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Research review paper

Bacterial and enzymatic degradation of poly(*cis*-1,4-isoprene) rubber: Novel biotechnological applications

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ABSTRACT

Poly(*cis*-1,4-isoprene) rubber is a highly demanded elastomeric material mainly used for the manufacturing of tires. The end-cycle of rubber-made products is creating serious environmental concern and, therefore, different recycling processes have been proposed. However, the current physical-chemical processes include the use of hazardous chemical solvents, large amounts of energy, and possibly generations of unhealthy micro-plastics. Under this scenario, eco-friendly alternatives are needed and biotechnological rubber treatments are demonstrating huge potential. The cleavage mechanisms and the biochemical pathways for the uptake of poly(*cis*-1,4-isoprene) rubber have been extensively reported. Likewise, novel bacterial strains able to degrade the polymer have been studied and the involved structural and functional enzymes have been analyzed. Considering the fundamentals, biotechnological approaches have been proposed considering process optimization, cost-effective methods and larger-scale experiments in the search for practical and realistic applications. In this work, the latest research in the rubber biodegradation field is shown and discussed, aiming to analyze the combination of detoxification, devulcanization and polymer-cleavage mechanisms to achieve better degradation yields. The modified superficial structure of rubber materials after biological treatments might be an interesting way to reuse old rubber for re-vulcanization or to find new materials.

1. Introduction

Polyisoprenoids are one of the eight major classes of biopolymers produced by living organisms (Linos and Steinbüchel, 2005). Together with cellulose, lignin and starch, natural rubber (NR) (one representative of polyisoprenoids) is one of the most widely used biopolymers. The chemical structure of polyisoprenoids consists of a methyl-branched carbon-carbon backbone and can be considered a derivative isoprene (2-methyl-1,3-butadiene) (Lynen et al., 1958). Based on their structural characteristics and their molecular weight, natural polyisoprenoids can be categorized into six groups: (1) terpenoids, (2) steroids, (3) carotenoids, (4) higher polyisoprenoid alcohols, (5) highly polymerized polyisoprenes, and (6) hybrid isoprenes (including ubiquinone, or coenzyme Q, and menaquinone, or vitamin K_2) (Swiezewska and Danikiewicz, 2005). Highly polymerized polyisoprenes include poly(*cis*-1,4-isoprene) (rubber) and its stereoisomer poly(*trans*-1,4-isoprene) (gutta-percha) (Fig. 1).

Natural rubber is an elastomer that is produced by coagulating and drying the latex of rubber plants (Steinbüchel, 2001). Approximately 2500 plant species are known to produce NR. However, the rubber tree *Hevea brasiliensis*, which grows only in subtropical climates, is by far the most relevant commercial source of this polymer (Nair, 2010). Rubber from *H. brasiliensis* represents 99% of the world market. Other sources are guayule (*Parthenium argentatum*) and Russian dandelion (*Taraxacum kok-saghyz*). Chemically, NR is a polymer of 2-methyl-1,3-butadiene, and also known as isoprene, a conjugated diene containing double bonds at alternate positions. Latex is a colloidal dispersion where various micrometric objects, mainly rubber particles and lutoids, are dispersed in the cytoplasmic serum (Bottier, 2020). The latex composition can vary depending on the source, seasonal effects, and the state of the soil. An average composition of latex is shown in Table 1.

Poly(*cis*-1,4-isoprene) is the main constituent of NR, corresponding to 90% of its dry weight. The composition and the molecular weight of this polymer varies depending on the source (Mooibroek and Cornish, 2000). The poly(*cis*-1,4-isoprene) synthesized by *H. brasiliensis* is a highly unsaturated polymer composed of three trans-isoprene units at the ω-terminus followed by several hundred to a few thousand *cis*-isoprene units, which corresponds to an average molecular weight of 10^6 Da (Nair, 2010). Poly(*cis*-1,4-isoprene) rubber is one of the most important biological materials of our modern society, since it is used for a variety of goods in daily life in manufacturing, medicine, household and transport. The usefulness of rubber-made products is due to its

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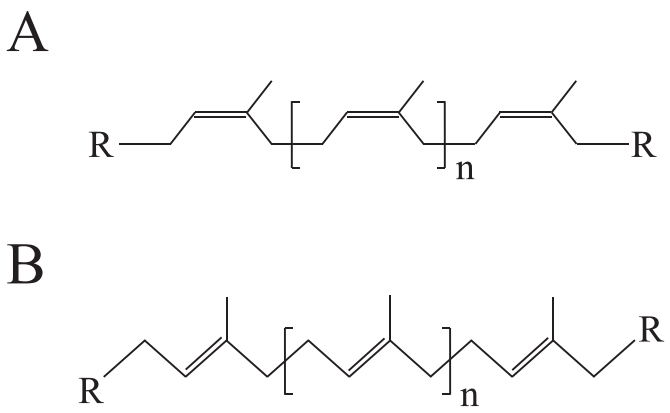

Fig. 1. Chemical structure of poly(*cis*-1,4-isoprene) (A) and poly(*trans*-1,4-isoprene) (B).

Table 1
Average latex composition (Ghosh, 2002).

Compound	Composition (% wt/wt)
Polyisoprene	30–40
Proteins	1.9–2.5
Carbohydrates	1–1.5
Lipids and related compounds	0.9–1
Inorganic components	0.4–0.6
Water	55–65

stability, elasticity, plasticity and insulating properties (Linos and Steinbüchel, 2005). The high demand and limited supply of rubber during the Second World War, forced the rubber industry to accelerate the production of synthetic rubber (SR) (Morton, 1981). The main raw material for producing SR are petrochemical feed-stocks, specifically crude oil. After the refining process of oil, the resulting product (naphtha) is combined with natural gas to produce monomers. SR is produced by the polymerization or polycondensation of these unsaturated monomers.

In 2017, the combined global annual production of SR and NR was 28.5 million metric tons (IRSG, 2018). The production grew around 75% over the last two decades. For many years, until 1990, two thirds of the total amount of consumed rubber was synthetic and one third NR. Since then, the proportion of SR consumption declined and nowadays, the production of the two rubber types differs only by 15%. An overview of the annual production and consumption of total rubber since the year 2012 is shown in Fig. 2. 92% of NR production takes place in Asia, with Thailand as the worldwide leader producing 4.2×10^6 tons in 2015. Indonesia is the second major producer with 3.1×10^6 tons, followed by Malaysia, India, Vietnam and China with a production of around 1×10^6 tons.

For many commercial applications, raw rubber is subjected to a vulcanization process in which the poly(*cis*-1,4-isoprene) chains are cross-linked either by heating in the presence of sulfur, as for the case with tires, by irradiation, by peroxidation, as in case of NR latex gloves (Berekaa et al., 2000; Stevenson et al., 2008) and several other cross-linking systems (Zhao et al., 2011). The formation of this network is a non-reversible chemical process. Vulcanized rubber possesses high tensile strength, hardness and abrasion resistance, it has great elastic properties and exhibits significantly enhanced durability compared to unvulcanized rubber.

The study of rubber-degrading microorganisms has been developed for more than 100 years, however, realistic biotechnological applications are very few until now. This work is focused on explaining the fundamentals about rubber biodegradation and how this knowledge can be applied for successful biotransformation of vulcanized and unvulcanized rubber materials.

2. Natural and synthetic rubber biodegradation

2.1. Rubber degrading microorganisms

The first report regarding decomposition of rubber by microorganisms was published in 1914, when Söhngen and Fol (1914) used highly-purified NR as a carbon source to prove the assimilation of the hydrocarbon chain by microorganisms. NR films were cultivated in glass bowls containing water and inorganic salts for several weeks at different temperatures. Two actinomycetes were identified and found to form colonies on the purified rubber material. In 1928, De Vries examined the possible decomposition of rubber hydrocarbon by fungi. Therefore, different *Aspergillus* and *Penicillium* species were inoculated in the presence of non-purified NR smoked sheets. After 5 years a weight loss of 30.9% of the NR was recorded. The first isolation of rubber-degrading bacteria using latex overlay plates was done by Spence and van Niel (1936). The growth of colonies was recognized by the formation of translucent halos in the opaque agar layer. A first report examining the biodegradation of vulcanized NR was presented by Blake and Kitchin (1949). The authors detected the decomposition of NR cables as a result of the microorganisms' actions. Kwiatkowska et al. (1980) reported the first biodegradation of vulcanized NR sheets by fungi (*Fusarium solani*), reaching weight losses of up to 40% of the initial weight. In 1985, Tsuchii et al. (1985) isolated the actinomycete *Nocardia* sp. strain 835A from soil. Several poly(*cis*-1,4-isoprene)-containing materials were tested in liquid cultures, reaching weight losses of up to 100% of the substrate. Tsuchii et al. (1990) treated polyisoprene latex with the extracellular crude enzyme of *Xanthomonas* sp.. Chromatographic analysis revealed the formation of low and high molecular weight oligomers. Different actinomycetes such as species of *Streptomyces*, *Amycolaptosis* and *Nocardia* were described to be able to metabolize NR as sole carbon source (Heisey and Papadatos, 1995). The ability of actinomycetes to degrade NR hydrocarbon, was confirmed by Jendrossek et al. (1997). After the isolation of a large number of Gram-positive and Gram-negative bacteria, it was found that the latex overlay technique was not suitable for the identification of clear zone-forming bacteria other than actinomycetes. Linos and Steinbüchel (1998) isolated strains belonging to the actinomycete genus *Gordonia*, which is considered as one of the most effective group of rubber-degrading bacteria. The *Gordonia* strains were isolated from stagnant water inside of deteriorating car tires, from soil of a rubber tree plantation and from sewage sludge.

