POTENTIAL APPLICATION OF PAPAIN ENZYME FROM PAPAYA LEAVES IN MAKING ANTIBACTERIA AND ANTIFUNGAL HANDWASH AND HAND SANITIZER

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POTENTIAL APPLICATION OF PAPAIN ENZYME FROM PAPAYA LEAVES IN MAKING ANTIBACTERIA AND ANTIFUNGAL HANDWASH AND HAND SANITIZER

NGUANG SUOK LING

Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering

Faculty of Chemical & Natural Resources Engineering
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DECEMBER 2013

SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering.

Signature :
Name of main supervisor : PROFESSOR MADYA NORDIN BIN ENDUT
Position : PROFESSOR MADYA
Date :

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

Signature :
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Date : JANUARY 2013

Dedication

Special dedication to my supervisor, my parents, brothers, friends, my fellow colleague and all faculty members for all your care, support, guidance and believe in me.

ACKNOWLEDGEMENT

With my great honour, I would like to thanks the following people and organisations;

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- My colleagues from Univeristi Malaysia Pahang who were respondents for this study. By their full support, guidance and co-operation, it only able to make this study possible to complete.

ABSTRACT

This paper presents antibacterial and antifungal study of papain enzyme and its potential in making hand wash and hand sanitizer. Papain enzyme could be obtained from papaya latex, fruits and leaves. In this research, papaya leaves chose for the papain extraction because it is biomass and easily to get the source. The extraction method used in this research is hot water extraction follow by the Folin & Ciocalteu papain analysis method. Before making the antibacterial and antifungal handwash and hand sanitizer, antibacterial and antifungal test was done in order to proof the antibacterial and antifungal properties of papain enzyme. Antibacterial and antifungal test were done by incubating the pathogens on the nutrient agar in incubator for three days then insert papain enzyme onto it then incubate for another three days to determine the mycelial growth of pathogens. A volume of 187mL of papain solution was extracted by 21.55g and 200mL water. During the papain enzyme analysis, dark blue solution formed when Folin & Ciocalteu reagent was added into the solution sample. The blue colour indicate the present of tpapain enzyme. Enzyme concentration was measured in 0.415μm while the enzyme activity is 0.2283unit/mL. It means that 10mL of the papain solution extracted contains 2.283 unit of papain enzyme. The mycelial growth of the *Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* bacteria and *Saccharomyces cerevisiae, Rhizopus spp. and Mucor spp*fungus were obviously decline 3 days after the papain solution added. Antibacterial and antifungal handwash and hand sanitizer were successfully produced at the end of this research.

ABSTRAK

Kajian ini membentangkan ciri-ciri anti-bakteria dan anti-kulat dalam enzim papain dengan potensi aplikasinya dalam pembuatan sanitizer tangan dan pencuci tangan. Enzim papain boleh didapati daripada 'latex' betik, buah dan daun betik. Dalam kajian ini, daun betik dipilih untuk pengekstrakan enzim papain kerana ia merupakan biojisim dan mudah didapati. Kaedah pengekstrakan yang diguna adalah 'Hot Water Extraction' diikuti dengan analisis papain 'Folin & Ciocalteu'. Sebelum membuat sanitizer tangan dan pencuci tangan, ujian anti-bakteria dan anti-kulat telah dijalankan untuk membuktikan ciri-ciri anti-bakteria dan anti-kulat yang terdapat dalam enzim papain. Ujian anti-bakteria and anti-kulat dijalankan dengan merangsangkan pathogen atas agar nutrien di dalam incubator selama tiga hari. Kemudian, enzim papain dimasukkan atas patogen dan dirangsangkan lagi selama tiga hari untuk menentukan pertumbuhan mycelial dalam patogen. Sebanyak 187mL larutan enzim papain telah diekstrak dari 200mL air. Semasa menjalankan analisis enzim papain, larutan biru tua menentukan larutan tersebut menpunyai komposisi enzim papain. Kepekatan enzim yang diukur melalui analisis enzim adalah 0.415μm dan aktiviti enzim pula ialah 0.2283unit/mL. Ini menunjukkan, dalam 10mL larutan enzim papain yang diekstrak, terdapat 2.283 unit enzim papain dalam larutan tersebut. Pertumbuhan mycelial *Staphylococcus aureus, Escherichia coli dan Bacillus subtilis bacteria dan Saccharomyces cerevisiae, Rhizopus spp. and Mucor sppfungus* telah menurun dengan jelas dari sudut pandangan penglihatan selepas tiga hari enzim papain ditambah. Anti-bakteria dan anti-kulat telah berjaya dibuat di akhir pengajian ini.

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LIST OF ABBREVIATIONS

AEBSF	4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride
ACN	Acetonitrile
BAEE	N-benzoyl-L-arginine ethyl ester
CATH	Class, architecture, topology, homologous superfamily
EDTA	Ethyl-diamine-tetra-acetic acid
E-64	Trans-Epoxysucciny-L-leucyl-amido(4-guanidino) butane; (L-3-trans-Carboxyoxiran-2-Carbonyl)-L-Leucyl-Admat
GMP	Good manufacturing practice
HCl	Hydrochloric acid
HWE	Hot water extraction
MAE	Microwave-assisted extraction
NaOH	Sodium hydroxide
PDE	Permitted daily exposure
PMSF	Phenylmethyl sulfonyl fluoride
PSE	Pressurized solvent extraction
SCFE	Supercritical fluid extraction
SCWE	Subcritical water extraction
SE	Soxhlet extraction
THF	Tetrahydrofuran
TLCK	Tosyllysine chloromethyl ketone hydrochlorid
TPCK	Technology pedagogical content knowledge
UV	Ultra violet

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Papaya belongs to *Carica*ceae family under kingdom Plantea. It originates from Mexico and South America. Papaya also called as pawpaw in Australia (Morton et al., 1987). The botanical name of papaya is *Carica* papaya. It is a small tropical tree with a straight stem marked by scars where leaves have fallen from it directly. There are many varieties of papaya, but the main varieties grown in the United State are Red Lady, Maradol, and various Solo types. According to Edison Frod (2011), to successfully grow a papaya tree, a frost free climate, lots of sunlight, lots of water and good soil needed. It is fast growing plant because it can grow up with fruits within 6 to 12 months.

Papaya fruits are melon-like, oval to nearly round, pyriform, or elongated club-shaped. They are 12-50cm long, and 10-20cm thick. The skin is waxy and thin but fairy tough. The fruit is varying size and weight, and can range from few 100g up to 10kg. (Augstburger et al., 2000). Christopher Columbus (2012) reputedly referred to the tropical fruit papaya as 'fruit of the angels'. It is rich in vitamin A, E, K and B, fiber, calcium, magnesium, phosphorus and zinc, as well as the essential nutrients lycopene, folate, lutein and enzymes.

Carica papaya plants produce natural compounds (annonaceous acetogenins) in leaf bark and twing tissues. The leaves are large, usually are 50-70cm in diameter, deeply palmately lobed, with seven lobes. Papaya leaf is an herbaceous tree with a stem of

spongy, soft wood that is hollow in the centre and bear melon-like fruit. Rajesh Sharma (2010) claims that papaya leaf is an excellent treatment for digestive disorders and extremely useful for any disturbance of the gastrointestinal tract. Efficacy papaya leaves has been widely used for traditional medicine in various countries particularly in a country where many overgrown papaya plants such as Indonesia, Vietnam and Australia. Although it is bitter, it is very good for the health of our bodies.

Several scientific investigations on the biological activities had been done through the leaves. Study shows the leaves are highly anti-humour and pesticidal properties. It was suggested that a potentially lucrative industry based simply on production of plant biomass could develop for production of anti-cancer drugs, pending Food and Drug Agency approval, and natural (botanical) pesticides (McLanghlin, 1992). The high level of natural self-defence compounds in the tree makes it high resistant to insect and disease infestation. (Peter, 1991). Atta, 1999 stated the fresh n green pawpaw leaf is an antiseptic, whilst the brown and dried pawpaw leaf is the best as a tonic and blood purifier. In addition, fresh and green papaya leaf has a therapeutic value due to its antiseptic quality. It cleans the intestines from bacteria. The tea made by pawpaw leaf promotes digestion and aids in treatment of ailments such as chronic indigestion, overweight and obesity, arteriosclerosis, high blood pressure and weakening of the heart.

Pawpaw leaf contains many active components that can increase the total antioxidant power in blood and reduce lipid peroxidation level, such as papain, chymopapain, crystatin, tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides and glucosinolates. (Otsuki, N et al., 2010). In French Guiana, both leaf and root are prepared in combination with Quassia amara, Euterpe oleracea and Citrus species for the treatment of malarial fever. (Vigneron et al., 2005). According to the folk medicine, papaya latex can cure dyspepsia and also applicable for external burns and scalds. Dried and pulverized leaves are sold for making tea; also the leaf decoction is admistered as purgative for horses and used for the treatment of genetic urinary system.

Papaya fruits are widely consumed in food industry, while the processing of papaya fruits, papaya leaf is not consumed in any industry and lastly the papaya leaf dried and fallen from the papaya tree which will yield the garbage from the papaya tree. In order to solve such problem, study on the papaya leaf had been done. From the previous study

done by Simmonne, (2005), papain enzyme from the papaya leaf yields the antibacterial and antifungal behaviour. As referring to research title, productions of antifungal hand wash and hand sanitizer by the natural enzyme are important for the bio green product which is more preferable than the chemical made product of hand wash and hand sanitizer. It is also environmental friendly and hazardless products which solve the garbage formation by papaya tree.

Papain enzyme can be obtained from papaya fruits, latex and roots. Papaya leaf has been chosen for the extraction for papain extraction because it provides the best potential for further commercial form. First, it is by-product of the papaya trees and low cost of raw materials which means we earn higher profit in selling our product. Papaya fruits consumed wisely in food industry, and it is wasted if the papaya fruit used only in extraction of papain enzyme since it contains a lot of nutrients and vitamins that beneficial to the consumer. While for papaya latex, the amount of latex produced by a papaya tree is not in large value, it is harder for us to extract the large amount of papain enzyme in the production of antifungal hand wash and hand sanitizer. Besides that, the cost for papain extraction from papaya latex is much higher compare with the extraction from papaya leaf. Papaya roots are the most important component for a papaya tree to survive. Once the roots are being cut, a papaya tree will die directly. It is a big waste of scarifying a papaya tree just for the papain enzyme extraction by papaya roots. As a conclusion, papaya leaf is the best choice and most preferable materials used for the papain enzyme extraction.

Normally, papain enzyme is obtained by extraction process. By mixing the chemical or physical or mechanical is the process of extraction. The extraction methods are Subcritical Water Extraction (SCWE), Microwave-Assisted Extraction (MAE), Soxhlet extraction(SE), Supercritical Fluid Extraction (SCFE), Pressurized Solvent Extraction (PSE) and Hot Water Extraction (HWE). All these method are applicable for papain enzyme extraction and for this research study, hot water extraction method is chosen for the papain extraction.

The most preferable extraction method for papain enzyme extraction is hot water extraction. Hot water extraction is and extraction method which is easy to be applied by industry without requiring expensive extraction equipment. In order to have higher amount of extraction, pre-treatment and ultrasonication process applied before proceed

with extraction process. This is because purpose of pre-treatment process is to breakdown the cell wall of the papaya leaves and ultrasonication is to disrupt the intracellular of the cell. In University Malaysia Pahang, we do have the equipment required for the hot water extraction. There are Daihan ultrasonic cleaner and BS-21 shaking water bath.

1.2 Motivation, problem statement and brief review

Many microbes are present in the intestinal tracts of humans and animals. These are known as fecal microorganism. Simmonne, (2005) claims that a person's hand arms, or fingers may contaminated with faecal microorganisms after using toilet. Papain enzyme from the papaya leaf yields the antibacterial and antifungal behaviour, the papain which is a sulfhydryl protease is one of the most commonly used enzymes in various industries including food, tanning and pharmaceutical industries (Prakash et al., 2009). A study by Kamalkumar et al., (2007) shows that the papain has been used in meat tenderizers and in face and hair care products. It is also increasingly being used in pharmaceutical preparations and in such diverse manufacturing applications as leather, wool, rayon and beer. With the evidence supported, papain enzyme plays antifungal and antibacterial role, it could be functional as bio green soap, hand wash and hand sanitizer which enable to remove and inhibit the growth of the fungi and bacteria.

There are several choices of selection in making the, hand wash and hand sanitizer, for example, aloe vera, peach, lemon and some other plant. In Malaysia, papaya is the common plant, which could be seen in every state. By referring to Edison Frod (2011), to successfully grow a papaya tree, a frost free climate, lots of sunlight, lots of water and good soil needed. It is suit to Malaysia climate. Papaya is fast growing plant because it can grow up with fruits within 6 to 12 months. By using the by-product of papaya tree, the papaya leaf is beneficial in business and environmental friendly compare to using the other fruits such as, peach and lemon. In addition, peach could not plant in Malaysia, we have to import from the other country, such as China. For lemon, it had been used wisely in food industry; it could be harder in getting the large amount of lemon in producing the other product. On the other hand, only papaya fruits being consumed, papaya leaf, the by-product of papaya tree usually not being used in any industry and died all the time. It could be easy in harvesting the large amount of papaya leaf in extraction of papain enzyme for making the antifungal products. Based on the traditional use of the papaya leaf by Neuwinger, (2000), it is used as treatment for

numerous maladies, ranging from gastrointestinal disorder to asthma and also as anthelmintic.

1.3 Objective

The following are the objectives of this research:

- Extract papain enzyme from papaya leaf
- Analyse the papain enzyme
- Study antifungal and antibacterial properties of papain enzyme
- Application of papain enzyme in production of antifungal and antibacterial handwash and hand sanitizer.

1.4 Scope of research

In order to achieve the objectives of this research study, several scope of study had been done. The scope in this research study is to make use of the waste disposal of papaya leaf which might cause the environmental problems. Throughout this research study, we have study the benefit of using the papaya leaf and also the papain enzyme's characteristic. In addition, we also study the extraction method of papain enzyme by hot water extraction. After that, we have to analyse the product of the extraction by Folin and Ciocalteu method to ensure that the product of extraction is papain enzyme.

Once we done the process of extraction, we will undergo antifungal test with *saccharomyces cerevisiae, Mucor spp and Rhizhopus spp.* species and antibacterial test with *staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* species. With this antifungal and antibacterial test, we can prove that papain enzyme is an antifungal and antibacterial enzyme. Next, we will apply the papain in making handwash and hand sanitizer. Apart from that, applications of antifungal papain enzyme in hand wash n hand sanitizer making could be done through this research study.

1.5 Organisation of this thesis

The structure of the reminder of the thesis is outlined as follow:

Chapter 2 provides a description of the applications of papain enzyme and general fact of papaya leaf, papain enzyme, hot water extraction and Folin and Ciocalteu method. A

general description of antifungal test is presented. This chapter also provides the calibration curve of papain enzyme and a brief discussion of the advanced experimental techniques available for hot water extraction and Folin and Ciocalteu method their applications and limitations for papain analysis. A summary of the previous experimental work on papaya leaf extraction is also presented. A brief discussion on the methods for handwash and hand sanitizer making are also provided.

Chapter 3 gives a review of the extraction, enzyme assay, antifungal test, handwash and hand sanitizer procedures. The procedures start with the sample preparation then follow by the pre-treatment process, ultrasonication then lastly by hot water extraction for the extraction part. For the Enzymatic assay, we using the Folin and Ciocalteu method, then follow by using UV spectrophotometer. Then, the experimental data are collected and compare with the calibration curve. Once the enzymatic assay was done, we proceed with the antifungal test for papain enzyme to study the antifungal property of papain enzyme. Lastly, papain enzyme is used to make antifungal handwash and hand sanitizer. All the full description of each step is discussed in this chapter.

Chapter 4 is devoted to the result obtained. Discussion of the result obtained is presented in this chapter. A calculation of extraction yield is performed in this chapter. Experiment data of the enzymatic assay are preform and comparison of the experimental data with the calibration curve is done in this chapter. Antifungal test on the different of the diameter growth of the fungal is shown and discussion made.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

This paper presents the experimental studies of papain enzyme property and the application of papain enzyme in making handwash and hand sanitizer. All the properties, specificity, characteristic, stability, structure, calibration curve, and setting of papain are discussed in literature review.

2.2 Introduction

Pawpaw (*Carica* papaya L.) is the most economically important fruit in the *Carica*ceae family (Oliver-Bever, 1986). Originally papaya is derived from the southern part of Mexico, *Carica* papaya is a perennial plant, and it is presently distributed over whole tropical area. It is an erect fast growing and usually unbranched tree or shrub. Although it is native to Central America, it has been transported to many parts of the tropics (Samson, 1986). The ripe fruit of the pawpaw plant is commonly consumed as food in different parts of the world. However, the unripen fruit is used as mild laxative, for diuresis, as galactogogue and as an abortifacient agent (Gill, 1992). Many parts of the plant are employed in the treatment of several ailments; for example the seed is used for expelling worms, and the seed and the roots are also used as abortifacient agent. The leaves (especially fallen ones) are used variously for the treatment of fever, pyrexia,

diabetes, gonorrhoea, syphilis, inflammation and as dressing for foul wounds (Gill, 1992). Some of the scientifically validated uses of *Carica* papaya include the abortifacient activity of the seeds (Oderinde et al., 2002), the effects of the seeds on germinal epithelium of the seminiferous tubules (Uche-Nwachi et al., 2001), the fruit juice for lowering blood pressure (Eno et al., 2000), the wound healing effects of the leaves (Starley et al., 1999; Mikhal'chik et al., 2004), and several other studies.

Figure 2-1 Papaya tree

2.3 Papaya Leaf

Pawpaw (*Carica* papaya L.) is the most economically important fruit in the *Carica*ceae family (Oliver-Bever, 1986). It is an erect fast growing and usually unbranched tree or shrub. Although it is native to Central America, it has been transported to many parts of the tropics (Samson, 1986). Gill, (1992) claims that the leaves, especially fallen ones are used variously for the treatment of fever, pyrexia, diabetes, gonorrhoea, syphilis, inflammation and as dressing for foul wounds.

