

DEGRADATION OF DEPROTEINIZED NATURAL RUBBER BY *GORDONIA* SP. ISOLATED FROM ENRICHMENT CONSORTIA

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Abstract. Biodegradation is a potential way of decomposing deproteinized natural rubber (DPNR). The enrichment consortia were demonstrated from a rubber processing factory waste. Nine DPNR-degrading bacteria were isolated from those consortia. The highest DPNR film weight loss in a mineral salt medium (MSM) was 43.92 ± 2.30 % after 30 days incubation using strain 5A1. The formation of aldehyde group during rubber degradation of 5A1 was determined using Schiff staining and Fourier Transform Infrared spectroscopy (FTIR) analysis. The 16S rRNA gene sequence, of 5A1 showed the highest identity with that of *Gordonia soli* CC-AB07. This is the first report to demonstrate a strong ability to degrade DPNR by *Gordonia* sp. isolated from a rubber processing factory waste in Viet Nam

Keywords: isolation, deproteinized natural rubber, degradation, *Gordonia* sp.

Classification numbers: 3.1.1.

1. INTRODUCTION

Natural latex is produced by more than 2000 plant species, with *Hevea brasiliensis* being the most important commercially. Latex extracted from the tree is coagulated and goes through several industrial processes to become natural rubber (NR). The *cis*-1,4-polyisoprene is the main constituent (> 90 % of dry weight) of NR [1]. NR is used for the manufacture of a wide range of items such as gloves, adhesives, and tires. However, more than 250 types of proteins can be found in latex, and 30 to 60 types of those are believed to cause allergic reaction in humans [2]. Thus, removal of protein from natural rubber latex is quite important. The deproteinized natural rubber (DPNR) is used to produce medical gloves such as surgical gloves, catheters, nursing products and contraceptive devices.

Despite its importance and diverse applications, the main downside of rubber products is the high contamination potential of soil and water. Most rubber materials are not recycled; burning of rubber waste to solves the problem of their accumulation in landfills but does not improve the ecological situation. Biodegradation of rubber waste could be one possible solution for this environmental problem. A variety of microorganisms that degrade rubber have been

isolated and characterized [3-19]. They were divided into two groups according to the growth type and other characteristics. Members of the first group form clearing zones around their colonies on latex overlay agar plates. Most representatives of this group show weak growth on the rubber. Member of the second group do not produce clearing zones on latex overlay agar plate, but they show strong growth on polyisoprene. Some well-known degrading bacteria belonging to this group such as *Gordonia polyisoprenivorans* strain VH2 and *G. westfalica* strain Kb1 were isolated and characterized [4-6]. The genus *Gordonia* plays an important role in bioremediation and biodegradation of persistent compounds. Up to now, *G. polyisoprenivorans* strain VH2 serves as a model organism from rubber degradation due to its genetic accessibility and efficient degradation of rubber [6, 7]. A novel rubber degrading *Gordonia* species, *G. paraffinivoran* strain MTZ041, isolated from compost, was able to use rubber as sole carbon source [8]. However, almost rubber degrading bacteria have been using natural or synthetic rubber as substrate for rubber degradation. Only *Nocardia facinica* strain NVL3 was mentioned that it grew well in the liquid media containing any of latex, synthetic rubber and DPNR also [9].

The degradation of DPNR with low nitrogen concentration impacts of NR has not been studied in detail. In this study the DPNR degrading bacteria were isolated from enrichment consortia obtained using the waste of rubber processing factory in Viet Nam. The isolate strain was selected based on the DPNR film weight. The DPNR degradation of this strain was determined by Schiff staining and FTIR analysis.

2. MATERIALS AND METHODS

2.1. Materials

High ammonia natural rubber latex containing about 60 % of dry rubber content (DRC) was provided by Viet Nam Rubber Latex Co., Ltd. (Viet Nam). Sodium dodecyl sulfate (SDS; 99 %) was purchased from Chameleon Reagent (Japan). Urea (99.5 %) was obtained from Nacalai Tesque (Japan).

