UNIVERSITY OF MILANO-BICOCCA

Department of Earth and Environmental Sciences

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BIODESULFURIZATION OF WASTE RUBBER: CHARACTERIZATION OF BACTERIAL STRAINS AND THEIR POTENTIAL USE

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FINAL DISSERTATION

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“D’accordo, gli esseri umani sono più intelligenti degli organismi unicellulari, però sono anche molto più delicati. Non appena fa un po’ freddo o un po’ troppo caldo, oppure le quotazioni in borsa subiscono un crollo inatteso, questa pesante complessità c’ indebolisce sul piano fisico e su quello mentale. I batteri sono sopravvissuti alle eruzioni vulcaniche e alle cadute di meteoriti; tollerano temperature estreme, si sentono a loro agio nelle caldissime sorgenti sottomarine come pure nell’Antartide, all’interno delle rocce o sul panino che mangiate a pranzo e, in genere, hanno meno grattacapi degli uomini. Sono il prodotto finale perfetto, insomma.”

Frank Schätzing - Il mondo d’acqua (2009)

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Chapter 1

Summary of the thesis

Rubbers are elastomers, either of natural or non-natural origin. In 1839, Charles Goodyear discovered the vulcanization process in which the addition of sulfur to rubber could effectively improve properties. Total world rubber production has increased to 24.3 million tons in 2010, a rise of 11.9% from 21.7 million tons in 2009. Large amount of rubbers are used as tires. However, vulcanized polymeric materials do not recycle easily; disposal of waste polymers is a serious environmental problem. End life tires can be mainly recovered through two ways: the recovery of material and the recovery of energy. The calorific value of end life tires allows their use as a source of energy in paper mills, cement works or in thermoelectric power plants. Most of these recovery techniques involve the separation of metallic and texture materials and a grinding process leading to significant reduction of the tire dimensions. Furthermore, the ground tire rubber (GTR) is not suitable to be processed in new blends due to the presence of the sulfur crosslink network that prevents the full compatibility with raw rubber leading to weak adhesion and deterioration of the final properties of new blends. In order to increase the compatibility with the raw rubber, end life tires must be devulcanized by breaking the three-dimensional crosslink network. In order to selectively break the sulfur crosslink network, several chemical, thermo-mechanical, and physical methods have been studied. However, selectivity is low and none of them seems to produce high quality reclaimed rubber. In recent years, many researchers have focused on the desulfurization of ground rubber with microorganisms, which exhibit biological activity towards sulfur and can oxidizing sulfur to sulfate by selectively breaking the sulfur crosslinks in rubber.

The general aim of the project is to develop a rubber biodesulfurization process for the product re-use. In particular, the project was divided into two main parts: i) isolation, selection and characterization of novel bacterial strains with desulfurization ability on

molecule model (dibenzothiophene - DBT), ii) optimization of the desulfurization parameters through the design of experiments techniques.

So far, no attempts have been made to isolate bacterial strains from GTR, or environmental samples exposed to tires. Indeed, tires could represent a harsh environments for microorganism due to toxic chemical additives and our hypothesis is that this kind of samples could have selected bacteria with desired desulfurizing activity and able to survive in tire environment.

For this reason, in **chapter 2**, new desulfurizing bacterial strains were isolated from water, soil and mud collected from tire landfill and directly from GTR, and we set up enrichment cultures aiming at isolating desulfurizing microorganisms. The desulfurizing ability of isolates was tested on a molecule model, DBT. Two promising strains were isolated and they were taxonomically classified. These strains are *Rhodococcus* sp. AF21875 and *Bacillus* sp. 8A1531. Furthermore, the bacterial community on GTR and community desulfurizing potential were described using Illumina sequencing of the 16S rRNA and real time PCR (qPCR) on 16S rRNA and DBT sulfone monooxygenase (*dsz*A), respectively.

In **chapters 3 and 4**, *Rhodococcus* sp. AF21875 was further characterized. Indeed, growth of this strain was observed in presence of different sulfur sources. The maximum of growth was observed in presence of DBT as sole sulfur source. On the contrary, the growth in presence of inorganic sulfur is lower and comparable to the growth in absence of sulfur source. This could indicate that this strain uses DBT as preferential sulfur source. For this reason, *Rhodococcus* sp. AF21875 genome sequencing was carried out to obtain further information. The genome sequencing showed that *Rhodococcus* sp. AF21875 is close to *Rhodococcus erythropolis.* Indeed, scaffolds of chromosomal contigs were predicted using *Rhodococcus erythropolis* PR4 , *Rhodococcus erythropolis* CCM2595 and *Rhodococcus erythropolis* R138 as reference genomes. At the current state of the analysis, three chromosome scaffolds and four putative plasmid contigs were obtained.

In the *Rhodococcus* sp. AF21875 genome, genes were found for biosynthesis of the sulfur-containing amino acids cysteine and methionine. Furthermore, genes involved

in desulfurization processes were observed. For example, the *ssu* operon responsible for organsulfur compounds, *tau* operon and *dsz* genes responsible for dibenzothiophene utilization were found

In **chapter 5** desulfurizing ability of the isolated strains *Rhodococcus* sp. AF21875 and *Bacillus* sp. 8A1531 and the reference strain *Gordonia. desulfuricans* 213E were evaluated on polymeric molecule through two level full factorial experimental designs. The experimental design was set up on model vulcanized-ground natural rubber (VGNR) in order to facilitate the analyses. The variables considered were the concentration of glucose as a source of carbon, the presence of DBT as a model sulfur compound for the desulfurization pathway induction and the initial biomass concentration. Treated VGNR was then characterised by a frequency sweep test and the fingerprinting method ARISA (automated ribosomal inter-genic spacer analyses) were used for microbiological characterization to test the persistence of the bacterial inoculum during the treatment. Viscosity decrease is related with the increasing content of short polymer chains. Short chains derive from cleavage of S-S, C-S or C-C bonds. VGNR treated with *Bacillus* sp. 8A1531 showed a complex viscosity significantly lower compared with the control experiments. For *Rhodococcus* sp AF21875 a high experimental variability was observed.

A final reduced model was obtained for *G. desulfuricans* 213E. The best combination of significant parameters was high concentration of carbon source, absence of DBT and a high OD. In this work, we highlighted the importance of microbiological analyses to assess the persistence of inoculated strains and the dynamics of the microbial communities hosted on the polymers. Indeed, we showed that several bacteria naturally colonize the vulcanized natural rubber, resisted to thermal and chemical pre-treatment and grew during incubations.

In the **chapter 6**, *Gordonia desulfuricans* 213E, *Rhodococcus* sp AF21875 and *Bacillus* sp. 8A1531 were tested to desulfurize GTR at bioreactor scale. ARISA were conducted on samples to detect the persistence of the inoculated bacteria. Furthermore, the bacteria abundance on GTR was determined by the quantification of the number of copies of the 16S rRNA gene and the *dsz*A gene was quantified to observe the

desulfurization potential ability of bacteria on GTR. Also in this case, ARISA characterization showed that there is a naturally community hosted on GTR ant it survived to thermal treatment. For this reason it is important to evaluate the persistence of the inoculated bacteria to connect the effects on GTR to this strain. *G. desulfuricans* 213E persisted in the bioreactor and *dsz*A copy number quantification showed increase during the experiment. In the bioreactor inoculated with *Rhodococcus* sp. AF21875 a different behavior was observed. Probably, in this case the addition of the inoculum reinforced the community harbored on GTR. In this case about 50% of the bacteria showed the presence of this gene, thus showing higher desulfurization potential ability. In the bioreactor inoculated with *Bacillus* sp. 8A1531, it was not possible to confirm the persistence of the strain because the ARISA profile of the strain was very similar to the community profile of GTR.

Finally, in the **chapter 7** general conclusions and the future perspective were reported.

Chapter 2

Isolation of new bacterial strains with desulfurizing ability

2.1 Abstract

Tires at the end of their life are generally stockpiled and came in contact with soil and water. Due to their particular chemical composition they could create a peculiar selective environment for microorganisms. Being interested in polymer biodesulfurisation our hypothesis is that microorganisms with desulfurizing activity are naturally enriched on exhausted tires which can constitute a suitable material for isolation of desulfurizing strains. In this paper, we investigated the microbial community present on ground tire rubber (GTR) by Illumina sequencing of the 16S rRNA gene. Real time PCR (qPCR) was conducted on two genes to evaluate the bacteria abundance (16S rRNA) and the abundance of desulfurizing bacteria (dibenzothiophene sulfone monooxygenase - *dsz*A) on GTR. Water, soil and mud were also collected from a tire landfill and GTR was used to set up enrichment cultures and to isolate desulfurizing bacteria. The microbial community biodiversity on GTR is low and the 55,8% is composed by *Rhodococcus*., Furthermore, the 17% of bacteria on GTR harbor the *dsz*A gene. From the enrichment cultures, 135 bacteria were isolated and screened for desulfurizing activity by Gibb's assay, resulting in 51 strains able to use dibenzothiophene (DBT) as sulfur source.

2.2 Introduction

In the last decades many studies were conducted on biodesulfurization (BDS). BDS is a biological process where sulfur is removed by microorganisms and/or enzymes from organic molecules. The majority of studies on BDS were focused on fuel as an alternative to hydrodesulfurization (HDS). Sulfur removal from fuels is important because sulfur dioxide is a waste product and in atmosphere causes environmental problem such as acid rain (Aminsefat et al 2012). Desulfurization should be carried out

without the degradation of the carbon molecular structure to preserve fuel calorific value. In the HDS process molecules such as dibenzothiophene (DBT) and alkyl substituted DBT are highly recalcitrant. For this reason, DBT is used as model molecule. Many studies aimed at finding new bacterial strains with DBT desulfurizing ability and at improving the fuel biodesulfurization (Davoodi-Dehaghani et al 2010, Aminsefat et al 2012). In literature, two different aerobic pathways are described for DBT biodesulfurization: i) destructive process or Kodama desulfurization, ii) specific oxidative BDS or 4S pathway (Srivastava 2012). Kodama pathway is considered a destructive process due to the cleavage of carbon-carbon bonds in benzene ring. Conversely, in the 4S pathway, the enzymes catalyze the oxidative C-S bond cleavage, without break the carbon skeleton.

Four enzymes are involved in 4S pathway: i) DBT monooxygenase (DszC), ii) DBT sulfone monooxygenase (DszA), iii) hydroxyphenyl benzene sulphonate desulfinase (DszB) and iv) flavin reductase (DszD) (Davoodi-Dehaghani et al 2010). This pathway was described for the first time in *Rhodococcus rhodochrous* IGTS8 (ATCC® 53968™) (Oldfield et al 1997). In IGTS8, *dsz* genes are located on a stable plasmid. In the following years many bacteria were isolated with this particular ability, for example *Microbacterium* sp. ZD-M2 (Li et al 2005), *Rhodococcus* sp Eu-32 (Akhtar et al 2009), *Stenotrophomonas* sp. NISOC-04 (Papizadeh et al 2011).

Until now desulfurizing bacteria have been isolated by different approaches. For example different environmental samples, like soil from the roots of a eucalyptus tree, were incubated in a medium with diesel or DBT as sulfur source (Akhtar et al 2009, Izumi et al 1994). In other cases, this kind of bacteria was isolated starting from contaminated samples with an elevated chemical stress, for example wastewater of dyeing industry (Rhee et al 1998, Chang et al 1998) or sludge sampled near mining areas (Chen et al 2008). However, in the majority of the works oil contaminated soil (Davoodi-Dehaghani et al 2010) or wastewater and sludge collected from petroleum refinery (Bhatia et al 2010) were used to isolate desulfurizing bacteria.

BDS could be not useful only to remove sulfur from fuels, but in the last year desulfurization process has been proposed for rubber devulcanization (Yao et al 2013, Li et al 2012b and Li et al 2011).

Vulcanization process is used to produce tires for improving mechanical proprieties owing to sulfur addition. Vulcanization makes the recycling more difficult and desulfurization is necessary to reuse the rubber. Microbial desulfurization represents an environmental friendly alternative to other methods that requires hazardous chemicals or energy-consuming operation conditions. In this process microorganisms with the ability to cleave the sulfur crosslink present in the vulcanized rubber are involved.

Recently, Li et al (2012a) used *Sphingomonas* sp. for ground tire rubber desulfurization. The strain showed biological activity on ground tire rubber and the process was improved with the surfactant addition in the medium to increase the affinity between bacteria and rubber (Hu et al 2014). Several studies were conducted with different chemolithotrophs able to use sulfur as source of energy*: Thiobacillus ferrooxidans, Thiobacillus thiooxidans, Thiobacillus thioparus*. In particular, *T. ferroxidans* (Jiang et al 2010) showed desulfurization ability without breaking the carbon chain and crosslink were oxidized to form sulfoxide and sulfone group. Previously, Romine and Romine (1998) evaluated the desulfurization process on ground tire rubber with *Sulfolobus acidocaldarius.* A pathway with sulfoxide/sulfone/sulfonate/sulfate as intermediates was proposed to metabolize the crosslink by authors with a mechanism is similar to “4S pathway”. A patent was written on devulcanization rubber by Christofi et al (2010). Authors used *Gordonia desulfuricans* 213E (NCIMB 40816) to treat ground tire (GTR). The sulfur quantity in the rubber decrease between 23% and 35%. Before the treatment benzothiophene (BTH) was added to the culture medium.

Tire is produced to last long, indeed it is infusible and resistant to the abrasion. For these reasons end of life tires were stockpile in landfill. In this way, tires enter the environment. The degradation is long and during this period the tires could became a selective environmental for bacteria thanks to the particular chemical tires

composition. Indeed, tires could represent a harsh environments for microorganism due to toxic chemical additives and our hypothesis is that this kind of samples could have selected bacteria with desired desulfurizing activity and able to survive in tire environment. After that, tire grinding and the storage of GTR could operate another section.

To date, all the strains tested for desulfurizing ability towards rubber were isolated from soil contaminated by oil shale (Kim et al 1999), soil of an iron mine (Yao et al 2013, Li et al 2011) or from soil of a coal mine (Li et al 2012a, Li et al 2012b). However, there is no evidence that devulcanization process involves the same pathway of DBT biodesulfurization and that tire constitutes a selective environmental. In literature, only *G. desulfuricans* 213E was isolated from soil contaminated with oil shale and it was able desulfurize BTH and also GTR. Indeed, tires could represent a harsh environments for microorganism due to toxic chemical additives and our hypothesis is that this kind of samples could have selected bacteria with desired desulfurizing activity and able to survive tire environment.

In this paper, the microbial community on GTR was described and desulfurizing bacteria were isolated and characterized from GTR and environmental samples collected from a tire landfill. In this way, we assessed whether these environments could have selected different populations compared with those reported in literature. In this work, the microbial community on GTR was described by Illumina sequencing of the 16S rRNA gene.

Furthermore, the qPCR was conducted on two genes: i) 16 rRNA to evaluate the abundance of bacteria on GTR and ii) DBT sulfone monooxygenase (*dsz*A) to estimate the potential desulfurizing activity on GTR. We also isolated bacterial strains from water, soil and mud collected from the tire landfill, from wastewater of a tire factory and directly from GTR. To reach this aim we developed and validated a specific HPLC method with fluorescence detection to quantify the concentration of 2 - hydroxybiphenyl (HBP) in order to evaluate the biodesulfurization capacity of isolates.

2.3. Materials and Methods

2.3.1 Amplification of the 16S rRNA gene, sequencing and sequence analyses

Total bacterial DNA was extracted using the FastDNA Spin for Soil kit (MP Biomedicals, Solon, OH, USA) from GTR sample according to the manufacturer's instructions. The V5-V6 hypervariable regions of the 16S rRNA gene were PCR-amplified and sequenced by MiSeq Illumina (Illumina, Inc., San Diego, CA, USA) using a 250 bp x 2 paired-end protocol. The multiplexed libraries were prepared using a dual PCR amplification protocol. The first PCR was performed in 3 x 75 µL volume reactions with GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA) and 1 µM of each primer. 783F and 1046R primers were used (Huber et al. 2007; Wang & Qian 2009) and the cycling conditions were: initial denaturation at 98°C for 30 s; 20 cycles at 98°C for 10 s, 47°C for 30 s, and 72°C for 5 s and a final extension at 72°C for 2 min. The second PCR was performed in 3 x 50 µL volume reactions by using 23 µL of the purified amplicons (Wizard® SV Gel and PCR Clean-up System, Promega Corporation, Madison, WI, USA) from the first step as template and 0.2 µM of each primer. Primers sequences contained the standard Nextera indexes (Illumina, Inc., San Diego, CA, USA) and the cycling conditions were: initial denaturation at 98°C for 30 s; 15 cycles at 98°C for 10 s, 62°C for 30 s, and 72°C for 6 s and a final extension at 72°C for 2 min. DNA quantity after the amplification was evaluated using Qubit® (Life Technologies, Carlsbad, CA, USA). The sequencing was carried out at the Parco Tecnologico Padano (Lodi, Italy). Each sequence was assigned to its original sample according to its barcode. Uparse pipeline was used for the following elaborations (Edgar 2013). Forward and reverse reads were merged with perfect overlapping and quality filtered with default parameters. Singletons sequences (i.e. sequence appearing only one time) were removed. Operational Taxonomic Units (OTUs) were defined clustering the sequences at a 97% of similarity and defining a representative sequence for each cluster. A subset of 750 random sequences was chosen from each sample and the abundance of each OTU was estimated by mapping the sequences of the sample against the representative sequence of each OTU at 97% of similarity. Taxonomic

classification at different ranks of the OTUs' representative sequences was obtained by RDP classifier (Cole et al., 2001).

2.3.2 16SrRNA gene and dibenzothiophene sulfone monooxygenase (*dsz*A) gene quantification

The bacteria abundance on GTR was determined by the quantification of the number of copies of the 16S rRNA gene. These number of copies are not directly associated to cell number due to the presence of multiple ribosomal operons in the bacterial genomes; however, they can be used to detect at relative shift in microbial biomass that is a likely constant bias in the number of copies (Bertolini et al. 2013). 466-bp fragment of the bacterial 16S rDNA (331–797 according to *Escherichia coli* position) was PCR-amplified with a universal primer set (Nadkarni et al. 2002). The PCR was performed in a total volume of 10 µL using the FluoCycleII Sybr reaction mix (Euroclone, Pero, Italy) with 0.3 µM (final concentration) of forward and reverse primers. The amplification was carried out under the following conditions: 95 °C for 4 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s, with acquisition of the fluorescence at the end of each 72 °C elongation step.

*dsz*A gene was quantified to observe the desulfurization potential ability of bacteria on GTR. This gene was involved in the DBT desulfurization (Alves et al 2007). Although, there is no evidence that *d*szA is involved in GTR desulfurization. PCR mix was prepared as previously described for the 16S rRNA gene and the amplification was performed under the following conditions: 95 °C for 4 min, followed by 40 cycles at 95 °C for 15 s, 65 °C for 30 s and 72 °C for 45 s, with acquisition of the fluorescence at the end of each 72 °C elongation step. *dsz*A primers are reported in the table 3. Fragments of interest were amplified from reference strains (*E. coli* K-12 substr. DH10B for 16S RNA gene and *Rhodococcus rhodochrous* IGTS8- ATCC 53968 for *dszA*) and cloned into the plasmid pCR2.1 (Life Technologies Italia, Monza, Italy) in order to prepare standards for calibration curves. The concentration of plasmidic DNA was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Serial

dilutions of the plasmidic DNA were included in triplicate in each run together with the samples.

2.3.3 Materials

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

Dibenzotiophene (DBT, assay 99 %), 2 -hydroxybiphenyl (HBP, assay 99 %) and the other chemicals including constituents of mineral salt medium (MSM) were purchased from Sigma Aldrich (Germany). The acetonitrile, water, ethyl acetate (HPLC grade) were obtained from Panreac (Spain). Tryptone, agar, yeast extracts and were obtained from Biolife (Italy).

2.3.4 Preparation of media

Sulfur free MSM (pH 7.0) (Chang et al 1998) was prepared by 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 $CaCl_2*2H_2O$, 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*½Ca 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_2PO4 0,2 g/L, NaCl 8,0 g/L, Na2HPO4 1,15 g/L), and 1 mL of sulfur-free trace metal solution (5 mg/L $MnCl_2$ $*6H_2O$, 0.5 mg/L H_3BO_3; 0.5 mg/L $ZnCl_2$; 0.5 mg/L $CoCl_2$ $*6H_2O$; , 0.46 mg/L $NiCl_2$ * $6H_2O$; 0.3 mg/L $CuCl_2$;, 0.1 mg/L $NaMoO_4$ $*2H_2O$; 1.49 mg/l $FeCl_2*4H_2O$, 0.003 mg/l $NaSeO_3$ and 0.008 mg/l Na_2WO) (Widdel et al., 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. LD solid media contained 1.5% agar.

2.3.5 Enrichment and isolation of DBT desulfurizing bacteria

DBT desulfurizing bacteria were enriched and isolated from different sample. Ten samples were collected from tire landfill in Italy (Ferrara), four samples were mud, five were soil and one were water. Two samples were collected from a tire factory. In particular wastewater of the factory was sampled in two different points. The enrichment cultures were set up in twice with these samples. Four enrichment cultures were set up with GTR. In total, twenty-four enrichment cultures were set up. One gram of soil, GTR or one milliliter of mud or water samples were suspended in 19 ml MSM. MSM used for enrichment of desulfurizing bacteria was supplemented with DBT as the sole source of sulfur. DBT was dissolved in ethanol (100 mM equal to 18426 ppm) and added to a sterilized MSM. Enrichment cultures were carried out in MSM containing 1 mM (equal to 184,26 ppm) DBT. Cultures were incubated at 30 °C for 7-10 days under shaking condition. Aliquot (1 ml) of turbid cultures was transferred into fresh 19 ml of MSM medium with DBT. After ten such sub-cultivation cultures were appropriately diluted, they have been plated onto LD agar plates and incubated at 30*C overnight. The individual colonies were picked up and isolates on the base of shape and color and further screened for their ability to grow on DBT.

2.3.6 Gibb's assay

Single colony of each isolate was inoculated in 20 mL LD and grown for 24 hours at 30°C with constant shaking. Aliquot (1 ml) of turbid cultures were transferred into fresh 19 ml of LD and grown overnight at the same condition above described. The cell suspensions were centrifuged at 5000 rpm for 10 min at 4°C to obtain cell pellets of each soil isolate. The cell pellets were washed with MSM to remove the trace of nutrient broth and centrifuged again. The cell pellets were suspended in MSM. The desulfurization ability of each soil isolate was checked by inoculating cell suspension with an optical densities equal to 0,1 into 20 mL of MSM supplemented with DBT (184,26 ppm) as the sole source of sulfur. The cultures were incubated at 30°C for 7 days under shaking condition. Two control experiments were also setup, one whit

Rhodococcus erythropolis IGTS8 (ATCC 53968), this strain is known for his DBT desulfurization ability (positive control) and the second one without bacteria (abiotic control). After 7 days of growth Gibb's assay was performed (Aminsefat et al 2012).

Gibb's assay was useful to find bacteria that can selectively remove sulfur from DBT and its derivatives. The test was used to detect and quantify 2-hydroxybiphenyl produced by the strain after incubation with DBT. 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 5 mL of 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 of 2,6-dichloroquinone-4-chloroimide dissolved in 1 mL of ethanol) was added to each sample and the reaction mixture was incubated at 30°C for 30 min. To separate the bacterial cells, the reaction mixture was centrifuged (4000 rpm, 4°C, 20 min) and the absorbance of the supernatant was measured at 610 nm (Beckman DU-640 Spectrophotometer). The standard curve was created using 2-hydroxybiphenyl versus Gibb's assay results.

2.3.7 Identification of isolates

Identification of strain, with DBT desulfurizing capability, was made by PCR amplification and sequencing of 16S rRNA gene. DNA was extracted from bacterial suspensions (Invisorb Genomic DNA kit III) and 16S rRNA gene fragments were PCR-amplified with the primers 27F (5'-AGAGTTTGATCCTGGCTAG-3') and 1492R (5'-CTACGGCTACCTTGTTACGA-3)'. PCR was performed in 80 µL reactions containing 20 µL of cellular suspension or 4 µL extracting 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 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 gene was 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-BLAST) to obtain the strain classification.

