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Production and application of thermostable protease 50a as liquid protein stain remover

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Abstract. Proteases are important enzymes involved in many vital physiological processes and has wide potential for industrial applications. Previously, thermostable alkaline protease 50a was reported and had potential as laundry detergent additive and eco-friendly enzymatic dehairing of animal hides. Thus, exploration of this enzyme potentials' need to be done in order to prove that it plays an important role in diverse industrial application. This study aimed to investigate the potential of the thermostable alkaline protease 50a as a protein stain remover. One formulation has been developed as liquid spray protein stain remover containing the enzyme and tested its ability to remove protein stain through washing performance. Four different types of fabric which were jersey, cotton, koshibo and crepe has been stained with blood as the protein source. Blood stain on the fabrics were then removed with four different solution; using distilled water only, distilled water with enzyme, formulated liquid spray with enzyme, and formulated liquid spray without enzyme. The formulated liquid spray with enzyme exhibited better removal bloodstains action by showing faintness of the blood stained on the fabrics after it was sprayed. The stains removal evaluation on different types of fabric with formulated liquid spray protein stain remover obtained are as followed order: crepe > jersey > cotton > koshibo. This study proved that the formulated liquid spray with addition of enzyme showed the most effective stain removal on crepe fabric.

1. Introduction

Proteases are enzymes that catalyse hydrolytic reactions in which protein molecules are degraded to peptides and amino acids [1]. They also digest proteinaceous stains such as keratin, blood, milk and gravy on fabrics [2]. From this reason, the use of protease, especially alkaline proteases in the detergent formulation is the most successful application. At the same time, the search for new sources of protease has never stopped [3,4]. Plants, animals and microorganisms are the sources of proteases. Among these sources, microbial proteases, especially from *Bacillus* sp. are the most preferred for commercial application due to large scale production with high enzymatic activity [5,6]. Microorganism can grow rapidly and can be genetically manipulated to generate new enzymes with desirable altered properties for various commercial applications [7]. Microbial proteases can be categorization into various classes, depending on the pH value and characteristics of the active site. Based on their pH optimum, microbial protease classified as acidic, neutral, and alkaline [8]. Among various types of microbial protease, alkaline serine proteases are preferred because they are generally active from neutral to alkaline pH [9, 10].

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Referring to the newly isolated bacteria from hot spring Lojing Highlands, Kelantan, Malaysia which is identified as *Bacillus subtilis* 50a and also classified as an alkaline protease and thermostable enzyme, it is really suitable for the production of detergent additive [11]. However, the use of wild strains produces limited quantities of desired proteases to be used in commercial application. Therefore, recombinant gene technology is applied in order to produce higher protease activity and increase the thermal stability of the strains towards enzyme inactivation. The improvements of thermal stability and proteases yield from this recombinant bacterial strain *E. coli* BL21 (DE3) pLysS harbouring 50a protease gene through molecular approach have led to the suitability towards the commercial and industrial requirement of protease. Thus this study aimed to investigate the potential of the thermostable alkaline protease 50a as a protein stain remover which added in formulation and tested its effectiveness through washing performance on four type of fabrics.

2. Methodology

2.1. Bacterial strain and production of thermostable alkaline protease 50a
A recombinant bacterium, *E. coli* strain BL21 (DE3) pLysS harbouring protease 50a gene that have been used in this study is capable of producing thermostable alkaline protease 50a. The wild type of this enzyme was previously isolated from Lojing Hotspring, Kelantan, Malaysia [11]. The thermostable alkaline protease 50a was produced in the medium containing 5.0 g/L of sorbitol, 20 g/L of tryptone, 2.0 g/L of calcium chloride and incubated at 30ºC, 240 rpm of agitation speed and 24 hour fermentation time after induction by 0.5 mM of isopropyl B-D- thiogalactopyranoside (IPTG). The cell was harvested by centrifugation at 10 000 rpm for 10 min. Then it was sonicated and supernatant was collected. The supernatant act as crude enzyme and was purified using heat treatment at 70ºC for 3 hours.

2.2. Determination of protease assay and protein content
Azocasein (0.5%) was prepared freshly and dissolved in 0.1 M Tris - 2.0 mM $CaCl_2$ - HCl buffer (pH 9). 0.1 ml of crude and purified enzyme were added into 1 mL of azocasein. Then they were incubated at 80˚C for 30 minutes. 10% of TCA was added and leaved at room temperature for 30 minutes. The samples were centrifuged for 10 minutes at 13 000 rpm. 1 ml of 1M NaOH was added in 1 ml of supernatant. Then it was read at 450nm. Protein content was measured spectrophotometrically at Abs 595 nm according to the Bradford method [12]. Bovine serum albumin (BSA) was used as a standard protein to construct standard curve.