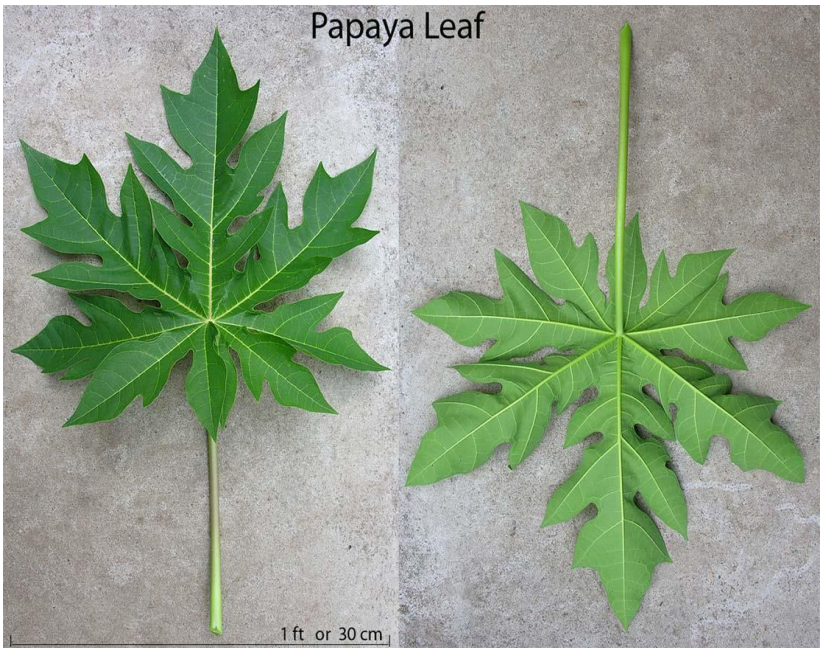

Figure 2-2 Papaya leaves

2.4 Cell membrane

Cell membrane is a biological membrane that separates the interior of the cells from the outside environment. It is selectively permeable to ions and organic molecules which control the movement of substances pass through the membrane. (Albert et al., 2002). The surrounding of the cell membrane is surrounded by phospholipid bilayer with embedded proteins. By referring to Belter, Cussler, & Wei-Shou., (1988), the basic envelop for Gram-negative cell, shown in the figure below, has three layers. The outer layer, about 8nm thick, consists of a polymer containing both protein and lipopolysaccharide. The second thinner layer, of peptidoglycan, exists in one form or another in virtually all species. Below this second layer is a gap, called the periplasmic space, which is also 8 nm thick. Enzymes are often located in this gap.

Gram-positive procaroytes are missing the first outer layer, but have both second peptidolycan layer and the periplasmic space. The third membrane, called the plasma membrane or the inner membrane is common to both Gram-positive and Gram-negative organisms. It consists largely of phospholipids, but also contains dispersed protein and metal ions. These lipid molecules have two parts, a hydrophobic part and hydrophilic part.

These three layers have different functions. The outer membrane and the peptidoglycan layer provide mechanical strength; it is their rupture. The weaker plasma membrane, the innermost layer controls the permeability of the cell, including transport of nutrients into the cell's interior and export of metabolites into the surrounding solution.

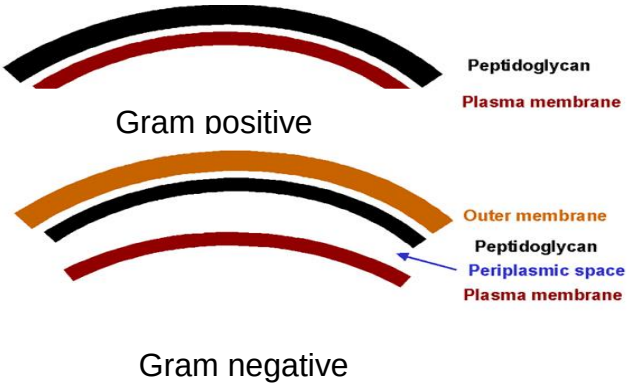

Figure 2-3 A schematic picture of cell wall by Fox, A., (2011)

2.5 Enzyme

Enzymes are protein in nature. Malvee, (2007) stated that, enzyme can be extracted from living tissues, purified and crystallized. Under controlled condition of isolation they retain their original level of activity and in some cases exhibit increased activity. Consequently purified enzyme can be used to carry out biochemical reaction outside the cell. This property of enzymes can be used in laboratory experiments and for commercial production of several important biochemical compound, drugs and industrial products. Therefore, enzyme research is an important area of biotechnology. For this research study the enzyme that we are going to study is papain enzyme which extracted from the papaya leaves.

2.6 Papain

Papain is a plant proteolytic enzyme for the cysteine proteinase family cysteine protease. Papain is found naturally in papaya (*Carica* papaya L.) manufactured from the latex of raw papaya fruits. Cohen et al. (1986) stated that it is very stable even at elevated temperature. Amri and Mamboya, (2012) stated that papain is able to break down organic molecules made of amino acids, known as polypeptides and thus plays a

crucial role in diverse biological processes in physiological and pathological states, drug designs, industrial uses such as meat tenderizers and pharmaceutical preparations.

Papain was a highly active endolytic cysteine protease from *Carica* papaya. It is stable in harsh conditions and active at low and high temperature. It also is less expensive than microbial enzymes beside has wide range of specificity and good thermal stability amongst other proteases. With such unique characteristics, papain has potential used in detergents. Papain can be chemically modified by different dicarboxylic enhydrides of citraconic, phthalic, maleic and succinic acids as Lysine residues are not a part of active site in papain. Abraham & Sangeetha, (2006) claims these anhydrides react with the ε-amino group of lysine residues and change its charges from positive to negative, leading to a shift in pH optima of the enzyme from 7 to 9.

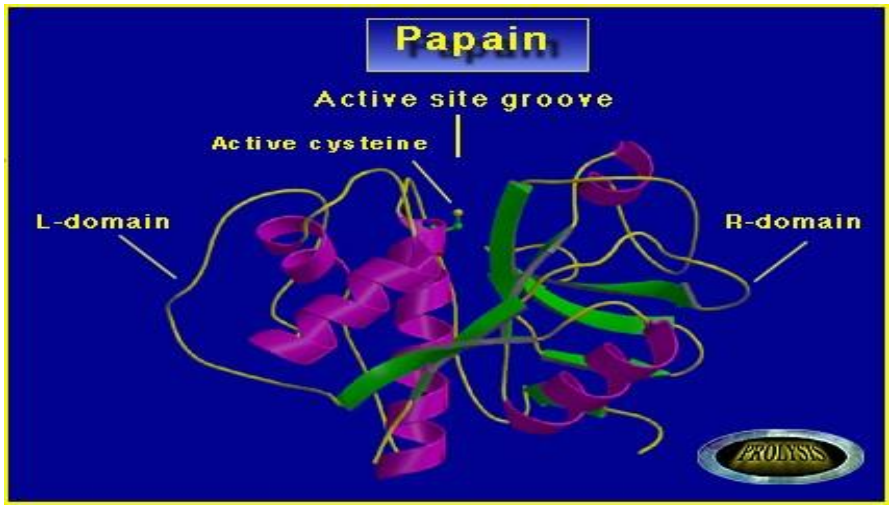

Figure 2-4 Papain structure by Calvero, (2007)

2.6.1 Papain specify

Papain has fairly broad specificity. It has endopeptidase, amidase and esterase acivities. Schechter and Berger, (1967) claims the active site consists of seven subsites (S1-S4 and S1'-S3') that can each accommodate one amino acid residue of a substrate (P1-P4 and P1'- P3'). Specificity is controlled by S2 subsite, a hydrophobic pocket that accommodates the P2 side chain of the substrate. Papain exhibits specific substrate preferences primarily for bulky hydrophobic or aromatic residues at this subsite. (Kimmel and Smith, 1954). Outside the S2 subsite preferences, there is a lack of clearly defined residue selectivity.

2.6.2 Papain composition

Papain is a single-chained polypeptide with three disulphide bridges and a sulfhydryl group necessary for the activity of the enzyme. It is expressed as an inactive precursor, prepropapain. By referring to Vernet et al. (1995) the formation of active papain requires several cleavage steps including an initial cleavage of the 18 amino acid preregion (the signal sequence), follow by further cleavage of the glycosylated 114 amino acid proregion.

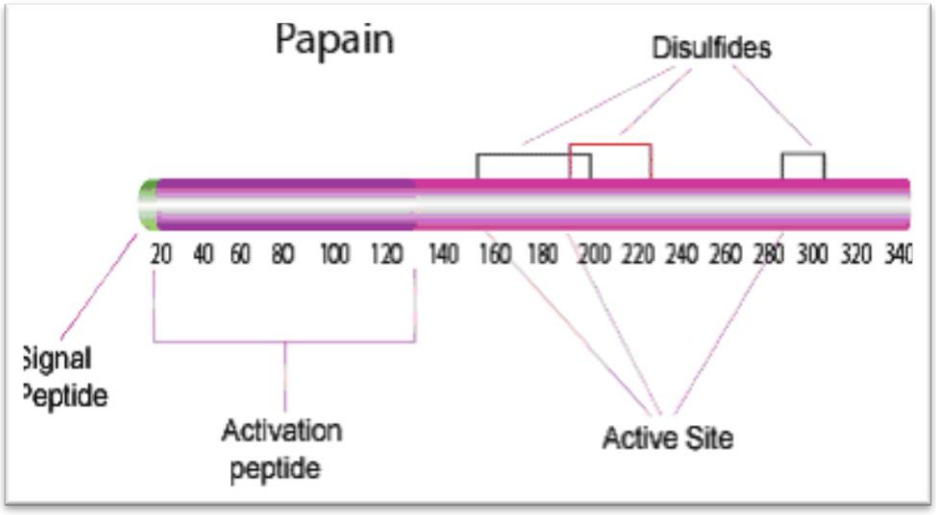

Figure 2-5 Papain composition by Margossian & Lowey, (1973)

2.6.3 Molecular Characteristics of papain

Azarkan et al., (2003) mention that mature forms of all papaya proteinases are between 212 and 218 amino acids, and exhibit a strong degree of homology. Maes et al., (1996) claims that X-ray structure analysis has shown that they adopt identical three dimensional folds.

2.6.4 Papain property

Protein Accession Number: P00784

CATH Classification:

- Class: Alpha Beta
- Architecture: Alpha Beta Complex
- Topology: Cathepsin B; Chain A

Molecular weight: 23.4kDa (Theoretical)

Optimal pH: 6.0- 7.0

Isoelectric Point: 8.88 (Theoretical)

Extinction Coefficient:

- 53610 cm-1M-1
- E1%, 280 = 22.88 (Theoretical)

Active Site Residues:

- Cysteine (C158)
- Histidine (H292)
- Asparagine (N308)

Activators:

- Cysteine
- Sulphide and sulphite
- Heavy metal chelating agents like EDTA
- N-bromosuccinimide

Inhibitors:

- PMSF
- TLCK, TPCK
- alpha2-macroglobulin
- Hg+ and other heavy metals
- AEBSF
- Antipain
- Cystatin
- E-64
- Leupeptin
- Sulfhydrl binding agents

- Carbonyl reagents
- Alkylating agent

All the papain properties adopt from Rozman-Pungercar et al., (2003)

Following are the data of papain activity toward pH, temperature and substrate concentration.

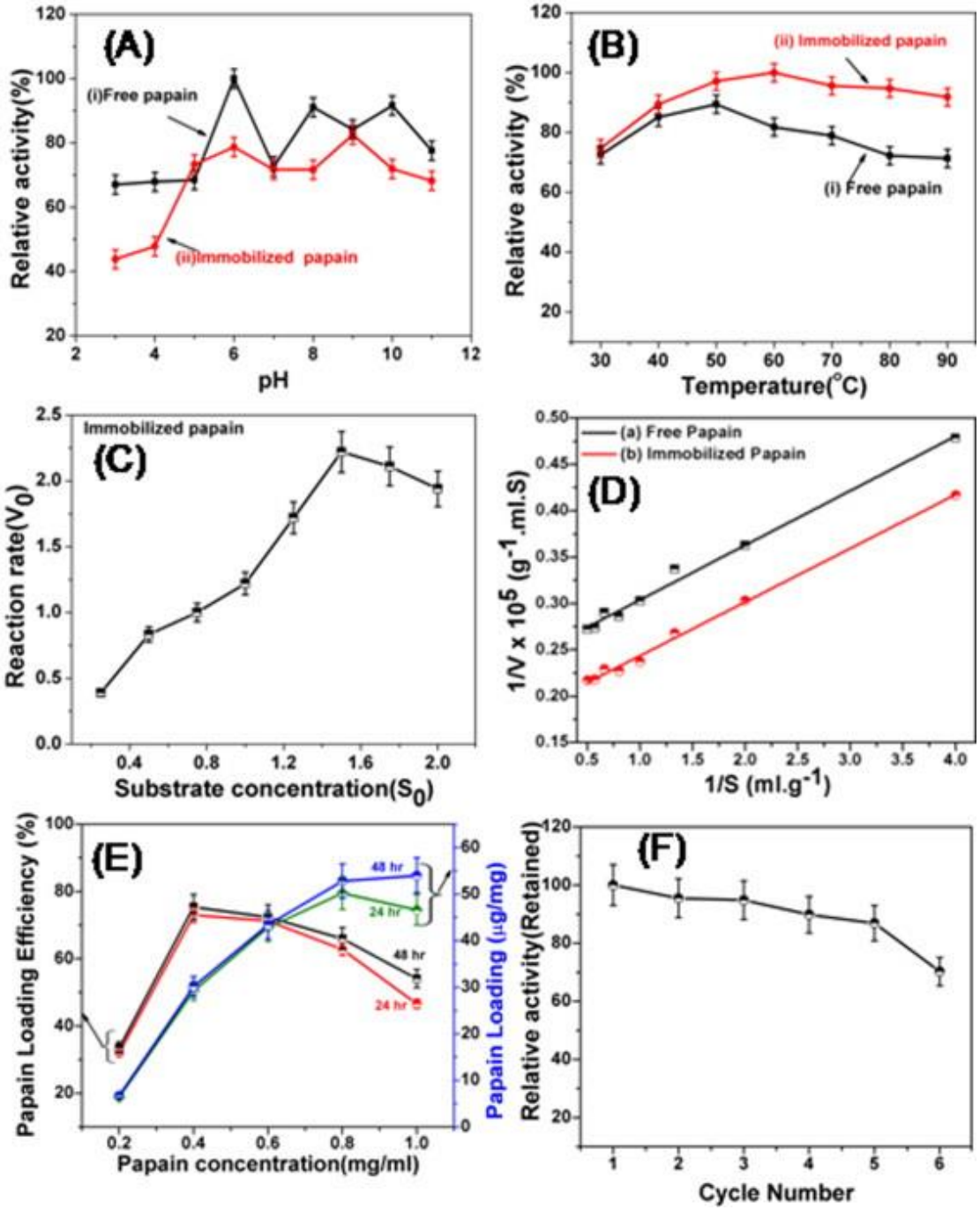

Figure 2-6 Papain activity toward parameters by Sahoo et al., (2013)

Data from Figure 2.6 are important for the setting for the extraction temperature and pH. In order to yield the higher extraction of papain enzyme, all the settings have to refer the data from Figure 2.6.

The unique structure of papain gives it the functionality that helps elucidate how proteolytic enzymes work and also makes it valuable for a variety of purposes. In the present review, its biological importance, properties and structural features that are important to an understanding of their biological function are presented. Its potential for production and market opportunities are also discussed.

2.6.5 Papain solubility and solution stability

Arnon, R., (1970) stated that papain is soluble in water at 10 mg/ml. Immediately prior to use, the enzyme is typically diluted in buffer containing ~5 mM L-cysteine. Activation/stabilizing agents include EDTA, cysteine, and dimercaptopropanol.

Although papain solutions have good temperature stability, the solution stability is pH dependent. Papain solutions are unstable under acidic conditions, i.e., at pH values below 2.8, there is a significant loss in activity. For the active enzyme in solution, the loss in activity is about 1-2% per day, probably as a result of autolysis and/or oxidation.

A common inactive form of papain obtained during isolation is a mixed disulfide formed between the active site sulfhydryl group of the protein and free cysteine. Papain solutions are stable to several denaturing agents, i.e., full activity is maintained after recrystallization in 70% methanol and in 8 M urea solutions. However, there is a significant loss in activity when papain is exposed to 10% trichloroacetic acid or to 6 M guanidine hydrochloride.

Papain enzyme is stable toward 2-8˚C for 6-12 months.

2.6.6 Papain thermal stability

A major limitation to industrial use of enzyme is their relative instability under operational condition, which involved exposure to extreme of temperature, pressure, pH, denaturant and organic solvents. The native structure of any protein is only marginally stable and will always fluctuate non-native state, the rate if which increases under extreme environmental condition. One of the major means of enzyme inactivation is thermal denaturation. There are several advantages in conducting an enzyme reaction at elevated temperature such as increase solubility of the substrate, decrease microbial

contamination, decrease viscosity and also increase reaction rate. The search for development of thermostable enzymes for use in the food and pharmaceutical industries is receiving considerable research interest. There are two options to increase the stability of a protein. The first one is to modify the solution condition and the second option is to modify the protein itself. The second option of protein modification is not recommended due to the cost factor.

Based on the previous research, the solution condition was modified to increase the stability of protein, the various substances that can act at high concentrations strongly affected stability of the folded protein. These substances are known as cosolvents and many of them are referred to stabilize the native structure of protein. (Prakash et al., 2009). Cosolvents like sugars and polyols have been known to have a profound effect on stability, structure and function of a protein. From the result, it showed that, cosolvent used namely sorbitol glycerol, sucrose and xylose can increase the thermal stability in a concentration dependent manner. These data suggested though all the cosolvents used in the above study tend to stabilize the protein against thermal denaturation. Besides that, we can store papain enzyme at 2-8˚C and do not freeze aqueous suspensions

2.6.7 Papain stability toward organic solvent

Water-miscible organic solvents can interact with enzyme and the water molecules associated with the protein structure but it can reduces the activity or stability of the enzymes. Using of aqueous organic solvent as extraction media can change the structural activity of papain due to the decreasing of hydrolytic activity and the number of active sites of papain. In the previous research, the stability of papain in aqueous organic solvents such as ethanol,1,4-dioxane, tetrahydrofuran (THF) and acetonitrile (ACN) were studied. For the result, the effects of the organic solvents on the activity of papain in aqueous solution were different. THF proved to be the most destabilizing solvent for papain while ACN, ethanol and 1,4-dioxane exhibited high stability in aqueous. Activity of the enzyme was decreased with organic solvent at concentration above 60% (Gupta, A., & Khare, S. K., 2005)

Papain should be activated before use to ensure maximum activity. The normal activation buffer is a solution containing 1.1mM EDTA, 0.067mM mercaptoethanol and 5.5mM cysteine-HCl. After 30 minutes in this solution, the enzyme is completely activated.

2.7 Application of papain enzyme

Based on Hasegawa, M., et al., (1987), papain is commonly used in cell isolation procedures where it has proven more efficient and less destructive than other proteases on certain tissues. For example, papain has been used to isolate viable, morphologically intact, cortical neurons from postnatal rats.2 Sigma's papain preparation (Product No. P4762) has been used for the isolation of smooth muscle cells.3,4 Papain was found to significantly increase the yield of viable smooth muscle cells while not affecting cell sensitivity to stimulants.

Limited papain digestion has proven useful for structural studies of enzymes and other proteins. (Margossioan, S. S., & Lowey, S., (1973). Papain is used in red cell serology to modify the red cell surface to enhance or destroy the reactivity of many red cell antigens as an adjunct to grouping, antibody screening, or antibody identification procedures. Papain has also been shown to be useful in platelet serology. (Lown, J. A., & Bale, B. J., 1995). Papain has also been used in the enzymatic synthesis of amino acids, peptides, and other molecules. (Rajesh, M., et al., 2003).