From the historical outline, it became evident that actinomycetes represent the predominant group of microorganisms able to degrade poly(*cis*-1,4-isoprene) rubber. A list of the most important rubber-degrading microorganisms from the last two decades is shown in Table 2. The literature points out that members of the CNM (*Corynebacterium*, *Nocardia*, *Mycobacterium*) group together with members of the genus *Gordonia* are the most potent rubber-degrading bacteria (Hiessl et al., 2012; Yikmis and Steinbüchel, 2012a). They belong to the group of non-clear zone-forming rubber-degrading bacteria and require direct contact with the rubber substrates (adhesive growth) (Linos et al., 2000). The other group of rubber-degrading bacteria form clear zones on rubber overlay latex plates. Representatives of this group are the actinomycetes *Streptomyces*, *Actinoplanes* and *Micromonospora* (Imai et al., 2011). Chia et al. 2014 isolated 13 microorganisms from aged latex. Analysis of 16S rRNA gene sequence revealed that most of those microorganisms corresponded to *Gordonia* and *Streptomyces* species, confirming the predominance of these actinomycetes in rubber degradation processes.

2.2. Rubber degrading enzymes

Three oxygenases have been described to be responsible for the

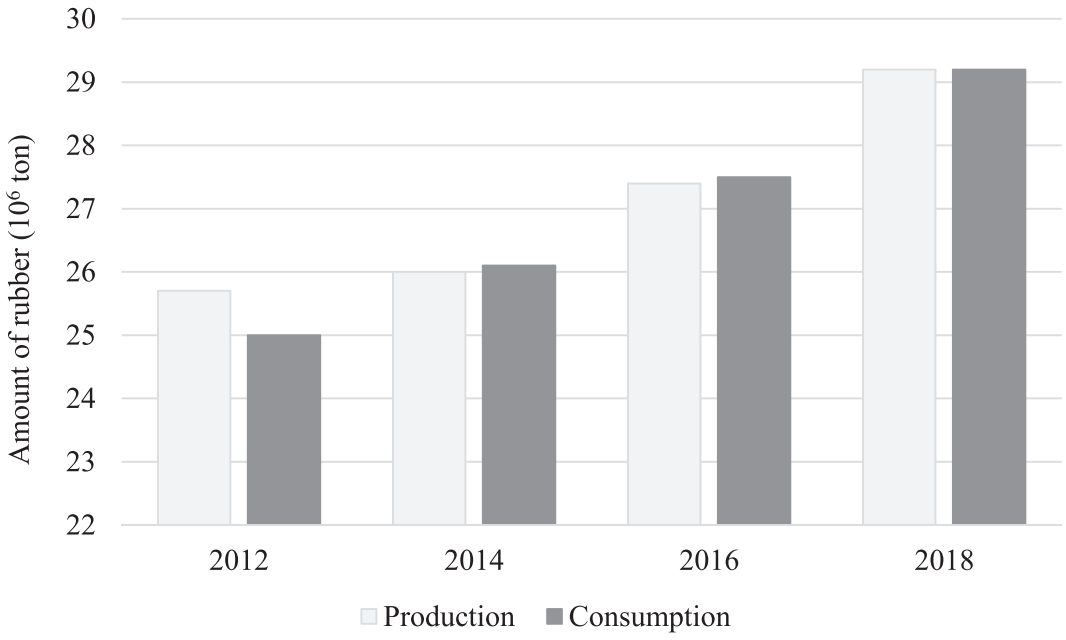

Fig. 2. Annual production and consumption of rubber (natural and synthetic).

primary extracellular cleavage of the polyisoprene carbon backbone. One of them is the latex clearing protein (Lcp), first detected in *Streptomyces* sp. K30 (Rose et al., 2005), and so far only identified in Gram-positive bacteria. Another enzyme is rubber oxygenase A (RoxA), first isolated and characterized from *Xanthomonas* sp. strain 35Y (Jendrossek and Reinhardt, 2003), present in Gram-negative clear zone forming bacteria. Recently, a third enzyme known as rubber oxygenase B (RoxB) was also isolated from *Xanthomonas* sp. strain 35Y and biochemically characterized (Birke et al., 2017).

The first purified rubber cleavage enzyme was RoxA, which was identified as a c-type diheme dioxygenase (Braaz et al., 2005; Jendrossek and Reinhardt, 2003). RoxA contains two heme centers that are covalently attached to RoxA via two heme-binding motifs (Jendrossek and Reinhardt, 2003). Recently, the three-dimensional structure of RoxA was elucidated revealing the essential amino acids of the active site (Birke et al., 2012; Seidel et al., 2013). RoxA homolog were identified in other Gram-negative bacteria such as *Haliangium ochraceum* and some *Mixobacteria*, but not in any Gram-positive rubber degrader (Birke et al., 2013). In contrast, *lcp* homologous genes were identified in *Gordonia* species (Bröker et al., 2008), in *Nocardia* species (Ibrahim et al., 2006), in *Actinoplanes* species and *Methylibium* species (Imai et al., 2011) and also in gutta percha-degrading bacteria

Table 2
Rubber degrading microorganisms identified from the year 2000.

Strain	Gram	Type of rubber degraded	Reference
Actinobacter calcoaceticus	−	Vulcanized rubber	Bode et al., 2001
Actinomadura sp.	+	Natural rubber latex	Ibrahim et al., 2006
Alternaria alternata	(fungus)	Natural rubber	Bosco et al., 2018
Amycolatopsis orientalis SY6	+	Latex dispersed in agar	Chengalroyen and Dabbs, 2013
Aspergillus niger	(fungus)	Natural rubber	Mohamed et al., 2017
Bacillus sp.	+	Natural latex rubber	Cherian and Jayachandran, 2009
Gordonia polyisoprenivorans VH2	+	Natural and synthetic rubber	Arenskötter et al., 2001; Berekaa et al., 2000; Hiessl et al., 2012
Gordonia polyisoprenivorans Y2K	+	Natural and synthetic rubber	Arenskötter et al., 2001
Gordonia westfalica	+	Natural and synthetic rubber	Linos et al., 2002
Methylibium fulvum HZ	−	Latex dispersed in agar	Chengalroyen and Dabbs, 2013
Methylibium sp. NS21	−	Latex dispersed in agar	Imai et al., 2011
Micromonospora aurantiaca W2b	+	Natural and synthetic rubber	Berekaa et al., 2000; Linos et al., 2000
Mycobacterium fortuitum NF4	+	Natural and synthetic rubber	Berekaa et al., 2000; Linos et al., 2000
Nocardia sp.	+	Vulcanized rubber	Ibrahim et al., 2006; Vivod et al., 2019
Sphingomonas sp	−	Ground tire rubber	Li et al., 2011
Penicillium chrysogenum	(fungus)	Natural rubber	Mohamed et al., 2017
Pseudomonas citronellolis	−	Synthetic rubber	Bode et al., 2000
Pseudomonas sp.	−	Natural and vulcanized rubber	Bosco et al., 2018; Roy et al., 2006
Pseudonocardia sp	−	Latex dispersed in agar	Chengalroyen and Dabbs, 2013
Rhizobacter gummiphilus sp	−	Natural rubber	Imai et al., 2013
Rhodococcus rhodochrous	+	Vulcanized rubber (glove)	Watcharakul et al., 2016
Rhodotorula mucilaginosa	(yeast)	Natural rubber	Bosco et al., 2018
Steroidobacter cummiodans sp		Natural rubber	Sharma et al., 2018
Streptomyces coelicolor	+	Vulcanized rubber (glove)	Bode et al., 2001, Bode et al., 2000
Streptomyces labedae	+	Natural rubber	Hesham et al., 2015
Streptomyces sp.	+	Latex, unvulcanized natural rubber	Chengalroyen and Dabbs, 2013; Gallert, 2000; Imai et al., 2011
Thermomonospora curvata	+	Latex dispersed in agar	Ibrahim et al., 2006
Xanthomonas sp.	−	Purified natural latex, synthetic rubber	Bode et al., 2001; Jendrossek and Reinhardt, 2003

(Warneke et al., 2007). The corresponding gene *lcp* was first identified in an UV-induced mutant strain of *Streptomyces* sp. K30, which was not able to form clear zones on latex-overlay plates. Complementation experiments using *lcp* restored this ability and thereby it was identified to be the responsible gene (Rose et al., 2005). Analysis of the amino acid sequence encoded by *lcp* from *Streptomyces* sp. strain K30 revealed a twin-arginine motif, thus indicating that Lcp is a substrate of the twin-arginine translocation (TAT) pathway (Yikmis et al., 2008).

Rubber degrading enzymes, RoxA, RoxB and Lcp, are secreted into the extracellular medium and cleave rubber at the double bonds of poly (*cis*-1,4-isoprene) via an oxidative reaction mechanism (Braaz et al., 2004). The three enzymes are able to cleave SR as well as NR and the cleavage products only differ in the number of intact isoprene units between the terminal ketone and aldehyde groups. Upon attack on rubber, RoxA releases low-molecular-mass degradation products, of which 12-oxo-4,8-dimethyltrideca-4,8-diene-1-al (ODTD) was identified as the major product (Braaz et al., 2005; Braaz et al., 2004). In contrast, Lcp cleaves rubber to multiple products corresponding mainly to oligo(*cis*-1,4-isoprene) molecules containing from four up to seven isoprene units (Jendrossek, 2014). This difference could be explained by the different cleavage mechanisms of RoxA and Lcp and the location of the active site inside the enzymes. For RoxA, an exo-type cleavage mechanism was postulated, which can explain the uniform spacing between two adjacent cleavage sites in the polymer. In contrast, an endo-type cleavage mechanism is proposed for Lcp, which might explain the variability of the different cleavage products (Jendrossek, 2014).

