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CHANGES OF BACTERIAL CONSORTIA IN ENRICHMENT OF DEPROTEINIZED NATURAL RUBBER WITH SAPA SOIL

Nguyen Hoang Dung, Nguyen Thi Thu Trang, Nguyen Thi Thanh, Pham Thi Quynh, Nguyen Tien Thanh, Nguyen Lan Huong*

School of Biotechnology and Food Technology, Hanoi University of Science and Technology, No. 1, Dai Co Viet, Hai Ba Trung, Ha Noi, Viet Nam

*Email: huong.nguyenlan@hust.edu.vn

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Abstract. The accumulation of rubber waste has become a global major environmental issue due to its adverse effects on ecology. Bioremediation is focused to minimize this problem. In this study, the degradation of deproteinized natural rubber (DPNR) by bacterial consortia enriched from Sapa soil, which has not historically related to rubber, was examined. The highest weight loss of 39.16 ± 1.95 % of DPNR film was detected in the sixth enrichment consortium after 30 days of incubation. The occurrence of hydroxyl groups in the film was observed by Fourier Transform Infrared Spectroscopy analysis. The changes in bacterial community in the consortia were determined by metagenomic analysis using 16S rRNA gene sequencing. The dominant phyla in all consortia were *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*, among them the phylum *Actinobacteria* was a key rubber-degrader in the consortia.

Keywords: bacterial consortium, degradation, deproteinized natural rubber.

Classification numbers: 3.1.1

1. INTRODUCTION

Natural rubber (NR) obtained from the latex of *Hevea brasiliensis* is a high polymer of *cis*-1,4-polyisoprene, which contains more than 90 % of *cis*-1,4-polyisoprene and less than 10 % of non-rubber constituent such as protein, phospholipids, etc. Although the protein content is only 1 - 2 % of latex [1], it is the cause of a latex allergy and may pose health risks on those who consume latex-derived products [2]. Therefore, removal of protein from NR is a requirement for deproteinized natural rubber (DPNR) products, especially for medical devices, gloves, and hypoallergic products. The rubber products must be scrapped and disposed at the end of duration of their uses. That waste represents a significant amount of solid waste as million tons of waste gloves are generated yearly, which takes a very long time for natural degradation. Serious environmental problems appear if the waste is burned or landfilled. Therefore, alternative treatment needs to be found for processing rubber waste to realize an eco-friendly method. Biodegradation of rubber waste is one way to solve this problem.

Microbial degradation of rubber has been investigated for more than 100 years [3]. Many rubber-degrading microorganisms were identified and characterized [4 - 5]. However, the studies

of rubber biodegradation have been directed to using single microorganism which still gave poor results. It was hypothesized that the degradation should be involved in a microbial community which consists of interaction among various microorganisms. Until now, only a few studies have been published on the rubber degradation capacity by microbial consortia. Nawong *et al.* reported that the degradation of NR gloves using a bacterial consortium isolated from rubber-contaminated soil in Songkhla, Thailand. It is shown that the rubber degradation capacity by consortium is higher than that of a single strain [6]. In our previous research, the biodegradation of DPNR was examined by bacterial consortia enriched from a rubber-processing factory's waste (soil, sludge, and wastewater) in Thanh Hoa. The change in bacterial composition during enrichment was analyzed, and the role of bacterial groups involved in the biodegradation was suggested. The phyla *Bacteroidetes* and *Proteobacteria* may play a role in the degradation of non-isoprene and the phylum *Actinobacteria* plays a key role in the degradation of rubber in the consortia [7]. Therefore, in this study Sapa soil, which had not been contaminated with rubber, was used for performing the enrichment experiment to compare the change and difference of bacterial community in the consortia enriched with contaminated samples. In addition, the DPNR degradation was demonstrated by enrichment consortia with Sapa soil**.** The weight loss and Fourier Transform Infrared Spectroscopy (FTIR) of the films during the enrichment were determined. The changes of bacteria community in the consortia were analyzed by partial sequencing of the 16S rRNA gene on the Illumina MiSeq platform, and the difference of bacterial community in the consortia is discussed.

2. MATERIALS AND METHODS

2.1. Materials

In this study, soil samples were collected from a mountain in Sapa (22º18'12'N, 103º46'30'E, Lao Cai, Viet Nam) with a depth of 20 to 40 cm and mixed well together, representing a composite sample of Sapa soil (S0). This soil is not contaminated with rubber.

