Isolation of Bacteria Able to Degrade Rubber Gloves and Cassava Starch Foam Blended with Natural Rubber Latex

Sirimaporn Watcharakul

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Microbiology

Prince of Songkla University

2012

Thesis Title Isolation of Bacteria Able to Degrade Cassava Starch Foam Blended with Natural Rubber Latex and Rubber Gloves

Author Miss Sirimaporn Watcharakul

Major Program Microbiology

Major Advisor:

…….
(Dr. Kamontam Umsakul)

Co-advisor:

……
(Dr. Brian Hodgson)

Examining Committee:

…….Chairperson
(Assoc. Prof. Dr. Duangporn Kantachote)

……
(Assoc. Prof. Dr.Varaporn Tanrattanakul)

……
(Asst.Prof. Dr. Suchada Chanprateep Napathorn)

……
(Dr. Brian Hodgson)

……
(Dr. Kamontam Umsakul)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Degree of Master of Science in Microbiology**.**

……..
(Prof. Dr. Amornrat Phongdara)
Dean of Graduate School

ชื่อวิทยานิพนธ์ การคัดเลือกแบคทีเรียย่อยสลายถุงมือยางและโฟมแป้งมันสำปะหลังผสมน้ำยางธรรมชาติ

ผู้เขียน นางสาวสิริมาภรณ์ วัชรกุล

สาขาวิชา จุลชีววิทยา

ปีการศึกษา 2554

บทคัดย่อ

จากการคัดเลือกจุลินทรีย์ที่มีความสามารถในการย่อยสลายยาง โดยสามารถใช้น้ำยางธรรมชาติเป็นแหล่งคาร์บอนและแหล่งพลังงานได้ทั้งหมด 119 ไอโซเลท พบ 78 ไอโซเลท สามารถสร้างวงใสรอบโคโลนีได้ในอาหาร Minimal Salt Medium (MSM) ผสมยางธรรมชาติ ทั้งยังสามารถย่อยสลายแป้งได้ เมื่อวัดขนาดรัศมีวงใสบนอาหาร MSM ที่มียางธรรมชาติ พบเพียง 9 ไอโซเลทที่มีขนาดวงใสมากกว่า 3 มิลลิเมตร

เมื่อศึกษาลำดับเบสของ 16s rRNA gene ของไอโซเลท CH13 ซึ่งมีขนาดรัศมีวงใสกว้างที่สุด พบว่ามีความเหมือนกับสายพันธุ์ *Streptomyces coelicolor* ถึง 99% จากการทดสอบการย่อยสลายพอลิเมอร์ พบว่าไอโซเลท CH13 เป็นสายพันธุ์ที่มีประสิทธิภาพในการย่อยสลายพอลิเมอร์ทั้งสองชนิดได้ดี เมื่อนำจุลินทรีย์มาบ่มกับถุงมือยางและพอลิเมอร์ โฟมผสม และทดสอบการย่อยสลายเบื้องต้นด้วย Schiff's reagent พบว่าตัวอย่างเปลี่ยนเป็นสีม่วง แสดงให้เห็นว่าเกิดหมู่แอลดีไฮด์ขึ้นจากการเสื่อมสภาพของพอลิเมอร์ เมื่อศึกษาภายใต้กล้อง Scanning Electron Microscopy (SEM) พบลักษณะการเกาะติดพื้นผิวของจุลินทรีย์ที่แตกต่างกันของพอลิเมอร์ทั้งสองชนิด บนผิวของพอลิเมอร์โฟมผสม จุลินทรีย์สามารถเจริญเติบโตได้ดีมีการเกาะติดบนผิวหน้าอย่างหนาแน่น

ในขณะที่บนผิวหน้าถุงมือยางจุลินทรีย์เกาะติดอย่างเบาบาง แต่อย่างไรก็ตาม ผิวหน้าพอลิเมอร์ ทั้งสองชนิดมีการเปลี่ยนแปลงอย่างเห็นได้ชัด จากการสังเกตพบว่า กลไกในการย่อยสลายพอลิเมอร์โดยการเกาะ ติดของจุลินทรีย์มีความแตกต่างกันเล็กน้อย โดยพื้นผิวของพอลิเมอร์ โฟมผสมนั้นจุลินทรีย์เกาะติดและแทรกผ่านผิวของพอลิเมอร์ ทำให้เกิดรูพรุนขนาดใหญ่ซึ่งเป็นสาเหตุการเสื่อมสภาพของพอลิเมอร์

เมื่อศึกษาการย่อยสลายพอลิเมอร์ โฟม ผสมและถุงมือยางด้วยวิธี Fourier Transform Infrared (FTIR) Spectroscopy พบว่ามีหมู่คาร์บอนิลเกิดขึ้นจากการแตกหักของพันธะคู่ (C=C) ในสายโซ่ของ Cis-1,4(isoprene) ชี้ให้เห็นถึงการเกิดปฏิกิริยา ออกซิเดชั่น นำไปสู่การแตกหักของพันธะคู่ ซึ่งเป็นขั้นตอนแรกของกระบวนการย่อยสลายยางธรรมชาติ

จากการตรวจสอบการย่อยสลายพอลิเมอร์ ทั้งสองชนิด โดยการวัดปริมาณคาร์บอนไดออกไซด์ที่ถูกปลดปล่อยออกมาในระยะเวลา 30 วัน ในอาหาร MSM ที่มี NH_4NO_3 เป็นแหล่งไนโตรเจนที่ความเข้มข้น 1 g/L พบว่าอัตราการปล่อยคาร์บอนไดออกไซด์ของพอลิเมอร์โฟมผสมและถุงมือยางสูงถึง 56.58 และ 10.24 เปอร์เซ็นต์ ตามลำดับ นอกจากนี้พบว่าในกระบวนการย่อยสลายพอลิเมอร์โฟมผสมในระยะเวลา 20 วัน พบว่าแป้งได้หายไปสูงสุดถึง 85 เปอร์เซ็นต์ ในระยะเวลา 20 วัน โดยแป้งได้ถูกเปลี่ยนไปเป็น Oligosaccharides จากนั้นถูกเปลี่ยนไปเป็น Free glucose โดย Amylolytic enzyme ซึ่งวิเคราะห์ได้จากวิธี Soxhlet extraction และการวิเคราะห์หาปริมาณ reducing sugar จากนั้นได้ศึกษาหาสภาวะที่เหมาะสมสำหรับการย่อยสลายพอลิเมอร์ทั้งสองชนิดในระยะเวลา 4 สัปดาห์ พบว่าอัตราการย่อยสลายพอลิเมอร์โฟมผสมและถุงมือยางสูงสุดที่ 96.8 และ 36.5% ตามลำดับ ที่ปริมาณเชื้อตั้งต้น 10^8 cells/mL อุณหภูมิ 30 องศาเซลเซียส พีเอช 7 และความเข้มข้นของ NH_4NO_3 10 g/L

Thesis Title	Isolation of Bacteria Able to Degrade Rubber Gloves and Cassava Starch Foam Blended with Natural Rubber Latex
Author	Miss Sirimaporn Watcharakul
Major Program	Microbiology
Academic Year	2011

ABSTRACT

A total of 119 rubber degrading bacteria were isolated using natural rubber latex as a sole source of carbon and energy. 78 of these isolates formed clear zones (translucent halos) around the colonies on a mineral salt medium containing NR and all degraded starch. 9 of these isolates that produced large radial zones of about 3.0 mm were checked for their stability for degrading NR. The 16s RNA sequence from isolate CH13, that consistently produced the largest clear zone size was 99% similar to *Streptomyces coelicolor*.

Degradation tests, showed that isolate CH13 was highly efficient at degrading both polymers. Polymers incubated with the bacterium and stained with Schiff's reagent produced a purple colour on these surface to provide evidence for the occurrence of degradation products containing aldehyde groups. SEM (Scanning Electron microscopy) observation revealed a different pattern of colonization by the isolate on the 2 different substrates. On the polymer blend, *S. coelicolor* CH13 produced an extensive coverage on the surface. In contrast growth on the rubber gloves strips was sparse. However, there were major changes to the surfaces of the rubber gloves and polymer blend. The strategies used by the bacteria to degrade the 2 polymers differed slightly in that direct penetration of the polymer was only achieved on the polymer blend. Strain CH13 adhered and merged into the polymer blend to form large holes and ultimately caused disintegration.

When fourier transform infrared spectroscopy was applied to the degrading polymers it was demonstrated, that after incubation occurred carbonyl groups were formed and double bonds were broken down (C=C) so the number of cis-1,4(isoprene) bonds decreased, to indicate that there had been an oxidative attack at the double bond which is normally the first metabolic step in the biodegradation of the NR.

Degradation of these polymers was verified by measuring the release of CO_2 over a 30 day period using a mineral salts medium with NH_4NO_3 as the nitrogen source at a concentration of 1 g/L when up to 56.58% of the polymer blend and approximately 10.24% of rubber glove were converted to CO_2. Furthermore, up to 85% of the starch in the cassava starch foam blend with natural rubber latex disappeared over 20 days during the degradation process. The starch was changed to oligosaccharides then free glucose using amylolytic enzymes as demonstrated by Soxhlet extraction result and analysis of reducing sugar levels.

Establishment of the optimum conditions for both polymers over a 4 week period showed that it was possible to achieve a percentage weight loss of 96.8 and 36.5 for the polymer blend cassava starch with NR latex and rubber gloves respectively with an initial inoculum size at 10^8 cells/mL. An incubation temperature of 30°C, a pH of 7 and NH_4NO_3 as a nitrogen source at a concentration of 10 g/L.

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my advisor Dr. Kamontam Umsakul for kindly providing me with the opportunity to pursue an MSc degree at Prince of Songkla University and also for her continuous support.

I am grateful to my co-advisor Dr. Brian Hodgson, Faculty of Pharmaceutical Sciences, Prince of Songkla University for his help and advice during my research and for valuable suggestions and cordial assistance on my thesis writing as well as for teaching me English conversation. Special thanks also to Assoc. Prof. Dr. Varaporn Tanrattanakul, Department of Materials Science and Technology, Faculty of Science, Prince of Songkla University, Thailand for supplying the new polymer and other materials for my research and for her generosity and useful comments. Special thank to Assoc. Prof. Dr. Duangporn Kantachote Head of the Department of Microbiology, Faculty of science, Prince of Songkla University. Songkhla, Thailand for her generosity of her time and comments.

Thanks to all the people in the Polymer Science Laboratory, The Department of Materials Science and Technology, Faculty of Science, Prince of Songkla University for their friendships and their support, especially Miss Wannapa Chumeka for her help and for teaching me all the chemical techniques. In addition, I thank members of the Environmental microbiology laboratory (BT 608) Prince of Songkla University with all my special friends for their warm friendship, companionship and help.

I also thank all my teachers in the Department of Microbiology, Prince of Songkla University for their generosity while teaching me.

Finally, I would like to express my deepest appreciation to my family for their love and support.

Sirimaporn Watcharakul

CONTENTS

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

ASW	=	Artificial Sea Water
°C	=	Degree Celsius
DMSO	=	Dimethyl sulfoxide
DO	=	Dissolve Oxygen
EC	=	Electrical Conductivity
EtOAc	=	Ethyl acetate
FTIR	=	Fourier Transform Infrared spectroscopy
g	=	Gram
MSM	=	Mineral Salt Medium
MeOH	=	Methanol
mg	=	Milligram
ml	=	Milliliter
Na_2SO_4	=	Sodium sulfate
NMR	=	Nuclear Magnetic Resonance
NR	=	Natural Rubber
OD	=	Optical Density
pm	=	Part Per Million
PMS	=	Phenazine methoxysulfate
TCBS	=	Thiosulfate Citrate Bile Salts Sucrose

LIST OF ABBREVIATIONS AND SYMBOLS (CONT.)

TLC	=	Thin Layer Chromatography
TSA	=	Tryptic Soy Agar
TSB	=	Tryptic Soy Broth
µm	=	Micrometer
µl	=	Microliter

CHAPTER 1

Introduction

There are very few aspects of our daily lives that now do not involve plastics. Plastic foams are just one of the many forms of plastics seemingly essential for our normal activities such as packaging, drink cartons and food containers. Foams from polystyrene, polyethylene, polyurethane etc. are macromolecules normally derived by synthesis from petroleum-based compounds. Foams are almost crystalline and their molecules have high molecular weights, and this makes attacks by enzymes from microbes very difficult. They are thus biodegraded very slowly or not at all and give rise to non-degradable waste polymers that have accumulated in the environment in large amounts causing a phenomenon in the oceans termed "white pollution". There is a need to produce alternative polymers that are more environmentally-friendly and biodegradable hence there is pressure to develop foams made from biopolymers, that can be made into biodegradable plastic foams.

Research workers in many countries are interested in developing biopolymers made from biodegradable plastic. In 2003 Carvalho *et al*. studied the characteristics of a number of thermoplastic starch/natural rubber polymer blends prepared directly from natural latex and corn starch. These polymer blends had a reduced modulus of tensile strength, and were less brittle than thermoplastic starch alone. Nevertheless, the shelf life of starch/natural rubber polymer blends can be virtually unlimited, and they are difficult to process. Thai research workers have recently improved the properties of cassava starch foams blended with natural latex. In their studies high concentrations of rubber were used. Although the blends had good properties such as high tensile strength and impact strength, conversely, they were still difficult to degrade. At present, research workers are developing new methods to degrade polymer blends. For instance in Brazil there has been research on the degradation of polychloroprene/natural rubber blends containing $FeCl_3 \cdot 6H_2O$ using the photo-Fenton process. They found that films containing $FeCl_3 \cdot 6H_2O$ are less stable than those without $FeCl_3 \cdot 6H_2O$; however the degradation rates were still too slow (Freitas *et al*., 2008). Other research has been

concerned with the biodegradation of polymers via microorganisms. Hamad *et al*. 2005 reported that the degradation of polyethylene was catalysed by *Brevibacillus borstelensis*, *Rhodococcus rubber* was isolated from soil by Siven *et al*., 2006. *Schlegelella thermodepolymerans* (Roman *et al*., 2004) also degraded poly(3-hydroxybutyrate) a biopolymer that it produces.

One thing of importance for any new polymers is to check the biodegradability of the materials they contain so they do not continue to add to the "white pollution". This shown to be slowly degraded are undesirable. It is possible that cassava starch foam blended with natural rubber latex is physiologically inert, even though rubber latex and starch are both natural products. However one "natural law" claims that any compound that is produced by living things can be degraded by living things as the enzymes that make them are simply catalysts. In theory one just needs to provide conditions where the reverse reaction to "synthesis" i.e. "degradation" is favoured and dominates "synthesis". This may occur by simply adding one new enzyme to the pathway. In the environment, NR/ST foams blended can be degraded by oxidative degradation to a mixture of alcohols, carboxylic acids, hydroxyl-carboxylic acids all glucose with a relatively lower molecular mass (Griffin *et al*., 1994) that can be used by many microorganisms as a source of energy.

Starch itself is one of the most commonly used energy sources for microbes and is readily mineralized. In theory polymer blends that include starch should therefore be degraded via microorganisms better than other managed wastes. Some enzymes released by microorganisms can split the back-bone of the polymer chains, to produce smaller molecules that are then assimilated aerobically to produce more microorganism instead of being mineralized to carbon dioxide. This reduces the amount of carbon dioxide released that might have resulted from mineralization. Incomplete degradation can also produce compost a value added product that will, when added to soil, have a beneficial effect on the environment. Degradation rates of any polymer can be greatly affected by its morphology as does the type of microorganism involved in this process. So producing the right polymer and isolating the right microorganism is the best way to be "green".

There has been an increased interest in the isolation and study of microorganisms able to degrade any new polymer and this information should

accompany its manufacture to ensure that the polymer will be environmentally-friendly, and have less impact on the ecology by being eliminated by biodegradation over a reasonable period of time. The goal of this work, was to isolate microorganisms able to readily degrade a new biopolymer, made by the Bioplastic Research Unit at Prince of Songkla University, i.e cassava starch foam blended with natural rubber latex.

The objectives of this study

1. To isolate and identify bacteria able to degrade cassava starch foam blended with natural rubber latex and rubber gloves.
2. To characterize the mechanisms involved in the degradation process.
3. To establish optimum conditions for the biodegradation rates.

Perceived advantages

Isolation of bacteria with an ability to degrade cassava starch foam blended with natural rubber latex and rubber gloves will improve the ability to ensure that these polymer foams can be degraded and removed from the environment.

The scope of this thesis

Develop methods for screening, isolating and identifying bacteria able to degrade cassava starch foam blended with natural rubber latex prepared at PSU, Bioplastics Research Unit. As the rubber might be the most difficult part to degrade, rubber gloves were also used to confirm that any isolated microbe could degrade NR products or even to facilitate enrichment for NR degrading microbes. Parameters investigated included changes in polymer morphology by SEM, examining starch loss by Soxhlet extraction and assays for appropriate starch degrading enzymes. Biodegradation of rubber was investigated by the detection of aldehyde groups in the polymers and change of molecular weight. Mineralisation was also investigated by measuring the release of CO_2 during the degradation process. Optimum conditions most for the critical the parameters were also investigated.

CHAPTER 2

Literature reviews

2.1 Biopolymers

The development of environmentally biodegradable polymers represents a very important goal in the drive towards responsible waste management and environmental protection.

2.2 Definitions

The definitions developed by the American Society for Testing and Materials (ASTM) for degradable, biodegradable, hydrolytically degradable and oxidative degradable plastics are probably the most widely accepted as written or in some slightly modified form.

Degradable plastic, a plastic designed to undergo a significant change in its chemical structure under specific environmental conditions resulting in a loss of some properties as measured by standard test methods appropriate to the polymer.

Biodegradable plastic, a degradable plastic in which the degradation results from the action of naturally-occurring microorganisms such as bacteria, fungi and algae.

Hydrolytically degradable plastic, a degradable plastic in which the degradation results from hydrolysis promoted either by chemical or enzymatic processes.

Oxidative degradable plastic, a degradable plastic in which the degradation results from oxidation.

Photodegradable plastic, a degradable plastic in which the degradation results from the action of natural sunlight (oxygen is also required).

2.3 Biopolymers from renewable resources

Recently scientists have started to look for plastics designed to be susceptible to microbial attack, to make them degradable in an active microbial environment. Biodegradable plastics or biopolymer have opened the way for consideration of new strategies of waste management for degrading wastes under environmental conditions (Augusta *et al.,* 1992; Witt *et al.,* 1997). Many renewable resources are of interest as alternatives for generating energy and as raw materials for producing biodegradable polymers and plastic products. The term "polymers from renewable resources" refers to natural products that are polymeric in character and produced by living organisms or can be converted to polymeric materials by conventional or enzymatic synthetic procedures (Scott, 2002) (Figure 1). Thus under that heading one can include natural polymers used as direct feedstock for plastic production as well as for artificial polymers obtained by chemical modification of preformed natural polymers.

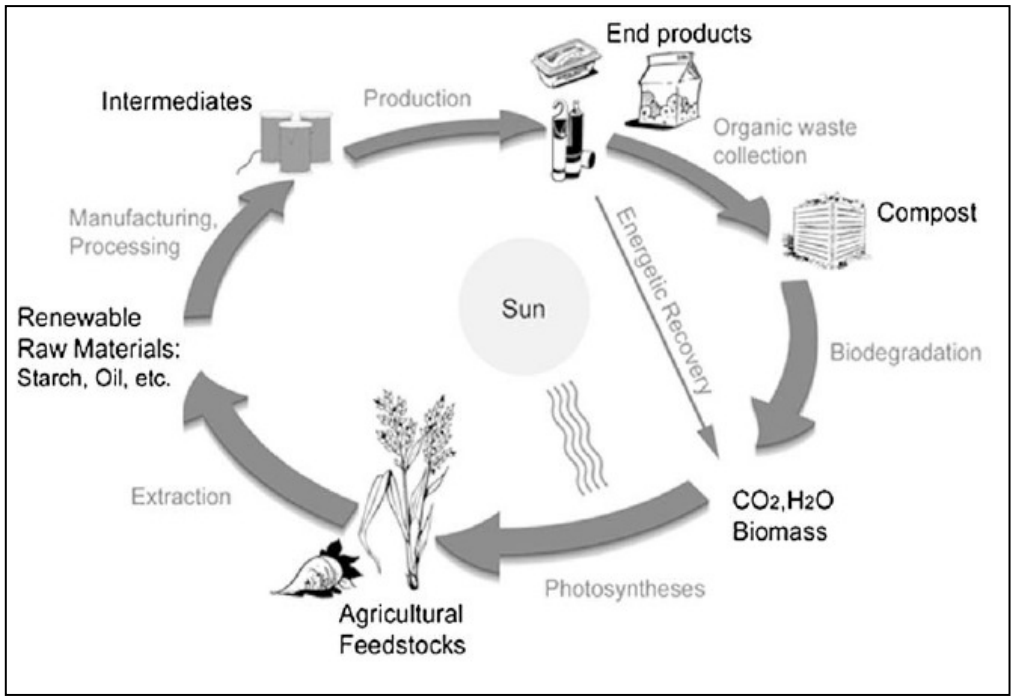


Figure 1. Life cycle of bioplastics

(Siracusa *et al*., 2008; www.european-bioplastics.org)

The biodegradable plastics: polyesters, polyhydroxyalkanoates (PHA), polylactides, polycaprolactone, aliphatic polyesters, polysaccharides and copolymers or blends of these, have been developed successfully over the last few years (Table 1), for example, Poly(3-hydroxyalkanoates) (PHA) are bacterial storage compounds of carbon and energy that can be accumulated in many bacterial cells such as *Schlegelella* sp. KB1a (Romen *et al*., 2004). The most know of these are poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate). The key properties of PHAs are their biodegradability, apparent biocompatibility, and their production from renewable resources. The global interest in PHAs is high as they can be used in different packaging materials, medical devices, disposable personal hygiene products and also have some agricultural applications as a substitute for synthetic polymers like polypropylene, polyethylene etc. (Ojumu *et al*., 2004: Lee, 1996).

Then there are NR-g-Poly methyl methacrylate-air dried sheets with cassava starch compounds (NR-g-PMMA–ADS–cassava starch compounds) that have been synthesized and used with the rubber component in a compounding formulation for packaging food (Nakason *et al*., 2005). Starch-based products can incorporate natural rubber latex to foam baked starch foams based on wheat, potato, and waxy corn starches (Shey *et al*., 2006). Bioplastics (biopolymers) obtained from the growth of microorganisms or from plants which are genetically engineered to produce such polymers are likely to replace currently used plastics at least in some fields (Lee, 1996). In the past 10 years, several biodegradable plastics have been introduced into the market. However, none of them is efficiently biodegradable in landfills. For this reason, none of the products has gained widespread use. At present, biodegradable plastic represents just a tiny proportion of the market as compared with conventional petrochemical derived materials. Bioplastics will become comparatively cheaper and competitive while oil prices continue to rise. Although not widely used today, plastic shopping bags can be made from polylactic acid (PLA) a biodegradable polymer derived from lactic acid. This is one form of a vegetable waste-based bioplastic.

Table 1. Uses of biodegradable plastics (Shah *et al*., 2008)

Plastics	Current Uses
Polyglycolic acid (PGA)	Specialized applications; controlled drug releases; implantable composites; bone fixation parts
Polylactic acid (PLA)	Packaging and paper coatings; other possibe markets include sustained release systems for pesticides and fertilizers, mulch films, and compost bags
Polycaprolactone (PCL)	Long-term items; mulch and other agricultural films; fibers containing herbicides to control aquatic weeds; seedling containers; slow release systems for drugs
Polyhydroxybutyrate (PHB)	Products like bottles, bags, wrapping film and disposable nappies, as a material for tissue engineering scaffolds and for controlled drug release carriers
Polyhydroxyvalerate (PHBV)	Films and paper coatings; other possible markets include biomedical applications, therapeutic delivery of worm medicine for cattle, and sustained release systems for pharmaceutical drugs and insecticides
Polyvinyl alcohol (PVOH)	Packaging and bagging applications which dissolve in water to release products such as laundry detergent, pesticides, and hospital washables
Polyvinyl acetate (PVAc)	Adhesives, the packaging applications include boxboard manufacture, paper bags, paper lamination, tube winding and remoistenable labels

PLA materials biodegrade quickly under composting conditions and do not leave toxic residue. However, bioplastics can have their own environmental impacts depending on the way it is produced (http://en.wikipedia.org). There is also an urgent need to develop efficient microorganisms to produce and degrade and their products to solve this crucial global issue of "white pollution" (Kathiresan, 2003).

In summary a small number of new bioplastics available that are known to be biodegradable and are being used for specific purposes.

2.4 Standard testing methods for degradation (Shah *et al*., 2008)

2.4.1 Visual observations

The evaluation of visible changes that can occur in plastics during degradation can be performed in many ways. Observations used to describe degradation include roughening of the surface, formation of holes or cracks, de-fragmentation, changes in color, or formation of bio-films on the surface. These changes do not prove the presence of a biodegradation process in terms of metabolism, but the parameter of visual changes can be used as a first indication of any microbial attack. To obtain information about the degradation mechanism, more sophisticated observations can be made using either scanning electron microscopy (SEM) or atomic force microscopy (AFM) (Ikada, 1999). After an initial degradation, crystals can appear on the surface; this could be explained by a preferential degradation of an amorphous polymer fraction, and erosion of the slower-degrading crystalline parts out of the material. In another investigation, (Kikkawa *et al*., 2002) used AFM micrographs of enzyme degraded PHB films to investigate the mechanism of surface erosion. A number of other techniques can also be used to assess the biodegradability of polymeric material. These include; Fourier transformed infrared spectroscopy (FTIR), differential scanning colorimetry (DSC), nuclear magnetic resonance spectroscopy (NMR), X-ray photoelectron spectroscopy (XPS), X-ray Diffraction (XRD), contact angle measurements and water uptake. Use of the following techniques are often used to follow the biodegradation of a polymer.

2.4.2 Weight loss measurements: determination of residual polymer

The loss of the polymer mass is widely measured in degradation tests (especially in field- and simulation tests), although no direct proof of biodegradation is obtained. Problems can appear with incorrect cleaning of the specimen, or if the material disintegrates excessively. The samples can be placed into small nets to facilitate recovery; this method is used in full-scale composting processes. A sieving analysis of the matrix surrounding the plastic samples allows for a better quantitative determination of the disintegration characteristics. For a finely distributed polymer samples (e.g., powders), the decrease in residual polymer can be determined by an adequate separation or extraction technique (polymer separated from biomass, or polymer extracted from soil or compost). By combining a structural analysis of the residual material and the presence low molecular weight intermediates, detailed information regarding the degradation process can be obtained, especially if a defined synthetic test medium is used (Witt *et al*., 2001).

2.4.3 Changes in mechanical properties and molar mass

In the case of visual observations, changes in material properties cannot be proved directly to be due to metabolism of the polymer material. However, changes in mechanical properties are often used when only minor changes to the mass of the test specimen are observed. Properties such as tensile strength are very sensitive to changes in the molar mass of polymers, which is also changed directly and acts as an indicator of degradation (Erlandsson *et al*., 1997). While, for an enzyme-induced depolymerization the material properties only change if a significant loss of mass is observed (the specimen becomes thinner because of the surface erosion process; the inner part of the material need not be initially affected by the degradation process, for an abiotic degradation process which often takes place over the entire material and includes the hydrolysis of polyesters or oxidation of polyethylenes) the mechanical properties may change significantly, although almost no loss of mass due to solubilization of the degradation intermediates occurs at this stage. As a consequence, these types of measurements are often used for materials where abiotic processes are responsible for the first degradation step (Breslin, 1993; Tsuji and Suzuyoshi, 2002).

2.4.4 CO_2 evolution/O_2 consumption

Under aerobic conditions, microbes use oxygen to oxidize carbon and form carbon dioxide as one of the major metabolic end product. Consequently, the consumption of oxygen (respirometric test) (Hoffmann *et al*., 1997) or the formation of carbon dioxide are good indicators for polymer degradation. Due to the normally low amount of other carbon sources present in addition to the polymer itself, when using synthetic mineral media, only a relatively low background respiration must be identified, and the accuracy of the tests is usually good. In particular, the type of analytical methods, especially for the determination of CO_2 have been modified. Besides conventional trapping of CO_2 in $Ba(OH)_2$ solution, followed by manual titration, infrared and paramagnetic O_2 detectors can also be used to monitor O_2 and CO_2 concentrations in the air stream. Although, the automated and continuous measurements have advantages, they also have disadvantages. For example, the exact air flow must be measured, the signals of the detectors must be stable for long periods of time and, if slow degradation processes are to be determined, the increase of CO_2 concentration or the fall in O_2 concentration to be detected is very small, thereby, increasing the likelihood of systematic errors. Under these circumstances, other concepts (e.g., trapping CO_2 in a basic solution, ±pH 11.5) with continuous titration or detection of the dissolved inorganic carbon (Pagga *et al*., 2001) may be useful alternatives. Other attempts to overcome problems with CO_2 detection are based on non-continuously aerated, closed systems. Here, either a sampling technique in combination with an infrared-gas analyzer (Calmon *et al*., 2000) or a titration system (Mueller, 1999) can be applied. Another closed system with a discontinuous titration method has been described by Solaro *et al.,* (1998). Tests using small closed bottles as degradation reactors and analyzing for the CO_2 in the headspace (Itavaara and Vikman, 1995) or by the decrease in dissolved oxygen (closed-bottle test) (Richterich *et al*., 1998) are simple and relatively insensitive to leakages, but may cause problems due to the low amounts of material and inoculum used. Although used originally in aqueous test systems for polymer degradation, CO_2 analysis was also adapted for tests in solid matrices such as compost (Pagga, 1998), and this method has now been standardized under the name, controlled composting test (ASTM, 1989; 1992). For

polymer degradation in soil, CO_2 detection proved to be more complicated than in compost because of slower degradation rates that led not only to long test durations (up to 2 years) but also low CO_2 evolution as compared to that from the carbon present in soil. One means of overcoming problems with background CO_2 evolution from the natural matrices compost or soil is to use an inert, carbon-free and porous matrix, wetted with a synthetic medium and inoculated with a mixed microbial population. This method proved practical for simulating compost conditions (degradation at ~60 °C) (Bellina *et al.,* 2000), but has not yet been optimized for soil conditions.

2.4.5 Radio labeling

In contrast to residue analysis, net CO_2 and $^{14}CO_2$ evolution measurements are simple, non-destructive and measure ultimate biodegradation. If appropriately ^{14}C labeled test material is available, the measurements and their interpretations are relatively straightforward. Materials containing a randomly distributed ^{14}C marker can be exposed to selected microbial environments. The amount of ^{14}C carbon dioxide evolved is estimated using a scintillation counter. This method is not subject to interference by biodegradable impurities or additives in the polymer. Biodegradability investigations using this technique for polymeric materials in different microbial environments show a high degree of precision and consistency (Sharabi and Von, 1993). However, labeled materials are expensive and not always available. The licensing and the waste disposal problems connected with radioactive work may also be a drawback.

2.4.6 Clear-zone formation

A very simple semi-quantitative method is the so-called clear-zone test. This is an agar plate test in which the polymer is dispersed as very fine particles within the synthetic medium agar; this results in the agar having an opaque appearance. After inoculation with microorganisms, the formation of a clear halo around the colony indicates that the organisms are at least able to depolymerize the polymer particles. This method is usually applied to screen for organisms that can degrade a certain polymer (Nishida and Tokiwa, 1993; Abou- Zeid, 2001), but it can

also be used to obtain semi-quantitative results by analyzing the growth of clear zones (Augusta *et al*., 1993).

2.4.7 Controlled composting test

The treatment of solid waste in controlled composting facilities or anaerobic digesters is a valuable method for treating and recycling organic waste material (Biological Waste Management Symposium, 1995). Composting of biodegradable packaging and biodegradable plastics is a form of recovery of waste that can cut the increasing need for new land filling sites. Only compostable materials (in other words biodegradable) can be recycled through biological treatment, since materials not compatible with composting could decrease the compost quality and impair its commercial value. The optimum environmental conditions for composting are the following: high temperature (58°C); aerobic conditions; proper water content (about 50%). Compost used as a solid matrix, requires a source of thermophilic microorganisms (inoculum), and a source of nutrients. The test method is based on the determination of the net CO_2 evolution, i.e. the CO_2 evolved from the mixture of polymer compost minus the CO_2 evolved from the compost control (blank) tested in a different reactor (Bellina *et al*., 1999). A very important requisite is that the packaging material under study must not release toxic compounds into the compost during degradation, which could hinder plants, animals, and human beings by entering the food chain (Tosin *et al*., 1998).

In summary there are a wide variety of standard methods available for use to measure biodegradation depending on purposes.

2.5 Bioplastic symbols

Bioplastic symbols that are stamped on products to help consumers identify products and packaging made from bioplastics are shown in Figure 2.

Figure 2. Symbols used to identify biopolymers

(http://www.machine marketplace.com/new-bioplastic-symbol.html)

2.6 Introduction to starch and natural rubber for use as biopolymers

2.6.1 Starch

Starch can be inexpensive, is available from many plants in Thailand and Asia. It is produced in excess of current market requirements. It is a major form of carbohydrate storage in many plants, and can be a cheap waste product from many commercial process. It is the principal component of most seeds, tubers and roots and is produced commercially from corn, wheat, rice, tapioca, sago and other sources. Most commercial starch is produced from corn that is comparatively cheap and abundant throughout the world. Wheat, tapioca and potato starch are produced on a smaller scale and at higher prices. Starch is totally biodegradable in a wide variety of environments and allows for the development of totally degradable products for specific market demands. In recent years this has resulted in a renewed interest in starch-based plastics. In the past, studies of starch esters and ethers wer abandoned for most applications due to the inadequate properties of these materials in comparison with cellulose derivatives. More recently, starch graft copolymers, starch plastic composites and starch itself have been proposed as alternative plastic materials so there has been a renewed interest in starch based polymers.

Starch is a polymer of the six-membered carbon ring of glucose units (glucopyranose). The majority of starch molecules (up to 100% in waxy starches, 72% in normal maize starch and 80% in potato starch) have a highly branched

structure, known as amylopectin. Amylopectin molecules are composed of chains of α-D-glucopyranosyl units joined by α-1-4 linkages with branches formed by joining these chains with α-1-6 linkages (Figure 3).

The branch chains are present in a double helical, and can produce crystalline structures. The average branch chain length is 20-30 glucosidic units with an average degree of polymerization (DPn) that ranges between $3x10^5$-$2.5x10^6$ (Mn $5x10^7$-$2x10^8$ Dalton) (BeMiller, 1994). The short branches in amylopectin are the source of the crystallinity of starch. The amylopectin structure is shown in Figure 3.

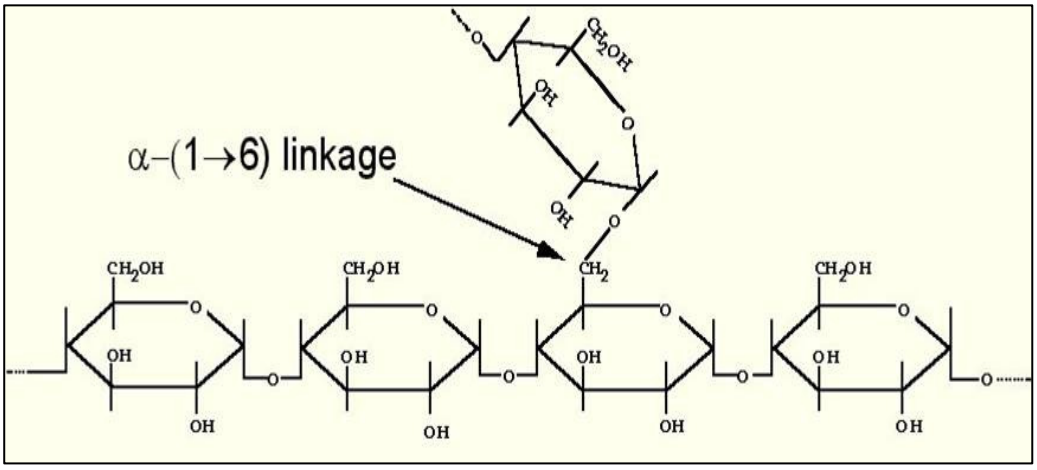


Figure 3. Schematic representation of amylopectin structure (http://www.cheng.cam.ac.uk/research/groups/polymer/RMP/nitin/Starchstructure)

The other constituent of starch is known as amylose, a primary linear molecule with very few branches; consisting of repeating units of 1-4 α-linked glucose monomers. In certain high amylose varieties, the amylose content can be as high as 50 or 70%. Its DPn is comprised of between 200-220 x 10^3 depending on the source and method of attainment. Amylose can have several conformations, its structure is shown in Figure 4.

The ratio of amylose and amylopectin in natural starch varies according to the starch source. The chemical structures of starch, such as the molecular size of the amylose, numbers and lengths of branch-chain amylopectin and the proportion of amylose to amylopectin do affect the functional properties of starch.

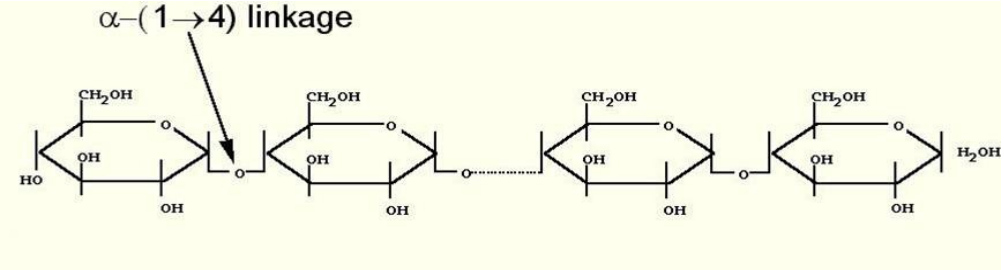


Figure 4. Schematic representation of amylose structure

(www.cheng.cam.ac.uk/research/groups/polymer/RMP/nitin/Starchstructure.html)

Amylopectin interacts with other molecules more effectively and amylose generates greater viscosity and gel strength.When the amylose content of the starch increases, the viscosity and gel strength of the starch paste also increases. Amylose alone and high-amylose maize starch, produce strong tough films. Starch is largely utilized, other than for food, especially in the U.S. and Brazil, for ethanol production by a fermentation process, that produces glucose as an intermediate product and many different end products. Starch also finds wide usage in several non-food sectors such as sizing and coating of papers, adhesives, thickeners, and as an environmentally friendly additive in composite materials. Appropriate selection of chemical reagents allows for the introduction of anionic or cationic charges into the starch molecules. The modification of starch and the properties and uses of such modified starches have been reviewed several times. Several polyhydroxide compounds have been developed from starch for industrial applications that are less expensive than comparable products made from crude oil. Starch can be easily converted to glucose from which a variety of cyclic and acyclic polyols. aldehydes, ketones, acids, esters and ether can be obtained. Recently a wood adhesive has been prepared from crosslinked cornstarch and polyvinyl alcohol with hexamethoxylmelamine in the presence of citric acid as a catalyst (Scott, 2002).

Modified starch finds several applications in industry, such as cationic starches for paper treatment. Thus starch is essential for paper making applications. Since starch is less crystalline than cellulose it is more susceptible to chemical modification and therefore more vulnerable to degradation during modification.

Research into starch modifications have mostly focused on improving the starch moisture resistance without losing its more favorable characteristics such as ease of degradation and its relatively low price.

Starch esters represent an important class of starch derivatives and this aspect has been recently reviewed by Tessler and Billmers (1996). Starch esters can be produced by an aqueous process, at low alkalinity under a controlled pH and low temperature reactions. However these processes usually achieve only a fairly low degree of substitution (DS). Starch acetates with a DS of 2.4 or higher cannot be biodegradable as they are more like cellulose, while intermediate DS acetate are more easily biodegradable. Most commercially used starch derivates have a DS of less than 0.2. Pure amylose starch is considered to be the most desirable precursor for starch-ester based thermoplastics since amylopectin has an adverse impact on the mechanical and physical properties of these derivatives. Starch can react with organic anhydrides in water to yield starch esters, such as starch acetate that has been produced commercially by this process (Scott, 2002).

