Isolation of a Novel Rubber Degrading Bacterium from a Consortium and Characterization of Its *lcp* Gene Products

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ชื่อวิทยานิพนธ์ การคัดแยกแบคทีเรียสายพันธุ์ใหม่ที่สามารถย่อยสลายยางจากกลุ่มจุลินทรีย์และจำแนกลักษณะของ *lcp* ยีนจากเชื้อที่แยกได้

ผู้เขียน นางสาวสิริมาภรณ์ วัชรกุล

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บทคัดย่อ

กลุ่มจุลินทรีย์ย่อยสลายยาง ST 608 ที่คัดแยกได้จากน้ำเสียจากกระบวนการผลิตยาง โดยทำการ เพิ่มจำนวนในอาหาร Mineral Salts Medium (MSM) ที่ใช้ถุงมือยางซึ่งเป็นตัวแทนของยางวัลคาไนซ์เป็นแหล่งคาร์บอนเพียงแหล่งเดียว เมื่อบ่มไปสามสัปดาห์ พบว่าถุงมือยาง มีการย่อยสลายอย่างเห็นได้ชัด ซึ่งแสดงให้เห็นถึงประสิทธิภาพการทำงานของกลุ่มจุลินทรีย์ย่อยสลายยาง โดยหลังการบ่มเป็นเวลา 30 วัน พบว่าน้ำหนักของยางหายไปมากถึง 50 เปอร์เซ็นต์ ซึ่งสอดคล้องกับผลการวัดปริมาณคาร์บอนไดออกไซด์ที่ถูกปล่อยออกมา 57 เปอร์เซ็นต์ โดยคำนวนจากปริมาณคาร์บอนทั้งหมดในตัวอย่าง จากการตรวจสอบการเสื่อมสภาพของชิ้นยาง โดยส่องผ่านกล้องจุลทรรศน์อิเล็กตรอน (Scanning Electron Microscope, SEM) พบว่ามีจุลินทรีย์เกาะติดผิวหน้าชิ้นยางจำนวนมากตรงพื้นที่ที่มีการย่อยสลายอย่างชัดเจน ซึ่งเป็นสาเหตุทำให้มีการเปลี่ยนแปลงทางโครงสร้างของยางจากการตรวจวัดด้วยวิธีการ Fourier Transform Infrared Spectroscopy Attenuated Total Reflectance (FTIR-ATR) พบว่า 1) พันธะคู่ในสายไอโซพรีนลดลงอย่างเห็นได้ชัด 2) มีหมู่แอลดีไฮด์และคีโตนเกิดขึ้นที่ปลายสายโซ่ 3) พบการสร้างพันธะของสารกลุ่มกรดไขมันและพอลิแซคคาไรด์จากเซลล์ของจุลินทรีย์

จากการตรวจวิเคราะห์กลุ่มจุลินทรีย์ ST608 โดยวิธีการ Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis (PCR-DGGE) พบจุลินทรีย์ 8 สายพันธุ์ที่มีบทบาทสำคัญ เมื่อบ่งชี้ชนิดด้วยยีน 16s rRNA พบว่าส่วนใหญ่เป็นจุลินทรีย์กลุ่มแอคติโนมัยซีทีส จากงานวิจัยชิ้นนี้แสดงให้เห็นว่ากลุ่มจุลินทรีย์ ST608 เป็นกลุ่มที่มีประสิทธิภาพสูงในการย่อยสลายยาง

ในขณะเดียวกันก็มีการเปลี่ยนแปลงของกลุ่มประชากรแบคทีเรียในขณะเกิดกระบวนการย่อยสลายด้วย

เนื่องจากองค์ความรู้เรื่องเอ็นไซม์ย่อยสลายยางและยีนที่เกี่ยวข้องยังมีเพียงเล็กน้อย โดยเฉพาะอย่างยิ่งในแง่ของความรู้ทางชีวเคมีที่เกี่ยวกับการแตกตัวของไอโซพรีนโดย Lcps (Latex Clearing Proteins) รวมไปถึงชนิดและหน้าที่การทำงานของโคแฟกเตอร์ ในงานวิจัยครั้งนี้ค้นพบแบคทีเรียย่อยสลายยาง *Rhodococcus rhodochrous* สายพันธุ์ RPK1 ได้มาจากกลุ่มจุลินทรีย์ ST608 ที่มีประสิทธิภาพสูงซึ่งน่าสนใจ และยังไม่มีการศึกษาถึงคุณสมบัติทางชีวภาพที่เกี่ยวข้องกับการย่อยสลายยาง ดังนั้นจึงมีการศึกษาการบ่งชี้ชนิดและอธิบายลักษณะคุณสมบัติของ Lcp_{Rr} โดยทำการคัดแยกยีนที่ทำหน้าที่เปลี่ยนรหัสการสังเคราะห์โปรตีนที่ทำให้ไอโซพรีนแตกตัว จากนั้นโคลนและให้แสดงออกในแบคทีเรีย *Escherichia coli* และทำโปรตีนที่ผลิตได้ให้บริสุทธิ์ เมื่อวัดค่ากิจกรรมจำเพาะของโปรตีน Lcp_{Rr} (Specific activity) ที่อุณหภูมิ 30 องศาเซลเซียส พบว่ามีค่า 3.1 U/mg ซึ่งสามารถย่อยสลายไอโซพรีนสายยาว ให้เป็นโมเลกุลของไอโซพรีนสายสั้นที่มีปลายเป็นคีโตนและแอลดีไฮด์ และยังพบว่าค่าความเป็นกรด-เบสที่เหมาะสมสำหรับการทำงานของโปรตีน Lcp_{Rr} ที่ pH 8 มีค่าสูงกว่าค่าความเป็นกรด-เบสของเอ็นไซม์ย่อยสลายยางตัวอื่นที่พบ (pH 7) เมื่อวิเคราะห์ Lcp_{Rr} ด้วย UVvis spectroscopy พบว่าโปรตีนชนิดนี้มีการดูดกลืนแสงที่ช่วงคลื่นจำเพาะของไซโทโครม และยังพบลักษณะเด่นโดยดูดกลืนแสงช่วงคลื่นที่ยาวกว่าโปรตีนย่อยสลายยางตัวอื่นที่พบ และยังไม่เคยมีรายงานมาก่อน นอกจากนี้ยังพบว่าโปรตีน Lcp_{Rr} เป็นโปรตีนที่มีฮีมชนิดบีเป็นโคแฟกเตอร์ (*b*-type heme) โดยทำการวิเคราะห์จาก 1) การวิเคราะห์โลหะ 2) การสกัดด้วยสารตัวทำละลาย 3) การวิเคราะห์ด้วยวิธี Bipyridyl 4) การตรวจวัดด้วย mass-spectrometry

ในการศึกษาครั้งนี้ชี้ให้เห็นถึงประสิทธิภาพการย่อยสลายถุงมือยางวัลคาไนซ์ของกลุ่มจุลินทรีย์ ST608 พร้อมทั้งบ่งชี้ชนิดของจุลินทรีย์ที่มีบทบาทสำคัญในกระบวนการ และยังแสดงให้เห็นถึงลักษณะคุณสมบัติทางชีวเคมีของโปรตีน Lcp_{Rr} ซึ่งแตกต่างจากโปรตีนย่อยสลายยางตัวอื่นๆ อีกด้วย

คำสำคัญ: การย่อยสลายทางชีวภาพ ยางธรรมชาติ กลุ่มจุลินทรีย์ลาเท็กเคลียริ่งโปรตีนบริสุทธิ์ แบคทีเรียย่อยสลายยาง โรโดคอคคัส

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Abstract

Rubber degrading consortium ST608 was enriched from a rubber processing waste pond using Mineral Salt Medium (MSM) supplemented with sterile rubber glove pieces, representative of a vulcanized rubber, as a sole carbon source. Substantial disintegration of the rubber pieces was visible within 3 weeks of incubation with the consortium, this indicated the presence of active rubber degrading microorganisms. The highest percentage weight loss of the rubber pieces over a 30 day period was 50%, and according to the CO_2 released, this accounted for 57% of the total available rubber carbon. The deterioration of rubber pieces was also observed by Scanning Electron Microscope (SEM), numerous microorganisms were associated with the areas of strong disintegration resulted in structural changes, that was detected by Fourier Transform Infrared spectroscopy Attenuated Total Reflectance (FTIR-ATR), which revealed (i) a significant decrease of the numbers of double bonds in the isoprene chains (ii) the formation of aldehyde-ketone groups (iii) the formation of different chemical bondings including fatty acid and polysaccharide of microbial cells. The bacterial consortium ST608 was analyzed by PCR-DGGE, and eight prominent strains were identified based on 16s rRNA genes with most isolates identified belonging to the actinomycetes group. This work clearly showed the potential of one bacterial consortium ST608 to biodegrade rubber while undergoing dynamic changes in the bacterial population. Some strains that played key roles throughout the experiment were identified.

Due to little is known about rubber degrading enzymes and their corresponding genes, in particular about the biochemistry of polyisoprene cleavage by Lcps and the types and functions of the involved cofactors. In this research study, one

of rubber degrading bacteria identified as a *Rhodococcus rhodochrous* RPK1, was derived from the potential consortium ST608, which has not been previously identified its biochemical properties. Thus, identification and characterization of properties of *lcp* gene was performed. A *lcp* gene of *R. rhodochrous* RPK1 that coded for a polyisoprene-cleaving latex clearing protein (Lcp_{Rr}) was identified, cloned, expressed in *Escherichia coli* and purified. Purified Lcp_{Rr} had a specific activity of 3.1 U/mg at 30°C and degraded poly(*cis*-1,4-isoprene) to a mixture of oligoisoprene molecules with terminal keto and aldehyde groups. The optimum pH of Lcp_{Rr} was higher (pH 8) than for other known rubber-cleaving enzymes (≈ pH 7). UVvis spectroscopic analysis of Lcp_{Rr} revealed a cytochrome-specific absorption spectrum with an additional feature at long wavelengths that has not been observed for any other rubber-cleaving enzyme. The presence of one *b*-type haem in Lcp_{Rr} as a co-factor was confirmed by (i) metal analysis, (ii) solvent extraction, (iii) bipyridyl assay and (iv) detection of haem-*b* specific m/z values via mass-spectrometry.

This study points the potential of bacterial consortium ST608 to degrade vulcanized rubber glove with identification of strains that played important roles in the process, and revealed the substantial differences in the active sites of Lcp_{Rr} proteins to other rubber degrading proteins.

Keywords: Biodegradation, Natural rubber, Consortium, Lcp,
Rubber degrading bacteria, Rhodococcus

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LIST OF ABBREVIATIONS AND SYMBOLS

ATR-FTIR: Attenuated total reflectance fourier transform infrared spectroscopy

cDNA: Complementary Deoxyribonucleic acid

CoA: Coenzyme A

DGGE: Denaturing gradient gel electrophoresis

GPC: Gel permeation chromatography

^{1}H-NMR: Proton magnetic resonance

HPLC: High performance liquid chromatography

kDa: Kilodalton

Lcp_{K30}: Latex clearing protein from *Streptomyces* sp. K30

Lcp_{Rr}: Latex clearing protein from *Rhodococcus rhodochrous*

Lcp_{VH2}: Latex clearing protein from *Gordonia polyisoprenivorans* VH2

MALDI-ToF: Matrix assisted laser desorption/ionization time of flight

MSM: Mineral salts medium

MW: Molecular weight

NR: Natural rubber

ODTD: 2-oxo 4,8-dimethyl-trideca-4,8-diene-1-al,

OxiA: Oxidoreductase A

OxiB: Oxidoreductase B

OxiAB: Oxidoreductase complex AB

PCR: Polymerase chain reaction

PHA: Polyhydroxyalkanoate

RoxA: Rubber oxygenase A

$RoxA_{xsp}$: Rubber oxygenase A from *Xanthomonas* sp.

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: Scanning electron microscope

TAE: Tris-acetate-EDTA buffer

V: Volts

(vol/vol): Volume by volume

% CO_2: Percent carbondioxide released

% (wt/vol) : % Weight by volume

% wt: % Weight

% (wt/wt): % Weight by weight

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CHAPTER 1

Introduction

Rationale and Background

During this century, environmental issues have been a focus of law, especially waste management, which has been of particular concern. There has been a growing interest in renewable resources using a base of biodegradable products. An alternative material widely used in many applications is natural rubber, which is a highly valuable source with superior physical properties (Tanaka and Sukdapipanich, 2001). One of the major problems with rubber products is that they cannot be recycled and even though it is a natural product, it is very difficult to degrade either biologically or chemically. As a consequence of these difficulties, huge amounts of waste rubber materials are accumulating around the world, on the landfill and in the ocean, and are one of the causes of environmental pollution (Shah et al., 2013). The International Rubber Study Group (IRSG) has reported that the total world rubber consumption is increasing annually up to a million tonnes, leading to further organic rubber waste problems (IRSG, 2016).

In addition modified rubber products contain various antioxidants, accelerator residues, stearic acid and other compounding ingredients from production processes cause leaching to environments and can cause the health problem in humans. One example is plasticizers, which is suspected to be an endocrine-disrupting activity that interferes with the reproductive system and normal development of animals and humans (Wang et al., 2003; Wang et al., 2004; Xu et al., 2005; Pan et al., 2009). Degradation of plasticizer, rubber vulcanization additive and other compounds are also concerned with the rubber degradation process with regard to biotechnological applications in rubber waste treatment. (Wever and Verachtert, 1997; Wang et al., 2003; Wang et al., 2004; Li et al., 2005; Xu et al., 2005).

One possible approach to deal with such waste problems are burning, deposits in landfill or bury in the soil. However, these methods can produce further

environmental pollution and health problems (Fattta et al., 1999). Thus, a better way to control this problem is to induce microbial transformation of rubber and their derivatives or plasticizer into useful products or even to completely degrade into CO_2 as a growth substrate (Bode et al., 2001; Braaz et al., 2004; Linos et al., 2000a; Rose and Steibüchel., 2005; Tsuchii et al., 1985; Tsuchii and Takeda., 1990). More recent experiments have revealed that the compounding ingredients of rubber products such as curing agents or antioxidants serve as growth substrates during the degradation process (Blake and Kitchin, 1949; Pan et al., 2009).

However, the biodegradation of rubber products in the environment is a result of microbial strategies for an effective availability of solid and hydrophobic substrate. Due to the million units of rubber have high molecular weights with their chemical weakness for microbial attack, being the C=CH bond the long closely aligned hydrocarbon chains make the rubber extremely hydrophobic, and difficult to degrade. However, it is thought that after the initial attachment and preliminary degradation, some bacteria can excrete detergent-like molecules that tend to separate the hydrophobic rubber chains, and open up access to more double bonds. This is especially, true for cross-linked structures such as rubber gloves that have been used as a typical rubber representative product and as a model for these experiments. However, in many cases the bacteria isolated with an ability to begin to attack the C=CH bonds and to degrade the rubber with an accumulation of products and substrates with aldehyde and ketone groups are unable to complete the process (Shah et al., 2013). To characterize these single bacteria, our experiments used rubber agar plates, which showed that at least two different strategies were involved, one resulted in the production of clearing zones and the other did not. Those that formed a clearing zone on rubber agar plates exhibited a slightly faster growth rate, and required cultivation for a period of an additional month (Bröker et al., 2004). Bacteria such as *Streptomyces* sp., a clearing zone producing strain, required a longer incubation period to obtain a reasonable cell mass (Rose et al., 2005). Hence bacteria with only an ability to initiate biodegradation are unable to complete the process.

One of a major approach is to study the rubber degrading enzymes and their corresponding genes. So far, there is very little known about biochemically characterization of rubber cleaving protein. Only two types of rubber-cleaving

enzymes have been identified, one is the rubber oxygenase RoxA from *Xanthomonas* sp. 35Y (Tsuchii and Takeda., 1990; Braaz et al., 2004) and it has been found only in Gram-negative bacteria (Birke et al., 2013). Extracellular dioxygenase RoxA secreted by *Xanthomonas* sp. 35Y, is characterized by its biochemistry, and can be classified as *c*-type dihaemdioxygenase. This enzyme cleaves poly(*cis*-1,4-isoprene) into a C_{15} compound with a terminal keto and aldehyde group (12-oxo 4,8-dimethyl-trideca-4,8-diene-1-al, ODTD) as the one major product (Braaz et al., 2005; Schmitt et al., 2010; Birke et al., 2012; Birke et al., 2013). The other rubber cleaving enzyme is a protein designated as latex clearing protein (Lcp) (Rose et al., 2005). It shares no significant sequence homology with RoxA, with cytochrome *c* peroxidases or with dihaeme 7,10-diolsynthases (Estupiñán et al., 2015), and it is present in Gram-positive rubber degrading bacteria and other Gram-positive Actinobacteria. Lcp was first described for *Streptomyces* sp. K30 (Rose et al., 2005). Lcp of *Gordonia polyisoprenivorans* VH2 and *Streptomyces* sp. K30, two well-studied Gram positive rubber degraders, oxidatively cleave poly(*cis*-1,4-isoprene) to products of different sizes of cleavage products (C_{20}-C_{35}) with the terminal keto and aldehyde end groups as in RoxA-generated ODTD (Ibrahim et al., 2006; Birke and Jendrossek, 2014; Hiessl et al., 2014). There have been different published reports of the cofactor and metal-contents of the Lcps from *Streptomyces* sp. K30 and of *G. polyisoprenivorans* VH2, and at present there are only two biochemically characterized Lcp proteins (Hiessl et al., 2014; Birke and Jendrossek, 2014).

This research provides the use of a microbial consortium that could be an interesting approach to achieve an effective biodegradation of rubber in a similar way to the microbial consortia of polymer degradation such as benzene or aromatic hydrocarbon which can be converted into many metabolites that other microorganisms probably use as their main source of the nutritions. The degradation of complex molecules is proposed to effectively degrade by bacterial consortia than an individual isolate because of the synergistic effect, which facilitate a co-operative degradation where one organism transforms its compound to the other products, that can be utilized by the other organisms (Tixier et al., 2002; Dejonghe et al., 2003; Ghazali et al., 2004; Ha et al., 2008; Lou et al., 2009; Sarkar et al., 2011). In addition, the analysis of a community of a microbial consortium revealed the potential

dominant strains, which play important roles throughout the process. One key isolate designed as RPK1, taxonomic analysis revealed that isolate RPK1 was a member of the genus *Rhodococcus* sp., a taxon that had not previously been identified as having the ability to utilise rubber as a sole source of carbon and energy, but that is well known for its members to have a high potential for the biodegradation of recalcitrant compounds (Martínková et al., 2009). Biochemical and biophysical characterization of the purified recombinant Lcp protein of *Rhodococcus rhodochrous* strain RPK1 revealed some properties not previously described for any other rubber-degrading enzyme.

Objectives

1. To investigate rubber degradation by bacterial consortium.
2. To analyse the bacterial consortium structure and identify key bacteria involved in the rubber degradation process.
3. To identify the corresponding genes of the selected key bacteria that play important roles in the rubber degradation process and characterize the biochemistry of purified rubber-cleaving enzyme.

Perceived advantages

Biodegradation shows more advantages in rubber degradation than chemical and physical processes (including burning) which produce harmful or toxic chemicals. This is especially true for the use of a microbial consortium containing various rubber utilizing strains with broad enzymatic efficiency to enhance the rate of the degradation process. This research will provide a useful new information and knowledge in the rubber degrading enzyme and corresponding genes to stimulate a basis for the “rubber biodegradation processes”.

CHAPTER 2

Literature review

This chapter provides a review of the literature on natural rubber biodegradation, including rubber waste disposal problem. One approach is to describe the microorganisms that are capable of degrading rubber, either in raw or in vulcanized state. Moreover, the current knowledge about the rubber degrading enzymes and their corresponding genes are summarized especially rubber oxidizing protein, Lcp and RoxA. In this context, biochemical characterization and structure of the important proteins are highlighted, considering with an activity of rubber biodegradation and approaches to optimize the degradation process. Analysis of rubber cleavage products e.g. primary cleavage mechanisms of polyisoprene and technical analysis are reviewed. It is generally intended to provide a stimulating basis for the rubber biodegradation.

Overview of rubber waste and its problem

The fast growing of civilization world, the use of rubber products have increased abundantly. These rubber products are then discarded in the environment after their limited lifetime, become rubber waste problems over the world. It is expected that the world total rubber consumption will grow up to 28.2 million tonnes in 2017, and will increase by an average of 3.1% per annum over 2016-2024, (IRSG 2016). It represents a serious environmental and economic issue for the sustainable development of the world. Even though the entire amount of rubber product is discarded, it needs a very long time for natural degradation due to the formation of the cross linked network by sulfur in polyisoprene, resulting in a stable vulcanized rubber, and the presence of stabilizers and other additives (Shah et al., 2013).

The disposal of rubber waste leads to many environmental pollutions (Adhikari et al., 2000). Many of the rubber additives, such as accelerators and antioxidants used in the rubber processing, may migrate to the environments, and

cause health problems in humans and animals. The plasticizer is suspected to be endocrine disrupting and affecting the normal development in humans and animals. Additives e.g. accelerators, antioxidants, and preservation materials can promote or inhibit biodegradation of rubber materials (Gomez and Moir, 1979; MacLaghlan et al., 1996; Wang et al., 2003a,b; Xu et al., 2005).

Moreover, the landfill for the safe disposal of rubber waste product become limited and indiscriminate disposals are causing water and land pollution problems in addition, burning of rubber waste generates large amounts of heat, smoke and maybe toxic gasses which is also a major factor for global warming and environmental issues (Shah et al., 2013; Stevenson et al., 2008).

Early investigation of the natural rubber biodegradation

Biodegradation of rubber by microorganisms have been reported since 1914 by Söhngen and Fol. The natural rubber films are modified for using as a source of carbon for microbial growth. The films were sterile and incubated for several weeks, with different temperatures. Only two Actinomycetes were presented and identified as an *Actinomyces elastic* and *Actinomyces fuscus*. In addition, the authors reported, the presence of holes in the rubber materials, with numerous of the bacterial cells, due to the adhering strategies of microorganisms on the materials (Söhngen and Fol, 1914).

Encouraged the results of Söhngen and Fol Söhngen, De Vries (1982) investigated the decomposition of rubber using fungi. The smoked rubber sheet was incubated with *Penicillium* sp. and *Aspergillus* sp., the biomass of the mold was increased and final weight loss of rubber was rised up to 30.9% after 5 years. The chemical analysis of the remaining rubber substrate after 5 years showed an increase of nitrogen concentration which indicated that fungi consumed rubber hydrocarbon rather than proteins (De Vries, 1928).

The first isolation of rubber degrading bacteria using latex overlay plates was performed by Spence and van Niel (1936). The mineral salts agar was overlayed by a thin layer of natural latex dispersing on mineral agar surface. Rubber degrading colonies were developed on overlay plates and some of colonies produced

clearing zone (translucent halos). The authors also used potassium nitrate instead of ammonium chloride as a nitrogen source in order to prevent latex coagulation after autoclave. In addition, they attempted to quantify the adhering biomass by dissolving the remaining latex clot after incubated with microorganisms. Analytical fractions of adhering biomass revealed that the growth was promoted by rubber carbon, not by the non-rubber constituents (Spence and van Neil, 1936).

Kalinenko (1938) isolated rubber degrading actinomyces and fungi using latex overlay technique and revealed that all isolates were able to utilize large quantities of latex rubber from dandelion plant. One of Actinomycetes produced large holes in rubber film (Kalinenko, 1938). In addition, Shaposnikov et al. (1952) confirmed the growth of Actinomycetes by weight loss experiments of thin rubber film (Shaposnikov et al., 1952). Nette et al. (1959) isolated several fungi and bacteria from pieces of contaminated rubber.

Zobell and Grant (1942) and Zobell and Beckwith (1944) isolated several rubber degrading strains such as Actinomyces, Proactinomyces, Micromonospora, Mycobacterium, Bacillus and Pseudomonas. These bacteria were incubated with thin rubber film and were then estimated the rubber oxidation by microbial oxygen consumption in liquid culture. They pointed out that a consumption of 3.3 mg of oxygen would be required to complete oxidize 1 mg of rubber to be $(C_5H_8)_x$. In other words, the complete mineralization from rubber to carbon dioxide can be described as equation $(C_5H_8) + 7H_2O \rightarrow 5CO_2+4H_2O$. The additional observation of increasing biomass and rubber metabolism was obvious (Zobell and Grant, 1942; Zobell and Beckwith, 1944).

Blake and Kitchin (1949) revealed the deterioration of natural rubber gum and rubber insulation of underground cables, caused by microorganisms referring to Actinomycetes and Micrococci. This vulcanized rubber linked together by sulfur bridges and several compounds that have been added during manufacture was biodegraded (Zobell and Beckwith, 1944; Zobell and Grant, 1942; Linos and Steinbüchel, 2001).

Microorganisms capable of rubber biodegradation

The biodegradation of rubber organic waste and other plastics waste using the microorganisms is one of the best options, that is environmental friendly and less harmful to humans and animal lifes. There by, the properties of several microorganisms which susceptible to natural rubber either in the vulcanized form have been examined. The published classification of rubber degrading microorganisms must consider their apparently direct or indirect involvement in the biodegradation process.

Rubber degrading bacteria

Many bacterial strains that are able to utilize rubber as a sole carbon and energy sources for growth have been identified (Shah et al., 2013; Rose and Steinbüchel, 2005; Rose et al., 2005). According to different strategies to degrade rubber, natural rubber degrading bacteria can be devied into two groups (Linos et al., 2000a). The first group is clearing zones forming bacteria (translucent halos) on latex overlay agar plates, indicating the secretion of rubber-cleaving extracellular enzyme. This is the first indication that they produce extracellular enzymes that can cleave the rubber chains. This group belongs to the mycelium-forming Actinomycetes such as Actinoplanes, Streptomyces and Micromonospora (Imai et al., 2011; Jendrossek et al., 1997; Shah et al., 2013). On the other hand, the second group is the bacteria that do not form halos, and exhibit adhesive growth with direct contact of the cells with the rubber material, and extensive disintegration of the substrate. The results showed that the most potent rubber degrading bacterial strain belongs to the genera Gordonia, Mycobacterium, and Nocardia (Arenskötter et al., 2001; Bröker et al., 2008; Linos et al., 1999; Linos et al., 2002; Linos and Steinbüchel, 1998; Tsuchii et al., 1995). This second group comprises of mycolic acid containing Actinobacteria belonging to the genera Gordonia, Mycobacterium and Nocardia. Some new rubber degrading bacteria belonging to the Corynebacterium, Nocardia, Mycobacterium group, such as *Gordonia polyisoprenivorans* strains VH2 and Y2K, *G. westfalica* strain Kb1, and *Mycobacterium fortuitum* strain NF4 were isolated (Arenskötter et al., 2001; Arenskötter et al., 2004; Linos et al., 2002). All rubber-degrading species described

are mesophilic species, exceptional for *Streptomyces albogriseolus* and *Streptomyces viridodiastaticus* which were able to grow at temperature up to 50°C (Gallert, 2000; Shah et al., 2013).

