International Biodeterioration & Biodegradation 63 (2009) 24–29

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Contribution to deterioration of polymeric materials by a slow-growing bacterium *Nocardia corynebacterioides*

Li Pan [a], Ji-Guang Gu [b,*], Bo Yin [c], Shu-Pei Cheng [d]

[a] Laboratory of Environmental Microbiology and Toxicology, School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, PR China
[b] Department of Ecology, Institute of Hydrobiology, Jinan University, No. 601 Huangpu Road, Guangzhou 10015, PR China
[c] Key Laboratory of Tropical Marine Environment Dynamics (LED), South China Sea Institute of Oceanography, Chinese Academy of Sciences, 164 Xingang Road West, Guangzhou 510301, PR China
[d] State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing 210093, PR China

ARTICLE INFO

Article history:
Received 12 May 2008
Received in revised form 19 June 2008
Accepted 20 June 2008
Available online 30 July 2008

Keywords:
Biodegradation
Biofilm
Bideterioration
Polymeric materials
Rubber product
Scanning electron microscopy
Pseudomonas aeruginosa
Alcaligenes xylosoxidans

ABSTRACT

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. These microorganisms showed different growth patterns in mineral salt media (MSM) supplemented with rubber strips or with the rubber extract. *A. xylosoxidans* T2, *P. aeruginosa* GP10 and *N. corynebacterioides* S3 reduced the weight of the rubber product by approximately 2.0, 4.0 and 5.3%, respectively, after 70 days of incubation with the rubber product in MSM. On average, 0.45 mg (water-soluble carbon) g^{-1} of the rubber product was released into solution phase after 7 days of incubation. Growth of *N. corynebacterioides* S3 was initially slower but exceeded the other two bacteria upon colonization of the polymer products. *N. corynebacterioides* S3 formed a dense biofilm on surfaces of the rubber product as observed under scanning electron microscopy. This study suggests that indigenous microorganisms in a range of environments are capable of degrading polymeric products through utilization of chemicals released from the polymer matrix, but the slow-growing microorganisms on material surfaces have a much more pronounced role in the materials deterioration.

1. Introduction

Polymeric products are widely used in our daily life. These rubber products mainly consist of *cis*-1,4-polyisoprene, other formulation ingredients such as sulfur, antioxidant and plasticizers to improve processing and manufacturing. These products contain a collection of many chemical components and their degradation is more complicated than the pure rubber or other polymeric materials. Degradation of them has been well documented (Heisey and Papadatos, 1995; Jendrossek et al., 1997; Linos and Berekaa, 2000; Berekaa and Linos, 2000; Arenskötter and Baumeister, 2001). Interestingly, almost all rubber degraders are Gram-positive bacteria and members of the actinomycetes (Linos and Steinbüchel, 1998; Linos et al., 1999, 2000; Berekaa and Berekaa, 2000; Leeflang, 1963; Tsuchii et al., 1985; Tsuchii and Takeda, 2001; Rook, 1955) except for one *Xanthomonas* strain (Tsuchii and Takeda, 1990), the only Gram-negative natural rubber degrader reported so far.

Because polymeric materials containing various polymerization by-products and also additives, the latter are not bound covalently to the matrix and may migrate to the adjacent environments. One example is the plasticizers that are suspected to be endocrine-disrupting and affecting the normal development in animals and humans. Degradation of plasticizers (Wang et al., 2003a,b, 2004; Li and Gu, 2006, 2007; Li et al., 2005; Xu et al., 2005, 2007a,b), rubber vulcanization accelerator, antioxidant is also the major focus of technological development in used rubber treatment (Bredberg et al., 2002; Christiansson et al., 2000; Wever and Verachtert, 1997) and ecological industrial management (Bauer and Herrmann, 1997). However, biodegradation of polymeric products in the environment is a result of physiochemical and biochemical interactions between responsible microorganisms and chemicals in the rubber products (Gu, 2003, 2007). Investigations on the biodegradation of polymeric materials by microorganisms isolated from different environments may provide insightful information in elucidating the extent of degradation by the diverse microbial population. In this study, microorganisms were isolated from different environments and their growth on polymer product and the product extract was further examined.

* Corresponding author. Tel.: +86 20 8838 1056; fax: +86 20 8522 0239.
E-mail address: gujiguang@163.com (J.-G. Gu).

0964-8305/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibiod.2008.06.003

2. Materials and methods

2.1. Polymeric product and culture medium

A new polymer bath mat manufactured in Malaysia for bathroom use was purchased from IKAE in Hong Kong and a used one of the same product was also obtained locally. Both of the mats were cut into strips with 5 × 10 mm of dimension and 3 mm thickness. The specific formulations for the two rubber mats were unknown due to the commercial confidentiality of the technical information.

