DECLARATION

I hereby declare that work embodies in this Report is the result of the original research except for the quotations and citations which have been duly acknowledge. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at University Malaysia Kelantan or at any other institutions.

_____

Student

Name :

Date :

I certify that the Report of this final year project entitled "EFFECT OF TAPZYME 50A FORMULATION ON DIFFERENT FABRICS IN DETERGENT SPRAY APPLICATION" by NOR SHAF REENA BINTI LIZAWARDI, matric number F15B0117 has been examined and all the corrections recommended by examiners have been done for the degree of Bachelor Applied Science (Bioindustrial Technology) with Honors, Faculty of Bioengineering and Technology, Universiti Malaysia Kelantan.

Approved by:

_____

Supervisor

Name :

Date :

ACKNOWLEDGEMENT

First and foremost, I would like to express my deepest appreciation to all those who provided me the possibility to complete this report. A special gratitude I give to my supervisor, Dr. Syed Muhammad Al-Amsyar Bin Syed Abd Kadir, Lecturer of Faculty Agro Based Industry at the University Malaysia Kelantan whose contribution in stimulating suggestions and encouragement, helped me to coordinate my project. Without her valuable comment and patience, this project definitely could not be completed successfully.

Next, I would like to thank and appreciation for my parents especially my mother, Siti Sahara Binti Nordin for her encouragement and uncountable support during my study. My grateful appreciation also for my fellow friends in Bioindustrial Technology programme including Nurul Fayyadhah Insyirah Binti Fauzi and Siti Zuriah Binti Zakaria for their great help throughout this project.

Finally, I would like to express my thanks to the UMK laboratory assistans, for their help and guidance especially in preparing the chemicals and operating the laboratory equipment while conducting this project. Last but not least, I would like to thank my friends for giving their co-operation and providing supportive atmosphere throughout the journey of this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

Abs	absorbance
BSA	Bovine Serum Albumin
$CaCl_2.2H_2O$	calcium chloride
IPTG	isopropyl-β-D-thiogalactopyronoside
LB-SMA	Luria Bertani - skimmed milk
NaCl	sodium chloride
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine
Tris-HCl	trisaminomethane hydrochloride

LIST OF SYMBOLS

pH	potential Hydrogen
˚C	degree Celcius
g	gram
mg	milligram
ml	millilitre
v/v%	volume per volume percent
U	Unit activity of enzyme
U/ml	Unit activity of enzyme per millilitre
w/v%	weight per volume percent
µg	microgram
rpm	revolution per minute
nm	nanometer
OD	optical density
min	minute
cm	centimetre
mM	millimolar
M	molar

Kesan Formulasi TAPzyme 50a Pada Fabrik Berbeza Dalam Aplikasi Detergen Semburan

ABSTRAK

Kajian ini melibatkan protease dari *E. Coli* BL21 (DE3) pLysS yang melindungi gen protease 50a, detergen semburan dan analisis prestasi keberkesanannya. Protease adalah enzim penting yang terlibat dalam banyak proses fisiologi penting dan mempunyai potensi luas untuk aplikasi perindustrian. Empat jenis semburan yang dilabelkan iaitu air suling, TAPzyme 50a, detergen dirumuskan semburan dan semburan dirumuskan tanpa TAPzyme 50a yang mempunyai pelbagai cara telah dirumuskan melalui kajian ini. Ujian penyingkiran kotoran darah telah dilakukan pada pelbagai jenis fabrik iaitu jersey, cotton, koshibo dan crepe. Percubaan ini telah membuktikan bahawa dengan penambahan TAPzyme 50a dengan detergen yang dirumuskan menunjukkan keberkesanan yang paling berkesan untuk kain crepe. Semasa kajian ini, darah manusia telah digunakan pada pelbagai jenis kain untuk melihat keberkesanan yang paling berkesan dengan menggunakan cara penyingkiran yang berbeza. Darah adalah campuran lengkap enzim, protein, sel dan bahan bukan organik. Ianya sesuai untuk kajian ini yang menggunakan TAPzyme 50a sebagai bahan dengan gabungan semburan dirumuskan semburan. Darah diatas kain telah dikeluarkan dengan air suling, TAPzyme 50a, semburan dirumuskan detergen dan semburan dirumuskan detergen tanpa TAPzyme 50a.Gabungan TAPzyme 50a dengan detergen yang dirumuskan menunjukkan tindakan penyingkiran darah yang lebih baik dengan memperlihatkan kepanikan kain crepe. Penilaian penyingkiran kotoran darah pada berbagai jenis kain dengan detergen yang dirumuskan semburan yang diperolehi adalah sebagai berikut: (Crepe > Jersey > Cotton > Koshibo), menunjukkan potensi aplikasi TAPzyme 50a dalam aplikasi detergen semburan. Kecerahan kain selepas penyingkiran prestasi keberkesanan diukur menggunakan kolorimeter.

Kata kunci: Enzim, Protease, Detergen Semburan, TAPzyme 50a

Effect Of TAPzyme 50a Formulation On Different Fabrics In Detergent Spray Application

ABSTRACT

This study involves protease from *E. Coli* BL21 (DE3) pLysS harbouring 50a protease gene, spray formulation detergent and its effectiveness performance analysis. Proteases are important enzymes that involved in many vital physiological processes and has wide potential for industrial applications. Four types of spray labelled namely distilled water, TAPzyme 50a, spray detergent and spray detergent without TAPzyme 50a. Stain removal bloodstains test were carried out on the different types of fabrics which are jersey, cotton, koshibo and crepe. This experiment had proved that with addition of TAPzyme 50a with the formulated detergent showed the most effectiveness to crepe fabric. During this study, the human bloods had used on different types of fabric to observe the most effectiveness by using different way of removal performance. Blood is a complete mixture of enzymes, proteins, cells and inorganic substances. It is suitable for this study that used TAPzyme 50a as a ingredient with combination of spray detergent. Bloostained on the fabrics were removed with distilled water, TAPzyme 50a, spray detergent and spray detergent without TAPzyme 50a. The combination of TAPzyme 50a with formulated detergent exhibited better removal bloodstains action by showing faintness of crepe fabric. The stains removal evaluation on different types of fabric with spray detergent obtained are as followed order: (Crepe > Jersey > Cotton > Koshibo), showed a great potential application of TAPzyme 50a in spray detergents application. The lightness of the fabrics after removal performance was measured by colorimeter.

Keywords: Enzyme, Protease, Spray Detergent, TAPzyme 50a

CHAPTER 1

INTRODUCTION

1.1 Background of study

In recent past, enzymes one of the important phenomenal in laundry detergent formulations. Enzymes are proteins that have catalytic functions necessary for the maintenance of life and activities that give certain biochemical reactions. Each of the enzymes works in a different environment as it changed in their properties. In living organisms all chemical reactions that occur depend on the catalytic action of enzymes. One example of the enzyme is protease. Proteases that are used for catalyze hydrolytic reactions whereby the protein particles are debased to peptides and amino acids.

In industrial enzymes, protease are the most important which contribute closely 40% of the total industrial market (Gupta *et al.,* 2002). According to Jonsson and Martin, (1965) that contemplated numerous parasitic societies for protease generation has been uncovered the media used and strain can influence the amount of proteases produced. Besides that, based on (Sumantha *et al*., 2006) proteases is essential gathering of chemicals created industrially in cleanser, protein, cowhide, meat and dairy ventures. Proteases generation can be found from various sources, for example, plants, creatures, and microorganisms. Among these sources, microbial proteases from genus Bacillus are the most general exploited industrial enzymes with major application in formulation of detergent (Godfrey *et al.,* 2001). Microbial proteases depend on the pH value and

characteristics of the active site. According to (Sumantha *et al*., 2006), microbial protease classified as acidic, neutral, and alkaline based on their pH optimum. The microbial proteases are subdivided into several groups which are serine protease, cysteine protease, aspartic protease, and metalloprotease (Kumar *et al.,* 2008) as per their side chain explicitness and useful gathering present at the dynamic site. Generally, alkaline serine proteases are preferred among various types of microbial protease because they are fundamentally dynamic from impartial to basic pH (Gupta *et al.,* 2002) that also digest proteinaceous stains which are blood, milk, keratin and gravy on fabrics (Kumar *et al.,* 2008).

Additionally, basic protease use can be found from various sources, for example, certain creepy crawlies, microbes, and organisms that relies upon different variables (Sellami-Kamoun *et al.,* 2008). Enzymes were produced from various *Bacillus sp.* from the beginning such as B. brevis (Banerjee *et al.*, 1999), *Bacillus sp.* SSR1 (Singh *et al*., 2001), B. *cereus* (Banik *et al.,* 2004), B. *licheniformis* RP1 (Sellami-Kamoun *et al.,* 2008), B. *licheniformis* U1 (Pravin *et al.,* 2014) have been recorded their usage in laundry detergent formulations. Formulation 'detergents' is the term applied to materials and products that offer the functions such as advance expulsion of material from a surface, scatter and settle materials in a mass framework according to Showell (2006) in Handbook of Detergents Part D. A detergent is an agent that use for cleansing purpose which somehow a synthetic substance other that soap was stated by (Dhakite *et al*., 2011).

Alkaline protease in detergent formulation is the most greatly application among all industry known as the largest market for using industrial enzyme (Saeki *et al*., 2007). Detergent are commonly use in daily life such as powder or liquids. Chemical that have been to use as a cleanser added substances ought to have the characteristics which are it ought to have a soluble pH and it ought to likewise be perfect with cleansers. (Anstrup *et*

al., 1984) the real utilization of cleanser perfect proteases is in clothing cleanser plans. Nowadays, many detergents were commercially in the international market which produced by the genus Bacillus for example, Dynamo ®, Era in addition to ®, (Procter and Gamble), Tide ® (Colgate Palmolive) that have contain proteolytic proteins. Just serine protease finds their application in cleanser plan (Kumar *et al*., 2008). Six groups of components of laundry detergent which are surfactants, manufacturers, catalysts, fading specialists, fillers and other minor added substances, for example, scattering operators, texture softening dirt, optical brighteners and color exchange hindering fixings (Kumar *et al.,* 2008) that depend on its function.

Alkaline protease-based detergent to enhance the cleaning ability of detergents and it also provide better cleaning properties and safe dirt removal. Therefore, process of cleaning depends on sort of stain, the organization of cleanser and the idea of the textures material utilized in the washing test. From that, this is perspectives in the assessment of catalyst execution. The detergent that consist enzymes have better cleaning characteristics as compared to synthetic detergents. Besides that, they are environment friendly and active at low washing temperature (Kumar *et al*., 1998) improve the fabric quality and keeping color bright (Hasan *et al.*, 2010).

1.2 Problem Statement

Due to the increasing industrial needs of protease, especially in industry such as processing of detergents, pharmaceuticals, food processing and production of leather nowadays led to the research focused on purification and characterization of protease production to support the increasing demand. According to (Singh *et al*., 2011) indicate

that the major target for commercial application is to reduce the cost of enzyme production and making the production economically feasible.

Protease production is the most widely used in detergent formulation that complex process with a significant relation to specific demands of the customers, economics, environmental concern and the availability of specific 'actives' for achieving the preferred functionality (Showell, 2006). Therefore, according to (Klein *et al.,* 2012) indicate the cost of producing enzymes is expensive and not efficient. There are a lot and various formulations for laundry formulations for laundry detergents despite that all of them carry out the same basic functions (Ainsworth, 1994). With there a lot of research of detergent formulations should follow the previous research to get the best of formulations that take time to stability in the formulation of an enzymatic detergent (Mabrouk *et al*., 2003) as well as to formulate an eco-friendly detergent formulation.

This study is focusing on the formulation of enzyme consist the protease known as TAPzyme 50a and studying the effectiveness of the formulated detergents through wash performance on different fabrics (ability to remove the stains). As the previous research the formulations of enzyme showed the different result in the different fabrics to remove the dirt that comes in many forms and it also takes time to remove or to clean the fabrics from stains. Besides, the use of protease in detergents has become the key ingredients instead of being minor additives (Nascimento and Martins, 2006). For the industrial application of detergents can reduce or even replace the usage of harmful chemicals in laundry detergents. In order to explore the application of protease in industrial application, influences of the protease activity on temperature, and pH should be investigate.

1.3 Objectives

The objective of this study is to investigate the formulation of enzymes for industrial detergent. This research was carried out with the following objectives:

i. To synthesize the TAPzyme 50a and spray formulation in detergent application.

ii. To identify the effectiveness of TAPzyme 50a formulation based on performance removing test bloodstains on different types of fabrics

1.4 Scope of Study

TAPzyme 50a production was measured in the optimum production medium from the potential bacteria strain *E.coli* BL21 (DE3) *pLsS* harbouring 50a protease was studied in this research. This research was conducted to fulfil the need of protease that has special characteristics of high yield, stable at high temperature and wide range of pH, in order to solve the limited yield of protease to fill the gap of the global demand of detergent application of using TAPzyme 50a. The alkaline protease from recombinant 50a strain has yet to be investigated in method which is heat treatment.

