RSC Advances

PAPER

Cite this: *RSC Adv.*, 2026, **16**, 26205

Deep eutectic solvent-assisted bacterial devulcanization, detoxification, and degradation of waste tyre rubber

Pritish R. Shukla, [a] Utpal Roy *[a] and Sunil Bhand [b]

Received 25th February 2026
Accepted 22nd April 2026

DOI: 10.1039/d6ra01664b

rsc.li/rsc-advances

The biodegradation of waste tyre rubber (WTR) is hindered by the presence of sulfur and additives, which limit microbial mineralization of the rubber polymers. To overcome this constraint, a hybrid chemi-biological approach is developed, wherein pretreatment with a reusable choline chloride/urea (ChCl/Ur) deep eutectic solvent (DES) is employed. Ground tyre rubber (GTR) is subjected to DES-assisted thermochemical pretreatment, followed by biological treatment using the *Rhodococcus rhodochrous* RPK1 bacterial strain in mineral salts medium for 28 days. The DES pretreatment significantly enhances biodegradation efficiency, resulting in a significant improvement over biological treatment alone. Structural, elemental, and thermal analyses confirm partial devulcanization, cleavage of sulfur crosslinks, removal of zinc additives, and reduced thermal stability. Crosslink density decreased by 43.6%, and Horikx analysis indicates a mixed degradation mechanism. The results demonstrate that DES pretreatment effectively reduces limiting factors, thereby improving rubber bioavailability. This hybrid strategy provides a viable framework for enhancing the biodegradation of WTR and supports a sustainable rubber recycling pathway.

1. Introduction

The accumulation of End-of-Life tyres (ELTs) has emerged as a persistent challenge in global solid waste management. With more than 1.5 billion tyres discarded annually worldwide,[1] waste tyres represent a large non-biodegradable stockpile. Their resistance to degradation is attributed to sulfur and other additives, which inhibit the natural degradation process.[2] As a result, conventional disposal practices such as landfilling and incineration pose serious environmental and public health risks. Tyre stockpiles act as breeding grounds for disease vectors and are susceptible to fires that release hazardous emissions, including CO_2, CO, SO_2, and polycyclic aromatic hydrocarbons (PAHs).[3] Although recycling approaches such as mechanical grinding and pyrolysis are practiced, these methods are often energy-intensive and generate products with limited economic value.[4] Consequently, the development of sustainable, low-energy engineering solutions for waste tyre management remains a priority.

Biodegradation has been explored as an alternative strategy for treating rubber waste. Certain microorganisms, particularly members of the phylum Actinobacteria (commonly referred to as actinomycetes), have evolved the ability to utilize rubber as a carbon source.[5] This process is mediated by extracellular enzymes such as latex clearing proteins (*Lcp*), which catalyze the oxidative cleavage of *cis*-1,4-polyisoprene double bonds.[6] The resulting oligo-isoprenoids are subsequently transported into the cell and metabolized *via* the β-oxidation pathway, leading to their mineralization into biomass, CO_2, and H_2O.[7] In parallel, microbial desulfurization enables the removal of sulfur from rubber.[8] Despite these capabilities, the biodegradation of tyre rubber remains inefficient due to mass-transfer limitations, diffusional constraints, and the inhibitory effects of toxic additives present in tyre such as zinc oxide, zinc stearate, benzothiazole derivatives, dicyclohexylamine, aromatic processing oils rich in PAHs, and antioxidants such as butylated hydroxytoluene (BHT) and *N*-phenyl-*N*′-isopropyl-*p*-phenylenediamine (IPPD).[9] The presence of sulfur-crosslinks restricts microbial adhesion and enzymatic accessibility. Additives such as activators, accelerators, and antioxidants further inhibit microbial growth, resulting in slower degradation rates that often require weeks or months to achieve mass loss.[10] Effective biological degradation, therefore, necessitates the integration of detoxification, devulcanization, and depolymerization processes.

To address these limitations, deep eutectic solvents (DESs) have emerged as promising pretreatment agents owing to their low volatility, biodegradability, selectivity, and effectiveness in solubilizing metal oxides and extracting sulfur-containing compounds from complex matrices.[11,12] By swelling the rubber matrix, DES pretreatment promotes the removal of toxic

[a]Department of Biological Sciences, Birla Institute of Technology and Science-Pilani, K. K. Birla Goa Campus, NH17B, Bypass Road, Zuarinagar, Goa, 403726, India. E-mail: uroy@goa.bits-pilani.ac.in
[b]Department of Chemistry, Birla Institute of Technology and Science- Pilani, K. K. Birla Goa Campus, NH17B, Bypass Road, Zuarinagar, Goa, 403726, India

additives (detoxification) and sulfur crosslinks (devulcanization), thereby enhancing microbial accessibility and subsequent biodegradation.[9]

In this study, a two-step hybrid process is developed in which a choline chloride/urea (ChCl/Ur) DES pretreatment is applied to enhance the bioaccessibility of rubber towards microbial degradation using a model rubber-degrading bacterium, *Rhodococcus rhodochrous* RPK1. The performance of this approach is evaluated through biomass formation, mass reduction, surface morphology, elemental composition, thermal behaviour, and the removal of sulfur crosslinks and toxic additives.

2. Experimental

2.1 Source of rubber and chemicals

GTR (400–600 μm) was obtained from a waste tyre recycling facility. ChCl and urea (>99% purity; HiMedia Laboratories Pvt. Ltd) were used as received. All microbiological chemicals and reagents were of analytical grade and used without further purification.

2.2 Bacterial strain and culture conditions

Rhodococcus rhodochrous RPK1 (DSM103064; DSMZ, Germany) was cultured in nutrient broth for inoculum preparation and transferred to 100 mL mineral salts medium (MSM; pH 7.0 ± 0.05, $Na_2HPO_4 \cdot 12H_2O$ (9.0 g L^{-1}), KH_2PO_4 (1.5 g L^{-1}), $H_8N_2SO_4$ (1.0 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.2 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.02 g L^{-1}), and Fe(III) ammonium citrate 1.2 mg L^{-1}) and were incubated in 250 mL flasks with 1% (w/v) untreated GTR or ChCl/Ur DES-pretreated GTR as a sole carbon source.[13]

2.3 DES preparation

ChCl and Ur were mixed at a 1 : 2 molar ratio and heated at 70 °C with continuous stirring until a clear, homogeneous deep eutectic solvent was formed.[11,12,14,15]

2.4 Pretreatment of GTR using ChCl/Ur DES

GTR was washed three times with 70% ethanol and vacuum-dried at 45 °C using a SpeedVac concentrator (Concentrator Plus, Eppendorf AG, Germany) to constant weight. The dried GTR was mixed with DES at a 1 : 40 (w/w) ratio, allowed to swell at room temperature for 24 h, and sonicated in a water bath at 37 kHz for 2 h, and then heated at 180 °C with stirring for 45 minutes. After treatment, the GTR was washed with 70% ethanol, filtered, and dried at 50 °C to constant weight.[11,12] Following the pretreatment step, the spent ChCl/Ur DES was separated from the treated GTR by filtration so that it could be directly reused in the next pretreatment cycle. As reported in our previous work (Shukla *et al.*, 2026),[15] the spent DES retained its partial eutectic character, as confirmed by ^1H and ^{13}C NMR and ATR-FTIR, and exhibited comparable devulcanization efficiency in the second treatment cycle, which supports solvent reusability. After two pretreatment cycles, when the spent DES could no longer be effectively reused, aerobic biodegradation constitutes an environmentally viable disposal route. The critical assessment of the sustainability and biodegradability of ChCl/Ur DES is comprehensively studied by Nejrotti *et al.* (2022),[16] based on the OECD 301D guidelines.[17]

2.5 Isothermal thermogravimetric analysis (TGA) of ChCl/Ur DES

Isothermal TGA was performed using a thermogravimetric analyzer (TA Instruments, Waters, SDT650 Trios) to evaluate the thermal stability or mass loss of the process components (GTR, ChCl/Ur DES, and ChCl/Ur DES-GTR mixture) at the pretreatment temperature (180 °C). Approximately 8–10 mg of each sample was placed in an alumina pan and analyzed under a N_2 atmosphere with a flow rate of 100 mL min^{-1}. The temperature program consisted of heating from room temperature to 180 °C at 10 °C min^{-1}, followed by an isothermal hold at 180 °C for 60 min. The mass change (%) was recorded as a function of time.

2.6 Cultivation of *R. rhodochrous* RPK1 with GTR

DES-pretreated and untreated GTR (1% w/v) were separately added to 100 mL MSM in 250 mL flasks and inoculated with a 10% (v/v) NB-grown culture (OD_{600} of 0.8–1.0). Cultures were incubated at 30 °C and 120 rpm for 28 days with biotic and abiotic controls. Cell growth and viability were monitored every 7 days by OD_{600} and colony-forming unit (CFU mL^{-1}) counts. Statistical differences were evaluated by one-way ANOVA at day 14 (maximum viability), with results reported as mean ± SD ($n = 2$) and significance set at $p < 0.05$.

The weight of rubber particles was calculated before (w_1) and after degradation (w_2) using eqn (i):

$$WL = [(w_1 - w_2)/w_1] \times 100 \quad \text{(i)}$$

where, WL: weight loss, %; w_1: initial weight of GTR before degradation, g; w_2: final weight of GTR after degradation, g.

2.7 Characterization of treated GTR

2.7.1 Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). ATR-FTIR spectra were recorded using a PerkinElmer Spectrum Two, U.S., to evaluate structural changes in GTR associated with detoxification, devulcanization and biodegradation, including –C–S–, –S–S–, Zn-stearate and –N–O– related bonds, C=C bending, and –CH of CH_2 and CH_3 stretching vibrations.[12] 2–5 mg of dried GTR were measured in the 400–4000 cm^{-1} wavenumber range at 4 cm^{-1} resolution, with 45 scans per sample.[18]

2.7.2 Field emission scanning electron microscopy (FE-SEM) and energy dispersive X-ray spectroscopy (EDS). Treated GTR particles were removed from cultures and fixed with a 2.5% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.4, for 24 h. The particles were washed with sterile double-distilled water and dehydrated for 10 min in graded ethanol (30%, 50%, 70%, 90% and absolute ethanol). Dehydrated GTR samples were subjected to critical point drying with liquid CO_2. The samples were then mounted on aluminium stubs using a copper strip, and gold sputtered.[19] Morphological changes on the rubber surface were examined using FESEM (FEI Quanta

250 FED) at 20 kV voltage acceleration coupled with EDS, which detected changes in the elemental composition of treated rubber particles at a microscopic level at 25 °C and one atmospheric pressure.[20]

2.7.3 Inductively coupled plasma- optical emission spectroscopy (ICP-OES). Total zinc content in GTR samples was quantified by ICP-OES following nitric acid digestion.[21] 0.1 g GTR was digested in 5 mL concentrated HNO_3 (68.9% v/v) for 24 h at room temperature, diluted to 250 mL with deionized water (2% v/v HNO_3), and filtered through a 0.2 μm GF + PVDF syringe filter. Zn analysis was performed using an Agilent ICP-OES (MY2221CQ01; Version 7.6.0.12121 with Firmware version 5590) system equipped with an autosampler and concentric nebulizer, with external calibration using multi-element standards in 2% HNO_3. Zinc was quantified at 213.857 nm, and concentrations (ppm) were converted to weight percentage using eqn (ii):

$$\text{Total zinc\% (w/w)} = \{[C \times V]/[m \times 1000\ (\text{mg g}^{-1})]\} \times 100 \quad \text{(ii)}$$

where, C: measured concentration (ppm); V: final volume of the solution (0.25 L); m: mass of the GTR (0.1 g).

2.7.4 Elemental (CHNS) analysis of treated GTR. Total carbon and sulfur contents were determined by CHNS elemental analysis using an Elementar Unicube analyzer. Approximately 2 ± 0.02 mg of GTR was combusted at high temperature, and carbon and sulfur percentages were quantified from the resulting CO_2 and SO_2 gases, respectively.[22]

2.7.5 Sol fraction, crosslink density, molecular weight between crosslinks, and Horikx plot anlaysis of treated GTR. The crosslink density of GTR was determined by equilibrium solvent swelling in *n*-hexane using the Flory-Rehner approach.[23,24] Following acetone extraction for 24 h, dried GTR samples (0.5 g) were swollen in *n*-hexane at 25 °C for 72 h, blotted, and weighed to determine the swollen rubber volume fraction (V_r), which was used to determine crosslink density (ν) using eqn (iii):

$$\nu = [-\ln(1 - V_r) - V_r - \chi V_r^2]/[V_1(V_r^{(1/3)} - V_r/2)] \quad \text{(iii)}$$

where, ν = crosslink density (mol cm^{-3}); V_r = volume fraction of rubber in the swollen network. V_1 = molar volume of *n*-hexane (131.6 cm^3 mol^{-1}); χ = Flory–Huggins interaction parameter for rubber/*n*-hexane system (taken as 0.45,[25] based on Hildebrand's solubility parameter).