A mineral salt medium (MSM) used in this study consisted of the following chemicals (mg l^{-1}): $(NH_4)_2SO_4$ 1000, KH_2PO_4 800, K_2HPO_4 200, $MgSO_4 \cdot 7H_2O$ 500, $FeSO_4$ 10, $CaCl_2$ 50, $NiSO_4$ 32, $Na_2BO_7 \cdot H_2O$ 7.2, $(NH_4)_6Mo_7O_{24} \cdot H_2O$ 14.4, $ZnCl_2$ 23, $CoCl_2 \cdot H_2O$ 21, $CuCl_2 \cdot H_2O$ 10 and $MnCl_2 \cdot 4H_2O$ 30 and the initial pH of the culture medium was adjusted to 7.0 with HCl or NaOH.

2.2. Enrichment, isolation and identification

Three enrichment cultures were established initially through the following procedures: 1.0 ml tap water or 1.0 g (wet weight) fresh sediment of Mai Po Nature Reserve in Hong Kong was transferred into two 250 ml Erlenmeyer flasks containing 100 ml of the sterile MSM described above and 3.0 g new rubber strips (autoclaved at 121 °C, 2 atm for 15 min). Similarly, 3.0 g used mat strips were added into a 250 ml Erlenmeyer flask containing 100 ml of sterile MSM directly. All Erlenmeyer flasks were incubated in an INNOVA 4340 Incubator Shaker (New Brunswick Scientific, NJ) at 150 rev min^{-1} and 30 °C in the dark. The subsequently enrichment transfer cultures were established in the following manner. One ml of the initial culture was transferred into 100 ml of MSM containing 3.0 g new mat strips (sterilized by autoclaving) after sufficient microbial growth monitored as OD_{600} spectrophotometrically. One hundred μl of the second enrichment culture after sufficient time of incubation were plated on Nutrient Agar (Difco Lab., Detroit, MI) plates for isolation of individual bacteria. After 24 h of incubation at 30 °C in the dark, single colonies with different colony morphologies were picked and streaked on newly made Nutrient Agar plates for purification under identical incubation conditions. The obtained pure cultures of bacterial isolates were subjected to Gram staining, biochemical tests using API 20NE Multitest System (bioMerieux, Marcy 1'Etoile, France) for Gram-negative bacteria and 16S rRNA gene analysis (described below).

2.3. Growth experiments

Individual isolates were further examined for their abilities in degrading polymer mat and growing with the mat as the sole source of carbon and energy. The overnight culture (100 μl) of each bacterial isolate in Nutrient Broth were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of MSM and 1.5 g new mat strip (sterilized by autoclaving), and incubated in an INNOVA 4340 Incubator Shaker (New Brunswick Scientific, NJ) at 150 rev min^{-1} and 30 °C in the dark. Bacterial growth in term of protein concentration in the culture medium was monitored during incubation and the procedures are further described in the following section in detail. Similarly, MSM containing 1.5 g mat strips was autoclaved at 121 °C, 2 atm for 15 min before the mat strips were removed and the extract was inoculated with 100 μl of bacterial culture and protein content in the culture was determined over time of incubation.

2.4. Protein assay

A modified Bradford method (Daniels et al., 1994) was used in monitoring the development of bacterial biomass during incubation in this study. From the aliquot samples taken regularly, 0.2 ml subsamples were transferred to 1.0 ml Coomassie Brilliant Blue reagent (100 mg Coomassie Brilliant Blue G250; 50 ml 95% ethanol; 100 ml 85% H_3PO_4; in 1 l of deionized water) and mixed thoroughly as described elsewhere (Cheung and Gu, 2005). After 5 min of reaction, A_{595} of the sample was measured on Spectrophotometer (Cintra 5, GBC Scientific Instrument, Australia). Concentration of standard protein and absorbance (Bovine serum albumin, Sigma Chemicals, St. Louis, MO) showed linear relationship between 2 and 50 μg ml^{-1}.

2.5. Weight loss after incubation with bacteria

In this experiment, each 250 ml Erlenmeyer flask containing 100 ml of MSM and 1.5 g new strips (sterilized by autoclaving) was inoculated with 100 μl of bacterial isolate culture grown in Nutrient Broth (Difco Lab., Detroit, MI). The flasks were incubated in an INNOVA 4340 Incubator Shaker (New Brunswick Scientific, NJ) at 150 rev min^{-1} and 30 °C in the dark. After 70 days of incubation NR strips were taken out, washed with ultra pure water and dried in an oven at 65 °C till constant weights were reached. Weight loss was taken as the difference between the initial and final weight over the period of 70 days. Due to the possible release of non-covalent bound chemical constituents from the products, such chemicals are also determined by organic carbon analysis as described in the followings.