2.6.2 Natural rubber

There are many plants that produce rubber-like substances as a milky colloidal suspension called latex, that consists predominantly of polyisoprene $(C_5H_8)_n$. However, only natural rubber from *Hevea braziliensis* and guayule rubber from *Parthenium argentatum* are used commercially. Natural rubber (NR) from *Hevea braziliensis* contains 93-95% *cis*-1,4-polyisoprene (Figure 5). NR, as a renewable natural resource, has many excellent properties, such as outstanding resilience, high strength and good processing-ability (Griffin *et al*., 1994). However, as an unsaturated polymer, NR will gradually degrade at a high temperature or when exposed to oxygen, ozone or ultraviolet light, which has a significant negative effect on its special applications (Zhou *et al*., 2001).

It was in 1860, that natural rubber was found (by dry distillation) to be made up of C_5H_8, isoprene units. In 1905, ozonolysis studies showed that the isoprene unit contained a double bond, but it was not until the 1920s –regarded as the early

period of polymer science, that natural rubber was concluded to be a giant molecule composed of isoprene units. The geometric isomerism of the isoprene units was determined by X-ray diffraction studies in 1942, i.e., the double bonds in natural rubber and guayule rubber are in the *cis*-configuration, whereas those in *Gutta percha* and balata rubber are in the *trans*-configuration (Figure 6). Early ^{1}H-NMR (nuclear magnetic resonance) studies on the geomertric isomerism of natural rubber concluded that natural rubber is composed exclusively of *cis*-isoprene units linked to each other by 1,4-addition. The content of other isomeric isoprene units is less than the limits of detection. Studies using ^{1}H-NMR and ^{13}C-NMR also showed that guayule rubber has almost the same geometric isomerism as that of Hevea rubber (Griffin *et al*., 1994).

Many naturally occurring polymers contain this same uninterrupted sequence of carbon atoms. The most studied and best understood of these is still natural *cis*-poly(isoprene) (NR), synthesized by the rubber tree *Hevea braziliensis*. An identical molecule can nowadays be synthesized in industrial chemical plants by addition polymerization from isoprene.

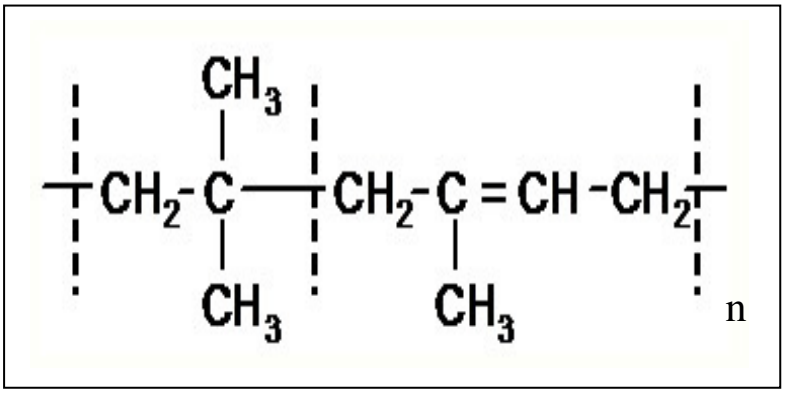
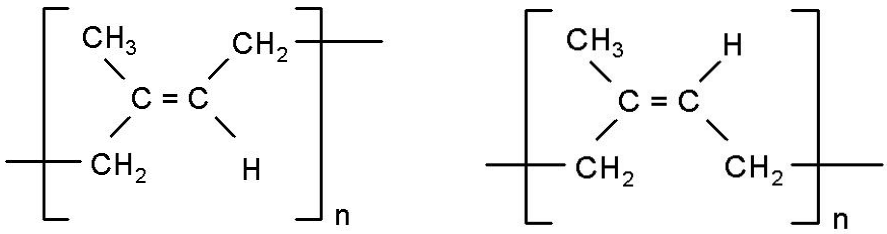


Figure 5. Basic structure of polyisoprene unit
(http://rubber.sc.mahidol.ac.th/rubbertech/terminology_3.htm)



trans-1,4 cis-1,4

Figure 6. Chemical structure of poly(*cis*-1,4-isoprene) and poly(*trans*-1,4-isoprene) (http://rubber.sc.mahidol.ac.th/rubbertech/terminology.htm)

Natural rubber was one of the first important industrial polymers. A particularly unique and desirable attribute of rubber was its rebound resilience even though this could be rapidly lost in a tropical environment. Furthermore, rubber latex products were attacked by microorganisms, which led to a more general loss of mechanical properties and to eventual bio-assimilation in the soil environment. To overcome these limitations of NR and to expand its application, modifications to NR was crucial. Various methods have been employed to modify NR. One of the ways involves a chemical modification, in which other groups or atom are introduced onto the NR molecular chains, and such derivatives include epoxidized NR (Saito *et al*., 2007; Ratnam *et al*., 2001), chloridized NR, grafted NR (Lu *et al*., 2002; Li *et al*., 2003), hydrogenated NR, cyclized NR and so forth (Xue and Fu, 2006). Another simple and economical way is to blend in intensifiers or fillers such as black carbon, white carbon, clay and calcium carbonate with NR (Saad *et al*., 1995; Gao *et al*., 2007). With the gradual loss of petroleum reserves and the higher demands for environmental protection, starch, as a natural, cheap, abundant and biodegradable resource, has also been identified as a useful molecule to link with NR (Yue *et al*., 2005; Nakason *et al*., 2005).

2.6.3 Polymer blends

Polymer blends or polymer mixtures are materials with two or more polymers blended together to create a new material with different physical properties . The rate of degradation of polymer blends is initially controlled by the degradation of

the more readily biodegradable component. The initial degradation process interferes with the structural integrity of the polymer and increases the surface area considerably for enzyme attack on more resistant areas. The exposure of the remaining polymer to microbes and secreted degradative enzymes is then enhanced. Work has been carried out to understand the degradation mechanisms of different polymer blends and their degradation products by microorganisms, for instance, starch/polyethylene blends, starch/polyester blends, starch/PVA blends, polylactic acid (PLA) (renewable resource) polyester and starch/rubber blends. The most studied and modified blend is starch/rubber. The mechanical properties of NR combined with starch are dominated by many factors, such as the dispersing ability, the interfacial combination and the type of starch. As the particle size of starch is very big (1000-5000 nm), and starch contains many hydroxyl groups with strong polarity, its compatibility with NR is poor. Hence, the mechanical properties of a composite prepared by direct loading of starch will deteriorate. A large amount of work has been conducted to improve the mechanical properties of starch/NR composites. In 1971s, Buchanan *et al*. prepared crosslinked starch with xanthide-styrene butadiene rubber (SBR) master batches. It was demonstrated that the crosslinked starch xanthide could improve the fatigue life and abrasion resistance, when used to partially replace carbon black in a premium tread for tyres (Buchanan *et al.,* 1971; Buchanan *et al.,* 1974). The Good year company employed modified corn starch which was called the biological filler polymer to partly replace traditional fillers like black carbon or white carbon to improve the properties of the tyres. Those tyres reinforced with corn starch possess many advantages, such as light quality, low rolling resistance, low noise and low emission of carbon dioxide (The Good Year Tire and Rubber company, 2007). Carvalho *et al.* (2003) prepared a starch/NR composite with rubber latex and starch paste by a co-coagulation method. It was found that the co-coagulation method can decrease the particle size of starch in NR to less than 1000 nm, and the mechanical properties of the composite is much better than the composites made by blending. Rouilly *et al*. (2004) prepared a composite by blending dimethylaminoethyl methacrylate grafted latex and starch. Nakason *et al*. (2005) synthesized a grafted copolymer by grafting NR with methyl methacrylate, and then blending the copolymer with NR and cassava starch. The results showed that the induction period

of cure for the composite decreased with the increase of cassava starch loading. The micrographs of starch and the composite are shown in Figure 7.

In summary the chemical and physical properties of starch and natural rubber before and after any chemical modifications are conductive to the formation of mixed polymers with many different properties.

2.7 Biodegradation of polymers: Mechanisms of structural modifications and polymer degradation

The deterioration of polymer surfaces is an interfacial process. It is controlled by the conditions prevailing directly at their surfaces. If microorganisms are involved in this process, they attach themselves to, and colonize, the surfaces in the form of biofilms. Thus, polymer biodeterioration can be considered as a biofilm problem.

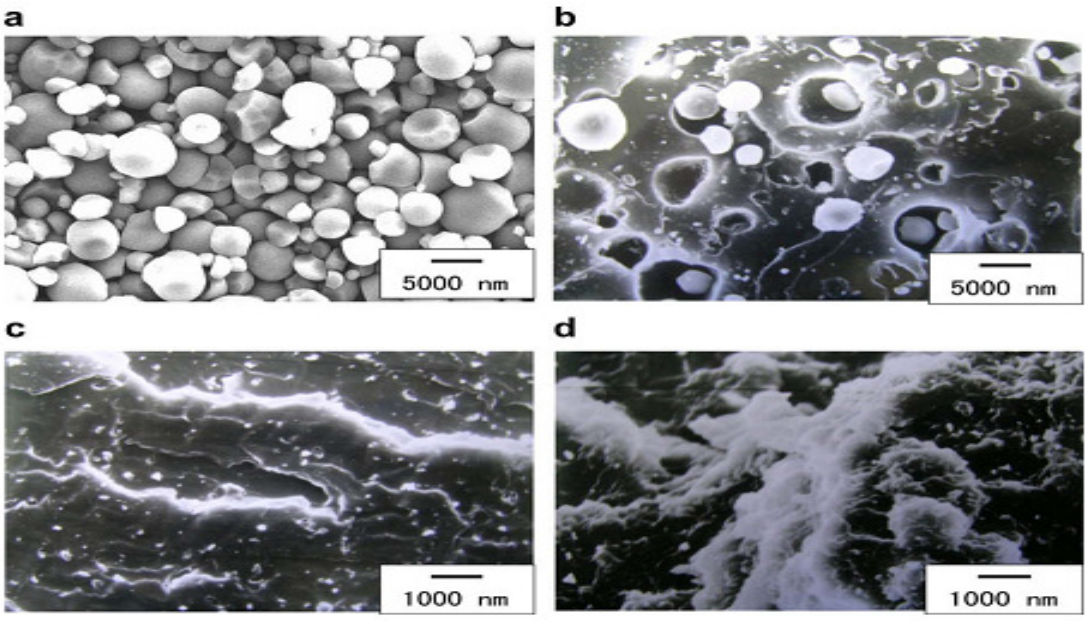


Figure 7. SEM micrographs of starch and composite: (a) unmodified starch; (b) unmodified starch (15 phr)/NR composites ; (c) modified starch (5 phr)/NR composite and (d) modified starch (15 phr)/NR composite. (Wang *et al*., 2009)

2.7.1 Biofilms

Microorganisms in biofilms display some particular features that are not shared with the same microorganisms in suspended form. In biofilms, the cells are embedded in a polymer matrix of their own origin, mainly consisting of polysaccharides and proteins (Costerton *et al.,* 1987). Biofilms contain mixed populations of bacteria, fungi, protozoa, and, if conditions allow, host even higher organisms in the food chain such as nematodes and larvae. They represent a very complex form of microbial life that is mainly characterized by a high degree of interaction between different types of organisms and by a more or less immobilized form of life. This allows the formation of stable aggregates in which synergistic effects can develop. In biofilms, the microbial cells can tolerate much higher concentrations of biocides than in suspension (LeChevallier *et al*., 1988). Another characteristic of biofilms is their heterogeneity. For example, as aerobic microorganisms in aerobic systems consume oxygen, anaerobic zones within biofilms can develop. These provide habitats for anaerobes that could not proliferate under the aerobic conditions prevailing in the water phase.

The requirements for the formation of biofilms are simple: a suitable surface, humidity, nutrients and microorganisms. As microorganisms are also ubiquitous, most of them live in an immobilized form in various types of biofilms, such as in soils, sediments and most industrial water systems (Costerton *et al.,* 1987). The adhesion to surfaces is a general microbial strategy for survival, especially in low nutrient environments (Marshall, 1985). Biofilms represent the oldest form of life on this planet (Schopf *et al*., 1983) and can be found in an extreme range of environments (Flemming *et al*., 1991). From an ecological point of view, life in a biofilm may offer important advantages to the cells. Among these are: (1) the possibility of forming stable microconsortia; (2) the facilitated exchange of genetic material; (3) the accumulation of nutrients from the bulk water phase; (4) protection against toxic substances and (5) protection against desiccation. A biofilm is mainly composed of water (80-95%), extracellular polymer substances (EPS) that contribute 85-98% of the organic matter, the microorganisms, entrapped organic and inorganic

particles (e.g. humic substances, debris, clay, silica, gypsum, etc.), substances sorbed to EPS, cells or particles and substances dissolved in the interstitial water. Thus, a chemical biofilm analysis can be quite misleading if a biofilm contains a lot of inorganic particles than the indicative character of the water content can be neglected. The distance between cell clusters can be substantial; it is predominantly filled with highly hydrated EPS. These, again, can surround void spaces such as pores, holes and channels (De Beer *et al*., 1994).

2.7.2 Forms of damage to polymers

There are different ways in which microorganisms can modify the structure and function of polymers. The undesired effects range from the mere presence of accumulated biomass as represented by biofilms, to the degradation of leached components, to direct attack and penetration. Some of the major damaging mechanisms by which microorganisms deteriorate synthetic polymers are summarized in Figure 8.

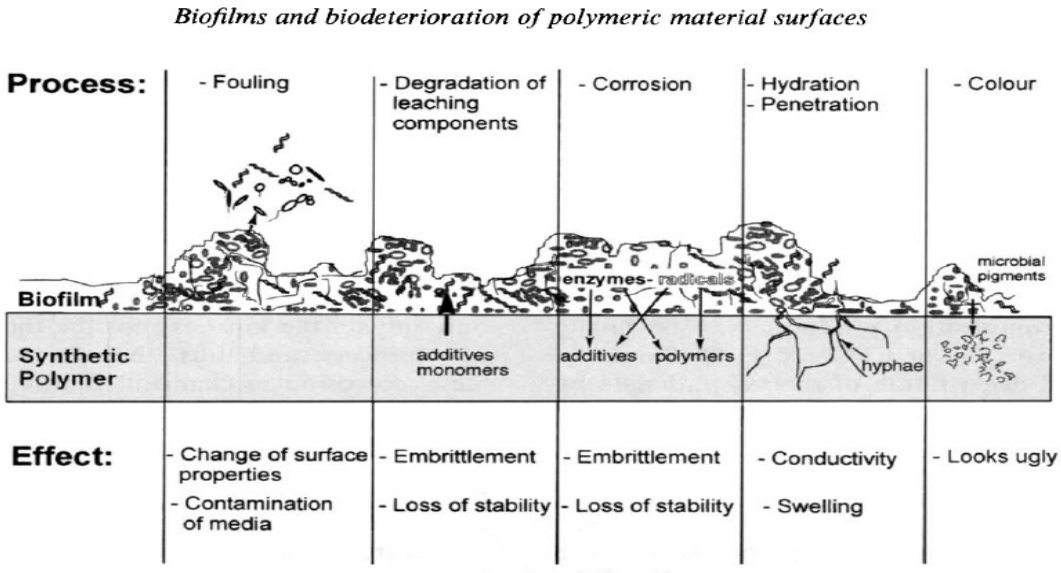


Figure 8. Some effects of biodeterioration on polymers (Flemming, 1998)

2.7.3 Fouling

Fouling is referred to as the unwanted deposition and growth of microorganisms on surfaces. In these cases, the polymer surface may be inert to microbial attack and merely provide a support for attached bacterial growth. Thus, original surface properties can be masked such as hydrophobicity. This can interfere with the function of the polymer, e.g. sulphur removal from power plant fumes. The resulting sulphate is precipitated with barium, and the precipitate is supposed not to attach to the walls but to accumulate at the bottom of the large reaction chamber, from where it can be removed automatically. Therefore, the walls of the reaction containers are coated with Teflon. It happened, however, that biofilms of sulphate reducing bacteria developed on the walls, giving their surfaces hydrophilic properties. This allowed the attachment of barium sulfate crystals that adhered over time so firmly that the reservoir had to be emptied, and the deposits had ‘to be removed mechanically from the walls in a costly and tedious procedure. If submersed in water, biofilms on plastic surfaces can contaminate the medium. An example is the production of ultrapure water in manufacture of electronical devices. This has been demonstrated for a large ultrapure water system of IBM equipped with PVDF piping colonized by biofilms that were identified as the major source of microorganisms in the process water (Patterson *et al.,* 1991). In reverse osmosis systems, biofilms develop on the surfaces of membranes. There, they form a gel layer that takes part in the separation process. They lead to an increase of the hydrodynamic resistance (transmembrane pressure drop) that in turn causes a decrease in permeate production and an increase in salt passage by concentration polarization, and in fluid friction resistance in cross-flow. These effects are termed as ‘biofouling’ and have been amply reviewed by Flemming and co-worker (Flemming *et al*., 1994) Figure 9 shows a biofilm on a reverse osmosis (RO) membrane that was used for the purification of river water. This biofilm has survived many cleaning and biocide treatment cycles.

2.7.4 Degradation of leached components

In order to cause a deterioration in the mechanical properties of a polymer, the microorganisms do not have to penetrate the polymer surface. Additives

and monomers may leach out, providing a food source for the attached biofilm. This is a very common type of interaction that takes place, for example, on plasticized Poly vinyl chloride (PVC) fittings such as those used for bathrooms, buildings or other purposes. The biological degradation of plasticisers has been known for a long time (Stahland Pessen, 1953). Cometabolic degradation of plasticisers has been demonstrated with fungal, bacterial and yeast isolates (Klausmeier, 1966). The plasticizer dibutyl adipate can be microbially degraded into adipic acid and n-butanol, by an extracellular esterase (Pantke, 1984). This process is accompanied by a lowering of the pH. The decomposition products may be finally metabolized to CO_2 or CH_4 and H_2O. Similarly, the microbial degradation of dioctyl phthalate by *Serratia marcescens* and *Bacillus megaterium* has been demonstrated (Williams and Dale, 1983).

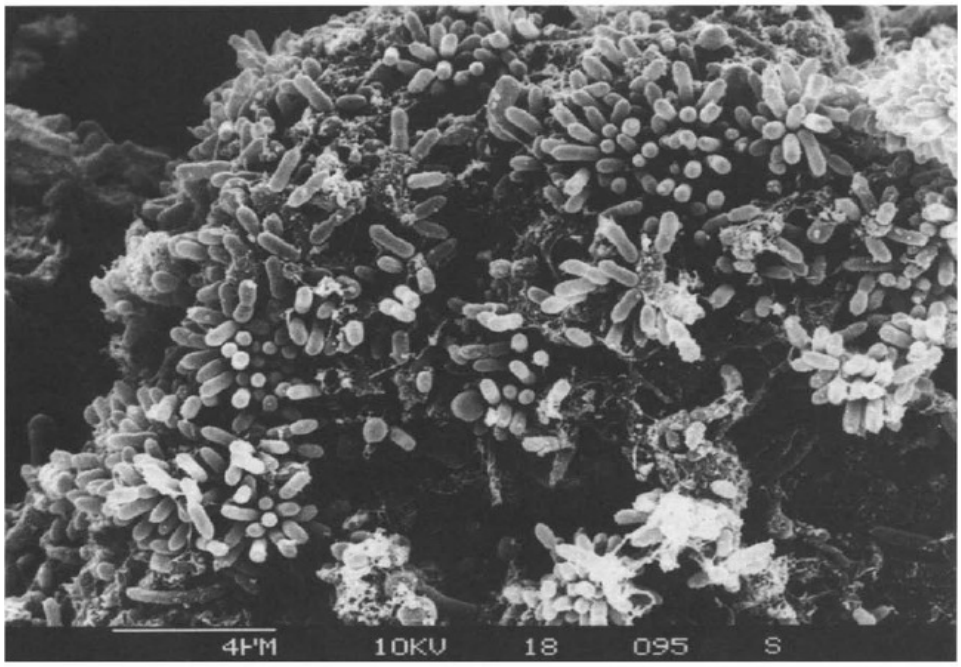


Figure 9. Scanning electron micrograph of a biofilm on an irreversibly blocked reverse osmosis membrane (Flemming, 1998)

As a result of plasticizer degradation, the remaining polymer material will suffer increased embrittlement, a loss of mechanical stability and lose its function as a barrier against water. As microbial degradation of the additive will create a

diffusion gradient in the bulk of the polymer mixture, the plasticizer concentration in the depth of the material will decrease. The damage caused by microbial mechanisms will indirectly affect water ingress, for example, the pores of concrete and the interior of buildings. In that case, water-related further damage may occur as well as other biodeterioration processes that could only be prevented by a limitation of water activity. In buildings, this can result in fungal growth in wallpaper, resulting in discolouration and increased release of fungal spores, which, in turn, can cause chronic allergical reactions. Specially educated dogs (‘mold dogs’) are used in order to localize such places.

Polymers used as additives in concrete can also be microbially attacked. In the case of high concentrations, they are degraded with the consequence of microbial contamination and mass development of biomass as observed in drinking water reservoirs (Morgenstern, 1982). Concrete additives in lower concentrations still can be used by microorganisms as soon as other corrosion mechanisms have made them bioavailable (Herb and Flemming, 1996). In this case, the microorganisms multiplied on the interior walls of drinking water reservoirs, formed pigments and led to an unhygienic appearance on the reservoir, that required expensive sanitation measures (Herb and Flemming, 1996).

2.7.5 Corrosion

Corrosion is an interfacial process and depends strongly on the parameters governing the microenvironment of the interface, such as pH conditions, redox potential and concentration of oxygen and salts. As pointed out earlier, microorganisms tend to form biofilms, i.e. ‘slime layers’, consisting of cells embedded in their highly hydrated, extracellular polymer matrix. The biofilm architecture gives rise to gradients that develop in pH value, redox potential, in the concentration of oxygen and salts. All these effects take place directly at the interfaces. Thus, biofilms can strongly influence corrosion relevant parameters locally, which are not reflected by data obtained from samples of a surrounding water phase. However, biofilms can only impact the kinetics, not the thermodynamics of a process, and thus, they do not represent a ‘new’ corrosion mechanism. Biofilms can

be very thin (3-5 pm) but can reach an average thickness of 50-150 pm. In some technical water systems, biofilm thickness may be in the range of centimeters, as is the case in paper manufacture or in waste-water treatment.

Polyurethanes are the best known type of polymers susceptible to microbial degradation. When used as insulation material for electrical equipment, this effect could lead to substantial damage. For example, between 1967 and 1969, the electrical system of Zurich airport was renovated. The insulation material, a polyurethane, was colonized by fungi that formed hyphae, penetrating the insulation. The entire system had to be renovated again after a short time (Pommer and Lorenz, 1985). Cancelled flights and loss of reputation added to these costs as an indirect damage. In an overview, Kay *et al.* (1993) report that a number of polycaprolactones have been shown to be degraded by a variety of bacterial and fungal isolates. Polyurethanes, derived from long chain polyesters were observed to be degraded to a greater extent than those manufactured from shorter-chain polyesters. IR-spectroscopy has provided direct evidence to show that the polyester segment of a polyester polyurethane is susceptible to microbial attack. In addition, polyester polyurethanes have been shown to be susceptible to commercially available lipases and also to purified esterase enzymes extracted from fungi. The involvement of extracellular hydrolytic enzymes in the microbial degradation of polyester polyurethanes has been suggested and, in view of the chemical structure of this polymer, the enzymes, esterases, ureases, and proteases have been implicated. The production of these enzymes has been reported by a number of fungal isolates capable of degrading the polyester polyurethane. The authors produced a considerable amount of evidence to indicate that the polyester segment of a polyester polyurethane was the main site for attack by fungi, utilizing inducible extracellular esterase enzymes.

Bio-corrosion of synthetic polymers is reported from medicine too. An example is the biological damage of medical-grade silicone rubber. This material is widely used for applications such as tubing, catheters, mammary and testicular implants, plastic reconstruction, encapsulation of the electronic components. In the case of voice prostheses, Neu *et al.* (1993) reported severe corrosion by yeasts. Not only did these organisms colonize the prosthetic surfaces and cause continuous

infections, but they were also associated with the mechanical deterioration of the material. The particular mechanism of destruction is still somewhat unclear. As there is no way known in which microorganisms directly hydrolyze silicone, participation of radical reactions have been discussed, where the radicals are provided by biological reactions (Williams, 1991). Free radicals are generated by reactions within activated eucaryotic cells, and they may play a role in free radical autoxidation of polymers (Williams and Zhong, 1991). Several types of oxy radicals are readily generated within tissues. In particular, superoxide radicals, $O_2\cdot$, various peroxy radicals $ROO\cdot$, and especially the hydroxy radicals $OH\cdot$ are very reactive species, and these may react with high rate constants, with many different substrates. The oxidation of several polymers may be initiated by such free radicals generated during the process of cell activation at a polymer surface in vivo. Hydrogen peroxide released from activated phagocytic cells is likely to be a particularly important intermediate, as its decomposition yields the extremely reactive hydroxy radical (Williams and Zhong, 1991). Such mechanisms may occur in biofilms as well; this might be one of the reasons that other synthetic rubbers and plastics, although generally considered as biologically inert, are in fact slowly degraded by microorganisms (Seal and Morton, 1986).

2.7.6 Hydration and penetration

Biofilms consist mainly of water (up to 95%) and, thus, provide an electrolyte that increases surface conductivity. This has led to unexpected failures of electrical equipment. For example, fungal growth on printed circuits affects the surface conductivity and causes short circuits; mold growth on computer motherboards has been reported, resulting in a failure of computer, radio and radar equipment in the tropics. Microbial colonization of control limit elements (Pantke and Patuska, 1984) has caused considerable damage, for instance, by overfilling fuel oil tanks. As a result of mold growth on refrigerator sealing (Frank, 1984) or plastic tubes in medical instruments (e.g. cooling water for dental appliances), health hazards may develop. Frank (1984) reports a case in which the plastic isolators of all emergency radio units in the life boats of a German freight vessel became conductive to fungal

and algal colonization. The ship ran into a gale in the South Atlantic and sank. The crew escaped in the life boats but could not be found in time because the radio units did not work any more, and they all died.

The conductivity of insulation material also can be provided by penetration of the polymer matrix which has been observed. Additionally, this leads to a decrease in mechanical stability. The mechanism by which fungal hyphae can penetrate synthetic polymer surfaces is still unclear as the required turgor is very high. A fungus has been described that can generate a turgor pressure of 8.0 megapascals (MPa). The appressorium of *Mugnaporthe grisea*, by this mechanism, penetrated non-biodegradable mylar membranes with a hardness range of 140- 250 MPa (Howard *et al.,* 1991).

2.7.7 Color

Biofilms can contain organisms that produce pigments. Some of these pigments, in particular those formed by some fungal strains, are lipophilic and tend to diffuse into a lipophilic polymer matrix. This can be observed on shower curtains and on PVC sealings in bathrooms, when humidity over a sufficient period of time is high enough to allow the growth of these organisms. This type of coloration cannot be removed by deep cleaning, looks appalling and leads to family conflicts about domestic hygiene and replacement costs. A relatively spectacular case of microbial pigmentation was reported by Frank (1984) when the tops of milk bottles, fabricated from synthetic polymer, turned red. The reason was prodigiosin, a dye produced by *Serratia murcescens*, which had colonized the bottle tops. This pigment had diffused into the material.

Obviously there are many possible ways which microbes can cause structural alterations to polymers probably more so with heteropolymers, even some that cannot be completely degraded.

2.8 Biodegradation of starch blends and natural rubber

2.8.1 Starch/polyethylene blends

Polyethylene is reported to be an inert polymer with strong resistance to microbial breakdown (Weiland *et al*., 1995). Biodegradation is decreased with an increase in molecular size (Tung *et al*., 1999). Linear paraffin molecules, below a molecular size of about 500 Da, can be utilized by several microorganisms (Albertsson and Karlsson, 1993). Scott (1990) concluded that microbial attack on polyethylene is a secondary process where an initial oxidation step results in the reduction of molecular size to a size sufficiently small for more rapid biodegradation to occur. Linking of readily biodegradable compounds, such as starch, to a low-density polyethylene matrix may enhance the degradation of the carbon–carbon backbone (Griffin, 1977). The biodegradability of starch/polyethylene blends, and chemically modified blends has been investigated (Johnson *et al*., 1993; Bikiaris *et al*., 1998). The aerobic and anaerobic biodegradability of starch-filled polymer blends has been evaluated. Carbon removal from a starch polyethylene blend was low compared to pure starch and the rate of removal was higher under aerobic conditions. Similar results were obtained by Chandra and Rustgi (1997) for the biodegradation of maleated linear low-density polyethylene starch blends in a soil environment containing a mixed fungal inoculum of *Aspergillus niger*, *Penicillium funiculom, Chaetomium globosum, Gliocladium virens* and *Pullularia pullulans.* Biodegradation of a starch polyethylene film containing a pro-oxidant and 6% starch showed evidence of polyethylene degradation in the presence of a lignin degrading bacterium of the genus Streptomyces and also in the presence of the white-rot fungus *Phanerochaete chrysosporium* (Lee *et al*., 1991). The rate of degradation of a starch-filled polyethylene depended on the starch content, and it was very sensitive to the environmental conditions and other ingredients in the formulation (Albertsson and Karlsson, 1993). Oxidation of impurities, such as fats and oils, would seem to be an important trigger for the biodegradation of polyethylene. The production of reactive oxygen species such as peroxides is a likely source of initiators of molecular breakdown although other radicals may be involved (Cornell *et al*., 1984). The

degradation of 11 commercially produced degradable starch/polyethylene blended compost bags was evaluated in a municipal yard waste compost site by chemical degradation, photodegradation and biodegradation of each product (Johnson *et al.*, 1993). The oxygen concentration at the surface of the films appeared to be the rate-limiting component. A pro-oxidant additive (transition metal) was critical in promoting the oxidative degradation of the polyethylene. Several researchers have investigated the use of modified starch in starch/low density polyethylene blends (Evangelista *et al*., 1991). The modified starches enhanced the miscibility and adhesion of starch in those blends. However, the poor biodegradability of modified starches leads to a very slow rate of biodegradation compared with the unmodified starch/polyethylene blends.

Other investigations have been carried out to evaluate the biodegradability of films containing starch, polyethylene (PE) and ethylene acrylic acid (EAA) (Shogren *et al.,* 1992). The results indicated that rapid and appreciable starch depletion led to deterioration of the mechanical properties of the films leaving a rather weak matrix which was prone to further physical disruption by biotic and abiotic factors.

2.8.2 Starch/polyester blends

Blends of starch and Polycaprolactone (PCL) are assumed to be completely biodegradable since each component in the blend is readily biodegradable as well as compostable (Tokiwa *et al*., 1994; Bastioli, 1994). Biodegradability of different grades of the commercial polyester Bionolle™ has been studied in activated sludge, soils and compost (Nishioka *et al.,* 1994). Bionolle™ 3000 was degraded more easily than Bionolle™ 1000 and Bionolle™6000. Moulds did not degrade Bionolle™ 6000 and some Gram-negative bacteria did not degrade Bionolle™ 1000. The degree of degradation mainly depended on the type of microorganism and their population. It is known that poly-(hydroxybutyrate) depolymerases and lipases are both capable of cleaving the ester bonds 4 poly-(hydroxyalkanoate) (Doi *et al*., 1992). Because of their structural similarity, these enzymes are expected to degrade Bionolle™. Biodegradation of Bionolle™ by compost has been studied and shown to

be complete (Jayasekara *et al*., 2005). Furthermore, the products were shown to be non-toxic to the earthworm, *Eisena fetida*. Blending of Bionolle with low cost starch has been investigated in order to improve its cost competitiveness whilst maintaining other properties at an acceptable level. It has been shown that the addition of a starch filler significantly improved the rate of degradation of the Bionolle™ component (Ratto *et al.,* 1999). A study of the biodegradation of cellulose, a poly(-caprolactone) starch blend and an aliphatic–aliphatic copolyester was undertaken in a ring test (Pagga *et al.*, 2001) involving several laboratories using standards like; ISO 14852 (1999), and ISO 14855. According to both criteria (the biological oxygen demand (BOD) and CO_2 released) the cellulose was more degraded than the poly(-caprolactone)-starch blend which in turn was more degraded (on average) than the copolyester.

2.8.3 Starch/PVA blends

The water-soluble synthetic polymer, polyvinyl alcohol (PVA) has excellent compatibility with starch and blends are expected to have good film properties. Several such blends have been developed and tested for biodegradable packaging applications and appear to have some potential for use (Tudorachi *et al.,* 2000). PVA and starch blends are assumed to be biodegradable since both components are biodegradable in various microbial environments. The processing and mechanical properties of starch/PVA blends have been well investigated (Park *et al.,* 1994; Bastioli, 1994; Haschke *et al*., 1998) but only a limited number of publications are available regarding the biodegradability of such blends. Data on the biodegradability of starch, PVA, glycerol and urea blends by bacteria and fungi isolated from the activated sludge of a municipal sewage plant and landfill indicated that microorganisms consumed starch and the amorphous region of PVA as well as the glycerol and urea plasticisers (Tudorachi *et al*., 2000). The crystalline region of PVA was unaffected. A biodegradability study of starch, PVA and glycerol blends has also been carried out by Park *et al*. (1994). ASTM D 5271-92 (1992) was used to evaluate the rate and degree of biodegradation of PVA blends which were reported to be biodegradable. In a separate study of PVA and poly- (3-hydroxybutyric acid)

blended films, the solubility of the PVA component in water was found to effect the biodegradability of the blend (Ikejima *et al.,* 1999).

2.8.4 Biodegradation of natural rubber, rubber product

During the past decades, several research investigations have been concerned with the microbial degradation of natural rubber and rubber products. In 1913, Söhngen and Fol (1914) isolated pure cultures of natural-rubber-consuming microorganisms from an enrichment culture in which pure rubber hydrocarbon was the main source of carbon. They prepared films of non vulcanized rubber of high purity by dissolving pieces of sheet rubber in benzene, separating the clear upper layer of the solution, and subsequently evaporating the benzene. It should be acknowledged that the thus prepared rubber still contained 0.1 percent nitrogen. However, it appears to be difficult to attain a lower nitrogen content without employing methods too complicated. Boggs and Blake (1936), in discussing the preparation of deproteinized rubber from latex, mentioned that even after repeated creaming or centrifuging of the latex the rubber still had a nitrogen content of 0.08 percent. Söhngen and Fol (1914) inoculated their thin rubber films, floating on an aqueous medium containing inorganic salts, with soil and observed the growth of colonies. The greater part of these colonies appeared to be Actinomycetes. After some time, the rubber under and around the colonies had disintegrated to such an extent that this could not be ascribed to the disappearance of impurities from the rubber. Two of the active strains isolated in pure culture were described as *Actinomyces elastica* and *Actinomyces fuscus*. A further contribution worth mentioning was made by Kalinenko (1938), who applied the technique of Spence and van Niel (1936) for the isolation of several rubber decomposing organisms. Among these organisms there were, besides *Actinomyces* species, also such molds as *Aspergillus oryzae* and a *Penicillium* species. Kalinenko claimed that all these cultures were able to consume large quantities of rubber in diluted latex. Moreover, he found that growth of one of the *Actinomyces* species on a thin film of purified natural rubber led to a perforation of this film. More direct evidence for a microbial attack of vulcanized rubber hydrocarbon was given by Blake, Kitchin and Pratt (1950, 1953, and 1955), who studied the deterioration of the rubber insulation of electric cables. They found that when the insulation had been buried for

some period of time in soil it lost its insulating properties, whereas in parallel tests this material, exposed to sterilized soil, remained practically unaltered. Synthetic rubber proved to be more resistant in the soil burial test. In 1997 Jendrossek *et al.* isolated 50 rubber-degrading bacteria using natural rubber latex as the sole source of carbon and energy. Out of those 50 isolates, 33 were identified as *Streptomyces* species and 8 as *Micromonospora* species. Screening of 1220 bacteria obtained from different culture collections revealed 46 additional rubber-degrading bacteria (Streptomyces 31 strains, Micromonospora 5, Actinoplanes 3, Nocardia 2, Dactylosporangium 1, Actinomadura 1, unidentified 3). All rubber-degrading isolates were identified as members of the actinomycetes, a large group of mycelium-forming Gram-positive bacteria. Interestingly no Gram-negative bacterium was isolated. In most strains expression of extracellular rubber-degrading enzymes was repressed by glucose and/or succinate. The reduction of the average molecular mass of solution-cast films of natural rubber from 640,000 to 25,000 in liquid culture upon bacterial growth indicates the participation of an endo-cleavage mechanism of degradation.

Tsuchii *et al.* (1985) investigated the microbial degradation of vulcanized natural rubber an actinomycete, *Nocardia* sp. strain 835A, grew well on unvulcanized natural rubber and synthetic isoprene rubber, but not on other types of synthetic rubber. Not only unvulcanized but also various kinds of vulcanized natural rubber products were more or less utilized by the organism as the sole source of carbon and energy. The thin film from a latex glove was rapidly degraded, and the weight loss reached 75% after a 2-week cultivation period. Oligomers with molecular weights from 10^4 to 10^3 were accumulated during microbial growth on the latex glove. The partially purified oligomers were examined by infrared and ^1H nuclear magnetic resonance and ^{13}C nuclear magnetic resonance spectroscopy, and the spectra were those expected for cis-1,4-polyisoprene with the structure, $OHC\text{-}CH_2\text{-}[\text{-}CH_2\text{-}C(\text{-}CH_3)=CH\text{-}CH_2\text{-}]n\text{-}CH_2\text{-}C(=O)\text{-}CH_3$, with average values of n of about 114 and 19 for the two oligomers. A second publication of Tsuchii and Takeda (1990) detected a rubber-degrading enzyme from a bacterial culture. It was found in the extracellular culture medium of *Xanthomonas* sp. strain 35Y which had been grown on natural rubber latex. Natural rubber in the latex state was degraded by the crude enzyme, and two fractions were separately observed by gel permeation chromatography of the

reaction products. One fraction was of higher molecular weight (HMW) with a very wide MW distribution from 10^3 to 10^5, and the other fraction was of lower molecular weight (LMW) with a MW of a few hundred. ^{1}H-nuclear magnetic resonance spectra of the partially purified degraded fractions were those expected of cis-1,4-polyisoprene mixtures with the structure of OHC-CH_2-(-CH_2-C(-CH_3)=CH-CH_2-)n-CH_2-C(=O)-CH_3, with average values of n of about 113 and 2 for HMW and LMW fractions, respectively. The LMW fraction consisted mostly of one component in gas-liquid chromatography as well as in gel permeation chromatography, and the main component was identified as 12-oxo-4,8-dimethyl trideca-4,8-diene-1-al (acetonyl diprenyl acetoaldehyde, $A_LP_2A_I$) by ^{13}C-nuclear magnetic resonance and gas chromatography-mass spectra. Not only the latices of natural and synthetic isoprene rubber, but also some kinds of low-MW polyisoprene compounds of cis-1,4 type, were degraded by the crude enzyme. The rubber-degrading reaction was found to be at least partly oxygenase catalyzed from the incorporation of ^{18}O into $A_LP_2A_t$ under an $^{18}O_2$ atmosphere.