According to Newkrik, M, M., et al., (1987), Fab and F(ab')2 antibody fragments are used in assay systems where the presence of the Fc region may cause problems. In these cases it is preferable to use only the antigen binding (Fab) portion of an antibody. Papain is used routinely for the preparation of Fab fragments from IgG. IgM may also be digested with papain resulting in high yields of homogeneous Fab preparations.

Papain cleaves antibodies into two Fab fragments, which recognize the antigen specifically with their variable region, and one Fc fragment.14 It cleaves above the hinge region containing the disulfide bonds that join the heavy chains, but below the site of the disulfide bond between the light chain and heavy chain. This generates two separate monovalent (containing a single antibody binding site) Fab fragments and an intact Fc fragment. The fragments can be purified by gel filtration, ion exchange, or affinity chromatography. Protocols for antibody digestion and purification of antibody fragments can be found in Antibodies:

In tissues such as lymph nodes or spleen, or in peripheral blood preparations, cells with Fc receptors (macrophages, monocytes, B lymphocytes, and natural killer cells) are present which can bind the Fc region of intact antibodies, causing background staining in areas that do not contain the target antigen. Use of Fab fragments ensures that the antibodies are binding to the antigen and not to Fc receptors. These fragments may also be desirable for staining cell preparations in the presence of plasma, because they are

not able to bind complement, which could lyse the cells. Fab fragments allow more exact localization of the target antigen, i.e. in staining tissue for electron microscopy.

Based on Sharmilee, (2010) study, papain's exfoliating qualities aid in the skins regenerative process by facilitating the removal of dead skin cells which in turn promotes healthier skin. It could use as a shower gel and shower soap to exfoliate and promote better general skin health. It is used as an extract to promote better skin health. It is used as a topical ointment for its ability to rapidly regenerate skin cells. It is used as a dietary supplement to promote better health. Again, it supports the papain enzyme are applicable in soap, hand wash and hand sanitizer making.

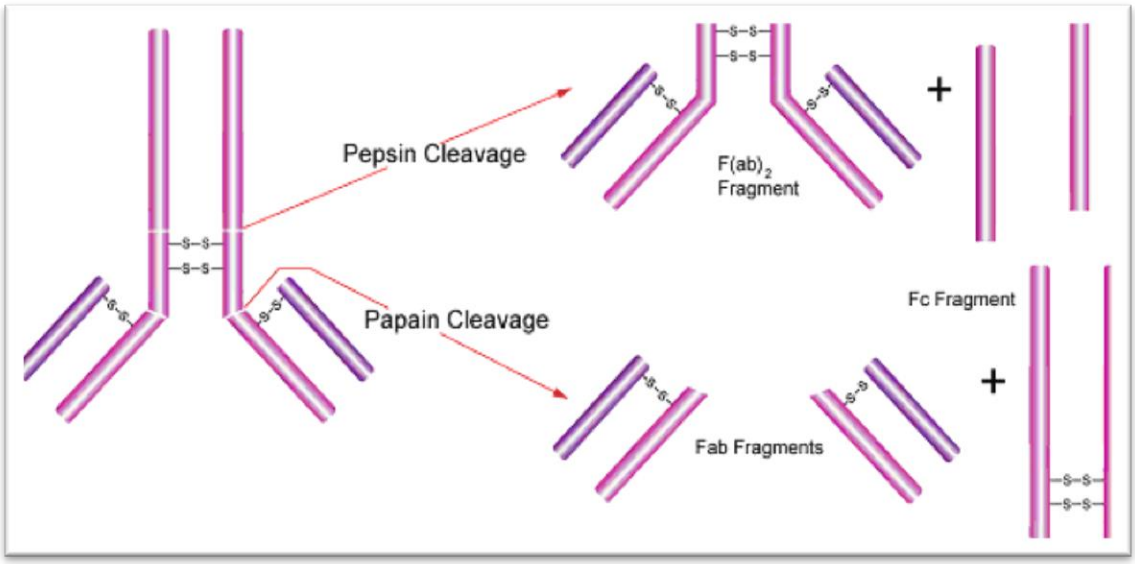

Figure 2-7 Papain Cleavage by Sepulveda et al., (1975)

2.8 Hot water extraction

The most environmentally friendly solvent that could be used for extraction of papain enzyme is water. Hot water extraction method is the simple, low cost and commercializes method for the extraction of papain enzyme from papaya leaf. In order to get better extraction, pre-treatment process, ultrasonication has to carry out first before conducting the extraction process.

2.8.1 Pre-treatment Process

Pre-treatment process is important to breakdown the cell wall of the papaya leaf. We seek to the rupture the cell wall solely to recover this protein. We want to remove only some of the layers to release specific enzymes. Mahmood, Sidik and Salmah, (2005) promoted pre-treatment process by cutting papaya fresh leaves into pieces and wash

with distilled water. Dried the cut leaves in oven at 50 ℃. The leaves were ground using a grinder. Then 50g of blended plant were weighted and placed into 100mL flask. The water was added in ratio 1:20.

2.8.2 Ultrasonication

According to Bar, (1987), in microbiology, ultrasound is primarily associated with the cell disruption (lysis) or disintegration. When sonicating liquids at the high intensities, the sound waves that propagate into the liquid media result in alternating high pressure (compression) and low pressure (rarefaction) cycle, with rates depending on the frequency. During the low pressure cycle, high intensity ultrasonic waves create small vacuum bubbles or voids in the liquid. When the bublles attain a volume at which they can no longer absorb energy, they collapse violently during a high pressure cycle. This phenomenon is termed cavitation. Shear forces break the cell envelope mechanically and improve material transfer. Ultrasound can have either destructive or constructive effect to the cell depending on the sonication parameters employed.

Under intense sonication enzymes or proteins can be released from cells or subcellular organelles as a result of cell disintegration. In this case, the compound to be dissolved into solvent is enclosed in an insoluble structure. In order to extract it, the cell membrane must be destructed. Cell disruption is a sensitive process, because the cell wall's capability to withstand high osmotic pressure inside. Good control of the cell disruption is required, to avoid an unhindered release of all intracellular products including cell debris and nuclei acids, or product denaturation. Ultrasonication servers as a well-controllable for cell disintegration. For this, the mechanical effects of ultrasound provide faster and more complete penetration of solvent into cellular materials and improve mass transfer. Ultrasound achieves greater penetration of a solvent into a plant tissue and improves the mass transfer. Ultrasonic waves generating cavitation disrupt cell walls and facilitate the release of matrix components.

The influence of continuous ultrasonic extraction to the yield of dispersed protein was demonstrated by Moulton & Wang, (1982). The sonication increased the recovery of dispersed protein progressively as the flake/ solvent ratio changed from 1:10 to 1:30. It showed that ultrasound is capable to peptize soy protein at almost any commercial throughput and that the sonication energy required was the lowest, when thicker slurries were used. The extraction of enzymes and proteins stored in cells and subcellular particles is an effective application of high-intensity ultrasound, as the extraction of

organic compounds contained within the body of plants and seeds by a solvent can be significantly improved. (Kim and Zayas, 1989). Ultrasound has a potential benefit in the extraction and isolation of novel potentially bioactive components, e.g. from non-utilized by-product streams formed in current processes.

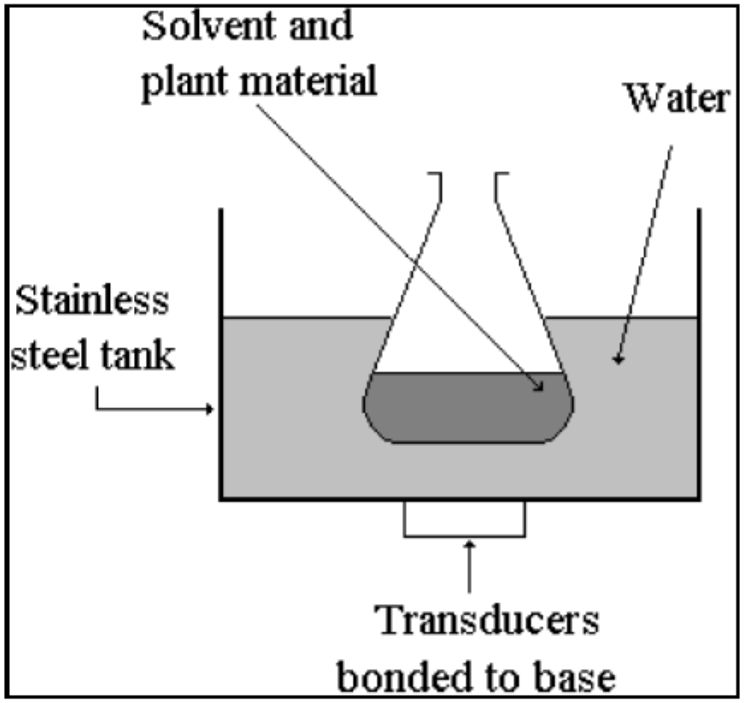

Figure 2-8 Experimental setup of cleaning bath by Vinatoru, (2001)

2.8.3 Folin Ciocalteu Method

Folin and Ciocalteu's phenol reagent does not contain phenol. Rather, the reagent will react with phenols and nonphenolic reducing substances to form chromogens that can be detected spectrophotometrically. It can also be used as a spray reagent in chromatographic procedures. Bary and Thrope, (1954) stated the color development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/ phosphotungstic acid complexes to form chromogens in which the metals have lower valence. The most common usage of this reagent is in the Lowry method for determining protein concentration (Lowry et al., 1951). In this method, protein is pre-treated with copper(II) in a modified biuret reagent (alkaline copper solution stabilized with sodium potassium tartrate). Addition of Folin and Ciocalteu's phenol reagent generates chromogens that give increasing absorbance between 550 nm and 750 nm. Normally, absorbance at the peak (750 nm) or shoulder (660 nm) is used to quantify protein concentrations between 1-100 µg/ml while absorbance at 550 nm is used to quantitate higher protein concentrations. In the absence of copper, colour intensity

would be determined primarily by the tyrosine and tryptophan content of the protein, and to a lesser extent by cysteine, and histidine. Copper (II) enhances color formation by chelation with the peptide backbone, thus facilitating the transfer of electrons to the chromogens. Peterson, (1979) claims that copper (II) has no effect on color formation by tyrosine, tryptophan, or histidine, but reduces that due to cysteine.

2.8.4 Extracting agent

The extraction solvent is important in the extraction process. It helps the extraction process run smoothly. The extraction solvent used according to the Good Manufacturing Practice (GMP) and the resultant residues or derivatives which could subsequently be present in technically unavoidable quantities should present no danger to human health. With the rules mentioned above, the extraction with other solvents condition of use has been specified for maximum residue limits in extracted foodstuffs of food ingredients. The United State Food and Drug Administration have Guidance for Industry list for the appropriate use of residual solvent. The solvent are divided it into three classes.

Class 1: Solvent which is known to cause unacceptable toxicities and should be avoided.

Class 2: Solvent associated with less serve toxicity.

Class 3: Solvent are the least toxic solvent should be used where practical.

For example, acetonitrile falls into class 2 with a permitted daily exposure (PDE) of 0.1mg/day and ethanol falls to class 3 because it has low toxic potential to humans and PDE of 50gm or more per day. These examples were cited by Maicu, (2008).

For hot water extraction, water is used as the extraction solvent. Water is non-toxic material and it is better to be used for extraction process compared to the other extraction solvent which has the toxicity no mater high or low.

2.9 Enzymatic assay

To study the enzymatic difference between laticifers and cultured cells of papaya by Yamamoto et al. (1986), the protein content were determined by method Bradford, (1976) using bovine serum albuminas a standard.

According to Ali, Segil and Azmi, (2002), enzyme activity of papin was determined by using N-benzoyl-L-arginine ethyl ester (BAEE) as substrate. The enzymatically

liberated N-benzoyl-L-arginine was titrated with 0.01M NaOH. One unit enzyme was taken to hydrolyze 1.0mmole of BAEE per min at pH 6.2 and 25˚C.

Besides that, activity of papain also can be determined by using casein as substrate. 5mL PBS for pH 7.5 and 1 mL casein solution of 10g/L were added to a 100mL conical flask. After enzymatic reaction at 40˚C for 15 min with gently stirring, 2mL CCl3COOH of 0.4mol/L was added into conical flask. The reaction mixture was statically equilibrated at room temperature for 10 min and then centrifuged. Five millilitres of 0.4 M Na2CO3 solution and 1mL of folin solution were added to a 50mL beaker including 1mL of filtrate. The beaker was incubated at 40˚C for 20 min. the absorbance of mixture was measured at 680nm. One unit of enzyme activity was defined as the tyrosine content formed per minute at 40˚C and pH 7.5. (Li, Xing, & Ding, 2007).

The content of extracted papain was determined by measuring the absorbance of the papain-loaded aqueous phase at 280nm on a UV-visible spectrophotometer (Jasco V-550, Japan). Timothy. R., Parsons, Maita. Y., & Lalli, C. M., (1984), explained that the extraction yield was calculated according to the standard curve. Pigment concentrations are calculated by taking the absorbance reading of the largest peak in a pure pigment spectrum, as characterized by a spectrophotometer, and dividing that absorbance value by the pigments specific absorption or molar absorption coefficients. Following equation denote the concentration equation.

$$Concentration\ of\ Pigment\ (mg/L) = \frac{Absorbance\ Units\ (Au)}{Specific\ Absorption\ Coefficient\ (L\ g^{-1}cm^{-1})} \times 1000$$

The derivations of these coefficients require extremely careful and highly analytical techniques but the end calculation is very simple. To obtain a specific absorption coefficient of a pigment, one would need to purify the pigment from all contaminants and either dry or crystallize the molecule. After purification, the resulting material needs to be dissolved in solvent and measured for absorbance. Keep in mind that absorbance measurements are solvent specific as well.

The specific absorption coefficient obtained from a spectrum of the pigment would serve as the universal standard for quantifying this specific pigment's concentration from any spectrophotometric measurement. Specific absorption coefficients of pigments

are usually given in volume per weight with respect to path length of the cuvette used (i.e. liter gram-1centimeter-1).

From the concentration of L-cysteine determined, the activity of papain was also performed in terms of Units, which was the amount on micromoles of cycteine equivalents released from casein per minute. The equation bellow denotes the activity of the papain enzyme.

$$\frac{Units}{mL}(Enzyme) = \frac{L - cysteine(\mu mole) \times A}{B \times C \times D}$$

Where A= Total volume (in millilitres) of assay

B= Time of assay (in minutes) as per the Unit definition

C= Volume of enzyme (in millilitres) of the enzyme used

D= Volume (in millilitres)

2.10 Amino acid correlates with papain

Papain is a cysteine protease of the peptidase C1 family. When protease digests casein, the amino acid cysteine is liberated along with other amino acid and peptide fragment. Folin's reagent will reacts with free cysteine to produce blue chromophore, which is quantifiable and measure as an absorbance value on the UV-Vis spectrophotometer. Cupp-Enyard, (2008) mentioned that absorbance value generated by the activity of the protease is compared to a standard curve. Form the standard curve, the activity of protease sample can be determined.

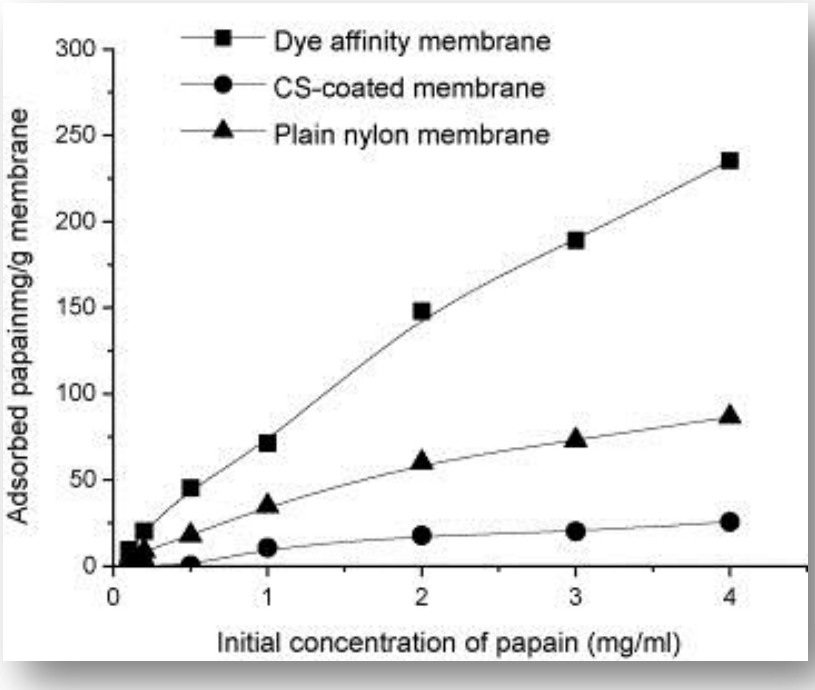

Figure 2-9 Graph of absorbance versus initial papain concentration by Nie & Zhu (2007)

2.11 Antifungal property of papain

Fungi are eukaryotic, non-chlorophyllous and heterotrophic organisms that depend on external nutrients and therefore live as saprophytes on non-living organic material, or as invasive pathogens in living tissue. They may live as parasites or symbionts of animals and plants under nearly all environmental conditions. Because they have the capability to grow under highly variable conditions, they are ubiquitous throughout most environments.

Fungi may have from a unicellular to a dimorphic or filamentous appearance. They are principally dispersed as sexual spores or asexual conidia, and are common components in the atmosphere. In addition, unidentifiable fungal hyphae fragments may be aerosolised in large numbers, which further increases the risk of human exposure through breathing.

More than 80 genera of the major fungal groups have been associated with symptoms of allergy. Most of these fungi are classified under Ascomycetes and Deuteromycetes with

a few in Basidiomycetes. Some of the most frequently occurring are Cladosporium, Penicillium, Aspergillus, Alternaria, and Aureobasidium.

The fungal spore counts in outdoor and indoor air vary considerably, depending on various environmental and other factors Fungi are highly variable, both in their morphology and in their antigenic makeup. With some fungi it is almost impossible to grow two consecutive cultures with similar antigenic profiles, although growing allergenic fungi in synthetically defined media has resulted in allergenic extracts which show less variability and demonstrate specific reactivity with patients.

Unlike many other airborne allergens, fungi are associated with a variety of illnesses besides IgE-mediated allergy. In contrast to pollen, fungi may cause adverse health effects in humans through other harmful immune response, by toxic or irritant effects, or by direct infection. The most prevalent immune disease caused by moulds is type I allergy (asthma and allergic rhinitis), but allergic bronchopulmonary mycoses, allergic sinusitis, hypersensitivity pneumonitis and atopic dermatitis may also occur. The prevalence of respiratory allergy to fungi is estimated at 20 to 30% among atopic individuals, and up to 6% in the general population.

These diseases may result from exposure to spores, vegetative cells, or metabolites of the fungi. In addition, as the fungal spores are small (usually less than 10 μm) a majority of them are capable of penetrating the lower airways of the lung and mediate allergic reactions. The conidia and fungal spores associated with immediate-type hypersensitivity are usually larger than 5 μm, while those associated with delayed-type hypersensitivity are considerably smaller, and can penetrate the smaller airways.

