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**Characterization of enzymes from desulfurizing
bacterial strains**

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Abstract

The environmental hazard posed by the accumulation of huge amounts of used tires might be partly relieved by the implementation of methods for recycling natural rubber (NR) from waste tires.

This approach requires rubber grinding and a process of devulcanization that breaks the sulfur-sulfur crosslinks among polymer chains. Several chemical or mechanical methods are already used to devulcanize ground-rubber. However, each of them has drawbacks related either to the lack of specificity or to the use of hazardous chemicals. It would therefore be desirable to develop processes in which selective and specific reactions are carried out in mild conditions of temperature and pressure, without the use of hazardous compounds. In this view, the use of biocatalysts could be a valuable and ecological alternative.

This study explores the possibility of applying enzymes to devulcanize rubber in a process of "biodevulcanization".

Since enzymes active in rubber devulcanization were not available at the beginning of this thesis, this research started with the analysis of microorganisms isolated from environmental samples contaminated with waste tires. The desulfuring properties of several bacteria were tested on the model substrate dibenzothiophene (DBT).

A first *in-vivo* screening of microorganisms allowed the selection of a new strain of *Rhodococcus* sp. referred as AF21875. This microorganism was studied with two aims: assessing the presence of a metabolic pathway for DBT desulfurization already described in other bacteria and identifying new metabolic abilities and enzymes.

In bacteria active in desulfurization, four enzymes co-operate in the reaction of desulfurization: DszA, DszB, DszC and DszD. The presence of the four corresponding *dsz* genes in the genomic DNA of *Rhodococcus* sp. AF21875 has been assessed. The four genes have been cloned in a strain of the bacterium *Escherichia coli* to allow for the production of recombinant Dsz enzymes.

The three recombinant proteins DszA, DszC and DszD are soluble and were successfully purified. More difficult was the production of DszB that is poorly expressed in any experimental condition. In view of a biotechnological application, structural and stability studies were carried out on DszA, DszC and DszD enzymes. In particular, we investigated secondary structure and heat stability by circular dichroism, while protein stability in the presence of different organic solvents was studied by spectrofluorimetry.

Enzymes activity on DBT was assessed by high performance liquid chromatography (HPLC) by detecting the formation of 2-hydroxybiphenyl (HBP), the reaction product of DBT desulfurization. The desulfurization activity of the four enzymes was then tested on vulcanized natural rubber using Rubber Process Analyzer and Fourier Transform Infrared Spectroscopy to detect chemical modifications induced by the enzymatic treatment. These analyses revealed minor changes. Other studies should be performed to attribute such modifications to desulfurization.

Overall, Dsz enzymes from *Rhodococcus* sp. AF21875 were found to be an interesting starting point for the application of protein engineering approaches aimed to improve not only their activity but also their stability.

A differential proteomic analysis of *Rhodococcus sp.* AF21875 was performed to identify enzymatic activities related to sulfur metabolism and different from Dsz proteins. Total proteins, extracted from cells grown either in the presence or in the absence of DBT, were separated by two-dimensional electrophoresis, showing that DBT induces a few changes in the proteome of *Rhodococcus sp.* AF21875. Three proteins, belonging to a metabolic pathway different from the Dsz one were identified by in-gel tryptic digestion and mass spectrometry.

Riassunto

L'enorme quantità di pneumatici fuori uso accumulati costituisce un grave problema ambientale. Per porvi rimedio si cercano soluzioni che mirano al riutilizzo della gomma naturale (NR), principale materia prima utilizzata nella produzione di pneumatici.

Prima di poter essere riutilizzata, la NR viene generalmente macinata finemente ottenendone un prodotto detto polverino. Il polverino viene quindi sottoposto ad un trattamento di devulcanizzazione, cioè alla rottura dei ponti trasversali zolfo-zolfo.

Diversi metodi chimici o meccanici sono utilizzabili per devulcanizzare il polverino. Tuttavia, ciascuno di essi presenta degli svantaggi legati alla scarsa specificità del target o alle condizioni del processo. Sarebbe quindi auspicabile la messa a punto di processi in cui reazioni specifiche siano realizzate in condizioni moderate di temperatura, pressione e senza l'utilizzo di sostanze pericolose. In questo scenario l'utilizzo di biocatalizzatori potrebbe costituire un'alternativa valida e di minor impatto ambientale.

Questo studio si occupa di verificare la possibilità di trattamenti enzimatici di "biodevulcanizzazione".

Non essendo noti enzimi in grado di devulcanizzare la gomma, questo lavoro di tesi è partito dall'analisi di microrganismi isolati da campioni ambientali e con potenziali proprietà desolforanti che sono state saggiate su un substrato modello, il dibenzotiofene (DBT).

Il primo *screening* sui microrganismi ha portato alla selezione di un nuovo ceppo di *Rhodococcus* qui indicato come sp. AF21875. L'attività desolforante di questo microrganismo è stata studiata con due approcci paralleli, da un lato verificando la presenza di geni codificanti

per enzimi della via metabolica di desolforazione del DBT e dall'altro individuando nuovi enzimi con abilità desolforanti.

In batteri attivi in processi di desolforazione, i geni *dsz* codificano per 4 enzimi che appartengono ad una stessa via metabolica: DszA, DszB, DszC e DszD. Nel DNA genomico di *Rhodococcus sp.* AF21875 è stata verificata la presenza dei quattro geni *dsz*, che sono stati clonati per consentire la produzione ricombinante delle proteine corrispondenti in un ceppo del batterio *Escherichia coli*.

Tre delle proteine, DszA, DszC e DszD, sono abbondantemente espresse in forma solubile e sono state purificate con successo. Più difficoltoso è risultato l'ottenimento della proteina DszB, espressa a basse concentrazioni. In previsione di un impiego biotecnologico delle proteine DszA, DszC e DszD è stata intrapresa l'analisi di alcune caratteristiche strutturali e di stabilità. In particolare, è stata analizzata la composizione della struttura secondaria e la stabilità al calore mediante dicroismo circolare; la stabilità in presenza di diversi solventi organici, attraverso spettrofluorimetria.

L'attività degli enzimi è stata monitorata mediante cromatografia liquida ad alta prestazione (HPLC) che consente di rilevare la formazione di 2-idrossibifenile (HBP) come prodotto finale di reazione su DBT. L'attività di desolforazione dei quattro enzimi è stata infine saggiata su gomma naturale vulcanizzata. Da analisi RPA (Rubber Process Analyzer) e FTIR (Fourier Transform Infrared Spectroscopy) è emerso che il trattamento enzimatico provoca modificazioni chimiche della gomma. Sebbene le analisi condotte evidenzino cambiamenti di modesta entità e non associabili univocamente ad un processo di desolforazione, gli enzimi individuati costituiscono un buon punto di partenza per approcci di ingegneria proteica volti a migliorare non solo

l'attività, ma anche la stabilità degli enzimi Dsz da *Rhodococcus sp.* AF21875.

Inoltre, per individuare nuove attività enzimatiche è stata eseguita un'analisi di proteomica differenziale delle cellule di *Rhodococcus sp.* AF21875. Da cellule cresciute in terreno privo o addizionato di DBT sono state estratte le proteine totali che sono state analizzate mediante elettroforesi bidimensionale. Quando *Rhodococcus sp.* AF21875 cresce in presenza di DBT, produce un *pool* di proteine che non si ritrovano tra le proteine espresse in assenza di DBT. Tre delle proteine di cui il DBT induce l'espressione sono state analizzate tramite digestione triptica in-gel e analisi di spettrometria di massa. Questa analisi ha permesso di identificare due enzimi che non sono coinvolti nel metabolismo dello zolfo ma appartengono ad una stessa via catabolica distinta da quella cui appartengono gli enzimi Dsz.

1. Introduction

1.1 Tire composition

A pneumatic tire is a high-performance, flexible, toroidal system filled with compressed air (Waddell et al., 1990). Tires are complex structures of interacting components that allow supporting the weight of the vehicle, cushioning irregular surfaces, providing traction for driving and braking, ensuring resistance and durability. All these performances depend on tires deformability features (Evans and Evans, 2006; Segatta Thomas J. et al., 1995; Waddell et al., 1990).

The first tire was exhibited in London in 1851 and had a solid rubber carriage. Since then the requirements of speed and comfort led to the development of modern tires (Waddell et al., 1990). Today each component is designed with specific properties for optimal efficiency and a unique, specific function (Evans and Evans, 2006).

It is possible to distinguish two major parts in a tire: the tread area, which physically touches the road surface, and the casing, which includes carcass plies, belts, bead and sidewalls (Figure 1) (Kohjiya and Ikeda, 2014; Waddell et al., 1990).

The tread is made up of several rubber materials. It protects the inner casing from hazards and impacts and guarantees the friction between the road and the tire. The surface of the tread usually has an engineered pattern, necessary to deal with the road surfaces in different weather conditions (Kohjiya and Ikeda, 2014; Lindenmuth, 2005).

The casing is made up of different layers of flexible fabrics cords encased in a matrix of rubber compounds, anchored around the steel wires with high tensile strength (Wong J. Y., 2001). Sidewalls protect the

inner casing from damages, the carcass is the main reinforcing layer which sustains internal air pressure and the belt constitutes the reinforcing layer between tread and carcass (Kohjiya and Ikeda, 2014).

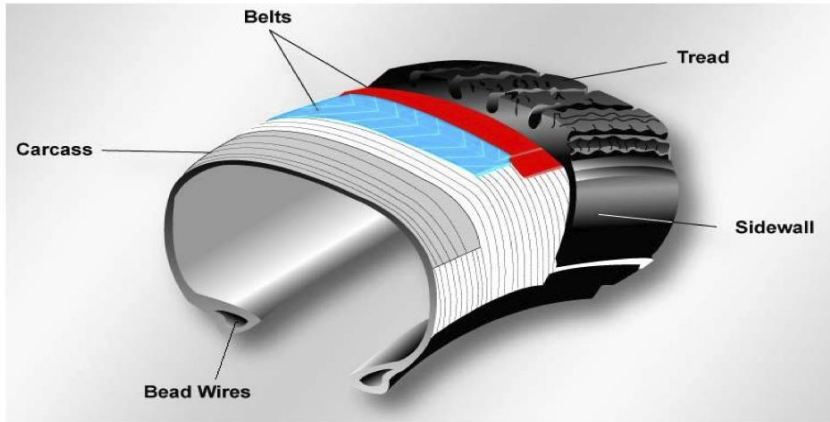


Figure 1- Anatomy of a tire

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A typical car tire is composed by about 60 different raw materials; to simplify we can describe a standard car tire or a truck one as composed by the materials reported in table 1 (Evans and Evans, 2006; Lindenmuth, 2005).

Ingredients	Car tire	Truck tire
Rubber elastomers	47%	45%
Carbon black	21.5%	22%
Metal	16.5%	25%
Textile	5.5%	-
Zinc Oxide	1%	2%
Sulfur	1%	1%
Additives	7.5%	5%

Table 1- Major components of tires
Modified from Evans and Evans, 2006

The number of materials increases if one considers that tires contain different kind of rubber compounds, reinforcing fillers such as different types of carbon black, clay, silica and a variety of chemicals and minerals. Chemicals are also added as antidegradants (i.e. antioxidants and antiozonants) to help protecting tires against deterioration by ozone, oxygen and heat, or as accelerators during the process of vulcanization. Tires also contain several types of textile (i.e. polyester and nylon) for reinforcement and numerous kinds and sizes of steel cables (Evans and Evans, 2006; Lindenmuth, 2005; Waddell et al., 1990). These materials are selected on the basis of their physical-chemical properties and their interactions with other components (Waddell et al., 1990).

Rubber confers the required viscoelastic properties (Evans and Evans, 2006). In general, rubbers used in tires are natural or synthetic elastomers (Figure 2). Synthetic elastomers are mainly produced by polymerization of butadiene and/or styrene-butadiene from mineral oil (Ahmed et al., 1996; Holst et al., 1998; James et al., 2005; Mark et al., 2005).

Different polymeric mixtures are selected to produce specific parts of a tire. For instance, sidewalls are generally made up of styrene-butadiene rubber that is more resistant to environmental hazards (i.e. ozone, oxygen, UV radiation and heat) (Lindenmuth, 2005; Wong J. Y., 2001).

Rubber composition varies also with the type of tire. In particular, to ensure higher resistance to abrasion and tearing, commercial vehicles such as trucks, busses and trailers require treads with a predominant fraction of natural rubber while mixtures of styrene-butadiene rubber and poly-butadiene rubber are commonly used for passenger tires treads (Ahmed et al., 1996; Halasa et al., 1989; Wong J. Y., 2001).

Natural rubber, whose amount varies between 45 and 80% is the major raw material in tires (TNRCC, 1999).

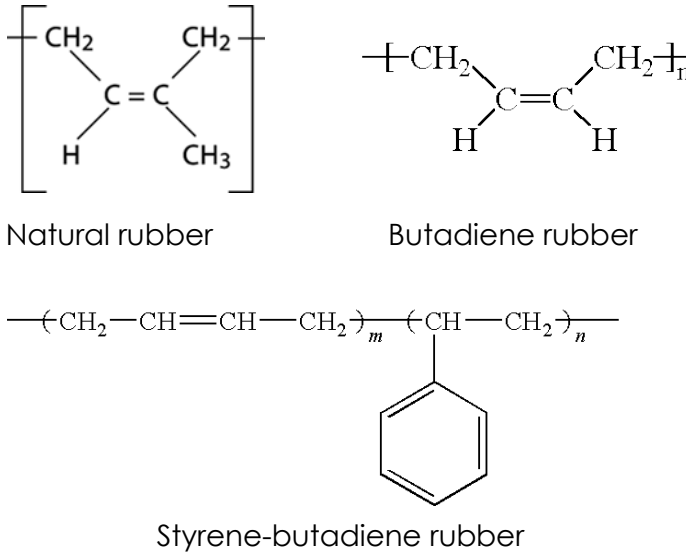


Figure 2 - Natural and synthetic rubber polymers in tire composition

1.2 Natural rubber

Natural rubber, or caoutchouc, has been known in Europe since the discovery of South America (Ahmed et al., 1996). The term natural rubber refers to a coagulated or precipitated product extracted as latex (milky exudations) from barks of trees belonging to several botanical families (i.e. *Hevea brasiliensis*, *L. kirkii*, *L. heudelotii*, *L. owariensis*, etc.) which grow mostly in tropical areas (Kohjiya and Ikeda, 2014; Rose and Steinbuchel, 2005).

The process of latex extraction consists in the bark incision and in the collection of the exudations into a small cup fixed to the tree trunk (Ahmed et al., 1996). This process is commonly called “tapping” and it’s usually carried out every two-three days, yielding up to 2,500 kg of natural rubber per year per hectare (Rose and Steinbuchel, 2005).

After tapping, rubber is recovered from the emulsion by coagulation with formic acid, washed, dried and compacted (Gent, 2014). After this treatment rubber is a sticky, milky colloid and viscous material (Rose and Steinbuchel, 2005).

Natural rubber mainly consists of poly(cis-1,4)-isoprene, a highly-unsaturated hydrocarbon polymer with a molecular mass in the range of about 10-10,000 kDa. Latex also contains minor impurities of other organic compounds (Fisher, undated; Greve, 2000; Holst et al., 1998; Kohjiya and Ikeda, 2014; Rose and Steinbuchel, 2005; Schmid et al., 2001; Schmidt et al., 2010). In the exudate, the isoprene polymers are present as particles of 3-5 μm in size, covered by a layer of proteins and lipids, which separate the hydrophobic rubber molecules from the hydrophilic environment (Rose and Steinbuchel, 2005).

Currently, leader in the production of rubber are Thailand, Indonesia, Malaysia, India, China, Sri Lanka and Vietnam. As the demand for natural rubber grows, Cambodia, Laos, Bangladesh and some African countries may further increase their production (Kohjiya and Ikeda, 2014).

The most important feature of rubber elastomers is their ability to undergo elastic deformations, to stretch and to return to their original shape in a reversible way (Mark et al., 2005). To gain this property is however necessary to treat rubber with a process of vulcanization.

1.3 Vulcanization

The vulcanization or "curing" process was invented by Charles Goodyear in 1839. This irreversible process promotes the change from a thermoplastic to a thermosetting solid material (Ahmed et al., 1996; Isayev and Oh, 2006; Long, 1985). The process consists in treating natural

rubber with elemental sulfur at high temperature (140–180°C); the material becomes non-sticky, more flexible, viscoelastic, strong and resilient while loses its plasticity. This is due to deep molecular changes consisting in the formation of a cross-linked molecular network (Fisher, undated; Lindenmuth, 2005; Mark et al., 2005). Sulfur-sulfur bonds spaced along the polymeric chains link together long rubber molecules. Depending on the polymeric structure of rubber and the vulcanization parameters applied either polysulfidic, disulfidic or monosulfidic crosslinks are formed (Holst et al., 1998) (Figure 3).

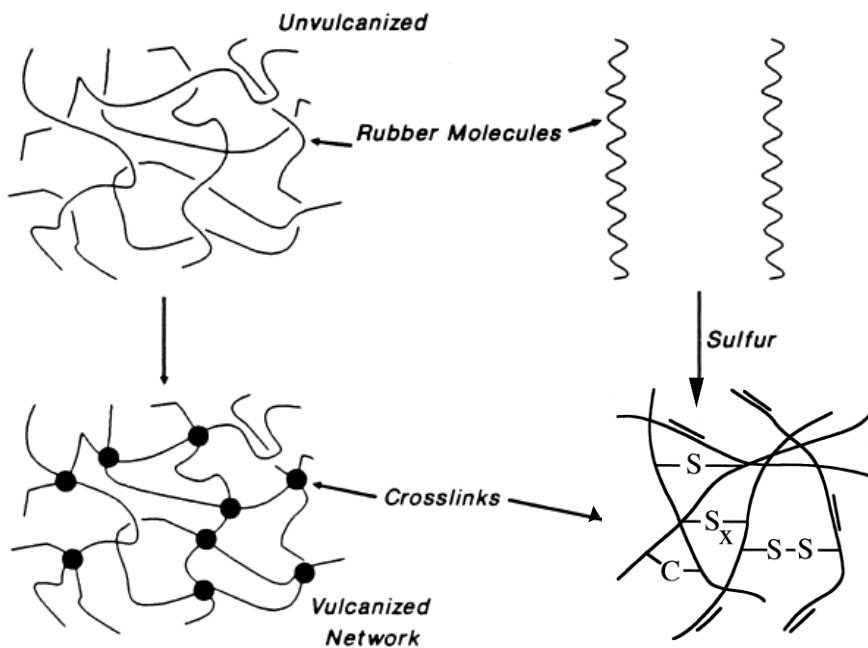


Figure 3 - Formation of bonds/polymer network after vulcanization (modified from Holst et al., 1998; Mark et al., 2005)

As a result of the formation of this network, besides the features listed ahead, rubber becomes essentially insoluble in any solvent (Mark et al., 2005).

