

Devulcanization of natural rubber vulcanizates by *Bacillus cereus* TISTR 2651

*B. Kaewpetch*¹, *S. Prasongsuk*², *S. Poompradub*^{3,4,5*}

¹Program in Biotechnology, Faculty of Science, Chulalongkorn University, 10330 Bangkok, Thailand

²Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, 10330 Bangkok, Thailand

³Department of Chemical Technology, Faculty of Science, Chulalongkorn University, 10330 Bangkok, Thailand

⁴Center of Excellence on Petrochemical and Materials Technology, Chulalongkorn University, 10330 Bangkok, Thailand

⁵Green Materials for Industrial Application Research Unit, Faculty of Science, Chulalongkorn University, 10330 Bangkok, Thailand

Received 25 February 2019; accepted in revised form 20 May 2019

Abstract. The ability of *Bacillus cereus* TISTR 2651 to perform biological devulcanization of natural rubber cured by conventional vulcanization (CV), semi-efficient vulcanization or efficient vulcanization was studied. Among the three curing systems, *B. cereus* TISTR 2651 could specifically devulcanize rubber products formed from the CV system the best, removing 26.44% of the sulfur within 20 days. Moreover, *B. cereus* TISTR 2651 could oxidize and desulfurize the sulfide crosslinks in the CV rubber and transform them to oxygen-containing sulfur-based groups via the 4S pathway. Additionally, the molecular weight, crosslink density and gel fraction of the CV rubber were also significantly decreased from the original levels. Horikx analysis was used to propose the mechanism of bacterial devulcanization. Finally, ground tire rubber (GTR) was devulcanized by *B. cereus* TISTR 2651 and the percentage of sulfur removal was about 27.98 for 20 days, where similar results were also obtained. Accordingly, *B. cereus* TISTR 2651 can be applied for the management of rubber waste, leading towards a solution of this environmental problem.

Keywords: rubber, material testing, bacillus cereus TISTR 2651, devulcanization, ground tire rubber

1. Introduction

The incremental production of natural rubber (NR) products, which are in the main as truck tires, automotive tires and gloves, has led to the global problem of their waste disposal, and so the requirement for their reuse or recycling is becoming of considerable concern worldwide [1, 2]. The three-dimensional network of sulfide linkages in vulcanized rubber is difficult to degrade in nature or decompose upon disposal [3, 4]. Accordingly, large quantities of rubber product waste are generated around the world and cause a global environmental problem and toxicity for living organisms. Therefore, rubber waste

management is an important issue to help resolve this environmental problem. Recycling of the vulcanized rubber is an alternative method that converts the rubber waste into value added products [5–7]. Devulcanization is one form of recycling, where the crosslinked bonds in the rubber chains are cleaved, and can be classified into the three broad processes of physical [8–14], chemical [12, 13, 15–20] and biological [12, 21–29] devulcanization processes. Physical devulcanization generally requires a high temperature or external energy for cleavage of the crosslinked bonds and so this process has a high cost and high energy consumption, challenging its

*Corresponding author, e-mail: sirilux.p@chula.ac.th
© BME-PT

economic and environmental viability. Moreover, the main rubber chains may be broken down during the processing [8–11].

Although chemical devulcanization appears more favorable due to its high selectivity in cleavage of the disulfide bonds, the chemical reagents used are typically toxic for the user and the environment [16]. In addition, the rubber waste needs to be immersed in an organic solvent to allow the chemical reagents to easily penetrate into the rubbery matrix and react with the crosslink bonds. Due to the difficulty of subsequent solvent removal and prevention of exposure to toxic chemicals [12, 15, 16], the advanced technique of using a non-toxic supercritical fluid (carbon dioxide, methane and water) has been applied instead, but this approach requires a high cost and energy consumption [4, 12, 17, 18].

Accordingly, biological devulcanization has been suggested as an alternative method for green technology. This process has a low cost and is environmentally friendly [12]. Several studies have confirmed that biological devulcanization is a potentially viable method to break sulfide crosslinks using selective microorganisms via various metabolic pathways [22–24, 30, 31]. The effect of *Gordonia amicalisa* on biological devulcanization of vulcanized synthetic rubber (vulcanized isoprene and vulcanized styrene butadiene rubber) was investigated [21]. The vulcanized styrene butadiene rubber was the specific substrate that could induce *G. amicalisa* to release desulfurizing enzyme on the devulcanization process [21]. *Sphingomonas sp.* was selected from coal mine soil and was then incubated with ground tire rubber (GTR) for 20 days. After devulcanization, the sulfur content was reduced 22.9% due to the breakdown of sulfide crosslinks [25]. *Thiobacillus ferrooxidans* from the soil of iron mine showed a strong sulfur oxidizing capacity under an acidic condition. After devulcanization with GTR for 30 days, the sulfur content was decreased by 52.8% [26]. Therefore, screening of microbes is important in order to obtain suitable efficient desulfurizing microorganisms. Since Thailand is located in a tropical biodiversity hotspot, there are many interesting resources to explore for potential new bacteria with the ability to break sulfide crosslinks in vulcanized rubber. In this study, *B. cereus* TISTR 2651 was isolated from sulfur-contaminated soil obtained from a coal waste dump area in Thailand. In our preliminary study, this strain

exhibited high efficiency to reduce sulfur content in dibenzothiophene as an organic sulfur compound model. Thus, the ability of this bacterium to perform desulfurization, especially for the devulcanization of NR vulcanizates (NV), was evaluated.

The aim of this study was to investigate the effect of *B. cereus* TISTR 2651 on the devulcanization of NR formed by the three different curing systems of conventional vulcanization (CV), semi-efficient vulcanization (SEV) and efficient vulcanization (EV). After devulcanization, the sulfur content was investigated using bomb calorimetry and elemental analysis. The change in the chemical structure and binding energy of the devulcanized rubber (DV) were examined by Fourier transform infrared spectroscopy (FT-IR) and X-ray photoelectron spectroscopy (XPS), respectively. The mechanism of breakage of the crosslink or main chain scission in DV by *B. cereus* TISTR 2651 was explained based on Horikx's equation. Finally, the devulcanization of GTR by *B. cereus* TISTR 2651 was also evaluated in order to determine the possibilities for the industrial application of *B. cereus* TISTR 2651.

2. Material and methods

2.1. Materials

The NR (STR 5L) used in this study was obtained from Thai Hua Chumphon Rubber Ltd., Chumphon, Thailand. *N*-cyclohexylbenzothiazole-2-sulfenamide (CBS), stearic acid, active zinc oxide (ZnO) and sulfur as a curing agent were purchased from Pan Innovation Ltd., Bangkok, Thailand. The powder form of GTR used in this study came from passenger car tires by fine grinding method (the mechanical grinding using steel rollers or blades at ambient temperature), with a particle size of 425–212 μm mesh size, was supplied by Union Pattanakit Ltd., Samut Prakan, Thailand. Toluene, tetrahydrofuran (THF), ammonium chloride (NH_4Cl) and absolute ethanol were obtained from Merck, Darmstadt, Germany. Glucose was purchased from Sigma-Aldrich, Saint Louis, USA. Magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), potassium dihydrogen phosphate (KH_2PO_4), barium chloride (BaCl_2), sodium hydroxide (NaOH) and methyl orange were obtained from Ajex Finechem, Auckland, New Zealand. Beef extract was supplied by Hi-media, Mumbai, India. All chemicals were analytical grade and commercially available.

