EXTRACTION OF PAPAIN ENZYMES FROM PAPAYA LEAVES

MASITA BINTI MD ISA

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

APRIL 2010

ABSTRACT

Due to importance of maximizing commercial value of papaya fruits, selection of papaya leaves as raw material in extracting papain enzymes is necessary to maintain the sales value of the fruit. The aim of the present study is to find out the best pre-treatment approach in extracting papain enzymes from papaya leaves as well as to determine the optimum parameter for the extraction process. The leaves were subjected to a pre-treatment approach followed by hot water extraction process and then, enzymatic analysis. Among various pre-treatment approaches, maximum amino acids concentration, 0.15 mol/liter was achieved when grinding incorporated with ultrasonication was applied. The optimum period for extraction was 4 hours while optimum temperature was at 65°C. At this optimum condition, 0.45 mol/liter amino acids was achieved after grinding incorporated with ultrasonication sample undergo extraction process while 0.41 mol/liter amino acids was achieved after enzyme treatment incorporated with ultrasonication sample undergo extraction process. Utilization of HPLC is highly recommended to get the actual yield of papain enzymes being extracted without being declared as other substances.

ABSTRAK

Melihat kepada kepentingan memaksimumkan nilai komersial buah betik, pemilihan daun betik sebagai bahan mentah dalam proses mengekstrak enzim papain adalah perlu bagi mempertahankan nilai harga jualan buah. Tujuan kajian ini adalah untuk mengetahui pendekatan pra-rawatan terbaik dalam mengeluarkan enzim papain dari daun betik serta untuk menentukan parameter optimum untuk proses ekstraksi. Daun dikenakan pendekatan pra-rawatan diikuti dengan proses pengestrakan menggunakan air panas dan kemudiannya analisis enzim. Di antara pelbagai pendekatan pra-rawatan, kepekatan maksimum asid amino, 0.15 mol / liter dicapai apabila gabungan proses kisaran dan ultrasonic diaplikasikan. Tempoh optimum untuk proses pengestrakan adalah selama 4 jam sementara suhu optimum adalah pada 65°C. Pada keadaan optimum, 0.45 mol / liter asid amino dicapai selepas sampel dari gabungan proses kisaran dan ultrasonik mengalami proses pengestrakan manakala 0.41 mol / liter asid amino dicapai selepas sampel dari gabungan proses rawatan enzim dan ultrasonik menjalani proses pengestrakan. Penggunaan HPLC sangat disarankan dalam mengetahui jumlah sebenar enzim papain yang berjaya diekstrak tanpa perlu dinyatakan sebagai molekul lain.

TABLE OF CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

mm	-	millimeters
º C	-	degree Celcius
WHO	-	World Health Organization
%	-	percent
g	-	grams
kg	-	kilogram
BPC	-	Biotechnology Performance Grade
CFTRI	-	Central Food Technological Research Institute
h	-	hour
kHz	-	Kilo Hertz
min	-	minute
MIBK	-	Methyl isobutyl ketone
DNA	-	Deoxyribonucleic acid
cm^2	-	Centimeter square
w/w	-	weight over weight
TCA	-	Trichloroacetic Acid
ml	-	milliliter
mM	-	milimolar
nm	-	nanometer
OD	-	Optical density
H_2O	-	water
A	-	Absorbance
HPLC	-	High-Performance Liquid Chromatography
BAEE	-	N-Benzoyl-L-arginine ethyl ester

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Carica papaya, which comes from Caricaceae family is a fruit native to eastern Central America and was cultivated long before the arrival of the Europeans; Spanish and Portuguese invaders took the fruit and quickly spread it to their other settlements. By 1513 and 1583, it was found growing in the West Indies and East Indies respectively. Its population growth into Africa and then spread through the Pacific islands as Europeans discovered it. By 1800, papaya was grown in all tropical regions, with Hawaii and South Africa now the main exporters. By referring to CRC World Dictionary of Plant Names, this non-seasonal fruit tree is also known as Betik (Malaya), Kepaya (Philippines), Mountain pawpaw (English), Wan Shou Kuo (China), Malulo (Congo), Fafy (Arabic), Mikana (Hawaii) and Namona (Paraguay). In addition, the plant is also described in a documented property forms and it act as analgesic, amebicide, antibacterial, cardiotonic, cholagogue, digestive, emenagogue, febrifuge, hypotensive, laxative, pectoral, stomachic and vermifuge (Anibijuwon and Udeze, 2009).

