ISOLATION AND CHARACTERIZATION OF RUBBER-DEGRADING BACTERIA FROM AGED LATEX

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ISOLATION AND CHARACTERIZATION OF RUBBER-DEGRADING BACTERIA FROM AGED LATEX

by

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LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABBREVIATIONS	FULL NAME
$(NH_4)_2SO_4$	Ammonium sulfate
BLAST	Basic Local Alignment Search Tool
$Ca(NO_3)_2$	Calcium nitrate
$CaCl_22\ H_2O$	Calcium chloride
CFU	Colony-forming units
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
$FeSO_47\ H_2O$	Iron sulfate heptahydrate
HCl	Hydrochloric acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRSG	International Rubber Study Group
K_2HPO_4	Dipotassium phosphate
KH_2PO_4	Potassium hydrogen phosphate
KOH	Potassium hydroxide
LB	Luria Bertani
Lcp	Latex clearing protein
$MgCl_2$	Magnesium chloride
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate
$MnSO_4$	Manganese sulfate
NA	Nutrient agar
$Na_2MoO_4 \cdot H_2O$	Sodium molybdate
NaCl	Sodium chloride

NB	Nutrient broth
NR	Nutrient rich
OD	Optical density
OsO_4	Osmium tetroxide
PCR	Polymer chain reaction
RNA	Ribonucleic acid
RoxA	Rubber oxygenase A
SEM	Scanning electron microscope
TAE	Tris-acetate ethylenediaminetetraacetic acid
UV	Ultra violet
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

LIST OF UNITS AND SYMBOLS

UNITS AND SYMBOLS	FULL NAME
%	Percentage
× *g*	Times gravity
℃	Degree Celcius
µg	Microgram
µL	Microliter
µM	Micromolar
bp	Base pair
g	Gram
h	Hour
kb	Kilo base pair
km	Kilometer
M	Molar
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
nm	Nanometer
Psi	Pounds per square inch
rpm	Rotation per minute
s	Second

V	Volt
v/v	Volume per volume
w/v	Weight per volume
α	Alpha
β	Beta
μm	Micrometer
ω	Omega

PEMENCILAN DAN PENCIRIAN BAKTERIA PENGURAI GETAH DARIPADA SUSU GETAH TERBIAR

ABSTRAK

Malaysia merupakan salah sebuah negara terbesar dalam pengeluaran getah. Pengurusan sisa-sisa getah memainkan peranan penting dalam berurusan dengan pengeluaran dan penggunaan yang tinggi di negara kita. Dalam usaha untuk mencari alternatif bagi pengurusan sisa-sisa getah semasa, bakteria pengurai getah telah dipencilkan daripada susu getah terbiar di tapak mendaki Bukit Jambul, Pulau Pinang. Di antara 13 pencilan yang diperolehi, pencilan CFMR-7 telah menunjukkan pembentukan zon jelas sekitar coloninya di atas agar perlapisan susu getah. Morfologi CFMR-7 dikaji di bawah mikroskop cahaya fasa-contrast dan mikroskop elektron pengimbas (SEM) menunjukkan bahawa ia adalah actinomycete yang mengandungi konidia. Oleh sebab itu, kajian penguraian telah dijalankan dengan mengkulturkan CFMR-7 bersama sarung tangan getah. Berat biojisim kering menunjukkan peningkatan lebih kurang 0.1 g/L sepanjang tempoh pengkulturan. Dalam pada itu, morfologi permukaan sarung tangan getah telah diperiksa dengan menggunakan SEM. Ia jelas menunjukkan bahawa permukaan sarung tangan getah yang dikultur dengan CFMR-7 selama 14 hari adalah lebih kasar berbanding dengan sarung tangan getah tanpa inocula. Selain itu, penjajahan CFMR-7 pada sarung tangan getah telah diperhatikan. Tanda-tanda penguraian enzim selanjutnya membuktikan bahawa pencilan ini mampu menguraikan sarung tangan susu getah. Urutan gen 16S rRNA CFMR-7 mendedahkan bahawa ia adalah *Streptomyces* sp. Selain itu, pengenalan urutan gen 16S rRNA daripada 12 pencilan lain mendedahkan bahawa pencilan CDRG-7 dan CFR-4 adalah 99% serupa dengan *Gordonia polyisoprenivorans* dan *Gordonia terrae*. *G. polyisoprenivorans* yang telah dilaporkan berupaya menguraikan

getah tanpa pembentukan zon jelas di atas agar perlapisan susu getah. Oleh sebab itu, satu set "degenerate primers" telah direka dari kawasan terpelihara gen-gen "latex clearing protein" (*lcp*) yang terdapat di pangkalan data NCBI. Amplifikasi serpihan DNA deraipada semua pencilan telah dilakukan dengan menggunakan set primer ini. Keputusan menunjukkan bahawa serpihan DNA dengan saiz dijangka (kira-kira 400 bp hingga 500 bp) telah berjaya didapati daripada *Streptomyces* sp. CFMR-7, *Gordonia* sp. CDRG-7 dan *Gordonia* sp. CFR-4. Keputusan menunjukkan bahawa DNA serpihan yang didapati daripada *Streptomyces* sp. CFMR-7 adalah 82% sama dengan *lcp* gen *Streptomyces* sp. K30; *Gordonia* sp. CDRG-7 adalah 99% sama dengan *lcp* gen *G. polyisoprenivorans* VH2; dan *Gordonia* sp. CFR-4 adalah 79% sama dengan *lcp* gen *G. westfalica* Kb1. Kesimpulannya, serpihan separa gen *lcp* *Streptomyces* sp. CFMR-7 dan *Gordonia* sp. CFR-4 menunjukkan perbezaan dengan gen-gen *lcp* yang dikenali dan mencadangkan bahawa bakteria ini adalah bakteria baru pengurai getah.

