ISOLATION AND IDENTIFICATION OF PROTEASE PRODUCING BACTERIA FROM VARIOUS SOURCES RELATED TO *Hevea brasiliensis*

by

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ADH decarboxylation of the amino acid arginine by arginine dihydrolase

AMY fermentation of amygdalin (glycoside)

ARA fermentation of arabinose (pentose sugar)

ATCC American Type Culture Collection

BSA Bovine serum albumin

CIT utilization of citrate as sole carbon source

DPNR Deproteinised natural rubber

DRC Dry rubber content

GEL test for the production of the enzyme gelatinase which liquefies gelatin

GLU fermentation of glucose (hexose sugar)

HNS Hydroxylamine neutral sulfate

H_2S production of hydrogen sulfide

INO fermentation of inositol (cyclic polyalcohol)

IND production of indole from tryptophan by the enzyme tryptophanase. Indole is detected by addition of Kovac's reagent.

LDC decarboxylations of the amino acid lysine by lysine decarboxylase

MAN fermentation of mannose (hexose sugar)

MEL fermentation of melibiose (disaccharide)

NRL Natural rubber latex

NRS Natural rubber serum

N10 Polyoxyethylene 10 phenol ether

NB Nutrient broth

OD Optical Density

ONPG test for b-galactosidase enzyme by hydrolysis of the substrate

	o- nitrophenyl-b-D- galactopyranoside
ODC	decarboxylations of the amino acid ornithine by ornithine decarboxylase
phr	Part per hundred rubber
pH	potential of Hydrogen
RHA	fermentation of rhamnose (methyl pentose sugar)
rpm	Rotation per minute
SAC	fermentation of sucrose (disaccharide)
SOR	fermentation of sorbitol (alcohol sugar)
TSB	Tryptic Soy Broth
TCA	Trichloroacetic acid
TDA	detection of the enzyme tryptophan deaminase
UPPA	Universal protein precipitating agents
URE	test for the enzyme urease
VFA	Volatile fatty acid
VP	the Voges-Proskauer test for the detection of acetoin (acetyl methylcarbinol) produced by fermentation of glucose by bacteria utilizing the butylene glycol pathway

LIST OF SYMBOLS

℃	Degree Celcius
%	percent
et al.	and other people
h	hour
µg	micro gram
µl	micro liter
w/v	weight per volume
H_2SO_4	Sulphuric acid
$FeSO_4$	Ferrous sulfate
$MgSO_4$	Magnesium sulfate
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO	Dipotassium phosphate
$(NH_4)_2SO_4$	Ammonium Sulphate
NH_4Cl	Ammonium chloride
NH_4NO_3	Ammonium Nitrate
NaOH	Sodium hydroxide

PEMENCILAN DAN PENGENALPASTIAN BAKTERIA PENGHASIL PROTEASE DARIPADA PELBAGAI SUMBER BERKAITAN *Hevea brasiliensis*

ABSTRAK

Penyaringan dan pemencilan bakteria penghasil protease telah dilakukan ke atas pokok getah *Hevea brasiliensis* yang merangkumi batang pokok, susu getah, getah skrap (getah beku), tanah di sekeliling pokok getah, susu getah yang dikumpulkan di kilang memproses getah, sisa getah yang terdapat pada tanah di keliling pokok getah dan cecair kumbahan dari kilang memproses getah. Sebanyak 15 pencilan telah menunjukkan hidrolisis pada agar 10 % susu tanpa lemak yang menandakan wujudnya penghasilan protease daripada bakteria yang dipencilkan. Daripada kesemua pencilan, tiga daripadanya dikenalpasti sebagai *Bacillus* sp. telah dipilih untuk kajian selanjutnya. Tiga pencilan ini dinamakan sebagai Strain K, Strain M dan Strain O. Strain K dan strain O telah menunjukkan suhu pertumbuhan setinggi 45 ºC dalam kadar pH yang luas iaitu dari pH 4 hingga pH 10. Hasil awal menunjukkan suhu 37 ºC dan pH 7 merupakan keadaan terbaik untuk pertumbuhan kedua dua strain ini. Strain M menunjukkan pertumbuhan yang optimum pada suhu 37 ºC dan pH 9. Kesemua strain menghasilkan protease paling tinggi pada 200 psm. Strain K menghasilkan protease tertinggi sebanyak 84 U/ml di dalam medium TSB apabila 1 % glukosa ditambah. Strain M menghasilkan protease tertinggi sebanyak 158 U/ml di dalam medium yang mengandungi 0.5 % glukosa, 0.75 % pepton, 0.5 % KH_2PO_4, 0.5 % $MgSO_4$ and 0.01 % $FeSO_4$. Kedua dua strain K dan M menunjukkan pertambahan penghasilan protease apabila diransang dengan pepton. Pencilan ini juga menunjukkan pertumbuhan dan penghasilan protease apabila dihidupkan di dalam serum getah asli. Namun