A feature of fungal allergies is the lack of clear evidence of disease, or of a well-defined pathology. There are various reasons for this diagnostic inadequacy, including heterogeneous disease symptoms and differences in routes and amount of exposure – but also the difficulties in characterisation and identification of the allergenic species.
Antifungal can be defied as destructive to fungi, or suppressing their reproduction or growth; effective against fungal infections. Myers, (2006) stated that, fungal infections are caused by microscopic organisms that can invade the epithelial tissue. The fungal kingdom includes yeasts, molds, rusts and mushrooms. Fungi, like animals, are

hetrotrophic, that is, they obtain nutrients from the environment, not from endogenous sources just like plants with photosynthesis. Most fungi are beneficial and are involved in biodegradation, however, a few can cause opportunistic infections if they are introduced into the skin through wounds, or into the lungs and nasal passages if inhaled. Papain has been used in pharmaceutical preparations of diverse food manufacturing applications as the production of high quality kunafa and other popular local sweets and pastries for quite a long time. Papain has been reported to improve meltability and stretchability of Nabulsi cheese with outstanding fibrous structure enhancing superiority in the application in kunafa, pizza and pastries (Abu-Alruz et al., 2009). Also as pharmaceutical products in gel based a proteolytic cisteine enzyme, papain presents antifungal, antibacterial and anti-inflammatory properties (Chukwuemeka and Anthoni, 2010).

2.11.1.1 Saccharomyces cerevisiae

Saccharomyces cerevisiae is an eukaryotic microbe. More specifically, it is a globular-shaped, yellow-green yeast belonging to the Fungi kingdom, which includes multicellular organisms such as mushrooms and molds. (Feldmann, H., 2010) Natural strains of the yeast have been found on the surfaces of plants, the gastrointestinal tracts and body surfaces of insects and warm-blooded animals, soils from all regions of the world and even in aquatic environments. Most often it is found in areas where fermentation can occur, such as the on the surface of fruit, storage cellars and on the equipment used during the fermentation process.

S. cerevisiae is famously known for its role in food production. Guo, et, al. (2008) stated that it is the critical component in the fermentation process that converts sugar into alcohol, an ingredient shared in beer, wine and distilled beverages. It is also used in the baking process as a leavening agent; yeast releasing gas into their environment results in the spongy-like texture of breads and cakes. Because of its role in fermentation, humans have known about and used *Saccharomyces cerevisiae* for a long time.

According to Kaeberlein, M. (2010), isolation of the species did not occur until 1938, when Emil Mrak isolated it from rotten figs found in Merced, California. Taking

advantage of its unique reproductive cycle, Robert Mortimer performed genetic crosses that used the isolated fig strain and other yeast strains obtained through other researchers. As a result, he created a new strain called S288c, which was then used as a parental strain in order to isolate most of the mutant strains currently used in research. Furthermore, this strain was then used to sequence the *S. cerevisiae* genome.

S. cerevisiae is also considered to be a "model organism" by scientists. Its big advantage is that it is both a unicellular and eukaryotic organism. Based on the reseach study by Tong, (2004), as a eukaryote, a majority of the yeast genes and proteins have human homologs, and a greater understanding of the yeast genome would also help scientists understand the human genome. Another advantage is its fast growth grate. On a normal yeast medium, it takes 90 minutes for the yeast population to double, and colonies are usually visible 2-3 days after placing them on fresh medium. Since the complete genome sequence is now available, mutants unique to eukaryotic organisms can now be expressed in an eukaryote as opposed to studying a similar gene in prokaryotes.

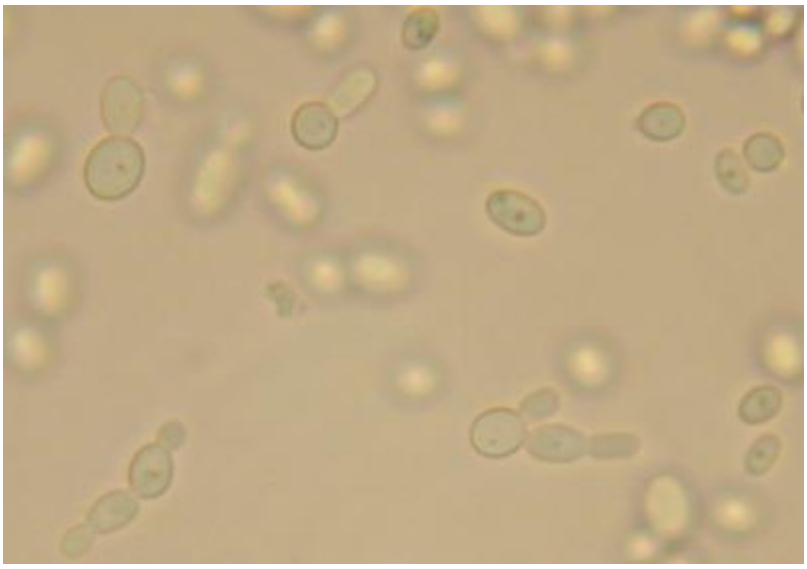

Figure 2-10 Microscope view of Saccharomyces cerevisiae

2.11.1.2 Mucor spp

Mucor spp is a dimorphic fungus belonging to the Zygomycete class that has been studied in the laboratory for 35 years. By referring to Subrahamanyam, A. (1983), researchers have been attracted to it mainly because of its dimorphism, light responses, high capacity for accumulating lipids, anaerobic and aerobic production of ethanol and gene silencing mechanisms (RNAi). In the last few years the rate of knowledge

acquisition in these fields has grown exponentially, and it is expected to grow even more in the future, due to the increasing number of molecular tools available to manipulate its genome, which allows the functional analysis of its genes. Zalar, et al. (2013), mention that *Mucor spp* is the fungus with the broadest molecular tool repertoire within the Zygomycota phylum. This includes genetic transformation using self-replicative plasmids, integrative transformation mediated by Agrobacterium, generation of knockout mutants and use of RNAi-based procedures to suppress gene function.

Mucor spp has also a biotechnological interest as a source of carotenes and lipids because it accumulates high levels of these compounds in the mycelium, has a good biomass production during submerged batch cultivation in bioreactors, it is able to use a wide range of carbon sources and it has demonstrated capacity to grow in industrial stirred-tank fermenters. (O'Donnell, et al.(1977) Nowadays, *Mucor spp* lipids have gained a special attention because they can be easily converted into biodiesel, suggesting that Mucor spp biomass could be an alternative to plant oils as a feedstock for biodiesel production.

The *Mucor spp* genome sequence will help the genomic, transcriptomic and proteomic approaches that will complement the current research carried out with this organism. It will help the identification and study of genes and proteins that participate in the processes described above and in the production of lipids useful for biodiesel production. In addition, the Mucor spp genome sequence will provide key information about the evolution of zygomycetes and other fungi.

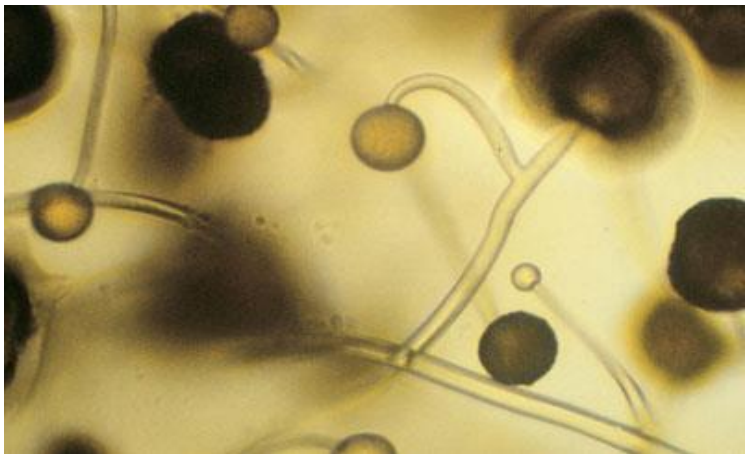

Figure 2-11 Microscope view of Mucor spp

2.11.1.3 Rhizopus spp.

Rhizopus spp. is the fungus commonly known as bread mould, and is the most common species of Rhizopus. It is found on old food and in soils, and even in children's sandboxes. The genus contains some 50 species, and bread mould is sometimes confused with species of Mucor or other species of Rhizopus such as Rhizopus oryzae. Rhizopus is closely related to Mucor and inhabits the same ecological niches. (Rohm, et al, 2010). The spores are dispersed in hot, dry weather.

Other typical microhabitats include fresh or decaying litter such as pine needles and leaves. Other known substrates are sweet potato, cold-stored strawberries, stewed fruits, and the nests, feathers and droppings of wild birds. The risk for occupational exposure is most likely to occur among food handlers during the storage, transfer and marketing of strawberries, peaches, cherries, corn and peanuts

It has been found to be prevalent in the atmosphere throughout the world, and is an important fungal allergen. It is found abundantly on damp walls, basement areas and on kitchen leftovers, and clinically an important fungus. Chinn& Diamond, (1982) stated that it has also been found in library and archive storage facilities, in the bioaerosol formed during conservative dental treatment, in silos storing imported wheat, in wheat mills, in bakery dust, in vegetables at the time of harvest, from sweet potato, applesauce, and strawberries, and from indoor and outdoor aerobiological studies.

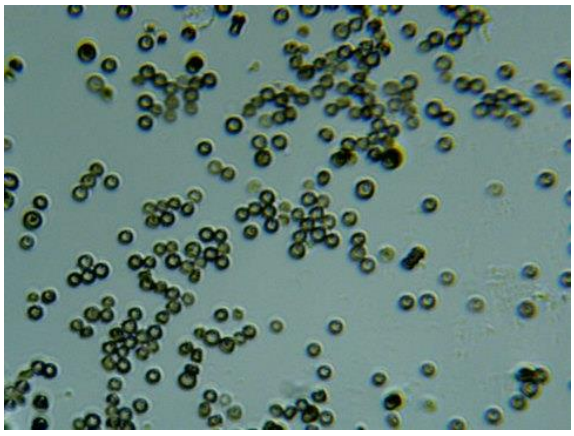

Figure 2-12 Microscope view of Rhizopus spp.

2.12 Antibacterial property of papain

Antibacterial is and agent which inhibits the growth or kills bacteria. Pawpaw (C. papaya L.) extracts have exhibited inhibitory effects on gram-positive bacteria and gram-negative bacteria. These organisms include: Bacillus subtilis, Escherichia coli, Salmonella typhi, Staphylococcus aureus, Enterobacter cloacae and Proteus vulgaris (Emeruwa, 1982). Following treatment with antibiotics, papaya juice helps to restore normal intestinal flora destroyed by the antibiotics for human. Pawpaw (C. papaya L.) peels serve as poultice for treatment of skin wounds that do not heal quickly. Pawpaw fruit stimulates milk production in weaning mothers, prevents constipation, nausea, morning sickness, motion sickness, cataract formation, chronic obstructive pulmonary disease, diverticulosis and hypertension. C. papaya L. contains enzymes such as papain, lipase and chymopapain. The papain and chymopapain can induce allergies when inhaled accidentally and Lipase, a hydrolase, is considered as a "naturally immobilized" biocatalyst.

2.12.1.1 Staphylococcus aureus

Staphylococcus aureus is a gram positive bacterium which appears under the microscope as spherical (coccus) organisms. According to previous research done by Hiramatsu, et al. (1997), it has been implicated as a causative agent in acute food poisoning episodes, toxic shock syndrome, impetigo, scalded skin syndrome, cellulitis, folliculitis and furuncles. It is a cause of systemic infections such as infective endocarditis, osteomyelitis, epiglottitis, and sinus infections amongst others. *S. Aries* is also responsible for many infections and systemic infections in the health care setting (nosocomial infections).

Iwase, et al (2010),stated that *Staphylococcus aureus* can cause illness toxin production as well as by infecting both local tissues and the systemic circulation. Disease transmission can occur by the following options:

Gastrointestinal: *Staphylococcus aureus* causes acute episodes of food poisoning via preformed enterotoxins. Food items likely to be infected with staphylococcal food poisoning include meat and meat products; poultry and egg products; salads such as egg, tuna, chicken, potato, and macaroni; bakery products such as cream-filled pastries, cream pies, and chocolate éclairs; sandwich fillings; and milk and dairy products.

Skin and hair infections: *Staphylococcus aureus* commonly colonizes many skin surfaces on the nasopharynx, and perineum; but can cause infection of these surfaces particularly if the cutaneous barrier has been disrupted or damaged.

Systemic infections: *Staphylococcus aureus* commonly causes infective endocarditis in IV drug abusers; osteomyelitis, sinus infections in the general population; and epiglottitis in young children.

Nosocomial infections: Methicillin resistant *Staphylococcal aureus* (MRSA) is a strain of the bacteria that is commonly implicated in nosocomial infections. Risk factors for MRSA colonization or infection in the hospital settings include prior antibiotic exposure, admission to an intensive care unit, surgical incisions, and exposure to infected patients.

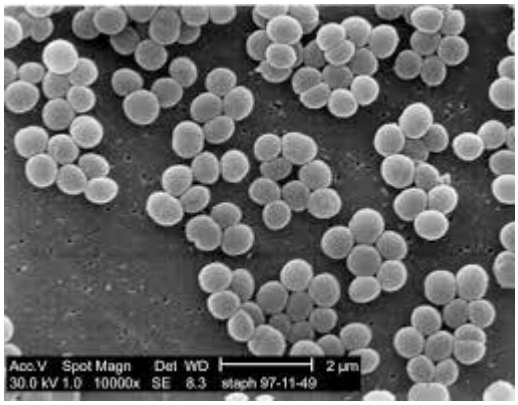

Figure 2-13 Microscope view of Staphylococcus aureus

2.12.1.2 Escherichia coli

Escherichia coli, it is also known as *E. coli*. It is a bacterium that is commonly found in the gut of endotherms (warm blooded organisms).

Several types of *E. coli* exist as part of the normal flora of the human gut and have many beneficial functions, such as the production of vitamin K2. This sentence was cited by Arifuzzaman, et al. (2006). They also prevent harmful bacteria, known as pathogenic bacteria, from establishing themselves in the intestine.

Normally, *E. Coli* strains pose no harm to human health, except for serotype O157:H7, which can cause food poisoning in humans and can become life-threatening. The patient will typically experience symptoms within three to four days after exposed to the bacteria. On the other hand, in some cases they may appear within a day or a week later. The individual may experience abdominal pain, diarrhea , nausea vomiting and fever.

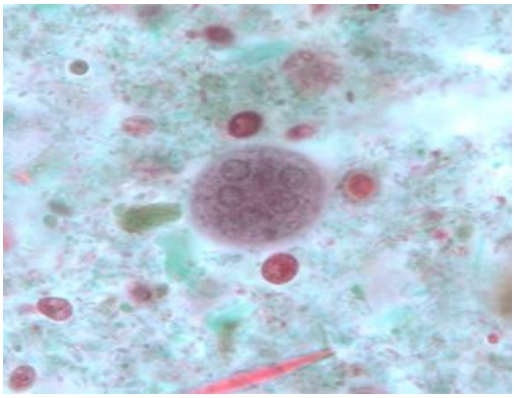

Figure 2-14 Microscope view of Escherichia coli

2.12.1.3 Bacillus subtilis

Bacillus subtilis is a Gram positive, spherical shaped bacteria, commonly found in soil. This bacterium is also known by the names hay bacillus, grass bacillus, or Bacillus globigii. Saito, Y., Taguchi, H., & Akamatsu, T. (2006) mention that *Bacillus subtilis* is an endospore forming bacteria, and the endospore that it forms allows it to withstand extreme temperatures as well as dry environments. Bacillus subtilis is considered and obligate aerobe, but can also function anaerobicly when in the presence of nitrates or glucose. Bacillus subtilis is not considered pathogenic or toxic and is not a disease causing agent. Bacillus subtilis has a flagellum which makes motility faster.

Since this bacterium is resistant to extreme temperatures, it can withstand high cooking temperatures. This is not to cause alarm, as it does not cause sickness if ingested. This bacterium can cause a stringy consistency in spoiled bread dough, if dough is exposed.

Bacillus subtilis is readily present everywhere; the air, soil and in plant composts. It is predicted that it spends most of it's time inactive and in spore form. When the bacterium is active though, it produces many enzymes. One enzyme contributes to the plant degradation process. Bacillus subtilis can also be found in the human body, mostly on

the skin or in the intestinal tract. However it is very rare for this bacterium to colonize in the human body.

Oggioni, et al. (1998) stated that along with enzymes, *Bacillus subtilis* also produces a toxin called subtilisin. Subtilisin can cause allergic reactions if there is repeated exposure in high concentrations. This only poses a risk to fermentation plants that use high quantities of subtilisin. Exposure restrictions have been imposed by OSHA for the factory setting and can be found on their website (Occupational Safety & Health Administration). Subtilisin is also used in laundry detergent. It has been known to cause allergic reactions after using such detergent, however only in large quantities.

There are several uses for *Bacillus subtilis* and the enzymes it produces. It can be used to create proteases and amylase enzymes. At one point *Bacillus subtilis* was widely used as a broad spectrum antibiotic. According to Pepe, et al. (2003) this was lost after the ability to produce cheaper, large-scale antibiotics. *Bacillus subtilis* can convert dangerous explosives into just compounds of nitrogen, carbon dioxide and water. The proton binding properties of the surface of this bacterium can also play a role in the degradation of radioactive waste. *Bacillus subtilis* also produces some fungicidal compounds, which are being investigated as control agents of fungal pathogens. It is currently being used as a fungicide for plant and ornamental seeds as well as various agricultural seeds.

Figure 2-15 Microscope view of Bacillus subtilis

2.13 Summary

This paper presents a literature study of papain properties such as, enzyme's absorbance, activity, efficiency and equipment setting for the experiment. In this chapter also mention all the bacterial and fungi properties and microscope view of it. Besides that, it provides the optimum condition and stability of the papain enzyme toward temperature, pH and organic solvent. Papain can be stored in 2-8˚C for 6 to 12 months.

CHAPTER 3

MATERIALS AND METHODS

3.1 Overview

Chapter 3 describes the materials and apparatus required during the experimental work. It also elaborates the procedures and steps included in conducting the experimental work of this research. In order to accomplish the objective of this research study, four main stages are going to carry out. In the first stage, extraction of the papain enzyme has to carry out. Extraction process includes the pre-treatment process, ultrasonication and lastly, extraction. Subsequently, the second stage is the analysis for papain enzyme from the extraction process. After that, it continues with the third stage, the study on the viability of papain enzyme application as hand wash and hand sanitizer. Last stage, fourth stage is the application of papain enzyme in making hand wash and hand sanitizer. This chapter also provides the systematic procedures to ensure that all the procedures can be systematically. Following are the general flow chart of this research.

3.2 Introduction

This chapter provide the guideline and the systematic flow of the procedures that has to be following in the laboratory work.