2.3.8 HBP quantitative analyses

For HBP quantitative analyses, the 10 mL of each culture medium was pH 2.0 acidified 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. All the experiments were set up in triplicates.

Stock solutions containing DBT/HBP ethyl acetate were prepared and stored at 4 °C. These solutions (1000 ppm for DBT and 100 ppm for HBP, respectively) were used to evaluate the interference of the two compounds, to prepare quality control (QC-S) and to prepare calibration curves (in the range between 0.005 ppm and 1.4 ppm). The QC-S were prepared adding only DBT and HBP to the culture medium at three different concentrations: 368.52 ppm, 184.26ppm and 92.13ppm for DBT and 17 ppm, 8.5 ppm and 4.3 ppm for HBP and they were especially used to evaluate the HBP recovery.

The HPLC system was an Agilent 1100 (Agilent Technologies, USA) equipped with a RP-C18 5-µ.m particle size column (150*4.6 mm, Vydac 201, Vydac, USA). Fluorimeter detector was used for the quantification of the bio-product. The DAD, coupled with the fluorimetry, was employed to determine the maximum excitation and emission wavelength for HBP. The isocratic mobile phase was water:acetonirile (50:50 v/v). The overall HPLC conditions and the optimal fluorescence wavelengths are summarized in table 1.

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

Table 1 Parameters of HPLC running

Parameter	Signal	concentration
LOD	0.52	0.001 ppm
LOQ	1.13	0.002 ppm

Table 2 LOD and LOQ values

Moreover, detection limit (LOD) and the limit of quantification (LOQ) were evaluated for the HBP as follow:

$$LOD = \ddot{S}_b + 3\sigma$$
$$LOQ = \ddot{S}_b + 10\sigma$$

Six repetitions of a blank sample were carried out without introducing the HBP.

$\hat{S}_b$ represents the average signal and sigma represents the standard error. These signals were used to calculate the limit concentrations.

2.3.9 Amplification of *dsz*A, *dsz*B, and *dsz*C genes

The plasmidic DNA (Pure Yeld Plasmid Miniprep System – Promega Corporation, USA) and the genomic DNA (Invisorb Genomic DNA Kit III – Invitek, Germany) were used as PCR template. The used PCR primers are reported in table 3 (Duarte et al. 2001).

PCR was performed in 80 µL reactions containing 4 µL extracting 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 (2001). Plasmidic DNA from *Rhodococcus 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 BLAST.

Gene	Primer	Sequence	Fragmente lenght (bp)
dszA	Forward	5'-TCG ATC AGT TGT CAG GGG -3'	547
	Reverse	5'-GGA TGG ACC AGA CTG TTG AG-3 '	
dszB	Forward	5'-ATC GAA CTC GAC GTC CTC AG-3'	422
	Reverse	5'-GGA ACA TCG ACA CCA GGA CT-3'	
dszC	Forward	5'-CTG TTC GGA TAC CAC CTC AC-3'	392
	Reverse	5'-ACG TTG TGG AAG TCC GTG-3'	

Table 3 Sequences of *dsz*ABC primers (Duarte et al. 2001)

2.4 Results

2.4.1 GTR bacterial community characterization

The GTR analyzed in this study was obtained by the pulverization at cryogenic temperature of whole truck tires. It had dimensions smaller than 0.4 mm (0,4-0,15 mm) (Mangili et al 2014). In the figure 1, the GTR surface is shown. The surface resulted smooth, this is due to the use of nitrogen steam hindered used during the milling process. Nitrogen steam hindered prevented a temperature increase, the degradation and oxidation of the sample.

The classification of the sequences was performed at the genus level to describe the community on GTR (Fig. 2). Only nine genera were retrieved: *Rhodococcus, Bacillus, Pseudomonas, Aeribacillus, Brevundimonas, Streptophyta, Stenotrophomonas, Paenibacillus, Fontibacillus.*

The 83,9% of bacteria belonged to Gram positive taxa. This group of bacteria was represented by two phyla, *Actinobacteria* and *Firmicutes.*

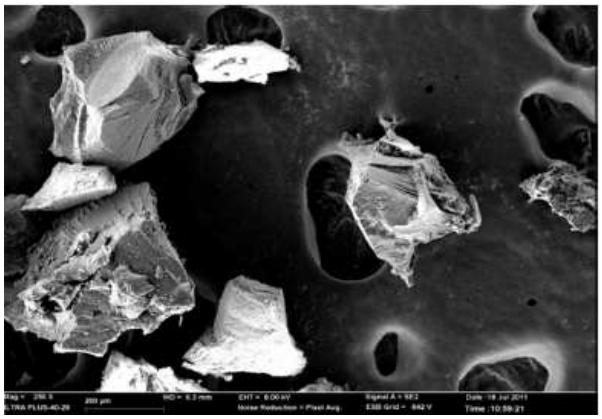

Figure 1 SEM picture of GTR particles (Mangili et al 2014)

Rhodococcus was the only genus belonging to *Actinobateria* and represented the 55,8% of the community. Among *Firmicutes* the following genera were represented: *Bacillus, Aeribacillus, Paenibacillus* and *Fontibcillus.* Leftover 15,1% of bacteria belong to Gram Negative taxa. *α-Proteobacteria* and *γ-Proteobacteria* were observed. Whitin *α-Proteobacteria* it was observed the genera *Pseudomonas* (9,9%) and *Stenotrophomas* (1%). *γ-Proteobacteria* was represented only one genera*: Brevundimonas* (4%). Finally, 1% of bacteria belonged to *Cyanobacteria.*

The number of copies of the 16S rRNA and *dsz*A genes were quantified. The copy number of ribosomal operons per g on GTR was 7,40(± 0,39)E+05 whereas the copy number of *dsz*A per g 1,27(± 0,54)E+05 (Fig. 3).

The *dsz*A gene is involved in the 4S pathway. This pathway is used by different microorganisms to desulfurize DBT to 2-hydroxybiphenyl without damage the carbon chain. Although, no evidence was reported in the literature of the involvement of this pathway in the desulfurization of GTR. However the presence of this gene may indicate a potential desulfurizing activity of community on GTR.

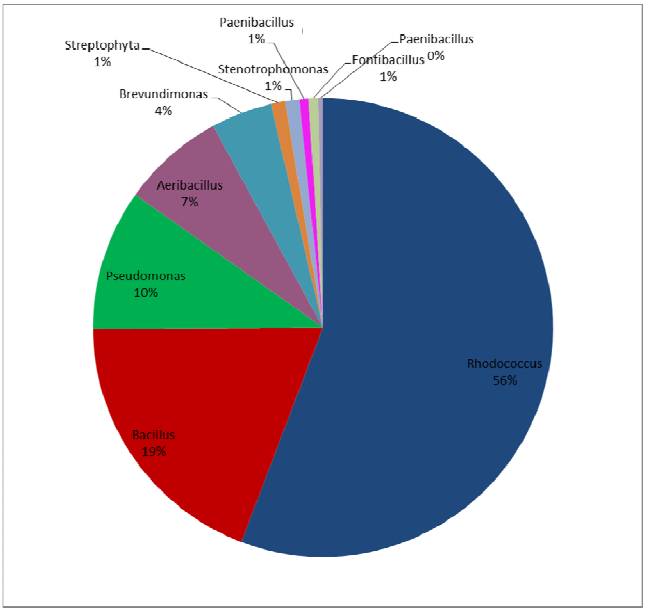

Figure 2 Taxonomic composition at genus level of the microbial communities on GTR

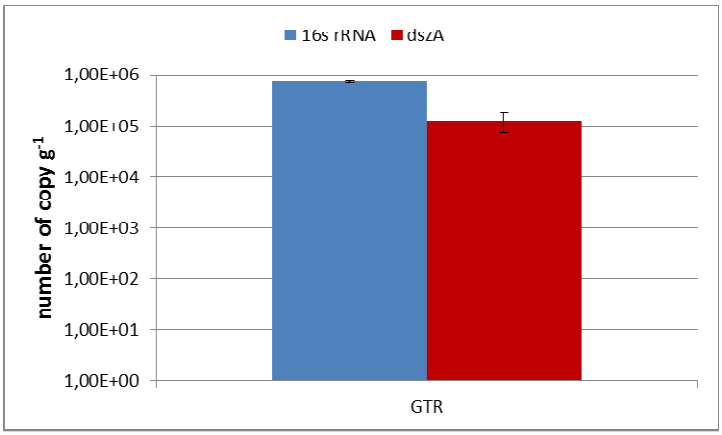

Figure 3 Number of copies per µL/g of GTR of the 16S rRNA gene and *dsz*A on GTR .

2.4.2 Isolation and identification of strains

Five samples of soil, three of water, four of mud samples and GTR were collected to isolate DBT desulfurizing microorganisms. After ten rounds of sub-cultivation, 135 pure strains were isolated on LD agar. After that, strains were screened for the desulfurizing ability. Gibb's assay was performed to select DBT desulfurizing strains. Isolates were inoculated into sulfur free MSM medium with DBT as sulfur source, and Gibb's assay were carried out after seven days of growth. Strains growth (OD600) was

measured (data not shown). The growth was observed for 84 isolates, but only 50 isolates were positives to Gibb's assay and able to desulfurize DBT.

After that, positive strains were taxonomically classified. The strains with desulfurization ability belonged to three different phyla: *Actinobacteria* (22 strains), *Firmicues* (25 strains), and *Proteobacteria* (3 strains). *Proteobactera* were represented only by three strains belonging to three different families: *Acetobacteraceae, Xanthomonadaceae, Caulobacteraceae*. Among Firmicutes, 21 isolates were *Bacillales* and 4 were *Lactobacillale*. The following genera were represented: *Bacillus*, *Fontibacillus*, *Paenibacillus*, *Staphylococcus*, and *Lactobacillus*. Among *Actinobacteria* both *Corynebacterineae* and *Micrococcineae* were isolated belonging to *Rhodococcus (*7 isolates*)* and *Microbacterium, (*15 isolates*)* genus.

The Gibb's assay and the taxonomic affiliation results were evaluated to choose the strains to be further investigated. In the choice of the strains the conservation of the maximum biodiversity among the stains was particularly considered along with the desulfurizing abilities. We chose seven strains for the following analyses and they are reported in table 5. In the table 5, the classification and the amount of HBP product calculated through Gibb's assay from each strain are also reported.

2.4.3 Reference strains

In this work, two reference strains were used, *R. rhodochrous* IGTS8 and *G. desulfuricans* 213E. They were selected for their known desulfurization ability (Oldfield et al 1997, Christofi et al 2010). *R. rhodochrous* IGTS8 was the first bacteria characterized with this ability. On the other hand, *G. desulfuricans* 213E was described as a strain able to desulfurize BTH but the authors reported that this strain does not grow on DBT as sulfur source (Kim et al 1999). On the contrary, we observed growth in presence of DBT as sulfur source. To confirm this ability, plasmidic DNA was extracted and *dsz* genes were amplified and sequenced (Fig. 4). BLAST results confirmed that both the reference strains harbor *dsz* gene on plasmid (Tab.4) while HPLC data showed that HBP production was equal to 15 ppm and 9,9 ppm for *R. rhodochrous* IGTS8 and *G. desulfuricans* 213E, respectively (Tab. 5).

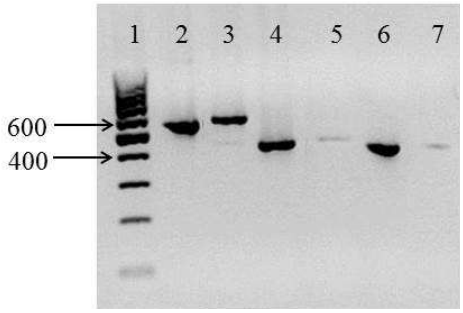

Figure 4. PCR of *dsz* genes in *Rhodococcus rhodochrous* IGTS8 and *G. desulfuricans* 213E. Lanes: 1 Marker, 2 *Rhodococcus rhodochrous* IGTS8 *dsz*A, 3 *G. desulfuricans* 213E *dsz*A, 4 *Rhodococcus rhodochrous* IGTS8 *dsz*B, 5 *G. desulfuricans* 213E *dszB,* 6 *Rhodococcus rhodochrous* IGTS8 *dsz*C, 7 *G. desulfuricans* 213E *dszC*.

Strain	Gene	ACCESSION NUMBER	DESCRIPTION	MAX IDENT
Gordonia desulfuricans 213E	dszA	gi|148470338|EF570781.1	Synthetic construct dibenzothiophene sulfone monooxygenase (dszA) gene, complete cds	99%
	dszB	gi|148470340|EF570782.1	Synthetic construct aromatic desulfinase (dszB) gene, complete cds	99%
	dszC	gi|170773988|EU527978.1	Mycobacterium goodii strain X7B dibenzothiophene monooxygenase (dszC) gene, complete cds	98%

Table 4.Analyzis of *dsz*ABC with BLAST

2.4.4 DBT desulfurization by isolates

To confirm the Gibb's assay results, we performed HPLC analyses. All the 7 strains grew on DBT (184,26 ppm) as sole sulfur source. Detectable HBP production was observed in *Microbacterium* sp. S2285D, *Rhdococcus* sp. AF21875, *Paenibacillus* sp. 5A304E, *Paenibacillus* sp. P2AT5 and *Paenibacillus* sp. 9A194C and *Bacillus* sp. 8A1531 (Tab. 6). On the contrary, the strain *Paenibacillus* sp. 10B1551 did not show HBP production (Tab. 6). *Microbacterium* sp. S2285D produced 15 ppm of HBP (Tab. 6).

This was the highest value of HBP production, but the standard deviation was high. The production of HPB by *Rhodococcus* sp. AF21875 was comparable with the HBP production by *G. desulfuricans* 213E. This strain produced 9 ppm of HBP (Tab. 6). *Paenibacillus* sp. 5A304E, *Paenibacillus* sp. P2AT5, *Bacillus* sp. 8A1531 and *Paenibacillus* sp. 9A194C produced less HBP, respectively 7 ppm, 8 ppm, 4 ppm and 3 ppm (Tab. 6). This analysis showed that *Rhodococcus* sp. AF21875 is a promising strain. The presence of *dsz*ABC were also investigated. The strain *Microbacterium* sp. S2285D did not show the presence of the three genes. On the other and the strains *Paenibacillus* sp. 10B1551, *Paenibacillus* sp. 5A304E and *Paenibacillus* sp. P2AT5 showed only the *dsz*A gene (Fig. 5). On the contrary, the strains *Bacillus* sp. 8A1531, *Paenibacillus* sp. 9A194C and *Rhodococcus* sp. AF21875 harbored all the genes for the desulfurization of DBT (Fig. 6 and Fig. 7).

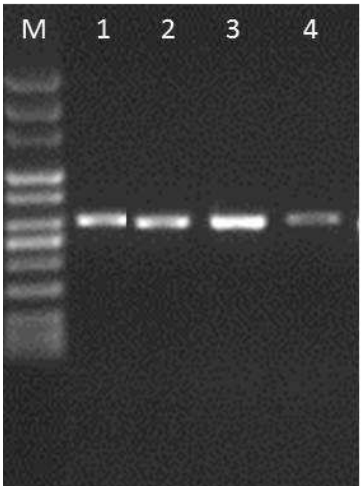

Figure 5 PCR of *dsz*A gene in *Bacillus* sp. 8A1531, *Paenibacillus* sp. 10B1551, *Paenibacillus* sp. 5A304E and *Paenibacillus* sp. P2AT5. Lanes: M Marker, 1 *Bacillus* sp. 8A1531 *dsz*A, 2 *Paenibacillus* sp. 10B1551 *dsz*A, 3 *Paenibacillus* sp. 5A304E *dszA*, 4 *Paenibacillus* sp. P2AT5 dszA

		BLAST			RDP		
Strains	Gibb's Results (ppm)	Description	Accession Number	Max Ident	Genus	confidence	Sample of origin
Rhodococcus rhodochrous IGTS8	15	*Rhodococcus* sp. IGTS8 16S ribosomal RNA, 23S ribosomal RNA, and 5S ribosomal RNA genes, complete sequence	gb|AF001265.1|	98%	*Rhodococcus*	100%	soil
Gordonia desulfuricans 213E	7	*Gordonia desulfuricans* 213E 16S ribosomal RNA gene, partial sequence	gi|265678432|NR_028734.1	99%	*Gordonia*	99%	soil contaminated with oil shale
S2285D	32	*Microbacterium hydrocarbonoxydans* strain : DSM 16089 16S ribosomal RNA, partial sequence	gi|343201537|NR_042263.1	95%	*Microbacterium*	46%	Soil of tire landilfill
AF21875	18	*Rhodococcus qingshengii* strain djl-6 16S ribosomal RNA, partial sequence	gi|343203016|NR_043535.1	99%	*Rhodococcus*	100%	wastewater of tire factory
5A304E	10	*Paenibacillus favisporus* strain GMP01 16S ribosomal RNA, complete sequence	gi|265678766|NR_029071.1	99%	*Paenibacillus*	100%	Soil of tire landilfill
8A1531	12	Bacillus circulans 16S ribosomaL	gi|343202436|NR_042726.1	92%	*Bacillus*	84%	Soil of tire landilfill
P2AT5	15	*Paenibacillus wynnii* strain : LMG 22176 16S ribosomal RNA, partial sequence	gi|343201518|NR_042244.1	98%	*Paenibacillus*	54%	GTR
9A194C	17	*Paenibacillus xylanexedens* strain B22a 16S ribosomal RNA, partial sequence	gi|343199088|NR_044524.1	96%	*Paenibacillus*	100%	water of tire landfill
10B1551	11	*Paenibacillus cineris* strain :LMG 18439 16S ribosomal RNA, partial sequence	gi|343201463|NR_042189.1	99%	*Paenibacillus*	99%	Soil of tire landfill

Table 5 Results of sequencing on selected strains and sample of origin

Strain	Repetition	HBP (ppm)	Error	HBP average (ppm)	Dev. Std.	St. Error
Rhodococcus rhodochrous IGTS8	A	11	1	15	3	2
	B	17	2			
	C	17	2			
Gordonia desulfuricans 213E	A	8,7	0,9	9,9	1	0,7
	B	10	1			
	C	11	1			
Microbacterium sp. S2285D	A	25	3	15	11	6
	B	16	2			
	C	3,4	0,3			
Rhodococcus sp. AF21875	A	12	1	9	4	2
	B	5,4	0,6			
	C	11	1			
Paenibacillus sp. 5A304E	A	5,1	0,5	7	2	1
	B	8,7	0,9			
	C	6,8	0,7			
Paenibacillus sp. P2AT5	A	5,2	0,5	8	3	2
	B	11	1			
	C	8,5	0,9			
Bacillus sp. 8A1531	A	0,56	0,06	4	6	4
	B	11	1			
	C	0,2	0,02			
Paenibacillus sp. 9A194C	A	-†	-	3	4	2
	B	2	0,3			
	C	7	1			
Paenibacillus sp. 10B1551	A	-†	-	-	-	-
	B	-†	-			
	C	-†	-			

† Below the quantification limit

Table 6 HBP production of selected strains

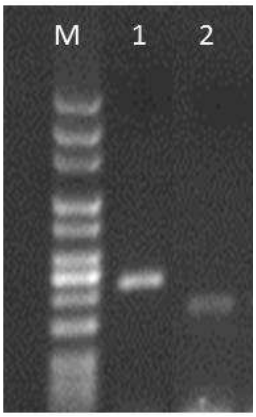

Figure 6 PCR of *dsz*B and *dsz*C gene in *Bacillus* sp. 8A1531. Lanes: M Marker, 1 *Bacillus* sp. 8A1531 *dsz*B, 2 *Bacillus* sp. 8A1531 dszC

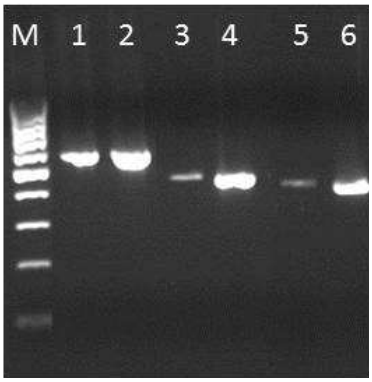

Figure 7. PCR of *dsz* genes in *Paenibacillus* sp. 9A194C and *Rhodococcus* sp. AF21875. Lanes: M Marker, 1 in *Paenibacillus* sp. 9A194C *dsz*A, 2 *Rhodococcus* sp. AF21875 *dsz*A, 3 in *Paenibacillus* sp. 9A194C *dsz*B, 4 *Rhodococcus* sp. AF21875 *dszB,* 5 in *Paenibacillus* sp. 9A194C *dsz*C, 7 *Rhodococcus* sp. AF21875 *dszC*

2.5. Discussion

In the last years, the biodesulfurization process has gained importance because both sulfur content in fuels regulations and the tire disposal in landfill regulations became more stringent. For these reasons is important to identify new desulfurizing strains. In particular, it is necessary to isolate bacterial strains unable to break C-C bond during the biodesulfurization process. In literature, 4S path was described. Through this pathway the carbon- sulfur bond was break without damage the carbon chain. Various

strains with this ability were isolated in the last years (Davoodi-Dehaghani et al 2010, Papizadeh et al 2011, Bahuguna et al 2011).

In the first part of the work, the GTR microbial community was characterized using by Illumina sequencing of the 16S rRNA.

To obtain GTR, tires underwent several processes. At the end of the life the tires was stocked in a landfill. In general, to recycle the tire the most used technique is the pulverization of tire. During this process, the metallic and texture materials were separated and the grinding was conducted only on the rubber part to reduce the material dimensions. The grinding process was carried out at cryogenic temperature. In this way, the main chains were not broken from high temperature could reach during the process. Finally, the GTR was stored before the following use. The genus *Rhodococcus* was the most abundant genus on GTR following by the genus *Bacillus*, together they composed the 75% of the bacterial community. However, a high number of bacteria was found on GTR through the qPCR analysis on 16 rRNA gene, but the most interesting result was the high percentage of the *dsz*A gene present on GTR. The biodesulfurization ability was described in many in Gram positive bacteria (Papizadeh et al 2011) and in particular in bacteria belong to mycolata. Results suggest that the exhausted tires are a selective environment, where few dominant populations were found and in particular the environment seems to favor the genus of *Rhodococcus*. Especially, the results seem to highlight the selection of bacteria with desulfurize ability. This could be related to the type of sulfur source. Indeed, the sulfur crosslink within tire could lead up to the selection of bacteria with desulfurizing ability considering the high quantity of this kind of sulfur source.

However, bacteria able to degrade natural and synthetic rubber were isolated (Shah et al 2013). This capability is not widespread among bacteria and seems to be limited to Gram positive actinomycetes (Jendrossek et al 1997). In particular, actinomycetes were resulted almost the only microorganisms able to biodegrade the natural rubber and to use the rubber hydrocarbon as a carbon source (Shah et al 2013). This kind of bacteria grew direct in contact with the rubber. Several strain of *Gordonia polyisoprenivorans* were isolated both from soil of a rubber tree plantation and from

fouled water inside a decayed automobile tire (Linos et al 1998 and Linos et al 1999). The *G. polyisoprenivorans* strains are deemed to be the most potent rubber-degrading bacteria (Rose et al. 2005). However other mycolic acid-containing *Actinobacteria* were found with ability as *Mycobacterium*, *Nocardia* and *Corynebacterium* (Arenskotter et al., 2001, Arenskotter et al. 2004; Shah et al 2013). Despite, none strains belong to the genus *Rhodococcus* was described in literature with this ability, we cannot exclude that the high presence of this genus was due to the presence of rubber hydrocarbon as a carbon source.