2.2. Preparation of NR vulcanizate (NV)

In order to investigate the effect of *B. cereus* TISTR 2651 on the devulcanization of NR, the formulation of rubber compounding with different curing systems is shown in Table 1. The content of NR, active ZnO and stearic acid was kept constant except the ratio of accelerator (*A*) to sulfur (*S*) [32]. Each curing system was designed as follows: CV with *A/S* = 0.33, SEV with *A/S* = 1.05 and EV with *A/S* = 3.75. All chemical agents were mixed using a two-roll mill (Labtech/RM150W, Hopkinton, USA) with a 0.5 mm roller spacing for 11 min at 50 °C. The cure characteristics of the rubber compounds were then measured according to ASTM D5289 using the moving die rheometer (TechPro/rheoTECH MD+, USA) at 150 °C with frequency of 1.7 Hz and amplitude of 0.5 degree. The cure properties were summarized in Table 1.

The rubber compounds were then cured at 150 °C by compression molding (Wabash/G30H15CX, Wabash, USA). The NV sheets obtained from the compression process at the optimum cure time were 15×15 cm² of 2 mm thickness. Subsequently, the NV sheets were crushed by a two-roll mill for five times at an ambient

Table 1. Formulation, in part by weight per hundred part of rubber [phr], for rubber compounding and cure properties of NR vulcanizates with different curing systems.

Ingredient [phr]	CV	SEV	EV
NR ¹	100.0	100.0	100.0
Active ZnO ²	5.0	5.0	5.0
Stearic acid	2.0	2.0	2.0
Sulfur	3.0	1.9	0.8
CBS ³ (Accelerator)	1.0	2.0	3.0
Total	111.0	110.9	110.8
Cure properties			
M_H^4 [dN·m]	6.40±0.09	7.79±0.16	6.32±0.19
M_L^5 [dN·m]	0.22±0.06	0.23±0.05	0.27±0.01
t_{s2}^6 [min]	6.83±0.12	7.85±0.18	10.33±0.23
t_{c90}^7 [min]	9.65±0.14	19.42±1.68	26.11±0.46

¹Natural rubber;

²Zinc oxide;

³N-cyclohexylbenzothiazole-2-sulfenamide;

⁴Maximum torque;

⁵Minimum torque;

⁶Scorch time was the time taken for a two-unit rise above the minimum torque;

⁷Optimum cure time was the time taken for attaining 90% of the maximum torque.

temperature to obtain small particle sizes and sieved through a molecular sieve to obtain a size range of 2.88–2.00 mm mesh size.

2.3. Devulcanization of NV

B. cereus TISTR 2651 was isolated from soil obtained from a coal waste dump in Chonburi Province, Thailand. This strain was gram-positive, rod-shape and aerobic bacterium. Its colony was white on Luria-Bertani (LB) agar medium. The 16S rRNA gene of this strain was revealed and had high similarity (94%) to those of *B. cereus* strain ATCC 14579, *B. cereus* strain JCM 2152, *B. cereus* strain CCM 2010, *B. cereus* strain NBRC 15305 and *B. cereus* strain IAM 12605 in the GenBank. This bacterium was submitted in the culture collection of the Thailand Institute of Scientific and Technological Research (TISTR). The culture medium for its cultivation was prepared as follows: MgCl₂·6H₂O (0.25 g), NH₄Cl (0.50 g), KH₂PO₄ (0.50 g), beef extract (0.50 g) and glucose (5.00 g) were dissolved in 1000 ml of distilled water. The pH of the culture medium was adjusted to 7.0 with 1 N NaOH, and then autoclaved at 121 °C for 20 min and allowed to cool before use.

Before bacterial treatment, 10 g ground NV was soaked in 100 ml of 75% (v/v) ethanol for 24 h in order to kill other microorganisms and then dried in the oven at 60 °C. Next, 2.5 g of ground NV was added into 100 ml of the culture medium containing a 24 h culture of *B. cereus* TISTR 2651. The initial concentration of the bacterium was 0.1 (OD₆₀₀) [25]. The culture was then incubated at room temperature (30±2 °C) with shaking (150 rpm) for 20 d. As a control, sterilized ground NV was likewise kept in culture medium as above for 20 d except without the *B. cereus* TISTR 2651.

2.4. Characterization of NV before and after devulcanization

2.4.1. Determination of the bacterial growth rate

The effect of *B. cereus* TISTR 2651 on the devulcanization process was detected by the growth rate of the bacterium during cultivation. The culture medium was sampled every 5 days for determination of the bacterial growth via a UV/VIS Spectrometer (UNICO/Model UV-2800, Dayton, USA) in terms of the optical density at a wavelength of 600 nm (OD₆₀₀).

2.4.2. Chemical properties of NV before and after devulcanization

The quantitative sulfur content of NV before and after devulcanization was examined by bomb calorimetry according to ASTM D3177. During the combustion process in the oxygen bomb calorimeter (Parr 6200, Moline, USA), the sulfur in sample was oxidized to sulfur trioxide which, in turn, combines with water in the bomb to condense as sulfuric acid. All parts in the bomb were then washed with methyl orange as a washing solution and the solution was collected for sulfur determination. The washing solution was adjusted to pH 5.5 with 2 M sodium carbonate and then neutralized by 1 M ammonium hydroxide solution until at pH 7. The sulfate content was determined in terms of barium sulfate (BaSO_4) by adding excess BaCl_2 .

The sulfur content was analyzed by an elemental analyzer (Thermo Scientific/FLASH 2000, USA). The sample (1–3 mg) was accurately weighed and mixed with an oxidizer (vanadium pentoxide) in a tin capsule. The sample was heated to 975 °C for 12 min under an oxygen atmosphere.

The functional groups of NV before and after devulcanization were analyzed by FT-IR using a Perkin Elmer spectrum one transform infrared spectroscope, Shelton, USA. The samples were analyzed in the range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} .

The surface elements and bonding state on the particle surface of rubbers were analyzed by XPS (Kratos/AXIS Ultra DLD, Manchester, UK) with the spectra of the samples being constructed from the narrow-high resolution scan.

2.4.3. Physical properties of NV before and after devulcanization

The powder rubber samples (0.4 g) were immersed in 100 ml of toluene for 72 h, then removed and the surface liquid was rapidly removed by blotting with tissue paper before they were weighed immediately. Subsequently, the rubber samples were dried in a vacuum oven at 80 °C until at constant weight.

The sol-gel fraction of the rubber sample was calculated according to Equations (1) and (2):

$$\text{Sol fraction [\%]} = \frac{W_0 - W_1}{W_0} \cdot 100 \quad (1)$$

$$\text{Gel fraction [\%]} = 100 - \text{sol fraction [\%]} \quad (2)$$

where W_0 and W_1 are the dried sample mass before and after swelling, respectively.

