respectively. The proteases activity were also observed to be increased in the presence of several ions such as Mn^{2+}, Fe^{2+}, Cu^{2+}, Mg^{2+}, and Co^{2+}. Whilst, the enzyme activity was marginally inhibited with the addition of Ca^{2+}, K^{+}, and Ni^{2+} with the residual activity of 85%, 81%, and 75%, respectively. Furthermore, the extracellular proteases have shown to be compatible with several Malaysia commercial liquid detergents, which could be beneficial for stain removal. The potential application of proteases in gelatine decomposition from used X-ray films was also determined in this article.

Keywords: Alkalophilic, *Bacillus lehensis* G1, proteases, stain removal, gelatine decomposition

INTRODUCTION

In Malaysia, the production of enzyme used to be considered almost non-existence, however, since Malaysian recognize the importance of enzymes, the development for enzyme industry becomes one of the national priorities in the area of biotechnology (Hii *et al.* 2012). Among the popular industrial enzymes is a protease. Protease is used as an additive in a variety of goods and processes such as in detergent, leather industry, photographic and biological-waste decomposition, medical treatment as well as the food industry (Bratanis *et al.* 2017, Baweja *et al.* 2016, Ray 2012).

Protease is an enzyme that hydrolysed the peptide bonds in amino acid residues of proteins or themselves and carried a vital role in various metabolic pathway such as in the process of immune system cascade amplification, vegetative growth, sporulation, activation of zymogen, as well as maintenance of the cellular protein pool (Esakkiraj *et al.* 2015, Li *et al.* 2013). Microorganisms generally produce a large array of protease, in the form of extracellular and/or intracellular (Jisha *et al.* 2013). Although proteases are continually produced worldwide, this enzyme is still insufficient to meet the growing demands of the industries. Thus, there has been a continuing exploration to find the new microbial sources of proteases with specific properties to fulfill the industrial enzyme requirements and to test their potential utilization in various industry (Jayakumar *et al.* 2012, Lagzian & Asoodeh 2012, Anbu *et al.* 2015).

Novel extracellular proteases from *B. lehensis* G1 that was isolated from a soil sample in rubber estate, Johor, Malaysia (Sulaiman *et al.* 2017), has yet to be investigated. *Bacillus* is an aerobic or facultatively anaerobic growth, Gram-positive, endospore-forming bacteria with a huge diversity of physiological properties and is mostly studied for industrial applications (Cihan *et al.* 2012, Jayakumar *et al.* 2012). Whilst, *B. lehensis* G1 is known as alkali-tolerant and able to degrade various type of substrates including cellulose, starch, agar and hydrocarbons (Blanco *et al.* 2012). In this study, an effort has been made to describe the biochemical properties of *B. lehensis* G1's proteases followed with the potential use of the enzyme in gelatine decomposition and detergent.

MATERIALS AND METHODS

Media and Culture Conditions

Horikoshi agar (Blanco *et al.* 2012) was adjust to pH 10 (using sterile 1 M Na_2CO_3) was used to routinely grow the *B. lehensis* G1. The extracellular proteases from *B. lehensis* G1 were produced in Horikoshi medium, incubated at 37°C and 250 rpm for 24 hours. The culture medium was centrifuged at 13,000rpm for 10 min at 4°C, and the cell-free supernatant was then concentrated using a centrifugal filter unit (Amicon Ultra, Millipore). The cell-free supernatant was used as the crude enzyme was stored at -20°C until further used.

Diffusion Plate Assay

Diffusion plate assay was performed by streaking *B. lehensis* G1 colonies on skim milk agar (2% Agar + 1% skim milk powder in 0.1 M Glycine-NaOH pH 10.0 buffer). The plate was incubated for 24 h and was observed for the development of halo-zone due to skim milk hydrolysis on the surface of the agar.

Biochemical Properties of *B. lehensis* G1's proteases

A standard enzyme assay was used to measure the *B. lehensis* G1's proteases activity in pH 10.0 of Glycine-NaOH buffer according to Joshi & Satyanarayana (2013). The protein concentration was measured using the Bio-Rad protein assay kit based on the Bradford technique with bovine serum albumin (BSA) as standard. One unit of *B. lehensis* G1's proteases activity is defined as the total of protease required to release 1 μmol of tyrosine per minute in the standard reactions.