ISOLATION AND CHARACTERIZATION OF RUBBER-DEGRADING BACTERIA FROM AGED LATEX

ABSTRACT

Malaysia is one of the largest rubber producing countries. Rubber waste management plays an important role to deal with high production and consumption of rubber in our country. In order to find an alternative for current rubber waste management, rubber-degrading bacteria were isolated from aged latex in Bukit Jambul hiking site, Penang. Among the 13 isolates obtained, isolate CFMR-7 showed clear zone formation around its colonies on the latex overlay agar. Morphology of CFMR-7 under phase-contrast light microscope and scanning electron microscope (SEM) showed that it is an actinomycete containing conidia. Degradation study was carried out by culturing CFMR-7 with latex glove. The dry biomass weight was increased approximately 0.1 g/L throughout the cultivation period. The surface morphology of the latex gloves was examined using SEM. It was clearly shown that surface of the latex glove cultivated with CFMR-7 for 14 days was rougher compared to that of latex glove without inocula. Besides, colonization of CFMR-7 on the latex glove was observed. The signs of enzymatic degradation further conclude that this isolate is able to degrade latex glove. The 16S rRNA gene sequence of CFMR-7 revealed that it is *Streptomyces* sp. Besides, the 16S rRNA gene sequence identification of two of the isolates revealed that isolates CDRG-7 and CFR-4 are 99% similar to *Gordonia polyisoprenivorans* and *Gordonia terrae*, respectively. *G. polyisoprenivorans* was reported to degrade rubber without clear zone formation on the latex overlay agar. Subsequently, a set of degenerate primers was designed from the conserved regions of the latex clearing protein (*lcp*) genes available in NCBI database. Amplification of DNA fragments from all the isolates was performed by using this set of primers. Results showed that

DNA fragment with expected size (approximately 400 bp to 500 bp) was successfully amplified from *Streptomyces* sp. CFMR-7, *Gordonia* sp. CDRG-7 and *Gordonia* sp. CFR-4. Sequencing results revealed that the amplified DNA fragments of *Streptomyces* sp. CFMR-7 shared 82% identidy with *Streptomyces* sp. K30 *lcp* gene; *Gordonia* sp. CDRG-7 was 99% identical to *G. polyisoprenivorans* VH2 *lcp* gene; and *Gordonia* sp. CFR-4 was 79% identical to *G. westfalica* Kb1 *lcp* gene. In conclusion, partial fragments of the *lcp* genes of *Streptomyces* sp. CFMR-7 and *Gordonia* sp. CFR-4 exhibited less identidy to the known *lcp* genes suggesting that these might be new rubber-degrading bacteria.

1.0 INTRODUCTION

Natural rubber is an ideal polymer for many applications. It is resistant to tear and abrasion as it has high elasticity. The superior properties of natural rubber have cause its widespread application as basic material for tires, latex gloves, pharmaceutical rubber articles, foam products and balloons. Statistical Summary of World Rubber Situation published by International Rubber Study Group (IRSG) reported that the global natural rubber production was 11 million tonnes while consumption was 10.9 million tonnes in year 2011 (IRSG, 2012). Malaysia, as one of the world's largest natural rubber producing countries, produced approximately 1 million tonnes of natural rubber in year 2011 while approximately 0.4 million tonnes of it were consumed domestically (Department of Statistics Malaysia, 2013).

In line with the high rubber consumption, efficient rubber disposal and recycling methods must be practically applied. The hydrophobic and resilient property of rubber cause a problem in rubber composting, and thus the recycling of rubber is an alternative to manage the solid waste of rubber. The methods of recycling rubber products are based on two concepts which are, the product reuse and the material reuse. The recovery processes to reuse the rubber products includes retreading, regrooving, and physical reuse. Chemical and thermal recovery processes such as chemical reclamation, pyrolysis, and combustion are used to recover the material. However, the existing methods of disposal and recycling of rubber products are difficult and less environmental friendly (Abraham *et al.*, 2011; Manual and Dierkes, 1997). Other than rubber waste management, the waste generated from the rubber producing industries must also be taken into consideration. Environmental problems associated with the processes in rubber production have been reported (Tekasakul and Tekasakul, 2006). To deal with the environmental problems

addressed, it is important to develop a more efficient and environmental friendly method for the rubber disposal and the waste generated from rubber industry.

Studies have shown that natural rubber can be degraded by some microorganisms (Heisey and Papadatos, 1995; Imai *et al*., 2011; Roy *et al*., 2006; Tsuchii and Takeda, 1990). The substrates that can be degraded and mineralized by these rubber-degrading microorganisms are not only limited to natural rubber, even vulcanized rubber (chemically cross-linked rubber) can be the substrates of biodegradation (Bode *et al*., 2001; Tsuchii *et al*., 1985; Tsuchii and Tokiwa, 2001). These studies have shown that the rubber-degrading microorganisms could provide a biotechnological solution to the problem of waste rubbers.

Microorganisms that are responsible for the degradation of natural rubber have been divided into two groups, which are the clear zone formers and the non-clear zone formers. Clear zone formers are able to grow and produce clearing zones on latex overlay agar. The second group of rubber-degrading microorganisms do not produce clearing zones on latex overlay agar. Instead, they grow adhesively on rubber substrates (Rose and Steinbüchel, 2005; Yikmis and Steinbüchel, 2012). To date, the molecular mechanism of rubber biodegradation is not fully understood. Thus far, only two key enzymes named rubber oxygenase A (RoxA) and latex clearing protein (Lcp) responsible for rubber degradation have been reported (Braaz *et al*., 2004; Rose *et al*., 2005). More enzymes associated with the rubber degradation are yet to be identified in order to understand its mechanism.

1.1 Objectives of study

It is important to understand the mechanisms of rubber degradation as well as to develop new strategy in dealing with rubber waste management problems in the

future. The ultimate goal of this project was to identify potential rubber-degrading bacteria from local environment for future studies. Therefore, the specific objectives of this study were:

1. To isolate and identify rubber-degrading bacteria from local rubber plantation.
2. To characterize the rubber-degrading isolates up to molecular level.

2.0 LITERATURE REVIEW

2.1 Natural rubber

Natural rubber, also called India rubber or caoutchouc, is a term refers to a type of coagulated product obtained from milky liquid called rubber latex. Generally, latex is a complex emulsion produced by many plants. It plays important role in plant defense system against herbivorous insects. It is usually exuded from the point of plant damage or injured tissue caused by insect or mechanical wounding (Konno, 2011). Rubber latex is specifically referring to the latex produced by rubber trees. It coagulates on exposure to air and mild heat to form rubber. (Sulz, 1888). Among the rubber trees, only latex from *Hevea brasiliensis* is focused and produced commercially which contributed to 99% of the world market (Wititsuwannakul and Wititsuwannakul, 2001).