As far as it is known, Linos *et al*. (2000) isolated a Gram-negative bacterium, strain AL98, from foul water inside a deteriorated car tire on a farmer's field in Münster, Germany. The strain was able to attack and cause some disintegration of natural rubber (NR), either in the raw state as NR latex concentrate or in the vulcanized state as in a NR latex glove, as well as raw synthetic cis-1,4-polyisoprene (IR). Surface investigation by scanning electron microscopy gave evidence for an adhesive growth behavior of the strain proceeding by colonizing the rubber surface, merging into the rubber and forming a biofilm prior to disintegration of the material. The solid glove substrate disappeared completely after a prolonged cultivation period as a result of continuous degradation. Taxonomic analyses of the strain, that was also based on an examination of the similarity of the complete 16S rRNA gene, revealed that strain AL98 was a strain of *Pseudomonas aeruginosa*. This is the first report of the isolation of a Gram-negative bacterium that exhibitied rubber decomposing properties. In a second publication of Linos *et al*. (2000), they investigated the strategies of rubber degrading-bacteria, and divided them into two groups from their degradation behavior. The first group grew only after direct contact with the rubber substrate and they produced considerable disintegration of the

material during cultivation. The second group consisted of the weaker rubber decomposers that did not grow by adhesion to the rubber substrates, as indicated by the formation of clear zones (translucent halos) around the bacterial colonies growing with NR dispersed in the mineral agar. Taxonomic analysis of four selected strains based on 16S rRNA similarity examinations revealed two *Gordonia* sp. strains, VH2 and Kb2, and one *Mycobacterium fortuitum* strain, NF4, belonging to the first group as well as one *Micromonospora aurantiaca* strain, W2b, belonging to the second group. A third publication of Linos *et al*. (2002), isolated *Gordonia westfalica* sp. Nov., as a novel rubber-degrading actinomycete.

Roy *et al*. (2004) did a comparative study of the biodegradation of di-cumyl peroxide (DCP) crosslinked and uncrosslinked natural rubber by *Pseudomonas* sp. A decrease in the organic carbon content along with the changes in the tensile strength of the treated rubber, of both the DCP crosslinked and uncrosslinked natural rubber, indicated that the rubber was utilized. A decrease in 60.88% MPa and 41.66% MPa was observed after five month's of treatment of the uncrosslinked natural rubber and the DCP crosslinked rubber, respectively. That biodegradation was more pronounced in the natural uncrosslinked rubber, was further confirmed by the formation of aldehydic compounds together with a decrease in the CH_2 stretching frequencies.

Studies by Bhatt *et al*. (2008) on the degradation of NR-PHA polymer blends were carried out over a period of 30 days using a soil isolate, *Pseudomonas* sp. 202. Extracellular protein concentrations as well as OD_{660} values were used to measure the growth of *Pseudomonas* sp. 202. The degradation of the blended plastic material, as evidenced by the percentage weight loss and the increase in the growth of organism was correlated with the amount of the polyhydroxyalkanoate (mcl-PHA) present in the samples. Growth of *Pseudomonas* sp. 202 resulted in a 14.63%, 16.12% and 3.84% weight loss of the PHA: rubber blends (natural, nitrile and pure butadiene rubber respectively).

Warneke *et al*. (2007) isolated a bacterium that degraded poly(trans-1,4-isoprene) (*gutta percha*). This is the first report of an axenic culture of a

bacterium capable of degrading gutta percha. From about 100 different habitats and enrichment cultures, six bacterial strains were isolated that utilized synthetic poly(trans-1,4-isoprene) as sole carbon and energy source for growth. All isolates were assigned to the genus Nocardia based on their 16S rRNA gene sequences. Four isolates were identified as strains of *Nocardia nova* (L1b, SH22a, SEI2b and SEII5a), one isolate was identified as a strain of *Nocardia jiangxiensis* (SM1) and the other as a strain of *Nocardia takedensis* (WE30). Degradation of poly(trans-1,4-isoprene) by these seven strains was verified in mineralization experiments by determining the release of CO_2. All seven strains were also capable of mineralizing poly(cis-1,4-isoprene) and to use this polyisoprenoid as a carbon and energy source for growth. Mineralization of poly(trans-1,4-isoprene) after 80 days varied from 3% (strain SM1) to 54% (strain SEI2b) and from 34% (strain L1b) to 43% (strain SH22a) for the cis-isomer after 78 days.

Cherian and Jayachandran (2009) detected microbial degradation of natural rubber latex by a novel species of *Bacillus* sp. SBS25. Pan *et al*. (2009) demonstrated a deterioration of rubber materials by caused by slow-growing bacteria. These bacteria, capable of degrading polymeric products, were isolated from several sources including tap water, sediment, and a deteriorated polymeric product. *Alcaligenes xylosoxidans* T2, *Pseudomonas aeruginosa* GP10, and *Nocardia corynebacterioides* S3 were able to utilize rubber products as a sole source of carbon and energy. *A. xylosoxidans* T2, *P. aeruginosa* GP10 and *N. corynebacterioides* S3 reduced the weight of a rubber product by approximately 2.0, 4.0 and 5.3%, respectively, after 70 days of incubation with the rubber product in mineral salts medium (MSM). On average, 0.45 mg (water soluble carbon) g^{-1} of the rubber product was released into the solution phase after 7 days of incubation.

2.8.5 Rubber-degrading genes

The basic molecular mechanism by which rubber is degraded is not known. Tsuchi and coworkers were the first research workers to isolate and identify low-molecular-mass oligo(cis-1,4-isoprene) derivatives with aldehyde and keto end groups from rubber-grown cultures of *Xanthomonas* and *Nocardia* species (Tsuchi *et*

al., 1985; Tsuchii and Takeda, 1990). It is assumed that degradation of the polymer backbone was initiated by oxidative cleavage of double bonds in the polymer chain. The resulting low-molecular-mass oligo(cis-1,4-isoprene) derivatives were then further degraded, presumably by β-oxidation. Analysis of the NR degradation products produced by *Streptomyces coelicolor* 1A and *Streptomyces griseus* 1D after 70 days of growth on latex gloves revealed an oligomer pattern similar to that observed for *Xanthomonas* sp. However, products with different end groups were detected (Bode *et al.,* 2000; Bode *et al.,* 2001).

Bröker *et al*. (2004) investigated the characterization of the 101-Kilobase-Pair Megaplasmid pKB1, isolated from the rubber-degrading bacterium *Gordonia westfalica* Kb1, they found that genes essential for rubber degradation were encoded by pKB1.

Braaz *et al.* (2004) detected a novel type of heme-dependent oxygenase that catalyzed the oxidative cleavage of rubber (Poly-*cis*-1,4-Isoprene). The enzyme was purified from the cell-free culture fluid of latex grown *Xanthomonas* sp. strain 35Y. This protein was identical to the gene product of a recently characterized gene cloned from *Xanthomonas* sp., as revealed by determination of its m/z values and sequencing of selected isolated peptides obtained after trypsin fingerprint analysis. The purified protein was specific for attacking natural rubber latex and chemosynthetic poly(cis-1,4-isoprene). Analysis of the amino acid sequence deduced from the cloned gene (roxA [rubber oxygenase]) revealed the presence of two heme-binding motifs (CXXCH) for covalent attachment of heme to the protein. Spectroscopic analysis confirmed the presence of heme, and approximately 2 mol of heme per mol of RoxA was found.

Rose *et al*. (2005) identified and characterized the genes for rubber degradation from *Streptomyces* sp. strain K30 that was responsible for formation of clear zone on natural rubber latex and poly(cis-1,4 isoprene). An open reading frame (lcp) of 1,191 bp was identified, which was preceded by a putative signal sequence and restored the capability to form clear zones on natural rubber latex in a non degrading mutant. Two ORFs putatively encoding a heterodimeric molybdenum hydroxylase (oxiAB) were identified downstream of lcp in *Streptomyces* sp. strain K30 strain that exerted a positive effect on clear zone formation and enabled the strain

to oxidize the resulting aldehydes. Heterologous expression of a fragment harboring lcp plus oxiAB in *S. lividans* TK23 resulted in the accumulation of aldehydes but only in the presence of 10 mM tungstate.

Ibrahim *et al.* (2006) identified poly(*cis*-1,4-isoprene) degradation intermediates during the growth of a moderately thermophilic actinomycetes on rubber and cloned a functional *lcp* homologue from *Nocardia farcinica* strain E1. Evidence was obtained that biodegradation of poly(cis-1,4-isoprene) was initiated by endocleavage, rather than by exocleavage. A gene (lcp) coding for a protein with high homology to Lcp (latex-clearing protein) from *Streptomyces* sp. strain K30 was identified in *Nocardia farcinica* E1. *Streptomyces lividans* TK23 expressing this Lcp homologue was able to cleave synthetic poly(cis-1,4-isoprene), confirming its involvement in initial polymer cleavage.

Bröker *et al*. (2008) investigated the genomes of the non-clearing-zone-forming and natural-rubber-degrading species of *Gordonia polyisoprenivorans* and *Gordonia westfalica* and detected genes that could express Lcp activity in *Streptomyces* strains. They found that *lcp*VH2 seemed not to be essential for rubber degradation in *G. polyisoprenivorans*.

In summary it seems that many strains of bacteria belonging to the order Actinomycetes, in the genera Actinomyces, Streptomyces, Nocardia and Gordonia can degrade NR and NR derivatives including starch/NR polymers and use the products as a sole source of carbon. A number of *Pseudomonas* spp. have also been identified that can degrade rubber because of the presence of a plasmid containing genes involved in rubber degradation. Degradation results in the production of low-molecular-mass oligo(cis-1,4 isoprene) derivatives with aldehyde or ketone end groups. An lcp (latex clearing protein) probably an oxygenase with bound heme groups has been studied. The observed rates of degradation are slow. There are still many aspects of rubber degradation that require further studies in order to improve degradation rates and establish the mechanisms involved.

CHAPTER 3

Materials and methods

3.1 Samples

NR latex concentrate was obtained from the Science and Technology Materials Department, Prince of Songkla university (PSU), Thailand and polymer blend (cassava starch foam blended with NR latex) from the Bioplastic Research Unit, PSU. NR latex gloves were from Siam Sempermed (Hadyai, Thailand).

3.2 Medium and growth conditions

A mineral salt medium (MSM) (liquid or solid with 1.5% agar) was used following Jendrossek *et al*. (1997), consisting of (g/L) $Na_2HPO_4 \bullet 12H_2O \bullet$(9.0), KH_2PO_4.(1.5), NH_4Cl (1.0), $MgSO_4 \bullet 7H_2O$ (0.2), $CaCl_2.2H_2O$ (0.02), Fe(III)[NH_4] citrate (0.0012), and a trace element solution (10000x, 0.1mL) pH 7. Natural rubber latex (NR) was added as a carbon source (0.6% v/v) in the form of latex prior to autoclaving to both liquid and solid MSM, (MSM-NR) (Jendrossek *et al*., 1997). This medium was supplemented with 0.05% yeast extract for the general cultivation stages (MSM-NR-YE). Sugars and organic acids were sterilized by filtration or autoclaving, respectively. Natural rubber latex was overlain on MSM (MSM-O-NR) for the selection of bacteria that formed clear zones on this selective medium (Ibrahim *et al*., 2006). The development of spores and the color of the aerial mycelium by isolated cultures was followed on glucose-yeast extract-malt extract medium (each 0.5%). The pH of all media was adjusted to 7.0 before autoclaving. All cultures were grown at 30°C and liquid cultures were in a shaking incubator at 120 rpm in the dark.

Liquid cultures in MSM-NR were carried out in 250-ml Erlenmeyer flasks. For carbon sources NR latex concentrate was added at 0.6% (v/v) to 100 ml MSM, or NR latex gloves and polymer blend were cut into small pieces of about 1x1 cm in diameter (Linos *et al*, 1999) to deliver 0.6 g of rubber gloves and 2.31 g of polymer blend (as 0.6% rubber). Sterilization by heat prior to inoculation was by

autoclave (121°C, 2 atm for 15 min.). Three kinds of latex agar plates were used: (1) MSM agar plates with NR latex (MSM-NR-P) (2) latex overlay plates (MSM-O-NR-P) and (3) MSM-NR-YE-P, culture for normal stock.

3.3 Enrichment and isolation

Soil samples (1.0 g soil, wet weight) were taken from different habitats associated with rubber factories in Hadyai, Thailand. The first enrichment was by precultivation in 100 mL MSM-NR-L in 250 mL flasks. For subsequent enrichment, transfer cultures were established in the following manner. One mL of the liquid enrichment culture after sufficient time of incubation were streaked on MSM-NR-P for isolation of individual bacteria. After 72 h of incubation at 30 °C in the dark, single colonies with different colony morphologies, but showing clearing zones, were picked and streaked on newly made MSM-NR-P, for purification under identical incubation conditions. The obtained pure cultures of bacterial isolates were all tested again for their ability to produce translucent halos around the colonies on MSM-NR-P (Pan *et al*., 2009).

3.4 Bacterial identification

Individual isolates obtained for their abilities to degrade NR were identified as follows. Identification methods included standard biochemical tests: such as staining methods, that included Gram stain, malachite green for spores, motility tests; haemolysis tests on blood agar, catalase test, oxidase test and test for amylase by growth on cassava starch agar and observing and measuring the clearing zone around the colonies after 24 h incubation.

Determination of 16s rRNA gene sequences was also performed to identify and classify isolates. Each strain was cultured for 2 days in Nutrient Broth. Genomic DNA was extracted by standard methods (Warneke *et al*., 2007). Primers used were 27F and 1389R to amplify the full length of 16S rRNA gene using PCR. This was carried out by the BIOTEC Culture Central Research Unit. The PCR amplification reaction and conditions used were optimum. Nucleotide sequences of

purified PCR products were determined with the Genetic Analyzer, and DNA nucleotide sequences of isolates were analyzed for identification using the data available from the GenBank database at The National Center for Biotechnology Information (Pan *et al*., 2009).

3.5 Weight loss of polymers after incubation with bacteria

In this experiment, each 250 mL Erlenmeyer flask containing 100 mL of MSM medium and precisely weighed 0.6 g rubber gloves strips or 2.31 g polymer blend (0.6% NR) (Waneke *et al.,* 2007) previously sterilized and dried at 60°C until a constant weight was achieved then placed into a desiccator for 24 h. Each flask was inoculated with a growing bacterial culture fluid of 10^9 cells/mL to provide a final number of 10^8 cells/mL of medium and incubated for 4 weeks. NR strips were then removed, washed with deionized water and dried at 60°C until the weight was constant. The dried samples were kept in the desiccator before being weighed (Hamad *et al.,* 2005). Weight loss was taken as the difference between the initial and final weight over the 4 weeks culture period (Pan *et al*., 2009).

3.6 Investigation of the degradation processes

Individual isolates were further examined for their abilities to degrade the blended polymer and rubber gloves by following their growth in liquid culture with the rubber polymer substrates or the polymer blend as their sole source of carbon and energy. Overnight cultures of each bacterial isolate on MSM-NR-P were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of MSM and 2.31 g of sterilized polymer strips or 0.6 g rubber gloves strips culture were incubated for up to 4 weeks (Pan *et al*., 2009).

3.6.1 Surface erosion of the starch/NR blended sheets and rubber gloves was observed by light microscopy and changes in the characteristic color of the polymers after incubation. Scanning Electron Microscopy (SEM) or light

microscopy or other suitable techniques was also employed depending on the initial observations.

3.6.2 Staining of cassava starch foam blended with natural rubber latex and rubber gloves with Schiff's reagent. The procedure used was as follows. The actively growing colonies of the chosen isolate on the rubber gloves and polymer blend surface were clearly visualized by staining with Schiff's reagent (Berekaa *et al*., 2005). The purple color produced by the reagent provided evidence that isoprene oligomers (In rubber gloves and polymer blend) containing aldehyde groups were produced and accumulated during the microbial degradation of rubber (Tsuchii *et al*., 1985). The purple color that developed over 10 min at room temperature was noted. The composition of the Schiff's reagent was as follows: 2 g of fuchsin dissolved in 50 mL of glacial acetic acid plus 10 g of $Na_2S_2O_5$ plus 100 ml of 0.1 N HCl plus 50 mL of H_2O (Linos *et al*., 2000).

3.6.3 Enzyme assay during degradation of the polymer blend**.** The presence of glucosidase enzymes by the bacteria was assayed by measuring free glucose levels and tests for an α-glucosidase enzyme activity in the culture supernatant over 20 days. Every 2 days, a sample was taken from the culture media with bacteria and polymer blend and centrifuged at 6000 rpm for 30 min (Procedure described by Wimmer *et al.,* 1997). The Somogyi-Nelson method was used to measure reducing sugars. The Somogyi-Nelson method is based on the absorbance at 500 nm of a colored complex formed between a copper-oxidized sugar and arsenomolybdate. The amount of glucose present was determined by comparison with a calibration curve using a spectrophotometer. Glucose standards were prepared using a 200 µg/mL solution in distilled water. 1.0 mL of culture supernatant (test) or distilled water (blank) was added into a test tube, with 1 mL of low-alkalinity copper reagent. Tubes were heated in a boiling water for 10 min. and instantly cooled (ice bath), then 1.0 mL of the arsenomolybdate reagent diluted to 5 mL with distilled water, was added and, stood for at least 15 min at room temperature. The solutions were transferred from the test tubes to cuvettes and the A_{500} of the sugar standards and solutions containing unknown amounts of sugar (If the A_{500} measured was greater than 1.0 OD unit, the samples were diluted further and the A_{500} measurement

repeated). The concentration of sugar present in the sample, was determined from the calibration graph of A_{500} versus weight (mg) of sugar (Chaplin and Kennedy, 1994). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 nmol of substrate per minute at 30°C and was expressed as U/mL.

Aliquots from the culture medium were also assayed for α-glucosidase activity by measuring the release of p-nitrophenol from p-nitrophenyl-α-D glucopyranoside (Sigma co., Ltd) as substrate. One mL of supernatant from the cell free culture fluid was added to 100 µL of 5 mM substrate solution tube (substrate was dissolved in 100 mM phosphate buffer pH 7.0) and incubated at 30°C for 30 min (Applied from Otieno *et al*., 2006; Scalabrini *et al*., 1998). The reaction was stopped with 200 µL of 1 M sodium carbonate solution and centrifuge at 6000 rpm for 10 min. The OD was then measured at 420 nm. The enzyme activity was calculated, one IU is defined as hydrolysis of 1 nmol of substrate per min at 30°C (Flachner *et al*., 1999).

3.6.4 Scanning electron microscopy (SEM) observation. Physical changes, of cubic pieces of the polymers with side lengths of about 1 cm, incubated with selected strains were observed by SEM. The polymer of cassava starch foam blended with natural rubber latex was present an MSM cultures (100 mL) containing 2.31 g of polymer blend had been that inoculated with 10 mL of fresh cell (final volume 10^8 cells/mL) of the respective strains grown in MSM medium with NR latex for 48 h. Polymer strip specimens after incubation with bacteria were taken and prepared for scanning electron microscopy (SEM) by an initial fixation in 2% glutaraldehyde followed by 1%OsO_4. Samples were dehydrated in a graded series of ethanol 30, 50, 60, 70, 80, 90% and absolute ethanol. The dehydrated samples were subjected to critical point drying with liquid CO_2 according to the standard procedure, the samples were mounted on aluminum specimen stubs using electrically conducting carbon and sputter-coated with approximately 15 nm gold with argon gas as the ionizing plasma. Imaging was performed with a scanning electron microscope (Pan *et al*., 2009; Linos *et al*., 2000) with a vacuum sputter device and examined at 25 kV under high vacuum conditions. Micrographs were recorded digitally (Warneke *et al*., 2007).

3.7 Determination of percentage mineralization

Evidence for the biodegradation of starch/NR foam and rubber gloves hydrocarbon chains to CO_2 was obtained by determination of the CO_2 released during the cultivation of cells in the presence of starch/NR foam or strips of rubber gloves as sole carbon sources. Experiments were carried out in tightly closed Erlenmeyer flasks and the CO_2 trapped as $BaCO_3$ from $Ba(OH)_2$ was measured. The flask contained 800 mL MSM medium, 0.6% (v/v) of rubber gloves or 18.48 g starch/NR foam blend and were inoculated with a spore suspension containing 10^9cells/mL to give a final concentration 10^8 cells/mL in the culture and pregrown for 48 h. before collecting the CO_2. Each test tube connected to the flask contained 15 mL of a 0.2 M $Ba(OH)_2$ solution. At each measuring point, the flask was aerated with CO_2 free air, and the test tube replaced by new tubes containing fresh $Ba(OH)_2$ solution. The remaining $Ba(OH)_2$ was then determined for each period by titration with 0.1 M HCl according to the following equation yielding the percentage mineralization. Phenolphthalein (20 µL of a 1%, w/v, solution in 2-propanol) was used as an indicator and the end point of titration was determined by a change of the color from magenta to colorless. A non-inoculated flask was treated in the same way as for the control (Warneke *et al*., 2007; Berekaa *et al*., 2005).

3.8 Soxhlet extraction

The amount of starch in the starch/NR blended samples that was lost during the degradation process was measured by the Soxhlet extraction method. Samples were dried weighed then ground before being placing a carefully weighed sample into a thimble holder and extracted by Soxhlet extraction with toluene at 110°C. This operation was repeated until extraction was completed. Complete extraction was determined by adding a drop of the extract solution into methanol until no precipitate was observed or the methanol remained clear. If precipitation did occur extraction was repeated for up to 10 h or until the solution remained clear when a drop of the extract solution into methanol. The residue after extraction was dried at 60°C until the weight was constant and kept in the desiccators (Chumeka, 2009).

3.9 Optimum condition for polymer biodegradation

3.9.1 Establishing the optimum inoculum size for biodegradation of rubber glove and polymer blend. A selected isolate was first activated in liquid MSM-NR and incubated at 30°C for 48 h. Inocula were prepared at 10^6 10^8 10^{10} cells/mL (final concentration) from the activated cultures, 10 mL of each concentration was transfered to 100 mL of MSM medium with polymer blends or rubber gloves as substrates. Culture were incubated at 30°C for 6 weeks. Weight loss was determined.

3.9.2 The effect of temperature on polymer degradation was investigated by incubating cultures at different temperature of 30°C, 35°C, 40°C as for the previous experiments.

3.9.3 The effect of nitrogen source, NH_4NO_3 was used as nitrogen source at concentrations of 0, 0.5, 1, 5 and 10 g/L each concentration was prepared in 100 mL of MSM medium with polymer blended or rubber glove. Optimum inoculums size used was that obtained from the previous experiment. Cultures were incubated at 30°C for 4 weeks. The percentage weight loss was recorded.

3.9.4 In the assay for optimum pH value, the basal medium was adjusted to different pH levels of 6.0, 7.0, 8.0 in 100 mL of MSM with polymer blend or rubber glove and the optimum inoculums size and temperature determined previously. All flasks were incubated at 30°C for 4 weeks and the percentage weight loss was determined.

CHAPTER 4

Results

4.1 Screening and isolation of rubber-degrading bacteria

Although many bacteria are able to hydrolyze starch, an ability to hydrolyze rubber is restricted to only a few genera, (see chapter 2). It was therefore decided to first try to select for bacteria able to degrade rubber rather than to test for their ability to degrade the new mixed starch/NR foam blend. Several environmental samples were obtained from different ecosystems in Songkhla province, Thailand after enriching for rubber degrading organisms by incubation in MSM-NR liquid medium (Linos and Steinbüchel, 1998) followed by streaked into MSM-NR plates. After 7 days of growth, 119 colonies were checked for their ability to grow with MSM-NR medium and 78 produced clearing zones (translucent halos) around their colonies (66%) and also grew with MSM-O-NR plates over 3 to 4 days (Table 2). These were selected and obtained as pure cultures of rubber-degrading bacteria (Linos *et al.,* 2000). When tested for their ability to degrade starch by growth on starch agar plates all 78 isolates degraded starch. From these 78 isolates that formed translucent haloes on MSM-NR plates 9 isolates that produced large radial zones on MSM-NR-P of >3.0 mm. were selected for further study (Table 3).

4.2 Bacterial identification and characterization of clear zone forming NR-degrading bacteria

Seventy-eight isolates from different ecosystems in East Songkhla province, Thailand formed a clearing zone on MSM-NR-P. Isolates were obtained from the sediment of waste, from a rubber factory, in fact from most sites and in some cases even after the soil extracts has been diluted 100-fold or 1000-fold. This indicated quite clearly that rubber degrading bacteria are part of most natural environments in the south of Thailand. For 9 isolates: A2, A7, A8, A25, E29, F5, F6, CH2 and CH13 (Table 3) the clear translucent zones around their colonies were >3.0

mm (after 3 days) and as they also produced clearings zones with a positive reaction to iodine on starch agar plates these were selected for further studies. Of these, 8 isolates were Gram-positive bacteria and only one was Gram negative. All produced catalase and were oxidase positive. This means that these bacteria contained cytochrome C oxidase and therefore utilized oxygen for energy production via an electron transfer chain. In the motility test only the Gram negative isolate was motile all others were not motile. Isolate F5 did not produce hemolysis on blood agar (Table 4).

Table 2. Screening NR-degrading bacteria from different ecosystems

Source	Total isolates	Total of clear zone producing isolates
Rubber plantation soil, Klong-hae, Hadyai	29	14
Rubber factory, Thakham, Hadyai	20	15
Rubber factory 1, Prik, Sadao	23	15
Rubber factory 2, Prik, Sadao	21	17
Rubber factory, Tha Pho, Sadao	15	10
Rubber plantations, Namom	11	7
Total	**119**	**78**

Table 3. Isolates producing clear zones around the colonies on MSM-NR-P and tested for starch hydrolysis (amylolytic)

No.	Isolates	Radius of clear zone (mm)	Starch hydrolysis (clearing zone)*
1	A1	0.5	+
2	**A2**	**5.0**	**+**
3	A4	1.0	+
4	A6	1.5	+
5	A7	4.0	+
6	**A8**	**4.0**	**+**
7	A9	1.0	+
8	A10	1.0	+
9	A11	1.0	+
10	A15	1.0	+
11	A18	1.5	+
12	A20	2.0	+
13	A21	0.25	+
14	A22	2.15	+
15	A23	2.05	+
16	A24	0.5	+

*+ amylolytic enzyme, – No amylolytic enzyme

Table 3. Continues

No.	Isolates	Radius of clear zone (mm)	Starch hydrolysis (clearing zone)*
17	**A25**	**3.55**	**+**
18	A26	0.45	+
19	A27	2.0	+
20	A30	2.0	+
21	A34	2.5	+
22	A35	1.0	+
23	A42	2.5	+
24	E1	1.0	+
25	E2	2.0	+
26	E5	0.5	+
27	E6	1.0	+
28	E7	1.0	+
29	E9	1.5	+
30	E11	0.5	+
31	E13	2	+
32	E14	1.5	+
33	E17	0.75	+

*+ amylolytic enzyme, – No amylolytic enzyme

Table 3. Continues

No.	Isolates	Radial of clear zone (mm)	Starch hydrolysis (clearing zone)*
34	E18	1.5	+
35	E19	1.0	+
36	E20	1.0	+
37	E22	0.55	+
38	E25	0.8	+
39	E26	1.55	+
40	E27	2.15	+
41	**E29**	**4.15**	**+**
42	E30	2.15	+
43	F2	1.0	+
44	F3	0.75	+
45	F4	0.5	+
46	**F5**	**3.0**	**+**
47	**F6**	**3.0**	**+**
48	F7	0.5	+
49	F8	0.5	+
50	F10	1.0	+
51	F11	0.6	+

*+ amylolytic enzyme, – No amylolytic enzyme

Table 3. Continues

No.	Isolates	Radial of clear zone (mm)	Starch hydrolysis (clearing zone)*
52	F15	0.4	+
53	F16	0.4	+
54	F18	0.75	+
55	F20	0.5	+
56	F22	1.2	+
57	G1	2.75	+
58	G3	2.84	+
59	G4	2.35	+
60	G5	2.1	+
61	G6	0.15	+
62	H1	1.0	+
63	H2	1.25	+
64	H4	1.0	+
65	H6	1.5	+
66	H7	0.65	+
67	H9	0.5	+
68	H11	0.5	+
69	H14	0.75	+

*+ amylolytic enzyme, – No amylolytic enzyme

Table 3. Continues

No.	Isolates	Radial of clear zone (mm)	Starch hydrolysis (clearing zone)*
70	H15	0.65	+
71	**CH2**	**3.0**	**+**
72	CH6	2.0	+
73	CH7	1.0	+
74	CH8	1.0	+
75	CH9	1.0	+
76	CH12	1.0	+
77	**CH13**	**6.0**	**+**
78	CH14	0.5	+

*+ amylolytic enzyme, – No amylolytic enzyme

4.3 Determination of 16s rRNA gene sequences to identify and classify the isolate

Because strain CH13 produced the largest translucent zone on MSM-NR-P (3 days, 6 mm) and grew well, it was selected for further taxonomic characterization by analysis of its 16S rRNA gene. The almost complete gene was sequenced consisting of 1500 nucleotides. According to the results of the Genbank data base search, the sequence revealed highest similarities of 99% to *Streptomyces coelicolor*. Strain CH13 was deposited in the culture collection of the Microbiology department, Prince of Songkla University, Thailand, as *Streptomyces coelicolor* CH13. All closely related bacteria also degraded rubber (Figure 10).

Table 4. Morphological and biochemical characteristics of bacterial isolates

Isolates	Gram stain	Catalase	Oxidase	Motility	Haemolysis	Radial of clear zone size (mm.)
A2	G-	+	-	+	α	5.0
A7	G+	+	-	-	α	4.0
A8	G+	+	-	-	α	4.0
A25	G+	+	-	-	α	3.55
E29	G+	+	-	-	α	4.15
F5	G+	+	-	-	α	3.0
F6	G+	+	-	-	α	3.0
CH2	G+	+	-	-	α	3.0
CH13	G+	+	-	-	α	6.0

4.4 Investigation of the biodegradation process

4.4.1 Colonization of rubber gloves and starch/rubber strips

The surfaces of the two polymers were examined by scanning electron microscopy (SEM) before and after incubation with *S. coelicolor* CH13 for 2 weeks (Figure 11 and 12). The uninoculated surface of the polymer blend was more smooth than the surface of the rubber gloves, (compare Figure 11A and 11B with 12E and 12F). Figure 11C and 11D shows bacterial cells merging into the polymer blend and causing it to disintegrate with large holes appearing. This began to occur after only 2 weeks. Growth on the NR glove is illustrated in the micrographs shown in the Figure 12. Figure 12G demonstrates the colonization of the surface of the NR glove material by *S. coelicolor* CH13 after 2 weeks, proceeding by producing specific colony craters

on the surface but the holes were much less prominent than for the polymer blend (compare Figure 11D with Figure 12H) even though the surface had an extensive cover of microbial growth (Figure 12G, 12H). SEM observation also revealed the different colonization patterns of the bacterium on these two materials. The bacterium had clearly penetrated the surface of the polymer blend (Figure 12H) leaving a surface film probably of the rubber component.

The color of the material changed after incubation with *S. coelicolor* CH13 especially the rubber glove culture that turned a brown color presumably due to the extensive surface colonization (Figure 12G). *S. coelicolor* CH13 had formed visible dark brown colonies on the surface of the material and there was an indication that the organisms may be producing extracellular enzymes due to the presence of surface holes in particular on the polymer blend. It was a general observation that the microorganisms attached to the polymer blend had a softer plumper appearance than those on the rubber gloves. This is obviously a reflection of the presence of the more readily degradable starch in the polymer blend.

4.4.2. Erosion of the surface of the polymers

The surfaces of the rubber gloves and polymer blend as seen by SEM after being incubated for a period of 4 weeks (non fixed bacterial cells with 2.5% glutaraldehyde), are presented in Figure 13 (I-L). In the control without bacteria cells (uninoculated) the surface was rough but not modified (Figure 13I, K) for both rubber glove strips and polymer blended strips. In contrast, *S. coelicolor* CH 13 caused large holes to appear in the rubber gloves (Figure 13J) and a similar observation was made on the polymer blend surface (Figure 13L). These erosion surfaces have been previously seen by Warneke *et al*. (2007). This confirmed that these isolates are potent polymer blend and rubber glove degrading bacteria.

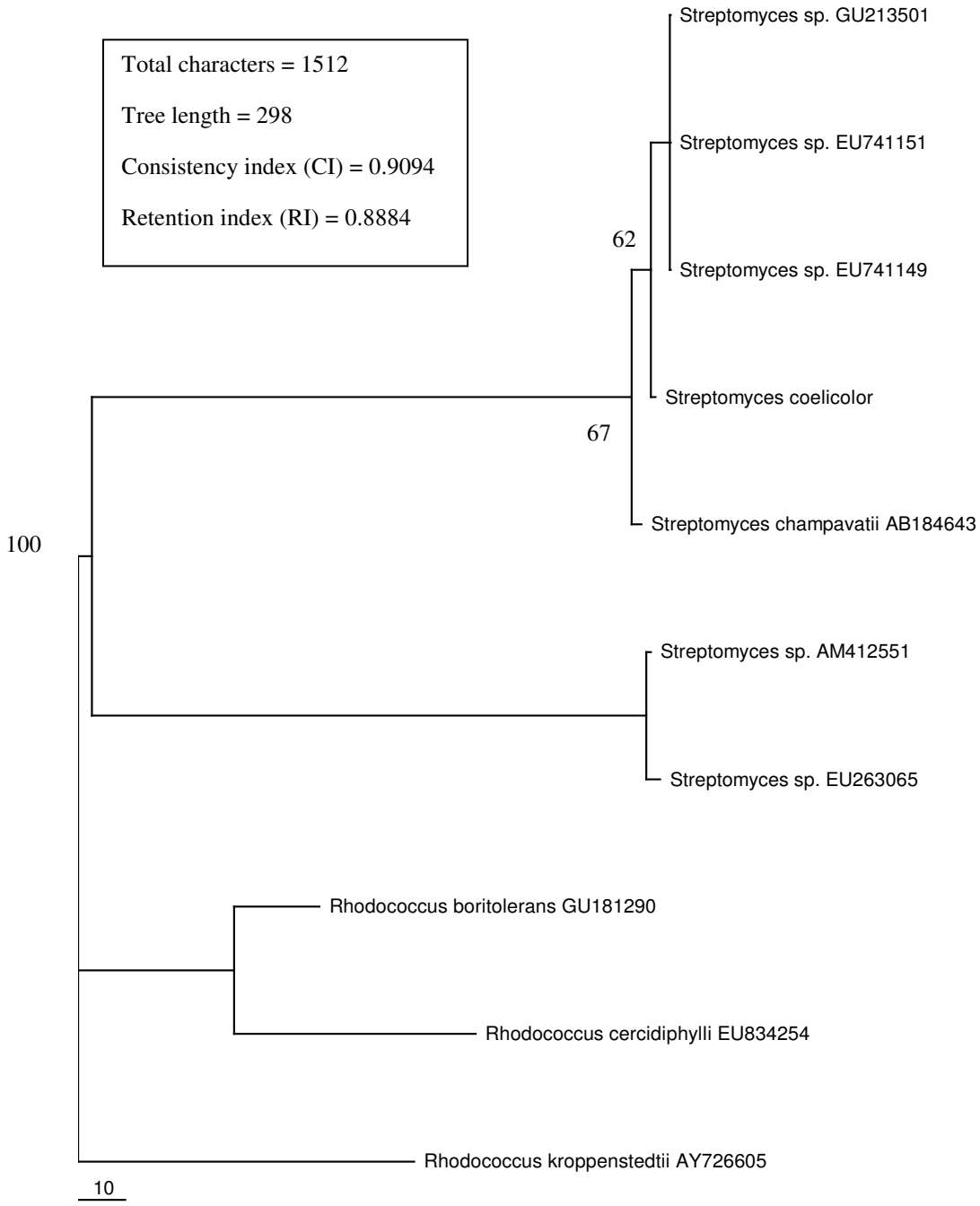


Figure 10. Maximum parsimony (MP) phylogenetic tree of *Streptomyces coelicolor* CH13. Bootstrap values were calculated from 1,000 resamplings using MP is shown at the respective nodes when the calculated values were 50% or greater

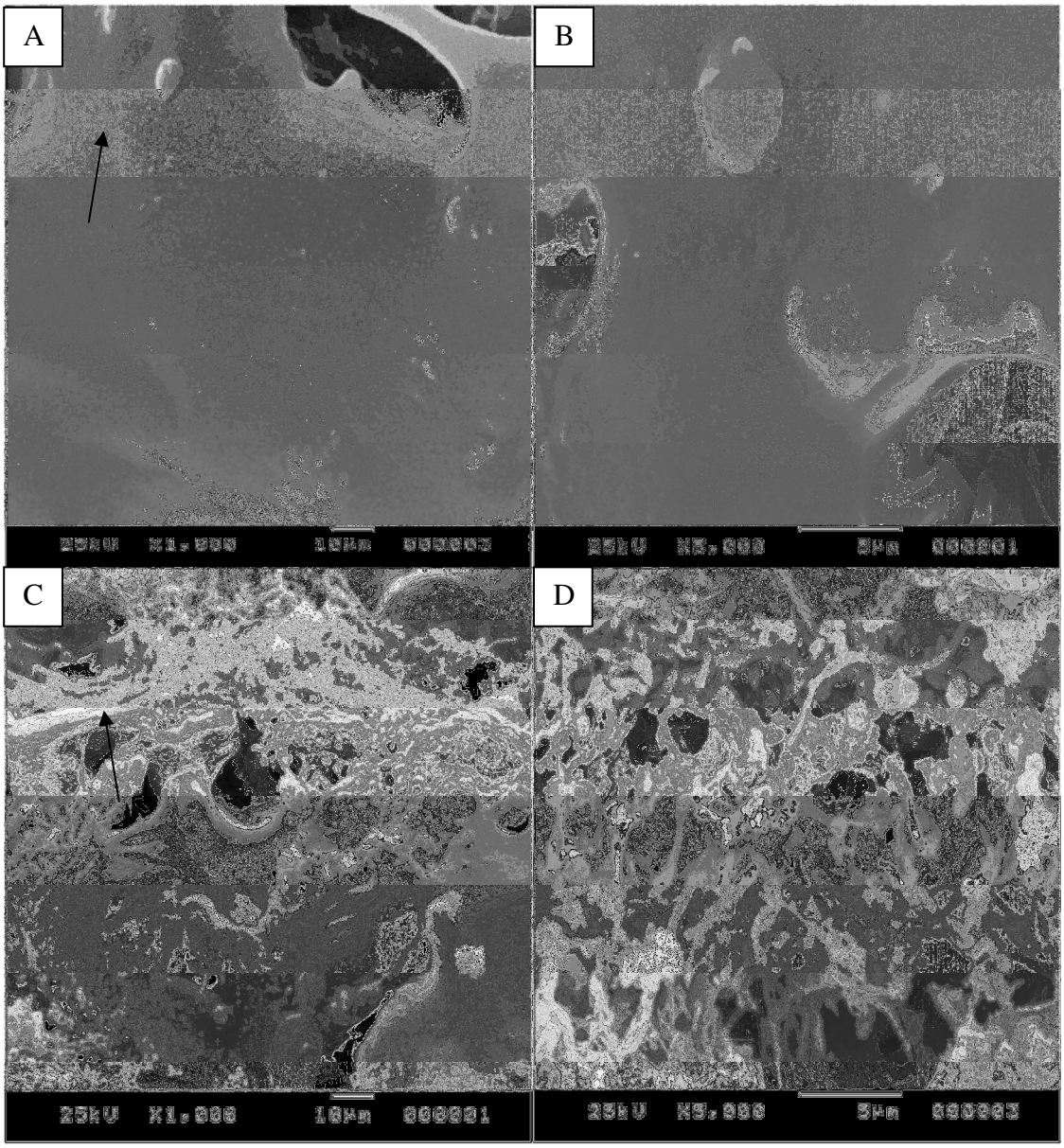


Figure 11. Scanning electron micrographs showing growth of *S. coelicolor* CH 13 on the polymer blend. A: Non-inoculated control showing polymer surface. B: Characteristics of the polymer blend surface control. C: Colonization of *S. coelicolor* CH13 on the polymer surface after 2 weeks. D: Characteristics of colonization at higher magnification after 2 weeks

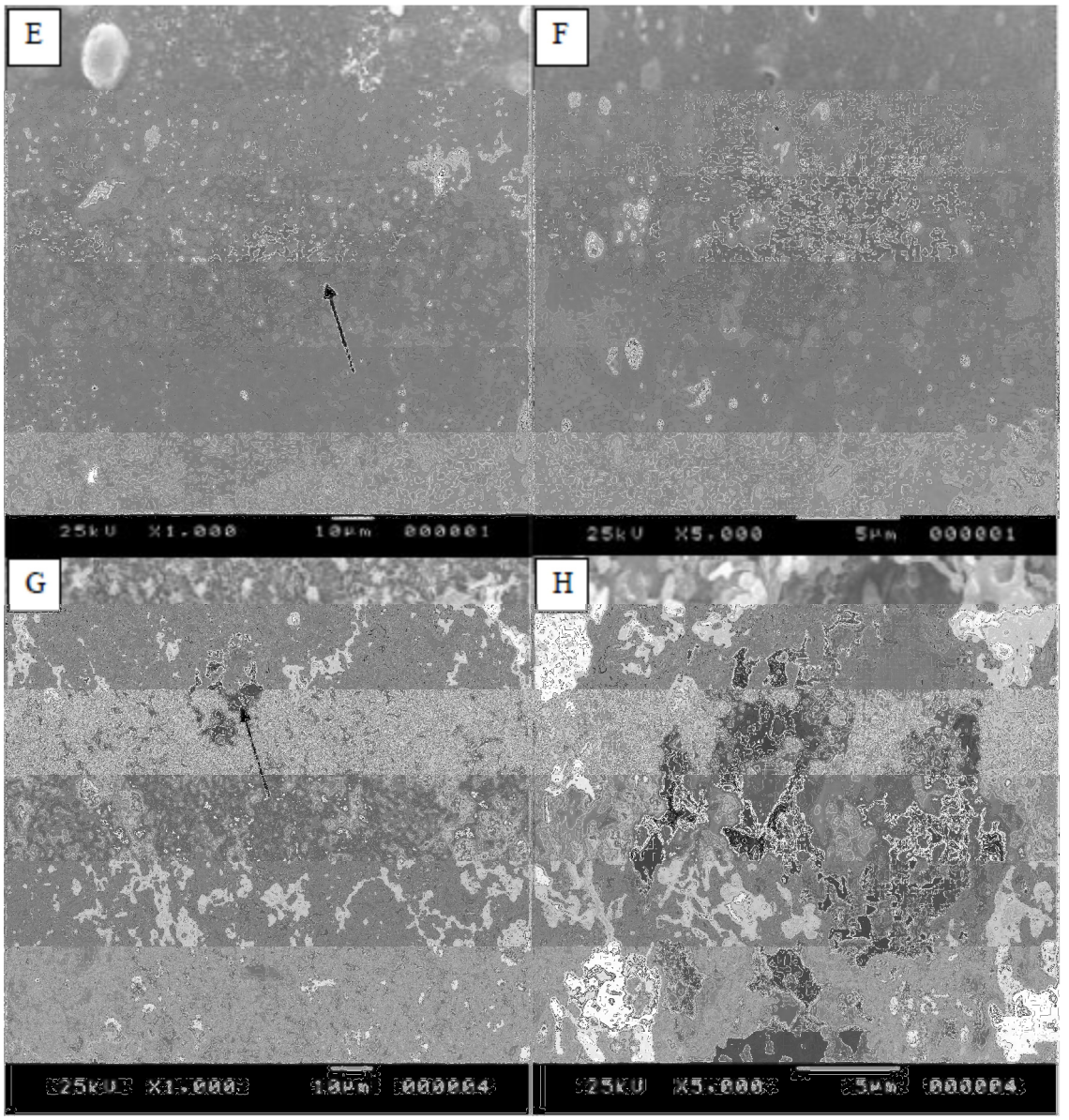


Figure 12. Scanning electron micrographs showing growth of *S. coelicolor* CH 13 on the rubber gloves. E: Non-inoculated control showing rubber gloves surface. F: Characteristics of the rubber gloves surface control. G: Colonization of *S. coelicolor* CH13 on the rubber gloves surface after 2 weeks. H: Characteristics of colonization at higher magnification after 2 weeks

4.3 Staining of NR latex gloves with Schiff's reagent

The partially degraded polymers after growth of *S. coelicolor* CH13 on the surfaces of NR latex gloves and polymer blend after cultivation in MSM liquid medium for 2 weeks were treated with Schiff's reagent. The purple color produced by the reagent on the materials provided evidence that isoprene oligomers containing aldehyde groups were being produced and accumulated during the microbial degradation, as previously described (Tsuchii *et al*., 1997).

