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Biodesulfurization of vulcanized rubber by enzymes induced from *Gordonia amicalisa*

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ABSTRACT

In this work, *Gordonia amicalisa* was used to treat two kinds of vulcanized synthetic rubbers, vulcanized isoprene rubber (v-IR) and vulcanized styrene butadiene rubber (v-SBR). The effects of microbial treatment on vulcanized rubbers were evaluated by changes of crosslinking density, sol fraction, morphology, element content and chemical group of treated rubber samples. The results showed that *G. amicalisa* can break C=C bond mainly on v-IR and S–S bonds mainly on v-SBR. After microbial treatment of 20 days, the crosslink densities of v-IR and v-SBR decreased by 13.7% and 22.1%, respectively. The carbon content on v-IR surface was decreased by 9.1%. FTIR analysis showed that C=C bonds transferred into C=O bonds, indicating the main chain scission. Regarding to the v-SBR, sulfur content on its surface was decreased by 22.9%, S–S bonds were broken and S=O producing, indicating the sulfur crosslink scission. Furthermore, the inducing mechanism of desulfurizing enzyme produce from *G. amicalisa* was investigated. v-SBR and v-IR were used as enzyme inducers, and dibenzothiophene was used as substrate reacting with desulfurizing-enzyme. The liquid chromatography and ultraviolet spectrum analysis showed that v-SBR was the specific substrates that can induce *G. amicalisa* to secret desulfurizing enzyme, making desulfurizing reaction happen.

1. Introduction

Vulcanized rubber products easily accumulate in the ecological environment because of its relatively stable three dimensional networks, resulting in a long-term environmental problem. For decades, many researchers used microwave, ultrasonic and chemical agents to recycle rubbers [1]. However, these methods could induce further environmental pollution and spend much energy. In recent years, microbial methods have attracted much interest because it has specific advantages compared to chemical and physical ones. It normally does not produce any harmful or toxic chemicals and often is not energy intensive [2–4].

Recently, many bacteria [5–7] has been isolated and used to break rubber crosslinked network. These microorganisms can be divided into two groups which follow different strategies to decrosslink rubber: rubber-degrading microorganisms [8] use C=C bonds on rubber surface as carbon source to gain energy, and rubber-desulfurizing microorganisms [2] gain energy by breaking sulfur linkage (S–C and S–S) bonds.

For decades, many studies [8,9] have been carried out on the biodegradation of rubber to investigate degradation products, reaction mechanism, and involved enzymes. The mechanism of rubber biodegradation was first proposed by Tsuchi and coworkers [10,11]: C=C bonds on natural rubber surface was oxidatively broken into oligomers with keto and aldehyde groups by *Xanthomonas* sp. strain 35Y. Steinbüchel and coworkers [12] confirmed that *Gordonia polyisoprenivorans* can degrade natural latex following the same mechanism. Two enzymes involving in the first step of breaking C=C bonds were identified: rubber oxygenase A (RoxA) [13]and latex-clearing protein (Lcp) [9]. The biochemical data and structure-function relationship of RoxA have been systematically investigated by Jendrossek and coworkers [14–16]. Now the research interest of rubber-degrading enzyme has transferred to Lcp [17,18].

Compared with the research on rubber-degrading microorganisms, the research on rubber-desulfurizing ones only have a fewer papers reported in last decades. Romine and coworkers [19] used *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, and *Sulfolobus acidocaldarius* to desulfurize waste rubber and investigated the

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mechanism of biodesulfurization by using dibenzothiophene. They reported that sulfur linkages on rubber surface were metabolized into sulfoxide/sulfone/sulfonate/sulfate, following a “4S” metabolic pathway. *Ceriporiopsis subvermispora* [20] was used to decrosslink natural rubber. Results showed that S—C bonds on rubber surface decreased while S—O bonds increased during cultivation period. Li et al. [21] reported that *Sphingomonas* sp. metabolized sulfide crosslinks on rubber surface through “4S” pathway. Yao et al. [7] proved that *Alicyclobacillus* sp. metabolized sulfur crosslinks on natural latex following the same pathway.

Until now, many studies of rubber biodesulfurization focus on finding high efficiency desulfurizing-bacteria and optimizing technical conditions of bioprocess. Desulfurizing-enzyme is a key to improving desulfurizing effect on rubber. Therefore, it is necessary to investigate the properties and producing mechanism of desulfurizing-enzyme. There is, however, no available report of desulfurizing-enzyme on rubber biodesulfurization.

In this work, two vulcanized rubbers, vulcanized isoprene rubber (v-IR) and vulcanized styrene butadiene rubber (v-SBR), were treated by *Gordonia amicalisa* for 20 days. The biodesulfurization and biodegradation effects on vulcanized rubbers were evaluated. Furthermore, the inducing mechanism of desulfurizing enzyme which was produced from *G. amicalisa* was investigated.