Currently, characterization of these isolates seem to use 16s rDNA similarities as their major emphasis, in addition some have used cell wall analysis, and fatty acid and lipid patterns (Yikmis and Steinbüchel, 2012). All rubber-degrading species described so far are mesophilic species, mainly Streptomycetes that are Gram-positive, aerobic, spore forming bacteria that are slowing growth in soil or water with branching and aerial mycelia. Many members of the genus Streptomyces possess the ability to secrete enzyme that can cleave the biopolymer chains, and produce more readily degradable metabolites for use as energy and nutrient sources (Schrempf, 2006).

Rubber degrading fungi

Investigation of rubber degradation by fungi was first examined by De Vries (1928). The taxonomic classification showed that they belong to Penicillium and Aspergillus groups. In 1938, Kalinenko (1938) also introduced *Aspergillus oryzae* and different *Penicillium* species as rubber degrading fungi, however no confirmation from other researchs has been further reported.

In addition, Kwiatkowska and co-worker (1980) found that *Fusarium solani* decreased weight loss of rubber, reaching up to 40% of initial rubber carbon after 91 days. Williams (1982) also isolated a fungal strain, e. g. *Penicillium variabile* from deteriorated rubber, causing weight loss and decrease of molecular weight of raw rubber (Kwiatkowska et al., 1980; Williams, 1982). Moreover, rubber smoked sheet was inoculated with spore in a humidity cabinet that leading to an increase in biomass on the rubber surface, as represented by cell protein determination. However, further increase in biomass and weight loss after 14 days could not be determined. The molecular weight was measured using viscosity analytical tool, the reduction in the molecular weight estimated up to 15% of polyisoprene after 70 days (Williams, 1982). Several rubber deteriorating fungi were isolated from mineral agar plates containing powdered NR as a sole substrate and also from the surface of deteriorated tyre materials. The fungal strains e.g. *F. solani, Cladosporium cladosporioides* and

Paecilomyces lilacinus were grown on the surface of rubber after 20 days of incubation. A relative reduction of the molecular weight up to 20% was detected using Gel permeable chromatography (GPC) (Borel et al., 1982).

In a recent study, Zeb (2009) isolated vulcanized rubber degrading fungal strain, *Paecilomyces variotii* strain SFA-25, from hot spring in Pakistan (Zeb, 2009). The SEM micrographs revealed the hyphal network of strain SFA-25 on the tyre pieces surface, and surface erosion was observed under the network. Fungi attack materials with a damaged or rough surface more successful than materials with smooth surfaces because propagules are retained more easily at the surface (Shah et al., 2013; Verran et al., 2000). Rough surfaces or those with cracks may trap nutrients and moisture more easily and provide favorable conditions for fungal attachment and growth. Once established on the surface, and deriving nutrients from components of the material or from debris accumulated at the surface, fungi usually start to penetrate into deeper layers through micro-cracks or damaged areas. The use of environmental carbon sources by microorganisms may enable them to attack a material more vigorously (Jenings and Lysek, 1996). Unfortunately, to date no progress has been provided.

Bacterial consortium

A consortium consisting of bacterial species with various physiologies was shown to have a higher rate of degradation than its individual members. These are considered to have several advantages for bioremediation such as greater stability and increased metabolic capability. These characteristics enable the consortium to overcome limitations for the complete biodegradation of toxic compound. The degradation efficiency of a microbial community depends on the stability of the constituent members and their ability to degrade or mineralize the target compounds (Davidson et al., 1994; Ambujoms, 2000). Nevertheless, in numerous degradative studies, uncharacterized biological microbial consortia have been used instead of characterized consortia. It is important from the perspective of further applications to identify bacteria in the consortium which are responsible for rubber degradation and this could lead to optimization of the degradation processes and the development of monitoring tools. Recently, there have been few reports on microbial consortia

capable of degrading organic compounds such as benzene, oil or phenol (Ambujom, 2000). They reported the consortium containing at least 10 members of a microbial community having eight phenol-degrading and two non-phenol degrading strains. These consortia could degrade 99% of a 500 mL/L of phenol over incubation period of 24 hour. These members included 4 genera, e.g. Bacillus, Pseudomonas, Rhodococcus and Streptomyces. The ability of a large number of members in a microbial consortium to maintain its stability with respect to its composition and effectiveness in phenol degradation has indicated its suitability for bioremediation applications (Ambujom, 2000). In another study, the presence of six morphologically different bacterial members that consisted predominantly Bacillus and P*seudomonas* spp. were able to degrade hydrocarbon contaminated soil such as diesel, crude oil and engine oil. All of them could effectively remove the medium chain and long chain alkanes of oil after 30 day of incubation period (Ghazali et al., 2004). However, rubber degradation by a consortium has not been reported. Thus, it will be an interesting approach to investigate rubber degradation by microbial consortia.

Biochemical analysis and catabolism of rubber degradation

The primary degradation reaction of *cis*-1,4-isoprene is an initial exposure the rubber to bacterial enzymes that can oxidize some double bonds and break the long chain hydrocarbon linkages to produce smaller more easy to degrade molecules. These enzymes are either excreted for the case of clear zone forming rubber degrading bacteria or attached to the bacterial cell walls in the group of bacteria that require direct growth on the rubber surface (Linos et al., 2000b; Linos and Steinbüchel, 2001).

In 1975, Cundell and Mulcock reported on the rubber degradation process, and hypothesised that the initial rubber oxidation step started from a monooxygenase, then C-O- and C=O- groups were formed indicating the deterioration of the rubber materials (Cundell and Mulcock, 1975). Moreover, Tsuchii and his team (1985) determined the degradation products of various kinds of rubber (vulcanized and non-vulcanized) during cultivation with *Nocardia* sp. 835A. The authors concluded that natural rubber is degraded by an oxygenase reaction, and may

also involve the scission of isoprene at the unsaturated double bond by enzymes in a similar way to that involved in squalene degradation, leading to the formation of aldehydes or ketones at each end of the split isoprene chains (Fig. 1) (Tsuchii et al., 1985).

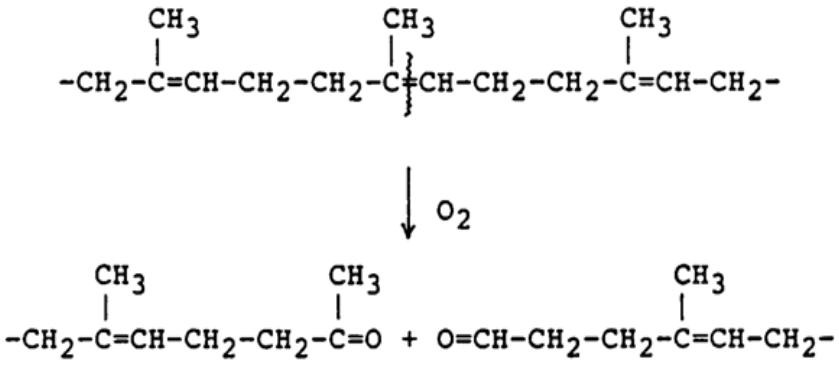

Fig.1 Schematic diagram represented the primary step of *cis*-1,4 isoprene biodegradation by *Nocardia* sp. 835A (Tsuchii et al., 1985).

Further investigation performed in the same laboratory (Tsuchii and Takeda, 1990) described the rubber degrading activity in the culture medium using *Xanthomonas* sp. strain 35Y which is also similar to the system using in *Nocardia* sp. 835A (Tsuchii et al., 1985). In addition, the authors attempted to cleave rubber with crude enzyme, and pointed out that the cleavage reaction was enzyme-catalyzed process. This evidence revealed that the enzyme reaction could be considered to be a two-step reaction. In the first step, the rubber polymer with a high molecular weight was degraded and converted to medium molecular weight (MW) hydrocarbon intermediates. The extracellular crude enzyme from strain 35Y had a high substrate specificity for *cis*-1,4-polyisoprene compounds and attacked high MW polymer chains rather than lower MW oligomers, 12-oxo-4,8-dimethyltrideca-4,8-diene-1-al was identified as one of the first degradation products. Moreover, Linos and their team (2000) confirmed Tsuchii's theories on the rubber cleavage mechanism by a *Gordonia* strain and found that not only aldehyde and ketone groups were detected but also the number of *cis*-1,4-isoprene bond decreased during the process, in addition, two different new functional groups were detected after being treated with

bacteria. This result indicated that an oxidative enzyme attacked at the double bond in the first metabolic step of the biodegradation process (Linos et al., 2000a; Linos and Steinbüchel, 2001).

In another experiment performed by Bode and coworker (2000), three degradation products from a liquid culture of *S. coelicolor* 1A with vulcanized rubber were identified as (6Z)-2,6-dimethyl-10-oxo-undec-6-enoic acid, (5Z)-6-methyl-undec-5-ene-2,9-dione, and (5Z,9Z)-6,10-dimethyl-pentadec-5,9-diene-2,13-dione. From this strong evidence, the authors proposed an oxidative pathway from poly(*cis*-1,4-isoprene) to methyl-branched diketones, as described in 4 steps (i) oxidation of an aldehyde intermediate to a carboxylic acid, (ii) one cycle β-oxidation, (iii) oxidation of the conjugated double bond resulting in a β-keto acid, and (iv) decarboxylation (Bode et al., 2000).

In an extensive investigation about the metabolites of rubber degradations in both Gram positive and Gram negative rubber degrading bacteria, a diketone derivates of oligo (*cis*-1,4-isoprene) was identified as a metabolic product, in addition the oxidative degradation pathway of poly(*cis*-1,4-isoprene) to acetyl-coenzyme A and propionyl-coenzyme A by *β*-oxidation was suggested for bacterial degradation of isoprene rubber (Bode et al., 2001).

According to all the evidences in the biochemical degradation reactions of polyisoprene rubber to the identification of metabolites, the “hypothetical pathway of rubber degradation” was established by Bode and his team (2001). The first step is the oxidative cleavage at the isoprene double bonds, with the formation of *cis*-1,4-isoprene oligomers such as 12-oxo-4,8-dimethyltrideca-4,8-diene-1-al and the presence of keto and aldehyde groups at the end of the split chains, these low molecular weight oligomers are utilized by bacteria and the aldehyde group is oxidized to the corresponding carboxylic acid. The acid is activated to a coenzyme A (CoA) ester and then passes though the *β*-oxidation cycle. The *α*-methyl groups accumulate as intermediates (Bode et al., 2000; Bode et al., 2001). However, more knowledge and evidences on the biochemical analysis, metabolite or end products of rubber requires further investigation.

Rubber degrading enzymes and corresponding genes: Lcp and RoxA

The natural rubber can be biodegraded, and used as a carbon and energy source for microorganisms, which has been known for more than a century. It was only a decade ago that the first polyisoprene specific rubber degrading enzyme was isolated (Braaz et al., 2004). The rubber degrading enzymes have been either isolated or characterized in biochemical details. Many indications have been provided in the literature for the occurrence of rubber degrading genes, and enzymes in catalyzing cleavage of rubber isoprene, including disintegration to cleavage products (Braaz et al., 2005; Birke et al., 2013; Birke and Jendrossek, 2014; Birke et al., 2015; Bode et al., 2001; Hiessle et al., 2012; Hiessle et al., 2014; Linos et al., 2000b; Yikmis et al., 2008; Yikmis and Steinbüchel, 2012; Watcharakul et al., 2016).

There are only two important enzymes involved in rubber degradation that have been isolated in an active form during this century. These two enzymes and their genes cleaving the isoprene backbone have been isolated and investigated recently, in particular the functions that could be assigned to the gene. One of the key enzymes is Lcp from *Streptomyces* sp. K30 (Rose and Steibüchel, 2005; Yikmis et al., 2012) and the other one is RoxA from *Xanthomonas* sp. (Jendrossek and Reinhardt, 2003; Braaz et al., 2004, 2005). Both of them form translucent halos when they cleave polyisoprene by an oxidative reaction pathway as the first step. However, these two enzymes use different cleavage mechanisms to degrade rubber devoid of any relevant amino acid similarities.

Rubber oxygenase A (RoxA)

Jendrossek and Reinhardt (2003) analyzed the amino acid sequences of an extracellular protein (~70 KDa) that was secreted from *Xanthomonas* sp. during growth with rubber. This sequence information was converted to cDNA and used as a DNA probe to amplify DNA fragment coding for the target protein, and resulted in a 2037 bp open reading frame that coded for an amino acid peptide of (74.6 KDa). Moreover, the amino acid sequence included two heme binding motifs (CXXCH) and a partial sequence was a conserved region of a diheme cytochrome *c* peroxidase in *Paracoccus denitrificans* and *Pseudomonas aeruginosa*. This is the first report to

identify the gene corresponding to the rubber specific protein (Jendrossek and Reinhardt, 2003).

Additional information on the extracellular protein excreted from the Gram negative *Xanthomonas* sp. 35Y was performed by Braaz and the team (2004). The purified protein was able to cleave both natural rubber and synthetic rubber by oxidative cleavages at the double bond of isoperene chains, the major cleavage product was identified as a 12-oxo-4,8- dimethyltrideca-4,8-diene-1-al (ODTD) and a minor metabolite that differed from the major by only the number of repeating units between the terminal functions, $CHO\text{-}CH_2$- and $–CH_2\text{-}COCH_3$. The authors determined that the mechanism of the oxidase enzyme was strictly dependent on the presence of oxygen and the reaction was inhibited by cyanide and carbon monoxide. The authors concluded that RoxA (Rubber oxygenase A) encoded for a rubber oxygenase protein in *Xanthomonas* sp. and this protein contained 2 mol of heme per mol of RoxA (Braaz et al., 2004).

The oxidative reaction mechanism of RoxA is well understood. RoxA has two *c*-type heme centers and more evidence from an electron paramagnetic resonance (EPR) spectra of the purified RoxA contained two low-spin Fe(III) heme centers. In the authors conclusion, RoxA is a novel type of diheme dioxygenase that is clearly different from a classical cytochrome *c*-peroxidases and is used as an exo-type cleavage mechanism to give the main end product 12-oxo-4,8-dimethyltrideca-4,8-diene-1-al (ODTD) (Birke et al., 2013; Birke and Jendrossek, 2014; Schmitt et al., 2010; Yikmis and Steinbüchel, 2012).

In recent study, Birke et al. (2013) identified RoxA-related sequences from members of the myxobacteria, namely, *Haliangium ochraceum*, *Corallococcus coralloides*, *Myxococcus fulvus*, and *Chondromyces apiculatus*, which revealed a function of enzymes and biochemical characterization. According to these results, all RoxA orthologs from these myxobacteria cleaved polyisoprene into 12-oxo-4,8-dimethyltrideca-4,8-diene-1-al (ODTD), as a cleavage product. The biochemical characterization also indicated that, the presence of two slightly different heme centers, and the structures of the RoxA orthologs have a high degree of similarity to the recently solved RoxA from *Xanthomonas* sp. Structure and several conserved residues shown obvious W_{302}, F_{317}, and a MauG motif at about H_{517} (Birke et al.,

2013). RoxA in Gram-negative bacteria other than Xanthomonas proves that RoxA is more common among rubber degrader.

Latex-clearing protein (Lcp)

All rubber degrading Actinomycetes seem to have at least one copy of a gene encoding a latex clearing protein (Lcp). This enzyme is considered to catalyze the initial oxidative cleavage of rubber within this group of Gram-positive bacteria (Birke et al., 2013; Ibrahim et al., 2006; Rose and Steinbüchel, 2005; Hiessl et al., 2014) and does not possess any similarity towards the amino acid sequence of RoxA. Lcp was first detected in *Streptomyces* sp. K30 by generating a chemically induced mutant that cannot form clear zones on latex overlay plates.

Latex clearing protein is considered to be a key enzyme in Gram positive rubber degrading bacteria. The corresponding genes were verified by Rose and team (2005). Three genes *lcp*, *oxi*A, *oxi*B seemed to exist as an operon as a polycistronic mRNA that was demonstrated in *Streptomyces* sp. strain K30. An oxidoreductase AB gene (*oxi*AB) was located directly downstream of the *lcp* encoded for a putative oxidoreactase enzyme, that functioned as an aldehyde dehydrogenase. The oxidoreductase complex (OxiAB) consisted of a large subunit (OxiB) with the possible function of binding the molybdopterin cytosine dinucleotide cofactor and of a small subunit (OxiA), responsible for the iron-sulfur cluster center. It was shown that the function of these genes resulted in the latex clearing proteins (Lcp) ability to cleave the double bonds in the isoprene chains to produce a smaller oligoisoprene or an aldehyde and ketone group that occurred at the initial step then, the aldehyde or ketone were oxidized by the oxidoreductase complex (OxiAB) (Bröker et al., 2008; Rose et al., 2005; Yikmis and Steinbüchel, 2012).

Recent study has verified the function of the *lcp* that is not essential for the clear zone forming phenomenon, many reports have shown that this gene is present in the nonclearing-zone-forming group such as *Gordonia* sp, or *Nocardia* sp. (Bröker et al., 2008).

The biochemical analysis of the Lcp has been reported, a purification procedure was developed for native Lcp. An extensive biochemical study has shown that the Lcp cleaves polyisoprene through an endo-type cleavage to tetra-C_{20} and

higher oligo-isoprenoids with aldehyde and keto end groups. In addition, Lcp is a member of the growing group of heme protein having different mechanisms to cleave rubber from the RoxA as shown in Table 1 (Birke et al., 2013; Birke et al., 2014; Birke et al., 2015).

Nevertheless, biochemical characterization of Lcp is enzymatically involved in the degradation of poly(*cis*-1,4-isoprene) and whether other enzymes are necessary for rubber disintegration by Actinobacteria are deficient.

Table 1. The biochemical characteristics of rubber oxidative cleavage enzymes, RoxA and Lcp (Birke et al., 2013 and Birke and Jendrossek, 2014)

Biochemical characters	**Gram positive rubber degrading bacteria**	**Gram negative rubber degrading bacteria**
Key enzymes	latex clearing protein	rubber oxygenaseA
Key genes	*lcp*	*roxA*
Gene length [bp]	~1224-1227	~2037
Molecular mass of key enzymes (apo/mature protein) [kDa]	~(44-45.2)/(41-42)	~74.7-71.5
Mechanism to cleavage isoprene	endo-type cleavage	exo-type cleavage
Metabolite products	tetra-C20, oligoisoprene/aldehyde and ketone terminal	ODTD
Major metal atoms in protein molecule	1 Fe	2 Fe
Heme type	*b*-type	*c*-type
Oxidation state of iron	Fe^{3+}	Fe^{3+}----O_2^-

Analysis of rubber degradation products

Analysis of the degradation products of disintegrating latex gloves revealed several compounds that could be separated by high-performance thin-layer chromatography. Three of the compounds isolated were identified by one-and two-dimensional ^{1}H NMR spectroscopy, as 2,6-dimethyl-10-oxo-undec-6-enoic acid, 5,6-methyl-undec-5-ene-2,9-dione and 5,9-6,10-dimethyl-pentadec- 5,9-diene-2,13-dione. Several Gram-positive and Gram-negative bacteria such as *Acinetobacter calcoaceticus*, *Xanthomonas* sp. and *P. citronellolis* (Bode et al., 2000) were reported to be rubber degraders by the reduction of molecular weight during degradation. An endo-type reaction was responsible for degradation of the polymer. However, the biochemical reactions, of the cleavage the polymer and the subsequent reactions leading to the central metabolism, are still unknown (Bode et al., 2001). An oxidative cleavage of the polymer for an initiation of microbial rubber degradation has been suggested (Tsuchii et al., 1985; Tsuchii and Takeda, 1990). This assumption based on the identification of acetonyl diprenyl acetoaldehyde and related oligomers in the culture fluid of a rubber-grown culture of *Xanthomonas* sp. 35Y or *Nocardia* sp. 835A. Bode and co-workers isolated three low molecular weight rubber degradation products by comparative HPTLC. In addition, low molecular weight degradation products were also detected (Bode et al., 2000; Bode et al 2001). The majority of the accumulated molecules could be degraded by *b*-oxidation, and propionyl-coenzyme A (CoA) would be generated. After two cycles of β-oxidation one isoprene unit would be metabolized to acetyl-CoA and propionyl-CoA. The last isoprene unit of the primary oxidation product containing the carbonyl function of the primary degradation product would be cleaved into acetyl-CoA and pyruvate. The principle degradability of R-methyl-carboxylates to acetyl-CoA and propionyl-CoA is known from the isoleucine degradation pathway. The propionyl CoA is a metabolite of rubber degradation, one would expect that rubber-degrading bacteria are generally able to grow with propionate as the sole source of carbon and energy. The β-oxidation pathway results in the sub-sequential cleavage of one isoprene unit into one molecule of acetyl-CoA and propionyl-CoA. Additional evidences for the involvement of a

functional β-oxidation pathway were obtained in *S. coelicolor* 1A by the selective inhibition of rubber degradation by the β-oxidation-specific inhibitor acrylic acid. However, the results could not indicate whether the identified metabolites 2,6-dimethyl-10-oxo-undec-6-enoic acid, 6-methyl-undec-5-ene-2,9-dione and 6,10-dimethyl-pentadec-5,9-diene-2,13-dione are dead end products or they can be further degraded. Enzymatic studies will be necessary to elucidate which of the proposed biochemical reactions is present in rubber degrading bacteria (Bode et al., 2001).

CHAPTER 3

Biodegradation of rubber and analysis of the community structure of a microbial consortium

Abstract

This study examines a microbial consortium, which was achieved by enriching a culture from rubber waste using MSM supplemented with rubber glove pieces as a sole carbon and energy source. Substantial disintegration of the rubber pieces indicated the presence of active rubber degrading microorganisms. Investigation of rubber degradation was performed by calculating the weight loss of rubber and the amount of CO_2 released. In addition the bacterial attachment behavior was observed by Scanning Electron Microscope (SEM), which showed numerous microorganisms adhered and penetrated into the materials, causing of rubber deterioration. Structural changes of rubber detected by Fourier transform infrared spectroscopy (FTIR-ATR), revealed the new formation of functional group and the decreased number of double bonds. The bacterial consortium ST608 was analyzed by PCR-DGGE, and some prominent strains were identified based on 16s rRNA genes with most isolates identified belonging to the actinomycetes group.

This research conclusively shows the potential of bacterial consortium ST608 to biodegrade rubber, it might be an alternative approach to provide the environmental solution for disposal of rubber waste.

Keywords: rubber biodegradation, bacterial consortium, rubber degrading bacteria

บทคัดย่อ

การศึกษานี้เป็นการศึกษาการทำงานของกลุ่มจุลินทรีย์ที่ได้มาจากน้ำเสียจากกระบวนการผลิตยาง โดยเลี้ยงบนอาหาร MSM ที่ใช้ถุงมือยางเป็นแหล่งคาร์บอน และพลังงานเพียงแหล่งเดียว การเสื่อม สภาพของถุงมือยางที่เกิดขึ้นแสดงให้เห็นถึงการทำงานของจุลินทรีย์ย่อยสลายยางที่มีประสิทธิภาพ การศึกษาการย่อยสลายยางทำโดยใช้วิธีวัดน้ำหนักยางที่หายไป และดูปริมาณคาร์บอนไดออกไซด์ที่ถูกปล่อยออกมา นอกจากนี้การศึกษาลักษณะการเกาะติดของจุลินทรีย์ ภายใต้กล้องจุลทรรศน์อิเล็กตรอน (SEM) พบว่ามีการเกาะของจุลินทรีย์จำนวนมากบนผิวหน้ายาง ทั้งยังแทรกเข้าไปในชิ้นยางซึ่งเป็นสาเหตุทำให้ยางเกิดการเสื่อมสภาพ การศึกษาลักษณะโครงสร้างที่เปลี่ยนไปของยางด้วยวิธี FTIR-ATR พบว่ามีการสร้างหมู่ฟังก์ชั่นใหม่ขึ้น ในขณะที่จำนวนพันธะคู่ลดลง ทั้งยังมีการวิเคราะห์กลุ่มจุลินทรีย์ด้วยวิธี PCR-DGGE และบ่งชี้ชนิดของสายพันธุ์ที่โดดเด่นโดยใช้ 16s rRNA gene ซึ่งพบว่าจุลินทรีย์ที่ได้ส่วนใหญ่เป็นกลุ่มแอคติโนมัยซีทีส

งานวิจัยนี้แสดงให้เห็นศักยภาพของกลุ่มแบคทีเรียย่อยสลายยาง ST608 และอาจจะเป็นทางเลือกที่ดีในการแก้ปัญหาด้านสิ่งแวดล้อมสำหรับการกำจัดของเสียจากยาง

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Introduction

This chapter presents an introduction to the investigation of rubber degradation, and summarizes the process of rubber biodegradation. The microorganisms that are capable of degrading *cis*-1,4-polyisoprene, which are the main constituents of natural rubber (NR), and the occurrence of the microbial degradation of rubber are described.

Different microbial strategies for an effective availability of the NR substrate are highlighted. In this context, factors affecting the rate of biodegradation and known approaches for the degradation process with specific regard to its application in rubber waste treatment are discussed. Analytical data concerning microbially caused surface and chemical mechanisms are presented to elucidate the primary cleavage mechanism of the polymer chain.