Nowadays industry employs many reagents and additives such as accelerators, activators, retarders, and pre-vulcanization inhibitors to improve the procedure of sulfur vulcanization (Holst et al., 1998; Lindenmuth, 2005; Mark et al., 2005).

The first process used required 5 hours at 140 °C to achieve the desired properties. In 1906 Oenslager discovered that aniline can act as an accelerator of the reaction. Due to its toxicity aniline was later substituted with thiocarbanilide.

The first delayed-action accelerators were introduced in 1925 with the development of 2-mercaptobenzothiazole (MBT) that remains substantially inert during compounding operations but gives a rapid rate of cure when the temperature of the mixture is raised. Since then different thiocarbamates were developed to enhance their delayed-action property (Bain and Dibbo, 1968; Mark et al., 2005).

The addition of zinc oxide or fatty-acids as activators of the process reduced further the time requested for vulcanization. Modern vulcanization procedures are accomplished in 1-3 minutes (Lindenmuth, 2005).

In general, thermal vulcanization may be considered the first-generation technology. Today alternative methods such as electron beams, UV radiation, ultrasonic waves may be used to catalyze the same reaction and are referred to as the second-generation technology (Akiba M. and Hashim A.S., 1997).

1.4 Recycling rubber tires

Every year a large amount of natural rubber is used; figure 4 reports the annual global production and the consumption of natural rubber from 1995 to 2012 with projection to 2020. We can observe that economic downturn from 2007 to 2009 reduced the rate of consumption but again from 2010 the consumption of rubber overcomes its supply (Kohjiya and Ikeda, 2014). This causes a great concern since synthetic rubber cannot fully replace natural rubber because it does not share the same properties and its synthesis requires oil-derived resources (Gregg and Macey, 1973; Sarbach, 1943).

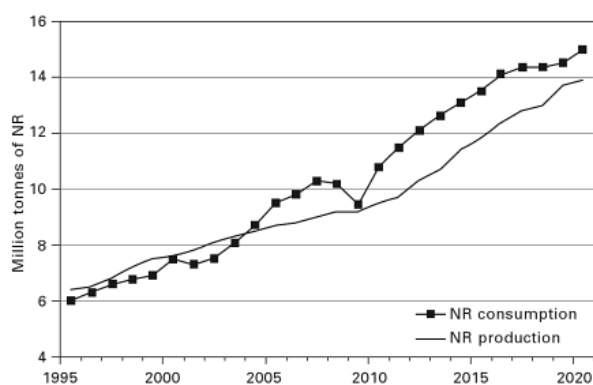


Figure 4 - Consumption and production of natural rubber from 1995, with projection to 2020 (Kohjiya and Ikeda, 2014).

Moreover environmental concern is growing concerning the large amounts the large amount of spent tires discarded each year. In 1977, approximately 70% of the scrap rubber were discarded in landfills (Adhikari et al., 2000). Used tires are a challenging problem, since they decompose very slowly (they have a virtually unlimited life span), are bulky, and floating to the top of the landfill. Stocked tires create serious fire dangers and provide a breeding ground for rodents and water-

incubators where pathogenic insects and bacteria can proliferate. (Ahmed et al., 1996; Isayev and Oh, 2006; Marin et al., 2004; Rajan et al., 2006).

In addition, environmental pollution caused by waste tires is becoming more and more serious and several countries have already banned landfilling of discarded tires (Adhikari et al., 2000; Fang et al., 2001; Srinivasan et al., 2008). The EU directive 1999/31/EC prohibits to landfill whole tires since 2003.

In this scenario, reusing or recycling waste rubber is relevant for protecting the environment, saving energy and industrial raw materials and reducing the manufacturing costs (Rajan et al., 2006).

Today the major use of scrap tires in the United States is burning them to generate energy. Indeed waste rubber is a high-value fuel with a calorific power similar to that of coal, approximately $3.3 \cdot 10^4$ kJ/kg (Fang et al., 2001). Unfortunately also this process contributes to air pollution (Isayev and Oh, 2006).

The robustness and overall features that make tires suitable for vehicles can sometimes be useful also for their secondary uses as whole scrap tires (Ahmed et al., 1996), for example as artificial reefs in coastal waters, breakwaters to protect the coast from erosion, dock fenders or crash barriers (tires are shock absorbers in harbors to protect ships or lining race-tracks to protect racing cars from crashing), coverings, containers, playground or other recreational surfaces (Ahmed et al., 1996; Isayev and Oh, 2006).

Parts or materials derived from tires can be also recycled rather than re-used. For example, sidewalls might be removed from treads and derived rubber cut in pieces become the basis for other new product (Ahmed et al., 1996; Marin et al., 2004).

The first step in recycling is grinding tires. This can be achieved by three different processes:

1. Rubber pieces are ground in a cracker mill at **ambient temperature**. Milled tires are reduced in irregular grains of 10 to 30 mesh with a rough surface. Multiple grinds can be used to further reduce the particle size. The significant amount of heat generated by the process can degrade rubber and if not cooled properly, cause combustion on storage (Ahmed et al., 1996; Isayev and Oh, 2006).
2. **Cryogenic grinding** uses low temperatures, cooling scrap tires in liquid nitrogen (-40°C) to convert rubber in a less viscous and more brittle material; in this way it is possible to obtain fine rubber grains or powder in a hammer mill. Grains have a regular shape and smooth sides. This process is more expensive than the previous one but better preserves rubber polymers from degradation (Ahmed et al., 1996; Isayev and Oh, 2006; Srinivasan et al., 2008; Zanetti and Genon, 2004).
3. **Wet-ambient grinding** is a modified ambient grinding process that reduces the size of particles by grinding rubber in a liquid medium. The advantage consists in the production of fine, smooth particles that can be easily further processed (Adhikari et al., 2000; Zanetti and Genon, 2004).

Rubber granulates can find different industrial applications: feedstock for the production of regenerated rubber, filling material in rubber compounds and others.

To lower manufacturing costs, ground rubber is used as a filling agent in compounds at a maximum percentage of 30%. Generally the products

obtained, such as mats or floor coverings, tubes, shoe soles, heels or plastic compounds, are not exposed to high performance demands.

Granulate rubber is also applied to build sports floors or to improve asphalt properties such as longevity of roads, crack resistance or against bleeding, noise reduction or decrease loss of force on wet surfaces (Ahmed et al., 1996; Isayev and Oh, 2006).

A relatively low percentage (10 weight %) of ground rubber can be employed as filler in new tires. In this case grains of recycled rubber do not interact with the virgin rubber and this might cause a decrease in tire tensile strength (Isayev and Oh, 2006).

In this view a very promising alternative is rubber reclaiming. Reclaiming is a procedure in which vulcanized waste rubber is converted, using mechanical, thermal energy or chemicals, into a state in which it can be mixed, processed and vulcanized again (Isayev, 2013).

1.5 Rubber devulcanization

The development of rubber recycling technologies would significantly reduce energy consumption and relieve the problem of scrap tires disposal and rubber waste (Isayev and Oh, 2006). The principle of rubber reclaiming is devulcanization.

Devulcanization is the process of breaking carbon-sulfur or sulfur-sulfur bonds of vulcanized rubber. Ideally, devulcanization should yield a product suitable for substituting virgin rubber, in terms of properties and in terms of cost of manufacture (Marin et al., 2004).

Different methods are known for rubber devulcanization, based on mechanical, microwaves, ultrasonic, chemical and biological approaches (Adhikari et al., 2000).

1.5.1 Mechanical processes

These technologies include mechanical and thermo-mechanical methods involving rubber grinding and a subsequent non-selective devulcanization based on high temperature and/or mechanical shearing (Figure 5). Heat is generated partially by electrical heating and partially by the friction of the grains. During this reaction, the weight of the grains drops down due to shearing and temperatures. The devulcanized rubber is extruded from the machine in a dry form ready for refining (Adhikari et al., 2000; Ahmed et al., 1996; Eldho et al., 2011; Isayev and Oh, 2006).

Thermo-mechanical recycling produces a combination of rubber degradation, breaking carbon-carbon bonds of rubber polymers, and of sulfur crosslinks cleavage (Rajan et al., 2006).

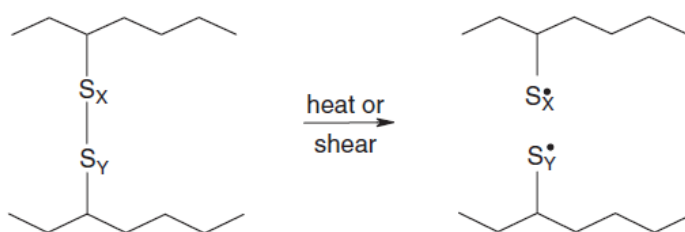


Figure 5- Mechanical and thermo-mechanical devulcanization mechanism

1.5.2 Microwave

Microwave technology applies a controlled dose of microwave energy at a specific frequency, sufficient to cleave quickly and uniformly sulfur-sulfur bonds. The material used in the microwave process must be polar enough to accept energy and to generate the heat necessary for devulcanization. On the basis of relative bond energies, it was deduced that scission of sulfur-sulfur and sulfur-carbon crosslinks with microwaves was preferred to scission of carbon-carbon bonds in the rubber

molecule, suggesting a more specific reaction (Adhikari et al., 2000; Ahmed et al., 1996; Isayev and Oh, 2006). Furthermore this method uses relatively little energy, requires little grinding and doesn't make use of chemicals.

However this treatment increases the temperature of the material very fast, reaching final temperatures of 260–350 °C that could affect rubber properties (Adhikari et al., 2000; Ahmed et al., 1996).

1.5.3 Ultrasonic method

Ultrasonic waves in the presence of pressure and heat can quickly break up the three-dimensional network of vulcanized rubber immersed into a liquid. Ultrasonic treatment modifies the properties of rubber that becomes soft and ready to be reprocessed, shaped and revulcanized. This process breaks down carbon-sulfur and sulfur-sulfur bonds. Structural studies of ultrasonically-treated rubber show that the process is accompanied by a partial degradation of the rubber chain. Nevertheless, ultrasonic devulcanization is very fast, simple, efficient and it is free of solvents and chemicals (Adhikari et al., 2000; Isayev, 2013; Marin et al., 2004).

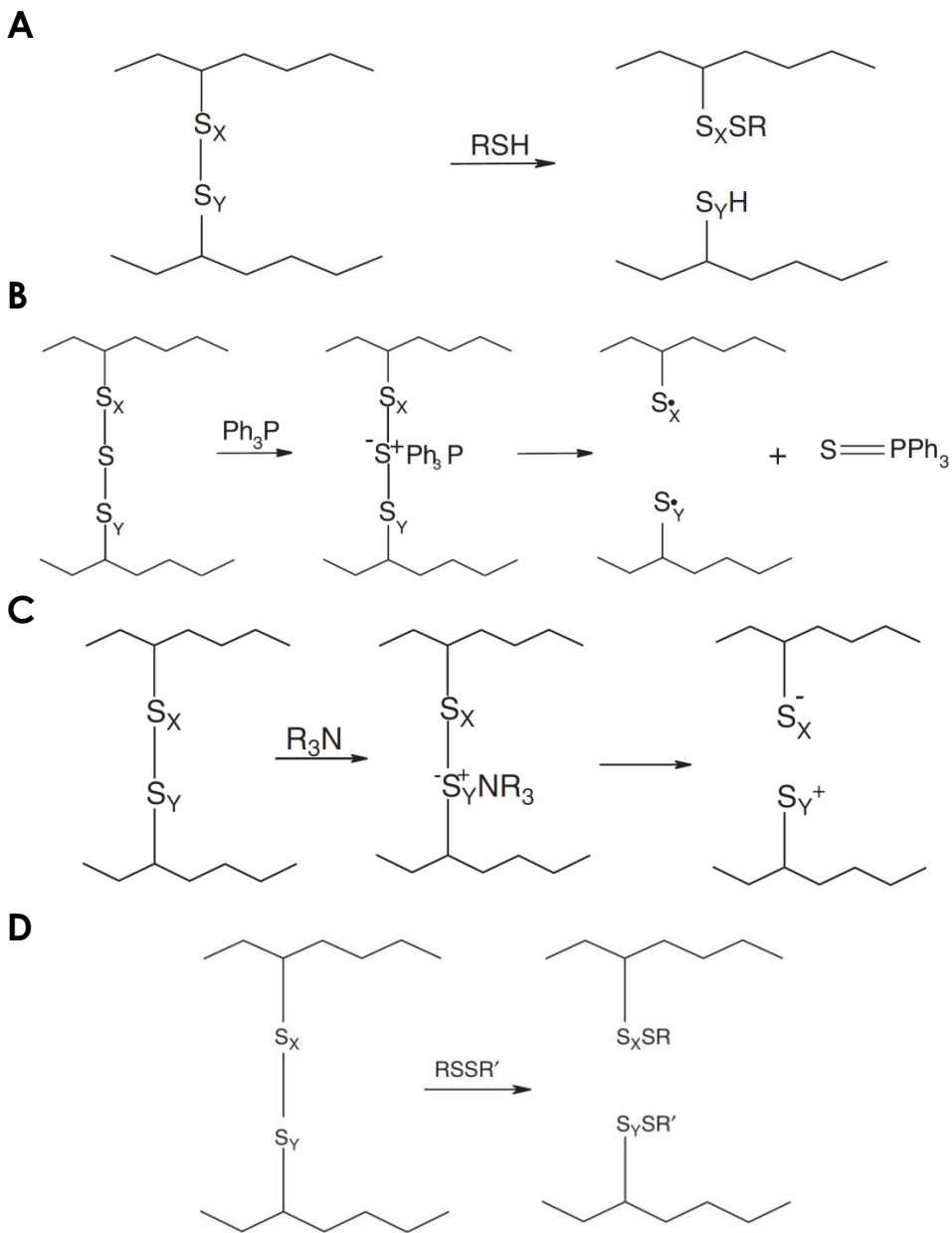


Figure 6 - Reaction mechanisms of devulcanization catalyzed by different chemical compounds: thiols **(A)**, triphenylphosphine **(B)**, nucleophilic substituent **(C)** and disulfides **(D)** (Modified from Rajan et al., 2006).

1.5.4 Chemical methods

Several organic or inorganic chemicals are employed to catalyze rubber devulcanization. These compounds induce the breakdown of sulfur crosslinks and interact with the radicals formed during the degradation of rubber.

The reaction in figure 6 describes the catalytic mechanisms of sulfur-sulfur cleavage by several chemical compounds. Disulfides, thiols, amines and mercaptans are the most common recycling chemicals (Rajan et al., 2006).

Thiols (Figure 6A) in combination with organic bases can selectively cleave sulfur crosslinks by substitution. Hexanethiol was found to cleave di- and polysulfidic crosslinks, while 2-propanethiol breaks only polysulfidic crosslinks in a nucleophilic reaction with piperidine as the base.

With mechanical treatment at high temperature organic monosulfides, disulfides or polysulfides (Figure 6D)(i.e. diphenyl disulfide, dibenzyl disulfide, diamyl disulfide) can promote an exchange reaction, breaking crosslinks (Adhikari et al., 2000; Rajan et al., 2006).

Triphenyl phosphine cleaves di- and polysulfidic bonds (figure 6B) and amines act on mono-sulfidic bonds (Figure 6C), both by nucleophilic substitutions.

Another method uses 2-butanol as the devulcanizing agent under high temperature and pressure. However, this process is extremely slow and requires separation of the devulcanized rubber from the solvent after the treatment (Marin et al., 2004).

Rubber can be also devulcanized by swelling in benzene with a sulfoxide compound like dimethylsulfoxide (DMSO). It is reported that these reagents cause selective scission of sulfur bonds. A disadvantage

of this process is the high toxicity of solvents employed (Eldho et al., 2011; Rajan et al., 2006).

Several solvents (i.e. toluene, naphthalene, benzene, cyclohexane) have been used to devulcanize rubber in the presence of inorganic sodium. The metal cleaves sulfur crosslinks at ca. 300°C in the absence of oxygen. Another proposed method uses an iron oxide phenyl hydrazine-based catalyst to reclaim powder rubbers and copper (I) chloride-tributylamine catalyst (Eldho et al., 2011; Rajan et al., 2006).

Overall, processes employing inorganic compounds are suitable for devulcanization of finely ground tire rubber that is also accompanied by a severe breakdown of the polymeric chains (Marin et al., 2004).

Often methods are mixed to improve devulcanization rate. For example chemicals and oils are frequently used in addition to the thermal and mechanical breakdown (Rajan et al., 2006).

For example, in the process called Pan (Fukumori et al., 2006; Myhre, 2005; Zhang et al., 2009), one of the oldest and most simple devulcanization procedures, ground rubber is mixed with water, oils and reclaiming agents, put into trays and heated to 180 °C with steam for 5–10 h. The reclaiming chemicals include a very complex mixture of solvents, softening oils, hydrocarbon resins, pine tar (Ahmed et al., 1996; Rajan et al., 2006).

1.5.5. Supercritical carbon dioxide

Supercritical carbon dioxide (scCO₂) is an excellent solvent for rubber materials and was used to facilitate the penetration and diffusion of devulcanization reagents into the grains. Rubber devulcanization in the presence of scCO₂ and chemicals has been performed at 140–200 °C for 60 min. Thiol-amine and diphenyl disulfide were found to be the most

effective among several devulcanizing reagents (Kojima et al., 2005; Kojima et al., 2004; Mangili et al., 2014; Rajan et al., 2006).

1.6 Technical challenges

Devulcanized rubber requires less time and energy to be re-processed than virgin rubber but it does not retain the same properties. In particular, reclaim affects mechanical properties like tensile strength, modulus, tear strength and storage modulus of rubber (Rajan et al., 2006).