3.3 Chemicals and raw materials

1. Papaya leaves
2. Sodium Acetata

3. Casein
4. Potassium Phosphate
5. Trichloroacetate acid
6. Sodium carbonate
7. Folin & Ciolcaltea's solution
8. Calcium acetate
9. Isopropyl alchohol
10. Lemon essential oil
11. Glycerine
12. Distilled water
13. Nutrient Agar

3.4 Apparatus and equipment

1. Daihan ultrasonic cleaner
2. BS-21 shaking water bath
3. Eppendorf refrigerated centrifuge 5810R
4. Hitachi U1800 UV-visible spectrophotometer
5. Stopwatch
6. 100mL beakers
7. Dropper
8. Filter clothes
9. Test tubes
10. Stainless steel spoon
11. Thermometers

3.5 METHOD OF RESEARCH

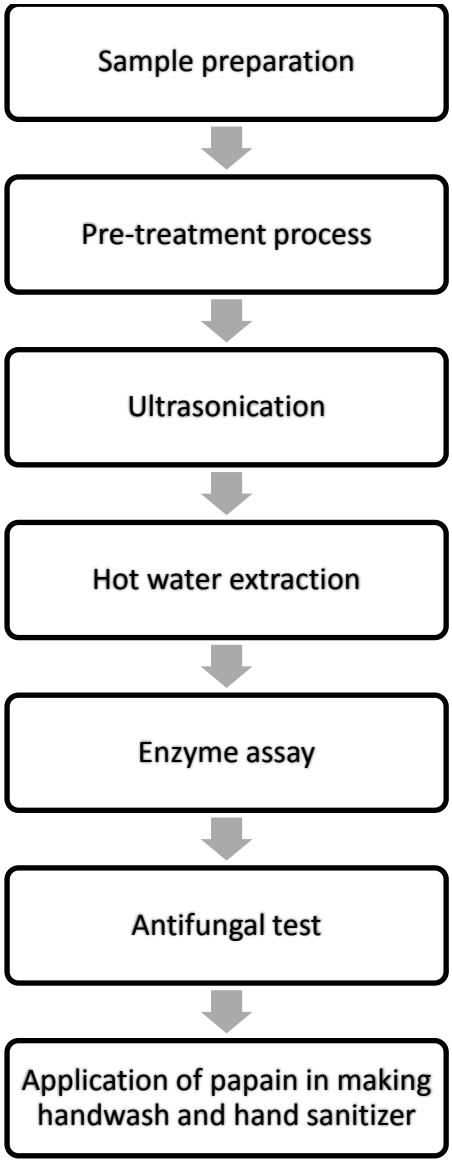

Figure 3-1: Flow chart of procedures

3.5.1 Sample preparation

The fresh green papaya leaves were used as the sample. Due to the complex cell wall matrix composed by the papaya leaf, pre-treatment process to be conducted in order to obtain the better extraction.

3.5.2 Pre-treatment process

Fresh papaya leaves were cut into size of 1cm and wash with distilled water. The cut papaya leaves were grinded. 10g of the grinded papaya leaves were weight and placed

into 100ml flask. Water poured into the flask with the ratio 1:0.5 to water: papaya leaves. The mixture was blended for 1 minute. (Mahmood et al., 2005)

3.5.3 Ultrasonication

Blended leaves were placed in Schott bottle and immersed in water for 1 hour with the frequency of 20, temperature of 20 ℃.

Figure 3-2 Ultrasonication process

3.5.4 Extraction process

The extraction method used is hot water extraction. Schott bottle containing sample from pre-treatment process was soaked into the shaking water bath and was extracted with 4 hours and temperature was set at 60 ℃, frequency at 20 kHz.

Figure 3-3 Hot water extraction process

3.5.5 Enzymatic assay

Cupp-Enyard, (2008) had perform the enzymatic analysis by using Folin & Ciocalteu method. Firstly, semi-solid sample from hot water extraction process was filtered using

filter cloth and the filtrate was collected. Then centrifuge the filtrate by using bench top centrifuge at 3250 rpm, temperature of 20℃ for 10 minutes. After 10 minutes, supernatant was collected for analysis. The enzymatic analysis method used is Folin & Ciocalteu method. Casein was set as the substrate in this method. 0.5mL sample in test tube mixed with 0.5mL enzyme diluents solution which is 10mM solution of sodium acetate. Then buffer with 5mM Calcium acetate, set the pH at 7.5 and added with 5mL of 0.65% (w/v) casein solution. All the solutions were mixed well and then incubated at 37℃ for 10 minutes. After 10 minutes, 5mL of 110mM trichloroacetic acid(TCA) was added and mixed well by swirling and incubate at 37℃ for 30 minutes to stop the reaction. Next, take 2mL of the solution out and mix with 5mL of 500mM sodium carbonate solution and 1mL of 0.5mM Folin & Ciocalteu's reagent. Mix the solution well and incubate at 37℃ for another 30 minutes. The procedure followed by remove test tube from the centrifuge and cooled to room temperature for few minutes. The dark blue solution appears indicate the present of papain enzyme. Lastly, read the sample in spectrophotometer at 660nm and compared reading with the calibration curve.

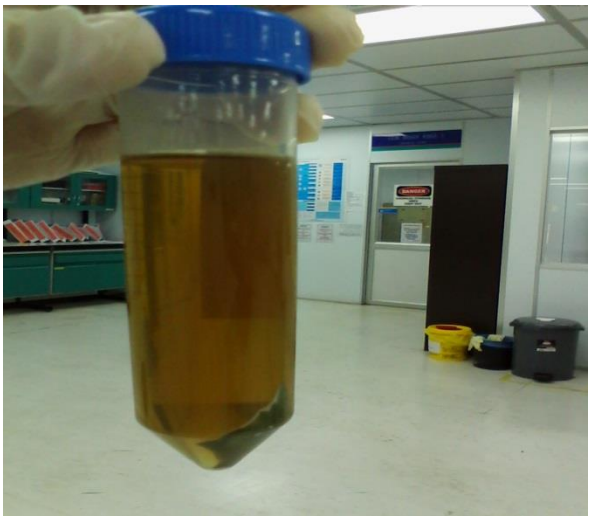

Figure 3-4 Supernatant and bottom layer, product of centrifugal effect.

3.5.6 Calculation of extraction yield

After the production of extraction collected, determine the gravimetrically and measure it with a measuring cylinder. The extraction yield done by Yahya et al., (2010) shows that the extraction yield is expressed as the percentage ratio of the mass of extracted material to the mass of grinded papaya leaves loaded in the closed vessel. The equation is shown below:

$$Extraction\ yield(\%) = \frac{Volume\ of\ extracted\ solution(mL)}{Volume\ of\ water\ added\ (mL)} \times 100$$

3.5.7 Determination on antibacterial and antifungal activity

Following the procedure of Nwinyi et al., (2010), firstly, place one disc (3mm diameter) of a 3 days old culture of the pathogens in each of the triplicates Petri-dishes (11 cm diameter) with 160mL Nutrient Agar medium and 3mL of the papain. The control experiments were set up with 3mL of sterile distilled water using same media. This is done in triplicate plates and incubated at room temperature for three days of mycelia extension of the cultures were determined by measuring culture along diameters and comparing with mycelial growth of the control. The difference in their diameter reflects the extent of inhibition by extracts (papain).

3.5.8 Application of papain enzyme in making handwash.

The method of handwash making used is promoted by Srinivasan, (2012). Firstly, boil the 120 mL water and measure out 100 mL. Then, stir the 10g of soap chips in the boiled water till completely dissolved. Use the stainless steel spoon to stir it. Mix 10 mL glycerine and 5mL of papain enzyme in a small cup. Then add glycerine-papain enzyme mix to soapy water after it cools about 60℃ and stir well. Add 8-10 drops of orange essential oil to the soap solution. Finally, pour the soap solution into squeeze bottle or dispenser.

3.5.9 Application of papain enzyme in making hand sanitizer

At first, mix the 60 mL of papain enzyme with 120 mL of isopropyl alcohol together until well blended. Add 8 to 10 drops of orange essential oil to the mixture for fragrance and stir well. Lastly, pour the finished hand sanitizer into an empty container and seal. This method was taken from Huffstetler, (2013).

3.6 Summary

This chapter explain all the steps and the setting for the extraction method and also the procedures taken to make antifungal handwash and hand sanitizer.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Overview

This chapter presents all the works and results done. It includes two parts, the first part is the extraction part by using a UV - spectrophotometer to study the enzyme activity and the second part is regarding the antifungal and antibacterial test by measuring the decrease of the size. The enzyme extracted is papain enzyme and the bacteria and fungi used in antibacterial and antifungal test are *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae, Rhizopus spp. and Mucor spp.*

4.2 Introduction

This chapter shows the pre-treatment process, ultrasonication and hot water extraction process that had been carried out as extraction process. For the enzyme analysis, a calibration curve plotted in order to measure enzyme activity. The antifungal and antibacteia test were carried out in laminar flow and the size of the pathogen was measured by using microscope.

4.3 Sample preparation& Extraction

Figure 4-1 Cut Papaya leaf

Mass of white plate: 5.66g

Mass of papaya leaves + white plate: 27.21g

Mass of papaya leaves: 21.55g

Water added: 200mL

Figure 4-2 Extracted solution

Extracted solution: 187mL

4.4 Calculation of extraction yield

$$Extraction\ yield(\%) = \frac{Volume\ of\ extracted\ liquid\ (mL)}{volume\ of\ water\ added\ (mL)} \times 100$$

$$Extraction\ yield(\%) = \frac{187}{200} \times 100$$

$$Extraction\ yield(\%) = 93.5\%$$

4.5 Factor affecting papain extraction

There are many factors affecting the amount of papain extraction. Some of the amount will release to the surroundings during the process of the extraction. Another factor is the temperature factor. High temperature will evaporate certain component inside the papaya leaf. Although the temperature set at the optimum temperature, 60˚C, it will still have the least amount of enzyme being killed or dentures. Apart from that, pH is factor affection the papain extraction. pH control the enzyme activity, when the pH set at the optimum pH of papain enzyme, it will react more effectively rather than other pH value. For example, when the pH set at 1, which is acidic condition and unfavorable for papain's activity, it will affect the amount of the extraction due to the unreactive papain enzyme in the extraction process. Based on the volume and yield calculated above, the hot water extraction is favorable for papain enzyme extraction. The reagent used in extracting papain enzyme is water. Water having a good heat conductivity, and pH 7. These characteristic will help in enzyme to be more reactive and favorable for enzyme remain active.

4.6 Enzyme analysis

L-cysteine declared as the papain enzyme in this research. Folin & Societies Phenol reacted with free cysteine to produce a blue colored chromophore (part of a molecule responsible for its color), which was quantified and measured as an absorbance value in the spectrophotometer. The more cysteine that was released from the casein, the more chromophores was generated. Absorbance values generated were compared to L-cysteine standard curve, which was generated by reacting known quantities of cysteine with the F-C reagent to correlate changes in absorbance with the amount of cysteine in

micromoles. From the standard curve, the concentration of the L-cysteine was determined.

Figure4-3 Dark blue solution of enzyme assay

To determine the enzyme activity, the following equations were needed to be determined.

ΔA660nm Absorbance = ΔA660nm Absorbance (Test) - ΔA660nm Absorbance (Test Blank)

ΔA660nm Absorbance (Test) = 1.396A - 1.072A

ΔA660nm Absorbance (Test) = 0.324A

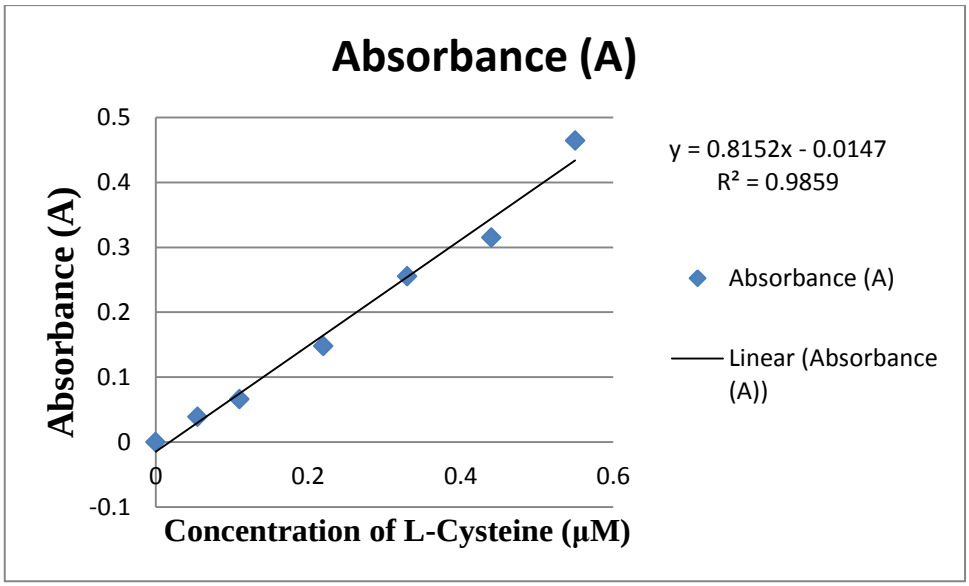

Figure 4-4 Standard Graph of Absorbance vs Concentration

By referring to Figure 4-4, we can calculate the concentration of L-Cysteine (µM) by using the following equation.

$y = 0.8152x - 0.0147$

$where\ y = 0.324A$

$0.324 = 0.8152x - 0.0147$

$0.3387 = 0.08152x$

$x = 0.415\mu M$

From the concentration of L-cysteine determined, the enzyme activity of papain was performed in terms of Units, which was the amount in micromoles of cysteine equivalents released from casein per minute.

To get the activity of enzyme in Units/mL, the following calculation was performed.

$$\frac{Units}{mL}(Enzyme) = \frac{\mu M(L-Cysteine) \times (11)}{(1) \times (10) \times (2)}$$

where;

11 = total volume (in millilitres) of assay

10 = time of assay (in minutes) as per the Unit Definition

1 = volume of enzyme (in millilitres) of enzyme used

2 = volume (in millilitres) used in Colorimetric Determination

$$\frac{Units}{mL}(Enzyme) = \frac{0.415 \times (11)}{(1) \times (10) \times (2)} = 0.2283 \frac{unit}{mL}$$

By comparing to the previous research done by Izzah, S., (2010),

Table 4-1 Enzyme activity in various temperature

Temperature (˚C)	Absorbance (x10 dilution factor)	Concentration of enzyme (µM)	Activity(unit/mL enzyme)
50	2.58	3.32	1.824
55	2.69	3.81	2.098
60	3.12	4.02	2.211
65	2.56	3.30	1.812
70	2.35	3.02	1.661

The different among the enzyme activity calculated and the literature value is

$$Difference\ (\%) = \left|\frac{Literature\ value - Calculated\ value}{Literature\ value}\right| \times 100\%$$

$$Difference\ (\%) = \left|\frac{0.2211 - 0.2283}{0.2211}\right| \times 100\%$$

$$Difference\ (\%) = 3.25\%$$

Since the difference is less than 5%, the calculated value of enzyme activity is accepted. It also means that in 10mL of the extracted solution, it contains of 2.283 units of papain enzyme.

4.7 Antibacterial and antifungal test

The three types of bacterial chosen for antibacterial test are *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis.* On the other hand, the three types of fungus chosen for antifungal test *are saccharomyces cerevisiae*, *Mucor spp* and *Rhizopus.* All of these bacterial and fungal are easy to be found in toilet, plants, and animals which approached to human very often. In this section, two steps were taken to determine the antibacterial and antifungal properties of papain enzyme. For the first step, photos taken on day 3 and day 6. Comparison among the sight view of the bacteria growth on day 3 and day 6 was done. By doing this, it still doesn't fully support on the antibacterial behaviour of papain enzyme. This is because they might be having unseen able bacteria exist. Double check by using microscope is more efficient and convinces to proof the antibacterial behaviour of papain enzyme.

4.7.1 Bacteria

4.7.1.1 Staphylococcus aureus

Sight view

Day 3

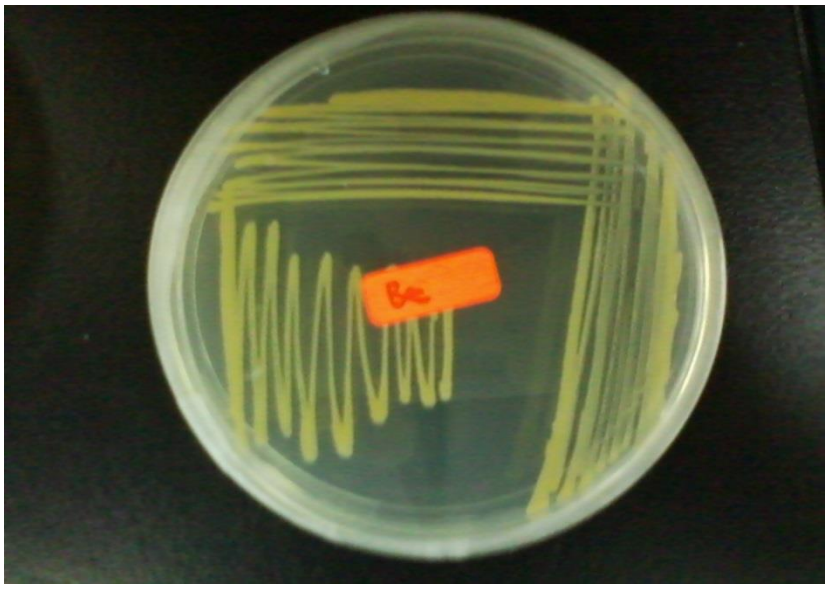

Figure 4-5 Sight view of Staphylococcus aureus at day three

At day 3, papain enzyme is placed into the petri dish.