The average molecular weight between crosslinks (M_c) was calculated from crosslink density (ν) using eqn (iv):

$$M_c = \rho/\nu \quad \text{(iv)}$$

where, ρ is the constant density of rubber (1.15 g cm^{-3}) for all the samples, so that variations in M_c reflect changes in network structure rather than density differences.

The sol fraction of GTR was determined by *n*-hexane extraction. Dried samples with an initial mass (m_i) were extracted under gentle agitation at room temperature for 3 days, rinsed with fresh *n*-hexane, and dried at 70 °C to a constant final mass (m_f). The sol fraction was calculated using eqn (v):

$$\text{Sol (\%)} = [1 - (m_f/m_i) \times 100]^{26} \quad \text{(v)}$$

Horikx analysis was used to determine the dominant degradation mechanism by correlating sol fraction with the relative decrease in crosslink density.[27] For each sample, sol fraction and crosslink density were plotted against the theoretical Horikx curves corresponding to ideal crosslink scission and main-chain scission, calculated according to the original equations and verified for rubber vulcanizates.[28,29]

2.7.6 Thermal and kinetics analyses. The thermal behaviour of untreated and treated GTR was investigated using a TA Instruments (Waters; SDT650 Trios). 5–10 mg of each sample was heated from 25 °C to 600 °C at 10 °C min^{-1} under nitrogen (100 mL min^{-1}).[30] Thermal decomposition kinetics were evaluated at three heating rates (β = 5, 10, and 20 °C min^{-1}). The first decomposition temperature peak (T_p) was determined from the derivative thermogravimetric (DTG) curve at each heating rate.[31] The activation energy was determined using the Kissinger method[32] from the linearized Kissinger eqn (vi), with corresponding R^2 values reported to fit quality.

$$\ln(\beta/T_p^{\,2}) = -(E_a/RT_p) + \ln(AR/E_a) \quad \text{(vi)}$$

where, β = heating rate (K min^{-1}), T_p = DTG peak, in K, E_a = activation energy (J mol^{-1}), R = gas constant (8.314 J mol^{-1} K^{-1}), A = Arrhenius factor (min^{-1}).

2.7.7 Detection of degraded products. Oligo-isoprenoid formation during rubber degradation was detected using Schiff's reagent as described by Linos *et al.* (2000).[33] Briefly, 10 mL of Schiff's reagent was added to 10 mL of the sample, mixed and incubated at room temperature for 5 min. The colour development was visually observed. Biotic and abiotic controls were treated under the same conditions. Additionally, LC-MS analysis was performed to identify degradation products. Cell-free supernatant from unpretreated GTR and ChCl/Ur DES-treated GTR containing *R. rhodochrous* culture was extracted by liquid–liquid extraction (LLE) using ethyl acetate at a sample : solvent ratio of 1 : 1 (v/v). The mixture was vigorously vortexed for 5 min, centrifuged at 10 000 rpm and 4 °C for 10 min to separate the phases. The organic phase containing the degradation products was collected and evaporated using a SpeedVac concentrator. The sample was extracted three times, reconstituted in 1 mL of the extraction solvent, and transferred into a 2 mL glass vial. The column was equilibrated with 50% methanol : water. The sample was analyzed using electrospray ionization (ESI) in full-scan mode (m/z = 100–1000). The data acquisition and processing were performed using Agilent Triple Quadrupole (V12.0.313.0). An injection volume of approximately 7 μL was used for analysis, and peaks corresponding to putative oligo(*cis*-1,4-isoprene) derivatives were interpreted based on previously reported fragmentation patterns.[34] Chromatograms and identified oligo-isoprenoids are provided in the SI as Table S3 and Fig. S2.

2.7.8 Total protein estimation. The total protein concentration in *R. rhodochrous* cultures containing untreated or DES-pretreated GTR was quantified by Lowry's method using Bovine

Serum Albumin (BSA) as the standard. Absorbance was measured at 740 nm, with protein levels monitored at 7 days intervals over a 4 weeks incubation period.[35,36]

2.7.9 Laccase and peroxidase enzyme activity assay. Laccase and peroxidase activities were assayed spectrophotometrically at 420 nm using 2,20-azino-bis(3-ethylbenzothiazoline 6-sulfonate) (ABTS). For laccase activity, 100 μL of the sample was added to a solution of 900 μL of 10 mM ABTS and 0.2 M sodium acetate (pH 5.0), while peroxidase activity was measured using 100 μL of the sample with 800 μL of 10 mM ABTS and 10 mM sodium acetate (pH 5.0), and 100 μL of 20 mM hydrogen peroxide (H_2O_2). Enzymatic activities were monitored on days 0, 14, and 28.[18]

2.8 Biocompatibility of ChCl/Ur DES with *R. rhodochrous* RPK1

2.8.1 Kirby–Bauer test. The inhibitory effect of ChCl/Ur DES on *R. rhodochrous* RPK1 was evaluated using a disc diffusion assay.[37] Cells grown in sterile NB at 30 °C under constant agitation for 36 h were spread on nutrient agar plates, and sterile 5 mm paper discs absorbed with 20 μL DES were placed on the agar surface. Plates were incubated at 30 °C for 36 h.[38,39] The extent of the inhibition zone was taken as an indicator of the antibacterial activity of the DES against the test microorganism.

2.8.2 MIC determination assay. The minimum inhibitory concentration (MIC) of ChCl/Ur DES against *R. rhodochrous* RPK1 and *Escherichia coli* ATCC 25922 was determined by a two-fold broth microdilution assay, according to the standard antimicrobial susceptibility testing protocols for liquid compounds.[40] Serial dilutions of DES were prepared in NB, inoculated with 1×10^6 to 1×10^7 CFU mL^{-1} in 96-well plates, and incubated at 30 °C for 24 h. The MIC was defined as the lowest DES concentration (mg mL^{-1}) completely inhibiting visible bacterial growth.

3. Results and discussion

3.1 Effect on the growth of *R. rhodochrous* RPK1

Cell biomass and viability were used to assess rubber bioavailability and the physiological response of *R. rhodochrous* RPK1 grown on GTR as the sole carbon source. Cultures containing DES-pretreated GTR, untreated GTR, and biotic controls were compared to evaluate the effect of pretreatment on growth dynamics and cell viability.

As shown in Fig. 1, cell biomass and viability reached a maximum on day 14, followed by a gradual decline, likely due to nutrient depletion or the accumulation of inhibitory metabolites. Cultures supplemented with DES-pretreated GTR consistently showed higher cell viability than those containing untreated GTR or the biotic control.[41] Cell counts increased from 4.25×10^7 CFU mL^{-1} to 1.92×10^9 CFU mL^{-1} within 14 days for pretreated GTR, whereas untreated GTR showed a maximum of 1.33×10^9 CFU mL^{-1}. One-way ANOVA of log-transformed CFU data showed a significant treatment effect ($p = 0.0013$), with cultures containing DES-pretreated GTR exhibiting a 43.87% higher cell viability than those with untreated GTR. This enhancement indicates improved microbial access to the rubber substrate, likely resulting from partial removal of inhibitory additives and loosening the sulfur-crosslinked network.[9]

R. rhodochrous degrades rubber primarily through the secretion of *Lcp* enzyme, which catalyzes the oxidative cleavage of *cis*-1,4-polyisoprene into oligo-isoprenoids[42] that are subsequently metabolized *via* β-oxidation.[5,13] The enhanced growth observed with DES-pretreated GTR therefore indicates that pretreatment facilitated enzymatic access to the polymer backbone. The limited growth observed with untreated GTR

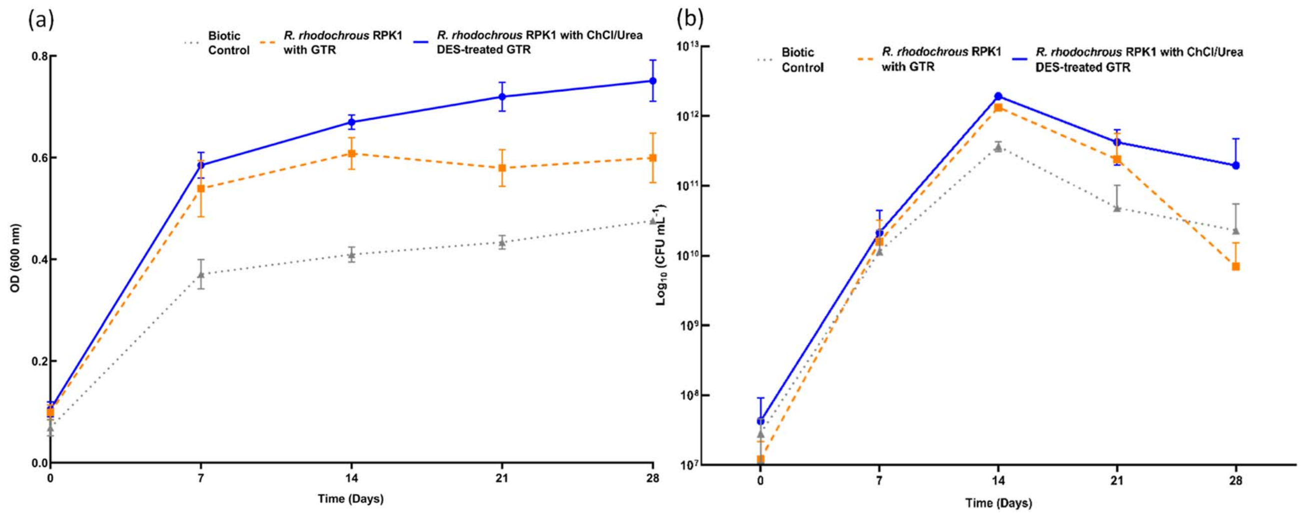


Fig. 1 Growth behaviour of *Rhodococcus rhodochrous* RPK1 in MSM supplemented with untreated GTR or ChCl/urea DES-pretreated GTR as the sole carbon source. (a) Cell biomass (OD at 600 nm). (b) Cell viability (CFU mL^{-1}). Values represent mean ± SD ($n = 2$); ($p = 0.0013$).

highlights the inhibitory effects of sulfur, fillers, and low-molecular-weight additives commonly present in tyre rubber. The partial removal of these components during DES pretreatment likely exposed polymeric chains and reduced enzymatic inhibition, thereby enhancing substrate bioavailability, consistent with previous reports by Altenhoff *et al.* (2019), Berekaa *et al.* (2000), and Chittella *et al.* (2025).[9,10,33]

3.2 ATR-FTIR analyses of treated GTR

The FTIR spectra (Fig. 2) revealed chemical changes in GTR following biodegradation as well as combination treatment. Compared to the abiotic control, a reduction in intensity at 669 cm^{-1}, corresponding to C–S stretching, was observed for DES-pretreated GTR, providing probable evidence of cleavage of sulfur linkages, whereas negligible change was observed in the biologically-treated GTR sample alone. Both *R. rhodochrous*-treated GTR, and pretreated and *R. rhodochrous*-treated GTR showed reduced intensity at 1538 cm^{-1}, associated with C=C stretching (unsaturated hydrocarbon backbone) and Zn- and N–O-related compounds,[11] suggesting partial polymeric oxidation and additive reduction. A minimal increase in intensity at 1630 cm^{-1} was observed, which might be corresponding to the C=C stretching of alkenes and aromatic groups, or conjugated C=O groups.[10,43] However, the change is minimal, and definitive reaction information cannot be drawn from this band alone. A slight increase in relative intensity was observed at 2360 cm^{-1} after treatments, which might suggest either the formation of a new unsaturated group or the formation of conjugated groups as a result of polymer backbone cleavage.[44] Reductions in symmetric –CH stretching of $-CH_3$ at 2916 cm^{-1} and –CH stretching of $-CH_2-$ at 2848 cm^{-1}, suggesting polymer degradation.[18]

A slight increase in broadening of the peak at around 3280 cm^{-1} was also observed in both treatments, reflecting the possible introduction of a hydroxyl group (from alcohols and carboxylic acids) or N–H stretching arising from oxidation and the presence of residual DES grafted onto the polymeric chain during treatment.[12] Overall, these changes in the FTIR spectrum suggested that DES pretreatment facilitated sulfur bond cleavage and partial additive removal, thereby aiding in increased susceptibility of GTR to biodegradation.