The optimum pH of *B. lehensis* G1's proteases were examined in a different pH of buffers which includes Citrate (pH 3–5), Tris (pH 6–8), and Glycine-NaOH (pH 9–11), respectively. Whilst, the effect of pH on the stability of *B. lehensis* G1's proteases activity were examined by pre-incubating the enzyme in the designated pH for 24 h. The residual activity was calculated using the standard enzyme assay.

The optimal temperature of *B. lehensis* G1's proteases were examined at different temperatures ranging from 25-90°C, and the enzyme activity was measured by standard assay procedure. Whilst, the temperature stability of the *B. lehensis* G1's proteases were carried out by pre-incubated the enzyme for an hour under the same temperature conditions.

Effect of Metals and Surfactants

Effect of metal ions on *B. lehensis* G1's proteases activity was determined using $MnSO_4$, NaCl, $CuCl_2$, $FeSO_4$, $NiCl_2$, $CoCl_2$, $ZnCl_2$, KCl, $CaCl_2$ and $MgSO_4$ at 5 mM of concentrations. For the inhibitory effect on *B. lehensis* G1's proteases, divalent chelator and surfactants such as ethylenediaminetetraacetic acid EDTA (1 mM), sodium dodecyl sulphate (SDS), Triton-X-100, Tween 20, and Tween 80 were used (1% v/v). Crude proteases were pre-incubated with aforementioned metal ions and surfactants at 37 ◦C for 1 h and then assayed for the residual activity using the standard assay.

Protease Identification

Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) was carried out at the Malaysia Genome Institute (National Institute of Biotechnology Malaysia). The peptide fragmentation results were given in FASTA sequence format was further subjected to align with *B. lehensis* G1's database (http://www.mgi-nibm.my/bacillus_lehensis_g1/).

Potential Application of *B. lehensis* G1's Proteases

The extracellular proteases from *B. lehensis* G1 were tested against the removal of gelatine on used X-ray films, its compatibility with commercial detergents and washing capability on blood-stained cloth. The used X-ray films were cut into small pieces (1 x 1cm) prior to incubating with crude proteases. The total of gelatine release into the solution was assayed at a 1 h time interval. For compatibility test; five commercial liquid detergents in Malaysia, such as Dynamo®, Breeze®, 5 CARE®, Tesco® and Tops® were boiled at 100°C for 10 minutes to deactivate their enzymes, and it was mixed with *B. lehensis* G1's proteases. The boiled detergents were initially assayed using standard protease assay to ensure that their enzyme was degraded. Then, the mixture of boiled detergents and *B. lehensis* G1' proteases were assayed for their residual activity and *B. lehensis* G1's proteases without detergents served as the control. Furthermore, cotton cloth was cut into pieces (4cm x 4cm) and stained with human blood. The cloth was allowed to air dry for 30 minutes. The removal of blood-stained was carried out by washing treatments using *B. lehensis* G1's proteases mixed with Tesco® liquid detergent at 40°C for 15 min using tap water. The result was visually measured. Stained cloth without added proteases served as the control.

RESULTS AND DISCUSSIONS

An initial search of proteases produced by the *B. lehensis* G1 was tested using skim milk agar. The colonies of *B. lehensis* G1 showed substantial halo-zones which indicated the extracellular proteolytic activity on the skim milk substrate (Figure 1). Then, the crude proteases were produced, followed by the examination of the enzyme properties. Activity assay of the extracellular proteases at different temperatures have shown that the optimal temperature of the enzyme was at 40°C. However, the enzymes still retained 65% of their activity at 50°C, as shown in Figure 2a. This finding showed that *B. lehensis* G1's proteases displayed a basal level of activity even at high temperature, which was between 70-80°C. Nevertheless, a further increase in the temperature caused a significant loss of the proteases activity. The thermos-stability of proteases was tested at different temperatures for an hour. Results showed that the enzymes were stable at room temperature and their optimal temperature with 100% activity remained (25°C-40°C). However, as shown in Figure 2b, the enzyme activity was decreased significantly from 20.1 to 4.95 U/mL when incubated at 60°C for 1 h. The temperature stability that is showed from *B. lehensis* G1's proteases is one of the important feature for an enzyme to be used in detergent industry (Pathak & Deshmukh 2012).