2.3. Liquid spray protein stain remover formulation and its performance
Liquid spray protein stain remover formulation containing purified thermostable alkaline protease 50a was designed based on the formulation as described in Table 1. PEG 400 was heated until melt. Sodium carbonate was prepared at room temperature. Final concentration of thermostable alkaline protease 50a at 3 U/mg has been used in this formulation. Then, all solution were mixed at 400 rpm for 5 min at room temperature. Water was topped up to make a final solution of 100 mL.

Table 1. Ingredients for preparing liquid spray protein stain remover formulation.

Ingredients
PEG 4000
Lavender oil
Thermostable alkaline protease 50a
Sodium carbonate
Tween 80
Distilled water

IOP Conf. Series: Earth and Environmental Science **596** (2020) 012012 doi:10.1088/1755-1315/596/1/012012

Aside from the formulated liquid spray with addition of the enzyme, three other solution was used as controls to compare the washing performance. They were distilled water, distilled water with enzyme added, and formulated liquid spray protein stain remover without the enzyme.

Fabrics namely crepe, jersey, cotton and koshibo were used in this study. 100 µL of blood sample was stained on clean fabrics pieces (5 x 5 cm), dried at 60ºC for overnight and kept at room temperature for 1 month. The formulated liquid spray protein stain remover and controls were sprayed 3 times (total volume 300 µL) on each stained cloth with the distance of 5 cm. Then they were rubbed for several times. Treated stained fabrics were measured the efficiency of stain remover by using colour meter. Colour of the blood stained were measured before and after been sprayed with liquid remover and were expressed as follow:

Stain removed = (W1-W2)/W1 x 100%:

W1= colour intensity before treatment
W2= colour intensity after treatment

3. Results and Discussion

3.1. Production of protease

Proteases are one of the standard ingredients in most of enzymatic detergent for over 60 years due to its capability to release of proteinaceous materials in stains such as those of milk, blood, grass, body fluids and food soils [13]. At the same time, proteases which contribute approximately 60- 65% of the total worldwide sale of enzymes in 1998 and in 2015, alkaline protease market size accounted for over 55% of total enzyme market share [14]. Thus, the search for novel detergent proteases has never stopped where is the *Bacillus subtilis* 50a was isolated and able to produce thermostable alkaline protease [11]. Now, the recombinant *E. coli* BL21 (DE3) pLysS act a host to carry the 50a protease gene has been utilised for production of thermostable alkaline protease 50a. Single step purification has been developed to purify the enzyme. Single step purification by using heat treatment is a strategy to obtain a yield with the highest catalytic activity of the enzyme. This purification step allowed the 50a protease enzyme to be correctly folded at the same time denaturing the mesophilic *E. coli* protein so that the only left protein was purely derived from the protease 50a gene. Table 2 showed purification summary of thermostable protease 50a. Specific activity has been set as concentration of the purified enzyme in formulation of protein stain remover.

Table 2. Purification summary of thermostable alkaline protease 50a

Method	Volume (ml)	Total activity (mg)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude	35	1778.84	2382.66	0.75	100	1
Heat treatment	35	3550.43	619.23	5.73	199.58	7.5

3.2. Liquid spray protein stain remover formulation and its performance

Four different type of fabrics: crepe, jersey, cotton and koshibo were chosen in this study and stained with 100 µL of blood. The blood spreaded rapidly across the surface of the crepe fabric compared to the other fabrics because crepe fabric is thinner than the other fabrics [15]. The bending in the presence of blood stained on fabric is also subjected to surface roughness. It may act as a sift for the particles to pass through. A softer surface allows a better spreading of blood on the fabrics [16]. The ability of blood stain to be removed were also can be influences by these factors. Based on Figure 1 the blood stain on the crepe

IOP Conf. Series: Earth and Environmental Science **596** (2020) 012012 doi:10.1088/1755-1315/596/1/012012

fabrics sprayed with formulated liquid spray with enzyme were almost invisible, suggesting that it was the easiest fabrics to be clean followed by jersey, cotton, and koshibo.