This problem depends on modifications of the main polymer chain of rubber that is often degraded during devulcanization, modifying the chemical-physical properties of the material. Indeed, the dissociation energies associated to C-C, C-S and S-S bonds are quite similar, as shown in Table 2. For this reason it is difficult to implement a specific process in which only S-S or S-C bonds are cleaved (Baranwal and Stephens, 2001).

Type of bond	Dissociation energy (kJ/mol)
-C-C-	352
-C-S-C- monosulfidic	285
-C-S-S-C- disulfidic	268
-C-S-S-S-C- polysulfidic	< 268

Table 2- Dissociation energy of chemical bonds in vulcanized rubber

Drawbacks of the devulcanization technologies described above are related to the high request of time, energy, solvents and reagents that are expensive, hazardous or polluting (Ahmed et al., 1996).

In this context, microbial devulcanization might be both an environmental friendly and a cost-effective alternative.

1.7 Microbial biodevulcanization

A number of industrial processes require desulfurization and the possibility to apply microorganisms to catalyze these reactions has been long studied. Microorganisms that metabolize sulfur during their growth have been investigated as for their ability to devulcanize ground rubber breaking S-S or C-S bonds (Li et al., 2012).

The use of microorganisms in rubber recycling offers many potential advantages, such as low energy consumption, ease of the process, low equipment requirements and no pollution (Yao et al., 2013). Some chemolithotrophic microorganisms, which derive energy from the oxidation of inorganic sulfur compounds, have been isolated for desulfurization of inorganic compounds (i.e. pyrite, copper sulfide) present in coal and in mineral oil (Ehrlich and Brierley, 1990; Oldfield et al., 1998; Olsson et al., 1994, 1995). Among them, strains of the genus *Thiobacillus* and some Archaea bacteria, such as *Sulfolobales* have been tested (Holst et al., 1998).

For example *Thiobacillus ferrooxidans*, isolated from the soil of an iron mine, was reported by Li and coworkers to be able to grow to a high biomass values in the presence of ground rubber and to desulfurize the rubber substrate (Li et al., 2011).

Christiansson demonstrated that *Thiobacillus ferrooxidans*, *Thiobacillus thioparus*, *Acidianus brierleyi* and *TH2 Lund*, a newly isolated Archaea, can break sulfur bonds in cryo-ground spent rubber in 20 days, oxidizing sulfur on the surface of the grains (Christiansson et al., 1998).

In 2003, Bredberg reported that the sulfur-oxidizing bacteria *Acidithiobacillus* and the sulfur-reducing *Pyrococcus furiosus* can cleave sulfur crosslinks in vulcanized rubber materials (Marin et al., 2004).

Some strains of *Pseudomonas*, *Rhodococcus*, *Paenibacillus* and *Bacillus* oxidize sulfur-organic molecules present in oil and coal, such as dibenzothiophene (DBT). These bacteria are chemoorganotrophic organisms that require organic compounds for growth (Konishi et al., 1997).

To quote a few examples, *Alicyclobacillus* sp. is reported to selectively break sulfur bonds on the surface of ground rubber without affecting the polymer chains and improving hydrophilic property of rubber. After 10 days cultivation, the content of sulfur decreased by 62.5% on ground-rubber while the content of oxygen increased by 34.9% (Yao et al., 2013).

Sphingomonas sp., isolated from coal mine soil, exhibited sulfur oxidizing capacity on rubber after 20 days cultivation (Li et al., 2012).

A few patents claim microbial desulfurization of rubber with whole bacterial cells.

Patent US 5597851 claimed the application of *Thiobacillus ferrooxidans*, *Rhodococcus rhodochrous* and *Sulfolobus acidocaldarius* for selective devulcanization that converted sulfur surface crosslinks to sulfoxide or sulfone groups (Bohm and Stephanopoulos, 2011; Romine and Romine, 1998; Romine and Snowden-Swa, 1997).

Others bacteria, such as *Bacillus sphaericus* ATCC n.53969 or *Rhodococcus rhodochrous* ATCC no. 53968 variants, were patented for their ability to react specifically on C-S bonds (Aggarwal et al., 2012; Kilbane and Woodstock, 1994).

In patent US 7737191B2, Christofi and coworkers used mycolata bacteria such as *Corynebacterium*, *Rhodococcus*, *Nocardia*, *Gordonia*, *Tsakamurella*, *Dietzia* and *Mycobacterium*, and in particular *Gordonia desulfuricans* strain SG213E for rubber treatment (Christofi et al., 2010).

Also Eukarya in particular white-rot and brown-rot fungi, such as *Ceriporiopsis subvermispora*, were studied for rubber devulcanization (Bredberg et al., 2002; Sato et al., 2004).

However, problems have to be addressed to make biological processes more effective, notably selectivity, sensitivity, rate of the reaction and temperature.

It was reported that the activity of some microbial cells can damage the polymer chain of poly-isoprene, thus affecting the chemical-physical properties of rubber material. Moreover, microorganisms can be sensitive to rubber additives and their growth be inhibited by soluble compounds released by rubber (Yao et al., 2013). In general, the rate of bacterial devulcanization is low because the reaction is limited to the surface layers of the elastomers (Marin et al., 2004). Ground rubber mixed with liquid culture medium forms a two-phase system where the two phases do not have any affinity (Li et al., 2011). To overcome this problem Bohm and coworkers described a process in which ground rubber was swelled in a solvent before treatment with microorganisms (Bohm and Stephanopoulos, 2011). With few exceptions represented by some extremophilic bacteria, microbial cells can grow in a range of temperatures that are largely below the temperatures used for chemical desulfurization. For this reason biodevulcanization cannot be accelerated by heating, unless thermophilic bacteria (optimal growth temperature 65-85°C) are used (Holst et al., 1998).

As an alternative to the use of whole cells, enzymes that catalyze desulfurization can be isolated from microorganisms or produced as recombinant proteins and directly used to cleave sulfur crosslinks.

1.8 Enzymes for industrial processes

Enzymes are proteins that catalyze under mild conditions all chemical reactions at the basis of the metabolism of living organisms (Marrs et al., 1999).

Enzymes can provide remarkable opportunities for industry to carry out effective and environmental friendly chemical conversions (Marrs et al., 1999; Posorske, 1984).

The advantages of using enzymes as industrial catalysts (biocatalysis) can be summarized as follow:

1. Selectivity of the reaction: enzymes modify specific chemical bonds, usually at specific sites on a molecule, which permits control of the products, minimizing also the formation of side products (Posorske, 1984; Schmid et al., 2001). We can distinguish among: substrate selectivity, the ability to act on a subset of compounds within a larger group of chemically related compounds; stereo-selectivity, the ability to act on a single enantiomer and regio-selectivity, the ability to act on one molecule site (Cherry and Fidantsef, 2003).

As a result, biocatalysts can be used in both simple and complex transformations without the need for blocking and deblocking steps that are common in organic synthesis (Schmid et al., 2001).

2. Mild reaction conditions: enzymes provide reasonable reaction rates under mild conditions, without request of toxic reagents. Enzymes typically function at ambient temperature, atmospheric pressure and neutral pH. Conversely chemical reactions may require high temperature or pressure to achieve suitable rates in a chemical process. Working under mild conditions results in significant savings in resources such as energy and water for the

benefit of both the industry and the environment (Cherry and Fidantsef, 2003; Kirk et al., 2002; Posorske, 1984).

3. Environmentally friendly - enzymes are proteins and therefore fully biodegradable. For this reason are not hazardous or polluting. In addition, enzymes-based processes generate less wastes because they use aqueous solutions and reduce solvent consumption.

Finally, enzymes require less energy, leading to lower costs and lower emissions of gases in the environment (Cherry and Fidantsef, 2003; Posorske, 1984).

The advantages inherent to biological catalysis have been exploited by a number of industries resulting in numerous applications in the food and pharmaceutical fields where high reaction selectivity on complex substrates is critical (Rozzell, 1999; Schmid et al., 2001).

The fruitful application of biocatalysis requires functional and stable enzymes. These enzymes can either be isolated from the nature repertoire or can be improved, in terms of activity and stability, by modern protein engineering.

To achieve this, it is important that innovations in enzyme technology and industrial process requirements are taken into account when looking for new enzymatic activities (Panke and Wubbolts, 2002). Thanks to advances in biotechnology, enzymes can be developed today for processes where no one would have expected an enzyme to be applicable just a decade ago (Kirk et al., 2002).

For example, "directed evolution", a methods based on random mutagenesis of gene sequences followed by selection of improved enzyme variants, can provide enzymes tailored for the production process. Enzymes can also be modified by chemical means, introducing

metals or modified cofactors, which might result in new catalytic activities (Marrs et al., 1999).

Moreover, developments in this field have shown that biocatalysts can be engineered to function in the presence of organic solvents maintaining activity and selectivity (Schmid et al., 2001).

It is important to underline that biocatalytic processes have still to be optimized and some hurdles to their industrial application, for example instability under reaction conditions and cofactors regeneration, remain to be overcome (Rozzell, 1999).

The growing use of industrial enzymes depends on constant innovation to improve their performances and reduce their costs. This innovation is driven by a rapidly increasing availability of natural enzymes, by recombinant DNA and fermentation technologies that allow this diversity to be produced at low cost, and by tools for protein modification that enable enzymes to be tuned to fit into the industrial market (Cherry and Fidantsef, 2003).

In the field of rubber treatment, different enzymes have been reported to catalyze rubber degradation, for example rubber oxygenase (RoxA) (Braaz et al., 2005; Braaz et al., 2004), lipoxygenase and linoleic acid or horseradish peroxidase/1-hydroxybenzotriazole, manganese peroxidase (MnP), laccase (Lac) (Enoki et al., 2003; Sato et al., 2003).

1.9 Biotechnology of biodesulfurization

Biodesulfurization is a biological process alternative to chemical ones in which sulfur removal from organic molecules is carried out by microorganisms as whole cells and/or enzymes, i.e. proteins extracted from them.

The biodesulfurization process investigated more in depth is sulfur removal from fossil fuel. This process has an important environmental impact because the combustion of sulfur-containing compounds emits sulfur oxides that can cause acid rains in the atmosphere (Aminsefat et al., 2012; Soleimani et al., 2007). In particular, sulfur dioxide is abundantly produced and can cause adverse effects also on human health such as respiratory illnesses. For these reasons great efforts are made to reduce the concentration of sulfur in diesel fuel and gasoline (Oldfield et al., 1998; Soleimani et al., 2007).

Desulfurization through a conventional process, known as hydrodesulphurization, requires high temperature and pressure but has some drawbacks because molecules, largely found in the heavier fractions of petroleum, such as dibenzothiophene (DBT) and alkyl substituted DBT are highly recalcitrant to desulfurization (Mc Farland, 1999; Monticello, 2000).

Some microorganisms are able to grow on sulfur-containing compounds, included DBT, breaking sulfur-carbon bonds and metabolizing sulfur. These microorganisms possess specific enzymes that catalyze these reactions, leading to the desulfurization of DBT.

In literature, two different aerobic metabolic pathways are described for DBT biodesulfurization: i) a destructive process or Kodama desulfurization, in which the carbon-carbon bonds of the benzene ring are cleaved (Figure 7) ii) a specific oxidative biodesulfurization pathway, known as "4S pathway" (Gupta et al., 2005; Srivastava, 2012). In the "4S pathway" (Figure 8), the oxidative cleavage of C-S bonds does not imply breaking of the carbon skeleton, producing 2-hydroxybiphenil (HBP) as the final product of the reaction (Davoodi-Dehaghani et al., 2010; Mc Farland, 1999; Monticello, 2000; Soleimani et

al., 2007). More in detail, the pathway requires the chain reactions of four enzymes, named DszA, DszB, DszC and DszD enzymes. The first enzyme is the flavin-dependent monooxygenase DszC which oxidizes DBT to DBT sulfoxide (DBTO) and then to DBT sulfone (DBTO₂). The second one is DszA, another flavin-dependent monooxygenase that acts on the C-S bond of DBTO₂ and produces 2'-hydroxybiphenyl-2-sulfinate (HBPS). Finally the DszB desulfonase converts HBPS to 2-HBP and sulfate. The FMN:NADH oxidoreductase DszD does not directly react with the substrate but is required for recycling FMNH₂, the coenzyme of monooxygenases (Akhtar et al., 2009; Mohebbali and Ball, 2008; Oldfield et al., 1997).

This pathway has been described for the first time in *Rhodococcus rhodochromus* IGTS8 (ATCC®53968™) where *dszABC* genes are located on a stable plasmid (Oldfield et al., 1997).

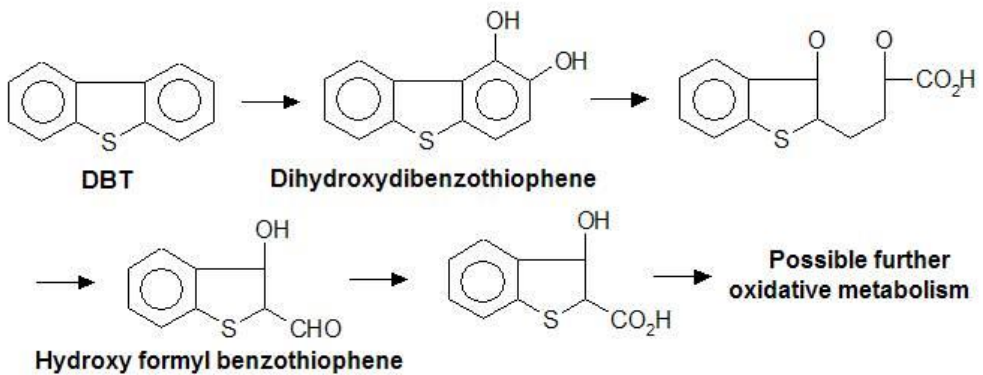


Figure 7 – Kodama enzymatic pathway on dibenzothiophene (DBT) (Ansari et al., 2007)

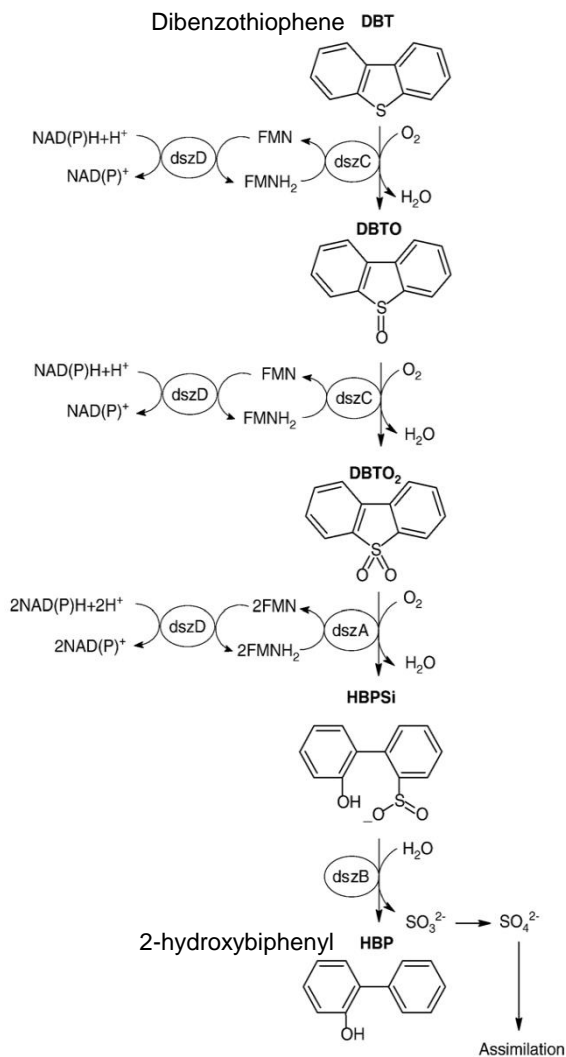


Figure 8 – The 4S metabolic pathway for DBT desulfurization (Soleimani et al., 2007)

2. Aim of the work and state of the art

An effective devulcanization process requires selective and specific reactions, carried out in mild conditions of temperature and pressure, without the use of hazardous compounds.

In this view, the employment of biological systems in general and of enzymes in particular could provide a valuable and environmental friendly approach.

This thesis work explores the possibility of applying enzymes for rubber devulcanization, either as the lone catalysts or as additives for bioprocesses in which also whole bacterial cells are employed.

Enzymatic devulcanization is still poorly described in the scientific and technical literature. Three patents cite the use of "enzymatic cocktails", without defining their specific composition (Bohm and Stephanopoulos, 2011; Romine and Snowden-Swa, 1997). To date, information about the conformational and catalytic properties of specific enzymes to be used for devulcanization is not available, nor are such enzymes commercialized by enzymes providers.

Therefore, this study proceeded by steps: the isolation of microorganisms with potential desulfurizing ability, the identification of specific enzymes, their production in recombinant and easy-to purify form and the study of their conformational stability. A mixture of the enzymes obtained was preliminary tested on dibenzothiophene and natural rubber.

3. Experimental work

This thesis addresses the identification, production and characterization of enzymes to be used as catalysts for the biodevulcanization of rubber from tires. For the sake of clarity, experiments and results are grouped in three major topics.

3.1 Isolation of novel bacterial strains with desulfurizing ability

3.2 Stability of desulfurizing enzymes towards temperature and organic solvents

3.3 Dsz enzymes desulfurizing activity on DBT and natural rubber as substrates

3.1 Chapter 1

Isolation of novel bacterial strains with desulfurizing ability¹

Background

In the last decades several studies addressed methods for the biodesulfurization of fossil fuels, to reduce the concentration of sulfur in diesel fuel and gasoline (Monticello, 2000; Oldfield et al., 1998; Soleimani et al., 2007). The majority of these studies was focused on finding previously unknown bacterial strains able to desulfurize dibenzothiophene (DBT), a recalcitrant compound abundant in the heavier fractions of petroleum (Aminsefat et al., 2012; Bhatia and Sharma, 2010; Davoodi-Dehaghani et al., 2010; Soleimani et al., 2007).

As already described (see 1.9 of introduction) two different aerobic metabolic pathways are described for DBT biodesulfurization: i) a destructive process or “Kodama desulfurization”, ii) a specific oxidative biodesulfurization pathway, known as “4S pathway” (Davoodi-Dehaghani et al., 2010; Mc Farland, 1999). The “4S pathway” and the four Dsz enzymes that catalyze the four reactions involved have been firstly described in *Rhodococcus rhodochrous* IGTS8 (ATCC®53968™). Later on other bacteria with the same metabolic pathway have been isolated, e.g *Microbacterium* sp. ZD-M2 (Li et al., 2005), *Rhodococcus* sp. Eu-32 (Akhtar et al., 2009), *Stenotrophomonas* sp. NISOC-04 (Papizadeh et al., 2011).