In literature few bacteria were described with desulfurizing ability and they are *Sphingomonas* sp (Hu et al 2014), *G. desulfuricans* 213E (Christofi et al 2010), *Alicyclobacillus* sp. *Thiobacillus ferrooxidans, Thiobacillus thiooxidans, Thiobacillus thioparus* (Christiansson et al 1998) and two *Archea, Sulfolobus acidocaldarius* (Romine and Romine. 1998) and *Pyrococcus furiosus* (Bredberg, et al 2001). Few genera were described and it is not possible to assess if the tire desulfurizing ability is widespread or restrict to few kind of microorganisms. However, it is interesting that none of genera described in literature within desulfurizing tire process were found in the microbial community of GRT.

In the second part of the work, we isolated 135 strains from different environmental samples. All this strains were tested for the desulfurizing ability. 84 strains grew in presence on DBT but only 51 isolates were positives to Gibb's assay and able to desulfurize DBT. This indicates that the only growth in presence of DBT is not sufficient to assert that the strains have desulfurizing ability. Indeed, Van Hamme (2004) reported that some strains were able to produce biomass on the trace sulfur found in medium components. Gibb's assay was a suitable tool for the initial screening of a large number of strains. However, false negative results are also possible with the Gibb's assay. This is due to the presence of phenolic compounds produced by microorganisms and so it was necessary, to support the results of this test, a more quantitative analysis to confirm the HBP presence (Papizadeh et al 2011). Indeed, the strain *Paenibacillus* sp. 10B1551 showed an HBP production equal to 11 ppm with the

Gibb's assay, but the HPLC analysis demonstrates that the HBP production was below the quantification limit.

We isolated 22 desulfurizing strains belonging to *Actinobacteria,* 25 strains to *Firmicues* and only 3 strains to *Proteobacteria*. These results confirm that biodesulfurization ability is preferentially present in GRAM positive bacteria (Papizadeh et al 2011). In addition, few genera were isolated. Excluding *Lactobacillus,* other species were already described for the biodesulfurization ability (Goindi et al 2002). Despite the high nutritional requirements of *Lactobacillus,* they are present in the soil (Chen et al 2005) and they have particular metabolic activity like tannin degradation (Jiménez et al 2014). The biodesulfurization capacity does not seem to be widely shared in the bacterial domain. On the contrary, this particular ability seems to belong at mycolata and few genera of Bacilli.

As a matter of fact, we used two mycolata strains as references: i) *R. rhodochrous* IGTS8, the first strain isolated able to break the C-S bond in DBT, and ii) *G. desulfuricans* 213E, a strain with desulfurize ability on tire. The latter strain was isolated by enrichment culture using as an inoculum a soil contaminated with oil shale and BTH as sole source of sulfur. This strains was described as able to growth in presence of BTH as sole sulfur source but unable to growth in presence of DBT as sulfur source (Kim et 1al 999). On the contrary, we observe that *G. desulfuricans* 213E is able to growth on DBT and it has the three genes for the DBT desulfurization, *dsz*A, *dsz*B and *dsz*C, on the plasmid. Furthermore, the analysis with HPLC showed that the production of HBP is equal to 9,9 ppm (±1). In literature, others bacteria were described as capable of desulfurizing both DBT and BTH with novel system of desulfurization (Bhatia et al 2010). *G. desulfuricans* 213E has likely both metabolic pathways.

In general, it was observed that in Gibb's assay the concentration of HBP resulted higher than in the HPLC results. This could due to the presence of interfering metabolites.

Among the isolated strains, *Rhodococcus* sp. AF21875 is a promising strain. The HBP production is comparable to the reference strains and it is more stable and with a faster growth than other strains.

Bacteria isolated from water, soil and mud collected from the tire landfill, from wastewater of a tire factory and from GTR are the same genera isolate from soil contaminated by oil and with DBT desulfurizing ability.

2.6 References

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Chapter 3
Genome announcement of *Rhodococcus* sp. AF21875

3.1 Abstract

Rhodococci are well known for their ability to catabolize organic pollutants and use organosulfur compounds as sulfur source. Here we present a whole genome shotgun of *Rhodococcus* species strain AF21875, an organism able of preferentially using dibenzothiphene over sulfate as sulfur sources. A wide variety of gene associated to organosulfur transport and assimilation were identified during genome annotation.

3.2 Introduction

Rhodococci are well known for their ability to metabolize a range of organic compounds (Larkin et al 2005). Members of genus *Rhodococcus* have been described for their ability of desulfurize a wide range of organosufur compounds (Van Hamme et al 2004, Lei B et al 1996). In the previous chapter was observed that desulfurizing bacteria isolated from water, soil and mud collected from the tire landfill, from wastewater of a tire factory and from ground tire belong to the same genera isolated from soil contaminated by oil.. For this reason we suggested that bacteria with desulfurize ability were suitable also for the ground tire desulfurization.

Rhodococcus sp. strain AF21875 has been isolated from wastewater of tire production factory and has the ability to produce biomass while exploiting dibenzothiophene as sole sulfur sources. Notably, no significant growth was observed when sulfate was provided as sole sulfur source. *Rhodococcus* sp. strain AF21875 is of potential interest for biodesulfurization of exhausted tires.

3.3 Material and methods

An IonTorrent PGM (Life Technologies Inc., Carlsbad, CA) and a single 316v2-chip was used for sequencing after extracting DNA from Rhodococcus sp. AF212875 cells. DNA was digested and sequencing adaptors ligated using an Ion Xpress Plus Fragment

Library Kit (Life Technologies Inc., Burlington, ON) according to the manufacturer's instructions. Adaptor-ligated DNA was size selected to a target length of 480 bp on a 2% E-Gel SizeSelect agarose gel, and Agencourt MAPure XP beads (Beckman Coulter, Mississauga, ON) were used for purification steps. An Ion Library Quantitation Kit was used to calculate the library dilution factor prior to amplification and enrichment with an Ion PGM Template OT2 400 kit on an Ion OneTouch 2 system. An Ion Sphere Quality Control Kit was used to quantify the percent enriched Ion Sphere Particles prior to sequencing with an Ion PGM 400 Sequencing Kit.

3.4 Results

In total, 2.43 million reads (mean length 219 bases) generated 532 Mb of data in Torrent Suite 4.2.1. These were assembled using SPAdes 3.1.0 (Bankevich et al 2012) (uniform coverage mode; kmers 21, 33, 55, 77, 99) into 52 contigs greater than 500 bp, giving a consensus length of 6,519,631 bp (largest contig 1,690,987 bp; N50 = 285,902 bp). The contigs were aligned with the close relative genomes using progressive Mauve (Darling et al 2010) and *Rhodococcus erythropolis* PR4 (= NBRC 100887) (GenBank Assembly ID: GCA_000010105.1) was found as the closest one. Based on this alignment, contigs were ordered and putative chromosomal contigs were retrieved. Scaffolds of chromosomal contigs were predicted by Ragout (Kolmogorov et al 2014) using *Rhodococcus erythropolis* PR4 , *Rhodococcus erythropolis* CCM2595 and *Rhodococcus erythropolis* R138 as reference genomes. Chromosome scaffolding resulted in three scaffolds of 456,521, 2,988,863 and 2,915,775 base pairs, respectively. Chromosome scaffolds and four putative plasmid contigs (70,079, 66,300. 64,415, 32,298 base pairs) were annotated using Prokka (Seemann et al 2014) (a customized database with proteins included in MetaCyc database was preferentially used during annotation) and Pathway tools was used to reconstruct metabolic pathways (Karp et al 2002).

Annotation generated 6,266 genes (22.6% of ORFs) including 56 RNA genes, with an overall GC content of 62.49%. 408 pathways were predicted as well as 2476 enzymatic reactions and 36 transport reactions. Sulfur-specific metabolic genes found in the

AF21875 genome include biosynthesis of the sulfur-containing amino acids cysteine and methionine, as well as genes with homologies to those in the *ssu* operon responsible for desulfurization or organsulfur compounds (Eichhorn et all 2001, Van der Ploeg et al 2001 and Kahnert et al 2000), *tau* operon and *dsz* genes responsible for dibenzothiophene utilization. Several genes included in central and peripheral catabolic pathways of aromatic hydrocarbons were found such as halobenzoates, phenols.

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Chapter 4
Phenotypic and genotypic evidences of sulfur metabolisms in *Rhodococcus* sp. AF21875

4.1 Abstract

Sulfur is an essential element for bacterial growth When bacteria are under sulfur-limiting conditions can utilize alternative sulfur sources as aliphatic sulfonates. The ssu and tau systems are involved in the uptake and utilization of a wide range of aliphatic sulfonates or taurine, respectively. In both systems an ABC transport system (*tauABC* and *ssuABC*) and an oxygenase system (*tauD* and *ssuDE*;) are present. Furthermore, several mycolata can utilize dibenzothiophene (DBT) and its derivatives as sulfur source. The aim of this work is characterize both phenotypically and genotipically some traits of the sulfur metabolisms in Rhodococcus sp. AF21875. *Rhodococcus* sp. AF21875 showed a preferential use of DBT as sulfur source compared with the inorganic sulfur and produced 10 ppm of HBP in 14 days. In AF21875 genome, both the assimilatory sulfate reduction pathways and the cysteine biosynthesis pathway has been detected. In addition, *tau*ABCD and *ssu*EADCB genes were annotated. Furthermore, a putative plasmid harboring *dsz*ABC genes was found.

4.2 Introduction

Sulfur is an essential element for all organisms (Ellis et al 2011). Indeed, it is a component of amino acids and enzyme cofactors (biotin, coenzyme A,) and it is also involved in a redox homeostasis process (Scott el al 2006). The favorite sulfur source of many bacteria is the cysteine which can be either assimilated from the environment or is synthesized from inorganic sulfate. Sulfate is the most abundant sulfur source in the environment and after the uptake into the cell, sulfur is transformed in adenosine 5'-phosphosulfate (APS) through the enzyme sulfate adenylyltransferase. Then the APS is converted in 3'-phosphoadenosine-5'-phosphosulphate (PAPS) by means of APS kinase. Sulfate moiety is reduced to sulfite by PAPS sulfotransferase. Sulfite is

converted in sulfide through the sulfite reductase. Finally, sulfide is incorporated into *O*-acetyl-L-serine (OAS) to yield L-cysteine (van der Ploeg et al 2001). Mclaren (1985) reported that the inorganic sulfur is only the 3-6% of total soil. For this reason, bacteria must have some way to acquire sulfur from the environment under sulfur-limiting conditions (Ellis et al 2011). In these cases, a set of sulfate starvation-inducible (SSI) proteins is expressed to utilize organosulfur compound as sulfur source. Within these proteins the ssu and tau systems are well characterized, in particular in *Escherichia coli* (van der Ploeg et al 2001). These systems are involved in the utilization of aliphatic sulfonates or taurine as sulfur sources, respectively (Kouzuma et al 2008). In both systems are present an ABC transport system (*tauABC* and *ssuABC*) and an oxygenase system (*tauD* and *ssuDE*;) (van der Ploeg et al 2001 – Fig. 4). The ssu system uses a broad range of linear aliphatic sulfonates as substrates, but no catalytic activity was observed with aromatic sulfonates, taurine, cysteate and methanesulfonate (Eichhorn et al. 1999). In this system is present an ABC transport system (*ssu*ABC), an alkanesulfonate monooxygenase flavin dependent (*ssu*D) and a FMN reductase (*ssu*E). ssuD catalyzes the release of corresponding aldehyde and sulfite through the unstable intermediate, 1-hydroxysulfonate (van der Ploeg et al 2001).

The best substrate of the tau operon is the taurine where t*au*D encode for the taurine dioxygenase which catalyzes the taurine oxygenation to instable intermediate, hydroxytaurine, and sulfite and aminoacetaldehyde are released (Ellis et al 2011 and van der Ploeg et al 2001). During the uptake of taurine an ABC transporter system is involved (*tau*ABC).

In addition to this alternative sulfur sources, several mycolata can utilize polyaromatic sulfur heterocycles (PASHs) such as dibenzothiophene (DBT) and its derivatives (Li et al 2005, Akhtar et al 2009 and Mohebali et al 2007). These compounds are found in fossil fuels and they are recalcitrant to hydrodesulfurization, a chemical process to reduce the sulfur content in fossil fuels. Biodesulfurization is considered an attractive technique. Particularly, the 4s pathway was described in literature to desulfurize DBT to 2-hydroxybiphenyl (HBP) via specific cleavage of the C–S bond without affecting their carbon skeletons. In this route are involved two monooxygenase flavin

dependent (DszC and DszA), a desulfinse (DszB) that releases sulfite and HBP and a flavin reductase (DszD) (Davoodi-Dehaghani et al 2010). *dsz* genes are located on a stable plasmid. The bacteria harboring this pathway, under sulfur limiting growth conditions, oxidize the sulfur heteroatom whitin DBT. The sulfur from DBT is released as sulfite and believed to integrate, at least partially, into the microbial biomass via sulfur assimilation pathways (Aggarwal et al. 2012).

In a previous work (see chapter 2) a promising bacteria with desulfurizing ability on DBT was isolated from process water in a tire factory. It is classified as *Rhodococcus* sp. AF21875 and it demonstrated an HBP production comparable with *Rhodococcus rhodochrous* IGTS8 (ATCC®53968™), the first strain discovered with this pathway (Oldfield et al 1997).

In this work, the growth of *Rhodococcus* sp. AF21875 in presence of different sulfur source was analyzed and the HBP production was quantified in order to evaluate the biodesulfurization capacity of strain. In addition, the *Rhodococcus* sp. AF21875 genome (see chapter 3 for methods) was sequences with the specific aim of investigating sulfur metabolism of this strain to improve the knowledge on organosulfur metabolism.

4.3 Materials and methods

4.3.1 Chemicals and reagents

Dibenzotiophene (DBT, assay 99 %), 2-hydroxybiphenyl (HBP, assay 99 %) and the other chemicals including constituents of mineral salt medium (MSM) were purchased from Sigma Aldrich (Germany). The acetonitrile, water, ethyl acetate (HPLC grade) were obtained from Panreac (Spain). Tryptone, agar, yeast extracts and were obtained from Biolife (Italy).

4.3.2 Microorganism and culture medium

The strain AF21875 was isolated form a wastewater of tire factory. The strain was cultured in a sulfur free mineral medium salt (MSM) (Chang et al 1998) that contained 10 g/L of glucose, 4.5 g/L of K_2HPO_4, 1.5 g/L of NaH_2PO_4, 2 g/L of NH_4Cl, 0.2 g/L of $MgCl_2$, 0.02 g/L of $CaCl_2*2H_2O$, 10 mL/L of vitamin (RPMI 1640-Sigma Aldrich-Germany), 5 mg/L $MnCl_2*6H_2O$, 0.5 mg/L H_3BO_3, 0.5 mg/L $ZnCl_2$, 0.5 mg/L $CoCl_2*6H_2O$, 0.46 mg/L $NiCl_2*6H_2O$, 0.3 mg/L $CuCl_2$, 0.1 mg/L $NaMoO_4*2H_2O$, 1.49 mg/l $FeCl_2*4H_2O$, 0.003 mg/l $NaSeO_3$ and 0.008 mg/l Na_2WO (Widdel et al., 1992). The strain grow at 30°C at pH 7.

4.3.3 Growth test on different sulfur sources

Growth of *Rhodococcus* sp. AF21875 was tested with different sulfur sources. The strain was grown for seven days at 30 °C with MSM and the following alternative sulfur sources: DBT (184,26 ppm), inorganic sulfur (SO_4^{2-}-650 ppm), DBT and SO_4^{2-}, without any sulfur source. Every condition was tested in triplicate and growth was estimated by optical density (OD) at 600 nm.

4.3.4 Quantitative analyses

The culture medium (10 mL) was acidified to pH 2 with 1 M HCl and the HBP was extracted with 20 mL of ethyl acetate. After that, the sample was put into a brandson ultrasonic bath for fifteen minutes at room temperature and each sample was centrifuged for five minutes at 4000 rpm. HBP was quantified using Agilent 1100 HPLC system (Agilent Technologies, USA) equipped with a RP- C18 5-µ.m particle size column (150*4.6 mm, Vydac 201, Vydac, USA). Fluorimeter detector was used for the quantification of the bio-product. The HBP excitation wavelength is 240 nm and the HBP emission wavelength is 340 nm. The isocratic mobile phase was water:acetonirile (50:50 v/v) an the flow rate was 1,6 mL/min. HBP concentration was calculated with a calibration curves (range 0.005 ppm and 1.4 ppm).

4.3.5 Molecular analyses

The strain, was identified by PCR amplification and sequencing of 16S rRNA gene. DNA was extracted from bacterial suspensions using Invisorb Genomic DNA kit III and 16S rRNA gene fragments were PCR-amplified. PCR was performed in 80 µL reactions containing 4 µL extracting DNA with 16 µL of water 2 U of Taq DNA Polymerase (Promega Corporation, USA) and 10 µM of each of following primer: 27F (5'-AGAGTTTGATCCTGGCTAG-3') and 1492R (5'-CTACGGCTACCTTGTTACGA-3)' 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.

On the other hand, the plasmidic DNA extracted by Pure Yeld Plasmid Miniprep System (Promega Corporation, USA) and used as PCR template to sequences the *dsz*ABC genes. The PCR primer sequences are the following: *dsz*A forward 5'-TCGATCAGTTGTCAGGGG -3', *dsz*A reverse 5'-GGATGGACCAGACTGTTGAG-3', *dsz*B forward 5'-ATCGAACTCGACGTCCTCG-3' *dsz*B reverse 5'-GGAACATCGACACCAGGACT-3' *dsz*C forward 5'-CTGTTCGGATACCACCTCAC-3' and *dsz*C *r*everse 5'-ACGTTGTGGAAGTCCGTG-3' (Duarte et al. 2001). PCR was performed in 80 µL reactions containing 4 µL extracting DNA with 16 µL of water, 2 U of Taq DNA Polymerase (Promega Corporation, USA) and 10 µM of each primer (Sigma- Aldich, Germany). Plasmidic DNA from *Rhodococcus rhodochrous* IGTS8 (ATCC 53968) was used as PCR templates for positive control. PCR products were purified using the Wizard- SV Gel and PCR purification kit (Promega Corporation, USA). The 16S rRNA and *dsz* genes were sequenced with ABI prism 310 CE systems by CIBIACI (Firenze, Italy). The obtained sequences were analyzed with the BLAST. The Ribosomal Data Project (RDP) was also used to analyze the 16S rRNA sequence.

4.3.6 Analyzed of predicted pathways

Whole genome shotgun of *Rhodococcus* species strain AF21875 was obtained through An IonTorrent PGM (see chapter 3). The genome sequence was analyzed genome using *pTools* (Karp et al., 2002).

4.4 Results

4.4.1 Growth characterization of AF21875

In the figure 1 the AF21875 growth curves in presence of different sulfur sources were reported. Maximum growth rate was observed in presence of DBT as sole sulfur source (Fig. 1 and Tab. 1). In particular, higher growth was reached after 48 hours and OD was equal to 1,46 (±0,086). After this peak, the strain entered its stationary phase. In presence of DBT and inorganic sulfur growth was similar to the previous with a peak after 48 hours and an OD equal to 1,2913 (±0,0511) (Fig. 1 and Tab. 1). Also, in the presence of inorganic sulfur (SO_4^{2-}) a peak after 48 hours was observed, but the OD is very low and equal to 0,4783 (±0,2959). Furthermore, the growth in presence of SO_4^{2-} was comparable with the growth in the medium without any sulfur sources.

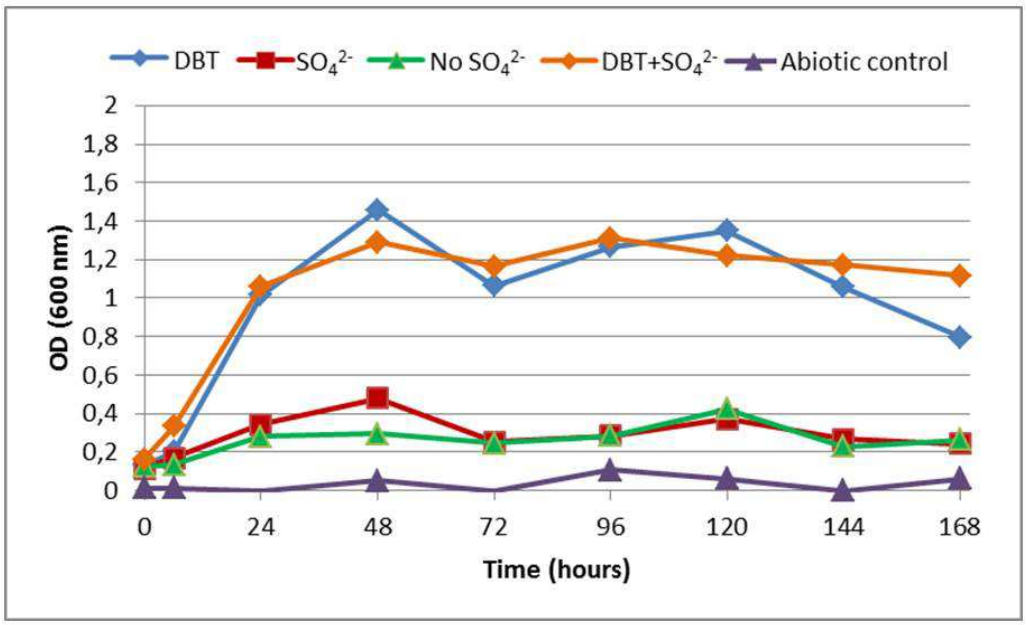

Figure 1 Growth of *Rhodococcus* sp. AF21875 with DBT, sulfur (So_4^{2-})′ without sulfur (No So_4^{2-}), or with DBT and SO_4 (DBT+ So_4^{2-}).

4.4.2 *Rhodococcus* sp. AF21875 desulfurization of DBT

The AF21875 desulfurization ability of DBT was evaluated using an HPLC analysis. The product of DBT desulfurization is the 2-hydroxybiphenyl (HBP) and the concentration of this compound was quantified in the culture medium. In the figure 2 AF21875 growth in presence of 184,26 ppm of DBT for 14 days was reported. The maximum of HBP production is 10 ppm and it was produced after 14 days of growth. On the other hand, the production of HBP continued despite the strain entered its stationary phase (Fig.2). The presence of HBP is important because it is the product of desulfurization through the 4S pathway, the enzymes catalyze the oxidative C-S bond cleavage, without break the carbon skeleton. For this reason, the presence of the four genes involve in this pathway was investigated. Furthermore, *Rhodococcus* sp. AF21875 was grown for seven days on DBT (5 mM) as carbon source and the optical density was measured (600 nm). Result demonstrated that this strain was unable to grow on DBT as carbon source (data not shown).

The plasmidic DNA was used to amplify the genes and to confirm the presence of this genes on a plasmid. The figure 3 showed the results of a PCR on *dsz*ABC genes. The strain AF21875 harbors the *dsz genes* on a plasmid (Fig. 3 and Tab. 3). The sequence of *dsz*A, *dsz*B and *dsz*C genes were aligned with the *Rhodococcus sp.* IGTS8 sequences present in gene bank and no difference was observed. Furthermore, the 16S rRNA sequence exhibited a similarity of 99% to *Rhodococcus qingshengii* (Tab. 4).