Day 6

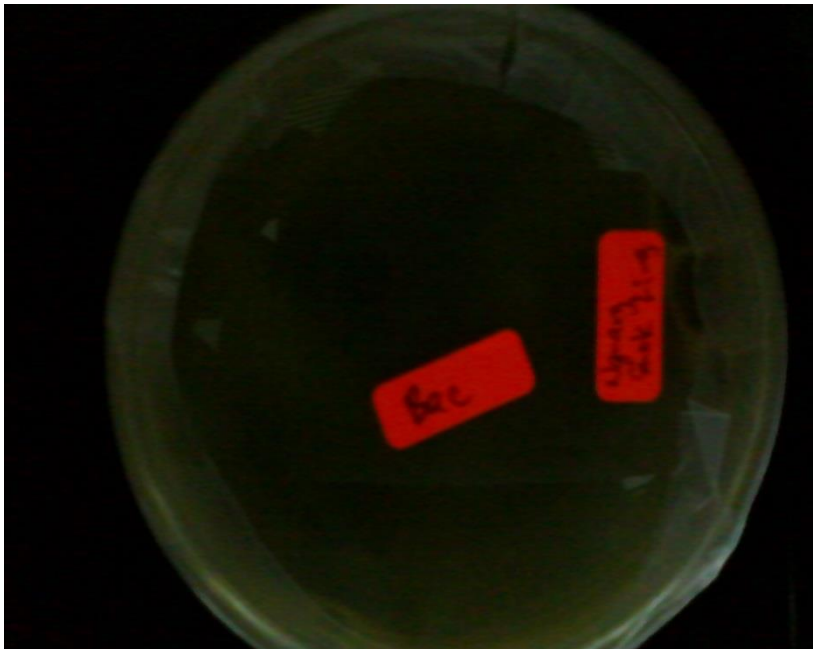

Figure 4-6 Sight view of Staphylococcus aureus after six day

Microscope view

Day 3

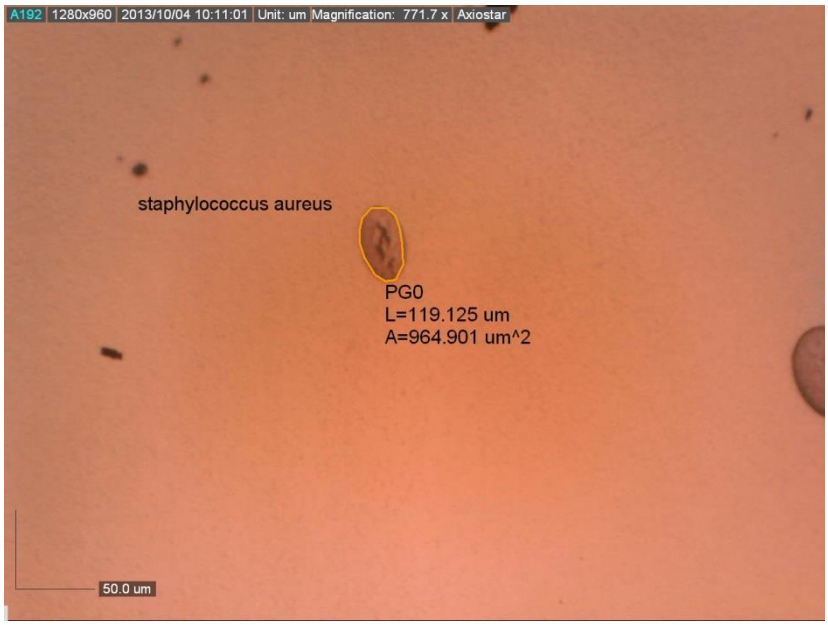

Figure 4-7 Microscope view of Staphylococcus aureus at day three

At day 3, papain enzyme is placed into the petri dish.

Day 6

Figure 4-8 Microscope view of Staphylococcus aureus at day six

Initial area=964.901μm^2

Final area= 365.536μm^2

Different= (365.536-964.901) μm^2= -599.365μm^2

4.7.1.2 Escherichia coli

Sight view

Day 3

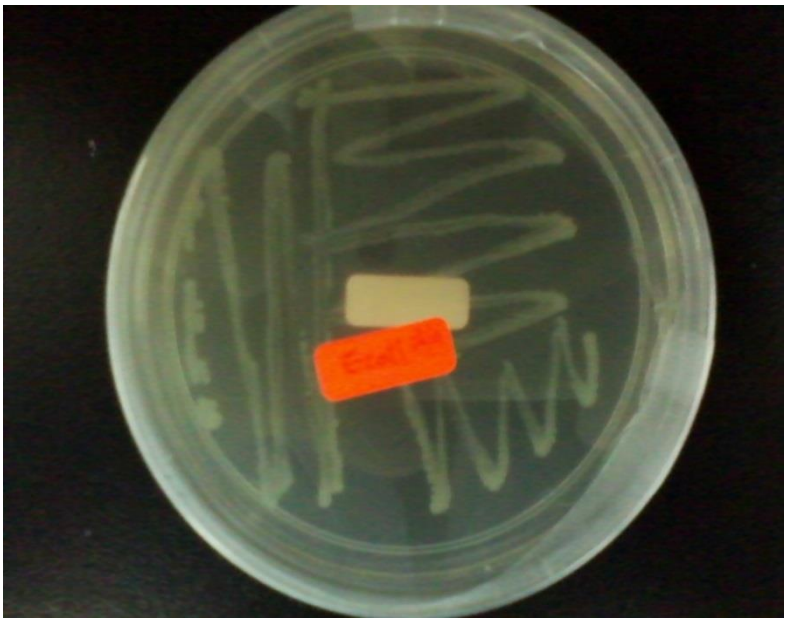

Figure 4-9 Sight view of Escherichia coli at day three

At day 3, papain enzyme is placed into the petri dish.

Day 6

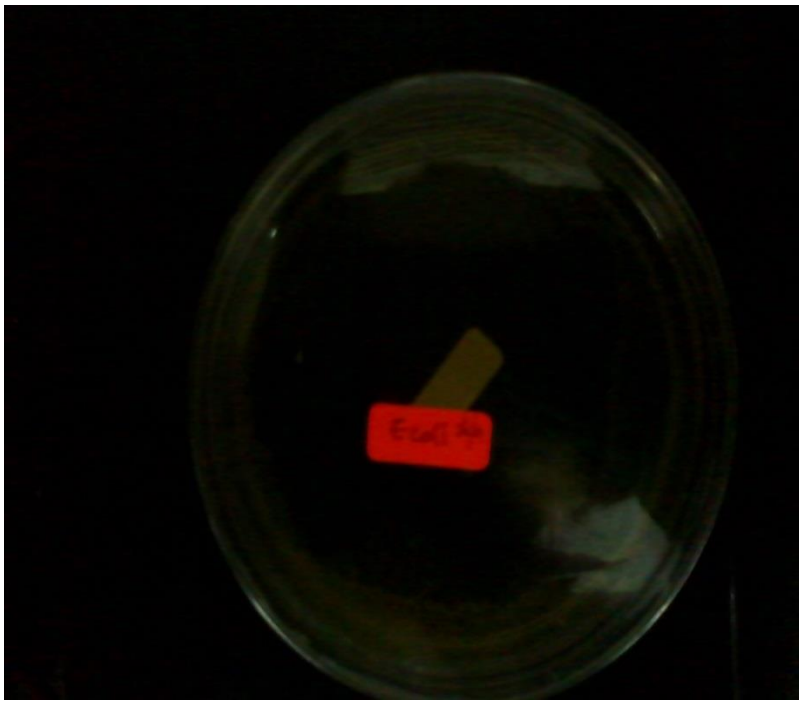

Figure 4-10 Sight view of Escherichia coli after six days

Microscope view

Day 3

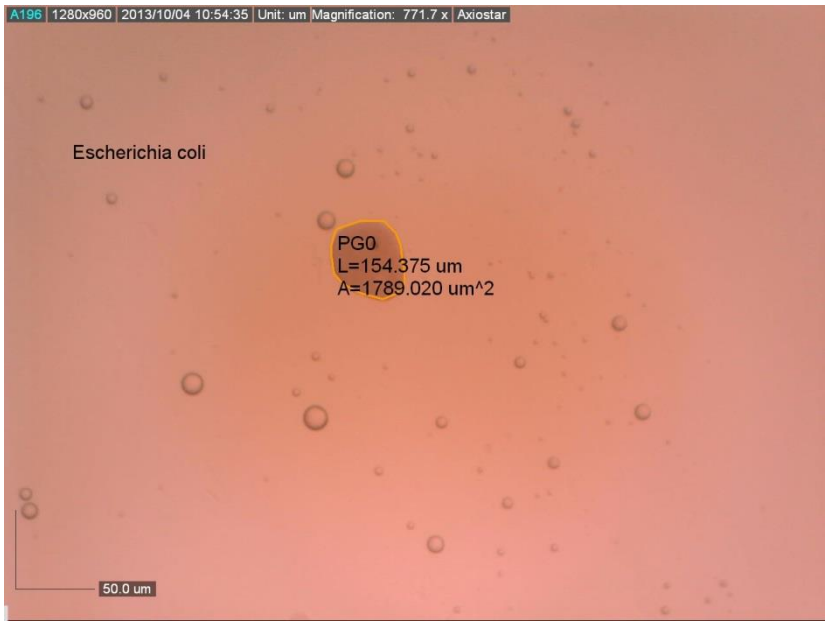

Figure 4-11 Microscope view of Escherichia coli at day three

At day 3, papain enzyme is placed into the petri dish.

Day 6

Figure 4-12 Microscope view of Escherichia coli at day six

Initial area=1789.020μm^2

Final area= 244.869μm^2

Different= (1789.020-244.869) μm^2= -1544.151μm^2

4.7.1.3 Bacillus subtilis

Sight view
Day 3

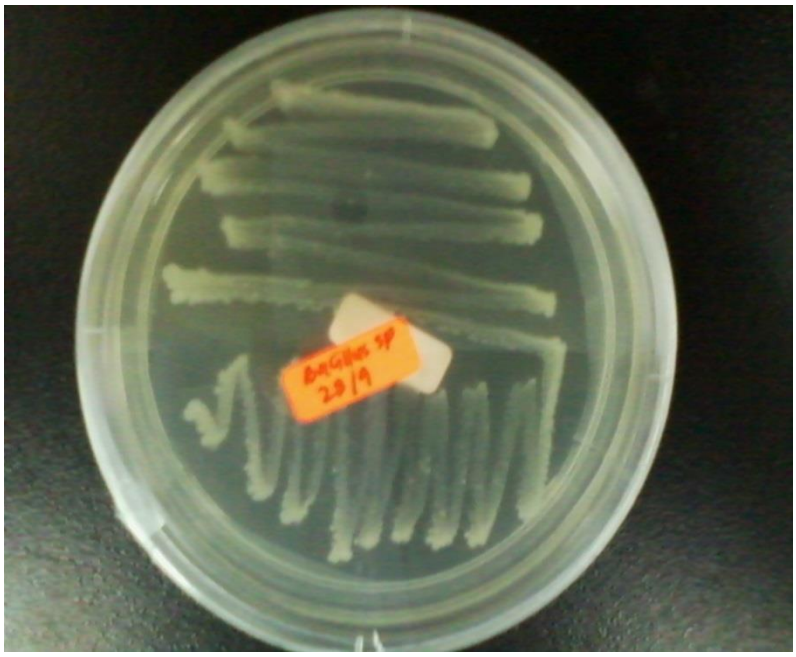

Figure 4-13 Sight view of Bacillus subtilis at day three

At day 3, papain enzyme is placed into the petri dish.

Day 6

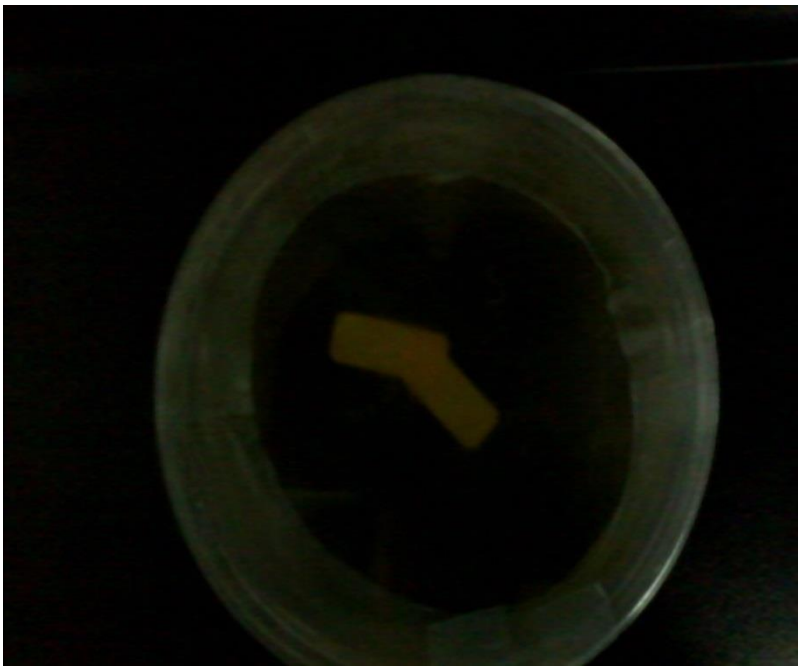

Figure 4-14 Sight view of Bacillus subtilis after six days

Microscope view

Day 3

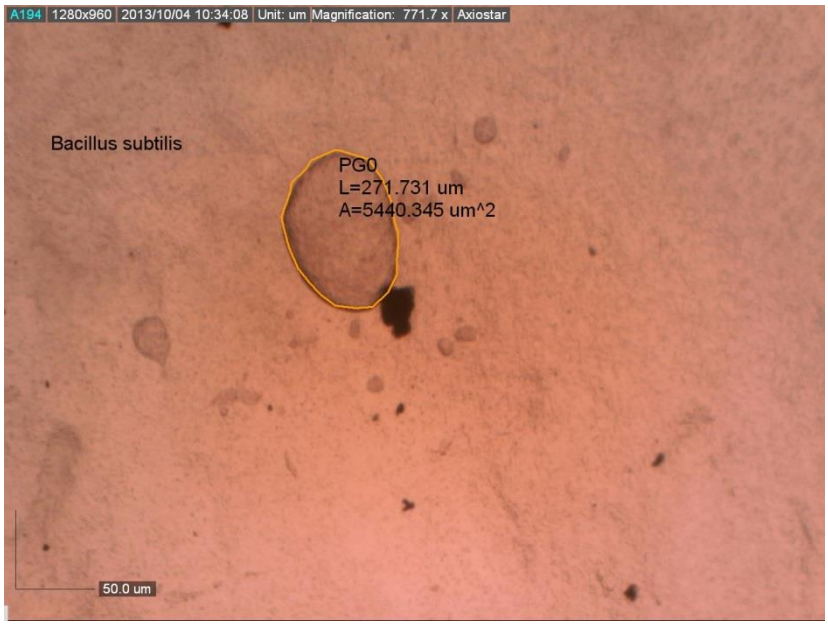

Figure 4-15 Microscope view of Bacillus subtilis at day three

At day 3, papain enzyme is placed into the petri dish.

Day 6

Figure 4-16 Microscope view of Bacillus subtilis at day six

Initial area=5440.345μm^2

Final area= 0μm^2

Different= (0-5440.345) μm^2= -5440.345μm^2

4.7.2 Fungal

4.7.2.1 Saccharomyces cerevisiae

Day 3

Sight view

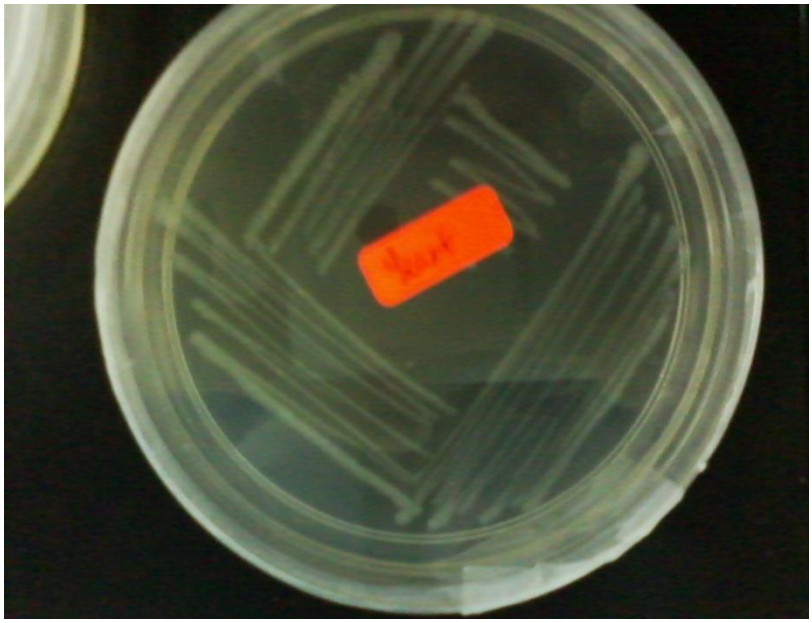

Figure 4-17 Sight view of Saccharomyces cerevisiae at day three

At day 3, papain enzyme is placed into the petri dish.

Day 6

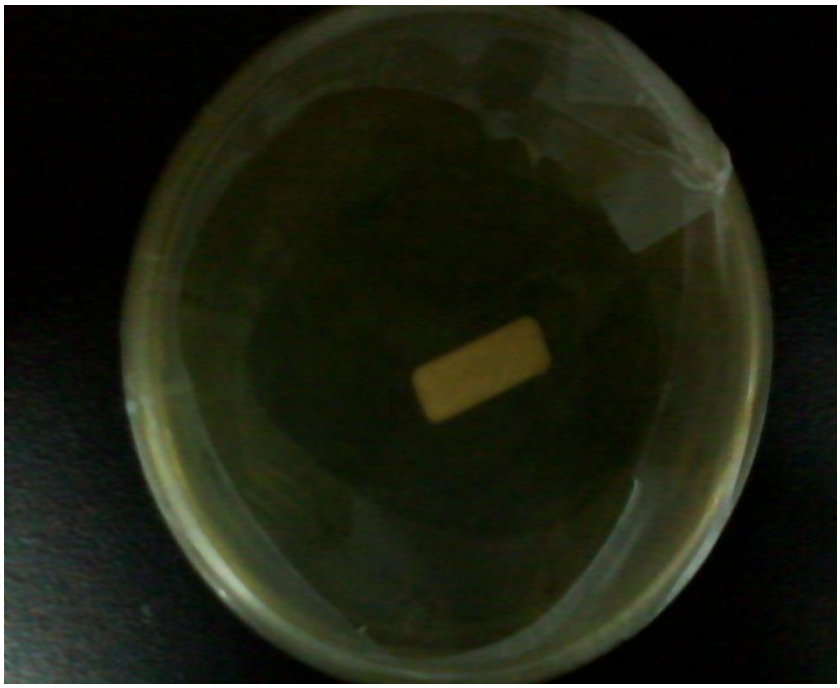

Figure 4-18 Sight view of Saccharomyces cerevisiae after six days

Microscope view

Day 3

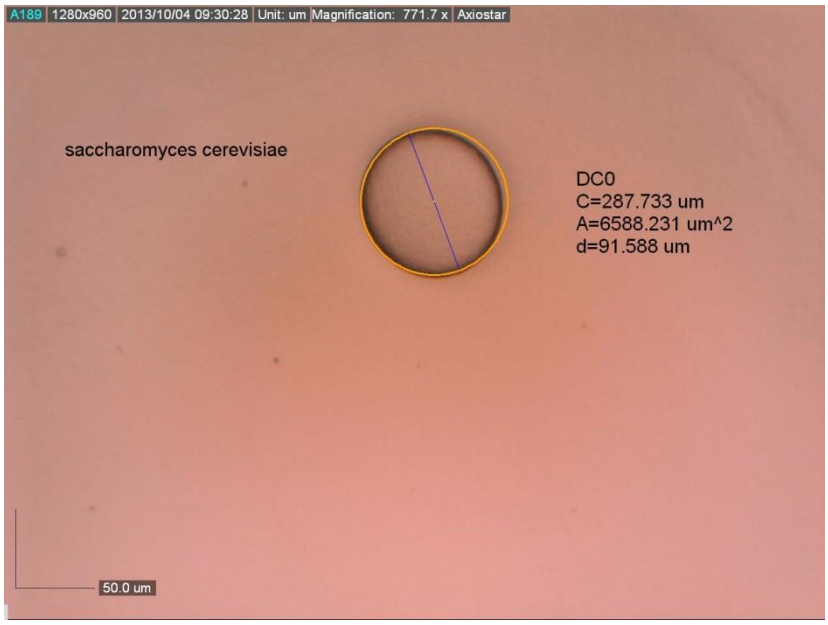

Figure 4-19 Microscope view of Saccharomyces cerevisiae at day three

At day 3, papain enzyme is placed into the petri dish.