Latex was vigorously mixed with water (1:1 w/w). DPNR was prepared by incubation of the latex with 0.1 % urea and 1 % SDS at room temperature for 60 min followed by centrifugation at 10000 rpm for 30 min. The cream fraction was re-dispersed in 1 % SDS solution, and it was washed twice by centrifugation to prepare the DPNR latex with 30 % DRC [20]. DPNR films were formed by spreading the DPNR onto Petri dishes and dried at 50 °C until constant weight. The rubber layers were separated from Petri dishes and cut into 1×1 cm square pieces (DPNR films).

Chemicals for minimal salt medium (MSM) grade were purchased from Merck (Germany). Chemicals for analysis of analytical grade were purchased from Merck (Germany), Sigma (Germany) and Wako (Japan).

2.2. Methods

2.2.1. Enrichment consortia and screening of DPNR-degrading bacteria

To establish the enriched consortia, 2 g of soil, 2 g of sludge and 40 mL of wastewater, which collected from a rubber processing factory in Viet Nam, were mixed and settled for 30 minutes, then 10 mL of suspension was collected into 250 mL flasks containing 90 mL of MSM and about 0.3 % of the rubber films as carbon sources. MSM contains 1 g KH_2PO_4 , 8.0 g

K₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.1 g NaCl, 20.0 mg CaCl₂·2H₂O, 18.3 mg FeSO₄·7H₂O, 0.8 mg MnSO₄·5H₂O, and 5 g NH₄NO₃ per liter. The enriched samples were incubated at 30 °C and 150 rpm on a shaker. Periodically after 14 days, 10 mL of those culture broths were transferred into the new MSM with DPNR films and further incubated under the same conditions above. The culture broths were assigned as enrichment with DPNR.

The rubber degrading bacteria were isolated from the consortia. DPNR-overlay agar plates used for cultivation of rubber-degrading strains were prepared by using MSM agar (1.5 % w/v agar) for the basal layer and a mixture of 0.3 % (v/v) DPNR in MSM agar for the overlay layer. After incubation at 30 °C for 7 days, the colonies were transferred into DPNR-overlay agar plates for further study.

The individual isolated strain was incubated in 20 mL of MSM supplemented with DPNR films (0.3 %, w/v) as a sole carbon source at 30 °C, 150 rpm for 30 days. Each experiment was performed in duplicate. After incubation, DPNR films were collected and washed with 0.1 M NaOH for 30 minutes followed by distilled water to remove attached biomass. Samples were then dried at 50 °C until a constant weight [3]. The weight loss of DPNR films was measured before (W1) and after degradation (W2). Weight loss (%) was calculated as follows:

$$\text{Weight loss} = \frac{W1 - W2}{W1} \times 100$$

2.2.2. Deproteinized natural rubber degradation by selected isolate

The selected strain was obtained based on the highest DPNR film weight loss after 30 days incubation. It was grown on a DPNR-overlay plate after 7 days and stained with Schiff's reagent. The purple colour produced by the reagent shows the evidence that polyisoprene oligomers containing aldehyde group was accumulated during the microbial degradation of rubber. Staining was performed as described previously [9].

The FTIR (Jasco-4600, Japan) analysis was done to detect the degradation of DPNR film after 30 days incubation by selected strain in MSM at 30 °C and 150 rpm on the basis of changes in the functional group. The DPNR films were mixed with KBr and made into a pellet, which was fixed to the FTIR sample plate. Spectra were taken at 400 to 4000 cm⁻¹ wave numbers for each sample. The correlation of absorption bands in the spectrum of an unknown compound with the known absorption frequencies were analyzed [10].

2.2.3. 16S RNA gene analysis

The total genomic DNA of the selected strain was extracted using a QIAamp DNA mini kit (QIAGEN). The 16S rRNA gene sequences were amplified using the bacterial universal primers named 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') [21]. The PCR reaction was carried out in a final volume of 20 µl containing 10 µl PCR Master mix 2x Promega Taq DNA Polymerase, 1 µl primer 10 µM 27F, 1 µl primer 10 µM 1492R, 1 µl of the DNA template and 7 µl MiliQ. The PCR was performed according to the following program: 2 min denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C; 1 min annealing at 50 °C; 1 min extension at 72 °C; and a final extension step of 2 min at 72 °C. The 16S rDNA gene was sequenced. The sequences analysis was carried out on an ABI Prism, 3100-Avant Genetic Analyzer (Hitachi, Japan).