After a cultivation period of 4 weeks with the Streptomyces strain the entire glove and polymer blend surface were colored, i.e. coated with bacteria whereas the noninoculated controls remained white also the starch polymer was stained probably because of the adsorbed Schiff's stain. This did, indicate that the deterioration of materials was via bacteria that adhered on the glove surface and polymer blend. However, the colonization ability of *Streptomyces* sp. CH13 was still low but increased during the incubation period. Rubber glove and polymer blend material were being used as carbon sources. This Schiff's tests performed after an incubation period of 4 weeks additionally revealed purple staining of small pieces of materials in the Streptomyces cultures, especially with strain CH13. This was a result of the initiation of the polymers disintegration process after the bacteria adhered to the materials and caused the biodeterioration. In our work the development of the purple color and disintegration was much more rapid (Figure 14).

4.4.4 Assay for the production of glucosidase enzymes

Investigation of the starch disappearance during the degradation process after incubation of the polymer blend with *Streptomyces* sp. CH13.

Hydrolysis of starch yields glucose which is an easily available substrate for bacterial growth. Starch hydrolysis by *Streptomyces* sp. CH13 had been revealed by the clearing zones and production of iodine staining areas on starch agar plates. The presence of glucose presumably reflected the production of amylolytic enzymes by the bacteria in the presence of the polymer blend, so the presence of

glucose was analyzed by the Somogyi-Nelson reaction. The amount of sugar released increased for up to 8 days reaching a maximum of about 530 µg/mL. This is only a minimum estimated value because some of the released glucose would be removed by the bacteria as a substrate for metabolism and growth. Between 8 and 14 days the glucose reduced slowly to 474 µg/mL. At this stage the amount of glucose being produced was similar to the amount being consumed. During this stage perhaps most of the readily hydrolysable glucosidic bonds had been broken leaving the less available bonds so the rate of glucose production declined while its metabolism by bacteria did not. This was followed by a rapid decrease of glucose to <54 µg/mL at day 20 (Figure 16).

The α-glucosidase activity was measured by the release of nitrophenol from p-nitrophenyl- α-D-glucopyranoside (Wimmer *et al.,* 1997) so this was a true estimate of the enzyme activity in the culture supernatant. This showed a slow but regular increase up to 65 U/mL on day 12. From days, 14 to 18 the enzyme increased at a fast rate up to 138.76 IU/mL when the glucose concentration was declining and then slowly decreased to 105.16 IU/mL at 20 days (Figure 16). It may be that this indicates the presence of different enzymes perhaps hydrolyzing different components of the starch which does consist of 20 % amylose and 80% amylopectin (Wilaiwan *et al.,* 1998). The hydrolysis of amylose was probably complete after 8 days whereas the total glucosidase enzyme continued to increase up to 18 days, as the glucose level decreased. This might be due to the production of the glucosidase being partially repressed by free glucose

4.5 To investigate the disappearance of starch after incubation

The disappearance of starch from the cassava starch foam blend with the NR latex polymer was investigated by the soxhlet extraction method. In the beginning the polymer foam blend containing 1.48 g starch in 2 g of polymer blend. One set of samples was removed from the incubation with *S. coelicolor* CH13 every 2 days over a period of 20 days. More than 70% of starch was removed quickly in the first 8 days and after 20 days more than 87% had been removed but at a much slower rate. This slower second rate may be due to the presenc of starch molecules that were

more difficult to degrade and may have required a different enzyme. As was expected the increase in glucose (Figure 16) was reflected by the increase of the amount of starch lost.

4.6 Mineralizations (% CO_2 release)

The polymer blend and rubber gloves were used as sole carbon sources for determining the ability of *Streptomyces* sp. CH13 to metabolize them and grow on: NR-latex concentrate, which constitutes > 90% dry weight of the NR latex gloves, and corresponds to the vulcanized NR, with crosslinks between the polymer chains, and the polymer foam blend, that constitutes > 60% of the dry weight of the rubber in the polymer. In all cases cultures were inoculated with cells adapted to use rubber. Figure 17A shows the time course of the mineralization of NR gloves and polymer blend substrates. For evaluation of the CO_2-release, it was assumed that the employed substrates consisted totally of carbon. After 30 days, 56% of the polymer blend was mineralized whereas only 10% of the rubber gloves had been mineralized. In addition, an initial increase of the viable cells was detected in the supernatant for each substrate (Figure 17B). In spite of the preferential adhesive growth behavior on the plastic sheets, there was an increase in the number of cells suspended in the medium during cultivation with the polymer blend and NR gloves.

Changes of bacterial population during the mineralization experiment are shown in Figure 17B. During both the polymer blend and rubber glove degradation bacterial numbers in the supernatant increased for up to 8 days, reaching a population of almost 10^9 cells/mL. It is possible that most of the bacteria became attached to the polymers surface to degrade, thus in the supernatant we detected only a fraction of the bacteria that did not adhere. This result indicates that there was sufficient nutrient in the initial medium to support this amount of growth. During this time the amount of sugar, a readily available nutrient, in the supernatant of the polymer blend was increasing through degradation of the polymer whereas with the rubber gloves there was no such substrate. This was reflected in the observation that the population in the polymer blend culture was maintained for another 10 days after which the glucose concentration became depleted (Figure 17B) and during this time

was able to generate the enzymes to degrade the rubber whereas in the case of the rubber gloves the bacterial numbers decreased rapidly until some more substrate became available from the small amount of rubber degradation at about 12 days. The degradation of the rubber gloves may have been compromised by the numbers of cross linkages in the vulcanized rubber. Perhaps this indicates that the bacterium rapidly loses viability when nutrients become limiting.

4.7 Fourier transform infrared spectroscopy (FTIR)

After microbial exposure, FTIR analysis of cassava starch foam blend with NR latex and NR glove indicated some reduction of the molecular sizes and the formation of new peaks with the breakdown of some bonds.

After incubating the foam blend St/NR with *S. coelicolor* CH13 for 4 weeks the intensity of the CH_2 band at 1455 cm^{-1} was decreased (Resolution 4). The presence of C-O (C-O-C, C-O-H) bonds for starch at peak 1155, 1082, 1023 cm^{-1} in the St/NR foam control disappeared after treatment with bacteria to indicate that many bonds had undergone enzyme-cleavage by the foam blend depolymerase. The presence of the C=C rubber bonds was denoted by the band at 1645 cm^{-1} as was the NR =C-H bond at 837, and 844 cm^{-1} both in the control and the foam treated with microbes. The microbial treated foam was also observed to have C=O bonds of carbonyl groups (aldehyde, ketone) at peak 1741 cm^{-1}, it is possible that some of the unsaturated double bonds (C=C) in NR were converted to C=O. This indicated that the foam blend after degradation by microbes lost many bonds to show breakdown in the polymer chain with the formation of new functional groups.

The presence of the C=C band at peak 1645 cm^{-1} of NR glove was significantly reduced during the microbial treatment. Also peaks form =C-H were observed at 875 cm^{-1} together with the formation of the C=O of carbonyl groups (ketone). This showed that the unsaturated double bond (C=C) was being converted to =C-H bond and C=O of the H-C=O of carbonyl group. In the same way, there was a decrease in the intensity of the band at 3000-3600 cm^{-1} of C-H bond after treating the NR glove with CH13. In addition to the presence of the CH_2 deformation of the peak 1456 cm^{-1} showed reduced breakdown and disappearance in the microbial treated NR

glove. This indicated that *Streptomyces coelicolor* CH13 was causing the polymer degradation (Table 5, Figure 18).

From this data, there was a significant loss of the intensity of the starch peak (Table 5) yet the unsaturated double bond (C=C) of NR, changed only a little so the rubber content is enriched during the degradation process as the starch is a preferred substrate for the bacteria and is quickly degraded and utilized by the bacteria. However, carbonyl groups are produced in the rubber component (it is possible both aldehyde and ketone form) from the oxidative breakdown of the unsaturated double bonds in the rubber so some rubber is being degraded but not completely after 4 weeks. However after 6 weeks 96% of the polymer blend was lost (Figure 24). The presence of starch has facilitated the breakdown of rubber that was initially 25% of the polymer blend. When the rubber gloves were the sole carbon source degradation of rubber was limited even though the many C=C bonds were being converted to C=O according to the FTIR spectra (Table 5, Figure 19). It is not known if the presence of the crosslinks in the vulcanized rubber affected its degradation.

4.8 Optimizing conditions for the biodegradation of polymer blend and rubber gloves

4.8.1 Effect of inoculum size on the bacteria degradation of the two rubber polymers

The effect of the inoculum size on the biodegradation of the two substrates was investigated using inoculum sizes of 10^6, 10^8, 10^{10} cells/mL. The highest percentage weight loss of the polymer blend and NR glove was 96.14% and 14.31% respectively, after 6 weeks of incubation using an inoculum size of 10^8 cells/mL. With inoculum sizes of 10^6, 10^{10} cells/mL, the percentage weight loss of polymers was lower (Figure 21). From the viable counts it would seem that the culture conditions used can only maintain a maximum population of 10^9 cells/mL. Most of the changes to population occurred in the first 2 weeks with an increase from 10^6 to 10^8 and 10^8 to 10^9 cells/mL. The initial population of 10^{10} cells/mL decreased

to 10^9 cells/mL. There was very little change in the population over the next 4 weeks (Figure 20). It is possible that the adhesion of bacteria on the polymers surface is limited. This process is presumably the first step in the microbial degradation of the polymer surface. The bacteria then release their enzymes to degrade and form a biofilm over the surface. The polymers surface became limiting when the bacteria increased much beyond 10^8 cells/mL and it was this initial concentration that showed the best degradation.

4.8.2 Effect of temperature

Being a mesophilic organism, *Streptomyces* sp. CH13 can grow over a range of incubation temperature and catalyse a good breakdown of the polymers. The maximum percentage weight loss of the polymer blend occurred at 30°C (87.3%), whereas at 35°C the weight loss was 70.9% and at 40°C it was only 65.2% (Figure 22B). For the rubber gloves the highest weight loss also occurred at 30°C (29.5%), followed by 35°C (19.1%) and 40°C (16.7%) (Figure 22A). Other workers have found that a temperature (>35°C) had an adverse effect on bacterial growth and enzymes produced for degradation of polymers (Thakur *et al.,* 2009).

4.8.3 Effect of the concentration of nitrogen source.

To evaluate this effect, the experiment was performed as follows: Temperature = 30 °C, pH = 7, and initial inoculums size 10^8 cells/mL with an incubation time of 4 weeks. The effect of nitrogen source present as NH_4NO_3 had a significant effect on the weight loss of the polymers. The nitrogen concentrations used were 0, 0.5, 1, 5 and 10 g/L and the percentage weight losses were 22, 43, 80, 90 and 95 for the polymer blend and 7, 15, 22, 30 of rubber glove respectively (Figure 23). The nitrogen source at a concentration of 10 g/L was found to provide the best conditions for the biodegradation of both polymers. In the previous experiment a concentration of 1 g/L was used. The change from 1 to 10 g/L increased the weight loss of the polymer from 80 to 90% whereas the increase for the rubber gloves was from 15 to 30%. We assume that this is a reflection of providing better optimum

conditions for growth of the bacteria. With the polymer blend the bacteria can obtain a rapid source of carbon from the starch but the nitrogen source is limiting whereas when the rubber glove is the only source of carbon both the carbon and nitrogen source is limited. Providing the rubber degrading bacteria with an extra more readily available carbon source with the rubber may be one way to increase the rate of rubber degradation.This may be available in normal soil.

4.8.4 Effect of pH

To study the effect of the pH of the culture medium on the weight loss of the polymers, the pH values of the medium were adjusted to 6, 7, and 8. The NH_4NO_3 concentration was 10 g/L and inoculums size was 10^8 cells/mL. At a pH of 6 the weight loss from the polymer blend after 4 weeks was (46.1%), at a pH of 7, it was 85.1% and at a pH of 8.0 it was 63.6% (Figure 24A and 24B). For the rubber glove the weight loss was 25.6% at pH 6, 34.4 % at pH 7 and 30.7% at pH 8. A pH of 7 was therefore chosen as the optimum for degradation by *S. coelicolor* CH13. This agreed with a report by Bystrykh *et al*. (1996) that *S. coelicolor* produced extracellular enzymes in cultures at around a pH value of 7.

4.8.5 Establishing the optimum conditions for polymer biodegradation

It is evident from the results of the previous investigations that the optimum conditions for polymer degradation over a 4 weeks period in a mineral salts medium by *Streptomyces* sp.CH13 are a pH of 7, an initial inoculum size of 10^8 cells/mL, with NH_4NO_3 at 10 g/L as a nitrogen source and a temperature of 30°C in a shaking incubator. Under these conditions a weight loss of 96.8% was achieved for the polymer blend and a 36.5% weight loss for the rubber gloves. The values obtained using optimum conditions have been compared with each of the parameters used (Figure 25A and 25B). Increasing the time of incubation to 8 weeks allowed for a slight increase in the degradation of the rubber gloves but the cells seemed to die (Figure 17B). If some method could be found to increase the viability during this

time, say by adding some other more readily available nutrient this may facilitate complete degradation.

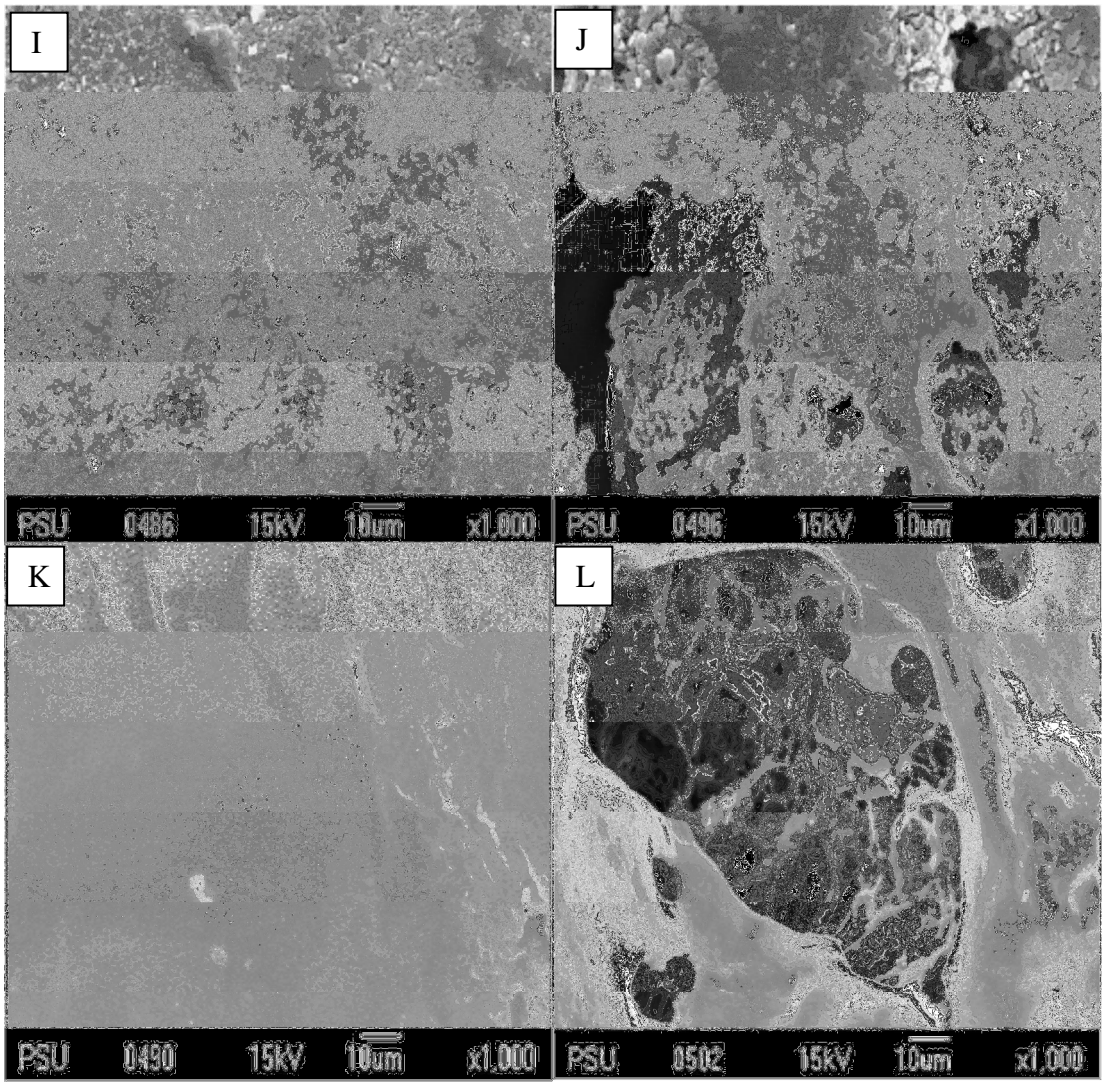


Figure 13. Scanning electron micrographs showing erosion surface of polymer blended strips and rubber gloves strips (I) Non-inoculated rubber glove control, (K) Non-inoculated polymer blend control, (J) Rubber glove strips incubated with *S. coelicolor* CH 13, (L) Polymer blend incubated with *S. coelicolor* CH 13 after 4 weeks

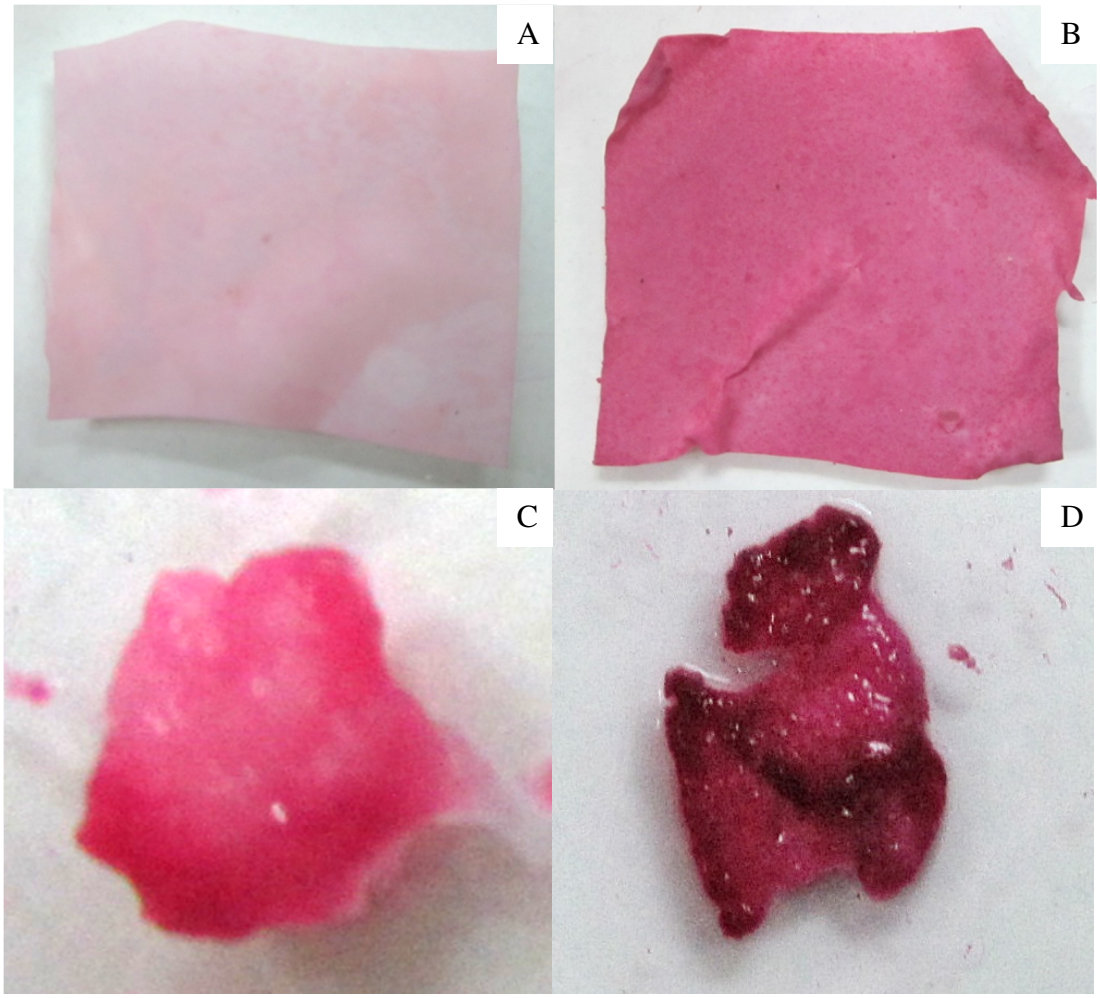


Figure 14. Shows the pieces of rubber gloves (B) and polymer blend (D) stained with schiff's reagent after incubation for 2 weeks compared with rubber glove control (A) and polymer control (C)

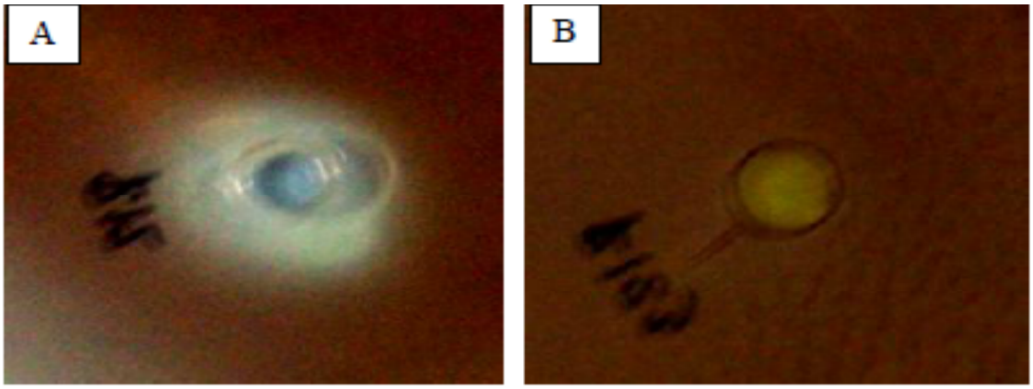


Figure 15. The clearing zone of starch hydrolysis by *Streptomyces coelicolor* CH13 production of iodine staining areas on a starch agar plate (A), compared with control (B)

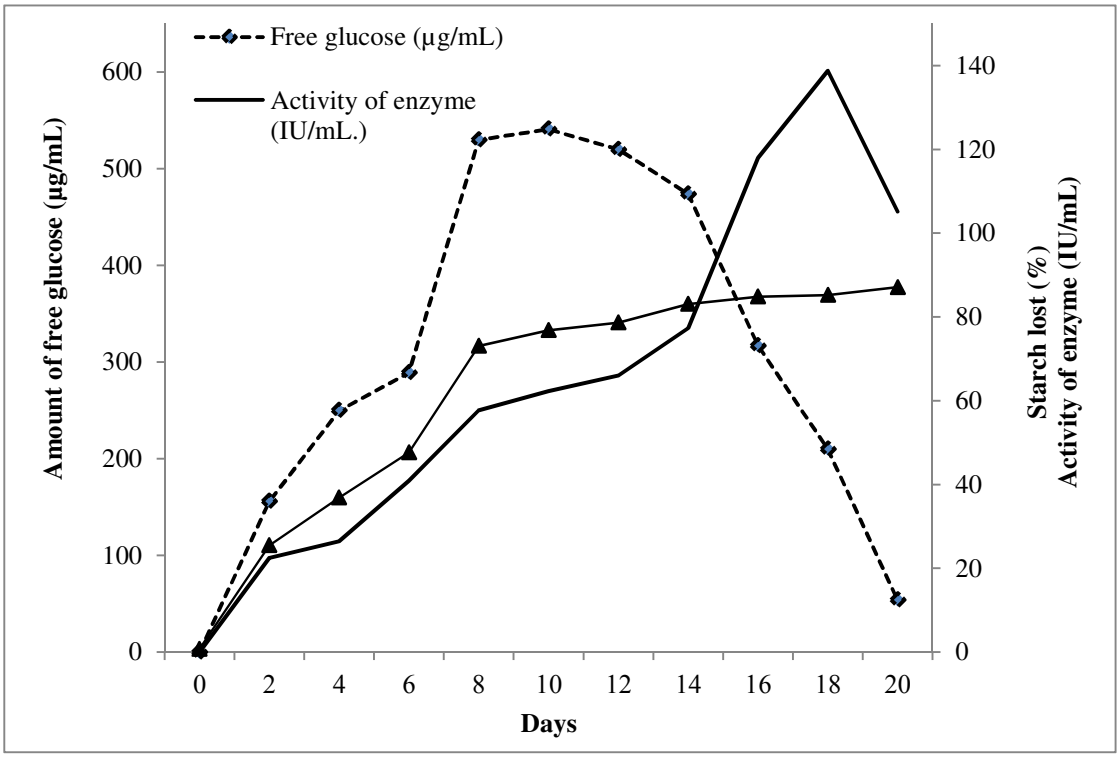
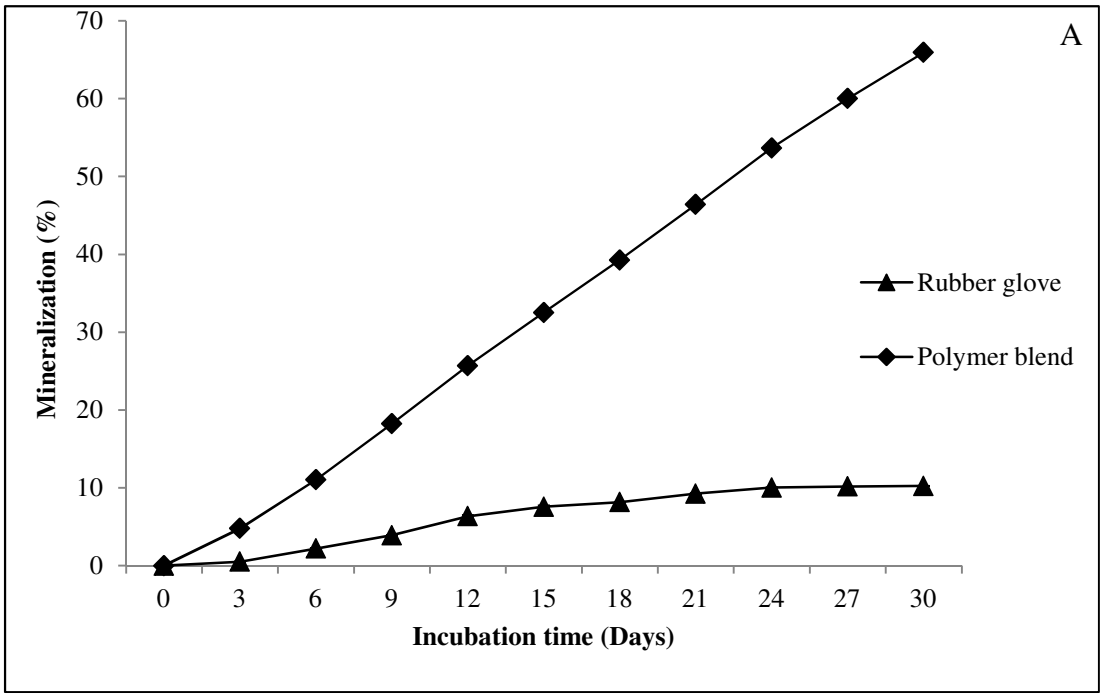


Figure 16. Showed the percentage of starch lost, enzyme activity and amount of free glucose when incubated polymer blend with *S. coelicolor* CH13 during 20 days



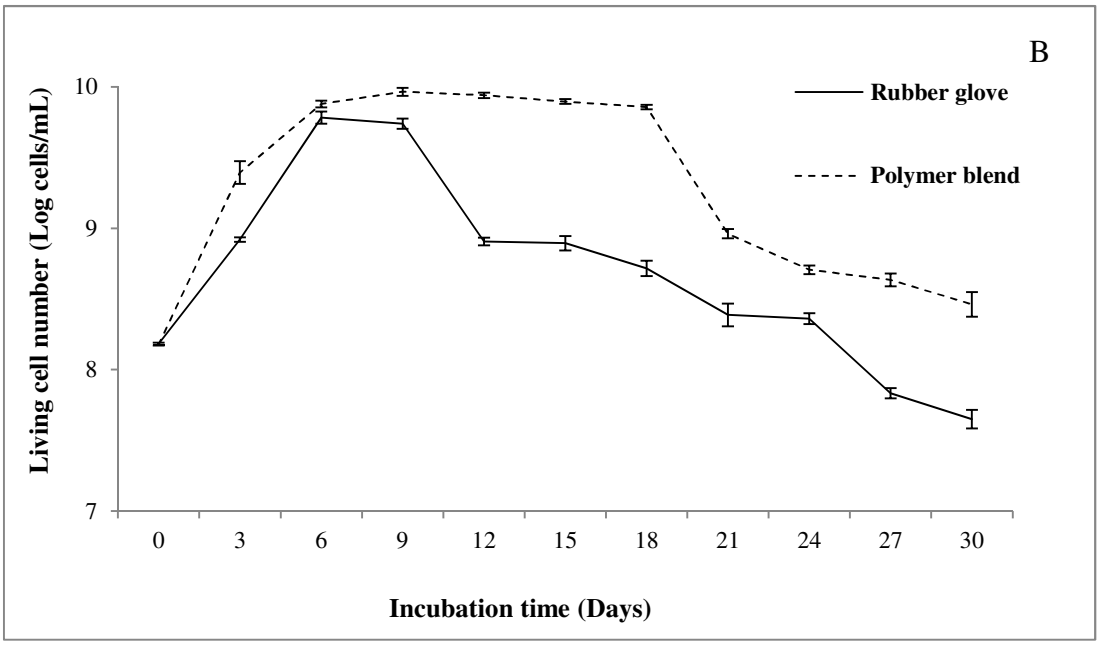


Figure 17. Percentage mineralization during growth of bacteria on polymer blend and rubber glove expressed as % CO_2 released from total carbon (A). Viable counts of suspended cells during cultivation (B)

Table 5. Wavenumbers (cm^{-1}) as observed in the FTIR spectra of polymer blend ST/NR and NR glove before and after incubation with *S. coelicolor* CH13 (Santos *et al*., 2005; Kaewtatip and Tanrattanakul, 2008; Tanrattanakul and Chumeka, 2009)

Materials	**Wavenumbers (cm^{-1})**	**Assignment**
NR	3434, 3443, 2920, 2921	O-H stretching
	2853, 2852	CH_2 symmetric stretching
	1770, 1718, 1741	C=O stretching (Carbonyl group)
	1677, 1645	C=C stretching
	1456, 1455	CH_2 deformation
	1316	CH_2 wagging
	1375	C-H deformation (CH_2)
	1090, 1080	C-CH_2 stretching
	837, 844, 875	=C-H wagging
Starch	3000-3600	O–H stretching
	1453, 1375	C-H deformation (CH_2 bending)
	1190–950	C–O stretching
	1155, 1082, 1080, 1023	C-O stretching (C-O-C, C-O-H)

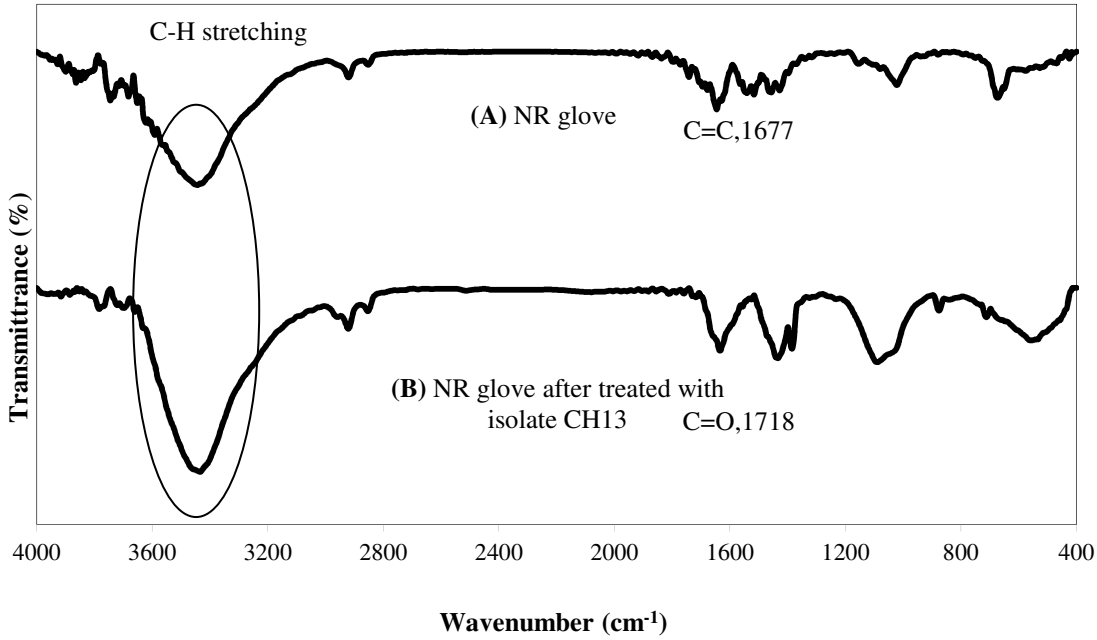


Figure 18.FTIR spectra NR glove (A), a NR glove control without bacteria incubated for 4 weeks (B), and after being incubated with *S. coelicolor* CH13 for 4 weeks

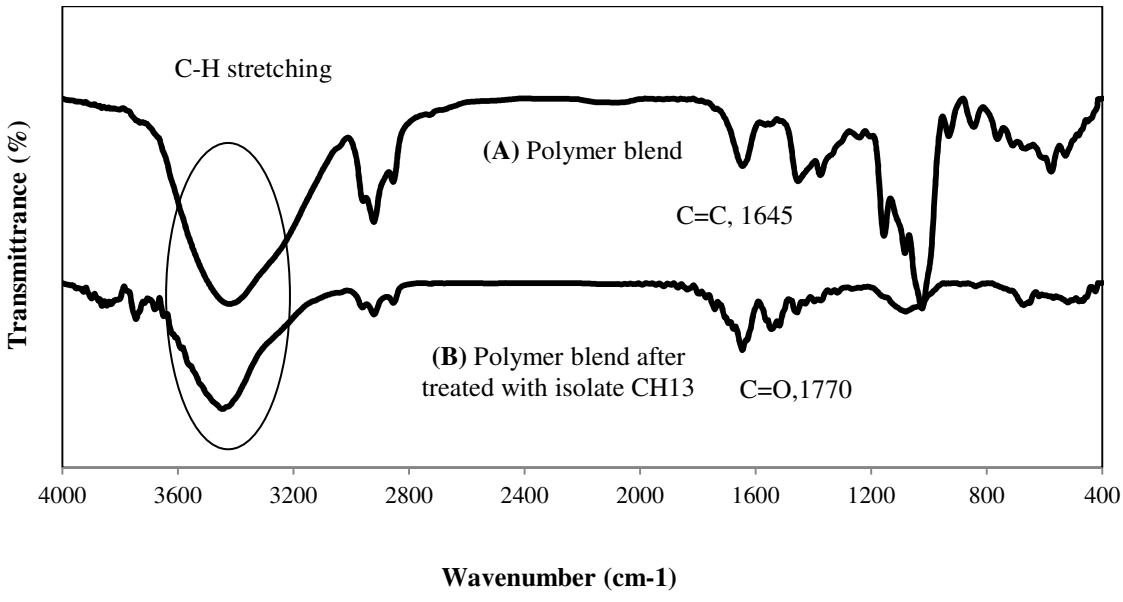


Figure 19. FTIR spectra cassava starch foam blend with NR latex (A), a cassava starch foam blend with NR latex control without bacteria incubated for 4 weeks (B), and after being incubated with *S. coelicolor* CH13 for 4 weeks