After obtaining the purified enzyme, the detergent formulation of ingredients for preparing detergent formulation were identify. The washing performed analysis were perform by using different fabrics pieces with different stains. Four different types of fabrics which are jersey, cotton, koshibo and crepe were used.

1.5 Significance of Study

Bacterial protease have been extensively studied due to its wide industrial application for example, cleansers, sustenances, pharmaceuticals, and others. Among acidic and neutral proteases, alkaline protease that produced by microorganism is the principle perspective from biotechnological point of view, and are examined not just in logical fields of protein science, protein designing yet additionally in connected fields (Jayasree *et al.*, 2011). Alkaline protease are stable, can resist and active under extreme condition such as in high pH and high temperature that are convenient for industrial application.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction of Enzymes

Enzymes are capable to change a specific compound into another at a high reaction rate. Enzyme is the agent that speeds up the reaction while not being modified within the process that only bind to specific molecules (substrate), with the basic mechanism by binding the substrate to the active site on the enzyme. Furthermore, the active site is the specific region of the enzymes that combines with the substrates which is undergoes a chemical reaction. When the substrate bind to the active site of enzyme, amino acids from the enzymes react with the substrate.

Products that can be used by the organism while the reaction changes the substrate. When the reaction is complete, the products leave active site of enzyme. The enzyme is unchanged by the reaction and can move on to combine with more substrate molecules (Sandhya *et al.,* 2006). In detergent industry, they have three types of enzymes that always been used such as lipase, amylase, cellulose. The protease enzyme hydrolysis reaction that remove the stains by acting on the proteins stains such as blood, ketchup and dairy products by breaking them into very simple units called peptide (Chaudhari, 2013). In laundry detergents manufactures, they have two types of detergent which are powders and liquids where in liquid detergent the enzyme stability

to prevent the degrading others enzymes that stable in the absence of water. Based on the previous research of liquid detergent, the suitability of an enzyme to be used which is the detergent at a high temperature because some characteristics of an ideal detergent enzyme are stable and active in the detergent solution and effective in a broad range of washing temperatures. Thermostable enzymes are stable and active at higher pH and temperature while made them a suitable candidate for laundry application (Gupta *et al.,* 2002). Thermostable protease also are useful in some industrial applications as stable under higher processing temperature can be used, with the result that the reaction rate is faster (Sellami-Kamoun *et al*., 2008).

Based on the enzyme application, industrial enzymes can be dividing into four major sorts such as food, feed, detergent and technical enzymes. Enzymes are very complex protein and their high degree of specificity as catalyst is display only in their native state. Specific conditions of pH, temperature, inhibitors and metal ions are required to fulfill the native confirmation. Thermostable proteases are useful in some industrial applications as stable under higher processing temperature can be used, with the result that the reaction rate is faster (Zhu *et al*., 2006). Protease secret from thermophilic bacteria with a particular interest and it has become increasingly useful in a variety of commercial applications.

Protease are useful and important components in industry, due to the increasing industrial demands, continuous research and development being continuous to focused on the search of which purification process that can meet need in commercial application and in order to solve the limited yield of protease to fill the gap of the global demand of protease. Protease has been considered as environmentally friendly as enzyme producers are suitable for commercial exploitation is non-toxic and non-pathogenic.

2.2 Sources of Protease

Protease can be found in plant, animals and microorganism. The most familiar protease in animal protease are trypsin, chymotrypsin, pepsin and renin. Chymotrypsin and trypsin enzyme was originally extracted from the pancreas while renin from calf stomach. Trypsin is the principle intestinal stomach related catalyst in charge of the hydrolysis of nourishment proteins. According to (Illanes, 2008) pointed out in his review that, pancreatin is a multi-enzyme that has been used traditionally in the tanning industry and as a digestive aid has been extracted from animal pancreas containing proteolytic, lipolytic and amylolytic activities.

Protease from plant sources has use in food industries and pharmaceutical applicationsa. According to (Tripathi *et al*., 2011) reported that most of the plant proteases have been delegated cysteine proteases, which are broadly utilized in a few procedures of nourishment industry. Papain and bromelain are two major plant proteases that important in the food industry (Nafi *et al*., 2013). Papain is known as cysteine isolated from the fruit of papaya. While, bromelain found in the juice of the stem and fruit of pineapple.

The most important source of protease enzymes compared to animal and plant are microorganisms (Banerjee *et al*, 1999). Microorganisms are selected for flexible proteolytic enzyme source. According to Krishna *et al*., (2009) microbial enzymes are versatile and easy to propagate on a large scale in short time by solid state or submerged fermentation. For various industries application, microbial proteases will grow rapidly and might be hereditarily controlled to produce new chemicals with a few properties changes that are alluring for applications (Aehle, 2007).

The examples of the microbial protease are bacteria and fungi. Bacteria are the most commercial proteases, mainly neutral and alkaline, are produced by organisms

belonging to the genus *Bacillus*. Bacterial neutral proteases are active in a narrow pH range (pH 5 to 8) and have relatively low thermo tolerance. While, bacterial alkaline proteases are characterized by their high activity at alkaline pH. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry.

Thus, although proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications.

2.3 Proteolytic enzyme

Proteolysis is a process of breaking down proteins into simpler compounds under the aid of proteases. Proteolysis breaks the polypeptide bond that link amino acids together. Proteases have evolved multiple times, and different catalytic mechanisms. Proteases can be found in animals, plants, fungi and bacteria. Protease is divided into three categories which are acidic, neutral or alkaline conditions.

Acidic protease usually found in mould, yeast, animal cells and rarely in bacteria. Acidic protease is enzyme that inactive at pH above 6.0 and exhibits maximum activity and stability in acid conditions (pH 2.0–5.0). This is because acid proteases have a low in basic amino acids and low in isoelectric point. Two types are widely used in the food and beverage industries which are from *Aspergillus*, which resemble pepsin and those from *Mucor*, which resemble rennin.

Neutral proteases are produced by most of the bacteria and fungi. According to Siddalingeshwara *et al*., (2010) neutral proteases were active in small range of pH and temperature and less fixed than alkaline protease. While (Sumantha *et al*., 2006) indicated that fungal neutral proteases are important to commercial fungal protease preparation which has various industries likes in food processing, protein modification, baking, animal feeds and pharmaceutical industries. This is because neutral proteases provide a high peptidase activity. One example of neutral fungal protease is *Aspergillus oryzae* (Sumantha *et al*., 2006).

Alkaline protease also can be obtained from fungi and bacteria. It best works optimally at pH 8 to 11. Alkaline protein can be obtained from *Bacillus* strain, *Streptomyces* strain and fungi strain. According to (Abidi *et al*., 2008) indicated that because of alkaline protease are stable, can resist and active under obsessive condition such as in high pH, high temperature, and in the presence of surfactant and oxidizing agents, they are suitable for industrial application. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry, leather processing, and food processing.

2.4 Classification of protease

There are two major groups of protease which are exopeptidases and endopeptidases. Exopeptidases is the ability to degrade peptide chain from their ends while endopeptidases is cleave internal peptides bonds (Sumantha *et al.,* 2006) also indicated that endopeptidase more important than to exopeptidase in industry. Endopeptidase have four groups which are serine protease, aspartic proteases, cysteine proteases and metalloproteases (Gupta *et al.,* 2002).

2.4.1 Serine proteases

The characterization of serine proteases are characterized by the presence of serine residue in their active site, usually a synergist set of three of serine, aspartate and histidine (Stoner et al., 2004). The triad is located in the active site of the enzyme, where catalysis occurs, and is preserved in all serine protease enzymes. According to (Kumar *et al*., 2002) indicated that these proteases are basically active from neutral to alkaline pH, with optimum pH between 7 to 11 and temperature between 50 to 70ºC. This protease has low molecular mass between 18 to 35 kDa and has broad substrate specificity. However, Gupta *et al*., (2002) indicated that some reports show a few exceptions by having high molecular mass like 90 kDa from *Bacillus subtilis (natto)*.

Most of the serine proteases are extremely thermostable, which make them suited to many applications that produced by *Bacillus* species was reported as one of the major industrial enzymes with increasing market demand (Calik *et al*., 2003). In humans, they are responsible for coordinating various physiological functions, including digestion, immune response, blood coagulation and reproduction.

2.4.2 Cysteine proteases

Cysteine proteases that known as thiol proteases that degrade proteins and about twenty families of cysteine proteases have been recognized. Cysteine proteases occur in both prokaryotes and eukaryotes. Cysteine proteases share a common catalytic mechanism that involves a nucleophilic cysteine thiol in a catalytic triad. The molecular mass of cysteine proteases are about 21 to 30 kDa and these proteases catalyst the hydrolysis of peptide, amide, ester, thiol ester and thiono ester bonds (Polaina and

McCabe, 2007). According to Sumantha *et al*., (2006) the optimum pH of cysteine proteases are between 2 to 3 and temperature in a range of 40 to 55ºC.

Generally, the cysteine proteases family is divided into exopeptidases and endopeptideases. Cysteine proteases are generally happen in fruits including the papaya, pineapple, fig and kiwi fruit. According to Polaina and McCabe, (2007) indicated that the best known cysteine protease is papain. The proportion of protease tends to be higher when the fruit is unripe. Cysteine proteases are used as an ingredient in meat tenderizers. Cysteine proteases are also used as feed additives for livestock to improve the digestibility of protein. Through the gut, they hydrolyse complex proteins into simple amino acids.

2.4.3 Aspartic proteases

Aspartic acid proteases are the endopeptidases that rely on aspartic acid residues for their catalytic activity. Aspartic proteases are peptidases and exhibit a wide range of activities and specification with a molecular mass about 30 to 45 kDa. Aspartic proteases are active at acidic pH in a range of 3 to 5 and strongly inhibited by pepstatin. The optimum temperature of these proteases is between range 40 to 55ºC. The vital sources of these proteases are from animal tissue (stomach), *Aspergillus*, *Mucor*, *Endothia*, *Rhizopus* and *Penicillium* (Sumantha *et al*., 2006).

There are aspartic acid residues that work together to promote a water molecule to invade the peptide bond on the active sites of aspartyl proteases. One of the aspartic acid residues will activate the water molecule by attracting the hydrogen atom of water. The other aspartic acid residue will polarize the carbonyl group on the peptide making it

easier to invade. One of the aspartic acids usually has a lower pKa value. Renin is an important member to this class of enzyme that supports the regulation of blood pressure.

2.4.4 Metalloproteases

A metalloproteinase, or metalloprotease, is any protease chemical whose synergist component requires a metal. A case of metalloproteases would be meltrin which assumes an essential job in the combination of muscle cells amid incipient organism improvement, in a procedure known as myogenesis. Most metalloproteases require zinc, yet some utilization cobalt. The metal particle is organization to the protein through three ligands. The ligands planning the metal particle can vary with glutamate, histidine, lysine, aspartate and arginine. The fourth coordination position is taken up by an effortlessly adjust water particle.

Metalloproteases are a class of hydrolases which cleave peptide bonds by the action of a water molecule which is activated by complexing to metal ions. These proteases generally active from pH between 5 to 7, temperature between 65 to 85ºC with molecular mass of 19 to 37 kDa (Sumantha *et al*., 2006). Thermolysin family which is extracellular metalloendoproteinase is the only metalloproteases that have achieved industrial application produced by the Gram-positive bacterium. Metalloproteinase inhibitors are found in numerous marine organisms, including fish, algea and bacteria.

2.5 Thermostable alkaline protease

Enzymes from thermophiles have an excellent characteristic of thermostability that is very helpful for numerous industrial processes. Yeh *et al*., (2010) pointed out in his review that, microorganisms that are characterized as extremophiles are worth of novel enzymes since their ability to adapt the extreme conditions of pH, temperature, high pressure and salinity. The thermophilic bacteria are organisms which are stable to heat and can grow at temperature 50ºC.

Thus, the alkalophiles are typically stable in the alkaline region but not stable at high temperature. High pH and heat stable organisms extraordinarily required and acceptable among the economic processes. The organisms that possess each characteristic of thermophiles and alkalophiles offer an excellent candidate for various industrial applications in most of the thermophilic alkalophiles have growth temperatures of more than 60ºC.

In industrial aplication, thermostable alkaline proteases have special advantages due to their stability at higher temperature which can increase the solubility of non-gaseous reactants and products, speed up the reaction rates, and also reduced occurrence of microbial contamination by mesophilic organisms (Purohit and Singh, 2011; Sookkheo *et al*., 2000). Furthermore, alkaline protease delivered by thermophilic and alkaliphilic bacilli can withstand at high temperature, pH, compound denaturing specialists, and in non-watery situations (Johnvesly *et al.,* 2001).

2.6 Type of fabrics

The analysis that distributed previous on the impacts of three textures on contact, weight, and anticipated bloodstains (Keenan, Ontario, & Supervisor, 2015). Based on the previous analysts found that harsh surface surfaces decline the qualities of evacuation when contrasted with the control, paper. Unpredictable spots was seen on 100% cotton, 65% polyester 35% cotton, and 85% polyester 15% cotton textures. From the recommended a reference accumulation of bloodstains on different textures be made.