Rubber latex is collected by making incisions into rubber tree trunk. It contains approximately 30 – 45% (wt/wt) of rubber polymeric materials, water, plant cellular materials such as proteins, carbohydrates, lipids, amino acids and other organic and inorganic compounds (Jacob *et al*., 1993; Ohya and Koyama, 2001). The rubber polymeric materials occur in the form of particles called rubber particles in latex. It appears as spherical shape or pear-like shape (from mature trees) covered by a complex layer of proteins and lipids to separate it from hydrophilic medium, as shown in Figure 2.1. Besides the complex proteins and lipids layer, triglycerides, sterols, tocotrienols and other lipids also found to combine with the particles (Ohya and Koyama, 2001).

Polymer of natural rubber, poly(*cis*-1,4-isoprene), is a member belonging to polyisoprenoid, which is one of the eight biopolymer classes that are classified according to their chemical structures. Other biopolymer classes include

polynucleotides, polyamides, polysaccharides, polyoxoesters, polythioesters, polyanhydrides and polyphenols (Steinbüchel, 2003). Natural rubber polymer consists of isoprene (C_5H_8) as monomer units. As shown in Figure 2.2, rubber polymer obtained from *H. brasiliensis* contains two isoprene units in the *trans* configuration at one end of long sequence of isoprene units in the *cis* configuration linked with other units at C_1 and C_4 (van Beilen and Poirier, 2007).

There are three groups of polyisoprenes suggested based on the structure at both chain ends of the polymer, the initiating terminal (ω-terminal) and the opposite end (α-terminal). The three groups are: (i) polyprenol type polymer with dimethylallyl group linked to 2 or 3 *trans*-isoprene units connected to sequence of poly(*cis*-1,4-isoprene) after ω-terminal [ω-(*trans*)$_{2-3}$-(*cis*)$_n$-α], (ii) natural rubber type polymer without dimethylallyl group linked two *trans*-isoprene units [ω'-(*trans*)$_2$-(*cis*)$_n$-α'], and (iii) wild rubber type polymer without *trans*-isoprene unit [ω'-(*cis*)$_n$-α] (Sakdapipanich, 2007; Yikmis and Steinbüchel, 2012).

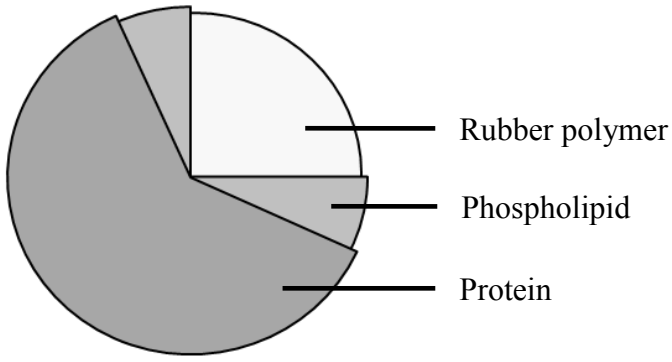


Figure 2.1. Schematic drawing of rubber particle surface (adapted from Ohya and Koyama, 2001).

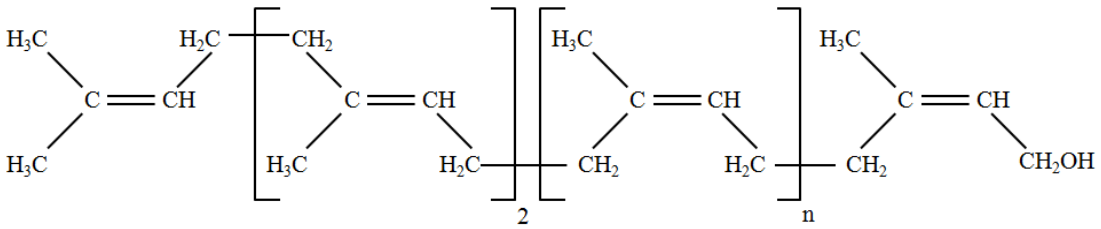


Figure 2.2. Structure of natural rubber [poly(*cis*-1,4-isoprene)]. Farnesyl diphosphate (FPP) is the initiating terminal molecule, two *trans*-isoprene units and sequence of *cis*-isoprene units with n refers to the number of repeating unit (adapted from van Beilen and Poirier, 2007).

2.2 Vulcanized rubber

Vulcanized rubber is a different form of natural rubber that undergone a chemical process called vulcanization. This process was first discovered by Charles Goodyear in 1839 and named after Vulcan, the Roman god of fire. Crude rubber or coagulated rubber obtained from latex drying process is soft and sticky. During the vulcanization, sulfur or other additives are added to crude rubber with heat to induce the three-dimensional cross-linking formation between the polymer chains. Poly(*cis*-1,4-isoprene) chains are covalently linked by sulfur bridges at double bonds of isoprene unit (Coran, 1978; Fisher, 1939). In contrast to crude rubber, vulcanized rubber exhibits improved material properties such as elasticity and strength and the appearance become non-sticky. Thus, the superior material properties have made vulcanized rubber to have widespread of applications (Kumar and Nijasure, 1997; Morawetz, 2000).

In general, vulcanization process comprises of three basic stages, which are mixing, molding and heating (Coran, 1978; Kumar and Nijasure, 1997). In the mixing stage, cross-linking agent such as sulfur as well as chemical additives such as activator, accelerator, anti-oxidant, color pigment, surfactant, and oils are mixed with

crude rubber. Next, molding is important to shape the rubber into desired products. The rubber mixture is poured into a mold before heating stage as it is impossible to shape cross-linked rubber. The last stage is heating the rubber mixture at 120 ºC to 200 ºC. In each cases of vulcanization, different temperatures and different chemical agents are employed to produce vulcanized rubber with different density of cross-linking as the material properties are closely associated with level of cross-linking (Fisher, 1939; Coran, 1978). Vulcanized rubbers that possessed different interesting properties such as heat-resistance, frost-resistance, oil or petroleum resistance and organic solvent resistance are widely producing nowadays.