The crosslink density of rubber samples was calculated by the Flory-Rehner equation [33], as shown in Equation (3):

$$\nu = \frac{-[\ln(1 - \nu_r) + \nu_r + \chi \nu_r^2]}{\nu_0 [\nu_r^{1/3} - \frac{\nu_r}{2}]} \quad (3)$$

where ν is the crosslink density [$\text{mol}/\text{cm}^{-3}$], ν_r is the volume fraction of the rubber network in the swollen gel, ν_0 is the molar volume of the toluene ($106.2 \text{ cm}^3/\text{mol}$) [34] and χ is equal to 0.393 and used as a rubber-solvent interaction parameter [34]. The percentage of devulcanization was calculated by using Equation (4) according to ASTM D 6814-02:

$$\begin{aligned} \text{Devulcanization [\%]} &= \\ &= \left[1 - \frac{\text{crosslink density devulcanized rubber}}{\text{crosslink density vulcanized rubber}} \right] \cdot 100 \quad (4) \end{aligned}$$

The molecular weight of the rubber samples was determined by gel permeation chromatography (GPC), performed on a Waters/2414 (MA) instrument using the refractive index as a detector, and equipped with Styragel HR5E 7.8×300 mm column (molecular weight resolving range of 2000–4000 000). The GPC column was eluted at 40 °C using THF at a flow rate of 1.0 ml/min and calibrated with polystyrene as a standard.

3. Results and discussion

3.1. Effect of *B. cereus* TISTR 2651 on the devulcanization of NV

In order to study the desulfurization in NV by *B. cereus* TISTR 2651, NVs formed from the three different curing systems (CV, SEV and EV) were examined. The growth rate of *B. cereus* TISTR 2651 over 20 days in culture medium with or without the addition of the respective NVs is shown in Figure 1. In medium alone the initial fast growth rate of the bacteria over the first 5 days then slowed down and after 10 days the net OD_{600} decreased with further culture time representing cell death. In contrast, the growth rate of *B. cereus* TISTR 2651 in the media with the different NVs was faster over the first 5 days than the control ($\text{SEV} > \text{CV} > \text{EV}$) and continued up to 10 (CV and SEV) or 15 (EV) d of culture before declining by 20 days. The higher and longer growth

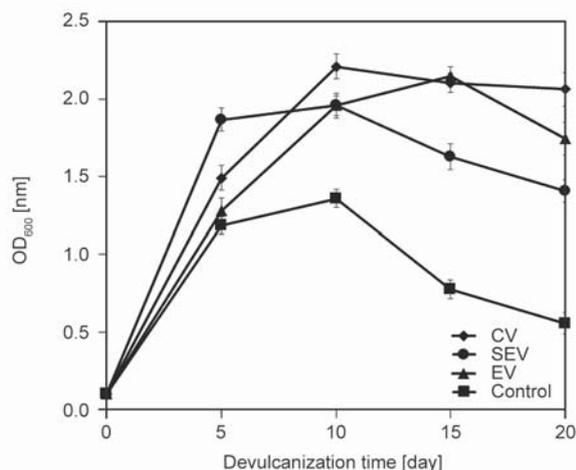


Figure 1. Growth rate of *B. cereus* TISTR 2651 during culture with *B. cereus* TISTR 2651 (devulcanization) for up to 20 days.

rate in the presence of the NVs confirmed that the hydrocarbon substances in NV could act as the nutrients for bacterial growth [30]. That the growth rate of *B. cereus* TISTR 2651 tended to gradually decrease after 10 days of culture may be because the destruction of the rubber network structure during its devulcanization may release elastomer or additives that inhibit the microbial growth [25], as well as entering death phase from the exhaustion of essential nutrients. With the longer culture (devulcanization) times (15–20 days) the proportion of dead to viable bacterial cells increased [30]. Elemental analysis was used to support that the hydrocarbon compounds in the respective NV could be used as nutrients for *B. cereus* TISTR 2651 growth. Table 2 shows the content of carbon (C), nitrogen (N), hydrogen (H) and sulfur (S) before and after devulcanization of the respective NVs for 20 days. Each element tended to be slightly decreased after devulcanization, except for the sulfur

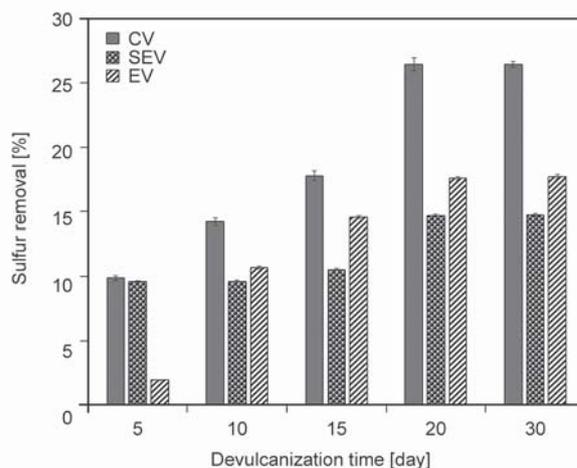


Figure 2. Representative percentage of sulfur removal in DVs, as determined by bomb calorimetry, during culture with *B. cereus* TISTR 2651 (devulcanization) for up to 30 days.

content that was significantly decreased by 10–16.4% after devulcanization, being highest in the NV formed by the CV system (CV-NV) at 16.4% sulfur removal. As an alternative approach to determine the sulfur content after devulcanization, NVs after various *B. cereus* TISTR 2651 cultivation (devulcanization) times were subjected to bomb calorimetry, with the results summarized in Figure 2. The percentage of sulfur removal increased with increasing *B. cereus* TISTR 2651 culture (devulcanization) times and a significant time-dependent increase in the sulfur removal level was clearly observed in the CV-NV samples. The removal of sulfur from the SEV-NV and EV-NV samples was also observed, but to a lesser degree. This result agreed with the elemental analysis. The optimum devulcanization time was 20 days as the sulfur removal level tended to be constant at culture times longer than 20 days. Accordingly, *B.*

Table 2. Elemental analysis of the vulcanized and devulcanized NR at a *B. cereus* TISTR 2651 culture (devulcanization) time of 20 days.

Element content [%]	CV		SEV		EV	
	NV ¹	DV ²	NV	DV	NV	DV
Carbon	79.41±0.12	71.06±0.34	80.14±0.54	77.79±0.56	80.81±0.48	79.63±0.77
Nitrogen	0.70±0.15	0.66±0.02	0.85±0.06	0.73±0.02	0.81±0.03	0.80±0.01
Hydrogen	10.87±0.29	9.73±0.23	10.90±0.28	10.54±0.18	11.01±0.34	10.81±0.22
Sulfur	2.76±0.21	2.31±0.14	2.09±0.27	1.88±0.11	1.24±0.23	1.08±0.14
Sulfur removal ³ [%]	–	16.36	–	10.05	–	12.90

¹Vulcanized NR;

²Devulcanized NR;

³Sulfur removal [%] = $(A - B)/A \cdot 100$, where *A* is the percentage elemental content of vulcanized rubber and *B* is the percentage elemental content of devulcanized rubber.

cereus TISTR 2651 can specifically devulcanize the CV-NV, which will be used to analyze for further study.

3.2. The metabolic pathway of *B. cereus* TISTR 2651 on the devulcanization process

The selective cleavage of sulfide crosslinks is necessary for the devulcanization process. To explain the metabolic pathway of *B. cereus* TISTR 2651 in the devulcanization of the CV-NV, the CV-NV after devulcanization was characterized by FT-IR analysis, with the results shown in Figure 3. Before devulcanization, the characteristic bands of cis-1,4-polyisoprene were observed at 2960–2850 cm^{-1} , attributed to the C–H saturated stretching [35]. The bands located at 1662 cm^{-1} were assigned to C=C deformations [21]. The stretching frequency of methyl-assisted conjugated double bonds due to a reversion was observed at 1540 cm^{-1} [36–38]. The bands at 1450 and 1380 cm^{-1} were ascribed to $-\text{CH}_2$ deformation [26], while the peak at 835 cm^{-1} was assigned to C–H bending [38]. An additional peak at 755 cm^{-1} was due to the C–S stretching of sulfide crosslinks in their structure [39, 40].