The papaya plant is a large herb that growth rapidly, producing a soft wood and its height can be reached of more than twenty-five feet. The plant did not consume much time to growth and bears fruit within a year, continuing to do so for another two years before the tree is cut down. The papaya tree needs a tropical climate, that is dry when cold and wet when warm, consequently, the best conditions for the tree to grow is at 25 °C, limited exposure to storm or winds, and have good

drainage as water-logging will cause the roots become rot and then kill the taproot within forty-eight hours. Besides, it does grow well in containers, making it an ideal house plant that can be put outside in the spring as soon as the fear of frost is past. This step is maybe the best solution that can be done in four season state as frost can kills the tree.

1.2 Problem Statement

A papain enzyme is abundance found in the leaves and the skin of the green fruits. Even though the quantity of papain enzyme is much higher in the latex from the skin of unripe fruit but the problem is it will affect the market value of the papaya. In order to collect the latex, three or four vertical incisions were made in the fruits with a sharp stainless steel instrument to a depth of 2 mm (Monti, 2000). Then, after two or three months the fruits are ripe and of course it still edible, but due to their scarred appearance, the sale value is decreases.

Common scenario in Malaysia, papaya tree is planted just to get its edible fruits but not the other part. Its leaves are commonly served as waste and it will be thrown away when the tree is cut off. Knowing that this leaves also have valuable enzyme that can be extracted and give profit, it is the best opportunity to create wealth from the waste and the most highlight opportunity is to create health from the waste.

Frequently, extraction process is done by using solvent but it will need further process in order to purify the extraction yield. It is safe and better to use green solvent as pre-treatment approach can help to recover the intracellular products. Besides, extraction of papain enzyme from the leaves has been established in other country but not in Malaysia. Thus, this is the idea to get the optimum condition for extraction process before the step for commercialize is proceed.

1.3 Objectives

The objectives of this study are to find out the best pre-treatment method and to determine the optimum parameter for extraction process.

1.4 Scope of Study

The scopes of this study are:

1. Enzyme treatment and grinding incorporate with ultrasonication will be applies as pre treatment.

2. Temperature will be use in hot water extraction is in the range 50º C – 70 ºC.

3. Period of extraction is in the range 1hour -6 hour.

1.5 Significance of Study

Hot water extraction is conventional method for extraction process and more often than not, it is not promised the much amount of extraction yield. In this research, pre-treatment is proposed as an idea to improve the efficiency of this conventional method so that the used of organic solvent in extraction process can be eliminated.

. Besides, this research can help to reduce the amount of waste and create a clean environment by utilizing the papaya leaves to create wealth from waste. Rather than dumping the leaves, it is the best solution to extract the leaves contains and converts it into valuable products.

In addition, previous studies have been proved that papain enzymes from papaya leaves can gives benefits to human health. It can be used as dengue cure, stomach and digestive order remedies and also gives chance for cancer patient to survive. Thus, this research is implementing the waste to health concept as papaya leaves have a valuable enzyme and can be utilized as medical product.

CHAPTER 2

LITERATURE REVIEW

2.1 Plant as Remedy

In an age when prescription drugs and over-the-counter medications are so plentiful and varied, we tend to forget that the vast number of these may well derive from common vegetation to be found in our local forests, bush, and wetlands. (Essortment.com, 2002). The reliance on herbal medicine continues to rise as the costs of conventional drugs increase and are becoming unaffordable by many in rural communities. In Nigeria, traditional healers and remedies made from plants play an important role in the health of millions of people especially in the rural areas (Rukangira, 2001). If ratios were to be compared between traditional practitioners and university trained doctors being patronized by the Nigerian populace; it is sure that there will be a tilt in high numbers, towards the traditional healers (Matthew, 2009). This consequently means that most of the populace are more exposed and disposed to taking traditional recipe as opposed to the Orthodox, refined medicine (Rukangira, 2001).