3.3 Morphological changes in treated GTR

Scanning microscopy was used to examine cell morphology, bacterial cell-rubber surface interaction, and surface changes in treated GTR, with micrographs recorded at 2500×, 5000×, 100 00×, and 250 00× magnifications (Fig. 4).[33]

Untreated GTR exhibited a smooth surface characteristic of vulcanized rubber, whereas biologically-treated and combination-treated samples showed increased surface roughness with raised clustered structures, including micropits, cracks, and holes,[45] as shown in Fig. 4. These features were relatively more pronounced in the combined DES-biological-treated samples, indicating increased surface degradation. These morphological changes were attributed to the biofilm-forming ability of *R. rhodochrous*, which adheres to the surface and utilizes GTR as the sole carbon source,[13] as shown in Fig. 3. The pits and holes indicated C–C bond breakage.[11,41]

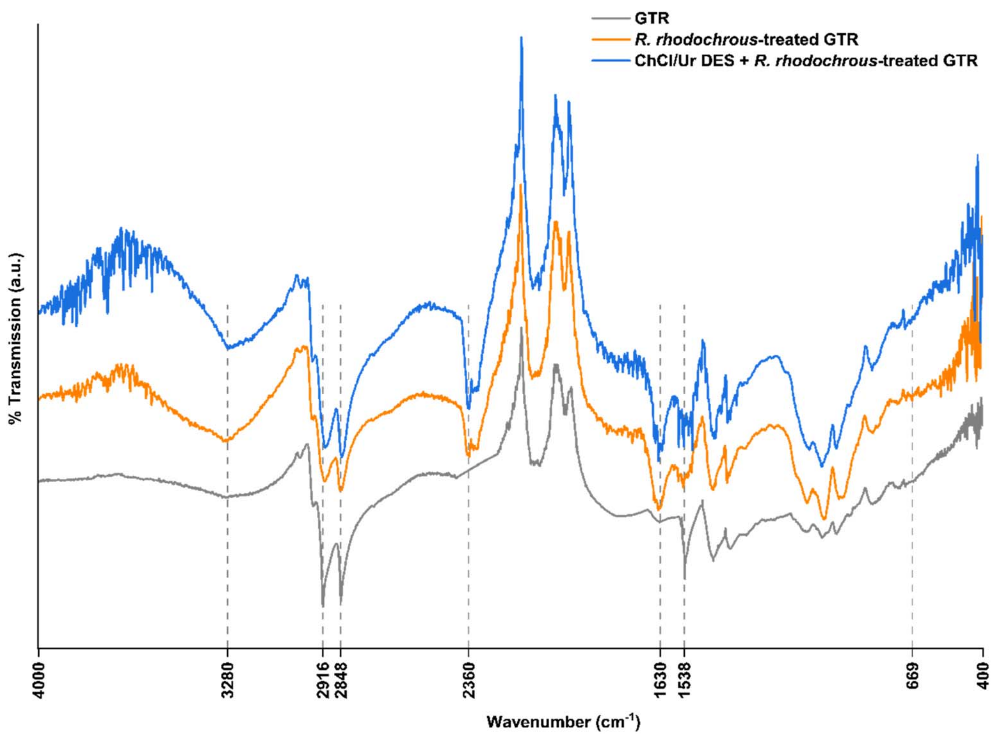


Fig. 2 ATR-FTIR spectra of GTR. Untreated GTR (grey), *R. rhodochrous*-treated GTR (orange), and ChCl/Urea DES-pretreated + *R. rhodochrous*-treated GTR (blue). Dashed vertical lines indicate key wavenumbers discussed in the text.

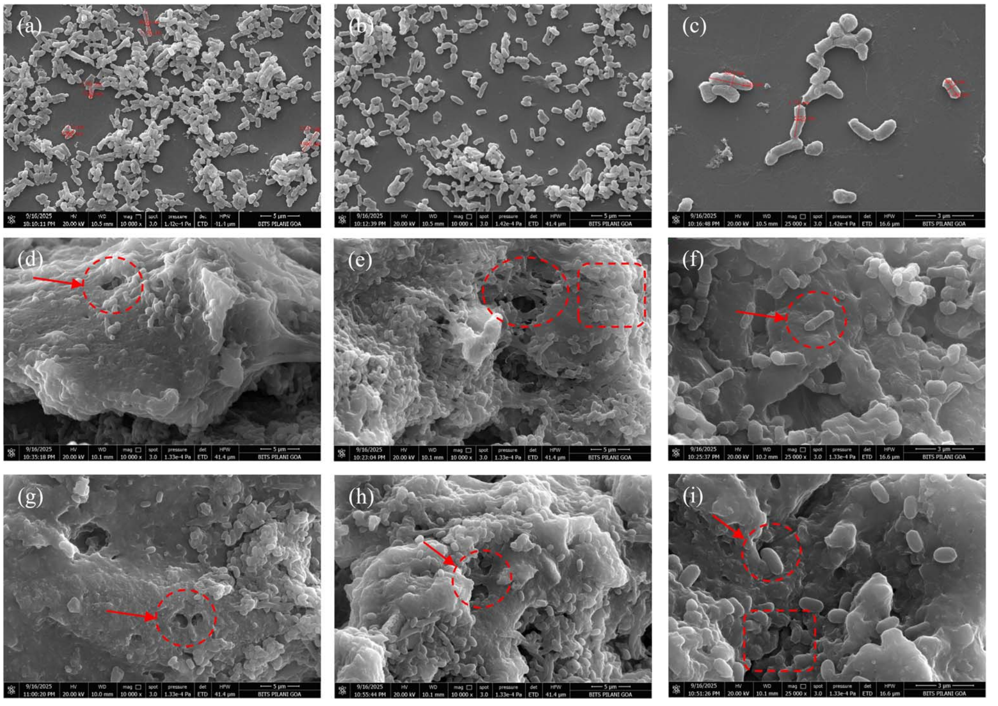


Fig. 3 Scanning electron micrographs of bacterial cell morphology, cell adhesion, and biofilm formation of *Rhodococcus rhodochrous* RPK1 on GTR during the biodegradation process. Shown are the biotic control featuring growth of short rods and ovoid cell shapes of size ~0.5–1 μm wide and 1–3 μm long, and arranged as single cells, pairs, and small clusters (a)–(c). Attachment of cells on the rubber surface with visible biofilm (extracellular matrix), cells in and around pits, cracks, and pores on the rubber (d)–(i). Dashed red circles and boxes represent bacterial cell adhesion, biofilm formation, cracks, and holes. Scale bars: 3 and 5 μm.

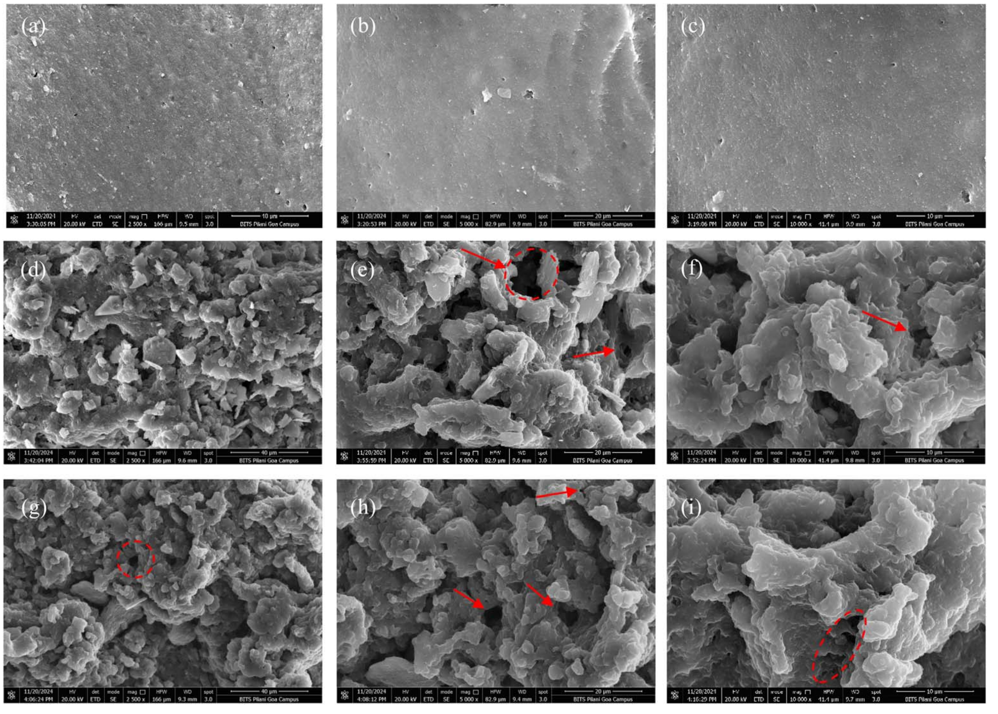


Fig. 4 Scanning electron micrographs of degraded GTR after completion of the biodegradation process. Shown are the untreated GTR featuring a smooth surface (a)–(c). *Rhodococcus rhodochrous*-treated GTR (d)–(f). ChCl/urea DES-pretreated + *Rhodococcus rhodochrous*-treated GTR (g)–(i). Dashed red arrows and circles represent micropits, cracks, and holes. Scale bars: 10, 20, and 40 μm.

Being oxidative in nature, the chemical pretreatment increased the porosity of the rubber surface, indicating that DES facilitated bacterial penetration into deeper layers, thereby increasing enzymatic activity.[20] SEM imaging was primarily intended to confirm surface structural changes qualitatively. However, these surface alterations are not sufficiently pronounced to be visually distinguished by surface morphology, as revealed in images. Hence, evidence distinguishing the extent of the effect of treatments is supported by EDS area-based analysis, ICP-OES zinc quantification, and CHNS elemental analysis.

3.4 Elemental composition changes in treated GTR

Elemental analysis (Table 1) was used to assess carbon utilization and changes in the elemental composition of GTR following chemical and biological treatments. The EDS area-based analysis revealed variations in carbon, sulfur, and zinc contents, reflecting polymer degradation and additive removal.[46,47] Bacterial treatment alone resulted in a 2.56 wt% decrease in carbon content, whereas the combination of DES pretreatment and biological treatment led to a substantially higher carbon reduction (12.67%), corresponding to a 4.94 increase in carbon utilization. An increase in oxygen content was observed in treated samples, indicative of an oxidation reaction. Sulfur removal was highest for the combined treatment, with sulfur content reducing from 4.05 wt% to 2.18 wt%, corresponding to a 46.17% reduction, as summarized in Table 1. These results indicate the hybrid effect of DES-assisted devulcanization and microbial activity, leading to enhanced sulfur reduction and polymer degradation compared to either treatment individually.[48]

Similarly, zinc content decreased by 68.45% following the combined DES-biological treatment, indicating effective detoxification of zinc-based additives. However, the Zn wt% increased from 0.8 wt% after DES-only treatment to 1.06 wt% after the combined treatment due to substantial carbon utilization by bacteria (12.53%), resulting in relative enrichment of inorganic zinc and silica. A concurrent increase in nitrogen content is attributed to residual microbial biomass or DES associated with the rubber surface.[11] Comparison with DES-only treatment showed that DES selectively reduced sulfur content by up to 35.8% without significantly affecting carbon content, demonstrating its selectivity for desulfurization and detoxification. This pretreatment increased accessibility of polyisoprene C=C bonds, thereby enhancing microbial utilization of rubber carbon. Consequently, the hybrid treatment achieved nearly twice the sulfur removal and significantly higher biodegradation efficiency than biological treatment alone, consistent with the involvement of *Dsz* enzymes and the sulfur-specific 4S desulfurization pathways.[49]

3.5 ICP analysis of zinc content

ICP-OES analysis showed that untreated GTR contained 1.94 ± 0.06 wt% zinc. Biological treatment alone resulted in an apparent increase to 2.25 ± 0.10 wt% Zn, suggesting microbial utilization of carbon (1.63% mass loss) rather than zinc, leading to relative enrichment of inorganic zinc. As *R. rhodochrous* is neutrophilic, it lacks the acid-producing capacity required to solubilize zinc oxides or zinc stearate, and the absolute zinc content therefore remained unchanged.[50] DES-only treatment reduced zinc content to 1.30 ± 0.17 wt%, achieving 33% removal efficiency, demonstrating the effectiveness of pretreatment in extracting zinc-related compounds.[51] Sequential DES pretreatment followed by biological treatment yielded a Zn content of 1.73 ± 0.11 wt%, showing initial zinc removal during pretreatment and subsequent concentration of residual Zn due to enhanced carbon mineralization, further indicating the role of DES-assisted detoxification for efficient biodegradation.