Hours	OD (600 nm)				
	DBT	SO_4^{2-}	No SO_4^{2-}	DBT+ SO_4^{2-}	Abiotic control
0	0,1320 (±0,0223)	0,1153 (±0,0125)	0,1250 (±0,0144)	0,1637 (±0,0391)	0,0127 (±0,0071)
6	0,2013 (±0,0693)	0,1687 (±0,0176)	0,1337 (±0,0225)	0,3343 (±0,0273)	0,0110 (±0,0200)
24	1,0137 (±0,1444)	0,3437 (±0,0336)	0,2823 (±0,0620)	1,0597 (±0,2222)	-0,0023 (±0,0059)
48	1,4560 (±0,0860)	0,4783 (±0,2959)	0,2953 (±0,0431)	1,2913 (±0,0511)	0,0493 (±0,0038)
72	1,0653 (±0,0031)	0,2537 (±0,0295)	0,2467 (±0,0152)	1,1650 (±0,1789)	-0,0020 (±0,0269)
96	1,2660 (±0,0282)	0,2830 (±0,0395)	0,2843 (±0,0301)	1,3100 (±0,1654)	0,1077 (±0,0766)
120	1,3507 (±0,4923)	0,3720 (±0,0789)	0,4243 (±0,1556)	1,2210 (±0,3443)	0,0600 (±0,0639)
144	1,0580 (±0,0917)	0,2657 (±0,0939)	0,2283 (±0,0526)	1,1723 (±0,2486)	0,0000 (±0,0000)
168	0,7959 (±0,0467)	0,2439 (±0,0164)	0,2640 (±0,0089)	1,1161 (±0,2195)	0,0625 (±0,0156)

Table 1 AF21875 growth at different times in presence of different sulfur sources. In the brackets, the standard deviations were reported

Time (days)	HBP (ppm)	es	OD_{600}	std
0	0	0	0,3662	0
3	6	3	1,1553	0,2184
7	5	1	1,424	0,1458
10	7	2	1,9949	0,4583
14	10	2	1,8733	0,0551

Table 2 *Rhodococcus* sp. AF21875HBP production at different times

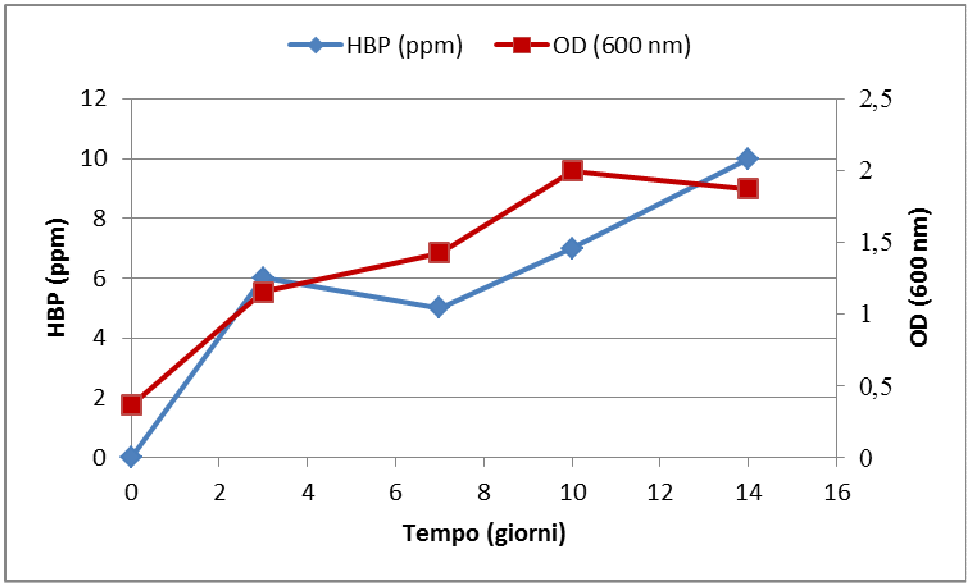

Figure 2. Growth of *Rhodococcus* sp. AF21875 in presence of DBT as sulfur source and HBP production

Gene	Accession Number	DESCRIPTION	MAX IDENT
*dsz*A	gi|148470338|EF570781.1	Synthetic construct dibenzothiophene sulfone monooxygenase (dszA) gene, complete cds	99%
*dsz*B	gi|148470340|EF570782.1	Synthetic construct aromatic desulfinase (dszB) gene, complete cds	99%
*dsz*C	gi|170773988|EU527978.1	Mycobacterium goodii strain X7B dibenzothiophene monooxygenase (dszC) gene, complete cds	99%

Table 3.Analyzis of *dsz*ABC with BLAST

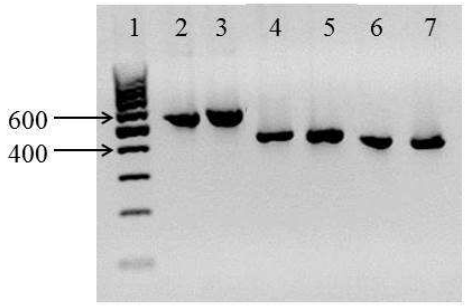

Figure 3. PCR of *dsz* genes in *Rhodococcus rhodochrous* IGTS8, and *Rhodococcus* sp. AF21875. Lanes: 1 Marker, 2 *Rhodococcus rhodochrous* IGTS8 *dsz*A, 3 *Rhodococcus* sp. AF21875 *dsz*A, 4 *Rhodococcus rhodochrous* IGTS8 *dsz*B, 5 *Rhodococcus* sp. AF21875 *dsz*B, 6, *Rhodococcus rhodochrous* IGTS8 *dsz*C, 7 *Rhodococcus* sp. AF21875 *dsz*C.

	BLAST			RDP	
Gibb's Results (ppm)	**Description**	**Accession Number**	**Max Ident**	**Genus**	**% di Conf**
18	*Rhodococcus qingshengii* strain djl-6 16S ribosomal RNA, partial sequence	gi|343203016|NR_043535.1	99%	*Rhodococcus*	100%

Table 4 Results of sequencing on selected strains

4.4.3 Predicted pathways of sulfur assimilation/utilization

As previously reported, metabolic pathways of *Rhodococcus* sp. AF21875 have been predicted based on the draft genome sequence using *pTools* (Karp et al., 2002). In the following paragraphs, the predicted pathways related to sulfur metabolism have been detailed.

Sulfur is an essential element for bacterial metabolism and can be assimilated from a wide range of sources. Among these sources, cysteine is the preferred one for many bacterial sources. Presence of cysteine and other reduced for of sulfur such sulfide and thiosulfate repress the uptake, activation and reduction of sulfate (van der Ploeg, 2001). When cysteine and inorganic sulfur are both absent from the growth medium, some microorganism can use alternative sulfur sources. In AF21875 genome, both the

assimilatory sulfate reduction pathways and the cysteine biosynthesis pathway has been detected.

Aliphatic sulfonates assimilation

Moreover, the annotation of the genome revealed the presence of the genetic potential of alternative organic sulfur sources, namely aliphatic sulfonats (alkanesulfonates and taurine), and thiophenes (dibenzothiphene and thiophene).
Indeed, in AF21875 genome several copies of the *tau*ABCD and *ssu*EADCB genes have been identified. As reported above, these gene clusters encodes an ABC-type transport system required for uptake of aliphatic sulfonates and a desulfonation enzyme. The general overview of uptake and desulfonation of taurine and alkanesulfonates is reported of figure 4.

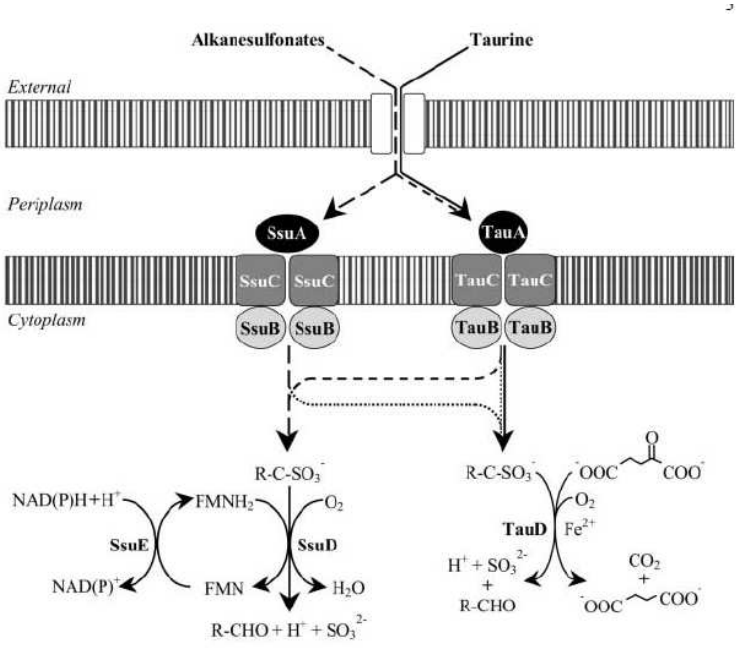

Figure 4 Uptake and desulfonation of taurine and alkanesulfonates in *E. coli*. (van der Ploeg et al 2001)

Particularly, AF21875 genome contains 5 copies of *ssu*A, *ssu*B and *ssu*D, 10 copies of *ssu*C and one copy of *ssu*E. *ssu*ABCD genes also localized contiguously in two predicted transcription units on the chromosome (Fig. 5).

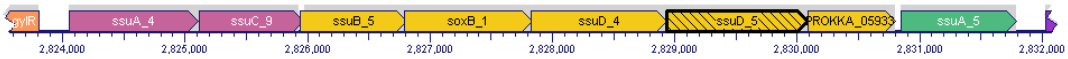

Figure 5 *ssu*ABCD transcription units

Regarding taurine assimilation, the annotation revealed the presence of 2 *tau*A copies, 6 copies of *tau*B and 5 copies of *tau*D. No predicted gene has been annotated as *tau*C. However, several genes annotated as *ssu*A of *ssu*C gene localized close to *tau*AB genes. The high homology between these genes for ABC transporters led to suppose possible mis-annotation.

Dibenzothiophene desulfurization

As already reported some microorganisms are able to use dibenzothiophene (DBT) and other thiophenes as a sole sulfur source. Consistently with amplification results, in AF21875 genome the complete DBT pathway (*dsz*ABC) has annotated on a putative plasmid in a single transcription unit (Fig. 6) and *dsz*C gene has been retrieved in 5 copies within the genome.

Figure 6 *dsz*ABC transcription units

Other sulfur related genes

The AF21875 genome contains also other partial pathways and genes for sulfur assimilation. Two copies of thiosulfate sulfurtransferase (*psp*E) are present in the genome. PspE is a periplasmic enzyme catalyzing the disproportionation of thiosulfate. Although the entire pathway for sulfolactate assimilation is not present in the genome, the presence of the gene coding for an S-sulfolactate dehydrogenase (*slc*C)

might indicate that sulfolactate could represent another possible catabolite of *Rhodococcus* sp AF21875.

It is worth noting that five copies of the gene encoding for a sulfoacetaldehyde reductase (IsfD) has been annotated. IsfD catalyzes the reduction of sulfoacetaldehyde to isethionate. This reaction could be part of a different taurine assimilation pathway than the one described above which consists in the conversion of taurine into sulfoacetaldehyde rather than in aminoacetaldehyde.

4.5. Discussion

Rhodococcus sp. AF21875 was isolated in a previous work (see chapter 2) from wastewater of a tire factory. In this paper we purposed a characterization of this strain focusing our attention on sulfur metabolism. In particular, genome sequence was analyzed seeking pathways of organic sulfur assimilation and utilization of these compounds as sulfur source. Furthermore, the desulfurizing ability was evaluated on DBT. DBT is used as a model compound for biodesulfurization of fossil fuels because it a recalcitrant compounds found after the conventional hydrodesulfurization treatment (Aminsefat et al 2012).

An interesting aspect is the particular growth of this strain in presence of different sulfur sources. Indeed, the maximum of growth was observed in presence of DBT as sole sulfur source. On the contrary, the growth in presence of inorganic sulfur is lower and comparable to the growth in absence of sulfur source. This could indicates that this strain uses DBT as preferential sulfur source. Akhtar and colleagues (2009) evaluated the ability of isolate *Rhodococcus* sp. Eu-32 to use different sulfur sources. An insignificant growth on sulfur was observed in comparison with the growth in presence of DBT. Also *Rhodococcus* sp. Eu-32 showed a growth in presence of sulfur or without sulfur were comparable. Indeed, Van Hamme (2004) reported that some strains were able to produce biomass on the trace sulfur found in medium components. However, Honda et al 1998 reported that in *R. erythropolis* IGTS8 sulfate promoted higher cell growth than DBT. Flux-based analysis of sulfur metabolism conducted on *R. erythropolis* IGTS8 showed an higher cell growth in presence of

sulfate than DBT (Aggarwal et al 2011). Furthermore, the same analysis conducted to study the phenotype in presence of DBT and sulfate together showed that the organism may grow faster when it fulfills a part of its sulfur needs via sulfate rather than DBT. This demonstrates that the strain prefers sulfate when both DBT and sulfate are present. Because sulfate yields a higher growth rate than DBT, the organism may use DBT only if sulfate is not present (Aggarwal et al 2011).

Aggarwal and colleagues (2011) suggested an explanation to the sulfur preference. During the DBT desulfurization through the 4S pathway, DBT was converted in dibenzothiophene sulfoxide, dibenzothiophene sulfone, hydroxyphenyl benzene sulfonate and finally this is converted into 2-hydroxybiphenyl (2-HBP) and sulfite (Fig.7) (Akhatr et al 2009). Sulfur metabolism converts sulfite to sulfate and then incorporated into the biomass precursors. When DBT is used as sulfur sources 4 moles NADH for mole of DBT are necessary and so more energy is requested (Aggarwal 2011). On the contrary, the strain does not need this extra NADH for metabolizing sulfate. In others words, microorganisms prefers sulfur as sulfur source because it is energetically less expensive compared to DBT for their growth. When DBT is the sole sulfur source or sulfur is not available more energy are consumed to obtain cysteine and so cells growth reduces.

Also in the case of taurine and alkanesulfonates, the presence of sulfur repress the *ssu* ant *tau* genes expression. In this case, regulation is better characterized. CysB protein is a transcriptional regulator that positively regulates the expression of the genes involved in the sulfate assimilation (Kredich et al 1996). The expression of the *cys* genes (genes involve in the sulfite reduction) was regulated by CysB and the coinducer *N*-acetylserine. The latter was obtained from *O*-acetylserine through a non-enzymatically reaction (Kredich et al 1996). *O*-acetylserine is synthesized by serine acetyltransferase, the activity of this enzyme is regulated through feedback inhibition by cysteine. In this way cysteine quantity in the cell regulates the expression of the *cys* genes. However CysB is also involved in the control of the expression of *tau* and *ssu* genes (van der Ploeg et al 2001). Indeed this protein controls the expression of cbl, a regulatory protein of *tau* and *ssu genes* expression (Fig. 8).

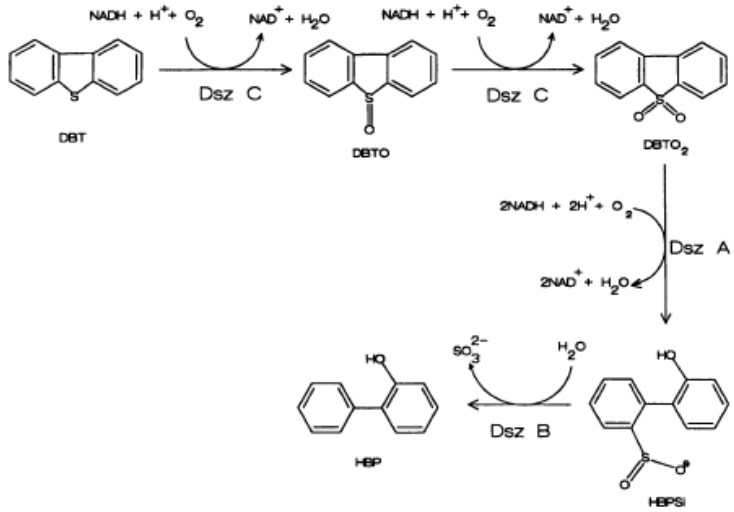

Figure 7 DBT desulphurization to HBP through 4S pathway by *Rhodococcus* sp. IGTS8. DBTO = dibenzothiophene sulphoxide, $DBTO_2$ = dibenzothiophene sulphone, HPBS = hydroxyphenyl benzene sulfonate, HBP = 2-hydroxybiphenyl (Oldfield et al 1997).

The desulfurization ability of AF21875 was tested and it showed an HBP production equal to 5 ppm in 7 days. The DBT consumption continued until 14 days, and the HBP concentration increased also in the stationary phase. The presence of this molecule indicates that the desulfurization proceeded through C-S breakage but not through C-C cleavage. This aspect is important for the biodesulfurization process both fuels and tire. Moreover, the presence of the genes *dsz*A, *dsz*B and *dsz*C confirms that this strain uses the 4S pathway for the DBT desulfurization. In addition, no growth was observed when DBT was used as carbon source. The sequences of these three genes were aligned with the respective sequences of *R. rhodochrous* IGTS8, no difference was observed. This confirms the conserved nature of *dsz* operon (Davoodi-Dehaghani et al 2010).

This strain has different pathways to assimilation and utilization of different organic sulfur. However, the most interesting aspect of AF21875 metabolism is the preference of DBT over sulfate as sulfur sources. Indeed, the DBT preferential utilization does not seem common within desulfurizing bacteria. To better characterize AF21875 is will be necessary to evaluate the growth in presence of taurine and different alkanesulfonate

to confirm that the predict pathway are complete and if also in these cases the bacteria prefer the organic sulfur than sulfate. In addition, it is necessary to investigate the expression pattern to understand this particular regulation of sulfur metabolism.

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Chapter 5
Experimental design approach to study biological devulcanization of ground rubber by *Gordonia desulfuricans* 213E, *Bacillus* sp. 8A1531 and *Rhodococcus* sp. AF21875

5.1 Abstract

In this study a two level full factorial experimental design was chosen to investigate biodesulfurization process of model vulcanized ground natural rubber (VGNR). In particular, the desulfurization ability of *Gordonia desulfuricans* 213E, *Bacillus* sp. 8A1531 and *Rhodococcus* sp AF21875 was tested. The aim of the experimental design was to investigate the influence on the process of concentration of glucose, the presence of dibenzothiphene (DBT) and the initial bacteria optical density (OD). The complex viscosity was chosen as experimental responses. Furthermore, automated ribosomal inter-genic spacer analyses (ARISA) was proposed to investigate the persistence of the inoculated strains during the experiments. A reduction of the complex viscosity was revealed in the VGNR treated with *Bacillus* sp. 8A1531 and *G. desulfuricans* 213E. The strain *Rhodococcus* sp. AF21875 showed a high dispersion of values. Only for the strain *G. desulfuricans* 213E a multiple linear regression was used for modeling the relationship between the response and the process variables. Reduced regression models were obtained considering only the significant variables and interactions. The glucose concentration and the DBT-OD interaction resulted the relevant parameters for the process. The fingerprinting showed the persistence of *G. desulfuricans* 213E and *Rhodococcus* sp AF21875. Conversely, no evidence of persistence was obtained for *Bacillus* sp. 8A1531 because the bacterial community in these samples was similar compared with the community in the non inoculated samples. These results highlight the importance to support the physic analyzes with microbiological methods to observe the bacterial persistence during the experiments.

5.2 Introduction

Natural rubber (NR) that mainly consists of poly-cis-1,4-isoprene is a natural polymer produced from *Hevea brasiliensis* tree and is one of the most used elastomers. In order to improve the mechanical properties and increase the elasticity and the resistance to the deformation required for tires and industrial applications, the NR is generally vulcanized (or cured) by heating between 120 and 200 °C in the presence of a vulcanizing (or curing) agent (Rader et al 2001). However, this three-dimensional chemical crosslink represents the main hinder to the rubber recycling, since the formation of this network is a non-reversible chemical process.

Moreover, waste vulcanized rubber products are rapidly increasing and they represent a disposal, health and environmental problem (Isayev et al 2014). In the past decades, there have been many attempts to the recycling of cured rubbers and several researches have been conducted in order to find an efficient devulcanization process that selectively brakes the tridimensional network. Most of the existing devulcanization processes for waste rubber involve mechanical, chemical, microwaves and ultrasonic technologies. However, the chemical ones are dangerous for the health and the environment since they require reactions that involve organic compounds oils and solvents. Thermal and mechanical ones require high amount of energy and generally are expensive. Moreover, the selectivity of all these processes is low and none of them seems to produce high quality reclaimed rubber, since the reduction of the crosslink density is always accompanied by a main chain breakage. (Isayev et al 2014, Rajan et al 2006, Adhikari et al 2000).

A microbial desulfurization was suggested in the last years as an alternative respect to methods that require hazardous chemicals or energetic operation conditions. In particular, microbial desulfurization is considered as a process safe for the environment, economic and more selective than mechanical and physical process, since many bacteria have the ability to cleave the sulfur-carbon bond. However, the biological treatment is limited to the surface, but able to generate more active sites for the re-vulcanization process (Li et al 2012b).

The majority of existing biodesulfurization researches has reported a microbial activity towards the sulfur-carbon bonds in dibenzothiophene (DBT), generally present as a chemical in fuel and therefore in the contaminated soils. Most of these studies pointed out on several microorganisms able to remove the sulfur content from the compound and use it as a micronutrient, without introduce any change in the carbon structure (Aminsefat et al 2012, Papizadeh et al 2011, Davoodi-Dehaghani et al 2010). In literature, this pathway was described and characterized for the DBT desulfurization and is known as "4S pathway" (Oldfield et al 1997).

Since the aim of the cured rubber bio-desulfurization is similar to the one studied for the DBT, several researches tried to extend and apply this technique to the rubber tire devulcanization, using the microorganism ability to selectively break the C-S bond. However, just few microorganism possess this ability towards waste rubber and ground tire rubber, due to their complex structure. A mechanism similar to "4S" was suggested by Romine and Romine (1997) for the desulfurization of ground tire (GTR) by *Sulfolobus acidocaldarius.* The sulfur crosslinks were metabolized into sulfoxide- sulfone- sulfonate- sulfate. Jiang (2010) reported the activity of *Thiobacillus ferrooxidans* on ground tire while, Li (2012a) used a chemoorganotroph bacterium, *Sphingomonas* sp., for ground tire rubber desulfurization.

Another chemoorganotroph was described in a patent by Christofi (2010). In this patent, the bacterium *Gordonia desulfuricans* 213E (NCIMB 40816) was employed for the desulfurization of vulcanized rubber. The sulfur quantity in the rubber decreased between 23% and 35%. Before the treatment, benzothiophene was added to the culture medium as a degradation pathway inducer.

In a previous work (see chapter 2) two promising bacteria with desulfurizing ability on DBT were isolated. These strains were isolated from a soil of landfill tire and from a process water in a tire factory and they are classified as *Bacillus* sp. 8A1531 and *Rhodococcus* sp. AF21875, respectively. As reported above, few articles are reported in literature on biodesulfurization of GTR (Li et al 2012a, Christofi et al 2010, Jiang et al 2010) and few aspects of the process are analyzed. Indeed, the effect of growth medium composition on desulfurizing activity has never been investigated. Another

important point is the examination of the persistence of the bacterial inoculum during the treatment. GTR is a material with an elevated porosity, and so sterilization is not completely efficient. Furthermore, GTR is an even more complex material with a variable composition.

For these reasons, in the present study, a statistical approach based on a full factorial experimental design was used to investigate how some process parameters might affect the complex viscosity (η*) of the model vulcanized-ground NR (VGNR) in order to facilitate the analyses, and therefore the desulfurization and degradation process on the rubber structure during microbial desulfurization. The variables considered were the concentration of glucose as a source of carbon, the presence of DBT as a model sulfur compound for the desulfurization pathway induction and the initial optical density (OD) of bcateria. We used *Gordonia desulfuricans* 213E as model organism and our isolates *Bacillus* sp. 8A1531 and *Rhodococcus* sp. AF21875. Treated VGNR was then characterised by a frequency sweep test and the fingerprinting method ARISA (automated ribosomal inter-genic spacer analyses) were used for microbiological characterization.

5.3 Materials and methods

5.3.1 Chemicals and reagents

The biological devulcanization was carried out on a model compound of vulcanized ground natural rubber (VGNR). NR was mixed at a two-roll mill as follows: 1 part per hundred of rubber (phr) powder N-Cyclohexylbenzothiazole-2- sulfenamide, 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 (the optimal time and temperature conditions). After the curing process, the NR was ground at room temperature in order to reach particle dimensions between 250 μm and 500 μm.

