

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

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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) $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (9.0), KH_2PO_4 (1.5), NH_4NO_3 (1.0), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.02), $\text{Fe(III)[NH}_4\text{] citrate}$ (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 30°C 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 30°C. 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 30°C 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, 45°C 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 30°C 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 65°C 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 H₂O (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 α-D-glucoside. Assay mixtures (1 mL) containing 5 mM substrate in 100 mM sodium phosphate buffer, pH 7.0, were incubated at 30°C. Reactions were stopped by addition of two volumes of 1 M Na₂CO₃. 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 30°C and was expressed as U.

Determination of the percentage mineralization

Evidence for the biodegradation of starch/NR foam and rubber gloves to CO₂ was obtained by determination of the CO₂ 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 CO₂ trapped as BaCO₃ 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 10⁹ 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 CO₂ 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.

$$\text{Mineralization (\% CO}_2\text{)} = \frac{\text{Required amount of HCl (mL)} \times 0.1}{\text{C content of amount of poly(1,4-isoprene) applied (mmol)} \times 2}$$

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 (10⁹ 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% OsO₄. 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 CO₂ 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 60°C for 1 hr. The solution was placed in a thimble holder and extracted by a Soxhlet extractor in toluene at 110°C. 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 60°C 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 30°C 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	<i>Streptomyces</i> 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 30°C for 3 days

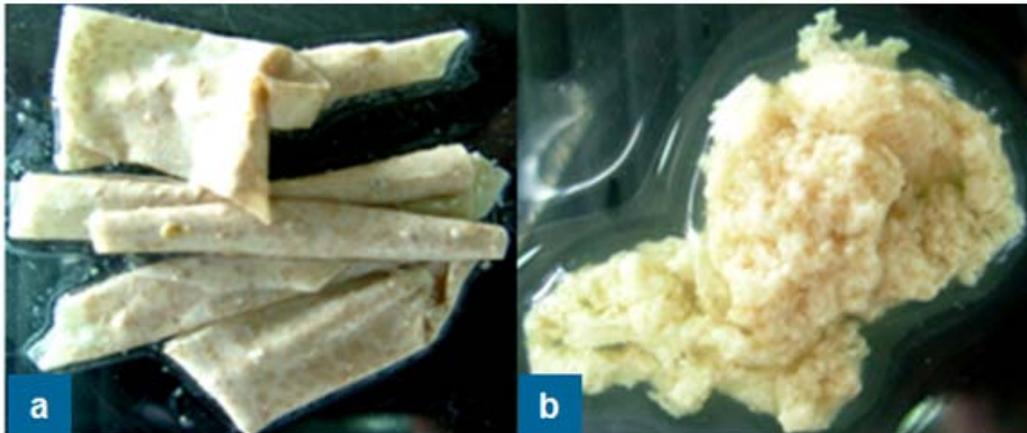


Fig. 1 Colonies of bacterial isolate CH13 on rubber gloves (a) and polymer blend (b) after incubated with *S. coelicolor* CH13 at 30°C for 4 weeks.

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.