The optimum temperature of proteases from different *Bacillus* strains and species has been reported to be varied. Three different *B. cereus* strain NS-2, SIU1, and TKU022 have demonstrated diverse optimal temperature with 40°C, 45°C, and 50°C, respectively (Singh *et al.* 2012, Liang *et al.* 2012, Bajaj *et al.* 2013). Several extracellular proteases showed a high optimal temperature at 60°C, such as *B. cereus*, *B. licheniformis* KBDL4 and *B. koreensis* BK-P21A (Saleem *et al.* 2012, Anbu 2013, Pathak & Deshmukh, 2012). Whilst, extracellular protease from hyperthermophilic *Bacillus* sp. MLA64 showed the highest protease activity at 95°C (Lagzian & Asoodeh 2012). Another study also showed that *B. circulans*, *B. clausii*, and *B. licheniformis* have a high optimal temperature which is 85°C, 70°C and 67°C, respectively (Lagzian & Asoodeh 2012, Benkiar *et al.* 2013).

The effect of pH on the *B. lehensis* G1's proteases activity was tested at various pH using appropriate buffers. Results exhibited that the proteases reached the highest activity at neutral pH and decreased of activity towards either lower or higher pH (Figure 2c). However, the enzymes were noticeably stable at different pH as the enzymes could retain nearly half of their activity at extremely low and high pH. The graph in Figure 2d showed that the proteases were stable at pH 6-8 with slightly decreased of the activity at the value of 20%. The residual activity was recorded at 81%, 77% and 61% at pH 8, 9 and 10, respectively. Generally, serine protease is classified as an alkaliphilic enzyme in several *Bacillus* species. For example, the serine protease from *B. brevis* MWB-01 - pH 8.0, *B. koreensis* BK-P21A - pH 9.0, *Bacillus* sp. MLA64 - pH 9.5, *B. circulans* DZ100 - pH 12.5 and *B. lehensis* - pH 12.8 (Olajuyigbe & Falade, 2014, Joshi & Satyanarayana 2013, Lagzian & Asoodeh 2012, Benkiar *et al.* 2013, Anbu *et al.* 2013). Meanwhile, metalloprotease is known as a neutral protease. Wang *et al.* (2013) have shown metalloprotease from *B. amyloliquefaciens* SYB-001 are neutral protease with an optimal pH of 7.0. This is similar to metalloprotease from *B. stearothermophilus* and *B. thuringiensis* (Ou & Zhu *et al.* 2012, Luo *et al.* 2013). This data is similar to our results of which metalloprotease behaved well at neutral pH. The neutral pH exhibited by *B. lehensis* G1 could be due to the abundant of metalloproteases as compared to the serine protease that is found to be secreted in the crude sample as showed by the mass spectrometry data. However, there is an exception for *B. cereus* TKU022 metalloprotease which prefers an alkali environment at pH of 10.0 (Liang *et al.* 2012).

