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Tailored Bioplasticizer for elastomeric compounds from sustainable biomass

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The major part of the content of this thesis
is under patent pending

Contents

2. The oleaginous yeasts *Rhodosporidium toruloides* and *Lipomyces starkeyi* as platforms for the production of rubber plasticizer oils

Glossary

6PPD	N'-phenyl-p-phenylenediamine
ACL	Citrate lyase
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BR	Butadiene rubber
C/N	Carbon-to-nitrogen ratio
CB	Carbon Black
CDW	Cell dry weight
CH_3OH	Methanol
$CHCl_3$	Chloroform
CO_2	Carbon Dioxide
DNA	Deoxyribonucleic acid
DSC	Differential Scanning Calorimetry
E’	Storage modulus
ELTs	End Life Tyres
FAMEs	Fatty acids methyl esters
FAS	Fatty acid synthase
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
HR	Homologous recombination
ICDH	Isocitrate dehydrogenase
IR	Poly-isoprene polybutadiene
ME	Malic enzyme
MES	Mild extracted solvate
M_H	Maximum torque

M_L	Minimum torque
mRNA	messenger RNA
MUFAs	Monounsaturated Fatty Acids
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NHEJ	Non-homologous-end-joining
NR	Natural Rubber
OD	Optical density
PCR	Polymerase chain reaction
phr	Part per Hundred Rubber
PPDs	Para-Phenylenediamines
PUFAs	Polyunsaturated Fatty Acids
SBR	Styrene butadiene rubber
SCOs	Single Cell Oils
SFAs	Saturated Fatty Acids
SR	Synthetic Rubber
T_{90}	Optimum cure time
TAG	Triacylglycerol
tanδ	Dissipative factor
Tg	Glass Transition Temperature
Tm	Melting point
T_s2	Scorch time
UDS	United States dollar

Introduction

1.1. Towards a sustainable tyre production

The tyre is an important part of the vehicle as it provides dampening and cushioning, traction, load carrying capacity, cornering force, minimizes vibrations and noise, and transmits driving and braking torque. The first pneumatic tyre was patented by William Thomson in 1847 [1], but the real popularity of tyres came later, due to the parallel growth of the modern automotive market. The possibilities related to tyre development such as travel faster and farther, the easiness of transportation of goods within shorter time, contributed to the extraordinary development of both the automotive and tyre market, that are today two driving fields in the global economy. As a confirmation of the success, the global tyre material market is expected to grow from USD 76.39 billion in 2018 to USD 96.47 billion by 2026 during the forecast period 2019-2026, according to the new report published by Fior Markets; almost 88% of the total number of tyres are nowadays produced by the most developed countries, including Europe, USA, Japan, China and India [2].

Nowadays, tyres are constituted by a very complex mixture of materials, each contributing with a particular function to the final performance. Unfortunately, these materials, together with the procedure needed to obtain tyres, have some drawbacks in terms of environmental impact. In particular, the main concern is that they cannot be decomposed to re-obtain the raw materials, and for some of them the source is getting scarce or inaccessible.

The impressive numbers in the annual tyre production involve *i)* the consumption of a large amount of natural resources and energy during the production phase and *ii)* the generation of a huge amount of discarded tyres (End of Life Tyres, ELTs), defined as tyres not suitable anymore to responding to the requirements for use on the road, due to tear or irreparable damages [3]. Thus, in the recent years, many efforts have been concentrated on the development of innovative techniques to handle ELTs [4,5]. In particular, it has been reported that, nowadays, ELTs are important resources for industries such as construction, automotive, cement, etc., and the European tyre and recycling industries are focused on increasing the value of secondary raw materials derived from discarded tyres [6].

In addition, some substances enclosed in tyres were shown to be easily released both during the production and use phase (due to tyre tread consumption), and finally disposal [7]. Moreover, recent scientific studies have highlighted that some of these substances can be toxic for the environment and can affect the whole ecosystem, especially in the urban areas. As a result, many companies in this field are nowadays focused on the (total or partial) reduction or substitution of the more toxic materials conventionally used in tyre production, based on risks assessment for each component.

Companies as Pirelli, Michelin, Goodyear, Continental have entered into important partnerships with biotech companies, all focusing on green chemistry to overcome the dependence on fossil resources [8].

In this thesis work, the above-mentioned aspects will be further discussed. More in detail, the project took place considering how and which tyre raw materials could be sustainably substituted: we focused our attention on plasticizers. The ones now in use are principally derived from petroleum, and therefore linked to a linear logic of production (take, make, dispose) which is very far from being sustainable. Moving from linear to circular economy, we suggested an alternative source for bio-based plasticizers, sustainable both in terms of final product and of its origin. Therefore, the thesis work is based on two main pillars: 1) the sustainable production of bio-plasticisers from renewable feedstock 2) tailoring bio-plasticisers to match tyre industry requirements.

1.2. Science and Technology of Rubber Compounds

1.2.1. Rubber Compounds and their ingredients

Rubbers, commonly known as elastomers, are an important class of polymers and one of the most utilized in material science and related technology. Peculiar properties of these materials, such as mechanical strength, flexibility, curing behavior, flexural properties, etc, make them significantly important. Filler particles and various other ingredients are added to the rubber matrices to not only increase the existing properties but also to attain new qualities, as colour, conductivity, etc. The two major constituents of rubber compounds are filler and rubber itself, where relative composition has to match the desired traits of the final products. Indeed, properties of the rubber compounds are largely affected by the nature of rubber and filler, by the dispersion of the filler in the rubber matrix and their compatibility in terms of filler-rubber interactions. [9–15].

Rubber compounds have many fascinating properties which are not present in other materials: abrasion resistance, high elasticity, and damping properties, to cite a few. Therefore, rubber compounds can match many and diverse requirements for applications in the sectors of automotive industry, railways, domestic appliances, electrical and electronics, civil and mechanical engineering.

The most common and important application of rubber compounds is in tyre manufacturing. Reduced rolling resistance, high mechanical properties, maximum traction in dry and wet environments, and optimized performance are the main objective to achieve in the tyre industry. The selection of one or more rubber, and the appropriate amount and type of filler make possible to obtain all the desired performances [16]. The rubber compounds developed in the tyre industry rely on five main elements: the polymers, the fillers, the vulcanization and stabilizer systems and the plasticizers.

1.2.1.1. Polymers

Polymers are the main ingredient of the rubber compounds: they can be either natural or synthetic. Polymers are high molecular weight materials, which are composed of long chains of molecules made up of repeated units of one or more types of monomers. These polymers chains are long and flexible, being composed from tens to thousands of monomer units. The rubber polymer chains, randomly distributed, are coiled and entangled between them, restricting their motion.

Crosslinked rubber polymers display combinations of very important properties, most significantly elasticity and resilience. They show the ability to return to their original shape after being deformed by stretching or compression and display a high level of extensibility.

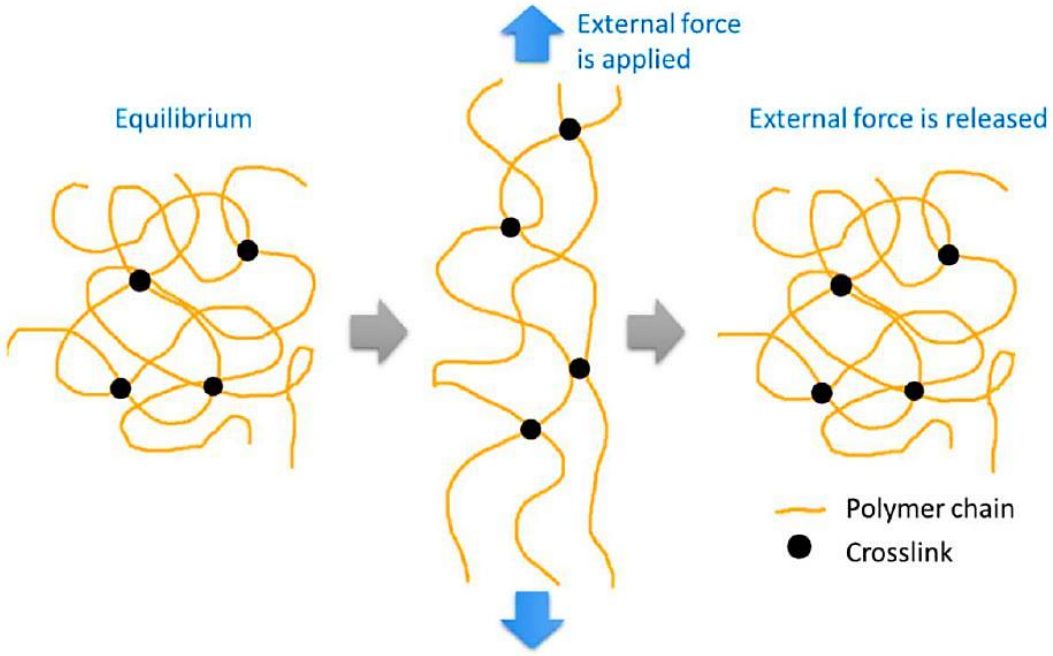

Fig.1. Schematic representation of elasticity of crosslinked polymer chains [17]

At molecular level, the polymer chains randomly distributed in the cured polymer present some crosslinkings: when stress is applied, the chains are uncoiled and aligned in the direction of applied force. When the stress is released, the chains gain their original random uncoiled shape as represented in Fig. 1. This characteristic results in some important properties, as for example high elongation at break, high elasticity, and low hardness [18].

The molecular weight and configuration of the constituent monomers have a significant effect on the physical properties of the polymers. Chain entanglements increase with increasing molecular weight, resulting in stiffness at high elongations [19–21]. Because the restricted rotation of the carbon bonds of the polymer chain causes an increase in the stiffness of the obtained product, polystyrene for instance is stiffer than polyethylene due to the steric hindrance of side groups [22]. Highly pure and regular structure of polymer chains increases the possibility of polymer segments to organize into crystalline domains, especially at high elongation, when the polymer chains align in the direction of the applied force, resulting in increased strength and stiffness [23–25]. Polymers are sensitive to temperature and show a change in elastic properties with thermal agitation. Generally, with a decrease in temperature, the stiffness increases, until the polymer gains a glass-like rigidity at a certain temperature, known as glass transition temperature, *Tg*. This results in an increase of hysteresis and internal viscosity. The size and number of side groups on the polymer

chains affect the glass transition temperature of the polymer. The *Tg* increase with increasing the size and number of side groups such as chlorine, benzene rings or vinyl groups, etc. Therefore, high hysteresis is related to a high glass transition temperature and consequently with a high friction and low air permeability [22].

Polymers can be classified into two general categories: natural rubber and synthetic rubber. Typically, natural rubber is obtained from the rubber tree, *Hevea brasiliensis*, and synthetic rubbers are prepared from different monomers produced from petroleum refining and cracking. Natural rubber accounts for about 46 % of total world rubber utilization, while the remaining are synthetic rubbers, out of which about 18 % is constituted by styrene butadiene rubber (SBR) [27]. In the tyre industry, up to 30 different kinds of synthetic rubber and 8 of natural rubber are used for the formulation of rubber compounds, and the 60% of total rubber is represented by synthetic rubber [28,29].

1.2.1.1.1. Natural Rubber

Natural rubber (NR) is a biopolymer of high commercial value with interesting properties such as high elasticity and resilience [30].

Chemically, it is composed of long polymeric chains of *cis*-1,4-poly(isoprene) and the chemical structure is shown in Fig. 2. About 300 to 70,000 isoprene molecules are linked together, leading to an average molecular weight between 100,000 and 1,000,000 g/mol [31–33].

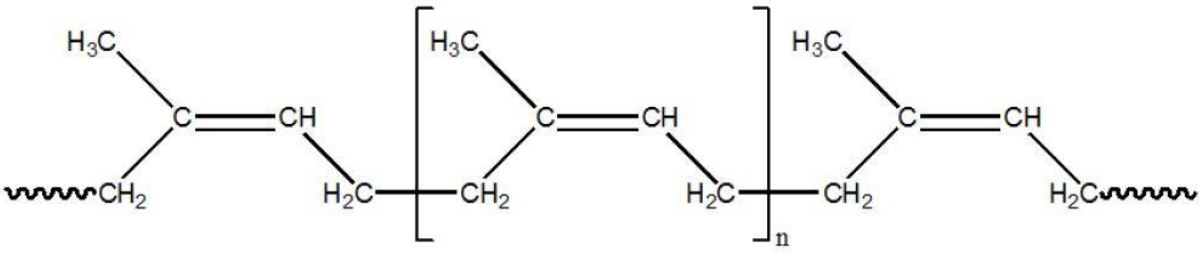

Fig.2. Chemical structure of natural rubber [*cis*-1,4-poly(isoprene)] [31].

Natural rubber (NR) is obtained in the form of a milky aqueous suspension, commonly known as NR latex, from the sap of many plants (up to 2500 species) [33]. Currently, high-quality NR in viable quantities with high commercial value is obtained from *H. brasiliensis* tree [34]. It is mainly in the conformation of *cis* isomer, while *trans* isomer can be found in the latex obtained from genus *Dichopsis*.

H. brasiliensis tree is indigenous from Brazil but it can be cultivated as a valuable crop in other tropical and subtropical counties. Thailand (35%), Indonesia (23%), Malaysia (12%), India (9%), and China (7%) are the main NR tree producers [35]. During the harvesting and processing, along with NR some other non-rubber components are also extracted. Consequently, the obtained latex contains about 94 % of NR and 6 % of non-rubber matter (about 2 % of resins, 1-2 % of proteins and phosphoproteins, 1 % of carbohydrate, 1 % of fatty acids, and 0.5 % of inorganic salts) [36]. It is believed that these components play their role in conferring the extraordinary characteristics of the NR [31]. The NR latex contains natural rubber particles in micron and sub-micron size, which are stabilized by the protein anions. These negatively charged particles form a stable colloidal suspension which protects the latex from coagulation. The exposure of NR latex to air can indirectly cause coagulation, very likely due to the decomposition of proteins by enzymes and bacteria. The stabilization of commercial NR latex is generally obtained by adding ammonia, which prevents the growth of microorganisms and maintains an alkaline environment [37,38].

NR, thanks to its outstanding properties, represents the ideal rubber material for many applications. The presence of double bonds in the chemical structure of NR provides the reactive sites for the attachment of functional groups, grafting or crosslinking. In addition, under high strain, the polymeric molecular chains align in the direction of the deformation, resulting in the formation of crystalline domains which act as a filler. This phenomenon increases the reinforcement of the material [39–41]. The high elasticity, resilience, low heat build-up, low hysteresis, good abrasion resistance, outstanding dynamic properties along with excellent processability, make NR ideal for the production of automotive tyres.

1.2.1.1.2. Synthetic Rubber

Synthetic rubbers (SR) are the artificial elastomers which are commonly synthesized from diverse monomers derived from petroleum. Isoprene, butadiene, styrene, propylene, ethylene, isobutylene, and acrylonitrile are the most commonly used monomers. The development of SR started in 20th century in order to fulfil the increasing demand of elastomeric materials and also to improve the final properties of the rubber compounds. Currently, there are more than 20 different varieties of SR, which can provide the desired set of properties required for a specific application [42]. Poly-isoprene (IR), polybutadiene (BR), styrene butadiene rubber (SBR), etc, are the general-purpose SR. These rubbers are characterized by the presence of chemical unsaturation (diene functionality) in their backbones, which makes them prone to be attacked by oxygen or ozone. The special purpose SR, in contrast to general purpose

SR, have inherent properties of resistance to high temperature, ozone, and oil depending on the repeating unit [43].

SBR is a copolymer of styrene and butadiene and the most commonly used SR. It has valuable properties such as high skid and abrasion resistance, high reinforcement, and, for certain grades, low cost. It can be synthesized by either solution or emulsion process. The solution process allows to obtain a final product with fine tunable properties if compared to the product obtained by emulsion process. Nevertheless, the latter displays higher tear strength and tensile strength, and easy processability, finding its applications in the tyre tread, breaker compounds of bias tires, ply, bead wire insulation, sidewalls, and conveyors belt covers. On the other hands, SBR obtained by solution process is commonly used in tyre tread of light truck and passenger tyres, large off-the-road tyres, and tyre tread of radial medium truck tyres [44] thanks to its better hysteresis profile.

60 % of SR world total production is consumed in the tyre industry. SBR accounts for the largest volume, which is 65 %, and butadiene rubber (BR) has the second largest contribution of SR used in tyres [45]. BR confers high abrasion resistance, cut growth resistance, and tread wear performance and it is commonly used in the tyre tread, sidewalls and some casing part of the tyre. A blend of BR with NR has been reported to improve the resistance of cut growth and better fatigue [48]. Regarding the microstructure, butadiene can take three configurations: *cis*-(1,4), *trans*-(1,4), and *vinyl*-(1,2) and relative percentage of these three isomers have a substantial effect on the performance of rubber compounds. High-*cis* BR (> 92 % *cis*) demonstrates improved abrasion resistance but their processing is difficult. High-*trans* BR (93 % *trans*) shows high toughness and crystallinity at room temperature and high-*vinyl* BR compounds displays superior wet traction and wet skid performance in tyre treads [44,46–49].

1.2.1.2. Reinforcements: Fillers

A wide variety of particulate fillers are used in the rubber industry for various purposes, among which the most important are: reinforcement, reduction in material costs and improvements in processing. Reinforcement is primarily the enhancement of strength and strength-related properties, abrasion resistance, hardness and modulus. In most applications, carbon black (CB) and silica have been used as the main reinforcing fillers that increase the adaptability of rubbers. When CB is compounded with rubbers, tensile strength, tear strength, modulus and abrasion resistance are increased. For this reason, CB has been extensively exploited in

numerous rubber engineering products. In general, a CB-reinforced rubber has a higher modulus than a silica-reinforced one. However, silica provides a unique combination of tear strength, abrasion resistance, aging resistance and adhesion properties. In tire treads, silica yields a lower rolling resistance at equal wear resistance and wet grip than CB [50].

There are some important properties of the fillers, such as chemical composition, particle size, particle shape, structure, aspect ratio, surface area, surface chemistry, and density, which can influence several aspects on the final performance [53,54]. In particular, the particle size significantly conditions the reinforcement of rubber compounds. Very small primary particles, with size ranging from 1 to 100 nm offer very high reinforcement and significantly enhance the mechanical properties of the rubber compounds [18].

In addition, the morphological characteristics such as surface area and structure of the fillers are significant for their reinforcing ability and for the interaction between filler and polymer. In fact, the surface area is directly related to the particle size of the filler. Then, small particle size, high surface area and the high surface area-to-volume ratio of filler improves the mechanical properties of the rubber compounds without altering their bulk properties such as density or light transmission [55–57]. The polymer chains are adsorbed on the filler surface either through physical interaction, such as Van der Waals forces, or chemical interactions by making a chemical bond with functional groups on the filler surface. A high surface area of the filler particles results in more polymers chains to be adsorbed on the surface, restricting the chains mobility under strain, leading to a high reinforcement of rubber compounds [18].

The structure is also an important property of the filler. The spherical filler primary particles are partially fused to form irregular chainlike or branched aggregates. During the processing of rubber compounds, these aggregates are unbreakable and act as reinforcing objects. The branched and chainlike structures of aggregates make them bulky and their effective volume in rubber compounds is much higher than the volume of aggregates, which determine the extent of strain amplification in deformable phase [55,68]. The shape of the filler also influences the reinforcement: filler with high aspect ratio shows the higher reinforcing effect than spherical filler [51].

1.2.1.3. Vulcanization System

Unvulcanized rubber is generally not very strong, as it does not regain its shape after a deformation. Vulcanization can be defined as a process that increases the retractile force and reduces the amount of permanent deformation remaining after removal of a deforming force. Thus, vulcanization increases elasticity while it decreases plasticity. The first commercial method for vulcanization has been attributed to Charles Goodyear. His process (heating natural rubber with sulphur) was first used in Springfield, Massachusetts, in 1841. Thomas Hancock used essentially the same process about a year later in England. Since those early days, there has been progress toward the improvement of the process and in the resulting vulcanized rubber articles. In addition to natural rubber, over the years many synthetic rubbers have been introduced. Also, in addition to sulphur, many other substances have been introduced as components of curing (vulcanization) systems. It is generally accomplished by the formation of a crosslinked molecular network (Fig.3). At molecular level, during the vulcanization, junctures (crosslinks) are formed along the rubber molecules and the average spacing between the crosslinks is represented in the form of molecular weight between the junctures, which range from 4,000 to 10,000 Daltons. Due to the formation of this network, it is no more possible for the rubber to be solubilized in a solvent or processed by any mean. Therefore, it is necessary that the final shape of the product is defined before the vulcanization step/process. [59]

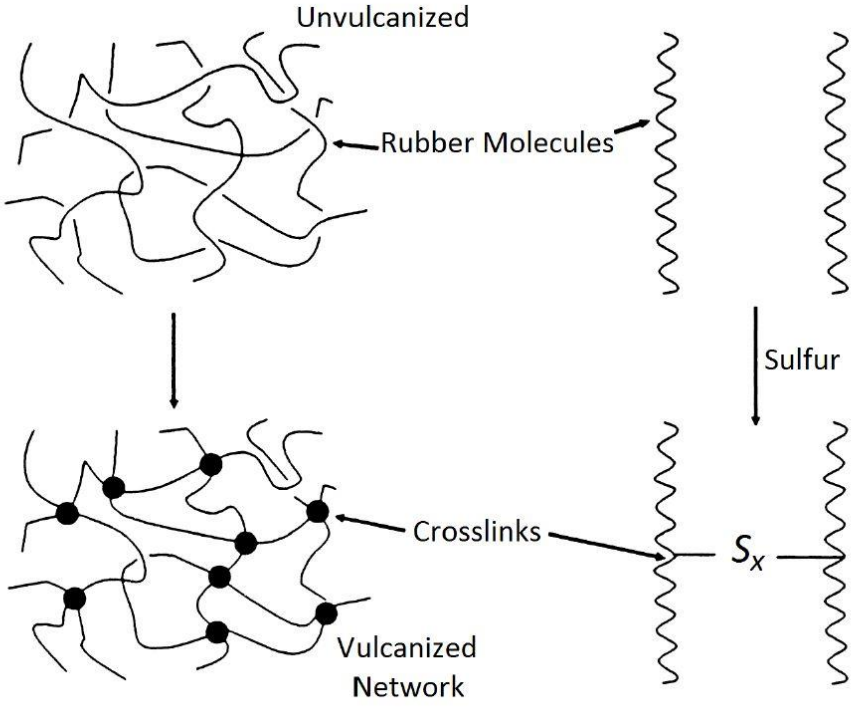

Fig.3. Schematic representation of crosslinks formation during vulcanization [54]

A vulcanization system in a rubber compound is composed of three main components; vulcanizing agents, activators, and accelerators. The main vulcanizing agent used are sulphur and peroxides.

Accelerators, able to reduce the vulcanization time, are usually organic compounds that increase the rate of vulcanization reactions and crosslink density. Based on their mechanism of action, they are classified as primary and secondary accelerators. Primary accelerators provide medium to fast curing and scorch delay while secondary accelerators are scorchy, and provide very fast cure [60]. The commonly used accelerators in vulcanization belong to the eight groups: thioureas, thiurams, thiazoles, guanidines, dithiocarbamates, sulfonamides, aldehydeamines and xanthates. In general, accelerators are used in combination with activators during vulcanization to gain the full potential of accelerators. Zinc oxide and stearic acid are the commonly used activators in the vulcanization system.