¹ Microbiological experiments were carried out by Valeria Tatangelo at DiSAT of Uni-MiB (Microbiology Laboratory, P.I. Prof. Giuseppina Bestetti).

Mass spectrometry analysis were performed by Dr. Carlo Santambrogio at Biotechnology and Biosciences Department of Uni-MiB (Mass Spectrometry Laboratory, P.I. Prof. Rita Grandori).

More recently, biodesulfurization has been proposed as a new tool for rubber devulcanization (Li et al., 2011, 2012; Yao et al., 2013). In microbial biodevulcanization the sulfur crosslinks present in rubber polymers are cleaved by specific enzymes present in the cells. Recently, *Sphingomonas* sp. has been used for the desulfurization of ground tire rubber (GTR) (Guangming et al., 2011; Li et al., 2012) and the process was improved by increasing the affinity between bacterial cells and rubber with the addition of surfactants in the medium (Hu et al., 2014). Several studies were performed with different chemolithotrophic strains, bacteria able to use reduced inorganic compounds as a source of energy, such as *Thiobacillus ferrooxidans*, *T. thiooxidans*, *T. thioparus*. It was shown, that *T. ferrooxidans* cells can oxidize rubber crosslinks to form sulfoxide and sulfone group without breaking the carbon chain (Guangming et al., 2010). Previously, Romine and coworkers exploited the desulfurization process on ground tire rubber with *Sulfolobus acidocaldarius* cells (Romine and Snowden-Swa, 1997).

The metabolic pathway proposed by the authors for biodesulfurization involves sulfoxide/sulfone/sulfonate/sulfate as intermediates produced with a mechanism similar to "4S pathway". However, no clear evidence about specific enzymes involved in devulcanization was published at that time. More recently, a patent on devulcanization of GTR claimed the use of *Gordonia desulfuricans* 213E (NCIMB 40816) (Christofi et al., 2010; Kim et al., 1999). In this patent was reported that after treatment the amount of sulfur in rubber decreases between 23% and 35%.

To date, all strains tested for rubber desulfurization were isolated from soil contaminated with oils but no evidence was provided that devulcanization involves the same reactions and enzymes described for desulfurization of DBT. *G. desulfuricans* 213E is the only strain isolated

from an oil contaminated soil able to desulfurize both benzothiophene and ground tire rubber (Chen et al., 2005). However, to date no attempts were made to isolate bacterial strains from ground tire rubber, or environmental samples exposed to fires. Indeed, tires could represent a harsh environments for microorganisms due to toxic chemical additives they contain. The working hypothesis of this part of the work was that contamination by ground tire rubber and fires would provide an environment where bacteria with desulfurizing activity are selected and from where they can be isolated.

In this view, we isolated bacteria from water, soil and mud collected from a tires landfill and we set up enrichment cultures aiming at isolating desulfurizing microorganisms. A novel strain, named *Rhodococcus* sp. AF21875 was isolated following this approach. In this study, ground tire rubber could not be used as the substrate to detect biodevulcanization, because of the lack of an easy analytical assay. For this reason, DBT was used instead. The final product of DBT desulfurization via the 4S-pathway is 2-hydroxybiphenyl (HBP) that is easily detectable using a colorimetric assay.

Besides assessing the ability of *Rhodococcus* sp. AF21875 cells to desulfurize DBT, a proteomic analysis was carried out with the aim of identifying unknown proteins, whose expression is induced when DBT is the sole source of sulfur during cells growth.

Experimental procedures

Chemicals and reagents

R. rhodochrous IGTS8 (ATCC 53968) was purchased from the American Type Culture Collection (ATCC) (USA) and *Gordonia desulfuricans* 213E (NCIMB 40816) was purchased from the Lebeniz Institut DSMZ-“German Collection of Microorganisms and Cell Culture”.

Dibenzothiophene (DBT, assay 99 %), 2-Hydroxybiphenyl (HBP, assay 99 %) and other chemicals were purchased from Sigma Aldrich (Germany). Acetonitrile, water, ethyl acetate (HPLC grade) were obtained from Panreac (Spain). Tryptone, agar, yeast extracts were obtained from Biolife (Italy).

Media for cells growth

Minimum salts medium (MSM) (pH 7.0) (Chang et al., 1998) was prepared dissolving 10 g of glucose, 4.5 g of K_2HPO_4 , 1.5 g of NaH_2PO_4 , 2 g of NH_4Cl , 0.2 g of $MgCl_2$, 0.02 g of $CaCl_2 \cdot 2H_2O$, 1 mL of vitamin (d-biotin 0,02 g/L, choline chloride 0,3 g/L, folic acid 0,1 g/L, myo-inositol 3,5 g/L, niacinamide 0,1 g/L, p-amino benzoic acid 0,1 g/L, d-pantothenic acid* $\frac{1}{2}Ca$ 0,025 g/L, pyridoxine*HCl 0,1 g/L, riboflavin 0,02 g/L, thiamine*HCl 0,1 g/L, vitamin B-12 0,0005 g/L, KCl 0,2 g/L, KH_2PO_4 0,2 g/L, NaCl 8,0 g/L, Na_2HPO_4 1,15 g/L), and 1 mL of sulfur-free trace metal solution (5 mg/L $MnCl_2 \cdot 6H_2O$, 0.5 mg/L H_3BO_3 ; 0.5 mg/L $ZnCl_2$; 0.5 mg/L $CoCl_2 \cdot 6H_2O$; , 0.46 mg/L $NiCl_2 \cdot 6H_2O$; 0.3 mg/L $CuCl_2$; 0.1 mg/L $NaMoO_4 \cdot 2H_2O$; 1.49 mg/l $FeCl_2 \cdot 4H_2O$, 0.003 mg/l $NaSeO_3$ e 0.008 mg/l Na_2WO) (Widdel and Bak, 1992) per 1 L of deionized water and autoclaved. Autoclaved rich medium (LD) contained 10 g peptone, 5.0 g NaCl, 5.0 g yeast extracts per liter deionized water.

Enrichment and isolation of DBT desulfurizing bacteria

DBT desulfurizing bacteria were enriched and isolated from soil, water, mud samples collected from the tire landfill in Italy (Ferrara), wastewater from Pirelli factories or directly from the GTR. One gram of soil, GTR or one milliliter of mud or water samples were suspended in 19 ml MSM. MSM used for enrichment of DBT desulfurizing bacteria was supplemented with DBT as the sole source of sulfur. DBT was dissolved in ethanol (100 mM) and added to sterilized MSM. Enrichment cultures were carried out in MSM containing 1 mM (184.2 ppm) DBT. Cultures were incubated at 30 °C for 7-10 days under shaking conditions. 1 ml aliquots were transferred into 19 ml of fresh MSM/DBT medium. After ten such sub-cultivation steps, cultures were appropriately diluted and plated onto LD agar plates and incubated at 30°C overnight.

Assay of desulfurizing activity (Gibb's assay)

Single colonies from each isolate were inoculated in 20 mL LD and grown for 24 hours at 30°C with constant shaking. Aliquots (1 ml) of cultures were transferred into 19 ml of fresh LD and grown overnight under the same conditions. Cell suspensions were centrifuged at 5000 rpm for 10 min at 4°C to obtain cell pellets of each isolate. The cell pellets were washed with MSM to remove traces of nutrient broth and pelleted again. Cell were resuspended in MSM. The desulfurization ability of each soil isolate was checked by inoculating cell suspensions (optical densities equal to 0,1) into 20 mL of MSM supplemented with DBT (1 mM - 184,26 ppm) as the sole source of sulfur. Cultures were incubated at 30°C for 7 days under shaking. Two control experiments were also set-up, one with *Rhodococcus erythropolis* IGTS8 (ATCC 53968) as the positive control, and the other without bacteria (abiotic

control). After 7 days of growth Gibb's assay was performed (Aminsefat et al., 2012).

The Gibb's assay was used to detect and quantify 2-hydroxybiphenyl produced upon incubation of the substrate DBT with bacterial cells. The Gibb's reagent (2,6-dichloroquinone-4-chloroimide) reacts with aromatic hydroxyl groups such as 2-hydroxybiphenyl to form a blue colored complex. The Gibb's assay was carried out as follows: the pH of the microbial culture was adjusted to 8.0 using 10% (w/v) sodium carbonate (Na_2CO_3). Then, 100 μL of Gibb's reagent (10 mg/mL dissolved in ethanol) was added to each sample and the reaction mixture was incubated at 30°C for 30 min and centrifuged (4000 rpm, 4°C, 20 min) to separate bacterial cells. The absorbance of the supernatants was measured at 595 nm (Beckman DU-640 Spectrophotometer). Different 2-hydroxybiphenyl concentrations were used to draw a calibration curve.

Identification of isolates

Identification of bacteria was by polymerase chain reaction (PCR) amplification and sequencing of 16S rRNA genes. DNA was extracted from bacterial cells (Invisorb Genomic DNA kit III) and 16S rRNA gene fragments were PCR-amplified with the primers 27F (5'-AGAGTTTGATCCTGGCTAG-3') and 1492R (5'-CTACGGCTACCTGTTACGA-3'). Polymerase chain reaction (PCR) was performed in 80 μL reactions containing 20 μL of cell suspension or 4 μL of DNA extracted from cells with 16 μL of water, 2 U of Taq DNA Polymerase (Promega Corporation, USA) and 10 μM of each primer (Sigma- Aldich, Germany). The cycle parameters were as follows: 10 min at 95 °C; 30 cycles of 30 s at 95 °C, 45 s at 55 °C, and 2 min at 72 °C; followed by a 5 min final extension step at 72 °C. PCR products were

purified using the Wizard-SV Gel and PCR purification kit (Promega Corporation, USA). The 16S rRNA genes were sequenced with ABI prism 310 CE systems by CIBIACI (Firenze, Italy). The 16S rRNA sequence analyzed by Ribosomal Data Project (RDP) and BLAST tool (Blast Alignment Tool – BLAST) to obtain the strain classification.

Quantitative analyses

For quantitative analyses, 10 mL of each culture medium were acidified up to pH 2.0 with 1 M HCl and then HBP was extracted with 20 mL of ethyl acetate. The sample was put into a Brandson ultrasonic bath for fifteen minutes at room temperature. After that, each sample was centrifuged for five minutes at 4000 rpm. Experiments were performed in triplicate.

Stock solutions containing DBT/HBP in ethyl acetate were prepared and stored at 4 °C. These solutions (1000 ppm for DBT and 100 ppm for HBP, respectively) were used to prepare quality control (QC-S) and calibration curves in the range between 0.005 ppm and 1.4 ppm. High-performance liquid chromatography (HPLC) was performed by an Agilent 1100 systems (Agilent Technologies, USA) equipped with a RP-C18 5- μ m particle size column (150*4.6 mm, Vydac 201, Vydac, USA). A fluorimeter detector was used for products quantification. Photo-diode array detection (DAD) coupled with fluorimetry was employed to determine the maximum excitation and emission wavelength for HBP. The isocratic mobile phase was water: acetonitrile (50:50 v/v). The overall HPLC conditions and the optimal fluorescence wavelengths are summarized below.

Flow rate	Pressure	Injection volume	Temperature	HBP excitation wavelength	HBP emission wavelength
1.6 ml/min	95 bar	3 µl	30 °C	240 nm	340 nm

Parameters of HPLC running

Detection of dszA, dszB, and dszC genes in bacterial DNA

Plasmidic DNA (Pure Yield Plasmid Miniprep System – Promega Corporation, USA) and genomic DNA (Invisorb Genomic DNA Kit III – Invitex, Germany) were used as templates for polymerase chain reaction. Sequence of the primers used for amplification is reported in table 1 (Duarte et al., 2001).

Gene	Primer	Sequence	Fragment length (bp)
dszA	Forward	5'-TCGATCAGTTGTCAGGGG-3'	547
	Reverse	5'-GGATGG CCAGA CTGTTGAG-3'	
dszB	Forward	5'-ATCGAACTCGACGTCCTCAG-3'	422
	Reverse	5'-GGAACATCGACACCAGGACT-3'	
dszC	Forward	5'-CTGTTCCGATAACCACCTCAC-3'	392
	Reverse	5'-ACGTTGTGGAAGTCCGTG-3'	

Table 1- Sequences of dszABC primers used for the amplification of genes (Duarte et al., 2001)

PCR was performed in 80 µL mixtures containing 4 µL purified DNA with 16 µL of water, 2 U of Taq DNA Polymerase (Promega Corporation, USA) and 10 µM of each primer (Sigma- Aldich, Germany). The dsz genes fragments were PCR-amplified as previously described (Duarte et al. 2001). Plasmidic DNA from *R. rhodochrous* IGTS8 (ATCC 53968) and *E. coli* TOP 10 were used as PCR templates for positive and negative controls, respectively. PCR products were purified using the Wizard- SV

Gel and PCR purification kit (Promega Corporation, USA). The *dsz* genes were sequenced with ABI prism 310 CE systems by CIBIACI (Firenze, Italy). The obtained sequences were analyzed with the bioinformatics tool BLAST (Basic Local Alignment search Tool-<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul et al., 1990).

Samples preparation for protein analysis

Rhodococcus cells grown for 7 days at 30°C in the presence of either 1 mM DBT or sulfate salts (2.5 mL/L of a sulfate stock solution obtained dissolving 10.75 g MgO, 2 g CaCO₃, 4.5 g FeSO₄, 1.44 g ZnSO₄, 1.12 g MnSO₄, 0.25 g CuSO₄, 0.28 g CoSO₄, 0.03 g H₃BO₃ with 25 mL MgSO₄ 1M, 25 mL FeSO₄ 36 mM, 51.3 mL HCl per 1L of distilled water at pH 7) were harvested by centrifugation and resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl pH 8). Cells extracts were mixed with 4 volumes of acetone and kept on ice for 1 hour. Proteins were precipitated by centrifugation and the pellets rinsed twice with acetone. Dried pellets were dissolved in solubilization buffer (SB) containing 7 M urea, 2 M thiourea and 4% (w/v) 3-[(3cholamidopropyl) dimethyl ammonio]- 1-propane sulphonate (CHAPS). Protein concentration was determined according to Bradford (Bio-Rad, California, USA) using bovine serum albumin as a reference.

Two-dimensional gel electrophoresis

300 µg of total proteins were dissolved in 300 µl SB, 60 mM dithiothreitol (DTT), 20 mM iodacetamide (IAA), 1% (v/v) carrier ampholytes (Bio-Rad, California, USA), glycerol and bromophenol blue (BBF). Isoelectrofocusing (IEF) was performed on a 17 cm Immobilized pH Gradient (IPG) strip (Bio-Rad, California, USA) pH 4-7. Gels were rehydrated for 1 hour and proteins focused at 20°C in a Protean IEF Cell

(Bio-Rad, California, USA) by applying a sharp voltage increase from 0 V to 10000 V (40-60000 V/hr). IGP strips were then equilibrated under shaking for 20 minutes in equilibration buffer EB1 (6M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS) in Tris HCl pH 6.8, 2% (v/v) DTT) and for 20 minutes in equilibration buffer EB2 (6M urea, 30% (v/v) glycerol, 2% (w/v) SDS) in Tris HCl pH 6.8, 2.5% (w/v) IAA). Strips were placed on the top of a 12% SDS-Polyacrilamide gel, embedded with agarose 0.5% (w/v) and Bromophenol blue (BBF) traces. SDS-PAGE was carried out at 30 mA/gel and gels were stained with GelCode Blue Stain Reagent (Pierce, Rockford, IL USA).

Mass spectrometry

Spots were excised from gels and destained by repeated washes with 50% acetonitrile (ACN) and 50% 50 mM ammonium carbonate. After destaining, gel fragments were dehydrated by pure ACN and then treated with 10 mM DTT in 100 mM ammonium carbonate for 30 minutes at 56°C. Gel slices were then dehydrated again with ACN and treated with 55 mM IAA in 100 mM ammonium carbonate for 20 minutes at room temperature (RT). After further dehydration gel pieces were incubated with 1µg/µL trypsin solution in 50 mM ammonium bicarbonate, for 1 hour at 4°C. Buffer was then removed and gel pieces were covered with 50 mM ammonium bicarbonate and incubated overnight at 37°C. After collecting supernatants, digested peptides were extracted through incubations in an equal volume of 5% formic acid and 10% ACN at 37°C. This step was repeated three times. Collected samples were then lyophilized, resuspended in 1% formic acid, and desalted by Zip Tip (Millipore, Billerica, MA, USA).

Electro Spray Ionization-Mass Spectrometry (ESI-MS) was carried out with hybrid Quadrupole-Time-of-Flight (q-TOF) mass spectrometer (QSTAR

ELITE, Applied Biosystems, California, USA) equipped with a nano-electrospray ionization sample source. Metal-coated borosilicate capillaries (Proxeon, Denmark) with medium-length emitter tip (1 μM internal diameter) were used for off-line analysis. The instrument was calibrated with a standard solution of Renin inhibitor (MH2 879.97 Da) and its fragment (MH 110.07 Da, Applied Biosystems, California, USA). Peptide identification was performed using the MASCOT software (Matrix Science, Boston, MA, USA) with following parameters: 2 missed cleavages, peptide tolerance 0.6 Da, MS/MS tolerance 0.6 Da, peptide charges 2+ and 3+. Spectra of tryptic peptides were acquired in the 400-1500 m/z range, with 1.0 sec accumulation time, ion-spray voltage 1100 V, declustering potential 80 V, with active Information Dependent Acquisition (IDA).

Results

Identification and characterization of bacterial strains

Twelve types of soil, water and mud samples from the tire landfill in Italy (Ferrara) and, waste water from Pirelli factories were collected to isolate DBT desulfurizing microorganisms. After ten rounds of sub-cultivation in MSM medium supplemented with DBT, 135 pure isolates were obtained. To assess desulfurizing activity, the isolated bacterial strains were grown in sulfur free MSM medium with DBT as sulfur source. *R. rhodochrous* IGTS8 (ATCC 53968), already known for its ability to break the C-S bonds in DBT, and *Gordonia desulfuricans* 213E, a strain with desulfurizing ability on tires, were both analyzed and considered as positive controls. Gibb's assays were carried out after seven days of growth. Growth on DBT medium was observed for 84 strains but only 51 out of them were able to desulfurize this substrate.