According to Bajaj & Jamwal (2013), metal ions are essential for certain enzymes to carry out their full catalytic activity. Metal ions could affect the enzymes in several manners which includes (i) accepting or donating electrons to activate electrophiles or nucleophiles, (ii) acting as electrophiles, (iii) preventing unwanted side reactions, (iv) forming coordinate bonds between enzyme and substrate, (v) holding the reacting groups in the specific three-dimensional conformation and (vi) stabilizing the catalytically active site of the enzyme. The effect of metal ions on *B. lehensis* G1's proteases were tested with the final concentration of 5 mM and the total activity as shown in Figure 3a. Interestingly, ion Mn^{2+} increased the proteases activity at 2.5-fold higher (254%), followed by Cu^{2+}, Fe^{2+} and Co^{2+} with 182%, 137% and 131%, respectively. Whilst, ion Ni^{2+}, Zn^{2+}, and K^{+} caused a slight reduction in proteases activity. Meanwhile, proteases activity remained unchanged with the addition of ion Mg^{2+}. Furthermore, EDTA that was known as the metalloprotease inhibitor (Olajuyigbe & Falade, 2014) was used in this study. The result has shown that *B. lehensis* G1' proteases have significantly loss their activity with the addition of 5 mM EDTA as shown in Figure 3a. This confirmed that there is an abundance of metalloproteases secreted by *B. lehensis* G1 as shown by the mass spec data. Surfactants such as Triton X-100, Tween 20, Tween 80, and SDS was also tested in this study. These surfactants are used to test the stability of proteases activity. In this work, the enzymes were not entirely affected by these surfactants as showed in Figure 3b. The crude enzyme showed high stability in the presence of SDS, retaining 100% of its activity after incubation at 37°C for an hour. Whilst, the residual activity of 80% and 78% retained by the enzyme with the addition of Tween 80 and Tween 20, respectively. However, incubation of enzyme with Triton X-100 significantly affected the enzyme with half of activity is inhibited. Similar result was showed by protease from *B. licheniformis* KBDL4 with high stability against an-ionic, non-ionic and oxidizing surfactant such as SDS and Tween 80. The stability of protease with the presence of surfactants is an important feature for detergent protease since the enzyme must be compatible and stable with all commonly used detergent compound such as SDS and Tween 80 that might be presence in the detergent formulation (Pathak & Deshmukh 2012).

In mass spectrometry, results showed there was a total mixture of 168 proteins includes enzymes, chaperones, ribosomal and uncharacterized proteins in the extracellular protein of *B. lehensis* G1. Whilst, identification of protease revealed six metalloproteases and a single serine protease in the sample. The identified proteases in the secreted crude sample and its properties were summarized in Table 1. According to the MEROPS database (https://www.ebi.ac.uk/merops/), the *B. lehensis* G1's metalloproteases were subgroup into four subtypes; M20, M23, M24, and M42 that are varied in their molecular structure, inhibitors, and conserved motif. However, in general, these metalloproteases are 'co-catalytic' with binding two metal ions; zinc or calcium. The metal ion is involved in the enzyme's catalytic activity, and the maintenance of the enzyme's structure rigidity (Ou & Zhu *et al.* 2012).

The second objective of this research is to find the potential applications of the proteases from *B. lehensis* G1. The application of proteases in silver recovery from X-ray film, its compatibility with Malaysia's commercial detergents as well as stain removal, were described in this paper. Various conventional methods have been applied for silver recovery from used photographic or X-ray film which includes burning the films, silver oxidation and using strong chemical solution for stripping the gelatine layer. However, these methods cause environmental pollution, hazardous, time-consuming and expensive. As an alternative step to extract the silver from the used X-ray film, the protease is used to break and release the silver by peeling the base coating from the surface of the X-ray film (Lakshmi & Hemalatha 2016). The proteolytic activity of the proteases on the gelatine decomposition on the used X-ray film was examined by measuring the amount of protein released into the solution at one-hour time intervals. The hydrolysis of the gelatine was initially slow on the first hour of incubation. However, the activity started to increase upon further incubation, as gelatine layer started to diminish from the surface of X-ray film. The proteases activity in the solution was recorded at 7.23 U/mL at 1h incubation. After 4 h of incubation, the activity was recorded at 11.23 U/mL and remained stationary, indicating that the hydrolysis of the remained gelatine (Figure 4a). The hydrolysed gelatine in the solution was verified by the changes of solution turbidity and the clearing of X-ray film (Figure 4b). The gelatine layer was almost hydrolysed entirely from the X-ray film after four hours, and this is showed by the clearing of the X-ray film (Figure 4c). According to Bholay *et al.* (2012), *B. pumilus* p1's alkaline protease was found to be able to degrade gelatine from waste photographic films with 0.4013 g of silver weight and thus provide benefits in silver recovery application. This is an alternative step to extract silver from X-ray film which is eco-friendly, non-hazardous, time and cost effective.