Next to the different cleavage mechanisms, RoxA and Lcp apparently represent completely different polypeptides without significant amino acid similarities (Yikmis and Steinbüchel, 2012b). While RoxA exhibits a molecular mass of 72 kDa and contain two heme groups (Braaz et al., 2005), the molecular mass of Lcp is 38 kDa with only one non-covalently bound heme (Birke et al., 2017). Even though RoxB shows a similar mass (70.3 kDa) compared to RoxA and is also classified as *c*-type diheme dioxygenase, the rubber cleavage mechanism is similar to that of Lcp, as a mixture of oligo-isoprenoid molecules was produced as degradation products.

2.3. Metabolism of poly(cis-1,4-isoprene) in bacteria

Since rubber is a high-molecular-weight polymer, it cannot be taken up by cells directly; the polymer first has to be cleaved extracellularly into low-molecular-weight compounds that can be transported across the cell membrane and used for the metabolism. The cleavage of rubber is performed by rubber oxygenases, as described before. The resulting low-molecular-weight compounds should be further oxidized, but the genes encoding the respective enzymes have not all been identified until now. In *Streptomyces* sp. strain K30, the aldehyde intermediates are oxidized via a heterodimeric molybdenum-dependent hydroxylase encoded by *oxiAB* (Rose et al., 2005), but *G. polyisoprenivorans* does not possess *oxiA* and *oxiB* homologues (Hiessl et al., 2012). The import of the intermediates across the cell wall is mediated by Mammalian Cell Entry (Mce) proteins, which may function as substrate-binding proteins. This mechanism was proposed based on the analysis of transposon-induced mutants of *G. polyisoprenivorans* when a mutant carrying the transposon insertion in a gene encoding the Mce protein was found to show a rubber-negative phenotype (Hiessl et al., 2012). In total, five Mce clusters are located in the genome of *G. polyisoprenivorans*, each of which comprises two genes encoding YrbE proteins, followed by six genes encoding Mce proteins (MceA-F). Once the rubber intermediates are taken up the cell, they are metabolized via β-oxidation. For a better understanding, a simplified version of the proposed metabolic pathway of poly(*cis*-1,4-isoprene) by Hiessl et al. (2012) is shown in Fig. 3. The resulting organic acids are converted to an acyl-CoA thioester by an acyl-CoA synthetase and further catabolized by an acyl-CoA dehydrogenase. The reduction of double bonds at even-numbered positions, followed by an isomerization, might occur via the enzymes 2,4-dienoyl-CoA reductase and enoyl-CoA isomerase, respectively, while the following hydration step is catalyzed by an enoyl-CoA hydratase. A 3-hydroxyacyl-CoA-dehydrogenase is responsible for the conversion of the hydroxyl derivate into the keto form.

A first β-oxidation cycle is complete when an acyl-CoA acetyltransferase releases acetyl-CoA. For the next β-oxidation cycle, only the (*S*)-isomer can serve as a substrate for the acyl-CoA dehydrogenase, and therefore the conversion of the (*R*)- into the (*S*)-stereoisomer by an α-methylacyl-CoA racemase (Mcr) is needed. As a result of the successive cycles of β-oxidation, propionyl-CoA and acetyl-CoA are consecutively released and further metabolized via the central metabolism. The length of the degradation products that are taken up into the cell will determine the number of cycles and the amount of released propionyl-CoA and acetyl-CoA.

3. Applications and alternatives for rubber recycling bioprocesses

As a consequence of the widespread use of rubber products and the difficulties in reusing rubber material, mainly in the form of tires, huge amounts of rubber waste are generated. This material could provide a great asset of raw material, instead of being environmental and health threats, if efficient methods for rubber recycling could be developed. Several methods have been proposed for reusing and recycling rubber materials, including mechanical, chemical, physical and a few biotechnological processes (Fang et al., 2001; Myhre and Mackillop, 2002; Stevenson et al., 2008; Yehia, 2004). One strategy to recycle rubber is to reduce the particle size by shredding and grinding. The three major processes for producing crumb rubber are mechanical grinding at ambient temperature, cryogenic grinding, and wet grinding. Crumb can be combined with freshly-produced virgin rubber and rebound with latex adhesives to produce new rubber. However, the physical performance of those generated products is somewhat deteriorated (Myhre and Mackillop, 2002). The presence of sulfur crosslink leads to a weak adhesion and deterioration of the final properties (Naskar et al., 2000). This factor has motivated the research on cost-effective in-situ regeneration or devulcanization of the scrap rubber to provide superior properties. In addition to direct recycling, waste rubber has other industrial applications. During the last years, it has been realized that the incorporation of tire crumb into asphalt pavement can be cost-effective and result in a road surface with improved properties and life span (Navarro et al., 2007). Other applications are the use of crumb rubber in flooring and play surfaces, in landfill and soil treatment, and in plastic blends and composites to produce thermoplastics (Myhre and Mackillop, 2002). The use of medium particle size tire crumb in thermoplastics shows a good potential, particularly for use in automotive components such as vehicle wheel well and trunk liners. Fine particle size crumb should be useful in the production of thermoplastic elastomer products. In this potential application, post-industrial waste such as ethylene-propylene-diene rubber (EPDM) scrap may be the preferred source of crumb (Wiessner et al., 2003).

As described above, during vulcanization, polyisoprene chains in rubber are covalently linked via sulfide bridges (mono-, di-, and polysulfidic bridges) involving as many as five sulfur atoms. Most of the properties of a rubber material depend on the type and quantity of cross-links formed between rubber polymers (Chaikumpollert et al., 2012). In comparison to untreated tire crumb, devulcanization of tire crumb rubber provides an improvement of the physical properties when either revulcanized alone or in blends with virgin rubber (Sabzekar et al., 2015). However, the physical performance, particularly tear strength, are again affected and additionally, the processing properties are changing (Punnarak et al., 2006). Lower performance and changed properties would be acceptable if the cost of the devulcanized rubber could be reduced. Accordingly, there is a need for focused studies on cost reduction of the devulcanization process.

The drawbacks of chemical and physical modification techniques

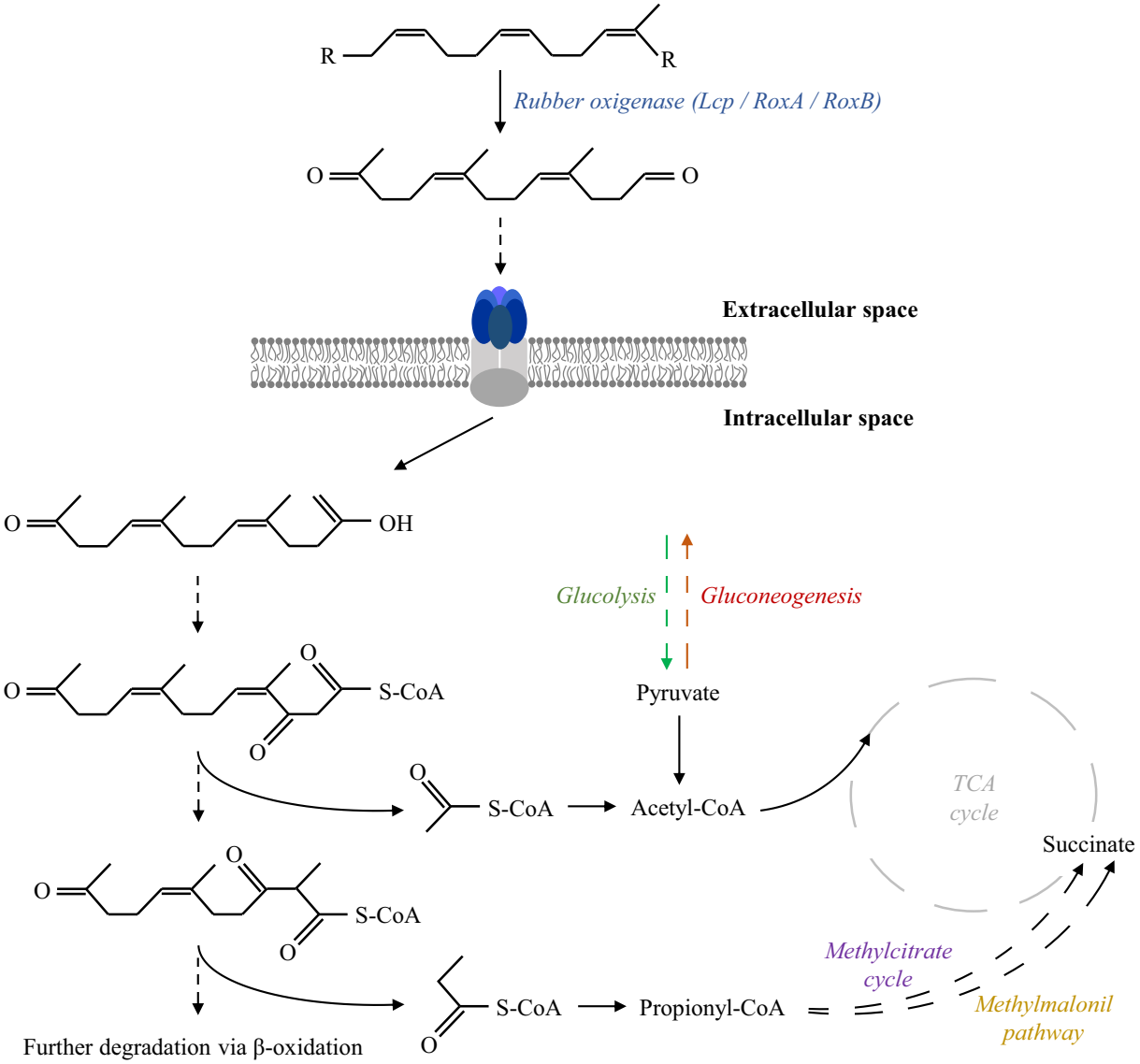

Fig. 3. Poly(*cis*-1,4-isoprene) metabolism in *G. polyisoprenivorans* VH2.

are the use of hazardous chemicals and the high energy requirements, respectively. Moreover, some of the alternatives to rubber treatment, such as the burning of scrap rubber, may not be an acceptable solution in the long term. Most rubber products are sulfur-vulcanized and protected with anti-degradants, producing sulfur and nitrogen oxides upon combustion (Bowman, 1975).