After devulcanization for 20 days, a peak at 1088 cm^{-1} (S=O stretching) was formed and the intensity of the peak at 1540 cm^{-1} was substantially diminished. This result suggested that *B. cereus* TISTR

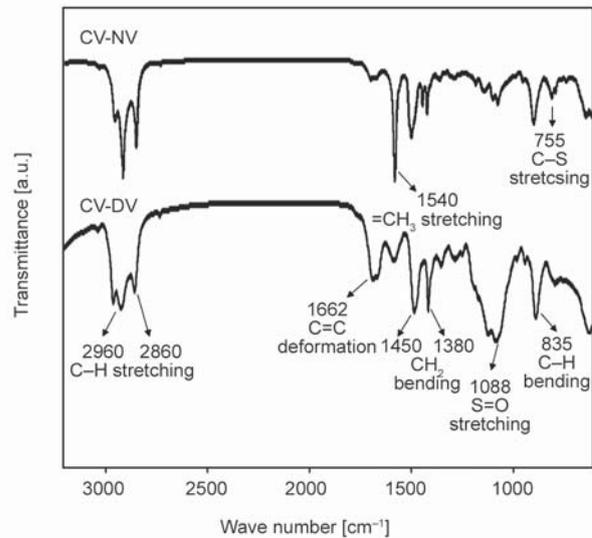


Figure 3. FT-IR spectra of the CV-NV rubber before and after culture with *B. cereus* TISTR 2651 (devulcanization) for 20 days.

2651 not only ruptured the partial main chains of rubber molecules but also oxidized the sulfide crosslinks via some metabolic pathway [21, 41]. Additionally, the band located at 1662 cm^{-1} (C=C deformations) had the high intensity in the devulcanized sample. It is indicated that after devulcanization by *B. cereus* TISTR 2651 some virgin rubber chains which are composed of cis 1,4-polyisoprene could be recovered. The XPS results showed that after devulcanization for 20 days, the intensity of C–C bond at 285 eV

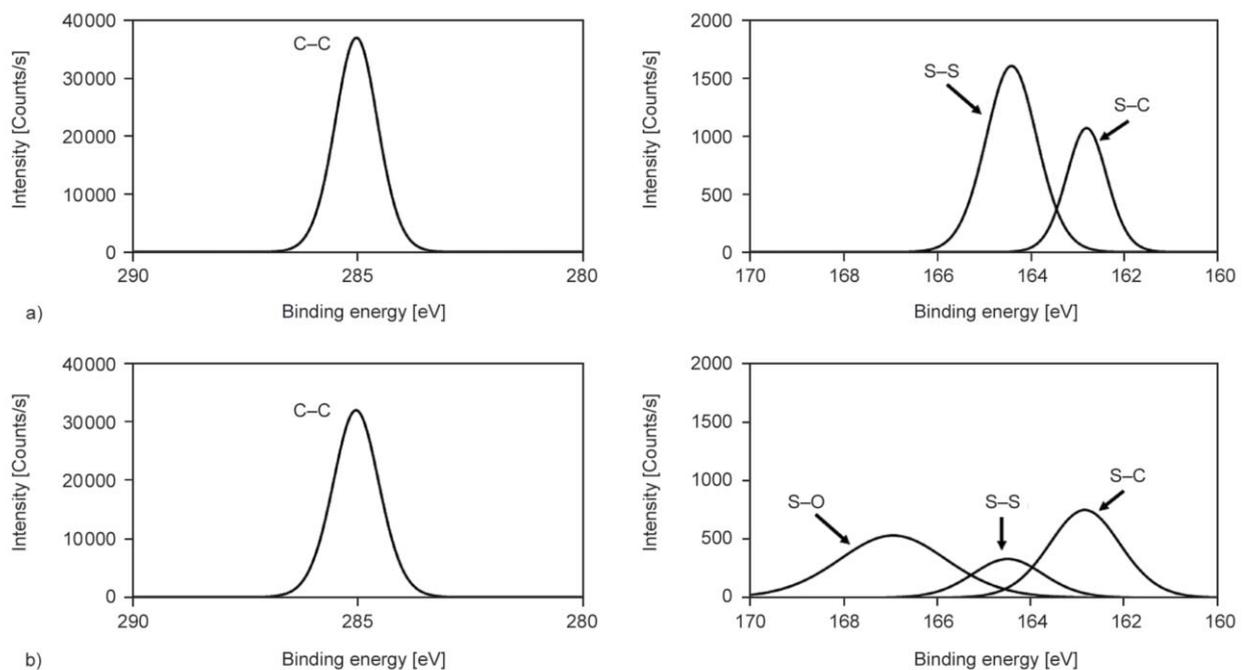


Figure 4. Narrow-high resolution scan of XPS spectra for (a) CV-NV and (b) CV-DV after culture with *B. cereus* TISTR 2651 (devulcanization) for 20 days.