The detergents compatibility study of *B. lehensis* G1' proteases have shown excellent stability after incubation with commercial detergents tested such as Dynamo®, Breeze®, 5CARE®, Tesco® and Tops®. This then may be applied as an additional component in the detergents formula. Results showed that proteases activities were retained for more than 85% of all the detergents tested, after being incubated at 40ºC for 1hour as shown in Figure 5. The effectiveness of the *B. lehensis* G1' proteases in removing the stain from stained cloth with human blood was showed in Figure 6. From five commercial detergents washing performance tested, only Tesco's detergent showed the lowest cleaning capability and therefore, *B. lehensis* G1' proteases were added to improve the washing performance. The blood-stained cloth that was washed with Tesco's detergent showed visible traces of blood stain retained on the cloth. However, after *B. lehensis* G1' proteases were added to the Tesco's detergent during the washing procedure, the blood stain was completely disappeared from the cloth. A relatively good wash performance was also demonstrated by the alkaline protease from *B. pumilus* MP27 and *B. subtilis* NCIM 2724 with the de-staining temperature at 50 °C and 37°C, respectively (Baweja *et al.* 2016, Parpalliwar *et al.* 2015). These results showed that the **p**roteases produced from *B. lehensis* G1 could be utilized as a detergent additive since it shows good stability in commercial detergent tested, thermostable and as well as having good washing performance.

CONCLUSION

In this article, the characterization and application of the extracellular proteases from *B. lehensis* G1 were investigated. The extracellular proteases have properties such as an optimum temperature of 40°C, optimum pH at 7.0, high thermostability and low sensitivity to pH. The neutral pH optimal, stability towards temperature, pH, and surfactants could make this enzyme potentially useful for industrial applications such as silver recovery and detergent industry. An evaluation of its potential for use in the food industry will be the subject of future work.

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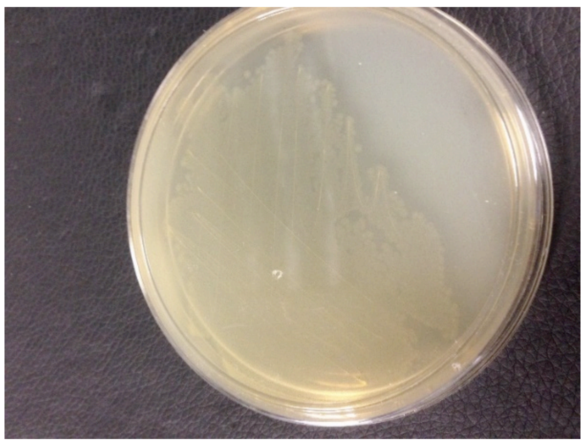

Figure 1. Skimmed milk agar plate showing secreted protease from B. lehensis G1 colonies; the zone of clearance around the colonies indicated proteolytic activity.