The gene sequences were aligned with published sequence in Genbank database using the basic local alignment search tool (BLAST) at the United State National Center of Biotechnology

Information. A phylogenetic analysis was performed with the Clustal W program using the neighbour joining method.

2.2.4. Nucleotide sequence accession number

The partial 16S rRNA nucleotide sequence of the selected strain has been deposited in the GenBank database.

3. RESULTS AND DISCUSSION

3.1. Screening of deproteinized natural rubber degrading bacteria

To enhance the growth of deproteinized natural rubber degrading bacteria, the enrichment consortia were demonstrated with five sub-transfers. The nine isolates obtained from enrichment consortia were able to grow on MSM agar containing DPNR as the sole carbon source. Only one of isolate, 1A2 formed a clearing zone around the colony and other isolates did not show the clearing zone around the colonies. However, all isolates grew well in the liquid MSM with DPNR films. The degradation of rubber by isolates was determined based on the DPNR film weight loss in Figure 1.

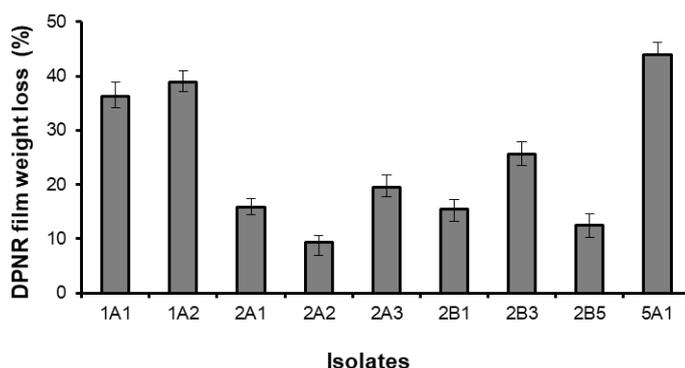


Figure 1. DPNR film weight loss after 30 days incubation with isolates.

After 30 days incubation, the weight loss of DPNR film by isolates varied from 9.31 ± 1.32 to 43.92 ± 2.30 %. The highest weight loss was obtained from the culture of strain 5A1. Nawong C. *et al.* isolated *Rhodococcus pyridinivorans* strain F5 from soil samples in Thailand, the weight loss of latex glove pieces for 30 days of incubation by F5 at 30°C was 9.36 % [10]. In another study Gallert C. showed the weight loss of latex gloves by *Streptomyces* sp. strain La7 for 32 days of incubation at 30 °C was 13.5 % [11]. Trang N. *et al.* reported that four NR degrading strains were isolated from waste in Cam Thuy of Viet Nam belonging to *Streptomyces* sp. The weight loss of NR pieces of strain NMD1, strain M4D1b, strain M4T1c, and strain T37 at 30 °C for 30 days were 4.28 ± 0.32 %, 3.11 ± 0.5 %, 2.05 ± 0.76 % and 2.43 ± 0.34 %, respectively [12]. It seems that strain 5A1 had strong ability to degrade DPNR.

3.2. Deproteinized natural rubber degrading bacteria by strain 5A1

In order to determine the rubber degradation products contain aldehyde groups, the accumulation of aldehydes on DPNR-overlay agar plate was examined by Schiff's reagent. The

growth of 5A1 for 7 days on DPNR-overlay medium and aldehyde intermediate by 5A1 on the medium were shown in Figure 2.

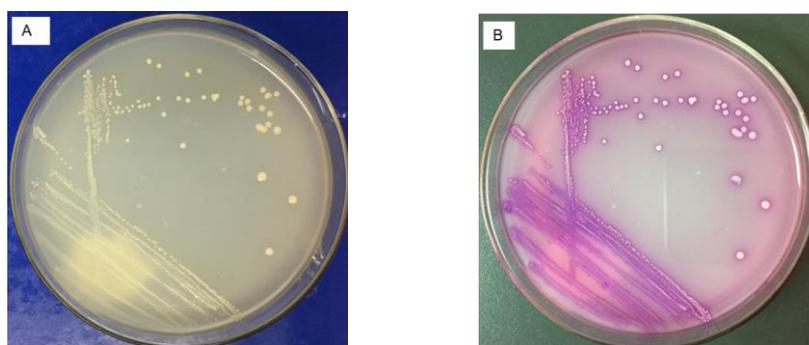


Figure 2. The growth of 5A1 for 7 days on DPNR-overlay medium (A), aldehyde intermediate by 5A1 on DPNR-overlay medium after staining with Schiff's reagent (B).