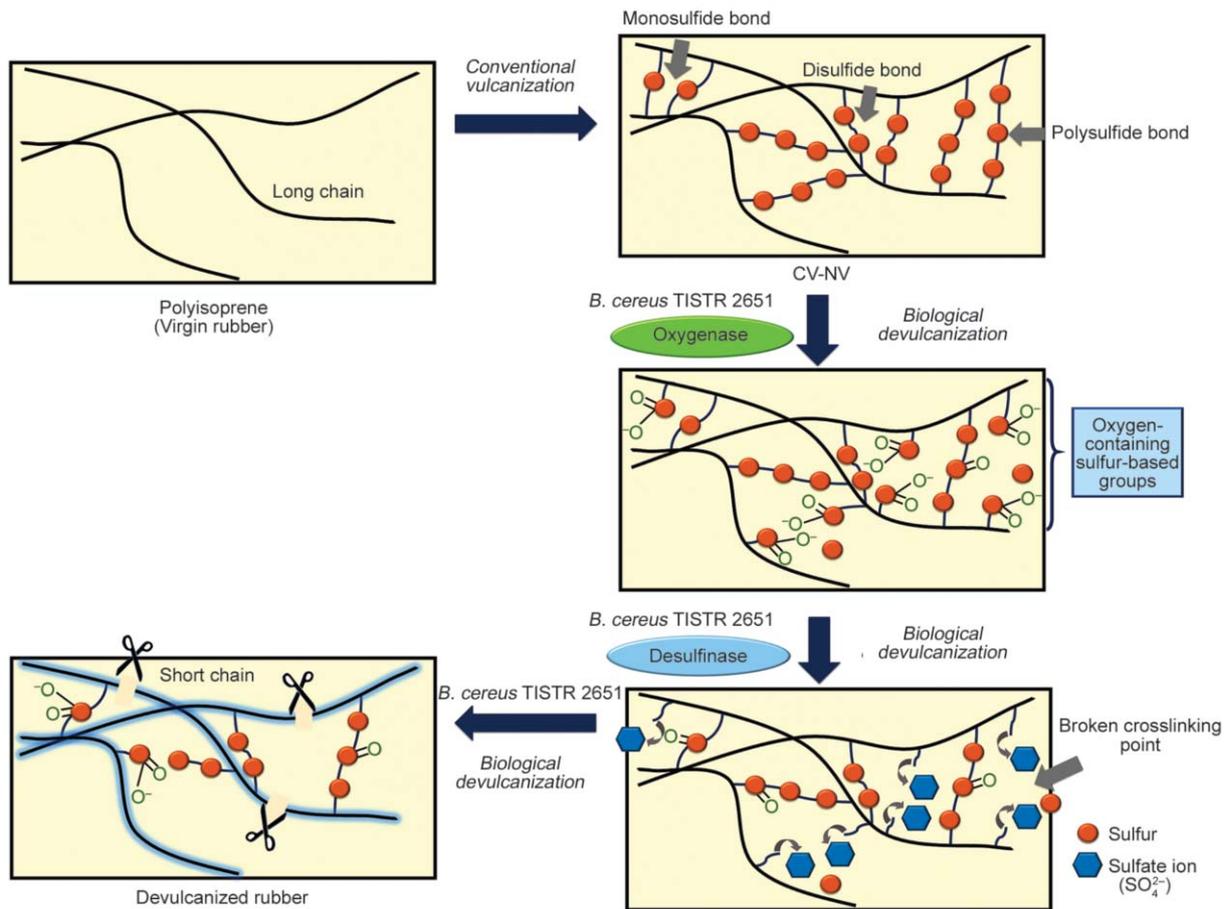


Figure 5. Biochemical process.

was slightly decreased due to the main chain scission. A decrease in the peak intensities of S–C (at 162.7 eV) and S–S (at 164.2 eV) were clearly observed, while the peak of the S–O bond appeared at 166.8 eV (Figure 4) [42]. This result supported that *B. cereus* TISTR 2651 causes the oxidation of sulfide crosslinks and that the sulfur may be converted to oxygen-containing sulfur-based groups [21]. Accordingly, the enzymes in the microorganism can catalyze the oxidation reaction, and the desulfurization pathway of *B. cereus* TISTR 2651 was proposed to be analogous to the 4S pathway as described in Figure 5 [24, 30, 31].

3.3. Physical properties of the DV

Table 3 summarizes the physical properties of the NV-CV rubber vulcanizates before and after culturing with *B. cereus* TISTR 2651 (devulcanization) for 20 days. After devulcanization, the number-average molecular weight (\bar{M}_n) and the weight-average molecular weight (\bar{M}_w) of the CV-DV were significantly decreased, while the polydispersity index (PDI) tended to be increased when compared to rubber

vulcanizates before devulcanization (CV-NV). This result may indicate that not only the main chain rubbers but also the sulfide crosslinks were cleaved by *B. cereus* TISTR 2651, where the various chain lengths of rubber chains obtained would result in a decreased crosslinking density and gel fraction of the CV-DV.

To confirm that the devulcanization occurred at the main chain or sulfide crosslinks, Horikx analysis was used. Horikx’s equation provides a theoretical relationship between the sol fraction generated after degradation of a polymer network and the relative decrease in the crosslink density [21, 43, 44].

When the main chain scission takes place, the relative decrease in the crosslink density is given by Equation (5) [43, 44]:

$$1 - \frac{\vartheta_f}{\vartheta_i} = 1 - \frac{(1 - S_f^{1/2})^2}{(1 - S_i^{1/2})^2} \quad (5)$$

where ϑ_i is the crosslink density of the NV, ϑ_f is the crosslink density of the DV, S_i is the sol fraction of the NV and S_f is the sol fraction of the DV.

When the crosslink scission occurs, the relative decrease in the crosslink density is given by Equation (6) [43, 44]:

$$1 - \frac{\vartheta_f}{\vartheta_i} = 1 - \frac{\gamma_f(1 - S_f^{1/2})^2}{\gamma_i(1 - S_i^{1/2})^2} \quad (6)$$

The terms γ_f and γ_i are calculated from Equation (7) [43, 44]:

$$S_f = \frac{1}{(1 - \gamma_f - \gamma_f S_f)^2}, S_i = \frac{1}{(1 - \gamma_i - \gamma_i S_i)^2} \quad (7)$$

where γ_i is the average number of crosslinked units per original chain and γ_f is the average number of crosslinked units per original chain after devulcanization [21, 43, 44].

Figure 6 shows the Horikx plot of the sol fraction against the relative crosslink density of DV at different devulcanization times. The solid curve corresponded to the main chain scission, and the dotted curve corresponded to crosslink scission. The experimental points for DV within 10 days of devulcanization were close to the crosslink scission curve, indicating that *B. cereus* TISTR 2651 had likely specifically broken the sulfide linkages in the crosslinked rubber chains. At 15 days of devulcanization, the experimental points were between crosslink scission and main chain scission curve, suggesting that after the crosslink scission was almost complete, the microorganism proceeded via a combination of crosslink scission and main chain scission in the neighborhood of crosslink sites. The breakdown of the main chain scission in DV by *B. cereus* TISTR 2651

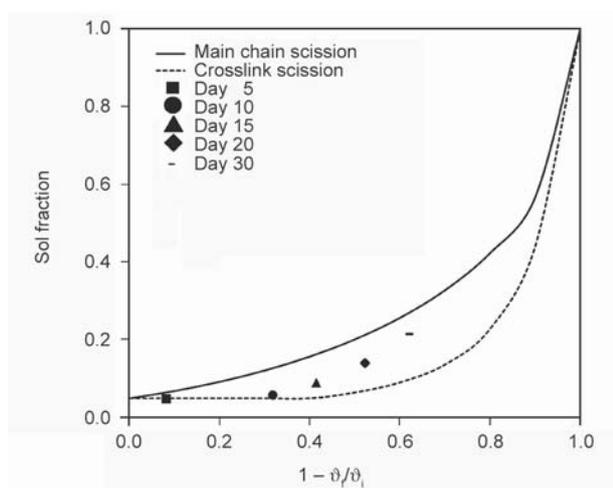


Figure 6. Horikx curves of CV-DV after culture with *B. cereus* TISTR 2651 (devulcanization) for different times.

was accomplished to use the hydrocarbon substance as the nutrients for its growth after more than 20 days of culture (devulcanization). Thus, it is important to note that the advantage of using *B. cereus* TISTR 2651 in this study was the selective breakage of the sulfide crosslinks within 10 days.

3.4. Devulcanization of GTR

For further potential application, the ability of *B. cereus* TISTR 2651 to devulcanize GTR was evaluated under the same conditions as CV-DV (Sections 3.1 to 3.3). The percentage of sulfur removal obtained from the bomb calorimetry analysis is summarized in Table 3. The sulfur content of the devulcanized (D)GTR was significantly decreased by about 27.98% from its original level after 20 days of culture. Accordingly, this result suggested that *B. cereus* TISTR 2651 could potentially be applied to remove sulfur from the vulcanized rubber of waste tires. The percentage of sulfur removal in the DGTR was comparable with that in CV-DV. Accordingly, the impurity or other chemical substances in GTR did not seemingly affect the level of devulcanization by *B. cereus* TISTR 2651.