The biodegradability of various types of rubber and rubber products is an important issue in term of the need to protect rubber goods against biological damage and deterioration, as well as to provide environmentally compatible means of disposal and recycling of rubber waste. During the past few decades, much attention has been paid to the protective aspects because of its economic relevance. Many applications of rubber-based biopolymers have been emphasized; due to rubber products are not easily biodegradable and having high average molecular weight of milliondaltons (Gu, 2007). Rubber product wastes tend to accumulate in large piles of unmanageable waste leading to critical environmental problems (Shah et al., 2013; Terzi et al., 2009), such as mountains of used tyres or other rubber waste products, that are usually disposed by incineration or landfill. However, the disposal rubber wastes on landfill or in the ocean generate many problems, including the leaching of plasticizer or additive compounds migrating to the environment, and may cause health problems in humans and animals (Gu, 2007; Gu et al., 1996).

Despite numerous reports and reviews concerning the biodegradation of rubber, the rate of rubber degradation is still slow and rarely completed (Ismail et al., 2013; Kanwal et al., 2015). Special emphasis of the degradation rate is given by Pan et al. (2009). They revealed the ability of the *Alcaligenes xylosoxidans* strain T2,

Pseudomonas aeruginosa strain GP10 and *Nocardia corynebacterioides* S3 to degrade rubber were rather poor. In subsequent reports, Tsuchii and coworkers concentrated on the *Nocardia* sp. strain 835A and found that the poor degradation rate was due to the microbial's remarkable rubber-degrading protein (Tsuchii et al., 1990, Tsuchii et al., 1996; Tsuchii et al., 1997).

Heisey and Papadatos (1995) found several Actinomycetes, including Streptomyces, Amycolatopsis and Nocardia strains, were able to metabolize highly purified NR as a sole carbon source but weight losses occurred after cultivated with vulcanized NR glove pieces were reached only 10% after 6 weeks (Heisey and Papadatos, 1995). One strain was classified as a *Gordonia polyisoprenivorans* Kd2, which represents a high potential of natural rubber degradation required extended over months to reach values of 20% for IR and 50% for NR of carbon released from rubber substrate as CO_2 during the cultivation time (Linos et al., 2000). However, the compounding ingradients had an effect on the susceptibility of rubber. In another experiment by Tsuchii et al. (1990), the effects of compounding ingredients on the growth of a strong rubber decomposing *Nocardia* sp. was tested. Their results revealed that biodegradability decreases when the sulphur content increases. It would be important and desirable to search for an effective combination of microorganisms because each microorganism have a different mechanism and substrate requirement therefore their combinations could be highly effective in the biodegradation of these unique polymers (Kanwal et al., 2015; Tsuchii et al., 1990).

The current researchs are generally seeking for different bacteria and fungi that can act synergistically, and play significantly roles on the rubber degradation process, which would be advantageous for rubber-waste management.

Bacteria with an ability to initiate biodegradation are unable to complete the process. Thus, developing of a microbial consortium is perhaps one option to complete the degradation process. The long term aim of this study is to develop a bacterial consortium that can degrade vulcanized rubber in a relatively short period of time and characterize the dominant strains that play key roles in the degradation process.

Materials and Methods

Bacterial enrichment culture

Rubber gloves used in this study were purchased from Top Glove Co., LTD., Thailand. Rubber latex was supplied from the Materials Science Department (Prince of Songkla University, Thailand). Sediments and soils from a several rubber waste ponds and rubber processing factories in Songkhla, Thailand were used as inocula to enrich the rubber degrading microorganisms in 1000 mL of Mineral Salt Medium (MSM, 9 g/L Na_2HPO_4 x 12 H_2O, 1.5 g/L KH_2PO_4, 1 g/L NH_4NO_3, 0.2 g/L $MgSO_4$ x 7 H_2O, 0.02 g/L $CaCl_2$ x 2 H_2O, 1.2 mg/L Fe(III) ammonium citrate supplemented with 1x1 cm pieces of sterilized rubber gloves as a carbon source. After 2 weeks of incubation at 30°C, one volume of culture was transferred to a fresh medium and inoculated for 4 weeks. Samples of the liquid cultur were taken on successive 6 day periods (0-30 days) to analyse microbial population and biodegradation. Rubber utilizing bacteria were isolated for further investigation using rubber agar plate. Substantial disintegration of new rubber pieces became visible indicated the presence of active rubber degrading microorganisms after 2 weeks of incubation. The bacterial consortium was maintained by transferring one volume of culture into new fresh medium every 4 weeks.

Degradation experiments and culture conditions

The consortium was obtained using an enrichment culture with rubber gloves as the sole carbon and energy source that was used thoughout the study. For each experiment, the microbial consortium was enriched in MSM with rubber pieces for a period of 7 days, and one volume of liquid culture was used as an inoculum. The investigations of rubber degradation were established using three replicates, in addition, a control experiment was always done pararelly in the same conditions without bacteria (abiotic control). All cultures were incubated using the conditions described above.

Aldehyde and ketone detection

Aldehydes and ketones were detected by staining rubber gloves with Schiff's reagent. This procedure was as follows: 1 mL of the Fuchsin reagent in a tightly stoppered bottle was added to the sample, and the purple color allowed to develop over a 10 min period at room temperature. Any excess reagent was then discarded, and 1 mL of 0.5% sulfite solution was added in order to suppress the nonspecific color reactions of the sample. The remaining purple color denoted the presence of aldehyde groups produced during the degradation of the rubber polymer. The composition of the fuchsin reagent has been described by Linos et al. (2000).

Growth experiments

A modified Bradford method for protein assay was used to monitor the development of bacterial biomass during incubation. One millilitre of liquid culture was centrifuged to harvest the bacterial cell mass, then 1 mL of Coomassie Brilliant Blue reagent (100 mg Coomassie Brilliant Blue G250; 50 mL 95% ethanol; 100 mL 85% H_3PO_4; in 1 L of deionized water) was added and mixed thoroughly (Cheung and Gu, 2005). After 5 minutes of reaction, the A_{595} of the sample was measured using a spectrophotometer. The amount of protein presented was used as a measure of the bacterial growth obtained from a standard protein calibration curve having a linear relationship from 0-50 µg/mL.

Weight loss of rubber after incubation

The percentage weight loss was measured in separate experiments, with degradation flasks set up in replicates. Aliquots of bacterial suspensions were transferred to MSM liquid culture with pieces of sterile rubber glove as the sole source of carbon, and were then incubated at 30°C. Some of the residual materials were taken at certain times during the degradation process over the period of 30 days, and the glove pieces were washed with sterile water and dried in an oven at 60°C until constant weights were obtained.

Scanning electron microscopy (SEM)

The behavior and characteristics of the bacterial consortium on the surface of the eroded rubber pieces were observed. Residual rubber material after treatment with a consortium for between 0 and 30 days after being incubated at 30°C were taken and prepared for examination by scanning electron microscopy (SEM). After an initial fixation in 2% glutaraldehyde and followed by 1% OsO_4, samples were then dehydrated in ethanol solutions with gradually increase of concentrations, and coated with gold-palladium (Pan et al., 2009) using a vacuum sputter device and examined with SEM at 20 kV under high vacuum conditions. Micrographs were recorded digitally.

Fourier transform infrared spectroscopy-attenuated total reflectance (FTIR-ATR)

Samples of the rubber glove pieces overgrown by the bacterial consortium ST608 were taken periodically over 6 days of degradation, the bacterial biofilm was removed from the surface by washing with sterile distilled water, then the glove pieces were dried and subjected to FTIR-ATR. Spectra were recorded by FTIR-ATR technique as described by Linos et al. (2000).

Mineralization

The consortium ST608 cultivated with rubber gloves as the sole carbon and energy were used for investigation of the release of CO_2 during the degradation of the rubber hydrocarbon chains by the bacteria. The determination of CO_2 was performed in a closed degradation system that precipitated the evolved CO_2 with $Ba(OH)_2$ to $BaCO_3$ as described in a previous report by Warneke et al. (2007). Determination was carried out in 300 mL of MSM supplemented with pieces of rubber, inoculated with a 10% inoculum and was then incubated for 4 weeks. The yield of CO_2 and the percentage of mineralization were obtained by titration with 0.1 M HCL according to the equation as described by Warneke et al. (2007).

DNA extraction

The bacterial consortium collected periodically was centrifuged from the liquid culture for DNA extraction. Total DNA of bacteria attached to the surface of pieces of rubber was directly extracted from surface of rubber pieces. Total DNA was extracted using the Power soil extraction kit (MoBio Laboratory, Inc). The purity and quantity of the extracted genomic DNA were determined by gel electrophoresis. The genomic DNA was stored at -20ºC for further experiments.

Primers were designed based on the 16s rRNA gene and then PCR was performed for producing 16s rRNA gene amplifications followed by identification using denaturing gradient gel electrophoresis (DGGE) analysis (Heuer et al., 1997). The sequences of the primers used are presented in Table 2. All primers were synthesized by Pacific Science CO., Ltd. The PCR conditions were optimized based on conventional conditions for 16s rRNA gene analysis over the annealing temperature range of 50-60°C; the temperature presented a sharp PCR product band of target size was selected. The PCR amplification was carried out using 30 cycles including an initial denaturation at 95ºC for 5 min, annealing for 30 sec, extension at 72ºC for 2 min with a final extension of 10 min. An aliquot of 5 µL of the PCR product was verified using electrophoresis on a 1.5% agarose gel at 100 V for 40 minutes. PCR was performed in a Bio-RAD T100^{TM} Thermal cycler.

DGGE was performed at a constant temperature of 60ºC in the running buffer of 0.5X TAE buffer at a constant voltage of 55V overnight (16 h). The acrylamide concentration was 8% with a denaturing gradient of 30 to 70% of denaturant (100% denaturant corresponded to 7 M urea plus 40% [vol/vol] of deionized formamide), then the gel was stained with ethidium bromide (0.5 mg/L) for 10 minutes and de-stained in deionized water.

Identification of bacterial species by sequencing of the DGGE bands

Each piece of gel that showed a strong DGGE band from the total community DNA was removed using a sterile scalpel and transferred into an Eppendorf tube containing 30 µL of sterile deionized water. The DNA of each band was left in the water at 4ºC overnight. After centrifugation at 10,000 rpm for 5 min, 10 µL of the solution was used as a DNA template for a PCR reaction using the same conditions as described above to unlock the primer GC-clamp. The amplified 16S rDNA was extracted from the agarose gel. Amplified PCR products were then sequenced (Muyzer et al., 1993).

Results and discussions

Enrichment and culture conditions

Several samples were collected from rubber contaminated sites; a rubber plantation, waste pond water from a rubber processing factory and sediment from rubber waste in Songkhla (Thailand) as shown in Fig. 2. Each of the samples was used as an inoculum to enrich effective rubber degrading bacterial groups over the 30 days of incubation. The experiment was performed in Mineral salts medium (MSM) supplemented with 1x1 cm of vulcanized rubber glove pieces as a sole carbon and energy source. The first enrichment showed a cloudy liquid culture with bubbles of gas on the top of the medium in shake flask after 2 weeks. This may due to the natural process of degradation in an aerobic liquid culture condition, the amount of dissolved gas excess from the respiratory process of degradation accumulated continuously during a long period that limited its water solubility, then the bubbles were formed in the liquid culture. This phenomenol confirmed the existence of living organisms as shown in Fig 3A (Smirnov and Berry, 2015). Then, two weeks later the pieces of rubber were transferred to fresh medium and were then incubated for 4 weeks. After that, it revealed a strong evidence of rubber deterioration of consortia, substantial disintegration of the new rubber pieces became visible. This indicated that active rubber degrading microorganisms were presented. Finally, one consortium designed as a consortium ST608 having a high rubber degrading activity in liquid culture and presenting a substantial disintegration of the rubber pieces within 4 weeks was obtained (Fig. 3C).

Even though the biodegradability of various rubbers and rubber products can be degraded by microorganisms, most experiments are performed with individual isolates that have long time intervals, thus it seems impossible to obtain complete biodegradation.

The preliminary investigations of biodegradation of rubber glove pieces by bacterial consortium revealed a potential alternative approach to achieve complete biodegradation of vulcanized rubber. Different microorganism involving in

a consortium, used difference strategies for an effective availability of hydrophobic substrate and accumulated metabolized products continuously during degradation process.

Fig. 2 Samples collection. Soil and rubber wastewater from various ecosystems were collected in Songkhla province, Thailand.

A number of different bacteria acting synergistically could play significant roles on the rubber degradation process for rubber-waste management. It would be important and desirable to find an effective combination of microorganisms with different mechanisms and substrate requirements that could be highly effective in the biodegradation of these unique polymers. Although there have been many studies focus on the isolation of bacteria that can attack rubber, there are insufficient reports on the biodegradation of rubber by microbial consortium. Previous effort identified individual bacterial strain well known as *Gordonia polyisoprenivorans* VH2. This strain is the most effective rubber degrader model, isolated from an automobile tyre (Linos et al., 1999) but takes an additional month to degrade rubber product. Although the use of single isolate to degrade rubber are more stable and easily to understand the process, it shows less powerful degradation than mixed culture.

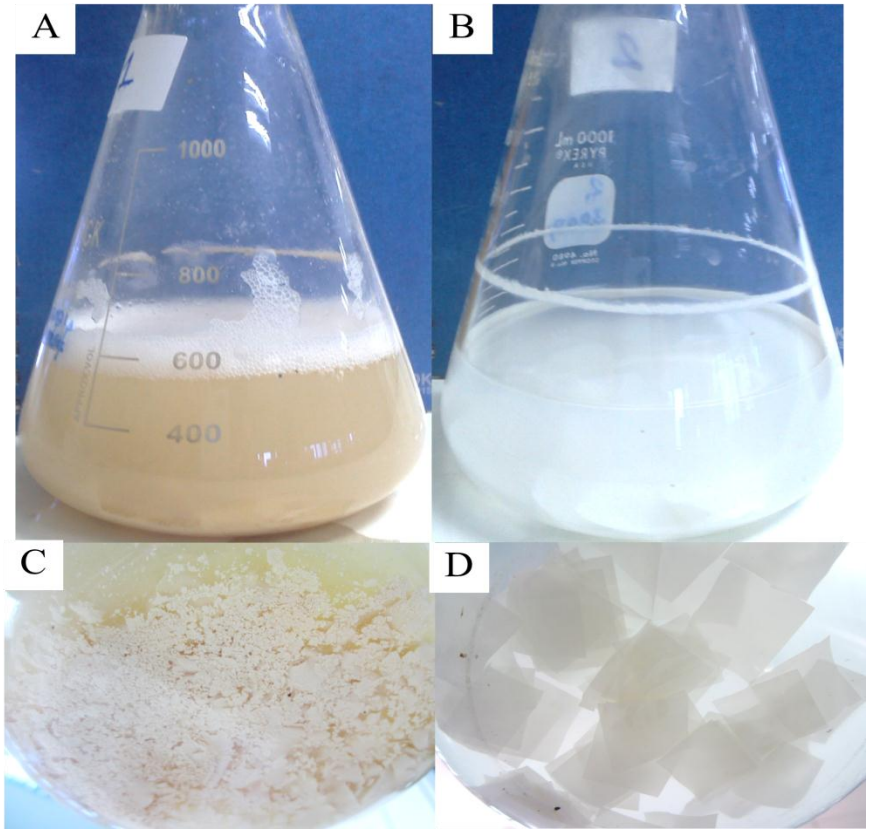

Fig. 3 The biodegradation of rubber gloves. Rubber glove pieces were incubated with the bacterial consortium for 2 weeks. Dissolved gas excess in the treated flask (A), was measured and compared with the uninoculated control (B). After 4 weeks of incubation, a substantial disintegration of rubber pieces was visible (C), whereas an untreated rubber did not significantly change (D).

Microbial strategies and surface analysis by scanning electron microscopy (SEM)

Surface erosion and colonization behavior of the rubber degrading bacterial consortium ST608 was investigated from the enrichment culture, the physical characteristics for initial observations were analysed by light microscopy and detail adhesion analysis by SEM as shown in Fig 5. The characteristics of the rubber glove pieces were monitored as shown in Fig. 6. After 18 days of the incubation period, the rubber glove pieces began to deteriorate and gradually showed almost

complete disintegration after 30 days. However, in the early stage of the experiment (2 weeks of incubation) only slight degradation was observed. During these primary stages of bacterial colonization on the surface, physical changes could be seen by naked eyes, but were not obvious. These findings were supported by staining rubber gloves with Commassie blue and Schiff's reagents (Fig. 4). Presumably during this time, the enzymes were being excreted to break the C=CH bonds of rubber and oxygen was introduced to enable the formation of the aldehyde and ketone groups at the end of isoprene chains produced the purple color as detected with Schiff's reagent (Birke et al., 2013; Birke and Jendrossek, 2014). During the early stages of degradation, there was no damage on rubber pieces or no detection of ketone groups, but the slow rate of degradation gradually accelerated over time presumably because of the growth of the attacking bacterial consortium.

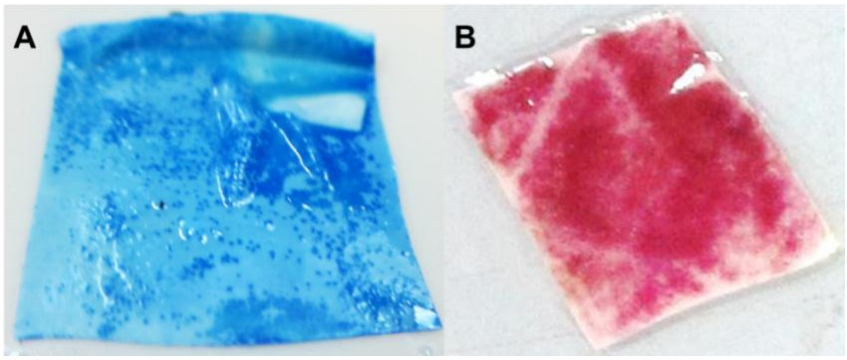

Fig. 4 Colonization of bacterial cells on NR latex gloves after being incubated with comsortium ST608 for 2 weeks. Numerous bacterial cells adhered to the rubber surface were observed by staining with commassie blue (A), and formation of aldehyde and ketone group during the degradation process was detected with Schiff's reagent (B).

SEM illustrates the behavior of microbial attachment on rubber surfaces. A section from the surface of a non-inoculated control is shown in Fig. 5A and 5B. Obviously, the control rubber materials did not disintegrate. The original surface of the rubber pieces was rough, so the bacteria could easily become attached. The section of rubber treated with consortium showed a direct contact of rubber degrading microorganisms as illustrated in Fig 5C and 5D. With regard to

colonization behavior of the bacteria after 4 weeks, cells were directly embedded and tended to produce mycelia corridors on the surface. They also penetrated into the material and had completely colonized the pieces with a condensed mycelia mass, as indicated on micrographs. In addition, after 4 weeks of incubation, the detailed analysis of bacteria at higher magnification was performed. An enormous number of microorganisms were huddled in eroded material holes with mycelia leading to a considerable disintegration of the rubber pieces. The various bacterial shapes of consortium ST608 presented in the biodegradation process are observed in Fig. 5E and 5F.

Microbial adhesion is a desirable and necessary step in the process of microbial biodegradation of polluting polymers. The hydrophobic interactions between microorganisms and materials are the important factors in the early stages of microbial adhesion (Klotz, 1990). The deterioration of rubber materials is triggered by adhering microorganisms that colonize on the rubber surface by bacterial biofilm, and consisting of various species of bacteria in consortium ST608.

The structure and function of materials can be damaged by bacteria in several ways, including: 1) coating the material surface 2) increasing the leaching of monomer out of the polymer matrix 3) attacking the polymer with enzymes and additives causing the loss of mechanical stability 4) accumulating of water and penetration of the polymer matrix with microbial filaments, causing swelling and increased conductivity 5) excretion of microbial pigments leading to colors in polymer (Flemming, 1998).

Such strong evidence concurs with a previous report by Linos et al. (2000). They investigated the surface of NR rubber and synthetic *cis*-1,4-isoprene after treating with Gram negative *Pseudomonas aeruginosa* which was identified as a potent rubber degrader by SEM. They also presented evidence of adhesive growth by colonization on a rubber surface, merging into materials, forming a biofilm, and accumulating aldehyde during colonization detected by Schiff's reagent. In a similar manner to other rubber degrading strains of the direct contact, non-clearing zone forming rubber degrading group grew adhesively on the surface of rubber and the most potent rubber degrading strains belong to the genera Gordonia, Actinobacterium and Nocardia (Yikmis and Steinbüchel, 2012).

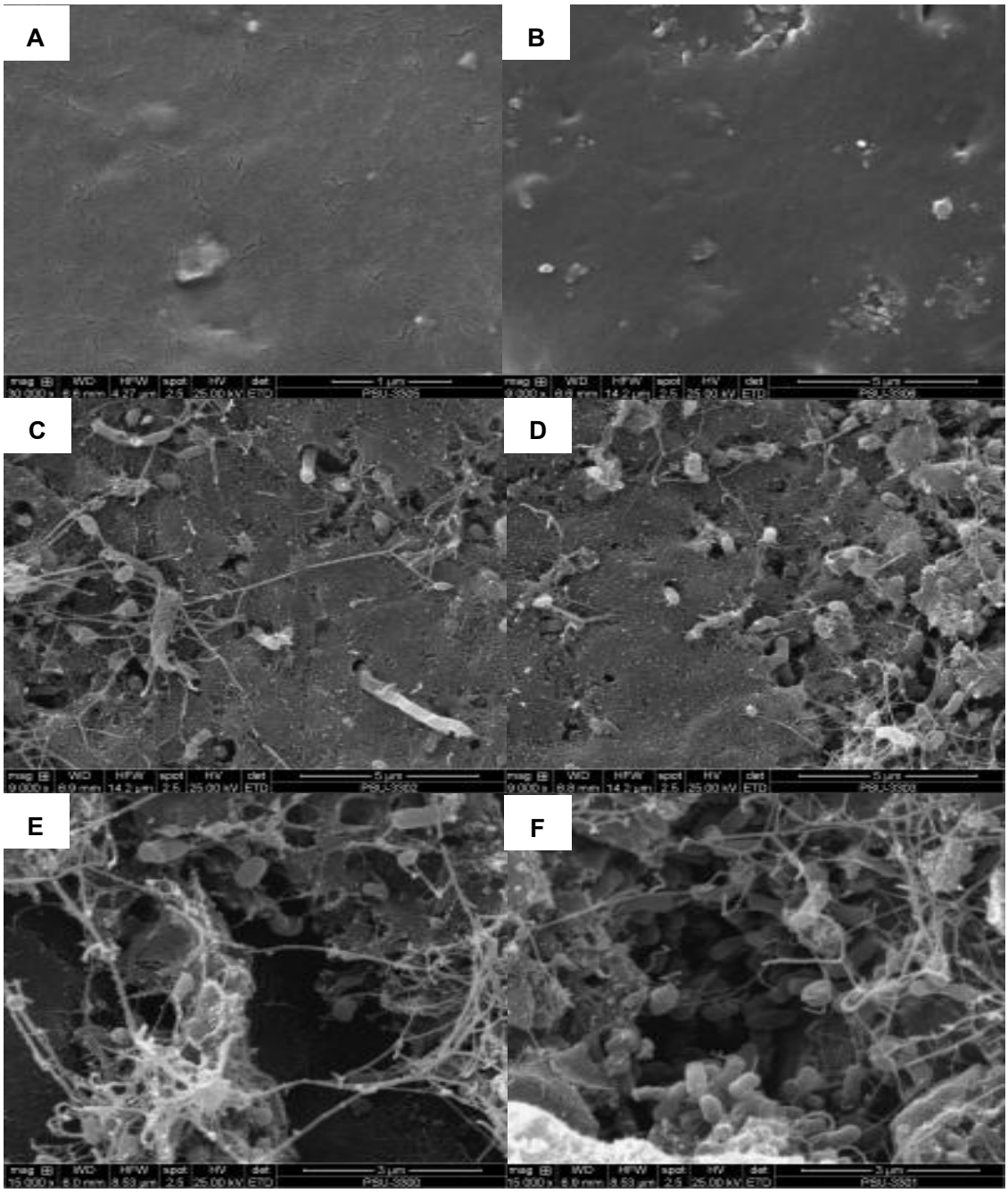

Fig. 5 SEM micrograph.The electron micrograph shows the growth of bacterial consortium ST608 on NR latex glove at different magnifications. (A, B): Non-inoculated control showing rubber surface. (C, D): Colonization of the rubber surface after 2 weeks, (E, F): Details of colonization of a rubber surface after 4 weeks. Bars corresponding to 1 µm (A), 3 µm (F, G) and 5 µm (B, D, E).

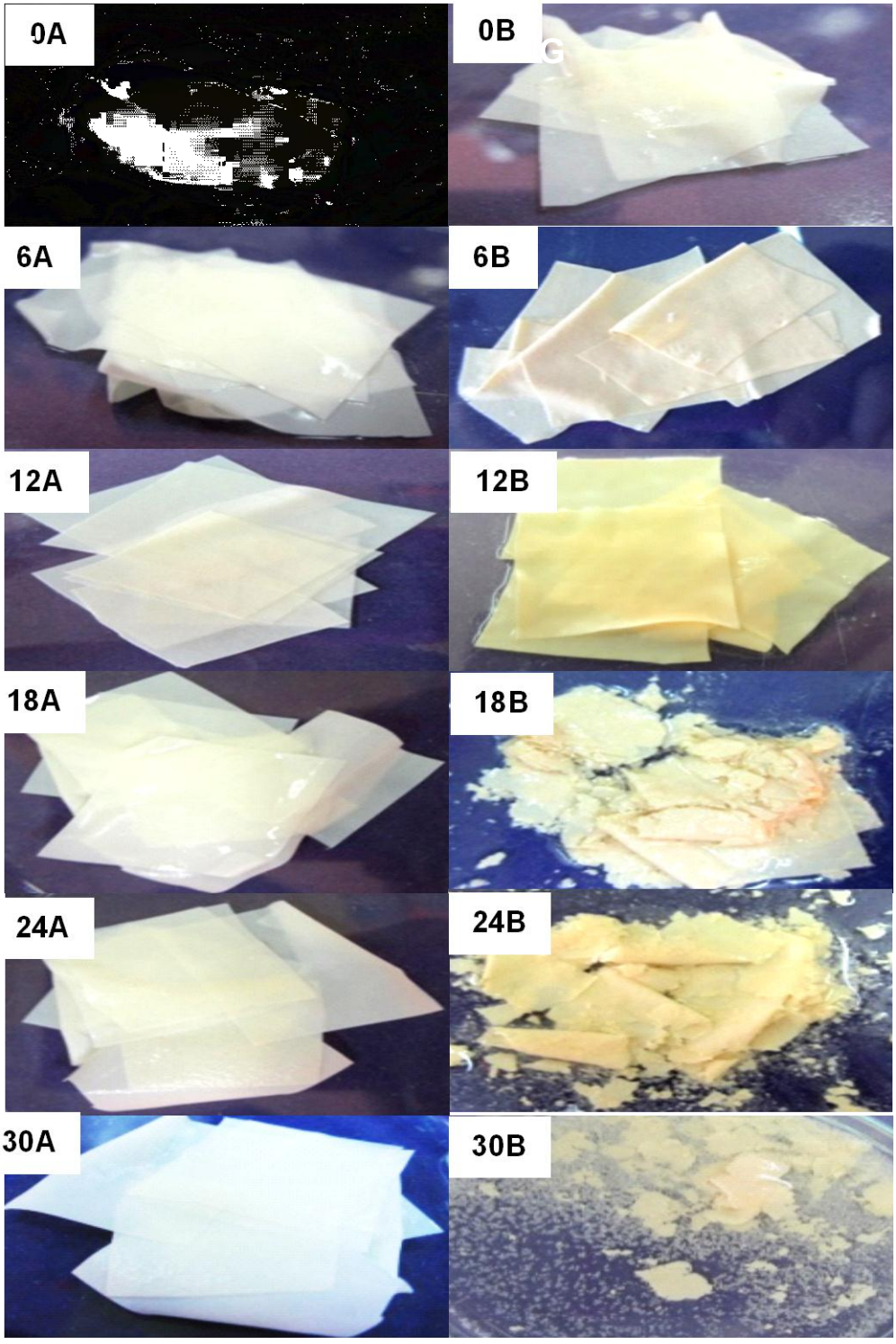

Fig. 6 The deterioration of rubber pieces. The rubber glove pieces were treated with microbial consortium ST608 for 0-30 days (0A-30A) compared with untreated rubber glove pieces (0B-30B).