Day 6

Figure 4-20 Microscope view of Saccharomyces cerevisiae at day six

Initial area=6588.231μm^2

Final area= 196.066μm^2

Different= (196.066-6588.231) μm^2= -6392.165μm^2

4.7.2.2 Mucor spp

Day 3

Sight view

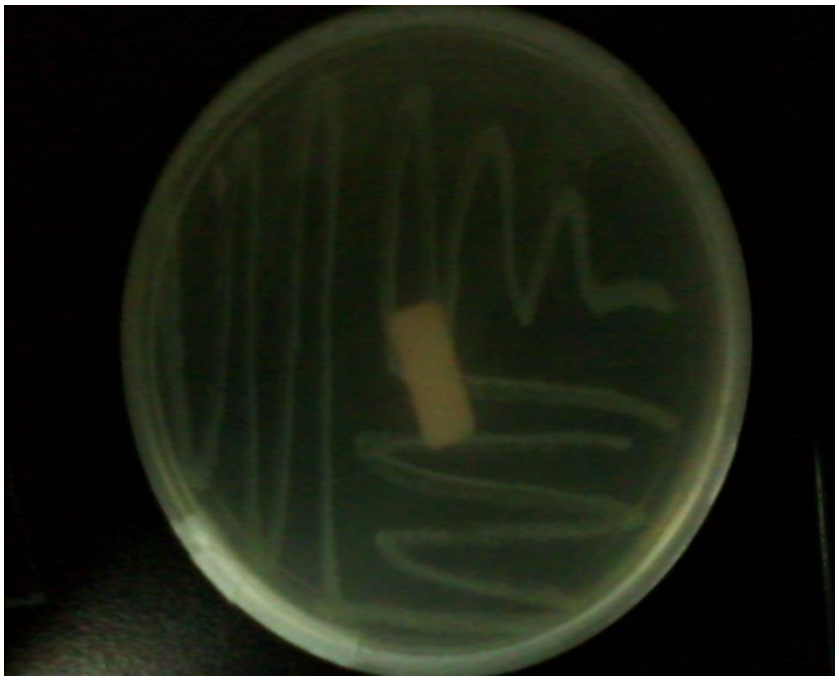

Figure 4-21 Sight view of Mucor spp at day three

At day 3, papain enzyme is placed into the petri dish.

Day 6

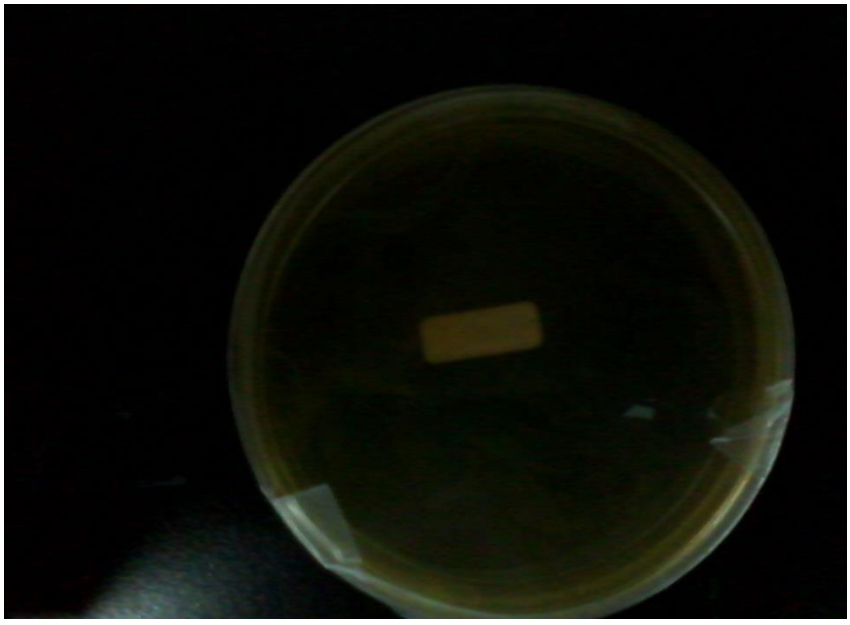

Figure 4-22 Sight view of Mucor spp at day six

Microscope view

Day 3

Figure 4-23 Microscope view of Mucor spp at day three

At day 3, papain enzyme is placed into the petri dish.

Day 6

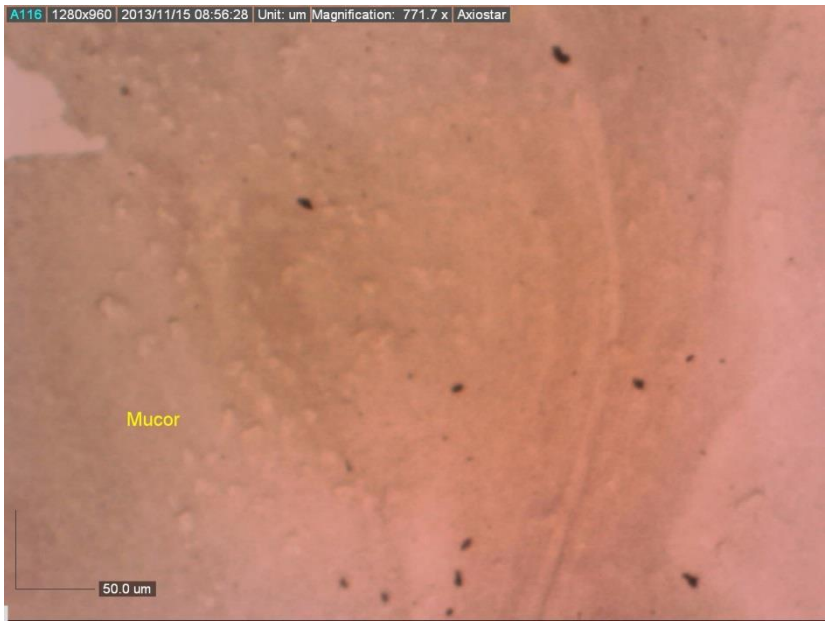

Figure 4-24 Microscope view of Mucor spp at day six

Initial area=899.331μm^2

Final area= 0μm^2

Different= (0-899.331) μm^2= -899.331μm^2

4.7.2.3 Rhizopus spp.

Sight view
Day 3

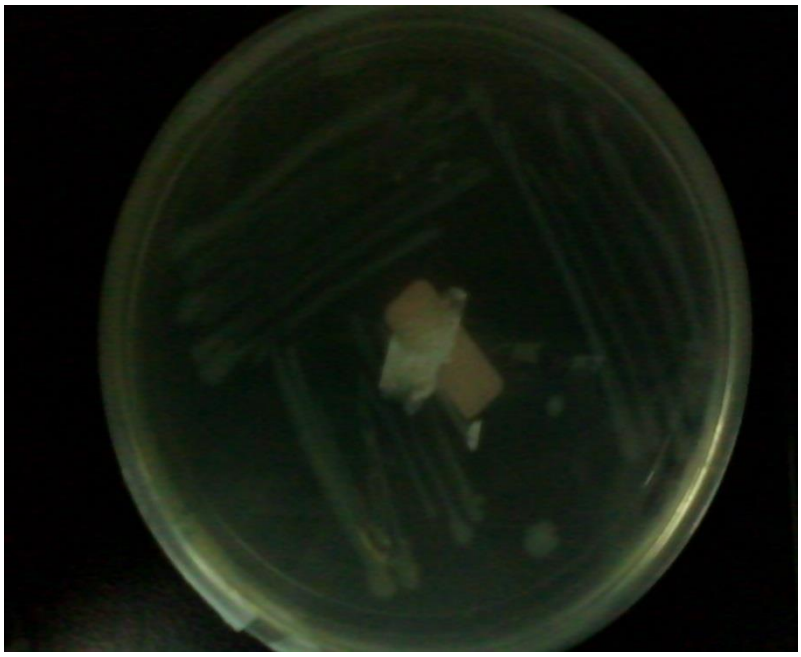

Figure 4-25 Sight view of Rhizopus spp. at day three

At day 3, papain enzyme is placed into the petri dish.

Day 6

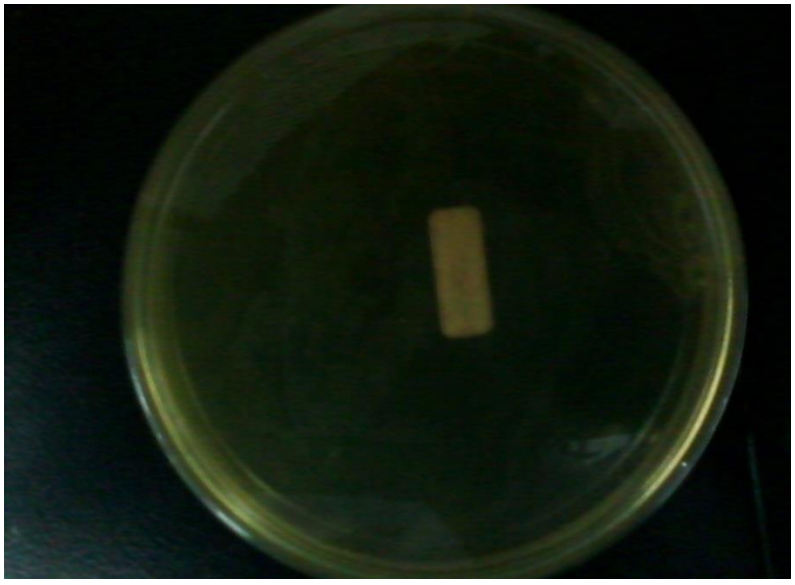

Figure 4-26 Sight view of Rhizopus spp. at day six

Day 3

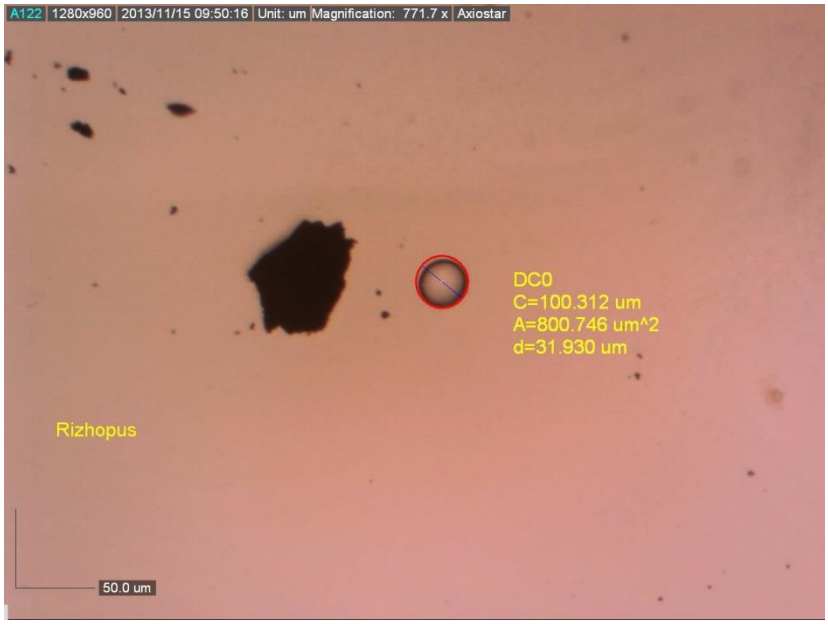

Figure 4-27 Microscope view of Rhizopus spp. at day three

At day 3, papain enzyme is placed into the petri dish.

Day 6

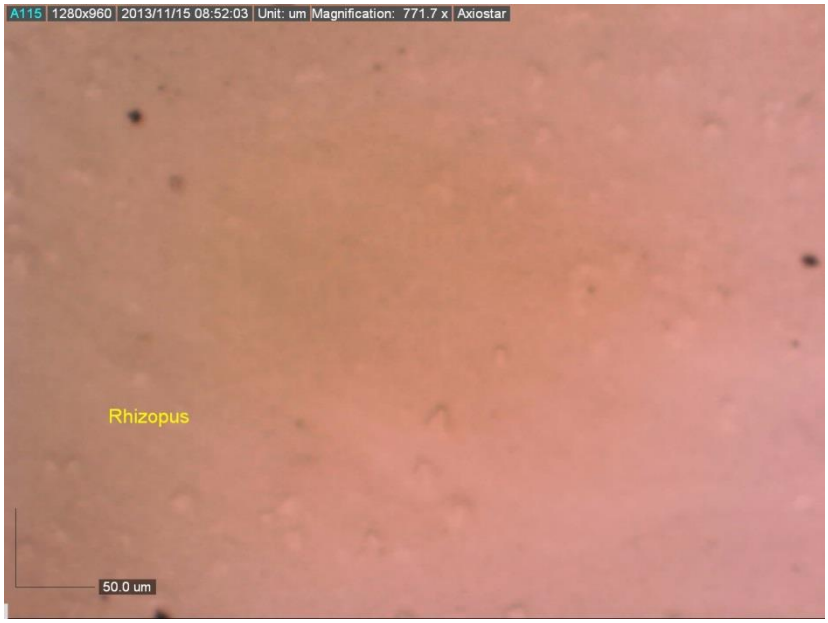

Figure 4-28 Microscope view of Rhizopus spp. at day six

Initial area=800.746μm^2

Final area= 0μm^2

Different= (0-800.746) μm^2= -800.746μm^2

Table 4-2 Summary table of size reduction of pathogens.

Bacteria	Area(μm^2)		Size reduction (μm^2)	Percentage reduction (%)
	Day 3	Day 6		
Staphylococcus aureus	964.901	365.536	599.365	62.12
Escherichia coli	1789.020	244.869	1544.151	86.31
Bacillus subtilis	5440.345	0	5440.345	100
Fungus				
Saccharomyces cerevisiae	6588.231	196.066	6392.165	97.02
Rhizopus spp.	800.746	0	800.746	100
Mucor spp	899.331	0	899.331	100

Based on the result obtained, we can conclude that papain enzyme yield the antibacterial property toward *Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* bacteria. However, for the fungus, papain enzyme yields the antifungul property toward *Saccharomyces cerevisiae, Rhizopus spp. and Mucor spp.* By referring to the sight view, we can see that with the papain enzyme placement, it could wipe off the three bacterial and fungal listed above. For microscope view, we can see that the decreasing of the area and the size of the bacteria. From the calculation, the different between the initial and final area for *Staphylococcus aureus* is a decrease of 599.365μm^2 which is the lowest reduce in size for bacteria, *Escherichia coli* shows a decrease of 1544.151μm^2 and *Bacillus subtilisbacteria* shows a highest decrease among the bacteria with a decrease of 5440.345μm^2. For fungal species, *Saccharomyces cerevisiae* shows the highest size reduction with a decrease of 6392.165μm^2. However, *Rhizopus spp.* shows the lowest size reduction of fungus with a decrease of 800.746μm^2 and *Mucor spp* shows a reduction of 899.331μm^2.

The antifungal and antibacterial property of papain may possibly due to the active compounds found within the leave of pawpaw which are glycosides and caricin. The other essential biologically active compounds that include alkaloids, carpaine, pseudocarpaine, flavanols, butanoic acid, tannins, linalool, benzlglucosinolate, cis and

trans-linalool, terpenoids, alpha- palmitic acid. Some of these compounds are effective super oxide antioxidants which enable to inhibit mycelial growth by reacting with cell wall components of these fungi. The low activity of the papain on the mycelial growth may possibly be due to the dense nature of the exudates. From the observation, after a few minutes papain bleeding exudates, it tends to thicken, thus it's becoming more gummy than before. This slows down the enzymes activity of papain on the mycelial growth of the fungi and bacterium isolated.

From previous research findings, it has been found that papain enzymes are found in pawpaw extracts having a target site on the cell wall of these isolated bacteria and fungi. The mode of this action may possibly be by an attack on the sugar residues on the cell of these fungal and bacterial species. According to Yoshio and Yoshio (1981), glucose was detected as a main sugar component in the cell wall of Mucor spp whereas in Rhizopus spp. glucosamine and N-acetylglucosamine were the major components. Hence the clue for remarkable inhibitory effects were exhibited by the extracts most probably attributed to this mode of action. Thus from the current findings, either of the extracts may be prepared to help stem the effects of post-harvest losses caused by these isolated fungi and bacteria.

Another major post-harvest diseases of C. papaya'anthracnose' effect has been controlled by prochlorazor priopiconazolle during transportation and storage. (Sepiah, 1993). On the other hand, some of the fungicides were utilized even have a residual effect on the treated fruits. Hot water treatments in combination with fungicides tend to improve the efficiency in controlling anthracnose. However, hot water dip treatments accelerate ripening in fruits (Paull, 1990); and the use of fungicide for extended periods may cause the emergence of fungicide – resistant strains of the fungus and bacteria. Then, a cost effective and reliable alternative is imperative.

Several attempts with limited results have also been experienced by the use of other physical means of C. papaya preservation (Sivakumaret al., 2002; Gamagae et al., 2004). From the previous finding, the possibility of the use of extracts of pawpaw (leaf extracts and papain) as a preserving the shelf life for all important fruit crops. Its approach to plant disease management is economically viable because it's less

environmental risk and can be exploited using biotechnological tools in industrial production which may substitute other synthetic fungicides and bacterium.

The bio-control potentiality of crude extracts of the plants, C. papaya has been well-established in the laboratory condition (Rawani et al. 2009). The highest mortality was recorded in C. papaya leaf extract. The phytochemical analysis of the plant extracts reveals the presence of several bioactive secondary metabolites that singly or in combinations may be responsible for the larval toxicity. As no mortality occurs in the non-target organisms (invertebrates), it can be summarized that all the plant extracts are safe to use in the aquatic ecosystem, though some toxicity of C. Collins in higher vertebrates has been reported (Sarathchandra and Balakrishnamoorthy 1998). As a conclusion, the crude extracts of C. papaya, M. paniculata, and C. collinus could be recommended for large-scale field trials and effectively used as potent larvicides in mosquito control programs too. Based on the previous study, C. papaya methanolic extract was of considerable and good larvicidal and pupicidal properties against the fungi and bacterial.

The mechanical function of papain is possible is through the cysteine-25 portion of the triad in the active site that attacks the carbonyl carbon in the backbone of the peptide chain freeing the amino terminal portion. When this occurs throughout the peptide chains of the protein, the protein will breaks apart. The mechanism by which it breaks peptide bonds involves deprotonation of Cys-25 by His-159. Asparagine-175 which helps to orient the imidazole ring of His-159 so that it allows this deprotonation process could take place. Although it is far apart within the chain of protein, these three amino acids are in close proximity due to the folder structure of the protein. It is though these three amino acids working together in the active site of the chains which provides this enzyme for its unique functions. Cys-25 then performs a nucleophilic attack on the carbonyl carbon of a peptide backbone (Menard et al., 1990; Tsuge et al., 1999). In the active site of papain, Cys -25 and His -159 are thought to be catalytically acting as a thiolate-imidazolium ion pair. Papain can be efficiently inhibited by peptidyl or non-peptidyl N-nitrosoanilines (Guo et al., 1996; 1998). The inactivation is due to the formation of a stable S-NO bond in the active site (Snitroso-Cys 25) of papain (Xian et al., 2000).