The FT-IR spectra of GTR before and after devulcanization (DGTR) by *B. cereus* TISTR 2651 are shown in Figure 7. The characteristic peak of GTR before devulcanization was similar to that in the CV-NV. After devulcanization for 20 days, the characteristic

Table 3. Physical properties of the vulcanized rubber before and after *B. cereus* TISTR 2651 culture (devulcanization) for 20 days.

Physical property	CV-NV ⁵	CV-DV ⁶	GTR ⁷	DGTR ⁸
\bar{M}_n ¹ [Daltons]	133 724	7 545	14 473	13 176
\bar{M}_w ² [Daltons]	156 182	11 263	19 680	18 247
PDI ³	1.17	1.49	1.35	1.38
Crosslink density · 10 ⁴ [mol/cm ³]	1.17	0.36	2.45	0.94
Devulcanization [%]	–	69.20	–	61.63
Sol fraction [%]	5.09	18.32	3.69	12.59
Gel fraction [%]	94.91	81.68	96.31	87.41
Sulfur removal ⁴ [%]	–	26.44	–	27.98

¹Number-average molecular weight;

²Weight-average molecular weight;

³Polydispersity index;

⁴Based on bomb calorimetry;

⁵Vulcanized NR with CV system;

⁶Devulcanized NR with CV system;

⁷Ground tire rubber;

⁸Devulcanized ground tire rubber

Remark: Uncured NR vulcanizate showed the \bar{M}_n 575 179 Daltons, \bar{M}_w 754 182 Daltons, and PDI 1.31 based on GPC analysis.

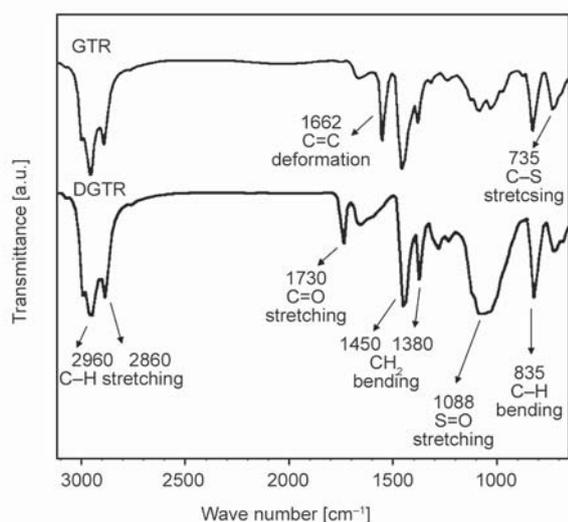


Figure 7. FT-IR spectra of GTR before and after culture with *B. cereus* TISTR 2651 (devulcanization) for 20 days.

peaks of S=O stretching (1088 cm^{-1}) and C=O stretching (1730 cm^{-1}) [21] were considerably increased. This result further supported that the sulfide crosslinks and the main chains of rubber were oxidized to form the oxygen-containing sulfur-based groups and carbonyl groups [21], and this may result in the decreased \bar{M}_n and \bar{M}_w values (Table 3). Moreover, the XPS results confirmed that the intensities of S–C and S–S bonds were significantly decreased whereas the peak of the S–O bond appeared after treatment (Figure 8). The main chain scission also

occurred due to the decrease in the intensity of C–C bond at 285 eV.

As seen in Table 3, the \bar{M}_n and \bar{M}_w values of GTR were smaller than those of CV-NV. This was because GTR was obtained by fine grinding process to produce the small particle sizes. Some of the rubbery chains were broken down during grinding, resulting in the decreased molecular weight of the polymeric material. After devulcanization, the \bar{M}_n , \bar{M}_w and PDI values of DGTR did not change significantly compared to CV-DV, due to the breakdown of rubbery chains as discussed before. In case of DGTR, molecular weight values did not decrease significantly during the bacterial treatment but the observed sol fraction seemed too high. The treatment might be effective only in the vicinity of the rubber surface and hence the devulcanization was not homogeneous. The polydispersity index (PDI) of CV-DV tended to be increased when compared to CV-NV. This result could be explained that after devulcanization the size distribution of rubber chains in CV-DV was more inhomogeneous than that in CV-NV. The crosslink density of two samples (CV-DV and DGTR) was rapidly decreased from the original CV-NV and GTR, respectively. The percentage of devulcanization, sol and gel fractions between them was nearly comparable. As a result, the biological process used in this study (using *B. cereus* TISTR 2651) can be applied to reduce rubber waste and to recovery virgin rubber

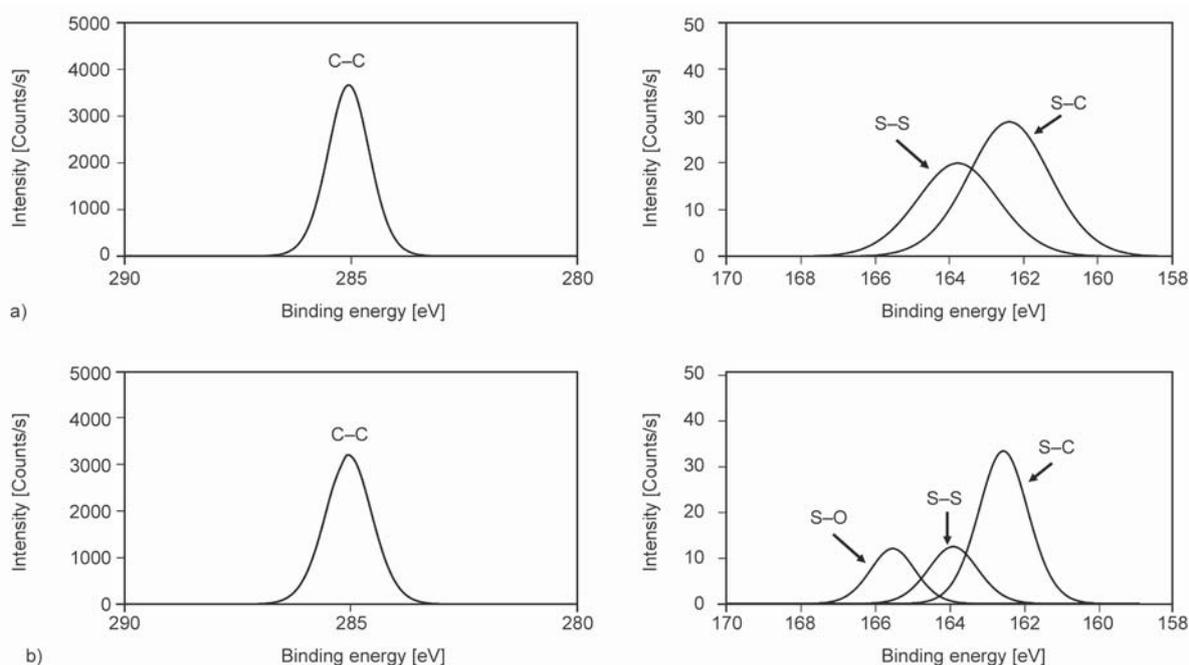


Figure 8. Narrow-high resolution scan of XPS spectra for (a) GTR and (b) DGTR after culture with *B. cereus* TISTR 2651 (devulcanization) for 20 days.

that can be used as a raw rubber material. The advantages of this process include it is a green process, low energy consumption and operates under a mild condition.