Investigation of the rubber degradation process

An investigation of the rubber degradation by the rubber degrading bacterial consortium from the enrichment process, included measurements of many parameters, the bacterial population growth, percentage weight loss, mineralization and FTIR-ATR spectroscopy analysis were detected.

The microbial population growth

In this experiment, it was impossible to apply the direct cell density method, because of the undissloved carbon source (vulcanized rubber), and sessile bacteria. Thus, an indirect determination of microbial growth was performed by total protein measurement using the Bradford assay. According to the spectroscopy analytical technique used to measure concentration of protein in solution both live and dead cell, Breadford is one of the high sensitivity, rapid and accurate techniques to estimate protein binded with the reagent and proteins with increased absorbancy at 595 nm under acidic condition (Kruger, 1996).

The sequences of total protein measurement at gradual periods of 0 to 30 days represented the growth of bacterial consortium attached on a rubber surface, and free bacteria in a liquid culture. A rubber glove was used as a representative of vulcanized rubber material, and acted as the sole carbon source (Fig. 7). A different pattern of bacterial growth is illustrated in Fig. 7. The total protein concentration (absorbance A_{595}) monitored as the growth of the bacterial consortium on a rubber surface did not present log phase. The protein concentrations of growth abruptly increased and reached the maximal value of 66.7 µg per piece of rubber in 6 days of incubation, which is the beginning stage of adhesion strategies to attach the material substrate in the process of microbial degradation (Klotz, 1990). After that, bacterial growth became a fairly stable colonization (6-24 days). It is possible that the available spaces on the surface were limited due to complete colonization of sessile bacteria. However, protein concentration reached a maximal value of 90.1 µg per piece of rubber at the end of the experiment in 30 days.

The concentration of protein in the liquid culture showed a small decrease from the beginning of 25.2 to 21.3 µg/mL while colonization occurred in 6 days. Then, the protein contentration slowly increased to 38.7 µg/mL. Some growing bacteria began to release from the surface, some metabolites encouraging the growth of other bacteria. After 12 days of growth, there was a rapid increased and reached the maximum value of 171.5 µg/mL in the liquid culture, and remained stable through the experiment for 30 days as shown in Fig. 7.

The microbial consortium had a growth pattern of strong adhesion of bacteria to rubber surface and this help facilitating efficient use of the available carbon compounds from rubber substrates, according to the Pan and co-worker report (2009).

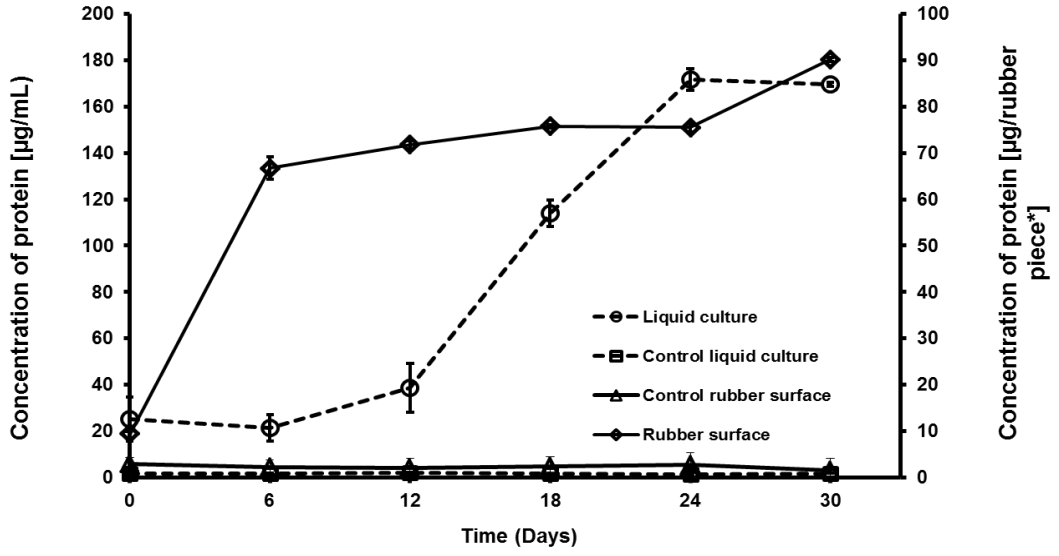

Fig. 7 Bacterial population growth**.** The growth of bacterial consortium was measured using the protein contents by Bradford assay. A different pattern of bacterial growth on the rubber pieces surface (diamonds) to that of a liquid culture (circles) compared with control rubber pieces surface (triangle) and liquid culture without bacteria (squares). Assays were performed with two technical replicates. Error bars indicate standard deviation.

Mineralization and weight loss

The evidence for rapid biodegradation of the hydrocarbon chains of the rubber gloves (polyisoprene) to CO_2 was obtained by measuring the respiratory CO_2 released during the cultivation of bacterial cells in the presence of rubber as the sole carbon source. Determination of the respiratory CO_2 was set up in air-tight closed system using $Ba(OH)_2$ to precipitate the released CO_2 from bacterial respiratory into $BaCO_3$ (white pellet form) as shown by the demonstrated system in a schematic Fig. 8. The experimental culture medium flask containing MSM supplemented with rubber glove pieces as substrates. Precipitate test tubes containing $Ba(OH)_2$ solution, and at each measuring point were replaced with new tubes of fresh $Ba(OH)_2$. Titration of the remaining $Ba(OH)_2$ was carried out using 0.1M HCL to calculate the CO_3^{2-} as $BaCO_3$ form using the equation in appendix C1 to obtain the percentage amount of CO_2 released. Phenolphthalein (20 mL of a 1%, w/v, solution in 2-propanol) was used as an indicator for titration, and at the end point of titration was determined by alteration of the colour from magenta to colorless. A non-inoculated Erlenmeyer flask was handled as same as the control.

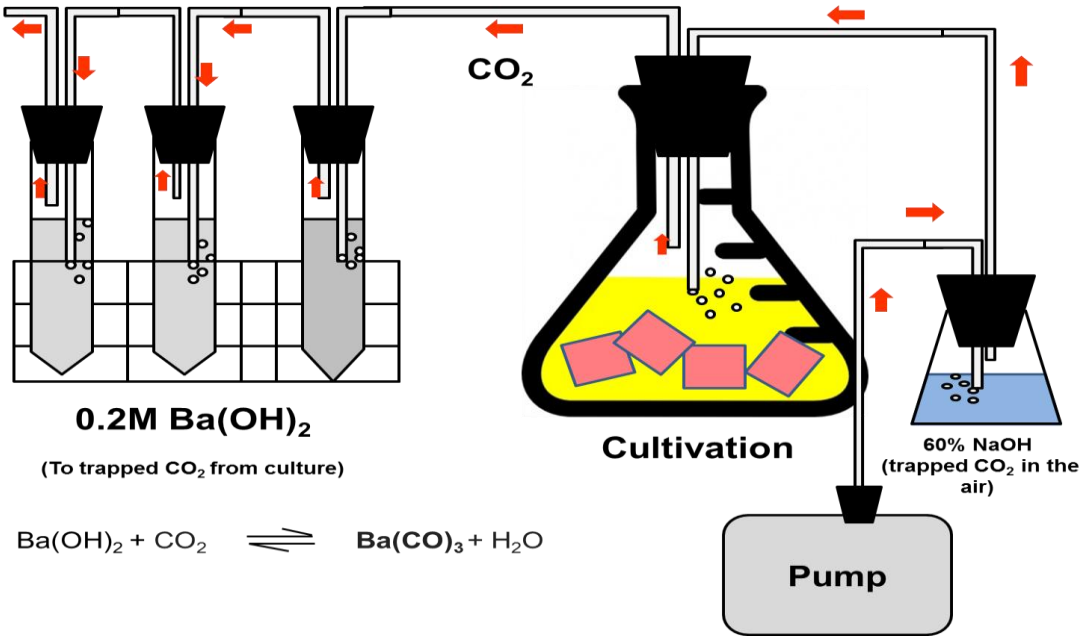

Fig. 8 The schematic of mineralization in the air-tight closed system.

The initial attack on the rubber gloves was most likely due to oxidative enzymes attacking the C=CH bonds to produce metabolites that could then be completely oxidised to CO_2.

During the 30 days of cultivation, values of 57% of the original carbon were converted to CO_2 by the bacterial consortium (Fig 9). In a similar manner, the growth of bacteria (total protein measurement) also increased in the same periods as determined by the Bradford assay (Fig 7). Hence, the original carbon content of the rubber gloves was converted to CO_2 and bacterial biomass. In addition, the percentage weight loss of rubber substrates after being treated with bacterial consortium for 30 days was about 50% compared with the control without bacteria. According to these results, it was assumed that the rubber substrates containing polyisoprene carbon as a nutrient were used as, 1) a source of carbon and energy for bacterial growth, 2) the increase in protein biomass 3) the release of CO_2. Obviously, the mineralization value of rubber material was higher than percentage weight loss of rubber. It is possible that the surface of the substrate material is accessible to microbial attack and complete cover by bacterial cells, thus the weight of the remaining rubber did include bacterial cell mass as determined in the bacterial population growth experiment, as was the case with solid biodegradable materials (Fig. 7).

This result concur with the present studies on the mineralization of natural rubber by co-culture between, *Bacillus cohnii* strain NBRC 15565 and *Brevundimonas naejangsanensis* strain BIO-TAS2-2, which were able to mineralize natural rubber latex at 50% and 10% concentrations of rubber latex to CO_2 released within 5 days of incubation (Muralidharan, 2016). However, in case of the individual isolate, *Nocardia takedensis* DSM 44801 could metabolize rubber carbon to CO_2 value of about 54% over 80 days or obtain double time as compared with consortia (Warneke, 2007). In the case of vulcanized natural rubber, it is very resistant to high temperatures and persists in the environment for very long time, the co-culture or consortium can possibly increase the degradation rate and precipitate more rapidly disintegrate than an individual isolate.

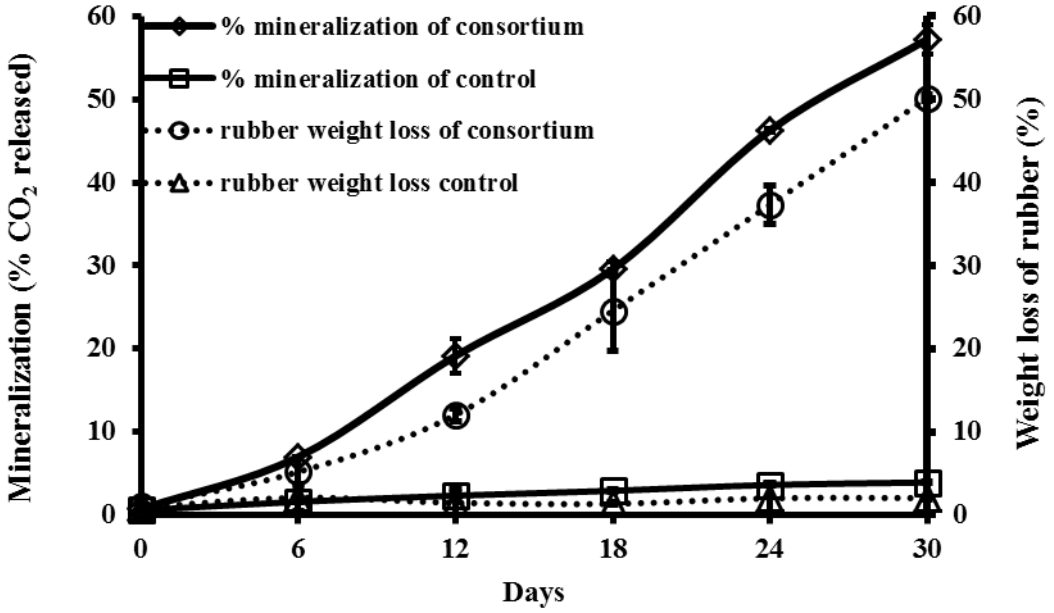

Fig. 9 CO_2 release and rubber weight loss during rubber degradation process

FTIR-ATR spectroscopy

The FTIR-ATR spectroscopy was applied to detect the changes in the functional groups of the polymer, and also detected the formation of a biofilm on the surface of the rubber gloves. The FTIR spectra of the NR latex gloves recorded in the transmission and ATR mode corresponded to the *cis*-1,4 double bonds in the polyisoprene chains in the region of 700-900 cm^{-1}. A relative decrease of the (=CH_2) band at 835 cm^{1} was observed for the rubber pieces after treating with bacterial consortium at interval times of 0, 6, 12, 18, 24 and 30 days, compared with the control without bacteria, and a polysaccharide (biofilm formation) band in the region of 900 to 1200 cm^{-1} referred to the most prominent bands (Linos et al., 2000) as shown in Fig 10A**.** The bands in the carbonyl groups region at 1650 to 1796 cm^{-1} slightly increased after being treated with bacteria, and the CH region from 2700 to 3000 cm^{-1} decreased (Fig 10B) in a similar manner with (=CH_2) *cis*-1,4 double bond in the polyisoprene chain. The spectra in Fig. 10A and Fig. 10B are presented as second derivatives of the FTIR spectra for a better interpretation and clarity of the results.

After microbial exposure, FTIR analysis of the rubber gloves indicated some reduction of the molecular sizes and the formation of new peaks with the breakdown of some bonds. The characterization confirmed that the structure of the rubber was changed after treatment with the bacteria and led to the deterioration of the polymer. The C=O stretching at 1796 cm^{-1}, showed that some of the unsaturated bonds (C=C) in the NR were possibly converted to C=O. A peak of C=O began to decline after being incubated with bacteria for 24 days (Fig 10). It is most likely that the bacteria metabolized the produced secondary metabolites during the degradation (Yikmis et al., 2008).

Most bacterial isolates that can attack rubber are Actinomyces, and they all seem to attack by oxidizing the C=CH bonds by introducing oxygen to form aldehyde and ketone groups, which are then presumably converted to carboxyl groups, to be further degraded by reactions like those involved in the metabolism of fatty acids. The initial attack seems to be performed by a common oxygenase (Linos et al., 2000) and some researchers have even identified potential rubber degraders by checking for the presence of this enzyme using molecular probes (Birke and Jendrossek, 2014). Most attempts to identify possible intermediates to clarify the degradation pathway have been successful (Bode et al., 2001). One possibility for the slowing down of the rate of degradation after the first attack is that the first intermediates could be toxic to the cells. Perhaps if a consortium of organisms was available to these first products, (that may be very hydrophobic) that prove to be metabolites for different bacteria, so an inhibition would not occur and the degradation could continue. Hence, we decided to test this possibility by establish a consortium.

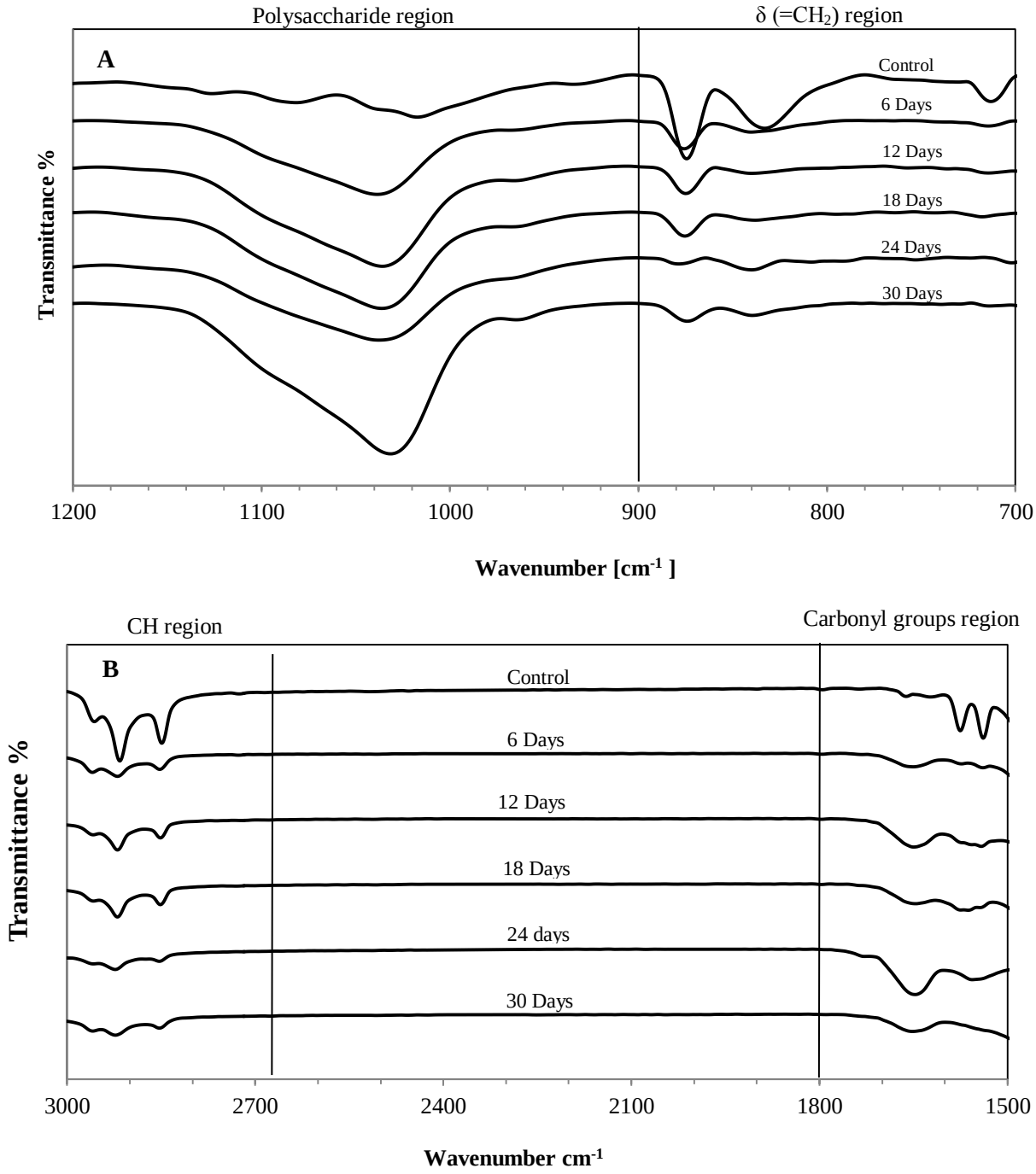

Fig. 10 FTIR spectra. The FTIR spectrum of rubber gloves in the region of 700 to 900 cm^{-1} comprising the ($=CH_2$) *cis*-1,4 double bond and polysaccharide band region from 900 to 1200 cm^{-1} (A). Second-derivative spectra of NR latex gloves in the region of 1500 to 3000 cm^{-1} comprising the carbonyl group region (-C=O) and CH region (B). A comparison of the non-inoculated (negative control) and the samples treated with the bacterial consortia.

The structure of rubber degrading bacterial community and bacterial dynamic changed over monitoring period

A consortium consisting of bacterial species with different physiologies show a higher rate of degradation than its individual isolate. This consortium is considered to have several advantages with regard to bioremediation, such as greater stability and increased metabolic capability (Ghazali et al., 2004; Bacosa et al., 2012). These characteristics enable the consortium to overcome limitations for the complete biodegradation of toxic compound or additives. The degradation efficiency of a microbial community depends on the stability of the constituent members as well as their ability to degrade or mineralize the target compound (Ambujoms et al., 2000). Nevertheless, in most of the biodegradation of rubber studies, uncharacterized biological microbial consortia have been used instead of characterized consortia. It is important from the perspective of further applications to identify bacteria in the consortium which are responsible for degradation and lead to optimization of degradation processes as well as development of monitoring tools. Now a few reports are available that show the microbial consortia are capable of degrading organic compounds (Zwolinski et al., 2000; Kanaly et al., 2000; Ambujan, 2001; Dejonghe et al., 2003; Ghazali et al., 2004; Ha et al., 2007; Lou et al., 2009; Bacosa et al., 2012). To overcome the severe limitations of culture-dependent methods in discovering bacterial diversity, molecular biological techniques have become increasingly popular. One of these molecular tools is the PCR amplification of variable regions of the genes encoding 16S rRNA (16S rDNA) by use of primers homologous to the conserved region. Subsequent electrophoretic separation of the PCR products in a polyacrylamide matrix over a denaturing gradient is a technique recently introduced in microbial ecology. The denaturing gradient can be achieved either chemically with urea and formamide in denaturing gradient gelelectrophoresis (DGGE). This technique is giving comparable fingerprints of microbial communities. The banding pattern represents the major constituents of the analyzed community, if present as minor constituents groups, may not be detected by molecular analysis. Thus, relatively less abundant but very important species may not be visible. The structure of a rubber degrading bacterial community, and bacterial dynamic changes

in degradation process were investigated using DGGE-PCR technique. It also used a specific PCR to amplify genes of actinomycetes that belong to rubber degrading bacteria to obtain fragments used in gradient gel analysis.

DNA extraction, primers design and 16s rRNA amplification of a rubber degrading bacterial community

The consortium was grown in shake flasks with MSM supplemented with pieces of rubber glove. Bacterial DNA was extracted from both sources of liquid culture and rubber gloves surfaces of each interval time sampling using the Power Soil Extraction Kit. The DNA concentrations of the extracts were measured by spectroscopy and were adjusted for PCR 16s rRNA gene amplification. Each band obtained at a different samplings time points of DNA showed a different amount of DNA yield that correlated to the result of the protein assay of bacteria. It might depend on the growth rate of bacteria during each period of time of the degradation process and a similar result was obtained for the total DNA isolated from the culture medium. However, both DNA sources gave an intensive band as shown in Fig 11. Total DNA from each band was then used as a template for 16s rRNA gene amplification (DNA concentration was adjusted using a spectrophotometer to the same amount of about 20 µg/µL). Analysis of the bacterial community was carried out during the rubber degradation using a combination of PCR with DGGE to identify individual bacteria in the community using primers for DGGE (Table 2). These primers enabled the detection of 16s rRNA gene of bacteria on the rubber surface (Fig. 12). Three primer pairs with GC clamp were designed based on 16s rRNA gene of the Actinomycetes groups (Heuer, 1997) and *E. coli* at a difference region of target gene, head (F243GC-R518), middle (F338GC-R518) and tail (F918GC-R1378). All PCR products were estimated by gel electrophoresis as illustrated in Fig 12 (A-C). A GC-rich sequence is attached to one primer of a pair to prevent complete melting during separation in denaturing gradient (Myers et al., 1985).

The Actinomycetes specific primer F243 in combination with reverse primers R513GC produced a DNA fragment at appropriate size of 310 bp with GC

clamp (Fig. 12A). Obviously, all of PCR products of the appropriate size were presented with all samples of bacteria on a rubber surface on each period (0-30 days), but absent with any of the control strain *E. coli* from the other bacterial taxa. It indicated that the primers F243 and R513 were specific for the Actinomycetes group that related to rubber degrading bacteria. Nevertheless, after separation of the PCR products in a polyacrylamide matrix over a denaturing gradient that was achieved with urea and formamide in gel electrophoresis (DGGE) based on GC-content, PCR products cannot be separated to a single band. It is possible that only a few bacteria were amplified and gave a high amount of products in gradient gel. These primers were community group specific because of the primer designed based on the Actinomycetes group.

Moreover, the second pair of primers were designed based on two conserved regions corresponding to *E. coli* gene nucleotide position 320-355 (primer 338F) and 505-535 (primer 518R), which have been extensively used for generating PCR products for DGGE analysis. This primer generate the PCR product size approximately 240 bp with GC clamp fragment for the most bacterial taxa as shown in Fig 12B. This primers match with the 16s rRNA gene of both Actinomycetes and *E. coli* that are represented by other genera as well as the DGGE profile obtained. This is shown in Fig. 12D. The bands seem to be completely separated on the acrylamide matrix. Each bands represent bacterial strain in the community.