Bacterial strains positive to Gibb's assay were taxonomically classified by 16S rRNA sequencing. Most of them belong to three different phyla: *Actinobacteria* (22 strains), *Firmicutes* (25 strains) and *Proteobacteria* (3 strains). *Proteobacteria* were represented by only three strains from three different families: *Acetobacteraceae*, *Xanthomonadaceae* and *Caulobacteraceae*. Among *Firmicutes*, 21 isolates were *Bacillales* and 4 were *Lactobacillales*. The following genera were represented: *Bacillus*, *Fontibacillus*, *Paenibacillus*, *Staphylococcus* and *Lactobacillus*. Among *Actinobacteria* both *Corynebacterineae* and *Micrococccineae* were isolated belonging to *Rhodococcus* (7 isolates) and *Microbacterium* (15 isolates) genus. Six strains were investigated with more detail (Table 2).

Strains	Gibb's Results (μM)	Classification
<i>Rhodococcus rhodocrous</i> IGTS8	15	<i>Rhodococcus</i>
<i>Gordonia desulfuricans</i> 213E	7	<i>Gordonia</i>
S2285D	32	<i>Microbacterium hydrocarbonoxydans</i>
AF21875	18	<i>Rhodococcus Qingshengii</i> strain dj1-6
5A304E	10	<i>Paenibacillus favisporus</i>
P2AT5	15	<i>Paenibacillus wynnii</i>
9A194C	17	<i>Paenibacillus xylanexedens</i>
10B1551	11	<i>Paenibacillus cineris</i>

Table 2- Gibb's results and classification of the six selected bacterial strains. *Rhodococcus rhodocrous* IGTS8 and *Gordonia desulfuricans* data are reported for comparison.

Since the Gibb's assay provides qualitative information only, samples obtained by incubating DBT with media containing the putative desulfurizing cells, were analyzed also by HPLC. Detectable HBP production was observed for *Microbacterium* sp. S2285D, *Rhodococcus* sp. AF21875, *Paenibacillus* sp. 5A304E, *Paenibacillus* sp. P2AT5 and *Paenibacillus* sp. 9A194C (Table 3).

Strain	HBP production (ppm)	Standard deviation
<i>Rhodococcus rhodocrous</i> IGTS8	15	3
<i>Gordonia desulfuricans</i> 213E	9.9	1
<i>Microbacterium</i> sp. S2285D	15	11
<i>Rhodococcus</i> sp. AF21875	9	4
<i>Paenibacillus</i> sp. 5A304E	7	2
<i>Paenibacillus</i> sp. P2AT5	8	3
<i>Bacillus</i> sp. 8A1531	4	6
<i>Paenibacillus</i> sp. 9A194C	3	4
<i>Paenibacillus</i> sp. 10B1551	-	-

† Below the quantification limit

Table 3- HBP production of selected strains measured by HPLC. Measures are performed in triplicate and standard deviations are reported for each bacterial strain.

This analysis shows that the production of HPB by *Rhodococcus* sp. AF21875 is comparable that by *G. desulfuricans* 213E chosen as positive control. Also *Microbacterium* sp. S2285D produced amounts of HBP but it was not further analyzed because growth on DBT could not be easily obtained.

Characterization of *Rhodococcus* sp. AF21875

AF21875 was classified as *Rhodococcus* because its 16S RNA shares 99% identity with that of *Rhodococcus qingshengii* (Table 2).

Amplification of plasmidic DNA purified from *Rhodococcus* sp. AF21875 cells with primers specific for the *dsz* genes, allowed to demonstrate that this bacterial strain harbors the *dszA*, *dszB* and *dszC* genes (Figure 1). The desulfurizing ability of *Rhodococcus* sp. AF21875 was tested after different time of growth in MSM medium supplemented with DBT as sole source of sulfur. The highest HBP production (10 ppm) was obtained after 14 days of growth (Table 4).

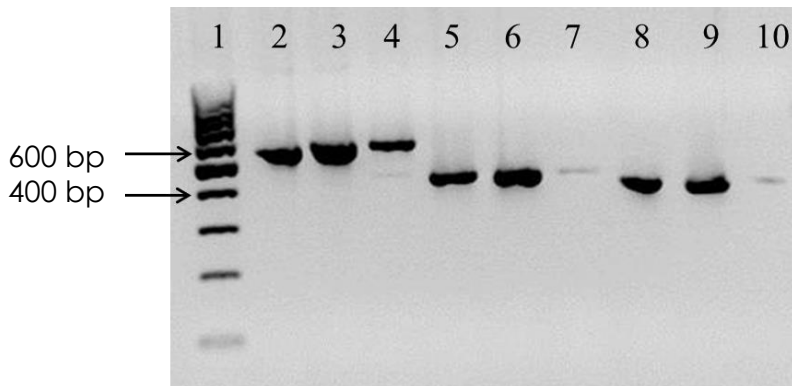


Figure 1- Amplification of *dszA*, *B* and *C* genes from selected bacteria. Lanes: 1 Marker, 2 *R. rhodochrous* IGTS8 *dszA*, 3 *Rhodococcus* sp. AF21875 *dszA*, 4 *G. desulfuricans* 213E *dszA*, 5 *R. rhodochrous* IGTS8 *dszB*, 6 *Rhodococcus* sp. AF21875 *dszB*, 7 *G. desulfuricans* 213E *dszB*, 8 *R. rhodochrous* IGTS8 *dszC*, 9 *Rhodococcus* sp. AF21875 *dszC*, 10 *G. desulfuricans* 213E *dszC*.

Time (days)	HBP (ppm)	Standard deviation
0	0	0
3	6	0.2184
7	5	0.1458
10	7	0.4583
14	10	0.0551

Table 4 - HBP produced by *Rhodococcus* sp. AF21875 grown for different time (0-14 days) in MSM supplemented with DBT. Measures were performed in triplicate and standard deviation is reported.

Proteomic analysis of *Rhodococcus* sp. AF21875 cells

To identify proteins whose expression is induced by DBT, we compared the proteome (i.e. the total proteins contained in the cell) of cells grown in the presence of 1 mM DBT as the carbon source with that of cells grown in media containing sulfate salts taken as the control. Electrophoresis was performed in two orthogonal directions to separate proteins on the basis of both their mass and their isoelectric point. Three proteins were found to be up-regulated by DBT (Figure 2) and identified by mass spectrometry. Proteins migrating as spot 1 and spot 2 were both identified as a N,N'-dimethyl-4-nitrosoaniline (NDMA)-dependent methanol dehydrogenases (ThcE). The protein mass of 46 kDa, determined by MS, is in agreement with the position of the spots in the gel. The slight difference in molecular weight and the difference in isoelectric point are both accounted for by the presence of covalent modifications (one alkyl group and two methyl groups) in the protein contained in spot 1. Spot 3 was identified as an S-ethyl dipropylcarbamothioate (EPTC)-inducible aldehyde dehydrogenase, by similarity with the same protein of *Rhodococcus erythropolis*.

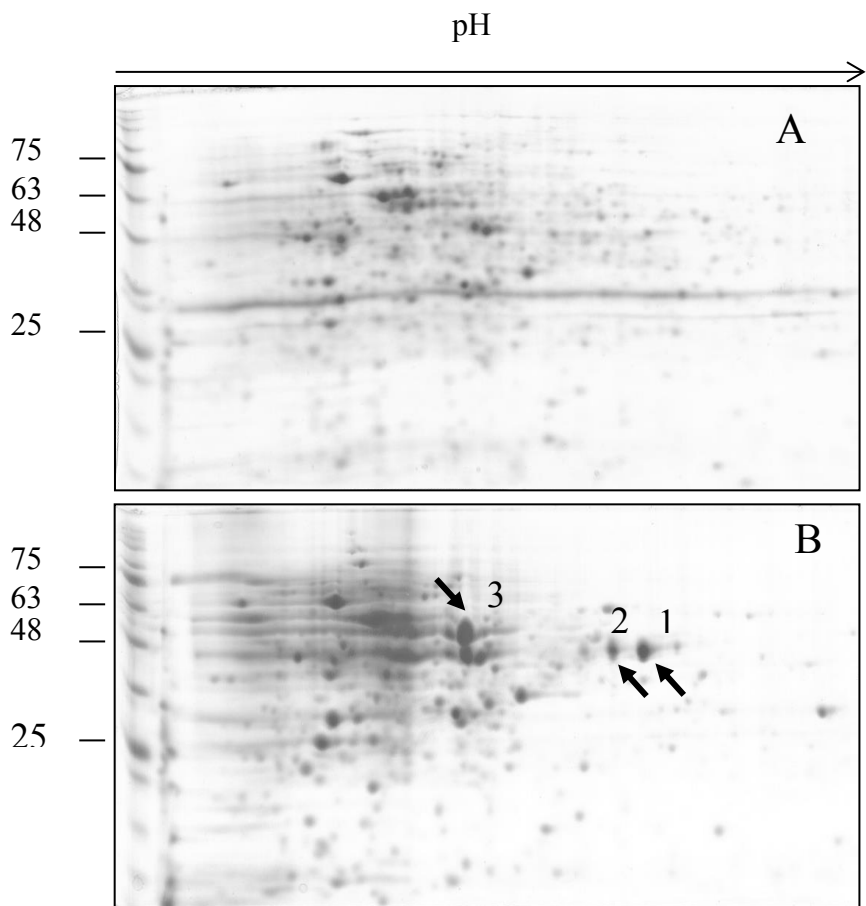


Figure 2 - Two-dimensional electrophoresis of protein extracted from *Rhodococcus* grown in the presence of either sulfate **(A)** or DBT **(B)** as the sulfur sources. Spots marked by arrows were excised and analyzed by mass spectrometry.

Discussion

In recent years, biodesulfurization has gained importance because laws regulating both, sulfur content in fuels and tires disposal in landfill became more severe. This prompted for the search of bacteria highly active in biodesulfurization in particular for cells whose metabolic activities cleave C-S yet preserving the C-C backbone of substrates. The so-called 4S pathway is described as an enzymatic process able to break carbon-sulfur bonds without damaging the carbon chain shared among a few bacterial species (Ashutosh et al., 2011; Davoodi-Dehaghani et al., 2010; Papizadeh et al., 2011).

In the first part of the work, 84 bacterial strains were isolated from samples contaminated with tires rubber. Out of them, 51 were able to desulfurize DBT. This indicates that growth on DBT medium does not necessarily imply that cells can desulfurize the substrate. Indeed, Van Hamme reported that some bacteria can produce biomass employing sulfur traces present in medium components as contaminants (Van Hamme et al., 2004). Gibb's assay was a suitable tool for the initial screening of a large number of strains, although false negatives were also detected, due to the presence of phenolic compounds produced by microorganisms. For this reason, the preliminary analysis was followed by HPLC to confirm the presence of HBP and to quantify its amount (Papizadeh et al., 2011).

The classification of strains isolated in the course of this work (*Actinobacteria* and *Firmicutes*) confirmed that biodesulfurization is typical of a few genera of gram-positive bacteria (Papizadeh et al., 2011), in particular *mycolata* and few species of *Bacilli*.

Rhodococcus sp. AF21875 catalyzed the conversion of DBT to HBP at levels comparable to those of the reference strains (*G. desulfuricans*

213E and *Rhodococcus rhodochrous* IGTS8). DBT consumption continued until 14 days of growth, and the HBP concentration increased also when the culture reached the stationary phase. Production of HBP together with the identification of *dsz* genes confirms that desulfurization proceeds through the 4S metabolic pathway, that does not affect C-C bonds, as already mentioned. This is important for the biodesulfurization process of both fuels and vulcanized rubber.

Despite the 4S pathway was described in different bacterial strains, only a few works addressed the transcriptomic and proteomic analysis of these cells, though these approaches might improve our understanding of the process, identifying proteins involved in the metabolism or uptake of DBT. Recently Wang and coworkers studied by transcriptomic analysis the genes expression in *Gordonia terrae* C-6 cultured in the presence of benzothiophene. Their study highlights that in the presence of benzothiophene not only monooxygenases and desulfinases are expressed but also others proteins related to the transmembrane transport, probably related to the transport of BT and its metabolites (Wang et al., 2013).

With the same purpose, we carried out a differential proteomic analysis of proteins produced in the presence of DBT. We found that DBT does not up-regulate the production of desulfuring enzymes, while it drives the increase of two proteins, N,N'-dimethyl-4-nitrosoaniline (NDMA)-dependent methanol dehydrogenases (ThcE) and S-ethyl dipropylcarbamothioate (EPTC)-inducible aldehyde dehydrogenase, not directly involved in sulfur metabolism. It has been already reported that ThcE production is induced in *Rhodococcus* sp. NI86/21 during assimilation, among others, of thiocarbamate and S-triazine herbicides and alcohols (i.e. ethanol, amino- or poly-alcohols) (Jaureguibeitia et

al., 2007; Nagy et al., 1995a; Nagy et al., 1995b). Furthermore, ThcE from *Rhodococcus* sp. NI86/21 has been identified as a subunit of NDMA-dependent alcohol oxidoreductase, an enzyme involved in the metabolism of different carbon sources (Nagy et al., 1995a).

Similarly, also ThcA has been found to be induced by EPTC in *Rhodococcus* sp. This enzyme acts preferentially on aliphatic aldehydes, but retains activity also on aromatic aldehydes (Nagy et al., 1995a). On this basis it is reasonable to hypothesize that DBT might induce the catabolic pathway activated by EPTC.

In conclusion, the proteomic analysis performed on *Rhodococcus* cells grown on DBT highlighted the increase of proteins not directly involved in sulfur metabolism. While such changes deserve more investigation to understand long range metabolic interactions, it is clear that the identified proteins are not of direct interest for devulcanization. For this reason, further research focused on Dsz enzymes only.

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3.2 Chapter 2

Stability of desulfurizing enzymes towards temperature and organic solvents²

Background

Biotechnology often takes advantage of the metabolic abilities of living beings that offer an astonishing repertoire of biocatalysts. Enzymes are exploitable in reactions of commercial and/or environmental relevance, either directly or upon improvement by protein or process engineering. In this context, enzymes evolved by bacterial strains to assimilate sulfur are of interest for application in the transformation of sulfur-containing chemical compounds, environmental decontamination and fossil fuels desulfurization (Mohebbali and Ball, 2008). Major researches are focused in reducing sulfur emission from combusted fossil fuels, according to increasingly strict environmental regulations. Major organosulfur contaminants of oils are aromatic compounds, such as benzothiophene, dibenzothiophene (DBT) and their alkylated derivatives. Desulfurization of crude and refined petroleum products is conventionally carried out by high-pressure and high-temperature hydrodesulphurization (HDS) (Babich and Moulijn, 2003; Furimsky and Massoth, 1999).

In the quest for biological processes alternative to the conventional ones, sulfur-metabolizing bacteria have been investigated. Strains of *Rhodococcus*, *Nocardia*, *Paenibacillus* and others were found able to desulfurize aromatic heterocycles, including DBT, via an aerobic

² The results of this chapter have been reported in the following publication: F. Parravicini, S. Brocca, M. Lotti, "Looking for catalysts for biodesulfurization – A study on stability of 4S-pathway enzymes towards temperature and organic solvents", submitted.

Bioinformatics analyses were performed at Institute of Technical Biochemistry, University of Stuttgart, in collaboration with Prof. J. Pleiss.

mechanism known as the 4S-pathway, in which desulfurization does not affect the carbon skeleton of substrates, as already described in the Introduction (Mc Farland, 1999; Mohebbali and Ball, 2008). Later on, the same metabolic route has gained attention also to catalyze rubber biodevulcanization. The 4S pathway converts DBT in non-sulfur compounds as 2-hydroxy-biphenyl (2-HBP), via subsequent reactions and involves four enzymes: two flavin-dependent monooxygenases (DszA and DszC), a desulfinase (DszB) and a FMN:NADH oxidoreductase, necessary to recycle the monooxygenases coenzyme (DszD) (Kilbane, 2006; Mc Farland, 1999). Desulfurization occurs through sequential oxidation of the sulfur moiety and cleavage of C–S bonds. The reaction is started by DszC which converts DBT to DBT sulfoxide (DBTO) and then to DBT sulfone (DBTO₂). DszA attacks the C-S bond of DBTO₂ and produces 2'-hydroxybiphenyl-2-sulfinate (HBPS), which is then converted to 2-HBP and sulfate by the DszB desulfinase. Finally, DszD recycles reduced flavins through the oxidation of NADH. Genetic studies demonstrated that *dszA*, *dszB* and *dszC* genes are plasmid-encoded and belong to the same operon (Li et al., 1996; Ma et al., 2006; Shavandi, 2013), while *dszD* is located on the bacterial chromosome (Matsubara et al., 2001). Though it was shown that whole bacterial cells can desulfurize diesel fuels (Yu et al., 2006; Zhang et al., 2007), at present microbial desulfurization is regarded as an interesting complementary technology, yet still not efficient enough to fully replace HDS (Mohebbali and Ball, 2008). In the following, the major issues to be solved are summarized, as they also limit application of biodevulcanization. To make the bioprocesses competitive, a number of major limitations have still to be solved, such as the inhibition of expression exerted in natural strains by end products and sulfate (Alves et al., 2005), the need of

cheap culture media to grow desulfurizing bacteria (Alves and Paixao, 2014), and cell sensitivity to temperature or to components of the reaction mixtures. Several strategies are under study to alleviate these hurdles, for example the use of solvent-tolerant (Tao et al., 2006) or thermostable (Konishi et al., 2000b) bacterial strains. Dsz genes have been overexpressed in heterologous hosts (Alves and Paixao, 2014), at higher copy number or under the control of strong promoters (Hirasawa et al., 2001; Li et al., 2007). Further improvements of the activity and stability of all involved enzymes might be achieved by protein and/or process engineering. Indeed, robustness of enzymes towards challenging reaction conditions is a major prerequisite to their industrial exploitation. As discussed in Chapter 3.1, a *Rhodococcus* strain was found to be active in biodesulfurization and the presence in its genome of dsz genes was demonstrated. In this part of the work, Dsz enzymes were produced as recombinant proteins from *E. coli* cells, purified and tested for their stability towards temperature and organic solvents, including swelling solvents used to swell natural rubber contributing to facilitate or to guarantee a deeper devulcanization (Loadman, 1998; Mcfarlane et al., 2013). We observed that Dsz proteins are rather unstable to organic solvents and heating and require to be stabilized before their use in biotechnological processes.