3.6 Detection of carbon and sulfur content changes by CHNS analysis

CHNS analysis further confirmed the extent of devulcanization and biodegradation, demonstrating that sulfur removal facilitates efficient rubber degradation (Fig. 5). Untreated GTR contained 88.66 wt% carbon and 2.34 wt% sulfur. Following DES pretreatment and biological treatment, total carbon content decreased by 12.57% (to 77.51 wt%) and sulfur content by 21.52% (to 1.84 wt%), indicating enhanced polymer mineralization. In contrast, biological treatment alone resulted in only 6.18% carbon and 16.87% sulfur reduction, reflecting the limited bioaccessibility of vulcanized GTR. DES treatment alone selectively reduced sulfur by up to 17.09% with a slight reduction in total carbon content (4.24%), confirming selective devulcanization. The values are given in Table S1 in the SI. The pretreatment increased twice the carbon utilization and approximately 1.28 times the sulfur reduction, thereby supporting the hypothesis that devulcanization facilitates the mineralization of rubber.[48] Previous studies have shown that pretreatments or microbial consortia can enhance rubber

Table 1 Elemental composition (wt%) of ground tyre rubber (GTR) before and after treatment, determined by EDS: untreated GTR, *R. rhodochrous*-treated GTR, ChCl/urea DES-pretreated + *R. rhodochrous*-treated GTR, and ChCl/Urea DES-treated GTR[a]

Name	C (wt%)	N (wt%)	O (wt%)	Si (wt%)	S (wt%)	Zn (wt%)
GTR	86.45	0.05	4.65	1.40	4.05	3.36
R. rhodochrous-treated GTR	84.23	0.70	10.55	1.40	3.10	2.06
DES-treated GTR	85.9	1.20	7.20	1.30	2.60	0.80
DES-pretreated + *R. rhodochrous*-treated GTR	75.50	1.72	16.18	1.55	2.18	1.06

[a] EDS values are given as weight percentage (wt%) on the particle surface; means of $n = 2$ measurements.

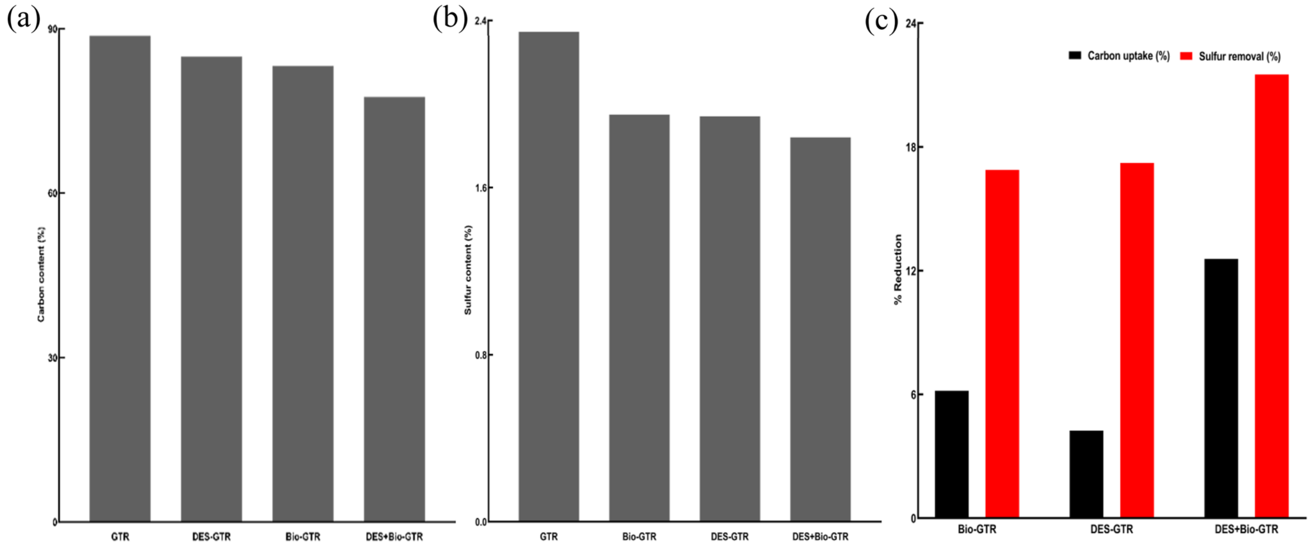


Fig. 5 Total carbon and sulfur contents of ground tyre rubber (GTR) before and after treatment, determined by CHNS elemental analysis. (a) Total carbon content (%). (b) Total sulfur content (%). (c) Percentage reduction in carbon utilization and sulfur removal across treatment conditions. GTR, untreated ground tyre rubber; DES-GTR, ChCl/Ur DES-treated GTR; Bio-GTR, *R. rhodochrous*-treated GTR; DES + Bio-GTR, ChCl/Ur DES-pretreated + *R. rhodochrous*-treated GTR.

desulfurization and degradation. Yao *et al.* (2013)[47] reported a 62.5% sulfur reduction in waste latex rubber using *Alicyclobacillus* spp. with Tween 80 as a cotreatment agent. Cui *et al.* (2016)[52] demonstrated improved desulfurization of GTR using mixed cultures of *Sphingomonas* and *Gordonia*, achieving a 32.4% sulfur reduction and a 9.5% decrease in crosslink density. More recently, Chittella *et al.* (2025)[9] showed that the DES pretreatment significantly enhanced the biodegradation of natural rubber gloves by *Klebsiella aerogenes*, with degradation increasing with pretreatment severity. Andler R *et al.* (2025)[53] further reported crosslink density reduction in vulcanized rubber blends following a sequential biological pretreatment and biodesulfurization. To date, no studies have reported the use of DES as a thermochemical pretreatment followed by subsequent *R. rhodochrous*-mediated biodegradation of GTR.

3.7 Change in crosslink density, average molecular weight between crosslinks, sol fraction, and Horikx plot analysis of treated GTR

Crosslink density of GTR was determined using the Flory–Rehner approach to evaluate the effectiveness of chemical and biological treatments. Untreated GTR exhibited a crosslink density of $9.61 \pm 0.16 \times 10^{-5}$ mol cm^{-3} and M_c of 1.20×10^4 g mol^{-1} (Table 2). Biological treatment with *R. rhodochrous* alone reduced the crosslink density to $8.09 \pm 0.29 \times 10^{-5}$ mol cm^{-3} (15.81% reduction), with a corresponding increase in M_c to 1.42×10^4 g mol^{-1}, consistent with the reported ability of *R. rhodochrous* to cleave C–S and S–S bonds in vulcanized rubber. DES pretreatment alone resulted in a greater reduction in crosslink density (26.84%) and increased M_c to 1.64×10^4 g mol^{-1}, indicating partial devulcanization.[12] The combined DES pretreatment and biological treatment produced the most pronounced effect, decreasing crosslink density to $5.44 \pm 0.12 \times 10^{-5}$ mol cm^{-3} (43.6% reduction) and increasing M_c to 2.11×10^4 g mol^{-1}, demonstrating a combined effect of chemical pretreatment and microbial action (see Table 2).

Devulcanization trends were further supported by sol fraction analysis, where untreated GTR exhibited a sol fraction of 6.33%, which increased marginally to 7.9% following biological treatment alone. In contrast, the combined treatment yielded a substantially higher sol fraction of 17.3%. These observations confirm that DES pretreatment enhances microbial devulcanization by increasing microbial accessibility of sulfur crosslinks and polymer chains, in agreement with previously published reports.[9,53]

Table 2 Crosslink density, average molecular weight between crosslinks (M_c), and percentage reduction in crosslink density of GTR samples determined by the solvent swelling method[a]

Name	Crosslink density (mol cm^{-3})	M_c (g mol^{-1})	% Crosslink density reduction
GTR	$9.61 \pm 0.16 \times 10^{-5}$	$1.20 \pm 0.02 \times 10^4$	—
R. rhodochrous-treated GTR	$8.09 \pm 0.29 \times 10^{-5}$	$1.42 \pm 0.05 \times 10^4$	15.81
DES-treated GTR	$7.03 \pm 0.25 \times 10^{-5}$	$1.64 \pm 0.06 \times 10^4$	26.84
DES-pretreated + *R. rhodochrous*-treated GTR	$5.44 \pm 0.12 \times 10^{-5}$	$2.11 \pm 0.05 \times 10^4$	43.6

[a] Values are mean ± standard deviation ($n = 3$).

To elucidate the dominant degradation mechanism, experimental data were analyzed using Horikx plots to distinguish selective sulfur crosslink scission from random polymer backbone cleavage.[27] The theoretical curves were calculated using an initial soluble fraction (S_i) of 6.33%.

Fig. 6 illustrates the relationship between the degree of devulcanization and the soluble fraction based on Horikx analysis. GTR treated with *R. rhodochrous* followed the theoretical curve for selective scission, indicating preferential cleavage of sulfur crosslinks. At a devulcanization degree of 15.8%, the theoretical soluble fraction for selective scission (7.8%) closely matched the experimental value, confirming minimal polymer backbone degradation,[8] consistent with the moderate increase in M_c observed for *R. rhodochrous*-treated GTR relative to untreated GTR (Table 2). In contrast, the hybrid treatment diverged from the selective–scission curve and shifted toward the intermediate region between the selective and random scission.[54] This shift correlates with the larger increase in M_c observed for the combined treatment and reflects partial cleavage of the polymer backbone in addition to sulfur crosslink scission. DES pretreatment reduced the initial crosslink density, thereby facilitating microbial access to the polymer matrix and promoting enhanced sulfur removal and carbon utilization.[55] As the experimental value remained below the theoretical random–scission curve, the combined treatment followed a mixed-mode scission pathway rather than achieving complete polymer breakdown, as shown in Fig. 6.

3.8 Isothermal analysis of ChCl/Ur DES

Isothermal TGA analysis was performed to evaluate the thermal behaviour (mass loss) of untreated GTR, ChCl/Ur DES, and the DES-GTR combined system at pretreatment temperature (180 °C) for 45 min. The thermograms are shown in Fig. 7. GTR exhibited negligible mass loss in the experiment, with 1.26% lost during the ramp and a further 1.14% during the 45 min isothermal hold at 180 °C, typical of the thermal stability of highly vulcanized tyre rubber. The ChCl/Ur DES showed a mass loss of 18.41% during the ramp phase, with a sharp decline observed above ∼130 °C, corresponding to the onset of urea volatilization (m.p. ∼132 °C).[11]

During the isothermal hold at 180 °C, an additional 20.11% mass loss was recorded, with the majority occurring within the first 10 minutes, after which the curve progressively flattened. The total mass loss over the entire experiment was approximately 38.53%, whereas the combined DES-GTR system exhibited 7.28% mass loss during the ramp phase and 21.98% during the isothermal hold, for a total of 29.2%. The observed mass loss at 180 °C is predominantly attributable to the volatilization or partial decomposition of urea from the DES, consistent with urea having a higher vapour pressure than choline, resulting in a shifted or altered eutectic nature of the solvent rather than complete structural collapse of the eutectic system. Notably, the isothermal mass-loss profile of the combined system was more gradual and sustained than the steep initial drop observed for DES alone, suggesting that DES impregnation within the GTR matrix affected the rate of urea volatilization. This observation is further supported by the increase in N (wt%) detected in the area-based EDS analysis of the treated GTR samples (Table 1).