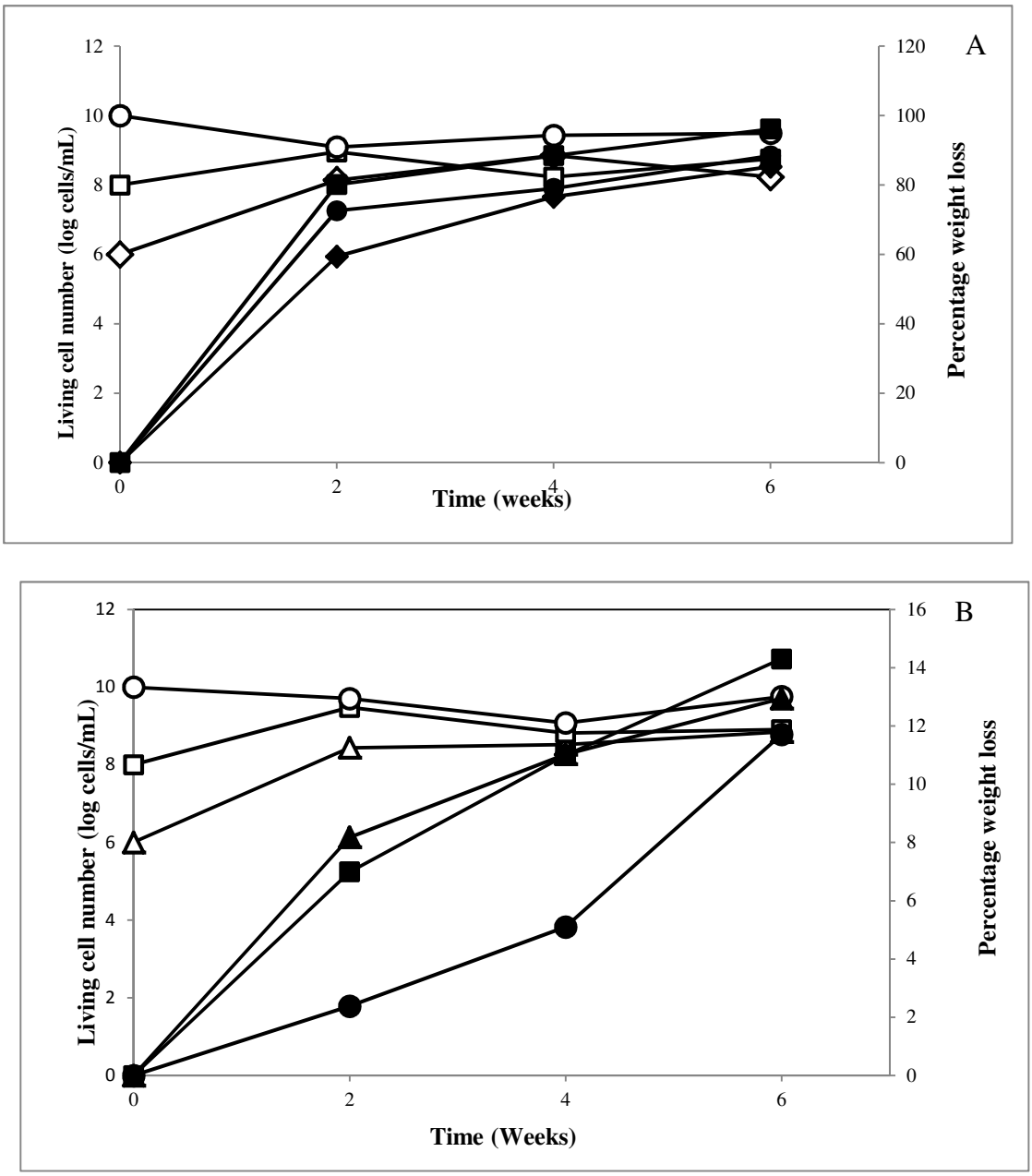
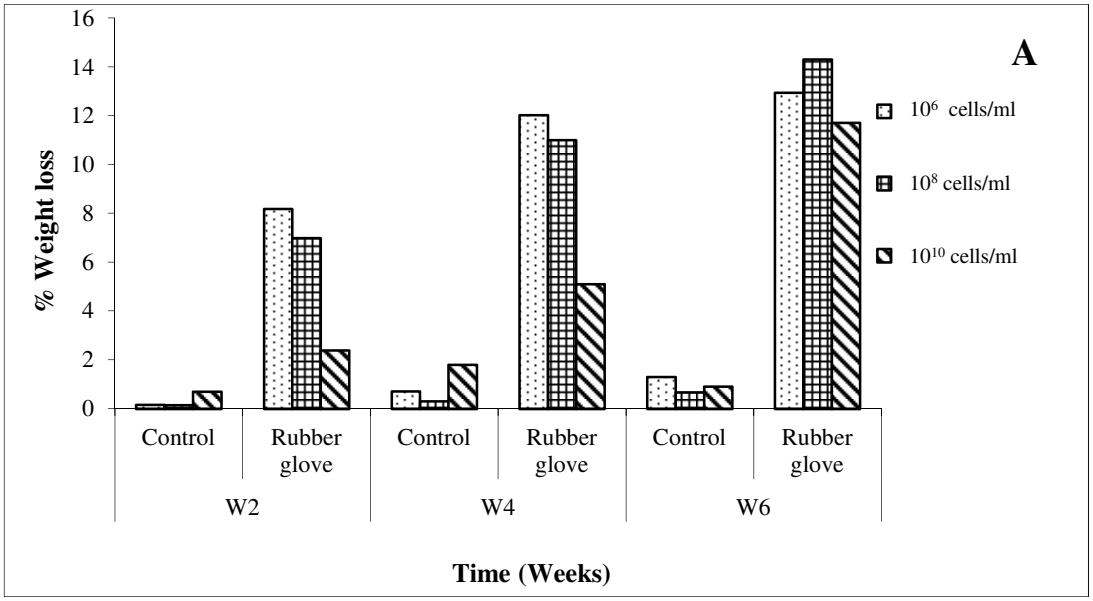


Figure 20. Effect of inoculum size on the percentage weight loss during growth of bacteria MSM containing (A) polymer foam blend (0.6% rubber w/v) with a defined inoculum size (◇) 10^6 cells/mL, (□) 10^8 cells/mL, (○) 10^{10} cells/mL and percentage weight loss inoculated with 10^6 cells/mL (◆), 10^8 cells/mL (■), 10^{10} cells/mL, (●) 10^{10} cells/mL. (B) Rubber gloves (0.6% rubber w/v) with a defined inoculum size (△) 10^6 cells/mL, (□) 10^8 cells/mL, (○) 10^{10} cells/mL and the percentage weight loss inoculated with 10^6 cells/mL (▲), 10^8 cells/mL (■), 10^{10}cells/mL (●)



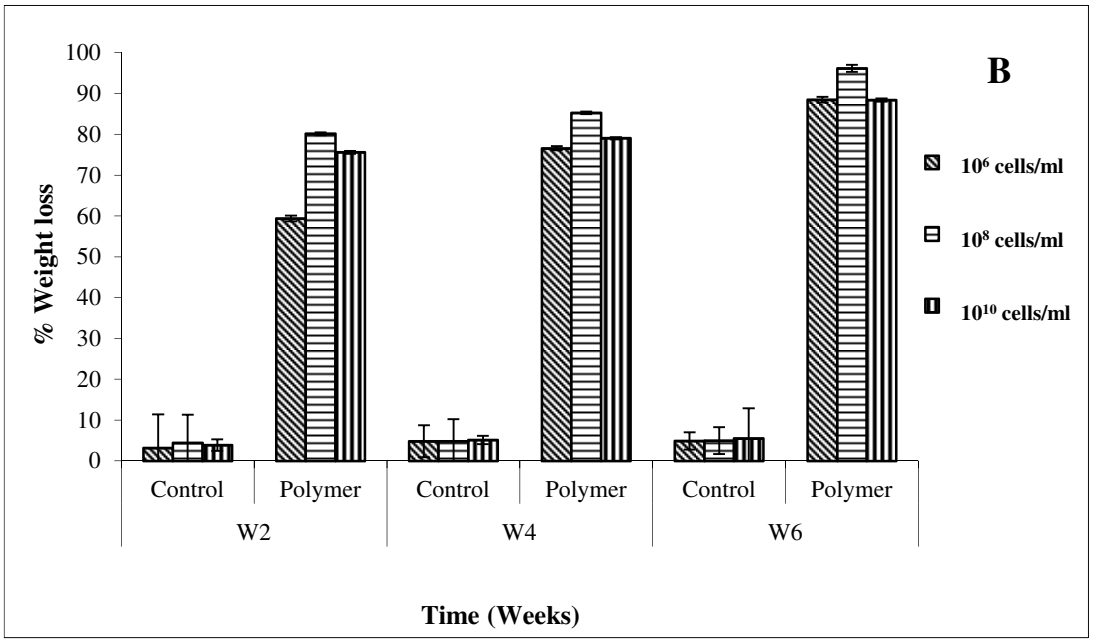


Figure 21. Inoculum size for optimum biodegradation rate of rubber gloves (A) and polymer blend (B) after an incubation time of 6 weeks

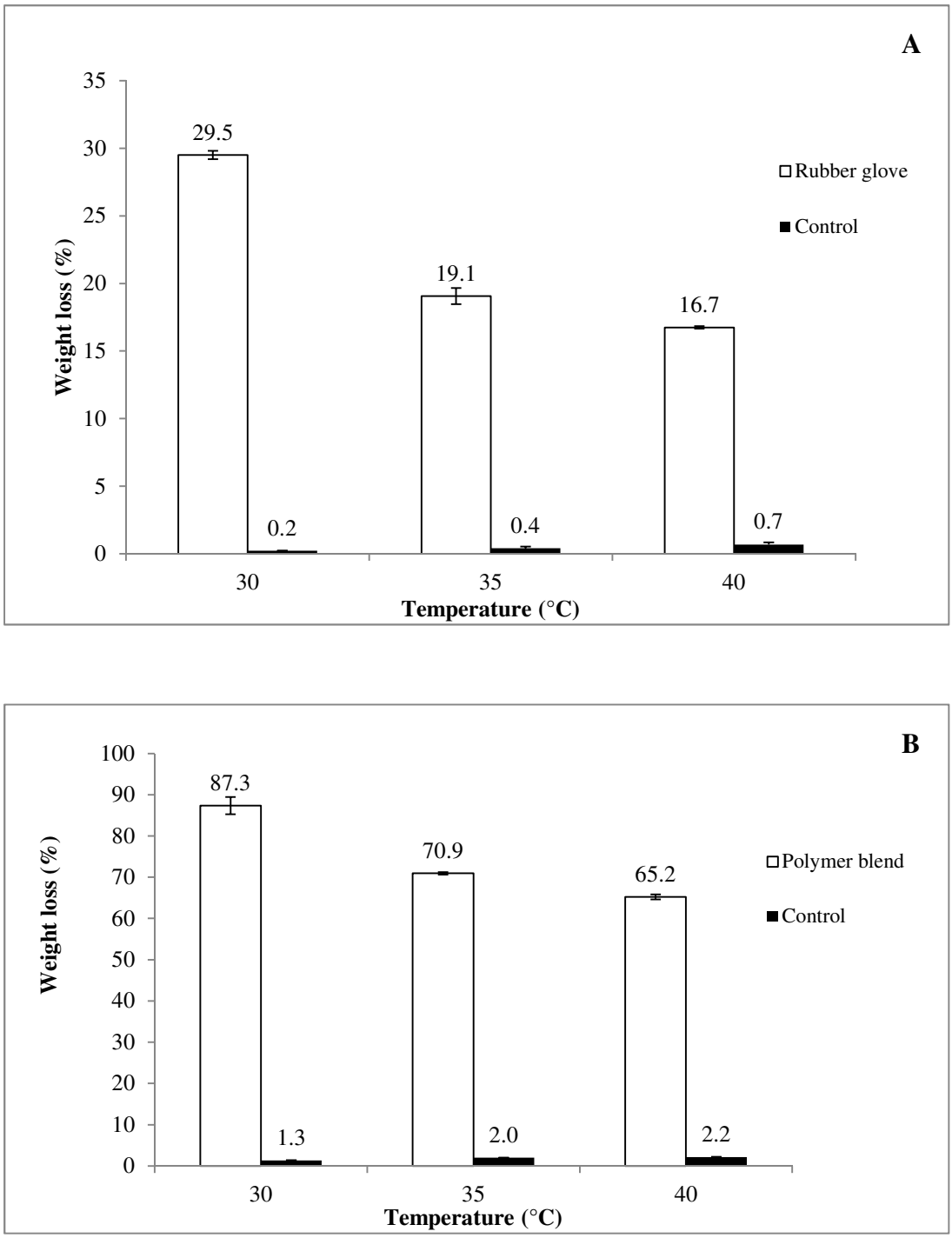


Figure 22. Effect of temperature of the MSM on the percentage weight loss of rubber glove (A) and polymer blend (B) by isolate CH13 after incubation for 4 weeks

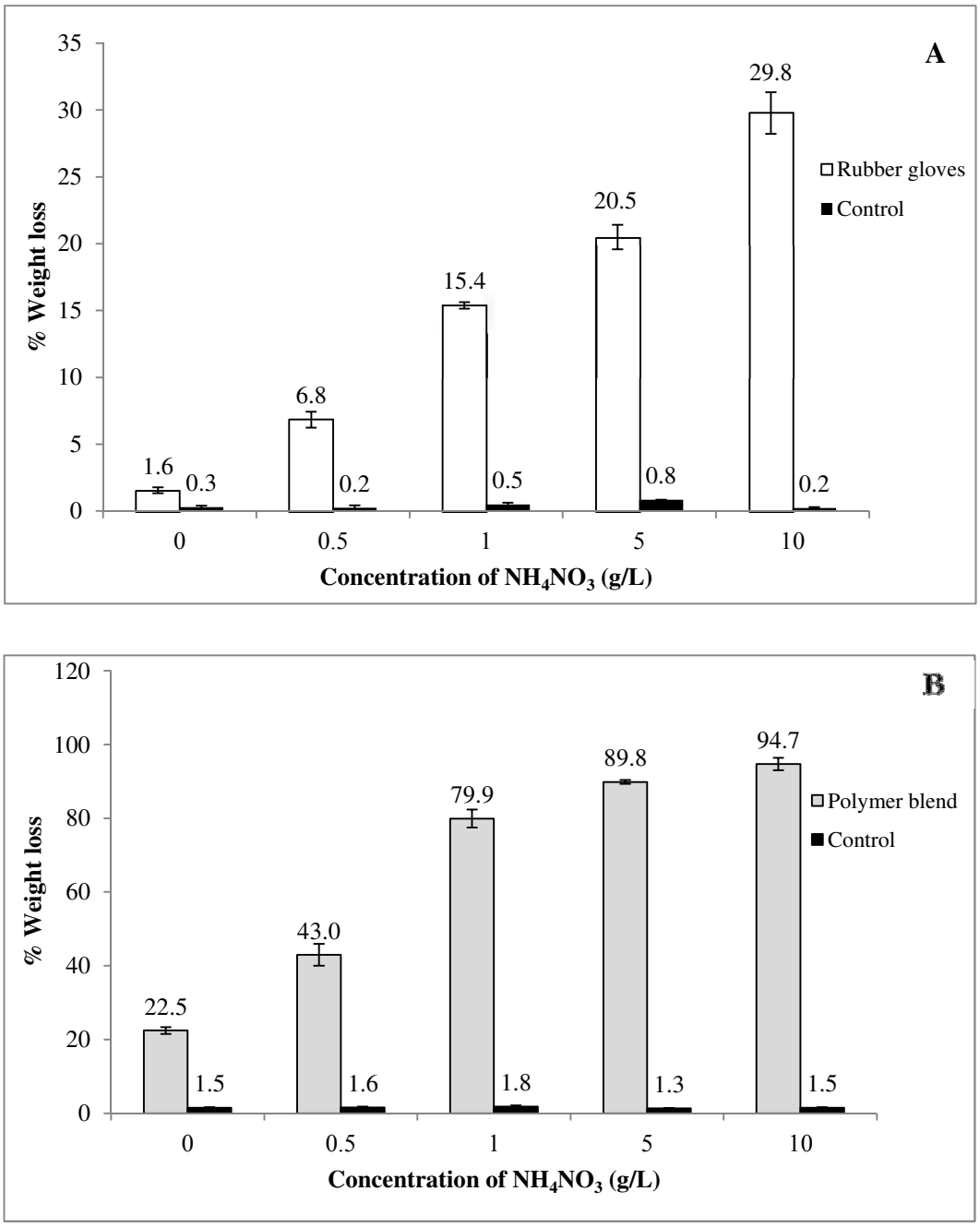


Figure 23. Percentage weight loss of rubber glove (A) and polymer blend (B) after incubation with isolate CH13 in media containing NH_4NO_3 as a nitrogen source in different concentrations

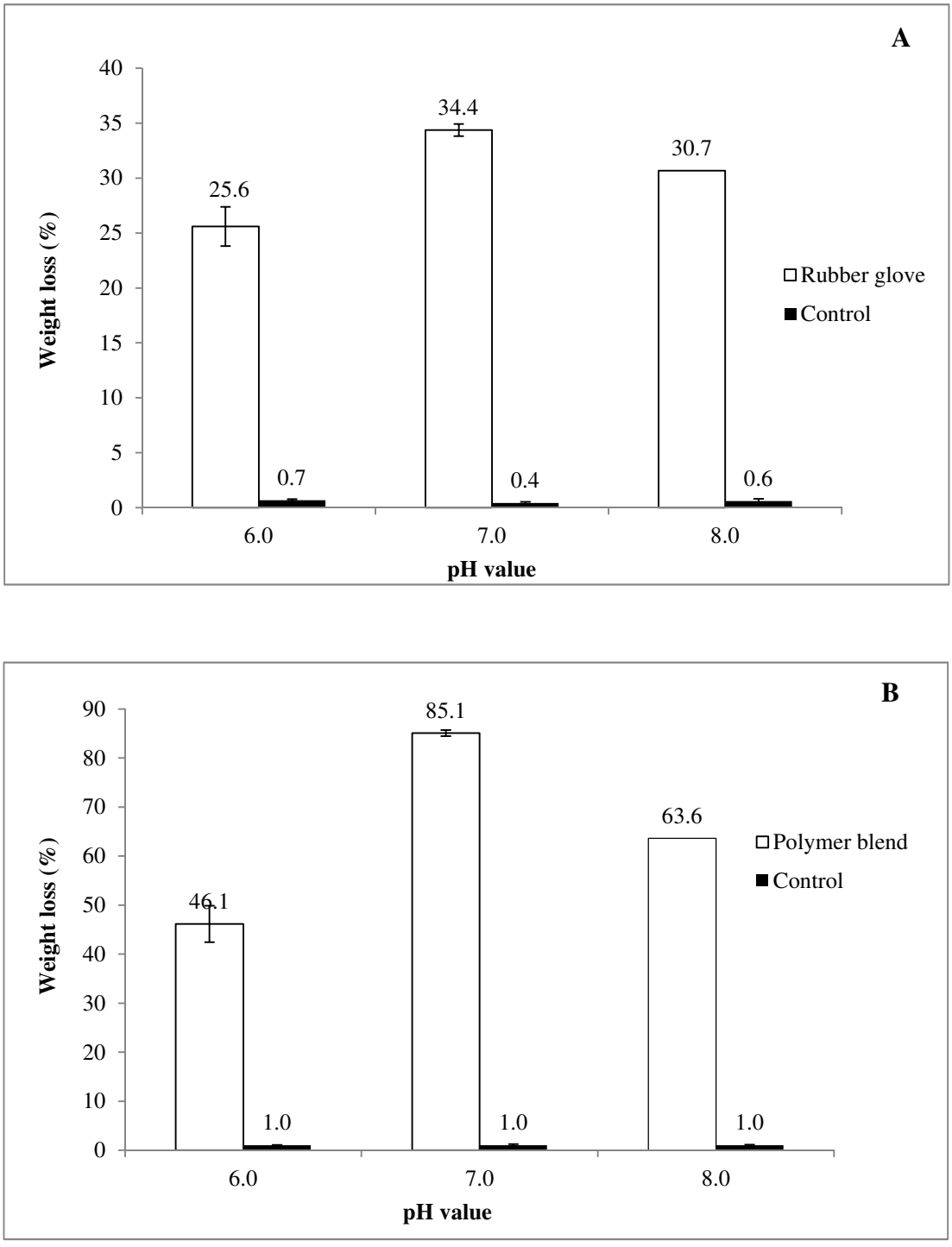


Figure 24. Effect of pH of the MSM on the percentage weight loss of rubber glove (A) and polymer blend (B) by isolate CH13 after incubation for 4 weeks

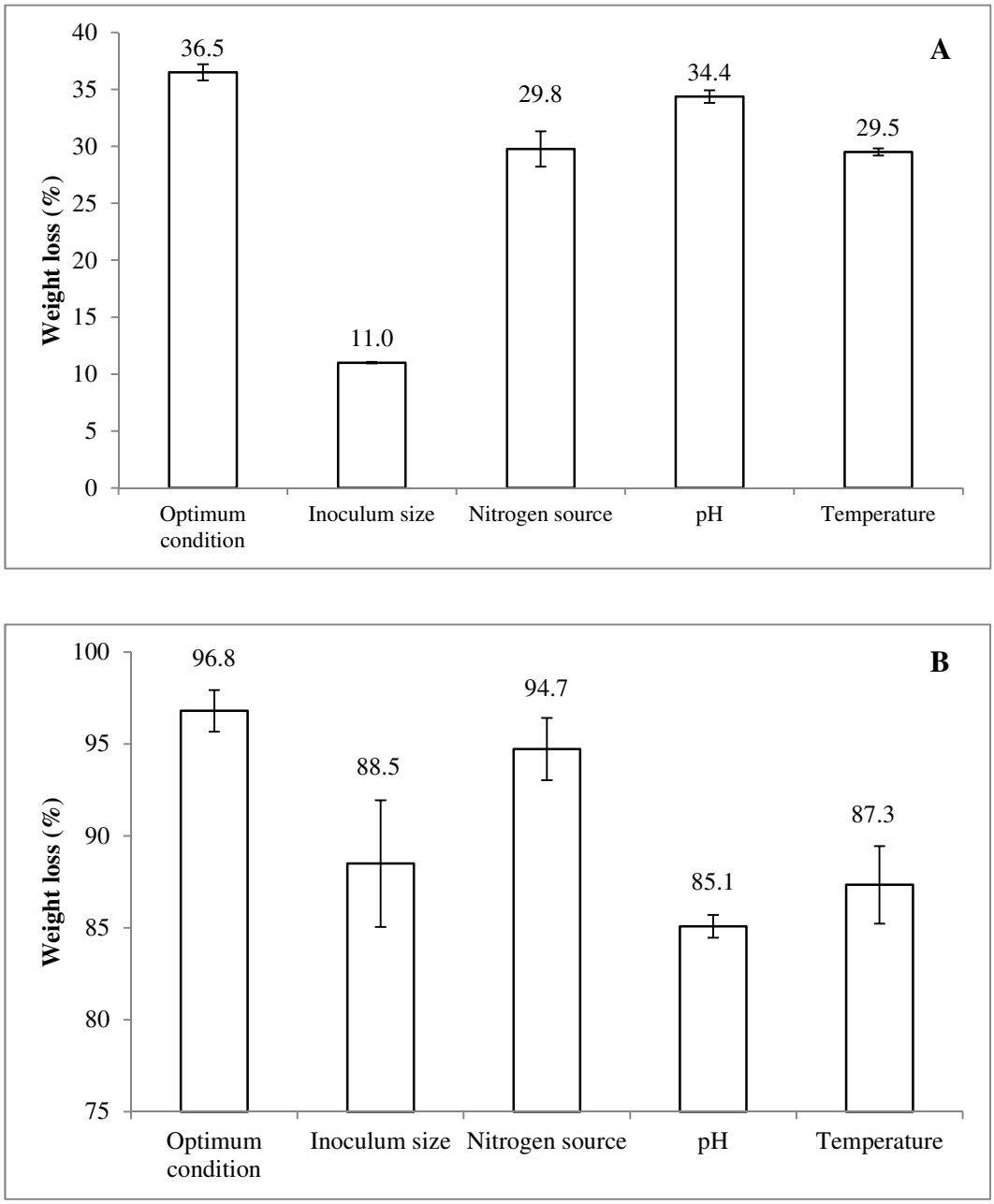


Figure 25. Weight loss with the optimum condition for rubber glove (A) and polymer blend (B) degradation by *S. coelicolor* CH13 compared with the weight loss obtained previously after incubation for 4 weeks

CHAPTER 5

Discussion

5.1 Screening, isolation and identification of rubber-degrading bacteria

The 119 natural rubber degrading-bacteria isolated were detected by their growth with NR latex as the sole source of carbon and energy. They were obtained from soil samples from *Hevea brasiliensis* plantations and from wastewater of a rubber producing company in Songkhla. It would seem that NR-degrading bacteria are widely distributed in nature. All except one of the NR-degrading strains found were Gram-positive bacteria. In the literature on the isolation of microbial rubber degradating bacteria from soil Gram-positive bacteria dominate (Heisey and Papadatos, 1995). Most belong to the genera of oxidative, Gram-positive, filamentous bacteria producing spores, such as Streptomycetes and Nocardia that can grow on or within their substrates. (Linos *et al.,* 2000; Jendroseek *et al.,* 1997). There has been one Gram-negative bacteriua, a Xanthomonas species that grew directly on natural rubber latex, (Tsuchii and Takeda, 1990), Also *Pseudomunas aeruginosa* AL98 was also reported by Linos *et al.* (1999) to degrade raw and vulcanized natural rubber as well as a synthetic cis-1,4-polyisoprene.

These 119 NR-utilizing bacteria were divided in two groups, 78 isolates produced halos on NR-containing media while the rest did not form a clearing zone around their colonies. These observation were similar to those obtained by Linos *et al.* (2000) who reported that there were two different strategies for bacteria to utilize rubber. The first group needed to have direct contact to the rubber substrate and became firmly attached to the rubber material so did not produce clearing zones (Spence and van Neil, 1936). This group belonged to the genera *Corynebacterium*, *Mycobacterium* and, *Nocardia*. The second group of rubber decomposers produced clear zones (translucent halos) around their colonies on media with NR dispersed in mineral agar. These mainly belong to the genus *Streptomyces*. As these are much easier to observe 9 strains that produced the biggest halos were selected for further

characterization studies. The isolate that formed the largest clear zone, CH13 was selected for more detailed characterization and to investigate the degradation process of the NR polymer.

Biochemical tests on isolate CH13 showed that it produced amylase and degraded the starch/NR mixed polymer. This isolate is a Gram positive bacterium producing catalase and oxidase enzymes, hence it most probably contained cytochrome C oxidase and utilized oxygen for energy production with an electron transfer chain and released CO_2 from its respiratory processes after utilizing the polymer molecules. It demonstrated incomplete hemolysis or α-hemolysis on blood agar probably caused by hydrogen peroxide production that also oxidized hemoglobin to green methemoglobin (George *et al.,* 2004). During sporulation it produced long chains of spores (rectiflexibiles type) that was typical of a *Streptomyces* species (Figure 26).

Taxonomic classification of the selected strain CH13, based on 16S rRNA similarity examinations, revealed it to be 99% similar to *Streptomyces coelicolor* and was therefore named *Streptomyces coelicolor* CH13.

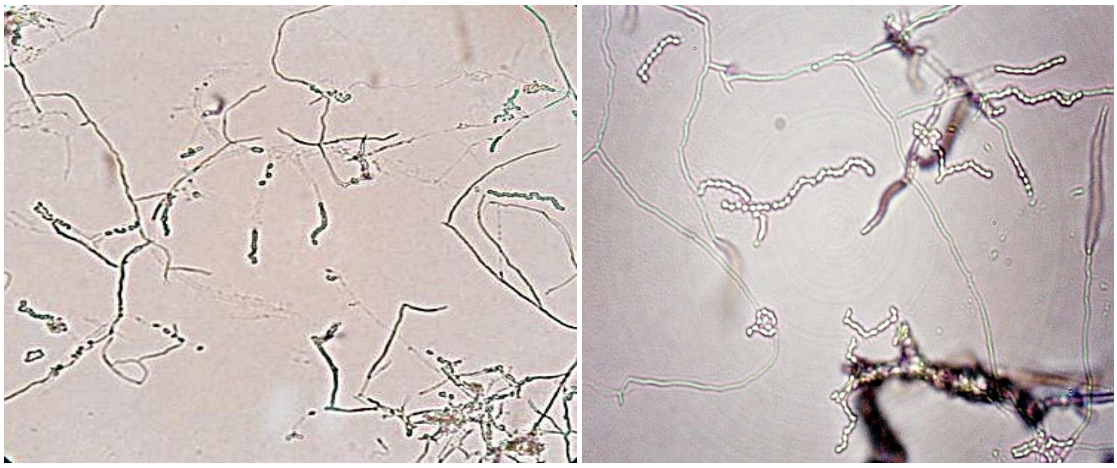


Figure 26. Show the sporulation of *Streptomyces coelicolor* CH13 that produced long chains of spores (rectiflexibiles type)

5.2 Investigation of the biodegradation process including:

5.2.1 Colonization of polymers

The colonization behavior was clearly seen by SEM observations. Mycelia were tightly attached to cover the surface with some being extensively embedded within the polymers (Figure s 11 and 12) and the color of the materials was changed after incubation. Isolate CH13 formed colony craters and large holes (Figure 13) causing erosions at the polymer surface and produced a bio-film on both polymers surfaces after a similar cultivation period. This indicated the immobilization of the cells in the polymers matrix. It is well known that bio-films often become embedded in a polymer matrix containing polysaccharides and proteins. This indicates an excretion of polymer-decomposing factors or enzymes by the bacteria that cause disintegration of the polymers. This adhesion to surfaces is a general microbial strategy for survival as well as for utilization of solid substrates. Flemming's report (1998) showed that direct and indirect damage by bacteria led to the deterioration of polymeric materials caused by; (1) adhering microorganisms that colonized the polymer surfaces; (2) increasing the leaching of monomers out of the polymer matrix; (3) attack by enzymes to degrade the polymer; (4) water accumulates and penetrates the polymer matrix together with microbial filaments that cause swelling; (5) excretion of lipophilic microbial pigments that led to changed colors in the polymer. This growth strategy of adhering and forming a bacterial biofilm can be very advantageous with respect to competition with other more faster-growing microorganisms. In the case of the *Streptomyces coelicolor* CH13, a rubber-degrading mechanism was obviously an additional characteristic. The colonization behavior resembles that of Pan's *Nocardia corynebacterioides* strain (2009), Linos's Actinomycetes (2000), and Tsuchii's *Nocardia* sp. strain 835A that have been studied in detail (Tsuchii *et al.,* 1985; Tsuchii *et al.,* 1987). SEM observation also revealed that the two different polymers were colonized differently. It was recognized that the bacteria that adhered to the starch/NR polymer had a softer appearance and were fatter and more cells were free and some were also more embedded within the polymer than those on rubber gloves. On the rubber gloves the bacteria seemed to be

harder and thinner and more firmly attached to the surface. It seemed that the starch was a preferred substrate, see also Berekaa *et al.* (2005).

5.2.2 Degradation products and deterioration of the polymer

Staining by Schiff's reagent was performed after cultivation of the *S. colicolor* CH13 on the NR latex gloves and polymer blend strips, (Figure 14). In both cases a magenta or red-purple color was clearly visualized and provided evidence for the presence of degradation products containing aldehyde groups. This has also been recently reported (Berekaa *et al*., 2000; Tsuchii *et al*., 1985; Linos *et al*., 2000). Rose *et al.* (2005) also detected aldehyde accumulation around the streptomyces strains that were growing on a latex overlay of agar plates by using the Schiff's stain.

Application of FTIR spectroscopy on the NR latex gloves and polymer blend being used as a sole carbon source after cultivation with *S. coelicolor* CH13 revealed insights into the biodegradation mechanism of the decomposition process. Spectra demonstrated a decrease in the number of *cis*-1,4 double bonds from the polyisoprene chain (Figure 18 and 19), and the appearance of ketone and aldehyde groups in the samples. All these observations can be interpreted as a consequence of the oxidative breakage of the rubber polymer chain length, thus leading to a change of the polymers. This indicates that the biodegradation mechanism described for the *Gordonia* strains as described by Linos *et al.* (2000) most likely occurred with strain CH13. This includes: scission of the polymer chain at the double bond by an oxygen attack to produce carbonyl groups with an aldehyde on the one side and a ketone on the other (Figure 27). A previous report from Tsuchii *et al.* (1985) showed that an analogous mechanism was proposed for the NR latex glove degrading *Nocardia* sp. strain 835A according to findings obtained after analysis of the extracted degradation products. Considering further data in the literature in which an analogous oxidative cleavage is known to occur after degradation of NR latex, either by extracellular enzymes by a *Xanthomonas* sp. (Tsuchii *et al*., 1990) or by an inorganic activation through molecular oxygen (Tangpakdee *et al.,* 1998) occurs as shown and leads to detection of aldehyde groups using the Schiff's reagent test on the surface of rubber on which a biofilm was produced by the strain. It is assumed that the first metabolic

step is common among all polymer-degrading bacteria, irrespective of their colonization strategy, and implies scission of the double bonds by enzymes producing activated oxygen (Figure 27).

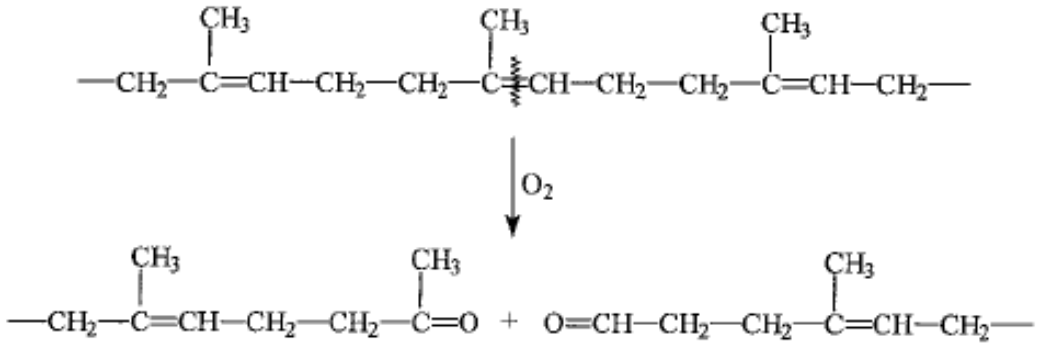


Figure 27. Schematic diagram of natural rubber degradation by strain 835A (Tsuchii *et al*., 1985)

5.3 Investigation of the disappearance of starch in the polymer blend

The strategy of bacteria to degrade polymers is to first adhere to its surface then to secrete enzymes that can initiate degradation by reducing the polymer chains lengths to eventually produce molecules that can be assimilated and converted by its metabolic processes to provide energy and precursors for its biosynthetic reactions so that it can grow. Evidence from scanning EM clearly showed that isolate CH13 became attached to the polymer surfaces and in the case of the polymer blend containing rubber latex and cassava starch caused a disintegration of the polymer sheets. As the major component of this polymer is starch and microbial degradation of starch normally leads to the production of oligosaccharides and eventually glucose, a readily available nutrient this is part of their strategy (Schlemmer *et al*., 2009). The loss of starch from the polymer was measured by the soxhlet extraction method and compared to the release of reducing sugars into the supernatant using the Somogyi-Nelson method, for measuring reducing sugar and the presence of an α-glucosidase enzyme measured by the liberation of nitrophenol from p-nitrophenyl α-D-glucoside.

The overall process could be divided into 3 separate phases, from 0 to 8 days, (phase 1) 8 to 14 days (phase 2) and 14 to 21 days (phase 3). It was not unexpected that the loss of starch closely corresponded to the increase in the reducing sugar component of the supernatant in phase 1. During this time the population of bacteria rapidly increased as did the loss of starch, the increase in the amount of free sugar and the amount of enzyme (Figure16). Towards the end of phase 1 the reducing sugar level increased most rapidly (8-10 days) probably due to the conversion of all the intermediate oligosaccharides to glucose. In phase 2 the bacterial population remained fairly constant and so did the level of free reducing sugar, the loss of starch proceeded at a much slower rate together with an increase in enzyme activity so the amount of glucose produced was about the same as the amount used by the bacteria. During phase 2 perhaps the growth of the bacteria became limited by the amount of available nitrogen in the medium (1 g/L). At this point, phase 3, 83 percent of the starch had been removed so the reducing sugar was metabolized more quickly than it was being produced and the amount of starch lost reached it's maximum of 87% but there was a large increase in the amount of enzyme. This indicated that formation of the enzyme was perhaps being controlled by the amount of available glucose ie a case of glucose repression (Figure 16 and 17 B).

The information is consistent with the following scenario. During phase 1 the bacteria multiplied, became attached to the substrate, and in the absence of any free substrate the hydrolytic enzyme was induced and converted the starch to oligosaccharides with a slower increase of free glucose. As the free glucose increased the rate of enzyme production decreased slightly until multiplication of the bacteria ceased at a time when the reducing sugar concentration and glucose was highest, (end of phase 1) and growth was being limited by the absence of available nitrogen so the rate of enzyme production decreased further. When the glucose was being rapidly used (phase 3), glucose repression was released and the enzyme level suddenly increased even in the absence of substrate so starch may not be an inducer of enzyme, just the absence of any suitable nutrient to grow. In the absence of glucose the bacteria lost viability and no further enzyme was produced. A similar mechanism of biodegradation was suggested by Zuchowska *et al.* (1998).

5.4 Mineralization of polymer blend and rubber glove substrate

During the aerobic biodegradation of organic materials, carbon dioxide and water are the final decomposition products of mineralization. The amount of carbon dioxide produced during the biodegradation of the test material was measured, and compared to the theoretical maximal amount and recorded as a biodegradation percentage. The process of biodegradation was shown in Figure 17A (Chapter 4) where carbon dioxide production was plotted against the time of cultivation. These experiments clearly revealed that both the polymers were being degraded by the isolate *S. coelicolor* CH13 but there was a marked difference between the two polymers in their degree of degradation. After 30 days 56.58% of the original carbon in the polymer blend was converted to CO_2 whereas only 10.24% of the carbon of the rubber gloves was converted to CO_2. This again demonstrated the ability and preference of the isolate to degrade starch rather than rubber. In both cases the population of bacteria achieved their highest level after about 1 week of incubation from 10^8 cells/mL at the start to 10^9 cells/mL for the polymer blend and 10^9 cells/mL for the rubber gloves. This population was maintained in the polymer blend for at least another week while the sugar concentration remained high whereas between 7 and 10 days with the rubber gloves the population fell to 8x 10^8 cells/mL. It was of interest that the population with the rubber blend also fell to 8x 10^8 cells/mL during a 3 days period when the reduction of the free glucose was occurring at its maximum rate from 300 to its minimum values of 0 µg/mL. It would seem that the isolate CH13 quickly lost viability when readily available substrates became limiting. A further exponential loss of viability occurred at about the same rate in both cases (Figure 17B). An increase in the number of cells suspended in the medium during cultivation that led to an increase biodegradation and higher mineralization rate was also described by Warnake *et al.* (2007); Linos *et al.* (2000); Berekaa *et al.* (2005).