Holbrook found that specific component of the bloodstains on surface on the control surface was the condition of the stain, not the degree of the stain (Rodenburg, 2016). The most insignificant component of shape twisting was seen on smooth surfaces which are 100% rayon and 100% nylon (Rodenburg, 2016). In view of Karger and Holbrook, most research deals with the enhancement, area, and portrayal of blood on surfaces. According to ask about on the portrayal of blood on diminish or structured surfaces was coordinated by

2.6.1 Different fabric compositions

Under the high power microscope can be recognized smoothly the actual widthwise and lengthwise structure of fibres such as the longitudinal (lengthwise) shape of some of the common fibres as visible under the high power microscope. The longitudinal view such as cotton, wool, silk and polyester fibres. Cotton consists of the feathery appendages of the seeds in a cotton plant, which assists in dispersing the seed by wind (Stoner et al., 2004). Cotton fibers would be made out of cellulose. Wool and fleece are the hair of sheep. Hair is composed of a protein called keratin, which is the same

protein that skin, nails, and feathers are made off. Polyester is synethetic, and although an ester is the result of a carboxylic acid (-COOH) combined with an alcohol (-OH), creating an ester group (Stoner et al., 2004).

More research ought to be directed looking at the presence of stain shapes on textures at various points of effect. A bigger gathering of texture types and stain perceptions ought to be made, maybe expanding upon Karger's concept of a reference library, (1998). Future research ought expand upon this library of information, as well as confirm the information. Different systems of splash reproduction ought to likewise be taken a gander at. As indicated by this examination created splash predictable with a high to medium speed affect. Low speed affect, purify, exchange, and other scatter types ought to be analyzed on textures. Other researcher thoughts incorporate which rae diverse separations to the objective, vertical versus level drying, recolor treated or pre-treated textures, upholstery textures, and more texture surfaces inside one texture piece (Keenan et al., 2015).

2.6.2 Different fabric textures

Texture arrangement alone did not influence the stains which are texture surface firmly affected shapes and attributes (Sellami-Kamoun et al., 2008). The rougher surface of the denim pants made a bigger number of stains with unpredictable shape (72%) and just 15% round/oval stains. The shirt had a marginally bigger level of round/oval stains (22%) and less stains of unpredictable shape (69%). Neither sort of articles of clothing demonstrated various characteristics to such a degree, to the point that the denim pants had satellite sprinkle on 2% of stains and the fabric had satellite on 1%, wicking on 1%, and weave effect on 2% of stains. The majority of the stains on the shirt expended just

underneath the best strands (Keenan et al., 2015). The stains on the denim pants did not show this trademark. These points of reference demonstrate the hugeness of focus more surfaces of surface of same synthesis.

2.6.3 Industrial application of protease

Proteases have a large variety of applications in industrial sectors such as food and feed industry, detergent industries, leather application and household waste and also in medical usage (Sookkheo *et al*., 2000). These enzymes have been used in a wide variety of applications for several years with satisfactory result. According to Abidi *et al*., (2011) protease enzyme accounted for 60% of worldwide sales of industrial enzymes.

2.6.4 Detergent industry

Proteases one of the standard elements of a wide range of cleansers going from those utilized for family unit washing to reagents utilized for cleaning contact focal points or dentures. The principal business protease is serine protease; subtilisin Carlsberg secluded from *Bacillus licheniformis* in 1947. This commercial enzyme with pH range of 6-10 and temperature range of 10 - 80ºC is also known as Alcalase Novo.

Detergent components contain different types of enzyme which work for different type of stains. According to Crutzen and Douglass, (1999); Anwar and Saleemuddin, (1998) indicated the type of strains of proteases are hydrolyse proteinaceous stain, amylase against starch and carbohydrate stain, and lipases are effective for oily and fat stains. High activity, stability over wide temperature range, alkaline pH and compatible

with various detergent components along with oxidizing agents and bleaches are the important characteristics of protease for the option in detergent industry.

CHAPTER 3

METHODOLOGY

3.1 Materials and Apparatus

Petri dish, inoculation loop, test tube, beakers, spatula, parafilm, pipette, micropipette, micropipette tips, magnetic stirrer, cuvette, falcon tube, microcentrifuge tubes, rubber gloves, face mask, Bunsen burner, SDS-PAGE set, glass rod, conical flask, aluminium foil, muslin cloth, media bottle.

3.2 Chemical and Reagents

Slant agar, Luria Bertani Broth, ampicillin, chloroamphenicol, NaOH, sorbitol, tryptone, $C_aCl_2.2H_2O$, saline, Basal medium, IPTG, lysis buffer, Tris-HCl, C_aCl_2 Azocasein, TCA, HCl, monopotassium phosphate, dipotassium phosphate, Acrylamide, APS, TEMED, PEG 4000, TAPzyme 50a, Na carbonate, Tween 80.

3.3 Experimental Procedure

3.3.1 Bacterial Strain and Screening of Protease Producing Strain

Recombinant bacterium, *E. coli* strain BL21 (DE3) pLysS that have been used in this study capable of producing thermostable protease 50 a (known as TAPzyme 50a) previously isolated from Lojing Hot Spring, Kelantan, was obtained from Faculty of Agro-Based Industry, University Malaysia Kelantan (Ibrahim and Yusoff, 2013). . The thermostable 50a protease gene from *Bacillus subtilis* 50a was subcloned into the pET22b(+) expression plasmid and then transformed into *E.coli* BL21 (DE3) *pLysS*. The bacterial strain was grown on Skimmed Milk (SM) and Luria Bertani (LB) agar supplemented that plates containing ampicillin (50 ug/mL) and chloroamphenicol (34 ug/mL) at room temperature (37˚C) for a day (24 hours). The plates were incubated overnight at 37ºC for screening of proteolytic activity by detection of clearing zones.

3.3.2 Stock Culture and Inoculum Preparation

The stock culture was prepared and maintained in 15% glycerol at -80˚C. After that, the stock culture was streaked and single colony of the growth was inoculated on Luria Bertani (LB) agar containing 50 µg/mL of ampicillin and 34 µg/mL of chloroamphenicol and incubated at 37ºC for 24 hours in horizontal/orbital incubator shaker of 150 rpm. The cells were harvested by centrifugation at 10,000 rpm, 4˚C for 10 min. The bacteria pellet was dissolved in saline (0.85% NaCl) to give an absorbance

reading of 0.5 at 600 nm. Inoculum 2.5% (2.5 mL) were inoculated into the optimum production medium.

3.3.3 Production of TAPzyme 50a

TAPzyme 50a production was carried out in the optimum production medium containing 5 g/L of sorbitol, 20 g/L tryptone, 2.0 g/L of calcium chloride ($CaCl_2.2H_2O$) and optimal culture conditions where the induction time was at OD_{600nm} 0.5, initial pH of medium at pH 8, incubation temperature 30ºC, 240 rpm of agitate speed and 24 hour fermentation time after induction.

3.3.4 Harvesting of Crude Protease

By using centrifugation, the culture was harvested by centrifuged at 10,000 rpm for 10 minutes at 4˚C. The supernatant was discarded and the cell pellet was resuspended in 0.1 M Tris-HCl, 2mM $CaCl_2$ buffer, pH 9 and further sonicated (2 minutes, 1 second pulse, 20% amplitude) to lyse the cell by using ultrasonification. After sonicated, the cell suspension was centrifuged at 10,000 rpm for 10 minutes at 4˚C. The cell pellet was discarded and the supernatant was collected as the crude protease enzyme.

3.3.5 Single step purification through heat treatment

The collected supernatant or crude TAPzyme 50a solution was put into hot water bath at 70ºC for 3 hours to precipitate the mesophilic *E.coli* proteins (Fu *et al.*, 2003). To remove the denatured contaminated proteins, the patially crude enzyme solution was

centrifuged at 10,000 rpm for 10 minutes. By polyacrylamide gel electrophoresis, the purity of the TAPzyme 50a determined and further assayed to determined protease activity.

3.3.6 Spray detergent formulation

Table 3.3: Ingredients for preparing spray detergent formulation

CHEMICALS	W/W (%)
PEG 4000	3.50
NA CARBONATE	25.0
TWEEN 80	20.0
TAPZYME	50.0
FRAGRANCE (LAVENDER OIL)	0.50
DISTILLLED WATER	1.00

3.3.7 Spray Detergent Performance Analysis

By using different fabrics pieces (5 x 5 cm) stained with human blood (Savitha *et al*., 2008), the washing performance analysis was performed. Several types of fabrics were used. The clean fabrics pieces (5 × 5 cm) were stained with 100 µL human bloods, dried at 60ºC overnight to fix the stain and kept at room temperature for one month. The following sets were prepared as below with the total volume 300 µL spray covered the bloodstained on fabric at the distance of spray detergent and stained cloth during the stain removal analysis was 5 cm to the spray bottles. The bloodstained on the fabrics were sprayed with the spray detergent and rubbed for several times. The observation made by comparing the treated stains within different types of fabrics and the controls. Clean fabric without bloodstained was taken as control.

i. Distilled water
ii. TAPzyme50a with distilled water
iii. Spray detergent containing TAPzyme 50a
iv. Spray detergent without TAPzyme 50a

3.4 Analytical Methods

3.4.1 Absorbance Reading Determination

Centrifuged at 10,000 rpm, 4˚C for 10 minutes of 2 mL of samples. Then, discard the supernatant and keep of the pellet. 2 mL of distilled water added to the pellet, vortexed and centrifuged again at 10,000 rpm, 4˚C for 10 minutes. After that, the absorbance was read at 600 nm using spectrophotometer.

3.4.2 Determination of Protein Concentration

The protein concentration was measured spectrophotometrically at wavelength of 595 nm according to the Bradford method. It is involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford, 1976). One of the most common used protein standards that give a color yield similar to that of the protein sample being assayed of protein standard. Based on function on the BSA standard curve (y=mx+c), the concentration of 50a protease samples are calculated by substituted the y-value with absorbance reading of respective 50a protease samples.

3.4.3 Preparation of Standard Curve BSA

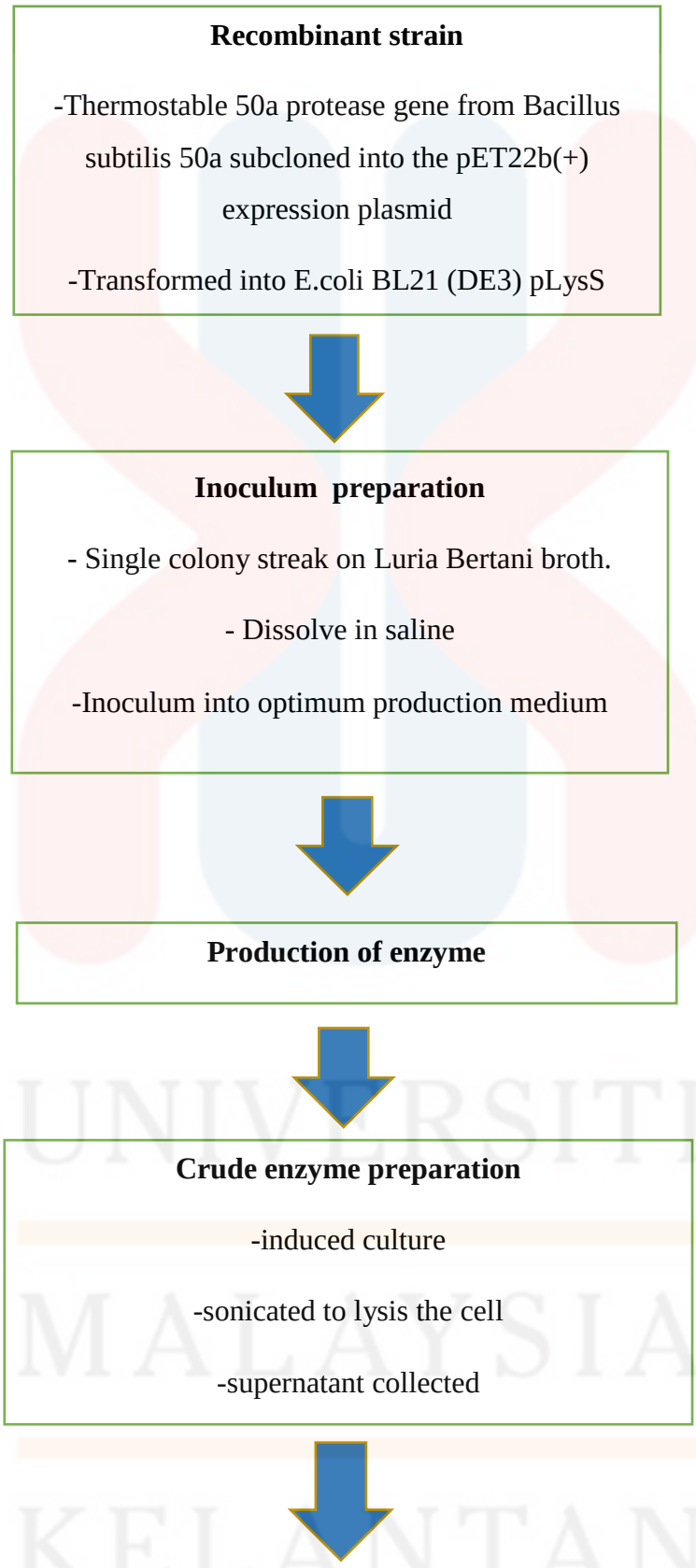
The absorbance reading of BSA standard solutions was obtained when the BSA standard curve was plotted. A straight line with the equation of (y=mx+c), where y=absorbance at 595nm and x=protein concentration while n, the best fit of the data were determined. If the absorbance of the test sample is outside of the absorbance range for the standards, the assay must be repeated with a more appropriate dilution (if any). The linear

range for the assay and for most spectrophotometers is 0.2 – 0.8 O.D. Units (Bradford Protein Concentration Assay, 2001).