2.3 Synthetic rubber

Synthetic rubber refers to any type of artificial polymer that possessed elastomeric property which similar to natural rubber. Same as natural rubber, synthetic rubber can be processed and vulcanized to form desired products. However, synthetic rubber is chemically synthesized by polymerizing petroleum-based precursors into homopolymer and copolymers with various material properties (IISRP, 2012; Street and Dillon, 1941). It can be produced in many different ways. Generally, the monomers of synthetic rubber are produced in the petrochemical industries using petroleum byproducts and natural gas. These monomers are then polymerized into desired synthetic rubber in the synthetic rubber industries, as shown in Figure 2.3 (IISRP, 2012).

Synthetic rubber has been discovered since 1870s and it was expensive at that time. The production of natural rubber dropped during the First and Second World Wars. United State of America faced natural rubber crisis during World War II when Japanese took over natural rubber production by invading Malaysia and the Dutch

East Indies. This triggered many involved countries to spend more effort on the development of synthetic rubber (IISRP, 2012; Yikmis and Steinbüchel, 2012).

Nowadays, synthetics rubbers are widely used in many economic sectors such as footware, automobiles, civil construction and plastics. Over 15 million tonnes of synthetic rubber produced in 2011 which contributed to 58% of total rubber production (IRSG, 2012). Furthermore, the world wide consumption of synthetic rubbers reached approximately 15 million tonnes in 2011, which showed the importance of synthetic rubbers to the world. Table 2.1 shows the main types of synthetic rubbers and its applications.

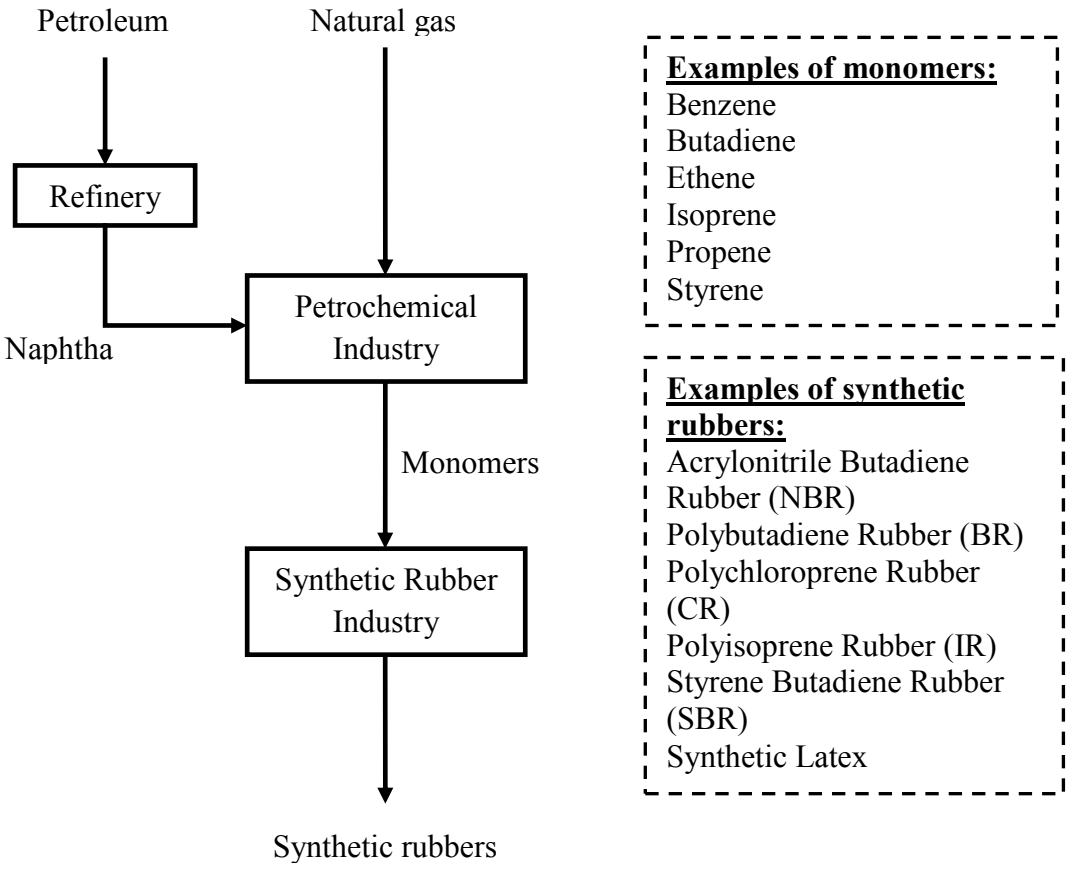


Figure 2.3. General synthetic rubber production process (adapted from IISRP, 2012).

Table 2.1. Applications for main types of synthetic rubbers (adapted from IISRP, 2012).

Name	Type of Rubber	Applications
SBR	Styrene-Butadiene	Asphalt modifications (SBR in solution), footware, adhesives, technical goods, tires and treads
BR	Polybutadiene	Footwear, technical goods, tires, treads and plastics modifications
NBR	Nitrile	Footwear, technical goods and plastics modifications
EPDM	Ethylene-propylene	Asphalt modifications, technical goods, tires and plastics modifications
IIR	Butyl	Adhesives, technical goods and tires
CR	Polychloroprene	Asphalt modifications, footware, adhesives and technical goods
TPR	Thermoplastic	Asphalt modifications, footware, technical goods and plastics modifications
Latex	Various types of latex	Asphalt modifications, footware, technical goods and treads

2.4 Rubber processing and its impact on the environment

Fresh rubber latex needs to be stored immediately before it is transported to rubber processing factories. It can be stored as five types of intermediate forms of rubber prior subjecting to downstream process. These intermediates products are ribbed smoked sheets, air dried sheets, block rubber, crepe rubber and concentrated rubber latex (Tekasakul and Tekasakul, 2006).