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

The strain *Gordonia desulfuricans* 213E (NCIMB 40816) was purchased from Lebeniz Institut DSMZ-"German Collection of Microorganisms and Cell Culture" (Germany). The acetone was obtained from Carlo Erba (Italy). Sulfur-free constituents of mineral salt medium (MSM) were purchased from Sigma Aldrich (Germany)**.**

5.3.2 Preparation of media

Sulfur free MSM (pH 7.0) (Chang et al 1998) was prepared by dissolving, 4.5 g/l of K_2HPO_4, 1.5 g/l of NaH_2PO_4, 2 g/l of NH_4Cl, and autoclaved at 121 °C for 30 minutes. Then, after sterilization by filtration, the following compounds were added: 0.2 g /l of $MgCl_2$; 0.02 g/l of $CaCl_2*2H_2O$; 10 ml/L vitamin (RPMI 1640-Sigma Aldrich- Germany); 5 mg/l $MnCl_2$ $*6H_2O$; 0.5 mg/l H_3BO_3; 0.5 mg/l $ZnCl_2$; 0.5 mg/l $CoCl_2*6H_2O$; 0.46 mg/l $NiCl_2*6H_2O$; 0.3 mg/l $CuCl_2$, 0.1 mg/l $NaMoO_4*2H_2O$; 1.49 mg/l $FeCl_2*4H_2O$; 0.003 mg/l $NaSeO_3$; 0.008 mg/l Na_2WO_4 (Widdel et al., 1992). Glucose was added according to table 2 as a carbon source after sterilization by filtration.

5.3.3 Design of Experiment

5.3.3.1 Experimental procedure

VGNR was extracted with acetone for 72 hours at room temperature, using a volume of 10 ml/g of rubber. Acetone was changed every 24 hours. After that, VGNR was dried under vacuum for 24 hours at 50 °C. This step was necessary to remove unreacted sulfur and vulcanizing reagents.

Each experiment of the experimental design was carried out in a flask with 10 g of VGNR, in 50 ml of final solution volume. Among the medium chemicals, the non-autoclavable ones were added to the flask only after the sterilization process.

All flasks were incubated under stirring at 30 °C for 14 days. At the end of the treatment, the treated VGNR was recovered and dried under vacuum for 24 hours at 50 °C. VGNR complex viscosity (η*) was chosen as experimental response. The complex viscosity (η*) was used as experimental response since it is influenced by the

NR structure and its change may result from both de-crosslinking and degradation of main carbon chain.

The samples showing the lowest viscosity were tested with molecular analyses and crosslink density.

5.3.3.2 Full factorial design

A two level full factorial experimental design (Box et al 1978, Eriksson et al 2008) was chosen to investigate the devulcanization process.

Table 1 shows maximum (+1), minimum (-1) and central (0) levels for each variable used in the present study. As a carbon source, glucose was used and DBT was added as a model sulfur compound for the desulfurization pathway induction. The third variable considered was the initial optical density and it was evaluated in terms of absorbance. Higher absorbance corresponds to the maximum concentration of bacteria and vice versa. These variables were chosen since were considered the most important parameters influencing the microorganisms growth.

Factor	**Min level**	**Max level**	**Central level**
Code	**-1**	**1**	**0**
Carbon Source Concentration (g/L)	10	20	15
DBT (mg/L)	0.00	3.12	1.56
Optical Density at 600 nm (OD) (AU)	0.1	1	0.55

Table 1 Factors and levels of the experimental design.

Eight experiments were carried out to investigate the experimental domain; three experiments were added to investigate the performance in the center of the experimental domain (Nc) and three experiments were added as control samples without the bacterial inoculum (N_A in table 3) to investigate the effect of the VGNR bacteria community lasting even after the sterilization. A fully randomized execution of the experiments was carried out in order to minimize the error due to the planning of the experiments. Experimental design was set up according to table 2.

The dependence of η*, on the factors was modeled applying the following equation (Box et al 1978):

$$y = \beta_0 + \sum_{i=1}^{n}\beta_i x_i + \sum_{i=1}^{n-1}\sum_{j=i+1}^{n}\beta_{ij} x_i x_j + \sum_{i=1}^{n-2}\sum_{j=i+1}^{n-1}\sum_{k=j+1}^{n}\beta_{ijk} x_i x_j x_k + \varepsilon \quad (1)$$

where β_0 is the constant term, β_i and β_{ij} are the regression coefficients, ε is the error, x_i and x_{ij} and x_{ijk} are the variables and n is their number. The coefficients were determined by multiple linear regression.

Experiment	Carbon Concentration (g/L)	OD (600 nm)	DBT (mg/l)	η*(Kpa.s)		
				***Gordonia desulfuricans* 213E**	***Bacillus* sp. 8A1531**	***Rhodococcus* sp. AF21875**
E1	10	0,1	0	3.01	2,25	3,03
E2	20	0,1	0	3.70	1,87	2,96
E3	10	1	0	2.40	2,28	2,95
E4	20	1	0	2.92	1,95	2,81
E5	10	0,1	3,12	2.76	2,01	2,30
E6	20	0,1	3,12	3.02	2,36	2,28
E7	10	1	3,12	3.11	2,47	1,65
E8	20	1	3,12	3.05	2,08	2,78
Center1	15	0,5	1,56	3.02	2,48	1,89
Center2	15	0,5	1,56	3.05	2,29	3,29
Center3	15	0,5	1,56	2.78	2,46	2,68

Table 2 Full factorial design and experimental response (complex viscosity)

Experiment	Carbon Concentration (g/L)	OD (600nm)	DBT (mg/l)	η*(Kpa.s)		
				***Gordonia desulfuricans* 213E**	***Bacillus* sp. 8A1531**	***Rhodococcus* sp. AF21875**
Control1	15	0	1,56	3.25	2,19	2,51
Control2	15	0	1,56	3.19	2,45	2,40
Control3	15	0	1,56	2.98	2,68	2,38

Table 3 Conditions and response of the controls

The analysis of variance (ANOVA) is normally used to determine which factors and which interactions have a significant influence on the process. In our 2^k factorial

designs, the use of ANOVA is questionable to discriminate whether the factors and interactions are real (Box et al 1978). Therefore, the relevance of the effects for the factors and interactions was also evaluated comparing each computed effect with the standard error (SE) through a t-test. The main and the interactive effects were calculated according to Box et al (1978).

The three N_c and three N_A were used to evaluate the experimental error and therefore the standard error for the effects by the following equations:

$$\sigma = \sqrt{\frac{N_C * \sigma^2_{N_C} + N_A * \sigma^2_{N_A}}{N_C + N_A}} \quad (2)$$

$$SE\ (effect) = \frac{2\sigma}{\sqrt{n}} \quad (3)$$

where n is the total number of runs of the two level factorial design and σ is the standard deviation of central experiments.

5.3.4 Complex Viscosity (η*)

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

As a response for the model, the value of η* was uniquely taken at the frequency of 30 Hz as show in Tab. 2, since the analysis was more stable at this frequency. Moreover, all the models obtained with the others frequencies gave the same significant factors and interactions with lower R^2 and Q^2.

5.3.5 Crosslink Density and Gel Fraction

In order to confirm that the devulcanization process took place, some samples (E3, E4, E5, E6 and control sample 1 of *G. desulfuricans* 213E design of experiment) were swelled in toluene and the crosslink density was calculated.

The crosslink density was determined according to ASTM D 6814-02 standard method through swelling measurements. About 1 g of each vulcanizate (W_1) was swelled in

cold toluene for 72 hours and the solvent was refreshed every 24 hours. After this period of time, the excess of solvent on the sample surface was removed with a paper towel and the swollen sample was weighed. Finally, the sample was dried in vacuum oven for 24 h and weighed again (W_2). The Flory-Rehner equation was used in order to calculate the crosslink density. The χ interaction parameter between rubber (NR) and swelling solvent (toluene) was equal to 0.39. The NR rubber density was 0.92 g/cm^3 (Valentín et al 2008)

The gel fraction was evaluated as:

$$Gel\ fraction\ (\%) = (W_2/W_1) * 100 \quad (5)$$

In order to investigate in more detail the relative effect of degradation of the main chain and of the crosslink network, the normalized gel fraction versus the normalized crosslink density was studied. The dependence of experimental normalized gel fraction versus normalized crosslink density was analyzed and compared to the Horikx function, that was derived from the statistical theory dealing with the gel fraction–crosslink density relationship (Horikx 1956, Huang and Isayev 2014).

5.3.6 DNA extraction

Total DNA was extracted from 0.5 g of VGNR (experiments E3, E4, E5 E6and Cotntrol1 of each design of experiment) and from 0.5 g of the VGNR after autoclave samples by using the FastDNA_SPIN for Soil Kit (MP Biomedicals, Solon, OH, USA). Also total DNA was extracted from a pure culture of *G. desulfuricans* 213E, *Bacillus* sp. 8A1531and *Rhodococcus* sp. AF21875 with ZR Soil Microbe DNA Miniprep kit (Zymo Research Corporation, Irvine, CA).

5.3.7 Automated ribosomal intergenic spacer analysis (ARISA)

ARISA is a PCR-based technique for the analyses of bacterial community structure and is based on amplification sizing of the16S-23S inter-genic region (Cardinale et al 2004). The 16S-23S inter-genic region has a heterogeneity in length and sequence. Every strain is characterized by the combination of the fragments of different length, usually

more than one due to the presence of multiple ribosomal operons in the bacterial genomes.

In the present study, this technique was used as to assess the persistence of the inoculated strain on VGNR. Ribosomal intragenic spacer was amplified by using both primer ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and primer ITSReub (5'-GCCAAGGCATCCACC-3') with a fluorescent probe (Cardinale et al 2004). PCR was performed in 40 µl containing 20 µl of 2X GoTaq Green Master Mix (Promega Corporation, Madison, WI), 5 µL of each primer (10 µM), 2 µl of DNA template and 8 µl of water. PCR program was the following: 94°C for 3 min, followed by 30 cycles at 94°C for 45 s, 55°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 7 min (Cardinale et al 2004). An aliquot of PCR reaction was analyzed by capillary electrophoresis using 2500 ROX (Applied Biosystems) as size standard for Peak Scanner (Applied Biosystems) analysis. Capillary electrophoresis results in an electropherogram where each peak represents an operative taxonomic unit (OTU).

The signed area of each peak was used to estimate the OTU relative abundance. A normalization was carried out to compare the OTU in different samples setting the sum of the all OTU in every sample equal to 10000. Finally, the OTU with a relative abundance minor than 1,5% were removed to reduce the noise. A principal component analysis (PCA) of the OTU relative abundance was performed using the STAMP software(Parks et al. 2014).

5.4 Results

5.4.1 Effect of the inoculum

The boxplots shown in Figure 1 report the distribution of the complex viscosity data obtained for the different inocula. The data of complex viscosity were normalized using the results from central points because there is a high difference between analyses carried out at different times. T-test revealed a significant reduction of the complex viscosity in the VNGR treated with *Bacillus* sp. 8A1531 compared with the uninoculated samples (p= 0.018). A lower viscosity was also observed in the design of

experiment with *G. desulfuricans* 213E compared to uninoculated sample, however the difference is not significant. The samples treated with *Rhodococcus* sp. AF21875 showed a high dispersion of values, thus its performance cannot be properly evaluated. The reason for this high experimental variability must be further investigated.

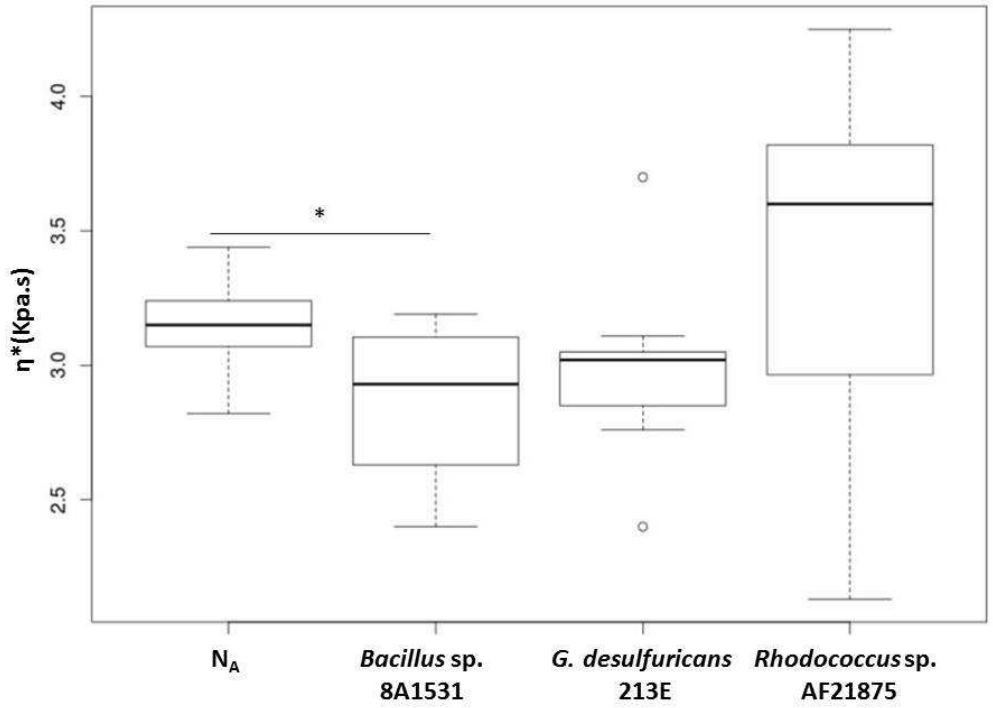

Figure 1 Boxplot of complex viscosity of samples without inoculum (N_A) and treated with *Bacillus* sp. 8A1531, *G. desulfuricans* 213 or *Rhodococcus* sp. AF21875 (* p= 0.018)

5.4.2 Regression models

The experimental conditions and the obtained responses are summarized in table 2. A preliminary regression model, evaluated for each response, was an interaction model including the four factors and all two-factor interactions and three factor interactions (Eriksson et al 2008). The ANOVA was carried out on this first model, thus it was possible to point out which factors and interactions were statistically significant. Tables 4, 5 and 6 show the results of ANOVA test for the response containing all the

factors and two-factor interactions. In both the experimental designs of *Bacillus* sp. 8A1531 and *Rhodococcus* sp. AF21875 no significant factors and interactions were identified (tab. 5 and 6).

This demonstrates that the growth parameters selected did not influence the devulcanization process. On the contrary, for *G. desulfuricans* 213E the significant factors C, OD, C*DBT and DBT*OD were identified. For this reason, the following analyses were conducted only on *G. desulfuricans* 213E data. The significance of factors and interaction was evaluated by estimation of effects and the standard error (SE) calculated as in Equations 2 and 3 and shown in Table 7.

Variable	Degree of Freedom	SS	P-value
Constant	1		<0.001
C	1	0.249	0.012
DBT	1	0.00101	0.793
OD	1	0.128	0.034
C*DBT	1	0.128	0.034
C*OD	1	0.03	0.201
DBT*OD	1	0.392	0.005
Residuals	4	0.0513	
Total	10	0.926	

Table 4 Analysis of variance for the interaction model of the responses of *G. desulfuricans* 213E experimental design.

In both cases, the terms whose P-value was higher than 0.05 were sequentially and systematically eliminated. The terms whose P value was lower than 0.05 were kept in the model since considered significant. Both these approaches identified the same significant factors and interactions.

Variable	Degree of Freedom	SS	P-value
Constant	1		<0.001
C	1	0,0703	0,338
DBT	1	0,0406	0,455
OD	1	0,0105	0,696
C*DBT	1	0,0561	0,386
C*OD	1	0,0595	0,373
DBT*OD	1	0,00061	0,924
Residuals	6	0,238	
Total	4	0,238	

Table 5 Analysis of variance for the interaction model of the responses of *Bacillus sp.* 8A1531 experimental design.

Variable	Degree of Freedom	SS	P-value
Constant	1		<0.001
C	1	0,101	0,588
DBT	1	0,938	0,148
OD	1	0,018	0,816
C*DBT	1	0,218	0,437
C*OD	1	0,146	0,52
DBT*OD	1	0,0008	0,961
Residuals	6	1,422	
Total	4	1,173	

Table 6 Analysis of variance for the interaction model of the responses of *Rhodococcus* sp. AF21875 experimental design.

A final reduced model was calculated by multiple linear regression for experimental response, considering only significant factors and interactions. Table 7 shows the scaled and centered regression coefficients and the coefficients of determination for the reduced model. The reduced regression model was statistically significant at 95% and without any lack of fit, considering the same probability (Box et al 1978, Myers and Montgomery 2002). Moreover the residual distribution, as shown in Figure 2, did not show evident anomalies. The normal distribution for the residuals was confirmed by the Shapiro-Wilk normality test at 99 % confidence level (Shapiro and Wilk 1965).

Variable	Estimated Effect	SE	P-value
Average	3.0	±0.1	<0.001
C	0.4	±0.1	0.016
DBT	-0.02	±0.1	0.851
OD	-0.3	±0.1	0.040
C*DBT	-0.3	±0.1	0.040
C*OD	-0.1	±0.1	0.374
DBT*OD	0.4	±0.1	0.016
C*DBT*OD	-0.04	±0.1	0.710

Table 7 *G. desulfuricans* 213E estimated effects and standard error (SE) calculated through N_c and N_A experiments (Eq. (2) and (3)).

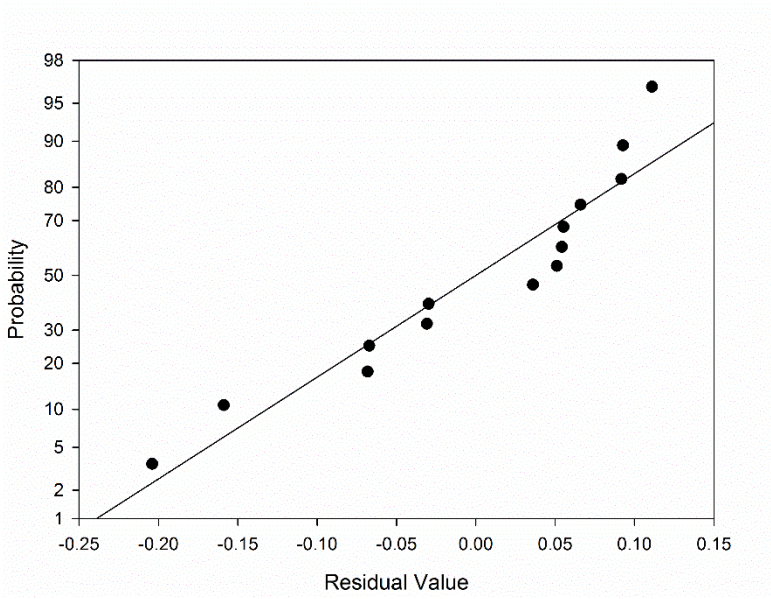

Figure 2. Normal probability plot of residualsVariable

From table 8 is also clear that DBT*OD and C resulted the most important variables, acting with a positive effect on the η*, on the other hand C*DBT and OD acts with a negative effect. The DBT resulted non-significant, however it was kept in the final model to preserve the hierarchy, since it is contained within significant higher-order interactions.

Variable	Estimated Effect	SE	R^2	R^2 adj	Q^2
Constant	2.98	0.03	0.89	0.82	0.60
C	0.18	0.04			
DBT	-0.01	0.04			
OD	-0.13	0.04			
C*DBT	-0.13	0.04			
DBT*OD	0.22	0.04			

Table 8 Regression coefficients and coefficients of determination.

In order to display the influence of the factors and interactions on the complex viscosity 3D-plot surfaces were analyzed and are shown in figure 3 (a, b and c) and figure 4 (a, b and c).

In particular, figure 3 shows a η* decreases alongside with a decrease of glucose as source of carbon from 20 g/L to 10 g/L and with a decrease of the DBT concentration from 0,312 mg/L to 0 g/L, especially at high value of OD. An increase in the medium initial bacteria concentration (OD) helps to reduce the rubber η*. When the bacteria were inoculated at high concentration of easily accessible source of carbon, they might preferentially use this compound without breaking the rubber carbon and the η* remain high.

The same behavior can be observed for the DBT. It is even clearer that when bacteria were inoculated with DBT as pathway inducer the bacteria may preferentially use this compound without breaking the rubber crosslinks and the η* remain high. Although the DBT was added at low concentration its effect was evident, since the sulfur is a micronutrient for the microorganisms and even a small amount of DBT is enough to avoid the rubber sulfur degradation.

From figure 4 the importance of initial OD related to the DBT concentration can be observed. As previously observed, higher OD was able to enhance the decrease of η*, however the importance of DBT as inducer of the desulfurization pathway, independently from the amount of carbon source and especially when OD is lower is evident. Indeed, in this case, the competition is low and the bacteria are more stimulated to induce the degradation pathway.

3D-plots and the final reduced model (Tab. 8) also show that, in order to induce bacteria to decrease the η*, it is necessary to keep the carbon source and DBT concentration at the lowest level and OD at the highest one. However, in this case we can observe that a main component for the η* reduction could be attributed both to the carbon and crosslink degradation. High concentration of C source can prevent the reduction of the η*, since the bacteria has enough carbon, limiting the rubber degradation. However, since the bacteria need sulfur to grow up and increase the biomass, a low DBT initial concentration, at high OD leads to a decrease in the η* due to utilization of sulfur crosslinks. It can be concluded that this condition maybe the most favorable one for a selective decrosslinking.

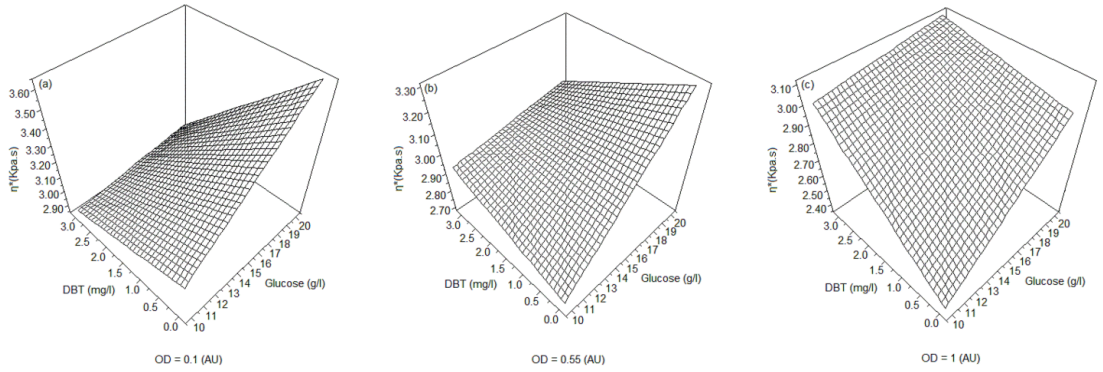

Figure 3 3-D plot for the experimental response as a function of DBT and Glucose.

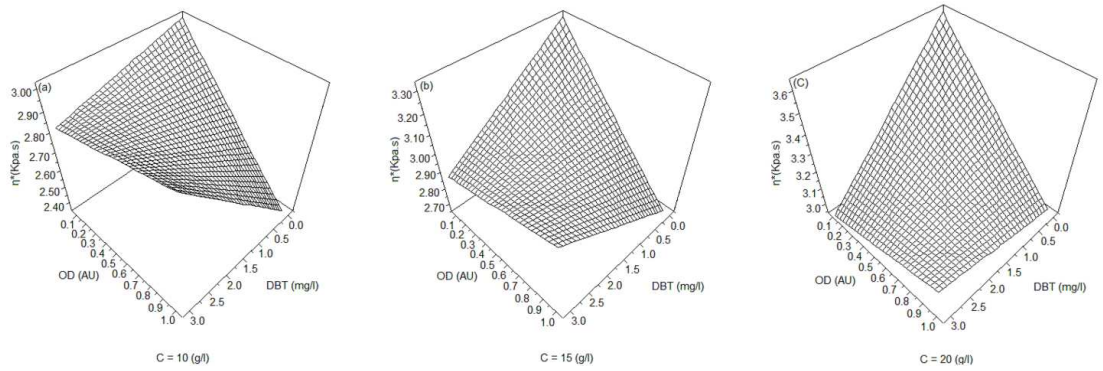

Figure 4 3-D plot for the experimental responses as a function of OD and DBT

Since it is difficult to determine the type of preferential bond breakage during the decrosslinking the Horikx function was used to analyze the relationship between the gel fraction and crosslink density. The analysis of soluble polymeric material (sol fraction) generated during the devulcanization process can be used to investigate the ratio between the main chain and crosslink scission (Huang and Isayev 2014). Figure 5 compares the experimental data (symbols) and the Horikx functions (Equation 6and 7).

$$1-\frac{v_{DVGNR}}{v_{VGNR}}=1-\frac{(1-(1-\zeta_{DVGNR})^{1/2})^2}{(1-(1-\zeta_{VGNR})^{1/2}{}^2} \quad (6)$$

$$1-\frac{v_{DVGNR}}{v_{IVGNR}}=1-\frac{\gamma_D(1-(1-\zeta_{DVGNR})^{1/2})^2}{\gamma_I(1-(1-\zeta_{IVGNR})^{1/2}{}^2} \quad (7)$$

where v_{IVGNR} and v_{DVGNR} are the crosslink density of initial and devulcanized VGNR, respectively.