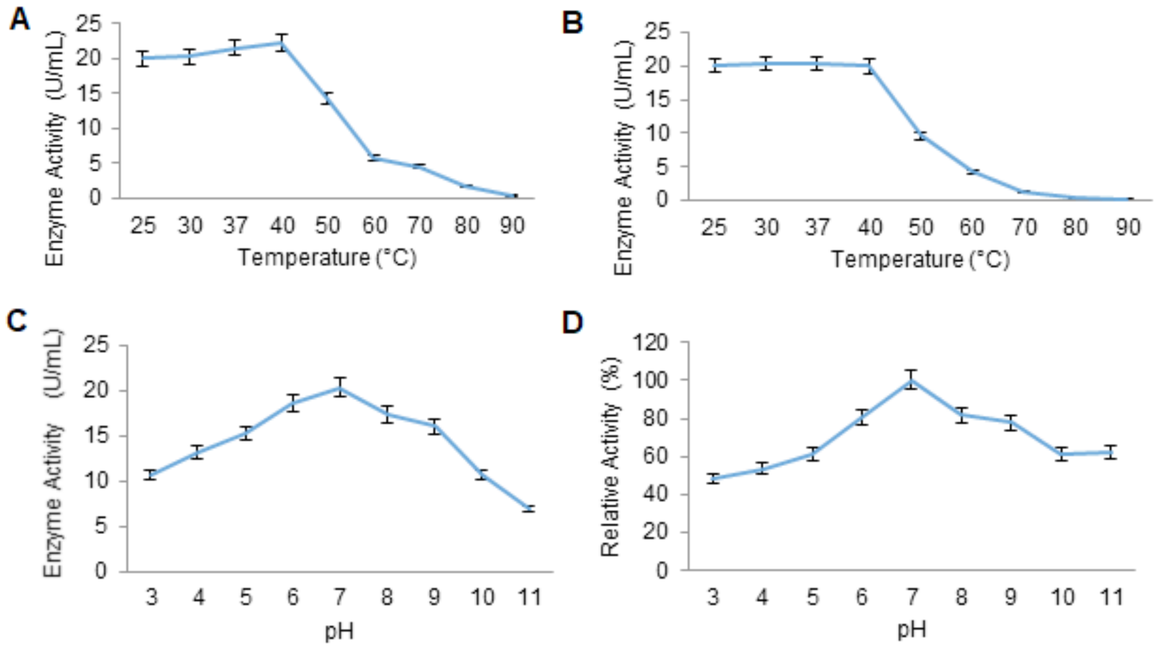

Figure 2. Optimal and stability tested for temperature and pH for extracellular proteases from B. lehensis G1. (A) optimum temperature, (B) thermo-stability, (C) pH optimum, and (D) pH stability. All the experiments were carried out in triplicate, standard error of 8.088E-003, p value of 0.0042, and standard deviation of 19.73. The error bars in the experiments indicate standard deviation (n=3).