The correlation coefficient (R) obtained from the constructed standard curve of BSA is a quantity that gives the quality of a least squares fitting to the original data. The better approximation when the closer the R value to 1. Generally, for a good linear approximation (Standard Curve Lab) when the an R-value greater than 0.97 (or and R value of 0.95 or higher).

3.4.4 Determination of Protease Assay using Azocasein as the Subtrate

To conduct protease assay, azocasein (0.5%) was prepared freshly on the day dissolved it in Tris-HCl buffer (pH 9). In each labelled test tube except 'control', 1 ml of azocasein is added and then pre-incubated for five minutes in 80˚C water bath. After that, 0.1 ml of samples is added to all test tubes including 'Control' and was incubated again at 80˚C for 30 minutes with 100 rpm shaking water bath. Summary of the research activities as shown in Figure 3.1.



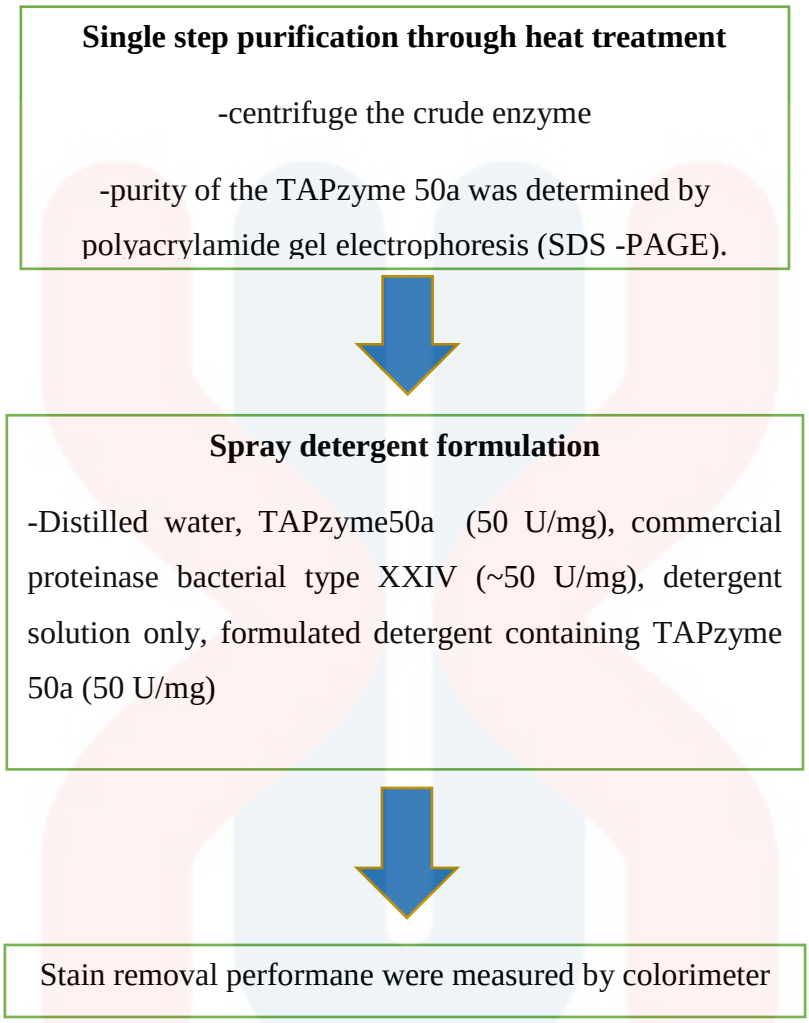


Figure 3.1: Flow chart of the research

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Production and Purification of TAPzyme 50a

To ensure the stability of *E.coli* BL21 (DE3) *pLysS* harboring alkaline 50a protease known as TAPzyme 50a. The plate that used to streak the recombinant strain is Luria Bertani- Skim Milk agar (LB-SMA). Luria broth (LB) is a nutrient rich media commonly used to culture bacteria in the lab. The LB-SMA supplemented with 50 µg/mL Ampicilin and 34 µg/mL Chloroamphenicol. The clearing zone formation around the colonies due to the protease produced by the culture hydrolysed the milk substrate in the LB-SMA when the plates were incubated overnight at 37^{o}C. The recombinant *E.coli* contain gene that resistant to Ampicillin and Chloroamphenicol and used in order to grow the recombinant. At the same time it continuous on production. The casein in the skim milk hydrolyzed by protease to forming the clearing zone. According to Sellami-Kamoun, (2008) hydrolysis indicates the clearing zone of protease producing organisms.

TAPzyme 50a production was measured in the optimum production medium consisted of sorbitol (5 g/L), tryptone (20 g/L), $C_aCl_2.2H_2O$ (2.0 g/L) that supplemented with 50 µg/mL of ampicillin and 34 µg/mL of chloroamphenicol. 200 mL of basal medium was inoculated with the 2.5% of seed culture at 37^{o}C, 150 rpm in

orbital incubator shaker. The absorbance was checked after 3 hours and the incubation was stopped when the absorbance reading at OD_{600nm} 0.5. Then, the induced culture with 0.5 Mm *isopropyl-ß-D-thiogalactopyronoside* (IPTG) to induced the lac operon and the recombinant gene expression in *E. coli*. The crude TAPzyme 50a produced intracellularly in *E. coli,* thus, sonication approach was applied. Then, it harvested after centrifugation process. The production of crude TAPzyme 50a that achieved at 101.43 U/mL of enzyme activity using azocasein assay. The crude TAPzyme 50a then purified using simple step of heat treatment method. The crude TAPzyme 50a was further incubated in water bath shaker with the temperature of 70^{o}C for 3 hours. The purified TAPzyme 50a was centrifuged to separate the denatured mesophilic *E. coli* protein. The activity and protein content of the crude and purified TAPzyme 50a was measured and summarized in Table 4.1.

The total protein content and total activity of the crude TAPzyme 50a was found to be 2383.66 mg and 1778.84 U, respectively with a specific activity of 0.75 U/mg. After heat treatment process, the specific activity of the TAPzyme 50a was increased to 5.73 U/mg with the purification fold of 7.50. The fold increase indicates that the heat treatment methods for purification of TAPzyme 50a was efficient. The recovery of the TAPzyme 50a also increased after the purification process with the value of 199.58% which showed that the protease was activated during heat treatment. In this line, the important role of temperature was proven as it can activate and inactivate the enzyme (Abou-Elela *et al*,. 2011).

Table 4.1: Purification summary of TAPzyme 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

Note: Total activity = starting volume (mL) x protease activity (U/mL)
Total protein = starting volume (mL) x protein content (mg/mL)
Specific activity = total activity (U) / total protein (mg)
Recovery = total activity of heat treatment/ total activity of crude x 100%
Purification fold = specific activity of heat treatment/ specific activity of crude

The Bradford protein assay is one of several simple methods commonly used to determine the total protein concentration of a sample. The method is based on the proportional binding of the dye Coomassie to proteins. Within the linear range of the assay (~5-25 mg/mL), the more protein present, the more Coomassie binds. Furthermore, the assay is colorimetric; as the protein concentration increases, the color of the test sample becomes darker. Coomassie absorbs at 595 nm. Although different protein standards used that chosen the most widely used protein as our standard - Bovine Serum Albumin (BSA). The serial of known protein concentration of BSA were at range between 5 ug/mL to 50 ug/mL with absorbance reading 595nm formed BSA standard curve. The absorbance reading of BSA known protein concentration and 50a protease sample unknown protein concentration obtained from Bradford assay. The 50a protease sample unknown protein concentration was calculated from substitution of average Abs_{595} reading into y-value of BSA standard curve function $y=0.0052_x$ (Figure 4.1). As the

Bradford protein assay showed a significant protein to protein variation, hence the calculated results are the estimation of protein concentration.

The best formulation was identified with containing of 50% (w/w) purified TAPzyme 50a. The formulated spray detergent containing TAPzyme 50a was further tested on its stain removal analysis. The bloodstains removal performance analysis was conducted using four different types of fabrics and the visual observation were illustrated in Figure 4.3. The positive control is the clean fabric and the negative control is the fabric that stained with 100 µL of human blood.

4.2 Spray detergent formulation

Spray detergent is formulated to remove the bloodstain from different types of fabric. Stain components with the suitable detergent enzyme are easily removed during the cleaning process, protease act by degrading the dirt into smaller and more soluble fragments on fabrics (Sakpal & Narayan, 2015). The effects of protease are very clear, and the enzyme cost is relatively low. A fresh proteinaceous stain on fabrics is generally not stubborn and can be removed simply. But, when the proteinaceous is dried on the surface fabrics it will become difficult to remove even with surfactants (Benluvankar *et al.,* 2016) . Four types of fabrics (jersey, cotton, koshibo and crepe) were sprayed with distilled water, TAPzyme 50a, spray detergent, and spray detergent without TAPzyme 50a, one by one. The different removal the bloodstains from the fabrics to various degree.

For the best result in removing a bloodstain from the fabrics was showed spray formulated detergent when the addition of TAPzyme 50a in detergent was better compared to TAPzyme 50a, distilled water and spray detergent without TAPzyme 50a. This is because the best removal was achieved with the detergent formulation in the

presence of TAPzyme 50a also these formulates have the ability to denature protein such as hemoglobin (Stoner *et al.*, 2004). Addition of TAPzyme 50a to detergents considerably increases the cleaning effect by removing protein stains such as blood (Hofmann *et al.*, 2018). For the blood stained crepe fabric it was observed that the best removal of stain was by the spray detergent in the presence of TAPzyme 50a. This formulation contains build, surfactant and fragrance that contain high of chelating to remove divalent cations responsible for water hardness and enhancing stain removal (Benluvankar *et al.*, 2016). Interestingly, bloodstains from crepe were removed better than jersry, cotton and koshibo by using spray detergent although blood penetrates into the crepe fabric more easily. This observation will be discussed in the next section.

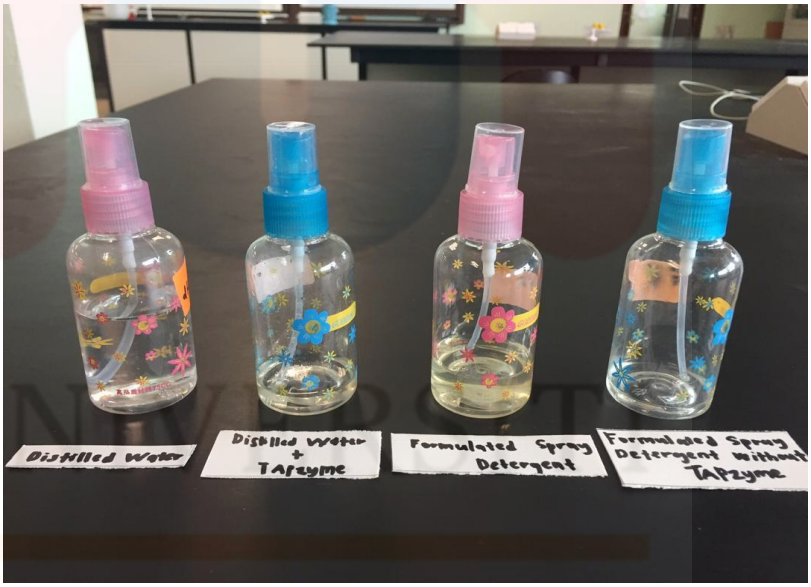


Figure 4.2: Four types of removal bloodstains were sprayed with 300 µL at 5 cm from the spray bottles to the fabrics

Table 4.2: Ingredients for preparing spray detergent

CHEMICALS	W/W (%)
PEG 4000	3.50
NA CARBONATE	25.0
TWEEN 80	20.0
TAPZYME	50.0
FRAGRANCE (LAVENDER OIL)	0.50
DISTILLLED WATER	1.00

4.3 Influence of removal bloodstains from fabrics and the effectiveness performance

A study was conducted to identify the effectiveness of TAPzyme 50a formulation based on the performance removal test onto different fabrics (jersey, cotton, koshibo and crepe). Before applying blood on textures, the textures washed with refined water before applying blood on the textures. At that point, every texture was sliced to a size of 5 × 5 cm. Blood was taken from three known undergraduate students in UMK, Jeli as volunteers. Every texture recolored with 100 µL of blood and left to dry at room temperature for a month. The distance across of bloodstain was appeared on the (Figure 4.4) subsequent to applying blood to the every texture. Each texture demonstrated an alternate breadth relying upon its capacity to retain the blood. In this circumstance, the texture that consumed more blood demonstrated a bigger width while texture that assimilated less blood demonstrated a littler breadth when the more prominent ingestion

held more blood and progressively helpful for discovery of the stain (Mushtaq *et al.,* 2015). For every texture with less assimilation, e.g. Bosky, blood remains as a coagulation at first glance and could be expelled by scratching (Cho *et al.,* 2015).