In the process of making ribbed smoked sheets and air dried sheets, the water-diluted rubber latex is mixed with formic acid to produce tofu-like slabs. There slabs are the squeezed into thin sheets using a squeezing machine which later

allowed to air dry. Ribbed smoked sheets are dried exposing it to smoke released from burning of rubberwood while air dried sheets are dried in a heating room (Tekasakul and Tekasakul, 2006). On the other hand, concentrated rubber latex is prepared by centrifugation. Ammonia treated latex is centrifuged to remove water and other impurities, which is called skim latex. Skim latex still containing 5 – 10% of rubber which can be recovered to make skim crepe or skim block by removing ammonia and adding sulfuric acid. The concentrated latex is later used to produce various rubber products such as latex glove.

Some environmental issues are identified from the rubber processing industries (Tekasakul and Tekasakul, 2006). Wastewater released from rubber drying process to produce ribbed smoked sheets and air dried sheets is acidic as formic acid is used in the process. Furthermore, ammonia-containing wastewater is also produced from the centrifugation of rubber latex. Report shown that the values of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total kjeldahl nitrogen (TKN), total phosphorus (TP) and sulfate in the wastewater are high (Tekasakul and Tekasakul, 2006).

In the process of drying rubber sheets using rubberwoods smoke, high concentration of smoke particles are released into the air. The smoke particles may cause environmental problems on the factory as well as nearby cities and residents. On the other hand, air pollution is also created from the latex concentrating industries. The ammonia solution used in these factories produces strong smell that can have adverse effects on health of workers and residents surrounding (Tekasakul and Tekasakul, 2006).

2.5 Rubber waste management

Waste rubber can be disposed as well as recycled into various products. Rubber is disposed as landfills in many countries, in example of United State, 58 % of rubber waste was subjected to landfills in 2006 (Abraham *et al.*, 2011). Landfill is not considered as a good method to manage waste rubber as it is thought to conserve resources and energy. Besides, sanitation may become concerns of landfill disposal of rubber is not managed properly especially in developing countries whereby their municipal waste management is often ineffective.

Recycling of waste rubber can be divided into three categories, which are product reuse, material reuse and energy reuse (Abraham *et al.*, 2011; Manual and Dierkes, 1997). In the first category, damaged rubber products such as tires are often being repaired by retreading and regrooving. This method is labor intensive and often used by many developing countries. Light vehicles tires can only be retreaded once while bus and truck tires can be retreaded at most six times. After retreading and regrooving, rubber products also subjected to secondary reuse as the next step of waste management (Abraham *et al.*, 2011; Practical Action, 2001). In this step, unwanted rubbers are used in many ways due to their shape, weight, and form, such as shock absorbers, tree guards, garden decorations etc.

In the category of material reuse, rubber products are broken down into different forms and reused to produce new products. This method is limited in developing countries as higher technology and sophisticated processes are needed to reclaim the materials. Rubber materials can be reclaimed through chemical and thermal processes (Abraham *et al.*, 2011; Manual and Dierkes, 1997; Practical Action, 2001). Chemical recovery process often includes heating waste rubber and treating it with chemicals. On the other hand, pyrolysis is employed in thermal

recovery process. Rubber waste will be heated in the absence of oxygen to decompose into gases and constituent parts. Other thermal recovery processes include high-pressure steam process and continuous steam process.

Other than materials, energy could be reclaimed from rubber products. Rubber products especially tires, consist high percentage of hydrocarbon which is a store of energy. The heat produced from incineration and pyrolysis of scrap rubber could be used to generate electricity and cement making process. An example, the Oxford Energy Company produces electricity by incinerating tires (Abraham *et al.*, 2011). Again, this method requires sophisticated technology.

2.6 Rubber degradation by fungi

Fungi play an important role in bioremediation. It is well known that fungus can degrade many types of complex organic compounds such as insecticides, coal tars, pentachlorophenol and heavy fuels. It releases enzymes to turn these organic compounds into basic elements by various mechanisms (Fomina *et al*., 2008). Although fungi are well known to be able to degrade many substances, however, reports of the biodegradation of rubber by fungi are scarce. Some studies revealed the ability to degrade rubber material by some strains of *Aspergillus*, *Fusarium* and *Penicillium* (Kwiatkowska *et al*., 1980; Roy *et al*., 2006; Williams, 1982). However, in another study, strains of these fungi were found to grow on surface of vulcanized rubber but unable to attack the rubber hydrocarbon in purified latex (Rook, 1955).

Several rubber-degrading fungi were reported by Borel and colleagues (1982). In this study, *Fusarium solani*, *Cladosporium cladosporioides*, *Phoma eupyrena* and *Paecilomyces lilacinus* were isolated using mineral agar containing powdered natural rubber from deteriorated tire and soil sample. Formation of mycelial layer on rubber

surface was observed after 20 days when the rubber material was cultivated with these isolates. Weight loss of the rubber material and decrease in rubber molecular weight confirmed the occurrence of rubber degradation.

2.7 Rubber degradation by bacteria

Studies of rubber degradation by bacteria are more intensive as compared to fungi. As mentioned in the previous chapter, rubber-degrading bacteria are divided into two groups based on their degradation strategies for natural rubber (latex overlay agar). The first group of rubber-degrading bacteria produces clear zones or halo around its colony on latex overlay agar. They release extracellular enzymes to degrade rubber materials. Generally, members which belong to this group are species of *Streptomyces*, *Actinoplanes*, *Micromonospora* and *Nocardia*. Two examples of clear zone formers are the well-studied strains *Streptomyces* sp. K30 (Rose *et al*., 2005) and *Xanthomonas* sp. 35Y (Braaz *et al*., 2004; Tsuchii and Takeda, 1990).

The other group of rubber-degrading bacteria requires direct contact with rubber materials and do not form clear zones on latex overlay agar. Some of the members do not even grow on latex overlay agar. Some members from this group degrade rubber very effectively, such as *G. polyisoprenivorans* VH2 and *G. westfalica* Kb1 (Linos *et al*., 2000). List of rubber-degrading bacteria are summarized in Table 2.2. Other than natural rubber, the highly cross-linked vulcanized rubbers as well as synthetic rubbers are able to be degraded by bacteria (Bode *et al*., 2000; Bode *et al*., 2001; Tsuchii and Tokiwa, 2001).

Table 2.2. Rubber-degrading bacteria mentioned (adapted from Rose and Steinbüchel, 2005).