The blood-stained fabrics has been sprayed with four different solutions which were distilled water, distilled water added with 50a enzyme, formulated liquid spray with 50a enzyme, and formulated liquid spray without 50a enzyme. Based on Figure 1 and Figure 2, formulated liquid spray with 50a enzyme showed the best performance as the protein stain remover since the blood stain on the fabrics especially on crepe were mostly removed and represents the highest percentage of blood stain removed. In general, enzyme detergents remove protein from clothes stained with blood far more effectively than non-enzyme detergent [17]. The addition of enzyme such as protease in detergent formulation is effective in removing proteinaceous stained since it can breakdown the protein into smaller polypeptides and make it easier to be removed.

A fresh proteinaceous stain on fabric or hard surface can be removed simply by cold water [18]. However, when the proteinaceous stain is dried, aged, or heated, it becomes more difficult to be remove even with surfactant because it coagulates and hinders the penetration of the cleaning liquor. In addition, protein residues may be oxidized and denatured due to the presence of oxygen, so the blood-stained becomes permanent [19]. Proteins are present in small amounts and are not completely removed by surfactants and bleaching systems [16]. Surfactants improve the wetting ability of water, loosen and remove stains and emulsify, solubilize or suspended stains in the wash [20]. The surfactants used approximately 15% to 40% of the total detergent formulation and account over half of its use in laundry detergents, household and personal care products [21]. Nevertheless, the addition of surfactant without the enzymes are not effective in removing protein stain. Thus, the addition of enzymes in the formulation are crucial in removing protein stain as they can digest the stain and make it easier to be removed. In this study, the total amount of surfactants used in liquid spray protein stain remover was around 20% of the whole ingredients which is less than ones normally used in the commercial detergent formulation. Even with lower volume of surfactants, the liquid spray was able to remove the blood stain effectively, suggesting that the addition of protease 50a facilitated in removing the stain.

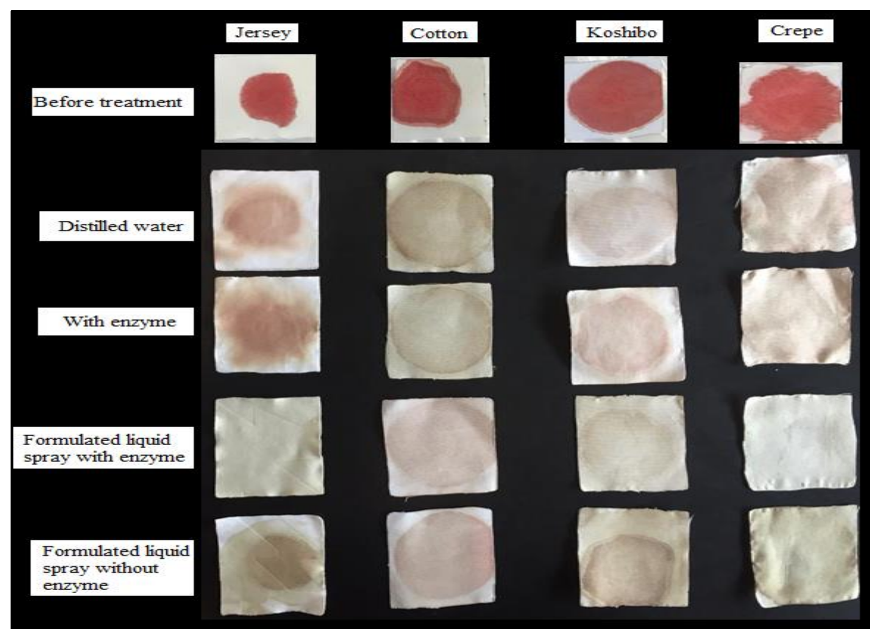

Figure 1. Comparison between four types of fabrics before and after treatment using different formulations liquid spray formulation