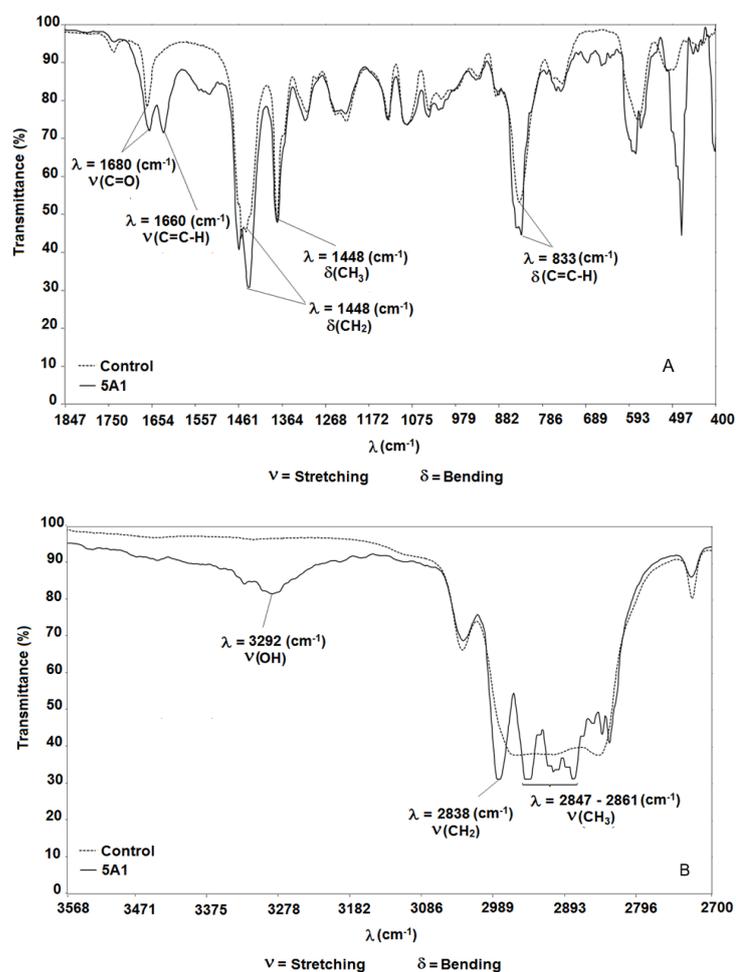


Figure 3. The FTIR spectra of DPNR film after 30 days incubation with 5A1. Spectra were taken at 400 - 1850 cm^{-1} wave numbers (A) and 2700 - 3600 cm^{-1} wave numbers (B).

A purple color remained around the colonies of 5A1 (Fig. 2B), this result suggests that 5A1 degrades rubber via intermediates with aldehyde as indicated by previous studies [6, 8, and 9].

The FTIR spectroscopy was applied to detect the changes in the functional groups of the polymer. The FTIR analysis of the DPNR films after incubation with 5A1 in MSM broth for 30 days showed various spectrum changes compared to the non-biotic control (Figure 3).

Characteristic peaks in DPNR films were found at 833 cm^{-1} representing -C=CH bending. On the other hand, the transmittance area at 1660 cm^{-1} was attributed to symmetric -C=CH stretching. The peaks at 1448 and 1375 cm^{-1} were assigned to C-H bending of CH_2 and CH_3 , respectively. In addition, the peaks appeared at 2838 and 2861 cm^{-1} corresponding to CH_2 and CH_3 stretching, respectively. The spectrum demonstrated a change in the signal area of CH_2 and CH_3 bonds in the polyisoprene chain. Moreover, the FTIR spectrum of DPNR films incubation with 5A1 showed very strong absorption peak at 1680 cm^{-1} indicating the presence of aldehyde and ketone (C=O) groups while samples of the control was not found. This result suggested that the break of functional groups like C=CH bonds to form aldehyde and ketone groups. This correlates with the result from staining with Schiff's staining on DPNR-overlay agar medium after growth of 5A1. Besides, another strong peak at 3292 cm^{-1} for OH stretching from the hydroxyl group was observed in the FTIR spectrum of the DPNR films after 30 days of incubation. Thus, the carbonyl group in the DPNR film was completely changed to the hydroxyl group due to the microbial treatment. The appearance of aldehyde, ketone, and hydroxyl groups from FTIR analysis indicated that some double bonds can be oxidized. The appearance of the peaks corresponding to aldehyde and ketone groups after incubation was also reported by Nawong C. *et al.* [10], Hiessl S. *et al.* [13], Bosco F. *et al.* [14], Vidya and Growther L. [15], Andler R. *et al.* [16].