High ammonia natural rubber latex (HANR) containing about 60 % of dry rubber content (DRC) (Vietnam Rubber Latex Co., Ltd., Viet Nam) was mixed with water (1:1 w/w) for making 30 % DRC latex, then deproteinized natural rubber latex was prepared by removing protein contained in the latex by the method described in our previous work [8]. DPNR latex sample was spread on Petri dishes (12 g/dish) and dried at 50 ℃? until a constant weight was achieved. The DPNR layers were separated from Petri dishes and cut into 1 × 1 cm square pieces (DPNR films).

The chemicals used in the experiments were purchased from Merck (Germany), Sigma (Germany), and Wako (Japan).

2.2. Methods

2.2.1. Enrichment consortia

The enrichment samples were prepared by vigorously mixing 5 g of soil with 70 mL of water and the mixture was then settled for 30 minutes, followed by collecting a volume of 10 mL from the suspension and putting into a 250 mL flask which contained 90 mL of Minimal salt medium (MSM) and 10 DPNR films (about 0.3 wt %) as a sole carbon and energy source. The composition of MSM medium was mentioned in Huong *et al.* [8]. After 30 days of incubation at

30 ºC and 150 rpm, 10 mL of each enrichment culture was transferred into new 250 mL flasks under the same conditions as mentioned above. The procedure was performed nine times and the bacterial consortia were assigned as S1 to S9 corresponding with each transferring, respectively. The DPNR films after nine incubations with consortia were assigned as DPNR-S1 to DPNR-S9, respectively. The DPNR film in uninoculated MSM was used as an abiotic control sample and designated as DPNR.

2.2.2. Degradation of DPNR films by the bacterial consortia

After 30 days of incubation, DPNR films were collected from each enrichment flask, then washed with 0.1 M NaOH for 30 minutes and dried at 50 ℃? until the weight remained constant. According to the method reported by Tsuchii *et al*. [9], the weight loss of DPNR films was calculated based on the difference in dry weight of the films before (W1, mg) and after incubation (W2, mg), as follows:

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

In order to detect the degradation of DPNR films based on the changes in the functional group, infrared spectra were recorded by Fourier transform infrared spectroscopy (FTIR) (Jasco-4600, Japan), as mentioned in a previous work of Nawong *et al*. [6].

2.2.3. Characterization of community structure in the consortia

The enrichment culture after 30 days of incubation with DPNR films was homogenized in a sonification bath for 10 min, then the DPNR films were removed. Biomass was collected from each enrichment consortium by centrifugation at 10000 rpm for 10 min. Fast DNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, USA) was used to extract genomic DNAs. In order to amplify the V4 region of the 16S rRNA gene, the Premix Ex Taq Hot Start solution (Takara Bio, Otsu, Japan) was used with a universal forward and reverse primers of Univ515F (5'-GTGCCAGCMGCCGCGGTAA-3′) and Univ806R (5'-GGACTACHVGGGTWTCTAAT-3′), respectively. The PCR conditions were as follows: a hot start at 94 °C for 3 min, 35 repeated cycles at 94 °C for 45 s, 50 °C for 1min and 72 °C for 1.5 min, and a final extension step at 72 °C for 10 min. The PCR products were purified from the MinElute PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications and quantified by a Qubit 3.0 fluorometer (Life Technologies, California, USA). The sequencing of the purified PCR products was performed on a Miseq Reagent Kit v2 and the Miseq system (Illumina, California, USA). The sequencing output data were analyzed using a QIIME software v.1.9.1 and classified into operational taxonomic units (OTUs) with a similarity level of more than 97 % based on the Greengenes Database v.13_8 [10].

The raw data obtained after sequencing were used to analyze alpha diversity using a QIIME pipeline (v.1.9.1) and the results are given in Table 1. The alpha diversity metrics were calculated for each sample using its exact sequencing depths (reads), Good's coverage, observed OTU, abundance-based coverage estimator (ACE), Chao1, Simpson, Shannon and phylogenetic diversity (PD) whole tree indicating the phylogenetic diversity [11 - 12].