As evident from Fig. 7, the thermal stability of GTR at 180 °C indicates that the changes observed in devulcanized GTR are attributable to DES- and biological-mediated devulcanization,

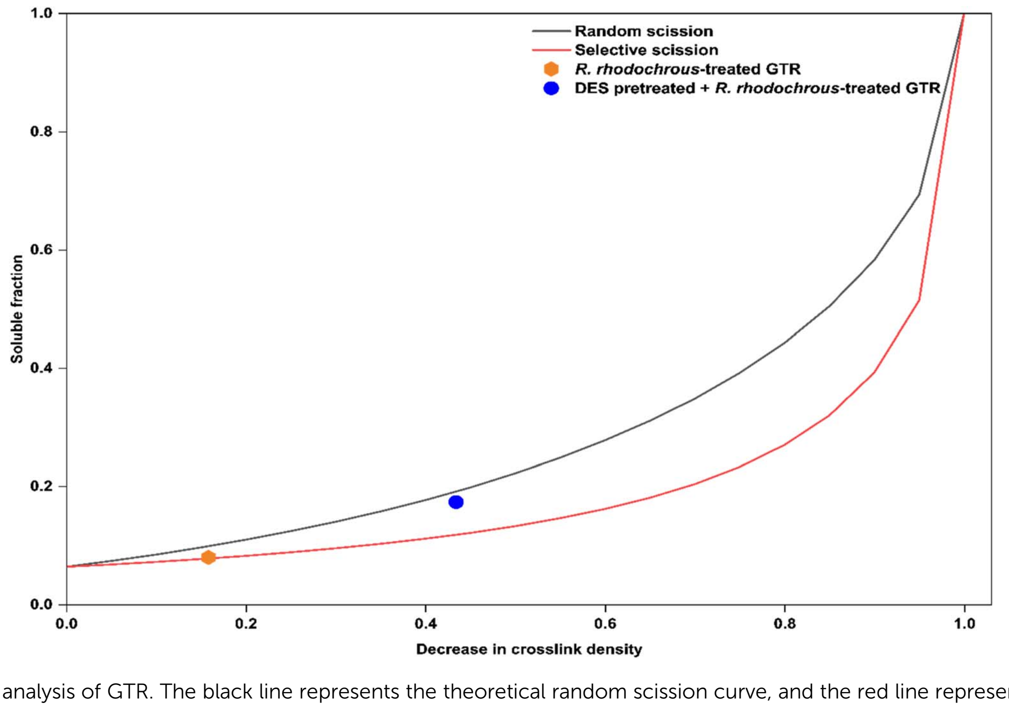


Fig. 6 Horikx plot analysis of GTR. The black line represents the theoretical random scission curve, and the red line represents the theoretical selective scission curve. The orange hexagon shows the experimental point for *R. rhodochrous*-treated GTR, and the blue circle shows the experimental point for DES-pretreated + *R. rhodochrous*-treated GTR.

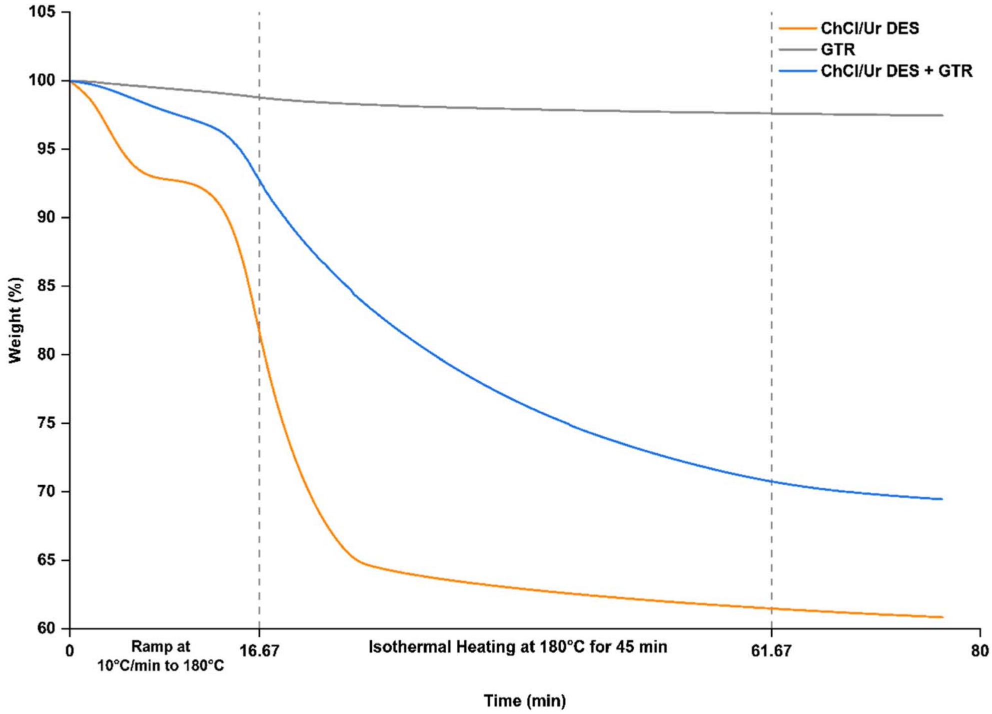


Fig. 7 Isothermal TGA profiles of GTR, ChCl:Ur DES, and DES-GTR mixture at 180 °C under N_2 atmosphere, showing ramp and isothermal stages.

not to thermal degradation of the rubber polymer backbone. The ChCl/Ur DES undergoes partial compositional change upon heating, predominantly through urea volatilization, but retains comparable functionality to support devulcanization in the second cycle.[15] Several studies indicated that the devulcanization of GTR occurs within the range of 140–200 °C.[11,12,56] Temperatures between 140 °C and 160 °C require longer treatment time, while temperatures above 180 °C increase the degree of devulcanization but drops the degree of selectivity. Therefore, 180 °C was chosen as the optimal temperature for the pretreatment process for a shorter period, to ensure effective devulcanization without completely degrading the DES in the process and not randomly affecting the polymeric backbone.

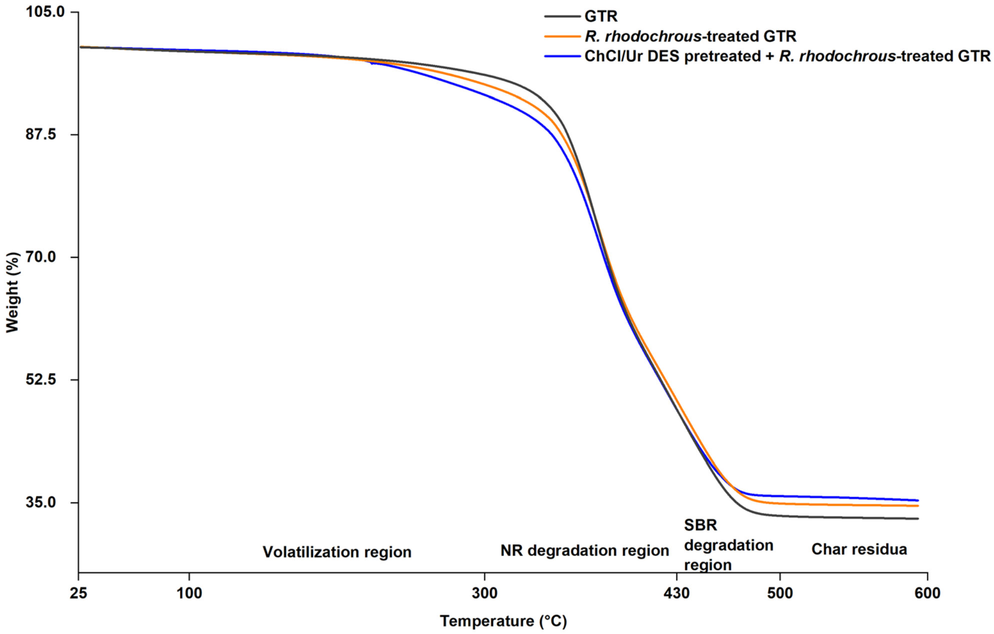
3.9 Thermogravimetric and kinetic analyses of treated GTR

Thermogravimetric analysis revealed four distinct mass-loss events of GTR samples. The first region (100–300 °C) corresponded to volatilization of additives and low-molecular-weight constituents, followed by natural rubber (NR) degradation between 300 °C and 430 °C, synthetic rubber (SBR) degradation between 430 °C and 500 °C, and char formation above 500 °C.[12,57] Overlap between the volatilization and NR degradation regions indicated the onset of polymer decomposition.

As shown in Fig. 8, treated GTR samples exhibited reduced thermal stability compared to untreated GTR. Onset temperatures corresponding to 2%, 5%, and 10% mass loss decreased following treatment, with the most pronounced reduction observed in combination treatment. This shift indicated enhanced degradation and lower thermal stability in the volatilization and NR degradation regions, consistent with partial devulcanization and polymer chain scission.[9] In contrast, the SBR degradation region and overall char content remained largely unchanged across treatments. A slight increase in char residue was observed in treated samples, likely due to differences in inorganic residual components and incomplete oxidation of polymer fragments, resulting in increased charred carbon content after breakdown of thermolabile components in the first two thermal regions.[48]

The E_a for thermal decomposition of GTR was determined using the Kissinger method applied to TGA data collected at heating rates (β) of 5 °C min^{-1}, 10 °C min^{-1}, and 20 °C min^{-1}. The corresponding kinetic parameters, including the pre-exponential factor (A) and coefficient of determination (R^2), are summarized in Table 3. Untreated GTR exhibited an E_a of 212.0 kJ mol^{-1}. The biological treatment alone reduced E_a to 198.4 kJ mol^{-1}, while DES pretreatment further reduced it to 187.7 kJ mol^{-1}. The lowest activation energy was observed for DES-pretreated + *R. rhodochrous*-treated GTR (176.9 kJ mol^{-1}), corresponding to a 35.1 kJ mol^{-1} (16.55%) reduction relative to untreated GTR.

High linear correlations were obtained for all Kissinger plots, with R^2 values greater than 0.995, confirming the reliability of the kinetic analysis.[58] The decrease in E_a across treatments indicates progressive weakening of the rubber network, consistent with partial devulcanization and polymer chain scission. The pronounced reduction in E_a for the combined treatment reflects greater disruption of sulfur crosslinks, thereby lowering the energy requirements for thermal degradation. Similar reductions in thermal stability following DES pretreatment have been reported previously,[11,59,60] whereas



Name	T (−2%)°C	T (−5%)°C	T (−10%)°C	T (−50%)°C	Char Residue at 600°C (%)
GTR	238	316.65	348.64	424.59	32.72
R. rhodochrous -treated GTR	220.47	294.57	341.265	429.89	35.573
DES pretreated+*R. rhodochrous* -treated GTR	215.7	276.495	332.425	426.11	34.898

Fig. 8 Thermogravimetric curves of ground tyre rubber (GTR). Shown are untreated GTR (dark grey), *R. rhodochrous*-treated GTR (orange). ChCl/urea DES-pretreated + *R. rhodochrous*-treated GTR (blue).

Table 3 Kissinger kinetic parameters for GTR samples

Name	T_p (°C)	E_a (kJ mol^{-1})	A (min^{-1})	R^2
GTR	364.97, 374.92, 386.74	212.0	6.9×10^{16}	0.9982
R. rhodochrous-treated GTR	364.11, 375.24, 387.29	198.4	5.5×10^{15}	0.9998
DES-treated GTR[15]	363.90, 374.46, 388.26	187.7	7.0×10^{14}	0.9951
DES-pretreated + *R. rhodochrous*-treated GTR	362.32, 374.78, 388.12	176.9	9.3×10^{13}	0.9999

biological treatment alone showed a limited impact due to the persistence of dense sulfur crosslinks.[48] The crosslink density profoundly affects the thermal stability of rubber samples.[61] The observed kinetic changes further support the role of DES pretreatment in enhancing the susceptibility of GTR to microbial degradation.[9,12]

3.10 Mass reduction measurement and Schiff's staining

The maximum mass reduction was observed for GTR subjected to DES pretreatment followed by biodegradation with *R. rhodochrous* RPK1, indicating that detoxification and partial devulcanization enhanced bacterial utilization of rubber. Compared to the abiotic control, *R. rhodochrous* alone achieved only 1.63% mass loss, whereas DES pretreatment increased mass loss to 12.53%, corresponding to a 7.68-fold increase in biodegradation efficiency (Fig. 9a and Table S2). This improvement is attributed to pretreatment-induced chemical modifications that promoted bacterial adhesion, thereby facilitating enzymatic attack on the rubber polymeric chains. Similar trends have been reported previously, including improved degradation by *Klebsiella aerogenes*,[9] while limited mass loss (∼1.63%) during biodegradation of untreated vulcanized rubber by *R. rhodochrous* RPK1 has also been documented by Andler *et al.* (2022).[41]

Schiff's staining indicated the presence of oligo-isoprenoids as degradation products in both treatment conditions. These compounds feature terminal aldehyde or ketone groups, which yield a purple or violet colour upon reaction with Schiff's reagent.[33] The detection of these products in both cases demonstrated that DES pretreatment did not inhibit microbial degradation activity but instead facilitated efficient rubber utilization, as shown in Fig. 9(b–e). Consistent with earlier studies, the formation of aldehyde-containing intermediates suggests an initial metabolic pathway in rubber-degrading bacteria.[36] Additionally, LC-MS analysis was performed to detect the presence of rubber-degradation products (oligo-isoprenoids) formed during treatment. The chromatograms and tentative peak assignments are provided in the SI as Table S3 and Fig. S2.