In a similar result of generating the PCR products using primer F918GC in combination with primer R1378, it generated the band of 16s rRNA gene of all DNA samples from bacterial consortium, *Gordonia polyisoprenivorans* (represent as an Actinomycetes), *E. coli* (represent as another genera) as expected the target size approximately 500 bp with GC clamp was obtained by gel electrophoresis as shown in Fig 12C. Unfortunately, the separation of these PCR products using the DGGE technique was not successful. It indicated that the primers F984GC combined with R518 might not be suitable to differentiate these environmental DNA samples, because only a few bacteria were detected and gave a high yield of PCR amplification with only long length PCR product, while the DGGE profile showd a thick-band without separation of these fragments (Fig. 12D). However, the attempts to change the gradient used did not improve in a better separation. One possible reason is the

limitation of the PCR product length for DGGE analysis, with denaturing gradient that can not be separated efficiently. The PCR products for the DGGE technique should be less than 500 bp to enhance the efficient resolution and analysis (Myers et al., 1985). Since the primers F984GC and R518 used in this experiment yielded long length PCR products (500 bp length) so they cannot be separated with the gradient. Therefore, the product fragment derived from the PCR amplification with primer F338GC and R518 generated the appropriated product length that allows for efficiently analysis. Consequencely, the template DNA of all environmental samples tested were amplifiable in the PCR-DGGE technique with primer F338FGC and 518R.

These results concur with the recent study by Heuer et al. (1997). They successfully classified the actinomycete bacterial community using specific primer for amplification 16S rDNA sequences.

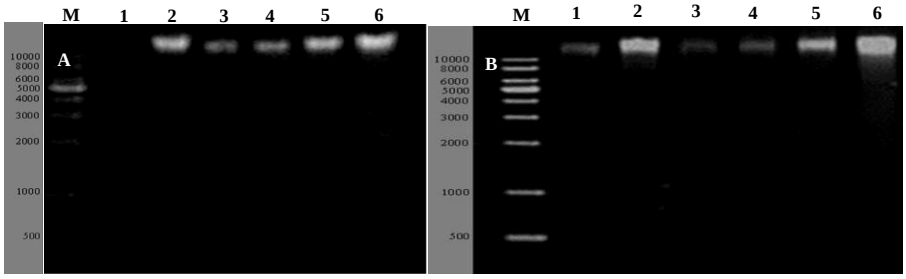

Fig. 11 gDNA of bacterial consortium 0 days (lane 1), 6 days (lane 2), 12 days (lane 3), 18 days (lane 4), 24 days (lane 5), 30 days (lane 6), (A) on rubber surface, and (B) in liquid culture

Table 2. Primers for 16s ribosomal RNA used in this study

Primers	Sequence '5-----3'	Ref.
27F	AGAGTTTGATCMTGGCTCAG	Heuer et al., 1997
1492R	TACGG(C/T)TACCTTGTTACGACTT	Heuer et al., 1997
F243	GGATGAGCCCGCGGCCTA	Heuer et al., 1997
R513GC	gc.-CGGCCGCGGCTGCTGGCACGTA	Huang et al., 2012
F338GC	gc.-ACTCCTACGGGAGGCAGCAG	Heuer et al., 1997
R518	ATTACCGCGGCTGCTGG	Heuer et al., 1997
F984GC	gc.-AACGCGAAGAACCTTAC	Myers et al., 1985
R1378	CGGTGTGTACAAGGCCCGGGAACG	Myers et al., 1985

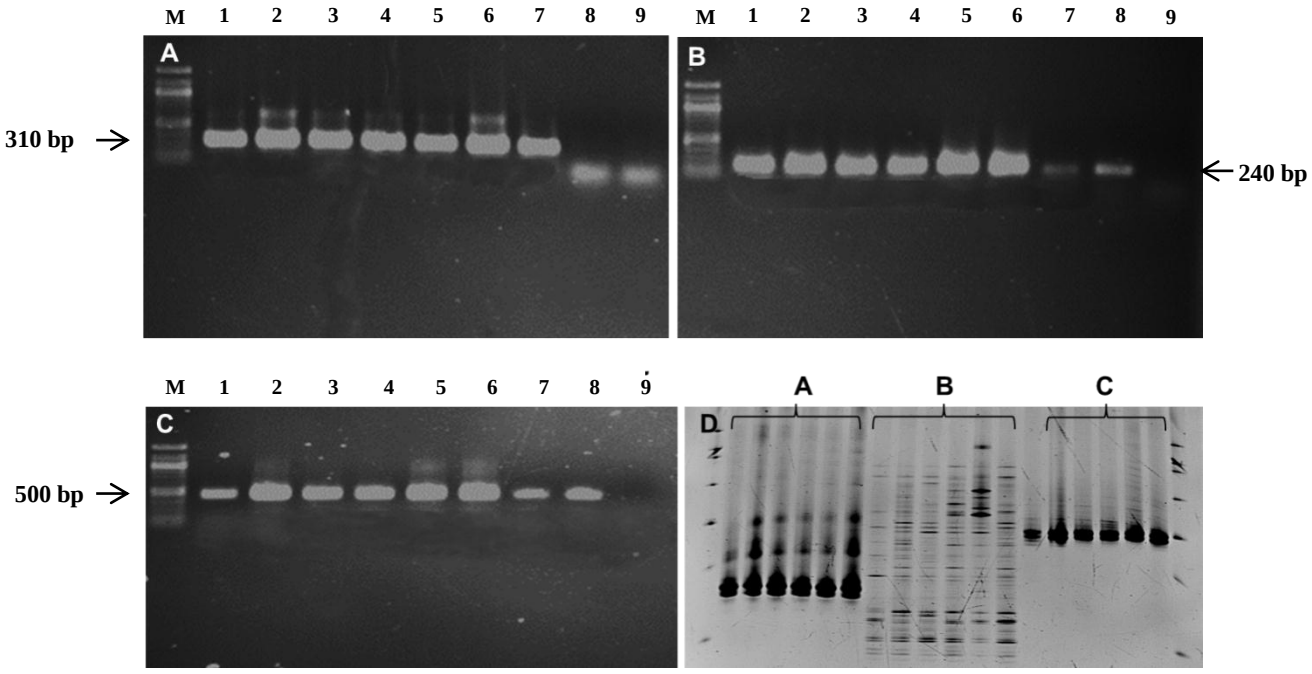

Fig. 12 The PCR products generated from different primer pairs of sample from 0 day (lane 1), 6 days (lane 2), 12 days (lane 3), 18 days (lane 4), 24 days (land 5), 30 days (lane 6), *Gordonia polyisoprenivorans* (lane 7), *E. coli* (lane 8) and negative control (lane 9), (A) F243GC/R513, (B) F338GC/R518, (C) F918GC/R1378, (D) DGGE profile of different three primers pairs.

Denaturing Gradient Gel Electrophoresis (DGGE) conditions and analysis

A primer pair of F338GC/R518 was used to obtain 16s rRNA gene fragment 338/518, which is suitable for gradient gel analysis as described above. PCR conditions for 16s rRNA gene amplification was performed followed by identification using denaturing gradient gel electrophoresis (DGGE) analysis. The PCR conditions were optimized based on conventional conditions for 16s rRNA gene analysis over the annealing temperature range of 50-60°C. The temperature that presented a sharp PCR product band of target size is 55°C. PCR amplification was carried out using 30 cycles, including an initial denaturation at 95ºC for 5 minutes annealing for 30 sec, extension at 72ºC for 2 min with a final extension of 10 min. An aliquot of the PCR product was verified using electrophoresis on a 1.5% agarose gel at 100 V for 40 min, and PCR was performed in a Bio-RAD T100TM Thermal cycler.

DGGE was performed at a constant temperature of 60ºC in the running buffer of 0.5X TAE buffer at a constant voltage of 55V overnight (16 h). The acrylamide concentration was 8% with optimization of a denaturing gradient gel 0 to 80% or 70 to 30%. The denaturing gradients of 30 to 70% of denaturant (100% denaturant corresponded to 7 M urea plus 40% [vol/vol] of deionized formamide) gave an appropriate result in the separation of PCR-DGGE product bands.

Analysis of the bacterial community during the rubber degradation by a combination of PCR with DGGE for identification of individual bacteria in the community using primers for DGGE (Table 2), detected the 16s rRNA gene fragments of approximately 240 bp with GC clamp. The DGGE profiles of the bacterial community with different incubation periods on the rubber surface and liquid medium are shown in Fig. 13. Although several bands were common between the two sources, the majority of the most intense bands from the bacterial population were derived more from the rubber surface (Fig. 13, column A-F) than from the liquid medium (Fig. 13 column G-L). These results indicate that the rubber degrading bacterial group used a specific adhesion strategy to colonize the surface of rubber pieces to cause rubber deterioration in a similar manner to the previous reports of Fleming (1998).

This strategy is an important approach for bacteria to effectively degrade polymeric materials (including rubber) by initially colonizing onto the polymer surface. The structure and function of the polymeric materials, or in this case rubber, can then be changed by enzymes that attack the biological origins of the polymer strands, and perhaps produce additives that lead to the loss of mechanical stability, or induce swelling of the fibers by penetrating the polymer matrix according to the SEM profile as mentioned above. However, both the direct and indirect strategy results in modification and degradation of the rubber pieces.

DGGE analysis of the microbial community structure

The pattern of microbial population changes is illustrated in Fig. 6. Significant changes in the bacterial population occurred on both the surface and in the liquid over the 30 days incubation period. At the beginning, before binding occurred,

no bands were observed on the rubber surface (Fig. 13 column A), while the liquid medium showed various bands from the initial inoculum (Fig. 13 column G). Some of these bands reappeared at day 6 attached to the rubber surface (Compare Fig. 13 column B and G). However after 6 days numerous different bands appeared from the rubber surface indicating that there had been considerable binding of different organisms but many did not survive until day 12 (Compare Fig. 13 column B and C). Over the same period there was little change in the liquid populations (Fig. 13 column G-I). At the beginning stage of the investigation (0-12 day in liquid medium), it was decided to try to identify just a few of the bands that were associated with bacteria that might play important roles in the degradation process. In particular, the most prominent population bands 4 represented the most dominant bacteria that were detected at all times from both solid and liquid so the organism associated with band 4 played a continuous role in the overall degradation process. From the rubber surface, bands 7 and 8 became absolutely dominant at day 24, so presumable these bacteria were responsible for the attack on the C=CH bonds and the release of nutrients, so the bacterial populations in the liquid also increased at 18, 24 and 30 days (Fig. 13 column J, K and L). Bands 7 and 8 from the rubber surface had disappeared at days 30 as the rubber pieces disintegrated and they never appeared in the liquid, so after removing the C=CH bonds they possibly died or could not compete with other bacteria for nutrients. In contrast bands 2, 3, 4 and 5 were obtained from both the solid and liquid sources throughout the experimental period. Band 1 that was prominent at day 0 of the incubation in liquid culture (Column G) reappeared at day 6 on the rubber surface then disappeared for prolonged periods in the liquid culture, however it reappeared after 6 days on the rubber surface (6 days, rubber surface) as a sessile bacterium, then faded from the process while band 3, 4 and 5 were prominent throughout the experiment (Fig. 13 column B-L). Band 2 (Fig. 13 column B-F) showed an intense band on the surface after 6 days to the end of experiment periods in contrast with its behavior in the liquid MSM medium that showed a thin band at the beginning then the intensity of band increased (Fig. 13 column F-L) from 0-30 days. It is possible that band 2 belonged to a sessile bacterium that used an adhesive strategy for attachment to the rubber and was then slowly released into the liquid culture.

For a preliminary identification of the microbes that appeared to be associated with the degradation, various bands were purified and their nucleotide base sequence determined and compared with the data base (Table 3). Band 4 was 98% similar to the 16s rRNA gene sequence of *Rhodococcus rhodochrous*. The 16s rRNA gene sequence for band 2 and 3 also obtained from both the material surface and liquid culture throughout the experiment were 99% identical to *Gordonia* terrae, and 98% to an uncultured bacterium and the band 5 was 100% similar to *Nocardia endophytica* strain KLBMP respectively. These predominating intensity bands were observed throughout all the incubation periods, and increased with the treatment time. In addition, the prominent band 1 was identified as being 98% similar to *Gordonia hongkongensis* HKU50 that intitially appeared only in the inoculum (Column G: 0 day), and the intensity of this band slightly appeared with the incubation period from liquid MSM culture medium, while band 6 was observed after 6 days of incubation period. This 16s rRNA gene sequence of band 6 showed a 98% similarity to the uncultured *Flavobacterium* sp. Most of the sequences examined belonged to Actinomycetes that are known to be part of the non-clearzone-forming rubber degrading bacterial group.

Overall, the present results show that the community of bacteria that adhered to the rubber surface, as seen from the DGGE profiles, were clearly involved in the dynamic changes, in both the bacterial populations and the rubber glove pieces from the beginning of the rubber degradation incubation process, and when they attacked the rubber surface and used it as a sole source of carbon and energy. During the middle steps of the incubation, some strains of the less dominating strains disappeared, so only the most strongly-rubber-degrading-bacteria or the dominant strains remained. However these dominant bacteria produced metabolites, most likely some long chain fatty acids or smaller molecules such as oligomers (8-10 unit) or other intermediate metabolites that were more readily used as carbon by other bacteria that were mainly seen in the liquid culture (Fig. 13).

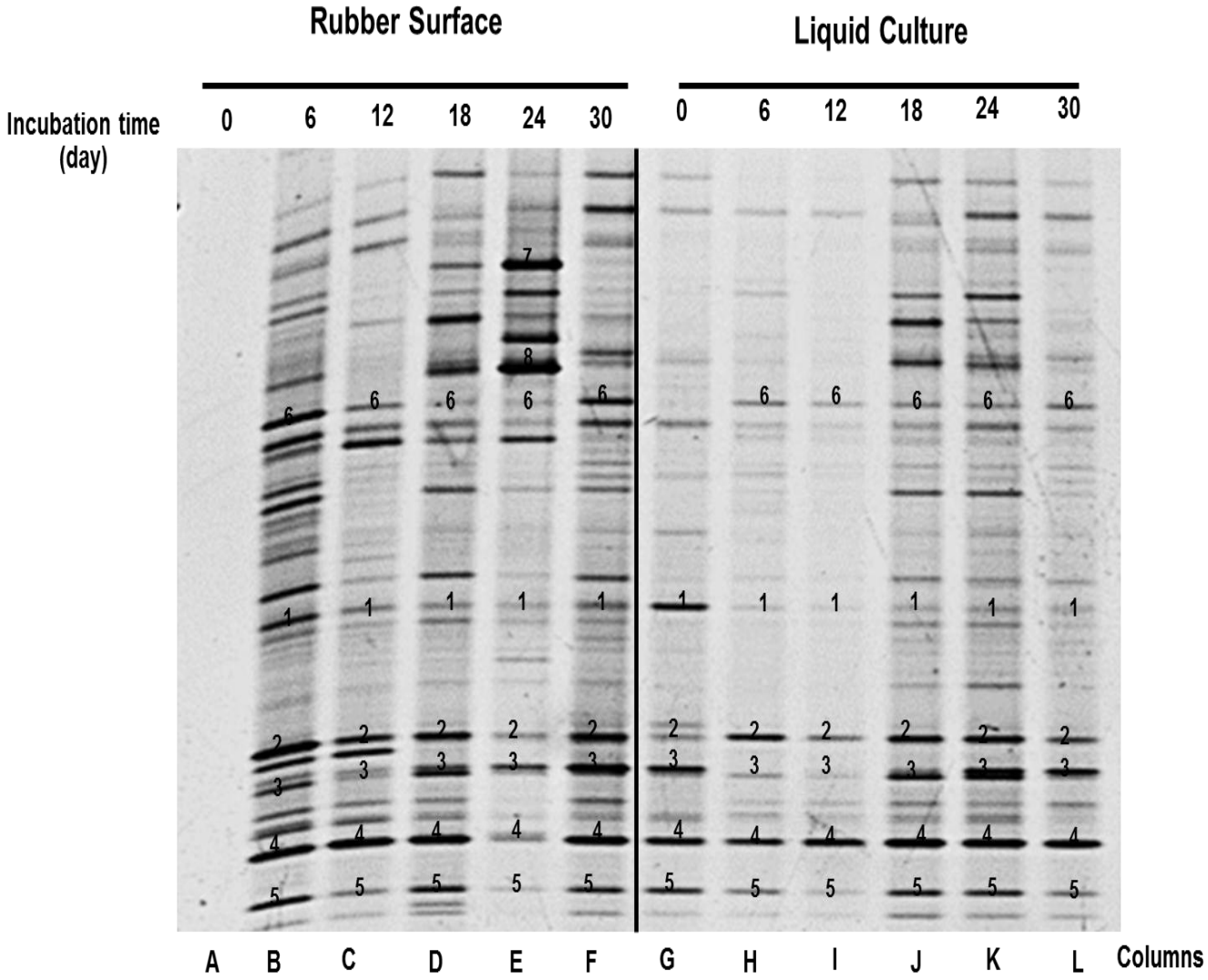

Fig. 13 DGGE profile. The pattern of the microbial community changes were identified by the DGGE technique during cultivation with rubber gloves pieces as a sole carbon source sampled at 0, 6, 12, 18, 24 and 30 days. The community of sessile bacteria on the rubber surface shown in first 6 columns and the community of bacteria in the liquid culture is shown in the second 6 columns. The numbers refer to the bands discussed in the text and their tentative identities in Table 3.

Table 3. Similarity of the representative 16s rRNA gene sequences from some of the DGGE bands from the rubber degrading consortium ST608

Band	Closet identify relative		% Identity
	Bacterial species	Accession No.	
1	*Gordonia* hongkongensis HKU50	KY584263	98
2	*Gordonia* terrae	KY584262	99
3	Uncultured bacterium	KY584261	98
4	*Rhodococcus rhodochrous*	KY584260	98
5	*Nocardia endophytica*	KY584259	100
6	Uncultured *Flavobacterium*	KY584258	98
7	*Rhodanobacter* sp. StRD750	KY584257	97
8	Uncultured Flavobacterium	JX270632.1	98

Conclusions

A bacterial consortium ST608 that was derived from a rubber waste factory was shown a substantial disintegration of vulcanised rubber gloves (Fig 3, 30B) that were used as a sole carbon and energy source in MSM medium over a period of 30 days. This clearly demonstrates for the first time that providing the right consortium, strongly disintegradation of vulcanized rubber can be achieved. As determined by 16s rRNA gene analysis at least 4 isolates and others played important roles in the degradation process with found throughout the experiment. The most dominant species are remained attached to the surface and growing in liquid culture. Others that were attached and also found growing in the liquid in addition to other different bacteria that were presumably feeding off the derived metabolites. Identification of the key bacterial species of this effective consortium ST608 and analysis of their metabolic behavior is now underway in our laboratory to obtain a clearer analysis of the processes involved.

CHAPTER 4

Identification of *lcp* gene and biochemical characterization of purified Latex clearing protein (Lcp) from *Rhodococcus rhodochrous* strain RPK1

Abstract

A new potential Latex clearing protein (Lcp_{Rr}) in Gram positive rubber degrading bacteria *Rhodococcus rhodochrous* RPK1 was identified in this study. DNA sequence analysis revealed one large open reading frame encoding for Lcp protein in *R. rhodochrous*. The determination of the activity of the purified Lcp_{Rr} using HPLC-based assay showed the optimum activity in phosphate buffer, higher than the other Lcp. UVvisible of the purified Lcp_{Rr} revealed a typical spectrum for heam which indicated that the Lcp_{Rr} is a heme-containing protein. Identification of protein type was performed by metal analysis, spectral analysis and MALDI-ToF analysis. The results demonstrated that Lcp_{Rr} is a *b*-type cytochrome which is accessible for external ligands and substrates, possessing a five-fold coordinated open state that is remarkable different from 6^{th} coordination site of heme in a closed state of Lcp_{K30}.

Keywords: Latex clearing protein (Lcp), Rubber oxygenase, Dioxygenase, Rhodococcus, Biodegradation

บทคัดย่อ

การศึกษาครั้งนี้ได้ทำการคัดแยกโปรตีน Latex clearing protein (Lcp_{Rr}) ชนิดใหม่จากแบคทีเรียย่อยสลายยางแกรมบวก *Rhodococcus rhodochrous* สายพันธุ์ RPK1 จากการวิเคราะห์ลำดับดีเอ็นเอแสดงให้เห็น Open reading frame ขนาดใหญ่สำหรับแปลรหัสโปรตีน Lcp ในเชื้อ *Rhodococcus rhodochrous* สายพันธุ์ RPK1 เมื่อตรวจวัดกิจกรรมการทำงานของโปรตีน Lcp_{Rr} โดยวิธี HPLC พบว่าโปรตีนชนิดนี้ทำงานได้ดีในฟอสเฟสบัฟเฟอร์ที่พีเอชสูงกว่า Lcp ชนิดอื่นอย่างเห็นได้ชัดเจน และจากการวิเคราะห์โปรตีน Lcp_{Rr} บริสุทธิ์ด้วยวิธี UV visible พบว่ามีการดูดกลืนคลื่นแสงที่ความยาวช่วงคลื่นเดียวกับฮีม (Heme) ซึ่งชี้ให้เห็นชัดว่า Lcp_{Rr} เป็นฮีมโปรตีน (Heme-containing protein) จากการบ่งชี้ชนิดของโปรตีนด้วยวิธีการวิเคราะห์โลหะ (Metal analysis) การตรวจวัดคลื่นแสง และ การวิเคราะห์ MALDI-ToF แสดงให้เห็นว่า Lcp_{Rr} เป็นไซโตโครมชนิดบี นอกจากนี้ยังพบอีกว่าสารตั้งต้นสามารถเข้าถึงแกนภายนอกของโครงสร้างโปรตีนได้อีกด้วย ซึ่งน่าจะเป็นโครงสร้างแบบ five-fold coordinated แบบเปิดซึ่งแตกต่างอย่างชัดเจนจากโครงสร้าง six-fold coordinated แบบปิดของ Lcp_{K30} ฮีมโปรตีน

คำสำคัญ: ลาเท็กเคลียริ่งโปรตีน **(**แอลซีพี**)** รับเบอออกซีจีเนส ไดออกซีจีเนส โรโคคอคคัส การย่อยสลายทางชีวภาพ

Introduction

This chapter provides the information about the rubber-degrading protein, and its corresponding genes of rubber degrading dominant strain isolated from the consortium ST608 (see chapter 3). Due to the rubber-degrading strain *Rhodococcus rhodochrous* RPK1 plays an important role in the rubber degradation process, and acts as a dominant species remaining attached to the rubber surface throughout the experiments, while the others were fed off after long incubation periods. Thus, the identification of the key bacteria of the effective consortium ST 608 was performed in this study. Multiple evidence showed that the rubber degrading protein, Latex clearing protein (Lcp) is responsible for the *lcp* gene in Gram positive rubber degrading bacteria, and many *lcp* genes in rubber degrading species have been described (Linos et al., 2000; Braaz et al., 2005; Rose and Steinbüchel, 2005; Yikmis et al., 2008; Yikmis and Steinbüchel, 2012a; Yikmis and Steinbüchel, 2012b). Although only a little knowledge of *R. rhodochrous* genomes is accessible, the biochemistry of polyisoprene cleavage by Lcp_{Rr}, the types of protein and functions of the cofactors involved are concealed.

It is well known that the initial microbial attack on rubber depends on the ability to produce and secrete the rubber-cleaving enzymes into the environment. Recently, only two types of rubber-cleaving enzymes have been discovered. One is the rubber oxygenase RoxA, that was first isolated from *Xanthomonas* sp. 35Y (Tsuchii and Takeda., 1990; Braaz et al., 2004), and so far this enzyme has been found only in Gram-negative bacteria (Birke et al., 2013). RoxA of *Xanthomonas* sp. 35Y is a *c*-type dihaem dioxygenase, and cleaves poly(*cis*-1,4-isoprene) into a C_{15} compound with a terminal keto and aldehyde group (12-oxo-4,8-dimethyl-trideca-4,8-diene-1-al, ODTD), as the major products (Braaz et al., 2005; Schmitt et al., 2010; Birke et al., 2012; Birke et al., 2013). The other rubber cleaving enzyme is a protein designated as a latex clearing protein (Lcp) (Rose and Steinbüchel, 2005). It shares no significant sequence homology with RoxA, with cytochrome *c* peroxidases or with dihaeme 7,10-diol synthases (Estupiñán et al., 2015), and it is present in Gram-positive rubber degrading bacteria, such as *Streptomyces* sp. K30 (Rose and

Steinbüchel, 2005) and other Actinobacteria. *G. polyisoprenivorans* VH2 and *Streptomyces* sp. K30, two well-studied Gram-positive rubber degraders, oxidatively cleave poly(*cis*-1,4-isoprene) to the different sizes but with the same keto and aldehyde end groups as in RoxA-generated ODTD (Ibrahim et al., 2006; Birke and Jendrossek, 2014; Hiessl et al., 2014).

Currently, there have been many reports published for the cofactor, and metal-contents of the Lcps from *Streptomyces* sp. K30 and of *G. polyisoprenivorans* VH2 (Hiessl et al., 2014; Birke and Jendrossek, 2014), however at present there are only two biochemically characterized Lcp proteins.

In this study, the dominant strain in consortium ST 608 (see chapter 3) was isolated as rubber degrading strains. Taxonomic analysis revealed that one strain was a member of the genus Rhodococcus, a full taxon that had not been previously identified as having the ability to utilize rubber as the sole source of carbon and energy but it had been well known for having a high potential for the biodegradation of recalcitrant compounds (Martínková et al., 2009). Biochemical and biophysical characterization of the purified recombinant Lcp protein of *Rhodococcus rhodochrous* strain RPK1 demonstrated some unexpected properties which are not previously described for any other rubber-degrading enzymes, in addition their properties were shared with the other characterized Lcp proteins.

Materials and Methods

Enrichment and isolation of rubber-degrading microorganisms

Sediment from a rubber waste pond at a rubber latex processing factory in Thailand (Namom rubber factory, Namom, Songkhla) was used as an inoculum to enrich the rubber-degrading microorganisms in a Mineral Salts Medium (MSM) supplemented with pieces of rubber gloves as a sole source of carbon and energy. After two weeks of incubation at 30°C, 0.1 volumes (without pieces of rubber) were transferred to a fresh medium and were incubated for an additional month. Substantial disintegration of the new rubber pieces became visible was indicated that the active rubber-degrading microorganisms were present as a consortium (see chapter 3). Several bacterial strains were isolated from this enriched culture by repeated purification by streaking on NB and LB agar plates. Each isolate was subsequently tested for its ability to degrade rubber in liquid MSM with rubber pieces as a carbon source. One isolate designed as isolate RPK1, remained attach to the rubber surface throughout the experiments (see DGGE analysis in chapter 3). This isolate might play an important role in the biodegradation process with a strong rubber-degrading activity thus it was selected for this study.