5.5 To establish optimum conditions for the biodegradation rates

5.5.1 Effect of inoculums size

The size of inoculum influenced the ability of *Streptomyces* sp.CH13 to degrade the polymer blend and rubber gloves in culture medium. The maximum percentage weight loss of polymers by the isolate was at an inoculums size of 10^8 cell/mL. This results are similar to those of Thakur *et al.* (2009), Grag and Nee lakantan (1981) who proved that the size of the inoculum can be an important factor in microbial degradation studies.

5.5.2 Effect of temperature

Streptomyces sp. CH13 is mesophilic organism growing and degrading rubber polymers best at 30°C. An increase to 40°C produced a significant decrease of activity. A similar finding was obtained by Liao *et al.* (2009) and Thakur *et al.* (2009) showed that the production of protein and enzymes involved with biodegradation of polymers was much better at 30°C than at 35°C.

5.5.3 Effect of different concentrations of nitrogen source

Although the strain grew and degraded polymers at all concentrations of NH_4NO_3 tested, the maximum biodegradation was obtained at a high concentration of 10 g/L. This indicated that during the first experiments when 1 g/L was being used nitrogen was a limiting factor for the growth and the enzymes used for degradation. Similar observations were reported by Khaoua *et al.* (1991) and Mansour *et al*. (1996).

5.5.4 Effect of pH

Although polymer biodegradation occurred at each of the pH values tested 6, 7, and 8, a pH of 7.0 allowed for the most degradation. This was similar to the observations of Brnakova *et al.* (2005) using *Streptomyces aureofaciens.* Another species of

Streptomyces had an optimum pH of 7.5 for the production of antimicrobial compounds as well as for growth (James *et al*., 1991) and an *S. coelicolor* culture produced, actinorhodin, an extracellular blue pigment-antibiotic best at a pH value of around 7.0 (Bystrykh *et al.,* 1996). Most *Streptomyces* spp. seem to operate best at producing extracellular biological active compounds at around pH 7.

5.5.5 The optimum conditions for biodegradation of the polymer blend and NR rubber gloves

From the above observations it seems that the optimum conditions for *Streptomyces coelicolor* CH13 to biodegrade the polymer blend and rubber glove in an MSM medium, are an inoculums size of 10^8 cells/mL, NH_4NO_3 at a concentration of 10 g/L, a pH of 7.0, and temperature at 30°C, in a shaking incubator. This achieved a maximum percentage weight loss of 96.8% for the polymer blend and 36.5% loss for rubber gloves over a 4 weeks incubation period.

These results establish quite clearly that this new polymer of starch/NR can be completely degraded by the new isolate *Streptomyces coelicolor* CH13 over a relatively short period of time. This also makes it clear that disposal of products made from this polymer in the normal environment would not lead to their accumulation as undegradable wasts. The presence of this isolate would facilitate a rapid rate of degradation. There are still many areas, particularly those associated with the mechanisms of degradation of NR that require further investigations. One particular example follows. As the maximum loss of weight from the rubber gloves was only 36.5% while for the starch/NR polymer the total weight loss was 96.8% the presence of starch seem to have facilitated the degradation of rubber. There must be some factor that limits the complete degradation of the rubber gloves. Perhaps this might have something to do with the observation that when strain CH13 runs out of readily available carbon substrate it dies or simply spores. In the case of rubber gloves, initially oxidation of the double bonds may produce some readily available carbon maybe from the ends of the chains but attack on many of the internal double bonds is restricted. The presence of starch mixed with the NR may simply aid access to these double bonds and provide substrate for microbial growth. These observations require further studies to achieve a better understanding of how to facilitate complete degradation of the rubber.

CHAPTER 6

Conclusions

In total, 119 bacterial strains capable of degrading NR latex were isolated from different regions in Songkhla province, Thailand. 78 strains belonged to the clear-zone-forming group of rubber-degrading bacteria; they produced translucent halos if cultivated on MSM containing NR latex. All 78 isolates degraded starch. From the 78 isolates that formed translucent halos, 9 isolates that produced large radial zones about 3.0 mm were tested for their stability to degrade NR polymers. One isolate, CH13 that produced the largest radial translucent halos of about 6.0 mm and was stable for rubber degradation was selected for further study.

The 16s rRNA gene sequences of strain CH13 had a 99% similarity to *Streptomyces coelicolor* so it was named *Streptomyces coelicolor* CH13. Investigation of the degradation process of a polymer blend of starch and NR and rubber gloves using SEM showed that CH13 colonized and produced a biofilm on both polymers. The CH13 isolate formed colony craters on the rubber glove surface and penetrated into the polymer blend material. It is well known that adhesion to surfaces is a general microbial strategy for survival as well as for utilization of solid substrates, especially in low-nutrient environments (Flemming, 1998). The actively growing colonies on the polymers surface were clearly visualized and produced a clear purple color, with Schiffs reagent thus providing evidence for the occurrence of degradation products linked to aldehyde groups. Application of FTIR spectroscopy indicated that the rubber glove and polymer blend were being used as a carbon source during cultivation of the Streptomyces strain. The spectra demonstrated a decrease in the number of rubber double bonds in the polyisoprene chain, and the appearance of carbonyl groups (ketone and aldehyde group). In addition, mineralization experiments showed that on the average, *Streptomyces* strain CH13 mineralized about 10.24% (wt/wt) of the rubber glove substrate and 56.58% (wt/wt) of polymer blend to CO_2 during a 4 week period. Furthermore 85% of the starch was lost after incubation with *S. coelicolor*

CH13 over 20 days. It was of interest that the loss in starch and the presence of the free glucose in the medium were a mirror image of each other.

In addition, *Streptomyces* sp. CH13 was a better degrader of NR polymers than previously reported by NR degrader bacteria. The optimum conditions for degradation by *Streptomyces* sp. CH13 over a 4 weeks incubation period were an initial inoculums size of 10^8 cells/mL, a concentration of 10 g/L NH_4NO_3 as nitrogen source, a pH of 7, and incubation temperature of 30°C. These conditions produced a 96.8 and 36.5 percentage weight loss of the polymer blend and rubber gloves respectively.

Bibliography

1. Abou-Zeid, D.M., Müller, R.J. and Deckwer, W.D. 2001. Anaerobic biodegradation of natural and synthetic polyesters, Dissertation, Technical University Braunschweig, Germany. Internet:http://opus.tu-bs.de/opus/volltexte. 246.

2. Albertsson, A.C. and Karlsson, S. 1993. Aspects of biodeterioration of inert and degradable polymers. International Biodeterioration and Biodegradation. 31: 70-161.

3. ASTM D4674e89. 1989. Standard test method for accelerated testing for color stability of plastics exposed to indoor fluorescent lighting and window-filtered daylight. ASTM International.

4. ASTM D 5271-92. 1992. Standard test method for assessing the aerobic biodegradation of plastic material in an activated-sludge-waste water treatment system. ASTM International

5. Augusta, J., Mfiller, R.J. and Widdecke, H. 1992. Investigations on the biodegradation of polyesters by isolated mesophilic microbes. Chemie Ingenieur Technik. 64: 410-415.

6. Augusta, J., Müller, R.J. and Widdecke, H. 1993. A rapid evaluation plate-test for the biodegradability of plastics. Applied Microbiology Biotechnology. 39: 673-678.

7. Bastioli, C. 1994. The starch based thermoplastics [Novamont]. Recycle '94-Davos Global Forum and Exposition. Davos, Switzerland.

8. Bellina, G., Tosin, M., Floridi, G. and Degli-Innocenti, F. 1999. Activated vermiculite, a solid bed for testing biodegradability under composting conditions. Polymer Degradation and Stability. 66: 65–79.

9. Bellina, G., Tosin, M. and Degli-Innocenti, F. 2000. The test method of composting in vermiculite is unaffected by the priming effect. Polymer Degradation and Stability 69: 113–20.

10. Be Miller, R.J. 1994. Carbohydrates In: Kirk-Othmer Encyclopedia of Chemical Technology, 4th Ed. Wilet & Sons, New York. 4: 911-948.

11. Berekaa, M.M., Barakaat, A., El-Sayed, S.M. and El-Aassar, S.A. 2005. Degradation of natural rubber by *Achromobacter* sp. NRB and evaluation of culture conditions. Microbiology. 54: 55–62.

12. Berekaa, M.M. and Linos, A. 2000. Effect of pretreatment of rubber material on its biodegradability by various rubber degrading bacteria. FEMS Microbiology Letters 184: 199–206.

13. Bhatt, R., Dishma, S., Patel, K.C. and Ujjval, T. 2008. PHA–rubber blends: Synthesis, characterization and biodegradation. Bioresource Technology 99: 4615-4620.

14. Bikiaris, D., Prinos, J., Koutsopoulos, K., Vouroutzis, N., Pavlidou, E. and Frangis, N. 1998. LDPE/plasticized starch blends containing PE-g-MA copolymer as compatibilizer. Polymer Degradation and Stability. 59: 91-287.

15. Biological Waste Management Symposium. 1995. Proceedings of the International Symposium on Biological Waste Management—A Wasted Chance? Hamburg: University of Essen and Technical University of Hamburg.

16. Blake, J.T., Kitchin, D.W. and Pratt, O.S. 1950. Failures of rubber insulation caused by soil microorganisms. American Institute of Electrical Engineers. 69: 748-754.

17. Blake, J.T., Kitchin, D.W. and Pratt, O.S. 1955. The microbiological deterioration of rubber insulation. Applied Microbiology. 3: 35-39.

18. Bode, H., Kerkhoff, K. and Jendrossek, D. 2001. Bacterial degradation of natural and synthetic rubber. Biomacromolecules. 2: 295-303.

19. Bode, H.B., Zeeck, A., Plückhahn, K. and Jendrossek, D. 2000. Physiological and chemical investigations on microbial degradation of synthetic poly(cis-1,4-isoprene). Applied and Environmental Microbiology. 66: 3680-3685.

20. Boggs, C.R. and Blake, J.T. 1936. Deproteinized rubber. Industrial & Engineering Chemistry. 28:1198-1202.

21. Braaz, R., Fischer, P. and Jendrossek, D**.** 2004. Novel type of heme-dependent oxygenase catalyzes oxidative cleavage of rubber (poly-*cis*-1,4-isoprene). Applied and Environmental Microbiology. 70: 7388-7395.

22. Breslin, V.T. 1993. Degradation of starch-plastic composites in a municipal solid waste landfill. Environmental Polymer Degradation. 1: 41-127.

23. Brnáková, Z., Godány, A. and Timko, J. 2005. An extracellular endo-deoxyribonuclease from S*treptomyces aureofaciens*. Biochimica et Biophysica acta. 1721: 116-123

24. Bröker, D., Arenskötter, M., Legatzki, A., Nies, D.H. and Steinbüchel, A. 2004. Characterization of the 101-kilobase-pair megaplasmid pKB1 isolated from the rubber-degrading bacterium *Gordonia westfalica* Kb1. Bacteriology. 186: 212-225.

25. Bröker, D., Arenskötter, M. and Steinbüchel, A. 2008. The genomes of the non-clearing-zone-forming and natural-rubber degrading species *Gordonia polyisoprenivorans* and *Gordonia westfalica* harbor genes expressing *Lcp* activity in Streptomyces strains. Applied Environmental Microbiology. 74 (8): 2288-2297.

26. Buchanan, R.A. 1974. Starch xanthide styrene–butadiene rubbers; Effect of prolonged water immersion. Starch-Stärke. 26(5): 72-165.

27. Buchanan, R.A., Kwolek, W.F., Katz, H.C. and Russell, C.R. 1971. Starch in rubber influence of starch type and concomitant variables in reinforcement of styrene–butadiene rubbers. Starch/Stärke. 23(10): 9-350.

28. Bystrykh, L.V., Fernander-Moreno, M.A., Herrema, J.K., Malportida, F., Hopwood, D.A and Dijkhuizen, L. 1996. Production of actinorhodin-related blue pigments by *Streptomyces coelicolor* A3(2). Bacteriology.178: 44-2238.

29. Calmon, A., Dusserre-Bresson, L., Bellon-Maurel, V., Feuilloley, P. and Silvestre, F. 2000. An automated test for measuring polymer biodegradation. Chemosphere. 41(5): 51-645.

30. Carvalho, A.J.F., Job, A.E., Alves, N., Curvelo, A.A.S. and Gandini, A. 2003. Thermoplastic starch/ natural rubber blends. Carbohydrate Polymer. 53(1): 9-95.

31. Chandra, R. and Rustgi, R. 1997. Biodegradation of maleated linear low-density polyethylene and starch blends. Polymer Degradation and Stability. 56: 185–202.

32. Chaplin, M.F. and Kennedy, J.F. 1994. Carbohydrate Analysis, 2th edn. New York: Oxford University Press.

33. Cherian, E. and Jayachandran, K. 2009**.** Microbial degradation of natural rubber latex by a novel species of *Bacillus* sp. SBS25 isolated from soil. International Journal of Environmental Research. 3(4): 599-604.

34. Cornell, J.H., Kaplan, A.M. and Rogers, M.R. 1984. Biodegradation of photo oxidized polyalkylenes. Applied Polymer Science. 29: 97-2581.

35. Costerton, J.W., Cheng, K.J.G., Geesey, G., Ladd, T.I., Nickel, J.C., Dasgupta, M. and Marrie, T.J. 1987. Bacterial biofilms in nature and disease. Annual Review of Microbiology. 41:435–464.

36. De Beer, D., Stoodley, P. and Lewandowski, Z. 1994. Liquid flow in heterogeneous biofilms. Biotechnology and Bioengineering. 44(5): 636-641.

37. Doi, Y., Kumagai, Y., Tanahashi, N. and Mukai, K. 1992. Structural effects on biodegradation of microbial and synthetic poly(hydroxyalkanoate). Biodegradable Polymers and Plastics. Cambridge: The Royal Society of Chemistry.

38. Erlandsson, B., Karlsson, S. and Albertsson, A.C. 1997. The mode of action of corn starch and aprooxidant systemin LDPE: influence of thermooxidation and UV-irradiationon the molecular weight changes. Polymer Degradation and Stability. 55: 45-237.

39. Evangelista, R.L., Nikolov, Z.L., Sung, W., Jane, J.L. and Gelina, R.J. 1991. Effect of compounding and starch modification on properties of starch-filled low density polyethylene. Industrial & Engineering Chemistry Research. 30: 1841.

40. Flachner, B., Bnumbauer, A. and Reczey, K. 1999. Stabilization of β-glucosidase in *Aspergillus phoenicis* QM 329 pellets. Enzyme and Microbial Technology. 24: 362–367.

41. Flemming, H.C. 1998. Relevance of biofilms for the biodeterioration of surfaces of polymeric materials. Polymer Degradation and Stability. 59: 309-315.

42. Flemming, H.C. and Geesey, G.G. 1991. Biofouling and biocorrosion in industrial water systems. Heidelberg. Springer. 3.

43. Flemming, H.C., Schaule, G., McDonogh, R. and Ridgway, H.F. 1994. Biofouling and biocorrosion in industrial water systems. Lewis Publishers Chelsea MI. 63.

44. Frank, H.K. 1984. Forum mikrobiol. 8: 339.

45. Freitas, R.A., Rubira, F.A. and Muniz, E.C. 2008. Degradation of polychloroprene/natural rubber (PCP/NR) blends by photo-Fenton process. Polymer Degradation and Stability. 93: 601-607.

46. Gao, C.Z., Dong, Y., Zhang, H.J. and Zhang, J.M. 2007. Utilization of distiller waste and residual mother liquor to prepare precipitated calcium carbonate. Clean Product. 15: 1419–1425.

47. George, R.C.R., Kenneth, J. and Kenneth, R. 2004. Sherris Medical Microbiology: An introduction to infectious diseases (4th ed.) McGraw Hill. 237.

48. Grag, S.K. and Neelakantan, S. 1981. Effect of culture factors on cellulose activity and protein production by *Aspergillus terreus*. Biotechnology and Bioengineering. 23: 9-1653.

49. Griffin, G.J.L. 1994. Chemistry and technology of biodegradable polymers. Chapman & hall: London.

50. Griffin, J.L. 1977. Biodegradable Synthetic Resin Sheet Material Containing Starch and a Fatty Material. Coloroll Limited, assignee. C08 J 003/20. United states patent 4016117.

51. Hamad, D., Geresh, S. and Sivan, A. 2005. Biodegradation of polyethylene by the thermophilic bacterium *Brevibacillus borstelensis*. Applied Microbiology. 98: 1000-1093.

52. Haschke, H., Tomka, I. and Keilbach, A. 1998. Monatsh Chem/Chemical Monthly. 12: 487-507.

53. Heisey, R.M. and Papadatos, S. 1995. Isolation of microorganisms able to metabolize purified natural rubber. Applied and Environmental Microbiology. 61: 3092-3097.

54. Herb, S. and Flemming, H.C. 1996. Dechema Monographs. 121-133.

55. Hoffmann, J., Reznicekova, I., Vaňkov, S. and Kupec, J. 1997. Manometric determination of biological degradability of substances poorly soluble in aqueous environments. International Biodeterioration Biodegradation. 39: 32-327.

56. Howard, R.J., Ferrari, M.A., Roach, D.H. and Money, N.P. 1991. Penetration of hard substrates by a fungus employing enormous turgor pressures**.** Proceedings of the national academy of sciences, USA. 88: 11281-12284

57. Ibrahim, E.M.A., Matthias, A., Luftmann, H. and Steinbüchel, A. 2006. Identification of poly(cis-1,4-Isoprene) degradation intermediates during growth of moderately thermophilic Actinomycetes on rubber and cloning of a functional *lcp* homologue from *Nocardia farcinica* strain E1. Environmental Polymer Degradation. 72(5): 3375-3382.

58. Ikada, E. 1999. Electron microscope observation of biodegradation of polymers. Environmental Polymer Degradation. 7: 197-201.

59. Ikejima, T., Yoshie, N. and Inoue, Y. 1999. Influence of tacticity and molecular weight of poly (vinyl alcohol) on crystallization and biodegradation of poly(3-hydroxybutyric acid)/poly(vinyl alcohol) blend films. Polymer Degradation and Stability. 66: 70-263.

60. ISO 14855. 1999. Determination of the ultimate aerobic biodegradability and disintegration of plastic materials under controlled composting conditions— Method by Analysis of Evolved Carbon Dioxide.

61. ISO 14852. 1999. Plastics—determination of the ultimate aerobic degradability of organic compounds in aqueous medium — Method by Analysis of Evolved Carbon Dioxide.

62. Itavaara, M. and Vikman, M. 1995. A simple screening test for studying the biodegradability of insoluble polymers. Chemosphere. 31: 73-4359.

63. James, P.D.A., Wards, C.E.D. and Dawso, M. 1991. The effects of temperature, pH and growth rate on secondary metabolism in *Streptomyces thermoviolaceus* grown in a chemostat. General Microbiology. 137: 1715-1720.

64. Jayasekara, R., Harding, I., Bowater, I. and Lornergan, G. 2005. Biodegradability of selected range of polymers and polymer blends and standard methods for assessment of biodegradation. Polymers and the Environment. 13: 51-231.

65. Jendrossek, D., Tomasi, G. and Kroppenstedt, R.M., 1997. Bacterial degradation of natural rubber: a privilege of actinomycetes?. FEMS Microbiology Letters 150: 179–188.

66. Johnson, E., Pometto, A.L. and Nikolov, Z.L. 1993. Degradation of degradable starch-polyethylene plastics in a compost environment. Applied and Environmental Microbiology. 59: 61-1155.

67. Kaewtatip, K. and Tanrattanakul, V. 2008. Preparation of cassava starch grafted with polystyrene by suspension polymerization. Carbohydrate Polymers. 73: 647-655.

68. Kalinenko, V.O. 1938. The role of Actinomycetes and bacteria in decomposing rubber. Mikrobiologiya. 17: 119-128.

69. Kathiresan, K. 2003. Polythene and plastics-degrading microbes from the mangrove soil. Revista de Biología Tropical. 51: 3–4.

70. Kay, M.J., McCabe, R.W. and Morton, L.H.G. 1993. Chemical and physical changes. International Biodeterioration & Biodegradation. 31: 209-255.

71. Khaoua, S., Librihi, A., Germain, P. and Lefebvre, G. 1991. Cephamycin C biosynthesis in *Streptomyces cattleya*: nitrogen source regulation. Applied Microbiology and Biotechnology. 35: 7-253.

72. Kikkawa, Y., Abe, H., Iwata, T., Inoue, Y. and Doi, Y. 2002. Crystal morphologies and enzymatic degradation of melt-crystallized thin films of random copolyesters of (R)-3-hydroxybutyric acid with (R)-3-hydroxyalkanoic acids. Polymer Degradation and Stability. 76: 78-467.

73. Klausmeier, R. 1966. The effect of extraneous nutrients on biodeterioration of plastics. Microbiological Deterioration. 23: 232-243.

74. LeChevallier, M., Cawthon, C.D. and Lee, R.G. 1988. Factors promoting survival of bacteria in chlorinated water supplies. Applied and Environmental Microbiology. 54: 649-654.

75. Lee, B., Pometto, A.L., Fratzke, A. and Bailey, T.B. 1991. Biodegradation of degradable plastic polyethylene by Phanerochaete and *Streptomyces* species. Applied and Environmental Microbiology. 57: 678-685.

76. Lee, S.Y. 1996. Bacterial polyhydroxyalkanoates. Biotechnology and Bioengineering. 49: 1-14.

77. Liao, D., Whitsel, E.A., Duan, Y., Lin, H.M., Quibrera, P.M. and Smith, R. 2009. Ambient particulate air pollution and ectopy the environmental epidemiology of arrhythmogenesis in Women's Health Initiative study. Toxicology and Environmental Health: A. 72: 30–38.

78. Linos, A., Berekaa, M.M., Steinbüchel, A., Kim, K.K., Spröer, C. and Kroppenstedt, R.M. 2002. *Gordonia westfalica* sp. nov., a novel rubber degrading actinomycete. International Journal of Systematic and Evolutionary Microbiology. 52: 1133–1139.

79. Linos, A., Berekaa, M.M., Reichelt, R., Keller, U., Schmitt, J., Flemming, H. C., Kroppenstedt, R.M. and Steinbüchel, A. 2000. Applied and Environmental Microbiology. 66: 1639-1645.

80. Linos, A. and Steinbüchel, A. 1998. Microbial degradation of natural and synthetic rubbers by novel bacteria belonging to the genus Gordona. Kautsch Gummi Kunstst. 51: 496–499.

81. Linos, A., Steinbüchel, A., Spröer, C. and Kroppenstedt, R.M. 1999. *Gordonia polyisoprenivorans* sp. nov., a rubber degrading actinomycete isolated from automobile tire. International Journal of Systematic and Evolutionary Microbiology 49: 1785–1791.

82. Li, S.D., Wang, C., Xu, K. and Peng, Z. 2003. Thermooxidative degradation of methyl methacrylate graft-natural rubber. Applied Polymer Science. 90: 32-1227.

83. Lu, G., Li, Z.F., Li, S.D. and Xie, J. 2002. Blends of natural rubber latex and methyl ethacrylategrafted-rubber latex. Applied Polymer Science. 85: 41-1736.

84. Mansour, F.A., El-Shirbiny, S.A. and El-Metwaly, N.A. 1996. Demethyltetracycline biosynthesis by *Streptomyces aureofaciens* Sub-species viridulans as influenced by medium composition Egyptian. Microbiology. 31: 35-221.

85. Marshall, K.C. 1985. Bacterial adhesion in oligotrophic habitats. Microbiology Science. 2: 321-326.

86. Morgenstern, H. 1982. Einflub von Polyvinylacetat-Zusatzen in Putzmortel auf die Schim melpilzbildung. Material Organismen. 17: 241-251.

87. Mueller, W.R. 1999. Sauerstoff und Kohlendioxid gleichzeitig messen. Labor Praxis. [September]. 8-94.

88. Nakason, C., Kaesaman, A. and Eardrod, K. 2005. Cure and mechanical properties of natural rubber-g-poly(methyl methacrylate)-cassava starch compounds. Materials Letters. 59 (29–30): 4020–4025.

89. Otieno, D.O., Ashton, J.F. and Shah, N.P. 2006. Evaluation of enzymic potential for biotransformation of isoflavone phytoestrogen in soymilk by *Bifidobacterium animalis*, *Lactobacillus acidophilus* and *Lactobacillus casei*. Food Research International. 39: 394-407.

90. Neu, T.R, Vander-Mei, H.C., Busscher, H.J., Dijk, F. and Verkerke, G.J. 1993. Biodeterioration of medical-grade silicone rubber used for voice prostheses: a SEM study. Biomaterials 14: 459-464.

91. Nishida, H. and Tokiwa, Y. 1993. Distribution of poly(β-hydroxybutyrate) and poly (ε-caprolactone) aerobic degrading microorganisms in different environments. Environmental Polymer Degradation. 1: 33-227.

92. Nishioka, M., Tuzuki, T., Wanajyo, Y., Oonami, H. and Horiuchi, T. 1994. Biodegradable Plastics and Polymers. Amsterdam: Elsevier Science. 90-584.

93. Ojumo, T.V., Yu, J. and Solomon, B.O. 2004. Production of polyhydroxyalkanoate a bacterial biodegradation polymer. African Journal of Biotechnology. 3: 18-24.

94. Pagga, U. 1998. Biodegradability and compose stability of polymeric materials in the context of the European packaging regulation. Polymer Degradation and Stability. 59: 6-371.

95. Pagga, U., Schefer, A., Muller, R.J. and Pantke, M. 2001. Determination of the aerobic biodegradability of polymeric material in aquatic batch tests. Chemosphere. 42: 31-319.

96. Pan, L., Gu, J.G., Yin, B. and Cheng, S.P. 2009. Contribution to deterioration of polymeric materials by a slow-growing bacterium *Nocardia corynebacterioides*. International Biodeterioration & Biodegradation. 63: 24-29.

97. Pantke, M. 1996. Microbially Influenced Corrosion of Materials-Scientific and Technological. Springer Berlin. 379.

98. Pantke, M. and Patuska, G. 1984: Mikrobiologische Schadensanalyse an Grenzwertgebern für Heizöltanks: Amts-und Mitt-Bl. Bundesanst Materialf. 14: 329-332.

99. Park, E.H., George, E.R., Muldoon, M.A. and Flammino, A. 1994. Thermoplastic starch blends with poly(vinyl alcohol): processability, physical properties, and biodegradability. Polymer News. 19: 8-230.

100. Patterson, M.K., Husted, G.R., Rutkowski, A. and Mayette, D.C. 1991. Ultrapure Water. May/June. 18.

101. Pommer, E.H. and Lorenz, G. 1985. Biodeterioration and Biodegradation of Plastics and Polymers. Biodeterioration Society, Kew, UK. 76.

102. Ratnam, C.T., Nasir, M., Baharin, A. and Zaman, K. 2001. The effect of electron beam irradiation on the tensile and dynamic mechanical properties of epoxidized natural rubber. European Polymer. 37(8): 76-1667.

103. Ratto, J., Stenhouse, P.J., Auerbach, M., Mitchell, J. and Farrell, R. 1999. Processing, performance and biodegradability of a thermoplastic aliphatic polyester/starch system. Polymer. 40: 88-6777.

104. Richterich, K., Berger, H. and Steber, J. 1998. The ‘two phase closed bottle test’ a suitable method for the determination of `ready biodegradability' of poorly soluble compounds. Chemosphere. 37: 26-319.

105. Romen, F., Reinhardt, S. and Jendrossek, D. 2004. Thermotolerant poly(3-hydroxybutyrate)- degrading bacteria from hot compost and characterization of the PHB depolymerase of *Schlegelellasp*. sp. KB1a. Archives of Microbiology. 182(2-3): 157-164.

106. Rose, K., Tenberge, K.B. and Steinbüchel, A. 2005. Identification and characterization of genes from *Streptomyces* sp. strain K30 responsible for clear zone formation on natural rubber latex and poly(cis-1,4-isoprene) rubber degradation. Biomacromolecules. 6: 8-180.

107. Rouilly, A., Rigal, L. and Gilbert, R.G. 2004. Synthesis and properties of composites of starch and chemically modified natural rubber. Polymer. 45(23): 7813–7820.

108. Roy, R.V., Mithu, D., Banerjee, R. and Bhowmick, A.K. 2005. Comparative studies on rubber biodegradation through solid-state and submerged fermentation. Process Biochemistry. 41: 181–186.

109. Scalabrini, P., Rossi, M., Spettoli, P. and Matteuzzi, D. 1998. Characterization of Bifidobacterium strains for use in soymilk fermentation. Food Microbiology. 39: 213-219.

110. Saad, A.L.G. and Younan, A.F. 1995. Rheological, mechanical and electrical properties of natural rubber-white filler mixtures reinforced with nylon 6 short fibers. Polymer Degradation and Stability. 50(2): 40-133.

111. Saito, T., Klinklai, W. and Kawahara, S. 2007. Characterization of epoxidized natural rubber by 2D NMR spectroscopy. Polymer. 48(3): 7-750.

112. Santos, K.A.M., Suarez, P.A.Z. and Rubim, J.C. 2005. Photodegradation of synthetic and natural polyisoprenes at specific UV radiations. Polymer Degradation and Stability. 90: 34-43.

113. Schlemmer, D., Sales, Maria, J.A. and Resck, I.S. 2009. Degradation of different polystyrene/thermoplastic starch blends buried in soil. Carbohydrate Polymers. 75: 58-62.

114. Schopf, J.W., Hayes, J.M. and Walter, M.R. 1983. Earth's earliest biosphere. Princeton University Press. 361-384.

115. Scott, G. 1990. Photo-biodegradable plastics: their role in the protection of the environment. Degradation and Stability. 29: 54-135.

116. Scott, G. 2002. Degradable polymers principles and applications 2 th. Kluwer academic publishers Dordrecht, Boston: London.

117. Seal, K.J. and. Morton, L.H.G. 1986. Chemical materials, Biotechnology. V. C. H, Weinheim, Germany. 8: 583-606.

118. Shah, A.A., Hasan, F., Hameed, A. and Ahmed, S. 2008. Biological degradation of plastics: A comprehensive review. Biotechnology Advances. 26: 246–265.

119. Sharabi, N.E. and Von B.R. 1993. Testing of some assumptions about biodegradability in soil as measured by carbon dioxide evolution. Applied and Environmental Microbiology. 59: 5-1201.

120. Shey, J., Imam, S.H., Glenn, G.M. and Orts, W.J. 2006. Properties of baked starch foam with natural rubber latex. Industrial Crops and Products. 24(1): 34–40.

121. Shogren, R., Thompson, A.R., Felker, F.C., Harry-O'Kuru, R.E., Gordon, H. and Greene, R.V. 1992. Polymer compatibility and biodegradation of starch-poly(ethyleneco-acrylic acid)-polyethylene blends. Applied Polymer Science. 44: 8-1971.

122. Siracusaa, V., Rocculib, P., Romanib, S. and Rosa, M.R. 2008. Biodegradable polymers for food packaging: a review. Trends in Food Science & Technology 19: 634-643.

123. Siven, A., Szanto, M. and Pavlov, V. 2006. Biofilm developement of the polyethylene-degradation bacterium *Rhodococcus rubber.* Application Microbial Biotechnology. 72: 52-346.

124. Sohngen, N.L. and Fol, J.G. 1914. Die Zeretzung des Kautschuks durch Mikroben. Centr. Bakteriol. Parasitenk.II Abt. 40: 87-98.

125. Solaro, R., Corti, A. and Chiellini, E. 1998. A new respirometric test simulating soil burial conditions for the evaluation of polymer biodegradation. Environmental Polymer Degradation. 5: 8-203.

126. Spence, D. and Van Niel, C.B. 1936. Bacterial decomposition of rubber in Hevea latex. Industrial & Engineering Chemistry. 28: 847-850.

127. Stalh, W.H. and Pessen, H. 1953. The microbiological degradation of plasticizers. I. Growth on esters and alcohols. Applied Microbiology. 1: 20-35.

128. Tangpakdee, J., Mizokoshi, A.E. and Tanaka, Y. 1998. Novel method for preparation of low molecular weight natural rubber latex. Rubber Chemistry and Technology. 71: 795–802.

129. Tanrattanakul, V. and Chumeka, W. 2009. Effect of Potassium Persulfate on graft co-polymerization and mechanical properties of cassava starch/Natural rubber foams. Applied Polymer Science. 116: 93-105.

130. Tessler, M.M., and Billmers, R.L. 1996. Preparation of starch esters. Environmental Polymer Degradation. 4: 85-89.

131. Thakur, D., Bora, T.C., Bordoloi, G.N. and Mazumdar, S. 2009. Influence of nutrition and culturing conditions for optimum growth and antimicrobial metabolite production by *Streptomyces* sp. 201. De Mycologie Médicale 19: 161-167.

132. The Goodyear Tire & Rubber company. 2007. Preparation of starch reinforced rubber and use thereof in tires. United State Patent and Trademark 7156137.

133. Tokiwa, Y. and Iwamoto. 1994. Biodegradable Plastics and Polymers. Amsterdam: Elsevier Science. 9-190.

134. Tosin, M., Degli, I.F. and Bastioli, C. 1998. Detection of a toxic product released by a polyurethane-containing film using a composting test method based on a Mineral Bed. Environmental Polymer Degradation. 6: 79–90.

135. Tsuchii, A., Suzuki, T. and Takeda, K. 1985. Microbial degradation of natural rubber vulcanizates. Applied and Environmental Microbiology 50: 965–970.

136. Tsuchii, A., Suzuki, T. and Takahara, Y. 1997. Microbial degradation of styrene oligomer. Agricultural Biology and Chemistry. 41: 21-2417.

137. Tsuchii, A. and Takeda, K. 1990. Rubber-degrading enzyme from a bacterial culture. Applied and Environmental Microbiology. 56: 268–274.

138. Tsuchii, A., Takeda, K. and Tokiwa, Y. 1996. Colonization and degradation of rubber pieces by *Nocardia* sp. Biodegradation **7:** 41-48.

139. Tsuchii, A., Takeda, K. and Tokiwa, Y**.** 1997. Degradation of the rubber in truck tires by a strain of *Nocardia*. Biodegradation **7:** 405-413.

140. Tsuji, H. and Suzuyoshi, K. 2002. Environmental degradation of biodegradable polyesters Poly(ε-caprolactone), poly[(R)-3-hydroxybutyrate], and poly(l-lactide) films in controlled static seawater. Polymer Degradation and Stability. 75(2): 55-347.

141. Tudorachi, C.N., Cascaval, M., Rusu, M. and Pruteanu, M. 2000. Testing of polyvinyl alcohol and starch mixtures as biodegradable polymeric materials. Polymer Test. 19: 99-785.

142. Tung, F.D., Wiles, M., Cermak, B.E., Gho, J.G. and Hare, C.W.J. 1999. Addcon'99 Conference, Prague, Czechoslovakia [October 27–28]. 207.

143. Wang, Z.F., Peng, Z., Li, S.D., Lin, H., Zhang, K.Z., She, X.D. and Fu, X. 2009. The impact of esterication on the properties of starch/natural rubber composite. Composites Science and Technology. 69: 1797-1803.

144. Warneke, S., Arenskötter, M., Tenberge, K.B. and Steinbüchel, A. 2007. Bacterial degradation of poly(*trans*-1,4-isoprene) (*gutta percha*). Microbiology. 153: 347-356.

145. Weiland, M., Daro, A. and David, C. 1995. Biodegradation of thermally oxidized polyethylene. Polymer Degradation and Stability. 48(2): 89-275.

146. Wilai, S., Khanjana, K. and Kranarong, S. 1998. Fine structure of Amylose, Amylopectin and functional properties of cassava starches extracted from Kasetsart 50.

147. Williams, D.F. 1991. Interfacial reactions in the degradation of polymers by cells and bacteria. Biofouling. 4: 230-255.

148. Williams, D.F. and Zhong, S.P. 1991. Are free radicals involved in the biodegradation of implanted polymer? Advanced Materials.3: 623-626.

149. Williams, G.R. and Dale, R. 1993. The biodeterioration of the plasticizer dioctyl phthalate. Biodet Bull. 19: 8-37.

150. Wimmer, B., Lottspeich, F., Ritter, R. and Bronnenmeier, R.K. 1997. A novel type of thermostable **α**-D-glucosidase from *Thermoanaerobacter thermohydrosulfuricus* exhibiting maltodextrinohydrolase activity. Biochemical. 328: 581-586.

151. Witt, U., Muller, R.J. and Deckwer, W.D. 1997. Biodegradation behavior and material properties of aliphatic/aromatic polyesters of commercial importance. Environmental Polymer Degradation**.** 5: 9-81.

152. Witt, U., Einig, T., Yamamoto, M., Kleeberg, I., Deckwer, W.D. and Muller, R. J. 2001. Biodegradation of aliphatic–aromatic copolyesters: evaluation of the final biodegradability and ecotoxicological impact of degradation intermediates. Chemosphere. 44: 99-289.

153. Xue, X.H. and Fu, X. 2006. Hydrogenation of natural rubber latex under normal pressure and properties of saturated rubber. Chinese Journal of Biological Control. 27(2): 95–98.

154. Yue, D.M., Zhao, H., Wu, Y.P. and Zhang, L.Q. 2005. Preparation of nano-starch particles and its dispersion in rubber. China Synthesis Rubber Industrial. 28 (6): 475.

155. Zhao, J.H., Wang, X.Q., Zeng, J., Yang, G. and Shi, F. 2001. Biodegradation of poly(butylene succinate-co-butylene adipate) by *Aspergillus versicplor*. Polymer Degradation and Stability. 90: 173-179.

156. Zuchowska, D., Steller, R. and Meissner, W. 1998. Structure and properties of degradable polyolefin–starch blends. Polymer Degradation and Stability. 60: 471–480.

Appendix

Appendix A

1. Media

1.1) Mineral Salt Medium (MSM)

Composition per liter:

$Na_2HPO_4 \cdot 12H_2O$	9.0 g
KH_2PO_4	1.5 g
NH_4Cl	1.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2 \cdot 2H_2O$	0.02 g
Fe(III) [NH_4]citrate	0.0012 g
Trace element solution (10,000X)	0.1 mL
Distilled water	1.0 L

1.2) Mineral Salt Medium (MSM) with NR latex (0.6% NR latex)

Composition per liter:

$Na_2HPO_4 \cdot 12H_2O$	9.0 g
KH_2PO_4	1.5 g
NH_4Cl	1.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2 \cdot 2H_2O$	0.02 g
Fe(III) [NH_4]citrate	0.0012 g
Trace element solution (10,000X)	0.1 mL
NR latex	0.6 %
Distilled water	1.0 L

1.3) Mineral Salt Medium (MSM) with NR latex (0.6% NR latex) with yeast extract

Composition per liter:

$Na_2HPO_4 \cdot 12H_2O$	9.0 g
KH_2PO_4	1.5 g
NH_4Cl	1.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2 \cdot 2H_2O$	0.02 g
Fe(III) [NH_4]citrate	0.0012 g
Trace element solution (10,000X)	0.1 mL
NR latex	0.6 %
Yeast extract	0.05%
Distilled water	1.0 L

1.4) Glucose Yeast extract Malt (GYM) Agar

Composition per liter:

Glucose	10 g
Yeast extract	5 g
Malt extract	5 g
Agar	18 g
Distilled water	1.0 L

1.5) Blood Agar (BA)

Composition per liter:

Blood agar base (Difco)	
Heart muscle, infusion form (Solids)	2.0 g
Pancreatic digest of casein	13.0 g
Yeast extract	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	1.0 L
Final pH 7.3±2	

1.6) Motility Test Medium

Composition per liter:

Motility test medium base (Difco)

Bacto tryptose	10.0 g
Sodium chloride	5.0 g
Agar	5.0 g
Distilled water	1.0 L

Final pH 7.2 ±0.2

1.7) Starch Agar

Composition per liter:

Starch agar base

Tryptone	10.0 g
Yeast extract	10.0 g
K_2HPO_4	5.0 g
Soluble starch	3.0 g
Agar	15.0 g
Distilled water	1.0 L

2. Chemicals

2.1) Crystal violet

Composition:

Solution A: Dissolved crystal violet 0.2 g in 95% ethanol alcohol 20 mL

Solution B: Dissolved ammonium oxalate 0.8 g in distilled water 80 mL

Mix Solution A into Solution B stand at room temperature for 24 h. then, filter the mixed solution.