Experimental procedures

Strains, growth media and materials

Escherichia coli strains DH5a TM (Thermo Fisher Scientific) and BL21 (DE3) (Novagen, Madison, USA) were used as the host for DNA amplification and for heterologous expression, respectively. *E. coli* cells were grown in low-salt Luria-Bertani (ls-LB) medium (10 g peptone, 5 g yeast extract, 5

g NaCl in 1 L water). Transformants of both strains were selected on agarized plates of Is-LB supplemented with 50 mg/L ampicillin and, when appropriate, with 5-Bromo-4-chloro-3-Indolyl- β -D-Galactopyranoside (X-Gal) 40 μ g/mL. Oligonucleotides were from Eurofin MWG (Alabama, USA), restriction enzymes and DNA ligase were from New England Biolabs (NEB, Ipswich, USA). Dibenzothiophene (DBT), 2-hydroxybiphenyl (2-HBP) and organic solvents were from Sigma Aldrich (Germany). The *Rhodococcus* sp. AF21875 strain was isolated from environmental samples and was a kind gift of G. Bestetti and A. Tatangelo, University of Milano-Bicocca. *Rhodococcus* cells were grown in minimal medium (10 g glucose, 4.5 g K₂HPO₄, 1.5 g NaH₂PO₄, 2 g NH₄Cl, 0.2 g MgCl₂, 0.02 g CaCl₂, 5 mg MnCl₂, 0.5 mg H₃BO₃, 0.5 mg ZnCl₂, 0.003 mg NaSeO₃, 0.008 mg Na₂WO₄, 1.49 mg FeCl₂, 0.5 mg CoCl₂, 0.46 mg NiCl₂, 0.3 mg CuCl₂, 1 mg NaMoO₄, 1 mL of vitamin solution in 1 L water) (Chang et al., 1998).

Amplification and cloning of dsz genes

Standard recombinant DNA techniques were applied according to Sambrook et al. (Sambrook and Russell, 2001). Genes *dszA*, *dszB*, *dszC* and *dszD* were amplified by PCR using 100 ng of total DNA extracted from *Rhodococcus* sp. AF21875 as a template and each primer at a concentration of 0.5 μ M. Oligonucleotide primers (Table SI) were designed on the basis of available sequences of the *Rhodococcus* *dsz* operon (gene bank accession number L37363.1). Each oligonucleotide contained restriction sites useful for the subsequent cloning of the amplified genes: NdeI/HindIII for *dszA* and *dszB*, NdeI/XhoI for *dszC* and *dszD*. Before their use in PCR oligonucleotides were phosphorylated in a reaction catalysed by polynucleotide kinase A (NEB), at 37 °C for 1 hour.

PCR was carried out in a volume of 50 μ L with 1 μ L of the Q5 @high-fidelity Pfull Ultra DNA polymerase (NEB), according to the manufacturer's instructions and applying the following temperature program: 30 s denaturation at 98 °C, 30 cycles of 10 s at 98 °C, 30 s at 61 °C, 30 s at 72 °C and a final extension step of 2 min at 72 °C. Amplified blunt-ended DNA was cloned into a SmaI-linearized pUC18 (Thermo Fisher Scientific) plasmid that, in the presence of X-Gal (5-Bromo-4-chloro-3-Indolyl- β -D-Galactopyranoside) allows for white-blue screening of transformed colonies. Each gene was separately back-cloned into pET22 (Novagen, Madison, USA) linearized with restriction enzymes (NdeI/HindIII for dszA and dszB, NdeI/XhoI for dszC and dszD). Constructs were checked by analysis of restriction patterns and by DNA sequencing (Primm, Milan, Italy).

Oligonucleotide	Sequence 5'-3'
DszA.for	CCG <u>C</u> ATATGACTCAACAACGACAAATGC
DszA.rev	CCAAAGCITTGAAGGTTGTCCTTGCAG
DszB.for	AGAC <u>C</u> ATATGACAAGCCGCGTCGAC
DszB.rev	ACCAAAGCITTCGGTGGCGATTGAGGCGCT
DszC.for	CCC <u>C</u> ATATGACACTGTCACCTGAAAAGCAG
DszC.rev	AAACTCGAGGGAGGTGAAGCCGGAATC
DszD.for	AAACATATGTCTGACAAGCCGAATGCC
DszD.rev	AAACTCGAGTTGACCTAACGGAGTC

Table S1- Oligonucleotides sequences. Restriction sites NdeI, XhoI and HindIII are underlined

Expression and purification of Dsz proteins

Transformed BL21 cells were grown over night in 50 mL of auto-induction ZYM-5052 medium (Studier, 2005) at 25 °C or 30 °C by shaking at 220

rpm and harvested by centrifugation at 1600 g at 4 °C. Cells were re-suspended in Purification Buffer (PB - 50 mM sodium phosphate pH 8.0, 300 mM NaCl and 10 mM imidazole) and lysed using a cell disruptor (Constant Systems Ltd) at 25000 psi. Insoluble proteins were sedimented by centrifugation for 15 min at 15000 g, at 4 °C. Recombinant, his-tagged proteins were purified from the supernatant by immobilized-metal affinity chromatography (IMAC) on Ni²⁺/NTA beads. To this purpose, the clear lysate was loaded on a column containing 1.5 mL of HIS-Select Nickel Affinity Gel (Sigma Aldrich, Germany) equilibrated with 3 mL of PB. The column was washed with 2 mL of PB and 2 mL of PB containing 20 mM imidazole and proteins eluted with PB containing 250 mM imidazole. Protein-containing fractions were exchanged to the final buffer (10 mM sodium phosphate, pH 7) by two consecutive gel filtrations on PD-10 columns (GE Healthcare, Little Chalfont, United Kingdom) according to the manufacturer's instructions. Protein concentration was determined by the Bradford protein assay (Bio-Rad, California, USA), using bovine serum albumin as a standard. SDS-PAGE was carried out on 14% acrylamide Laemmli gels (Laemmli, 1970) stained with GelCode Blue (Pierce, Rockford, USA) after electrophoresis. Broad-range, pre-stained molecular-weight markers (GeneSpin, Milan, Italy) were used as standards.

Circular dichroism spectroscopy

CD spectra were recorded by a spectropolarimeter J-815 (JASCO Corporation, Easton, USA) in a 1-mm path-length cuvette at room temperature. Samples were in 10 mM sodium phosphate buffer, pH 7. Measurements were performed at variable wavelength (190 - 260 nm) with scanning velocity 20 nm/min and data pitch 0.2 nm. Spectra were

averaged over three acquisitions, and smoothed by the Means-Movement algorithm (Sreerama and Woody, 2000). Unfolding of 0.150 mg/ml protein solutions was monitored recording consecutive spectra at temperatures in the range 20°C- 95°C. Thermal ramps were obtained in the range of 20 to 95°C with increments of 1°C/min. The decrease in CD signal was monitored at 195 nm.

Fluorescence Spectroscopy

Protein samples were re-suspended in 10 mM sodium phosphate buffer, pH 7, and fluorescence emission spectra were measured on a Cary Eclipse Fluorescence Spectrophotometer (Varian), with excitation at 290 nm and emission range 300 – 450 nm, employing a 1-cm path-length quartz cuvette. To detect structural changes induced by organic solvents, DszA, DszC and DszD were incubated under continuous agitation at room temperature with methanol, acetonitrile, hexane and toluene at 5, 10 or 20% for 3 hours. Fluorescence emission was measured at different times. All measurements were made in triplicate.

Sequence analysis and structural modeling

DNA sequence analysis were performed using BLAST (Altschul et al., 1990) either with the megablast algorithm, optimized for highly similar sequences or by discontinuous megablast, optimized to study dissimilar sequences.

The 3D model of DszA was built using SWISS-MODEL (Arnold et al., 2006; Biasini et al., 2014). Template search was performed against the SWISS-MODEL template library with BLAST (Altschul et al., 1990) and HHBlits (Remmert et al., 2012). In this approach, models are built based on the target-template alignment using Promod-II. Coordinates conserved

between the target and the template are copied from the template to the model. Insertions and deletions are re-modeled using a fragment library. Side chains are then rebuilt. Finally, the geometry of the resulting model is optimized by using a force field. The global and per-residue model quality was assessed using the QMEAN scoring function (Benkert et al., 2011).

Results

Sequence analysis

Genes of *Rhodococcus* AF 21875 encoding DszA, DszB, DszC and DszD were amplified by PCR from total DNA using primers designed on the basis of the corresponding *Rhodococcus* sp. sequences available in databases and sequenced. BLAST analysis of the four deduced amino acid sequences identified a small group of hits sharing very high identity with the queries in strictly related bacterial genera, mainly *Rhodococcus* sp, *Brevibacillus*, *Nocardia* and *Gordonia*. Outside this narrow group of microorganisms, the sequence coverage dropped abruptly from 95-80% to 25-5% along with the total alignment score. This observation may be accounted for by evolutionary constraints and suggest that *dsz* genes were shared only recently among a restricted group of *Mycolata*. Interestingly enough, in the only case of DszD, sequences sharing highest identity all belonged to *Rhodococcus* species, while identity with sequences from other genera did not exceed 70% even in the case of strict phylogenetic relatedness (*Nocardia*). This might depend on the different location of the genes in the bacterial genome. Indeed, *dszA*, *dszB* and *dszC* have been found on small plasmidic DNA (Denome et al., 1994; Piddington et al., 1995) and therefore are expected to be

more easily exchanged among species, while *dszD* is on the bacterial chromosome (Matsubara et al., 2001). It is also worth to mention that DszD is the less specific out of the four enzymes and that in microorganisms other than *Rhodococcus* its activity could be provided by other oxidoreductases.

3D model of the DszA monooxygenase

While the 3D structure of DszB, C and D proteins are available in PDB (accession number 2DE2, 4JEK 4DOI), the structure of DszA is not reported. Therefore, a model of DszA was built by homology, as a support for the conformational analysis performed in this work. BLAST analysis revealed that DszA belongs to the superfamily of flavin-containing mono-oxygenases (EC number 1.14.13.8). Highest identity with proteins of known 3D structure was shared with a nitrilotriacetate monooxygenase (PDB code 3sdo.1.A, 41% identity) and with the alkane monooxygenase LadA from *Geobacillus thermodenitrificans* Ng80-2 (PDB code 3b9o.1.A, 37% identity). Both structures were used as templates to generate a DszA model by SWISS MODEL (Arnold et al., 2006; Biasini et al., 2014). The model obtained on the LadA template turned out to be the most reliable according to a simple structure assessment procedure based on GMQE (0.66) and QMEAN4 score (-6.56). A further advantage of the LadA-based model was that it allowed to locate the flavin mononucleotide (FMN) cofactor. The protein conforms to a TIM-barrel scaffold from which different extensions protrude. The most significant are a long extension (E) formed by a loop and a short alpha-helix, probably involved in DszA dimerization as LadA (Li L. et al, 2008), and a large pocket (P) formed by alpha helices $\alpha 11$ - $\alpha 14$ (DszA numbering). This pocket is assumed to build the substrate

binding region and the catalytic core of the enzyme (Fig. 1A). The structural alignment (Fig. 1B) highlighted a large insertion in the region corresponding to the LadA substrate binding site (residues 320-360). This feature is consistent with the activity of DszA on aromatic substrates (Ohshiro et al., 1999), bulkier than the long-chain, linear alkanes accepted by LadA (Feng et al., 2007). Analysis of sequence and structural alignments also highlighted that residues of LadA involved in FMN binding are mostly conserved also in DszA (Asp59, Ser 138, Ala 228, Leu 229, Ser 230, Phe 246, Phe 373 and Tyr 160), and suggest a similar binding mechanism.

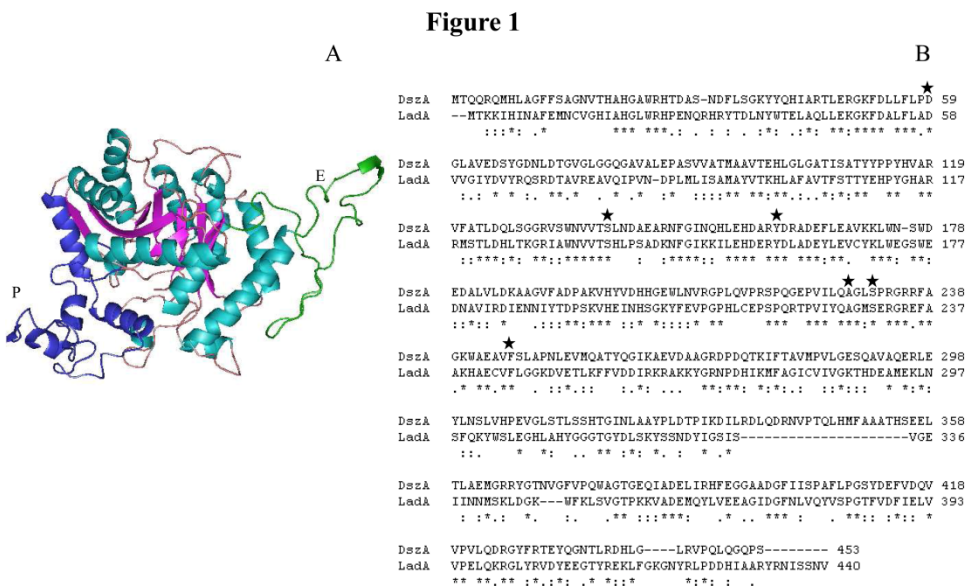


Figure 1. Three dimensional model of DszA. **A)** homology model based on the LadA structure. The E loop is marked in green and the pocket P is in blue. **B)** sequence alignment of DszA and LadA. Residues involved in FMN cofactor binding are highlighted by stars.

Figure 2

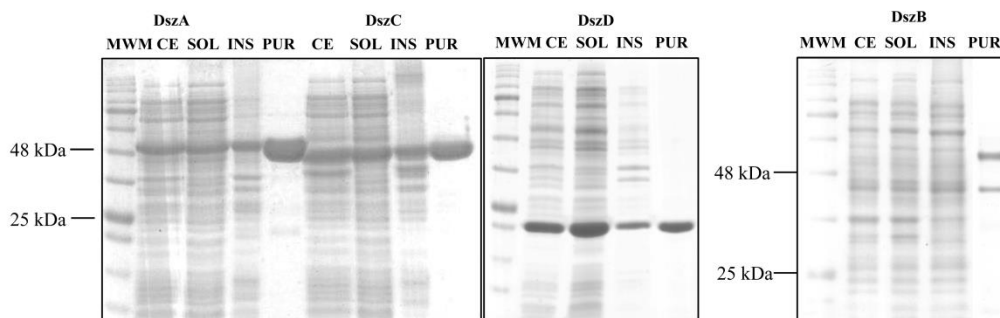


Figure 2 - Production and purification of recombinant Dsz protein. Expression was at 30°C for Dsz A, C and D and at 25°C for DszB. CE; crude extract, SOL: soluble protein fraction, INS: insoluble protein fraction, PUR: proteins purified by IMAC; MWM: molecular weight markers.

Production of recombinant Dsz proteins and stability analysis

Amplified gene sequences were cloned in the expression vector pET22 and used to transform *E. coli* BL21 cells. Expression was in auto-induction ZYM-5052 medium (Studier, 2005) at different temperatures. SDS-PAGE analysis revealed the presence of proteins with the expected molecular mass (48 kDa for DszA and DszC; 45 kDa for DszB and 22 kDa for DszD). At 30°C, DszA, DszC and DszD were produced as soluble proteins, and were easily purified by IMAC affinity chromatography (Fig. 2). The DszB protein was more difficult to be obtained because of its low expression, aggregation propensity and for the presence of contaminants in the purified samples. For these reasons, structural analysis of DszB could not be performed.

The model of DszA described in this work and the available 3D structures of DszC (Duan et al., 2013) indicate that the two monooxygenases prevalently contain alpha-beta secondary structures. Accordingly, the

far-UV CD profiles of DszA and DszC show negative minima at 209 and 220 nm and a positive maximum at 195 nm, typical of alpha-beta structures (Figure 3A, B). DszD mainly contains beta structures with a few small α -helices (PDB entry 3PTF), as also indicated by the overall lower intensity of the ellipticity signal (Figure 3C) and by deconvolution data (not shown).

Thermal unfolding of DszA, DszC and DszD was induced by heating protein solutions from 20°C to 95°C and monitored by circular dichroism in the far-UV (Figure 3A-C), while unfolding transitions were recorded at fixed wavelength of 195 nm (Figure 3D). A progressive unfolding was observed for all the enzymes, as highlighted by the loss of signal at 195 nm. In the case of DszA and DszC we also observed a decrease of the peak at 220 nm (Figure 3A, B). In the DszC spectra, the isodichroic point at ~ 207 nm (Figure 3B) was consistent with a two-state transition of α/β structures (Figure 3D, dotted line). This protein unfolded with an apparently cooperative process, with a melting temperature of 48°C. DszA unfolding is a multi-stage process characterized by two different melting temperatures, at ~45°C and ~87°C (Figure 3D, continuous line). The denaturation profile of DszA (Figure 3D, continuous line) hints the existence of at least one conformational intermediate induced by heating at 40-50 °C and stable at 50-75°C. This intermediate has still a very high content of secondary structure (Figure 3A, continuous line) and is highly resistant to heating ($T_m = 87^\circ\text{C}$). This particular behavior may suggest a two-step unfolding, the first one involving loss of quaternary structure (DszA is dimeric) (Doble and Kumar, 2005; Stapleton and Singh, 2002), and the second monomers unfolding. The reversibility of thermally-induced denaturation proceeds apparently until the restoration of this unfolding intermediate, as suggested by the good

overlap of spectra obtained at 50-60°C and after refolding. The denaturation of DszD did not show any isodichroic point and resulted in a final spectrum with low ellipticity at 195 nm and intense negative ellipticity at 202 nm, overall suggesting that at 95°C the protein was almost fully unfolded (Figure 3C). DszD showed a low cooperative and barely distinguishable multi-stage denaturation process (Figure 3D, dashed lines). For all enzymes, unfolding was at different extent reversible, since samples were able to partially recover secondary structure when re-cooled to 20°C (Figure 3A-C, dashed lines).