Bacterial strains, plasmids and culture conditions

The bacterial strains, plasmids and oligonucleotides used in this study, are presented in Appendix Table 1. Rubber degrading bacteria *R. rhodochrous* strain RPK1 was grown with a nutrient broth (NB) medium or in mineral salts medium (MSM, 9 g/L Na_2HPO_4 x 12 H_2O, 1.5 g/L KH_2PO_4, 1 g/L NH_4NO_3, 0.2 g/L $MgSO_4$ x 7 H_2O, 0.02 g/L $CaCl_2$ x 2 H_2O, 1.2 mg/L Fe(III) ammonium citrate with solid rubber pieces or with rubber latex milk and were then inubated at 30°C. Pieces (1 cm x 1 cm) of heat-sterilized vulcanized rubber (0.6 % [wt/vol]) (Siam Top Glove Co., LTD), were added to the sterile MSM for the enrichment and growth of *R. rhodochrous*. Plasmid-haboring recombinant *E. coli* strains were grown with LB medium at 22°C or

37°C in the presence of the appropriate antibiotics. Polyisoprene latex was kindly provided by Weber and Schaer, Hamburg (Germany) and was 3 washed for 3 washing steps in 0.1% (wt/vol) Nonidet P40 before used for purification of Lcp_{Rr}, recombinant *E. coli* cells were grown in LB medium supplemented with 0.1% (wt/vol) L-rhamnose at 22°C. Utilization of carbon sources was examed on mineral salts agar with separately filter-sterilized carbon sources at these following final concentrations (sugars, sugar alcohols and sugar acids at 0.5% [wt/vol], sodium acetate [0.25%, wt/vol], sodium benzoate [0.1%, wt/vol]). Volatile compounds (alkanes) were applied by adding a quantity of 100 µL to a sterile filter paper placed in the lid of a petri disk. The plates were sealed with parafilm and were then incubated separately at 30°C.

Cloning and heterologous expression of *lcp*$_{Rr}$, and determination of the 16S rRNA gene sequence of the isolate RPK1

The *lcp*$_{Rr}$ gene was amplified using the chromosomal DNA from *R. rhodochrous* strain RPK1 as DNA template and the oligonucleotides Lcp_{Rr}-complete_for and Lcp_{Rr}-complete_rev as the PCR primers. Takara Primestar DNA polymerase was used as the proof-reading polymerizing enzyme. The DNA sequence of the product was determined, and it had been then deposited under the accession no. KU140417. Alternatively, the coding sequence of mature Lcp_{Rr} was amplified from chromosomal DNA, using Lcp_{Rr}-mature-PstI_for and Lcp_{Rr}-mature-HindIII_rev as the primers. The DNA products were purified by agarose gel electrophoresis, and were then cleaved with restrictive enzymes e.g. PstI and HindIII. The cleaved products were ligated into plasmid pUC9::*lcp*$_{K30}$ that had been cleaved by the same restriction enzymes. The coding sequence for strep-tagged *lcp*$_{Rr}$ was cut using HindIII and NdeI, and was subsequently ligated into the expression plasmid p4782.1 and transformed to competent *E. coli* JM109 cells.

A part of the 16S rRNA gene of the isolate RPK1 was PCR-amplified using the primers 16S-universal_for and 16S-universal_rev. The DNA sequence of the resulting PCR product was determined (1412 bp), and demonstrated a strong similarity to the 16S rRNA genes of several *Rhodococcus* sp. strains. The 16S rRNA gene sequence of the isolate RPK1 was determined after PCR amplification using the

primers (16S-Rr-complete_for and 16S-Rr-complete_rev), that were specific for the known 16S rRNA gene sequences of *R. rhodochrous* strains reported in the NCBI data base, and it is now available under the accession no KU140418.

Purification of Lcp_{Rr}, Lcp_{K30} and of $RoxA_{Xsp}$

Purification of the rubber oxygenase of *Xanthomonas* sp. 35Y ($RoxA_{Xsp}$) and latex clearing protein Lcp_{K30} was performed as described previously (Birke et al., 2015). Both of $RoxA_{Xsp}$ and Lcp_{K30} were used for comparison of some properties, sharing with the Lcp_{Rr} proteins (Schmitt et al., 2010; Hiessl et al., 2014).

Lcp_{Rr} was purified as followed: eight individual 600 mL LB cultures in 3 L Erlenmeyer flasks were inoculated each with 0.02 volumes of a seed culture of *E. coli* JM109 harboring the plasmid p4782.1::lcp_{Rr} that had been grown with the same medium. It was important that the presence of L-rhamnose (0.1%, wt/vol) at the beginning in the main cultures was enhanced the yield of the expressed Lcp_{Rr} protein. Cells of the main culture were then harvested by centrifugation after approximately 20 hours of growth at 22°C and were immediately used for protein purification. The cell pellet was resuspended in 100 mM potassium phosphate buffer, pH 7.7, containing 150 mM sodium chloride (KPN, 2 mL KPN/g cell wet weight). A soluble cell extract was prepared by two French press steps, and subsequent centrifugation at 40,000 g for 40 min. The supernatant (about 60 mL) was directly applied to a 10 mL Strep-Tactin HC gravity flow column that had been equilibrated with KPN buffer. The column was washed with at least five volumes of KPN buffer before the Lcp_{Rr} protein was eluted by about 30 mL of 5 mM desthiobiotin dissolved in KPN. Lcp_{Rr}-containing fractions were combined, and desalted by running through a G25 Sephadex (26/160) Hiprep desalting column (53 mL bed volume), that had been equilibrated with 1 mM potassium phosphate (KP) buffer, pH 7.0 and subsequently concentrated to 1-2 mL using ultrafiltration (10 kDa cut-off). Remaining impurities were removed by chromatography on a Superdex 200 column (16/600, equilibrated with 1 mM KP, pH 7) at a flow rate of 1 mL/min. Combined Lcp_{Rr}-containing fractions were ultrafiltrated (10 kDa cut-off) and were then concentrated to approximately 1.5 mL. Aliquots of the

purified Lcp_{Rr} protein were stored on ice for about three days (Lcp_{K30} up to one week) or shock-frozen with liquid nitrogen, and stored at -70°C for further study.

Determination of the cytochrome type of Lcp_{Rr}

The heme type of Lcp_{Rr} was determined by the bi-pyridyl assay as described by Hiessl et al. (2012). Purified $RoxA_{Xsp}$, cytochrome *c* (horseheart, type III, Sigma, St. Louis, USA) (both *c*-type cytochromes) and hemoglobin (*b*-type) (bovine, Sigma, St. Louis, USA) were used as the controls for known *c*-type and *b*-type cytochromes, respectively. The respective protein stock solution of about 25 µL (4-8 mg/mL) was added to 975 µL solution A (100 mM sodium hydroxide, 20% (vol/vol) pyridine, 0.3 mM potassium ferricyanide). Subsequently, 2-5 mg sodium dithionite were added, and the spectrum of the reduced cytochrome was recorded. The absorption maxima of the resulting α-bands were characteristic for *b*-type (556 nm), and *c*-type (550 nm) cytochromes. Bi-pyridyl-heme complexes of α-type cytochromes absorb at 584-588 nm. Additional assays for determination of the heme type were performed using extraction of heme by acidic acetone, and a matrix assisted laser desorption ionization time of flight (MALDI-ToF) analysis as previously described (Birke et al., 2015).

Assay of Lcp activity

An HPLC-based assay for Lcp_{Rr}-derived polyisoprene degradation products, was used for most routine assays: poly(*cis*-1,4-isoprene) latex was diluted with 100 mM KP buffer, pH 7, to 0.2% (assay volume 0.7 mL), and was incubated in the presence of the purified Lcp protein for 2 hours at a temperature as indicated (for routine assay at room temperature [23°C]). For inhibition studies, the corresponding compound was added and gently solubilized in the reaction mix before the enzyme was added (final inhibitor concentration 1 mM). The products were extracted with 1 mL ethyl acetate (in a 2 mL Eppendorf tube), dried, and dissolved in 100 µL methanol. Aliquots were applied to an RP8 HPLC column (12 x 4 mm, 5 µm particle size, 0.7 mL/min) with water (A), and methanol (B) as mobile phases. The

concentration of B was increased from 50% (vol/vol) to 100% (vol/vol) within 15 min; products were detected at 210 nm. The C_{35} product peak (at about 23 min) was used for quantification and compared to a control without inhibitor. Alternatively, activity of Lcp_{Rr} was assayed by determination of the rate of oxygen consumption in an OXY-4 mini apparatus (PreSens, Regensburg, Germany) as described previously (Birke et al., 2015). Triplicates and controls without Lcp_{Rr} or with heat-inactivated Lcp_{Rr} were recorded simultaneously. A stability assay was performed by incubation of the purified Lcp_{Rr} protein in the assay buffer at 37°C for variable time periods. The remaining activity of the protein was determined as described above.

Other techniques (protein determination)

The concentration of protein solutions was determined by the bicinchoninic acid (BCA) method. The concentrations of the purified rubber-cleaving enzymes were determined from the molar extinction coefficients of Lcp_{Rr}, Lcp_{K30} and $RoxA_{Xsp}$: Lcp_{Rr}, ε_{407} = 9.5 x 10^4 M^{-1} cm^{-1}, Lcp_{K30}, ε_{412} = 8.0 x 10^4 M^{-1} cm^{-1}, and $RoxA_{Xsp}$, ε_{406} = 2.06 x 10^5 M^{-1} cm^{-1}. Separation of proteins was performed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) under reducing (2-mercaptoethanol) conditions. The metal content of the purified Lcp protein was determined using inductively coupled plasma-MS (ICP-MS) by the Spuren-Analytisches Laboratory Dr. Baumann (Germany). Fuchsin staining of polyisoprene degradation products was performed, by addition of a 1% Fuchsin solution (0.5 g Fuchsin, 12.5 mL acetic acid, 2.5 g $Na_2S_2O_3$, 0.2 ml HCl (37%) and 37.5 ml H_2O) to the Lcp assay mixture. Staining of the cells for poly-3-hydroxybutyrate [P(3HB)] and polyphosphate was performed as described previously (Tumlirsch et al., 2015).

Results and discussions

Screening, isolation and identification of rubber-degrading bacteria in consortium ST608

Rubber-degrading bacteria strain RPK1 was isolated from the consortium ST608 (see chapter 3), which substantially disintegrated pieces of rubber gloves when incubated together in MSM. A liquid culture of inoculum flask was taken as a series period (0, 6, 12, 18, 24, 30 days of incubation) for bacterial cultivation on a rubber latex agar plate. Several bacterial strains were presented from this enrichment medium especially, a yellow colony which was presented at all interval periods as shown in Fig. 14. The colony presented throughout the experiment was repeated purified by streaking on rubber latex agar plates. Each isolate was subsequently tested for rubber-degrading ability in liquid MSM with rubber pieces as a carbon source. One isolate, designated as isolate RPK1, was found in all of the incubation periods and acted like a dominant strain showed a high rubber-degrading activity in liquid culture as observed by a strong disintegration of the rubber pieces within four weeks (see appendix Fig. 4).

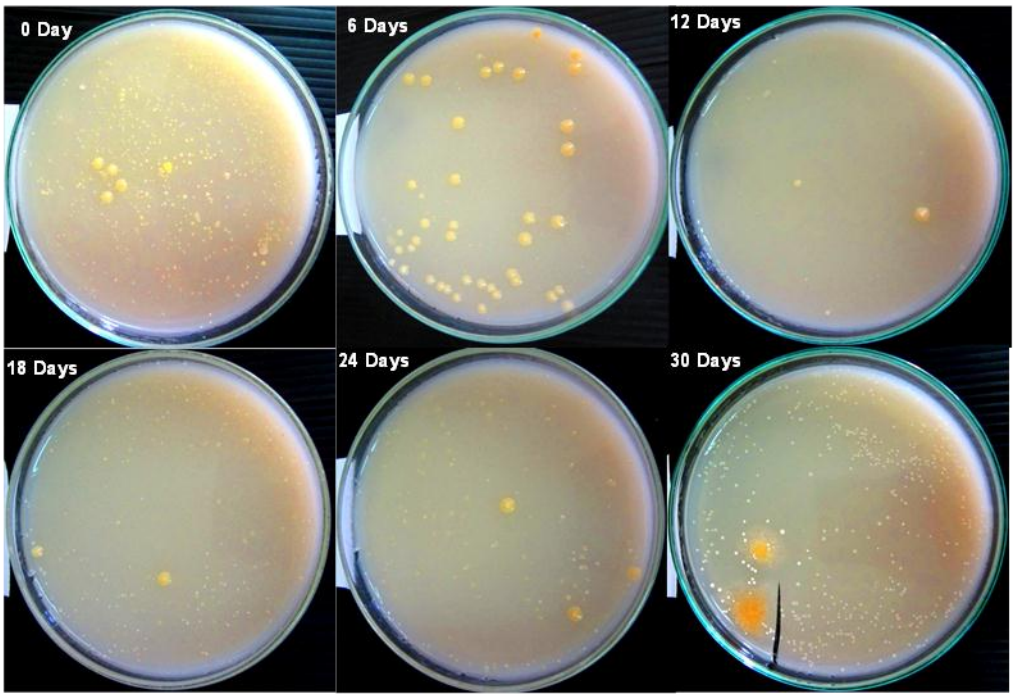

Fig. 14 The cultivation of rubber-degrading bacteria for 7 days from consortium ST608 on rubber latex agar plate at different times.

Isolate RPK1 did not form clearing zones on an opaque polyisoprene latex mineral salts agar, whereas known clear zone formers bacteria such as *Xanthomonas* sp. 35Y (Tsuchii et al., 1990), or the *Streptomyces coelicolor* strain 1A (Jendrossek et al., 1997) formed relatively large clearing zones. Isolate RPK1 has been interested because of the development of the remarkable intense orange to red color of their colonies on NB agar after prolonged incubation (Fig. 15A).

Microscopic examination of the cells showed the presence of non-motile cells. Depending on the growth phase, the cells were coccoid (cells from late stationary phase), rod-shaped (cells from early and late log phase) or long rods (up to 1 x 5 µm), partially branched and star-like in exponentially growing cultures (Fig. 15B, 15C,15D, 15E). Isolate RPK1 was catalase positive and was stained positive by the Gram-staining procedure. It grew well at 43°C (but not over 45°C) and also tolerated in the presence of 3% NaCl (in NB). Strain RPK1 was able to accumulate Nile red stainable storage compounds such as polyhydroxyalkanoates (PHAs) or triacylglycerols and to synthesize polyphosphate granules. RPK1 utilized complex media (NB, LB medium) and grew on MSM with D-mannitol, fructose, acetate, benzoate or octane as a single carbon source. Glucose, sucrose, gluconate, pentane, petroleum or pyridine could not be utilized for growth. Polymers such as polyhydroxybutyrate (PHB), casein or starch were also not utilized by RPK1. These characteristics, in combination with the red color of the colonies, and the variable morphology of the cells, suggested that isolate RPK1 could be a member of the genus Rhodococcus.

To verify this assumption, the DNA-sequence of the PCR-amplified 16s rDNA (accession No. KU140418) was determined. The 16S rDNA was 99.7% and 99.2% identical to *Rhodococcus* MK3027 and *R. rhodochrous* MTCC11081, respectively. According to the biochemical and morphological data, the isolate RPK1 was classified in a member of the species *R. rhodochrous*. It differed from rubber degrading *Xanthomonas* sp. 35Y (Tsuchii, 1990, Braaz, 2004), *Streptomyces* sp. K30 (Rose et al., 2005), and other rubber degrading streptomycetes (Jendrossek et al., 1997) by the inability to produce clearing zones on opaque polyisoprene latex agar. Previously, bacteria with strong rubber-degrading activity without a clearing zone

formation were isolated and identified as *Gordonia polyisoprenivorans* or *Gordonia westfalica* (Bröker et al., 2008).

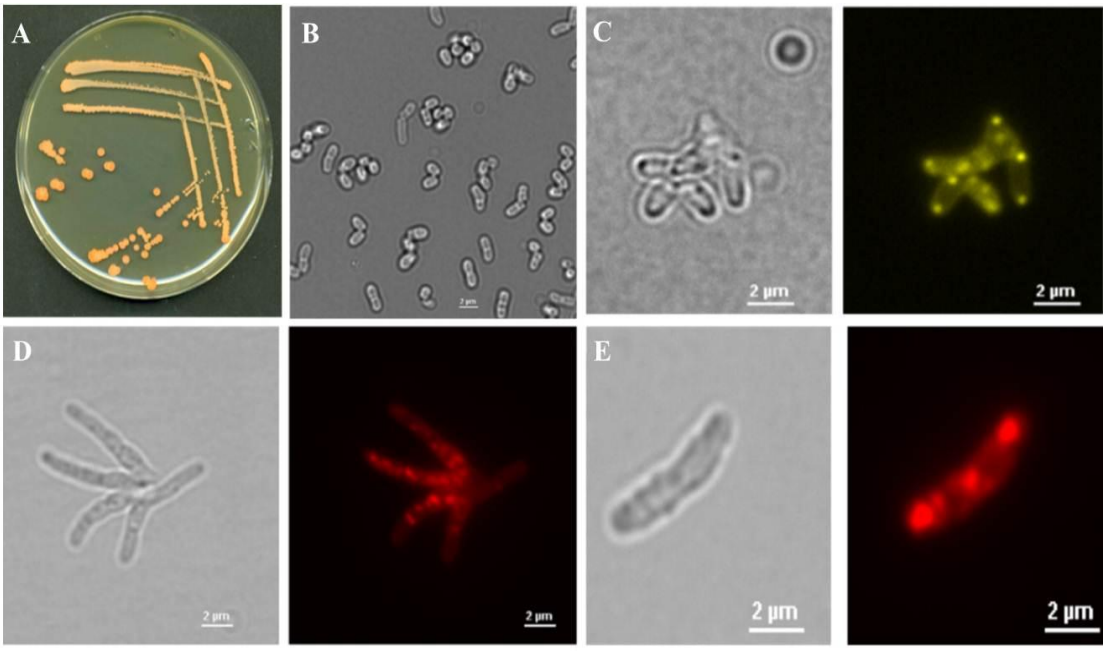

Fig. 15 Features of *R. rhodochrous* RPK1. (A) Formation of red-coloured colonies of *R. rhodochrous* RPK1 when grown NB agar; (B) morphology of stationary of *R. rhodochrous* RPK1 cells in bright field microscopy; (C) *R. rhodochrous* RPK1 cells when grown on NB medium supplemented with acetate (bright field and fluorescent image staining with DAPI using DAPI-polyphosphate-specific emission filters); (D) *R. rhodochrous* RPK1 cells when grown on NB medium supplemented with acetate (bright field and fluorescent image staining with Nile red); (E) *R. rhodochrous* RPK1 cells when grown on NB medium supplemented with acetate (bright field and fluorescent image staining with Nile red).

Identification of the gene for the latex clearing protein in *R. rhodochrous* RPK1

Reccently, the tested rubber degrading Actinobacteria have at least one gene coding for a so-called latex clearing protein (*lcp*) that is supposed to be responsible for the initial oxidative attack of the polyisoprene carbon backbone (Rose et al., 2005; Hiessl et al., 2012; Yikmis et al., 2012; Nanthini, 2015). Remarkably, non-latex-clearing zone formers such as *G. polyisoprenivorans* also have *lcp* genes (Broker et al., 2008). Alignment of the amino acid sequences of Lcp proteins of the

different species revealed the conserved regions within the Lcp amino acid sequence (Hiessl et al., 2014). Moreover, the *lcp* genes in the genomes of *Rhodococcus* strain MTCC11081, *Rhodococcus* sp. MK3027 and *Rhodococcus* sp. ARG-BN062 were identified hypothetically by a screening of the published genome sequences for the presence of *lcp* sequences. The deduced amino acid sequences of these hypothetical Lcp proteins included the DUF2236 domain, containing the central part of most, but not all Lcp proteins (Hiessl et al., 2014). Two oligonucleotides based on the upstream and downstream region of the *lcp* genes of these *Rhodococcus* strains were generated (Lcp_{Rr}-PstI_for and Lcp_{Rr}-HindIII_rev), and a PCR reaction was performed with chromosomal DNA of *R. rhodochrous* strain RPK1. A 1.5 Kbp DNA fragment was obtained and its DNA sequence was determined (accession number KU140417). Analysis of the DNA sequence revealed one large open reading frame of 1227 bp coding for a peptide of 408 amino acids (45.2 KDa). The deduced amino acid sequence showed strong similarities to postulated Lcp proteins of *R. rhodochrous* and of several *Rhodococcus* sp. strains (81 to 99 % identical amino acids). A high degree of similarities was also detected to many other putative Lcp proteins of genome-sequences bacteria, including that of many Streptomycetes and other Actinobacteria. The amino acid sequence of Lcp_{Rr} was compared with that of the only two other biochemically characterized Lcp proteins, a 70% (76%) identity (similarity) and a 57% (66%) identity (similarity) were determined to Lcp_{VH2} of *G. polyisoprenivorans* (Bröker et al., 2008) and to Lcp_{K30} of *Streptomyces* sp. K30 (Rose et al., 2005), respectively (Fig. 16, appendix Fig. 1). A 30 amino acid long sequence of the N-terminus of Lcp_{Rr} was predicted to code for a signal peptide enabling secretion of the protein. The molecular mass of the predicted mature protein was 42.2 KDa.

		Identity [%]		
		Lcp_{Rr}	**Lcp_{K30}**	**Lcp_{VH2}**
Similarity [%]	**Lcp_{Rr}**	100	57	70
	Lcp_{K30}	66	100	52
	Lcp_{VH2}	76	60	100

Fig. 16 An identity and similarity values of the purified and biochemically characterized Lcp proteins

Expression and purification of Lcp_{Rr}

The DNA sequence coding for the Lcp_{Rr} signal peptide was replaced by a Strep-tag coding sequence and the modified gene was cloned under control of an L-rhamnose-dependent promoter into p4782.1 vector and subsequently transformed to *E. coli* JM109. The Lcp_{Rr} protein was purified by two subsequent chromatographic steps from soluble cell extracts that had been prepared from the combined cells of a 5-L of *E. coli* (p4782.1::*lcp_{Rr}*) culture after grown in a LB medium supplemented with 0.1 % L-rhamnose at 22°C for 20 h. A yield of approximately 7.7 mg purified Lcp_{Rr} protein (5.3 mg/mL in BCA assay) was obtained after concentration of the Superdex 200 eluate through ultrafiltration. The Lcp proteins of S*treptomyces* sp. K30 (Lcp_{K30}) and rubber oxygenase RoxA of *Xanthomonas* sp. strain 35Y ($RoxA_{Xsp}$) were also purified and were then used for comparison purposes. All purified proteins were separated by SDS-PAGE and checked for the purity. As shown in Fig. 17, Lcp_{Rr}, Lcp_{K30} and $RoxA_{Xsp}$ were almost homogenous (≥ 97%). Lcp_{Rr}, similar to Lcp_{K30}, migrated at slightly higher apparent molecular masses (50 and 47 kDa) as deduced from the gene sequences (43 and 42 kDa, respectively). Most remarkably, concentrated Lcp_{Rr} had a brownish color. This was in sharp contrast to the red color of concentrated solutions of Lcp_{K30} or of $RoxA_{Xsp}$ and suggested substantial differences of the Lcp_{Rr} in comparison to the other rubber-cleaving proteins.

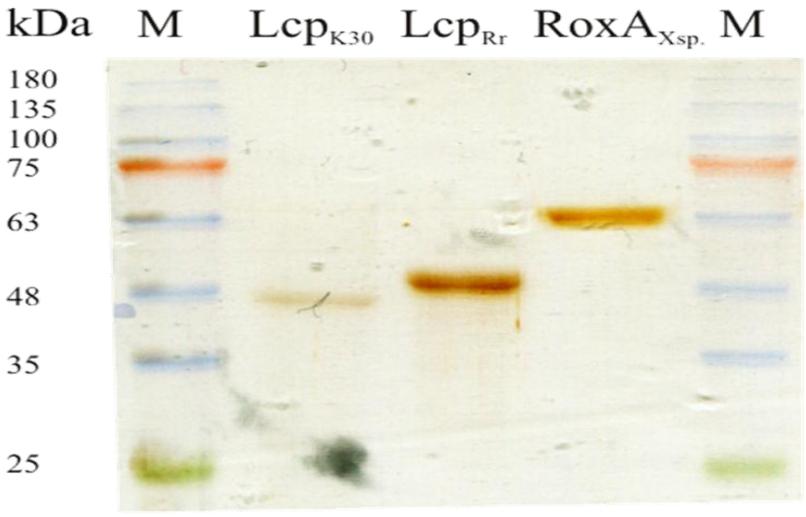

Fig 17 SDS-PAGE of the purified Lcp_{Rr}, Lcp_{K30} and RoxA. Purified Lcp_{Rr}, Lcp_{K30} and $RoxA_{Xsp}$ proteins were separated by reduction of SDS-PAGE and subsequently staining with silver. KDa values of marker proteins are indicated.

Biochemical properties of the Lcp_{Rr}

The purified Lcp_{Rr} protein was investigated for rubber cleaving activity using the oxygen consumption and the HPLC-based rubber cleavage product assay. The oxygen consumption assay confirmed that the Lcp_{Rr} cleaved poly(*cis*-1,4-isoprene) latex in an oxygen-dependent manner; specific activities of 0.9 U/mg and of 3.1 U/mg were determined for Lcp_{Rr} at pH 8 and at 23°C and 30°C, respectively. Variable data were determined for the specific activity of Lcp_{Rr} at 37°C, possibly because of a decreasing stability of the Lcp_{Rr} protein at higher temperatures. The HPLC (Fig. 18A) and Fuchsin assay (Fig. 18B) revealed that Lcp_{Rr} produced the same mixture of polyisoprene cleavage products (C_{20} and higher oligo-isoprenoids with terminal keto and aldehyde groups), that had been determined for Lcp_{K30}. 12-oxo-4,8-dimethyltrideca-4,8-diene-1-al (ODTD), was only detectable in trace amounts for Lcp_{Rr} or for Lcp_{K30} but was the main product of the $RoxA_{Xsp}$-derived rubber cleavage products. Determination of the activities of purified Lcp_{Rr} at different pH values using the HPLC-based product assay, showed a pH optimum around pH 8 (Fig. 20), which was about one pH unit higher than the pH optimum, that had been previously determined for $RoxA_{Xsp}$ or for Lcp_{K30} and Lcp_{VH2} (Braaz et al., 2004; Hiessl et al., 2014; Birke and Jendrossek, 2014). The stability of both the Lcp preparations decreased upon incubation in buffer at 37°C (Fig. 19B). In accordance with these results, the concentration of rubber degrading products in an *in vitro* latex cleavage assay with Lcp_{Rr} or Lcp_{K30} increased for only 4-8 hours (Fig. 19A). $RoxA_{Xsp}$, on the other hand, was much more stable and continuously produced ODTD molecules for up to 70 h (Schmitt et al., 2010).