begitu, untuk mendapatkan pertumbuhan dan penghasilan protease yang lebih bermakna, serum getah asli perlu diperkayakan dengan medium basal dan glukosa. Apabila supernatan daripada strain M yang mengandungi protease diaplikasikan pada susu getah, terdapat penurunan lebih dari 80 % kandungan nitrogen pada getah kering dalam masa 72 jam tempoh aplikasi. Proses pengemparan getah asli sebelum diaplikasikan dengan supernatan yang mengandungi protesea telah dapat meningkatkan lagi tahap penguraian protein pada getah asli. Protease yang dihasilkan oleh strain ini juga menunjukkan kestabilan pada N10 and HNS, yang berindak sebagai pengstabil dalam pemprosesan getah.

ISOLATION AND IDENTIFICATION OF PROTEASE PRODUCING BACTERIA FROM VARIOUS SOURCES RELATED TO *Hevea brasiliensis*

ABSTRACT

Screening and isolation of proteolytic bacteria were carried out from *Hevea brasiliensis* tree including from bark, fresh latex, coagulated rubber, soil around the tree, latex from processing tank, residual latex on soil and effluent from effluent treatment pond. Fifteen isolates showed hydrolysis on 10 % of skim milk agar, indicating protease production from these isolates. Among the isolates, three were identified as *Bacillus* sp. and were selected for further study, designated as Strain K, Strain M and Strain O. Strain K and Strain O showed growth temperatures as high as 45 ºC within a broad pH range of 4 to 10. Preliminary results showed that both bacterial isolates grew best at 37 ºC and pH 7. Strain M showed optimal growth at 37 ºC and pH 9. All isolates produced the highest protease activity at 200 rpm. Strain K produced the highest protease activity around 84 U/ml in a TSB medium with 1 % glucose added. Strain M produced the highest protease activity around 158 U/ml in a medium containing 0.5 % glucose, 0.75 % peptone, 0.5 % KH_2PO_4, 0.5 % $MgSO_4$ and 0.01 % $FeSO_4$. Both of these strains showed an incremental in enzyme production when induced with peptone. These isolates also showed growth and protease activity in natural rubber serum. However to obtain significant growth and protease production, natural rubber serum needs to be supplemented with basal medium and glucose. When the supernatant of Strain M containing proteases were applied to latex, there was a reduction of almost 80 % of the nitrogen content in the dry rubber within 72 hours. Centrifugation process of the latex was found to be a good method in improving the latex condition for better

degradation of protein. The enzymes also showed good stability in the presence of N10 and HNS, which work as stabilizer.

CHAPTER 1- INTRODUCTION

1.1 Protease

Proteases (proteinases, peptidase or proteolytic enzymes) are a large group of enzymes, ubiquitous in nature and found in a wide variety of microorganisms. Proteases also known as peptidyl-peptide hydrolases (EC 3.4.21-24 and 99), are industrially useful enzyme (Qasim *et al.*, 2003). They are molecules of relatively small size and compact, spherically structures that catalyze the peptide bond cleavage in proteins (Polgar, 1989). Basically, there are six classes of proteases, which are serine proteases, threonine proteases, cycteine proteases, aspartic acid proteases, metalloproteases and glutamic acid proteases (Beynon and Bond, 1989). Proteases break peptide bonds between amino acids of proteins by cleave at peptide bond involves making an amino acid residue that has the character of a polarized peptide bond (serine, cysteine and threonine peptidases) or a water molecule (aspartic acid, metallo- and glutamic acid peptidases) nucleophilic so that it can attack the peptide carbonyl group. One way to make a nucleophile is by a catalytic triad, where a histidine residue is used to activate serine, cysteine or threonine as a nucleophile. These enzymes are important in a number of diverse and crucial biological processes. For example, they involve in the regulation of metabolism and gene expression,

enzyme modification, pathogenecity and the hydrolysis of large proteins to smaller molecules for transport and metabolism (Rao *et al*., 1998). Proteases are also further subdivided into exopeptidase and endopeptidase. Exopeptidase enzymes cleave peptide bonds at the amino or carboxy-terminus and endopeptidase cleave peptide bonds internally in a polypeptide (Beynon and Bond, 1989).