2.2.4. Statistical analysis

Principal Component Analysis (PCA) were performed in the XLSTAT program (Addinsoft, XLSTAT Version 2020.3.1.1000), aiming at finding the relationship between the relative abundances of dominant phyla and the weight loss of DPNR films in each consortium.

3. RESULTS AND DISCUSSION

3.1. Degradation of DPNR films by the bacterial consortia

Sapa soil samples were not historically associated with natural rubber and DPNR, therefore, to enrich the growth of rubber degrading bacteria in soil, the enrichment consortia were demonstrated by incubating soil with DPNR films for 30 days and continuously transferred nine times. The degradation of DPNR films by the consortia was shown in Figure. 1.

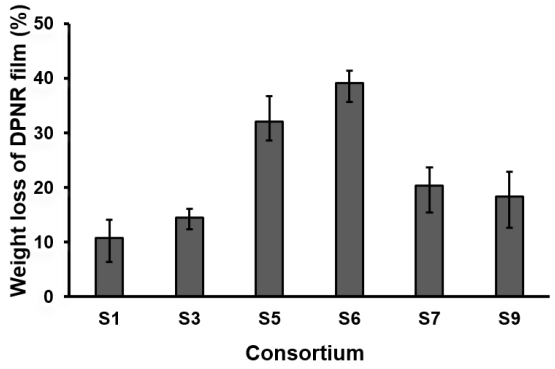

Figure 1. DPNR film weight loss after 30 days of incubation by the consortia

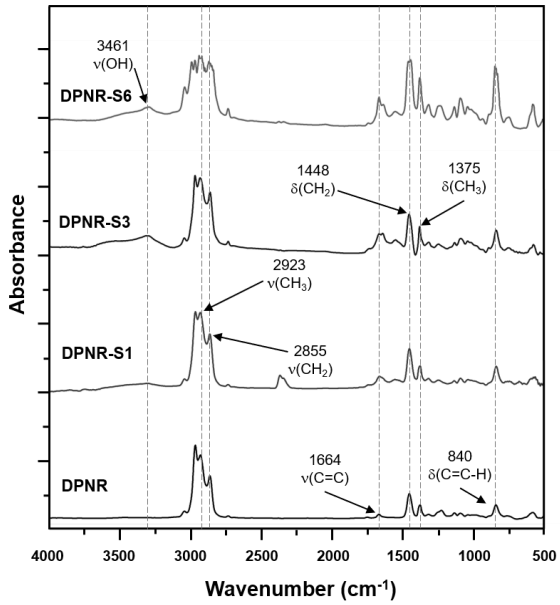

Figure 2. FTIR analysis of DPNR films after 30 days of incubation by the consortia. Abiotic control sample as DPNR. The DPNR films after incubation with the first, third and sixth consortia as DPNR-S1, DPNR-S3 and DPNR-S6, respectively.

The weight loss of DPNR film in each consortium after 30 days of incubation reached 10.77 - 39.16 %. The highest weight loss was obtained in the sixth enrichment consortium (39.16 ± 1.95 %). These results confirmed the degradation of DPNR by consortia enriched from Sapa soil. However, the weight loss was significantly decreased in S7 and S9, which suggests that changes in the bacterial community structure during enrichment could affect the DPNR degradation. Nawong *et al*. also reported that the weight loss of natural rubber glove was obtained at 18.38 - 59 % by a bacterial consortium enriched from contaminated soil after 30 days of incubation [6]. In our previous work, a DPNR film weight loss of 48.37 % was obtained in the fourth enrichment consortium after 14 days of incubation. Since that consortium was enriched from a rubber-processing factory's waste [7], it is clear that the ability of DPNR degradation by consortia enriched with contaminated rubber waste was higher than that enriched from Sapa soil.

Besides the weight loss of DPNR film, the changes in the functional groups of the polymer were analyzed by FTIR. The FTIR spectra of DPNR films after 30 days incubated with the consortia and the control sample were shown in Figure 2.