Lcp_{Rr} is a *b*–type cytochrome and remarkable difference to LcpK30

The purified Lcp_{Rr} had a brownish colour whereas the Lcp_{K30} was red. Appendix Fig 5 showed a comparison of the UVvis spectra of the purified Lcp_{K30} and Lcp_{Rr} proteins in the oxidized state and in the dithionite-reduced state. Lcp_{Rr} (and Lcp_{K30}) as isolated, both proteins showed similar absorption maxima at 407 (412) nm and at 535 (544) nm that are typical for haem containing proteins in the oxidised state.

However, the purified Lcp_{Rr} had an additional broad absorption maximum around 645 nm. The absorption band at 645 nm was absent in Lcp_{K30} and in other biochemically characterized RoxA proteins such as $RoxA_{Xsp}$ and $RoxA_{Cco}$ (Birke et al., 2013). When the Lcp preparations were chemically reduced by the addition of sodium dithionite, the absorption bands at 407 (412) nm and 535 (544) nm were shifted to 428 (430) nm and 560 (562) nm. A comparison of the reduced spectra of both Lcp proteins showed differences in the Q-bands (500 – 600 nm). Apparently, Lcp_{Rr} is far less pronounced in this region than Lcp_{K30}. Nevertheless, these data corresponded to the Soret and Q-bands that are typical for haem-containing proteins and strongly indicated that Lcp_{Rr} is a haem-containing protein. The band around 645 nm, however, was not changed by the addition of dithionite. To confirm that Lcp_{Rr} is a haem-containing protein and to determine its haem type, a metal analysis and a spectral analysis by the haem-bipyridyl assay were performed (Appendix Fig. 3). 6.5 µg Fe/mL Lcp_{Rr} protein solution (5.3 mg protein/mL) were determined. This corresponded almost perfectly with a stoichiometry of one atom Fe per one Lcp_{Rr} molecule. It was of interest that low amounts of copper (2.8 µg/mL) were also identified and corresponded to 0.36 atoms Cu per one Lcp_{Rr} molecule. Zinc was detected at the detection limit (0.1 µg/mL) and Nickel was below the detection limit (<0.1 µg/mL); other metals (vanadium to zinc tested) were also not detected in significant amounts. Divalent cations such as magnesium or calcium were not present (below the detection limit of 0.1 µg/mL) (Watcharakul et al., 2016). An absorption maximum of 556 nm was determined using the bipyridyl assay for Lcp_{Rr} and for haemoglobin that was used as a *b*-type cytochrome control protein (Appendix Fig. 3). These results indicated the presence of a *b*-type haem in Lcp_{Rr}. In contrast to the covalently linked *c*-type cytochromes, the haem groups of the *b*-type cytochromes are not covalently linked to the peptide chain and can be therefore extracted by an acid solvent extraction (Morrison and Horie, 1965). Acid solvent extraction of the purified Lcp_{Rr} yielded a coloured supernatant and a non-coloured precipitate. In contrast, solvent extraction of the *c*-type cytochromes such as $RoxA_{Xsp}$ or of other commercially available cytochrome *c* enzymes yielded a non-coloured supernatant and a red precipitate which is corresponding with the covalent attachment of porphyrin to the polypeptide. MALDI-ToF analysis of the purified Lcp_{Rr} resulted in the identification of ions with

m/z values of 616 (data not shown) which is typical for haem *b* (Yang et al., 2013). All these results indicated that, Lcp_{Rr} is a *b*-type cytochrome similar to Lcp_{K30} (Birke et al., 2015). In addition, MALDI-ToF analysis of Lcp_{Rr} also revealed an ion species with *m/z* values of 619 besides that of 616 which could correspond to a verdo-haem (Andreoletti et al., 2009). As the activity of the purified Lcp_{Rr} rapidly and substantially decreased during storage, the haem species with *m/z* value of 619 could represent a haem degradation product of the inactivated Lcp_{Rr} (Watcharakul et al., 2016).

Lcp_{Rr} is accessible for external ligands

Previous studies on the rubber oxygenase, RoxA had revealed that the active haem site in RoxA had only one axial amino acid ligand. The other axial ligand was a dioxygen molecule that was stably bound to haem in a Fe^{3+}——O_2− transition state. The oxygen molecule in RoxA could be partially removed by the addition of imidazole, thereby moving the negative charge from the oxygen molecule to the iron atom (Fe^{2+}). This charge transfer resulted in a small visible change of the UVvis spectrum as observed by an increase of the absorption of the Q-bands at 549 nm (Birke and Jendrossek, 2014; Hiessl et al., 2014; Schmitt et al., 2010). When imidazole was added to the dithionite-reduced RoxA, substantial increases in the Soret and Q-bands were determined compared to the reduction of RoxA bands without imidazole (Birke et al., 2015; Schmitt, 2012). The increase in absorption was interpreted as the result of the binding of the imidazole molecule to the (now) free sixth (axial) coordination site of the haem iron. An analog experiment with purified Lcp_{K30} was performed and showed that there was a significant difference between both Lcp proteins: addition of imidazole to the dithionite-reduced Lcp_{K30} had no effect on the UVvis spectrum and there was no detectable increase of the Q-bands. This indicated that the 6^{th} coordination site of the haem apparently was not accessible for imidazole and the Lcp_{K30} protein was present in a “closed state”. Binding of the substrate (polyisoprene) would therefore require a conformational change of the Lcp_{K30} structure. In contrast, addition of imidazole to the dithionite-reduced Lcp_{Rr} protein resulted in a substantial increase of the Soret- and Q bands (Appendix Fig. 5)

and this can be explained by the binding of imidazole to the reduced haem. Similar results were obtained when both the Lcp proteins were treated with mercaptoethanol: no change of the UVvis spectrum was determined for Lcp_{K30} while the prominent changes were detected for the Lcp_{Rr} protein (Appendix Fig. 2). In conclusion, the Lcp_{Rr} seems to rest in a different conformation in their as isolated states. Whereas the Lcp_{K30} was in a six-fold coordinated "closed" state, the haem group of Lcp_{Rr} was readily accessible to external ligands and substrates, and this indicated a five-fold coordinated "open" state. Further evidences can be found in the UVvis spectra of five-fold coordinated myoglobin in the oxidised (met myoglobin) and reduced (desoxy-myoglobin) state. The UVvis spectra of the latter proteins showed similarities to the corresponding spectra of Lcp_{Rr}, particularly in the region of the less pronounced Q-bands of the reduced Lcp_{Rr} compared to Lcp_{K30} as well as in the 645 nm region in the oxidised state (Schenkman et al., 1997). The presence of the 645 nm absorption band in Lcp_{Rr} might be also explained by a charge transfer phenomenon of a charged residue/ion in close neighbourhood to the haem group in Lcp_{Rr} and in its absence in Lcp_{K30} (Pond et al., 1999). Unfortunately, only the RoxA structure (Seidel et al., 2013) but no Lcp structure was available to obtain direct support for our assumption.

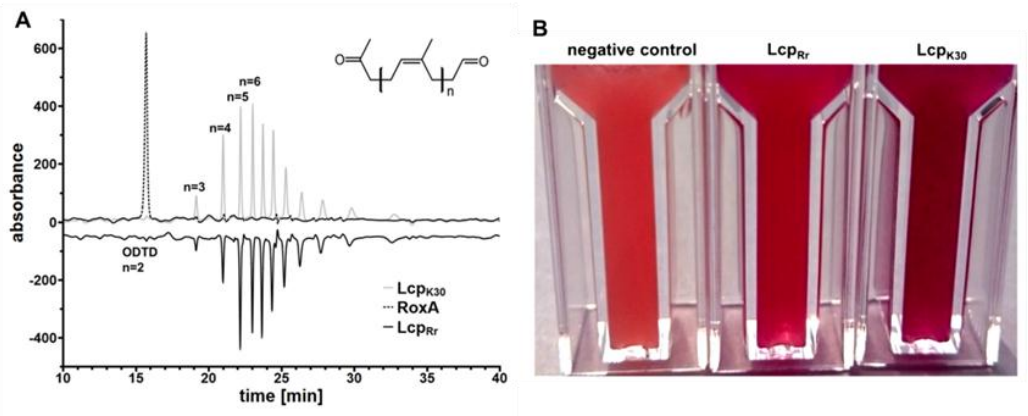

Fig. 18 Activity assays of $Lcp_{Rr.}$ (A) HPLC-product assay. Purified Lcp_{Rr} after size exclusion chromatography was used in the assays. Graphs for purified Lcp_{K30} and purified $RoxA_{Xsp}$ are given for comparison. (B) Detection of aldehyde products of Lcp-degraded polyisoprene by Fuchsin assay. Polyisoprene latex in KP buffer, pH 8 was incubated with 4 µg/mL of the purified Lcp_{Rr}, 4 µg/mL of the purified Lcp_{K30} or without enzyme (control) incubated at 30°C for 1 h. Development of pink color after addition of Fuchsin solution confirmed the formation of carbonyl groups in degradation products by Lcp_{Rr} and Lcp_{K30}

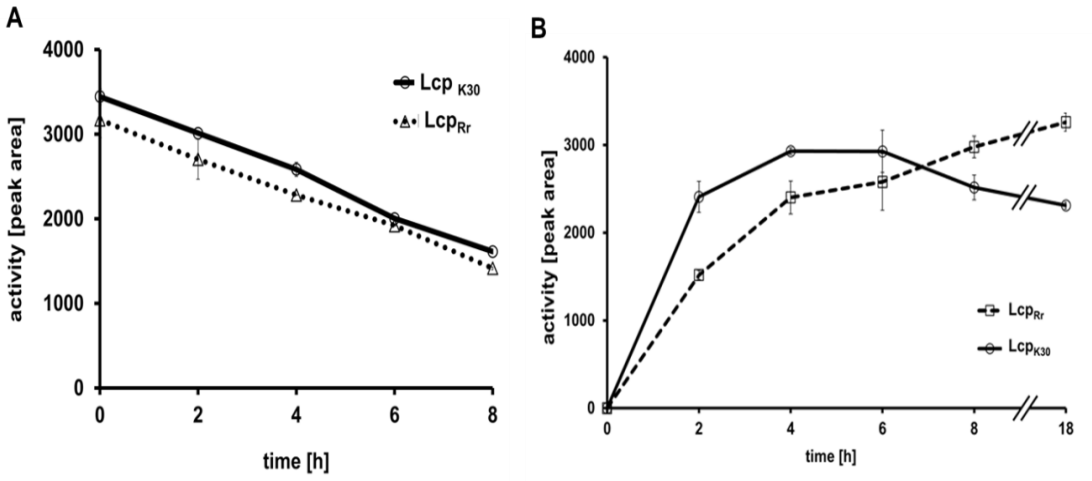

Fig. 19 Stability of Lcp_{Rr} and Lcp_{Rr} and product formation. Lcp proteins were incubated in the presence of polyisoprene latex for 0 to 8 h at room temperature and the amount of the products was determined by HPLC (A). Lcp proteins were incubated at 37°C for up to 18 h before the standard activity assay was performed (B).

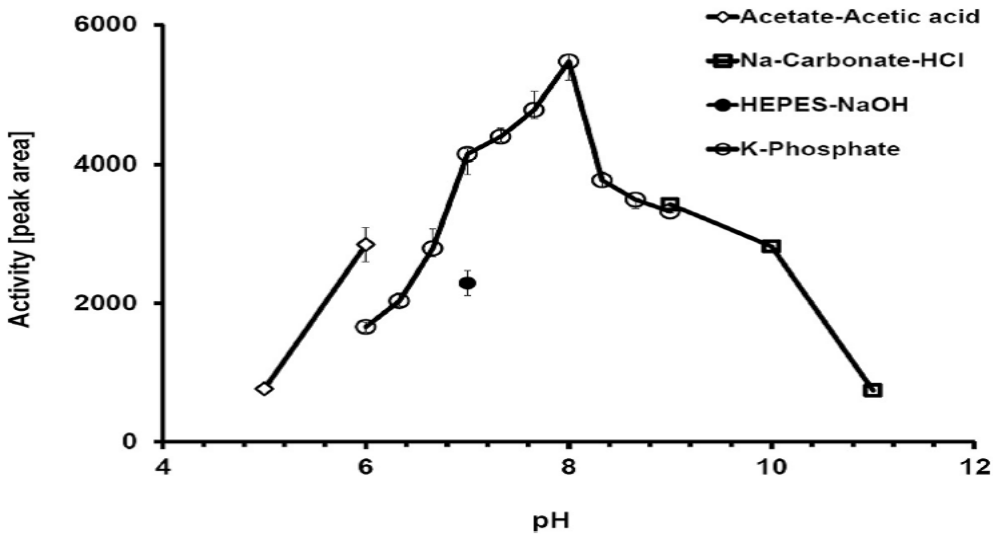

Fig. 20 pH optimum of the Lcp_{Rr}. The pH optimum was determined using the HPLC-based product assay in a pH range of 5 to 11 using acetate buffer (pH 5 - pH 6, diamonds), phosphate buffer (pH 6 - pH 9, open circles), carbonate buffer (pH 9 – pH 11, squares), or HEPES (pH 7, closed circle). Assays were performed with two biological and two technical replicates. Error bars indicate standard deviation

Lcp_{Rr} is insensitive to most chelating inhibitors.

Metal-dependent proteins are often inhibited by chelating compounds. Therefore, a variety of known chelator compounds were tested for an effect on the activity of Lcp_{Rr} using the HPLC-based activity assay. EDTA, tiron, or phenanthroline had no significant effect on its activity (Fig. 21). Ethyl xanthogenate partially inhibited Lcp_{Rr} by about 40% similar to $Lcp_{K30,}$ but different to Lcp purified from *G. polyisoprenivorans* (Lcp_{VH2}) that completely inhibited Lcp_{VH2} at 2 mM xanthogenate (Hiessl et al., 2014). The only compound that had a strong effect on the activity of the Lcp_{Rr} was the metal chelator diethyl dithiocarbamate (82% inhibition, Fig. 21). However, as in the case of diethyl dithiocarbamate had no effect on the UVvis spectrum of Lcp_{Rr} and this excludes a direct effect of the inhibitor to the heme site Carbon monoxide, which completely inactivated by RoxA, had no effect on the UVvis spectrum of Lcp_{Rr} as isolated and this finding was in agreement with the presence of an oxidized (Fe^{3+}) heme center. Carbon monoxide had no inhibitory effect on polyisoprene cleavage during the HPLC-based product assay when sufficient oxygen was also present in the assay mixture. However, when Lcp_{Rr} was incubated in a carbon monoxide-saturated and oxygen-free buffer before added to an oxygenated polyisoprene latex assay solution, a lag phase of Lcp_{Rr} dependent oxygen consumption was observed in the oxygen consumption assay. However, the polyisoprene-cleaving activity recovered within 20 to 30 min during incubation and exposure of the assay solution to air. The same result was obtained when Lcp_{K30} was exposed to carbon monoxide. Addition of carbon monoxide to dithionite-reduced Lcp_{Rr} or Lcp_{K30} had (minor) visible effects on the UVvis spectra as revealed by a small increase of the α-band of Lcp_{Rr} and Lcp_{K30} upon addition of carbon monoxide. The effect of carbon monoxide on the UVvis spectrum of Lcp was reversible by addition of a dioxygen atmosphere and indicated that the binding of carbon monoxide to the chemically reduced heme group in Lcp was reversible. This is in contrast to $RoxA_{Xsp}$, which binds carbon monoxide irreversibly and completely inhibits the activity of RoxA without recovery. An apparent consequence of our data is that the hemes of Lcp proteins undergo a Fe^{3+} to Fe^{2+} reduction and the reduced Lcp proteins presumably are sensitive to carbon monoxide.

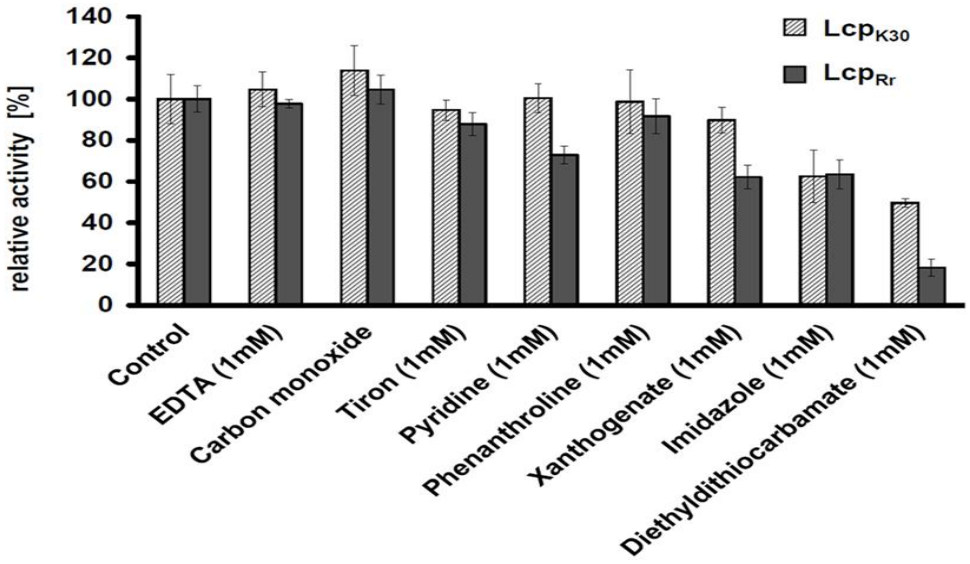

Fig. 21 Inhibition of Lcp_{Rr} by potential inhibitors

lcp genes are frequently present in the genomes of Actinobacteria (Yikmis et al., 2012) and many rubber degrading species have been described in this group (Jendrossek et al., 1997; Linos et al., 2000; Linos et al., 2002; Arenskötter et al., 2001; Imai et al., 2011; Nanthini et al., 2015). Most of the rubber degrading Actinomycetes such as *Streptomyces* sp. K30 (Rose et al., 2005), *Streptomyces coelicolor* 1A, and many others are able to perform clearing zones on opaque polyisoprene latex agar. However, several of the most potent rubber degraders do not produce clearing zones and apparently need close contact to the degraded rubber materia. Two well known rubber degrading *Gordonia* species e.g. *G. polyisoprenivorans* and *G. westfalica* and also the strain used in this study e.g. *R. rhodochrous* RPK1 belong to this group of non-clearing zone formers. One might speculate that Lcp proteins of non-clearing zone formers constitute a group having an open conformation with free access to the active site and that the other Lcp proteins that confer clearing zone formation ability to the Lcp-producing strains have a closed form. Prototype of the first group would be Lcp_{Rr} while the prototype of the latter would be Lcp_{K30}. It will be necessary to biochemically investigate more Lcp proteins and to elucidate the structure of Lcp proteins as well as to find more evidences for or against this hypothesis.

Conclusions

This study extends the list of rubber-degrading non-clearing zone formers by latex clearing proteins (Lcp) to the genus Rhodococcus (besides Gordonia) and raises the question as to whether the designation “latex clearing protein” is well-chosen. Rubber oxygenase B (RoxB) would be an appropriate alternative. However, the designation Lcp has been used in several previous publications and has also been used for many annotated genes in genome-sequenced Actinobacteria. Re-classification of Lcp as RoxB therefore could be confusing.

Isolation and characterization of the Lcp protein of *R. rhodochrous* RPK1 in this study shows that the current studies, Lcp proteins differ in some spectroscopic features and/or in spatial arrangements of their metal ions/cofactors and indicate the presence of two or even more subgroups of Lcp proteins. It will be necessary to study more Lcp proteins to reveal the complete variability of rubber degrading enzymes present in rubber-degrading organisms.

CHAPTER 5

Concluding Remarks

This concluding chapter summarizes the contributions of this thesis to the literature, and discussed in this final chapter in order to integrate the conclusions drawn and suggests potential directions of future work on the biodegradation of rubber, using a microbial consortium with the biotechnological processes that would be usedful for rubber waste treatment.

One of the major findings of this research work was to demonstrate an almost complete degradation of the vulcanized rubber gloves within 4 weeks, by bacterial consortium ST608, which was derived from a rubber waste pond. This clearly demonstrates that an effective consortium can accelerate the rubber degradation process, and provides strong evidence for complete degradation, indicating the presence of a potent rubber degrading bacteria.

Analysis of the structure community of consortium ST608, based on the 16s rRNA gene revealed the key bacteria which appeared throughout the experiment. One strain was identified as a *Rhodococcus rhodochrous* RPK1, and this was considered as the dominant strain in consortium. The biochemical properties of protein were examined to obtain a better understanding of the catabolism process of isoprene.

The whole *lcp* gene, which is responsible for the latex clearing protein sequence was identified. The results provide a lot of information, which has not been previously reported. In particular, the Lcp protein from *Rhodococcus rhodochrous* RPK1 can be classified as a heme protein *b*-type cytochrome, with five-fold “open” state external ligands and as a protein structure, which is readily accessible to external ligands and substrates. Our data point to substantial differences in the active sites of Lcp protein obtained from different rubber degrading bacteria. It will be necessary to solve the structure of Lcp proteins to corroborate our results.

This research strongly suggests that the use of microbial consortium can improve the performance of the rubber degradation process, and may be the best

approach for more efficient treatment of rubber waste in the future. However, more knowledge with regard to the responsible genes and biochemical properties of rubber degrading enzymes are needed to understand the mechanisms of the bacterial processes, which in turn will improve the efficiency of rubber waste biodegradation.

Suggestions for future research

1. The use of natural bacterial consortium is a high potential to treat rubber waste, while the stability of its degradation ability is difficult to control. It depends on the growth conditions of each member of a microbial community, which play significant roles in the degradation process, and may depend on the presence of other species or strains to be able to survive when the source of energy is limited and confined to complex carbons. A possible way to improve this state is to identify the key microorganisms in the natural consortium and investigate their appropriate conditions then, re-mixed each key strain that has a synergistic effect on the rubber degradation process.

2. This research is the first identification of the chemical and biophysical characteristics of the purified Lcp protein of the *Rhodococcus rhodochrous* strain RPK1. It will be necessary to biochemically investigate more Lcp proteins and to elucidate the structure of the Lcp proteins to find more evidences for or against this hypothesis.

3. This research is only relevant with regard to Lcp from the *Rhodococcus rhodochrous* strain RPK1. The representative of key bacteria in this rubber degradation process was investigated its biochemical properties and was solved some structure to understand of the degradation mechanisms. However, there has been insufficient research about the rubber-degrading enzymes of other microorganisms. It is also necessary to investigate more Lcp proteins from others strains, in order to reveal the variability of rubber degrading enzymes present in rubber-degrading organisms. Further research should include an optimization of the suitable conditions to enhance the activity of enzymes, so that maximum rubber degradation can be achieved.

4. Biotechnological processes can be designed to benefit from microbial degradation of rubber to produce useful products, rather than using chemical and physical processes. This might include using genetic engineering to develop more efficient rubber degrading strains. However, biotechnological specialists must consider a variety of different methods and designs. The problem with rubber waste at present is contamination by impurities, such as additives used in manufacturing processes that might be toxic to many microoraganisms, for example, sulfur in a vulcanization system. In addition, some compounds are added to prevent microbial attack of the product during use. Compound ingredients need to be selected with a satisfactory level of microbiological resistance.

The number of biodegradation processes available for rubber waste is still very limited, and microbial recycling is probably the most investigated option, although it is not yet in commercial use. However, the biotechnological method is the most promising solution to the future increases in rubber waste.

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APPENDIX

A1. Report of microbial identification by 16s rDNA sequence analysis

LOCUS	KU140418 1412 bp DNA linear BCT 20-APR-2016
DEFINITION	*Rhodococcus rhodochrous* strain PRK1 16S ribosomal RNA gene, partial sequence.
ACCESSION	KU140418
VERSION	KU140418.1
SOURCE	*Rhodococcus rhodochrous*
ORGANISM	*Rhodococcus rhodochrous* Bacteria; Actinobacteria; Corynebacteriales; Nocardiaceae; Rhodococcus.
REFERENCE	1 (bases 1 to 1412)
AUTHORS	Watcharakul, S., Roether, W., Birke, J. and Jendrossek, D.
TITLE	Biochemical and Spectroscopic Characterization of Purified Latex Clearing Protein (Lcp) from newly Isolated Rubber Degrading *Rhodococcus rhodochrous* Strain PRK1 Reveals Novel Properties of Lcp
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 1412)
AUTHORS	Watcharakul, S., Roether, W., Birke, J. and Jendrossek, D.
TITLE	Direct Submission
JOURNAL	Submitted (12-NOV-2015) Institute for Microbiology, University of Stuttgart, Allmandring 31, Stuttgart, BW 70329, Germany
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
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ORIGIN

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1261	actcgacccc	gtgaagtcgg	agtcgctagt	aatcgcagat	cagcaacgct	gcggtgaata
1321	cgttcccggg	ccttgtacac	accgcccgtc	acgtcatgaa	agtcggtaac	acccgaagcc
1381	ggtggcccta	acccccctcgt	gggatggagc	cg		

A2. Report of microbial identification by *lcp* gene sequence analysis

LOCUS	KU140417 1451 bp DNA linear BCT 20-APR-2016
DEFINITION	*Rhodococcus rhodochrous* strain PRK1 latex clearing protein gene, complete cds.
ACCESSION	KU140417
VERSION	KU140417.1
SOURCE	*Rhodococcus rhodochrous*
ORGANISM	*Rhodococcus rhodochrous* Bacteria; Actinobacteria; Corynebacteriales; Nocardiaceae; Rhodococcus
REFERENCE	1 (bases 1 to 1451)
AUTHORS	Watcharakul, S., Roether, W., Birke, J. and Jendrossek, D.
TITLE	Biochemical and Spectroscopic Characterization of Purified Latex Clearing Protein (Lcp) from newly Isolated Rubber Degrading *Rhodococcus rhodochrous* Strain PRK1 Reveals Novel Properties of Lcp
JOURNAL	Unpublished
REFERENCE	1 (bases 1 to 1451)
AUTHORS	Watcharakul, S., Roether, W., Birke, J. and Jendrossek, D.
TITLE	Direct Submission
JOURNAL	Submitted (12-NOV-2015) Institute for Microbiology, University of Stuttgart, Allmandring 31, Stuttgart, BW 70329, Germany
COMMENT	##Assembly-Data-START## Sequencing Technology :: Sanger dideoxy sequencing ##Assembly-Data-END##
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WGPFIAVREGLLPLPLAPDAYWTFDELLRQFVLLYMSELRPISIEIPEYNNPNY
P"

ORIGIN

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181	cacaggcccg	accctggagg	tggtcgcccg	caggatcgat	accgggtacg	gggagcggtg
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1441	gtcgtcggcg	a				

B. Media and Reagents

Media

1. Mineral Salt Medium (MSM)

MSM consists of the following components in g/L: MSM, 9 g/l Na_2HPO_4 x 12 H_2O, 1.5 g/L KH_2PO_4, 1 g/L NH_4NO_3, 0.2 g/L $MgSO_4$ x 7 H_2O, 0.02 g/L $CaCl_2$ x 2 H_2O, 1.2 mg/L Fe(III) ammonium citrate. Carbon source e.g. rubber gloves pieces (0.6% wt/vol) or latex milk (o.6% vol/vol) is sterilized together with medium in an autoclave at 121℃, 15 psi for 20 min. In particulary, the latex overlay agar using MSM as a based, and overlay the top with fresh latex milk (with ammonia), Leave plate at room temperature to evaporate ammonia for 24 h.

