

Research Article**Purification and Characterization of Surfactant-Stable Protease from *Bacillus Licheniformis*: A Potential Additive for Laundry Detergent**Vivi Mardina¹ and Faridah Yusof^{2*}¹Department of Mathematics and Natural Sciences,
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ABSTRACT:

This study purified and characterized the protease from *Bacillus licheniformis* that was cultured in skim latex serum fortified media. Ammonium sulphate precipitation and ion exchange chromatograph was employed in purification steps with the enzyme activity increase to 2.28 fold of purification compare to the crude enzyme. Assessment of the purified protein by SDS PAGE showed a single band with molecular mass of about 47 kDa. The enzyme was stable at temperature range of 35 °C to 65 °C and also at pH 6.0 and 7.0 for 60 min. The presence of Mn²⁺ and Ca²⁺ ions in the produced protease stimulated strongly the activity of the enzyme by 176.65% and 119.07% respectively, while inhibitory effects were found in the presence of Cu²⁺, Zn²⁺, Mg²⁺, and EDTA. The enzyme exhibited their stability toward surfactants (Triton X100, Tween 20, SDS), solvents (acetone, chloroform, hexane and toluene), oxidizing agent (H₂O₂) and Tesco Everyday Value[®] detergent with the residual activity around 80%. It also demonstrated the removal activity of blood stain completely with supplementation of the 7 mg/ml detergent solution. The established characteristics of the enzyme indicated their potentiality for detergent application.

Keywords: *Bacillus licheniformis*, Skim latex serum, Metalloprotease, thermostable, surfactant stable protease,**[I] NTRODUCTION**

Purification and characterization are necessary and critical step in order to analyze the function, physical properties, and amino acid sequence of a specific protein [1]. Proteases constitute the largest portion of these purified proteins for therapeutic and industrial application [2]. Recently, the ability of microbial proteases for the production of extracellular enzyme has stimulated interest in their exploitation for large scale production [3]. Among them, *Bacillus* genus is receiving more attention due to technological and economic reasons. In the technical productions, they showed the outstanding properties such as fast growing, simple culturing for large scale production, and

metabolically vigorous to secrete large amount of protein directly into the fermentation medium that help simplify the purification steps [4]. Moreover, in the economic perspective, micro and macro nutrients for the growth of *Bacillus* genus can be provided easily by using low cost media to enhance the yield [5, 6]. One of the potential substrate for culturing the *Bacillus* genus is skim latex serum [7].

Skim latex serum is a liquid waste from rubber factory that is rich in various organic compounds and has been proved to be an important basal media for various fermentation processes [8]. The previous report [7]covered the valorization strategy of skim latex effluent as

a basal medium to cultivate *Bacillus licheniformis* (ATCC 12759) for extracellular protease production by Response Surface Methodology. Therefore, this study will continue to report the downstream processing for partial purification, characterization and application of the enzyme for detergent industry.

[II] MATERIALS AND METHODS

2.1. Protease Production

The microorganism was grown for 24 h on skim latex serum based media (70 %, v/v). It was supplemented by 1 % (w/v) LB broth, and 1 % (w/v) galactose. The culture condition was set at agitation of 75 rpm, pH 7, and temperature at 35 °C. The cells were harvested by centrifugation at speed of 10,000 xg for 10 minutes at 4 °C. The supernatant was collected as the starting point for purification.

2.2. Protease Assay

Proteolytic activity was assay by casein digestion method that was described by Mardina et al [7]. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg/ml tyrosine in 1 minute under the assay condition.

2.3. Protein Determination

The amount of total protein was determined by Bradford method with bovine serum albumin as a standard protein. Each experiment was performed in triplicate and the average value was then calculated after correction with the corresponding blank.

2.4. Purification Procedure

15 ml protease enzyme in the cell free supernatant was fractionated by 100% ammonium sulfate overnight at 4 °C with constant stirring. The precipitate was recovered by centrifugation for 15 min at 4 °C and 10,000 xg. It was re-suspended in 8 ml of 50 mM Tris-HCl buffer, pH 8 and dialyzed against the same buffer.