2.6. Total organic carbon analysis

To assess the water-soluble total organic carbon (TOC) concentration from the mat strips in solution phase, 100 g strips were extracted in 1 l of MSM amended with 400 mg l^{-1} hexavalent chromium to effectively inhibit the growth of microorganisms from potential contamination (Gu and Cheung, 2001) and incubated under the identical conditions as the bacterial growth experiments described above. Over time of incubation, aliquot samples were taken at a regular time interval and stored frozen for further analysis. TOC concentration in 6.0 ml sample was measured on a Shimadzu TOC-5000A Total Organic Carbon Analyzer. Glucose and Na_2CO_3 were used as standards for calibration of total carbon and inorganic carbon, respectively. Concentration of standards and the corresponding peak areas showed linear relationship between 2 and 200 mg l^{-1}.

2.7. Scanning electron microscopy

Polymer strip specimens after bacterial growth experiment were taken and prepared for scanning electron microscopy (SEM) examination following the procedures of initial fixation in 2% glutaraldehyde and then 1% OsO_4. Samples were dehydrated in a series of ethanol solutions with gradual increase of concentrations, critical-point dried in liquid CO_2, and coated with gold-palladium as described elsewhere previously by Gu et al. (1998). The samples were observed under a Leica Cambridge S440 scanning electron microscope (Cambridge, UK).

2.8. Analysis of 16S rDNA gene sequence

Genomic DNA of strain S3 cultured overnight in Nutrient Broth (Difco Lab., Detroit, MI) was extracted as described by Wang et al. (1996). Primers used to amplify the full length of 16S rRNA gene were those as described elsewhere (Cello et al., 1997). PCR amplification reaction and conditions were also previously described elsewhere in more detail (Cheung and Gu, 2005). Nucleotide sequence of purified PCR product was determined with the ABI $P^{®}_{RISM}$ 3100 Genetic Analyzer (Applied Biosystems, RRC-West, MBRB 3260). DNA nucleotide sequence of strain S3 was analyzed with the data available from the GenBank. For the construction of a phylogenetic tree, sequences of the most closely related were included in the comparison. The similarity values for these sequences were transformed into phylogenetic distance values that compensate for multiple substitutions at any given site in the sequence. The phylogenetic dendrogram was constructed with the neighbor-joining method (Saitou and Nei, 1987) using the PHYLIP package. The 16S rDNA sequence of S3 was deposited in GenBank with accession number AY742898.

3. Results and discussion

3.1. Isolation and characterization

Tap water, sediment and the used mat bath mat were used as a source for isolation of bacteria capable of growth on mat for source of carbon and energy in enrichment process. Among the bacterial isolates obtained from the three sources, three isolates including *Alcaligenes xylosoxidans* strain T2 from tap water, *Pseudomonas aeruginosa* strain GP10 from used mat product and *Nocardia corynebacterioides* S3 from mangrove sediment, were most active in utilizing the mat products as sole source of carbon and energy. These three strains were then used in subsequent comparative investigation to substantiate their growth and utilization of the organic carbons from the polymeric mat. Both morphological and biochemical characteristics of these three strains are summarized in Table 1. Further 16S rRNA gene sequence analysis of a region between nucleotides 782 and 1321 confirmed strain S3 to be highly similar to *Nocardia corynebacterioides* (99%).

3.2. Growth in the culture medium with NR

A. xylosoxidans T2, *P. aeruginosa* GP10 and *N. corynebacterioides* S3 showed quite different growth patterns in MSM supplemented with mat strips during incubation (Fig. 1). *P. aeruginosa* GP10 started to grow without any apparent lag phase. Protein concentrations of *P. aeruginosa* GP10 culture medium increased and reached the maximal value of 64.0 μg ml^{-1} in 38 days of incubation. After that, bacterial growth declined gradually probably due to the insufficient supply of available organic carbon in the culture medium. Similarly, after an apparently longer lag phase of 15 days, protein concentration of *A. xylosoxidans* T2 culture began to increase and reached the maximal value of 38.4 μg ml^{-1} in the culture medium after 47 days of incubation. Protein contents of both *P. aeruginosa* GP10 and *A. xylosoxidans* T2 decreased in similar manner after reaching