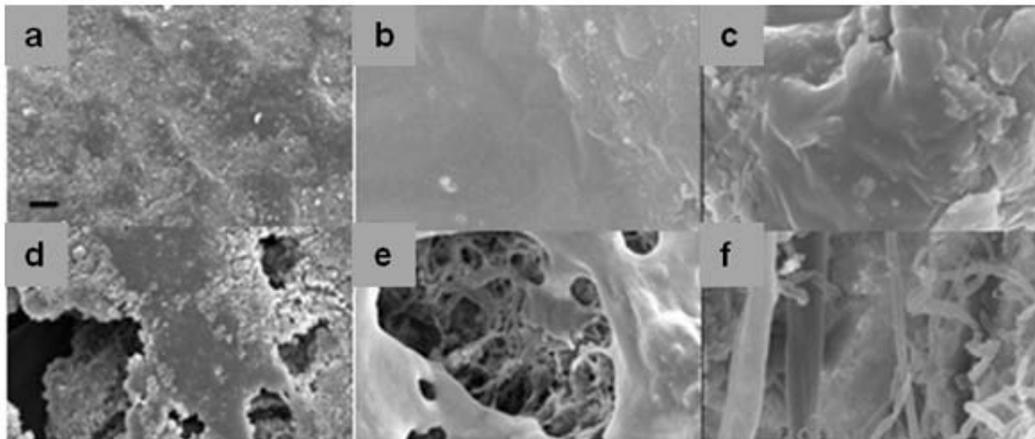


Fig. 2 Scanning electron micrographs showing erosion of the surface of polymer blended strips and rubber gloves strips. (a) Non-inoculated rubber glove; (b, c) Non-inoculated polymer blend; (d) Rubber glove strips incubated with *S. coelicolor* CH13 at 30°C for 4 weeks; (e, f) polymer blend incubated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

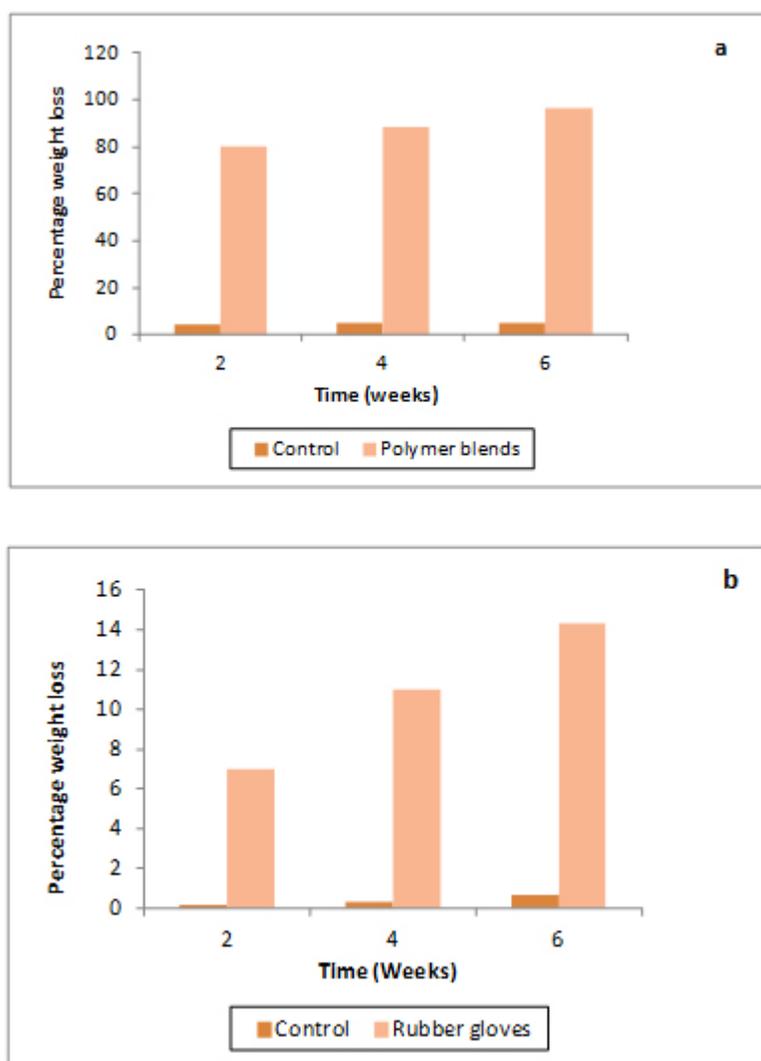


Fig. 3 The percentage weight loss of polymer blends (a) and rubber gloves (b) after incubation with *S. coelicolor* CH13 at concentration of 10^8 spores/mL at 30°C for 6 weeks.

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 $540.8 \mu\text{g mL}^{-1}$ 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 is not fully known. It is assumed that degradation of the polymer backbone is initiated by oxidative cleavage of the double bonds in the polymer chain (Braz et al. 2004). There has been intensive ongoing molecular work on the process (Braz et al. 2004; Braz et al. 2005; Ebaid et al. 2006; Bröker et al. 2008).