The intensity of the typical peaks of −C=C-H (at 840 cm^{-1}), -CH_3 (at 1375 cm^{-1}), -CH_2 (at 1448 cm^{-1}), -C=C (at 1664 cm^{-1}), and -OH (at 3461 cm^{-1}) groups increased in these films incubated with the consortia (such as DPNR-S1, DPNR-S3 and DPNR-S6) compared to the abiotic control (DPNR). At these peaks, the absorptions at 840 cm^{-1} corresponding to −C=CH out-of-plane bending, which can only be attributed to the *cis*-1,4 isoprene monomer, increased for the DPNR films incubated with consortia and significantly increased for DPNR-S6. Simultaneously, the signals at the 1448 and 1375 peaks that characterize the methyl and methylene groups increased and sharply increased at DPNR-S6. These changes were also observed in a study of Roy *et al*. [13], who described these as a sign of rubber degradation [13]. Bosco *et al*. reported that the oxidation of natural rubber could be confirmed by the appearance of signals at about 3300 - 3400 cm^{-1}? due to the formation of hydroxyl groups after four months incubated with *Rhodotorula mucilaginosa* [14].

3.2. Characterization of community structure in consortia

Table 1. The the alpha-diversity indices in enrichment consortia and Sapa soil.

Consortia	Reads	Good's coverage	OTU	ACE	Chao1	Shannon	Simpson	PD whole_tree
S0	29399	0.99 (0.99 ± 0.0003)	414 (336 ± 5.0)	1690.9 (1501.4 ± 128.43)	1347.1 (1192.3 ± 131.56)	1.10 (1.10 ± 0.01)	0.26 (0.26 ± 0.002)	38.4 (33.1 ± 0.81)
S1	35524	0.98 (0.95 ± 0.0004)	848 (612 ± 11.0)	3723.0 (2738.8 ± 214.92)	3154.6 (2665.8 ± 399.85)	4.55 (4.54 ± 0.01)	0.88 (0.88 ± 0.001)	57.2 (45.7 ± 0.81)
S3	28723	0.99 (0.99 ± 0.0000)	347 (347 ± 1.0)	1220.1 (1218.4 ± 8.08)	1141.5 (1141.6 ± 12.21)	4.34 (4.34 ± 0.00)	0.92 (0.92 ± 0.000)	25.0 (25 ± 0.02)
S5	45565	0.99 (0.99 ± 0.0004)	420 (326 ± 9.0)	1501.1 (1205.3 ± 87.78)	1501.9 (1134.8 ± 126.7)	3.59 (3.58 ± 0.01)	0.85 (0.85 ± 0.001)	26.6 (22.3 ± 0.49)
S6	39815	0.99 (0.98 ± 0.0005)	618 (464 ± 9.0)	2228.5 (1772.7 ± 138.64)	2020.4 (1699.8 ± 187.54)	4.41 (4.41 ± 0.01)	0.92 (0.92 ± 0.000)	35.4 (29.1 ± 0.64)
S9	52789	0.99 (0.99 ± 0.0005)	543 (337 ± 11.0)	1887.4 (1310.8 ± 143.90)	1773.3 (1180.3 ± 117.06)	4.04 (4.03 ± 0.01)	0.89 (0.89 ± 0.001)	30.7 (22.4 ± 0.40)

Total DNAs extraction and sequencing of 16S rRNA gene fragments of enrichment consortia were conducted by the NGS method on an Illumina analysis system to evaluate the diversity of the bacterial community. After filtering low-quality sequences, the number of reads obtained in each sample varied from 28723 to 52789, the alpha diversity indices were calculated for both the original number of reads and the cutoff of 22000 reads. The Good's coverage

indexes for both the original reads and the cutoff reads were achieved at over 98 % in all samples, except at 95 % for a cutoff read in S1 (Table 1). The alpha diversity metrics of bacteria were highest in S1, lowest in S0, and higher than those from the other consortia. Among the enrichment consortia, the bacteria community in S6 was less diverse than that in S1, while the weight loss of film in S6 was the highest. Nawong *et al*. showed that the rubber degradability of mixed culture of top five strongest isolates was higher than that of all ten isolated rubber-degrading strains [6]. Nguyen *et al.* reported that the consortia with lower values of diversity indices degrade rubber more effectively [7]. It was suggested that the presence of more diverse microbes could increase either substrate competition or by-product inhibition to rubber-degrading bacteria [15].