The biodegradability of rubber and rubber products plays an important role in providing environmentally compatible solutions for the disposal and recycling of rubber waste. According to Bredberg et al. (2005) several important aspects must be considered for an efficient biotechnological treatment of rubber: development of new microorganisms and enzymes, efficient reactor designs, optimization of time in the reactor, partial degradation of the polymer backbone, detoxification of rubber, synthesis of new products from recycled rubber, and reuse of recycled rubber.

3.1. Heterologous synthesis of poly(cis-1,4-isoprene) rubber degrading enzymes

The use of the rubber degrading enzyme Lcp has been studied as a potential alternative for poly(*cis*-1,4-isoprene) rubber degradation. For this, the strain *Escherichia coli* C41 has been used as host strain for the heterologous expression of genes encoding Lcp1 from G. polyisoprenivorans VH2. The first study regarding heterologous expression of $lcp1_{VH2}$ in *E. coli* reported a final protein concentration of only 2 mg l^{-1} (Hiessl et al., 2014). Higher $Lcp1_{VH2}$ concentrations were not reached, mainly due to low protein solubility and large amounts of aggregated protein in form of inclusion bodies. Studies have shown that fermentation variables such as temperature, pH, oxygen availability and nutrient composition can affect transcription, translation, proteolytic activity, secretion, production levels, and stability of the target protein. In addition, the selection of the strain, the cultivation strategy and the use of engineered target proteins are relevant factors for increasing the protein solubility. Therefore, it was necessary to establish a simple and effective method for purifying large amounts of the enzyme at low costs and to optimize the cultivation conditions for high-yield production of $Lcp1_{VH2}$.

Several aspects, steps and operations are involved in the enzyme synthesis process (Fig. 4). Those operations must be analyzed to improve the overall yields of soluble and active protein. A first step is focused on the selection and/or design of the cultivation medium and the use of an auto-induction medium (AIM) instead of Luria Broth –

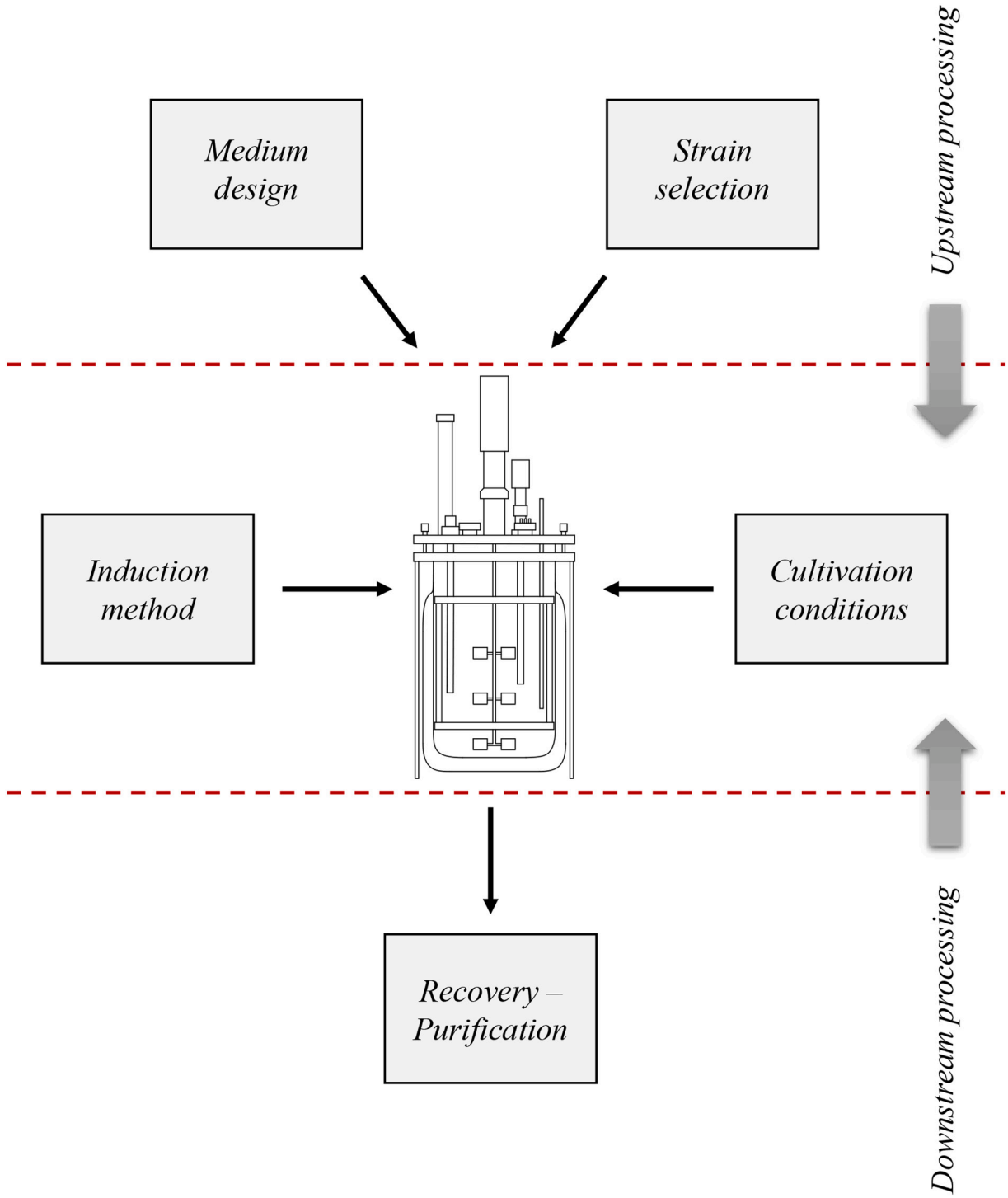

Fig. 4. Relevant operations for the improvement of Lcp production.

Isopropyl β-D-1-thiogalactopyranoside (LB-IPTG) system for cultivation. Inducible systems, *T7* or *lac*-based promoters (*tac*, *trc*, *lac*, *lacUV5-T7 hybrid*, etc.), which can be effectively induced by the addition of IPTG, are the most frequently used methods (Donovan et al., 1996). However, studies showed that high concentration of IPTG can inhibit cell growth and recombinant protein production (Choi et al., 2000; Yim et al., 2001). It should also be mentioned that the IPTG-inducible production system may not be desirable for the large-scale production of recombinant proteins because of the high cost of IPTG (Chauhan et al., 2001). Andler and Steinbüchel (2017) showed that the use of AIM increased the cell density 4-fold compared to LB-IPTG medium and, consequently, higher $Lcp1_{VH2}$ production yields were achieved. Another challenge is to establish a simple and cheap purification process for achieving large amounts of active enzyme. The purification of $Lcp1_{VH2}$ by ammonium sulfate precipitation considerably reduced costs and operational time while reaching 50–60% of protein purity. The use of AIM for *E. coli* C41::*lcp1*$_{VH2}$ cultivation and salting-out precipitation with ammonium sulfate for protein purification reduced the associated costs approximately 100-fold in comparison with the previous study, and a 30-fold higher $Lcp1_{VH2}$ concentration was reached (60 mg l^{-1}) (Andler and Steinbüchel, 2017). However, large amounts of non-soluble protein was still lost due to the formation of inclusion bodies. A crucial

optimization step needed in order to increase the expression of soluble protein is focused directly on the fermentation process (Fig. 4). The literature suggests several strategies to overcome this problem, which could be classified into strategies with or without engineering of the target (Sørensen and Mortensen, 2005).

Strategies avoiding target modification include cultivation at reduced temperatures, selection of a specific strain, modification of the cultivation strategy, and including molecular chaperones. Altenhoff et al. (2020) performed a fed-batch fermentation in 2-l bioreactors for the production of $Lcp1_{VH2}$. Despite the fact that high cell dry weights (~60 g L^{-1}) were reported, due to the high amounts of C-sources added to the medium (glucose or glycerol), only a 3.7-fold higher Lcp production yield was reached compared to the report by (Andler and Steinbüchel, 2017). Considering that the purification system was an affinity chromatography with a nickel-charged affinity resin using and the chelating ligand nitrilotriacetic acid (Ni-NTA) connected to a fast protein liquid chromatography (FPLC) system, the associated production costs were higher than previous established processes. The low Lcp concentration (0.2 g L^{-1}) might be explained due to the solubility problem of $Lcp1_{VH2}$ and the high loss in the soluble fraction. Andler et al. (2019a) studied two crucial parameters for improving the solubility of the target protein ($Lcp1_{VH2}$), the cultivation temperature and the agitation rate were, reaching up to 99.6% of solubility in cultures at 10 °C and 150 rpm. However, at low temperatures the production rates were negatively affected and long cultivations times were needed. As a process, the optimal condition was found to be 25 °C and 300 rpm, reaching $Lcp1_{VH2}$ concentrations of 0.38 g l^{-1}.