1.2 Protease production

Protease production is an inherent property of all organisms and these enzymes are generally constitutive. However at times they are partially inducible.

1.2.1 Nutritional factors

Extracellular protease production in microorganisms is highly influenced by media components, for instance the variation in C/N ratio, presence of some easily metabolizable sugar, such as glucose and presence of metal ion such as Fe^{3+}, $Ca^{2+,}$ Mg^{2+}, Na^{+}, Zn^{2+} and Cu^{2+} (Ellaiah *et al*., 2003; Adinarayana, *et al*., 2003). These metal ions protect the enzyme against thermal denaturation and play a vital role in maintaining the active formation of the enzyme at higher temperature (Gupta *et al*., 2002a).

1.2.2 Physical factors

Besides nutritional factors, several other physical factors such as aeration (Rahman, 2005), inoculum density, pH (Chen *et al*., 2002), temperature (Votruba *et al*., 1991)) and incubation time (Beg *et al*., 2003) also affect the amount of protease produced (Qasim *et al*., 2003).

1.2.3 Strain factor

The amount of protease produced also varies greatly with strains used, the existence of distinct protease from different strains, which catalyze the same reactions allow flexibility in choice of fermentation conditions since these different enzymes may have different stabilities and different pH and temperature optima (Manachini and Fortina 1998; Gupta *et al*., 2002a).

1.2.4 Sporulation factors

Protease are largely produced during stationary phase and generally regulated by carbon and nitrogen stress. Moreover protease is known to be associated with the onset of stationary phase, which is marked by the transition from vegetative growth to sporulation stage in spore formers.

Sporulation in bacilli starts in the early stationary phase, which is considered to be a transitional period of preparation for sporulation (Msadek *et al*., 1993). However, sometimes its duration exceeds the duration of

logarithmatic growth phase (Balaban *et al*., 2003). In this period, cells secrete large amount of hydrolytic enzymes, including protease, which are excreted from the cells before and after initiation of sporulation (Errington, 1993).

Therefore, protease production was often related to the sporulation stage in many bacilli, such as *B. subtilis* and *B. intermedius*. It was reported that *Bacillus intermedius* 3-19 secretes two alkaline proteinases, glutamyl endopeptidase and thiol-dependent serine proteinase, in the early stationary phase (Balaban *et al*., 1993; Leshchinskaya *et al*., 1997). (Sharipova *et al*., 2002) also reported that *Bacillus intermedius* cells from the early stationary growth phase secrete extracellular protease, 75 % of which is subtilisin-like-thiol-dependent protease and 10 % of which is glutamyl endopeptidase.

Pero and Sloma (1993) also mention that spore-forming bacteria have an array of regulatory systems some of which act to suppress protease formation until the appropriate development stage is reached, often at the end of rapid or exponential growth. With these reported proof, it is believed that sporulation in bacilli correlates with the synthesis of alkaline protease.

This evolutionarily acquired feature acts to repress the metabolically expensive formation of enzymes if the nutritional conditions make their formation superfluous. This condition is an example of how the microorganism

avoids metabolic ‘burdens’. Moreover, in the reported literature, the protease production is also corresponded with growth and some are not (El Mansi *et al*., 2007).

1.3 Bacterial protease in the industrial setting

Proteases have become the most important industrial enzymes accounting for nearly 60 % of total worldwide enzyme sales (Ardinaraya *et al*., 2003). These enzymes have diverse applications in a wide variety of industries, such as in detergent, leather depilation and softening, laundry, silk degumming, food, rubber and waste processing industries (Olajuyigbe and Ajele, 2005). Proteases also can be used in the hydrolysis of fibrous protein such as horn, feather, and hair converting them to useful biomass. The table below shows the application of some protease producing microbe in the industrial setting.

Other potential industrial applications of protease include their use in peptide synthesis, in resolution of racemic mixtures of amino acids, and in hydrolysis of gelatin layers of X-ray films for silver recovery (Anvar and Saleemuddin, 1998; Kumar and Takagi, 1999). These showed a good sign of world demand for enzymes especially protease as shown in Table 1.1.