3.11 Total protein estimation

Since rubber biodegradation is enzyme-mediated, total protein concentration was used as an indicator of microbial activity and

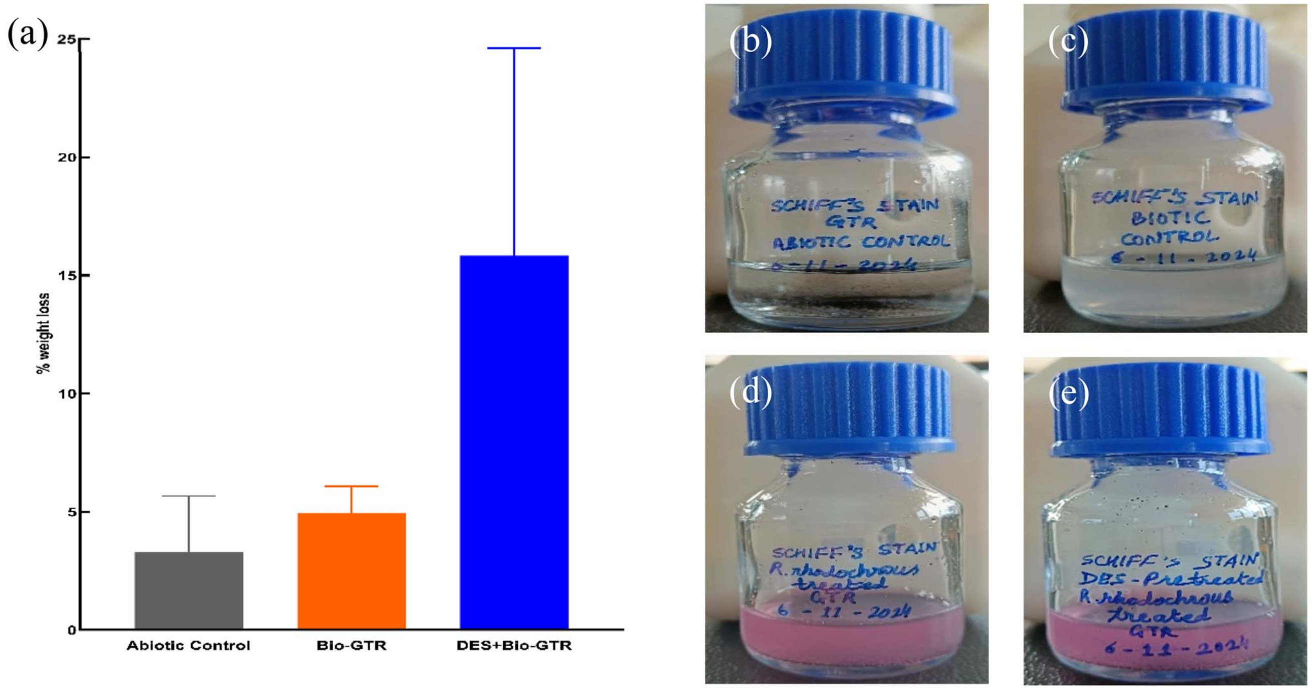


Fig. 9 Percentage weight loss and Schiff's staining of treated ground tyre rubber (GTR). (a) Weight loss (%) for ChCl/urea DES-pretreated + *R. rhodochrous*-treated GTR (DES + Bio-GTR), *R. rhodochrous*-treated GTR (Bio-GTR), and abiotic control in MSM medium at 30 °C after 28 days. (b–e) Schiff's staining of (b) abiotic control, (c) biotic control (MSM without GTR), (d) *R. rhodochrous*-treated GTR, and (e) ChCl/urea DES-pretreated + *R. rhodochrous*-treated GTR, showing the development of pink colouration corresponding to oligo-isoprenoid formation.

rubber utilization. Protein levels were quantified using Lowry's method with bovine serum albumin as the standard.[36] At day 0, protein concentrations were similar across cultures. Protein concentrations increased over time in all cultures. However, DES-pretreated GTR consistently supported higher protein levels. The maximum protein concentration (0.43 mg mL^{-1}) was observed at day 28 for pretreated GTR, compared with 0.38 mg mL^{-1} for untreated GTR and the control (Fig. 10). Two-way ANOVA revealed significant effects of treatment ($F = 12.79$, $p < 0.001$) and incubation time ($F = 3.57$, $p < 0.05$). The higher protein levels observed for DES-pretreated GTR are attributed to reduced sulfur content and additive toxicity, which enhanced accessibility of the rubber matrix and supported increased enzymatic activity and biomass formation.[9] Several bacteria and

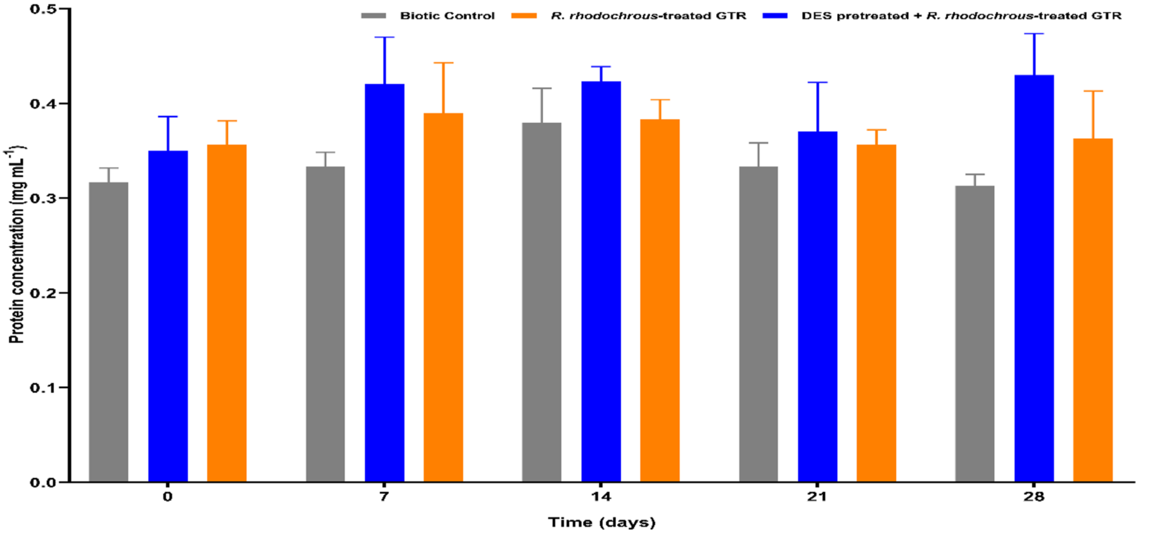


Fig. 10 Total protein concentration in *Rhodococcus rhodochrous* RPK1 cultures with untreated GTR, ChCl/urea DES-pretreated GTR, and biotic control, determined by Lowry's method. Values represent mean ± SD ($n = 3$). Two-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$) was performed for statistical analysis.

fungi have demonstrated increased protein content when rubber served as a substrate.[62] *Bacillus pumilus* and *Bacillus subtilis* cultures showed maximum protein concentrations of 0.086 μg mL^{-1} and 0.101 μg mL^{-1}, respectively, when cultured with NR for 7 and 8 weeks, respectively, at 37 °C.[18,36] The production of rubber-degrading enzymes and variation in protein content depend on pH, temperature, bacterial strain, and the total duration of the biodegradation process.[9]

3.12 Laccase and peroxidase enzyme activity assay

Laccase and peroxidase activities increased markedly during the biodegradation period and were sustained throughout incubation (Fig. 11). In cultures containing DES-pretreated GTR, *R. rhodochrous* exhibited a 105.5-fold increase in laccase activity and a 99.5-fold increase in peroxidase activity at day 28 relative to enzyme levels at day 0 in the biotic control. In contrast, cultures containing untreated GTR showed substantially lower increases, with 32.5-fold and 40-fold enhancements in laccase and peroxidase activities, respectively.

These enzymes are known to decompose ligninolytic compounds,[18] which are considered part of the enzyme mediator system, and function as secondary enzymes.[62] The presence of laccase and peroxidase is also a response to the presence of aromatic compounds in the GTR. Multiple studies provided evidence for the presence of laccase and peroxidase enzymes in the oxidation of C═C bonds in rubber, in addition to *Lcp*, *Rox*A, and *Rox*B.[63] Nayanashree and Thippeswamy (2015)[36] reported the highest laccase and peroxidase activities of 0.0138 IU and 0.0142 IU, respectively. Similarly, fungi, *Trametes versicolor* and *Pleurotus ostreatus*, were found to be able to secrete laccase and peroxidase to degrade vulcanized rubber particles.[18,64] The observations on protein concentration and enzyme activities indicate that increased protein synthesis is associated with enhanced biodegradation activity.

As observed, while the DES pretreatment increased microbial mineralization of GTR, bioaccessibility remains constrained by residual crosslinks, inorganic fillers, and diffusional limitations within the rubber matrix. These limitations may be addressed by further reducing GTR particle size to increase surface area and enhance microbial adhesion,[34] or by using microbial consortia or mixed cultures to achieve a synergistic effect.[65] Recombinant strains overexpressing rubber oxygenases (*Lcp*, *Rox*A/*Rox*B) could enhance the depolymerization of partially devulcanized GTR.[66] The process integration in bioreactor systems with controlled aeration and continuous nutrient supplementation could further improve enzyme production and substrate accessibility. These directions provide a pathway to overcome the limitations to advance scalable biological valorization of waste tyre rubber.

3.13 Biocompatibility of ChCl/Ur DES with *R. rhodochrous* RPK1

DESs are often regarded as non-toxic solvents; however, their biocompatibility must be validated for specific biological systems. Accordingly, the toxicity of ChCl/Ur DES was evaluated against *R. rhodochrous* RPK1 (Fig. S1) and *E. coli* ATCC 25922 using disc diffusion and MIC assays. No inhibition zone was observed around DES-impregnated discs following incubation at 30 °C, indicating that ChCl/Ur DES was non-toxic to *R. rhodochrous*.[38] This observation agrees with earlier studies showing that amine-based DESs do not inhibit bacterial growth.[67]

MIC analysis further confirmed the low toxicity of the DES. As shown in Table 4, the MIC was ∼256 mg mL^{-1} for *R. rhodochrous* and >128 mg mL^{-1} for *E. coli*, indicating weak

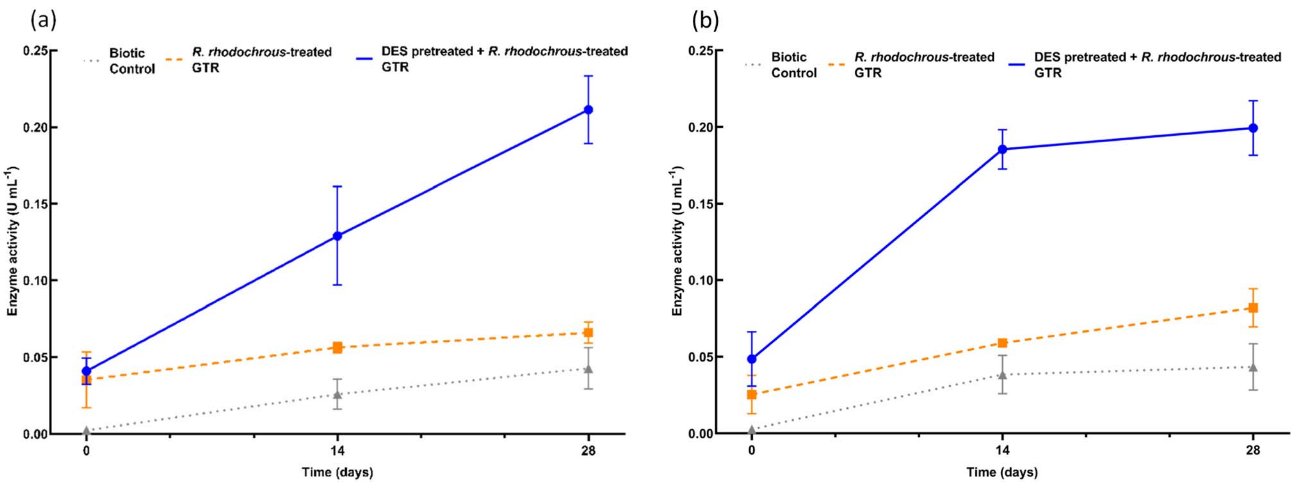


Fig. 11 Extracellular enzyme activities in *Rhodococcus rhodochrous* RPK1 cultures grown with untreated ground tyre rubber (GTR), ChCl/urea DES-pretreated GTR, or biotic control. Laccase activity (a) and peroxidase activity (b) are expressed in U mL^{-1} over 28 days. Values represent mean ± SD ($n = 3$). Two-way ANOVA followed by Tukey's *post hoc* test ($p < 0.05$) was performed for statistical analysis. Two-way ANOVA confirmed significant effects of treatment and incubation time on both enzymes ($p < 0.0001$) and a significant treatment × time interaction ($p < 0.00001$), indicating treatment-dependent temporal regulation of enzyme production.