The dialysate was applied on the column chromatography that was performed by self-assembled liquid chromatography system. The system consists of peristaltic pump (Master Flex Console Drive), chromatography column (1.5 by 10 cm) and fraction collector. The bed column

volume was determined using the following equation:

$$\text{Bed column volume} = \pi \times \text{radius}^2 \times \text{height} \dots \dots \dots (1)$$

The procedure involved equilibration the DEAE-sepharose column by 53 ml of 150 mM Tris-buffer, pH 8 (5 column volume) and 7 ml sample injection. The collections were begun as soon as after the sample injection. Subsequently, 4 column volume of washing buffer was pumped into the column and considered as first the elution buffer (150 mM Tris-buffer + 1 M NaCl) and any protein eluted in this period was considered purified or bound protein. A total of 36 tubes with 2 ml elution per tube were collected for further analyses that include reading absorbance at 280 nm and protease activity.

2.5. Characterization of Protease

2.5.1. Molecular Mass Determination

The purity and relative molecular weight of the purified enzymes were estimated by 5% native and 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The molecular mass (using a standard molecular weight marker) was determined by SDS-PAGE. The protein bands were visualized by 0.05% solution Coomassie Brilliant Blue R-250 (Sigma).

2.5.2. Effect of pH on Activity and Stability of the Enzyme

Optimum pH for the protease activity was carried out by performing the assay with various pH buffers (pH 3 to 9).

The following buffer systems (50 mM) were used: glycine-HCl buffer for pH 3, citrate phosphate buffer for pH 4 and pH 5, phosphate buffer for pH 6 – 7, TrisHCl for pH 8 and glycine-NaOH for pH 9. All runs were carried out in triplicate and relative activity was reported.

pH stability of the enzyme was also determined by measuring the residual activity after 1h incubation the enzyme without substrate at 37°C in the mentioned buffers. Control was determined without incubation the enzyme with any buffers.

All experiments were performed in triplicates.

2.5.3. Effect of Temperature on Activity and Stability of the Enzyme

The optimal temperature for protease activity was investigated by varying the incubation temperature of the assay from 35°C to 75°C at pH 8 for 10 min. All experiment were carried out in triplicate and relative activity was measured.

While, the thermal stability was examined by incubating the enzyme without substrate in water bath at various temperature ranging from 35 °C to 75 °C for 60 min. The residual activity was measured at 20 min, 40 min and 60 min incubation respectively. The non-heated enzyme was considered as a control. All runs were conducted in triplicates.

2.5.4. Effect of Metal Ion on Protease Activity

Effect of various metal ions at 10 mM on the enzyme activity was investigated by preincubating enzyme and metal ion solution with ratio 1:2 for 60 min at 37 °C. The examined ions include the following corresponding salts: NaCl, KCl, CaCl₂, MgSO₄, MnSO₄, CuSO₄, and ZnSO₄. The residual activity was measured by standard assay condition. Control was determined without incubation the enzyme with any ions. All experiment was conducted in triplicate.

2.5.5. Effect of Enzyme Inhibitors on Protease Activity

In order to determined the type of protease, the effect of different inhibitors was studied using ethylene diamine tetra acetic acid (EDTA), phenil methyl suphonyl fluoride (PMSF), β-mercaptoethanol in the concentration of 5mM and pepstatin with concentration of 1mM. The enzyme was preincubated with these inhibitors for 60 min at 37 °C. The proteolytic activity was measured under standard assay conditions and all runs were performed in triplicate.

2.5.6. Effect of Surfactants and Solvents on Stability of Protease

Stability of the protease against different surfactant/ solvents at 1% concentration was determined by assaying the enzyme in the presence of each solvent at 37 °C for 30 min using casein as substrate. They included Tween 20, Triton X-100, SDS, acetone, hexane,

toluene, chloroform and hydrogen peroxide (H₂O₂). 1 ml of 1% (v/v) the above mentioned solvents were added to 0.5 ml the enzyme and incubated for 30 min at 37 °C. Control had no solvent in reaction mixture. All the reactions were performed in triplicate.

2.6. Detergent Compatibility

The compatibility of the enzyme with commercial detergent was investigated using Tesco Everyday Value[®] detergent that does not contain any enzyme. 1 ml of 7mg/l the detergent solution was incubated with 0.5 ml the enzyme sample for 30 min at 37 °C. The enzyme activity of pretreatment enzyme with the detergent was compared to the control that absent from any commercial detergent. Residual activity was measured by the protease assay and reported as a result.