The weave of the fabric, water wicking, chemistry, and chemical modification can influence the absorption of blood on the fabrics (Castro *et al.,* 2015). Based on (Figure 4.3), crepe had the largest stain size, followed by koshibo, cotton and jersey. The blood spread across the surface of the crepe more than for any other fabrics, for that this spreading engulfed surrounding the surface. A possible explanation could be that more blood spreading over the surface fabric because of the crepe fabric is thin compared to others fabric (Wu, *et al.,* 2018). In fact, the bending in the presence of bloodstains on textures is subject to texture sponginess and surface (Hofmann et al., 2018). Textures are additionally utilized as channels to evacuate particles, including the cells. In this way, cells could be sifted through and just blood serum could enter into the yarn (Keenan et al., 2015). Aside from that, the snugness of a weave or sew could influence the relocation of cells.

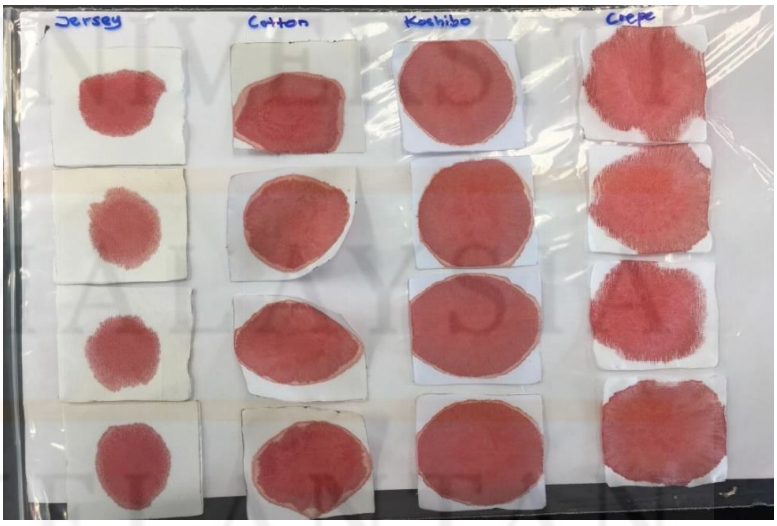


Figure 4.3: Appearance of different types of fabric after stained with 100 µL of human blood

Before the removing of stains performance, the fabrics were measured the visuality of the color on the basis of hue, chroma and lightness (L*a*b) by using the color meter. For each fabric were sprayed three times and agitate by hand at room temperature. During agitation, the fabrics were well agitated by hand in order to remove the amount of blood. This specific washing technique adopted was the one frequently used in Pakistan for the removing stains on the clothes (Keenan *et al.*, 2015). For removing the bloodstains on the different fabrics, one type of fabric washed with four type of removal which are distilled water, TAPzyme 50a, spray formulateddetergent and spray formulateddetergent without TAPzyme 50a). A positive control, for each type of fabrics containing a bloodstain also removed with distilled water. After removing the bloodstains for all fabrics, fabrics were left to dry at room temperature for 24 hours. After removing stains, the visibility of stains was noted. Some stains still visible to the naked eye and could be seen easily. After removing the stains, bloodstains on some fabrics were not visible.

4.4 Stain Removal Performance Test

Between the formulated detergent include the distilled water alone and TAPzyme 50a, jersey is the most hardness to remove the bloodstains because of the weave of the fabric different compared to crepe, koshibo and cotton. The quality and strength of yarn are affected by the removing bloodstains performance (Mushtaq, Rasool, & Firiyal, 2015). The effectiveness of spray formulated detergent and TAPzyme 50a to remove the bloodstain showed almost same result at the end but with the addition of TAPzyme 50a was combine with formulated detergent is more effective compared to TAPzyme 50a alone because it contains builder and surfactant (Olsen & Falholt, 1998). A fresh proteinaceous stain is generally not stubborn and very easy to remove just only used a

cold water. But, when the proteinaceous stain is dried and aged that is becoming very difficult to remove even with a surfactant as mentioned by Crutzen and Doughlass (1999).

In general, enzyme detergents remove protein from clothes soiled with blood far more effectively than non-enzyme detergents (Sakpal & Narayan, 2015). Moreover, the application of enzymes in detergents enhances the detergents ability to remove tough stains and also makes detergent eco-friendly. Apart from that, for the best result to remove the bloodstain from fabric still requires the joint action of the TAPzyme 50a, the surfactant system, and mechanical agitation (Schroeder *et al.,* 2006). Besides that, TAPzyme 50a needs a small amount of calcium for the sake of stability. A fresh proteinaceous stain on fabric or hard surface is not stubborn and can be removed simply by cold water (Castro *et al.,* 2015). But, when the proteinaceous soil is dried, aged, or heated, that it becomes more difficult to remove even with surfactant because it coagulates and hinders the penetration of the cleaning liquor (Mushtaq *et al.,* 2015). In addition, protein residues may be oxidized and denatured due to the presence of oxygen, so that the bloodstain becomes permanent (Mushtaq *et al.,* 2015). Proteins are present in small amounts and are not completely removed by surfactants and bleaching systems (Hofmann *et al.,* 2018).

In fact, blood behaviour can be predicted and reproduced since the blood is an elastic, non-Newtonian fluid and obeys fluid dynamic laws. Previous studies in textile science indicated that finishing treatments applied to fabrics and laundering of fabrics alter the wettability and wicking of the blood into the fabrics, by changing the relationship between how the liquid interacts with the fabric and the surface energy of the fibres (Castro *et al.,* 2015). Moreover, the thickness of the fabrics may also decrease due to disperse of the surface and it will decrease in the stain size or diameter (Keenan *et al.,* 2015). Apart from that, the ability to absorb the bloodstain is decreased.

The compatibility removal performance can be evaluated by adding enzyme into formulated detergent at a certain concentration (Kumar and Bhalla, 2004). Moreover, the use of enzyme as a detergent additive is not a straightforward practice because enzyme issensitive to some detergent ingredients during the storage of the product (Crutzen and Douglass, 1999). Besides, an ideal detergent protease must be compatible and stable to all commonly used detergent components such as surfactants, builders and other additives that might be contained in the formulation (Gupta *et al.,* 1999; Kumar and Takagi, 1999). Overall, the measurement of the lightness of spray formulated detergent were clearly very closed to the TAPzyme 50a compared to distilled water alone and spray formulated without TAPzyme 50a.

Figure 4.4: a) Four pieces of jersey before removal performance

Note: (A) Distilled water, (B) TAPzyme 50a, (C) Spray detergent, (D) Spray detergent without TAPzyme 50a

Figure 4.4: b) Four pieces of jersey after removal performance

Note: (A) Distilled water, (B) TAPzyme 50a, (C) Spray detergent, (D) Spray detergent without TAPzyme 50a

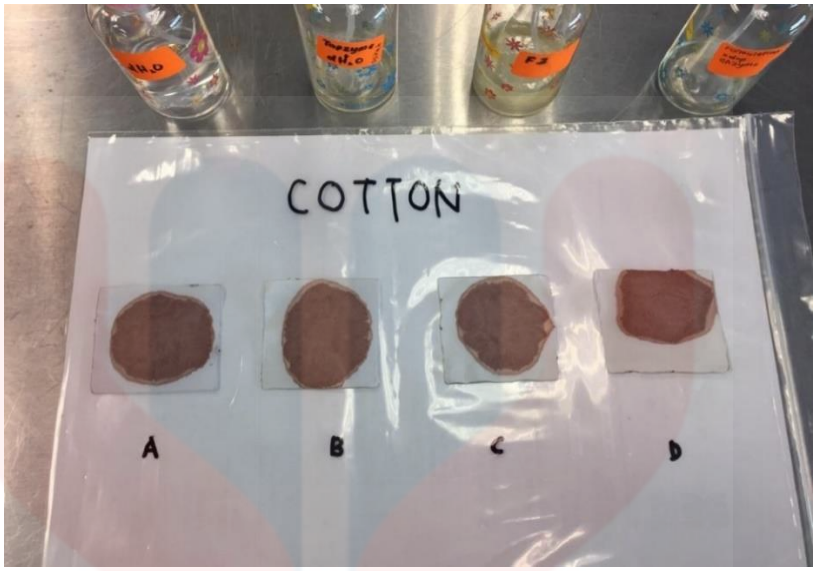


Figure 4.5: a) Four pieces of cotton before removal performance

Note: (A) Distilled water, (B) TAPzyme 50a, (C) Spray detergent, (D) Spray detergent without TAPzyme 50a

Figure 4.5: b) Four pieces of cotton after removal performance

Note: (A) Distilled water, (B) TAPzyme 50a, (C) Spray detergent, (D) Spray detergent without TAPzyme 50a

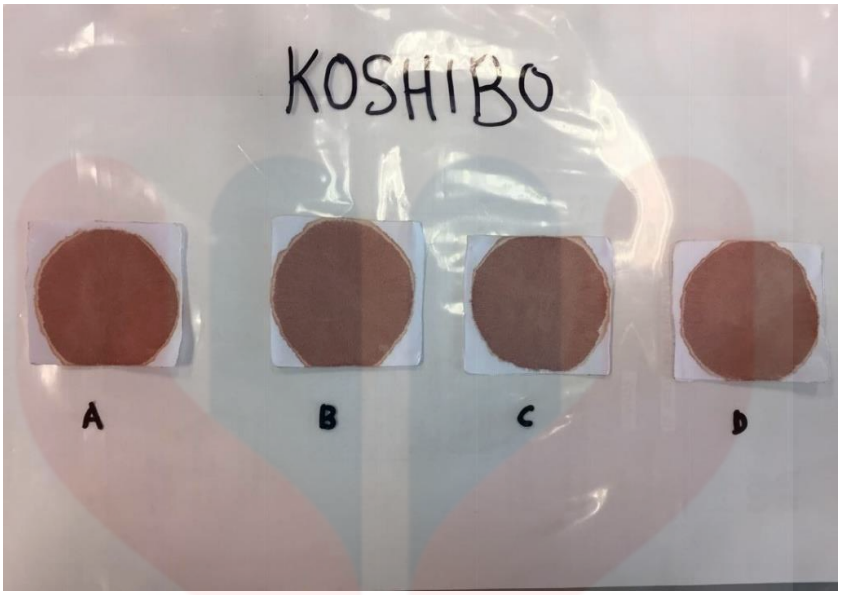


Figure 4.6: a) Four pieces of koshibo before removal performance

Note: (A) Distilled water, (B) TAPzyme 50a, (C) Spray detergent, (D) Spray detergent without TAPzyme 50a

Figure 4.6: b) Four pieces of koshibo after removal performance

Note: (A) Distilled water, (B) TAPzyme 50a, (C) Spray detergent, (D) Spray detergent without TAPzyme 50a

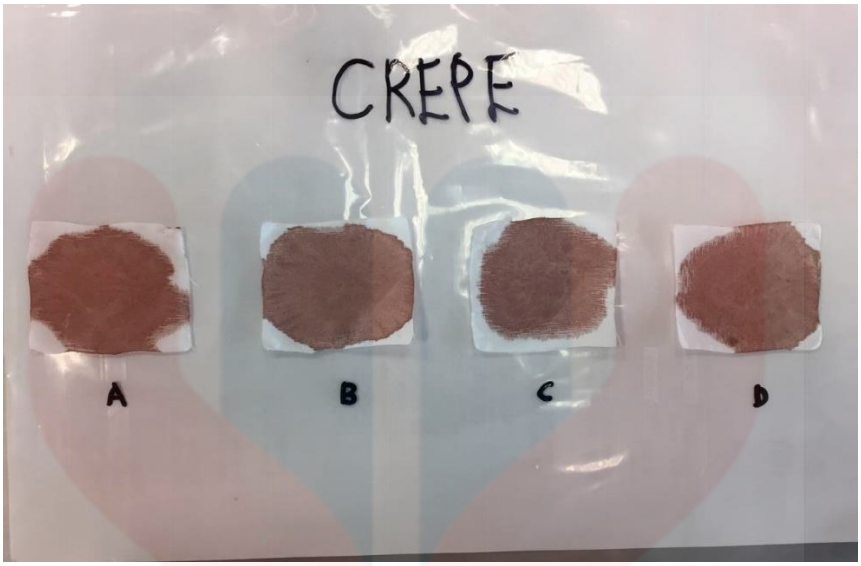


Figure 4.7: a) Four pieces of crepe before removal performance

Note: (A) Distilled water, (B) TAPzyme 50a, (C) Spray detergent, (D) Spray detergent without TAPzyme 50a

Figure 4.7: b) Four pieces of crepe after removal performance

Note: (A) Distilled water, (B) TAPzyme 50a, (C) Spray detergent, (D) Spray detergent without TAPzyme 50a