For strategies involving engineering of the target protein, the use of fusion protein technology and the screening for soluble variants have been reported to have been successful (Sørensen and Mortensen, 2005). An interesting result was obtained with the use of N utilisation substance protein A (NusA) as a fusion partner, which enhanced the solubility of $Lcp1_{VH2}$ in cultures at 30 °C (5.7-fold higher solubility than without the tag) and also enhanced the production yields. A final concentration of 1 g l^{-1} of the fusion protein NusA-His-$Lcp1_{VH2}$ was achieved with oxygenase activity in the presence of polyisoprene latex. As there was no need for removing the fusion tag or further time-consuming chromatographic purification steps, this method resulted in an affective, simple and cheap process to produce large amounts of soluble and active $Lcp1_{VH2}$ (Andler et al., 2019a). A final fourth step was also investigated in this study, which was focused on the non-soluble protein fraction (inclusion bodies). The literature points out that the renaturation of protein from inclusion bodies can present several drawbacks, and the search for proper reconstitution conditions can be very laborious and time consuming (Sørensen and Mortensen, 2005). Nevertheless, in some cases, the synthesis of proteins as inclusion bodies and using in vitro solubilization and refolding techniques is more convenient (Singh and Panda, 2005). Considering that 1.34 g $Lcp1_{VH2}$ l^{-1} (87% of the total production) is discarded as insoluble protein in cultivations of *E. coli* C41::*lcp1*$_{VH2}$ at 30 °C, it was necessary to consider the in vitro solubilization and refolding. The overall yield of refolded and active enzyme obtained from the inclusion bodies of *E. coli* was 55%. Considering the low ratio of soluble to non-soluble fraction in cultivations at high temperatures, this might be considered as a potential method to improve the final concentration of $Lcp1_{VH2}$. However, further optimization of the refolding step will be necessary to increase the production yields. The presented results establish the large-scale production of $Lcp1_{VH2}$ with optimization of the cultivation medium, the fermentation process parameters, and the purification method.

3.2. The use of rubber degrading enzymes for the cleavage of poly(cis-1,4-isoprene) rubber

Besides the extensive knowledge about the molecular structure, the biochemical functions and the poly(*cis*-1,4-isoprene) cleavage mechanisms of rubber degrading enzymes (Jendrossek, 2014), only a few studies regarding the use of pure or partially purified rubber oxygenases have been published, mainly about Lcp form *Gordonia polyisoprenivorans* VH2.

A cell-free bioprocess was established as an alternative for a total or partial degradation of poly(*cis*-1,4-isoprene) rubber (Andler et al., 2018a). For this, a 200-ml enzyme reactor was designed in order to provide a stable system with temperature and aeration control under sterile conditions. The cleavage of the backbone of rubber particles by $Lcp1_{VH2}$ was studied and characterized. The most effective cleavage was achieved by repetitive additions of 50 μg of $Lcp1_{VH2}$ ml^{-1}, obtaining total degradation of 0.2% (*w*/w) polyisoprene particles after five days of incubation. Isolation of the oligo(*cis*-1,4-isoprene) molecules was achieved by using silica gel column chromatography and a relative quantification of the isolated products was performed by high performance liquid chromatography-mass spectrometry (HPLC-MS) after derivatization with 2,4-dinitrophenylhydrazine (2,4-DNPH). Gel permeation chromatography (GPC) analysis showed an increased amount of degradation products corresponding to the enzyme addition pulses (Andler et al., 2018a). Downstream processing of the degradation products showed a defined distribution, with molecules comprising 1 to 11 isoprene units and exhibiting an average molecular weight of 885 g mol^{-1}. Oligo(*cis*-1,4-isoprene) molecules containing five to six isoprene repetitive units were found in higher concentrations. Longer incubation periods or the addition of larger amounts of $Lcp1_{VH2}$ did not cause a detectable change of the product pattern (Andler et al., 2018a).

Poly(*trans*-1,4-isoprene) rubber, commonly known as gutta-percha, is also possible to degrade using rubber cleaving enzymes. Vivod et al. (2019), incubated Lcp from *Nocardia Nova* SH22a, which has the ability to cleave both polyisoprene isomers. Large amounts of His_6-tagged Lcp_{SH22a} were synthesized using AIM (Andler and Steinbüchel, 2017) and for the purification of the enzyme, immobilized metal affinity chromatography (IMAC) was performed. After incubation of Lcp_{SH22a} with poly (*trans*-1,4-isoprene) rubber, oligo(*trans*-1,4-isoprene) molecules were extracted with pentane, derivatized with Girard-T reagent and analyzed by Electrospray Ionization-Mass Spectrometry (ESI-MS). Interestingly, the spectra showed only two oligoisoprenoid corresponding to molecules containing 2 and 4 isoprene repetitive units in their structure. The difference on the cleavage mechanism comparing both isomers might be related to the substrate specificity according to the authors.

Recently, a novel poly(*cis*-1,4-isoprene) rubber biotransformation method was reported, using a multi-phase enzymatic reactor (Andler et al., 2020). The system was focused on the continuous migration of oligo(*cis*-1,4-isoprene) degradation products from the aqueous phase (0.2 M Tris-Buffer pH 7.0) to the organic phase (ethyl acetate or pentane). $Lcp1_{VH2}$ was added into the aqueous phase every 24 h for 5 days, reaching biotransformation yields between 42 and 52% depending on the enzymatic reactor design and the extraction solvent. The enzyme showed a slight activity decrease in the presence of the organic solvents compared to the absence of them. However, the method is showing an interesting procedure for continuous extraction of degradation products, avoiding liquid-liquid extraction or solid phase extraction as reported previously (Andler et al., 2018a).

3.3. Rubber as carbon source for fermentative processes

The biodegradation of polyisoprene would be complete if the microorganisms are incorporating acetyl-CoA and propionyl-CoA, as the final metabolites of β-oxidation, into the central metabolism (Hiessl et al., 2012). This was confirmed by the growth of *G. polyisoprenivorans* VH2 when polyisoprene was the sole carbon source in the cultivation medium. The focus of this study was the detection, characterization and quantification of the corresponding oligo(*cis*-1,4-isoprene) molecules obtained by in vivo degradation. A cultivation strategy using the mutant strain *G. polyisoprenivorans* strain TH5, which was unable to take up the intermediates oligo-isoprenoid molecules into the cell cytoplasm

for further degradation, was established (Andler et al., 2018b). Oligo-isoprenoid molecules accumulated in the extracellular space due to the cleavage of polyisoprene by Lcp. Due to the inability of this mutation to grow on polyisoprene as sole carbon source, a daily addition of propionate to the cultivation medium was necessary. Only after eight days of cultivation it was possible to detect the formation of degradation products corresponding to oligo(*cis*-1,4-isoprene) molecules containing three to ten isoprene units. Longer incubation periods did not result in the formation of smaller molecules such as mono or di-isoprenoids. In fact, a repetitive pattern indicating oligo(*cis*-1,4-isoprene) molecules contained five to six isoprene units were found in the highest concentration. Similar results were obtained using Lcp from *Streptomyces* K30 in experiments containing latex polyisoprene (Jendrossek, 2014; Birke et al., 2015; Braaz et al., 2005; Röther et al., 2016). An *endo*-cleavage mechanism is proposed for Lcp, as several sizes of degradation products are obtained. In contrast, RoxA cleaves polyisoprene by an *exo*-cleavage mechanism, producing mainly one product, known as ODTD (Braaz et al., 2005). However, the mass distribution of the degradation products suggests that Lcp has a specific cleavage mechanism that is not as random as was thought. The isolation and characterization of the synthesized oligo-isoprenoid molecules containing aldehyde and ketone terminal groups might be interesting for the manufacture of new products with properties and characteristics not described so far.

Although polyisoprene could be used as carbon source for the growth of several microorganisms, very few reports exist on this subject. In a previous study, the possibility of transferring and expressing foreign genes for polyhydroxyalkanoate (PHA) synthesis in *G. polyisoprenivorans* VH2 was studied (Arenskötter et al., 2003). For this experiment, hexadecane was used as a sole carbon source and the formation of medium chain length PHA (mcl-PHA) was detected by gas chromatography (GC) analysis. A similar study was presented for the biosynthesis of PHA utilizing polyisoprene as sole carbon source in order to find recycling methods for rubber waste materials and alternative carbon sources for the production of PHA (Andler et al., 2019b). Cultivations with recombinant *G. polyisoprenivorans* VH2 harboring pAK68 (*c*ontaining *phaCAB* from *Ralstonia eutropha*) led to the intracellular accumulation of known short chain length PHA (scl-PHA), as the copolymer poly(3HB-*co*-3 HV). A novel polymer was synthesized by a strain expressing *phaC1* from *Pseudomonas aeruginosa*. Oligo-isoprenoid molecules coming from the metabolism of polyisoprene were shown to act as precursors for the synthesis of PHA. The results showed the synthesis of PHA containing different oligo-isoprenoid molecules of different chain length on its structure. This suggest the synthesis of a novel polymer containing oligo-isoprenoid molecules coming from the β-oxidation as reported by Hiessl et al. (2012). The production of biomass or different metabolic products might represent an interesting recycling process. The biosynthesis of high-value compounds coming from industrial waste or by-products is crucial for biosustainability.