2.7. Evaluation of washing performance using blood stain

20 µl chicken blood was poured to clean pieces of pure white cotton clothes. It was allowed to 10 min in room temperature before drying at 80 °C for 5 min in hot air oven. The stained clothes pieces that had sizes around 4 x 4 cm² were incubated at 60 °C in different following sets:

- Conical flask with distilled water (100 ml) + stained cloth as a control
- Conical flask with distilled water (100 ml) + stained cloth + 5 ml Tesco Everyday Value[®] detergent(7mg/ml)
- Conical flask with distilled water (100 ml) + stained cloth + 5 ml Tesco Everyday Value[®] detergent (7mg/ml) + 3 ml crude enzyme.

The destaining activity of protease was observed for 10 min and 30 min incubation respectively.

[III] RESULTS

3.1. Enzyme Purification and Molecular Weight

Summaries of the purification scheme and chromatography profile in this study are presented in Table 1 and Figure 1 respectively. Approximately 1.42 fold purification of the crude enzyme was achieved with 73.32 % recovery and 11.06 U/mg specific activities. The intensity of band produced showed a single band with molecular mass of about 47kDa.

Purification step	Total activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude enzyme	21.867	2.80	7.81	100	1
Ammonium sulphate precipitation	16.033	1.45	11.06	73.32	1.42
Dialysis	15.40	1.26	12.22	70.42	1.56
DEAE sepharose	14.6		17.81	66.76	2.28

Table: 1. Purification Scheme for *Bacillus licheniformis* protease.

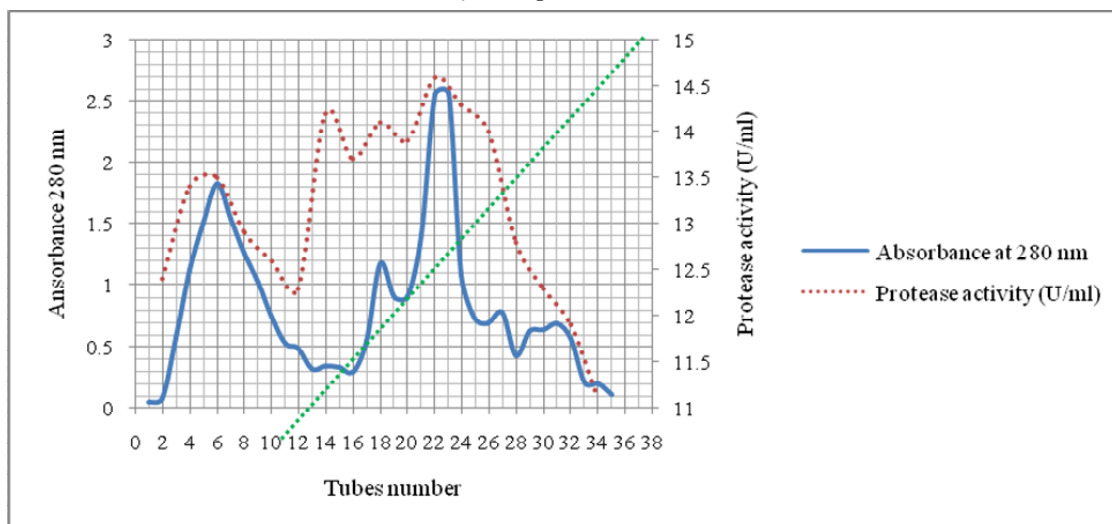


Fig: 1. Chromatography of *B. licheniformis* protease on DEAE-sepharose. The column (1.5x10 cm) was equilibrated with 1.5 M Tris-buffer (pH 8) loaded with enzyme preparation and eluted with a linear gradient (0 up to 1M NaCl) at a flow rate 2ml/min.