ζ_{IVGNR} and ζ_{DVGNR} are, the gel fraction of initial and devulcanized, respectively. γ_I and γ_D are the crosslinking index before and after the devulcanization process. The value of M_n is available for the natural rubber STR-20 and therefore it was possible to calculate this parameter (Rajan et al 2006).

Equation 6 was used to plot the relationship between the normalized gel fraction and the normalized crosslink density of devulcanization VGNR in the case of the only main chain breakage. Equation 7 was used in case of the only crosslinks cleavage. Using these equations it was possible to describe the type of preferential bond breakage during devulcanization process.

Figure 5 shows the normalized gel fraction versus the normalized crosslink density of the VGNR biodevulcanization. The solid and dotted lines in Figure 5 show the breakage of only main chains and only crosslinks, respectively.

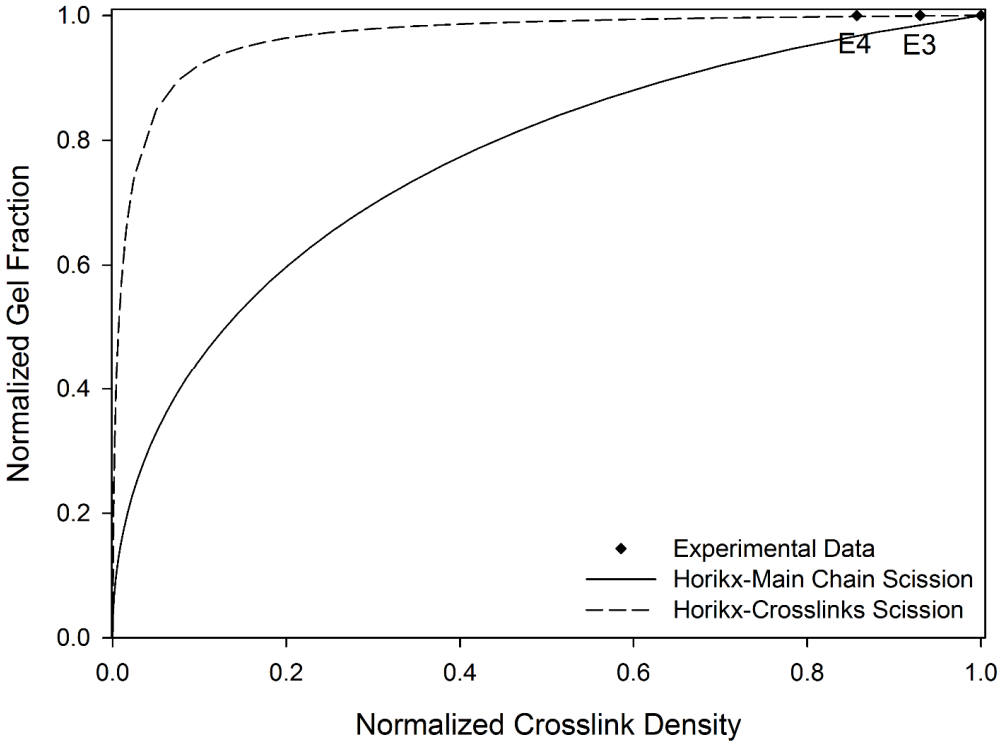

Figure 5 Normalized gel fraction as function of normalized crosslink density compared to the Horikx functions.

Some devulcanized samples show a decrease in the crosslink density without showing a decrease in the gel fraction (Figure 4). In particular, it can be observed that E3 and E4 samples show a slight decrease in the crosslink density and these two points coincide with the dotted Horikx function. In both these experiments the DBT concentration was kept at the lowest value. Therefore, it can be concluded that the biological treatment preferentially cleaves the crosslink network and the biodevulcanization is a selective process. However, all the data points are located in the area close to the value 1 for both the normalized crosslink density and normalized gel fraction.

5.4.3 Microbial characterization

ARISA was carried out on the conditions E3, E4, E5 and E6 (Tab. 2) and on VGNR after acetone extraction and autoclave treatment. Since in literature the ribosomal operons sequences of *G. desulfuricans* 213E, *Bacillus* sp. 8A1531, *Rhodococcus* sp. AF21875 has

never been reported and the number and the length of 16S-23S intergenic region in unknown, ARISA was also conducted on a pure culture of these three strains.

VGNR and GTR are complex materials with an elevated porosity. These polymeric materials are colonized to microbial populations during storage, transport or grounding. Indeed, chemical (acetone extraction) and thermal (autoclave) treatments were applied to reduce the bacterial continent. However, these treatments did not lead to a complete sterilization of the materials. Furthermore, sterilization treatment is a process not applicable at industrial scale. For these reasons, an effective treatment should assure the persistence of the inoculated bacteria and microbial monitoring should be used to observe, also, the survival of inoculated strain during the competition with the naturally bacterial populations hosted on VGNR or GTR.

Actually, some ARISA fragments were detected in the VGNR sample after acetone and autoclave treatment (Figure 6). Although we cannot exclude that these fragments are due to the amplification of DNA from dead organisms. The fingerprinting profile of the uninoculated samples (control1) after incubation revealed that live microbial populations actually resisted to chemical and thermal treatments on VGNR. Particularly, the fragment 596 base pairs (bp) was shared between GNR and Control1.

Furthermore, in the figure 6 a difference in the communities of samples VGNR and Control1 was observed. This indicates a change in the community during the experiment and it confirms the presence of bacterial survive to the sterilization treatments.

5.4.3.1 *G. desulfuricans* 213E

In the figure 7 the characteristic fragments of *G. desulfuricans* 213E are reported. These fragments were actually found in samples E3, E4, E5 and E6 (Figure 8). This demonstrates that the *G. desulfuricans* 213E persisted under the four tested conditions of the experimental design. However, in E4 and E6 samples the fragment 596 bp, typical of the autochthonous rubber microbial community, was also detected. From figure 6 can be also observed that 582 bp fragment is the second abundant fragment in the Control1 sample and it was also present at 35 % in all the other

treated samples (Fig. 8). This demonstrate that, despite the inoculum addition, others bacterial populations grew.

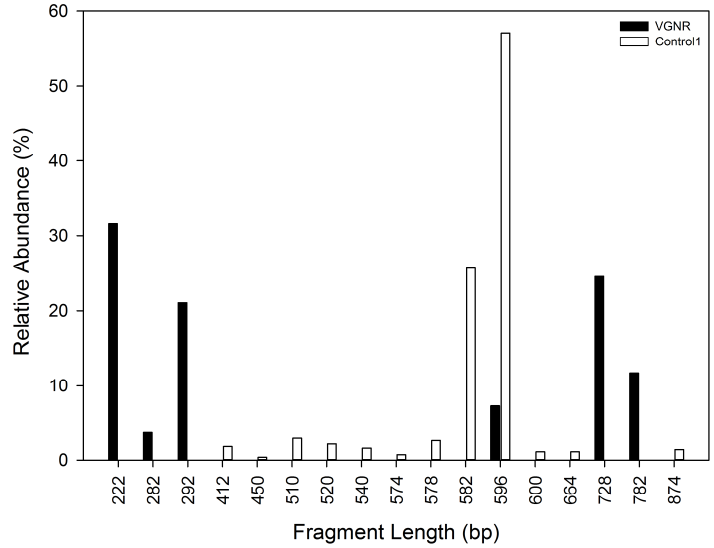

Figure 6 Control1 and VGNR ARISA profile

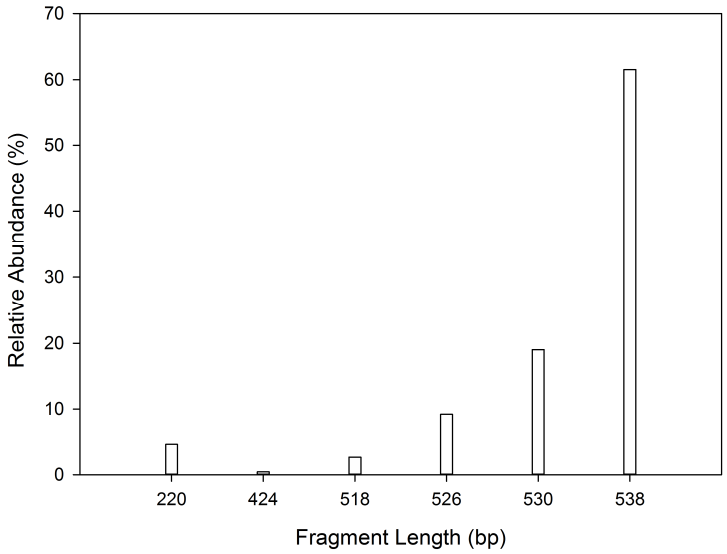

Figure 7 *G. desulfuricans* 213E ARISA profile

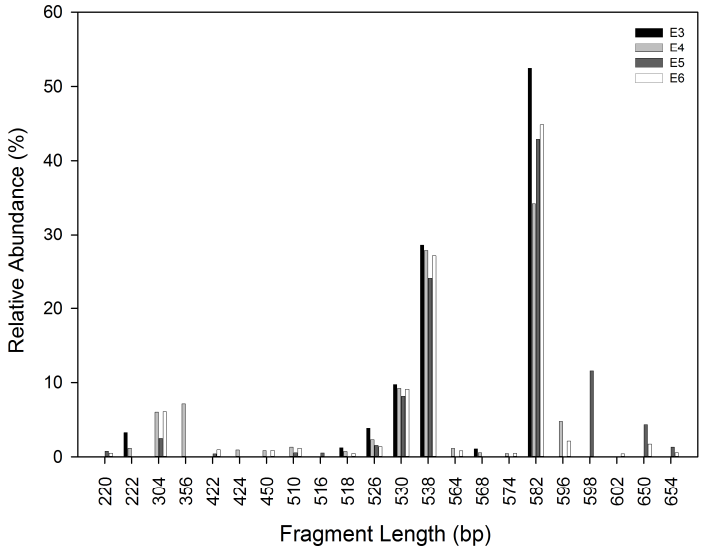

Figure 8 ARISA profile of the experiments 3 (E3), experiments 4 (E4), experiments 5 (E5), experiments 6 (E6) of *G. desulfuricans* 213E design of experiments

5.4.3.2 *Bacillus* sp. 8A1531

The characteristic fragments of this strain are 304, 422, 450, 510, 564, 582, 596, 648 and 652 bp (Fig. 9). The most abundant fragments are 304, 510, 582 and 648 bp. Several characteristic fragments of *Bacillus* sp. 8A1531 have the same length of fragments found in the control1 (450, 510, 582 and 596 bp) and VGNR sample (596 bp). For this reason it was not possible to disentangle between inoculated strain and the naturally population hosted on the VGNR.

In the sample E3, E4, E5 and E6 the most abundant fragment was 582 bp (Fig. 10). Also the fragment 596 bp was recovered in all samples. Despite, characteristic fragments 304 and 510 bp are present at the end of the experiments in all experiments, it is not possible to assert that the strain persisted up to the end of the design of experiment.

5.4.3.3 *Rhodococcus* sp. AF21875

The characteristic fragments of *Rhodococcus* sp. AF21875 are 410, 412, 424 and 426 bp (Fig. 11). The lasts two are the more abundant fragments. Unlike the previous analyses, the most abundant fragment in the treated VGNR is not 582 bp (Fig. 12). Indeed, the most abundant fragment is 220 bp for the sample E3, 556 bp for the

sample E4, 426 for the samples E5 and E6. In the sample E3 no characteristic fragment of AF21875 strain was found. This could indicate that the strain do not persist under this condition. In the samples E4, E5 and E6 the characteristic fragments of *Rhodococcus* sp. AF21875 (424 and 426 bp) were found. This indicates that the strain persisted in these three different conditions.

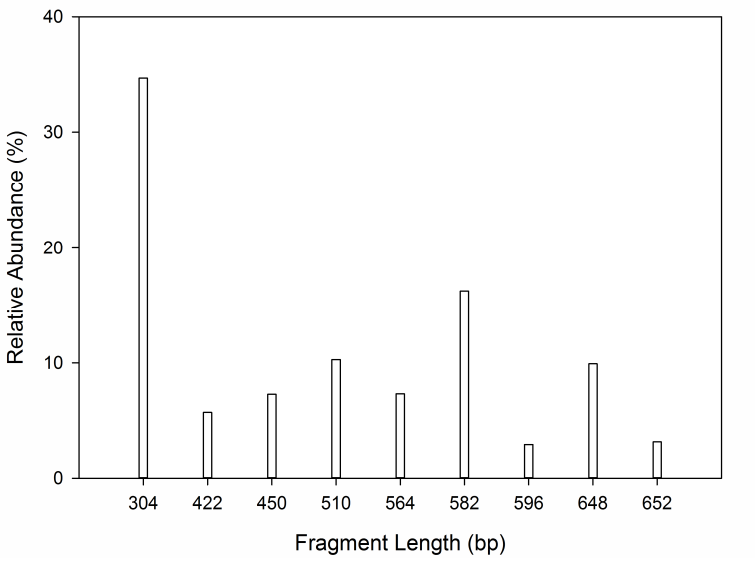

Figure 9 *Bacilllus* sp. 8A1531 **A**RISA profile

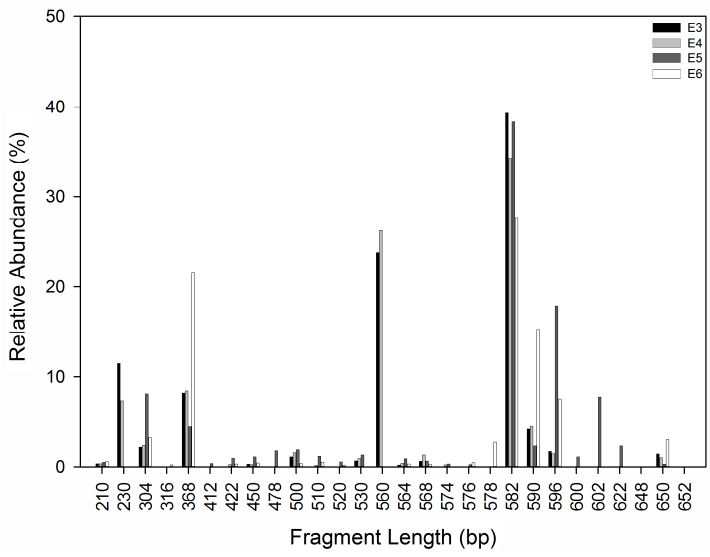

Figure 10 ARISA profile of the experiments 3 (E3), experiments 4 (E4), experiments 5 (E5), experiments 6 (E6) of *Bacillus* sp. 8A1531 design of experiments

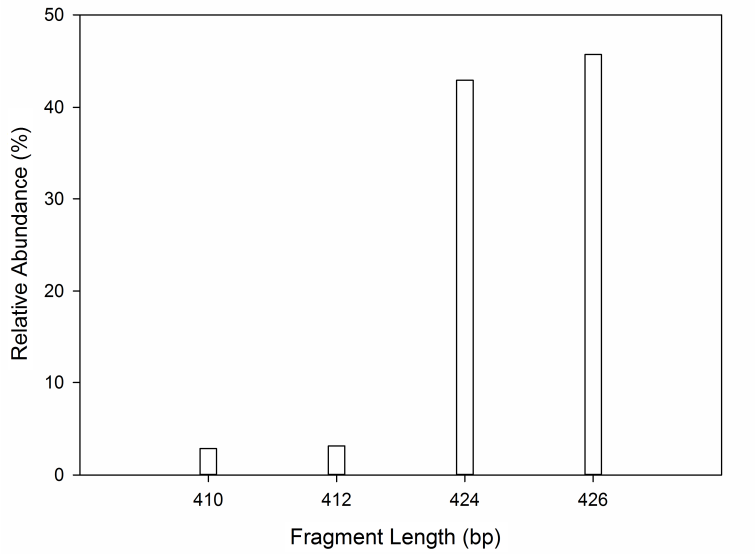

Figure 11 *Rhodococcus* sp. AF21875 and VGNR ARISA profile

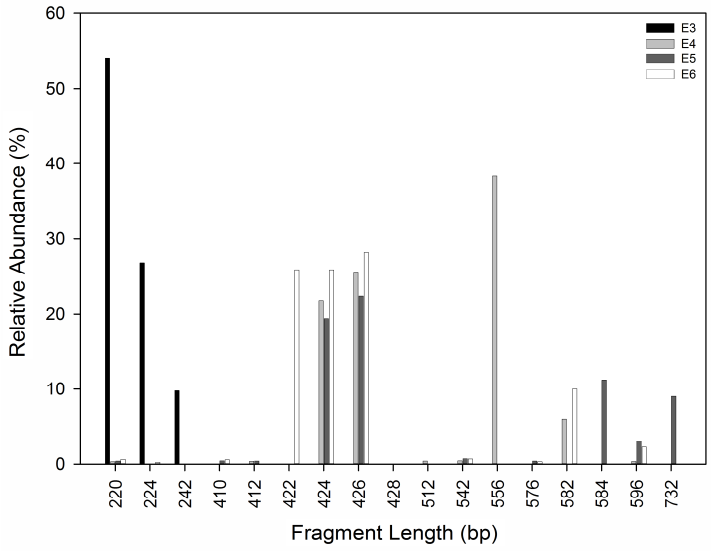

Figure 12 ARISA profile of the experiments 3 (E3), experiments 4 (E4), experiments 5 (E5), experiments 6 (E6) of *Rhodococcu* sp. AF21875 design of experiments

5.4.3.4 Microbial community structures

ARISA profiles were also used to infer β-diversity among the microbial communities. PCA revealed that the VGNR collected from different experiments formed three separate clusters (Fig. 13). These data confirmed that the microbial communities developed in the three sets of experiments were different. Furthermore, the cluster is

close to the strain inoculated profile in each set of experiments. Moreover, the contol1 profile is close to the sample inoculated with *Bacillus* sp. 8A1531. This confirms that it is not possible to assert that the strain persisted till the end experiment.

The community on the VGNR is similar to the community found in the experiments inoculated with *Rhodococcus* sp. AF21875. We also observed a shift in the VGNR bacterial community during the experiments without inoculum. Indeed, the community profile of the Control1 is closer to the profile of *Bacillus sp. 8A1531* than to VGNR community profile.

5.5 Discussion

In literature some papers reported the biodesulfurization of ground tire rubber (Hu et al 2014, Christofi et al 2010). Nevertheless, in none of these works a design of experiment was carried out to study the best condition, and parameters that could influence the bacterial desulfurization ability. For this reason, biodesulfurization of VGNR was testetd in this study by a full factorial experimental design. Biodesulfurization process was carried out with three bacterial strains: i) *G. desulfuricans* 213E ii) *Bacillus* sp. 8A1531 iii) *Rhodococcus* sp AF21875. The first strain was described in a patent on biodesulfurization of ground tire (Christofi et al 2010). For this reason, this strain was used as reference strain. Furthermore, in the patent the growth conditions were not reported in details, therefore we tested this strain to obtain more information on its growth condition for the desulfurization of vulcanized polymeric material. Other two strains have been isolated from soil in a tire dumpsite and from process water in a tire factory and have the ability to desulfurize DBT, thus being promising for the desulfurization of rubber structure.

Therefore, in the present study, growth parameters potentially affecting the complex viscosity of VGNR were studied. Furthermore, the frequency sweep analysis was supported with a microbiological analysis to assess the persistence of the inoculated strain during the experiments.

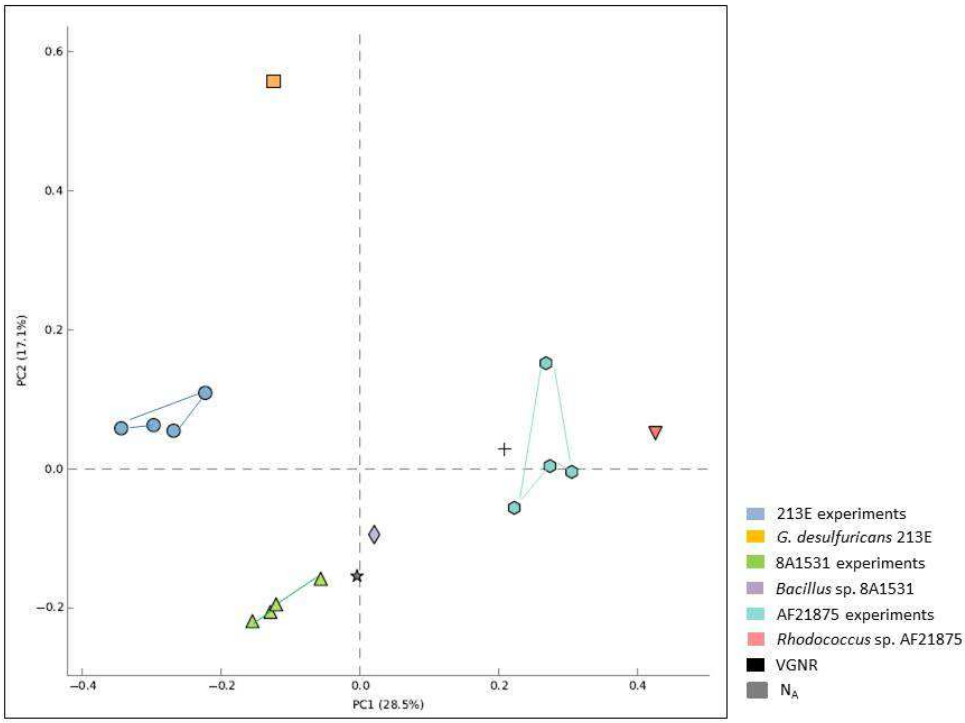

Figure 13 Principal Component Analysis (PCA) of the OTU relative abundance. Design of experiments inoculated with *G. desulfuricans* 213E (213E experiments- dark blue circles *),* Design of experiments inoculated with *Rhodococcus* sp. AF21875 (AF21875 experiments - blue hexagon); Design of experiments inoculated with *Bacillus* sp. 8A1531(8A1531 experiments - green triangles); pure culture of *Bacillus* sp. 8A1531 (*Bacillus* sp. 8A1531 - violet diamond), pure culture of *Rhodococcus* sp. AF21875 (*Rhodococcus* sp. AF21875- pink triangles), pure culture of G. desulfuricans 213E (G. desulfuricans 213E- orange square), VGNR (VGNR- black cross), N_A (non inoculated experiment- grey star). Cluster 1 =213E-E3, 213E-E4, 213E-E5, 213E-E6; Cluster 2 = AF21875-E3, AF21875-E4, AF21875-t5, AF21875-E6; Cluster 3 = 8A1531-E3, 8A1531-E4, 8A1531-E5, 8A1531-E6.

VGNR treated with *Bacillus* sp. 8A1531 showed η* significantly lower compared with the control experiments. For *G. desulfuricans* 213E a lower viscosity was observed but the difference is non significant compared to the non inoculated samples. For *Rhodococcus* sp AF21875 a high experimental variability was observed.