Bacterium	Source
Clear zone formers:	
Actinoplanes sp.	Jendrossek *et al*., 1997
Micromonospora aurantiaca W2b	Linos *et al*., 2000
Streptomyces sp. La7	Gallert, 2000
Streptomyces sp. K30	Rose *et al*., 2005
Xanthomonas sp. 35Y	Tsuchii and Takeda, 1990
Non clear zone formers:	
Gordonia polyisoprenivorans VH2	Linos *et al*., 2000
Gordonia polyisoprenivorans Y2K	Arenskötter *et al*., 2001
Gordonia westfalica Kb1	Linos *et al*., 2002
Mycobacterium fortuitum NF4	Linos *et al*., 2000
Nocardia farcinica S3	Ibrahim *et al*., 2006

2.8 Latex clearing protein (Lcp)

Latex clearing protein was first identified in *Streptomyces* sp. K30 by Rose and colleagues (2005). In that study, UV-induced mutants that were defective in rubber degradation were screened and identified. An open reading frame was identified from a cloned DNA fragment of *Streptomyces* sp. K30. It restored the ability of rubber-degrading negative mutants and also enabled *S. lividans* TK23, to form clear zone on latex overlay agar. This open reading frame was named latex

clearing protein (*lcp*) gene. Later, a *lcp*-homologue gene was identified in *Nocardia farcinica* E1 (Ibrahim *et al*., 2006). Cloning of the *lcp*-homologue revealed its function in *S. lividans* TK23.

Subsequent study on *Streptomyces* sp. K30 *lcp* found that its amino acid sequence containing a twin-arginine motif indicating that Lcp probably is transported extracellularly from *Streptomyces* sp. K30 via twin-arginine translocation (Tat) pathway (Yikmis *et al.*, 2008). Further study proved this assumption when evidence of Lcp being secreted out by *Escherichia coli* using Tat pathway was found in the same work. Besides, the transcription of *lcp* was found to be induced by poly(*cis*-1,4-isoprene) which further conclude that Lcp is involved in rubber degradation. The latest study about the *Streptomyces* sp. K30 *lcp* was conducted by Yikmis and Steinbüchel (2012). This study successful demonstrated for the first time of knocking out *lcp* in *Streptomyces* sp. K30. Furthermore, heterologous expression of *lcp* in *S. lividans* and *S. erythraea* enabled them to degrade synthetic poly(*cis*-1,4-isoprene). This study proved that Lcp is involved in initial polymer cleavage.

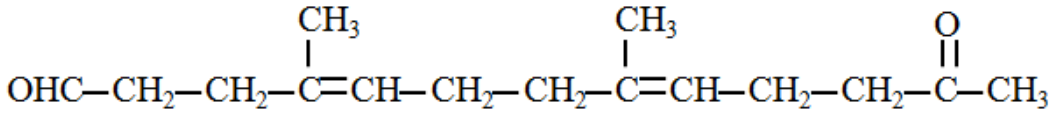
Other than *Streptomyces* sp. K30 and *N. farcinica* E1, *lcp* was also found in non-clear zone formers in particular, *G. polyisoprenivorans* VH2 and *G. westfalica* Kb1 (Bröker *et al*., 2008). Recently, genome sequencing revealed that *G. polyisoprenivorans* VH2 containing two *lcp* genes. Gene deletion study found that both *lcp* genes are functioning which indicating that both genes are involved in rubber degradation (Hiessl *et al*., 2012).

2.9 Rubber oxygenase A (RoxA)

RoxA was reported as a novel type of diheme dioxygenase after the extensive study in its structure and activity (Braaz *et al*., 2005; Schmitt *et al*., 2010). It was first

identified from *Xanthomonas* sp. 35Y, one of the only two gram-negative rubber-degrading bacteria, before *lcp* was found (Braaz *et al*., 2004). Study revealed that RoxA releases a low-molecular-mass oligoisoprene unit (12-oxo-4,8-dimethyltrideca-4,8-diene-1-al) as main degradation product when the purified protein degrades both natural rubber latex and synthetic poly(*cis*-1,4-isoprene) under *in vitro* conditions. Besides, a homologous series of minor compounds with terminal functions, $CHO\text{-}CH_2\text{-}$ and $\text{–}CH_2\text{-}COCH_3$ was also detected (Braaz *et al*., 2004). Figure 2.4 shows the structure of rubber degradation products produce by RoxA.

A



B

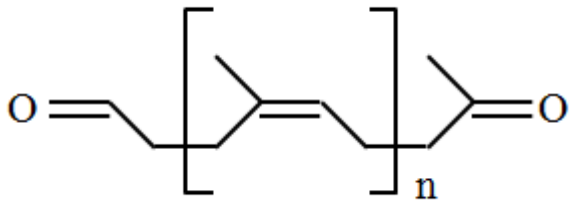


Figure 2.4. Proposed structure of rubber degradation products produced by RoxA. A, structure of 12-oxo-4,8-dimethyltrideca-4,8-diene-1-al; B, general structure of minor degradation products, with n = 1 to 5. (Adapted from Braaz *et al*., 2004)

2.10 An oxidoreductase, OxiAB

OxiAB is an oxidoreductase first found in *Streptomyces* sp. K30 when *lcp* was identified (Rose *et al*., 2005). The genes encoding the oxidoreductase *β*-subunit (*oxiB*) and the oxidoreductase *α*-subunit (*oxiA*) was found in the downstream sequence of *lcp* in *Streptomyces* sp. K30 (Figure 2.5). The gene encoding OxiAB

was also found homologous with a heterodimeric molybdenum hydroxylase. Heterologous expression of *oxiAB* with *lcp* in *S. lividans* TK23 revealed the involvement of OxiAB in rubber degradation. Aldehydes intermediates were found to accumulate when tungstate, a specific inhibitor of molybdenum hydroxylases, was added into culture medium. Therefore, OxiAB is postulated to involve in rubber degradation, whereby, it probably oxidizes aldehyde intermediate produced by Lcp.

In the subsequent study, *oxiAB* was found to be expressed only when poly(*cis*-1,4-isoprene) was present (Yikmis *et al*., 2008). This result further concludes that it is involving in rubber degradation. However, in contrast to *Streptomyces* sp. K30, genome sequencing found that *oxiAB* is missing in the genome of *G. polyisoprenivorans* VH2 (Hiessl *et al*., 2012).