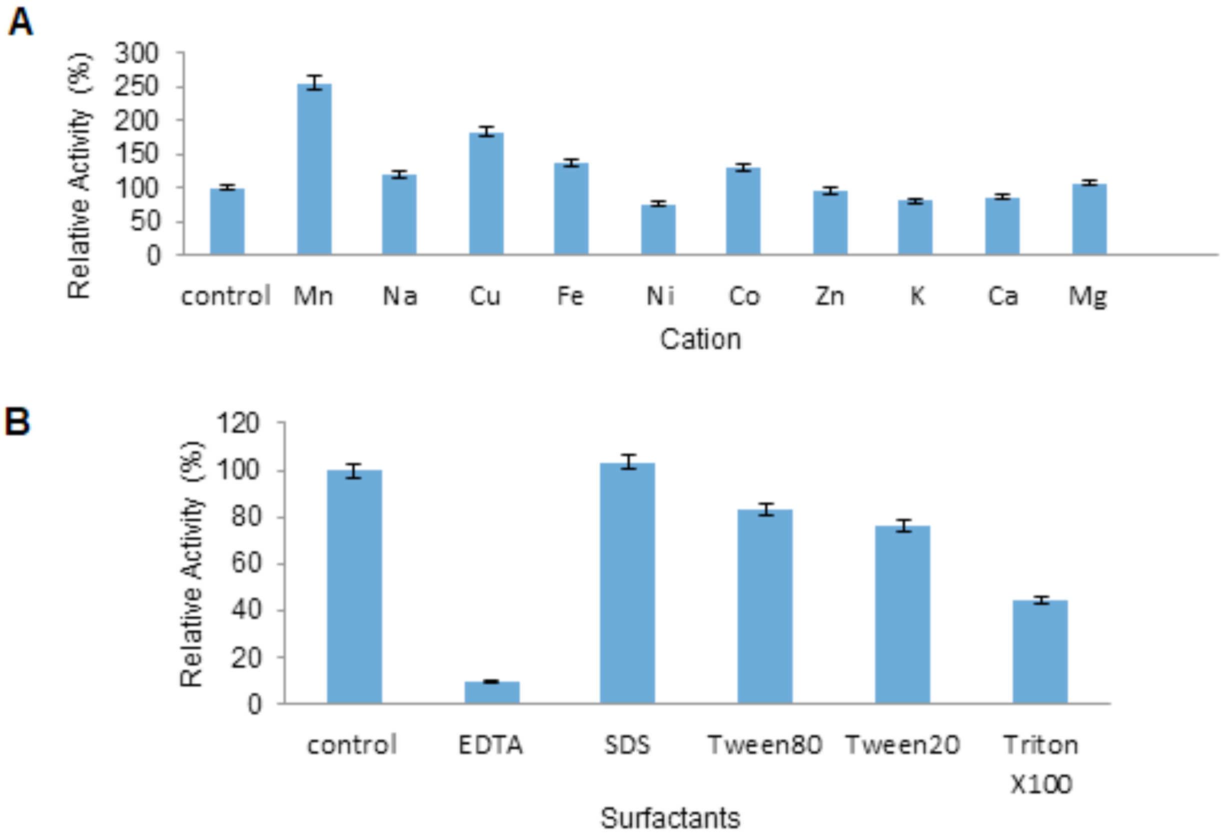

Figure 3. Effect of (A) cation and (B) surfactant for extracellular proteases from B. lehensis G1. All the experiments were carried out in triplicate, standard error of 8.088E-003, p value of 0.0431, and standard deviation of 21.90. The error bars in the experiments indicate standard deviation (n=3).

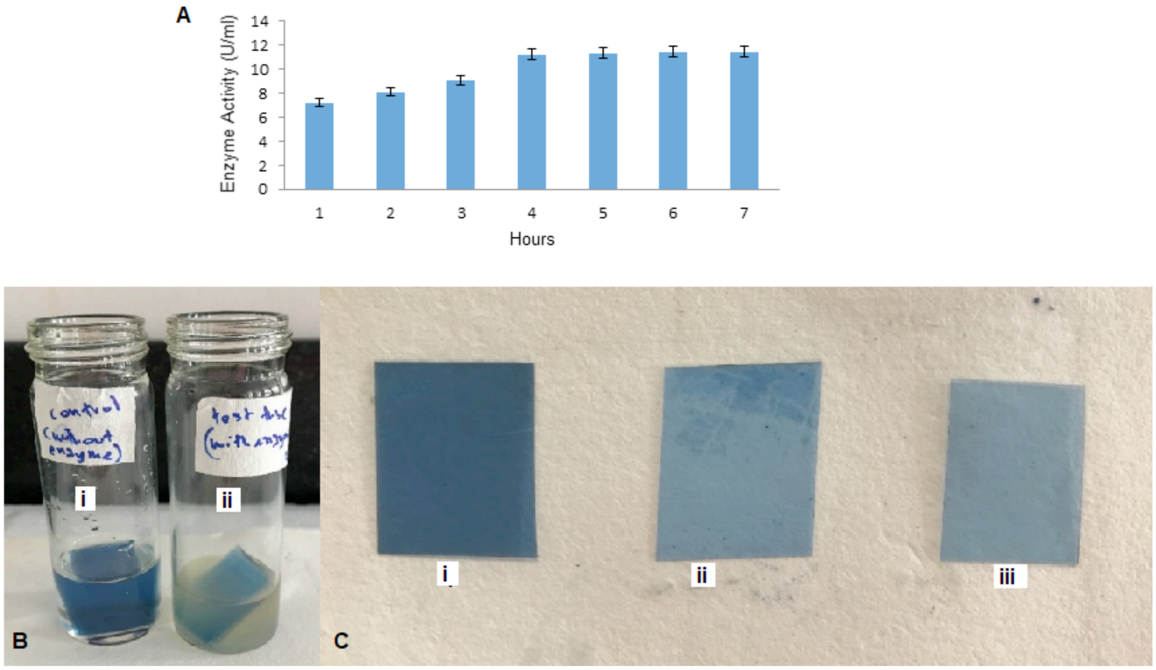

Figure 4. Application of proteases on removal of gelatine layer from X-ray films. (A) Protease activity through clearing of gelatine on the surface of X-ray film tested in 7 hours, (B) Changes of solution turbidity as gelatin release into solution, (i) control, without enzyme, (ii) test subject, and (C) Clearing of gelatine on the surface of X-ray film (i) control without enzyme, (ii) test subject after 2h of incubation, (iii) test subject after 4h of incubation. The error bars in the experiments indicate standard deviation (n=3).