Table 4 Microplate absorbance readings (OD_{600}) showing the effect of ChCl/urea DES on the growth of *Rhodococcus rhodochrous* RPK1 and *Escherichia coli* ATCC 25922[a]

DES concentration	256 (mg mL^{-1})	128 (mg mL^{-1})	64 (mg mL^{-1})	32 (mg mL^{-1})	16 (mg mL^{-1})	8 (mg mL^{-1})	4 (mg mL^{-1})	2 (mg mL^{-1})	GC	MC
R. rhodochrous	0.41 ± 0.11	0.55 ± 0.07	0.51 ± 0.004	0.56 ± 0.048	0.58 ± 0.03	0.70 ± 0.115	0.62 ± 0.027	0.58 ± 0.084	0.66 ± 0.037	0.04
E. coli	0.06 ± 0.019	0.29 ± 0.113	0.55 ± 0.107	0.59 ± 0.029	0.79 ± 0.018	0.80 ± 0.007	0.75 ± 0.133	0.64 ± 0.046	0.91 ± 0.046	0.02

[a] GC = growth control; MC = media control.

bactericidal activity and supporting biocompatibility. These values are consistent with previous reports on the limited antimicrobial effects of DESs.[39,40,68] Consequently, the non-toxicity of ChCl/Ur DES toward *R. rhodochrous* enabled the effective implementation of the hybrid chemi-biological approach, thereby enhancing the overall potential for rubber biodegradation.

4. Conclusions

The experimental results presented herein demonstrate that the limited biodegradability of waste tyre rubber (WTR) is strongly influenced by sulfur crosslinks and toxic additives, which restrict microbial accessibility to the polymeric backbone. By applying a ChCl/Ur DES pretreatment, this study demonstrates reductions in these limiting factors, establishing a link between partial devulcanization, detoxification, and improved biodegradation response. The increase in biodegradation efficiency, the reduction in crosslink density, and the substantial removal of zinc-related compounds collectively indicate that the ChCl/Ur DES functions as a partial devulcanization and detoxification agent, improving rubber bioavailability, consistent with a shift in the Horikx plot toward a mixed degradation mechanism. These findings suggest that a hybrid chemi-biological approach represents a promising early step contributing towards the exploration of sustainable alternatives to conventional WTR management methods such as pyrolysis, incineration, and landfilling. Substantial further development, including process optimization, scale-up studies, and the evaluation of alternative DES formulations and microbial systems, is necessary for future steps. The presented results provide a basis for future investigations aimed at enhancing degradation efficiency and advancing sustainable waste tyre management strategies.

Author contributions

Pritish R. Shukla: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing- original draft, review & editing, and visualization, Utpal Roy: conceptualization, formal analysis, data curation, writing-review & editing, validation, resources, supervision, project administration, and funding acquisition, Sunil Bhand: resources, project administration, and funding acquisition.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability

All relevant data is available from the corresponding author upon reasonable request.

Supplementary information (SI): tables related to CHNS analysis, weight reduction measurements, and LC-MS-based identification of oligo-isoprenoid mass distributions, along with figures showing the disc diffusion assay and LC-MS spectra of extracted oligo-isoprenoids. See DOI: https://doi.org/10.1039/d6ra01664b.

Acknowledgements

We are grateful for the project funding support from Birla Carbon CSR, and Anusandhan National Research Foundation (ANRF), Department of Science and Technology (DST), Government of India, and Confederation of Indian Industry (CII) for awarding the Prime Minister's Fellowship for Doctoral Research to the first author. We thank our institute's Central Sophisticated Instrumentation Facility (CSIF) for providing imaging and elemental data analysis support, Prof. Vivek Rangarajan, Department of Chemical Engineering, for his valuable guidance, and the Chemical Engineering Sophisticated Instrumentation Laboratory (CESI) for providing characterization facilities.

References

1 B. Han, D. Kumar, Y. Pei, M. Norton, S. D. Adams, S. Y. Khoo, *et al.*, Sustainable transformation of end-of-life tyres into value-added products using thermochemical processes, *Carbon Res.*, 2024, **3**(1), 73, DOI: 10.1007/s44246-024-00167-4.

2 C. Guajardo-Flores, J. Rojas, Y. Baldera-Moreno, F. Adasme-Carreño, D. Kasai and R. Andler, New insights on poly(cis-1,4-isoprene) rubber degradation through enzymatic kinetics and process improvement, *Front. Bioeng. Biotechnol.*, 2025, 13, DOI: 10.3389/fbioe.2025.1593339.

3 W. A. Laftah and W. A. Abdul Rahman, A comprehensive review of tire recycling technologies and applications, *Mater Adv.*, 2025, **6**(15), 4992–5010, DOI: 10.1039/D5MA00463B.

4 M. S. Abbas-Abadi, M. Kusenberg, H. M. Shirazi, B. Goshayeshi and K. M. Van Geem, Towards full recyclability of end-of-life tires: challenges and opportunities, *J. Cleaner Prod.*, 2022, **374**, 134036, DOI: 10.1016/j.jclepro.2022.134036.

5 R. Andler, Bacterial and enzymatic degradation of poly(cis-1,4-isoprene) rubber: novel biotechnological applications, *Biotechnol. Adv.*, 2020, **44**, 107606, DOI: 10.1016/j.biotechadv.2020.107606.

6 D. Jendrossek, G. Tomasi and R. M. Kroppenstedt, Bacterial degradation of natural rubber: a privilege of actinomycetes?, *FEMS Microbiol. Lett.*, 2006, **150**(2), 179–188, DOI: 10.1111/j.1574-6968.1997.tb10368.x.

7 F. Bosco and C. Mollea, Biodegradation of Natural Rubber: Microcosm Study, *Water, Air, Soil Pollut.*, 2021, **232**(6), 227, DOI: 10.1007/s11270-021-05171-7.

8 Y. Li, S. Zhao and Y. Wang, Microbial desulfurization of ground tire rubber by *Thiobacillus ferrooxidans*, *Polym. Degrad. Stab.*, 2011, **96**(9), 1662–1668, DOI: 10.1016/j.polymdegradstab.2011.06.011.

9 H. Chittella, L. W. Yoon, S. Ramarad and Z. W. Lai, Biodegradation of deep eutectic solvent pretreated natural rubber gloves by *Klebsiella aerogenes*: a sustainable approach to rubber waste management, *Biochem. Eng. J.*, 2025, **213**, 109569, DOI: 10.1016/j.bej.2024.109569.

10 A. L. Altenhoff, J. de Witt, R. Andler and A. Steinbüchel, Impact of additives of commercial rubber compounds on the microbial and enzymatic degradation of poly(cis-1,4-isoprene), *Biodegradation*, 2019, **30**(1), 13–26, DOI: 10.1007/s10532-018-9858-5.

11 R. Saputra, R. Walvekar, M. Khalid, K. Shahbaz and S. Ramarad, Effective devulcanization of ground tire rubber using choline chloride-based deep eutectic solvents, *J. Environ. Chem. Eng.*, 2019, **7**(3), 103151, DOI: 10.1016/j.jece.2019.103151.

12 H. Ramezani, F. Scarpa, Q. Zhang, W. Ji, A. Khorramshokouh, S. Rochat, *et al.*, Green and sustainable devulcanization of ground tire rubber using choline chloride–urea deep eutectic solvent, *RSC Sustainability*, 2024, **2**(8), 2295–2311, DOI: 10.1039/D4SU00079J.

13 S. Watcharakul, W. Röther, J. Birke, K. Umsakul, B. Hodgson and D. Jendrossek, Biochemical and spectroscopic characterization of purified Latex Clearing Protein (Lcp) from newly isolated rubber degrading *Rhodococcus rhodochrous* strain RPK1 reveals novel properties of Lcp, *BMC Microbiol.*, 2016, **16**(1), 92, DOI: 10.1186/s12866-016-0703-x.

14 A. P. Abbott, D. Boothby, G. Capper, D. L. Davies and R. K. Rasheed, Deep Eutectic Solvents Formed between Choline Chloride and Carboxylic Acids: Versatile Alternatives to Ionic Liquids, *J. Am. Chem. Soc.*, 2004, **126**(29), 9142–9147, DOI: 10.1021/ja048266j.

15 P. R. Shukla, U. Roy, S. Bhand and R. R. Yaragal, Sustainable devulcanization and detoxification of worn-out tires using a reusable green solvent, *Results Eng.*, 2026, 109656, DOI: 10.1016/j.rineng.2026.109656.

16 S. Nejrotti, A. Antenucci, C. Pontremoli, L. Gontrani, N. Barbero, M. Carbone, *et al.*, Critical Assessment of the Sustainability of Deep Eutectic Solvents: A Case Study on Six Choline Chloride-Based Mixtures, *ACS Omega*, 2022, **7**(51), 47449–47461, DOI: 10.1021/acsomega.2c06140.

17 Organisation for Economic Co-operation and Development (OECD), Test No. 301: Ready Biodegradability, *OECD Guidelines for the Testing of Chemicals, Section 3*, OECD Publishing, Paris, France, 1992, DOI: 10.1787/9789264070349-en.

18 R. Andler, V. D'Afonseca, J. Pino, C. Valdés and M. Salazar-Viedma, Assessing the Biodegradation of Vulcanised Rubber Particles by Fungi Using Genetic, Molecular and Surface Analysis, *Front. Bioeng. Biotechnol.*, 2021, **9**(761510), DOI: 10.3389/fbioe.2021.761510.

19 S. P. Braga, A. P. dos Santos, T. Paganini, D. Barbosa, G. W. C. Epamino, C. Morais, *et al.*, First report of cis-1,4-polyisoprene degradation by *Gordonia paraffinivorans*, *Braz. J. Microbiol.*, 2019, **50**(4), 1051–1062, DOI: 10.1007/s42770-019-00143-w.

20 C. Valdés, C. Hernández, R. Morales-Vera and R. Andler, Desulfurization of Vulcanized Rubber Particles Using Biological and Couple Microwave-Chemical Methods, *Front. Environ. Sci.*, 2021, **20**, 9, DOI: 10.3389/fenvs.2021.633165.

21 S. Li, T. Q. Tran, B. Ji, A. S. Brand and W. Zhang, Optimization of Zn Leaching Recovery from Tire Rubber and High-Purity ZnO Production, *J. Sustain. Metall.*, 2025, **11**(1), 406–423, DOI: 10.1007/s40831-024-00981-5.

22 R. Roychand, R. J. Gravina, Y. Zhuge, X. Ma, J. E. Mills and O. Youssf, Practical Rubber Pretreatment Approch for Concrete Use—An Experimental Study, *J. Compos. Sci.*, 2021, **5**(6), 143, DOI: 10.3390/jcs5060143.

23 P. J. Flory and J. Rehner, Statistical Mechanics of Crosslinked Polymer Networks II. Swelling, *J. Chem. Phys.*, 1943, **11**(11), 521–526, DOI: 10.1063/1.1723792.

24 S. Paul, M. Rahaman, S. K. Ghosh, A. Katheria and N. C. Das, Rubber compounds from pilot-scale supercritical carbon dioxide assisted devulcanized rubber: curing characteristics, mechanical and thermal properties, *Spec. Polym.*, 2025, **6**(2), e70003, DOI: 10.1002/pls2.70003.

25 E. Díez, G. Ovejero, M. D. Romero and I. Díaz, Polymer–solvent interaction parameters of SBS rubbers by inverse gas chromatography measurements, *Fluid Phase Equilib.*, 2011, **308**(1–2), 107–113, DOI: 10.1016/j.fluid.2011.06.018.

26 L. E. Alonso Pastor, K. C. Núñez Carrero, J. Araujo-Morera, M. Hernández Santana and J. M. Pastor, Setting Relationships between Structure and Devulcanization of Ground Tire Rubber and Their Effect on Self-Healing Elastomers, *Polymers*, 2021, **14**(1), 11, DOI: 10.3390/polym14010011.

27 M. M. Horikx, Chain scissions in a polymer network, *J. Polym. Sci.*, 1956, **19**(93), 445–454, DOI: 10.1002/pol.1956.120199305.