2. Materials and methods

2.1. Materials

Isoprene rubber was provided by Qingdao Yikesi New Material Co., Shandong, China. Styrene-butadiene rubber was supplied by Qilu Branch of China Petrochemical Co., Shandong, China. Carbon Black N330 was provided by Dolphin Carbon Black Development Co., Tianjin, China. Dibenzothiophene (DBT), 2-hydroxybiphenyl (2-HBP), reduced nicotinamide adenine dinucleotide (NADH) (purity $> 98\%$), methanol and ethyl acetate (chromatography grade) were provided by Shanghai Sigma-Aldrich Co., Shanghai, China. Other chemicals were of analytical grade.

2.2. Preparation of vulcanized isoprene and styrene-butadiene rubber

The vulcanized isoprene rubber (v-IR) and vulcanized styrene butadiene rubber (v-SBR) were prepared by the following steps: raw rubber was masticated on the two-roll mill, blended with additives for 10 min. The basic formulation is shown in Table 1. About 35 g of compounded rubber stock was placed in a mold and pressed by the platens press (Model XLB-DQ, Shanghai, China). The samples were cured at 143 °C and 150 °C for IR and SBR, respectively. The optimum cure times was measured by the Oscillating Disk Rheometer (Model P3555B, Beijing, China).

After vulcanization, v-IR and v-SBR were cut into 2 × 2 × 2 mm cubes for bioprocess.

Table 1
Formulation of IR and SBR vulcanizates (phr).

	IR	SBR
Raw rubber	100	100
Carbon black	50	50
Zinc oxide	4.0	4.0
Stearic acid	2.0	2.0
Antioxidant RD	1	1.5
Accelerator CBS	1.2	1.0
Accelerator TMTD	–	0.2
Sulfur	2.25	1.8

2.3. Biodesulfurization process

Gordonia amicalisa (CICC No. 20664) was supplied by China Center of Industrial Culture Collection. The culture medium contained (per liter) 4 g glucose, 2 g NH_4Cl, 2.44 g KH_2PO_4, 5.57 g Na_2HPO_4, 0.2 g $MgCl_2$, 0.04 g $CaCl_2$, 0.04 g $FeCl_3 \cdot 7H_2O$, 0.008 g $MnCl_2 \cdot 4H_2O$, 0.001 g $ZnCl_2$, 0.004 g $CoCl_2 \cdot 6H_2O$, 0.001 g $AlCl_3 \cdot 6H_2O$, 0.001 g $CuCl_2 \cdot 2H_2O$, 0.001 g H_3BO_3 and 0.001 g $NaMoO_4 \cdot 2H_2O$. The medium solution had been autoclaved at 115 °C.

G. amicalisa was cultured in 250 mL Erlenmeyer flask in a shaker incubator (temperature was 30 °C and stirring speed was 200 rpm). Each flask was filled with 50 mL medium. The inoculum amount was 10% (v/v), cultivation temperature was 30 °C, and stirring speed was 200 rpm.

Before microbial treatment, v-IR and v-SBR were immersed in 75% ethanol (v/v) for 72 h in order to kill the microorganisms and remove harmful additives. Detoxicated v-IR and v-SBR were filtered out and dried in a sterile cabinet. After 3-day-incubation, v-IR and v-SBR were added into flasks. The adding amount was 5% (w/v). After microbial treatment for 20 days, desulfurized samples were filtered out, washed by distilled water and dried at room temperature. The control samples were immersed in uninoculated medium for 20 days.

2.4. Detection desulfurizing-enzyme induced from G. amicalisa

To detect how the desulfurizing-enzyme was induced from *G. amicalisa* cell, v-IR and v-SBR were used as enzyme inducers. Firstly, *G. amicalisa* was cultured in liquid medium as described previously. After three-day-cultivation, detoxicated v-IR and v-SBR were added into medium with *G. amicalisa* for two-day treatment. Then the rubbers were removed and the cultured solution was centrifuged at 5000 r/min for 15 min. The supernatant liquid, which may contain desulfurizing-enzyme, was harvested and stored at 4 °C before use.

DBT was used as the substrate of desulfurizing-enzyme to test desulfurization activity by measuring the decreasing amount of DBT [23,24]. The standard reaction mixture contained 0.1 mol/L potassium phosphate buffer (pH 7.0), 0.1 mmol/L DBT, 0.3 mmol/L NADH and a suitable amount of supernant which contained enzyme. The volume of mixture was 10 mL. This mixture was reacted at 30 °C and 200 r/min for 72 h. After reaction, 1 mol/L HC1 was added to terminate the reaction. Thereafter, 10 mL of ethyl acetate was added to extract residual DBT and DBT metabolite. The ethylacetate layer was collected and centrifuged. The supernatant was analyzed by HPLC and UV-vis spectrum.

2.5. Characterizations

2.5.1. Sol and gel fractions

0.5 g rubber samples (W_0) were immersed in acetone for 48 h to remove the polar substrates, following immersed in toluene for 72 h to extract soluble part in rubber [22]. Then the swelled-rubber samples were taken out of toluene solution and dried at 40 °C in a vacuum oven until no significant weight loss <0.1%. The sol and gel fractions were calculated by the following equation:

$$\text{Sol fraction} = \frac{W_0 - W_1}{W_0} \times 100\% \tag{1}$$

$$\text{Gel fraction} = \frac{W_1}{W_0} \times 100\% \tag{2}$$

where W_0 is the initial weight of the rubber and W1 is the dried