Table 1.1: Application of protease producing microbes in the industrial setting

Species	Source	pH	Industrial application
Streptococcus sp.	Bacteria	8	Dairy/cheese production
Bacillus stearothermophilus	Bacteria	9.5	Detergents and heavy duty laundry powder
Tritirachium album (proteinase T)	Fungus	9-12	Laundry detergents formulations
Tritirachium album (proteinase R)	Fungus	7-10	Laundry detergents formulations
Conidiobolus coronatus (alkaline proteinase B)	Fungus	9.7	Resolution of racemic mixtures of D,L-phennyl alanine and glysine
Bacillus sp. Y (BYA)	Bacteria	10-12.5	Detergents formulations
Bacillus lichenformis (Alcalase)	Bacteria	8.2	Catalyst for N-protected amino acids
Bacillus sp. (AH-101)	Bacteria	12-13	Dehairing/ leather industry

1.4 Protease producing microbes

Microorganisms are attractive sources of protease because of their biochemical diversity and the ease with which enzyme production may be increased by environmental and genetic manipulation (Shika *et al*., 2007). The use of microorganism to produce enzyme has a number of technical and economic advantages and in presently this has become the predominant mode of enzyme production.

Among the various proteases, bacterial proteases are the most

significant, compares with animal, moulds, yeast and fungal proteases. Among bacteria, *Bacillus* species are specific producers of extracellular protease (Han and Damodara, 1998; Puri *et al*., 2002; Huang *et al*., 2003; Olajuyigbe and Ajele, 2005), that accounting for about 35 % of the total microbial enzyme sales (Outtrup, 1990).

1.5 Protease producing bacteria.

Bacteria represent an attractive source of protease as they can be cultured in large quantities in a relatively short time by establish fermentation method, and they can produce an abundant, regular supply of the desired product (Rao *et al.*, 1998; Hasan *et al*., 2006). Normally, microbial protease are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compares to the protease obtained from plants and animals.

The rapid growth of bacteria and the limited space required for cell cultivation are characteristics to represent a good source of enzymes (Sally, 2000). Moreover microbial proteins have a longer shelf life and can be stored under less than ideal condition for weeks without significant lost of activity (Gupta *et al*., 2002a). Besides that, bacterial protease can be genetically manipulated to generate new enzymes for various applications easily (Shika *et*

al., 2007). However, although protease production is an inherent property of all organisms, only those microbes that produce a substantial amount of extracellular protease have been exploited commercially. Of these, strains of *Bacillus* sp. dominate the industrial sector. Table 1.2 shows some of the reported species of *Bacillus* that can produce protease.

1.6 *Bacillus* strain as a suitable protease producing bacteria.

Currently, *Bacillus* sp. is one of the most widely used bacteria for the production of commercial enzymes. The commercial producers of protease are summarized in Table 1.3. A myriad of *Bacillus* sp. from many different exotic environments had been explored and exploited for protease production, but most potential alkaline protease producing bacilli are strains of *B. licheniformis, B. subtilis, B. amyloliquifacients* and *B. mojavensis* (Kalisz, 1998; Rao *et al*., 1998; Kumar and Takagi, 1999; Gupta *et al*., 2002a). And among the *Bacillus* sp., *Bacillus subtilis* is the main group that is used in enzyme industry (Gupta *et al*., 2002b). The reason for this is their high pH and temperature stability. Alkaline protease which has either a serine center or metallo-type is exhibiting a wide pH range of pH 6 to pH 13. These high pH and wide pH range are important in the industrial applications.

Bacillus sp. also have a high optimal temperature that generally lies within 55 ºC to 60 ºC (Ferrero *et al*., 1998; Banerjee *et al*., 1999; Jasvir *et al*., 1999; Joo *et al*., 2003). These high optimal temperatures is important for industrial applications as many works are performed under high temperature condition, such as leather processing, detergents, peptide synthesis, food industry and biotechnological applications (Haki and Rakshit, 2003). *Bacillus* sp. produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates. *Bacillus* strains also have the ability to secrete industrially significant proteases which are stable and compatible with various detergent components (Yang *et al*., 2000; Christiansen and Nielsen, 2002).

Bacillus sp. produces an endospore that allows it to endure extreme conditions of heat and desiccation in the environment. *Bacillus* sp. is a suitable protease producer as this strain can grow under a wide range of conditions and produce a variety of proteases with low degree of virulence to human health (Edberg, 1991). *Bacillus* sp. also not considered as a plant pathogen (Claus and Berkeley, 1986) and can utilize de-ammoniated latex effluent as its carbon source for growth (Omar *et al*. 2000; Omar, 2002). These characteristic have put *Bacillus* sp. as a suitable protease producer in the enzyme industry as well as in rubber industry. Table 1.2 shows some of the reported species of *Bacillus*

that can produce protease.