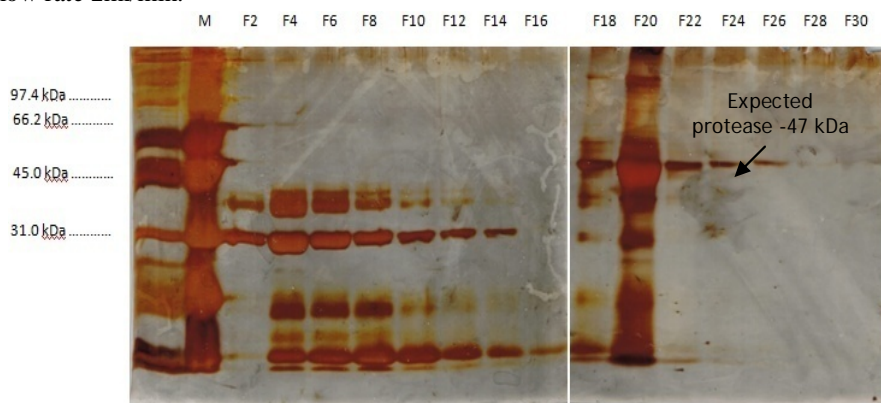


Fig: 2. SDS-PAGE analysis of collected fraction from ion exchange chromatography. M: marker, F2-F10: inactive fraction in first washing, F12-F30: fraction in elution phase, F20 – F26: fraction with high protease activity.

3.2. pH Optimum and pH Stability

The effect of pH on protease activity was carried out using a variety of buffer ranging of pH 3 to pH 9. An optimum pH for protease activity was attained at pH 7 (Figure 3). The pH stability profile reported that the *B. licheniformis* protease was highly stable within the pH values ranged from 6 to 8 and retained about 90 % of its activity after incubation at the pH range for 30 min (Figure 3).

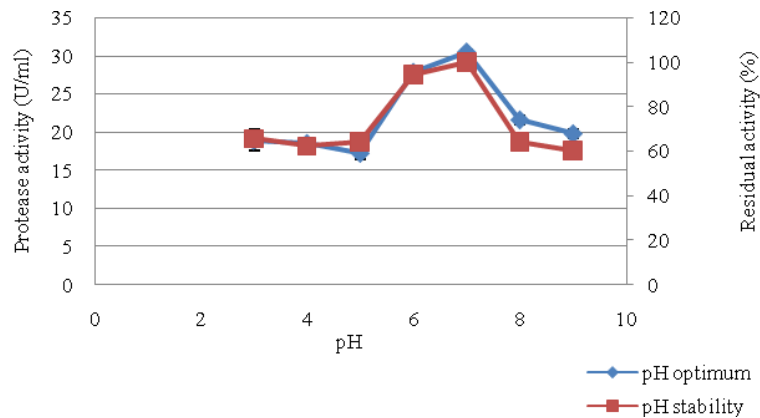


Fig: 3. Effect of pH and stability on the enzyme activity. The maximum activity at pH 7 was taken as a control (100 %), (*value in figure represented as mean \pm SD).

3.3. Temperature Optimum and Thermal Stability on Protease Activity

The effect of different temperature on protease activity was determined from 35 °C to 75 °C. As shown in Figure 4, the *B. licheniformis* protease was active between 35°C to 75 °C with an optimum around 65 °C.

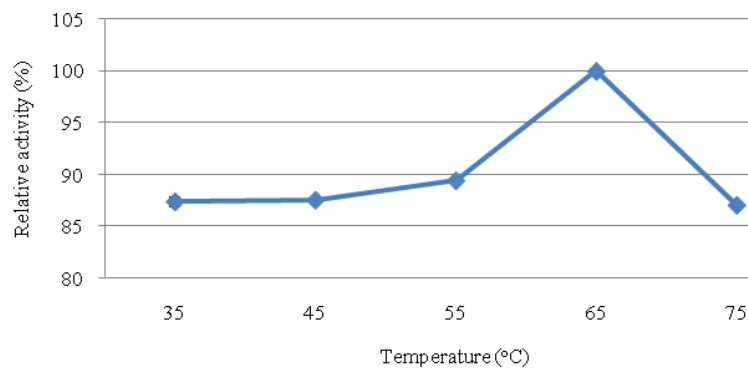


Fig: 4. Effect of temperature on *B. licheniformis* protease activity.

The maximum activity of enzyme at 65 °C was taken as a control (100 %) (*value in figure represented as mean \pm SD). Moreover, the thermal stability of the enzyme was examined by incubating the enzyme without substrate at selected temperature ranging from 35 °C to 65 °C. The thermo stability profile (Figure 5) indicated that the enzyme was stable up to 65 °C and more than 88 % residual activity was maintained even after incubation for 60 min.