Vulcanization greatly influences the properties of the vulcanizate. In particular, hysteresis decreases by increasing the crosslink density. Hysteresis represents the deformation energy that is lost as heat. At low crosslinking, the properties linked with breaking energy, such as tear strength, fatigue life, and toughness, increase but additional crosslinking causes a decrease in these properties. An increase of crosslink density and hysteresis cause an increase in the properties that are related to energy-at-break. Since hysteresis decreases with increasing the crosslink density, so these properties are maximized at some intermediate crosslink density [59,61].

1.2.1.4. Stabilizer Systems

The unsaturated nature of natural and synthetic rubbers provides them unique viscoelastic properties, although these rubbers containing carbon-carbon double bond are more prone to attack by ozone, oxygen, and thermal degradation. In order to protect vulcanized rubber from ozone, oxygen, heat, and light, antioxidants or antiozonants are added to rubber compounds to limit the degradation of vulcanized rubber products.

Antioxidants protect the rubber from oxidation by reacting with oxygen and free radicals. Antioxidants are generally grouped in primary and secondary antioxidants; primary antioxidants act as a chain terminator and secondary antioxidants act as hydroperoxide decomposer. Three types of antioxidants, i.e. phenolic, aromatic amine and hydroperoxide decomposing antioxidants are commonly used.

Antiozonants protection mechanism of rubber is described by four principal theories. The first theory suggests the competitive reaction of antiozonants with rubber for ozone. The second theory describes the formation of a protective layer of ozonized antiozonant on the rubber surface, which protects the further attack of ozone. The third theorizes the reaction of antiozonants with ozonized rubber fragments and restoring the rubber chains by relinking them. The fourth postulates the formation of Criege zwitterions from the ozonized products.

Along with antioxidants and antiozonants, waxes are also frequently used in rubber compounds as protective agents. The solubility of the waxes is very poor in the rubber, so they migrate to the surface of rubber products and protect them from the attack of ozone by making a protective layer on the surface [46,59].

1.2.1.5. Plasticizers and Special Compounding Ingredients

The primary ingredients of rubber compounding are polymers, fillers, vulcanization system and stabilizer system. In addition to these, some secondary ingredients are also added to rubber compounds: the most important class is that of plasticizers, i.e. oils and resins. Depending of the compounds, they allow to obtain different final products.

The plasticizers are added to enhance the flexibility and processing of the rubber compounds. In most cases, plasticizers cause a decrease in tensile strength and static modulus but an improvement in low-temperature flexibility and elongation at break.

In addition, different kind of resins, such as processing or extending resins, curing resins, and tackifying resins can also be also added to rubber compounds. They provide an improvement in tensile strength, tack, dispersion of filler, and resistance to fatigue and cut growth [46,62].

In technical applications, it is common also the use of coloring agents.

1.2.1.5.1. Plasticizers

Plasticizers are organic substances which play some important roles in formulating rubber. They soften the rubber compound, reducing modulus and hardness, increase elongation, and in some cases, improve low temperature flexibility. They also improve flow in extrusion and moulding by making the uncured compound less elastic and reducing viscosity and friction. At low levels, plasticizers aid in the dispersion of fillers. At higher amounts, they reduce uncured compound viscosity, often lower the overall

raw material compound cost, by extending the polymer with less expensive oil and filler and reduce vulcanizate stiffness [63].

They can be divided into primary and secondary plasticizers: the first ones are mainly used to reduce the viscosity of the compounds, being able to solubilize the elastomers, and then act as lubricants between the polymer chains to improve flow. Secondary plasticisers have lower solubility in the polymer matrix and can be used in combination with primary plasticisers to enhance their effect. To determine the amount of plasticizer that could be added to elastomer it is important to evaluate the compatibility. In particular, primary plasticizers (those that swell the polymer) can be used in large quantities, whereas the secondary are used in limited quantities. From a physico-chemical perspective, solubility depends from enthalpic and entropic contributions: while the latter is almost always negative, i.e. favourable to mixing, the former is typically neutral for substances of similar polarity and more and more positive (opposing mutual solubility) for substances of different polarity. Thus, the choice of a primary plasticizer must be matched to the polymer; that is, nonpolar plasticizers generally find use in nonpolar elastomers, whereas polar elastomers use polar plasticizers (Fig.4) [64,65,66]. From a technological perspective, achieving a good compatibility can avoid the “sweatout” or exudation of oil to the surface of rubber usually most pronounced after vulcanization [67]. In addition, related to compatibility of oils petroleum-derived with rubber, they can be also considered their aromatic content [66].

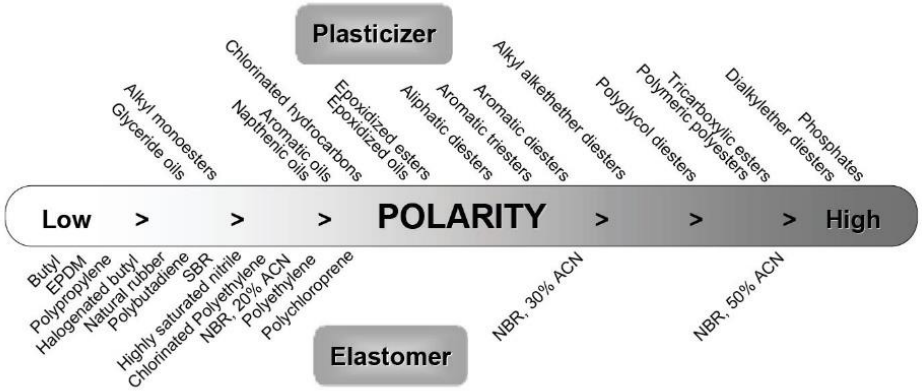

Fig.4. Graphical representation of the different degree of polarity between plasticizer and elastomer [62].

Focusing on the tyre industry, rubber plasticizers can be generally classified as mineral oils (petroleum based), synthetic plasticizers, or vegetable oils and other natural products. Mineral oils are further classified by their content of aromatic, paraffinic, and naphthenic hydrocarbons, which tune their properties, together with their molecular weight (some typical molecular structure are shown in Fig.5) [68,69].

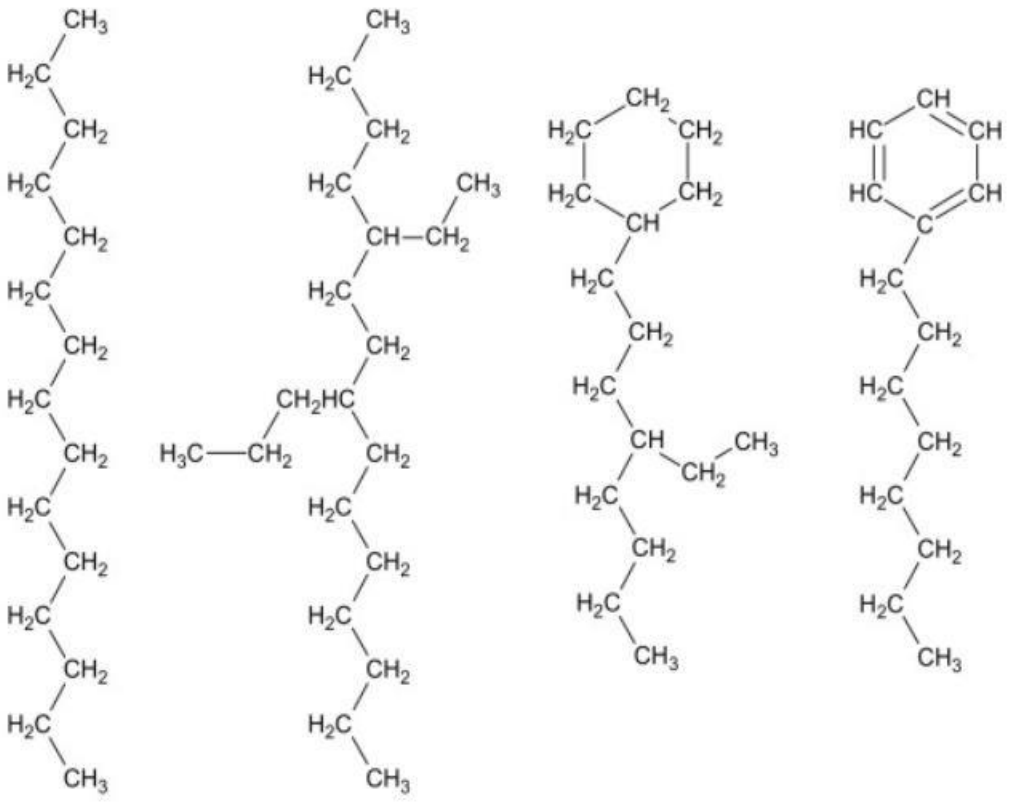

Fig.5. Examples of structures of some hydrocarbon molecules present in oils: (a) paraffin (alkane); (b) branched paraffin (alkane); (c) naphthenic; (d) aromatic [60].

All mineral oils are nonpolar and relatively cheap, so they are widely used in nonpolar general-purpose elastomers such as NR, polyisoprene (IR), polybutadiene (BR), styrene butadiene (SBR), butyl (IIR), and ethylene propylene (EPR or EPDM) [57]. On the 13th of February 2004, the Council of the European Communities (2005) adopted a proposal aimed at banning the use and marketing of PAH-rich extender oils from the 1st of January 2010 [70]. Thus, currently available mineral oils do not include significant levels of regulated Polycyclic Aromatic Compounds – they can be classified into several classes as a function of process and specific components: mild extract solvates (MES), treated distillate aromatic extracts (TDAE) and naphthenic oils (NAP), Residual Aromatic Extract (RAE). Some naturally occurring oils have been found to be suitable alternatives to the presently available mineral oils in the market: the main class is that of vegetable oils, including rapeseed oil, bean oil, castor oil, coconut oil [66].

1.3. From fossil-based to bio-based plasticizers: Moving toward a bioeconomy

Since fossil carbon resources are non-renewable within the time of their utilization, their depletion could cause price volatility [71], as well as risks in their supply due to the increasing demand of energy and goods that is directly correlated with world population increment [72]. Altogether, these limitation highlights that this economic model, which is well-described by "linear economy" (Fig. 6), is no longer sustainable. In order to reduce the dependence on fossil oil, a change to a more sustainable economic system is required.

In this contest, the alternative to the linear fossil-based economy is represented by the "circular economy" (Fig. 6), which aims to realize a closed loop material flow in the whole economic system through a 3R approach (Reduce, Reuse, Recycle), minimizing environmental deterioration and wastes but without restricting the economic growth or social and technical progress [72].

Fig.6. Linear *versus* circular model of production Source: PYXERA Global (www.pyxeraglobal.org).

The concept of circularity is well represented by bio-based economy, which mainly relies on a green chemistry and biorefinery. The biorefinery is defined by the International Energy Agency (IEA) as the "sustainable processing of biomass into a spectrum of marketable products and energy" [71]. Indeed, differently from fossil sources, biomasses are both more widespread worldwide and renewable within the time of their consumption. Thanks to the latter characteristic, a higher energy security can be achieved, leading to a more sustainable socio-economic growth [73].

In this context, it is possible to identify *first generation biomasses*, *i.e.* edible biomasses which include sugar, starch, vegetable oils or animal fats. In particular,

vegetable oils are environment friendly, biodegradable, less costly and readily available.
Vegetable oils consist primarily of triacylglycerols (TAG). TAG, also termed triglycerides, are classified as neutral lipid consisting of glycerol molecules with three long chain fatty acids attached at the hydroxyl groups via ester linkages. The fatty acids in vegetable oils are all of similar length, between 14 and 22 carbons long, with varying levels of unsaturation. A particularly interesting variation to the normal vegetable oils is castor. Castor oil is a triglyceride, but the fatty acids are almost exclusively ricinoleic acid, an 18-carbon monounsaturated fatty acid with a hydroxy branch at the 12th carbon [74]. Due to this composition, they can be used in many applications replacing petrochemical derivatives (oleochemicals, biofuel, etc) [75, 76]. Focusing the attention on tyre industry, several research data show the use of pure vegetable oils as plasticizers in rubber with different degree of success [77-86].
Unfortunately, the use of first generation biomasses cause several concerns: direct and indirect (land use) competition with food chain, availability depending of soil fertility and per hectare yields. Moreover, the effective savings of CO_2 emissions and fossil energy consumption are limited by the high energy input required for crop cultivation and conversion [87]. In addition, they are subjected to seasonality and affected by climate.

Oleaginous microorganisms are able to accumulate microbial lipids, also known as Single cell oils (SCOs), also starting from *second generation biomasses, i.e*. a number of biobased intermediates or waste streams, such as, for instance, low-cost sugars and biodiesel-derived glycerol as feedstock. The microbial fatty acids profile is similar to most plant oils and animal fats and this could make them an alternative for replacing some conventional resources (Fig.7) [88].

Oil source	Relative % fatty acid composition							Other
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	
Oil seeds								
• Soybean		11		4	24	54	7	
• Rapeseed		4		2	62	22	10	
• Sunflower		7		5	19	68		
Tree fruit and Kernels								
• Palm		44		4	38	10	1	C4-C10(1%);C12:0(1%)
• Palm Kernel	16	8		3	15	2		C4-C10(1%);C12:0(1%)
• Coconut	18	9		3	6	2		C4-C10(4%);C12:0(48%)
• Olive		13	1	3	72	10	1	C20(l%)
Microorganisms								
A. Yeast								
• *Rhodosporidium toruloides*		18	3	3	66			C23:0(3%);C24:0(6%)
• *Rhodotorula glutinis*		37	1	3	47	8		
• *Lipomyces starkeyi*		34	6	5	51			
B. Fungi								
• *Mortierella isabellina*		29		3	55	3	3	
• *Mucor circinelloides*		22		4	38	10	15	
• *Pythium ultimum*		15		2	20	16	1	C4-C10 (7%); C20:1 (4%); C20:4 (15%); C20:5 (12%)
C. Bacteria								
• *Rhodococcus opacus*				3–19	6–74			
• *Acinetobacter calcoaceticus*		28	20	6	35			C12 (6%)
D. Microalgae								
• *Schizochytrium linacinum*	3–4	56–60		1–4				C22:5:2 (4-6%); C22:6 (29-35%)
• *Chorella* SP		7–19	10-9	1–4	8–9	1–14	16–19	C15 (5%); C16:2 (11%)
• *Chaetoceros muelleri*	18–40	5–40		0–25	0–4	0–5	0–5	C12 (6–20%); C16:2 (0–8%)
E. Thraustochytrids								
• *Schizochytrium* sp. *SR2I*	4	55		1				C22:5(6%),C22:6(30%)
• *Thraustochytrium aureum*	3	8			16	2	2	C20:4(3%), C22:6(52%)
• *Skeletonema costatum*	17	17	11		2	1		C18:4 (6%), C20:% (41%), C22:6 (7%)

Fig.7. Vegetable and microbial oils composition [89].

1.3.1. Oleaginous microorganisms

Oleaginous microorganisms are so defined as they can accumulate SCO at least up to 20% of their cell dry weight as lipid content. The oleaginous property is distributed among specific genera and species of microalgae, fungi (yeasts and molds) as well as bacteria [90].

The life cycle of oleaginous microorganisms can be characterized by three distinct physiological phases, namely the balanced growth phase, the oleaginous phase, and the reserve lipid turnover phase.

During the balanced growth phase, in which all nutrients are found in excess in the growth environment, the oleaginous microorganisms convert the carbon source into cell mass, rich in proteins and polysaccharides, while restricted quantities of lipids, mainly polar lipids such as phospholipids and glycolipids that are essential for the construction of cell membranes, are synthesized. Then, following the balanced growth phase *de novo* lipid accumulation occurs during the oleaginous phase, triggered through unbalanced growth conditions, where carbon source must be in excess whereas at least one of essential nutrient, usually nitrogen (but also sulphate, phosphate, or magnesium) is depleted. The lipids produced are stored as TAG into the lipid bodies, a specialized organelle for neutral lipid storage.
After the exhaustion of the extracellular carbon source, or due to low uptake rate, the oleaginous microorganisms utilize their own storage lipids as energy source for maintenance purposes or as intracellular carbon source for the production of new lipid-free material, provided that essential nutrients are available in the growth medium. However, the addition of carbon source in the growth medium during lipid turnover may suppress, partially at least, the lipid degradation [91].

1.3.1.1. Single cells oils (SCOs) accumulation mechanism

The accumulation mechanism is common among the different oleaginous microorganisms. TAG begins when a non-carbon nutrient, such as sulphur, oxygen, phosphorus, and especially nitrogen, becomes growth limiting. In nitrogen limited conditions, the consensus hypothesis for lipogenesis activation begins with a ∼95% decrease in (AMP) concentration due to increased activity of AMP deaminase [90,91].

The depletion of AMP is responsible of an inhibition of the mitochondrial NAD^+-dependent isocitrate dehydrogenase (NAD^+-ICDH), enzyme allosterically activated by AMP. ICDH inhibition is critical in signalling the onset of lipogenesis and is responsible of the accumulation of its precursor (isocitrate) into the mitochondrial compartment. Aconitase works to convert the accumulated isocitrate into citrate, which is exported to the cytoplasm where it is cleaved by the ATP-citrate lyase (ACL), a key enzyme for lipogenesis present in all oleaginous microorganisms [91].
The acetyl-CoA is then directed to the novo fatty acids synthesis in the fatty acid synthase (FAS) complex. The products of the FAS complex, palmitoyl-CoA, and stearoyl-CoA, are shuttled to the endoplasmic reticulum where they are used to produce TAGs, or undergo NADPH-dependent desaturation or/and a two carbon

elongation before being directed to TAGs synthesis (Fig.8). The supply of NADPH needed for lipid synthesis in oleaginous microorganisms can be provided by different sources including $NADP^+$ dependent isocitrate dehydrogenase, the oxidative pentose phosphate pathway, or the malic enzyme (ME) [92].

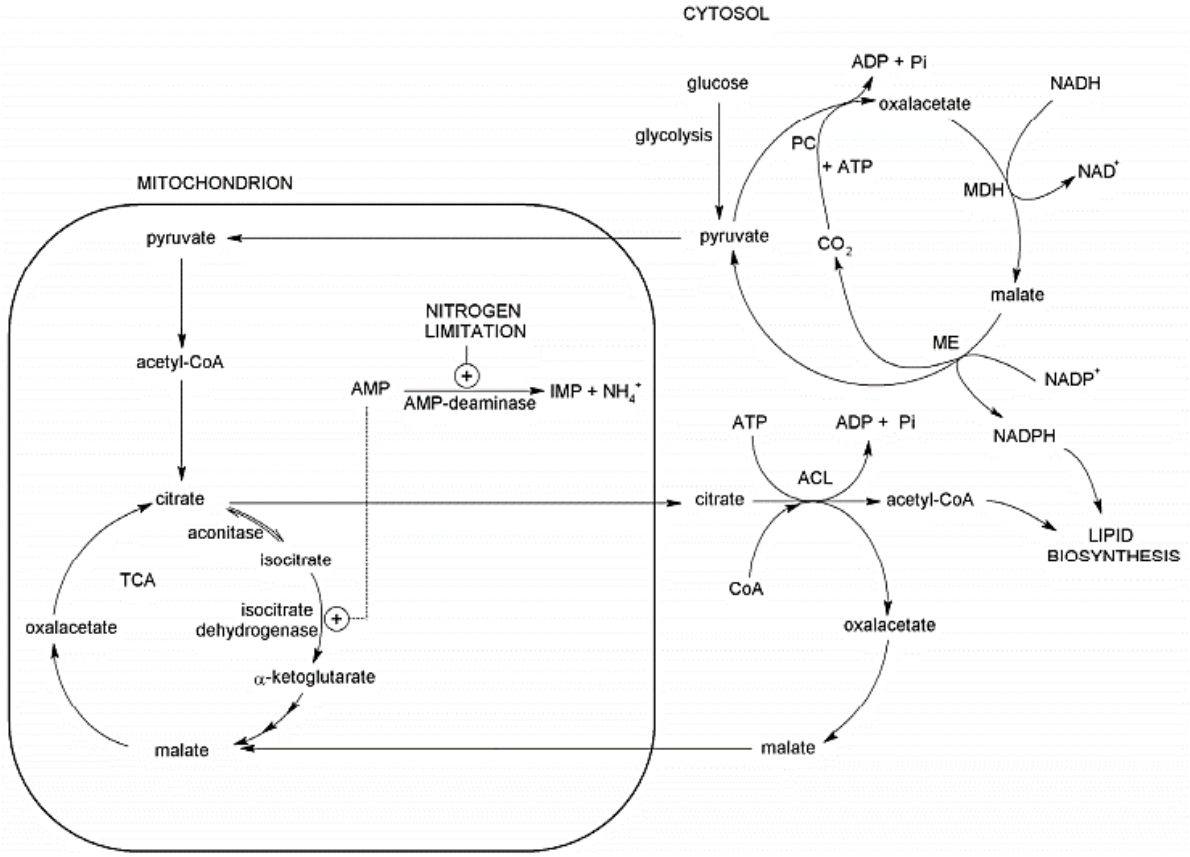

Fig.8. Lipid biosynthetic pathway [93].

1.3.2. Oleaginous yeast as cell factories

Most of the oleaginous yeasts are able to accumulate lipids at levels of more than 40% of their dry weight and as much as 70% under nutrient-limiting conditions. This confers them a unique physiology as suitable candidates for the production of biofuels and bio-based oleochemicals with diverse applications as lubricants and supplements in food, feed and cosmetic industries [94,95]. However, the lipid content and fatty acids profile differs between species. The best-known oleaginous yeasts are typically found in the following genus: *Candida*, *Cryptococcus*, *Lipomyces*, *Rhodosporidium*, *Rhodotorula*, *Rhizopus*, *Trichosporon* and *Yarrowia* [89]. Oleaginous yeasts are attractive hosts for lipids production over other oleaginous microorganisms, such as filamentous fungi and microalgae, because they are easy to cultivate, tolerate low pH, resist to viral infection, are suitable for animal feed co-

product and are able to metabolize a large variety of raw materials, and finally they are generally easy to cultivate in large fermenters [90,92].

1.3.3. Factor affecting SCOs accumulation and composition

C/N ratio has been found to have the major impact on oil accumulation in oleaginous microorganisms [96]. C/N ratio greater than 20 is needed for oleaginous microorganisms to trigger lipid production, and in many cases the range is between 50 to 100, but values can be even higher [97].

In addition to that, other parameters can affect not only the titre of microbial oil, but also the lipid composition.

Temperature

Temperature has clearly an effect on cell growth, but it is also reported to affect the final lipid profile [98]. A temperature lower than the optimal usually corresponds to an increase in unsaturated and short chain fatty acids, for example by increasing the ratio of linoleic acid to oleic acid [99].

pH

The initial pH of the culture medium also plays a critical role on lipid synthesis. A decrease of lipid production was exhibited when the pH was below 4.0 and above 8.0. Moreover, the optimum pH for each oleaginous yeast for biomass production and hence single cell oil is also influenced by the different carbon sources available in the growth medium [98].