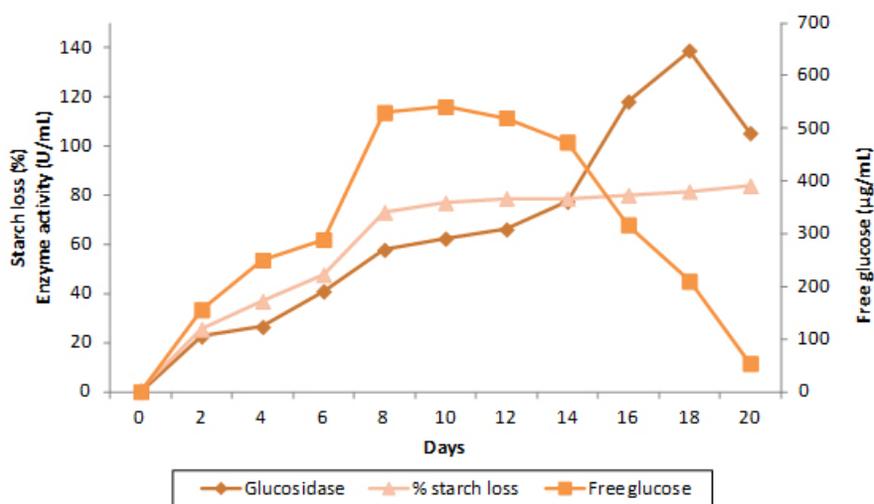


Fig. 4 Percentage starch loss, enzyme activity and amount of glucose present in culture supernatant after incubating polymer blends with *S. coelicolor* CH13 over 20 days period.

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 CO₂ whereas only 10% of the carbon of the rubber gloves was converted to CO₂. 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 2×10^8 at the start to 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 8×10^8 cells/mL. It was of interest that the population with the polymer blend also fell to 8×10^8 cells/mL during day 15th to 18th when the reduction of the free glucose was occurring at its maximum rate from 500 to 53 $\mu\text{g/mL}$ (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).

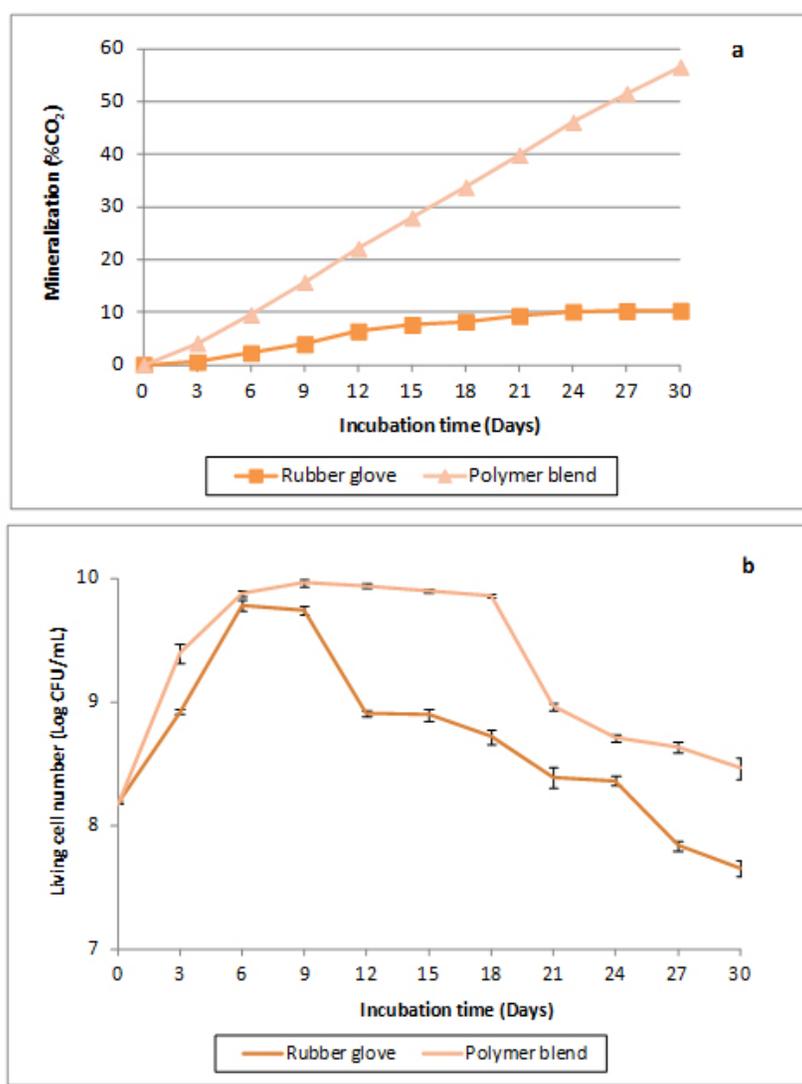


Fig. 5 Percentage mineralization during growth of bacteria on polymer blend and rubber gloves expressed as % CO₂ released from total carbon (a); Viable counts of suspended cells during cultivation (b).