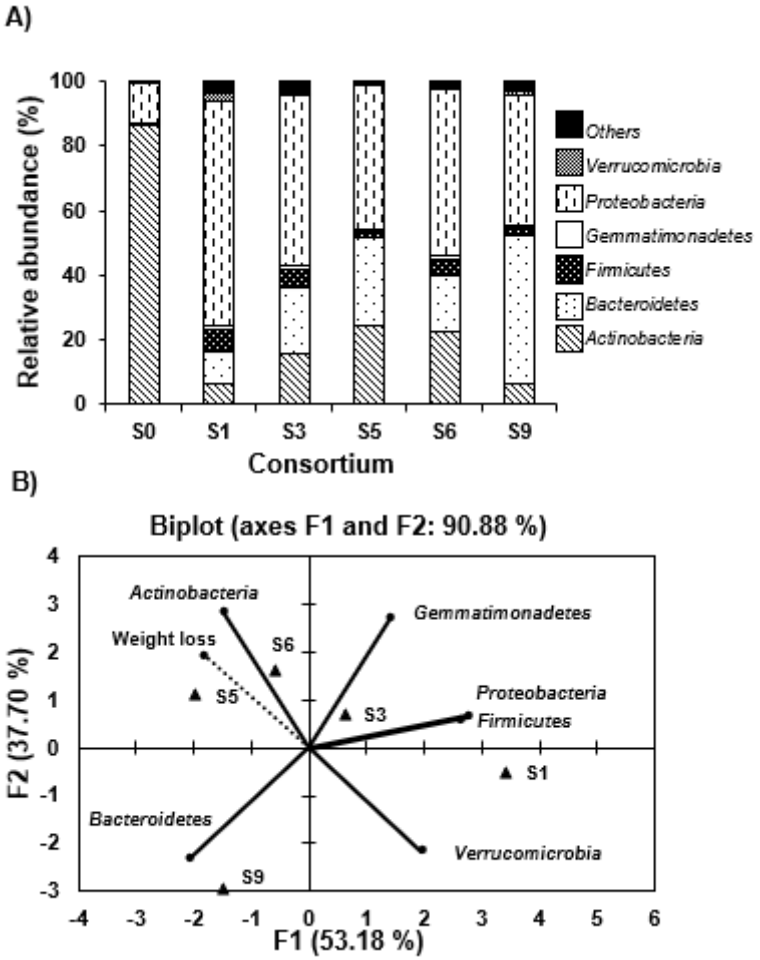

Figure 3. Dominant phyla in the enriched consortia. Relative abundances of dominant phyla in Sapa soil and the consortia (A). Principal component analysis biplot based on the relationship between the relative abundance of dominant phyla and the weight loss of films in each consortium (B).

After 30 days of incubation with DPNR, the changes in bacterial community in the consortia were shown in Fig. 3A, the dominant phyla were *Actinobacteria* (6.18 - 86.57 %), *Bacteroidetes* (0.06 - 46.23 %), and *Proteobacteria* (12.55 - 69.42 %). However, the relative abundances of the dominant phyla in the consortia were more diverse than that in Sapa soil sample (S0). It could be explained that S0 is the mountain soil with a low organic carbon content

(3 - 4 % by dry weight; data not shown) and has not been historically associated with rubber. The abundances of *Proteobacteria* and *Bacteroidetes* showed a drastically increase from 12.55 % in S0 to 69.42 % in S1 and 0.06 % in S0 to 46.23 % in S9, respectively. In addition, other phyla appeared during the enrichment process, such as *Fimicutes*, *Gemmatimonadetes* and *Verrucomicrobia*, while the abundance of *Actinobacteria* drastically decreased from 86.57 % in S0 to 6.18 % in S9. The dominant of *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* was also detected in the consortia enriched by a rubber-processing factory's waste in Thanh Hoa [7].

The abundances of phyla in each consortium were used as variables, which were shown together with the weight loss of the films during the enrichment process in Fig. 3B. The biplot was performed at about 90.88 % of the total variability of data. The weight loss of DPNR films and the abundance of *Actinobacteria* in S5 and S6 consortia were positively correlated, while the abundances of other phyla were not or negatively correlated. Therefore, the PCA analysis had demonstrated that *Actinobacteria* was a key rubber-degrader in the consortia. This also corresponds to the results in previous reports [7]. Besides that, the relative abundances of *Actinobacteria* in S5 and S6 were higher than that in other consortia, which may explain why the weight loss of films in those were higher than that in others (Fig. 1). The *Bacteroidetes* and *Proteobacteria* are the most abundant phyla in the soils [14] and in the consortia enriched with NR and DPNR [7]. They may play a role in the degradation of other compounds than polyisoprene such as protein or lipid during enrichment process [7].