3.3. Phylogenetic analysis using 16S RNA gene sequence

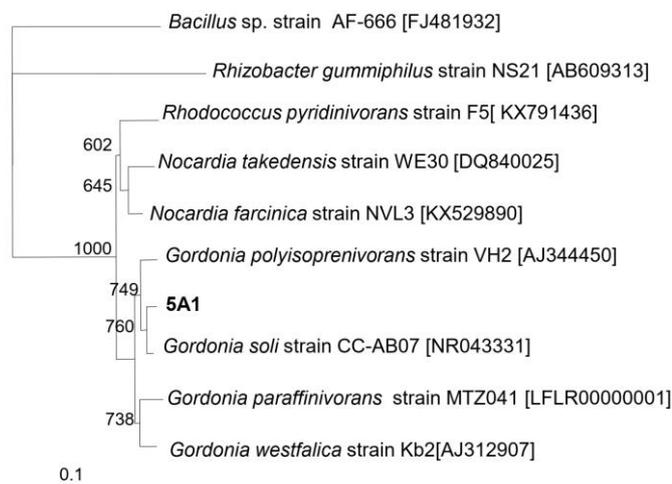


Figure 4. Phylogenetic tree of 16S rRNA gene sequences of 5A1 with the other known rubber degrading bacteria. The tree was constructed by the neighbor-joining method with bootstrap analyses for 1000 replicates and the bar shows 0.1 substitutions per nucleotide position.

The 16S rRNA gene sequence of 5A1 was determined, and shown the highest similarity to that of *Gordonia soli* CC-AB07 (99 %). The isolates was named *Gordonia sp. strain 5A1* and

submitted to GenBank under the accession number MN545427. The phylogenetic tree of 16S rRNA gene sequences of 5A1 with the other known rubber degrading bacteria was constructed by the neighbor joining method in Figure. 4.

G. polyisoprenivorans strain VH2 was first the genus *Gordonia* isolated from soil of a rubber tree plantation had been reported mainly because of its ability to degrade natural and synthetic rubber [17] and the genome of VH2 was sequenced and annotated to elucidate the degradation pathway [6]. *G. westfalica* strain Kb2 was isolated from foul water held inside a deteriorated automobile tire found on a farmer's field in Germany. Strain Kb2 was able to solubilize and mineralize natural rubber substrates and synthetic cis-1,4-polyisoprene [5]. *G. paraffinivorans* MTZ041 isolated from a compost, which was observed as the formation of biofilm-like structures on natural and synthetic rubber [8]. The 16S rRNA gene sequence of strain 5A1, consisting of 1405 nucleotides, was compared to the member of the genus *Gordonia*. It showed similarity 97 % with the 16S rDNA sequences of *G. polyisoprenivorans* strain VH2, *G. westfalica* strain Kb2, and *G. paraffinivorans* MTZ041.

4. CONCLUSIONS

In the present study, the enrichment consortia showed the potential to enhance the growth of deproteinized natural rubber degrading bacteria. Nine DPNR degrading bacteria were isolated. *Gordonia* sp. strain 5A1 demonstrated the highest rubber degrading activity among all isolates. The formation of aldehyde groups during degradation of DPNR by 5A1 was observed using Schiff staining and FTIR analysis. Further studies are necessary to elucidate the *Gordonia* sp. 5A1 with other isolates and the bacterial community in consortium. The synergistic interaction of microorganisms will create an alternative approach to perform rubber degradation.

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