Table 1.2: Protease producing *Bacillus* species (Kumar and Takagi, 1999).

Bacillus sp. and their strains
Bacillus alcalophilus ATCC 21522
B. alcalophilus
B. alcalophilus subsp. *halodurans* KP1239
B. amyloliquefaciens
B. circulans
B. coagulans
B. firmus
B. intermedius
B. lentus
B. licheniformis
B. proteolyticus
B. pumilus
B. sphaericus
B. subtilis
B. subtilis var.*amylosacchariticus*
B. thuringiensis
Bacillus sp. Ya-B

Table 1.3: Commercial producers of alkaline protease (Kumar and Takagi, 1999).

Organism	Trade names	Manufacturer
Bacillus licheniformis	Alcalase	Novo Nordisk, Denmark
Alkalophilic *Bacillus* sp.	Savinase, Esperase	Novo Nordisk, Denmark
Alkalophilic *Bacillus* sp.	Maxacal, Maxatase	Gist-brocades, The Netherlands
Alkalophilic *Bacillus* sp.	Opticlean,Optimase	Solvay Enzymes GmbH, Germany
Alkalophilic *Bacillus* sp.	Proleather	Amano Pharmaceuticals Ltd., Japan
Aspergillus sp.	Protease P	Amano Pharmaceuticals Ltd., Japan
Protein engineered variant of Savinase	Durazym	Novo Nordisk, Denmark
Protein engineered variant of alkalophilic *Bacillus* sp.	Maxapem	Solvay Enzymes GmbH, Germany

1.7 Protein in latex.

Hevea brasiliensis is a specific name of rubber tree. When the bark of rubber tree is cut, white and milky fluid will flow out. This white and milky fluid is called latex or natural rubber latex (NRL). It contains 30 % to 40 % of the rubber hydrocarbon particles suspended in a serum together with a few percent of other non-rubber substances such as proteins, amino acids, lipids, carbohydrates, sugars, and some metal (non-rubber fraction), and the remaining major component is water (Ong *et al*., 1998). Protein present in fresh *Hevea brasiliensis* latex to the extent of approximately 1 % to 1.5 % by weight, of which about half is associated with the rubber.

After the latex has been coagulated with acid, a commercial sample of dried rubber typically contains about 1.5 % protein. This protein and other non rubber components absorb water and give rise to a variety of undesirable effect such as reduced modulus and increased creep under load in the finished article. Therefore, it is important to reduce the protein contents of rubber subsequently coagulated from the latex.

A protein contented in natural rubber latex also considered to be a causative agent and can cause immiediate (I type) allergy such as dyspnea or urticaria when contact with the skin or mucosa (Nutter, 1979).

The protein also can cause variation in quality and vulcanization properties of the natural rubber product because the kind and quantity of the protein vary depending on the locality and production season of the latex (Cherrappathanathu, 1977).

1.8 Deproteinase Natural Rubber (DPNR).

In rubber industry, DPNR is produced as one of a modified natural rubber. The process of DPNR processing is still being improved within these ten years. DPNR is a purified form of natural rubber with lower levels of nitrogen and ash contents that confers improved performance when used in engineering applications. By using an alkaline protease, proteins in natural rubber latex are hydrolyzed into water soluble forms that can be washed off during subsequent processing (Ichikawa *et al*., 1993). The end result is a rubber grade with lower values in creep, water absorption, compression set and stress relaxation in comparison with other rubbers. The removal of these non-rubber components confers special attributes to the rubber which enhance its value in certain specialized applications.

There are two principal grades of DPNR available, which is DPNR-CV and DPNR-S. The viscosity of DPNR-CV stabilized between 60-70 Mooney

units. DPNR-S has no viscosity stabilization feature but the initial viscosity is between 70-80 Mooney units. DPNR-S has a very much less storage hardening than the normal natural rubber (Fahmi, 2006).

1.9 DPNR production

Deproteinised Natural Rubber (DPNR) is a purified form of natural rubber (NR) from which most of the ash and protein components have been removed. In Malaysia, the rubber is produced under very closely controlled conditions at the Rubber Research Institute Experimental Station, Sungai Buloh, Selangor. It contains about 96% rubber hydrocarbons compares to about 93% for normal natural rubber grades.