Aeration

The aeration is another important parameter not only for yeast growth but also for total lipid accumulation and composition. For example, under oxygen limited conditions, the glyceride fraction varies highly and the amounts of phospholipids and sterol decrease. These variations directly result in increasing amounts of saturated fatty acids, which become the main component of lipids. On the other hand, in aerated culture, the cellular viability consequently increases, and free fatty acids are oxidized to unsaturated fatty acids because yeast cells need unsaturated fatty acids for growth [97].

1.3.4. Current application of microbial oils

Biodiesel production

In the last decades, the global biodiesel production started raising mainly due to the European Union (EU) Renewable Energy Directive (RED) that requires 10% of all transport fuels to be delivered from renewable sources by 2020 in every Member State being more than 85% of the RED transport target expected to come from biofuels (Biodiesel is the main biofuel in the EU transport sector, with a 78.2% share of total consumption, by energy, according to Eurostat 2013). This demand started raising many concerns related with the sustainability of its production, mainly the biodiesel of first generation, obtained using food crops. Due to their high percentage of oleic acid, oleaginous yeasts represent a favourable alternative for the production of biodiesel, allowing to overcome all the sustainability issues related with first-generation biodiesel production [99]. Since the intracellular lipids accumulated by oleaginous yeasts are majorly composed of TAGs, they can be extracted and chemically transformed into fatty acids methyl esters (FAMEs) (Biodiesel) and glycerol (crude) [100]. As reported in literature, different classes of oleaginous microorganisms have been investigated for biodiesel production using industrial waste, for instance the yeasts *Rhodosporidium toruloides, Yarrowia lipolytica, Rhodoturola glutinis, Rhodoturola graminis* are able to accumulate lipids using molasses and crude glycerol as carbon sources, as well as *L. starkeyi* using lignocellulosic biomasses. [101, 102].

Oleochemicals

Oleochemicals are defined as a class of aliphatic compounds industrially-derived from animal or vegetable lipids. Oleochemical products can be classified according to the chain length, the terminal reductive state (e.g. acid, aldehyde, ester, olefin, alcohol, alkane), and any modifications (e.g. branching, unsaturation, hydroxyl-, cyclopropane-) to the main chain. These chemical features dictate the value and the end uses of each oleochemical. For instance, long-chain fatty acids (C16-C18) have a long history of use as soaps, while medium-chain fatty acids (C8-C12) have found additional uses as herbicides, precursors to lubricants, and polymer additives. Long-chain fatty acid methyl-esters (FAMEs) and fatty acid ethyl-esters (FAEEs) are the main components of commercial biodiesels that provide the energy for combustion. In addition to be used as fuels, medium-chain FAMEs, FAEEs, and methyl ketones (MKs) can be used as flavors and fragrances. Fatty alcohols (FAOH) are used to produce laundry detergents, industrial lubricants and surfactants, medicines, and personal care products. Long-chain alka(e)nes (ALKs) can be blended with liquid transportation fuels and serve as “drop in” replacements for petroleum-derived alkanes in fuel,

solvent and other chemical applications. Long- and medium-chain α-olefins are used to produce detergents and plasticizers, and as monomers for elastomers used in automotive parts. In addition, very-long-chain oleochemicals such as fatty alcohols, e.g. 1-docosanol (C22), and fatty waxes, e.g. Jojoba oil, are widely used in lubricants, detergents, polymers, photographic film-processing agents, coatings, cosmetics and pharmaceuticals. These wide-ranging applications, particularly biodiesel, are driving continued growth of the oleochemical industry [103]. Many microorganisms appear as promising platforms for the production of different valuable chemicals [104], and in particular the oleaginous yeasts, *R. toruloides*, *L. starkeyi*, *Y. lipolytica* are known for the production of oils used for lubricants production, especially thanks to the high amount of MUFAs, in particular oleic acid, which guarantees greater oxidative and temperature resistance [105].

Other applications

In addition to the application describe above, microbial oils can be used for the production of biopolymers, as substitute of petroleum products. For instance, polyols obtained from microorganisms are used in car and biomedical industry due to their flexibility and resistance [99]. Furthermore, some oleaginous yeasts could be used as platform to PUFAs production widely used as food additive and nutraceuticals [106-108].

1.3.5. Metabolic engineering to modulate fatty acids composition

The oils synthesized by oleaginous yeasts mainly contain stearic (18:0), oleic (18:1) and linoleic (18:2) acids together with palmitic acid (16:0), which were also the most represented fatty acids in the plant oils which means that they could be used for the same purpose [967].

	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Rapeseed oil	3	–	1	64	22	8
Soya oil	12	–	3	23	55	6
Sunflower oil	6.40	0.01	2.90	17.70	72.90	–
Jatropha oil	14.70	0.65	6.75	40.05	36.60	0.15
Cocoa butter	23.31	0.95	24.51	28.74	3.93	–
Palm oil	43.03	0.19	4.31	39.47	10.82	0.29
Cryptococcus curvatus	18	–	16	50	16	–
Yarrowia lipolytica	15	2	11	47	21	3
Rhodosporidium toruloides	20	1	15	47	13	3
Rhodosporidium toruloides 68-264	12	0.4	20.9	54.2	5.6	1
Cryptococcus victoriae 10-939	21	5.5	20.5	42.5	6.6	0.8
Lipomyces starkeyi	37	4	6	49	1	–
Rhodotorula glutinis	23.80	5.90	2.00	54.80	10.70	1.70

Fig.9. Relative mass percentage of the main fatty acids present in some seed oil and single cell oils [adapted from 99].

Genetic engineering of oleaginous yeasts is a powerful tool for tailoring both the quantity and quality of produced lipids [109]. In particular, metabolic engineering is a process for optimizing native metabolic pathways and regulatory networks or for assembling heterologous metabolic pathways for production of targeted molecules using molecular, genetic and combinatorial approaches. The purpose of the metabolic engineering is to generate a cell factory that produces cost-effective molecules at industrial scale [110].

The overexpression and/or deletion of gene encoding enzymes involved in the elongation and/or desaturation of fatty acids can allow to obtain SCOs with a composition tailored for chemicals or biofuels [111]. In order to obtain oil with high levels of oleic acid, used to develop biolubricants, hydraulic fluids, and oils for electrical transformers, the “pink-yeast” *R. toruloides* has been modified by the simultaneous overexpression of elongase (*ELO1*) and deletion of Δ12 desaturase (*FAD2*), obtaining an increase in oleic acid content of 23% compared to the parent strain [112], as well as it was also shown that the overexpression of the endogenous gene *LsELO2* in *L. starkeyi* BY4741 led to an increase of oleic acid content [113]. A similar result was obtained by the overexpression of *OLE1* gene in *R. toruloides* in order to produce oil for biodiesel industry [114].

Among the different oleaginous yeasts, *R. toruloides* and *L. starkeyi* represent promising platforms for SCOs production, especially for their ability to grow on a wide range of residual biomasses (Fig.10), in line with the circular economy concept [115].

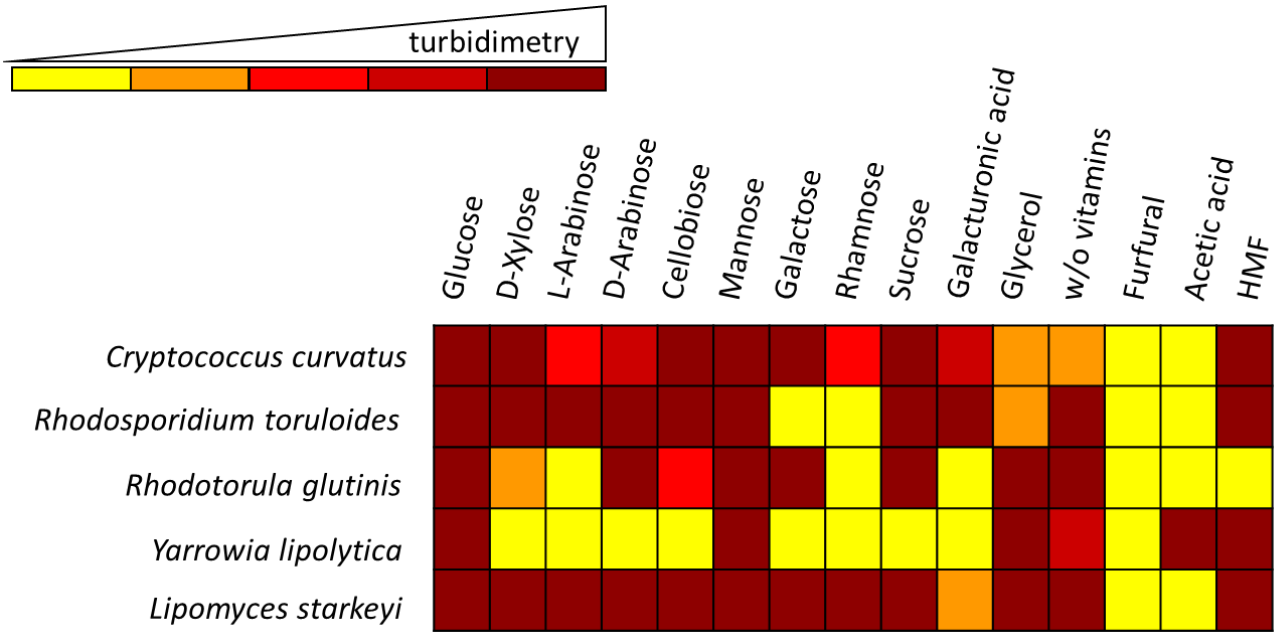

Fig.10. Turbidimetry scale of the oleaginous yeasts *C. curvatus, R. toruloides, R. glutinis, Y. lipolytica, L. starkeyi* grown on different feestocks and in the presence of inhibitors (furfural, acetic acid, HMF) [adapted from 115].

1.3.6. *Rhodosporidium toruloides*

The oleaginous yeast *Rhodosporidium toruloides*, now also known as *Rhodotorula toruloides*, is a red, heterothallic, bipolar yeast that belongs to *Pucciniomycotina* (Basidiomycetes). *R. toruloides* is a natural producer of carotenoids (which are responsible for the red color of the cells and provide antioxidant properties) and lipids (up to 65% of dry cell weight, CDW) in the form of triacylglycerols (TAGs) under nitrogen-limited conditions. All these compounds are industrially relevant: carotenoids are used as colorants, antioxidants, or vitamin A precursors and are therefore important for the food, feed, and pharma companies; lipids and lipid-derived compounds are good substitutes for petroleum-derived platforms for the production of fuels and chemicals [112].

In the 1950, the first studies on *R. toruloides* as a potential biotechnological microorganism started: its preferable traits are the capacity to grow to high cell density and its ability to utilize a wide range of carbon (sugars, lignocellulosic and sludge hydrolysate) and nitrogen sources, which can be potentially exploited to produce high-value products from low-cost substrates [110, 116, 117]. Moreover, the robustness and tolerance towards inhibitory compounds has been well demonstrated

[108, 109, 118]. Over the years, several complete genome sequences of the strain have been published, but the absence of efficient and reliable genetic manipulation tools have been a major obstacle in *R. toruloides* development as workhorse. The first transformation protocol for *R. toruloides* based on polyethylene glycol (PEG)-mediated protoplasts was published in 1985, but it was found to be inefficient and the transformants unstable. The most commonly used transformation methods are based on *Agrobacterium tumefaciens*-mediated transformation (ATMT). More recently, electroporation methods have been described as well as lithium acetate/polyethylene glycol (PEG)- mediated chemical.
In the near future, the development of efficient synthetic biology tools for DNA assembly, such as Golden Gate cloning, or genome editing, such as CRISPR/Cas9, is expected to boost metabolic engineering approaches in this yeast [112].

1.3.7. *Lipomyces starkeyi*

The oleaginous yeast *Lipomyces starkeyi* is an ascomycetous yeast belonging to the order Saccharomycetales, and the genome analysis suggests it is homothallic. It is a unicellular with an ovoid/round shape that form mucoid colonies with white/cream colour. *L. starkeyi* was first described by Robert Starkey (Starkeyi 1946) [119].
It is able to proliferate aerobically and can metabolize an extensive range of carbon and nitrogen sources but is incapable of fermentative metabolism [120, 121]. Among carbon sources, it is able to assimilate a wide range of feedstocks, including low cost substrate [122, 123]. *L. starkeyi* is one emerging species that has garnered attention for its exceptional potential for biotechnological application [120]. This is due to its ability to accumulate lipids over 70% of its cell biomass under defined culture conditions [124]. The genome sequence of *L. starkeyi* has already been determined [125], as well as different transformation methods have been investigated such as the use of lithium acetate [126], spheroplasts [127] and *A. tumefaciens* [128]. Nevertheless, the transformation frequency is still very low, and an optimized procedure for targeted DNA modification and insertion needs to be established [121].
It is important to consider that the introduction in this yeast of DNA fragment is mainly dependent of non-homologous-end-joining (NHEJ) mechanism, rather than homologous recombination (HR). Consequently, even medium-small size fragments (about 2000 bp) show a very low frequency of integration. Recently, it has been demonstrated that the deletion of some proteins involved in the NHEJ (such as *KU70, KU80, or LIG4*) can increase the frequency of HR caused by reduction of the random integration of DNA fragments [129].

Among the low cost substrates, recently, it has been demonstrated in our group the ability of the strains *R. toruloides* DSM444 and *L. starkeyi* DSM70295, selected for this study, to grow on residual feedstocks, in particular on *Camelina sativa* [130], and sugar beet pulp [131] hydrolizates and crude glycerol [118]. In that context, for this study we have chosen the waste of biodiesel industry, crude glycerol.

1.3.8. Second generation feedstock: Crude glycerol

Crude glycerol, generated as a by-product during biodiesel production, consists mainly of glycerol, methanol, and salts.

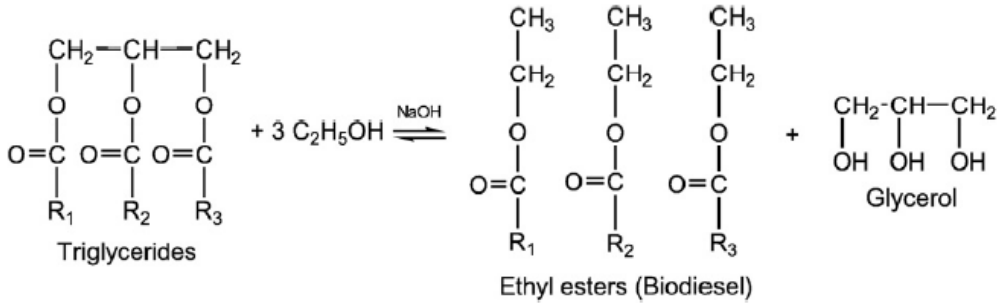

Fig.11. Trans-esterification of a triglyceride using NaOH as catalyst to obtain biodiesel [129].

Together with the wide application of biodiesel, enormous quantities of crude glycerol are likely to be generated in the near future. Albeit refined glycerol would be a valuable product, the purification process is always costly because of the presence of impurities in crude glycerol. In some European countries, crude glycerol is even treated as a new kind of industrial wastewater. Therefore, the use of crude glycerol as a substrate for lipid production by oleaginous microorganisms would provide considerable benefits in terms of economy and environment [132-134].

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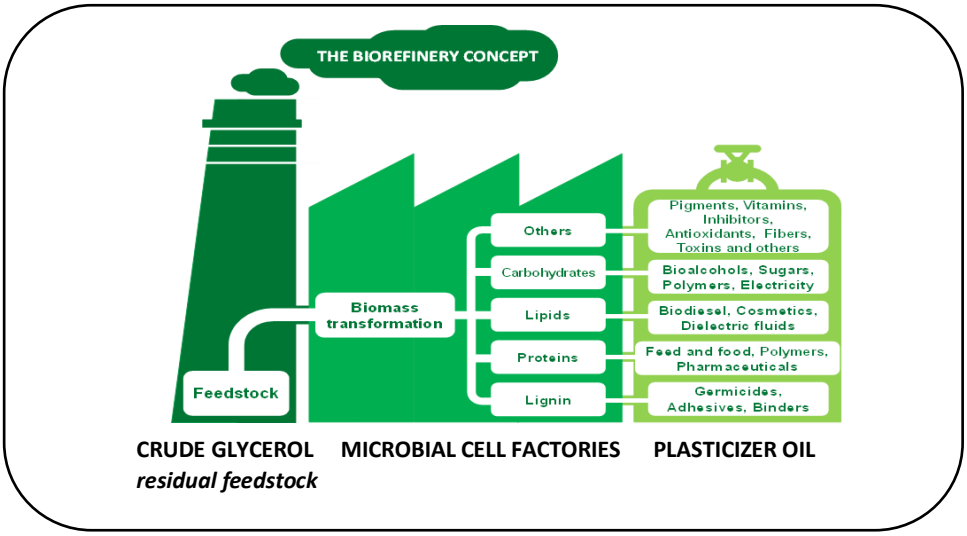
Outline of this study

As widely described in the introduction, nowadays there is an increasingly stringent need to replace mineral oils coming from distillation of petroleum and plants oils used in tyre compounding. To this purpose, a possible alternative is provided by oleaginous yeasts, which are able to accumulate oils with a composition very similar to vegetable oils, and which can be customised either by optimizing cultivation parameters and yeast hosts, or by engineering them. The research described in this thesis focuses on understanding the potential use of microbial oils in tyre compounds, as well as the study of oils properties affected by their composition that can influence the compatibility with specific elastomers. **Chapter 1** describes how the oleaginous yeasts *Rhodosporidium toruloides* and *Lipomyces starkeyi* were fermented on a residual feedstock, crude glycerol, a bio-product of biodiesel industry. After that, the two obtained microbial oils, the MICRO-OIL1 and MICRO-OIL2, having a different fatty acids composition, were used to prepare elastomeric compounds. The microbial oil compounds characterization suggested an applicability of these oils in tyre compounds, which adopted a behaviour similar to conventional oil used to this aim (IT 102020000006688).

An additional study confirmed that the presence of carotenoids in the MICRO-OIL1 did not affect the good results obtained before.

Chapter 2 describes a compatibility test developed by us to study and score the combination oil-elastomer, to be able to choose the best formulations. The test was used to select a further compound test recipe for MICRO-OIL2. In detail, the mixture prepared with the MICRO-OIL2 and the elastomer SLR3402 showed a DSC curves that suggested a good miscibility, later confirmed by compounds characterization. In addition, among all the compounds, the one prepared with the vegetable oil, AP-88® (Cargill), showed the best value of tensile strength, maybe due to its composition rich in monounsaturated fatty acids. Although the overall static and dynamic properties of AP-88® compound were inferior to the properties of MICRO-OIL2 compound, the improvement in ultimate properties related to higher monounsaturated fatty acids led to the attempt to increase such component in MICRO-OIL2, developed in the next chapter. Indeed, in **Chapter 3** a metabolic engineering approach was applied to obtain a strain, *Lipomyces starkeyi-OLE1-FAD2*, able to produce a microbial oil with a customized fatty-acids profile. Because of the novelty and the applicability of the obtained results, the work was valorised with the deposit of a patent application (IT102020000006715).

Thesis organization



Rhodosporidium toruloides | ***Lipomyces starkeyi*** | ***Lipomyces starkeyi-OLE1-FAD2***

MICRO-OIL1 | **MICRO-OIL2** | **MICRO-OIL4**

Production (fermentation process) and extraction of Microbial oils (MICRO-OIL1 and MICRO-OIL2) (Chapter 2)

- Replacement in rubber compounds (NR/BR based) of MES and vegetable oils with MICRO-OIL1 and MICRO-OIL2
- Effect of carotenoids in rubber compounds

Production (fermentation process) and extraction of Microbial oils (MICRO-OIL4) (Chapter 4)

- Replacement in rubber compounds (SBR based) of MES and vegetable oils

Compatibility tests between different elastomers and oils (Chapter 3)

- Compatibility elastomers/oils
- Rubber compounding using the chosen elastomer and oils

Conclusions and future perspectives (Chapter 5)

2

The oleaginous yeasts Rhodosporidium toruloides and Lipomyces starkeyi as platforms for the production of rubber plasticizer oils

Patent application (PA):
PA1: CASTELLANI LUCA (IT); GIANNINI LUCA (IT); GUERRA SILVIA (IT); BRANDUARDI PAOLA (IT); DI LORENZO RAFFAELLA DESIRE' (IT). “Produzione di mescole elastomeriche comprendenti oli ad azione plastificante ottenuti a partire da cellule microbiche oleaginose”
Publication info: IT 102020000006688

2.1. Introduction

Rubber formulations and compounding is a complex technology involving the selection and blending of elastomers and other ingredients to meet the desired final product requirements in terms of performance, processing, environmental impact and cost [1]. Plasticizers play important roles in rubber formulation: they can improve processing by lowering the compound's viscosity and they can aid the flexibility of the formulated rubber at low temperature [1,2]. Petroleum-based mineral oils, obtained from distillation process, are conventionally used as plasticizers in rubber compounding. Because of the rapid depletion in world petroleum reservoirs together with the uncertainty in petroleum supply due to political and economic reasons, the necessity of alternatives plasticizers sources is evident [3]. Vegetable oils can be suitable candidates as plasticizer oils, especially because of their composition (mixture of triglycerides, TAGs), which makes them prone to chemical modifications [2]. Unfortunately, vegetable oils are considered as first generation feedstocks in competition with food industry. Moreover, there are concerns related to the supply chain, as they depend on seasonal crops, which in turn also depend on climate and its possible instability. Because of that, the attention was in the last decades redirected to consider the single cell oil (SCO) produced by oleaginous yeasts [4,5] as sustainable source of oil.

Single cell oil (SCO) is commonly defined as neutral storage lipids accumulated by oleaginous eukaryotic microorganisms, such as yeast, molds and microalgae, and is often synonymous with lipid, oil and triacylglycerol (TAG) [6]. Most oleaginous yeasts can accumulate lipids over 40% of their dry weight and up to 70% under nutrient-limiting conditions. In general, the limiting nutrient in the culture medium is nitrogen, essential for the biosynthesis of proteins and nucleic acids. The use of different carbon/nitrogen ratio can influence the lipid accumulation, depending however by the nature of microorganisms. Because the formulation of culture media is one of the most important steps for lipids accumulation, it is important to optimize the nutrients in terms of composition and costs. The utilization of cheap hydrophilic and hydrophobic substrates such as agro-industrial wastes, cheese whey, molasses and waste cooking oil have been reported in literature [7-9].

This chapter shows the use of a waste by-product of biodiesel industry, crude glycerol, as carbon source for oleaginous yeast fermentation. We used two oleaginous yeasts, *Rhodosporidium toruloides* and *Lipomyces starkeyi* for their ability to accumulate high amounts of SCOs [10]. The gas chromatography analysis of the obtained oils showed a lipid composition, rich in saturated and monounsaturated fatty acids, similar to that of vegetable oils, making them suitable as plasticizer for rubber compounding. The microbial oils obtained, MICRO-OIL1 and MICRO-OIL2, have been tested in rubber mixture as substitute for petroleum and vegetable derived-oils, in order to assess and compare the performance of the final compounds.