A final reduced model was obtained only for *G. desulfuricans* 213E; for the other strains no significant models resulted. For *G. desulfuricans* 213E the model indicates

that the lowest η*is obtained when the carbon source is minimum (10 g/L), the DBT is absent and the initial OD is equal to 1 but in this case the η* reduction might be attributed both to the carbon and crosslink degradation.

Therefore, for the future experiments it is advised to use a high concentration of carbon source, absence of DBT and a high OD. This condition is the most favorable one for a selective decrosslinking.

It is difficult discern between the S-S or S-C and C-C breakage during the treatment the Horikx function was used to analyze the samples treat with *G. desulfuricans* 213E with the lowest η*. The results showed that during the biological treatment the crosslink network was broken. This demonstrated that the biodevulcanization is a selective process.

However, it is clear that the biological devulcanization is a low yield process limited to the surface due to the bacteria dimensions unable to penetrate the rubber matrix.

Yao and colleagues (2013) reported the biodesulfurization process on waste latex rubber (WLR) with *Alicyclobacillus* sp.. Latex rubber is more simple than GTR, indeed it has low additive contents and low biological toxicity. Unlike GTR, for natural rubber and WLR it is possible to draw the Horiks function. Also in this case, the main chains remained undamaged and sulfur bonds were broken. This confirm that biodesulfurization was an effective and selective process. Nevertheless, this method is not sufficiently sensitive to note the difference between the samples.

In literature only five bacterial strains were reported with desulfurization ability on rubber: *Thiobacillus* sp*., Thiobacillus ferrooxidans*, *Alicyclobacillu*s sp., *Sphingomonas* sp. and *G. desulfuricans* 213E.

In these works, the authors attend to structural analysis of devulcanized rubber. In particular, the surface chemical groups devulcanized GTR were analyzed with Fourier Transform Infrared Spectroscopy-Attenuated Total Reflectance (FTIR-ATR) and X-ray Photoelectron Spectroscopy (XPS) and water contact angle. Through FTIR-ATR the chemical groups on the surface of the rubber, before and after the treatment, was analyzed (Yao et al 2103) but the surface elements and the bonding state on the rubber surface exposed to the liquid medium were analyzed by XPS. On the contrary,

the wettability of rubber surface was measured with the water contact angle (Li et al 2012b). After treatment, it was found by XPS that the frequency of S-S bonds and S-C bonds were decreased during the treatment and the formation of S-O bonds was observed on the surface of devulcanized rubber (Yao et al 2013). In general (Li et al 2012b, Yao et al 2013 and Li et al 2012a), the XPS results showed that oxygen content on the surface of devulcanized rubber increased. The oxidization reaction might be catalyzed by enzymes in the microorganisms, and this improve the surface activity and reprocessing ability of desulfurated rubber. These results suggested that a like 4S pathway was used by microorganisms during this process (Li et al 2012a). This was also confirmed by the FTIR analyses. Indeed, this analysis highlighted that the peak of C-H and C-H_2 bond did not change after the treatment (Li et al 2012a). On the contrary, the S-C peak disappears and the peak of S-O increases (Yao eta al 2013). These results confirm that some crosslinked sulfur bonds on the surface of rubber were broken to form sulfone groups.

Through the water contact angle analysis was observed that rubber surface property changed gradually from lipophilic to hydrophilic (Yaho et al 2013). Probably, this was due to the oxidization desulfurization reaction on the surface of devulcanized rubber (Li et al 2012b).

However, in previous works the attention were point out mainly to the rubber structure while only the growth of bacteria was assessed as microbiological analyses. Indeed, the bacterial community and persistence analyses of the inoculated strains were not conducted.

For this reason, in our work we combined the frequency sweep analysis with fingerprinting method ARISA to characterize the microbiological community.

Microbiological analyses confirmed that bacterial populations resisted to thermal and chemical pre-treatment and grew during incubations. Despite that, in the presence of *G. desulfuricans* 213E and *Bacillus* sp. 8A1531 η* is lower than the control. Therefore, for *Bacillus* sp. 8A1531 the growth parameters selected did not influence the devulcanization process. Furthermore, the community fingerprinting analysis that *G. desulfuricans* 213E and *Rhodococcus* sp. AF21875 persisted in all the experiments. For

the strain *Bacillus* sp. 8A1531, it is not possible to assert that the strain persist up to the end of the design of experiment, because its community is similar to the non-inoculated samples. For *Bacillus* sp. 8A1531 is necessary to repeat the experimental design wider range of conditions or take in consideration other growth parameters, while for *Rhodococcus* sp. AF21875 is necessary to investigate experimental variability with another experimental design. In this paper, it is highlights the need to monitor the bacterial community in the experiment and in no inoculated sample to observe the persistence of inoculated strain. It, should be investigated the presence of desulfurize ability in the bacterial community harbors on GTR.

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Chapter 6

Characterization of microbial community during a GTR desulfurization process

6.1 Abstract

The strains *Gordonia desulfuricans* 213E, *Rhodococcus* sp. AF21875 and *Bacillus* sp. 8A1531A are bacteria with desulfurizing capability; they were tested in a GTR biodesulfurization process. Three different bioreactors were set up in order to curry out the process in a controlled environmental. A community fingerprinting, automated ribosomal inter-genic spacer analyses (ARISA), were conducted on sample collected after different times during the experiment to detect the persistence of the inoculated bacteria and to compare the community of different bioreactors. Furthermore, the abundance of total bacteria was estimated through qPCR and the quantification of the number of copies of the 16S rRNA gene. The catabolic *dsz*A genes were, also, quantified in order to estimate the biodesulfurization potential. The community fingerprinting analysis showed persistence of *Gordonia desulfuricans* 213E, for others two strains it was not possible to confirm the persistence. This is due to the presence of the same fragments also in the GTR before the treatment. Furthermore, a change in the community was observed in all the three bioreactors. In particular the communities tend to became similar to the GTR community. Nevertheless, in all three bioreactors an increase of *dsz*A was observed. This could be indicating a selection of bacterial with this ability inside the bioreactor. A microbial characterization is therefore suggested to monitor the change in community and the persistence of the inoculated strain.

6.2 Introduction

In vulcanization process, long chains of the rubber molecules are linked together by crosslinks of one or more sulfur atoms. These crosslinks modify the properties of the rubber improving elasticity and strength to the polymer (Flory 1953). The formation of this network is a non-reversible chemical process. Consequently, this three-dimensional crosslinks impedes the tire recycling.

This chemical process, united with increasing of tire production, represents an important environmental problem (Amari et al 1999). The grinding of waste tire is one of the most used recycling techniques: the process consists in the pulverization of waste rubber after rubber separation from metallic and texture parts of tire. In this way ground tire (GTR) is obtained, however its introduction in new blends with virgin rubber results in bad properties. This is due to the presence of the sulfur crosslink that leads to a weak adhesion and deterioration of the final properties (Naskar et al 2000). Devulcanization of GRT is therefore necessary to increase the quality of the final product. In literature, different devulcanization processes, such as chemical, mechanical and thermo-mechanical (Adhikari et al 2000 and Rajan et al 2006), have been proposed in order to increase the compatibility between GRT and natural rubber in the new blends. Nevertheless, the selectivity of these methods is low, also they consume a large quantity of energy, require toxic reagents, and cause secondary pollution.

In recent years microbial desulfurization has been proposed as alternative process. Indeed, this process is safe for environmental, economic and more selective than mechanical and physical process. The first study of biodesulfurization of GTR was conducted in 1990 by Torma and Raghavan. In this experiment, *Thiobacillus ferrooxidans* and *T. thiooxidans* was added to the rubber material containing 15.5% sulfur and the sulfate concentration was measured at final time equal to 350 ppm. The studies on chemolithotrophs were continued until 2011. Indeed, Li and colleagues (2011) observed that GTR devulcanized, by *T. ferrooxidans,* filled NR composites had

better mechanical properties and lower crosslink density than the non devulcanized GTR filled NR composites.

Starting form 2010, also the chemoorganotrophs have been studied. when *Gordonia desulfuricans* 213E (NCIMB 40816) was described (Christofi et al 2010). After the treatment with this strain the sulfur content varied between 23% and 35% respect to the original content and when this devulcanized GTR was blended with virgin rubber, the sample showed good tensile strength. Yao et al (2013) reported the desulfurization effect of *Alicyclobacillus* sp. on waste latex rubber. The toxic effect of this kind of rubber on *Alicyclobacillus* sp. was not conspicuous and the addition of the surfactant Tween 80 was used to increase the contact of waste latex rubber and *Alicyclobacillus* sp. to improve the desulfurization. Recently, Li (2012) used *Sphingomonas* sp. to ground tire rubber desulfurization. This strain showed biological activity on ground tire rubber and the supplement of surfactant, during the process, improves the ground tire rubber desulfurization (Hu et al 2014). In all these articles, the desulfurization process was analyzed through the following analyses: i) X-ray photoelectron spectroscopy analyzer (XPS) to characterize the sulfur bonding states; ii) Fourier transform infrared spectrometry (FTIR spectra) to analyze the chemical groups on the surface of GTR; iii) sol fraction, it represents the soluble polymer fraction extracted by a suitable solvent, an increase of this parameters is due to the higher quantity of short chain; iv) crosslink density and iv) mechanical proprieties. Although the biodevulcanization process requires the addition of bacterial inocula in the process reactor with the contemporary presence of GRT autochthonous microbial community, in all the aforementioned works only basic microbiological monitoring was applied. Typically, only bacterial growth was measured by optical density at 600 nm (OD) or as dried weight and the estimation of bacterial attachment on GTR surface with the SEM. To the best of our knowledge no studies addressed neither the persistence of the inoculated bacterial strain nor the dynamics of the microbial communities during treatment.

In the present study, *Gordonia desulfuricans* 213E, *Rhodococcus* sp AF21875 and *Bacillus* sp. 8A1531 were selected as inocula for GTR desulfurization. Automated

ribosomal inter-genic spacer analyses (ARISA) were conducted on samples collected during treatment. Furthermore, the bacteria abundance on GTR was determined by the quantification of the number of copies of the 16S rRNA gene and the *dsz*A gene was quantified to estimate the abundance of desulfurizing bacteria on GTR.

6.3 Materials and methods

6.3.1 Chemicals and reagents

Gordonia desulfuricans 213E (NCIMB 40816) was purchased from Lebeniz Institut DSMZ-"German Collection of Microorganisms and Cell Culture". Constituents of mineral salt medium (MSM) were purchased from Sigma Aldrich (Germany)**.**

The ground truck-tire rubber (GTR), used in the present study, had dimensions smaller than 0.4 mm and it was a cryo-ground rubber from whole truck tires. The rubber fraction of truck was mainly composed by natural rubber (NR) and synthetic rubber (Butadiene Rubber, BR and Styrene-Butadiene Rubber, SBR) (Mangili et al 2014).

6.3.2 Preparation of media

Sulfur free MSM (pH 7.0) (Chang et al 1998) was prepared by dissolving, 4.5 g/l of K_2HPO_4, 1.5 g/l of NaH_2PO_4, 2 g/l of NH_4Cl, and autoclaved at 121 °C for 30 minutes. Then, after sterilization by filtration, the following compounds were added: 20 g/L of glucose, 0,2 g /l of $MgCl_2$; 0,02 g/l of $CaCl_2*2H_2O$; 10 ml/L vitamin (RPMI 1640 Vitamins Solution-Sigma Aldrich- Germany); 5 mg/l $MnCl_2*6H_2O$; 0,5 mg/l H_3BO_3; 0,5 mg/l $ZnCl_2$; 0,5 mg/l $CoCl_2*6H_2O$; 0,46 mg/l $NiCl_2*6H_2O$; 0,3 mg/l $CuCl_2$, 0,1 mg/l $NaMoO_4*2H_2O$; 1,49 mg/l $FeCl_2*4H_2O$; 0,003 mg/l $NaSeO_3$; 0,008 mg/l Na_2WO_4 (Widdel et al., 1992).

6.3.3 Desulfurization process

A bioreactor (Biostat B, Sartorius Stedim Biotech S.A) was set up to test the ability of *Gordonia desulfuricans* 213E, *Rhodococcus* sp. AF21875, *Bacillus* sp. 8A1531 to desulfurize GTR. In previous work (see chapter 5), indications of activity was obtained

by design of experiments. The following conditions were used to set up the bioreactors containing *Gordonia desulfuricans* 213E, *Rhodococcus* sp. AF21875. The final volume of the medium was 1,5 L. The growth medium was the sulfur free MSM with an initial glucose concentration equal to 20 g/l and 150 g of GTR as sulfur source. The tests were conducted for ten days at 500 rpm, 30°C and pH 7. The bioreactor was previously autoclaved with GTR and the MSM. After that, thermo-labile components of the medium were added and the bacteria were inoculated at OD equal to 1. Glucose concentration was monitored every 2 days and added only if the concentration was found lower than 10 g/l. GTR was sampled after the addition of the inoculum, after 5 days and at the end of the experiment (10 days). In the bioreactors with *Bacillus* sp. 8A1531 initial glucose concentration was equal to 10 g/l, also the glucose concentration was not monitored.

6.3.4 DNA extraction

Total DNA was extracted from 0,5 g of untreated GTR and from 0,5 g of the GTR treated at different times using FastDNA_SPIN for Soil Kit (MP Biomedicals, Solon, OH, USA). Total DNA was extracted from a pure culture of *G. desulfuricans* 213E, *Bacillus* sp. 8A1531and *Rhodococcus* sp. AF21875 by using ZR Soil Microbe DNA Miniprep kit (Zymo Research Corporation, Irvine, CA).

6.3.5 Automated ribosomal intergenic spacer analysis (ARISA)

Ribosomal intragenic spacer was amplified by using primers ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') labelled with fluorescent probe (Cardinale et al 2004). PCR was performed in 40 µl containing 20 µl of 2X GoTaq Green Master Mix (Promega Corporation, Madison, WI), 5 µL of each primer (10 µM), 2 µl of DNA template and 8 µl of water. PCR program was the following: 94°C for 3 min, followed by 30 cycles at 94°C for 45 s, 55°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 7 min (Cardinale et al 2004). An aliquot of PCR reaction was analyzed by capillary electrophoresis using 2500 ROX (Applied Biosystems) as size standard for Peak Scanner (Applied Biosystems) analysis. Capillary

electrophoresis results in an electropherogram where each peak represents an operative taxonomic unit (OTU). A principal component analysis (PCA) of the OTU relative abundance was performed using the STAMP software (Parks et al. 2014).

6.3.6 Dibenzothiophene (DBT) sulfone monooxygenase (*dsz*A) gene and 16SrRNA gene quantification

The bacteria abundance on GTR was determined by the quantification of the number of copies of the 16S rRNA gene. These number of copies are not directly associated to cell number due to the presence of multiple ribosomal operons in the bacterial genomes; however, they can be used to detect at relative shift in microbial biomass that is a likely constant bias in the number of copies (Bertolini et al. 2013). A 466-bp fragment of the bacterial 16S rDNA (331–797 according to *Escherichia coli* position) was PCR-amplified with a universal primer set (Nadkarni et al. 2002). The PCR was performed in a total volume of 10 µL using the FluoCycleII Sybr reaction mix (Euroclone, Pero, Italy) with 0.3 µM (final concentration) of forward and reverse primers. The amplification was carried out under the following conditions: 95 °C for 4 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s, with acquisition of the fluorescence at the end of each 72 °C elongation step. *dsz*A gene was quantified to observe the desulfurization potential ability of bacteria on GTR. This gene was involved in the DBT desulfurization (Alves et al 2007). Although, there is no evidence that *d*szA is involved in GTR desulfurization, all the inoculated strains harbors *dsz* genes. PCR mix was prepared as previously described for the 16S rRNA gene and the amplification was performed under the following conditions: 95 °C for 4 min, followed by 40 cycles at 95 °C for 15 s, 65 °C for 30 s and 72 °C for 45 s, with acquisition of the fluorescence at the end of each 72 °C elongation step. Fragments of interest were amplified from reference strains (*E. coli* K-12 substr. DH10B for 16S RNA gene and *Rhodococcus rhodochrous* IGTS8- ATCC 53968 for *dszA*) and cloned into the plasmid pCR2.1 (Life Technologies Italia, Monza, Italy) in order to prepare standards for calibration curves. The concentration of plasmidic DNA was measured with a

NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Serial dilutions of the plasmidic DNA were included in triplicate in each run together with the samples.

6.4 Results

6.4.1 Strains Persistence

The bacterial community present on GTR before, during and after the treatment was characterized by ARISA. The strains persistence is revealed by the presence of the characteristic fragments at the final time. In the figure 1 the characteristic fragments of *G. desulfuricans* 213E compared with the fragments present in GTR before the treatment are reported. *G. desulfuricans* 213E characteristic fragments are 220, 518, 526, 530 and 538 bp. The lasts three are the more abundant fragments. The characteristic fragments of GTR untreated are 294, 300, 304, 408,412, 424, 426, 450, 510, 564, 568 and 744. Clearly, no fragments of *G. desulfuricans* 213E are present among the GTR fragments (Fig. 1). In figure 2, ARISA fragments of G. *desulfuricans* 213E are reported for three different times. Remarkably, both fragments typical of the pure strain (530 an538) and of the GTR (294, 426 and 450) were retrieved in the sample collected immediately after the inoculation (213E-B). Other fragments (354, 356 and 528) were observed no belonging to G. *desulfuricans* 213E and GTR. Conversely, in the following time (213-M) only one inoculum fragment was detected and only three fragments detected were from GTR. However, new fragments were observed (262,452, 540, 566, 584 and 598). In the final time (213-F) the characteristic fragments of *G. desulfuricans* 213E were observed; particularly the fragment 538 bp is the most abundant one. The characteristic fragments of GTR were observed and also the other fragments found in the previous time. The inoculated strains persist during the experiment.

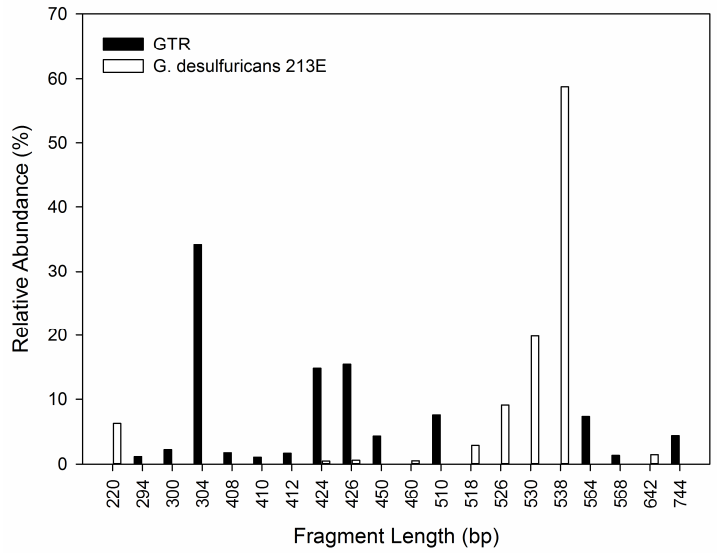

Figure 1 ARISA profile of bioreactor inoculated with *G. desulfuricans* 213E and GTR profile

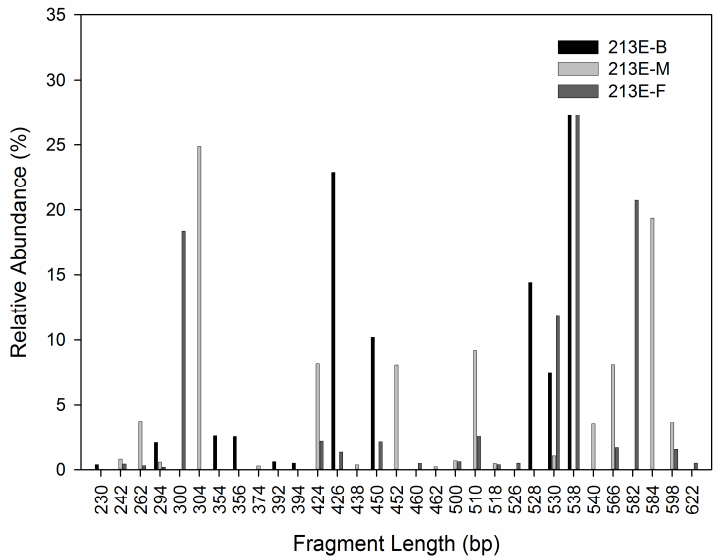

Figure 2 ARISA profile of bioreactor inoculated with *G. desulfuricans* 213E at different times: beginnig time (213E-B), medium time (213E-M) and final time (213E-F)

The characteristic fragments of *Rhodococcus sp.* AF21875 are long 410, 412, 424 and 426 base pairs (Fig. 3). The lasts two are the more abundant fragments. In this case the characteristic fragments are also found in the GTR.

For this reason it is not possible to discern between inoculated strain and the naturally population hosts in the GTR. After the inoculum (AF21875-B), all four characteristic

fragments were observed (Fig. 4). In addition to the fragments 424 and 426, also the GTR fragment 510 was observed. Other fragments were observed (224,366, 374, 538, 566 and 592). After 5 days of growth (AF21875-M), only the characteristic fragments long 424 and 426 are found. The GTR characteristic fragments (304,450 and 510) and the new fragments (474 and 584) were observed. The more abundant fragment was the fragment 592. At the final time (AF21875-F) the *Rhodococcus sp.* AF21875 characteristic fragments were found and they were the more abundant fragments. However, it is not possible to assert that the strain persisted up to the end of experiment.

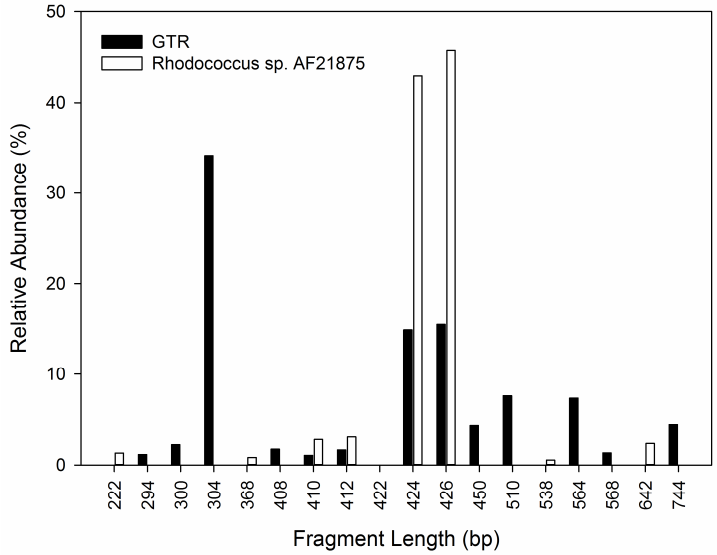

Figure 3 ARISA profile of bioreactor inoculated with *Rhodococcus* sp. AF21875 and GTR profile

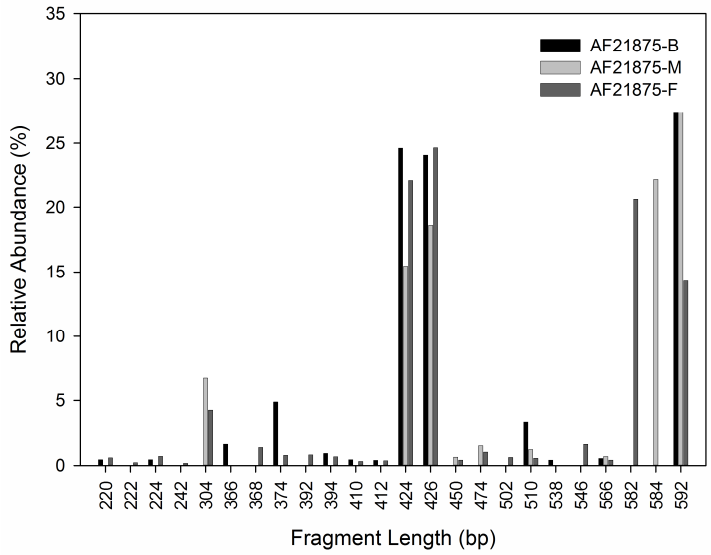

Figure 4 ARISA profile of bioreactor inoculated with *Rhodococcus* sp. AF21875 at different times : beginnig time (AF21875-B), medium time (AF21875-M) and end (AF21875-F)

The characteristic fragments of *Bacillus* sp. 8A1531 are long 304, 422, 450, 510, 564, 582, 596, 648 and 652 base pairs (Fig. 5). The most abundant fragments are 304, 510, 582 and 648. Also in this case, it was observed that more fragments are superimposed to GTR fragments (Fig. 5). In the beginning of the experiment, the more abundant fragment was 230, it does not belong neither to *Bacillus* sp. 8A1531 nor GTR (Fig. 6). In half of the experiment (8A1531-M), more characteristic fragments of *Bacillus* sp. 8A1531 (304, 422, 510 and 582) and the GTR fragment 426 were observed. Other new fragments were also observed (460, 530 568,756 and 784). At the end of the experiments (8A1531-F), the more abundant fragment of *Bacillus* sp. 8A1531 and the new fragments (874 and 538) were observed. Also in this case, it is not possible to assert that the strain persisted up to the end of the experiment.