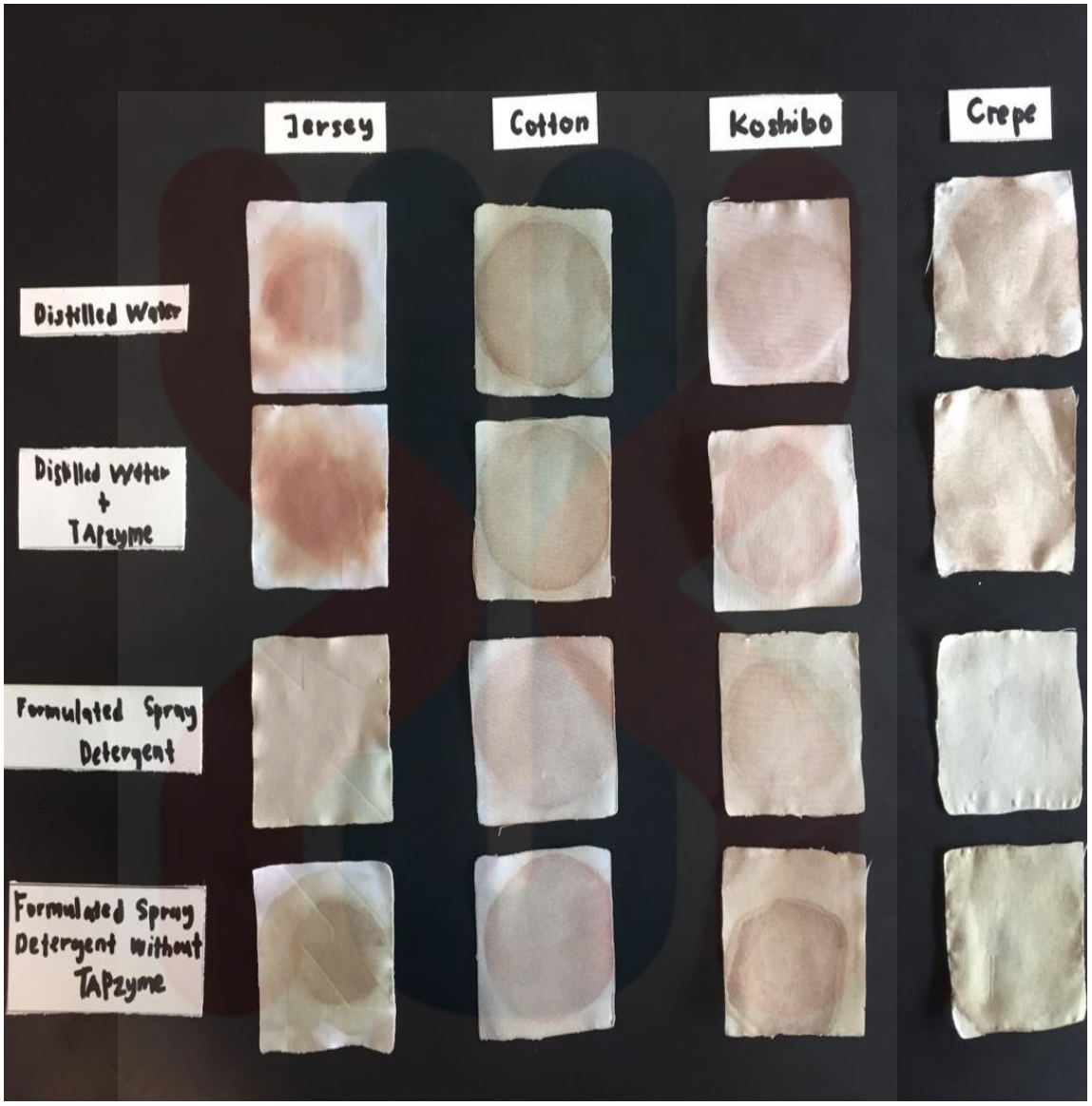


Figure 4.8: Comparison between all types of fabric after removal performance

Among the four types of removal bloodstains in this study, spray detergent with TAPzyme 50a showed the best performance in removing the bloodstains on crepe cloth with presence of TAPzyme 50a and also surfactants (Tween 80) are the active cleaning agents that play an important role in extracting stains from the fabrics. Surfactants improve the wetting ability of water, loosen and remove stains and emulsify, solubilize or suspended stains in the wash (Ainsworth, 1994). According to Schroeder *et al*., (2006), surfactants comprising from 15% to 40% of the total detergent formulation and account

over half of its use in laundry detergents, household and personal care products. In this study, the total amount of surfactants used in spray detergent only 20% of the whole ingredients, where the amount of surfactants used in spray detergent are less than ones normally used in the commercial detergents formulation. Hence, it is relevant and possible to get such that the outcomes through this study.

Although that, it can be seen from the result that the koshibo fabrics were not completely clean even after being treated with the spray formulated detergent supplemented with TAPzyme 50a. This is may be due to the types of fabric. Moreover, koshibo is categorized as a fabric based on filaments formed by chemical solution and squeeze through fine screens, then spun into threads and yarns for fabrics allows direct attraction of water molecules thus, large portion of water quantity being absorbed by koshibo (Quiz, n.d.). This process cause direct sorption of the water with bloodstains to adhere strongly on koshibo fibres. Nevertheless, the bloodstains were hardly removed from the koshibo fabric if removed with the spray detergent only. This is may be due to lack of effectiveness of the detergent formulated. A combination surfactant system usually give a better results detergency performance than the composition containing single surfactant, thus various combination surfactants system have been developed for detergent compositions for different uses (Schroeder *et al.*, 2006).

Moreover, they also stressed that laundry detergents as of late contain certain blend of various sorts of surfactant to reinforce the cleaning execution ability and stay gentle to the skins (Sakpal & Narayan, 2015). From this study, the spray formulated detergent are in conformity with the stated reports which include mixture of nonionic surfactant (Tween 80).

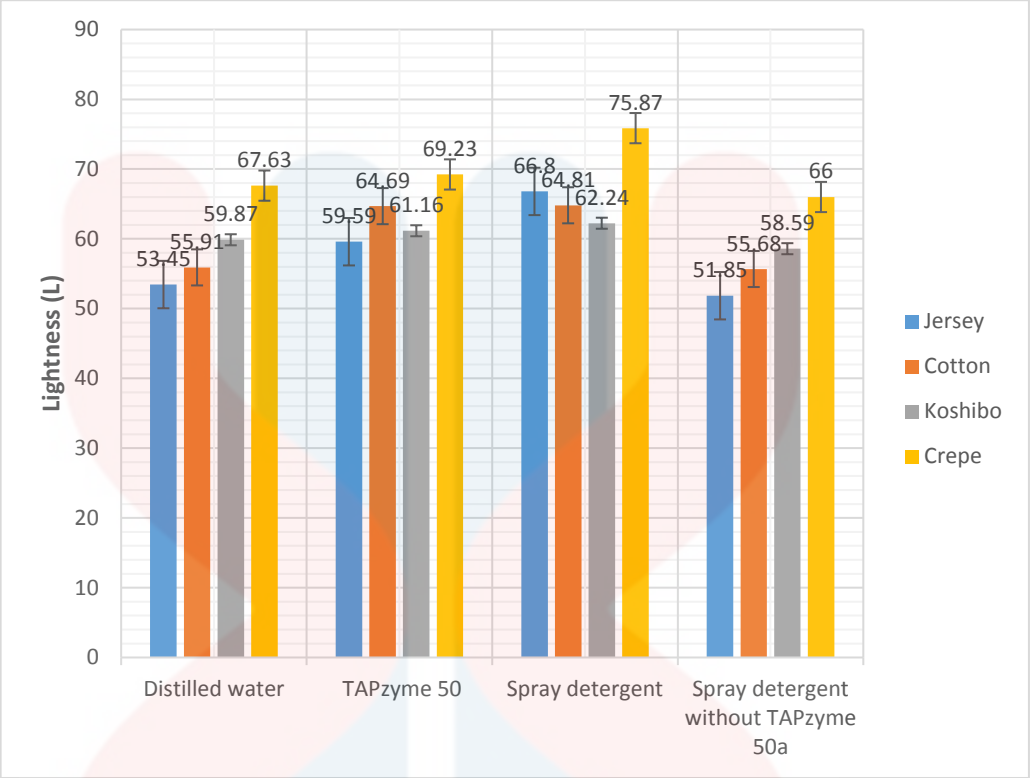
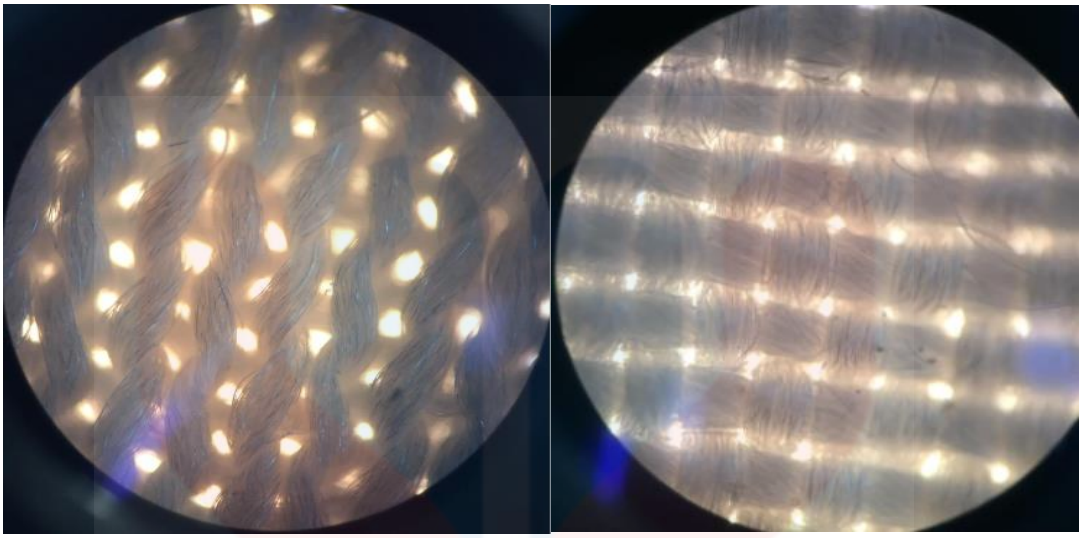


Figure 4.9: Color meter reading of different types of fabric after removal performance

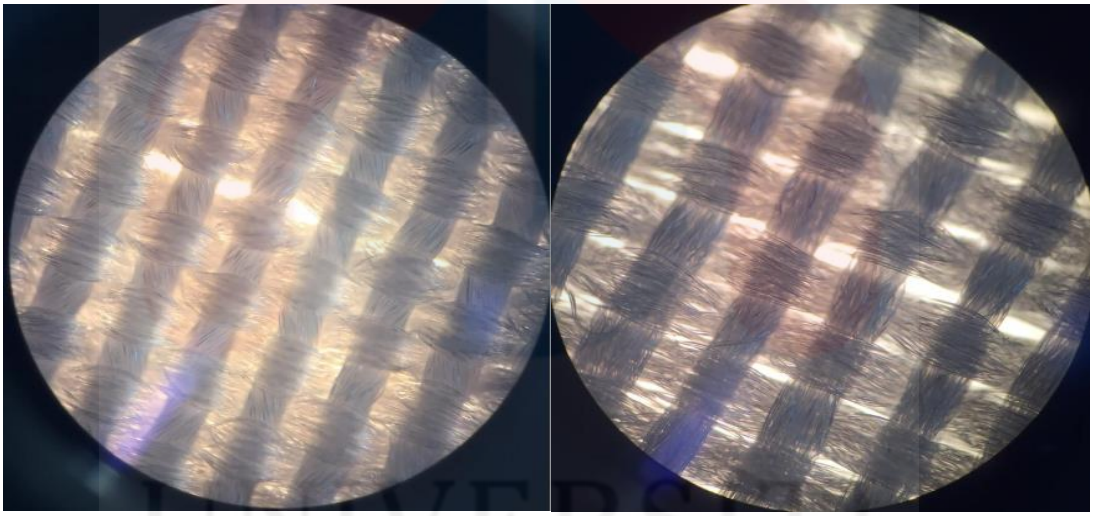
4.5 Different fabric compositions

For this study that had used different types of fabric which are jersey, cotton, koshibo and crepe. Jersey is a knit fabric that originally made of wool, but now is made of wool, cotton and synthetic fibers (Quiz, n.d.). This fabric also can be a very stretchy single knitting that usually light-weight. Knitting is a method that yarn is form to create a fabric, in other used of many types of garments. Different types of yarn which are fibre type, texture, and twist. Cotton is a soft, fluffy staple fiber is almost pure cellulose (Mushtaq *et al.,* 2015). The fiber is most often spun into yarn and used to make a soft. Moreover, it is the most widely used natural fiber cloth and the fibers are hollow and upon close inspection feature twisted ribbon. This fibers are woven or knit into fabric that means of the two most common method: plain weaves and twill weaves.