In the second part of this chapter we investigated the possible antiozonant/antioxidant effect of MICRO-OIL1 because of the carotenoids contained therein (Fig.1). Carotenoids are natural pigments which are synthesized by plants and are responsible for the bright colours of various fruits and vegetables. There are several dozens of carotenoids in the food that we eat, and most of these carotenoids have antioxidant activity [11]. Also microorganisms are able to produce this kind of molecules, in particular the "pink yeast" *R. toruloides* is able to naturally accumulate carotenes and xanthophylls, such as β-carotene, torulene, and torularhodin [12]. During tyre compounding antiozonants/antioxidant are often added to avoid rubber failure. Over the course of time mechanical-dynamic (fatigue), oxidation processes, temperature, exposition to UV light (photo oxidation) and to aggressive media lead to irreversible changes in the physical and chemical properties of an elastomer material, often resulting in premature functional failure of the component concerned. In particular, it has been reported that one of the factors causing tyre failure is the surface crack development due to an ozone attack. Ozone induces crack initiation at the surface which then propagates as a result of flexing. The Para-PhenyleneDiamines (PPDs) represent a prominent example of antiozonants. They are not only able to protect from ozone damages but also to improve resistance to fatigue, oxygen, heat and metal ions [13, 14].

Fig.1. MICRO-OIL1 produced by *R. toruloides*

Compounds containing MICRO-OIL1 produced by the pink yeast *R. toruloides* were prepared and compared with compounds prepared with the addition of the conventional antioxidant, 6PPD.

2.2. Experimental part

2.2.1. Materials and Methods

2.2.1.1. Strain and Media

R. toruloides (DSM4444) and *L. starkeyi* (DSM70295) were purchased from DSMZ. Yeasts were stored in cryotubes at −80 °C in 20 % glycerol (vv^{-1}).

The composition of the inoculum and fermentation medium (MeOL) was per liter: 1 g of yeast extract (0.114 g of nitrogen), 1.31 g of $(NH_4)_2SO_4$ (0.278 g of nitrogen), 0.95 g of Na_2HPO_4, 2.7 g of KH_2PO_4, 0.2 g of Mg_2SO_4 $7H_2O$, 0.04 g. After the pH was adjusted to 5.5 using NaOH 4 M, the medium was supplemented with a 100X trace mineral stock solution consisting of (per liter): 4 g $CaCl_2$ $2H_2O$; 0.55 g $FeSO_4$ $7H_2O$; 0.52 g citric acid; 0.10 g $ZnSO_4$ $7H_2O$; 0.076 g $MnSO_4$ H_2O; and 100 µL 18 M H_2SO_4 [10].

Yeast extract was provided by Biolife Italiana S.r.l., Milan, Italy. All the other reagents were provided by Sigma-Aldrich Co., St Louis, MO, USA.

Glycerol, both crude and pure, was used as carbon and energy source. Following the protocol described in [15] for the calculations of the carbon-to-nitrogen ratio (C/N), a carbon content in glycerol of about 39.1 %, and a nitrogen content of 21.2 and 11.4 % in ammonium sulfate and yeast extract, respectively, were assumed.

2.2.1.2. Raw material

Crude glycerol derived from industrial biodiesel production out of palm oil. The stock had 80 % content of glycerol and appeared as a dark brown liquid. In the text, reported crude glycerol concentrations ($g\ L^{-1}$) refer to the HPLC measured glycerol. Composition analyses of this stock were conducted: the original sample was diluted 50-fold and passed through 0.22 µm filter. Crude glycerol was autoclaved at 120° C and left to settle about 24 hours. The top, dark red layer, mainly consisting of free fatty acids (FFAs), was removed.

2.2.1.3. Microbial oil preparation: Fed-batch fermentation cultures

Yeasts were revived from cryo-preserved stocks stored at −80 °C and grown on YP-Glycerol agar plates. One colony was used to inoculate 1 L flasks with 200 mL of the culture medium and seed cultures were placed on a rotary shaker at 25 °C and 220 rpm about 3 days. Exponential phase shake flasks cultures were used to inoculate bioreactors to a final optical density (OD_{660}) of 3.0. Briefly, cells were centrifuged at 6000 rpm for 5 min, washed twice with water, and finally resuspended in 40 mL of sterilized water, to be added into fermentors.

The fed-batch experiments were conducted in 2.0 L bioreactors (Sartorius Stedim BIOSTAT® Bplus, Germany). For the bioreactor cultivations, the aeration rate, agitation, and temperature were set to 1 vvm, 300 rpm (in cascade to 25 % of dissolved oxygen), and 25 °C, respectively. The pH was maintained by automatic pumping of 4 M NaOH.

The cultivation was designed to have two phases: 1) *"batch phase"* to allow cells adaptation and growth; 2) *"pulse fed-batch phase"* to promote lipids accumulation.

The final glycerol concentration set was 100 g/L, divided in the two phases. Bioreactor experiments were started with a working volume of 1.0 L and with 60 gL^{-1} as initial concentration of pure glycerol, resulting in a ratio C/N of 35:1. When the residual concentration of glycerol reached about 20 g/L the pulse fed-batch started, using a solution comprising ~40 g/L of crude glycerol as sole nutrient source.

The time of pulse was set based on the glycerol uptake rate ($gL^{-1}h^{-1}$). The highest glycerol uptake rate on pure glycerol was taken as reference to determine the starting point of the pulse phase.

When needed, antifoam emulsion (Sigma-Aldrich, MO, USA) was added to prevent excess foam formation. Aliquots were collected at regular intervals to evaluate the concentration of main extracellular metabolites (HPLC analysis), optical density (OD660), cell dry weight (CDW) and lipid content and composition (gas chromatography).

2.2.1.4. Analytical methods

The optical density was measured at 660 nm (OD_{660}) with a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation). Samples collected at different times were centrifuged at 14000 rpm for 10 min. The supernatants were filtered (0.22 µm filter) and glycerol concentrations were HPLC determined using a Rezex ROA-Organic Acid (Phenomenex). The eluent was 0.005 N H_2SO_4 pumped at 0.5 mL min^{-1} and column temperature was 40 °C. Separated components were detected by a refractive-index detector and peaks were identified by comparing with known standards (Sigma-Aldrich, St Louis, MO, USA).

Biomass was harvested by centrifugation of the culture samples at 4000 rpm for 10 min. The pellets were then washed twice with distilled water and dried at 40 °C (Concentrator 52301, Eppendorf, Germany) until a constant weight was obtained.

Additional biomass was also preserved for cellular lipids and fatty acids analysis.

2.2.1.5. Microbial oil preparation: extraction and analysis of composition

To determine the lipid content in yeast cells, lipids were extracted, based on the method of Bligh and Dyer [16] and Folch et al. [17], with modifications, and then analyzed by GC. Briefly, cells were centrifuged at 8000 rpm for 15 min and washed twice with distilled water. Cells were then resuspended in a volume of HCl 2 M (1:1) and chemically disrupted putting on thermostatic bath for at 95 °C for 1h. Then, $CHCl_3$:CH_3OH (2:1) solution was added to the mixture cells:HCl (ratio 1:1), mixed by vortex or inversion and centrifuged at 10000 rpm for 10 min. After centrifuge, of the 3 phases, the organic phase on the bottom was collected; 10 ml of chloroform were added, mixed again by vortex or inversion, and centrifuged at 10000 rpm for 10 min. Finally, the organic phases on the bottom were collected and left overnight under chemical hood for the evaporation. For derivation of methyl esters from fatty acids a 0.4 M solution of KOH-methanol was added to oil, the mixture was vortexed and placed in a thermobath at 60 ° C for 1 hour. After a volume of BF_3 -methanol (14% w / w) (1:1) was added, mixed by vortex and placed for one hour in a thermobath at 60 ° C; finally, n-hexane was added to extract the fatty acids methyl esters (FAMEs). Fatty acid methyl esters were analysed by gas chromatography (SAVI LABORATORI & SERVICE S.r.l. via Roma, 80 46037 Roncoferraro (MN) Italy) according to the methods (UNI EN ISO 12966-1:2015 + UNI EN ISO 12966-2:2011 + UNI EN ISO 12966-4:2015).

2.2.1.6. Compounding

2.2.1.6.1. Materials for rubber compounding

Natural rubber (STR20) was purchased from SRI Trang Agroindustry; Polybutadiene (BR 60 Mooney 63) is produced by Versalis; Ultrasil 7000, which is commonly used in the tyre industry, was from Evonik, bis[3-(triethoxysilyl)propyl] tetrasulfide (TESPT) 50% supported on carbon black (N234) 50% was obtained from Evonik, soluble sulfur was from Zolfidustria Srl, zinc oxide was from Empils-zinc, stearic acid was from SO.G.I.S. INDUSTRIA CHIMICA S.p.A, 2,2,4-trimethyl-1,2-dihydroquinoline (TMQ) was from LANXESS GmbH, N-(1,3-Dimethylbutyl)-N'-phenyl-p-phenylenediamine (6PPD) was obtained from Eastman Chemical Co. The accelerator tetrabenzylthiuram disulphide (TBZTD) and Vulkacit® NZ/EGC (N-tert-butyl-benzo-thiazyl sulfenamide (TBBS)) were from LANXESS GmbH. Mild extracted solvate (MES) was purchased from ENI SPA, CASTOR OIL (Radia 6132®) was produced by Oleon, AP-75 and AP-88 were obtained from Cargill®. MICRO-OIL1 and MICRO-OIL2 have been produced in this study.

2.2.1.6.2. Natural Rubber-Butadiene-based NR-BR compounds

The NR-BR compounds were prepared by physically mixing the elastomers (natural and butadiene rubber) with other ingredients (filler system, vulcanization system, antioxidants and accelerators) using a Brabender internal mixer with a total volume of 50 ml and filling coefficient of 0.9. The compounds were prepared using microbial and vegetable oils or mineral oil, as reference. The final content of oils for each compound was 20 phr. The formulation of the compounds is shown in Table 1.

Ingredients	NR-BR MICRO-OIL1	NR-BR MICRO-OIL2	NR-BR CASTOR OIL	NR-BR AP-75	NR-BR AP-88	NR-BR MES
Polymers						
STR20	70	70	70	70	70	70
BR 60 Mooney 63	30	30	30	30	30	30
Filler system						
Ultrasil 7000	55	55	55	55	55	55
TESPT+N234	8.8	8.8	8.8	8.8	8.8	8.8
Oils						
MICRO-OIL1	20	-	-	-	-	-
MICRO-OIL2	-	20	-	-	-	-
CASTOR OIL	-	-	20	-	-	-
AP-75	-	-	-	20	-	-
AP-88	-	-	-	-	20	-
MES	-	-	-	-	-	20
Vulcanization system						
Soluble sulfur	2	2	2	2	2	2
Zinc oxide	2.5	2.5	2.5	2.5	2.5	2.5
Stearic acid	1.5	1.5	1.5	1.5	1.5	1.5
Antioxidant and accelerators						
TMQ	1	1	1	1	1	1
6PPD	1.5	1.5	1.5	1.5	1.5	1.5
TBZTD	0.2	0.2	0.2	0.2	0.2	0.2
TBBS	1.8	1.8	1.8	1.8	1.8	1.8

Table 1: Recipe formulation expressed in phr (part per hundred rubber)

The NR-BR compounds were prepared in two steps. The first step lasted 10 min. The internal mixer temperature was set at 60 °C and rotor speed at 60 rpm. During the

first three minutes, the polymers were added into the mixer, and then filler was added and mixed for the next 5 min. The temperature of the mixer was raised to 135 °C in the next 2 min. Stearic acid and zinc oxide were added at 8 min, mixed for next two min increasing the temperature up to 140° and at min 10 the mixing was stopped. The second step was performed at 60 °C for 11 min at a rotor speed of 60 rpm. The masterbatch from the first step was added during the first 4 minutes. Then, antioxidants, soluble sulphur, and accelerators were added at min 4 and mixed for the next 7 min. The mixing was stopped at 11 min. At the end of both steps, the prepared compounds were passed three times through a two-roll mill at 40 °C for complete homogenization.

2.2.1.7. Effects of carotenoids in rubber compounds

The effect of carotenoids in rubber compounds was tested preparing the NR-BR MO1 (MICRO-OIL1) compounds with or without 6PPD, basically following the receipt formulations described above. The formulation of the prepared compounds is shown in Table 2.

Ingredients	NR-BR MO1 WITH 6PPD	NR-BR MO1 WITHOUT 6PPD
Polymers		
STR20	70	70
BR 60 Mooney 63	30	30
Filler system		
Ultrasil 7000	55	55
TESPT+N234	8.8	8.8
Oils		
MICRO-OIL1	20	20
Vulcanization system		
Soluble sulfur	2	2
Zinc oxide	2.5	2.5
Stearic acid	1.5	1.5
Antioxidant and accelerators		
TMQ	1	1
6PPD	1.5	-
TBZTD	0.2	0.2
TBBS	1.8	1.8

Table 2: Recipe formulation expressed in phr (part per hundred rubber)

2.2.1.8. Compounds characterization

2.2.1.8.1. Vulcanization of NR-BR compounds

The vulcanization curves of the prepared NR-BR compounds were recorded using a Moving Die Rheometer (RPA 2000, Alpha Technologies). The measurements were performed at a temperature of 150 °C, running time of 30 min, and rotor frequency of 1 Hz. Vulcanization parameters, such as maximum torque (M_H), minimum torque (M_L), scorch time (T_s2), and optimum cure time (T_{90}) were obtained from the vulcanization curves.

2.2.1.8.2. Tensile mechanical analysis: Static properties

Tensile mechanical properties, such as tensile strength, elongation at break, and stress at different percentage of elongation (10, 50, 100 and 300%) were measured by tensile mechanical analyses by Pirelli's laboratories. Before performing the analysis, the samples were pressed in 2 mm thick sheets by a two-roll mill and vulcanized in a hydraulic press at 151 °C for 25 min. The vulcanized samples were conditioned in the measuring environment for 24 h. Samples for analysis were prepared by die-cutting the vulcanized sheets to obtain dumbbell-shaped specimens of standard dimensions. Tensile mechanical analyses of the prepared dumbbell-shaped specimens were performed by using a Zwick/Roell tensile testing machine. The measurements were performed according to ISO 37 and UNI 6065 standards. The stress-strain curves were recorded by progressive straining of samples. For each material, five samples were measured, and the average value was reported.

2.2.1.8.3. Tensile mechanical analysis after thermal aging: Static properties

Tensile mechanical properties after thermal aging, such as tensile strength, elongation at break, and stress at different percentage of elongation (10, 50, 100 and 300%) were measured by tensile mechanical analyses, as mentioned above, after 168 hours at 70°C.

2.2.1.8.4. Dynamic mechanical analysis: Dynamic properties

Dynamic-mechanical properties were measured by Pirelli's laboratories using an Instron dynamic device in the traction-compression mode according to the following methods. A test piece of the crosslinked elastomeric composition having a cylindrical form (length = 25 mm; diameter = 12 mm) and kept at the prefixed temperature (10, 23 and 70 °C) for the whole duration of the test, was compression-preloaded up to a 25% longitudinal deformation with respect to the initial length and then submitted to a dynamic sinusoidal strain having an amplitude of 3.5% with respect to the length

under preload, with a 100 Hz frequency. The dynamic-mechanical properties are expressed in terms of dynamic storage modulus (E') and loss factor (tanδ) values. The tanδ value is calculated as a ratio between loss (E'') and storage modulus (E').

2.3. Result and discussion

2.3.1. Fed-batch fermentation of *R. toruloides* and *L. starkeyi*

The oleaginous yeasts *R. toruloides* and *L. starkeyi* were cultivated in fed-batch mode in MeOL medium [10] with glycerol (pure and crude) as carbon and energy source to evaluate growth and SCOs accumulation. Fermentations were performed at 25 °C, pH 5.5 and monitoring glycerol consumption. The inlet gas flow rate was maintained constant at 1 vvm and stirring was set in cascade to 25 % of dissolved oxygen to ensure fully aerobic conditions.

The cultivation was designed to occur in two phases: 1) "*batch phase"* on pure glycerol (till 20 g/L of residual glycerol) to allow cell growth; 2) "*pulse fed-batch phase"* where the medium containing only crude glycerol was fed to reach again 60 g/L to promote allow cell adaptation and oils accumulation. The batch phase was started with an initial biomass high enough to allow a rapid accumulation and a faster shift to the production phase (see Materials and Methods for details).

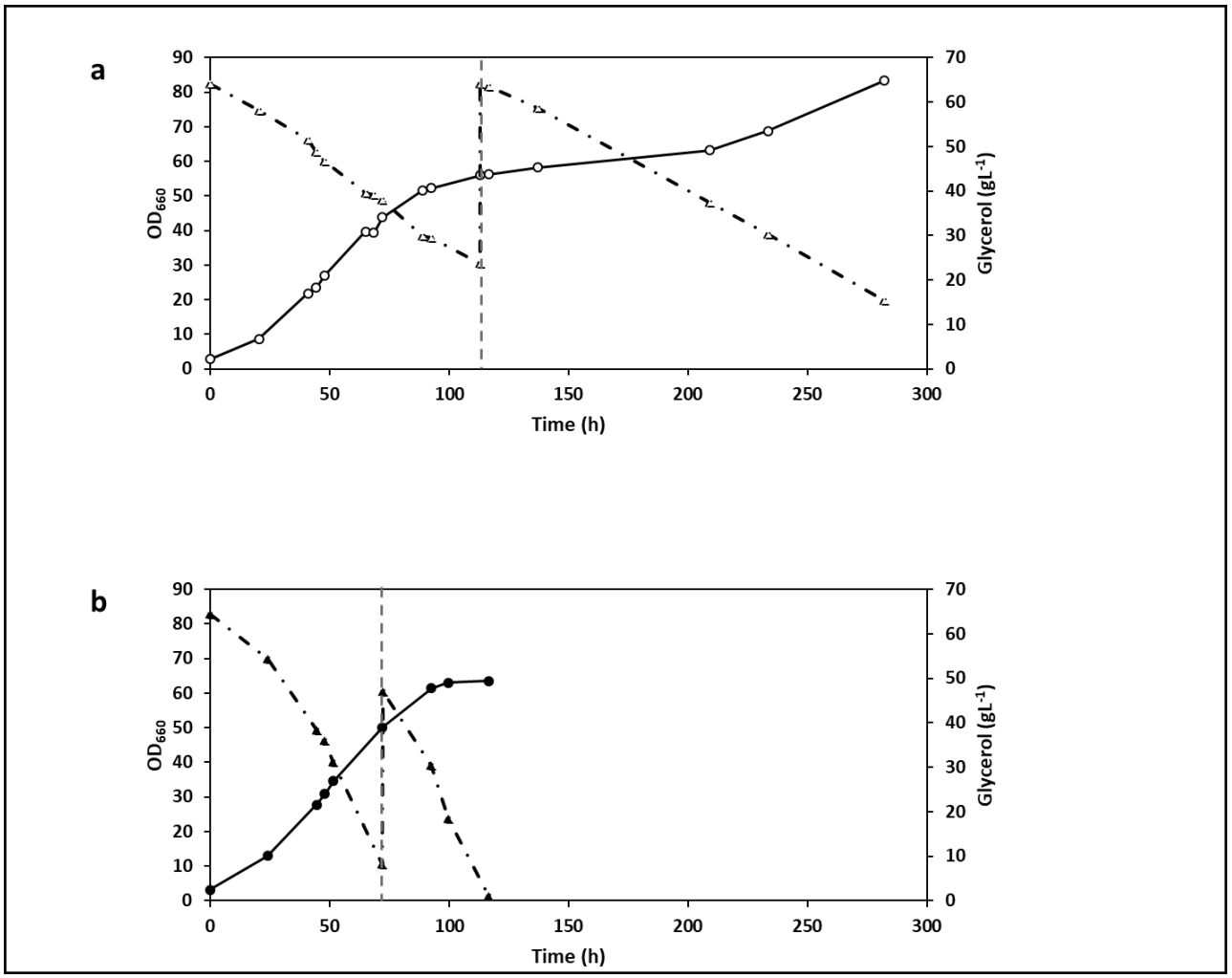


Fig.2 Glycerol consumption and growth (OD, optical density) profiles of *R. toruloides* (**a**) and *L. starkeyi* (**b**) under fed-batch pulse cultivation (grey dashed lines). Growth (OD_{660}, continuous line) and glycerol consumption profiles (g L^{-1} , dashed line) of *R. toruloides* (**a**) and *L. starkeyi* (**b**). Data represent the mean of three independent assays.

Using the pulse fed-batch strategy, above described, the strains were able to growth, but with some difference in terms of fermentation kinetic: *R. toruloides* is slower than *L. starkeyi* (as shown Fig.2). This corresponds to glycerol uptake rate during the batch phase corresponding to 0.35 $gL^{-1}h^{-1}$ and 0.78 $gL^{-1}h^{-1}$, respectively. After the pulse, it was also confirmed that on crude glycerol *L. starkeyi* was much faster compared to *R. toruloides* (1.03 $gL^{-1}h^{-1}$ and 0.29 $gL^{-1}h^{-1}$), confirming the results obtained in our previous work [10].

Strain	Maximum DW (gL^{-1})	Maximum oil content (gL^{-1})	Lipids (%)
R. toruloides	36.5	21.1	57.8
L. starkeyi	41.4	19.5	47.2

Table 3. Comparison of parameters related to biomass and lipid production among *R. toruloides* and *L. starkeyi*.
Data shown are the mean of three independent experiments where the deviation from the mean value was less than 5 %.

Table 3 reports the cell mass (maximum DW) and lipid content (maximum oil content) of these two yeasts. The highest biomass production was achieved in *L. starkeyi* (41.4 gL^{-1}), compared to *R. toruloides* where we obtained 36.5 gL^{-1}. Conversely, for *R. toruloides* we obtained the higher lipid content and percentage, 21.1 gL^{-1} and 57.8%, compared to *L. starkeyi*, 19.5 gL^{-1} and 47.2%, confirming also in this case our previous results [10].

2.3.2. Gas chromatography analysis: MICRO-OIL1 and MICRO-OIL2

MICRO-OIL1 and MICRO-OIL2, produced by fed-batch fermentation by the oleaginous yeasts *R. toruloides* and *L. starkeyi,* were hydrolyzed and transmethylated. The resulting fatty acid methyl esters (FAMEs) were then analysed by gas chromatography. The fatty acids profiles of MICRO-OIL1 and MICRO-OIL2, together with those of three vegetable oils, CASTOR OIL, AP-75 and AP-88 are shown in Table 4.

% (wt/wt)	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)	Ricinoleic acid (C18:1OH)	SFAs	MUFAs	PUFAs
MICRO-OIL1	30.0	1.0	10.0	37.0	16.0	-	-	42.0	38	20
MICRO-OIL2	30.6	4.2	6.6	53.7	2.9	-	-	38.9	58	3.1
CASTOR OIL	-	-	-	4-5	-	-	85-90	1-2	89-95	4-5
AP-75	2-6	-	1-5	71-81	7-17	2-4	-	3-11	71-82	9-21
AP-88	3-4	-	2-3	84	8	-	-	6-10	84	6-10

Table 4. Gas chromatography analysis of MICRO-OIL1 (*R. toruloides*) and MICRO-OIL2 (*L. starkeyi*) compared to the vegetable oils (CASTOR OIL, AP-75 and AP-88).
SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids, PUFAs, polyunsaturated fatty acids.
Data shown are the mean of three independent experiments where the deviation from the mean value was less than 5 %.