It is also a known fact that the Orthodox drugs are refined from extracts of many of these medicinal plants. This is why the traditional medicine has some success story. In all countries of the world, there exists traditional knowledge related to the health of humans and animals (Matthew, 2009). The importance of traditional medicine as a source of primary health care was officially recognized by the World Health Organization (WHO) in the primary health care declaration of Alma Ata (1978) and has been globally addressed since 1976 by the traditional medicine

program of the WHO (Rukangira, 2001). The program defined traditional medicine as: “the sum total of all the knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusive on practical experience and observation handed down from generation to generation, whether verbally or in writing” (Rukangira, 2001).

As an alternative to pharmaceuticals, many consumers are enlisting the aid of a good defense - herbal remedies. According to the World Health Organization, 80 percent of the world's people rely on plants for their medicines and their use has skyrocketed in Europe and America in recent years, due primarily to concerns about booming health care costs and fears over the side effects of conventional medicines. According to Health and Nutrition Breakthroughs, the overuse of antibiotics in Western medicine has also encouraged a revival of herbal remedies, as more and more bacterial strains become immune to antibiotic drugs (Rembert, 2009).

Referring to Anibijuwon and Udeze (2009), there is no plant without medicinal value. Papayas are an excellent fruit for antioxidants, containing not only vitamin E, but also more vitamin A than carrots, and more vitamin C than oranges. It is also an excellent source of calcium, potassium, iron, B vitamins, and proteins. The active compounds are normally extracted from all plant parts, but the level of the compounds differs in different parts of the tree, the age of the tree and also the sex of the tree. For example, phenolic compounds are found to be higher in male trees than female trees and almost no papain found in fully ripe fruit but there is abundance found in the leaves and green fruit. In addition, female and hermaphrodite trees yield cruder papain than male trees and older fruit yields more than younger fruit. However, the activity of the papain is higher in the extracts from the younger fruit than the older fruit. Thus, parts known to contain the highest concentration of the principles are preferred to therapeutic purposes and it can be either be the leaves, stems, barks, roots, bulks, woods, flowers, fruits or the seeds (Kafaru, 1994).

2.2 Papaya Leaf

Papaya leaf, the huge fingered leaves form a spiral similar to those of the palm tree is an excellent treatment for digestive disorders and extremely useful for any disturbances of the gastrointestinal tract. At the same time, papaya leaves is often used in restaurants and is the major recipe in commercial meat tenderizes. Just take the leaves and wrapped the meat with it.

The medicinal folk used the leaves poultice onto nervous pains and elephantoid growths. The leaf smoked for asthma relief in various remote areas. In Indonesia, papaya leaves are used as feed for animals after parturition. Two leaves boiled in water fed every 2 days for 1 week to tenderize their flesh (Stacey Chillemi). It also has been reported that papaya leaf extracts is used as a profilaxis against malaria, though no studies on this use could be found in literature (Satrija *et al.*, 1994).

Papaya leaf juice is claimed to have reversed cancer in many people living on the Gold Coast in Australia. Harold W. Tietze in his book Papaya The Medicine Tree, describes how to make the juice and tells the stories of many cancer survivors who reportedly used the juice to get rid of their cancer. The book contains the following report that was published in the Gold Coast Bulletin "PawPaw Cancer Plea Bears Fruit". Gold coast gardeners have responded to an appeal by cancer victims desperate to find supplies of pawpaw leaves and the Gold Coast man who, 14 years ago, first exposed the leaves as a possible cure for cancer has been tracked down to a Labrador (Gold Coast) nursing home. The story of how Stan Sheldon cured himself of cancer by drinking the boiled extract of pawpaw leaves was first told in the Gold Coast Bulletin in 1978 (Xiamen inc, 2009). Following quote is taken from a letter that has been written by R.J.W:

"*… I was inspired to send some leaves to a few people dying from cancer. The first, a banana grower aged 40, had two operations on his bladder for cancer which did not prevent metastasis. I placed him on a very simple diet consisting of zero junk food, fresh living food with no preservatives, white flour, sugar, colourings*

or additives and told him to "stuff a handful of pawpaw leaves into a saucepan and fill with water. Boil, simmer for one hour and drink it till it comes out of your ears. He did so and five weeks had no trace of cancer whatsoever." (Xiamen inc, 2009).