2.2) 95% Ethyl alcohol (Decolorizing solvent)

2.3) Gram stain iodine (Mordant)

Composition:

Dissolved iodine powder 1.0 g and Potassium iodide 2.0 g in distilled water 300 mL.

2.4) Safranin (Counterstain)

Composition:

Dissolved safranin O 25% w/v in 95% ethyl alcohol 10 mL add distilled water to100 mL

2.5) Catalase test reagent

Composition:

Dissolved 35% H_2O_2 8.6 g in 1000 mL of distilled water

2.6) Phenolphthalein

Composition:

Dissolved phenolphthalein 1.0 g in 50 mL of 95% ethyl alcohol

2.7) 60% Sodium hydroxide

Composition:

Dissolved NaOH 60 g in 100 mL of distilled water

2.8) Somogyi reagent

Composition:

Solution A: Dissolved KNa 12 g and Na_2CO_3 24 g in 250 mL of distill water

Solution B: Dissolved $Cu_2SO_4 \cdot 5H_2O$ 4 g in 50 mL of distilled water

Solution C: Dissolved anhydrous Na_2SO_4 180 g in 500 mL of distilled water and boil

Pour solution B into solution A and invert for, gentle mixing add $NaHCO_3$ 16 g into mix solution

Pour the solutions into a volumetric flask 1000 mL, then pour solution C and distilled water (keep for 1 week before use)

2.9) Nelson reagent

Composition:

Solution D: Dissolved ammonium molybdate 25 g in 450 mL of distilled water then slowly add 21 mL of sulfuric (96%)

Solution E: Dissolved $Na_2HAsO_4 \cdot 7H_2O$ 3 g in 25 mL of distilled water

Pour solution E into solution A in volumetric flask 1000 mL add 525 mL of distilled water

2.10) 5 mM p-nitrophenyl glucoside (Substrate)

Composition:

Dissolved p-nitrophenyl glucoside 0.0015 g in 1 mL of 100 mM sodium phosphate

2.11) p-nitrophenol (standard solution)

Composition:
Stock solution; Dissolved p-nitrophenol 1mg in 1 mL of 100 mM sodium phosphate buffer

Prepare a standard concentration of 50, 100, 150, 200, 250 µg/mL in 100 mM sodium phosphate buffer

2.12) 100 mM sodium phosphate buffer

Composition:

Solution A: Dissolved Na_2HPO_4 14.2 g in 1000 mL of distilled water

Solution B: Dissolved NaH_2PO_4 12 g in 1000 mL of distilled water

Pour solution A into solution B, Mix well

2.13) Sodium carbonate (Stopping reagent)

Composition: Dissolved Na_2CO_3 105.99 g in 1000 mL of distilled water

2.14) Schiff's reagent

Composition:

Solution A: Dissolved 10 g of $Na_2S_2O_5$ in 50 mL of acetic acid

Solution B: Pour 15 mL of 0.1 M HCL into 50 mL of distilled water

Pour solution A into solution B and then add 2 g of fuchsin into the mix solution

Appendix B

Dissolved p-nitrophenol 1 mg in 1 mL of 100 mM sodium phosphate buffer for stock solution

Prepare a standard concentration of 50 µg/mL by adding 0.05 mL of stock ρ nitrophenol solution to 0.95 mL of 100 mM sodium phosphate buffer

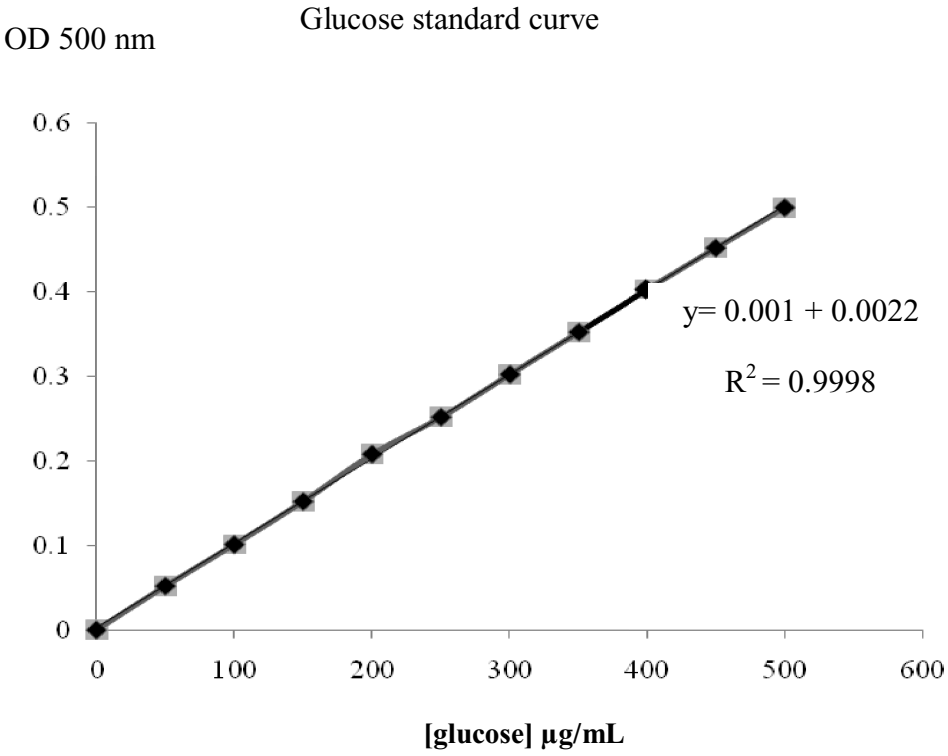
Prepare a standard concentration of 100 µg/mL by adding 0.10 mL of stock ρ-nitrophenol solution to 0.90 mL of 100 mM sodium phosphate buffer

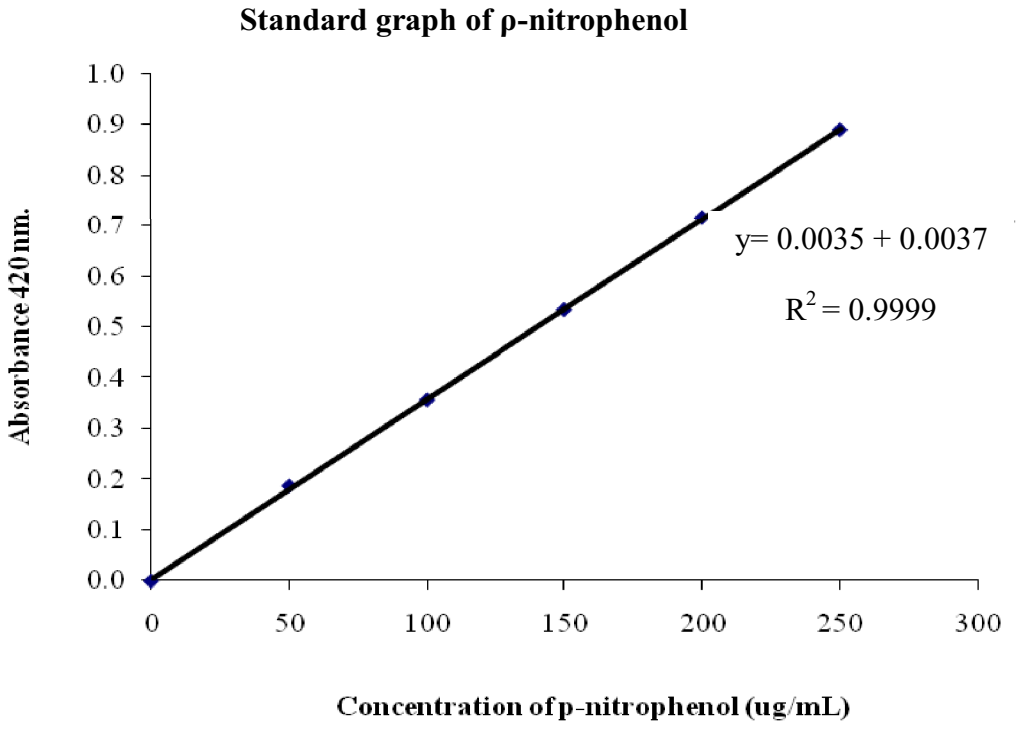
Prepare a standard concentration of 150 µg/mL by adding 0.15 mL of stock ρ-nitrophenol solution to 0.85 mL of 100 mM sodium phosphate buffer

Prepare a standard concentration of 50 µg/mL by adding 0.20 mL of stock ρ-nitrophenol solution to 0.80 mL of 100 mM sodium phosphate buffer

Prepare a standard concentration of 50 µg/mL by adding 0.25 mL of stock ρ-nitrophenol solution to 0.75 mL of 100 mM sodium phosphate buffer

100 mM sodium phosphate buffer for blank absorbance measure at 420 nm.


Glucose standard curve
OD 500 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
0
100
200
300
400
500
600
y= 0.001 + 0.0022
R2 = 0.9998
[glucose] µg/mL


Standard graph of ρ-nitrophenol
Absorbance 420 nm.
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
50
100
150
200
250
300
y= 0.0035 + 0.0037
R2 = 0.9999
Concentration of p-nitrophenol (ug/mL)

Appendix C

Electronic Journal of Biotechnology ISSN: 0717-3458
http://www.ejbiotechnology.info
DOI: 10.2225/vol15-issue1-fulltext-10 RESEARCH ARTICLE

Biodegradation of a blended starch/natural rubber foam biopolymer and rubber gloves by *Streptomyces coelicolor* CH13

Sirimaporn Watcharakul[1] · Kamontam Umsakul[1] ✉ · Brian Hodgson[2] · Wannapa Chumeka[3] · Varaporn Tanrattanakul[3]

1 Prince of Songkla University, Faculty of Science, Department of Microbiology, Hat-Yai, Songkhla, Thailand

2 Prince of Songkla University, Faculty of Pharmaceutical Sciences, Hat-Yai, Songkhla, Thailand

3 Prince of Songkla University, Faculty of Science, Department of Materials Science and Technology, Hat-Yai, Songkhla, Thailand

✉ Corresponding author: kamontam.u@psu.ac.th
Received August 23, 2011 / Accepted December 13, 2011
Published online: January 15, 2012

Abstract

Background: The growing problem of environmental pollution caused by synthetic plastics has led to the search for alternative materials such as biodegradable plastics. Of the biopolymers presently under development, starch/natural rubber is one promising alternative. Several species of bacteria and fungi are capable of degrading natural rubber and many can degrade starch. **Results:** *Streptomyces coelicolor* CH13 was isolated from soil according to its ability to produce translucent halos on a mineral salts medium, MSM, supplemented with natural rubber and to degrade starch. Scanning electron microscope studies showed that it colonized the surfaces of strips of a new starch/natural rubber biopolymer and rubber gloves and caused degradation by forming holes, and surface degradation. Starch was completely removed and polyisoprene chains were broken down to produce aldehyde and/or carbonyl groups. After 6 weeks of cultivation with strips of the polymers in MSM, *S. coelicolor* CH13 reduced the weight of the starch/NR biopolymer by 92% and that of the rubber gloves by 14.3%. **Conclusions:** This study indicated that this bacterium causes the biodegradation of the new biopolymer and natural rubber and confirms that this new biopolymer can be degraded in the environment and would be suitable as a 'green plastic' derived from natural sources.

Keywords: biodegradation, biopolymer, natural rubber, starch, *Streptomyces coelicolor*

INTRODUCTION

Plastic foam is the material most often disposed of that, because of its resistance to biodegradation remains forever in the environment as a pollutant and is now creating huge areas of "white pollution" on the land and in the oceans. This problem has for long activated research into finding or producing alternative synthetic materials, and recently a search for versatile, multipurpose, biodegradable plastics (Steinbüchel, 2001; Yu et al. 2006). Recently there have been efforts to produce biopolymers from agricultural products that could be developed and promoted as green renewable resources. One particular example is a cassava starch foam blended with natural rubber latex (NR). In theory it should be environmentally friendly because both components of the polymer are natural biodegradable compounds that form polymers (Tanrattanakul and Chumeka, 2009). Cassava starch and rubber are abundant and low cost sources, with large numbers of possible applications particularly in the food and packaging industries. Several research groups have been attempting to improve the versatility of starch/NR polymers (Carvalho et al. 2003; Wang et al. 2009) including their ability to be biodegraded. In 2006 Shey et al. (2006) incorporated natural rubber latex into baked starch foam bases made from wheat, potato and waxy corn starch. They showed that the natural rubber latex component increased

polymer flexibility and the moisture resistance of baked starch foams. The new polymer used in this study was derived from the work of Tanrattanakul and Chumeka (2009). The graft polymer was produced by suspension and melt blending. NR acts as an impact modifier for thermoplastic starch and enhances the flexural modulus of this new polymer blend. Their study clearly showed that the mechanical properties and impact strength of starch/NR polymers were improved. Even though polymers with a higher amount of NR content provided even higher impact strength, their biodegradability decreased as the natural rubber content increased. The content of this polymer was 65% starch 35% NR. In this work we have investigated its potential for biodegradation.

Because rubber is elastomeric and consists of molecules with a high average molecular weight of nearly a million, it is slow to degrade. There is normally an inverse relationship between the carbon-chain length of a polymer and its biodegradability and any C chain longer than 500 is extremely difficult to biodegrade (Tanrattanakul and Chumeka, 2009). Biodegradation of biopolymer products in the environment is complex involving interactions between environmental factors and the physical and biochemical interactions of microorganisms (Gu, 2003) so there are many parameters that need to be considered. Microbial degradation is the most significant and influential process in the mineralization of persistent organic pollutants (Seo et al. 2007). Research involved in the biodegradation of NR has mainly focused on screening for bacteria with ability to degrade natural rubber compounds. Many bacteria and actinomycetes able to degrade pure natural rubber (poly cis-1,4-isoprene) and chemically treated or synthetic rubber have been reported. These include *Nocardia* sp. (Warneke et al. 2007), *Amycolatopsis* sp. (Heisey and Papadatos, 1995), *Pseudomonas* sp. (Linos et al. 2000; Roy et al. 2006), *Gordonia* sp. (Linos et al. 2002), *Streptomyces* sp. (Rose et al. 2005), *Xanthomonas* sp. (Braaz et al. 2004), *Bacillus* sp. (Cherian and Jayachandran, 2009), *Achromobacter* sp. (Berekaa et al. 2005). Previous studies have also shown that actinomycetes play the most important role in decomposing natural rubber waste materials (Tsuchii and Takeda, 1990; Heisey and Papadatos, 1995; Linos and Steinbüchel, 2001).

There is much interest in the isolation and study of microorganisms able to degrade any new biopolymer and we believe that this information should accompany its sale to ensure that when the polymer is purchased the buyer knows that it will be environmentally-friendly, and have a low impact on the ecology by being eliminated through biodegradation over a reasonable period of time. In this report, special emphasis is given to biodegradation of a new biopolymer, cassava starch foam blended with natural rubber latex and compared with the biodegradation of rubber gloves. The isolation of a bacterial strain CH13, identified as a representative of the species *Streptomyces coelicolor* and its ability to degrade both polymers, is reported.

MATERIALS AND METHODS

Medium and growth conditions

Soil samples were taken from different sites associated with rubber factories and their wastes in Hatyai, Thailand. For the enrichment process soil samples were suspended in Erlenmeyer flasks containing a minimal salts medium (MSM) plus a trace elements solution consisting of (g/L) $Na_2HPO_4.12H_2O$ (9.0), KH_2PO_4 (1.5), NH_4NO_3 (1.0), $MgSO_4.7H_2O$ (0.2), $CaCl_2.2H_2O$ (0.02), Fe(III)[NH_4] citrate (0.0012), and 0.1 mL of the trace element solution (10000x, modified from Jendrossek et al. (1997). The added carbon source for initial isolations was natural rubber latex (NR) 0.6% (v/v) added to both liquid and solid MSM (MSM plus 1.5% (w/v) agar) prior to autoclaving. At other times the carbon sources were strips of rubber gloves or the new polymer blend. Liquid cultures were incubated on a horizontal rotary shaker at 150 rpm 30ºC for 5 days. In addition solid MSM media was overlain with 7 mL of NR latex as sole carbon source to detect colonies producing translucent haloes caused by degradation of the water-insoluble polymer (Ibrahim et al. 2006).

Cassava starch foam blended with natural rubber latex (35%) was supplied by the Bioplastic Research Unit, Prince of Songkla University, Thailand. Rubber gloves were from the Siam Sempermed company, Songkhla Thailand.

Isolation of natural rubber degrading bacteria

Samples from various ecosystems and enrichment cultures were diluted with sterile mineral medium and mixed well then 0.1 mL of each dilution was spread on MSM agar plates with an NR layer as the sole carbon source and incubated at 30ºC. Colonies producing translucent halos were purified by alternating transfers to MSM/NR media and yeast extract plates for further characterization.

Bacterial identification of the best strains able to degrade NR were characterized and identified as follows

Identification methods for the best polymer degrading isolates included morphology and basic biochemical tests. 16s rRNA gene sequence analysis was also performed to identify and classify the isolates. Genomic DNA was extracted by standard methods (Warneke et al. 2007). The primers used were 27F and 1389R to amplify the full length of the 16S rRNA gene. This was carried out at BIOTEC Culture Central Research Unit, Thailand. Nucleotide sequences of purified PCR products were determined with the Genetic Analyzer, and DNA nucleotide sequences of isolates were checked for identification using the data available from the GenBank database at The National Center for Biotechnology Information.

Optimum conditions for the rate of biodegradation

One isolate, strain CH13 that produced the largest halos on MSM/NR rubber plates was selected. The optimum inoculum size, temperature, pH and amount of NH_4NO_3 for degrading rubber gloves and polymer blend were determined from cultures incubated in 100 mL of MSM liquid medium supplemented with strips of polymer blend or rubber gloves as sole carbon sources and shaken for 4 weeks. The initial inoculum for all cultures was a culture grown in MSM medium with NR latex at 30ºC for 48 hrs. To check optimum inoculum size, cell numbers were adjusted to produce final concentrations of 10^6 10^8 10^{10} CFU/mL. The optimum pH values were obtained in a series of MSM media with pH values of 6, 7 and 8, the temperatures chosen were 35, 40, 45ºC and the NH_4NO_3 concentrations were 0.5, 1.0 and 10 g/L.

Investigation of the degradation process

The process of degradation of the blended polymer and rubber gloves was further investigated by first observing physical changes to the strips of the polymers by light microscopy and SEM. Other experiments were used to determine the weight loss of preweighed rubber strips, CO_2 production for the percentage mineralization, the loss of starch determined by extraction and the production of reducing sugars together with detection of aldehyde groups produced through microbial oxidation of the double bond components of the polyisoprene chains, changes in the molecular weight of the polymer chains and the presence of glucosidase enzymes.

Weight loss

An overnight culture on MSM agar with added yeast extract and an NR latex overlay were inoculated into 200 mL of MSM supplemented with 5.17 g of starch/NR blended strips or 1.8 g (0.6% w/v rubber glove strips and incubated at 150 rpm at 30ºC for up to 6 weeks (Pan et al. 2009.)

After 4 weeks of incubation NR strips were removed, washed with water and dried in an oven at 65ºC until constant weights were reached. The weight loss was taken as the difference between the initial and final weight over the period of 4 weeks.

Staining with reagents to detect degradation products

Schiff's reagent. Treated starch/NR blend and rubber glove strips were stained with Schiff's reagent (Linos et al. 2000). A pink to dark red colour indicated the presence of aldehyde or carbonyl groups.

Detection with 2, 4-DNP reagent. 1 mL of 2, 4-DNP reagent was added to the sample, and the yellow precipitate that developed over 1-2 min at room temperature was noted. Any precipitating yellow colour

denoted the presence of aldehyde groups produced during the degradation of the polymers. The composition of the 2, 4-DNP reagent is as follows: 3 g of 2, 4-dinitrophenyl hydrazine dissolved in 15 mL of sulfuric acid plus 70 mL of 95% ethanol plus 20 mL of H_2O (Ehrlich et al. 1948).

Amylase assay

The glucosidase enzyme activity in the culture was investigated. The presence of reducing sugars released from the starch by any glucosidase enzymes were determined by the method of Nelson-Somogyi (Somogyi, 1952). Glucosidase activity itself was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl a-D-glucoside. Assay mixtures (1 mL) containing 5 mM substrate in 100 mM sodium phosphate buffer, pH 7.0, were incubated at 30ºC. Reactions were stopped by addition of two volumes of 1 M Na_2CO_3. The absorbance of the liberated *p*-nitrophenol was measured at 420 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 nmol of *p*-nitrophenol per minute at 30ºC and was expressed as U.

Determination of the percentage mineralization

Evidence for the biodegradation of starch/NR foam and rubber gloves to CO_2 was obtained by determination of the CO_2 released during the cultivation of cells in the presence of starch/NR foam or strips of rubber gloves as sole carbon sources. Experiments were carried out in tightly closed Erlenmeyer flasks and the CO_2 trapped as $BaCO_3$ from $Ba(OH)_2$ was measured. The flask contained 300 mL MSM medium, 2% (w/v) of starch/NR foam or rubber gloves and were inoculated with 10^9 CFU/mL of culture grown for 48 hrs on NR/MSM agar plates. Each test tube was connected to the flask contained 15 mL of a 0.2 M $Ba(OH)_2$ solution. At each measuring point, the flask was aerated with CO_2 free air, and the test tube replaced by new tubes containing fresh $Ba(OH)_2$ solution. The remaining $Ba(OH)_2$ was then determined for each period by titration with 0.1 M HCl according to the following equation, yielding the percentage mineralization.

$$\text{Mineralization (\% } CO_2) = \frac{\text{Required amount of HCl (mL) x 0.1}}{\text{C content of amount of poly(1,4-isoprene) applied (mmol) x2}}$$

Phenolphthalein (20 µL of a 1%, w/v, solution in 2-propanol) was used as an indicator and the end point of titration was determined by a change of the colour from magenta to colourless. A non-inoculated flask was treated in the same way as for the control (Warneke et al. 2007).

Scanning electron microscopy (SEM) observation

Changes to the surface structures of the treated samples of starch/NR foam and rubber gloves by strain CH13 was verified by SEM. The starch/NR foam was cut into cubic pieces with a side length of about 1 cm. MSM (300 mL) containing 5.17 g of starch/NR foam pieces was inoculated with 30 mL of CH13 cells (10^9 CFU/mL) grown for 48 hrs in MSM with NR latex. The starch/NR foam after incubation with CH13 was removed at various times and prepared for examination by SEM. Samples were initially fixed in 2% glutaraldehyde and then 1% OsO_4. Samples were then dehydrated in a series of ethanol solutions with a gradual increase of concentrations. The dehydrated samples were subjected to critical point drying with liquid CO_2 according to a standard procedure. Subsequently, samples were coated with gold-palladium (Pan et al. 2009) with a vacuum sputter device and examined with the scanning electron microscope (JSM-5800LV, JEOL) at 20 kV under high vacuum conditions (Warneke et al. 2007). Micrographs were recorded digitally.

Soxhlet extraction of starch

The loss of starch in the starch/NR blend samples was determined during the degradation process by using a Soxhlet extraction method. Samples were dissolved by stirring in toluene at 60ºC for 1 hr. The solution was placed in a thimble holder and extracted by a Soxhlet extractor in toluene at 110ºC. This operation was repeated until extraction was completed. Completed extraction was determined by adding a drop of the toluene into methanol until no precipitate was observed or the methanol was clear. If precipitation occurred the process was repeated again for up to 10 hrs until there was no precipitate. The remaining solution was then evaporated and dried at 60ºC and weighed (Tanrattanakul and Chumeka, 2009).

Intrinsic viscosity-average molecular weight of the polymer

The investigation was carried out on the starch/NR blend and rubber gloves samples. Evaluation of the viscosity provided an estimate of the average molecular weight on a capillary Ubbelohde viscometer. The total sample was dissolved in toluene solvent and filtered. The solutions (15 mL) were placed in the Ubbelohde viscometer. The intrinsic viscosity system was established in a water bath at 30ºC and the rate and time of flow was used to calculate the limiting viscosity number and its relationship to the molecular weight of the polymer (Polymer Chemistry Lab, PSU).

RESULTS AND DISCUSSION

Isolation, identification and characterizations

Soil samples from different ecosystems in Songkhla province, Thailand were screened for NR and starch-degrading bacteria. Isolates that could grow well on both latex agar medium and liquid medium were selected. The isolate CH13 was selected because of its most rapid growth and was initially identified as a *Streptomyces* sp. CH13 (Table 1) based on its morphological and biochemical properties. *Streptomyces* sp. CH13 used rubber latex as carbon source and grew well with latex agar medium. Natural rubber degrading microorganisms had previously been divided into two groups that followed different strategies to degrade rubber. The first group formed translucent halos on solid media containing dispersed latex particles and excreted rubber-cleaving enzymes like polyisoprenoid oxygenase (Linos et al. 2000) that cleave the double bonds in the rubber backbone to initiate rubber degradation (Tsuchii and Takeda, 1990; Rose et al. 2005). The second group did not form translucent halos because they became strongly adhered to and only grew on the surface of the rubber so rubber degrading enzymes were not released into the medium (Linos et al. 2000). *Streptomyces* sp. CH13 formed a clear zone around its colonies and produced an extracellular α-glucosidase enzyme activity. This is in agreement with observations of Bode et al. (2000), who found that degradation of rubber by *S. coelicolor* produced clearing zones on opaque latex agar. This indicated that it could degrade both the rubber and starch in the biopolymer foam blend. This strain CH13 was then used for further investigations of its degradation potential of the polymer foam rubber blend and rubber gloves. The identity of *Streptomyces* sp. CH13 was confirmed, by a full length 16s rRNA gene sequence analysis, as *Streptomyces coelicolor* with a high similarity of 99%. This isolate is therefore referred to as *S. coelicolor* CH13.

Investigation of the biodegradation process

Colonization of the bacterial isolate CH13 on the polymer blend and rubber glove strips after incubation for 4 weeks (Figure 1a, 1b) was examined. The isolate grew and became attached to the surface of the rubber gloves and polymer blend strips. Many holes were present on the surfaces.

Table 1. Morphological and biochemical characteristics of bacterial isolate CH13 capable of growing on MSM containing NR latex.

Microorganism	*Streptomyces* sp. CH13
Source	Soil
Colony	Round, Rough White-yellow
Catalase	Positive
Oxidase	Negative
Amylase	Positive
Motility	Negative
Radial zone on rubber agar	5 mm
Morphology	Gram-positive, grayish-yellow mycelium, spore forming, present translucent halo around the colonies on MSM containing natural rubber latex after incubation at 30ºC for 3 days

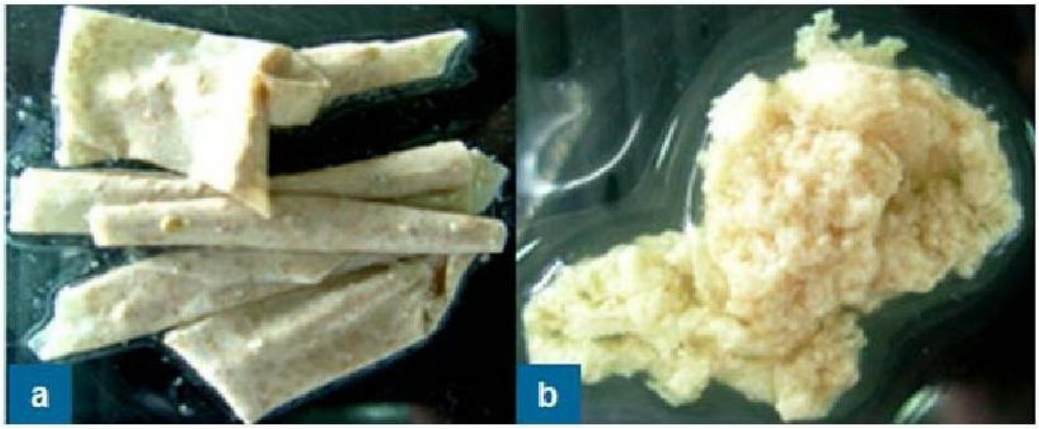


Fig. 1 Colonies of bacterial isolate CH13 on rubber gloves (a) and polymer blend (b) after incubated with *S. coelicolor* CH13 at 30ºC for 4 weeks.

SEM observation also revealed a different pattern of colonization by the isolate on the 2 substrates. On the polymer blend, *S. coelicolor* CH13 produced an extensive coverage of the surface. In contrast growth on the rubber gloves strips was sparse. However, major changes to the surfaces of the rubber gloves and polymer blend (Figure 2d, e, f) with many holes were evident on the surfaces compared to the observations of the incubated but non-inoculated samples that remained intact (Figure 2a, b, c). *S. coelicolor* CH13 also exhibited some filamentous growth (Figure 2f). The colour of the samples also changed (data not shown) after incubation, especially the culture of the polymer blend, which became coloured with yellow-brown and yellow-white colonies on its surface.

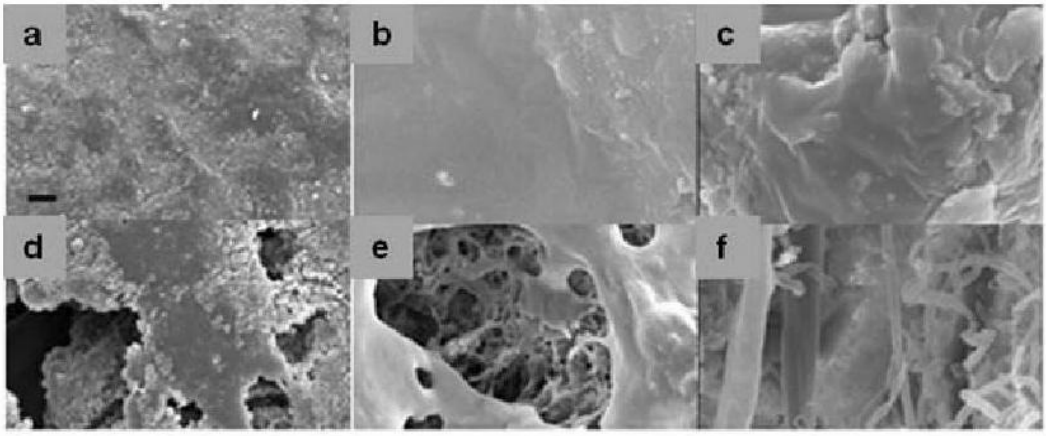


Fig. 2 Scanning electron micrographs showing erosion of the surface of polymer blended strips and rubber gloves strips. (a) Non-inoculated rubber glove; (b, c) Non-inoculated polymer blend; (d) Rubber glove strips incubated with *S. coelicolor* CH13 at 30ºC for 4 weeks; (e, f) polymer blend incubated with *S. coelicolor* CH13 at 30ºC after 4 weeks. (Bar: 10 µm).

The percentage weight loss of the polymer

The percentage weight losses of the polymer blend treated with *S. coelicolor* CH13 in 3 separate samples was 82.15, 86.12 and 92.14 over the time period of 2, 4 and 6 weeks, (Figure 3a) compared to the sterile control that showed only a < 5 % weight loss over the same time period. The weight loss

of the rubber gloves was much less over the same period with a 6.99, 11.00 and 14.31% weight loss. These results indicated that *S. coelicolor* CH13 was capable of readily utilizing the polymer blend of NR and starch (see enzyme assay) and was also able to degrade the rubber gloves but at a much slower rate. As the initial polymer blend consists of 65% starch and 35% NR, the presence of starch has facilitated the degradation of rubber as the total weight loss over the 6 weeks period for the blend was up to 92% and according to the chloroform extraction process 7% of the starch remained. We assume that in this case the starch is metabolized first and acts as a growth substrate for the bacteria so that the bacteria population increases rapidly and the rate of degradation of NR is therefore increased. In the absence of starch on the rubber gloves strips, growth substrates are produced only slowly hence the much reduced rate of degradation. Recalcitrant polymers like rubber or other polymer products can be degraded by some groups of microorganisms (Sabev et al. 2006; Bhatt et al. 2007; Warneke et al. 2007; Pan et al. 2009). There are many differences in weight loss during biodegradation with different isolates and different polymer products used as a source of carbon and energy (Bode et al. 2001; Pan et al. 2009). This is the first report of a stimulation of the rate of NR biodegradation by the presence of another substrate in this case starch as a part of an NR/starch co polymer.

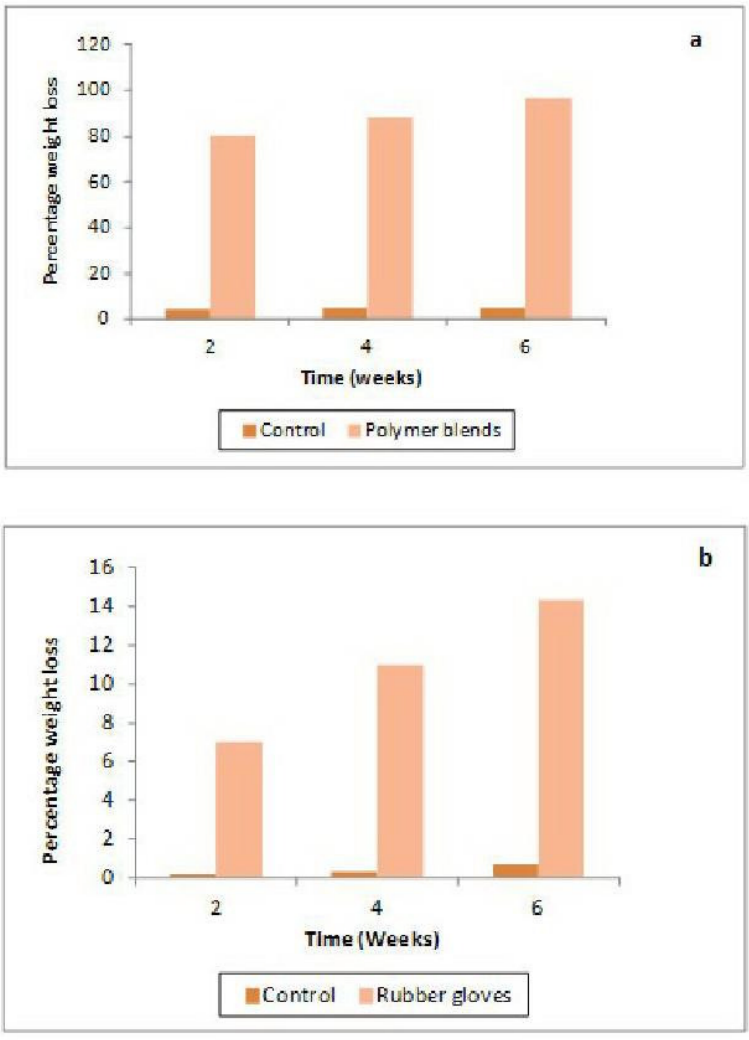


Fig. 3 The percentage weight loss of polymer blends (a) and rubber gloves (b) after incubation with *S. coelicolor* CH13 at concentration of 10^8 spores/mL at 30ºC for 6 weeks.

Staining and enzyme assay

The SEM observations indicated that the microorganisms were capable of producing enzymes that degraded the polymer for use as a growth substrate (Bode et al. 2001; Pan et al. 2009). Analysis of rubber degradation by *S. coelicolor* showed that it was able to cleave the carbon backbone of poly(cis-1,4-isoprene) (Bode et al. 2001). Staining with Schiff's reagent indicated that aldehydes were present around the colonies of *S. coelicolor* CH13. This correlates with the results from 2, 4-DNP staining. The development of a yellow colour on both polymers was observed indicating that the bacterium was able to begin the rubber degradation and reduce the average molecular weight of rubber.

The breakdown of starch will require production of a glucosidase enzyme and oligosaccharides followed by glucose are expected to be released into the medium. The presence of reducing sugar in the medium was measured in the culture of *S. coelicolor* CH13 with polymer blend. Reducing sugar residues measured as glucose reached values of 540.8 µg mL^{-1} after 10 days of incubation (Figure 4). The assay for glucosidase reached its highest activity of 138.7 U mL^{-1} after 18 days, and then decreased rapidly (Figure 4). The hydrolysis of starch obviously occurs rapidly. Based on these results *S. coelicolor* CH13 could utilize almost all the starch in the polymer blend after 8 days. These results were supported by the determination of starch. Starch decreased rapidly over the first 8 days and continued to decrease slowly after that. The total weight loss of 92% (w/w) of polymer blend clearly shows that this bacterial isolate could utilize natural rubber in the polymer after the starch disappeared. The changes in molecular weight of the samples before and after degradation were also investigated by the determination of the intrinsic viscosity. There was a 2.5 fold decrease in the molecular weight of the material extracted with toluene. This indicated that *S. coelicolor* CH13 was able to cleave the carbon backbone of poly(cis-1,4-isoprene). Our preliminary results from Fourier transform infrared spectroscopy (FTIR) strongly confirmed the degradation of natural rubber (data not shown). The basic molecular mechanism by which rubber is degraded isnot fully known. It isassumed that degradation of the polymer backbone is initiatedby oxidative cleavage of the double bonds in thepolymer chain (Braaz et al. 2004). There has been intensive ongoing molecular work on the process (Braaz et al. 2004; Braaz et al. 2005; Ebaid et al. 2006; Bröker et al. 2008).

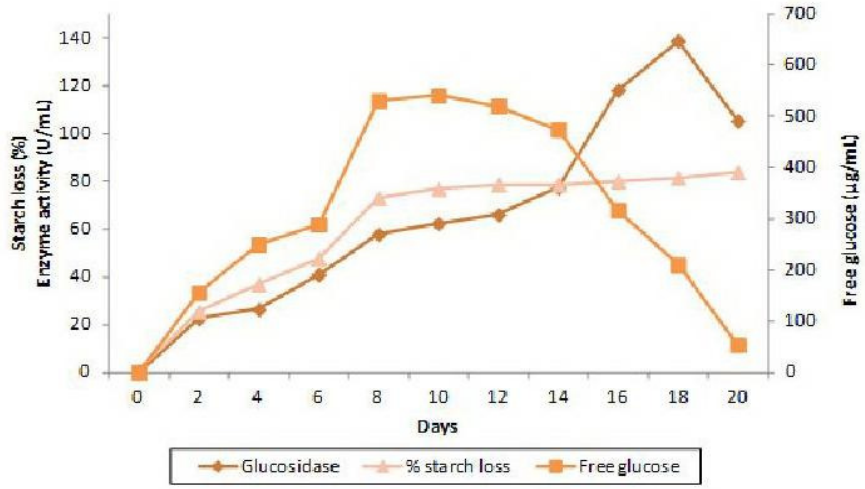


Fig. 4 Percentage starch loss, enzyme activity and amount of glucose present in culture supernatant after incubating polymer blends with *S. coelicolor* CH13 over 20 days period.