28 M. A. L. Verbruggen, L. van der Does, W. K. Dierkes and J. W. M. Noordermeer, Experimental validation of the Charlesby and Horikx models applied to de-vulcanization of sulfur and peroxide vulcanizates of NR and EPDM, *Rubber Chem. Technol.*, 2016, **89**(4), 671–688, DOI: 10.5254/rct.16.83776.

29 S. Seghar, L. Asaro and N. Aït Hocine, Experimental Validation of the Horikx Theory to be Used in the Rubber

Devulcanization Analysis, *J. Polym. Environ.*, 2019, **27**(10), 2318–2323, DOI: 10.1007/s10924-019-01513-z.

30 T. Kleps, M. Piaskiewicz and W. Parasiewicz, The Use of Thermogravimetry in the Study of Rubber Devulcanization, *J. Therm. Anal. Calorim.*, 2000, **60**(1), 271–277, DOI: 10.1023/A:1010134315762.

31 A. M. Ramírez Arias, J. C. Moreno-Piraján and L. Giraldo, Kinetic Study of Waste Tire Pyrolysis Using Thermogravimetric Analysis, *ACS Omega*, 2022, **7**(19), 16298–16305, DOI: 10.1021/acsomega.1c06345.

32 H. E. Kissinger, Variation of peak temperature with heating rate in differential thermal analysis, *J. Res. Natl. Bur. Stand.*, 1956, **57**(4), 217, DOI: 10.6028/jres.057.026.

33 A. Linos, M. M. Berekaa, R. Reichelt, U. Keller, J. Schmitt, H. C. Flemming, *et al.*, Biodegradation of *cis*-1,4-Polyisoprene Rubbers by Distinct Actinomycetes: Microbial Strategies and Detailed Surface Analysis, *Appl. Environ. Microbiol.*, 2000, **66**(4), 1639–1645, DOI: 10.1128/AEM.66.4.1639-1645.2000.

34 R. Andler, S. Hiessl, O. Yücel, M. Tesch and A. Steinbüchel, Cleavage of poly(cis-1,4-isoprene) rubber as solid substrate by cultures of *Gordonia polyisoprenivorans*, *New Biotechnol.*, 2018, **44**, 6–12, DOI: 10.1016/j.nbt.2018.03.002.

35 L. OliverH, R. NiraJ, A. L. Farr and R. RoseJ, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 1951, **193**(1), 265–275, DOI: 10.1016/S0021-9258(19)52451-6.

36 G. Nayanashree and B. Thippeswamy, Biodegradation of Natural Rubber by Laccase and Manganese Peroxidase Enzyme of *Bacillus subtilis*, *Environ. Processes*, 2015, **2**(4), 761–772, DOI: 10.1007/s40710-015-0118-y.

37 H. M. Bedair, T. M. Samir and F. R. Mansour, Antibacterial and antifungal activities of natural deep eutectic solvents, *Appl. Microbiol. Biotechnol.*, 2024, **108**(1), 198, DOI: 10.1007/s00253-024-13044-2.

38 M. Marchel, H. Cieśliński and G. Boczkaj, Thermal Instability of Choline Chloride-Based Deep Eutectic Solvents and Its Influence on Their Toxicity-Important Limitations of DESs as Sustainable Materials, *Ind. Eng. Chem. Res.*, 2022, **61**(30), 11288–11300, DOI: 10.1021/acs.iecr.2c01898.

39 K. Radošević, M. Cvjetko Bubalo, V. Gaurina Srček, D. Grgas, T. Landeka Dragičević and I. Radojčić Redovniković, Evaluation of toxicity and biodegradability of choline chloride based deep eutectic solvents, *Ecotoxicol. Environ. Saf.*, 2015, **112**, 46–53, DOI: 10.1016/j.ecoenv.2014.09.034.

40 I. Juneidi, M. Hayyan and M. A. Hashim, Evaluation of toxicity and biodegradability for cholinium-based deep eutectic solvents, *RSC Adv.*, 2015, **5**(102), 83636–83647, DOI: 10.1039/C5RA12425E.

41 R. Andler, C. Guajardo, C. Sepúlveda, V. Pino, V. Sanhueza and V. D'Afonseca, Biodegradation of rubber in cultures of *Rhodococcus rhodochrous* and by its enzyme latex clearing protein, *Biodegradation*, 2022, **33**(6), 609–620, DOI: 10.1007/s10532-022-09998-7.

42 P. Srivastava, S. Subramanian and N. Manickam, Biochemical and molecular mechanisms of *Rhodococcus rhodochrous* IITR131 for polyethylene terephthalate degradation, *J. Appl. Microbiol.*, 2025, **136**(1), lxae312, DOI: 10.1093/jambio/lxae312.

43 S. N. S. Hapuarachchi, S. R. Kariyapper, M. B. D. M. D. Gunawardana, S. Egodage and T. U. Ariyadasa, Biodegradation of natural rubber latex by a novel bacterial species isolated from soil, in *2016 Moratuwa Engineering Research Conference (MERCon)*, IEEE, 2016, pp. 293–296, DOI: 10.1109/MERCon.2016.7480156.

44 Y. Wang, L. Jiao, C. Zhao, W. Dong, W. Gong and D. Dong, The impact of biodegradable plastics on methane and carbon dioxide emissions in soil ecosystems: a Fourier transform infrared spectroscopy approach, *Sci. Rep.*, 2025, **15**(1), 7678, DOI: 10.1038/s41598-025-90322-7.

45 M. M. Berekaa, A. Linos, R. Reichelt, U. Keller and A. Steinbüchel, Effect of pretreatment of rubber material on its biodegradability by various rubber degrading bacteria, *FEMS Microbiol. Lett.*, 2000, **184**(2), 199–206, DOI: 10.1111/j.1574-6968.2000.tb09014.x.

46 B. Rosso, E. Gregoris, L. Litti, F. Zorzi, M. Fiorini, B. Bravo, *et al.*, Identification and quantification of tire wear particles by employing different cross-validation techniques: FTIR-ATR Micro-FTIR, Pyr-GC/MS, and SEM, *Environ. Pollut.*, 2023, **326**, 121511, DOI: 10.1016/j.envpol.2023.121511.

47 C. Yao, S. Zhao, Y. Wang, B. Wang, M. Wei and M. Hu, Microbial desulfurization of waste latex rubber with *Alicyclobacillus* sp, *Polym. Degrad. Stab.*, 2013, **98**(9), 1724–1730, DOI: 10.1016/j.polymdegradstab.2013.06.002.

48 M. G. Aboelkheir, P. B. Bedor, S. G. Leite, K. Pal, R. D. Toledo Filho and F. Gomes de Souza, Biodegradation of Vulcanized SBR: A Comparison between *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Streptomyces* sp, *Sci. Rep.*, 2019, **9**(1), 19304, DOI: 10.1038/s41598-019-55530-y.

49 A. Bagchi and P. Srivastava, Genetic and metabolic engineering approaches for enhanced biodesulfurization of petroleum fractions, *Front. Bioeng. Biotechnol.*, 2024, 12, DOI: 10.3389/fbioe.2024.1482270.

50 G. M. Gadd, Metals, minerals and microbes: geomicrobiology and bioremediation, *Microbiol.*, 2010, **156**(3), 609–643, DOI: 10.1099/mic.0.037143-0.

51 S. Khare, A. Singhal, S. Rallapalli and A. Mishra, Bio-chelation for sustainable heavy metal remediation in municipal solid waste compost: a critical review of chelation technologies, *Environ. Sci. Pollut. Res.*, 2025, DOI: 10.1007/s11356-025-36368-6.

52 X. Cui, S. Zhao and B. Wang, Microbial desulfurization for ground tire rubber by mixed consortium-*Sphingomonas* sp. and *Gordonia* sp, *Polym. Degrad. Stab.*, 2016, **128**, 165–171, DOI: 10.1016/j.polymdegradstab.2016.03.011.

53 R. Andler, C. Cancino, F. González-Arancibia, J. Rojas, R. Castro, D. Kasai, *et al.*, Bio-desulfurization of different vulcanized rubber blends: an ecofriendly approach for end-of-life tires waste pollution, *J. Environ. Chem. Eng.*, 2025, **13**(2), 115598, DOI: 10.1016/j.jece.2025.115598.

54 L. Asaro, M. Gratton, S. Seghar and N. Aït Hocine, Recycling of rubber wastes by devulcanization, *Resour., Conserv.*

Recycl., 2018, **133**, 250–262, DOI: 10.1016/j.resconrec.2018.02.016.

55 M. Vahdatbin, P. Hajikarimi and E. H. Fini, Devulcanization of Waste Tire Rubber *via* Microwave and Biological Methods: A Review, *Polymers*, 2025, **17**(3), 285, DOI: 10.3390/polym17030285.

56 D. Á. Simon and T. Bárány, Microwave Devulcanization of Ground Tire Rubber and Its Improved Utilization in Natural Rubber Compounds, *ACS Sustain. Chem. Eng.*, 2023, **11**(5), 1797–1808, DOI: 10.1021/acssuschemeng.2c05984.

57 X. Zhang, P. Saha, L. Cao, H. Li and J. Kim, Devulcanization of waste rubber powder using thiobisphenols as novel reclaiming agent, *Waste Manage.*, 2018, **78**, 980–991, DOI: 10.1016/j.wasman.2018.07.016.

58 R. Wang, C. Xie, L. Zeng and H. Xu, Thermal decomposition behavior and kinetics of nanocomposites at low-modified ZnO content, *RSC Adv.*, 2019, **9**(2), 790–800, DOI: 10.1039/C8RA09206K.

59 R. Saputra, R. Walvekar, M. Khalid, N. M. Mubarak and M. Sillanpää, Current progress in waste tire rubber devulcanization, *Chemosphere*, 2021, **265**, 129033, DOI: 10.1016/j.chemosphere.2020.129033.

60 R. Saputra, R. Walvekar, M. Khalid, C. T. Ratnam, N. M. Mubarak and S. Dharaskar, Devulcanisation of ground rubber tyre by novel ternary deep eutectic solvents, *J. Mol. Liq.*, 2020, **306**, 112913, DOI: 10.1016/j.molliq.2020.112913.

61 A. P. Mathew, S. Packirisamy and S. Thomas, Studies on the thermal stability of natural rubber/polystyrene interpenetrating polymer networks: thermogravimetric analysis, *Polym. Degrad. Stab.*, 2001, **72**(3), 423–439, DOI: 10.1016/S0141-3910(01)00042-8.

62 A. Basik, J. J. Sanglier, C. Yeo and K. Sudesh, Microbial Degradation of Rubber: Actinobacteria, *Polymers*, 2021, **13**(12), 1989, DOI: 10.3390/polym13121989.

63 M. Enoki, Y. Doi and T. Iwata, Oxidative Degradation of *cis*- and *trans*-1,4-Polyisoprenes and Vulcanized Natural Rubber with Enzyme-Mediator Systems, *Biomacromolecules*, 2003, **4**(2), 314–320, DOI: 10.1021/bm025678g.

64 K. Bredberg, Microbial detoxification of waste rubber material by wood-rotting fungi, *Bioresour. Technol.*, 2002, **83**(3), 221–224, DOI: 10.1016/S0960-8524(01)00218-8.

65 S. Weyrauch, B. Seiwert, M. Voll and T. Reemtsma, Long term biodegradation study on tire and road wear particles and chemicals thereof, *Sci. Total Environ.*, 2025, **975**, 179240, DOI: 10.1016/J.SCITOTENV.2025.179240.

66 J. Birke and D. Jendrossek, Rubber Oxygenase and Latex Clearing Protein Cleave Rubber to Different Products and Use Different Cleavage Mechanisms, *Appl. Environ. Microbiol.*, 2014, **80**(16), 5012–5020, DOI: 10.1128/AEM.01271-14.

67 B. Y. Zhao, P. Xu, F. X. Yang, H. Wu, M. H. Zong and W. Y. Lou, Biocompatible Deep Eutectic Solvents Based on Choline Chloride: Characterization and Application to the Extraction of Rutin from *Sophora japonica*, *ACS Sustain. Chem. Eng.*, 2015, **3**(11), 2746–2755, DOI: 10.1021/acssuschemeng.5b00619.

68 Q. Wen, J. X. Chen, Y. L. Tang, J. Wang and Z. Yang, Assessing the toxicity and biodegradability of deep eutectic solvents, *Chemosphere*, 2015, **132**, 63–69, DOI: 10.1016/j.chemosphere.2015.02.061.