2.3 Beneficial of Papain

Cysteine protease hydrolase enzyme is the extract from papaya that is known as the papain enzyme and this enzyme is one of the special compounds that give much benefit for health effect. This protein-dissolving substance is an excellent remedy for stomach and digestive disorders. It also has the ability to dissolves dead tissues without damaging living cells and a partial quote from *Papaya leaves for Cancer* highlight this enzyme has been proven in labs and clinical studies to eat away the protein fibrous coating on cancer cells so that your body can kill the cancer. Hence, no wonder this enzyme is established as a weapon to cure cancer.

The application of papain was extensively investigated through research and recent research indicates that papain enzymes used in tropical folk medicine to heal cuts and wounds, truly has healing properties. This claimed has been proved through the paper of Mahmood *et al*. (2005). His group has tested the efficacy of papain on wound healing potential in rats. Four groups of male *Sprague Dawley* rats each consists of six animals were experimentally wounded in the posterior neck area. The wound on the rats Group 1, 2 and 3 was treated with blank Vaseline, 5 and 10% Vaseline, respectively. On the other hand, as a reference, group 4 is treated with Solcoseryl jelly. Group 2, 3 and 4 have shown accelerate wound healing potential compared to group 1. These results strongly document that papain accelerates the wound healing process, thus, it may be the reason for the patient used this enzymes to reduce swelling after surgery.

In some parts of the world, papain is applied in veterinary area as vermifuge, anthelmintic and amoebacide that eliminates worms and other parasites (Chillemi) or in other simple daily words, it is used as de-wormers. This can be proved by

reviewing research conducted by Satrija *et al*. (1994) that tested the efficacy of papain against *Ascaris suum* in 16 pigs. The result showed that at doses 4- and 8-g/kg BW treatments significantly decreased 99% the egg per gram produced and the number of adult worms by 80 and 100%, respectively. Satrija *et al*. also had carry out a study that indicates that papain is effective against *Ascaridia galli* in chickens.

Papain also has been identified as antimicrobial and Anibijuwon and Udeze (2009) had conducted this research against some human pathogenic bacteria such as *Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli, Streptococcus mutans, Staphylococcus aureus* and *Proteus mirabilis*. The minimum inhibitory concentration showed that papain has potential to inhibit growth such pathogenic bacteria.

Generally, papain promised much benefits for us although not so many research is conducted to prove all these beneficial. The powdered papain enzyme extract is made into a paste and often used to treat jelly fish, bee and yellow jacket stings and stingray wounds (Nutritional Supplement, 2007). It is also has been practiced at Trinidad to treat scorpion bites. The action of the enzyme breaks down the toxin and the venom. This enzyme has also been found to be very effective in treating lower back pain, sprains and strains and also an excellent aid to keep the skin healthy. Also, it helps boost immune system function and is believed to be helpful in shingles, allergies and tumors (Nutritional Supplement, 2007).

2.4 Toxicity

The common established disadvantage of papain is decreasing infertility. In trials with rats, it caused infertility and irregular oestrous cycle to female rats while male rats had decreased sperm motility. The oral doses also decreased testis mass and sperm count. The fertility of the male and female rats came back to normal within 2 months (60 days) after the treatment were stop (Cornell University Department of Animal Science). In addition, papain might cause abortions shortly

after conception as it apparently dissolves a protein (s) responsible for adhering the newly fertilized egg to the wall of the uterus (Cornell University Department of Animal Science). By knowing this fact, pregnant women should avoid from taking papain in order to save the baby life.

Precaution step must also being taken by person who takes blood medication (Sulfinpyrazone) or blood thinning medication (Coumadin, aspirin,etc.). Before taking herb that consist papain enzyme, consult with physicians as it may have anticoagulant properties. Besides, individual that have latex allergy problem should never take papaya in all its forms as it will cause anaphylactic shock. Ensure to keep papaya powder in safe place as inhaling papaya powder which is high in the enzymes papain and chymopapain can induce allergies (Viable Herbal Solutions).

At the first place, the use of papain enzyme is very safe. However, it is the best approach to consult a physician before someone includes nutritional supplements of any kind in the diet. This warning should be specially heeded by all potential individual as stressed above.