Consistently with the data previously obtained in the lab [10], the fatty acid profiles obtained for the two yeasts showed that the most represented are the long-chain fatty acids, in particular palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic acid (C18:2), as also recently reviewed in [17]. Focusing the attention on the individual

percentages of MICRO-OIL1 and 2, it is possible to highlight some interesting differences among the two yeasts fermented in this study. In *R. toruloides* the percentage of 18:2 was about ten times more of the percentage observed in *L. starkeyi*. Also the percentage of oleic acid was significantly different: the highest was observed in *L. starkeyi* (~ 53.7%), while in *R. toruloides* was about 37%.
Consequently, the total distribution of saturated, monounsaturated and polyunsaturated fatty acids is significantly different, especially regarding the content of PUFAs (20% for MICRO-OIL1 and only 3.1% for MICRO-OIL2) as well as for MUFAs (38% and 58%, respectively). These results, together with the low amount of SFAs for both strains, confirm that these microorganisms are able to accumulate oils very similar to vegetable oils, that can be used in substitution to diverse oleochemicals [19]. However, the composition of vegetable oils, reported in Table 2 used in this study as vegetable oil reference, is quite different. We chose CASTOR OIL for the high content of ricinoleic acid (85-95%) that make it versatile for application in the chemical industry [20]. On the other hand, in the sunflower oils, AP-75 and AP-85, the most represented fatty acid is oleic acid, 71-82% and 84%, respectively, and it is already known for its application for bio-based lubricant production [20].
As the oil composition was judged as promising to be tested for plasticizer properties, we performed series of ten (10) liter fermentation for each strain, obtaining a yield oil/glycerol ($Y_{o/g}$) of about 0.20 and reaching a production of about 200 g of oil per fermentation. These quantities have been used for preparing the oil batched used for all the experiments described below.

2.3.3. Natural Rubber-Butadiene formulations: NR-BR compounds

2.3.3.1. Compounding

The NR-BR compounds were prepared by using the MICRO-OIL1 and 2, together with the vegetable oils (CASTOR OIL, AP-75 and AP-88) and the mineral oil MES, as references. The final content of oils for each compound is 20 phr. The compounds were prepared in two steps. The temperature was raised up to 140 °C during the first step to create a chemical bond between the filler and silane coupling agent. The vulcanizing agents were added in the second step, which was performed at lower (60°C) temperature to avoid the pre-vulcanization of the rubber compounds.

2.3.3.2. Characterization of NR-BR compounds

2.3.3.2.1. Vulcanization characteristics

The prepared NR-BR compounds were vulcanized for 30 min and change in torque was recorded versus time. The vulcanization curves give a complete picture of the overall kinetics of vulcanization. The vulcanization curves of the compounds are shown in Fig. 3. Some of the vulcanization parameters, such as scorch time (T_S2), optimum curing time (T_{90}), minimum (M_L) and maximum (M_H) value of torques are shown in Table 5.

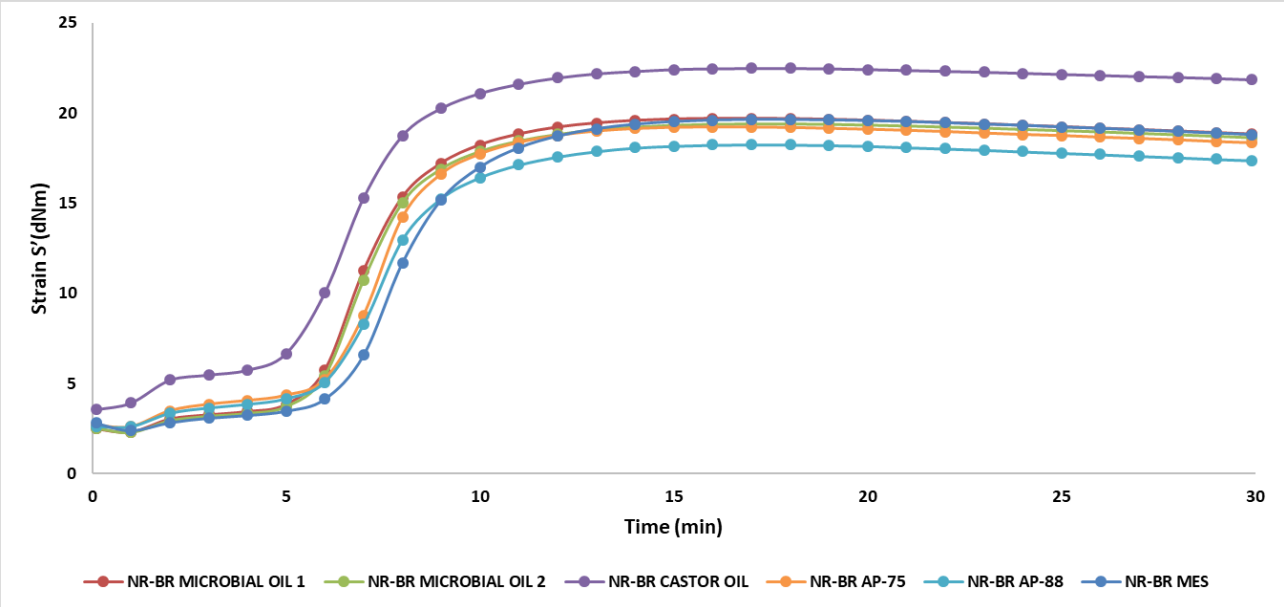


Fig.3. Vulcanization curves of the prepared NR-BR compounds

Parameters	NR-BR MICRO-OIL1	NR-BR MICRO-OIL2	NR/BR CASTOR OIL	NR-BR AP-75	NR-BR AP-88	NR-BR MES
T_s2 (min)	5.36	5.51	3.06	5.26	5.47	6.19
T_{90} (min)	9.66	9.77	9.34	9.81	10.30	10.83
M_L (dNm)	2.21	2.24	3.49	2.48	2.46	2.40
M_H (dNm)	19.69	19.40	22.49	19.23	18.25	19.64

Table 5: Vulcanization parameters of the prepared NR-BR compounds

The vulcanization curves were obtained by plotting the torque (Strain S' (dNm)) over time.

The torque is proportional to the elastic modulus at low strains. As the vulcanization is carried out at high temperature, the effect of viscous modulus on torque is negligible. The increase in torques is representative of vulcanization and proportional to the number of crosslinks formed. The time elapsed before the beginning of vulcanization, the rate of vulcanization once it starts and extent of crosslinking are the important parameters of the vulcanization process. Scorch time (T_s2) represents the start of vulcanization reactions, as visible from a sudden increase in torque, and must be sufficient to allow the mixing, flowing, and shaping of the rubber products before vulcanization. Once the crosslinking starts, the process must be fast with a controlled crosslinking degree [22]. Table 5 shows that the scorch time of the compounds prepared with the MICRO-OIL1 and 2 is almost the same of AP-88, and significantly shorter compared similar to MES; only CASTOR OIL shows a distinctive reduction of this parameter. Similarly, T_{90}, the time required to reach the optimum value of crosslinking, also shows a small decreasing trend for MICRO-OIL1 and 2 together with the vegetable oils, CASTOR OIL and AP-88, compared to MES.

Although the observed differences are small, these results show that the use of MICRO-OIL1 and 2 are somehow affecting the crosslinking; reduce the time for initiation of vulcanization and curing time.

Results show, also, that the value of both minimum torque (M_L), that represent the value of torque before the start of vulcanization reactions, is lower for the compounds containing microbial oils compared to all the reference oils. The lower minimum torque, M_L, signifies that the viscosity is lower and, consequently, points to an improvement of material processability (good plasticizing action) [23]. The values corresponding to the maximum torque (M_H), that give an indication of the cross-linking density of the fully vulcanized rubber [24], are almost the same for all the compounds, except for the one with CASTOR OIL, that is higher compared to the other. The peculiar behaviour of CASTOR OIL, having also lower plasticising effect than all other tested oils, could be attributed to its higher polarity and potential interaction with the silica filler that could indicative a faster reversion of the vulcanization curve compared to the other compounds.

2.3.3.2.2. Static and dynamic properties of compounds

Tensile mechanical and dynamic analyses were performed to determine the properties of the compounds (Table 6).

	NR-BR MICRO-OIL1	NR-BR MICRO-OIL2	NR/BR CASTOR OIL	NR-BR AP-75	NR-BR AP-88	NR-BR MES
Density (g/cm^3)	1.121	1.125	1.151	1.140	1.134	1.137
STATIC PROPERTIES						
M10 [MPa]	0.51	0.48	0.61	0.48	0.45	0.51
M50 [MPa]	1.27	1.20	1.24	1.14	1.08	1.29
M100 [MPa]	2.17	2.04	1.86	1.86	1.79	2.31
M300 [MPa]	10.21	9.49	7.67	8.78	8.30	10.93
Tensile strenght [MPa]	20.4	20.2	11.5	20.1	20.6	20.2
DYNAMIC PROPERTIES						
E' (10°C) [MPa]	7.135	6.9	8.246	6.79	6.243	6.826
tanδ (10°C)	0.198	0.198	0.24	0.202	0.199	0.201
E' (70°C) [MPa]	5.713	5.725	6.1	5.684	5.684	5.86
tanδ (70°C)	0.107	0.104	0.136	0.119	0.118	0.109

Table 6: Static and dynamic properties of butadiene-based compounds

Static properties are measured as described in the paragraph 2.2.1.8.2., in order to measure the modulus at different elongation (10% to 300%), and the tensile strength. The modulus gives information about the strength of the vulcanized rubber at specific elongation value, whereas the tensile strength indicates how much force it takes to deform and fracture the rubber. We started the analysis comparing the values obtained for the reference oil MES with that of vegetable oils (CASTOR OIL, AP-75 and AP-88). CASTOR OIL, except for the modulus at 10% of elongation, showed values lower compared to MES oil, indicating a worse performance. Also, sunflower rich-oleic acid oils, AP-75 and AP-88, allow to obtain compounds with moduli at 10, 50, 100 and 300% of elongation lower than MES, but similar Tensile strength. Vegetable oils negatively affect the modulus at 300% elongation (M300), in fact the three compounds reach maximum 80% of the reference value. Conversely, the NR-BR compounds prepared with MICRO-OIL1 and MICRO-OIL2, object of this study, showed static properties, especially the tensile strength, closer to the compound prepared with mineral oil MES.

We also measured the dynamic properties, in particular the storage modulus (E') and tanδ at low temperature, 10°, and 70°. These are the main properties that describe the viscoelastic behaviour of compounds. From these properties, it is possible to derive the mechanical response of rubber under dynamic loading conditions (*e.g.* simulating the operative conditions of a tire). Here, we measured the storage modulus to get information about the elasticity of compounds and the tanδ that represent the energy lost in a cyclic deformation, related to grip, rolling resistance and fuel consumption.

The values corresponding to dynamic properties of the compounds containing MICRO-OIL1 and MICRO-OIL2, confirm that the microbial oils allow to obtain compounds very similar to the reference mineral oil, MES. In particular, the storage moduli (E') at 10° and 70° are the same of MES, while tanδ values are slightly lower. In addition, as well as for static properties, the compounds prepared with the vegetable oils, AP-75 and AP-88, showed dynamic storage moduli (E') lower than those of compounds with MES oil, and higher tanδ values. It is important to highlight that the low values of tanδ at 70°C observed by comparing the compounds containing the microbial oils and those with vegetable oils, could predict a lower rolling resistance of tyre including the former, with consequently lower fuel consumption and reduced CO_2 emissions (Patent application number: IT 102020000006688)

2.3.4. Comparison between rubber compounds including carotenoid-enriched microbial oil (MICRO-OIL1) with or without the additional contribution of antiozonant 6PPD in respect to properties

2.3.4.1. Compounding

NR-BR compounds were prepared using the MICRO-OIL1, with or without 6PPD. The final content of oil for each compound is 20 phr. The compounds were prepared in two steps. The temperature was raised to 140 °C during the first step to create a chemical bond between the filler and silane coupling agent. The vulcanizing agents were added in the second step, which was performed at low temperature to avoid the pre-vulcanization of the rubber compounds, as high temperature was used in the first step.

2.3.4.2. Characterization of MICRO-OIL1-NR-BR compounds

1.3.4.1.1. Static properties of compounds before and after thermal aging

Tensile mechanical analysis was performed to determined static compounds properties before and after thermal aging (Table 7).

STATIC PROPERTIES					
Compound Name	MO1 with 6PPD	MO1 W/O 6PPD		MO1 with 6PPD	MO1 W/O 6PPD
M50 [MPa]	1,39	1,45	THERMAL AGING 168H 70°C	1,53	1,57
M100 [MPa]	2,46	2,62		2,78	2,92
M300 [MPa]	11,30	11,42		12,46	12,65
Tensile strenght [MPa]	20,18	20,48		19,83	19,32
Elongation at break [MPa]	460,91	460,91		432,88	438,91

Table 7: Static properties of MICRO-OIL1-NR-BR compounds

*MO1: MICRO-OIL1

**All the compounds are NR-BR based (see par.2.2.1.7. for recipe details).

Thermal aging causes both crosslinking and chain-scission. Cross-linking usually causes the increase in modulus and the decrease in extensibility of the material. Rubbers are susceptible to oxidative ageing due to their unsaturated carbon–carbon double bonds in the backbone [25].

In order to verify if the presence of carotenoids contained in MICRO-OIL1 could substitute the conventional antioxidant/antiozonants, *e.g.* 6PPD, we determined tensile mechanical properties before, as reference, and after thermal aging at 70° for 168 h, and measuring the effects of aging by the changes in elongation modulus, tensile strength and elongation at break.

Table 7 shows the static properties obtained for the compounds prepared with MICRO-OIL1 as plasticizer, with the addition or not of the antioxidant/antiozonant 6PPD, before and after thermal aging.

The NR-BR based compounds prepared with MICRO-OIL1 with or without 6PPD before aging (see the two columns on the left in the tables) showed static properties especially the elongation at break, similar to the each other, regardless of the presence or not of 6PPD, indicating the same performance [20].

After thermal aging (values reported in the two right columns in Table 6), the values obtained are very similar.

The results suggest that the presence of carotenoids in the MICRO-OIL1 allows to maintain the same static properties that are obtained by adding an antioxidant/ antiozonant agent, and therefore it can be concluded that carotenoids contained in MICRO-OIL1 might have a protective action against thermal aging.

2.4. Bibliography

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3

Physical properties of Microbial oils: can they suggest us the best elastomer to match?

3.1. Introduction

The glass transition temperature (*Tg*) is one of the most important thermophysical properties of amorphous polymers. In the highly viscous region above the *Tg*, polymeric materials are soft and rubbery, whereas below the *Tg*, polymers are hard and brittle. The glass transition is a complex process that is affected by a number of factors, including heating rate, ageing history, morphology, molecular weight. In fact, the true nature of the glass transition is not well understood [1]. One or more of the thermal analytical methods such as differential scanning calorimetry (DSC), derivative DSC, thermomechanical analysis (TMA), dynamic mechanical analysis (DMA), and, more recently, dielectric analysis (DEA) are generally used to determine *Tg* of polymers [2]. Thermal analyses of petroleum-derived mineral oils are usually executed because it is reported that the crystallization of paraffins can lead to the clogging of pipelines during the transportation of paraffinic crude oils [3]. In addition, DCS analysis has been widely used to have valuable information on melting and crystallization of vegetable oils and animal fats, and it is commonly used for monitoring phase transition of TAG mixtures or for evaluating the effects of minor components on the crystallization of oils and fats [4].

Considering the important information that can be extrapolated from thermal analysis, in this chapter we performed as first a compatibility study for some selected elastomers and oils (mineral, vegetable and microbial). It consisted of: *i)* preparation of different oil/elastomer mixtures; *ii)* DSC analysis of the latter, to determine the shifting and/or overlapping of *Tg* curves.

In the second part, taking into account the results obtained, we prepared compounds and determined tensile and dynamic properties.

3.2. Experimental part

3.2.1. Materials and Methods

3.2.1.1. Oils

Mild extracted solvate (MES) was purchased from ENI SPA; AP-88 was obtained from Cargill®. MICRO-OIL1 and MICRO-OIL2 have been produced in this work (see Chapter 2 for details).

3.2.1.2. Elastomers

Two different styrene-butadiene rubber (SBR) were used: *i)* SLR 4630, characterised by a medium styrene content (25%) and 63% of vinyl out of the total Butadiene, extended with 37.5 parts of TDAE oil obtained from Trinseo and *ii)* SLR 3402, characterised by a low styrene content (15%) and 30% of vinyl out of the total Butadiene (without any extension oil) from Trinseo.

3.2.1.3. Physical properties of oils

Physical properties of oils were measured by standard techniques by Pirelli's laboratories.

Melting point (Tm, °C) and glass transition temperature (Tg, °C) were measured by Differential Scanning Calorimetry (DSC), starting from a temperature of -140°C to +60°C.

Iodine value (or iodine number) was determined using the Wijs solution (containing iodium monochloride (ICl) dissolved in acetic acid (CH_3COOH)). Iodium monochloride reacts with unsaturated fatty acids in the oil, then the addition of potassium iodide (KI) determines the release of iodium that did not react, measured by titration with sodium thiosulfate ($Na_2S_2O_3$) (ISO 3961).

3.2.1.4. Compatibility test

The compatibility between the different oils and elastomers was evaluated by dissolving them in the same solvent, chloroform, to obtain solutions containing different amount of oils or elastomers (5 per each test, Table 1). After this, we mixed the solution to obtain different ratio oil/elastomer, and we let evaporate the solvent in stove at 80°C overnight. Then, the glass transition temperature (*Tg*) of the different mixtures have been measured using DSC (Mettler DSC 823e Calorimeter), according to ISO 28343.

Briefly, the pans containing the samples (range quantity from 5 mg to 20 mg) have been placed in the cell of the instrument set at a temperature of 25°C. An increase of the latter to 60°C at a rate of 30°C/min were carried out to remove any thermal history of the sample. After, the samples were cooled to -120°C at a cooling rate of 10°C/min and maintained at this temperature for 5 minutes. Then, the thermograms were recorded during the last heating step from -120°C to 60°C at 20°C/min. There may be cases where the glass transition is followed by a peak of crystallization and subsequent fusion. (For the analysis the nitrogen gas flow was adjusted according to the instrument manufacturer's recommendations).

Mixture	Oil amount (% wt/v)	Elastomer (% wt/v)
1	0	100
2	25	75
3	50	50
4	75	25
5	100	0

Table 1: Ratio oil/elastomer used for compatibility test

3.2.1.5. Compounding

3.2.1.5.1. Materials for rubber compounding

Natural rubber (NR, SIR20) was purchased from ANEKA BUMI PRATAMA, SED, Indonesia, styrene butadiene rubber (SBR, SLR3402) is produced by Trinseo, Ultrasil 7000, a silica grade commonly used in the tyre industry, was from Evonik, bis[3-(triethoxysilyl)propyl] Tetrasulfide (TESPT) 50% supported on carbon black (N234) 50% was obtained from Evonik, soluble sulfur was from Zolfindustria Srl, zinc oxide was from Empils-zin, stearic acid was from SO.G.I.S. INDUSTRIA CHIMICA S.p.A, 2,2,4-trimethyl-1,2-dihydroquinoline (TMQ) was from LANXESS GmbH, N-(1,3-Dimethylbutyl)-N'-phenyl-p-phenylenediamine (6PPD) was obtained from Eastman Chemical Co. The accelerator tetrabenzylthiuram disulphide (TBZTD) and Vulkacit® NZ/EGC (N-tert-butyl-benzo-thiazyl sulfenamide (TBBS)) were from LANXESS GmbH. Mild extracted solvate (MES) was purchased from ENI SPA, AP-88 was obtained from Cargill®. MICRO-OIL2 have been produced in this study.

3.2.1.5.2. Styrene-butadiene-based formulations: NR-SBR compounds

The NR-SBR compounds were prepared by physically mixing the elastomers (natural and polybutadiene rubber) with other ingredients (filler system, vulcanization system, plasticizers, antioxidants and accelerators) using the Brabender internal mixer with a total volume of 50 ml and filling coefficient of 0.9. The formulation of the prepared compounds is shown in Table 2.

Ingredients	NR-SBR MICRO-OIL2	NR-SBR AP-88	NR-SBR MES
		Polymers	
SIR20	15	15	15
SLR3402	85	85	85
		Filler system	
Ultrasil 7000	60	60	60
Silane JH75S	9.6	9.6	9.6
		Oils	
MICRO-OIL2	20	-	-
AP-88	-	20	-
MES	-	-	20
		Vulcanization system	
Soluble sulfur	1.3	1.3	1.3
Zinc oxide	2	2	2
Stearic acid	2	2	2
		Antioxidant and accelerators	
TMQ	2	2	2
6PPD	2	2	2
TBZTD	2.3	2.3	2.3
TBBS	0.6	0.6	0.6

Table 2: Recipe formulation expressed in phr (part per hundred rubber)

The NR-SBR compounds were prepared in three steps. The first step was performed in 7 min. The internal mixer temperature was set at 140 °C and rotor speed at 60 rpm. During the first minute, the polymers were added into the mixer, and then the filler was added and mixed for the next 4 min. Then, stearic acid and oil were added at and mixed for the next two minutes, and after 7 min the mixing was stopped. The second step was performed at 90 °C for 2 min at a rotor speed of 60 rpm. antioxidants and zinc oxide were added to the masterbatch obtained from the first step, and mixed for the next 2 min. The mixing was stopped after 2 min. During the final step, the temperature has been decreased to 80°C, and the masterbatch was mixed with accelerators and soluble sulphur for two minutes. At the end of the three steps, the compounds were passed three times through a two-roll mill at 40 °C for further homogenization.

3.2.1.6. Compounds characterization

3.2.1.6.1. Tensile mechanical analysis: Static properties

Tensile mechanical properties, such as tensile strength, elongation at break, and stress at different percentage of elongation (10, 50, 100 and 300%) were measured by tensile mechanical analyses by Pirelli's laboratories. Before performing the analysis, the samples were pressed in 2 mm thick sheets by a two-roll mill and vulcanized in a hydraulic press at 151 °C for 25 min. The vulcanized samples were conditioned in the measuring environment for 24 h. Samples for analysis were prepared by die-cutting the vulcanized sheets in five dumbbell-shaped specimens of standard dimensions. Tensile mechanical analyses of the prepared dumbbell-shaped specimens were performed by using a Zwick/Roell tensile testing machine. The measurements were performed according to ISO 37 and UNI 6065 standards. The stress-strain curves were recorded by progressive straining of samples. For each sample, five measurements were performed, and the average value was reported.

3.2.1.6.2. Dynamic mechanical analysis: Dynamic properties

Dynamic-mechanical properties were measured by Pirelli's laboratories using an Instron dynamic device in the traction-compression mode according to the following methods. A test piece of the crosslinked elastomeric composition having a cylindrical form (length = 25 mm; diameter = 18 mm) and kept at the prefixed temperature (10, 23 and 70 °C) for the whole duration of the test, was compression-preloaded up to a 20% longitudinal deformation with respect to the initial length and then submitted to

a dynamic sinusoidal strain having an amplitude of 7.5% with respect to the length under preload, with a 100 Hz frequency. The dynamic-mechanical properties are expressed in terms of dynamic storage modulus (E') and loss factor (tanδ) values. The tanδ value is calculated as a ratio between loss (E'') and storage modulus (E').