2. LB (Luria-Bertani) Liquid/Solid medium (LB)

NR consists of 10 g/L peptone, 5 g/L of yeast extract 5 g/L of NaCl.

Reagents

1. Schiff's reagent

Dissolve 0.5 g of Fuschine (Rosaniline hydrochloride) in 250 mL of warm distilled water. Cool, saturate with SO_2 (solution becomes colourless or pale yellow) and dilute to 500 mL with water (Linos et al., 2000).

2. Bradford Solution

Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 mL 95% ethanol, add 100 mL 85% (wt/vol) phosphoric acid. Dilute to 1 Lwhen the dye has completely dissolved, and filter through Whatman #1 paper just before use. The Bradford reagent should be a light brown in color. (Optional) 1 M NaOH (to be used if samples are not readily soluble in the color reagent (Cheung and Gu, 2005)

C. Additional figures

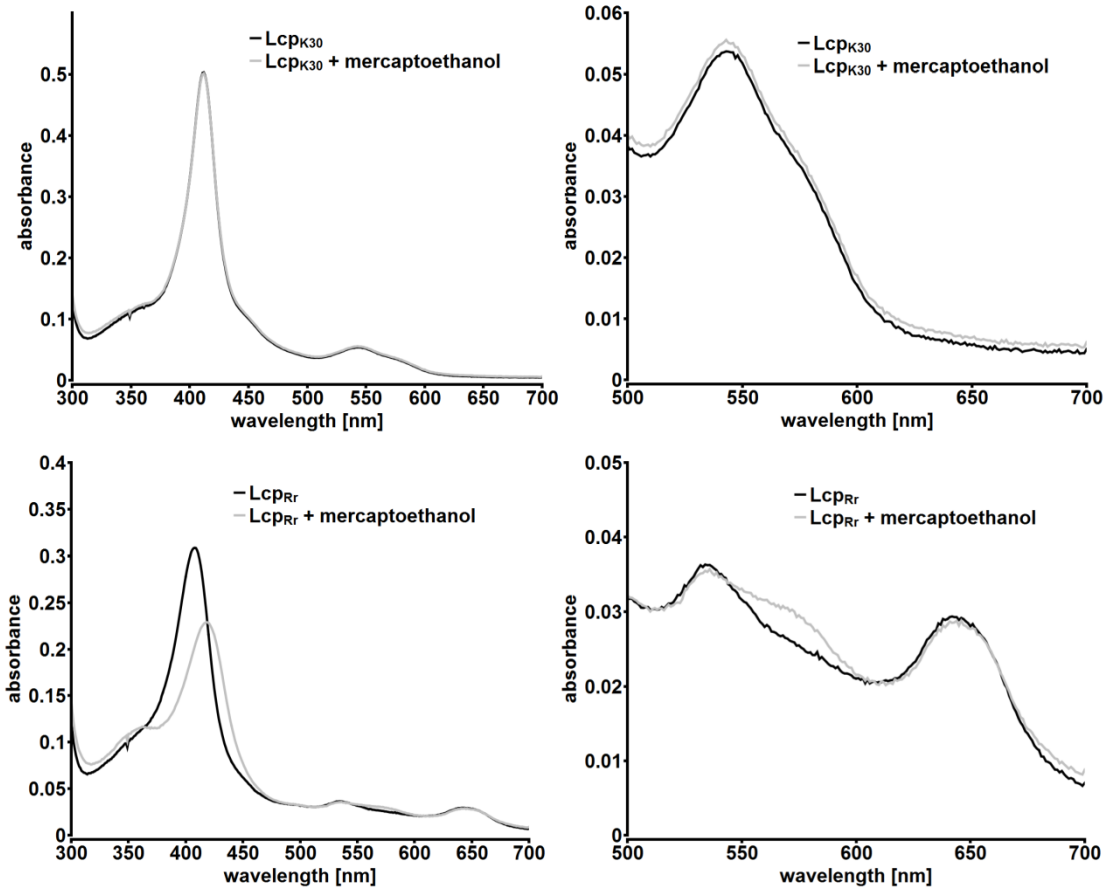
C1. Formula for Mineralization

$$\text{Mineralization } (\%CO_2) = \frac{\text{Required amount of 0.1 HCL (mL)} \; x \; 0.1}{(\text{Content of amount of poly}(1,4-\text{isoprene})\text{applied (mmol)} \text{x } 2}$$

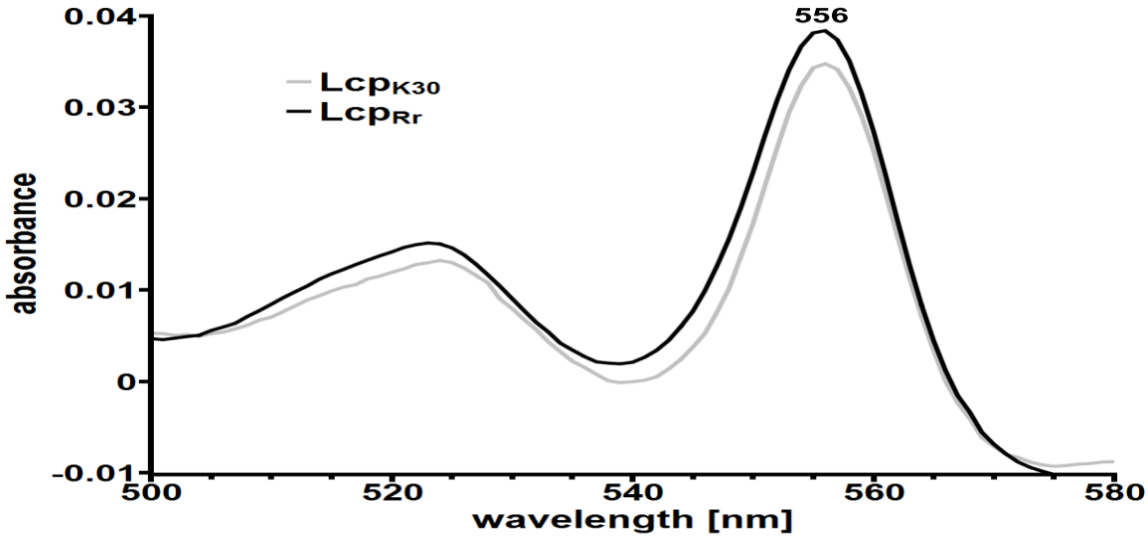
C2. Figures

```
LcpRx       -RPWRWSPAGSIPGTGSGADPRQVWDNEADPLVASLLERGDVPRVNELLRTWKKNSQPLP
LcpK30      RPLWTWSPSASVAGTGVGVDPEYVWDEEADPVLAAVIDRGEVPAVNALLKQWTRNDQALP
LcpVH2      ---------NSIPGVGTGADPRTIFDDEADQVIARVIDSGKVVEVNNLLNQWRTNSQPLP
                     *: *.* *.**. ::*:*** ::* ::: *.*  ** **. *  *.* **

LcpRx       EGLPNDLRDFMEHARQLPTWADPAKLETAVRFNEKRGLYLGVLYGFVSGMMSTVIPKEAL
LcpK30      GGLPGDLREFMEHARRMPSWADKAALDRGAQFSKTKGIYVGALYGLGSGLMSTAIPRESR
LcpVH2      AGLPPELRDFIEHARQLPSWTDRGKLAAAVRFNQRRGTYLGVLYGFASGMMSTVIPKEAR
             *** :**:*:****::*:*:* . *  ..:*.: :* *:*.***: **:***.**:*:

LcpRx       AVYYSKGGANMKGRISKTAKLGYDIGSRNAYLGDGEMIVTSVKTRLVHAAVRHLLPQSSH
LcpK30      AVYYSKGGADMKDRIAKTARLGYDIGDLDAYLPHGSMIVTAVKTRMVHAAVRHLLPQSPA
LcpVH2      AVYYSKGGWDLKDRISKTAKLGYDIGALNAYQPDGEMVVTCVKTRMAHAGVRHLLPKSAH
            ******** ::* **:***:****** :**  .*.*:**.****:.**.******:*

LcpRx       WSNMA-PEDVPISQHDMMVTWHSLPTSVMRTLKEWKVPIPAAEADGFLHSWQVSAHMLGI
LcpK30      WSQTSGGQKIPISQADIMVTWHSLATFVMRKMKQWGVRVNTADAEAYLHVWQVSAHMLGV
LcpVH2      WVRSA-PEEKPISQADIMVTWHSLPTTVMRNLEKWKVPLPADESEGFLHSWQVTAAMLGV
            * . :  :. **** *:******* * ***.:::* * : : ::::.:** ***:* ***:

LcpRx       EDQYIPATWDDAESQAQEALDPILAPTPEGVKLADMLLDLGKGLDFTLLTRPILGALTRF
LcpK30      SDEYIPATWDAANAQSKQVLDPILAHTPEGEALTEVLLGIVAELDAG-LTRPLIGAFSRY
LcpVH2      QDQYIPNSWATADSQAKQVLDPILAWTPEGQKLADMLLDLGMDLDLTLLSRPILGALTRF
            .*:*** :*  *::*:::.****** ****  *:::** :   **   *:**::**::*:

LcpRx       ALGDEIADWLNIPREPVWGTLLDVAWGPFIAVREGLLPLPLAPDAYWTFDELLRQFVLLY
LcpK30      TLGGEVGDMIGLAKQPVLERLIATAWPLLVAFREGLIPLPAVPAVLWTLEEALRKFVLLF
LcpVH2      MLGDKIADWLHIPREPVWTPLLETAWGPYVAVREGLLNAGLPRETYWLFDEFLRQFVLFY
             ** ::.* : : ::**   *: .**   :*.****:       . * ::* **:***::

LcpRx       MSELR-PISIEIPEYNNPNYP
LcpK30      LSEGR-RIAIDIPDVNRPS--
LcpVH2      MAELRMPISIQIPYFNNPNYN
            ::* *  *:*:**  *.*.
```

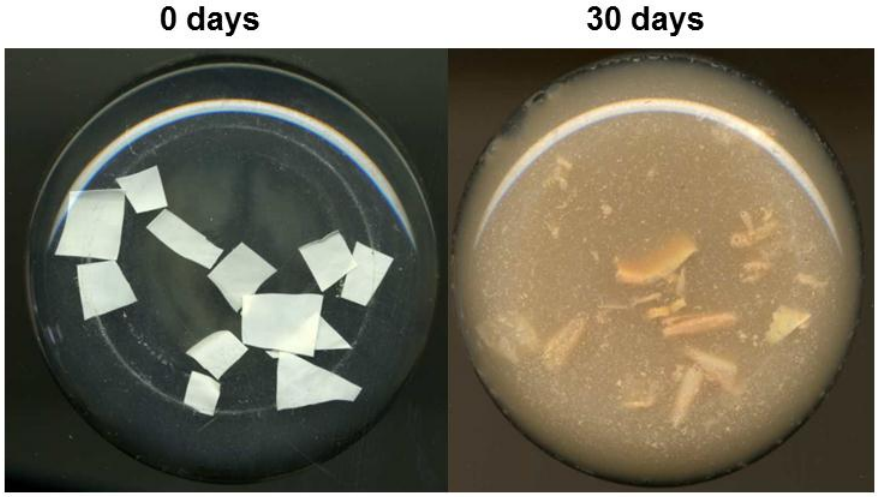
Additional Fig. 1: Amino acid sequence alignment of biochemically characterized Lcp proteins (without signal peptide sequences). Asterics, colons and dots indicate identical or similar residues, respectively.



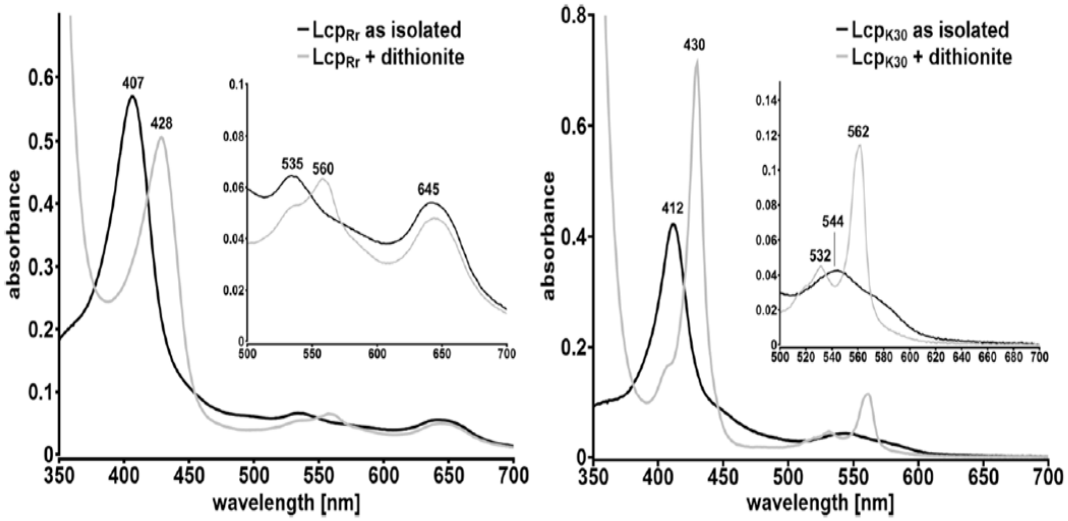
Additional Fig 2: UVvis spectra of Lcp_{K30} and Lcp_{Rr} in the presence of mercaptoethanol. Note, a change of the spectrum in the presence of mercaptoethanol only in case of Lcp_{Rr} but not in case of LcpK30.



Additional Fig 3: Bipyridyl assay of Lcp_{Rr} (black line) in comparison to LcpK30 (grey line). Note, the presence of an absorption maximum at 556 nm typical for a *b*-type cytochrome.



Additional Fig 4: Degradation of rubber pieces by *R. rhodochrous* RPK1 after 0 and 30 days of incubation in shaking flasks with mineral salts medium at 30 °C.

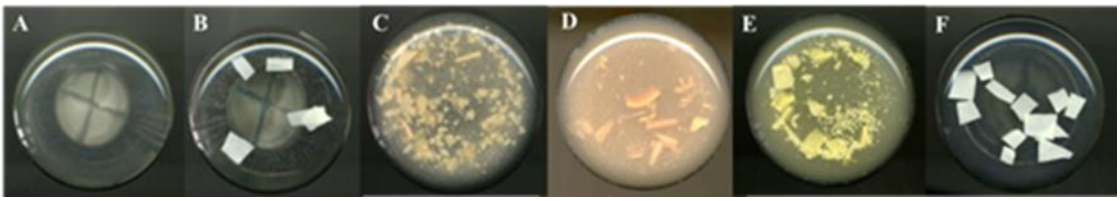


Additional Fig. 5 UVvis spectrum of Lcp_{Rr} and Lcp_{K30} as isolated (black lines) and after reduction with dithionite (grey lines). Both Lcp proteins show a prominent band at 407 (412 nm in case of Lcp_{K30}) that is characteristic for porphyrines. After reduction with dithionite a characteristic shift of the α-band to 428 (430) nm as well as an increase in the Q-band region (560/562 nm) was observable. Assays were repeated at least three times with two separate protein batches. A typical experiment is shown.

D Additional Experiments

D1. Taxonomic Identification of Key Rubber Degrading Bacterial Isolate In Degradation Process

Isolate N1, N2 and N3 were found at all interval period in the degradation process, which did not form clearing zones on an overlay rubber agar plate. These characteristics in combination with the 16s rRNA gene sequence, revealed that the isolate N1, N2 and N3 were identified to *Rhodococcus rhodochrous* strain RPK1 99.7% identity, *Gordonia* sp. R3 100% identity and *Pseudomonas* sp. DG17 96% identity, respectively.



Appendix Fig. 1 Rubber degradation in liquid MSM medium supplemented with sterile rubber pieces incubated for 30 days (A) MSM medium with mixed culture (negative control), (B) MSM medium with sterile rubber pieces (negative control), (C) inoculated with mixed culture, (D) inoculated with isolate N1, (E) inoculated with isolate N2, (F) inoculated with isolate N3.

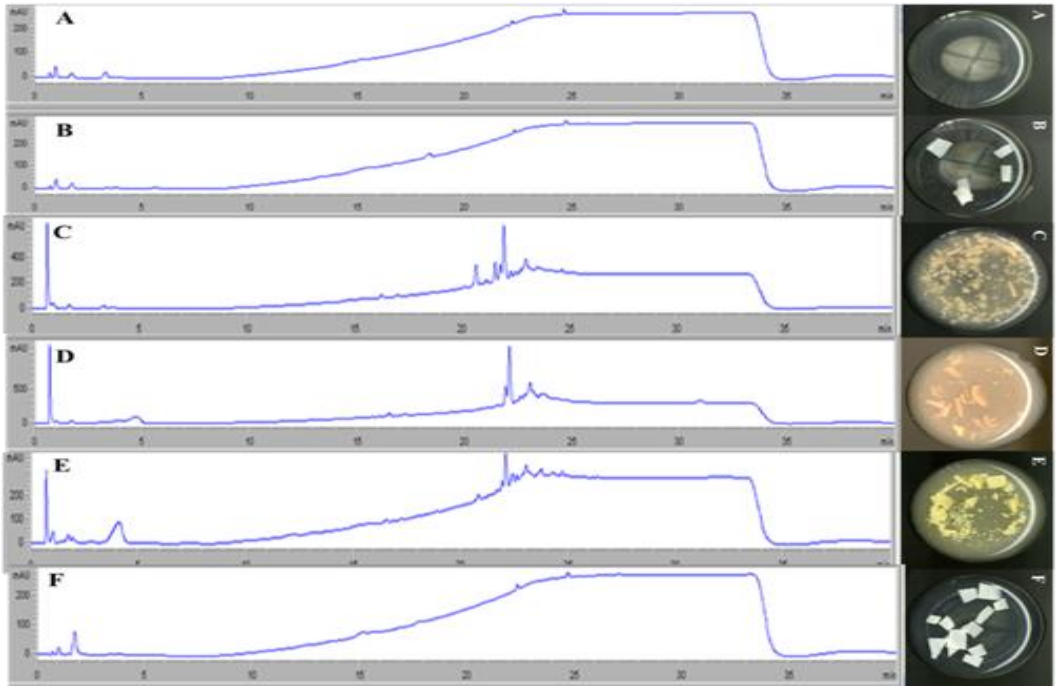
D2. Biodegradation of vulcanized rubber gloves by bacterial mixed cultures.

There are amazing examples of consortium ST608 that readily degrade vulcanized rubber with high potential ability (Appendix Fig. 1). However, it is difficult to mornitor their synergistic reaction between each strain. Thus, for preliminary experiment, we designed to identify the key strain that found throughout the degradation process and detected it cleavage products via HPLC, compared the data with cleavage products of purified Lcp and ODTD (Birke et al., 2015).

Rubber cleavage product during degradation process was determined via HPLC assay (Watcharakul et al., 2016). On the rubber degradation process, microbial secretes rubber degrading enzyme to attack the C=CH bonds of isoprene and degrade rubber with accumulation of products (Shah et al., 2013), which was presented as a large series of degradation products of different chain lengths of carbon, and the tetra-isoprenoid analog (C_{20} oligo-isoprenoid) is the smallest main end product (appendix Fig. 2C) compared with HPLC peak of purified protein cleaving rubber as described in previous report (Watcharakul et al., 2016; Birke et al., 2015). The cleavage products were detected in flask of bacterial mixed culture (appendix Fig. 2C), isolate N1 (designed as a RPK1 after taxonomically identified) (appendix Fig. 2D) and isolate N2 (appendix Fig. 2E), while isolate N3 (appendix Fig. 2F) did not give any of cleavage product as a similar results with negative control in appendix Fig. 2A and appendix Fig. 2B. Moreover, the flask of bacterial mixed culture obviously produced a series of different carbon chain lengths (appendix Fig. 2C) as similar products with activity of purified Lcp (the areas of the first four product peaks at 18.8 to 22.9 min, corresponding to C_{20} to C_{35} terpenoids) as described in previous report (Watcharakul et al., 2016; Birke et al., 2015), while the flask of isolate N1 and N2 were found only C_{35} as major cleavage product (at the area of the 22.9-min peak corresponding to C_{35} hepta-isoprenoid) (appendix Fig. 2D, 2E).

These results indicate that, the potential of bacterial mixed culture significantly is higher than the single isolate, which were observed by physical characteristic of rubber and cleavage products obtained. It is revealed that the synergim of rubber degrading bacteria, in which each different bacterium relies, to some extent, on the abilities of others for survival and together, they facilitate access to all the double bonds hidden within a hydrophobic barrier and also metabolize and accumulating growth inhibitors. Our study have no evidence of rubber disintegration when used of single isolate N3 (*Pseudomonas* sp.) to degrade rubber (appendix Fig. 2F). In the mixed culture flask, isolate N3 was found throughout the experiment for 30 day, which was observed by bacterial plate count on rubber agar plate (Fig. 13). It is possible that the isolate N3 is a weak rubber degrading bacteria and required additional month to degrade rubber and might be one of strains that utilized rubber products during the process to accelerate the rate of degradation. According to

previous study (Linos et al., 2000), they found that *P. aeruginosa*, which is one of the rubber decomposing properties and did not produce any clearing zones (translucent halos) on latex overlay plates as same as a rubber decomposer actinomycetes.



Appendix Fig. **2** Rubber cleavage products detection via HPLC-based incubation with bacteria for 30 days, (a) negative control without rubber glove, (b) negative control without bacteria, (c) mixed culture, (d) isolate N1, (e) isolate N2, (f) isolate N3.

Appendix Table 1: Bacterial strains, plasmids and oligonucleotides used in this study

Strain or plasmid	**Relevant characteristics**	**Reference**
E. coli JM109	Plasmid storage and expression of *lcp*	
E. coli XL1-blue	Transformation strain	Stratagene
Rhodococcus rhodochrous RPK1	Wild type strain, degrades rubber	this study
pUC9::*strep-lcp*$_{K30}$ (SN5339)	cloning vector for *lcp*$_{K30}$, Ap^r	Birke et al., 2015
pUC9::*strep-lcp*$_{Rr}$ (SN5759)	cloning vector for *lcp*$_{Rr}$, Ap^r	this study
p4782.1 (SN3513)	Mobilizable broad host range expression vector, Km^r	Altenbuchner, 1992
p4782.1::*strep-lcp*$_{K30}$ (SN5496)	coding sequence of *strep-lcp*$_{K30}$ under rhamnose promoter control, Km^r	Birke et al., 2015
p4782.1::*strep-lcp*$_{Rr}$ (SN5760)	coding sequence of *strep-lcp*$_{Rr}$ under rhamnose promoter control, Km^r	this study
Oligonucleotides		
Lcp$_{Rr}$-complete_for	GCAGAATCCACATGTCCT	
Lcp$_{Rr}$-complete_rev	CGACAAACCCACAGATGA	
Lcp$_{Rr}$-mature-PstI_for	GGGCCTGCAGCGGCCCTGGAGGTGGTCGCC	
Lcp$_{Rr}$-mature-HindIII_rev	CCGGTAAGCTTTCAGGGATAGTTGGG	
16S-universal-for	GAGTTTGATC(A/C)TGGCTCAG	
16S-universal-rev	GG(C/T)TACCTTGTTACGACT	
16S-Rr-complete_for	CTGGCGCGGTGCTTAAC	
16S-Rr-complete_rev	CAGTAATTCCGGACAACG	

Kanamycin resistance (Km^r), Ampicillin resistance (Ap^r)

VITAE

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List of Publication and Proceeding

Watcharakul, S, Röther, W., Birke, J., Umsakul, K., Hodgson, B., and Jendrossek, D. 2016. Biochemical and spectroscopic characterization of purified Latex Clearing Protein (Lcp) from newly isolated rubber degrading *Rhodococcus rhodochrous* strain RPK1 reveals novel properties of Lcp. *BMC Microbiology* 16(92): 1-13. (Published)

Watcharakul, S., Umsakul, K., and Brian Hodgson. 2016. Biodegradation of vulcanized rubber. *International Conference on Research & Innovation in Food, Agriculture and Biological Sciences (RIFABS-16)*. Phuket, Thailand. December 12-13, 2016. (Proceeding)

Watcharakul, S., Umsakul, K, Röther, W., Birke, J and Jendrossek, D. (2016). Properties of the Latex Clearing Protein from Rhodococcus rhodochrous (LcpRr). *15th International Symposium on Biopolymers (ISBP2016)*. Madrid, Spain. September, 26-28, 2016. (Abstract)