2.5 Production of Papain

Conventionally, papain enzyme is produced from the milky latex obtained from the skin of the green fruit. Fruits that have nearly reached their full size but are still green give the highest latex yield and normally, latex yields are greatest in the first twelve months of tapping. In the second year, the yields are about 65% of the first year's diminishing more in each following year. This is because, the size and the number of fruits, as well as the latex yield decrease with age and height of trees. In many plantations the economic age limit is around two and a half years.

2.5.1 Time of Latex Collection

Collecting the latex should be done during the morning hours or on misty, cloudy days or after a good rain, generally in seasonal periods when warm temperatures and humid conditions coincide. Tapping the fruit at this time is conducive in getting the higher yields of latex milk. Collecting latex from the trees during times of hot, dry, and/or windy weather conditions would not only be a wasted effort, but would considerably weaken the whole stand of trees. Tapping is also unadvisable at low temperatures.

2.5.2 Fruits to be Tapped

The oldest fruits (lowest on the trunk) alone should be tapped while they are still entirely green. For various reasons it is unadvisable to extract latex from all the green fruits on the trunk. As mention above, fruits that have nearly reached their full size but are still green give the highest latex yield. If the younger, small fruits higher on the trunk are tapped at the same time as the old fruits, growth will be arrested and they will ripen prematurely. The whole tree will be greatly weakened by the repeated, excessive loss of fluid. When a fruit, irrespective of its size, develops yellow patches on its skin, its sap pressure, its latex quantity, and the enzyme concentration diminishes rapidly. A fully yellow fruits contains little latex and almost no enzymes (Becker, 1995).

2.5.3 Tools for Tapping

The incisor, used for making the incisions in the skin of the fruits, simply consists of a stick or stake to which part of a common, double-edge safety razor blade is attached. The blade is inserted in a stake of light, tough, well-planed wood eight to ten mm. wide and thick enough to be stable. The length depends upon the height of the fruit. No more than three mm of blade should be exposed, the sharp

edge pointing backward. If the stick is taken from suitable wood, it will hold the blade firmly. If not, the blade may be cemented into the slit by using a waterproof household cement, or by tying thin silk or nylon thread around the stake near the blade. Any sharp edges on the head of the stake should be rounded off (Becker, 1995).

2.5.4 Manufacturing Process

White milky latex of green and fully grown papaya fruits is collected in the early morning by making deep longitudinal cuts by stainless steel or wooden sharp knives. Latex is collected in stainless steel trays while latex coagulated in the surface of the fruits is scrapped and collected in the trays. A fruit is tapped about 6 times in the course of 16 days. This latex is passed through 50 mesh sieves to remove dirt and then it is mixed with potassium metabisulphate and spread on trays and dried in a vacuum shield drier at a temperature of about 55°C for 4-5 hours.

The dried product is packed in air-tight containers and stored in a cool, dry place. It should be kept in flake form as powdering decreases the stability of the product during storage. Dried flakes are powdered and diluted with lactose powder to get BPC grade papain. Plastic containers should be used to pack crude papain flakes or powder as metal containers would result in loss of enzyme activity. Transportation is also very critical as papain has to be kept below 20°C temperature or else its shelf life is reduced. With proper storage and handling, its shelf life is 5-6 months.

Recovery of BPC grade papain is in the range of 25% to 30%. In other words, 100 kgs. of good quality latex is required to produce 25-30 kgs. of BPC grade papain. CFTRI, Mysore, has developed the technical knowhow for the product. The process flow chart is as below:

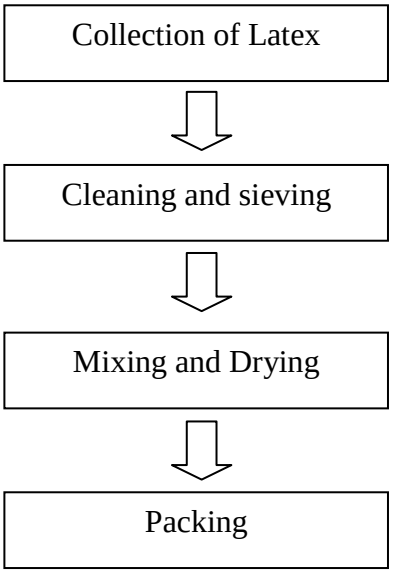

Figure 2.1: Manufacturing process of BPC grade papain

2.6 Pre-treatment

Papain is an intracellular enzyme which is surrounded by a cell wall barrier consists of cellulose, hemi-cellulose and pectin. The disruption process which is known as pre-treatment need to be done to break the barrier so that the release of intracellular contains is increases. Pre-treatment can be done by some option corresponding to the different types of cells and the problems involved in isolating the enzymes (Golker, 2004). There are two groups of option that can be chosen either physical methods or chemical methods.