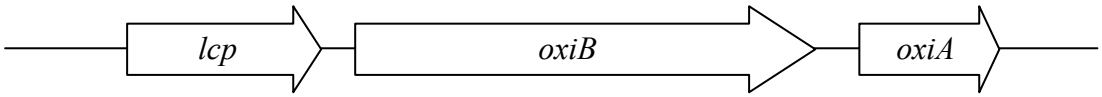


Figure 2.5. Molecular organization of the genes of *Streptomyces* sp. K30 involving in rubber degradation. *lcp*: latex clearing protein, *oxi*B: oxidoreductase *β*-subunit, *oxiA*: oxidoreductase *α*-subunit. (Adapted from Rose *et al*., 2005)

3.0 MATERIALS AND METHODS

3.1 General methods

3.1.1 Weighing

All the materials and chemicals were weighed by *Shimadzu AX120* (Shimadzu, Japan) *Sartorius BL160* (Sartorius, Germany) electronic balances.

3.1.2 pH determination

The pH of all media used was measured by using *Mettler Toledo 320* (Mettler-Toledo, Switzerland) pH meter calibrated with pH 4.0 and pH 7.0 buffers.

3.1.3 Sterilization

All the media and materials that needed to be sterilized were sterilized using *HIRAYAMA HI CLAVE*^{TM} *HVE-50* (Hirayama, Japan) autoclave machine operated at 121 °C, 15 Psi for 15 min. Heat-sensitive materials such as trace elements and antibiotics were filter-sterilized by using *Minisart*® *CE* (Sartorious, Germany) cellulose acetate membrane with the pore size of 0.22 μm.

3.2 Isolation of rubber-degrading bacteria

3.2.1 Latex purification

Crude latex was reported containing approximately 30 – 45% (wt/wt) of rubber polymeric materials, proteins which attached on rubber polymeric materials and dissolved in water (0.5 – 2.5%), phospholipids (0.6 – 2.8%), ash fraction mainly magnesium and potassium phosphate (0.2%), tocotrienols (0.09%) and water. Latex purification process aimed to reduce the amount of impurities. However, proteins attached on rubber material might also serve as carbon and nitrogen sources for bacterial growth (Jacob *et al*., 1993; Ohya and Koyama, 2001). In this process, crude

latex was collected from a rubber tree in Bukit Jambul hiking site (N5º20'35.80''; E100º17'0.31'') in Penang island, Malaysia. The freshly tapped crude latex was transported immediately to laboratory and was washed three times with 0.002% Tween 80 (in ratio of one to one) at 19320 × *g* for 10 min at 4 ºC using *Kubota High Speed Refrigerated Centrifuge 6500* (Kubota, Japan) with AG508CA rotor.

The top layer, which is a layer of latex cream as shown in Figure 3.1B, from each centrifugation was collected and was used for the next centrifugation. The bottom layer of each centrifugation was discarded. The final latex cream was mixed with approximately equal volume of 0.002% Tween 80 and was sterilized by autoclaving. The purified latex was stored at 4 ºC.

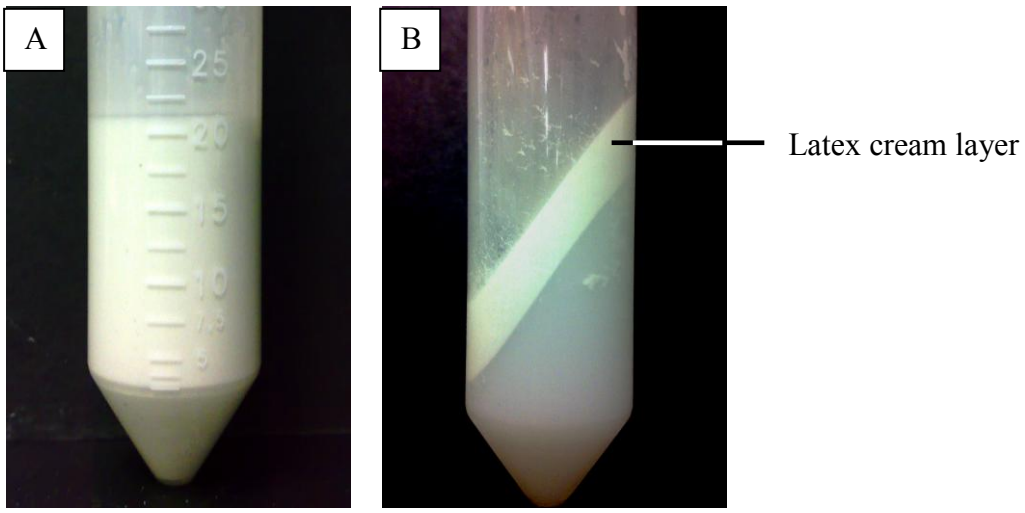


Figure 3.1. Pretreatment of crude latex. A, crude latex before centrifugation; B, latex cream layer separated from the crude latex after centrifugation.

3.2.2 Cultivation medium

Latex overlay agar was used in isolation and maintenance of the isolates. It was prepared by the overlay technique described by Braaz *et al.* (2004) with some

modifications. During the agar preparation, a bottom layer of mineral salts agar was allowed to solidify in a petri dish then was overlaid with the same agar supplemented with 0.2% (v/v) purified latex.

The composition of mineral salts medium used in latex overlay agar followed the recipe described by Heisey and Papadatos (1995) as listed in Table 3.1. All the components were weighed and were dissolved in distilled water. The pH of the medium was adjusted to 7.0 by adding either HCL or KOH. Trace elements solution and $MgSO_4 \cdot 7H_2O$ solution were prepared separately and were sterilized by filter sterilization and heat sterilization respectively. Both solutions were added aseptically into the medium before used. For agar preparation, 15 g/L of bacteriological agar powder was added into the medium before sterilization by autoclaving. Trace elements solution and $MgSO_4 \cdot 7H_2O$ solution were added into the medium once the medium has cooled to approximately 50 ºC. Subsequently, the agar mixture was stirred well before it was poured into petri dishes.

Table 3.1. The composition of mineral salts medium (Heisey and Papadatos, 1995).