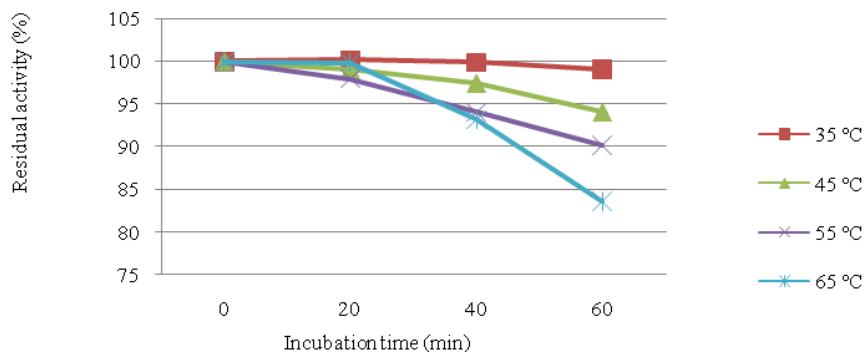


Fig: 5. Effect of temperature on the thermo stability of *B. licheniformis* protease.

3.4. Effect of Metal Ions on Protease Activity

This study investigated the effect of various metal ions including Mn^{2+} , Mg^{2+} , Na^+ , K^+ , Cu^{2+} , Zn^{2+} , and Ca^{2+} . The results are presented in Table 2. Mn^{2+} and Ca^{2+} showed strong activation of the enzyme activity, while Cu^{2+} , Zn^{2+} , and Mg^{2+} showed slightly decrease effect on the proteolytic activity. The present of Na^+ , K^+ ion at concentration of 10 mM increased the activity of the enzyme by 4.86 % and 5.6% respectively.

Metal ions	Concentration	Residual activity (%)
$MnSO_4$	10 mM	176.65 ± 0.781
NaCl	10 mM	104.86 ± 0.655
KCl	10 mM	105.06 ± 0.802
$CuSO_4$	10 mM	95.33 ± 0.964
$ZnSO_4$	10 mM	95.91 ± 0.351
$CaCl_2$	10 mM	119.07 ± 0.05
$MgSO_4$	10 mM	98.64 ± 0.608
Control		100 ± 0.416

(*value in figure represented as mean ± SD).

Table: 2. Effect of various metal ions on *B. licheniformis* protease.

3.5. Effect of Enzyme Inhibitors on Protease Activity

The effect of enzyme inhibitors on protease activity was exhibited in Figure 6.

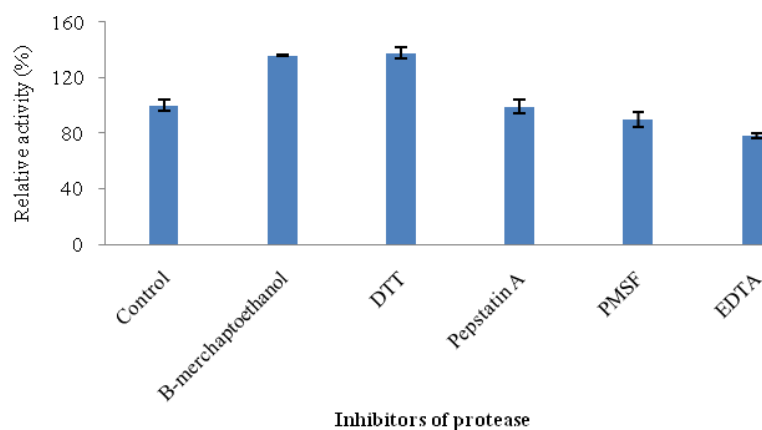


Fig: 6. Effect of various protease inhibitors on the protease activity from *B. licheniformis*.

Among the protease inhibitors studied, EDTA (metallo enzyme inhibitor) at concentration 5 mM revealed an inhibitor effect on protease activity with relative activity of 78.04 %. The study also observed that the activity of enzyme was reduced in the presence of PMSF (serine protease inhibitors) indicating small percentage of serine protease present in the culture supernatant.