4. Conclusions

B. cereus TISTR 2651, isolated from sulfur-contaminated soil at a coal waste dump area in Thailand, was used to devulcanize rubber products (NV formed from CV, EV or SEV curing systems and GTR) under a mild condition. This bacterium could not only oxidize the sulfide crosslinks of rubber products but also ruptured the partial main chains during devulcanization. The percentage of sulfur removal by this microorganism was significantly decreased for the CV-NV samples. Other physical properties of the CV-NV after devulcanization for 20 days, such as the \bar{M}_n , \bar{M}_w , crosslink density and gel fraction, were significantly decreased compared to before devulcanization. From the Horikx analysis, the dominant devulcanization mechanism of *B. cereus* TISTR 2651 was crosslink scission which took place at an early stage of devulcanization time (within 10 days). Although the GTR was comprised of many curing substances that could potentially inhibit the devulcanization, the efficiency of *B. cereus* TISTR 2651 to break sulfide crosslinks was no different from that with CV-DV. Accordingly, *B. cereus* TISTR 2651 can be used as microorganism for the biological devulcanization of vulcanized NR products to solve the rubber waste problem produced at a large scale in the rubber industry.

Acknowledgement

The financial support for this project was provided by the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund).

References

- [1] Hirata Y., Kondo H., Ozawa Y.: Natural rubber (NR) for the tyre industry. in ‘Chemistry, manufacture and applications of natural rubber’ (eds.: Kohjiya S., Ikeda Y.) Woodhead Publishing, London, 325–352 (2014). <https://doi.org/10.1533/9780857096913.2.325>
- [2] Rattanapan C., Suksaroj T. T., Ounsaneha W.: Development of eco-efficiency indicators for rubber glove product by material flow analysis. *Procedia – Social and Behavioral Sciences*, **40**, 99–106 (2012). <https://doi.org/10.1016/j.sbspro.2012.03.167>
- [3] Coran A. Y.: Vulcanization. in ‘The science and technology of rubber’ (eds.: Mark J. E., Erman B., Roland C. M.) Academic Press, Massachusetts, 337–381 (2013).
- [4] Asaro L., Gratton M., Seghar S., Hocine N. A.: Recycling of rubber wastes by devulcanization. *Resources, Conservation and Recycling*, **133**, 250–262 (2018). <https://doi.org/10.1016/j.resconrec.2018.02.016>
- [5] Sienkiewicz M., Kucinska-Lipka J., Janik H., Balas A.: Progress in used tyres management in the European Union: A review. *Waste Management*, **32**, 1742–1751 (2012). <https://doi.org/10.1016/j.wasman.2012.05.010>
- [6] Myhre M., Saiwari S., Dierkes W., Noordermeer J.: Rubber recycling: Chemistry, processing, and applications. *Rubber Chemistry and Technology*, **85**, 408–449 (2012). <https://doi.org/10.5254/rct.12.87973>
- [7] Isayev A. I.: Recycling of natural and synthetic isoprene rubbers. in ‘Chemistry, manufacture and applications of natural rubber’ (eds.: Kohjiya S., Ikeda Y.) Woodhead Publishing, Amsterdam, 395–435 (2014). <https://doi.org/10.1533/9780857096913.3.395>
- [8] de Sousa F. D. B., Scuracchio C. H., Hu G-H., Hoppe S.: Devulcanization of waste tire rubber by microwaves. *Polymer Degradation and Stability*, **138**, 169–181 (2017). <https://doi.org/10.1016/j.polymdegradstab.2017.03.008>
- [9] Mangili I., Lasagni M., Huang K., Isayev A. I.: Modeling and optimization of ultrasonic devulcanization using the response surface methodology based on central composite face-centered design. *Chemometrics and Intelligent Laboratory Systems*, **144**, 1–10 (2015). <https://doi.org/10.1016/j.chemolab.2015.03.003>
- [10] Si H., Chen T., Zhang Y.: Effects of high shear stress on the devulcanization of ground tire rubber in a twin-screw extruder. *Journal of Applied Polymer Science*, **128**, 2307–2318 (2013). <https://doi.org/10.1002/app.38170>
- [11] Aoudia K., Azem S., Hocine N. A., Gratton M., Pettarin V., Seghar S.: Recycling of waste tire rubber: Microwave devulcanization and incorporation in a thermoset resin. *Waste Management*, **60**, 471–481 (2017). <https://doi.org/10.1016/j.wasman.2016.10.051>
- [12] Mangili I., Lasagni M., Anzano M., Collina E., Tatangelo V., Franzetti A., Caracino P., Isayev A. I.: Mechanical and rheological properties of natural rubber compounds containing devulcanized ground tire rubber from several methods. *Polymer Degradation and Stability*, **121**, 369–377 (2015). <https://doi.org/10.1016/j.polymdegradstab.2015.10.004>
- [13] Zedler Ł., Klein M., Saeb M. R., Colom X., Cañavate J., Formela K.: Synergistic effects of bitumen plasticization and microwave treatment on short-term devulcanization of ground tire rubber. *Polymers*, **10**, 1265/1–1265/17 (2018). <https://doi.org/10.3390/polym10111265>