3.3. Result and discussion

3.3.1. MICRO-OILs physical properties

Thermal analysis carried out by DSC analysis can give information about the solid-liquid change phase of materials. It is widely reported in literature, that for vegetable oils it is difficult to assess the glass transition temperature (*Tg*) and the melting peak, due to the great variety among TAGs, as their principal constituents. In addition, these properties can be also influenced by the thermal scan used to perform the thermal analysis [4]. The iodine value is a measure of the degree of unsaturation of fatty acids. This value for oil and fat is defined as weight of iodine absorbed by 100 part of weight of sample. It is often used to determine the quality of vegetable oils used in food industry, after particular storage conditions [5].

In this study, we determined the thermal properties: glass transition temperature (*Tg*) and melting peak of microbial oils. Since the difficulties to determine thermal properties of vegetable oils, that showed a composition similar to microbial oils, we decided to perform the DSC analysis using as scan temperature the range from -140° to +60°, the same used for the mineral oil (MES) and AP-88, in order to do a comparison. In addition, we also measured the iodine value of microbial oils, comparing them to the vegetable oil oleic acid-rich, AP-88.

	Glass transition temperature (°C)	Melting peak (°C)	Iodine value gI2/100g
MICRO-OIL 1	-88.7	10	65.3
MICRO-OIL2	-80.7	8	55.5
MES	-60	-	-
AP-88	-97	-5	84.6

Table 3: Physical properties of the oils used in this study

Table 3 showed the physical properties evaluated in this study. Starting from glass transition temperature *Tg*, microbial oils and vegetable oil are characterized by a

significantly lower *Tg* compared to the value measured for MES, probably due to their composition. We suppose that the different *Tg* values obtained between the microbial oils, where the *Tg* of MICRO-OIL1 is slightly lower compared to MICRO-OIL2, is due to the major content of polyunsaturated fatty acids. Conversely, the melting peaks of microbial oils are higher compared to AP-88.

The iodine value related to unsaturation degree confirmed the fatty acids compositions (see Chapter 2 par. 2.3.2.). The values of obtained for MICRO-OIL1 is higher than those of MICRO-OIL2, due to higher polyunsaturated fatty acids contents and carotenoid too, but both are lower compared to vegetable oils AP-88, probably due to high oleic acid content of the latter.

3.3.2. Compatibility test

One of the most important parameters for the selection of a plasticiser-polymer combination is their compatibility. If an incompatibility between the polymer and the plasticiser exists, the latter will not work effectively as primary plasticiser and will be more prone to migration. As a consequence, a change of the tyre compound properties may occur. The plasticiser's mechanism of action, according to the free volume theory, relies on the interposition of lubricant molecules between the polymer chains, thus reducing the forces between the polymer chains and increasing their mobility [6,7], which lower the cohesive forces between the polymer chains, and so the chain mobility is increased. For this reason, the glass transition temperature *Tg* may be decreased and the elasticity of the polymers is enhanced [8].

Due to the importance of compatibility between elastomers and oils (plasticizers), we performed a compatibility test based on the curves obtained by DSC analysis, focusing the attention on *Tg* peaks overlapping/shifting on curves. Table 4 showed the glass transition temperature (*Tg*) of elastomers and oils.

Elastomer	Tg °C	Oil	Tg °C
SLR 3402	-62	MES	-60
SLR 4630	-30	MICRO-OIL1	-88.7
		MICRO-OIL2	-80.7
		AP-88	-97

Table 4. Elastomers and oils glass transition temperature (Tg).

In literature, the use of vegetable oils with a composition similar to microbial oils, is reported, especially in tyre compounding with butadiene (BR) and styrene-butadiene

(SBR) as elastomers, (already tested in this study, see Chapter2 for details) [9]. Therefore, we decided to perform the compatibility test using two (2) SBR elastomers with different compositions as well as different *Tg*, in order to understand the best combination elastomer/microbial oil. Therefore, we prepared and analysed different mixtures oils/elastomers (as showed in the Table 1 Materials and Methods). The mixtures obtained by mixing the elastomer SLR4630 and the different oils showed distinct *Tg* for the polymer and microbial oils: then we hypothesized that our oils were not compatible with the former polymer. (See supplementary files).

The Fig. 1, 2, 3 and 4 below showed the DSC curves obtained mixing SLR3402 and the different oils (MICRO-OIL1, MICRO-OIL2, AP-88 and MES, respectively).

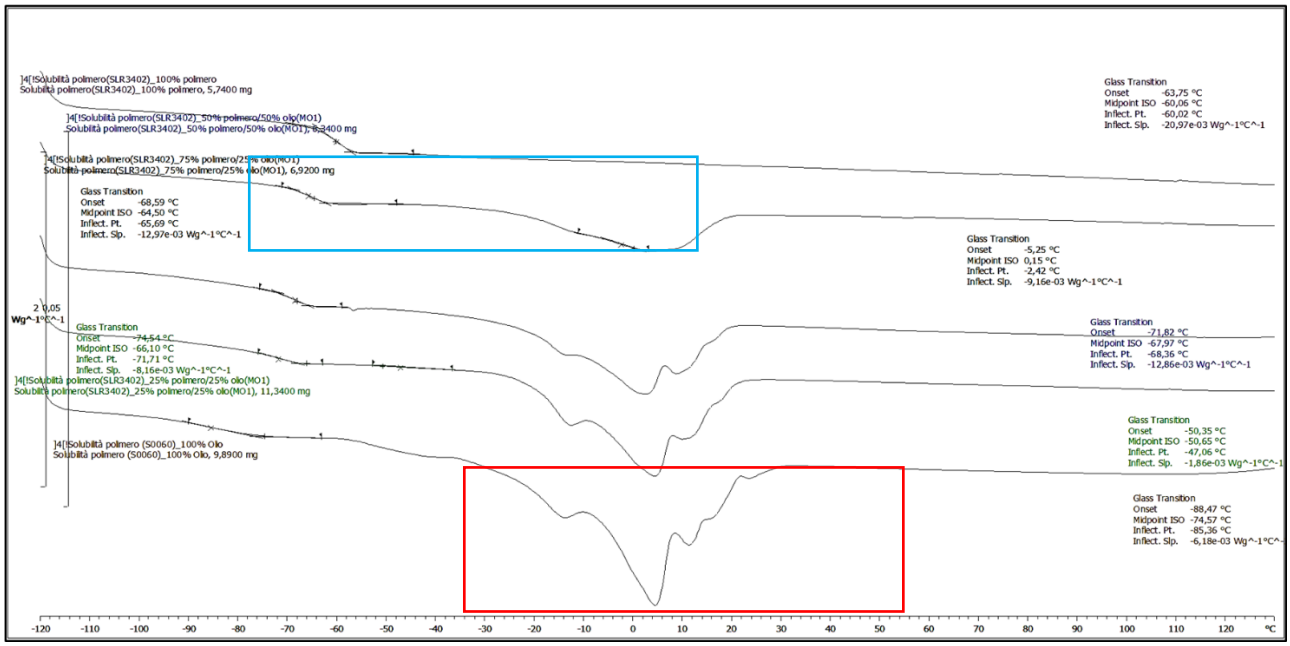

Fig.1. DSC curves of mixture between the elastomer SLR3402 and the microbial oil MICRO-OIL1. For every panels, from the top to the bottom: 100% E, 75% E /25% O, 50% E /50% O, 25% E /75% O, 100% O. Thermal scan from -120 to +60, method P30. O: oil; E: elastomer.
*These data are only for internal use.

As already discussed in this chapter, microbial oils are very heterogeneous due their composition: for this reason, the DSC curves corresponding to 100% of MICRO-OIL1 showed different peaks (Fig1. red box). In addition, starting from the bottom of the Fig.1, decreasing the oils percentage in the mix, it is possible to observe that the peaks of oils seem to disappear, however not completely, in fact also the curve corresponding to the lowest percentage of MICRO-OIL1 (blue box), still showed the different elastomer and oil *Tg*, that can suggest an incompatibility between them.

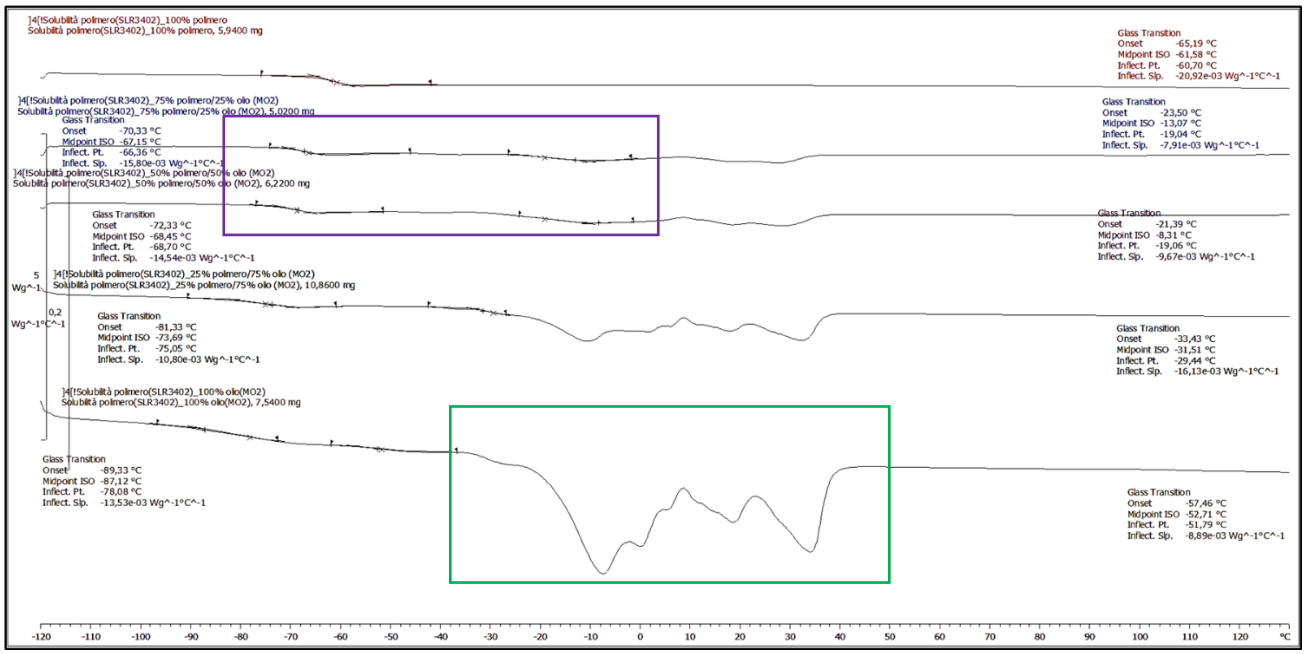

Fig.2. DSC curves of mixture between the elastomer SLR3402 and the microbial oil MICRO-OIL2. For every panels, from the top to the bottom: 100% E, 75% E /25% O, 50% E /50% O, 25% E /75% O, 100% O. Thermal scan from -120 to +60, method P30. O: oil; E: elastomer.
*These data are only for internal use.

The Fig.2 showed the DSC curves obtained by mixing SLR3402 with the MICRO-OIL2. As well as for the MICRO-OIL1, also the curve corresponding to 100% of oil showed different peaks (green box). Anyway, conversely to that observed in the Fig.1 for the MICRO-OIL1, here decreasing the oil content, the *Tg* of the MICRO-OIL1 and SLR3402 were almost overlapped (see purple box), using the ratio elastomer: oil 75 to 25 and 50 to 50, which allowed us to hypothesize a compatibility between them.

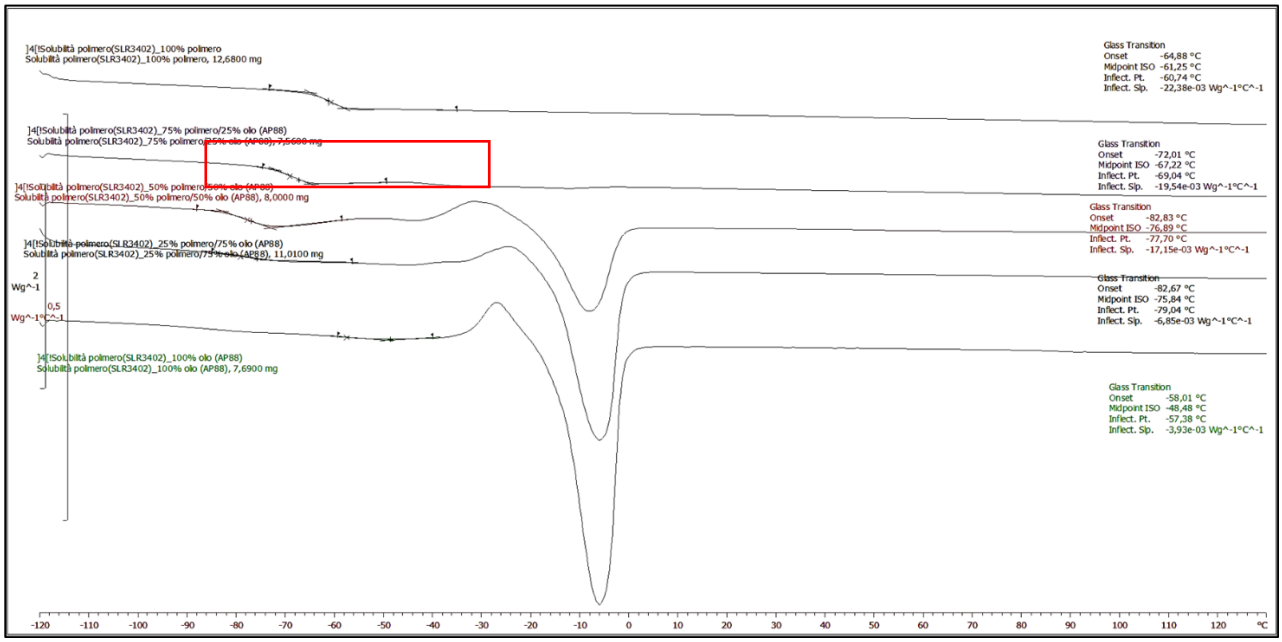

Fig.3. DSC curves of mixture between the elastomer SLR3402 and the vegetable oil AP-88. For every panels, from the top to the bottom: 100% E, 75% E /25% O, 50% E /50% O, 25% E /75% O, 100% O. Thermal scan from -120 to +60, method P30. O: oil; E: elastomer.
*These data are only for internal use.

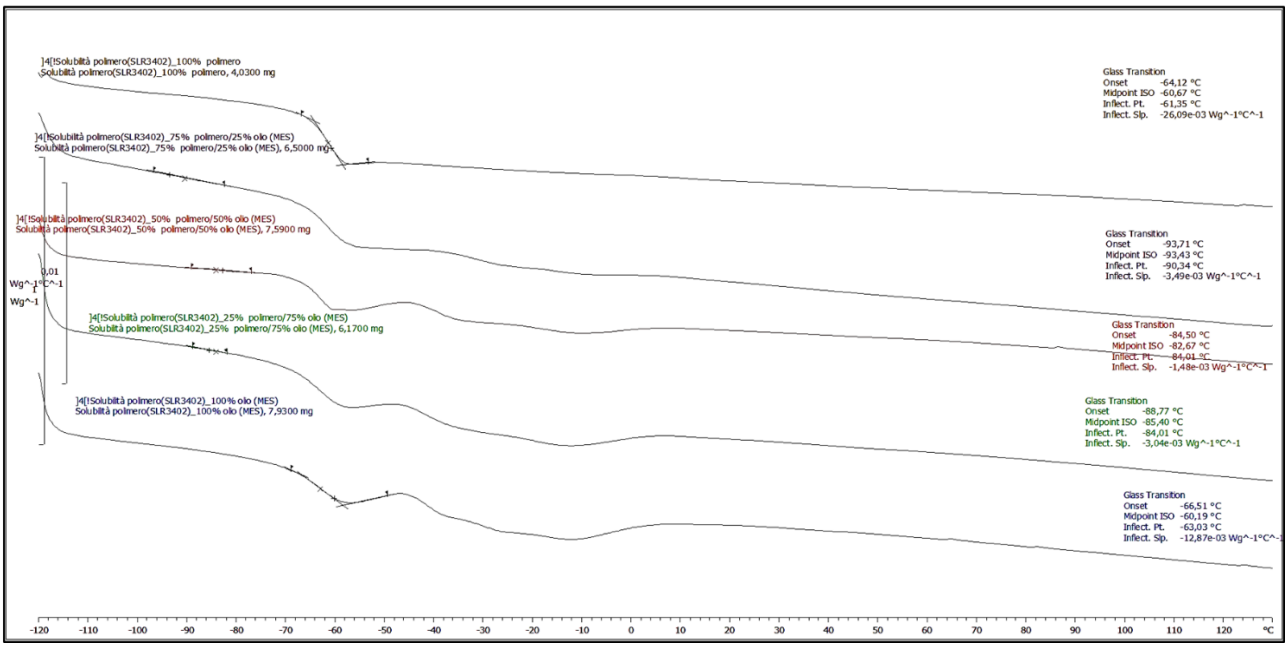

Fig.4. DSC curves of mixture between the elastomer SLR3402 and the mineral oil MES. For every panels, from the top to the bottom: 100% E, 75% E /25% O, 50% E /50% O, 25% E /75% O, 100% O. Thermal scan from -120 to +60, method P30. O: oil; E: elastomer.
*These data are only for internal use.

The Fig.3 and 4 showed the curve obtained for the two oils, the mineral oil MES and the vegetable oil AP-88, respectively. The vegetable oil, AP-88, seems to be compatible with SLR3402 in the ratio 25% to 75% (red box Fig.3). On the other hand, for the mineral oil, MES, due their very closely *Tg*, it resulted difficult to provide a hypothesis about compatibility.
In respect to the results obtained in this compatibility tests, it seems that the best candidate for the mixing is MICRO-OIL2. Therefore, the second part of this chapter was dedicated to the preparation of compounds, using MICRO-OIL2, AP-88 and MES as plasticizers.

3.3.3. Styrene-butadiene-based NR-SBR compounds

3.3.3.1. Compounding

The NR-SBR compounds were prepared using the MICRO-OIL2, and the vegetable (AP-88) and mineral oil (MES), as references and styrene-butadiene-based elastomer (SLR3402). The final content of oil for each compound is 20 phr. The compounds were prepared in three steps as described in previous chapter. The temperature in the first step was set to 140 °C to create a chemical bond between the filler and silane coupling agent. During the second and third steps, performed at 90° and 80°, respectively, to avoid the pre-vulcanization of the rubber compounds, the vulcanizing agents, accelerators and antioxidants were added.

3.3.3.2. Characterization of the NR-SBR compounds

3.3.3.2.1. Static and dynamic properties of compounds

Starting from the results obtained from the compatibility test, we decided to prepare compounds with the low-styrene elastomer SLR 3402 mixed with natural rubber (NR) and the other ingredients (see materials and methods for the formulations receipts).

In addition, according with compatibility results, we decided to prepare the formulations only for MICRO-OIL2, which showed a good compatibility with the elastomer. As reference we used MES and AP-88, already described in the second chapter.

	NR-SBR MICRO-OIL2	NR-SBR AP-88	NR-SBR MES
STATIC PROPERTIES			
M10 [MPa]	0.72	0.60	0.74
M50 [MPa]	1.69	1.42	1.69
M100 [MPa]	2.65	2.24	2.74
M300 [MPa]	9.16	8.55	11.07
Tensile strenght [MPa]	11.05	16.32	14.4
DYNAMIC PROPERTIES			
E' (23°C)	8.8	7.3	8.7
tanδ (23°C)	0.183	0.174	0.188
E' (70°C)	7.6	6.5	7.7
tanδ (70°C)	0.124	0.132	0.135

Table 5: Static and dynamic analyses for determining the properties of compounds.

As already shown in the first chapter, in order to evaluate the performance of NR-SBR compounds prepared with the different oils, we performed tensile mechanical (static properties) and dynamic analysis (dynamic properties).

The NR-SBR compounds prepared with MICRO-OIL2 showed moduli at 10, 50 and 100 %, except for 300% of elongation similar to the compound prepared with mineral oil MES, and better than the vegetable oil AP-88, indicating the resilience of the compound. In addition, we obtained values of tensile strength not very different from mineral oil, but lower compared to vegetable oil AP-88, as already demonstrated also for others vegetable oils [9,10].

Dynamic analysis gives information about the elasticity expressed by storage modulus (E') and dissipation of energy (tanδ). The analysis was performed at two different temperatures, in order to better evaluate the compound performance in different stress and temperature conditions. It is also important to highlight that the value of tanδ at 70°C is related to rolling resistance and fuel consumption: therefore, lower values could predict a lower rolling resistance of tyre with consequent lower fuel consumption and reduced CO_2 emissions.

The values corresponding to dynamic properties confirmed that MICRO-OIL2 allows to obtain compounds with performances very similar to the reference mineral oil, MES. In particular, the storage moduli (E') at 23°and 70° are the same of MES, while tanδ values are slightly lower, especially at 70°. On the other hand, the compounds prepared with the vegetable AP-88, showed lower moduli that could indicate a worse elasticity, while tandδ at 70° is higher compared MICRO-OIL2.

These results suggest: *i)* a good compatibility between the MICRO-OIL2 and the elastomer SLR3402, especially in terms of dynamic behaviour; *ii)* the possibility to evaluate a MICRO-OIL further enriched in monounsaturated fatty acids, in order to mimic some of the interesting static properties of the compound obtained with the vegetable oil AP-88.

3.4. Supplementary files

3.4.1. Compatibility test

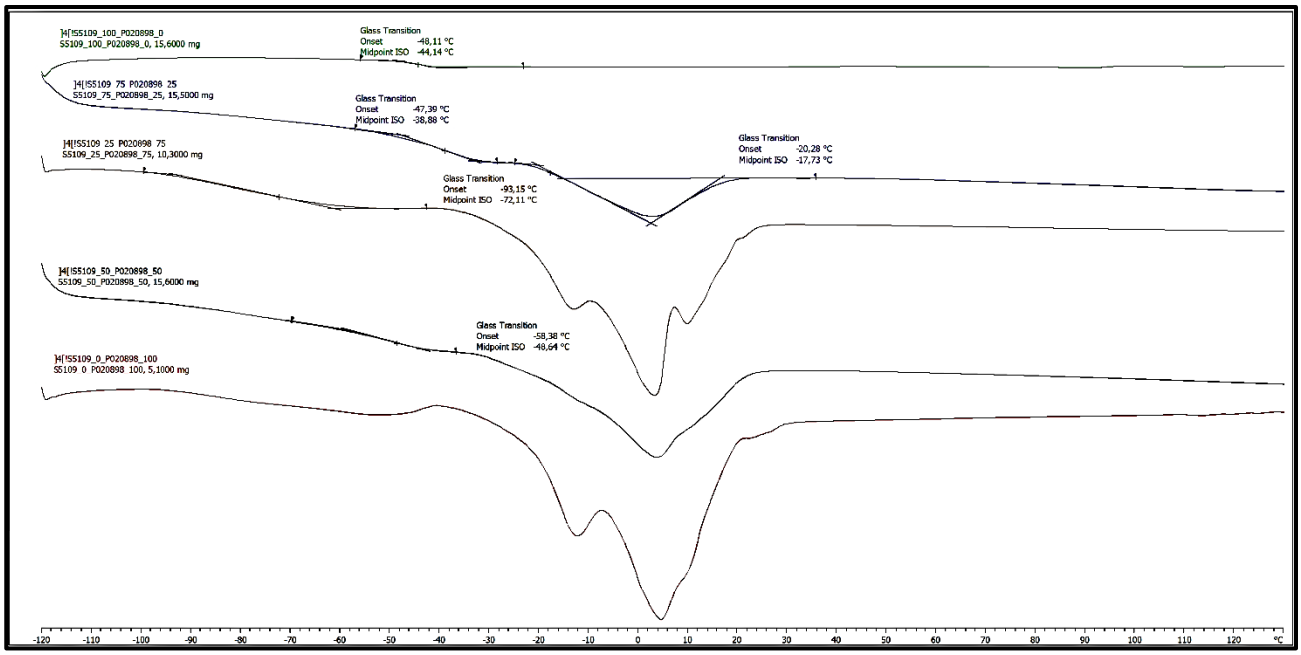

Fig.S1. DSC curves of mixture between the elastomer SLR4630 and the microbial oil MICRO-OIL1. For every panels, from the top to the bottom: 100% E, 75% E /25% O, 50% E /50% O, 25% E /75% O, 100% O. Thermal scan from -120 to +60, method P30. O: oil; E: elastomer.