Not only stability to temperature but also the enzymes robustness towards solvents is of relevance in processes where the biocatalysts can be challenged by harsh reaction conditions. Therefore we investigated the structural stability of Dsz proteins toward solvents of common use. In these experiments proteins unfolding was monitored by fluorescence spectroscopy since the signal is less affected by the solvent than in CD spectroscopy. To this end, proteins were incubated in the presence of methanol, acetonitrile, hexane and toluene at 5%, 10% and 20% v/v concentrations and analyzed at different times through the emission spectra of intrinsic tryptophan fluorescence. Spectra were recorded after 5, 30, 60 and 180 minutes of incubation for each concentration. In Figure 4 we report spectra obtained in the presence of 20% v/v solvents, after 3 hours of incubation. Lower solvents concentrations and shorter incubation induced similar though milder effects. Data are therefore not reported. Overall, solvents caused a red shift of the maximum peak of tryptophan fluorescence, reflecting the increased solvent accessibility of these residues. Dsz proteins were differently affected by each assayed solvent, e.g., the red shift was more evident for DszD, more

sensitive to all solvents, and almost undetectable for DszC. Data on red shifts are summarized in Table 1.

Solvent	Redshift (nm)		
	DszA	DszC	DszD
Acetonitrile	6	1	15
Methanol	5	1	5
Hexane	1	3	5
Toluene	3	2	6

Table 1- DszA, DszC and DszD redshifts of the maximum peak after 3-hour incubation in 20% v/v acetonitrile, methanol, toluene and hexane. The shifts indicate averages between three distinct measurements on different proteins preparations.

The intensity of tryptophan fluorescence increases or decreases, depending on the solvent features, concentration and duration of exposition. Overall, the fluorescence intensity increases when a protein expands in a more loose structure and tryptophan residues become more exposed to the solvent (Kamatari et al., 1996; Uversky et al., 1997), while loss of signal intensity is often due to protein aggregation. We observed that hexane and toluene induce an increase of fluorescence intensity in the case of DszA, even upon short-time incubation (5 min, spectra not shown). Prolonged expositions to acetonitrile and methanol induced an intense increase of the tryptophan fluorescence of DszD. Toluene caused a marked decrease of the signal upon long incubation times, at whatever concentration. Overall, our observations of intrinsic fluorescence and thermal stability profiles support the conclusion that DszC is the most stable of Dsz enzymes considered.

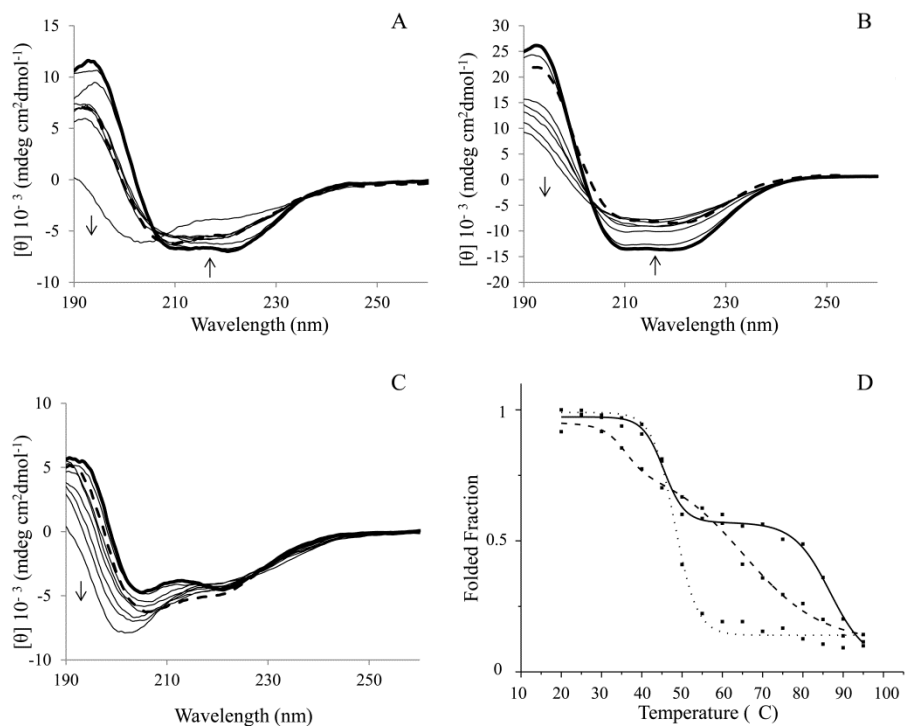


Figure 3 - Heat denaturation of Dsz proteins. **A**, **B** and **C**) far-UV spectra at 20°C (bold lines), 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 95°C (continuous lines) and 20°C after denaturation (dashed lines) of DszA (A), DszC (B) and DszD (C). **D**) Kinetics of thermal denaturation of DszA (continuous line), DszC (dotted line) and DszD (dashed line) heated in the temperature range from 20°C to 95°C measured at 195 nm.

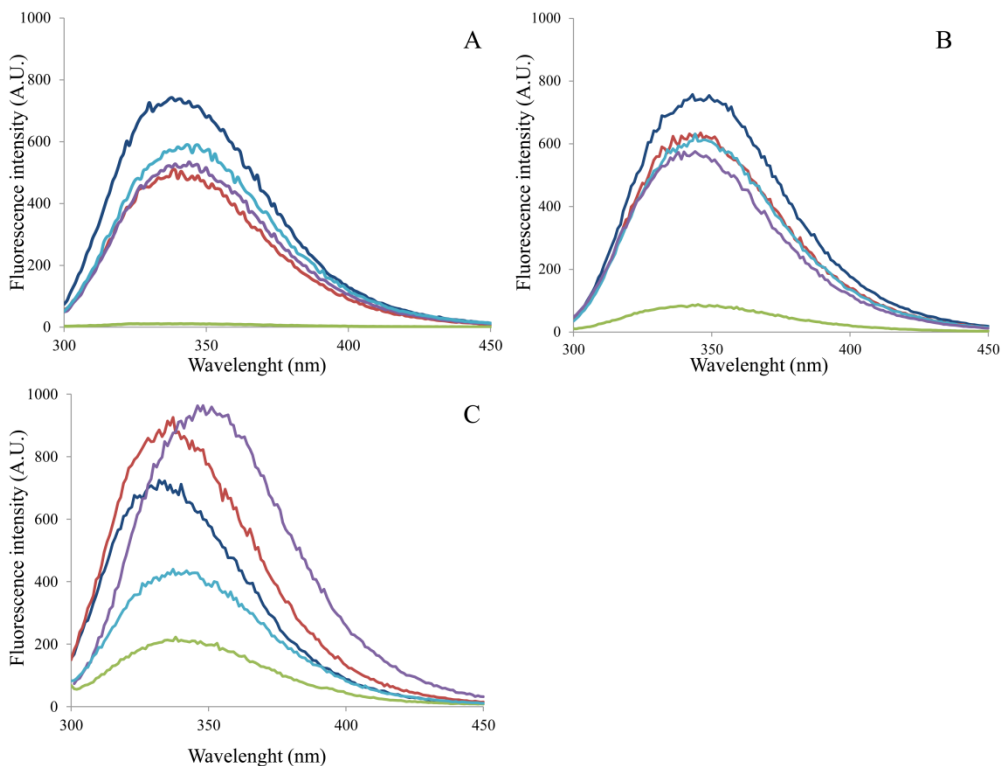


Figure 4- Unfolding of Dsz proteins in the presence of 20% v/v of organic solvents after 3 h of incubation: acetonitrile (purple), toluene (green), methanol (cyan) and hexane (red). Intrinsic fluorescence emission spectra of DszA (**A**), DszC (**B**) and DszD (**C**). Samples without solvents (dark blue) show that DszA has a maximum of fluorescence emission at 338 nm, DszC at 343 nm while DszD at 332 nm.

Discussion

Major issues still open for the implementation of Dsz-based biodesulfurization concern the overall activity and the stability of the biological system that depends on: i) robustness and metabolism of bacterial cells, ii) activity and specificity of each enzyme, tuning and coordination of enzymatic steps, iii) stability of the enzymes involved.

i) Strains tolerant to temperature yet able to desulfurize DBT have been described, for example *Klebsiella* and *Paenibacillus* species (Bhatia and Sharma, 2012; Konishi et al., 2000a). For an effective use of whole cells biocatalysts, a deeper understanding of the overall metabolism of desulfurizing enzymes is necessary. As recently pointed out by Aggarwal and colleagues (Aggarwal et al., 2012), besides the pathways already considered, also steps to incorporate sulfur in the biomass can be critical. Along this rational, a comprehensive transcriptomic analysis of *Gordonia* strains showed that growth on benzothiophene up-regulates 135 genes that are mainly, but not exclusively, monooxygenases and desulfinases (Wang et al., 2013).

ii) Dsz genes have been cloned and expressed in host, in some cases upon engineering copy number or structure of the operon or promoters (Li et al., 2008; Li et al., 2007). Biological systems can be further improved and validated through a precise measure of the intracellular enzymes level. It is also important to assess the possible interference of the gene products with cell metabolism. For example, it would be not surprising if the overexpression of a desulfinase would harm cells or if a too high level of monooxygenases would deplete the cell of FMN and NAD cofactors. Proteomics and transcriptomic studies may help to shed some lights on these issues. Not last, one has to consider that high enzymes levels not always correspond to high activity (Gonzalez-

Montalban et al., 2007) and that effects of protein and metabolic engineering on the highly-connected metabolism network might be unpredictable.

iii) As shown in this work, Dsz enzymes from *Rhodococcus* are not really robust neither to temperature nor to solvents. Indeed, desulfurizing bacteria did not evolve to operate in the conditions that might be necessary in industrial processes, neither are their proteins. If whole-cell catalysts are used, enzymes are partly protected from solvents (cells are not), but not from temperature. Stabilized variants might be reintroduced in the original strains or in host cells by themselves resistant to the reaction conditions, eventually mixing different enzymes from different sources, or supplied to the reaction mixture as supplements. In this last hypothesis, the topic of stability is still more relevant. Moreover, it has to be pointed out that the cellular localization of Dsz proteins is not yet fully clear. Some authors reported that biodesulfurization activity can be associated with the cell envelope suggesting that desulfurization could involve the outer surface of cells (Kilbane and Le Borgne, 2004).

The aim of this work was to highlight possible weaknesses of each enzyme, for what concern structural stability so as to be able to target them individually either by site-directed mutagenesis or by directed evolution. We found that the three enzymes investigated behave differently at high temperature and in the presence of high solvent concentration. Worth to be mentioned are the differences between the two monooxygenases DszC and DszA. Both bind flavine mononucleotide as the coenzyme and are organized in a quaternary structure, tetrameric for the former (Zhang et al., 2014) and dimeric for the latter (Doble and Kumar, 2005; Stapleton and Singh, 2002). Heat-induced denaturation, however, seems to proceed through different

pathways. This information could be of some relevance in selecting the strategies of stabilization, in particular on the choice of focusing on the robustness of the tertiary structure or on the strength of monomers association. Not last, it has been suggested that stable enzymes can be more easily evolved since they are able to tolerate favorable but destabilizing amino acid substitutions during the pathways of directed evolution (Tokuriki and Tawfik, 2009). In this view, stable structures could be better suited for any modification aiming at improving specificity and activity. Accordingly to our results, Dsz enzymes are stable at temperature below the 35 °C and sensitive to solvents, particularly toluene. To test enzymes activity on rubber, we have to take into account that it's not possible to use swelling solvents, during the reaction, not even at low concentration and that temperature of the reaction have to be controlled.

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3.3 Chapter 3

Dsz enzymes desulfurizing activity on DBT and natural rubber as substrates³

Background

Dsz enzymes convert dibenzothiophene (DBT) in the final product 2-hydroxybiphenyl (HBP) through four subsequent reactions described in introduction (see 1.9 in introduction). The activity of all four enzymes is necessary for the correct development of the reaction (Raheb and Hajipour, 2011).

In this part of the work, we tested the activity of mixtures of purified Dsz enzymes first on their natural substrate dibenzothiophene (DBT) and then on vulcanized ground natural rubber (VGNR). Indeed, VGNR has a composition similar to that of ground tire rubber but simpler, not including carbon black and other raw materials that can be present in tires in variable amount. These features make VGNR a substrate suitable to test enzymatic treatments with analytical approaches simpler than those required to study vulcanized ground tire rubber.

Evaluation of the rate of rubber devulcanization is a challenging task, from both a biochemical and rheological point of view. From the biochemistry side, rubber is an insoluble material and there are no easy-to-use assays to detect the extent of its chemical modifications. We applied Fourier transform infrared spectroscopy (FT-IR) in attenuated

³ Rubber rheological analyses were carried out by Dr. Paola Caracino at Pirelli laboratories.

Fourier Transform Infrared Spectroscopy (FT-IR) and High Performance Liquid Chromatography (HPLC) analysis were performed in collaboration with Ivan Mangili at DISAT of Uni-MiB (Chemistry Laboratory, P.I. Prof. Marina Lasagni).

total reflectance (ATR). By this approach raw data can be rapidly obtained but a careful and time-consuming post analysis is required.

From the rheological side, rubber has mechanical properties that are intermediate between those of an elastic solid and a viscous liquid. It is expected that desulfurization impacts on these features. By using an oscillatory rheometer is possible to quantify both the viscous-like and the elastic-like properties of rubber. More in detail, such a device measures viscosity of samples when a sinusoidal shear deformation is induced. The probed time scale is determined by the frequency of oscillation, ω , of the shear deformation. In this technology, a sample is placed between two plates, the top plate remains fixed while the bottom plate rotates, imposing a time-dependent sinusoidal shear ($\gamma(t)$). The time-dependent stress $\sigma(t)$ is quantified by measuring the torque that the sample imposes on the top plate, revealing differences between materials (Figure 1A). If the material is an elastic solid, the sample stress is proportional to the strain deformation and the stress is always in phase with the applied sinusoidal deformation. In contrast, if the material is a viscous fluid, the applied strain and the measured stress are out of phase. Viscoelastic materials, such as rubber, show a response that contains both in-phase and out-of-phase contributions (Figure 1B). The viscoelastic behavior of the system at different oscillation frequencies (ω) is characterized by the storage modulus, $G'(\omega)$, and the loss modulus, $G''(\omega)$, which respectively characterize the solid-like and fluid-like contributions to the measured stress response. The stress response of a viscoelastic material is given by $\sigma(t)=G'(\omega)\gamma_0 \sin(\omega t)+ G''(\omega)\gamma_0 \cos(\omega t)$. (Baranwal and Stephens, 2001; Phewthongin et al., 2005; Wyss et al., 2007).

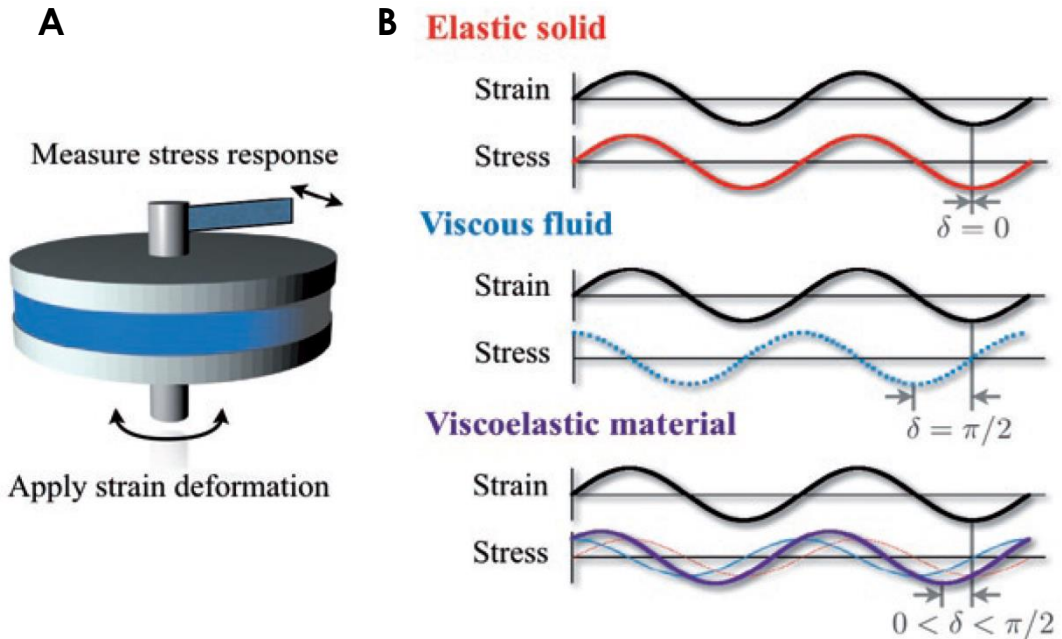


Figure 1 – **A)** Schematic representation of a rheometry setup. The sample (blue) is placed between two plates. **B)** Stress response to oscillatory strain deformation for an elastic solid (red), a viscous fluid (dotted blue) and a viscoelastic material (violet). δ indicates the phase shift with respect to the applied strain deformation (Wyss et al., 2007).

Experimental procedures

Chemicals, reagents and culture media

Natural rubber (NR) in a solid state was mixed at a two-roll mill as follows: 1 phr (1 part per hundred rubber) N-Cyclohexylbenzothiazole-2-sulfonamide, 1 phr sulfur, 5 phr zinc oxide and 2 phr stearic acid, based on 100 phr of NR. After mixing, the rubber sheet was cured for 18 min at 150 °C and at high pressure. After the curing process, the NR was ground at room temperature in order to obtain particles of size ranging between 250 μm and 500 μm .

To remove unreacted sulfur and vulcanizing agents, vulcanized ground natural rubber (VGNR) was extracted with acetone for 72 hours at room temperature. 10 ml of solvent were used for every g of material and acetone was changed every 24 hours. VGNR was then dried under vacuum for 24 hours at 50 °C.

N-Cyclohexylbenzothiazole-2-sulfenamide was Vulkacit CZ/EG-C from (Lanxess, Germany); stearic acid was Stearina TP8 from Undesa (Spain); sulfur was from Zolfoindustria (Italy), zinc oxide was from Zincol (Italy) and NR was STR20 – 1,4 cis polyisoprene from Von Bundit (Thailand).

Dibenzothiophene was purchased from Sigma (Germany), acetone was obtained from Carlo Erba (Italy). Acetonitrile, water, ethyl acetate (HPLC grade) were obtained from Panreac (Spain). Tryptone, agar and yeast extracts were obtained from Conda (Spain).