- [14] Seghar S., Asaro L., Rolland-Monnet M., Hocine N. A.: Thermo-mechanical devulcanization and recycling of rubber industry waste. *Resources, Conservation and Recycling*, **144**, 180–186 (2019).
<https://doi.org/10.1016/j.resconrec.2019.01.047>
- [15] Sabzekar M., Chenar M. P., Mortazavi S. M., Kariminejad M., Asadi S., Zohuri G.: Influence of process variables on chemical devulcanization of sulfur-cured natural rubber. *Polymer Degradation and Stability*, **118**, 88–95 (2015).
<https://doi.org/10.1016/j.polymdegradstab.2015.04.013>
- [16] Ghorai S., Bhunia S., Roy M., De D.: Mechanochemical devulcanization of natural rubber vulcanizate by dual function disulfide chemicals. *Polymer Degradation and Stability*, **129**, 34–46 (2016).
<https://doi.org/10.1016/j.polymdegradstab.2016.03.024>
- [17] Liu Z., Li X., Xu X., Wang X., Dong C., Liu F., Wei W.: Devulcanization of waste tread rubber in supercritical carbon dioxide: Operating parameters and product characterization. *Polymer Degradation and Stability*, **119**, 198–207 (2015).
<https://doi.org/10.1016/j.polymdegradstab.2015.05.017>
- [18] Mangili I., Oliveri M., Anzano M., Collina E., Pitea D., Lasagni M.: Full factorial experimental design to study the devulcanization of ground tire rubber in supercritical carbon dioxide. *The Journal of Supercritical Fluids*, **92**, 249–256 (2014).
<https://doi.org/10.1016/j.supflu.2014.06.001>
- [19] Thaicharoen P., Thamyongkit P., Poompradub S.: Thioalicyclic acid as a devulcanizing agent for mechanochemical devulcanization. *Korean Journal of Chemical Engineering*, **27**, 1177–1183 (2010).
<https://doi.org/10.1007/s11814-010-0168-9>
- [20] Zhang X., Saha P., Cao L., Li H., Kim J.: Devulcanization of waste rubber powder using thiobisphenols as novel reclaiming agent. *Waste Management*, **78**, 980–991 (2018).
<https://doi.org/10.1016/j.wasman.2018.07.016>
- [21] Hu M., Zhao S., Li C., Wang B., Fu Y., Wang Y.: Bio-desulfurization of vulcanized rubber by enzymes induced from *Gordonia amicalisa*. *Polymer Degradation and Stability*, **128**, 8–14 (2016).
<https://doi.org/10.1016/j.polymdegradstab.2016.02.017>
- [22] Su T., Su J., Liu S., Zhang C., He J., Huang Y., Xu S., Gu L.: Structural and biochemical characterization of BdsA from *Bacillus subtilis* WU-S2B, a key enzyme in the ‘4S’ desulfurization pathway. *Frontiers in Microbiology*, **9**, 1–11 (2018).
<https://doi.org/10.3389/fmicb.2018.00231>
- [23] Cui X., Zhao S., Wang B.: Microbial desulfurization for ground tire rubber by mixed consortium-*Sphingomonas sp.* and *Gordonia sp.* *Polymer Degradation and Stability*, **128**, 165–171 (2016).
<https://doi.org/10.1016/j.polymdegradstab.2016.03.011>
- [24] Davoodi-Dehaghani F., Vosoughi M., Ziaee A. A.: Bio-desulfurization of dibenzothiophene by a newly isolated *Rhodococcus erythropolis* strain. *Bioresource Technology*, **101**, 1102–1105 (2010).
<https://doi.org/10.1016/j.biortech.2009.08.058>
- [25] Li Y., Zhao S., Wang Y.: Microbial desulfurization of ground tire rubber by *Sphingomonas sp.*: A novel technology for crumb rubber composites. *Journal of Polymers and the Environment*, **20**, 372–380 (2012).
<https://doi.org/10.1007/s10924-011-0386-1>
- [26] Li Y., Zhao S., Wang Y.: Microbial desulfurization of ground tire rubber by *Thiobacillus ferrooxidans*. *Polymer Degradation and Stability*, **96**, 1662–1668 (2011).
<https://doi.org/10.1016/j.polymdegradstab.2011.06.011>
- [27] Linh D. V., Huong N. L., Tabata M., Imai S., Iijima S., Kasai D., Anh T. K., Fukuda M.: Characterization and functional expression of a rubber degradation gene of a *Nocardia* degrader from a rubber-processing factory. *Journal of Bioscience and Bioengineering*, **123**, 412–418 (2017).
<https://doi.org/10.1016/j.jbiosc.2016.11.012>
- [28] Tatangelo V., Mangili I., Caracino P., Bestetti G., Collina E., Anzano M., Branduardi P., Posterl R., Porro D., Lasagni M., Franzetti A.: Microbial desulfurization of ground tire rubber (GTR): Characterization of microbial communities and rheological and mechanical properties of GTR and natural rubber composites (GTR/NR). *Polymer Degradation and Stability*, **160**, 102–109 (2019).
<https://doi.org/10.1016/j.polymdegradstab.2018.12.021>
- [29] Aboelkheir M. G., Visconte L.Y., Oliveira G. E., Filho R. D. T., Souza F. G.: The biodegradative effect of *Tenebrio molitor* Linnaeus larvae on vulcanized SBR and tire crumb. *Science of The Total Environment*, **649**, 1075–1082 (2019).
<https://doi.org/10.1016/j.scitotenv.2018.08.228>
- [30] Mohebbi G., Ball A. S.: Biodesulfurization of diesel fuels – Past, present and future perspectives. *International Biodeterioration and Biodegradation*, **110**, 163–180 (2016).
<https://doi.org/10.1016/j.ibiod.2016.03.011>
- [31] Dinamarca M. A., Ibacache-Quiroga C., Baeza P., Galvez S., Villarroel M., Olivero P., Ojeda J.: Biodesulfurization of gas oil using inorganic supports biomodified with metabolically active cells immobilized by adsorption. *Bioresource Technology*, **101**, 2375–2378 (2010).
<https://doi.org/10.1016/j.biortech.2009.11.086>
- [32] Poompradub S.: Soft bio-composites from natural rubber (NR) and marine products. in ‘Chemistry, manufacture and applications of natural rubber’ (eds.: Kohjiya S., Ikeda Y.) Woodhead Publishing, London, 303–324 (2014).
<https://doi.org/10.1533/9780857096913.2.303>

- [33] Flory P. J., Rehner J.: Statistical mechanics of cross-linked polymer networks I. Rubberlike elasticity. *Journal of Chemical Physics*, **11**, 512–520 (1943).
<https://doi.org/10.1063/1.1723791>
- [34] Sheehan C. J., Bisio A. L.: Polymer/solvent interaction parameters. *Rubber Chemistry and Technology*, **39**, 149–192 (1966).
<https://doi.org/10.5254/1.3544827>
- [35] Jana G. K., Mahaling R. N., Das C. K.: A novel devulcanization technology for vulcanized natural rubber. *Journal of Applied Polymer Science*, **99**, 2831–2840 (2006).
<https://doi.org/10.1002/app.22984>
- [36] Ng S-C., Gan L-H.: Reaction of natural rubber latex with performic acid. *European Polymer Journal*, **17**, 1073–1077 (1981).
[https://doi.org/10.1016/0014-3057\(81\)90030-6](https://doi.org/10.1016/0014-3057(81)90030-6)
- [37] Baker C. S. L., Gelling I. R., Newell R.: Epoxidized natural rubber. *Rubber Chemistry and Technology*, **58**, 67–85 (1985).
<https://doi.org/10.5254/1.3536059>
- [38] Jana G. K., Das C. K.: Recycling natural rubber vulcanizates through mechanochemical devulcanization. *Macromolecular Research*, **13**, 30–38 (2005).
<https://doi.org/10.1007/BF03219012>
- [39] Hirayama D., Saron C.: Chemical modifications in styrene–butadiene rubber after microwave devulcanization. *Industrial and Engineering Chemistry Research*, **51**, 3975–3980 (2012).
<https://doi.org/10.1021/ie202077g>
- [40] Aoudia K., Azem S., Hocine N. A., Gratton M., Pettarin V., Seghar S.: Recycling of waste tire rubber: Microwave devulcanization and incorporation in a thermoset resin. *Waste Management*, **60**, 471–481 (2017).
<https://doi.org/10.1016/j.wasman.2016.10.051>
- [41] Chou L-H., Yang C-K., Lee M-T., Shu C-C.: Effects of partial oxidation of crumb rubber on properties of rubberized mortar. *Composites Part B: Engineering*, **41**, 613–616 (2010).
<https://doi.org/10.1016/j.compositesb.2010.09.009>
- [42] Sato S., Honda Y., Kuwahara M., Kishimoto H., Yagi N., Muraoka K., Watanabe T.: Microbial scission of sulfide linkages in vulcanized natural rubber by a white rot basidiomycete, *Ceriporiopsis subvermispora*. *Biomacromolecules*, **5**, 511–515 (2014).
<https://doi.org/10.1021/bm034368a>
- [43] Horikx M. M.: Chain scissions in a polymer network. *Rubber Chemistry and Technology*, **29**, 1166–1173 (1956).
<https://doi.org/10.5254/1.3542617>
- [44] Joseph A. M., George B., Madhusoodanan K. N., Alex R.: Effect of devulcanization on crosslink density and crosslink distribution of carbon black filled natural rubber vulcanizates. *Rubber Chemistry and Technology*, **89**, 653–670 (2016).
<https://doi.org/10.5254/rct.16.84819>