ARISA profiles were also used to infer β-diversity among the microbial communities. PCA revealed that the communities on GTR collected from different bioreactors formed three separate clusters (Fig. 7). These data confirmed that the microbial communities developed in the three bioreactors were different. Furthermore, for *G. desulfuricans* 213E and *Rhodococcus* sp. AF21875 each cluster is close to the strain inoculated in that bioreactor, thus confirming that the addition of these inocula

completely unbalanced the microbial community present on the GTR. In the bioreactor inoculated with *G. desulfuricans* 213E, a shift in the bacterial community occurred during the experiment. Indeed, in the sample collected immediately after the inoculum (213E-B) the community fingerprinting is very closed to the profile of the pure strain.

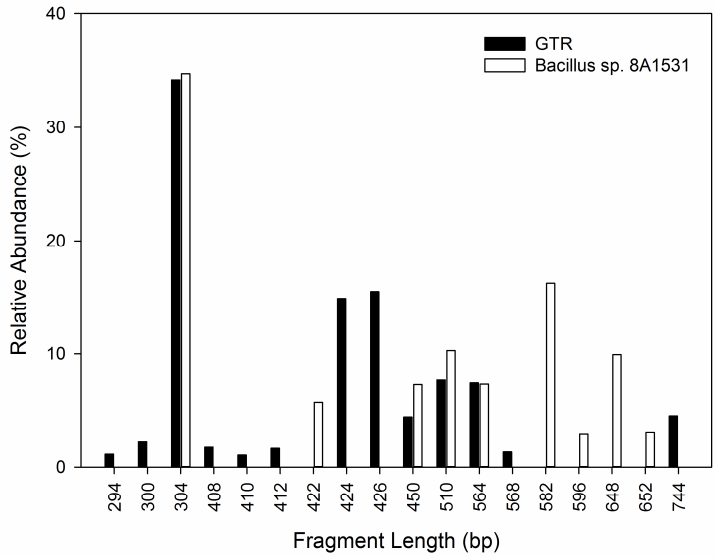

Figure 5 ARISA profile of bioreactor inoculated with *Bacillus sp.* 8A1531 and GTR profile

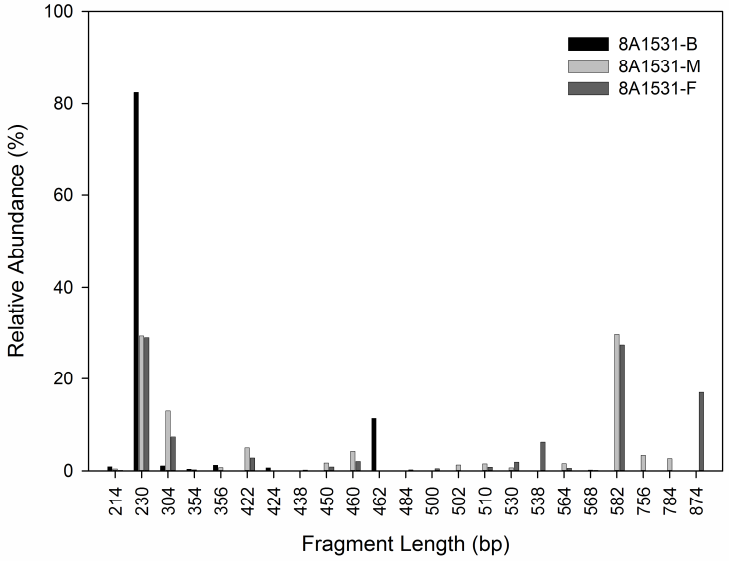

Figure 6 ARISA profile of bioreactor inoculated with *Bacillus sp.* 8A1531 at different times: beginnig time (8A1531-B), medium time (8A1531-M) and final time (8A1531-F)

After the inoculum, the community changed and tent to the original autochthonous GTR community as revealed by the fact that sample 213E-M is close to GTR sample. However, afterward the community fingerprint became again more similar to pure culture profile.

In the reactor inoculated with *Rhodococcus* sp. AF21875 a linear shift of the community fingerprint was observed with the community profile of the sample immediately after inoculation close to pure culture profile and the community structures approaching the original autochthonous GTR community in the following experimental times.

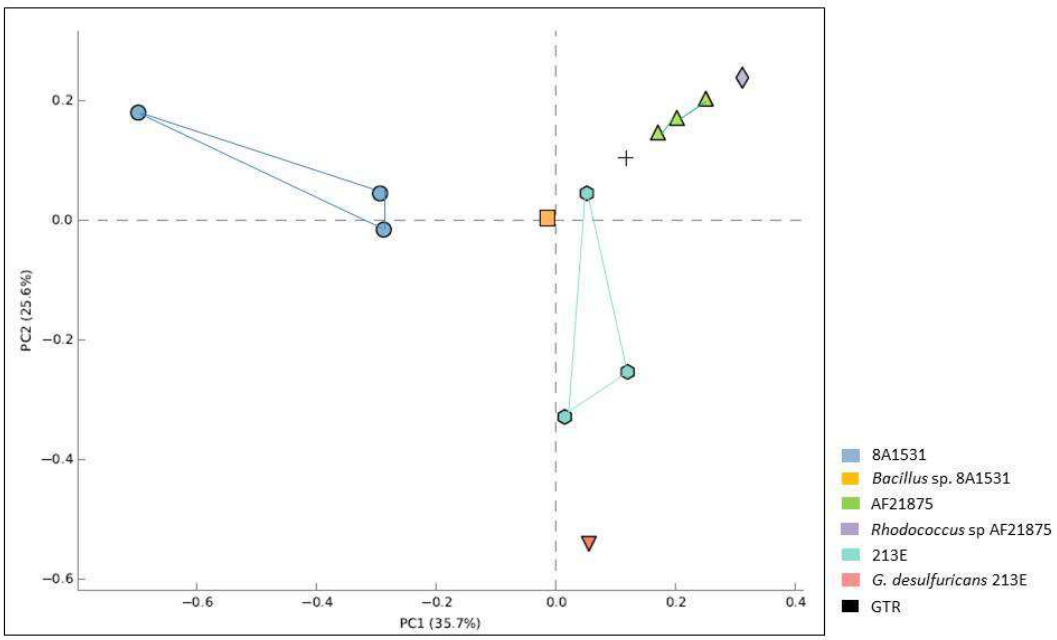

Figure 7 PCA Principal Component Analysis (PCA) of the OTU relative abundance. Bioreactors inoculated with *Rhodococcus* sp. AF21875 (AF21875-green triangles); Bioreactors inoculated with *Bacillus* sp. 8A1531(8A1531-dark blue circles); Bioreactors inoculated with *G. desulfuricans* 213E (213E- blue hexagon), pure culture of *Bacillus* sp. 8A1531 (*Bacillus* sp. 8A1531 -orange square), pure culture of *Rhodococcus* sp. AF21875 (*Rhodococcus* sp. AF21875- violet diamond), pure culture of G. desulfuricans 213E (G. desulfuricans 213E- pink triangles), GTR (GTR- black cross). Cluster 1 =213E-B, 213E-M, 213E-F; Cluster 2 = AF21875-B, AF21875-M, AF21875-F; Cluster 3 = 8A1531-B, 8A1531-M, 8A1531-F.

Conversely, the microbial community profile in the reactor after the inoculum with *Bacillus* sp. 8A531 was distant from the pure culture profiles while during the treatment the community profiles got closer to the pure culture. This behavior can be due to the fact that the DNA was extracted directly from cell attached GTR. *Bacillus* sp. 8A1531 is less hydrophobic than others two strains and it needs more time to adhere to the GTR. However in this case we would have expected that the community profile after inoculum were close to allochthonous GTR profile. This is not the case and we do not have a clear explanation for that. During the experiment the community tent to *Bacillus* sp. 8A1531 profile.

6.4.2 Gene copy quantification

The number of copies of the 16S rRNA gene was quantified. The copy number of ribosomal operons per g on GTR before the treatment was $7,4*10^5$. When the inoculum was added into bioreactors it increased of four orders of magnitude in the case of the samples 213E and AF21875 while the copy number of ribosomal operons per g increased only of two order of magnitude in the sample. 8A531 (Tab. 1 and Fig. 9).

In the experiment with 213E the highest bacterial density was reached at the intermediate time while a decreasing of one order of magnitude was observed at the final time. Probably, this was due to the depletion of some the component of the culture medium (Tab.1).

During the treatment with AF21875 the copy number of ribosomal operons remained constant until the intermediate sampling (AF21875-M) and increased up to 1,16E+10 (AF21875-F) at the end of the experiment was observed. Conversely, in the bioreactor inoculated with 8A1531, the abundance of bacteria increased one order of magnitude in the sample at the intermediate sampling (8A1531-M) and then remained constant until the end of the experiments.

The *dsz*A gene is involved in the 4S pathway. This path is used by different microorganisms to desulfurize DBT to 2-hydroxybiphenyl without damage the carbon chain. In literature, no evidence is reported of the involvement of this pathway in the

desulfurization of GTR. However, the presence of this gene may indicate a potential desulfurizing activity. All tested strains have this gene. On GTR before treatment, there was a copy number of *dsz*A per g equal to $1,27*10^5$. In the treatment with 213E and AF21875 the copy number of *dsz*A increase after the inoculum until $3,6+10^6$ and $2,50+10^8$, respectively. In the treatment with 8A1531, the copy number of *dsz*A per g remains constant, despite the inoculum addition. During the treatment with 213E the copy number of *dsz*A decreased of an order of magnitude at the intermediate time and then increased up to 2,35E+07 at the end of the experiments. This was consistent with ARISA profile, which suggested the G. *desulfuricans* 213E was outcompeted by the native GTR population only at the beginning of the treatment while it increased its abundance in the bioreactor in the second part of the treatment. In the treatment with AF21875 the copy number of *dsz*A per g increased until 2,65E+09 at the intermediate time and then it remained constant. Despite the increased of copy number of *dsz*A, this data are in contrast with the ARISA analyses, where the relative abundance of this strain is higher at the end of the treatment than in the previous sampling. Probably, the increased of copy number of *dsz*A was due to the growth of others bacteria present in the bioreactor. Indeed, in the PCA a shift of the bacterial community toward the GTR profile was observed. In the treatment with 8A1531 an increase of the copy number of *dsz*A, was observed only between the intermediate sampling and the end of the experiment.

		16S rRNA	*dsz*A
	Time	**copy number*µL/g**	**copy number*µL/g**
GTR		7,40(0,39)E+05	1,27(0,54)E+05
Gordonia desulfuricans 213E	B	7,91(1,68)E+09	3,62(1,13)E+06
	M	1,60(0,37)E+11	2,47(0,87)E+05
	F	2,13(0,28)E+10	2,35(0,88)E+07
Rhodococcus sp. AF21875	B	6,91(0,94)E+09	2,50(0,57)E+08
	M	3,80(1,01)E+09	2,65(0,11)E+09
	F	1,16(0,15)E+10	6,26(1,48)E+09
Bacillus sp. 8A1531	B	4,71(0,72)E+07	5,71(1,59)E+05
	M	1,01(0,11)E+08	2,07(1,08)E+05
	F	4,05(0,19)E+08	1,41 (0,48)E +07

Table 1 Number of copies per µL/g of GTR of the 16S rRNA gene and *dsz*A detected by qPCR into different bioreactors after inoculum (B), in the medium(M) and after (F) the treatment and on the GTR without inoculum (GTR). In the brackets there is the standard deviation of each sample.

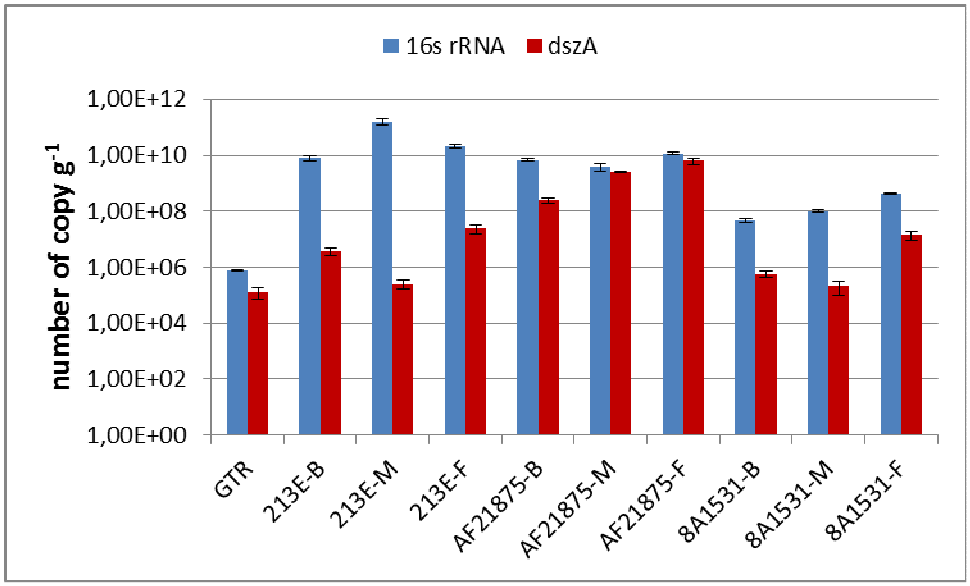

Figure 9 Number of copies per µL/g of GTR of the 16S rRNA gene and *dsz*A detected by qPCR into different bioreactors, after inoculum (B), in the medium (M) and after (F) the treatment and on the GTR without inoculum (GTR).

6.5 Discussions

Recently, biodesulfurization was suggested as an alternative and environmental friendly method for the devulcanization of GTR (Hu et al 2014, Christofi et al 2010). In these works the main proprieties considered were the chemico-physical characteristic of the devulcanizad GTR, and the final proprieties of the new bland with devulcanizad GTR. The only considered microbiological parameter was the growth measured by OD or microscopically evaluated on GTR.

Therefore, in this study of GTR biodesulfization molecular microbiological techniques were used for monitoring the bacterial community structure and the persitence of the inocula. In particular, three strains were tested: i) *G. desulfuricans* 213E ii) *Rhodococcus* sp. AF21875 iii) *Bacillus* sp. 8A1531. Bioreactors were set up according to the indications showed by a full factorial experimental design (see chapter 5). The bacterial community at different times was characterized by ARISA. Furthermore, 16s rRNA gene and *dsz*A catabolic gene were quantified by qPCR. In the chapter 5, it was demonstrated that bacterial populations resisted to thermal and chemical pre-treatment and grew during incubations. Also in this case, ARISA characterization showed that there is a naturally community hosts on GTR ant it survived to thermal treatment. Indeed, it was observed a change of bacterial community in all three bioreactors. In particular, bacterial community shifts towards the GTR bacterial community. This indicates that the bacterial populations survived to autoclave sterilization and growth in the bioreactors. For this reason it is important to evaluate the persistence of the inoculated bacteria to connect the effects on GTR to this strain. A community change was observed in the bioreactor inoculated with *G. desulfuricans* 213E. The first shift was observed between the initial time and the medium time. At the beginning the community is close to *G. desulfuricans* 213E. However, the community shift toward to GTR community. This could indicate an adaptation period for *G. desulfuricans* 213E at the new condition in particular to the chemical stress due to the GTR chemical residual. This is also reported by Li et al (2011). They observed a biomass decrease for the next 7 days when the GTR was added into the medium. The same behavior was also observed in a GTR biodesulfurization process with

Sphingomonas sp (Li et al 2012). Indeed, Li et colleagues (2012) observed that the growth curve of microorganisms showed a difference between the growth in presence of glucose or GTR and glucose. When glucose was added without GTR, the growth of microorganisms was rapid and reached a maximum on the 8th day. Conversely, when GTR with glucose was added into the medium, the growth activity slightly decreased. Nevertheless, an increase of bacterial abundance between the beginning and the medium time was observed. This shows a competition process between *G. desulfuricans* 213E and native community hosted on GTR. The last, probably, are better adapted to the chemical stress compare to *G. desulfuricans* 213E. However, a second change in community was observed, indeed at the final time the community is again close to *G. desulfuricans* 213E profile.

Conversely, different trend was observed in the bioreactor inoculated with *Rhodococcus* sp. AF21875. In this bioreactor, only a shift toward the GTR community was observed. The *Rhodococcus* sp. AF21875 population is closer to GTR profile than *G. desulfuricans* 213E population. Indeed in the ARISA analysis of characteristic fragments of *Rhodococcus* sp. AF21875 were also found in GTR profile. Probably, GTR community strains close to *Rhodococcus* sp. AF21875 were already present. The bacterial abundance showed an increased during the experiment. Probably, in this case the addition of the inoculum may have increased the abundance of mycolata harbored on GTR. The same behavior was observed for the *dsz*A gene. In this case it was observed the highest value of *dsz*A copy number (6,26E+09 copy number*µL/g). In this bioreactor about 50% of the bacteria showed the presence of this gene, and this point up a higher desulfurization potential ability.

In the last bioreactor, *Bacillus* sp. 8A1531 was inoculated. Also in this case, it was observed a bacterial community changes. Differently, in this case the community profile showed a shift toward the profile of *Bacillus* sp. 8A1531. One possible explanation is that the DNA, extracted directly from the GTR, was not representative of the microbial community in the bioreactor. Indeed, *Bacillus* sp. 8A1531 is less hydrophobic than others two strains, and so it is likely that it needed more time to adhere to the GTR. In this bioreactor the lowest values both of bacteria abundance

and *dsz*A copy number were found. Probably, this was due to the lower glucose concentration, leading to lower bacterial growth. GTR community profile is close to the profile of *Bacillus* sp. 8A1531 and *Rhodococcus* sp. AF21875. Indeed, in the second chapter it was observed that the more abundant genera on GTR are *Rhodococcus* and *Bacillus*. This explain because the GTR community profile is close to the profile of *Bacillus* sp. 8A1531 and *Rhodococcus* sp. AF21875. Moreover, an elevated value of *dsz*A number of copy (1,27E+05 copy number*µL/g) was found on GTR. This could indicate a desulfurization potential ability of bacteria hosted on GTR. As previously remarked (chapter 5), GTR biodesulfurization process should be monitored also through microbial analyses and the role of *dsz*A in the GTR biodesulfurization should be investigated.

6.6 References

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Chapter 7
Final Remarks

In the last years, the interest in developing biodevulcanization is increasing because it is considered as a safe process for the environment; it is economical and selective. Aim of this work was to contribute both to development of the treatment technology and to increase the knowledge about microbial process involved in biodevulcanisation.

In this project vulcanized ground natural rubber and ground tire (GTR) biodevulcanization were optimized. New desulfurize strains were isolated. Particularly, the strain *Rhodococcus* sp. AF21875 showed a particular sulfur metabolism, for this reason sulfur metabolism of this strain was further investigated through genome sequencing. Furthermore, this works helps to suggest aspects to be further investigated.

For the first time a microbial community adapted and selected on end life tire was observed. The desulfurize potential of this community may be further studied. In this work we analyzed only one sample of GTR. It will be interesting compare different samples of GTR obtain with different techniques of grinding.

It is aslo necessary unveil the mechanisms of biodevulcanization and suitable model molecules are necessary to investigate these biodesulfurization pathways. These studies could aslo take profit from further genomic and transcriptomics analyses of bacterial strain with different variety of sulfur metabolisms. In fact, before our sequencing, only one genome sequence of desulfurizing bacteria (*Rhodococcus* sp. JVH1) was reported in literature. This strain is unable to desulfurize dibenzothiophene but it desulfurize benzothiophene and alkyl benzothiophenes. Interestingly, another strain, which prefers organic sulfur source to sulfate, was recently described. A comparative genomics and trascriptomics could be performed to investigate the regulation and the difference in the sulfur metabolism.

In this work, we highlighted the importance to support the chemico-physical analyses with microbiological methods in order to observe the bacterial persistence during the

experiments. In fact, VGNR and GRT host autochthonous bacterial communities that potentially compete with the inoculated strains during the treatment.

Scientific Production

Articles

- Franzetti A., **Tatangelo V**., Gandolfi I., Bertolini V., Bestetti G., Diolaiuti G., D'Agata C., Mihalcea C., Smiraglia C. and Ambrosini R. **"Bacterial community structure on two alpine debris-covered glaciers and biogeography of *Polaromonas* phylotypes"** The ISME Journal (2013),pp 1-10,

- **Tatangelo V**., Franzetti, A., Gandolfi I., Bestetti G., **"Effect of preservation method on the assessment of bacterial community structure in soil and water samples**" FEMS Microbiology Letters (2014) 356 (1), pp. 32-38,

- Daghio M., **Tatangelo V.,** Franzetti A., Gandolfi I., Papacchini M., Careghini A., Sezenna E., Saponaro S., Bestetti G., "**Hydrocarbon degrading microbial communities in bench scale aerobic biobarriers for gasoline contaminated groundwater treatment"** (submitted),

- **Tatangelo V.,** Mangili I., Caracino P., Bestetti G., Collina E., Lasagni M., Franzetti M, "**Experimental design approach to study biological devulcanization of ground rubber by *Gordonia desulfuricans* 213E strain"** (submitted),

- **Tatangelo V.,** Van Hamme JD, Bottos EM, Bestetti G, Franzetti A. **"Whole Genome Shotgun Sequence of *Rhodococcus* species strain AF21875"** (in preparation).

List of Communications

- Gandolfi I., Franzetti A**., Tatangelo V**., Bertolini V., Ambrosini R. e Bestetti G., **"Molecular characterization of microbial communities present in debris covered of two Alpine glaciers",** poster at "29th SIMGBM National Meeting - September 21-23 2011, Pisa, Centro Congressi CNR",

- **Tatangelo V.** and Bestetti G., **"Biodesulfurization of waste rubber: characterization of bacterial strains and their potential use",** poster at EMBO Workshop on Microbial Sulfur Metabolism **-** April 15-18 2012, Noordwijkerhout The Netherlands,

- **Tatangelo V.**, Franzetti A., Bestetti G**.** "**Biodesulfurization Of Waste Rubber: Characterisation Of Bacterial Strains And Their Potential Use"** poster at IX Incontro di dottorandi in Ecologia e Scienze Ambientali, 15-18 Aprile 2013, Milano,

- **Tatangelo V.**, Franzetti A., Bestetti G. **"Biodesulfurization Of Waste Rubber: Characterisation Of Bacterial Strains And Their Potential Use"** poster at "30th Meeting of SIMGBM - September 18th-21st 2013, Ischia,Italy".

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"Ma la cosa più importante dell'esperienza è che il viaggiatore riesce a pensare a tante cose assieme, e tutte apparentemente opposte."
Montero Glez – Quando la notte obbliga (2009)

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"Succede che il vento soffia così forte da cancellare il numero di scarpe. E che al suo passaggio infuriato strappa via i pois dei fazzoletti, porta con se latrati di cagna e baci perduti per sempre. E succede anche che il mare si increspa e che, salato com'è, investe la costa e gioca con gli umori del viaggiatore."

Montero Glez – Quando la notte obbliga (2009)