Mineralization of polymer blend and rubber glove substrate

During the aerobic biodegradation of organic materials, carbon dioxide and water are the final decomposition products. The amount of carbon dioxide produced during the biodegradation of the test material was measured, and compared to the theoretical maximal amount and recorded as a biodegradation percentage. The process of biodegradation is shown in Figure 5a, where carbon

dioxide production was plotted against the time of cultivation. These experiments clearly revealed that both the polymers were being degraded by the isolate *S. coelicolor* CH13 but there was a marked difference between the two polymers in their degree of degradation. After 30 days 60% of the original carbon in the polymer blend had been converted to CO_2 whereas only 10% of the carbon of the rubber gloves was converted to CO_2. This again demonstrated the ability and preference of the isolate to degrade starch rather than rubber. In both cases the population of bacteria achieved their highest level after about 1 week of incubation from 2 x 10^8 at the start to at 10^9 for both samples. This population was maintained in the polymer blend for at least another week while the sugar concentration remained high whereas between 7 and 10 days with the rubber gloves the population fell to 8 x 10^8 cells/mL. It was of interest that the population with the polymer blend also fell to 8 x 10^8 cells/mL during day 15th to 18th when the reduction of the free glucose was occurring at its maximum rate from 500 to 53 µg/mL (Figure 4). It would seem that the isolate CH13 quickly lost viability when readily available substrates became limiting. The decreases in population might also contribute to the preferential adhesive growth on the substrates. The exponential loss of viability occurred at about the same rate in both cases (Figure 5b). An increase in the number of cells suspended in the medium during cultivation that led to an increase in biodegradation and a higher mineralization rate was also described by Linos et al. (2000); Berekaa et al. (2005) and Warneke et al. (2007).

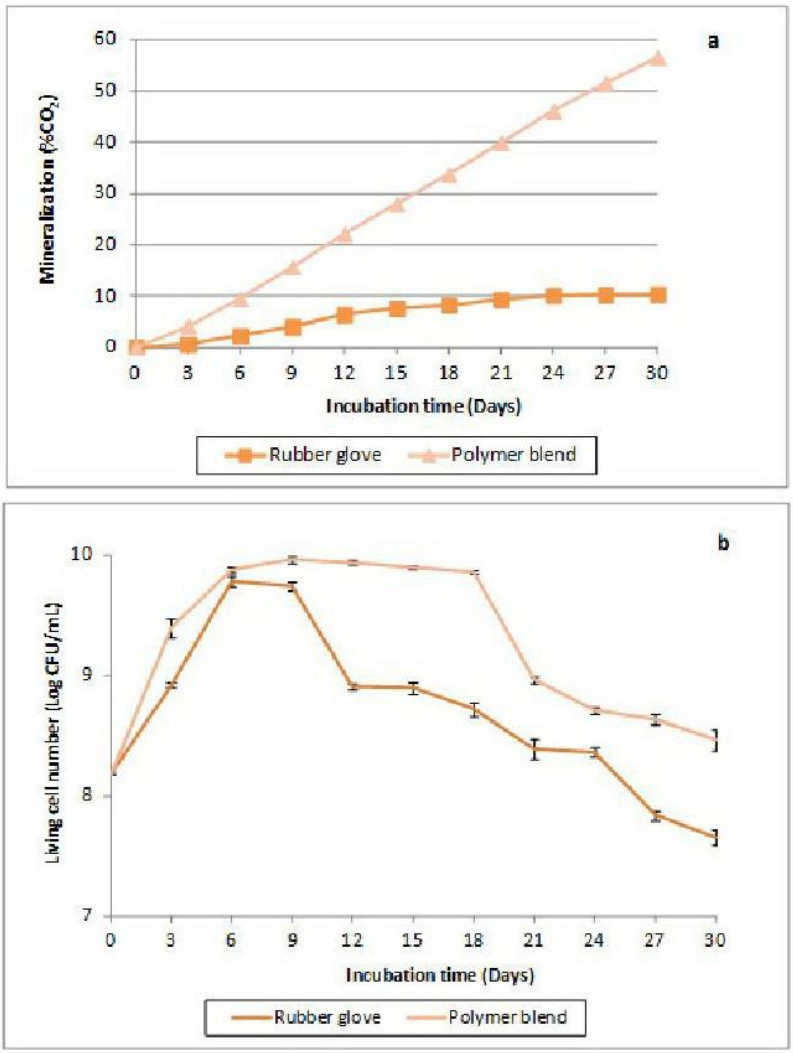
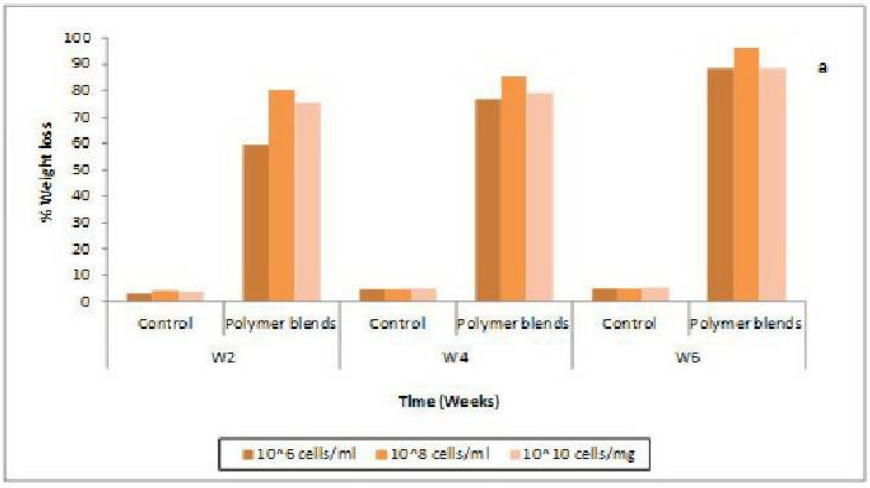


Fig. 5 Percentage mineralization during growth of bacteria on polymer blend and rubber gloves expressed as % CO_2 released from total carbon (a); Viable counts of suspended cells during cultivation (b).

Optimum conditions for the rate of biodegradation

Effect of inoculum sizes. The effect of changes to the rate of degradation of the polymer blend using different inoculum sizes showed that, in the system using, a concentration of *S. coelicolor* CH13 of 10^8 spores/mL was optimum for the biodegradation process as measured by the percentage weight loss of materials. After 2, 4 and 6 weeks the percentage weight loss of the polymer blend was 80.15, 85.25 and 96.14 respectively (Figure 6a). In contrast, inoculating with 10^6 and 10^{10} spores/mL produced a 59.39, 76.59, 88.6 and 75.59, 79.03, 88.40% weight loss respectively, after 2, 4, 6 weeks. The sterile control had only a < 5.0% weight loss in the same conditions. The weight loss was greater over the first 2 weeks than over the succeeding 2 week periods. The weight loss of the rubber gloves with an inoculums size at 10^8 spores/mL for the same time periods was 6.99, 11.00 and 14.31%. With an inoculum size of 10^6 and 10^{10} spores/mL, the weight loss was 8.19, 12.03, 12.95 and 2.39, 5.10, 11.72%, in 2, 4, 6 weeks respectively. The sterile control exhibited a weight loss of < 1.8% (Figure 6b). These results showed that the percentage weight loss of both the polymer blend and the rubber gloves increased for at least 6 weeks with all inoculum sizes. During that time the numbers of bacteria increased to a final 10^{10} spores/mL (data not shown) so an inoculum of 10^{10} spores/mLhad less opportunity to grow and therefore degrade the polymer.



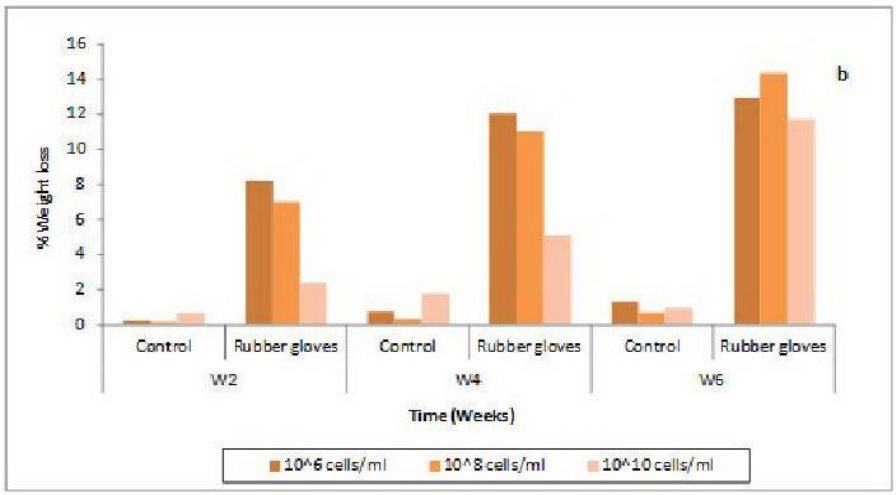


Fig. 6 The percentage weight loss of polymer blends (a) and rubber gloves (b) after incubation with *S. coelicolor* CH13 at different inoculum concentrations of 10^6, 10^8, 10^{10} cells/mL at 30ºC for 6 weeks.

There are many reports of finding antibiotics from *S. coelicolor* (Kim et al. 2004; Willems et al. 2008). It is possible that the production of antibiotics from a higher inoculum size could restrict further growth. The initial inoculum size of 10^8 reached the final 10^9 spores/mL within 2 weeks. This means that these growth conditions only allow for restricted growth potential so the best degradation occurred with an inoculum size of 10^8 spores/mL of *S. coelicolor* CH13. Perhaps this might change if the culture conditions were modified by changing the nutrient composition such as the N source to 10 g/L instead of the 1 g/L used in many of the experiments.

Effect of nitrogen source concentration. Although the strain grew and degraded polymers at all concentrations of NH_4NO_3 tested the maximum biodegradation was obtained at a high concentration of 10 g/L. The nitrogen source at a concentration of 10 g/L was found to provide optimum conditions for the biodegradation of both polymers. In most of the previous experiments a concentration of 1 g/L was used. The change from 1 to 10 g/L increased the weight loss of the polymer from 80 to 90% whereas the increase for the rubber gloves was from 15 to 30%. We assume that this is a reflection of providing more optimum condition for growth of the bacteria. With the polymer blend the bacteria can obtain a rapid source of carbon from the starch but the nitrogen source is limiting whereas when rubber is the only source of carbon both the carbon and nitrogen source is limited. Providing the rubber degrading bacteria with an extra more readily available carbon source together with the rubber may be one way to increase the rate of rubber degradation.

Effect of pH and temperature. Polymer biodegradation occurred at each of the pH values tested 6, 7, and 8. A pH of 7.0 allowed for the most degradation of 85% weight loss compared to 46.1% and 63.6% at pH 6 and 8 respectively. The maximum percentage weight loss of the polymer blend occurred at 30ºC (87.3%), whereas at 35ºC the weight loss was 70.9% and at 40ºC it was only 65.2%. For the rubber gloves the highest weight loss also occurred at 30ºC (29.5%), followed by 35ºC (19.1%) and 40ºC (16.7%). An increase to 40ºC produced a significant decrease of activity.

From the above observations it seems that the optimum condition for *Streptomyces coelicolor* CH13 tobiodegrade the polymer blend and rubber glove in an MSM medium, is an inoculums size of 10^8 spores/mL, NH_4NO_3 at a concentration of 10 g/L, a pH of 7.0, and temperature at 30ºC, in a shaking incubator. This achieved a maximum percentage weight loss of 96.8% for the polymer blend and 36.5% for the rubber gloves over a 4 week incubation period.

Investigation of the disappearance of starch in the polymer blend

The strategy of bacteria to degrade polymers is to first adhere to its surface then to secrete enzymes that can initiate degradation by reducing the polymer chain lengths and eventually produce molecules that can be assimilated and converted by its metabolic processes to provide energy and precursors for its biosynthetic reactions so that it can grow. Evidence from scanning EM clearly showed that isolate CH13 became attached to the polymer surfaces and in the case of the polymer blend containing rubber latex and cassava starch caused a disintegration of the polymer sheets. As the major component of this polymer is starch and microbial degradation of starch normally leads to the production of oligosaccharides and eventually glucose (Schlemmer et al. 2009) a readily available nutrient, the loss of starch from the polymer was measured by the soxhlet extraction method and compared to the release of reducing sugars into the supernatant using the Somogyi/Nelson method, for measuring reducing sugar and the presence of an α-glucosidase enzyme measured by the liberation of nitrophenol from p-nitrophenyl α-D-glucoside. The overall process could be divided into 3 separate phases, from 0 to 8 days, (phase 1) 8 to 14 days (phase 2) and 14 to 21 days (phase 3). It was not unexpected that the loss of starch closely corresponded to the increase in the reducing sugar component of the supernatant in phase 1. During this time the population of bacteria rapidly increased as did the loss of starch, the increase in the amount of free sugar and the amount of enzyme (Figure 4). In phase 2 the bacterial population remained fairly constant and so did the level of free reducing sugar, the loss of starch proceeded at a much slower rate together with the increase in enzyme activity so the amount of glucose produced was about the same as the amount used by the bacteria. During phase 2 perhaps the growth of the bacteria became limited by the amount of available nitrogen in the medium (1 g/L). At this point, phase 3, 78% of the starch had been removed so the reducing sugar was metabolized more quickly than it was being produced until the amount of starch lost reached it's a maximum of 83% and there was a huge increase in the amount of enzyme until day 18 after which the enzyme decreased. This indicated that the formation of the enzyme was perhaps being controlled by the amount of available glucose as a case of glucose repression.

The information is consistent with the following scenario. During phase 1 the bacteria multiplied, became attached to the substrate, and in the absence of any free substrate the hydrolytic enzyme was induced and converted the starch to oligosaccharides with a slower increase of free glucose. Towards the end of phase 1 the reducing sugar level increased most rapidly (6-8 days) probably due to the conversion of all the oligosaccharides to glucose. As the free glucose increased the rate of enzyme production decreased slightly until multiplication of the bacteria ceased at a time when the reducing sugar concentration and glucose was highest, (end of phase 1) so the rate of enzyme production decreased further. When the glucose was being rapidly used (phase 3) glucose repression was released and the enzyme level suddenly increased even in the absence of substrate so starch may not be an inducer of the enzyme, just the absence of any suitable nutrient to grow. In the absence of glucose the bacteria lost viability and no further enzyme was produced. The glucosidase enzyme increased over the first 2 days and then increased slowly over the next 12 days when the rate of starch loss was increasing, then increased rapidly over the next 4 days when the glucose concentration was rapidly decreasing and the amount of starch left in the polymer was very low.

CONCLUDING REMARKS

S. coelicolor CH13 degraded the new starch/NR polymer, natural rubber and a natural rubber product (rubber gloves) effectively. The results from SEM, weight loss and percentage mineralization strongly confirmed the degradation of natural rubber. *S. coelicolor* CH13, or a similar organism could, in the future, contribute to a biotechnological solution for degrading rubber product wastes, in which microbial degradation would be combined with physicochemical methods in order to work efficiently. Further improvements to the degradation conditions will be needed using a fractional experimental design. Intensive studies on the molecular mechanism of rubber degradation by such isolate and its requirements are being further investigated.

ACKNOWLEDGMENTS

We thank the Bioplastic Research Unit, Faculty of Science, Prince of Songkla University for providing polymer blends samples.

Financial support: This research was supported by the Thai Government's budget, Fiscal year 2553 and by a grant from Prince of Songkla University.

REFERENCES

BEREKAA, M.M.; BARAKAAT, A.; EL-SAYED, S.M. and EL-AASSAR, S.A. (2005). Degradation of natural rubber by *Achromobacter* sp. NRB and evaluation of culture conditions. *Polish Journal of Microbiology*, vol. 54, no. 1, p. 55-62.

BHATT, R.; SHAH, D.; PATEL, K.C. and TRIVEDI, U. (2007). PHA-rubber blends: Synthesis, characterization and biodegradation. *Bioresource Technology*, vol. 99, no. 11, p. 4615-4620. [CrossRef]

BODE, H.B.; ZEECK, A.; PLÜCKHAHN, K. and JENDROSSEK, D. (2000). Physiological and chemical investigations into microbial degradation of synthetic poly(*cis*-1,4-isoprene). *Applied and Environmental Microbiology*, vol. 66, no. 9, p. 3680-3685. [CrossRef]

BODE, H.B.; KERKHOFF, K. and JENDROSSEK, D. (2001). Bacterial degradation of natural and synthetic rubber. *Biomacromolecules*, vol. 2, no. 1, p. 295-303. [CrossRef]

BRAAZ, R.; FISCHER, P. and JENDROSSEK, D. (2004). Novel type of heme-dependent oxygenase catalyzes oxidative cleavage of rubber (Poly-*cis*-1,4-Isoprene). *Applied and Environmental Microbiology*, vol. 70, no. 12, p. 7388-7395. [CrossRef]

BRAAZ, R.; ARMBRUSTER, W. and JENDROSSEK, D. (2005). Heme-dependent rubber oxygenase roxA of *Xanthomonas* sp. cleaves the carbon backbone of poly(*cis*-1,4-Isoprene) by a dioxygenase mechanism. *Applied and Environmental Microbiology*, vol. 71, no. 5, p. 2473-2478. [CrossRef]

BRÖKER, D.; DIETZ, D.; ARENSKÖTTER, M. and STEINBÜCHEL, A. (2008). The genomes of the non-clearing-zone-forming and natural-rubber-degrading species *Gordonia polyisoprenivorans* and *Gordonia westfalica* harbor genes expressing Lcp activity in *Streptomyces* strain. *Applied and Environmental Microbiology*, vol. 74, no. 8, p. 2288-2297. [CrossRef]

CARVALHO, A.J.F.; JOB, A.E.; ALVES, N.; CURVELO, A.A.S. and GANDINI, A. (2003). Thermoplastic starch/natural rubber blends. *Carbohydrate Polymers*, vol. 53, no. 1, p. 95-99. [CrossRef]

CHERIAN, E. and JAYACHANDRAN, K. (2009). Microbial degradation of natural rubber latex by a novel species of *Bacillus* sp. SBS25 isolated from soil. *International Journal of Environmental Research*, vol. 3, no. 4, p. 599-604.

EBAID, M.; IBRAHIM, A.; ARENSKÖTTER, M.; LUFTMANN, H. and STEINBÜCHEL, A. (2006). Identification of poly(*cis*-1,4-isoprene) degradation intermediates during growth of moderately thermophilic actinomycetes on rubber and cloning of a functional *lcp* homologue from *Nocardia farcinica* strain E1. *Applied and Environmental Microbiology*, vol. 72, no. 5, p. 3375-3382. [CrossRef]

EHRLICH, G.; TAYLOR, H.E. and WAELSCH, H. (1948). The effect of surface-active substances on the fuchsin reaction of higher fatty aldehydes. *The Journal of Biological Chemistry*, vol. 173, p. 547-551.

GU, J.D. (2003). Microbiological deterioration and degradation of synthetic polymeric materials: Recent research advances. *International Biodeterioration & Biodegradation*, vol. 52, no. 2, p. 69-91. [CrossRef]

HEISEY, R.M. and PAPADATOS, S. (1995). Isolation of microorganisms able to metabolize purified natural rubber. *Applied and Environmental Microbiology*, vol. 61, no. 8, p. 3092-3097.

IBRAHIM, E.M.A.; ARENSKÖTER, M.; LUFTMANN, H. and STEINBÜCHEL, A. (2006). Identification of poly(*cis*-1,4-Isoprene) degradation intermediates during growth of moderately thermophilic actinomycetes on rubber and cloning of a functional *lcp* homologue from *Nocardia farcinica* strain E1. *Applied and Environmental Microbiology*, vol. 72, no. 5, p. 3375-3382. [CrossRef]

JENDROSSEK, D.; TOMASI, G. and KROPPENSTEDT, R.M. (1997). Bacterial degradation of natural rubber: A privilege of actinomycetes? *FEMS Microbiology Letter*, vol. 150, no. 2, p. 179-188. [CrossRef]

KIM, H.B.; SMITH, C.P.; MICKLEFIELD, J. and MAVITUNA, F. (2004). Metabolic flux analysis for calcium dependent antibiotic (CDA) production in *Streptomyces coelicolor*. *Metabolic Engineering*, vol. 6, no. 4, p. 313-325. [CrossRef]

LINOS, A.; REICHELT, R.; KELLER, U. and STEINBÜCHEL, A. (2000). A gram-negative bacterium, identified as *Pseudomonas aeruginosa* AL98, is a potent degrader of natural rubber and synthetic *cis*-1,4-polyisoprene. *FEMS Microbiology Letters*, vol. 182, no. 1, p. 155-161. [CrossRef]

LINOS, A. and STEINBÜCHEL, A. (2001). Biodegradation of natural and synthetic rubber, In: KOYAMA, T. and STEINBÜCHEL, A. eds. *Biopolymers*. Wiley-VCH, Weinheim, Germany. p. 321-359.

LINOS, A.; BEREKAA, M.M.; STEINBÜCHEL, A.; KIM, K.K. SPROER, C. and KROPPENSTEDT, R.M. (2002). *Gordonia westfalica* sp. nov., a novel rubber-degrading actinomycete. *International Journal of Systematic and Evolutionary Microbiology*, vol. 52, no. 4, p. 1133-1139. [CrossRef]

PAN, L.; GU, J.G.; YIN, B. and CHENG, S.P. (2009). Contribution to deterioration of polymeric materials by a slow-growing bacterium *Nocardia corynebacterioides*. *International Biodeterioration & Biodegradation*, vol. 63, no. 1, p. 24-29. [CrossRef]

ROSE, K.; TENBERGE, K.B. and STEINBÜCHEL, A. (2005). Identification and characterization of genes from *Streptomyces* sp. strain K30 responsible for clear zone formation on natural rubber latex and poly(cis-1,4-isoprene) rubber degradation. *Biomacromolecules*, vol. 6, no. 1, p. 180-188. [CrossRef]

ROY, R.V.; DAS, M.; BANERJEE, R. and BHOWMICK, A.K. (2006). Comparative studies on crosslinked and uncrosslinked natural rubber biodegradation by *Pseudomonas* sp. *Bioresource Technology*, vol. 97, no. 18, p. 2485-2488. [CrossRef]

SABEV, H.A.; HANDLEY, P.S. and ROBSON, G.D. (2006). Fungal colonization of soil-buried plasticized polyvinyl chloride (pPVC) and the impact of incorporated biocides. *Microbiology*, vol. 152, no. 6, p. 1731-1739. [CrossRef]

SCHLEMMER, D.; SALES, M.J.A. and RESCK, I.S. (2009). Degradation of different polystyrene/thermoplastic starch blends buried in soil. *Carbohydrate Polymers*, vol. 75, no. 1, p. 58-62. [CrossRef]

SEO, S.J.; KEUM, Y.S.; HARADA, R.M. and LI, Q.X. (2007). Isolation and characterization of bacteria capable of degrading polycyclic aromatic hydrocarbons (PAHs) and organophosphorus pesticides from PAH-contaminated soil in Hilo, Hawaii. *Journal of Agricultural and Food Chemistry*, vol. 55, no. 14, p. 5383-5389. [CrossRef]

SHEY, J.; IMAM, S.H.; GLENN, G.M. and ORTS, W.J. (2006). Properties of baked starch foam with natural rubber latex. *Industrial Crops and Products*, vol. 24, no. 1, p. 34-40. [CrossRef]

SOMOGYI, M. (1952). Notes on sugar determination. *Journal of Biological Chemistry*, vol. 195, p. 19-23.

STEINBÜCHEL, A. (2001). Perspectives for biotechnological production and utilization of biopolymers: Metabolic engineering of polyhydroxyalkanoate biosynthesis pathways as a successful example. *Macromolecular Bioscience*, vol. 1, no. 1, p. 1-24. [CrossRef]

TANRATTANAKUL, V. and CHUMEKA, W. (2009). Effect of potassium persulfate on graft co-polymerization and mechanical properties of cassava starch/natural rubber foams. *Journal of Applied Polymer Science*, vol. 116, no. 1, p. 93-105. [CrossRef]

TSUCHII, A. and TAKEDA, K. (1990). Rubber-degrading enzyme from a bacterial culture. *Applied and Environmental Microbiology*, vol. 56, no. 1, p. 269-274.

WANG, Z.F.; PENG, Z.; LI, S.D.; LIN, H.; ZHANG, K.X.; SHE, X.D. and FU, X. (2009). The impact of esterification on the properties of starch/natural rubber composite. *Composites Science and Technology*, vol. 69, no. 11-12, p. 1797-1803. [CrossRef]

WARNEKE, S.; ARENSKÖTTER, M.; TENBERGE, K.B. and STEINBÜCHEL, A. (2007). Bacterial degradation of poly(*trans*-1,4-isoprene) (*Gutta percha*). *Microbiology*, vol. 153, no. 2, p. 347-356. [CrossRef]

WILLEMS, A.R.; TAHLAN, K.; TAGUCHI, T.; ZHANG, K.; LEE, Z.Z.; ICHINOSE, K.; JUNOP, M.S. and NODWELL, J.R. (2008). Crystal structures of the *Streptomyces coelicolor* TetR-like protein ActR alone and in complex with actinorhodin or the actinorhodin biosynthetic precursor (*S*)-DNPA. *Journal of Molecular Biology*, vol. 376, no. 5, p. 1377-1387. [CrossRef]

YU, L.; DEAN, K. and LI, L. (2006). Polymer blends and composites from renewable resources. *Progress in Polymer Science*, vol. 31, no. 6, p. 576-602. [CrossRef]

Appendix D

Proceedings The 2nd CMU Graduate Research Conference
การจัดการประชุมเสนอผลงานวิจัยระดับบัณฑิตศึกษา มหาวิทยาลัยเชียงใหม่ ครั้งที่ 2 วันที่ 24 พฤศจิกายน 2553

Isolation of bacteria able to degrade new polymer from cassava starch foam blended with natural rubber latex.

Sirimaporn Watcharakul*, Brian Hodgson**, Varaporn Tanrattanakul***, Kamontam Umsakul****

Department of Microbiology, Faculty of Science, Prince of Songkla University

Abstract

The aim of this study was to isolate bacteria from soil that degraded a new natural rubber/starch biopolymer and rubber gloves. From about 6 different habitats, 78 isolates produced translucent halos on Minimal Salt Medium (MSM) with natural rubber. One isolate, CH13 that produced the largest translucent halo of about 3 mm did degrade the polymer blend and rubber gloves. This isolate was very closely related to *Streptomyces coelicolor* (100%) based on its 16s rRNA gene sequence. This bacterium produced surface erosion of strips of the new biopolymer and rubber gloves after an incubation period of 4 weeks in MSM. This study indicates that this bacterium can cause the biodegradation of the new biopolymer and confirms that this biopolymer will be degraded in the environment and is suitable as a green plastic from natural sources.

Key words: Rubber degrading bacteria, *Streptomyces coelicolor*, Polymer blended-degrading bacteria

1. Introduction

Plastics, normally derived by synthesis from petroleum-based compounds, are now essential for daily life. Unfortunately after disposal they do not degrade and create many environmental hazards

This problem has led to the search for alternative biodegradable plastic materials or biopolymers (Steinbüchel, 2001). Biopolymers obtained from agriculture are in principle available from renewable resources. Of the biopolymers presently under development, cassava starch foam blended with natural rubber latex is the most promising product. (Tanrattanakul and Chumeka, 2009) Cassava starch and rubber are abundant and low cost resources, each with a large number of applications such as packaging, industrial products and the food industry.

The major drawback of cassava starch foam blended with natural rubber latex is its slow degradation rate. This is because the rubber component is elastomeric, with a high average molecular weight of nearly a million and as a result is hard to degrade biologically (Tanrattanakul and Chumeka, 2009). Most research on biodegradation has screened for bacteria able to degrade pure natural rubber and chemically treated or synthetic rubber. For instance, Warneke *et al.* (2007) isolated 6 strains of *Nocardia* spp. that utilized natural rubber as a sole source of carbon and energy for growth. The most extensive investigation was done by Tsuchii and co-workers (1985). They isolated a *Nocardia* sp. (strain 835A), able to degrade natural rubber, synthetic rubber and cross-linked natural rubber.

There has been an increased interest in the isolation and study of microorganisms able to degrade any new biopolymer and this information should

* Master student in Microbiology, Faculty of science, Prince of Songkla university e-mail: snep_p@hotmail.com

**Lecturer Dr., Faculty of Pharmacy, Prince of Songkla university e-mail: bhdoc01@yahoo.com

***Lecturer Assoc. Prof. Dr., Department of Science and material technology Faculty of science, Prince of Songkla university e-mail: varaporn.t@psu.ac.th

**** Lecturer Dr., Department of microbiology, Faculty of science, Prince of Songkla university e-mail: Kamontam.u@psu.ac.th

accompany its manufacture to ensure that the polymer will be environmentally friendly, and have less impact on the ecology by being eliminated by biodegradation over a reasonable period of time. The goal of this work is to isolate microorganisms able to readily degrade a new biopolymer i.e. cassava starch foam blended with natural rubber latex. As the rubber might be the most difficult part to degrade, rubber gloves are also used for an enrichment process.

Methodology

1. Source of polymers Cassava starch foam blended with natural rubber latex (35%) was supplied by the Bioplastic Research Unit, Prince of Songkla University, Thailand. Rubber gloves were Siam Sempermed company, Songkha Thailand.

2. Medium and growth conditions. Soil samples were taken from different sites associated with rubber factories and their wastes in Hatyai, Thailand. For the enrichment process soil samples were suspended in Erlenmeyer flasks containing a minimal salts medium (MSM) plus trace elements solution (Jendrossek *et al*., 1997) and natural rubber latex (NR) 0.6% (v/v) was added as a carbon source to both liquid and solid MSM (MSN plus 1.5% (w/v) agar) prior to autoclaving. Liquid cultures were incubated on a horizontal rotary shaker at 150 rpm 30°C for 5 days. In addition solid MSM media was overlain with 7 ml of NR latex as carbon source to detect colonies producing translucent haloes caused by degradation of the water-insoluble polymer (Ibrahim *et al*., 2006).

3. Isolation of natural rubber degrading microorganisms. Samples from various ecosystems and enrichment cultures were diluted with sterile mineral medium and mixed well. 0.1 ml of each dilutions were spread on MSM plates with an NR layer as the sole carbon source and incubated at 30°C. Colonies with translucent halos were purified by alternating transfers to MSM media with NR and yeast extract plates.

4. Bacterial identification. The strains that produced the best degradation of NR were identified as follows.

4.1 Standard identification methods including staining reactions such as Gram stain, patterns of utilizing various polymers and carbon sources, motility tests, haemolysis tests on blood agar, catalase test, oxidase test and a test for amylase with a cassava starch substrate.

4.2 Determination of 16s rRNA gene sequences to identify and classify isolates.

Each strain was cultured for 2 days in Nutrient Broth. Genomic DNA was extracted by standard methods (Warneke *et al*., 2007). The primers used for producing PCR products were 27F and 1389R to amplify the full length of the 16S rRNA gene. This was carried out at the BIOTEC Culture Central Research Unit. The PCR amplification reaction and conditions used were standard optimum conditions. Nucleotide sequences of purified PCR products were determined with the Genetic Analyzer, and DNA nucleotide sequences of isolates were analyzed for identification using the data available from the GenBank database at The National Center for Biotechnology Information (Pan *et al*., 2009).

5. Investigation of the degradation process. Individual isolates were further examined for their abilities to degrade the blended polymer and rubber gloves by investigating growth with the two rubber substrates as the sole source of carbon and energy. Overnight cultures of each bacterial isolate in MSM with rubber latex were inoculated into 250 ml Erlenmeyer flasks containing 200 ml of MSM and

Proceedings The 2nd CMU Graduate Research Conference
การจัดงานประชุมการประชุมนำเสนอผลงานวิจัยระดับบัณฑิตศึกษา มหาวิทยาลัยเชียงใหม่ ครั้งที่ 2 วันที่ 24 พฤศจิกายน 2553

3.42 g of new polymer strips (0.6% rubber) or 1.2 g of rubber gloves strips all size 2x2 cm. (0.6% rubber) (sterilized by autoclave), and incubated on an incubator shaking at 150 rpm, 30°C for up to 4 weeks (Pan *et al.*, 2009).

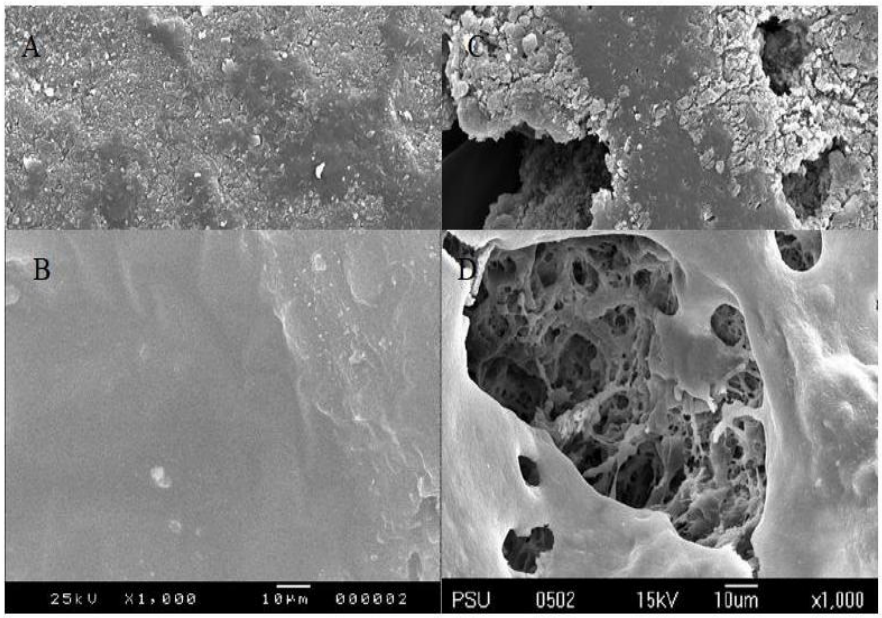
6.4.1 Surface erosion of the starch/NR blended sheets was observed by SEM and any observed colors changes of these polymers were recorded.

Results and Discussion

1. Isolation of rubber-degrading bacteria.

Among the bacterial isolates obtained from the six samples of soil from different ecosystems in Songkhla province, Thailand were 118 isolates capable of growth on MSM medium with NR, and 78 of them produced translucent halos around the colonies. Previously isolated rubber degrading microorganisms had been separated into two groups that used different strategies to degrade rubber. The first group formed translucent halos on solid media containing dispersed latex halos on solid media containing dispersed latex particles, and included the excretion of rubber-cleaving enzymes like polyisoprenoid oxygenase (Linos *et al.*, 2000) that cleaved the double bonds in the rubber backbone for initiation of rubber degradation (Tsuchii *et al.*, 1990). The second group did not form translucent halos because they attached to the surface of the rubber particles (Linos *et al.*, 2000).

From all the 78 isolates that formed translucent haloes six isolates produced large zones of about 3-6 mm (Table 1). All these 78 isolates produced the amylase enzyme that hydrolyzed starch in this new polymer.



2. Bacterial identification

Six isolates were Gram-positive bacteria. All produced the catalase enzyme that degraded hydrogenperoxide to water and oxygen and all produced the oxidase enzyme.

This means that these bacteria contain cytochrome C oxidase and therefore utilize oxygen for energy production with an electron transfer chain. In the motility test all six isolates were non motile. Isolate F5 did not produce hemolysis on blood agar (Table 1). The full length 16s rRNA gene sequence analysis of isolate CH 13 was highly similar to *Streptomyces coelicolor* (100%).

Figure 2. Translucent halos produced by *Streptomyces coelicolor* CH 13 on Minimal Salt Medium agar mixed with natural rubber latex

3. Surface erosion of the starch/NR blended sheets

The surface of rubber gloves and polymer blend as seen by SEM after being incubated for a period of 4 weeks, are presented in figure 1 (A-D). In the control without bacteria cells (unioculated) the surface was rough but not modified (Figure A, C) for both rubber glove strips and polymer blended strip. In contrast, *S. coelicolor* CH 13 cells caused large holes to appear in the rubber gloves (Figure B) and a similar observation was made on the polymer blended surface (Figure D) These erosion surfaces have been previously seen by Warneke *et al*. (2007). Thereby confirming that these isolates are also potent polymer blend and rubber glove degrading bacteria.

Conclusion

This study has shown that bacteria can be isolated from soil with properties similar to *Streptomyces coelicolor* and the potential to degrade the new NR/starch biopolymer and rubber gloves. This bacterium is capable of causing surface erosion and is effective in degrading the rubber materials tested.

Acknowledgements

I am very grateful to the Bioplastic Research Unit, Faculty of Science, Prince of Songkla University, Thailand. Thanks are extended to Miss Wannapa Chumeka for advice concerning polymer laboratory techniques and her help with polymer analysis. Many special thanks for students in the applied science laboratory for help during this study.

References

[1] Ibrahim, E.M.A., Matthias, A., Luftmann, H. and Steinbüchel, A. (2006). Identification of Poly(cis-1,4-Isoprene) Degradation Intermediates during Growth of Moderately Thermophilic Actinomycetes on Rubber and Cloning of a Functional *lcp* Homologue from *Nocardia farcinica* Strain E1. Environmental polymer degradation. 72(5): 3375-3382.

[2] Jendrossek, D., Tomasi, G. and Kroppenstedt, R.M., (1997). Bacterial degradation of natural rubber: a privilege of actinomycetes?. FEMS Microbiology Letter. 150: 179-188.

[3] Linos, A., Berekaa, M.M., Steinbüchel, A., Kim, K.K., Sproer, C. and Kroppensted, R.M. (2002). *Gordonia westfalica* sp. nov., a novel rubber-degrading actinomycetes. International journal of systematic and evolutionary microbiology. 52:1133-1139.

[4] Pan, L., Gu, J.G., Yin, B. and Cheng, S.P. (2009). Contribution to deterioration of polymeric materials by a slow-growing bacterium *Nocardia corynebacterioides*. International biodeterioration & biodegradation. 63: 24-29.

[5] Steinbüchel, A. and Koyama, T. (2001). Biopolymer, Polyisoprenoids. Wiley-VCH verlag GmbH, Weinheim: Germany.

[6] Tanrattanakul, V. and Chumeka, W. (2009). Effect of Potassium Persulfate on graft co-polymerization and mechanical properties of cassava starch/Natural rubber foams. Applied polymer science. 116: 93-105.

[7] Tsuchii, A., Suzuki, T. and Takeda, K. (1985). Microbial degradation of natural rubber valcanizates. Applied and environmental microbiology. 50(4): 965-970.

[8] Tsuchii, A. and Takeda, K. (1990). Rubber-degrading enzyme from a bacterial culture. Applied and environmental microbiology. 50(1): 269-279.

[9] Warneke, S., Arenskötter, M., Tenberge, K. B. and Steinbüchel, A. (2007). Bacterial degradation of poly(*trans*-1,4-isoprene) (gutta percha). Microbiology. 153: 347-356.

Table 1. Morphological and biochemical characteristics of bacterial isolates

Isolates	Gram stain	catalase	oxidase	Motility	haemolysis	Size of clear zone (mm.)
A7	G+	+	-	-	α	4.0
A8	G+	+	-	-	α	3.5
F5	G+	+	-	-	-	3.0
F6	G+	+	-	-	α	3.0
CH2	G+	+	-	-	α	3.0
CH13	G+	+	-	-	α	5.0

VITAE

Name Miss Sirimaporn Watcharakul

Student ID 5210220090

Educational Attainment

Degree	Name of Institution	Year of Graduation
B. Sc.	Microbiology	2008

Scholarship Awards during Enrolment

1. Graduate School Songklanakarin Scholarship 2009-2011
2. Teacher assistant (TA) fund From Faculty of Science
3. JSPS (Japan Society for the Promotion of Sciences) Exchange Program for East Asian Young Researchers, 'Collaborative research on development of useful microbial resources in tropical environments with South-East Asian young researchers'

List of Publication and Proceeding

Publication

1. Watcharakul, S., Umsakul, K., Hodgson, B., Chumeka, W. and Tanrattanakul, V. 2012. Biodegradation of a blended starch/natural rubber foam biopolymer and rubber gloves by *Streptomyces coelicolor* CH13. Electronic Journal of Biotechnology. 15(1).

Proceeding

1. Watcharakul, S., Umsakul, K., Hodgson, B. and Tanrattanakul, V. 2010. Isolation of bacteria able to degrade new polymer from cassava starch foam blend with natural rubber latex. Proceeding of 2010 2nd CMU Graduate Research Conference Proceedings, 25-26 November, 2010, Chiangmai, Thailand. 466-470.