3.4. Poly(cis-1,4-isoprene) rubber surface modification

To increase the interactions between ground rubber particles with the virgin rubber or other materials, a surface modification process for ground rubber is necessary in order to make the polymer chains on the particle surfaces more flexible and mobile to facilitate renewed interparticle bonding during revulcanization (He et al., 2016). Some surface activation processes actually devulcanize the surface layer of the crumb particle. To reduce the concentration of sulfur crosslinks in vulcanized rubber, several methods have been applied, among them the use of mechanical shear, heat and other energy input, and a combination of chemicals such as oils, accelerators, amines, etc. A technique that has been successfully employed to recycle crumb rubber is to simply rebind the particles using an adhesive binder such as polyurethane precursors, liquid polymers, oligomers, resin adhesives, virgin polymers, and rubber curatives (Crespo et al., 2009).

Besides the formation of oligo-isoprenoid molecules, the Lcp-mediated conformational changes on the polyisoprene surface, might represent a potential method to improve the material properties for rubber recycling methods. Analysis by attenuated total reflectance - Fourier-transform infrared spectroscopy (ATR-FTIR) confirmed that the surface of the polyisoprene particles was altered after incubation with $Lcp1_{VH2}$. In fact, carbonyl groups (C = O) were detected after three days of incubation, indicating the presence of partially cleaved chains of the polymer (Andler et al., 2018a). It is known that crumb rubber has poor interface compatibility with inorganic materials (He et al., 2016). This is due to the hydrophobic surface of rubber in contrast to the hydrophilic nature of inorganic materials. The structural changes on the polyisoprene surface, in particular the presence of carbonyl groups, will contribute for decreasing the hydrophobicity of the rubber material and an increase in the adhesion between rubber and materials with hydrophilic nature. An improved interface bonding facilitates the mixture of rubber with other materials, which can be considered as a potential recycling process.

3.5. Rubber detoxification and desulfurization

In general, synthetic materials as rubber and plastic waste are resistant against microbial attack. This can be explained due to the lack of capable enzymes to attack their structure because of the short time of presence in nature evolution (Ali Shah et al., 2008), and the numerous toxic compounds, which are part of the product manufacturing process. For achieving successful microbial rubber degradation, pre-treatments such as microbial detoxification and microbial desulfurization/devulcanization must be performed.

3.5.1. Rubber bio-detoxification

An important pre-treatment for rubber degradation should focus on the detoxification of compounds or additives that inhibit microbial growth, including zinc oxide, zinc salts and several other additives (Bredberg et al., 2002). A good candidate is the fungus *Recinicium bicolor*, which is able to detoxify cryo-ground tire rubber (CGTR), allowing other bacteria to grow on the rubber more successfully (Stevenson et al., 2008). Several fungal species have the ability to degrade certain aromatic compounds found in rubber and have been successfully used to detoxify rubber material. This is for example the case in white rot fungal species, which were found to have the ability to biodegrade the aromatic polymeric dye polyvinylamine sulfonate anthrapyridone in the presence of CGTR (Bredberg et al., 2002). *R. bicolor* is considered to be the most effective fungus for treating pure CGTR. The fungal ability for reducing solid waste, specifically polymeric materials like plastics, has been demonstrated showing a great bioremediation potential (Sánchez, 2020). Bacterial detoxification has been proposed as well, specifically for the vulcanization accelerator 2-mercaptobenzothiazole (MBT). Species of *Rhodococcus*, *Corynebaterium*, and *Pseudomonas* as well as *Escherichia coli* also exhibit the ability to break down or biotransform MBT (Haroune et al., 2004).

Biotechnological processes have been proposed for rubber recycling that do not include harmful or toxic chemicals and are normally not energy intensive. Nevertheless, microorganisms are in many cases sensitive to chemical substances, including rubber additives. According to (Altenhoff et al., 2019), antioxidants, such as *N,N′*-di-aryl-*p*-phenylenediamine (DAPD/DTPD), N-(1,3-dimethylbutyl)-*N′*-phenyl-*p*-phenylenediamine (6PPD) and N-isopropyl-*N′*-phenyl-*p*-phenylenediamine (IPPD) are the most interfering group of additives for microbial and enzymatic degradation of rubber. The accelerators ZBEC-70 (a dithiocarbamate) and CBS-80 (a thiazole) were also found to inhibit cell growth; however, $Lcp1_{VH2}$ was not significantly affected under low concentrations of these additives.

3.5.2. Rubber bio-desulfurization

Desulfurization or devulcanization of polyisoprene rubber can be achieved with sulfur-oxidizing bacterial species or sulfur-reducing

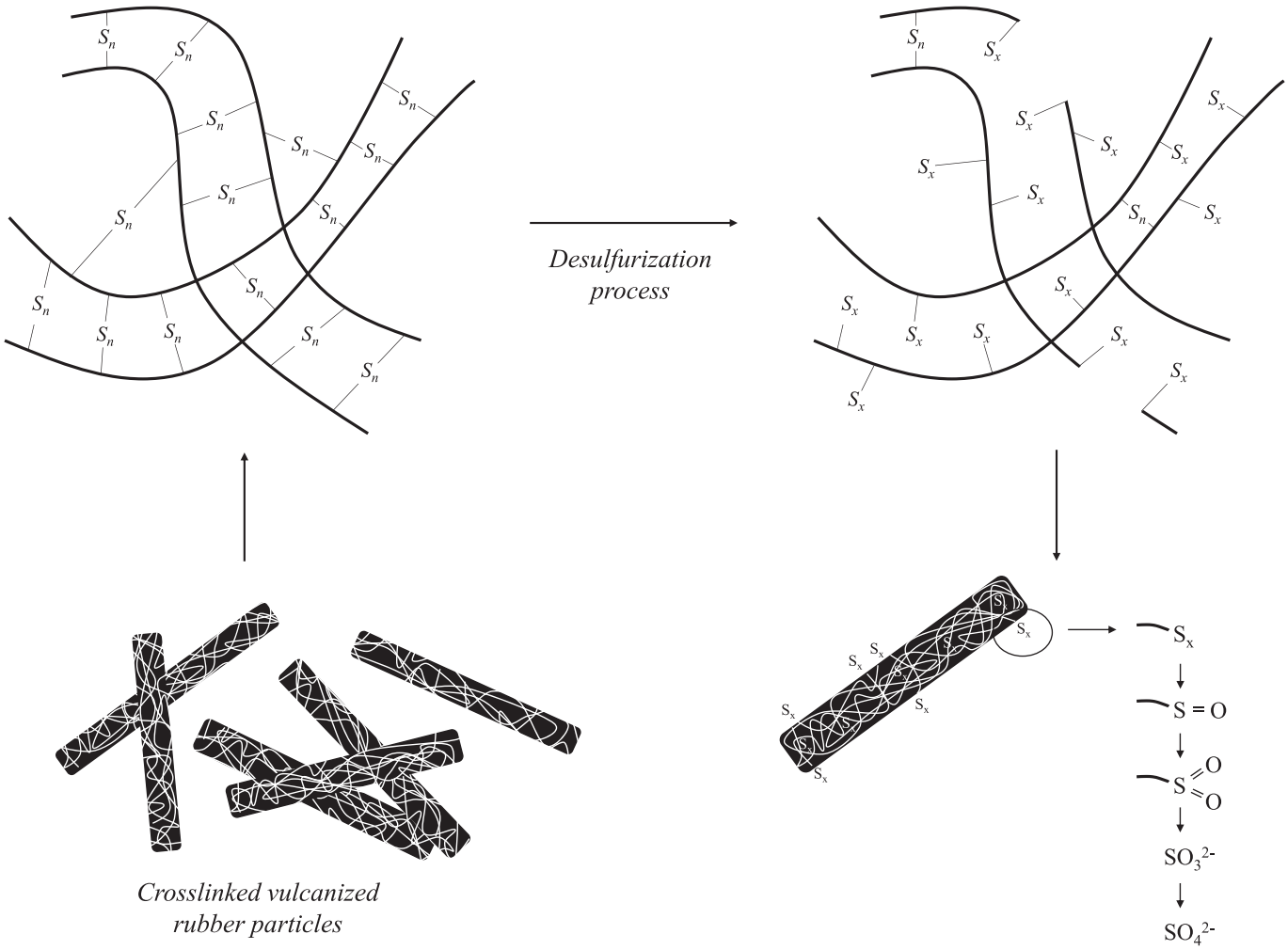

Fig. 5. Proposed desulfurization process of vulcanized rubber particles using sulfur-oxidizing microorganisms and the formation of sulfate ion.

archaea. Microorganisms able to cleave sulfur-sulfur and sulfur-carbon bonds have been discovered and can be used to devulcanize waste rubber. The main metabolic pathway described for biological desulfurization using dibenzothiophene (DBT) as a model substrate is the 4S pathway encoding by dszABC operon, described for the first time by Oldfield et al. (1997). The pathway requires the chain reactions of four enzymes: DszA, DszB, DszC and DszD. (Chandra Srivastava, 2012; Gupta et al., 2005; Khosravinia et al., 2018). A proposed desulfurization process is shown in Fig. 5, where cross-linked sulfur networks in vulcanized rubber particles are presented, as well as, the biological devulcanization mechanism and the sulphate release pathway.