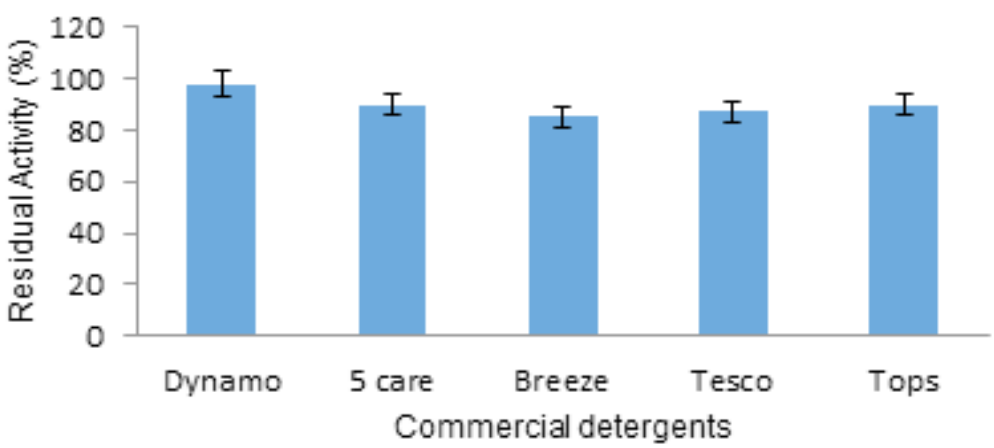

Figure 5. Compatibility test of extracellular proteases with commercial detergents in Malaysia. All the experiments were carried out in triplicate, standard error of 8.088E-003, p value of 0.031, and standard deviation of 19.80. The error bars in the experiments indicate standard deviation (n=3).

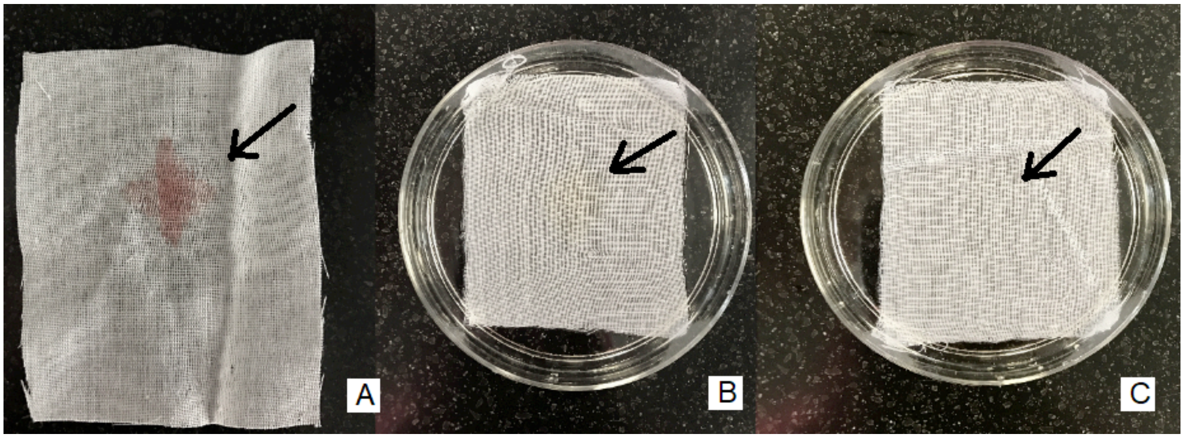

Figure 6. Clearing of blood stain on the cloth using Tesco® detergent and B. lehensis G1's proteases. (A) control (without washing step), (B) stained cloth in 10 min washed (Tesco® detergent only), and (C) effective clearing of stained cloth in 10 min washed (Tesco® detergent with added B. lehensis G1's proteases).

Table 1. Mass spectrometry results for extracellular proteases of B. lehensis G1.

No	BleG1	Proteases Name	Size (kDa)	Calculated pI	Type of proteases	MEROPS
1	3821	Peptidase	49.8	5.03	Metalloprotease	M20
2	2767	Aminopeptidase	38.6	5.40	Metalloprotease	M42
3	2462	Peptidase	38.8	5.06	Metalloprotease	M24
4	2675	Aminopeptidase	38.2	5.39	Metalloprotease	M42
5	1563	Tripeptidase	39.8	5.62	Metalloprotease	M20
6	3825	Oligopeptidase	67.4	5.57	Serine protease	S9A
7	3033	Metalloprotease	51.9	4.72	Metalloprotease	M23