*These data are only for internal use.

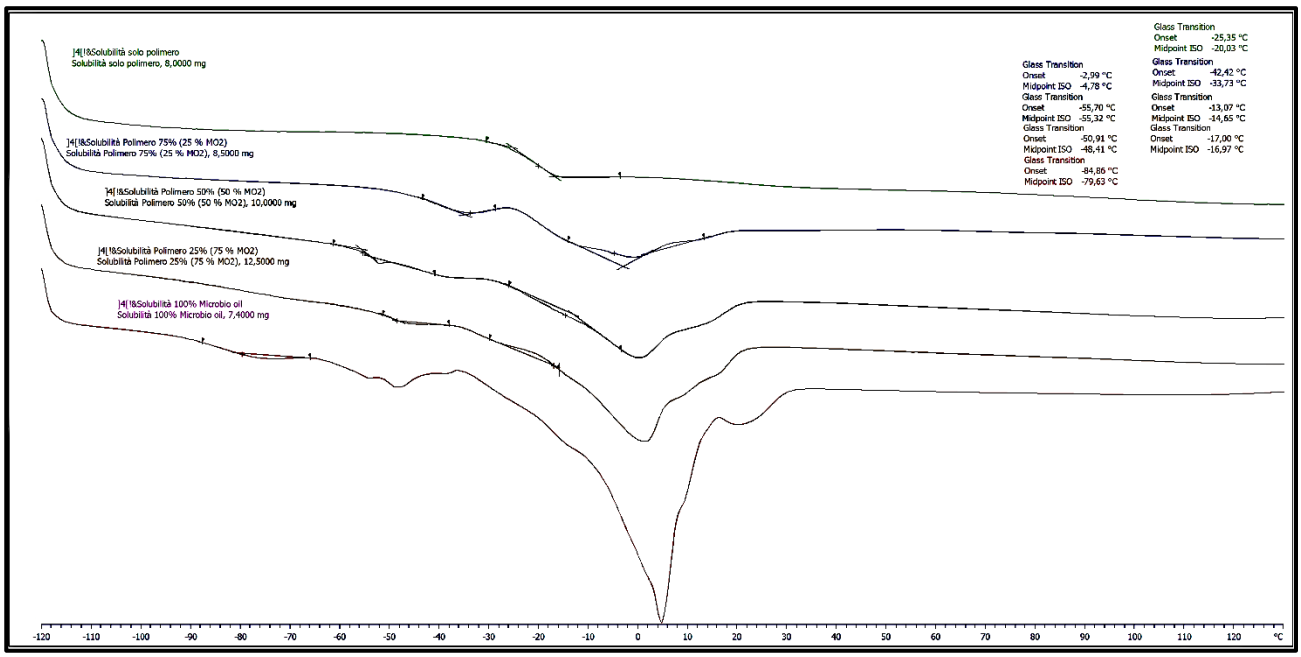

Fig.S2. DSC curves of mixture between the elastomer SLR4630 and the microbial oil MICRO-OIL2. For every panels, from the top to the bottom: 100% E, 75% E /25% O, 50% E /50% O, 25% E /75% O, 100% O. Thermal scan from -120 to +60, method P30. O: oil; E: elastomer.

*These data are only for internal use.

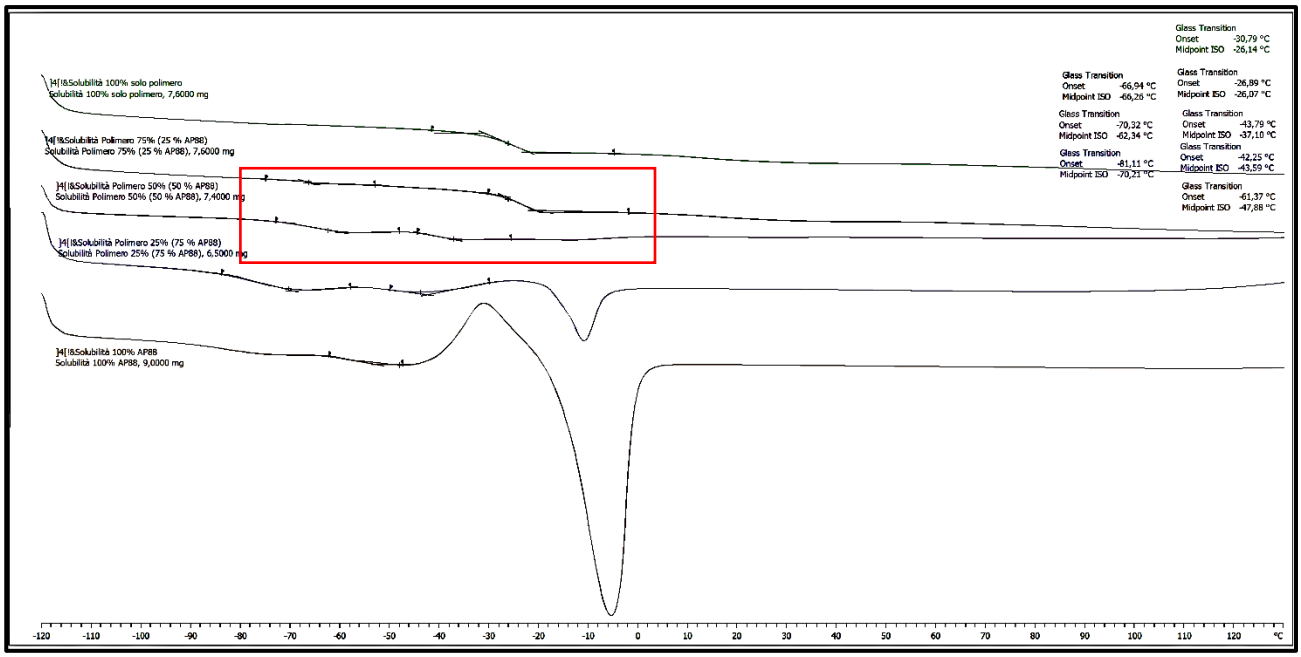

Fig.S3. DSC curves of mixture between the elastomer SLR4630 and the vegetable oil AP-88. For every panels, from the top to the bottom: 100% E, 75% E /25% O, 50% E /50% O, 25% E /75% O, 100% O. Thermal scan from -120 to +60, method P30. O: oil; E: elastomer.
*These data are only for internal use.

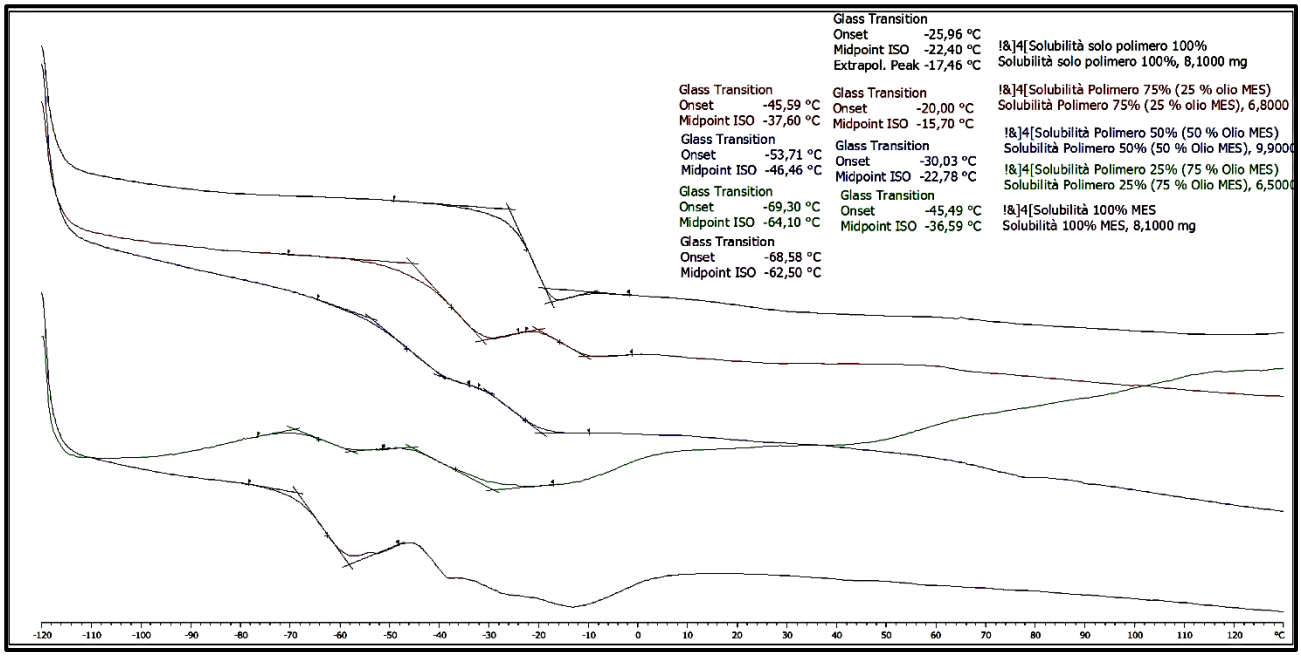

Fig.S4. DSC curves of mixture between the elastomer SLR4630 and the mineral oil MES. For every panels, from the top to the bottom: 100% E, 75% E /25% O, 50% E /50% O, 25% E /75% O, 100% O. Thermal scan from -120 to +60, method P30. O: oil; E: elastomer.
*These data are only for internal use.

The Fig.S1., S2., S3. and S4. show the DSC curves obtained by mixing SLR4630 and the different oils object of this study (MICRO-OIL1, MICRO-OIL2, AP-88 and MES, respectively). Overall, it seems that the curves corresponding to MICRO-OIL1 and MICRO-OIL2 mixed with the elastomers, maintain their characteristic trends, also when they are used in lower percentage compared to the elastomer, suggesting incompatibility between them. The same behaviour has been observed for the mineral oil MES, conversely the vegetable oil AP-88 seems be more versatile, in fact starting from the bottom it is possible to observe that decreasing the oil content the *Tg* of the AP-88 and SLR4630 were almost overlapped (see red box), using the ratio elastomer: oil 75 to 25 and 50 to 50, which allowed us to hypothesize a compatibility between them.

3.5. Bibliography

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4

Metabolic engineering of the oleaginous yeasts *Lipomyces starkeyi* for tailoring plasticizer oil production

Patent application (PA):
PA1: CASTELLANI LUCA (IT); GIANNINI LUCA (IT); GUERRA SILVIA (IT); BRANDUARDI PAOLA (IT); DI LORENZO RAFFAELLA DESIRE' (IT). “Produzione di mescole elastomeriche comprendenti oli ad azione plastificante ottenuti a partire da cellule microbiche oleaginose”
Publication info: IT 102020000006688

PA2: BRANDUARDI PAOLA (IT); DI LORENZO RAFFAELLA DESIRE' (IT). “Procedimento di ingegnerizzazione di un microorganismo oleaginoso”
Publication info: IT 102020000006715

4.1. Introduction

The use of Vegetable oils in many industrial applications has attracted the attention of researchers and industries due to their potential as substitutes to petrochemical derivatives, because they are environmentally friendly, biodegradable, potentially less costly and readily available compared to mineral oils.
Among many others (see [1] for a recent review), application of vegetable oils as additive in polymer processing has been reported. Palm, soya bean and sunflower oils have demonstrated the possibility to be used as alternative processing aids and activators in carbon black filled natural rubber [2-4]. It has been reported elsewhere that vegetable oils, in compounds prepared with different amount of them, can act as coupling agent between carbon black and rubber interfaces, due to an interaction mediated by unsaturated bonds present in vegetable oils, leading to the enhancement of elasticity in cured compounds depending of oil quantity [5]. Furthermore, monounsaturated and polyunsaturated fatty acids, characterized by unsaturated bounds, can be the substrate for chemicals modification to obtain epoxidized vegetable oils, used as process aids in rubber compounding [2,6-8]. However, vegetable oils are in competition (direct or indirect) with food, subjected to seasonality, climate instability and presenting all the limits related to intensive agriculture. This led to the search for valuable alternatives [9]: single cell oils (SCOs), obtained from oleaginous microorganisms, can be exploited. In the case of Ascomycetes yeasts, genetic tools, as metabolic engineering, can be applied and offer the possibility to change the fatty acids profile of SCOs [10,11]. Despite genetic manipulation are possible, the amenability to that of *L. starkeyi* is limited, as it does not present homologous recombination (HR) for DNA fragments and repair, and only in the last years some efficient engineering tools has been developed [12-15].
In this chapter, we describe a metabolic engineering approach used to modify the fatty acids profile of the oleaginous yeast *Lipomyces starkeyi*: we have chosen to overexpress the genes encoding for two enzymes (Δ9 and Δ12 desaturase) involved in the unsaturation of fatty acids, discovering that in this yeast, under the tested conditions and parameters, we unexpectedly obtained a shift towards monounsaturated instead of polyunsaturated fatty acids. As the obtained oil, named MICRO-OIL4, presents traits of interest for being compounded with elastomers for tyre production, we tested and analysed the data of the compounds prepared with the synthetic elastomer SLR3402, following the same recipe showed in Chapter 3 and used for MICRO-OIL2.

4.2. Experimental part

4.2.1. Materials and Methods

4.2.1.1. Strain and Media

L. starkeyi DSM70295 was purchased from DSMZ. Yeasts were stored in cryotubes at −80 °C in 20 % glycerol (vv^{-1}).

The composition of the inoculum and fermentation medium (MeOL) was per liter: 1 g of yeast extract (0.114 g of nitrogen), 1.31 g of $(NH_4)_2SO_4$ (0.278 g of nitrogen), 0.95 g of Na_2HPO_4, 2.7 g of KH_2PO_4, 0.2 g of Mg_2SO_4 $7H_2O$, 0.04 g. After the pH was adjusted to 5.5 using NaOH 4 M, the medium was supplemented with a 100X trace mineral stock solution consisting of (per liter): 4 g $CaCl_2$ $2H_2O$; 0.55 g $FeSO_4$ $7H_2O$; 0.52 g citric acid; 0.10 g $ZnSO_4$ $7H_2O$; 0.076 g $MnSO_4$ H_2O; and 100 µL 18 M H_2SO_4 [16].

Yeast extract was provided by Biolife Italiana S.r.l., Milan, Italy. All the other reagents were provided by Sigma-Aldrich Co., St Louis, MO, USA.

Glycerol, both crude and pure, was used as carbon and energy source. Following the protocol described in [17] for the calculations of the carbon-to-nitrogen ratio (C/N), a carbon content in glycerol of about 39.1 %, and a nitrogen content of 21.2 and 11.4 % in ammonium sulfate and yeast extract, respectively, were assumed.

4.2.1.2. Crude glycerol

Crude glycerol derived from industrial biodiesel production out of palm oil. The stock had 80 % content of glycerol and appeared as a dark brown liquid. In the text, reported crude glycerol concentrations ($g\ L^{-1}$) refer to the HPLC measured glycerol. Composition analyses of this stock were conducted: the original sample was diluted 50-fold and passed through 0.22 µm filter. Crude glycerol was autoclaved at 120° C and left to settle about 24 hours. The top, dark red layer, mainly consisting of free fatty acids (FFAs), was removed.

4.2.1.3. Yeast strains, transformation and selection

The parental strain *L. starkeyi* DSM70295 was used for the overexpression of the genes *LsOLE1* and *LsFAD2*, using the expression cassette shown in Fig. 1, obtained by excision from plasmid pLS02-*LsOLE1* and pLS04-*LsFAD2*, using the restriction enzyme EcoRI-HF and XhoI, respectively. (See supplementary files for details).

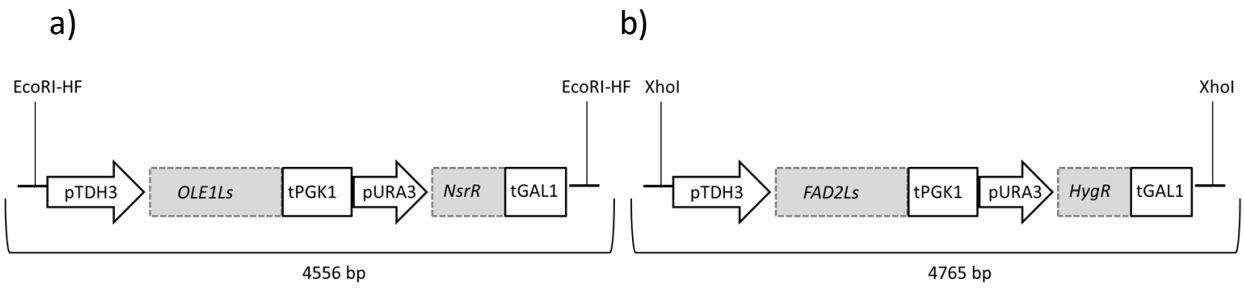


Fig.1 Expression cassette for LsOLE1 **a** and LsFAD2 **b** overexpression. Both the cassettes have been constructed using the endogenous promoters (pTDH3 and pURA3) and terminators (tPGK1 and tGAL1), and the selection markers, Nsr^R (Nourseothricin) for LsOLE1, and Hyg^R (Hygromicin) for LsFAD2. (bp: base pairs).

Yeast transformations were performed according to the protocol reported in [12] and the strains were transformed with the constructs above-described. The obtained clones were screened by PCR using the following conditions: 30 sec at 98 °C, 35 cycles (each consisting of 10 sec at 98 °C, 30 sec at 63 °C and 1 min at 72 °C), were carried out, followed by 2 min at 72 °C. The control primers, *LsTDH3*-fw (5'- ATAATAATCC GAACTGCCGC-3'), *LsOLE1* cntr-rev (5'- CAACTACCAT GGGGAAGATG-3') and *LsFAD2* cntr-rev (5'- CAAGTATACCGACATCGGAGATTA) were designed on the promoter pTDH3 sequence and into *LsOLE1* and *LsFAD2* gene sequences (length of amplified fragments: 700 and 709 bp, respectively. Data not shown).

4.2.1.4. Real-time quantitative PCR

Genomic DNA was extracted from the transformant strains, L. starkeyi-*OLE1*-*FAD2*, and from the parental strain, as control, following the standard protocol [18]. RNA was extracted using the kit ZR Fungal/Bacterial RNA Miniprep (Zymoresearch/The epigenitics company). The retrotrascription to obtain cDNA was performed using the kit iScript™ cDNA Synthesis (Bio-Rad Laboratories,Inc.). The obtained genomic DNAs and the cDNAs were used to perform a real-time quantitative PCR using SsoFast™ EvaGreen® Supermix with Low ROX (Bio-Rad Laboratories,Inc.) with the specific primers *LsOLE1*-RT-fw (5'-CCACTTCTTGACTGCCATGC-3'), *LsOLE1*-RT-rev (5'-GAGGAACCAGATGAGCCACT-3'), *LsFAD2*-RT-fw (5'- GATCGCGACTTTGGCTTCAT-3') and *LsFAD2*-RT-rev (5'- GAATTCGCGACGAGTAGTGG-3'), according to the manufacturer's instructions. Results obtained were normalized using actin as housekeeping gene (gene copy number and expression ratio=1).

4.2.1.5. Batch cultures

Yeasts were revived from cryo-preserved stocks stored at -80°C and grown on YP-Glycerol agar plates. One colony was used to pre-inoculate 500 mL flasks with 100 mL

of the culture medium (MeOL) supplemented of 15 gL^{-1} as initial concentration of pure glycerol, resulting in a medium with a balanced C/N ratio. After, exponential phase shake flasks cultures were used to inoculate 250 mL Erlenmeyer containing 50 mL of liquid medium (MeOL), using as carbon and energy sources, pure glycerol to a final concentration of 100 gL^{-1}. The pH value of the medium was adjusted to 5.5 with NaOH 4 M. Shake flasks experiments were performed in triplicates on a rotary shaker at 25 °C and 220 rpm. The cultures were initiated upon 45 mL of the cultivation medium inoculated with 5 mL of the seed culture to a final absorbance (OD_{660}) of 3.0. Growth was monitored by regularly measuring optical density (OD_{660}) over time and by determining the cell dry weight (CDW) at 0 and 216 h (final point), every 24h. The supernatant of these samples was used for the HPLC analysis.

4.2.1.6. Microbial oil preparation: Fed-batch fermentation cultures

Yeasts were revived from cryo-preserved stocks stored at −80 °C and grown on YP-Glycerol agar plates. One colony was used to inoculate 1 L flasks with 200 mL of the culture medium and seed cultures were placed on a rotary shaker at 25 °C and 220 rpm about 3 days. Exponential phase shake flasks cultures were used to inoculate bioreactors to a final optical density (OD_{660}) of 3.0. Briefly, cells were centrifuged at 6000 rpm for 5 min, washed twice with water, and finally resuspended in 40 mL of sterilized water, to be added into fermentors.

The fed-batch experiments were conducted in 2.0 L bioreactors (Sartorius Stedim BIOSTAT® Bplus, Germany). For the bioreactor cultivations, the aeration rate, agitation, and temperature were set to 1 vvm, 300 rpm (in cascade to 25 % of dissolved oxygen), and 25 °C, respectively. The pH was maintained by automatic pumping of 4 M NaOH.

The cultivation was designed to have two phases: 1) "*batch phase"* to allow cells adaptation and growth; 2) "*pulse fed-batch phase"* to promote lipids accumulation.

The final glycerol concentration set was 100 g/L, divided in the two phases. Bioreactor experiments were started with a working volume of 1.0 L and with 60 gL^{-1} as initial concentration of pure glycerol, resulting in a ratio C/N of 35:1. When the residual concentration of glycerol reached about 20 g/L the pulse fed-batch started, using a solution comprising 40 g/L of crude glycerol as sole nutrient source.

The time of pulse was set based on the glycerol uptake rate ($gL^{-1}h^{-1}$). The highest glycerol uptake rate on pure glycerol was taken as reference to determine the starting point of the pulse phase.

When needed, antifoam emulsion (Sigma-Aldrich, MO, USA) was added to prevent excess foam formation. Aliquots were collected at regular intervals to evaluate the concentration of main extracellular metabolites (HPLC analysis), optical density (OD660), cell dry weight (CDW) and lipid content and composition (gas chromatography).