3.6. Effect of Solvents on Protease Stability

The effect of various organic solvents on the enzyme stability is summarized in Table 3. The *B. licheniformis* protease was highly stable in the present of surfactants (Triton X100, Tween 20, SDS), organic solvents (acetone, chloroform, hexane and toluene), and oxidizing agent (H_2O_2).

Chemicals	Concentration	Relative activity
SDS	1 % (v/v)	182.76 ± 1.150
Tween 20	1 % (v/v)	97.25 ± 0.208
Triton X-100	1 % (v/v)	97.06 ± 0.1
Hexane	1 % (v/v)	90.51 ± 0.306
Toluena	1 % (v/v)	105.01 ± 4.015
Acetone	1 % (v/v)	84.75 ± 0.436
Cloroform	1 % (v/v)	82.75 ± 0.473
H ₂ O ₂	1 % (v/v)	93.75 ± 0.4
Control*		100 ± 0.306

*Control contains the enzyme with 50 mM Tris buffer at pH 8.

Table: 3. Effect of Detergents and Solvents on Protease Activity.

3.7. Detergent Compatibility

Prior to applying *B. licheniformis* protease in removal blood stain, the potential of this enzyme was examined for its compatibility with commercial detergent formulation. The study was selected Tesco Everyday Value[®] Detergent that does not contain any detergent and the finding was displayed in Figure 7. The enzyme was compatible with commercial detergent as the activity retained 78 % after 30 min incubation at 37 °C.

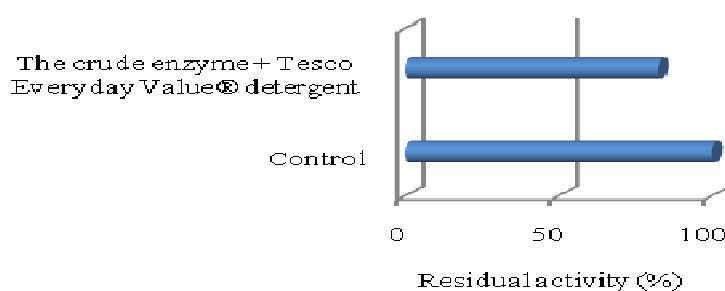


Fig: 7. Compatibility of the protease from *Bacillus licheniformis* with Tesco Everyday Value[®] detergent.

3.8. Washing Performance Analysis

Stain removal ability of crude enzyme was analyzed using cotton cloths stained with blood. The treatment of stained cloth with detergent supplemented with crude enzyme gave a better stain removal over the wash performance employing detergent alone (Figure 8).

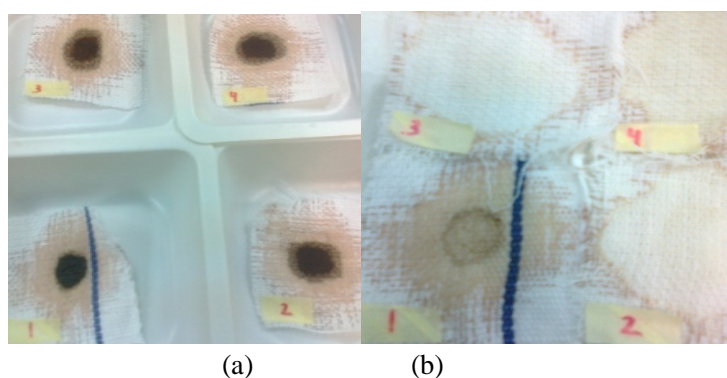


Fig: 8. Application of the enzyme in removal of blood stain.

(a) The stained clothes after drying. (b) The stained clothes after 30 min incubation at 60 °C (1: control, 2: stained cloth + detergent only, 3: stained cloth + detergent + the protease, 4: stained cloth + enzyme only).