In this study, the cotton fabric that had used is plain weaves. Koshibo polyester fabric is a midweight woven fabric. These fabric based on filaments formed by chemical solution and squeeze through fine screens, then spun into threads and yarns for fabrics. Filaments feasible kinked to produce textures resembling natural materials such as cotton and wool and left smooth to produce silk like results (Castro *et al.,* 2015). The appearance of a garments also affected by the weight of the yarn that can describes the thickness of the spun fibre (Mushtaq *et al.,* 2015). So, from the process removal the bloodstains from the fabrics it can conclude the thicker the yarn, the more visible and apparent stitches will be the thinner the yarn and the finer the texture (Mushtaq *et al.,* 2015).



(a) (b)



(c) (d)

Figure 4.10: Image of jersey (a), cotton (b), koshibo (c) and crepe (d) under the microscope

The actual widthwise and lengthwise structure of fibres cannot be seen with naked eyes but can be recognized easily under the high power microscope (Hofmann *et al.*, 2018). Here is the longitudinal (lengthwise) shape of some of the common fibres as visible under the microscope. Figure 4.9 shows the longitudinal view of some fibres. Addition,

the shape of bloodstain changed by the wicking pattern that controlled by the spacing between yarns and capillaries formed between fibers (Mushtaq *et al.*, 2015). The interaction of the blood and fabric when compared to the textile science investigations cited is a complicated and dynamic process (Cho *et al.*, 2015). Blood is a complex mixture and dissolved substances in plasma (Wu *et al.*, 2018). So, through thickness and over the surfaces were responsible for creating the bloodstains. Moreover, the fibre content in the fabric specimens affected the final bloodstains formed (Castro *et al.*, 2015). Based on the figure under the microscope the yarn knitted of the jersey is different from the other fabrics, so that when removal performance using the control (distilled water) is the most hardest because the jersey had the most thickness compared to other fabrics. Twists given to fibre strands for formation of a yarn. It is possible that this used the double layer such as 'S-twist' (clockwise), the quality and strength of yarn is affected by the number of twist.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In conclusion, in this study engages with the formulation of an enzymatic detergent containing TAPzyme 50a as the detergent additives for spray detergent. Four types of removal bloodstains performance with different ingredient through this experiment and the result showed that TAPzyme 50a is the most effectiveness with the spray detergent. Furthermore, the addition TAPzyme 50a with spray detergent showed better removing performance compared to TAPzyme 50a alone, distilled water or spray detergent without TAPzyme 50a. The combination of TAPzyme 50a and spray detergent are effective in removing the bloodstains from white crepe. The TAPzyme 50a was produced using optimization medium was characterized for its potential used as a detergent additive. It is proved by removing performance using bloodstained on the four different fabric. The visual observation revealed that the addition of TAPzyme 50a in detergent provided better result.

5.2 Recommendation

As for suggestion, the further study should carry out by using different surfactants that possibly more effective for detergent. Apart from that, the proposed formulation in this research should be improved in terms of composition. Besides, future research should focus more on formulating an ecofriendly detergent and should be cost-effective and safer to use. In addition, for a more accurate removing performance, the measurement using the whiteness indices suggested (Stoner et al., 2004). There are numerous conceivable approaches to expand upon this exploration later on. More texture types necessary to be anatomize.

Just four textures were decided for this examination of time requirements. For the future research condition expand upon this library of information, as well as confirm the information. Moreover, different instruments that can impact of expulsion bloodstains need to likewise be taken a gander at. This examination about the diverse sorts of textures on how the adequacy with expansion of the protein fundamental to be analyzed on textures. On the past thoughts incorporate the diverse separations to the objective, vertical versus even drying positions, recolor treated or pre treated textures, upholstery textures, and more texture surfaces inside one texture piece.

REFERENCES

Abidi, F., Limam, F. & Nejib, M.M. (2008). Production of alkaline proteases by *Botrytis cinerea* using economic raw materials: Assay as biodetergent. Process Biochemistry, 43, 1202-1208.

Aehle, W. (2007). Enzyme in industry: Productions and applications (3rd Ed.). Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA.

Anwar, A. & Saleemuddin, M. (1998). Alkaline Proteases: A review. Bioresource Technology, 64, 175-183.

Banerjee, U.C., Sani, R.K., Azmi, W. & Soni, R. (1999). Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. Process Biochemistry, 35, 213-219.

Banik, R.M. & Prakash, M. (2006). Purification and characterization of laundry detergent compatible alkaline protease from *Bacillus cereus*. Indian Journal of Biotechnology, 5, 380-384.

Benluvankar, V., Priya, S. E., & Gnanadoss, J. J. (2016). Medium Formulation and its optimization for increased protease production by Penicillium sp . LCJ228 and its potential in blood stain removal, *4*(01), 20–26.

Boshale, S.H., Rao, M.B., Deshpande, V.V. & Srinivasan, M.C. (1995). Thermostability of high-activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20). Enzyme and Microbial Technology, 17, 136-139.

Calik, P., Celik, E., Telli, I.E., Oktar, C. & Ozdemir, E. (2003). Protein-based complex medium design for recombinant serine alkaline protease production. Enzyme and Microbial Technology, 33, 975-986.

Castro, T. C. De, Taylor, M. C., Kieser, J. A., Carr, D. J., & Duncan, W. (2015).

Systematic investigation of drip stains on apparel fabrics : The effects of prior-laundering , fibre content and fabric structure on final stain appearance. *Forensic Science International*, *250*, 98–109.

Cho, Y., Springer, F., Tulleners, F. A., & Ristenpart, W. D. (2015). Quantitative bloodstain analysis : Differentiation of contact transfer patterns versus spatter patterns on fabric via microscopic inspection, *249*, 233–240.

Gupta, R., Beg, Q.K. & Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. Applied Microbiology Biotechnology, 59, 15-32.

Haddar, A., Sellami-Kamoun, A., Fakhfakh-Zouari, N., Hmidet, N. & Nasri, M. (2010). Characterization of detergent stable and feather degrading serine protease from *Bacillus mojavensis* A21. Biochemical Engineering Journal, 51, 53-63.

Hofmann, M., Adamec, J., Anslinger, K., Bayer, B., Graw, M., Peschel, O., & Schulz, M. M. (2018). Detectability of bloodstains after machine washing.

Ibrahim, N.A & Yusoff, N. (2013). Thermostable alkaline serine protease from thermophilic *Bacillus species*. International Research Journal of Biological Sciences, 2(2), 29-33.

Illanes, A. (2008). Enzyme Biocatalysis: Principles and applications. Netherlands: Springer Jain, D., Pancha, I., Mishra, S.K., Shrivastav, A. & Mishra, S. (2012).

Johnvesly, B. & Naik, G.R. (2001). Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus sp*. JB-99 in a chemically defined medium. Process Biochemistry, 38,139-144.

Keenan, H. K., Ontario, P., & Supervisor, I. (2015). A Comparison of Bloodstains on Fabric : Characteristics of Impact Spatter, (605), 1–45.

Klein-Marcuschamer, D., Oleskowicz-Popiel, P., Simmons, B. A., & Blanch, H. W. (2012). The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnology and Bioengineering*, *109*(4), 1083–1087.

Kumar, C.G. & Takagi, H. (1999). Research review paper: microbial alkaline protease: from a bioindustrial viewpoint. Biotechnology Advances, 17,561-594.

Kumar, D., Savitri, Thakur, N., Verma, R. & Bhalla, T.C. (2008). Microbial protease and application as laundry detergent additive. Research Journal of Microbiology, 3(12), 661-672.

Mushtaq, S., Rasool, N., & Firiyal, S. (2015). Detection of dry bloodstains on different fabrics after washing with commercially available detergents, *0618*(October).

Olsen, H. S., & Falholt, P. (1998). The Role of Enzymes in Modern Detergency, *1*(4)

Purification and characterization of haloalkaline thermoactive, solvent stable and SDS-induced protease from *Bacillus sp*: A potential additive for laudry detergents. Bioresource Technology, 115, 228-236.

Quiz, S. (n.d.). Fibre to Fabric, 18–25. Rodenburg, P. (2016). the Right.

Sakpal, H. C., & Narayan, G. (2015). Thermostable alkaline protease from Bacillus sp and its potential applications, *10*(5), 58–67.

Sandhya, C., Sumantha, A. and Pandey, A. (2006). Proteases. In Pandey, A., Webb, C., Soccol, C.R. & Larroche, C., Enzyme Technology (pp.319-329). New Delhi: AsiaTech Publications Inc.

Sumantha, A., Larroche, C. & Pandey, A. (2006). Microbiology and industrial biotechnology of food-grade protease: A perspective. Food Technology Biotechnology, 44(2), 211-220.

Sellami-Kamoun, A., Haddar, A., Ali, N. E. H., Ghorbel-Frikha, B., Kanoun, S., & Nasri, M. (2008). Stability of thermostable alkaline protease from Bacillus licheniformis RP1 in commercial solid laundry detergent formulations. *Microbiological Research*, *163*(3), 299–306.

Stoner, M. R., Dale, D. A., Gualfetti, P. J., Becker, T., Manning, M. C., Carpenter, J. F., & Randolph, T. W. (2004). Protease autolysis in heavy-duty liquid detergent formulations: Effects of thermodynamic stabilizers and protease inhibitors. *Enzyme and Microbial Technology*, *34*(2), 114–125.

Wu, J., Michielsen, S., & Baby, R. (2018). Impact Spatter Bloodstain Patterns on Textiles, (July), 1–9.

APPENDICES

STAIN: BLOOD

FABRICS	Distilled water	TAPzyme 50a	Spray detergent	Spray detergent without TAPzyme 50a
JERSEY	L : 49.38 a : 16.82 b : 17.27	L : 48.83 a : 16.17 b : 16.78	L : 48.43 a : 16.10 b : 16.49	L : 49.76 a : 15.82 b : 16.41
COTTON	L : 49.77 a : 18.93 b : 17.52	L : 50.46 a : 18.10 b : 17.97	L : 49.85 a : 18.21 b : 17.50	L : 48.06 a : 18.50 b : 17.46
KOSHIBO	L : 49.24 a : 20.46 b : 17.98	L : 49.41 a : 20.37 b : 17.89	L : 51.02 a : 19.67 b : 18.24	L : 50.45 a : 19.61 b : 18.05
CREPE	L : 52.38 a : 20.31 b : 19.02	L : 53.06 a : 19.71 b : 18.60	L : 50.57 a : 19.71 b : 17.93	L : 53.08 a : 19.09 b : 18.00

Figure A1: Summary of color meter reading of different types fabrics before removal performance

STAIN: BLOOD

FABRICS	Control (distilled water)	TAPzyme	Spray formulated detergent	Spray formulated detergent without TAPzyme
JERSEY	L : 53.45 a : 15.12 b : 19.43	L : 59.59 a : 12.70 b : 18.56	L : 66.80 a : 4.70 b : 21.81	L : 51.85 a : 10.14 b : 20.87
COTTON	L : 55.91 a : 14.62 b : 18.49	L : 64.69 a : 9.95 b : 19.33	L : 64.81 a : 6.56 b : 22.75	L : 56.68 a : 11.59 b : 24.73
KOSHIBO	L : 59.87 a : 12.86 b : 17.66	L : 61.16 a : 12.12 b : 17.32	L : 62.24 a : 7.66 b : 23.34	L : 58.59 a : 9.42 b : 25.44
CREPE	L : 67.63 a : 6.10 b : 27.42	L : 69.23 a : 9.99 b : 22.14	L : 75.87 a : 2.91 b : 21.40	L : 66.00 a : 12.53 b : 22.12

Figure A2: Summary of color meter reading of different types fabrics after removal performance