Sulfur-oxidizing bacteria, specifically of the genus *Thiobacillus*, *Rhodococcus* and *Sulfolobus* have been applied successfully to oxidize disulfide linkages (Holst et al., 1998; Romine and Romine, 1998). The sulfur-reducing Archaeon *Pyrococcus furiosus* has also been studied regarding this potential application (Bredberg et al., 2001). Desulfurization of ground tire rubber (GTR) by *Gordonia desulfuricans* and *Rhodococcus* sp. was studied by Tatangelo et al. (2019), as well as the characterization of the autochthonous communities during this process. The biodesulfurization potential was measured by the expression of *dszA* over time. Aboelkheir et al. (2019a) compared the desulfurization potential of three bacterial strains: *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Streptomyces* sp. After a 4-weeks cultivation experiment using self-prepared vulcanized butadiene-styrene rubber (SBR), the biodegradation effect was evaluated by different analysis including FTIR-ATR, thermogravimetric analysys (TGA), differential scanning calorimetry (DSC) and scanning electron microscopy / energy dispersive x-ray spectroscopy (SEM/EDS). The results pointed out *Streptomyces* sp. as the most effective one in terms of biodevulcanization. An interesting structural change in rubber samples after cultivation with all the bacteria was the enhancement in wettability properties, which was measured by the contact angle using water as a solvent. This will allow the rubber surface to be more hydrophilic as polarity tends to increase after oxidation of the degraded surface. Likewise, FTIR results showed different modifications of the polar groups on the rubber surface like the appearance of the C═O stretching band at 1735 cm^{-1}, which might be associated to the steraric acid used during rubber compounding (Aboelkheir et al., 2019a; Aboelkheir et al., 2019b).

In a recent study, *Bacillus cereus* TISTR 2651 was also investigated removing up to 26.4% of the sulfur present in NR vulcanizates (Kaewpetch et al., 2019). Li et al. (2012) evaluated the desulfurization of ground tire rubber in liquid cultures of *Sphingomonas* sp., and the resulted desulfurated ground tire rubber (DGTR) was evaluated in terms of physical and mechanical properties. After preparing SBR-DGTR composites, the swelling value was higher than those of SBR vulcanizates and a lower crosslinked density was calculated due to the decrease of the sulfur content. Both are proper indicators of a successful desulfurization process. A more recent report compared the devulcanization potential of different bacteria such as *Thiobacillus ferrooxidans*, *Gordonia westfalica*, *Gordonia polyisoprenivorans*, *Gordonia alkanivorans*, *Nocardia* sp., *Amycolaptosis sulphurea* and *Pseudomonas putida*. The sulfur reduction of the tire matrix was studied, reducing the total sulfur

content of the studied ground tire by 6–21% depending on the strain in use (Ghavipanjeh et al., 2018).

After the detoxification and devulcanization of rubber, two alternatives might be possible; recycling or degradation. If degradation is preferred, rubber can be utilized as carbon source by rubber-metabolizing microorganisms such as *Gordonia* species or *Streptomyces* species. The other alternative, as explained above, is the bioconversion of the polymer to oligo-isoprenoid molecules after incubation with rubber cleaving enzymes (Lcp or RoxA). A multi-step bioremediation process approach where rubber can first be detoxified, devulcanized, and then recycled or biodegraded with the methods developed in this study can be established as a potential rubber recycling alternative.

4. Techniques for rubber degradation analysis

Several techniques have been used for detecting and quantifying rubber transformation or rubber degradation after biological treatments. Some of the most employed methods include: i) staining procedures to identify the formation of carbonyl groups present at the terminal groups of oligo(cis-1,4-isoprene) degradation products (Fig. 6A), ii) nuclear magnetic resonance (NMR) for identifying the structure and conformation of degradation products (Fig. 6B), ESI-MS for the analysis of the molecular mass of the oligo(*cis*-1,4-isoprene) when endocleavage enzymatic mechanisms are performed (Fig. 6C), GPC for detecting the mean molecular mass of the initial polymer, and the bioconversion of the polymer to the corresponding degradation product (Fig. 6D), and FTIR for the identification of the functional groups of the rubber structure and appearance of carbonyl groups after bioconversion (Fig. 6E). From the described methods, ESI-MS might have some advantages due to the high analytical sensitivity and the detailed spectra which indicates the number of isoprene units of each oligo-isoprenoid. The latest become relevant if an endocleavage mechanism is applied, for instance, when Lcp or RoxB are studied as catalysts. For successful detection using ESI-MS, the oligo-isoprenoids must be derivatized prior to the analysis with Girard reagent T to form a hydrazone conjugate harboring a precharged quaternary ammonium moiety, which facilitates the detection of the resulting conjugate by positive-ion ESI-MS. Instead, NMR, GPC and FTIR do not require pretreatments but the information of the resulting degradation products is usually limited.

In the search of quantitative analysis of the level of rubber biodegradation in terms of the biodeterioration of the polymer and/or the formation of the corresponding oligomers an HPLC-MS method was established by Andler et al., 2018a. The oligo(*cis*-1,4-isoprene) molecules were derivatized with 2,4-DNPH and separated by a RP C18 column with a gradient method using water with formic acid (0,1% *v*/v) and methanol. The area of each peak was calculated and compared to the total area in the chromatogram and the total mass of extracted product. From the initial mass of the polymer (2.00 g) a total amount of 1.58 g corresponding to the oligo(*cis*-1,4-isoprene) molecules was calculated and the highest concentration was found for the product containing 5 isoprene units in its structure with 0.337 g L^{-1}.

Röther et al., (2017) established a protocol for detecting oligo-isoprenoid molecules based on HPLC using a RP-8 reversed phase column and a gradient method with a mixture water-methanol as mobile phase. The chromatograms identify the length differences between the degradation products by using of Lcp or RoxA as catalysts as described above.

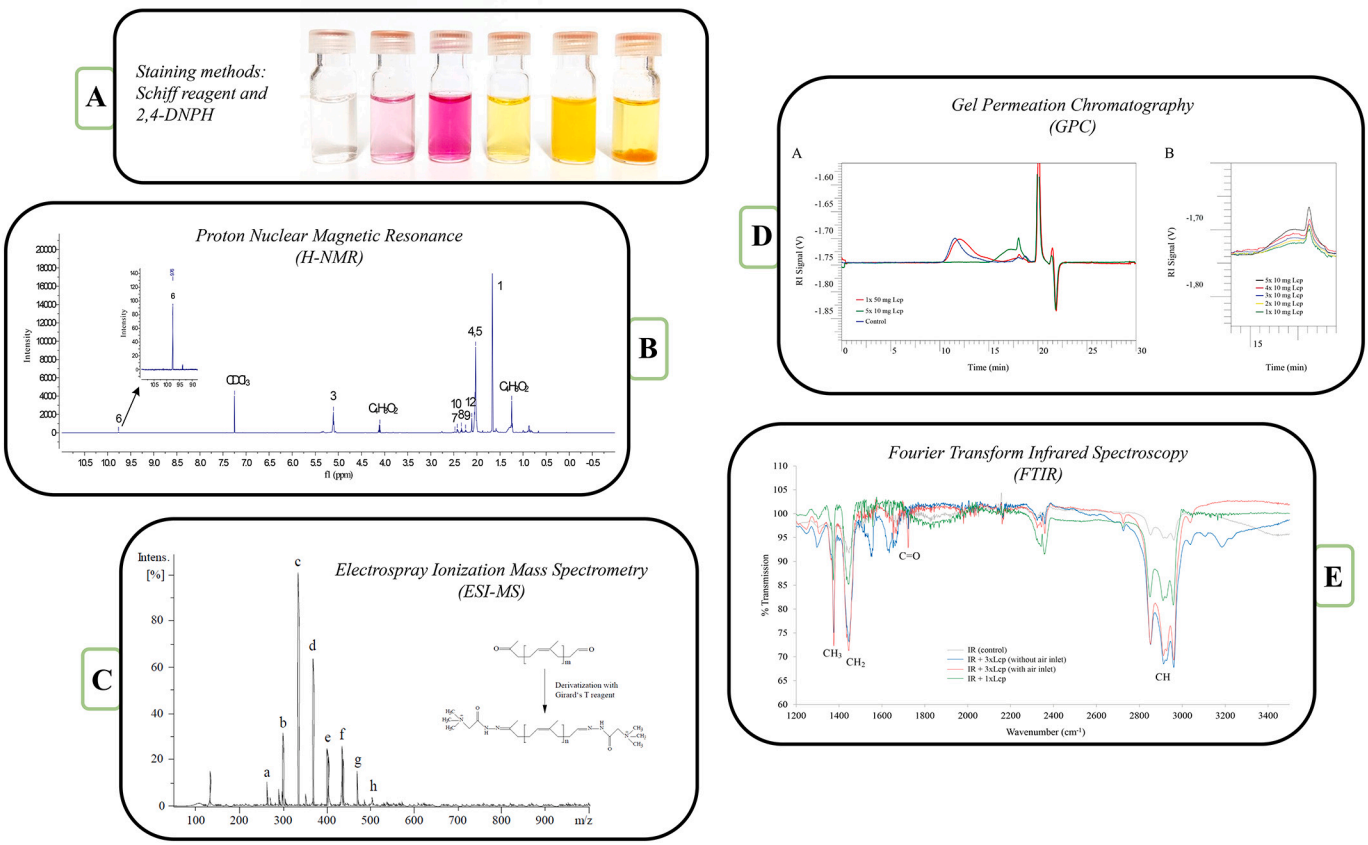

Fig. 6. Methodologies for detecting poly(*cis*-1,4-isoprene) rubber degradation. A) Qualitative analysis by staining aldehyde groups with Schiff reagent and carbonyl groups with 2,4-Dinitrophenilhydrazyne. B) Proton analysis of degradation products by Proton Nuclear Magnetic Resonance (H-NMR). C) Identification of oligo(cis-1,4-isoprene) rubber after incubation with Lcp by Electrospray Ionization-Mass Spectrometry (ESI-MS). D) Mean molecular mass of poly(*cis*-1,4-isoprene) before and after the incubation with Lcp by Gel Permeation Chromatography (GPC). E) Superficial analysis of the functional groups of poly(*cis*-1,4-isoprene) before and after the incubation with Lcp by FTIR.