[IV] DISCUSSION

Bacillus licheniformis (ATCC 12759), a commercial bacterial is well known to produce extracellular protease enzymes. Its secretion is greatly influenced by medium composition as well as culture condition [9]. In this study, the bacteria were cultured in skim latex serum fortified media as reported in the previous investigation [7]. This is the first report of purification and characterization of protease from *Bacillus licheniformis* that was cultivated in skim latex serum effluent. The study succeeded to purify the protein in a simple purification procedure involving ammonium sulphate precipitation, dialysis and ion exchange chromatography (self-assembled liquid chromatography system). The enzyme activity increase to 2.28 fold of purification compare to the crude enzyme. Assessment of the purified protein by SDS PAGE showed a single band with molecular mass of about 47 kDa. Most of the neutral protease from *Bacillus* strain was reported to have a molecular mass of between 36 and 52 kDa [10, 11, 12, 13, and 14]. The proteolysis activity of protease was monitored at different pH in the range of 3 to 9 and temperature of 35 – 65 °C. The enzyme showed a responsible activity within the pH value ranged from 6 to 8, with optimum activity at pH 7 (Figure 3). Based on the optimum pH, this study categorized that the produced enzyme was extracellular neutral protease [15]. On the other hand, the protease was stable up to 65 °C and more than 88 % residual activity was maintained even after incubation for 60 min. Thermo stability of protease is an important characteristic in industrial enzymes particularly laundry detergent. Researchers [16, 17 and 18] have recommended one criterion that should be fulfilled by protease is active and stable under thermophilic condition (60

°C). Thus, the thermostable enzyme in this study suggested the potential application in laundry detergent.

The stimulatory effect on protease activity was observed in the presence of Mn^{2+} and Ca^{2+} , while inhibitory effects were found in the presence of Cu^{2+} , Zn^{2+} , Mg^{2+} (Table 2), and EDTA (Figure 6). This data might classify that the enzyme was neutral independent metallo protease or metallo protease. Most of metallo protease require zinc ion for catalytic mechanism (contain zinc ion at active site). However, this study observed that the presence of Zn^{2+} weakly inhibited the activity of enzyme. Researchers [19, 20 and 21] reported that metallo protease from *Bacillus cereus* BG1, *B. cereus* WQ9-2, and *Aeromonas veronii* were significantly inhibited by Zn^{2+} and Cu^{2+} . The similar matter with this study could be explained that the excess of zinc in zinc peptidases can inhibit the optimum activity of the enzyme [21]. The stability of the enzyme in the presence of EDTA (78.04 % residual activity) actually is advantageous for utilization of this enzyme as detergent additive since some of detergents included high amount of chelating agents as water softeners [14, 22].

The effect of detergent and solvents on the activity of protease was observed. Generally, more than 82 % the activity was retained in all chemicals tested (surfactants, organic solvents, and oxidizing agent). The stimulatory effect was found in the presence of SDS and toluene by 182.76 % and 105.01 %, respectively. These results are agreement with earlier reports [23, 24, 25]. The stability of this protease towards SDS is significant because SDS stable enzymes are also not generally available except for a few *Bacillus clausii* I-52 and *Bacillus sp* [24]. Sodium Dodecyl Sulphate (SDS) is anionic detergent that has a negatively charge polar head group. It is effective at emulsifying oil, clay soil, and fabric softener residue. Whereas, toluene is non-polar solvent that may minimize, prevent and inhibit crystallization of certain ingredients in the aqueous composition. It is often used in heavy

liquid detergent [26]. Thus, the excellent properties of the enzyme toward SDS and toluene are particularly suitable for solid and liquid laundry detergent [24, 26].

In addition, the *B. licheniformis* protease showed compatibility with Tesco Everyday Value[®] detergent as the activity retained about 80% after 30 min incubation at 37 °C. Researcher [27] reported that *Bacillus cereus* that showed detrimental effect to EDTA and MnCl₂ was stable for 3 h at 50 °C with residual activity more than 60 % in the presence of local detergent such as Ariel[®], Persil[®], Bonex[®] and Sunlight[®].

Furthermore, combination of the enzyme with Tesco Everyday Value[®] detergent in washing performance significantly improved the cleansing of the blood as shown in Figure 8.

[V] CONCLUSION

The compatibility of the characterized protease with surfactants, solvents, oxidizing agent, and commercial detergent confirmed its potential application in laundry industry. It was supported by the ability of enzyme to degrade proteinaceous material in the stains completely with supplementation of the detergent as shown in wash performance test (Figure 8). The protease was also stable at temperature range of 35 °C to 65 °C and at pH 6 and 7 for 60 min. Thus, the produced enzyme could be potentially used for recovering high quality and value added product from skim latex serum effluent.

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