The changes of dominant genera of the phylum *Actinobacteria* in Sapa soil and the consortia were shown in Fig.4.

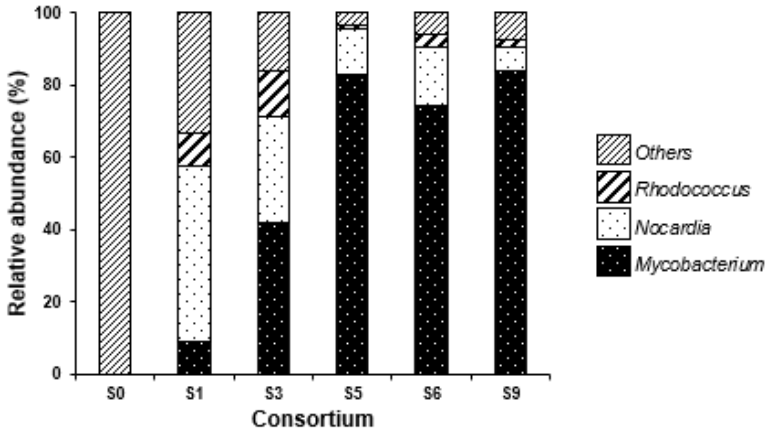

Figure 4. Relative abundance of dominant genera in phylum *Actinobacteria* in Sapa soil and the consortia.

Although the abundance of phylum *Actinobacteria* in S0 was clearly observed (Fig 3A), only the dominant family *Micrococcaceae* was detected, and it absolutely disappeared during enrichment process. The composition of the phylum *Actinobacteria* at the genus level in the consortia was shown in Fig. 4. In the first enrichment (S1), the relative abundance of *Nocardia* was 48.75 %, while that of *Mycobacterium* and *Rhodococcus* was 9.10 % and 9.19 %, respectively. However, the abundance of *Mycobacterium* increased significantly with subsequent enrichments and presented the most dominant genus in S5, S6, and S9, whereas the abundances of *Nocardia* and *Rhodococcus* decreased dramatically compared to those in S1. It is suggested that *Mycobacterium* was a key rubber-degrading bacterium in the consortia. Nguyen *et al.* also reported that *Mycobacterium* and *Gordonia* were found to be the most dominant and key players

in the enrichment of consortia with a rubber-processing factory's waste [7]. However, *Gordonia* was not detected in the consortia enriched with Sapa soil, which could affect the weight loss of DPNR compared to that in previous research.

Besides the abundance of *Mycobacterium* in the consortia, the proportion of each dominant genus in the community was different. The relative abundance of *Mycobacterium* was 74.40 % in S6, lower than that in S5 (83.21 %) and S9 (84.04 %). However, the relative abundances of *Norcardia* and *Rhodoccoccus* were 16.40 % and 3.58 %, respectively, in S6 and higher than those in S5 (12.66 % and 6.40 %) and S9 (0.94 % and 2.32 %). This supports our finding that the degradation of DPNR in S6 was higher than that in S5 and S9. In most of published studies related to rubber degradation, the most potential rubber degrading bacteria belonged to *Actinobacteria* phylum such as *G. polyisoprenivorans* VH2 [16], *Gordonia* sp. 5A1 [8], *M. fortuitum* NF4 [17], *N. farcinica* NVL3 [18], *R. rhodochrous* RPK1 [19], and *R. pyridinivorans* F5 [6] were reported, however the bacterial community structure in the consortia is needed in further investigations.

4. CONCLUSIONS

The consortia enriched by Sapa soil were able to degrade DPNR films. Based on the results of weight loss and FTIR analysis, the highest DPNR biodegradation by the enrichment of consortium was obtained at the sixth enrichment. The dominant phyla in all consortia were *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*. The genus *Mycobacterium* in the phylum *Actinobacteria* was the dominant and had more influence on the DPNR degradation in the consortium. Further studies are necessary to elucidate roles of each bacterial group and their ratio to form stable consortia with highly effective degradation of rubber.

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Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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