4.8 Potential application of papain enzyme in making hand wash and hand sanitizer

Due to the antifungal and antibacterial properties yield by papain enzyme is suitable for washing and cleaning purpose. A study by Kamalkumar et al., (2007) shows that the papain has been used in meat tenderizers and in face and hair care products. It also means papain enzyme is gentle and not harmful for the skin. It is believed the presence of papain enzyme, microbial enzymes has a wide range of specificity and good thermal stability amongst other proteases. With such unique characteristics, papain has potential used in detergents. Papain enzyme has a long history of being used to treat sports injuries, other causes of trauma and allergies (Dietrich, 1965). Fortunately papain has a proven track record in managing all of these conditions with clinical evidence of significant benefits for use of papain protease enzyme in cases of sports injury. It has previously been reported that minor injuries healed faster with papain proteases than with placebos.

Furthermore, athletes using papain protease supplements were able to cut recovery time from 8.4 days to 3.9 days (Trickett, 1964; Dietrich, 1965). Papain also has been successfully used to overcome the allergies associated with leaky gut syndrome, hypochlorhydria (insufficient stomach acid) and intestinal symbiosis like gluten intolerance. Papain has previously been reported to have significant analgesic and anti-inflammatory activity against symptoms of acute allergic sinusitis like headache and toothache pain without side effects (Mansfield et al., 1985). Papain can be chemically modified by different dicarboxylic enhydrides of citraconic, phthalic, maleic and succinic acids as Lysine residues are not a part of the active site in papain. Abraham & Sangeetha, (2006) claim these anhydrides react with the ε-amino group of lysine residues and change its charges from positive to negative, leading to a shift in pH optima of the enzyme from 7 to 9.

As referring to research title, productions of antifungal hand wash and hand sanitizer by the natural enzyme are important for the bio green product which is more preferable than the chemical made product of hand wash and hand sanitizer. It is also environmental friendly and hazardless products which solve the garbage formation by papaya tree. In making the hand wash, was insert at the temperature at 60˚C because it

is the optimum temperature of the enzyme reactivity and to prevent the enzyme damage from the high temperature.

4.9 Summary

From the result and discussion done, we can conclude that the papain enzyme could be extracted by hot water extraction, papain enzyme yield antibacterial and antifungul properties and it could be used in making handwash and hand sanitizer.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

5.1.1 Extraction yield and volume

A slight changes in the temperature, extracting time, pH, or many more variations of moisture could affect the measured amount of extracted material. Based on the previous research done by Grimm, et al., (2001), a mere change on the product quantity from extraction might due to the operation condition of the process. With optimum temperature, extracting time, pH and speed, the papain enzyme are extracted successfully by using the hot water extraction method which exceeds the first objective of this research study.

The volume and yield of the papain enzyme by the hot water extraction is 187mLwhich is 93.5% of the original volume of water added. The high volume and yield can conclude that the method of extraction is favorable for papain extraction. This is because the reagent used in hot water extraction is water. Water has the characteristic of good heat conductivity and having pH of 7. pH 7 is favorable for enzyme to remain active and easily being extracted due to the high reactivity at the temperature of 60˚C.

5.1.2 Analysis of papain enzyme

During sample preparation for spectrophotometer analysis, blue colour appeared. By referring to the Folin & Ciocalteu method in papain analysis, blue colour indicates the present of papain enzyme. After the enzyme analysis done by UV spectrophotometer, the absorbance reading collected at ΔA660nm is 0.324A. The concentration of the papain enzyme extracted is 0.415μM. The concentration was measured by using the calibration curve plotted. The enzyme activity calculated is 0.2283unit/mL. It also means that in 10mL of the extracted solution, it contains 2.283 units of papain enzyme. By referring to the previous research study at the extracting temperature of 60˚C and four hours extracting time, the enzyme activity tabulated is 0.221unit/mL. The error difference between the current extracted enzyme activity and previous study is 3.25%. Isserlis, L. (1918) stated that the margin error in a research data should not exceed 10% consider acceptable. By saying so, we can conclude that the extraction of papain enzyme yields an accurate and precise result. The second objective of this research study, papain enzyme analysis was achieved.

5.1.3 Antifungal and antibacterial test

Antifungal and antibacterial are the phenomena that the growth of pathogens being reduced or inhibited. From this research study, papain enzyme yields the antibacterial property toward *Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* bacteria being wiped off. On the other hand, toward the fungus, papain enzyme reduces the size of *Saccharomyces cerevisiae*, and wipe off *Rhizopus spp. and Mucor spp*. From the sight view, the papain enzyme placement could wipe off the all three bacterial and fungal listed above. For microscope view, we can see that the decreasing of the area and the size of the bacteria. From the calculation, the difference between the initial and final area for *Staphylococcus aureus* is a decrease of 599.365μm^2 which is the lowest reduction in the size of bacteria, *Escherichia coli* shows a decrease of 1544.151μm^2 and *Bacillus subtilis* shows a highest decrease among the bacteria with a decrease of 5440.345μm^2. For fungal species, *Saccharomyces cerevisiae* shows the highest size reduction with a decrease of 6392.165μm^2. However, *Rhizopus spp.* shows the lowest size reduction of fungus with a decrease of 800.746μm^2 and *Mucor spp* shows a reduction of 899.331μm^2. Based on the comparison among the difference of initial and final area(size) of the pathogen, we can conclude that papain enzyme yields the

antifungal and antibacterial property and yet the third objective of this research study was achieved.

5.1.4 Potential application of papain enzyme in making handwash and hand sanitizer

In application of papain enzyme in making handwash and hand sanitizer, we can conclude that the both handwash and hand saitizer produced yield the properties of the handwash and hand sanitizer. For the handwash made by papain enzyme, it yield the soapy feeling towards hand and also yield bubble when washing the hands with it. On the other hand, for the hand sanitizer made by papain enzyme, it also yields the hand sanitizer property such as the hand could be dried in a few seconds times. A dry and clean hand could be resulted after applying the hand sanitizer. Thus, the last objective in this research, application of papain enzyme in production of antifungal and amtibacterial handwash and hand sanitizer was achieved.

5.2 Recommendation

In the laboratory work of extracting the papain enzyme, the apparatus and equipments used in hot water extraction should be cleaned first before starting the experiment. For example, the equipment of shaking water bath should wash away all the dirt left by using some chemicals. Besides that, the schott bottle must be closed with its lid in order to prevent the other materials fall into it during the extraction process. Apart from that, during the centrifuge separation, the solution should not exceed the 80% of the solution level in a centrifuge tube.

Further enzyme analysis should be done to determine the content in the papin solution extracted. For example, GCMS and FTIR analysis should be carried out for the component content in the papain solution so that we could justify the cysteine is the components that really inhibit the growth of fungi and bacteria. Furthermore, with the GCMS and FTIR analysis, we could study the concentration of the enzyme as well which is more accurate by using the uv-spectrophotometer.

For the antipathogen test, more bacterial and fungus should be introduced so that more antipathogen could be study by papain enzyme. During the antipathogen test, it should be done triplicate for every single species of pathogen. It is important to carry out triplicate for each species because it might have some errors or mistakes during handling procedure. For example, the agar might be contaminated if exposed to the environment. Every procedure deal with the pathogen must be done in a closed system which is under the laminar flow in the lab.

Hand wash and hand sanitizer produced should undergo the proper packaging and another test of the antibacterial test by using the violet light so that it is more convincing to the people on the reliability on the papain made antipathogen hand wash and hand sanitizer. Another recommendation of these products is the lifespan of the papain enzyme in the hand wash and hand sanitizer. Due to the biomass made product, it is hard to control the lifespan of the enzyme. Apart from that, the colour for the hand sanitizer is brownish which is not appropriate for a hand sanitizer colour. Colour adjustment should be done in order to have better colouring.

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APPENDICES

Appendix A

Preparation of reagents for enzymatic analysis

Reagents used:

1. A: 50mM Potassium Phosphate Buffer, pH 7.5
2. B: 0.65% Casein
3. C: 110mM Tricholoacetic acid, 6.1N
4. D: 0.5mM Folin & Ciocalteu's Phenol reagent (F-C)
5. E: 500mM Sodium Carbonate (Na_2CO_3)
6. F: 10mM Sodium Acetate Buffer with 5mM Calcium Acetate, pH 7.5 at 37˚C. (Enzyme Diluent)
7. G: 1.1mM L-Tyrosine Standard
8. H: Protease Enzyme solution

Reagent preparation:

1. A: 50mM Potassium Phosphate Buffer, pH 7.5

   Prepare using 11.4mg/ml of potassium phosphate dibasic, trihydrate in deionized water and pH was adjusted with 1M HCl.

2. B: 0.65% (w/v) Casein Solution

   0.65% (w/v) casein solution is prepared by mixing 6.5mg/ml of casein in 50mM potassium phosphate buffer. Gradually increase the solution temperature with gentle stirring to 80-85˚C for 10 minutes until a homogenous dispersion was achieved. It is

very important not to boil the solution. The pH was then adjusted if necessary with 1M NaOH and 1 M HCl.

3. C: 110mM Trichloroacetaic acid (TCA), 6.1N

Prepared by diluting a 6.1N stock 1:55 with deionized water. Trichloroacetic acid is a strong acid and should be handled with care.

4. D: 0.5mM Folin & Ciocalteu's Phenol Reagent (F-C)

Prepare 0.5 mM Folin & Ciocalteu's Phenol Reagent (F-C) by diluting 1:3 with deionized water. The solution reacted with cysteine to generate a measureable colour change that directly related to the activity of protease. Folin's phenol reagent is an acid and should be handled with care.

5. E: 500mM Sodium Carbonate (Na_2CO_3)

Prepare 500mM sodium carbonate using 53mg/ml of anhydrous sodium carbonate with deionized water.

6. F: 10mM sodium Acetate Buffer with 5mM Calcium Acetate, pH 7.5 at 37˚C (Enzyme Diluent)

This solution used to dilute enzyme solutions.

7. G: 1.1mM L-Cysteine standard (Standard Solution)

Prepared using 0.13mg/ml L-cysteine in deionized water and heated gently until the cysteine dissolves. As with casein, do not boil this solution. L-cysteine standard was allowed to cool to room temperature. This solution used to make standard curve.

8. H: Protease Enzyme Solution

Immediately before use, 0.5ml protease was dissolve in 0.5ml enzyme diluent solution

Appendix B

Preparation of standard curve

A standard curve was prepared by pipetting the following reagents into suitable vials (in millilitres):

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Blank
Reagent G	0.05	0.10	0.20	0.30	0.40	0.50	0.00
Deionized water	1.95	1.90	1.80	1.70	1.60	1.50	2.00
Reagent E (Na_2CO_3)	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Reagent D (F-C)	1.00	1.00	1.00	1.00	1.00	1.00	1.00

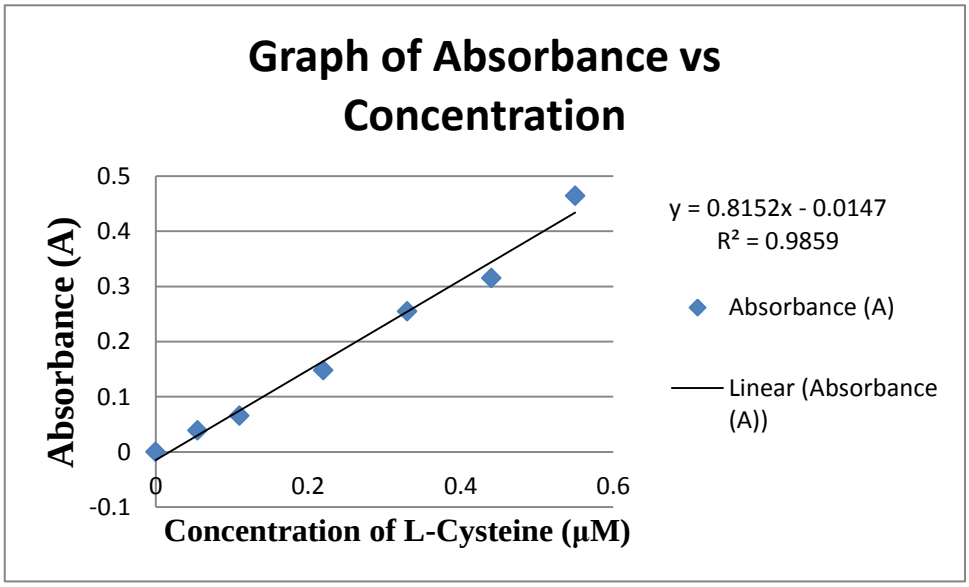
The solution was mixed by swirling and incubated at 37˚C for 30 minutes. Then, they were allowed to cool to room temperature. The absorbance was read at 660nm for each of the vials in suitable cuvettes.

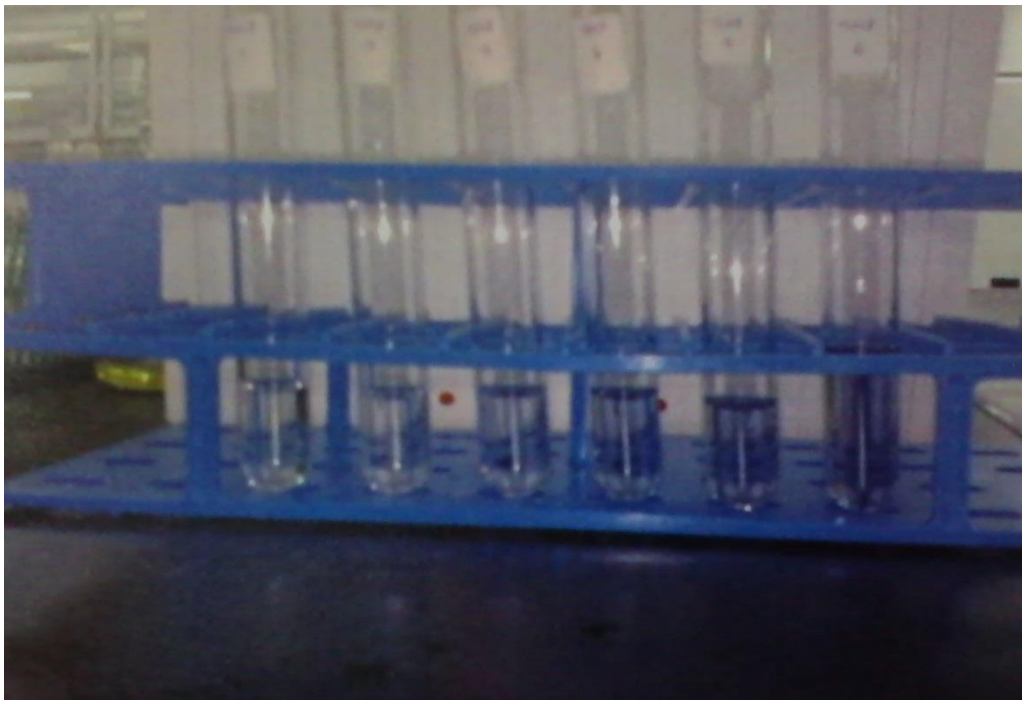
The standard curve was created using a graphing program to plot the changes in absorbance of the standard on Y-axis versus the amount in micromoles (μM) for each of 5 standards on the X axis.

Concentration of L-Cysteine (µM)	Absorbance (A)
0	0
0.055	0.039
0.11	0.066
0.22	0.148
0.33	0.255
0.44	0.315
0.55	0.464

Absorbance reading for L-Cysteine standard curve

A standard graph was plotted.





The colour development of standard curve

A best line fit and corresponding slope equation was generated. The slope equation was y= 0.8152x - 0.0147. The result in the micromoles of L-Cysteine liberated during this particular proteolytic reaction could be obtained by inserting the absorbance value for one of the best test sample into the slope equation.

Appendix C

Calculation of enzyme activity

To get the activity of enzyme in Units/mL, the following calculation was performed.

$$\frac{Units}{mL}(Enzyme) = \frac{\mu M(L - Cysteine) \times (11)}{(1) \times (10) \times (2)}$$

where;

11 = total volume (in millilitres) of assay

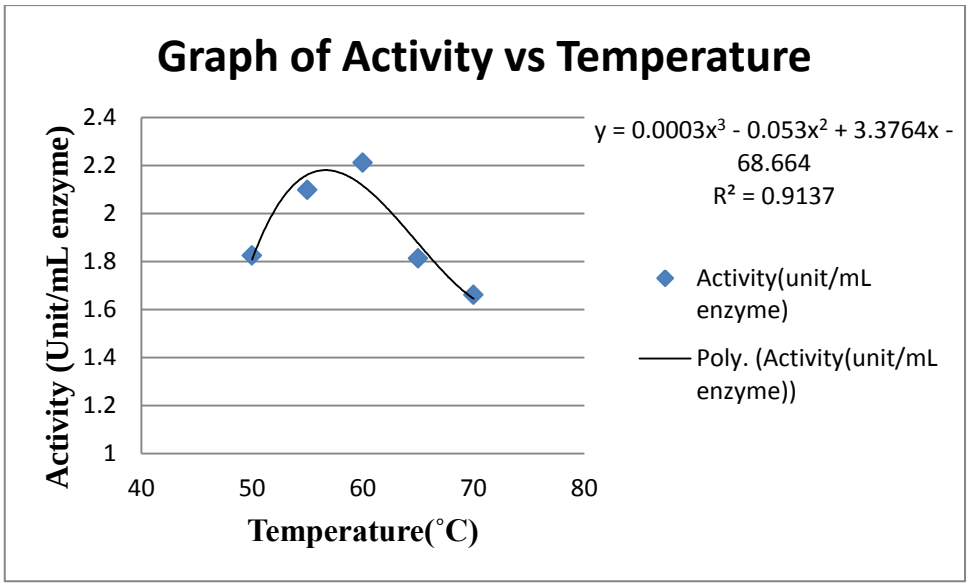
10 = time of assay (in minutes) as per the Unit Definition

1 = volume of enzyme (in millilitres) of enzyme used

2 = volume (in millilitres) used in Colorimetric Determination

Temperature (˚C)	**Absorbance (x10 dilution factor)**	**Concentration of enzyme (µM)**	**Activity(unit/mL enzyme)**
50	2.58	3.32	1.824
55	2.69	3.81	2.098
60	3.12	4.02	2.211
65	2.56	3.30	1.812
70	2.35	3.02	1.661

(Izzah. S., 2010)


Graph of Activity vs Temperature
Activity (Unit/mL enzyme)
2.4
2.2
2
1.8
1.6
1.4
1.2
1
40
50
60
70
80
Temperature(˚C)
y = 0.0003x³ - 0.053x² + 3.3764x - 68.664
R² = 0.9137
Activity(unit/mL enzyme)
Poly. (Activity(unit/mL enzyme))