Ammonia, a non-ionic surfactant, and Hydroxylamine Neutral Sulphate (HNS) are added into bulked field latex to enhance the latex stability. Alcalase is used to degrade the protein in the latex. The ammoniated and stabilize latex is allowed to react with the Alcalase for 72 hours in a stainless steel conical bottom reaction tank. After the completion of the enzymatic hydrolysis reaction, the reacted latex is neutralized with dilute formic acid and coagulated by steam in a specially designed steam column coagulator. The resultant coagula are then processed in a continuous fashion through a series of creepers and finally

chopped into small crumbs in a shredder. The crumbs are pumped through a static screen before entering the dryer boxes. The wet crumbs are then dried at about 85 °C for about 6hours to 8 hours in a hot air dryer. After cooling, the golden colored crumbs are weighed, pressed, baled and packed to SMR standards (Fahmi, 2006).

1.10 DPNR applications.

When DPNR is compounded using a soluble efficient vulcanization system, DPNR demonstrated low creep and stress relaxation, low water absorption, low compression set and a more consistent modulus when subjected to conditions of variable humidity (Khoo, 2003). Therefore, DPNR is suitable for a niche market where the requirements for such properties are very stringent. Table 1.4 shows the application of DPNR based on its special properties. In the local market, the application of DPNR is important in the manufacture of hydraulic engine mounts (hydromounts) for the automobile industry. Hydromounts will gently reduce or absorbed the vibration in engine car. Therefore just a negligible vibration will be transfer to the main body car compartment even when the road surface is poor (Khoo, 2003).

Another application is in large shock absorbers for Deltawerken in the Netherlands. These large shock absorbers have to withstand prolonged contact with seawater and yet must not absorb too much seawater to cause corrosion in the embedded steel plates (Khoo, 2003). Moreover, the creep of the rubber should be minimal because of the very long expected service life that more than 100 years. For these reasons, DPNR is preferred over the normal natural rubber.

Table 1.4: Areas of DPNR application

Features	Application
Low stress relaxation and low creep.	Hydromounts, seals, joint rings, large shock absorbers, and suspension bushes helicopter rotor bearings.
Low water absorption.	Underwater applications, large shock absorbers.
Good dynamic properties.	Anti-vibration mounting and surge fenders.
Low protein and low ash.	Medical, pharmaceutical and food application.

1.11 Status of the problem in DPNR producing.

Many attempts have been made in the past to produce commercial quantities of DPNR at a reasonable price and quality to meet the needs of consumers. Recently, Alcalase is used in the processing of DPNR in Rubber

Research Institute of Malaysia. The Alcalase reaches an optimum activity of 90 % at 60 ºC. From the preliminary study of DPNR processing factory in Rubber Research Institute of Malaysia, revealed that they had spent high cost for buying the protease. Therefore, by finding other source of protease, it might cut the production costs of DPNR and hopefully can increase the production rate as well.

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1.12 Isolation of protease producing bacteria

It is reported that in year 2005 the global proteolytic enzyme demand will increase dramatically to 1.0 – 1.2 billon dollars. The present enzymes toolbox is not sufficient to meet most of the industrial demands. Therefore, researchers have diverted their attention for isolation and characterization of enzymes from natural and human made environment of desired product.

Considering the high demand of proteases and knowing the richness and biodiversity of our local environment, it is very likely that there is a local alkalophilic *Bacillus* which can grow in a milky latex condition in the alkaline and natural condition. Isolating such a species, will be of great value to the enzyme industry for different applications, especially for the rubber industry. With this as main objective, natural rubber serum (NRS) of *Hevea brasiliensis* and other

related sources of rubber industry working area that is under highly alkaline condition and in natural condition are screened for these strains. Isolation and screening of microorganisms from naturally occurring alkaline habitats or from alkaline wastewater is expected to provide new strains producing enzyme that active and stable in highly alkaline conditions. At the same time, it is restricted mainly to aerobic and phototrophic organisms which are able to grow on relatively simple and chemically defined media. Therefore, this study is an initial work performed on such species isolated and an initial step for further optimization processes.

1.14 Objectives of study

The objectives of this research are as follows:

i. To isolate and identify protease producing bacteria from various sources related to *Hevea brasiliensis*

ii. To search for a suitable medium for growing the bacterial culture for protease production.

iii. To investigate the ability of crude protease produced by the selected bacterial strains to degrade protein in natural rubber latex.

iv. To study the ability of the natural rubber serum (NRS) that can serve as a growth medium for protease production by the selected bacteria strains.