Production of recombinant Dsz enzymes

E. coli BL21(DE3) cells transformed with pET22[DszA], pET22[DszB], pET22[DszC] and pET22[DszD] were grown over night in 50 mL of auto-induction ZYM-5052 medium (Studier, 2005) at 25 °C or 30 °C by shaking at 220 rpm to induce proteins expression. Cells containing each one of the four plasmids were harvested by centrifugation at 1600 g at 4 °C, re-suspended in Reaction Buffer (RB - 25 mM sodium phosphate pH 7.5, 100 mM NaCl) and lysed using a sonicator (Branson digital sonifier) at 20% of amplitude. 10 cycles of 10 s were applied to ice-cooled samples with 10 s stops between cycles. Crude cells extracts from the four strains were mixed in equal ratio to obtain a balanced mixture of the four enzymes and used to set up *in-vitro* reactions.

SDS-PAGE was carried out on 14% acrylamide Laemmli gels (Laemmli, 1970), stained with GelCode Blue (Pierce, Rockford, USA) after

electrophoresis. Broad-range, pre-stained molecular-weight markers (GeneSpin, Milan, Italy) were used as standards.

Enzymes reaction on DBT

4 mg of crude proteins extracts as quantified by Bradford assay, were incubated in RB buffer at 30 °C for different times in the presence of 0.1 mM (18.42 ppm) DBT as the substrate and 3 mM nicotinamide adenine dinucleotide (NADH), 10 µM Flavin mononucleotide (FMN) as cofactors. Negative controls were set up without substrate.

After 1, 2 and 3 hours, 1 mL of the reaction mix were collected, extracted with ethyl acetate and analyzed by High-performance liquid chromatography (HPLC).

Sample extraction and HPLC analysis

1 mL of each reaction mix was acidified to pH 2.0 with 1 M HCl and then DBT and HBP were extracted with 2 mL of ethyl acetate. Samples were incubated into a Brandson ultrasonic bath for fifteen minutes at room temperature and then centrifuged for five minutes at 4000 rpm.

Stock solutions containing HBP in ethyl acetate were prepared and stored at 4 °C. These solutions (100 ppm HBP) were used to prepare quality control (QC-S) and to obtain calibration curves in the range between 0.005 ppm and 1.4 ppm. HPLC was performed by an Agilent 1100 system (Agilent Technologies, USA) equipped with a RP-C18 5-µm particle size column (150*4.6 mm, Vydac 201, Vydac, USA). A fluorimeter detector was used for the quantification of HBP. The photo-diode array detection (DAD), coupled with the fluorimeter, was employed to determine the maximum excitation and emission wavelength for HBP. The isocratic mobile phase was water:acetonitrile (50:50 v/v). The overall

HPLC conditions and the optimal fluorescence wavelengths are summarized below:

Flow rate	Pressure	Injection volume	Temperature	HBP excitation wavelength	HBP emission wavelength
1.6 ml/min	95 bar	3 μ l	30 °C	248 nm	340 nm

Rubber treatment

50 ml of the enzyme mixture described above was used to treat 9 g of VGNR at 30°C for 3 hours. At the end of the reaction, rubber was separated from cellular debris, which are more dense, by centrifuging 5 minutes at room temperature at 5000 rpm. Then, rubber was separated from the reaction mixture using a Büchner funnel. VGNR was then washed with 500 mL of deionized water to remove proteins and reagents and dried under vacuum for 24 hours at 50 °C.

Rheological analysis

Rheological characterization was performed with a Rubber Process Analyzer RPA 2000, Monsanto. The frequency sweep tests were carried out at 100 °C, at a strain amplitude of 20 % and within a frequency range of 0.1 - 30 Hz.

Fourier Transform Infrared Spectroscopy (FT-IR)

Fourier transform infrared spectra with a Nicolet iS10 FT-IR Spectrometer with the attenuated total reflectance (ATR) mode were carried out on VGNR at room temperature with a 4 cm^{-1} resolution and 32 scans signal average. Spectra were recorded from 4500 to 650 cm^{-1} .

X-link density

The crosslink density was measured on treated and untreated samples of VGNR.

Crosslink density and evaluation of the devulcanization percentage were carried out according to standard test method for determination of percent devulcanization of crumb rubber based on crosslink density (ASTM D 6814-02) through swelling measurements. Toluene was chosen as a solvent considering the Hildebrand solubility parameter that provides a numerical estimate of the degree of interaction between materials, particularly used in predicting swelling of polymers by solvents (Chandrasekaran, 2010). 1 g of VGNR was let to swell in cold toluene for 72 h and the solvent was refreshed every 24 h.

The Flory-Rehner equation was used in order to calculate the crosslink density (Valentin et al., 2008; Yao et al., 2013):

$$-\ln(1 - v_2) + v_2 + X_1 v_2^2 = V_1 n [v_2^{1/3} - v_2/2]$$

v_2 indicates the volume fraction of polymer in the swollen system, V_1 the molar volume of the solvent, n is the number of crosslinks (mole/cm³), and X_1 is the Flory solvent-polymer interaction parameter.

The interaction parameter between rubber and the swelling solvent, $X_1 = 0.39$, was chosen considering rubber as polymer and toluene as solvent. V_1 for toluene is 106.3 cm³ /mole. This equation allows the calculation of the number of crosslinks (n) from swelling data (Croll, 2010; Nelson, 1983).

Results

Activity of Dsz enzymes on DBT

DszA, DszC and DszD enzymes were produced in *E. coli* BL21 cells grown overnight at 30°C in autoinduction medium (Studier, 2005) while DszB protein was obtained at 25°C, since this temperature supported additives higher yields of production. After cell lysis, the presence of Dsz proteins was assessed by SDS-PAGE on total cell extracts (data not shown).

Reactions were set up with 0.1 mM DBT (18.42 ppm) as the substrate and 4 mg of Dsz enzymes mixture containing cofactors (3 mM NADH and 10 µM FMN). Reactions were incubated for 1, 3 and 18 h at 30°C. Different additives such as DTT or Triton X were tested in the reaction buffers. HPLC separation of the reaction products revealed small amounts of HBP (Figure 2). The best results were obtained after 1 hour reaction (0.61 µg/µL- 1.23 ppm) in reaction buffer without additives.

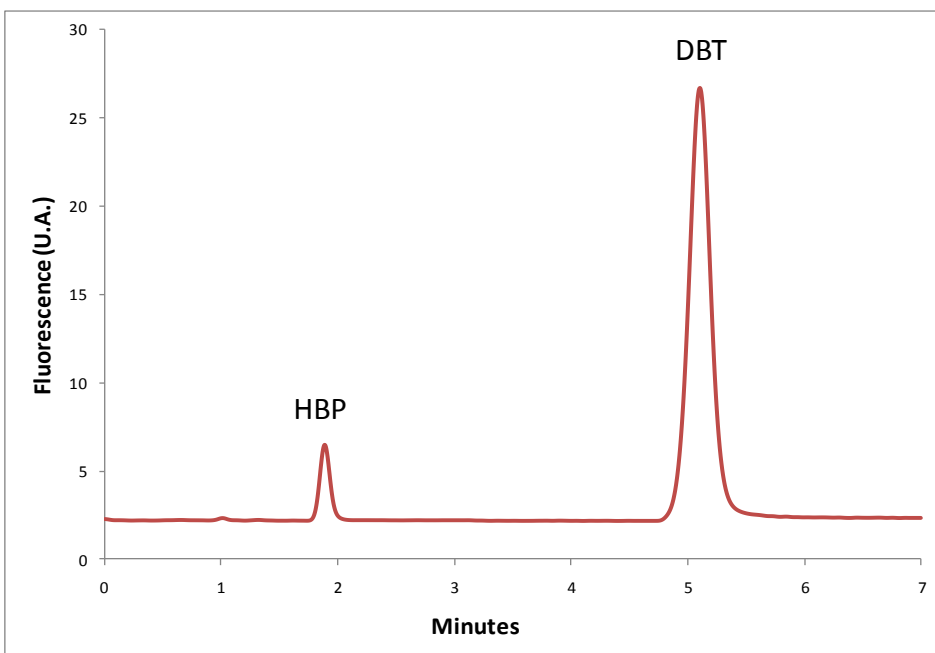


Figure 2 – Product of the reaction of DBT with a mixture of Dsz enzymes after 1 hour incubation

Enzyme activity on VGNR

The same enzymatic mixture was then preliminary exploited for the devulcanization of vulcanized ground natural rubber (VGNR). The reaction mixtures were prepared as reported in experimental procedures. After 3 hours of incubation, rheological and chemical changes were analyzed with different methods.

Rheological analysis with RPA (Rubber process analyzer) allowed to measure the storage modulus, $G'(\omega)$ that describes the viscoelastic behavior of treated and untreated VGNR (Figure 3).

$G'(\omega)$ of treated VGNR is lower than that of the negative control suggesting that rubber has been modified by the enzymes and has partially lost its elasticity.

To investigate the chemical modifications occurred after enzymatic treatment, rubber was analyzed by FT-IR spectroscopy (Figure 4). Spectra obtained before and after the treatment showed the typical peaks of natural rubber: CH and CH₂ bands at 833 cm⁻¹ and 1450 cm⁻¹, the sulphate and sulfone bands at 1081 cm⁻¹ and 1360 cm⁻¹ respectively, at 1658 cm⁻¹ we observe the cis(C=C) band.

As a matter of fact, the spectra of treated and untreated rubber were quite similar, with the only peak at 1658 cm⁻¹ which was slightly higher in the treated rubber sample. This corresponds to an increase of the cis double carbon-carbon bonds. However, we are aware that this modification might be not associated to the treatment with Dsz enzymes. The hypothesis that rubber was not affected by enzymatically devulcanization is further corroborated by the intensity of the FTIR peaks of sulphate and sulfone (1081 and 1360 cm⁻¹) that was similar for the two samples and by measurement of X-link density. Indeed, differences between X-link density of treated and non-treated rubber are hardly detectable (0.14 mmol/cm³).

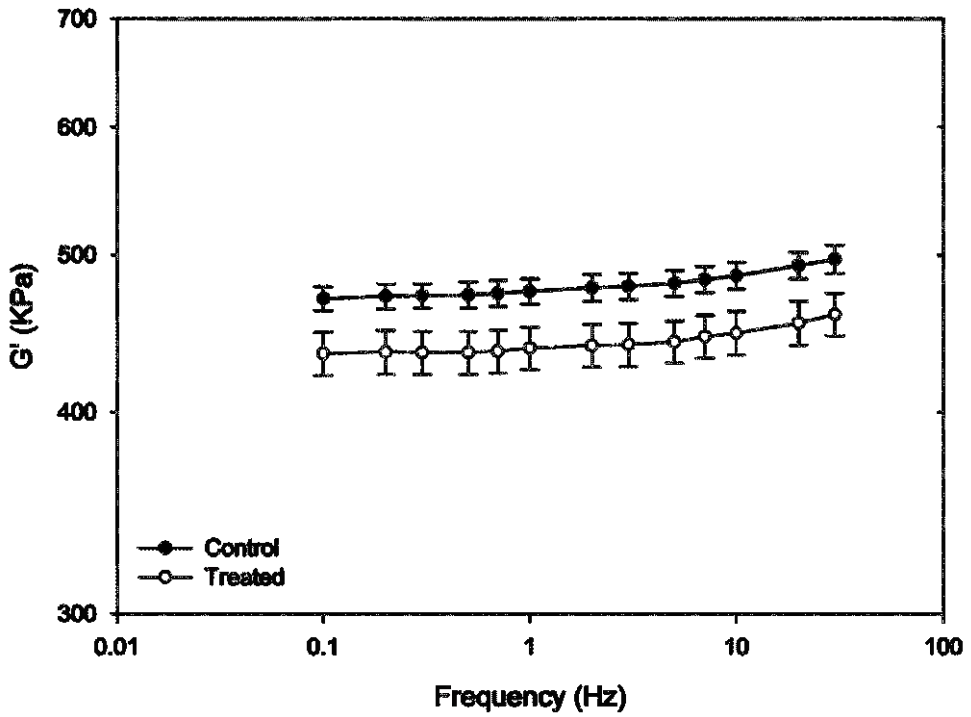


Figure 3 – Storage modulus (G') of treated (empty circles) and untreated (filled circles) rubber at different frequencies

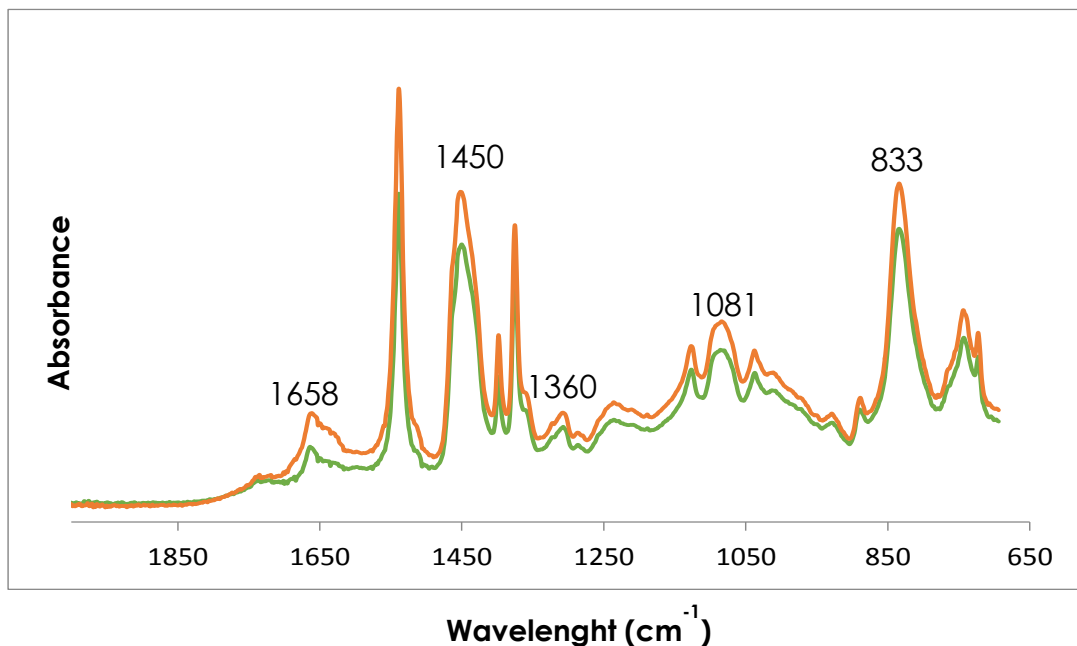


Figure 4 – FTIR spectra of treated (orange) and untreated (green) VGNR. Peculiar peaks are indicated.

Discussion

Our data suggest that enzyme-treated rubber undergoes only subtle physical and chemical modifications. On this basis it can be hardly decided whether Dsz enzymes act on rubber through the same pathway proposed for DBT, and even if they catalyze the same order of reactions. Moreover, the FTIR spectra of treated and non-treated samples show only subtle differences in the peaks of sulphone or sulfate. Overall, we do not have experimental evidence that enzymatic desulfurization has occurred on natural rubber. It should be also considered that the sensitivity of cross-link density analysis might be too low to reveal subtle differences of surface de-reticulation and making it difficult to obtain comparable data (Valentin et al., 2008). In this view

the application of other techniques such as X-ray photoelectron spectroscopy (XPS) (Hammer, 2007) or Multiple quantum (MQ) Nuclear magnetic resonance (NMR) experiments (Valentin et al., 2008) could be taken into account. With XPS can be obtained information from rubber surfaces; while MQ NMR is more sensitive to analyze sulfur cross-link distribution (Hammer, 2007; Valentin et al., 2008).

On the other hand we found that the mixture of the four Dsz enzymes catalyzed the desulfurization of DBT. Although the reaction rate (1.23 ppm of HBP produced in 1 hour) is too low to be applied to an industrial process, it is higher than that observed in microbial processes, where catalysis is carried out with whole cells. Indeed, the activity of *Rhodococcus* AF21875 (see Chapter 3.1) produces 10 ppm of HBP in fourteen days of cultivation, while ~1.2 ppm are obtained in 1 hour via the enzymatic reaction. The reaction performed *in-vitro* with enzymatic mixtures stops after one hour. Our hypothesis is that at least one of the Dsz enzymes involved in the reaction is inactivated within one hour.

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4. Conclusions and perspectives

This work was intended as a first approach to study *in vitro* the activity of desulfurizing enzymes on sulfur-containing compounds of different complexity and provided promising though preliminary results. It also highlighted a few major issues that have to be faced in future projects: i) the need of optimized conditions to reach higher yields in the desulfurization of DBT and rubber; ii) the need of a sensitive method for the detection of devulcanization rate; iii) the requirement for pre-treatments of the rubber substrate.

The low reaction yield observed with the DBT substrate may be related to the poor level of DszB. Indeed, this enzyme catalyzes the rate-limiting step in the 4S pathway (Reichmuth et al., 2000), but is produced by transformed (recombinant) *E. coli* cells in amounts lower than the other Dsz enzymes. A higher production of DszB might be obtained by co-expressing chaperons or proteins that may help its proper folding and solubility (Harper, 2011). A protein engineering approach, based on rational or random mutagenesis, might help to improve DszB amount and activity by changing its amino acid sequence. For instance, it was shown that a double mutant (Y63F and Q65H) of DszB from *Rhodococcus erythropolis* KA2-5-1 exhibits improved catalytic activity and heat stability (Ohshiro et al., 2007). Another opportunity is offered by the technique of DNA shuffling that, mixing different fragments of genes encoding for similar proteins from different organisms, was successfully applied to increase the activity of other dsz enzymes (Coco et al., 2001). Also the activity of flavin oxidoreductase (DszD) is fundamental for FMNH₂ regeneration for DszA and DszC monooxygenases. To enhance the efficiency of the whole process, DszD activity might be replaced

with more performing heterologous oxidoreductases, obtained from bacteria different from *Rhodococcus*, already known to catalyze the same reaction (Galan et al., 2000; Reichmuth et al., 2000).

Finally, it is of relevance to detect reagents that may inhibit the enzymes. For instance intermediates of the pathway (Reichmuth et al., 2000), elemental sulfur (Ohshiro et al., 1996) or some metals (Ohshiro et al., 1999).

Our results also suggest that requirement of pretreatments to make devulcanized rubber more accessible to biological catalysts.

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