Compounds	Concentration
K_2HPO_4	8.00 g/L
KH_2PO_4	1.00 g/L
$(NH_4)_2SO_4$	0.50 g/L
$MgSO_4 \cdot 7H_2O$	0.20 g/L
NaCl	0.10 g/L
$Ca(NO_3)_2$	0.10 g/L
Trace elements:	
$CaCl_2 2\ H_2O$	0.02 g/L
$FeSO_4 7\ H_2O$	0.02 g/L
$Na_2MoO_4 \cdot H_2O$	0.50 mg/L
$MnSO_4$	0.50 mg/L

3.2.3 Sampling site and samples

Latex samples were collected from Bukit Jambul hiking site (N5º20'35.80''; E100º17'0.31'') in Penang island, Malaysia. Bukit Jambul hiking site has a small rubber plantation which is still producing latex. It is located about 4 km away from Universiti Sains Malaysia which eased the transportation of the samples to laboratory.

Various latex samples were collected as listed in Table 3.2. All the latex samples were collected by using sterile forceps and were kept into sterile 50 mL centrifuge tubes. Interestingly, a container filled with aged latex was found under a rubber tree. Figure 3.2 shows the appearance of the container. Aged latex cream in

the container was collected using a sterile spatula and stored in a sterile tube. Surface of the aged latex in container was swabbed by a sterile cotton bud and then immersed in 1 mL sterile distilled water in a test tube. All the samples were transported to laboratory and processed immediately.

Table 3.2: Latex samples collected from Bukit Jambul hiking site.

Sampl.	Description
Aged dried latex on trunk	Collected from the old tapping area on rubber tree trunk, latex was dried more than 2 days
Dried latex on trunk	Collected from the new tapping area on rubber tree trunk, latex was dried within 2 days
Aged dried latex on ground	Dried latex on sandy ground under rubber tree
Aged latex in container	Latex that collected in a container over a period of time
Surface of the aged latex in container	Latex that collected in a container over a period of time

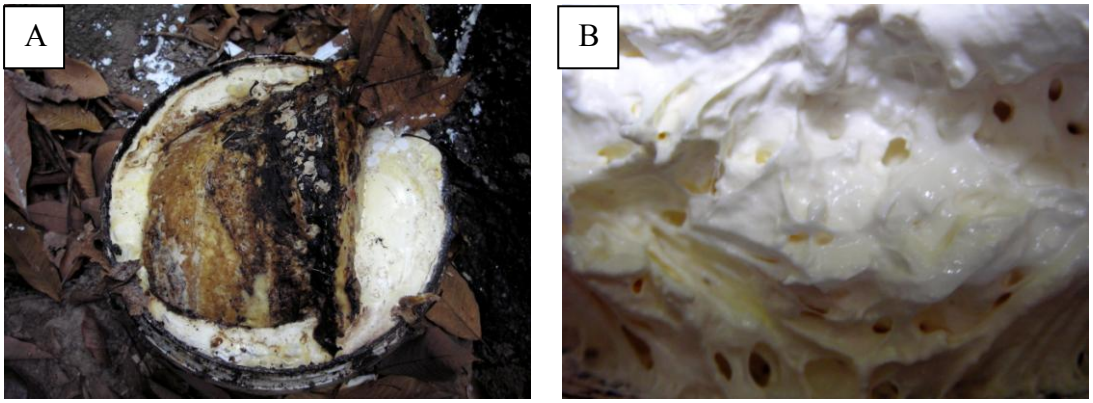


Figure 3.2. Appearance of aged latex in container. A, surface of the aged latex; B, aged latex in the container.

3.2.4 Enumeration and isolation of bacteria

Approximately 1 g of the aged dried latex samples were mixed with 9 mL of sterile distilled water. The samples were mixed thoroughly by a vortex. Subsequently, the samples were serially diluted to dilution of 10^{-9}. Water of the aged latex in container and the test tube containing cotton bud swabbed with aged latex surface were serially diluted with sterile distilled water to dilution of 10^{-9}. Approximately 100 µL of undiluted samples and samples with dilution of 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} were plated on latex overlay agar by spread plate method. All the plates were incubated at 30 ºC for 7 days. The numbers of bacterial colony grew on each plate were recorded. Bacterial colonies with different colony morphology were picked and streak on a new plate. Pure isolates were obtained by culturing individual colony several times on fresh agar. The colony-forming unit (CFU) per g or mL of original sample was calculated from the following equation (Madigan and Martinko, 2006):

Number of bacteria present in 1 g of the solid sample (CFU) = Number of colony × Dilution factor × 90

Number of bacteria present in 1 mL of the liquid sample (CFU) = Number of colony × Dilution factor × 10

3.2.5 Maintenance of pure isolates

Most of the pure isolates were streaked on nutrient agar (NA) instead of the latex overlay agar for maintenance purpose as the isolates grow faster on NA. Isolates were subcultured on fresh NA weekly for short term maintenance. The composition of nutrient broth (NB) is listed in Table 3.3. All the components were dissolved in distilled water and sterilized. The initial pH of nutrient agar was

adjusted to 7.0. Approximately 15 g/L of agar was added for the preparation of NA. For long term storage purpose, 20% (v/v) glycerol stocks of all isolates grown to exponential phase were prepared by adding glycerol to the cultures grown in NB at 30 ºC, 200 rpm on a rotary shaker for 12 h – 16 h. Subsequently, glycerol stocks were stored at -80ºC.

Table 3.3. The composition of NB (Himedia, India).

Compound	Concentration (g/L)
Peptic digest of animal tissue	5.0
NaCl	5.0
Beef extract	1.5
Yeast extract	1.5

3.3 Phenotypic characterization of isolates

3.3.1 Gram's staining

Standard Gram's staining procedures were performed. Fresh culture of isolates grew on NA was smeared and heat-fixed on a glass slide. The sample was flooded with crystal violet for 30 s. Excessive crystal violet was removed by holding the slide vertically and rinsing with some iodine solution. It was then flooded with iodine solution for 30 s followed by rinsing with water to remove crystal violet and iodine solution. After that, sample was flooded with 95% ethanol for 5 s and immediately rinsed with water. Subsequently, safranin was applied on the smear for 30 s for counter staining purpose. The slide was then rinsed with water and blotted dry. The slide was observed under a light microscope.