CHAPTER 2- MATERIALS AND METHODS

2.1 Isolation of protease producing bacteria.

Samples were taken directly from the *Hevea brasiliensis* tree including from the bark, fresh latex, coagulated rubber, soil around the tree, latex from processing tank, residual latex on soil and effluent from effluent treatment pond. These samples were serial diluted with distilled water and the dilutions were pipetted on the skim milk agar. Skim milk agars containing peptone (0.1 % w/v), NaCl (0.5 % w/v), agar (2.0 % w/v), and skim milk (10 % w/v) were used as selected medium to isolate protease producing microbes. Here are the details of each samples sampling method.

i) Bark.

The outer thick layer that near the panel of rubber tree barks from three different rubber trees were collected. The samples were mashed and 1 g of the mashed sample was diluted in 9 ml of distilled water. One hundred microlitres of 10^{-2} and 10^{-5} diluted sample were transferred onto the skim milk agar surface and were spread uniformly with a sterile glass spreader.

ii) Fresh latex.

Fresh latex from three different rubber trees was collected and one ml of each samples were diluted in 9 ml of distilled water. One hundred microlitres of 10^{-2} and 10^{-5} diluted sample were transferred onto the skim

milk agar surface and were spread uniformly with a sterile glass spreader.

iii) Coagulated rubber.

Coagulated rubbers that have been left after two days in rubber cups from three different rubber trees were collected. The inner and the outer part of the coagulated rubbers we cut and mixed together. The samples were minced into a small portion before diluted in Phosphate Buffer Saline (PBS). One milliliter of each samples in the Phosphate Buffer Saline were diluted in 9 ml of distilled water. One hundred microliters of 10^{-2} and 10^{-5} diluted sample were transferred onto the skim milk agar surface and were spread uniformly with a sterile glass spreader.

iv) Soil.

Soil around the rubber trees of three different rubber trees plantation area was collected. Each sample was mix smoothly and 1 g of the sample was diluted in 9 ml of distilled water. One hundred microliters of 10^{-2} and 10^{-5} diluted sample were transferred onto the skim milk agar surface and were spread uniformly with a sterile glass spreader.

v) Latex from processing tank.

Latex that had been stored in the processing tank of rubber factory for 72 hours was collected and one milliliter of the samples was diluted in 9 ml

of distilled water. One hundred microliters of 10^{-2} and 10^{-5} diluted sample were transferred onto the skim milk agar surface and were spread uniformly with a sterile glass spreader.

vi) Residual latex.

Residual latex on soil from three different rubber trees was collected. The residual latex was cut into a small portion and was diluted in Phosphate Buffer Saline (PBS). One milliliter of each samples in the Phosphate Buffer Saline were diluted in 9 ml of distilled water. One hundred microliters of 10^{-2} and 10^{-5} diluted sample were transferred onto the skim milk agar surface and were spread uniformly with a sterile glass spreader.

vii) Effluent.

Effluent from effluent treatment pond of rubber processing was collected and one milliliter of the sample was diluted into the 9 ml of sterile distilled water. One hundred microliters of 10^{-2} and 10^{-5} diluted sample were transferred onto the skim milk agar surface and were spread uniformly with a sterile glass spreader.

Plates were incubated at 37 ºC for 24 hours (Ardinaraya *et al*., 2003). These microorganisms were mesophilic bacteria. Normally for bacteria species involved in biodegradation, there are more active in temperature ranging from

approximately (15 ºC to 40 ºC), in this research 37 ºC was used as considering the higher temperature that near 40 ºC in the latex processing factory. Protease enzyme that excreted from the cell degraded the skim milk based on their ability to proteolytically break downs proteins to peptones or to coagulate milk to form a clear zone. Therefore, a clear zone of skim milk hydrolysis gave an indication of protease producing microbes. To confirm that clearing was a result of casein hydrolysis, the plates were flooded with mercuric chloride solution that acts as protein precipitants. Remain clearances were presumptive evidence of protein hydrolysis (Azma, 2005). The purity of the isolated bacteria was ascertained through repeated streaking. A sterilized loop that had been heated until red-hot and cooled in air briefly was used to transfer the clear zone colony onto the sterile skim milk agar. By streaking over the skim milk agar, the isolated bacteria will spread out. The initial streak and subsequent streaks were made and the loop was been resterilized between streaks. Clear zones colonies of isolated bacteria will appear after incubation. By repeating picking and restreaking of a well isolated colony, a pure culture can be obtained.