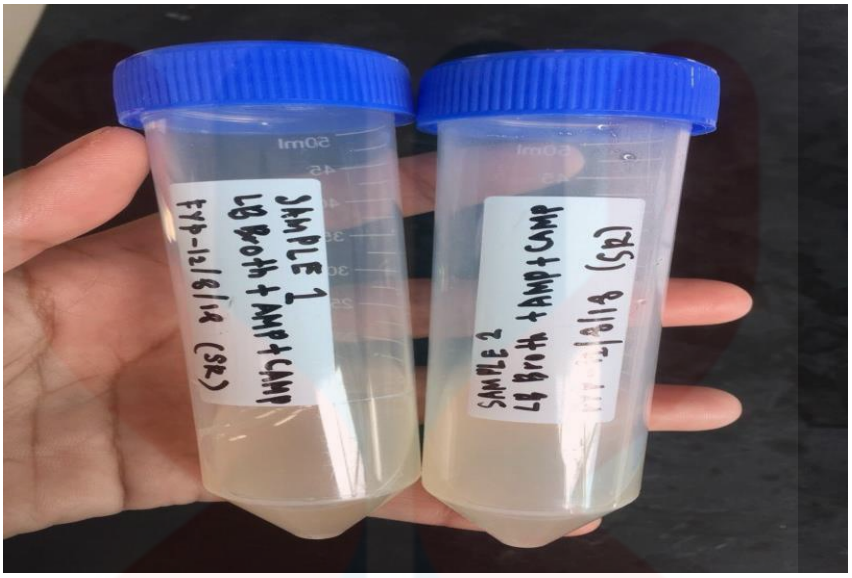


Figure A3: Two samples of combination of Luria Bertani Broth, AMP and CAMP

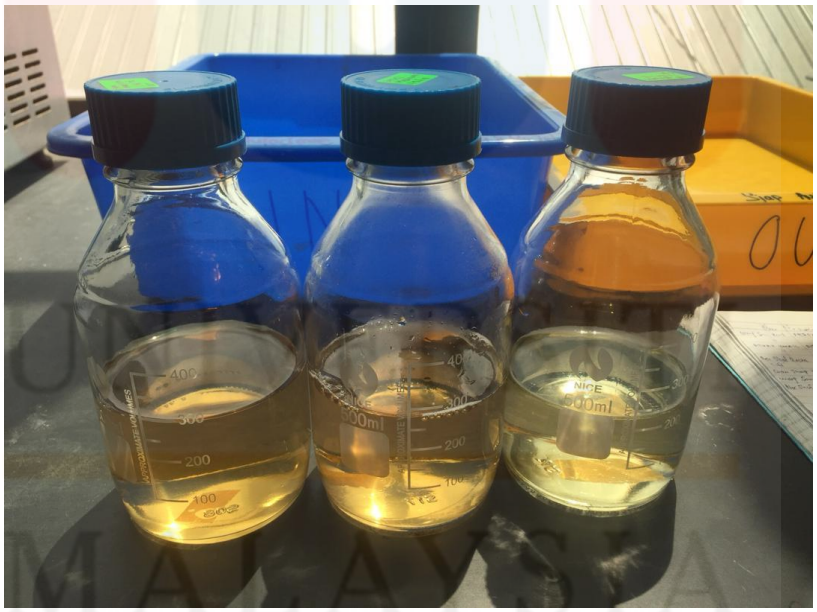


Figure A4: Three samples of basal medium before autoclave

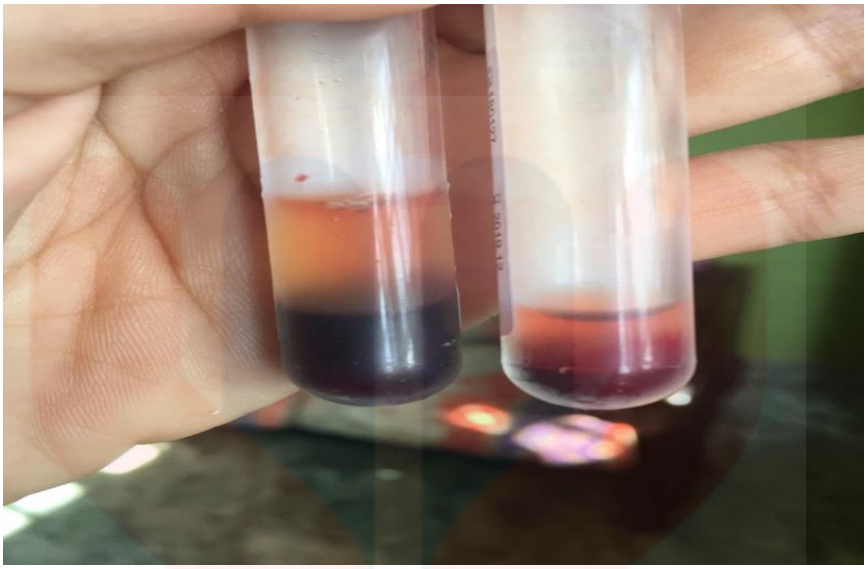


Figure A5: Blood samples were collected from volunteers UMK students were kept in Anti-coagulant EDTA tube and placed it in refrigerator

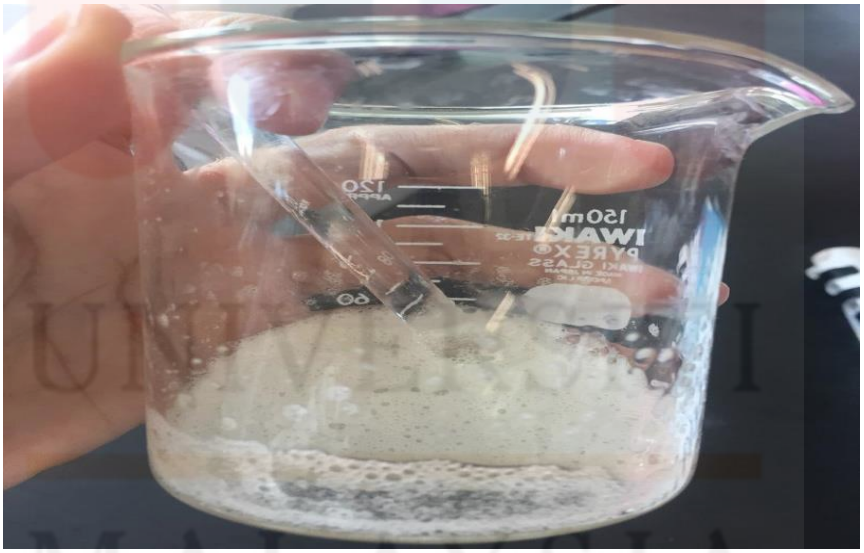


Figure A5: Preparation of formulated detergent

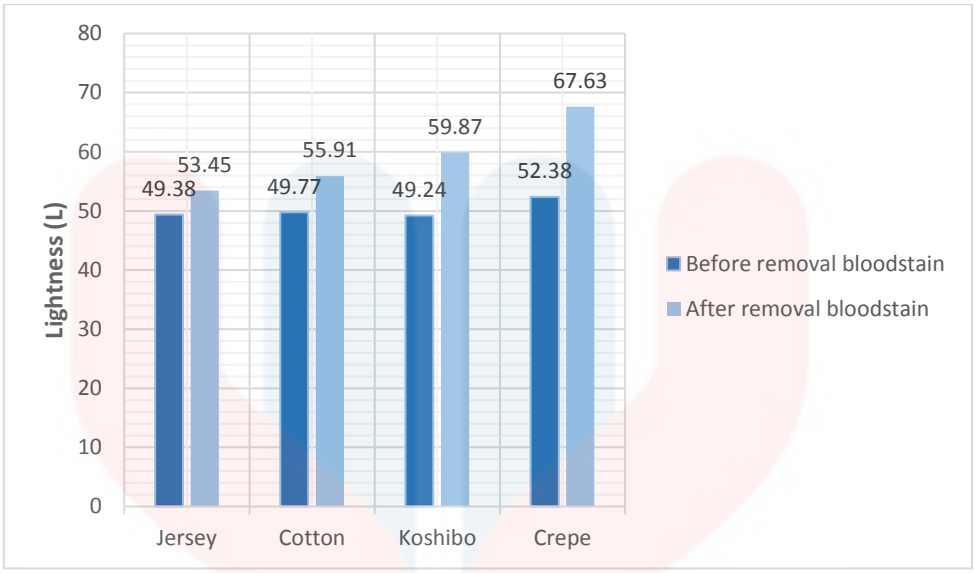


Figure A6: The lightness of different fabric by using distilled water removal bloodstains

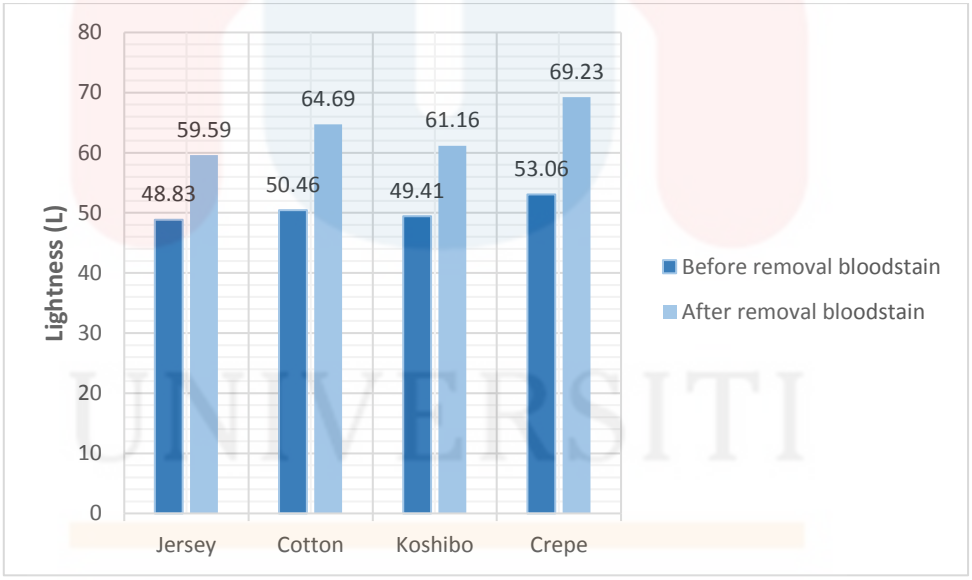


Figure A7: The lightness of different fabric by using TAPzyme 50a removal bloodstains

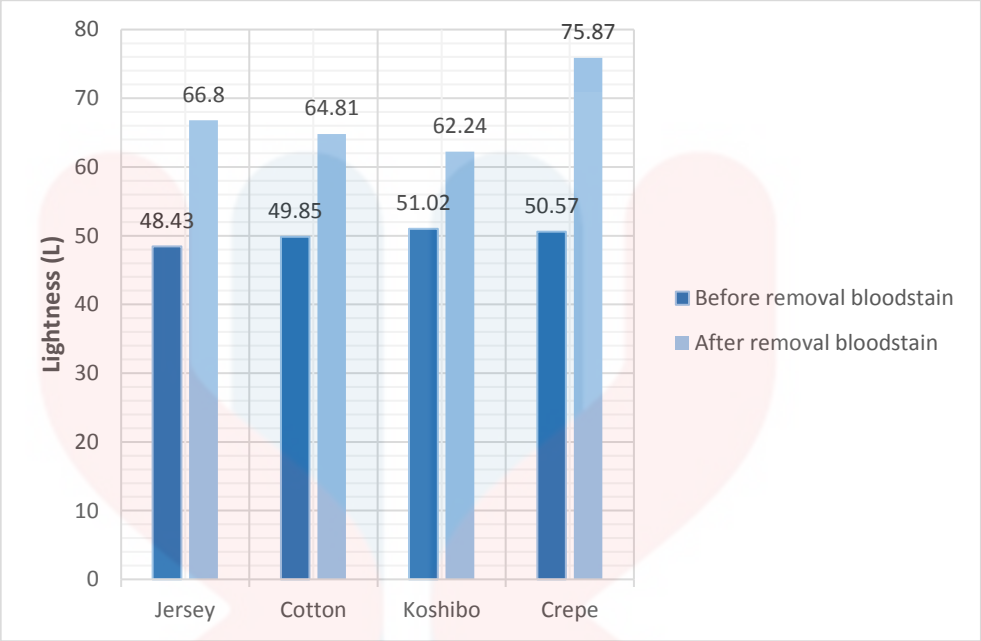


Figure A8: The lightness of different fabric by using spray detergent removal bloodstains

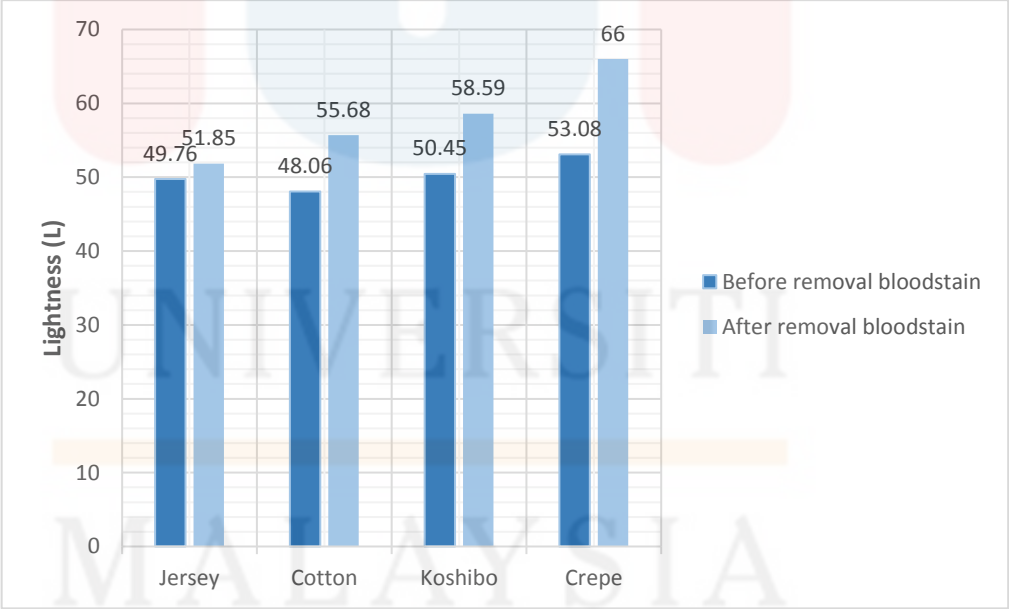


Figure A9: The lightness of different fabric by using spray detergent without TAPzyme 50a