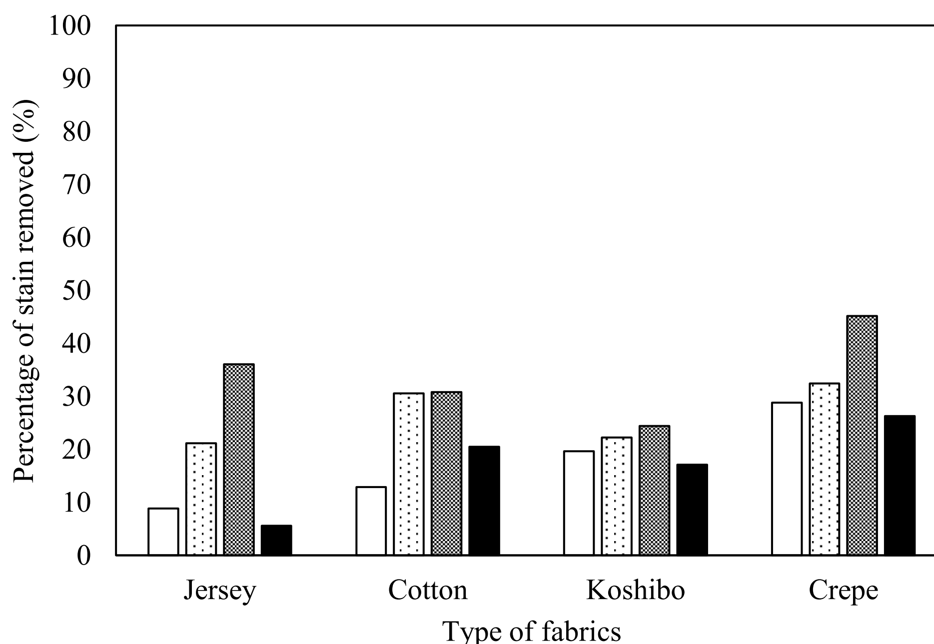

Figure 2. Percentage of stain removed using ☐ distilled water, ☐ with enzyme, ▩ formulated liquid spray with enzyme and ■ formulated liquid spray without enzyme onto jersey, cotton, koshibo and crepe fabric.

4. Conclusion

This study demonstrates the production of the thermostable alkaline protease 50a and its application in the formulation of liquid spray protein remover. The findings revealed that this enzyme offers a promising potential as additive in the formulation as evidenced by its performance in removing blood stain on four different fabrics. Further study could be performed in enhancing the formulation of the liquid spray protein remover to improve the efficiency of the washing performance. In addition, the application of the formulated liquid spray could be further extended on other fabrics to access the washing performance.

References

References

[1] Rathakrishnan P, Nagarajan P and Kannan R 2011 *Chem. Ind. & Chem. Eng. Q. (Online)* **17** 215-22
[2] Saeki K, Ozaki K, Kobayashi T and Ito S 2007 *J. Biosci. Bioeng.* **103** 501-8
[3] Romdhane I, Fendri A, Gargouri Y, Gargouri A and Belghith H 2010 *Biochem. Eng. J.* **53** 112-20
[4] Jellouli K, Ghorbel-Bellaaj O, Ayed H, Manni L, Agrebi R and Nasri M 2011 *Process Biochem.* **46** 1248-56
[5] Yang Y, Jiang L, Zhu L, Wu Y and Yang S 2000 E. *J. Biotechnol* **81** 113–18
[6] Beg Q, Saxena R and Gupta R 2002 *Biotechnol. Bioeng***.** **78** 289–95
[7] Kuhad R, Gupta R and Singh A 2011 *Enzyme Res* 1- 10
[8] Sumantha A, Larroche C and Pandey A 2006 *Food Technol. Biotechnol.* **44** 211-20
[9] Gupta R, Beg Q and Lorenz P 2002 *Appl. Microbiol. Biotechnol.* **59** 15-32
[10] Singhal P, Nigam V and Vidyarthi A 2012 *Int. J. Adv. Biotechnol. Res.* **3** 653–69
[11] Ibrahim, N and Yusoff N 2013 *Res. J. Biol. Sci.* **2** 29-33
[12] Bradford M 1976 *Anal. Biochem*., **72** 248-54
[13] Kumar D, Savitri, Thakur N, Verma R and Bhalla T 2008 *Int. Res. J. Microbiol*. **3** 661-672

IOP Conf. Series: Earth and Environmental Science **596** (2020) 012012 doi:10.1088/1755-1315/596/1/012012

[14] Global Market Insight (2016, November 16) Protease Market Size, Industry Analysis Report, Regional Outlook, Application Development Potential, Price Trends, Competitive Market Share and Forecast, 2020-2026. Retrieved from https://gminsights.com/industry-analysis/proteases-market
[15] Wu J, Michielsen S and Baby R 2018 *J. Forensic Sci.* **64** 1-9
[16] Miles H, Morgan R and Millington J 2014 *Sci. Justice* **54** 262-66
[17] Sakpal H and Narayan G 2015 *J. Pharm. & Biol. Sci.***10** 58–67
[18] Castro T, Taylor M, Kieser J, Carr D and Duncan W 2015 *Forensic Sci. Int.* **250** 98–109
[19] Mushtaq S, Rasool N and Firiyal S 2015 *Aust J Forensic Sci* **48** 1-8
[20] Bajpai D and Tyagi V 2007 *J. Oleo Sci*. **56** 327-40
[21] Scheibel J 2004 *J Surfactants Deterg* **7** 319-328