2.6.1 Pre-treatment option

Physical methods are targeted more towards cell wall disruption while the chemical methods are mainly used for destabilizing the cell membrane. There is some example for both methods:

Table 2.1: Physical and chemical method for pre-treatment approach

Physical methods	Chemical methods
Disruption in bead mill	Disruption using detergents
Disruption using a rotor-stator mill	Disruption using enzymes
Disruption using French press	Disruption using solvents
Disruption using ultrasonic vibrations	Disruption using osmotic shock

The combination between enzyme treatment and grinding with ultrasonication is applied in enhancing the amount of papain enzyme recovery. Enzyme treatment integrated with ultrasonication is a combination of chemical and physical methods while grinding incorporated with ultrasonication is a combination of physical methods. By this, the best approach is determined based on the amount of papain enzyme recovery.

2.6.2 Enzyme treatment

In the paper written by Shweta Shah (2004), extraction with enzyme pretreatment and sonication have been reported as an efficient procedure for obtaining oil from Jatropha seed kernels. The oil yield percentage is increases to 97% when sonication is applied before enzyme pretreatment compare to only 92% yield obtained from enzyme pretreatment without sonication. Pinelo (2008) also claimed that the use of cell wall degrading enzymes can improve the extraction of phenols as the total phenol release was generally higher when enzymatic maceration was applied. The main reason for recovery increasing with the use of enzymes is that the enzymes disrupt the integrity of the cell walls; as a result, the extraction is more efficient (Li *et al.*, 2006). Based on these reasons, enzymatic maceration is being chosen as one of the pre treatment approach for this study.

2.6.2.1 Cellulase

The plant cell walls constituents which are cellulose, hemi-cellulose and pectin can be hydrolyzed by using cellulase, beta-glucosidase and pectinase enzyme, respectively. It have been proved that beta-glucosidase is the most effective enzyme for extracting taxanes from needles of T.Chinensis but, it is not affordable. Hence, by considering the economics effect, cellulase was chosen for the treatment of needles (Zu *et al.*, 2009). This idea can be applied for extracting intracellular products from the plant source including papaya leaves.

The best condition for cellulose to work is at its optimum temperature, 50°C (Dr. A. Aboul-Enein, 2010). The parameter for enzymatic approach is determined based on the previous study conducted by Zu *et al.* (2009) as cellulase is utilized in the research.

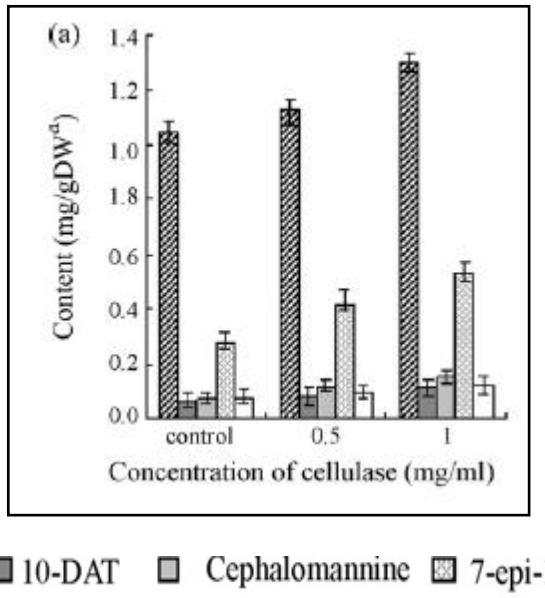

Figure 2.2: Comparison between different concentrations of cellulase in extraction of different plant (Zu *et al*., 2009)

This study was used 1-3% of concentration for cellulose and it was identified that 3% concentration of cellulase give the best yield.