Another widely used technique for analyzing rubber surface changes is by scanning electron microscopy (SEM). Bacterial colonization, surface crashes or erosion, formation of holes and defragmentation are possible to detect when using SEM. Linos et al. (2000) showed micrographs of NR latex gloves and synthetic polyisoprene-coated aluminum pieces from liquid cultures of different actinomycetes showing clear colonization, disintegration of rubber and biofilm formation by the adhesive growth of some strains such as *Gordonia* sp. In another report, the rubber degradation performance by different bacterial and fungi species using latex and rubber as sole carbon source was investigated. The appearance of cracks on the surface of the latex particles and cell-shaped cavities by bacteria, and a dense exopolysaccharide (EPS) matrix by fungi was able to visualize by SEM (Vilanova et al., 2014). Morphological modifications can be performed by SEM coupled to Energy Dispersive Spectroscopy (EDS) (Aboelkheir et al., 2019a; Aboelkheir et al., 2019b) Although this technique can offer a rapid elemental analysis of carbon, sulfur, oxygen and other elements, it has some limitations regarding the capacity of offering precise quantitative element concentrations. The analysis is focused only on the surface of the micrograph and not on the whole molecule surface.

5. Future perspectives in rubber biodegradation

Biotechnological processes have been proposed for rubber recycling that do not include harmful or toxic chemicals and are normally not energy intensive. However, microorganisms are in many cases sensitive to chemical substances, including rubber additives. According to the literature and the results obtained in our Laboratory, a single bacterium, fungi or enzyme cannot achieve high biodegradation or biodeterioration yields of vulcanized rubber. The development of a multistage process including detoxification, desulfurization-devulcanization and total or partial biodegradation will be necessary for an effective rubber waste recycling procedure (Fig. 7).

Despite the fact that biotechnological approaches have several advantages for rubber degradation, the use of living organisms catalyzing solid and impure substrates lead to slow reactions (long incubation times), and usually low degradation yields. Therefore, the use of partially purified enzymes can significantly improve the overall productivity. Enzymes should be synthesized in a large scale volume, with highly efficient expression systems and with cost-effective recovery methods in order to establish an affordable process for large amounts of rubber waste. Screening of all the necessary enzymes that are involved for achieving a total rubber degradation process must be performed. Moreover, the analysis of the enzymatic activity of these enzymes coming from different organisms (bacteria, fungi, yeast) in the presence of vulcanized rubber particles will be necessary for choosing the most effective catalyzing agent.

Oligo(*cis*-1,4-isopene) molecules, the degradation products obtained after the cleavage of poly(*cis*-1,4-isoprene) backbone by rubber oxygenases, have interesting potential for searching new rubber-based materials. By scaling-up the depolimerization process it would be possible to produce large amounts of ODTD (if RoxA is applied) or oligomers with different lengths according to the number of isoprene units (if Lcp or RoxB is applied). A pending study would be to analyze the physico-chemical properties of the degradation products and the relation with their molecular mass. Anyhow, some characteristics of the degradation products are showing competitive advantages compared to old poly(*cis*-1,4-isoprene) for recycling. The shorter isoprene chain length compared to the initial polymer and the presence of oxygen functional groups on rubber surface can improve the interfacial adhesion between rubber and polar polymers (Fazli and Rodrigue, 2020). On the other hand, after biotransformation the rubber surface is altered, which is also decreasing hydrophobicity of the material surface. Another interesting missing research approach would be the analysis of mixing the biologically treated rubber with fresh vulcanized rubber improving the poor current yields, which has been exploited by chemical and physical methods (Aoudia et al., 2017; Garcia et al., 2015; Tripathy et al., 2002) or mixing it with other materials as universal filler. A potential industry for the use of old rubber is the building

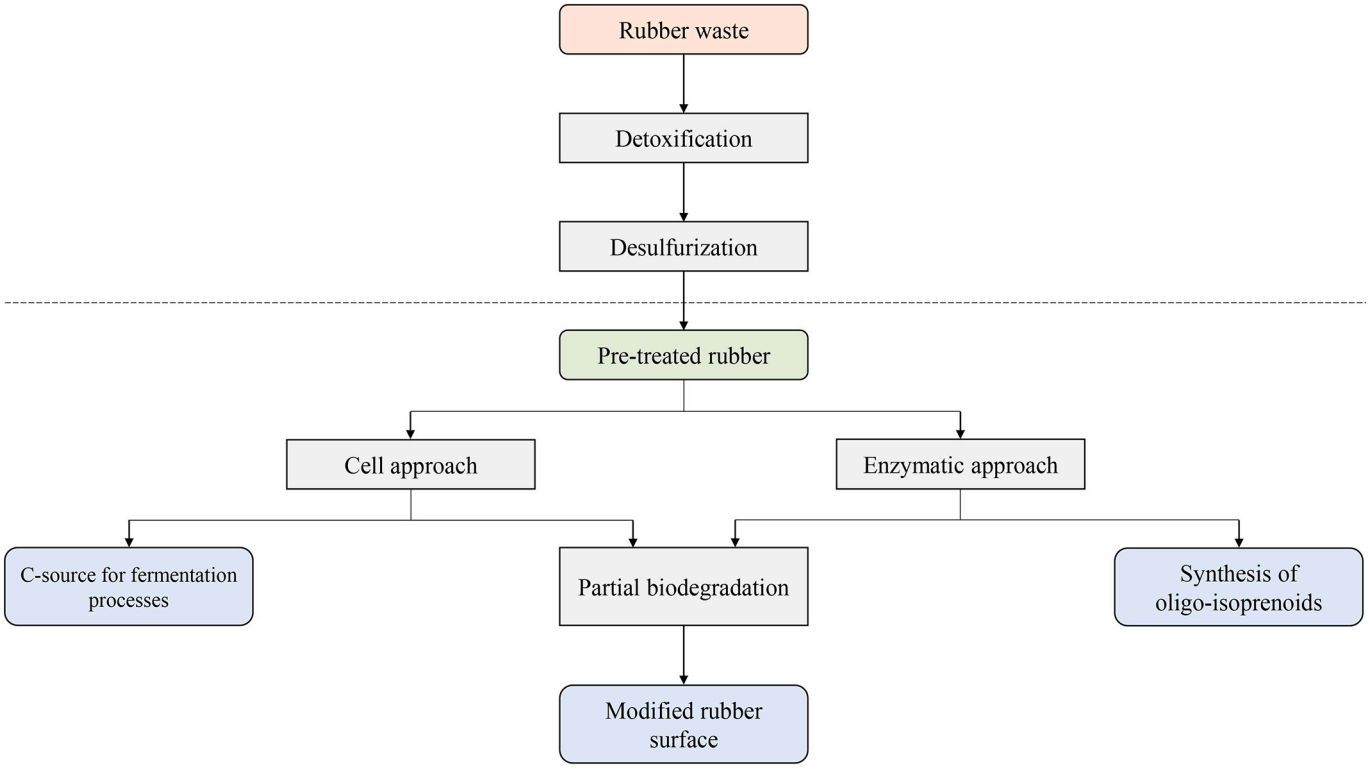

Fig. 7. Multi-stage bioprocess for rubber recycling.

sector, finding applications such as the mixture with concrete (Issa and Salem, 2013; Pelisser et al., 2011; Thomas et al., 2016), as insulation material (Maderuelo-Sanz et al., 2011; Zhao et al., 2010) and as drainage layer in extensive green roofs (Rincón et al., 2014). Biologically treated rubber could improve physical rubber interactions with the different polar materials found for construction purposes.

Another consideration to improve yields and productivities for rubber degradation treatments would be the combination of sustainable chemistry methods also called environmentally compatible chemistry (Veitía and Ferroud, 2015), with the biotechnological approaches discussed within this report. Several considerations must be taken into account as the use of safer solvents, reduction of by-products, decrease the energy requirements to accomplish environmental and economic aspects, the use of catalytic processes, and many others (Hafez et al., 2013). Specifically, the use of catalytic agents for the oxidation of some rubber additives coming from the vulcanization process can be very effective and less time consuming compared to biological methods. Last reports have shown that cleavage of poly(*cis*-1,4-isoprene) rubber using rubber oxygenases, mainly Lcp, has been effective in terms of the reaction performance and the need of relatively short times (Andler et al., 2018a; R. Andler et al., 2019a). However, the activity of this enzyme is sensitive to several rubber additives like *N,N′*-di-aryl-*p*-phenylenediamine (DAPD/DTPD), N-(1,3-dimethylbutyl)-*N′*-phenyl-*p*-phenylenediamine (6PPD) and N-isopropyl-*N′*-phenyl-*p*-phenylenediamine (IPPD) (Altenhoff et al., 2020) that need to be removed. So far, the proposed biotechnological processes for detoxification and desulfurization of vulcanized rubber are slow and the yields are poor. One of the aspects that must be addressed in the near future for scientist of the field.

6. Conclusion

The use of microorganisms in rubber recycling processes offers many potential advantages, such as low energy consumption, process ease, low equipment requirements and no pollution. The major challenge for a competitive rubber biodegradation process is the complex composition of rubber materials, finding toxic components that inhibit cell growth and the activity of the enzymes. Within the last two decades several studies have been focused on the basic research of rubber biodegradation and only a few reports aim to find technical applications. A multi-step bioprocess involving pretreatments such as detoxification and desulfurization processes are crucial for the final cleavage of poly (*cis*-1,4-isoprene), where rubber oxygenases such as Lcp have shown interesting results for the biotransformation of the polymer. Moreover, the optimization of each treatment and its subsequent scale-up would provide rubber products with specific modifications on the structure surface, which is necessary for the improvement of the reactivity properties in terms of possible mixtures with hydrophilic-based materials. Novel biotechnological applications for polymers transformation or degradation are the next step for sustainable recycling of rubber solid waste and other synthetic polymers.

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