2.2 Screening of protease producing microbes.

The isolated protease producing bacteria were then grown in the nutrient broth medium at 37 °C, 200 rpm for 24 hour. The culture was centrifuged (11 000g, 30 min, 25 °C) to remove the bacterial cells. Ten microliters of the clear supernatant were spotted on the sterile filter paper and put on the surface of skim milk agar at alkaline (pH 8.5) and neutral condition (pH 7). After 24 hours incubation time, the plates were examined for zones of clearing in positions where the filtrate was spotted. The screening on alkaline skim milk agar was been investigated on purpose to see the enzyme performance of selected isolates on alkaline condition.

2.3 *Bacillus* isolation.

One hundred milligrams of each sample were weight and placed each into a separate screw cup culture tube of sterile TSY + 2 % glucose medium in a good aseptic technique. The caps were replaced loosely and the tubes were placed into an 80 ºC water bath for 10 minutes. This step killed all the vegetative bacteria but did not affect the *Bacillus* endospore. The tubes then were removed from the water bath and were allowed to cool. Using a sterile loop, a loop full of each heat-treated tube was directly streaked onto a medium

agar plate. This procedure allowed for direct isolation of *Bacillus* under aerobic condition.

2.4 Maintenance of the culture.

Samples from the clear zone colonies were streaked onto the nutrient agar. After 24 hours incubation, the single colony was restreaked onto the skim milk agar to confirm the proteolytic activity. This single colony was grown in the nutrient broth. After 24 hours incubation, 20 % glycerol stock was prepared and poured onto the culture. The glycerol culture suspension was then dispensed into the Eppendorf tubes and stored in the freezer at -40 °C. The glycerol work as a cryoprotectant penetrated into the cells and protects them by reducing the severity of dehydration effects and preventing ice crystal formation. As a cryoprotectant agent, glycerol was water soluble and cell-membrane permeable, had a low melting point, and can substitute for water as a hydration shell. Therefore, glycerol can reduce the severity of dehydration upon freezing of water. Hence, freezing and formation of external ice crystal was depressed. As ice crystal outside the cell continue to form, the solute concentration increase and water begins to migrate out of the cell. Eventually, all of the water crystallizes as pure ice, leaving a concentrated solute that finally freezes with

only little formation of crystals that would damage the cell (Joseph *et al*., 1999). These bacterial stocks can be preserved for long periods for its continued use.

2.5 Preparation of the inoculums.

A loop-full of culture from agar plate of each selective strain that produced clear zone on skim milk agar were transferred into 50 ml of sterile nutrient broth media and incubated on a rotary shaker at 200 rpm (rotation per minute) and 37 °C for 24 hours. Although these bacteria are mesophilic that normally have optimum growth at 30 °C, the incubation temperature was 37 °C as considering the higher temperature that near 40 °C in the latex processing factory. After 24 hours of incubation, the samples of pre-culture were taken and read for optical density. From the optical density, the biomass of the strains was calculated using the standard curve for indirect measurement of bacteria biomass. Approximately 1% (v/v) of cell suspension with cell biomass 1.0 g/L was used as inoculums.

2.6 Generating a standard curve for indirect measurement of bacteria biomass.

Before using turbidity as an estimation of cell number, a standard curve

must first be prepared for each strain to be studied, relating some direct measurement of cell number (microscopic or viable count) or mass (dry weight) to the indirect measurement obtained from turbidity (Madigan *et al*., 2003a).

2.6.1 Cell dry weight determination.

Three empty aluminum weighing pans were dried in an oven at 100 ºC for 24 hours. After that, the aluminium weighing pans were weighed and stored in a desiccators lined with Drierite (anhydrous $CaSO_4$). The bacterial culture was transferred to the centrifuge tubes. After centrifugation at 9000 g for 10 minutes at 4 °C, the clear broth was discarded carefully and the cell pellet was scraped from the centrifuge tubes and transferred into the weighing pans in 1 ml, 2 ml and 3 ml, respectively. The wet weight of the culture was measured immediately. Then, the cells in the aluminum weighing pans were dried in an oven at 100 ºC. It will take 24 hours to dry the samples completely, depending on the oven temperature and the thickness of the paste. After the cells were completely dry, the pans with the cells were weighed. The difference in the weight was calculated and the average of cell dry weight was expressed in g/L. The cell dry weight was divided to the dilution factors. Then the cell dry weight of each dilution was plotted against absorbance of serial dilutions.