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 inoculum 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/mL had less opportunity to grow and therefore degrade the polymer.

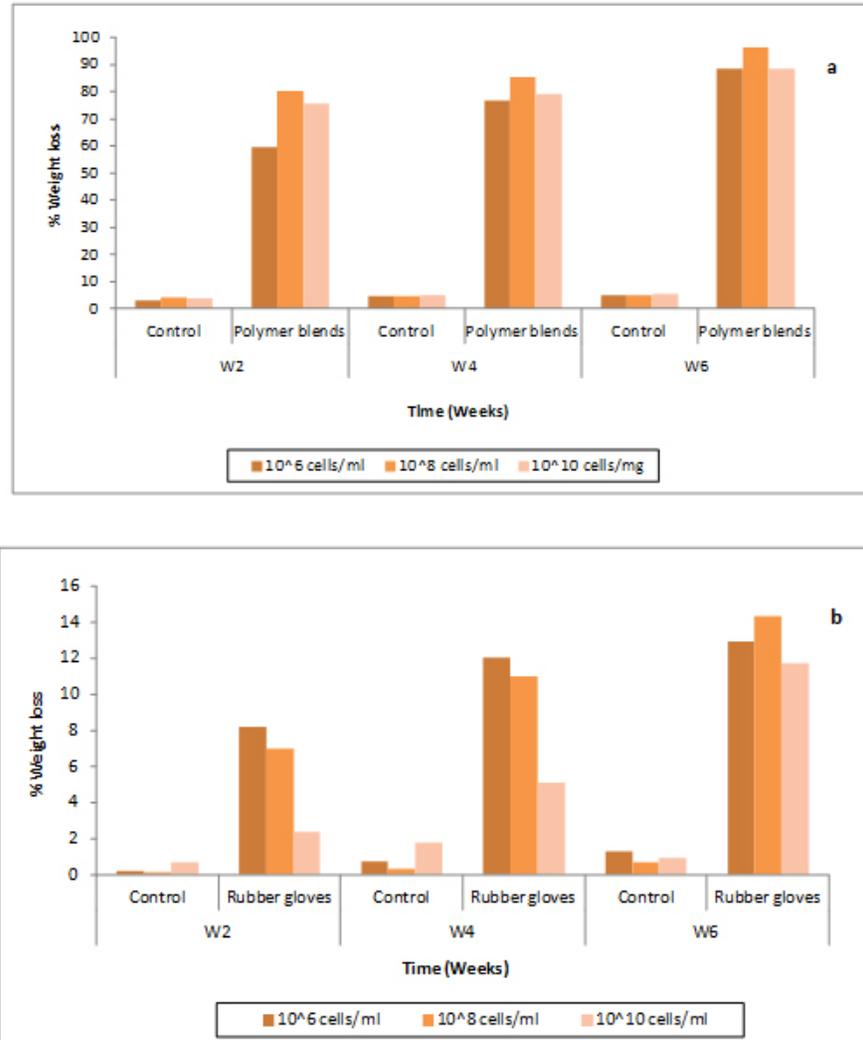


Fig. 6 The percentage weight loss of polymer blends (a) and rubber gloves (b) after incubation with *S. coelicolor* CH13 at different inoculum concentrations of 10^6 , 10^8 , 10^{10} cells/mL at 30°C for 6 weeks.

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 10^5 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 30°C (87.3%), whereas at 35°C the weight loss was 70.9% and at 40°C it was only 65.2%. 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%). An increase to 40°C produced a significant decrease of activity.

From the above observations it seems that the optimum condition for *Streptomyces coelicolor* CH13 to biodegrade the polymer blend and rubber glove in an MSM medium, is an inoculum size of 10^8 spores/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% 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 its 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.

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