4.2.1.7. Analytical methods

The optical density was measured at 660 nm (OD_{660}) with a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation). Samples collected at different times were centrifuged at 14000 rpm for 10 min. The supernatants were filtered (0.22 µm filter) and glycerol concentrations were HPLC determined using a Rezex ROA-Organic Acid (Phenomenex). The eluent was 0.005 N H_2SO_4 pumped at 0.5 mL min^{-1} and column temperature was 40 °C. Separated components were detected by a refractive-index detector and peaks were identified by comparing with known standards (Sigma-Aldrich, St Louis, MO, USA).

Biomass was harvested by centrifugation of the culture samples at 4000 rpm for 10 min. The pellets were then washed twice with distilled water and dried at 40 °C (Concentrator 52301, Eppendorf, Germany) until a constant weight was obtained.

Additional biomass was also preserved for cellular lipids and fatty acids analysis.

4.2.1.8. Microbial oil preparation: extraction and analysis of composition

To determine the lipid content in yeast cells, lipids were extracted, based on the method of Bligh and Dyer [19] and Folch et al. [20], with modifications, and then analyzed by GC. Briefly, cells were centrifuged at 8000 rpm for 15 min and washed twice with distilled water. Cells were then resuspended in a volume of HCl 2 M (1:1) and chemically disrupted putting on thermostatic bath for at 95 °C for 1h. Then, $CHCl_3$:CH_3OH (2:1) solution was added to the mixture cells:HCl (ratio 1:1), mixed by vortex or inversion and centrifuged at 10000 rpm for 10 min. After centrifuge, of the 3 phases, the organic phase on the bottom was collected; 10 ml of chloroform were added, mixed again by vortex or inversion, and centrifuged at 10000 rpm for 10 min. Finally, the organic phases on the bottom were collected and left overnight under chemical hood for the evaporation. For derivation of methyl esters from fatty acids a 0.4 M solution of KOH-methanol was added to oil, the mixture was vortexed and placed in a thermobath at 60 ° C for 1 hour. After a volume of BF_3 -methanol (14% w / w) (1:1) was added, mixed by vortex and placed for one hour in a thermobath at 60

° C; finally, n-hexane was added to extract the fatty acids methyl esters (FAMEs). Fatty acid methyl esters were analysed by gas chromatography (SAVI LABORATORI & SERVICE S.r.l. via Roma, 80 46037 Roncoferraro (MN) Italy) according to the methods (UNI EN ISO 12966-1:2015 + UNI EN ISO 12966-2:2011 + UNI EN ISO 12966-4:2015).

4.2.1.9. Physical properties of MICRO-OIL4

MICRO-OIL4 standard physical properties were measured in Pirelli's laboratory. Melting point (Tm, °C) and glass transition temperature (Tg, °C) were measured by Differential Scanning Calorimetry (DSC), starting from a temperature of -140°C to +60°C.

Iodine value (or iodine number) was determined using the Wijs solution (containing iodium monochloride (ICl) dissolved in acetic acid (CH_3COOH)). Iodium monochloride reacts with unsaturated fatty acids in the oil, then the addition of potassium iodide (KI) determines the release of iodium that did not react, measured by titration with sodium thiosulfate ($Na_2S_2O_3$) (ISO 3961).

4.2.1.10. Compounding

4.2.1.10.1. Materials for rubber compounding

Natural rubber (NR, SIR20) was purchased from ANEKA BUMI PRATAMA, SED, Indonesia, styrene butadiene rubber (SBR, SLR3402) is produced by Trinseo, Ultrasil 7000, which is commonly used in the tire industry, was from Evonik, bis[3-(triethoxysilyl)propyl]-tetrasulfide (TESPT) 50% supported on carbon black (N234) 50% was obtained from Evonik, soluble sulfur was from Zolfindustria Srl, zinc oxide was from Empils-zinc, stearic acid was from SO.G.I.S. INDUSTRIA CHIMICA S.p.A, 2,2,4-trimethyl-1,2-dihydroquinoline (TMQ) was from LANXESS GmbH, N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine (6PPD) was obtained from Eastman Chemical Co. The accelerator tetrabenzylthiuram disulphide (TBZTD) and Vulkacit® NZ/EGC (N- tert-butyl-benzothiazole sulphonamide (TBBS)) were from LANXESS GmbH. Mild extracted solvate (MES) was purchased from ENI SPA, AP-88 was obtained from Cargill®. MICRO-OIL4 have been produced in this work.

4.2.1.10.2. Styrene-butadiene-based formulations: NR-SBR compounds

The NR-SBR compounds were prepared by physically mixing the elastomers (natural and polybutadiene rubber) with other ingredients (filler system, vulcanization system, antioxidants and accelerators) using Brabender internal mixer with a total volume of 50 ml and filling coefficient of 0.9. The formulation of the prepared compounds is shown in Table 1.

Ingredients	NR-SBR MICRO-OIL4	NR-SBR AP-88	NR-SBR MES
		Polymers	
SIR20	15	15	15
SLR3402	85	85	85
		Filler system	
Ultrasil 7000	60	60	60
Silane JH75S	9.6	9.6	9.6
		Oils	
MICRO-OIL4	20	-	-
AP-88	-	20	-
MES	-	-	20
		Vulcanization system	
Soluble sulfur	1.3	1.3	1.3
Zinc oxide	2	2	2
Stearic acid	2	2	2
		Antioxidant and accelerators	
TMQ	2	2	2
6PPD	2	2	2
TBZTD	2.3	2.3	2.3
TBBS	0.6	0.6	0.6

Table 1: Recipe formulation expressed in phr (part per hundred rubber)

The NR-SBR compounds were prepared in three steps. The first step was performed in 7 min. The internal mixer temperature was set at 140 °C and rotor speed at 60 rpm. During the first minute, the polymers were added into the mixer, and then filler system was added and mixed for next 4 min. Then, stearic acid and oil were added at

and mixed for next two minutes, and at 7 min the mixing was stopped. The second step was performed at 90 °C for 2 min at a rotor speed of 60 rpm. The masterbatch from the first step was added of antioxidants and zinc oxide, and mixed for the next 2 min. The mixing was stopped at 2 min. During the final step, the temperature has been decreased to get 80°C, and the masterbatch was mixed with accelerators and soluble sulfur for two minutes. At the end of the three steps, the prepared compounds were passed three times through a two-roll mill at 40 °C for further homogenization.

4.2.1.11. Compounds characterization

4.2.1.11.1. Tensile mechanical analysis: Static properties

Tensile mechanical properties, such as tensile strength, elongation at break, and stress at different percentage of elongation (10, 50, 100 and 300%) were measured in Pirelli's laboratory by tensile mechanical analysis. Before performing the analysis, the samples were pressed in 2 mm thick sheets by a two-roll mill and vulcanized in a hydraulic press at 151 °C for 25 min. The vulcanized samples were conditioned in the measuring environment for 24 h. Samples for analysis were prepared by die-cutting the vulcanized sheets in five dumbbell-shaped specimens of standard dimensions. Tensile mechanical analyses of the prepared dumbbell-shaped specimens were performed by using a Zwick/Roell tensile testing machine. The measurements were performed according to ISO 37 and UNI 6065 standards. The stress-strain curves were recorded by progressive straining of samples. For each sample, five measurements were performed, and the average value was reported.

4.2.1.11.2. Dynamic mechanical analysis: Dynamic properties

Dynamic-mechanical properties were measured in Pirelli's laboratory using an Instron dynamic device in the traction-compression mode according to the following methods. A test piece of the crosslinked elastomeric composition having a cylindrical form (length = 25 mm; diameter = 18 mm) and kept at the prefixed temperature (10, 23 and 70 °C) for the whole duration of the test, was compression-preloaded up to a 20% longitudinal deformation with respect to the initial length and then submitted to a dynamic sinusoidal strain having an amplitude of 7.5% with respect to the length under preload, with a 100 Hz frequency. The dynamic-mechanical properties are expressed in terms of dynamic storage modulus (E') and loss factor (tanδ) values. The tanδ value is calculated as a ratio between loss (E'') and storage modulus (E').

4.3. Result and discussion

4.3.1. Overexpression of *LsOLE1* and *LsFAD2* in *L. starkeyi*

The strain that overexpresses *LsOLE1* and *LsFAD2* was constructed using the expression cassettes (see Fig.1 Materials and Methods) and the transformation protocol described in [12]. As it was already described in the Introduction of that chapter, the absence of homologous recombination (HR) for DNA fragments and repair in *L. starkeyi* impairs the possibility of targeted integration and determines a low efficiency in terms of number of transformants (we have obtained overall few units of transformants per µg of DNA). Then, we selected one transformant strain of *L. starkeyi-OLE1-FAD2,* and in order to verify the presence of supernumerary genes, we performed real-time PCR on genomic DNA confirming that it contains two copies of both the genes, *LsOLE1* and *LsFAD2* in its genome (data showed in supplementary data, Fig S7). By RTq PCR on cDNA we determined that mRNA levels were approximately 4-fold higher for *LsOLE1* (Fig. 2A) and 2 to 3-fold higher for *LsFAD2* than those of control cells (Fig. 2B).

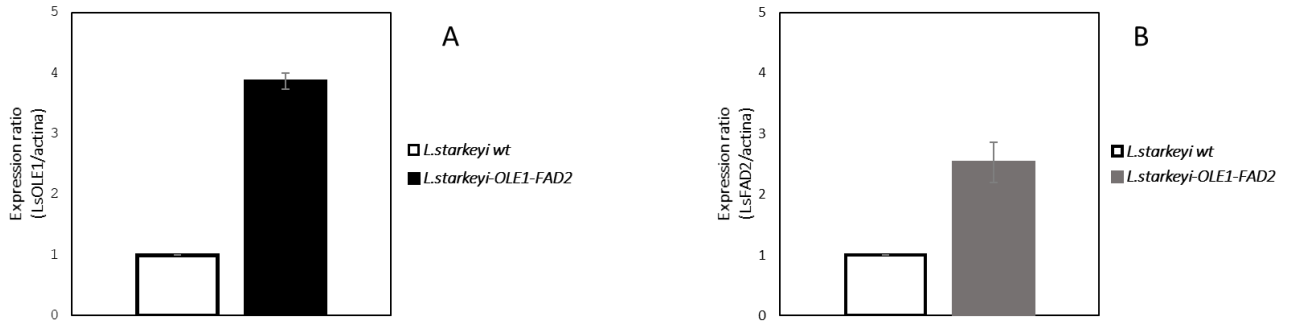


Fig.2. Expression levels of *LsOLE1* and *LsFAD2* genes. A) Expression levels of *LsOLE1* in control cells (empty bars) and cells overexpressing *LsOLE1* (black bars). B) Expression levels of *LsFAD2* in control cells (empty bar) and cell overexpressing *LsFAD2* (grey bar) analysed during exponential phase. The expression level of the actin gene was defined as unity. Error bars indicate standard deviation, n = 3

4.3.2. Effect of metabolic engineering on the growth of *L. starkeyi*

To characterize the effects of *LsOLE1* and *LsFAD2* overexpression on the biomass accumulation, substrate consumption and oil accumulation, first we grew the wild type strain and the engineered strains in flask-batch culture (small scale) in MeOL media (composition described in Materials and Methods section). For the experiments, pure glycerol was used as sole carbon and energy source at the concentration of 100 gL^{-1}. Cultivations were performed at 25°, 220 rpm and followed for 216 h, monitoring biomass (optical density), substrate consumption (pure glycerol), and oil production (chemical extraction). As previously reported [16],

medium formulated with 100 gL^{-1} of pure glycerol well sustained the growth of control strain, but also of the engineered strain selected for this study.

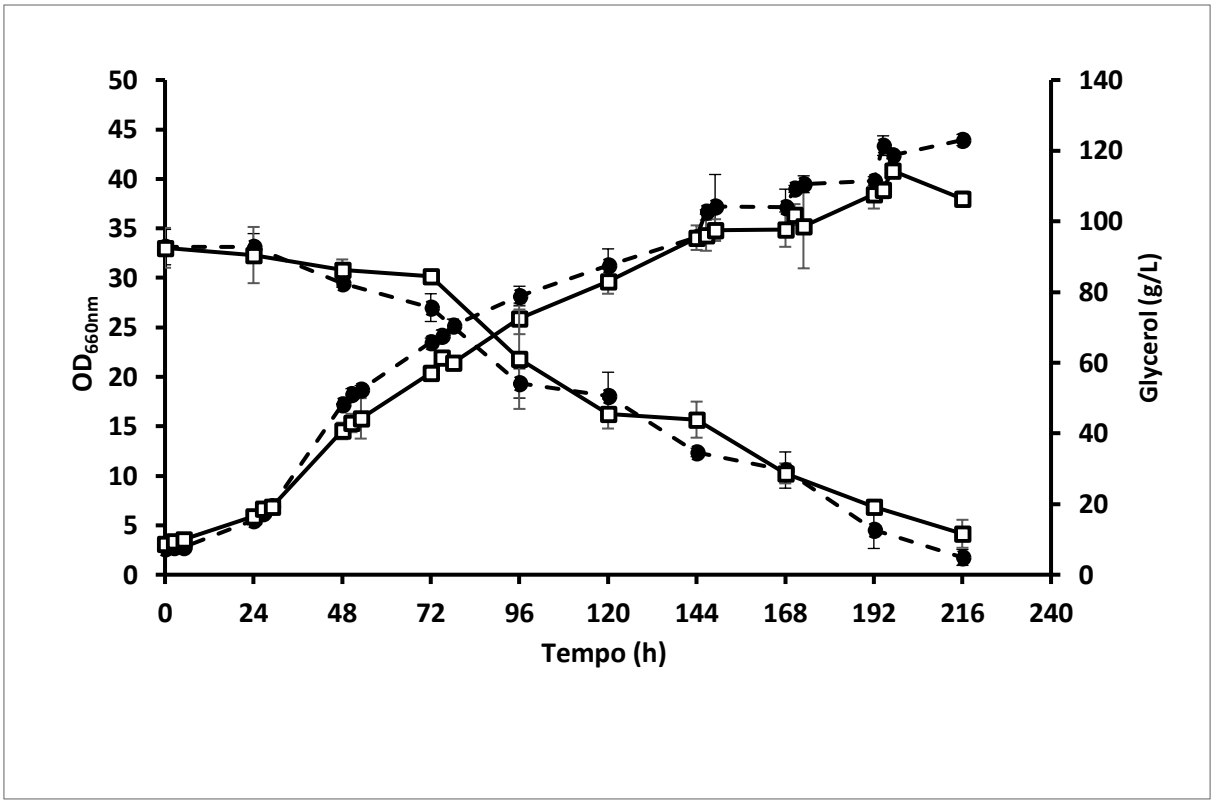


Fig.3. Growth curves (OD_{660nm}) and glycerol consumption (Glycerol gL^{-1}) of *L. starkeyi* wt (dashed lines and ●) and *L. starkeyi-OLE1-FAD2* (continuous lines and □) in shake flasks cultivated on 100 gL^{-1} of pure glycerol as sole carbon source, at 25 °C and 220 rpm. Data are mean ± standard deviation (error bars) of three independent assays.

As shown in Fig.3, almost identical OD profiles, glycerol consumption and oil accumulation were observed. We can conclude that the double overexpression did not affect the main parameters here analysed, including the oil production.

4.3.4. Fed-batch fermentation for MICRO-OIL4 production

Since we demonstrated that the engineering of *L. starkeyi* did not influence the biomass as well as the oil accumulation during shaking flasks cultivation, we also considered the bioreactor cultivation using feeding strategy already proposed for the parental strain and described in Chapter 2.

Briefly, strains were cultivated at 25 °C, pH 5.5 and monitored until glycerol exhaustion. The inlet gas flow rate was maintained constant at 1 vvm and stirring was set in cascade to 25 % of dissolved oxygen to ensure fully aerobic conditions.

The cultivation was designed to have two phases: 1) "*batch phase"* on pure glycerol (till 20 g/L of residual glycerol) to allow cells adaptation and growth; 2) "*pulse fed-batch phase"* where the medium was fed to reach again 60 gL^{-1} of glycerol (crude versus pure) to promote oils accumulation.

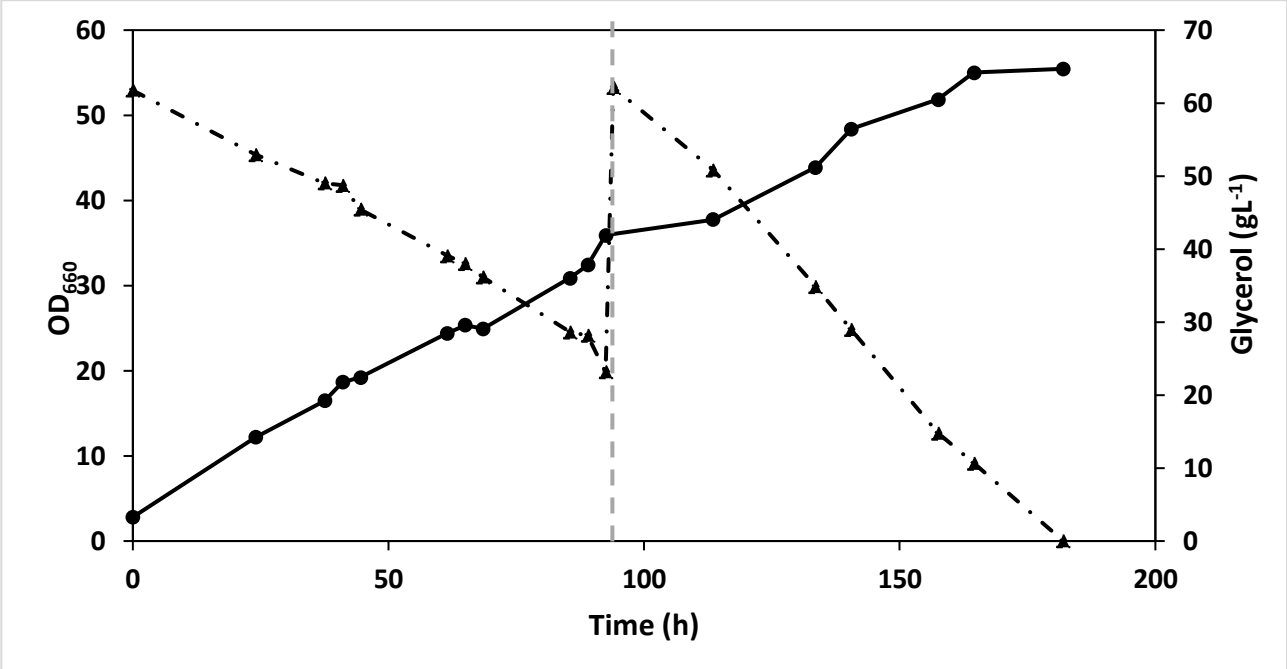


Fig.4. Glycerol consumption and growth profiles of *L. starkeyi-OLE1-FAD2* under fed-batch pulse cultivation (grey dasched lines). Growth (OD_{660}, continuous line) and glycerol consumption profiles (g L^{-1}, dashed line) of *L. starkeyi-OLE1-FAD2*. Data are mean of three independent assays.

The strain L. starkeyi-OLE1-FAD2 accumulates biomass in bioreactor (Fig.4) slower compared to the parental strain (see chapter 2). The glycerol uptake rate was 0.37 $gL^{-1}h^{-1}$ for the initial phase of growth (till 68 hours from the inoculum), whereas when the concentration of glycerol reached 20 gL^{-1} (at about 92h), the glycerol uptake rate corresponded to 0.41 $gL^{-1}h^{-1}$. After the glycerol pulse, the cells were able to consume the substrate much faster compared to the batch phase, reaching an uptake rate of 0.71 $gL^{-1}h^{-1}$. Therefore, with this feeding strategy, we could compensate the slower initial growth of strain *L. starkeyi-OLE1-FAD2*, even when using crude glycerol as here described.

Strain	Maximum DW (gL^{-1})	Maximum oil content (gL^{-1})	Lipids (%)	
L. starkeyi *	41.35	19.5	47.2	Chapter2
*L. starkeyi-OLE1-FAD2***	41.4	24.6	59.5	This study

Table 2. Comparison of parameters relate to biomass and lipid production among *L. starkeyi** and *L. starkeyi-OLE1-FAD2***. Data shown are the mean of three independent experiments where the deviation from the mean value was less than 5 %.

*parental strain

*engineering strain

Table 2 reports the cell mass (maximum DW) and lipid content (maximum oil content) of the *L. starkeyi OLE1-FAD2* strain. The biomass production for the engineered strain corresponds to 41.4 g/L of CDW, similar to the value of 41.35 g/L obtained for the parental strain. Conversely, the oil content (19.5 gL^{-1}) and the lipid percentage (47.2 %) were markedly inferior for the parental compared to the engineered strain, 24.65 gL^{-1} and 59.5% respectively. At the best of our knowledge, this is the first time that the engineering strain here described was cultivated in bioreactor using an industrial waste as carbon source. In addition, in order to obtain a major quantity of oil to perform the compounding rubber experiments, we make also for this strain ten (10) liter fermentations, obtaining a yield oil/glycerol ($Y_{o/g}$) of about 0.25 and reaching a production of 250g of oil per fermentation. These quantities have been used for all the experiments described below.

4.3.5. Microbial oil preparation: extraction and analysis of composition

The MICRO-OIL4 produced by fed-batch fermentation of *L. starkeyi-OLE1-FAD2* was hydrolyzed and transmethylated. The resulting fatty acid methyl esters (FAMEs), were then analysed by gas chromatography. The fatty acids percentages obtained for the MICRO-OIL4 are shown in the Table 3 below together with MICRO-OIL 1 and 2, and AP-88 (see Chapter 2 for details).

% (wt/wt)	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)	SFAs	MUFAs	PUFAs
MICRO-OIL1	30.0	1.0	10.0	37.0	16.0	-	42.0	38	20
MICRO-OIL2	30.6	4.2	6.6	53.7	2.9	-	38.9	58	3.1
MICRO-OIL4	28.3	5.9	4.7	55.1	4.2	-	34.6	61.0	4.4
AP-88	3-4	-	2-3	84	8	-	6-10	84	6-10

Table 3. Gas chromatography analysis of MICRO-OIL4 (*L. starkeyi-OLE1-FAD2*), compared to MICRO-OIL1 (*R. toruloides*) and MICRO-OIL2 (*L. starkeyi*) and the vegetable oil AP-88.
SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids, PUFAs, polyunsaturated fatty acids.
Data shown are the mean of three independent experiments where the deviation from the mean value was less than 5 %.

Oleic acid was predominantly represented in all the strains, but with different percentage. *OLE1* and *FAD2* genes, overexpressed in *L. starkeyi OLE1-FAD2* codify for two desaturase, delta-9 and delta-12 desaturase, involved in the production of MUFAs and polyunsaturated fatty acids (PUFAs), respectively.
It has been reported that increasing the activity of delta-9 desaturase in the oleaginous yeast *R. toruloides* lead to oleic acid rich oil [21].

In addition, Matsuzawa et al. [22] have tested the overexpression of *FAD2* gene in a *L. starkeyi* strain deleted to promote homologous recombination (LsΔlig4, OGURO 2017), obtained significantly differences compared to wild type in linoleic acids amount.
Taking into account these dates, the expected composition for the MICRO-OIL4 obtained from *L. starkeyi-OLE1-FAD2* was more in the direction of polyunsaturated fatty acid, but we instead obtained more monounsaturated ones. In particular, the combined overexpression lead to 61 % of MUFAs with only 4.4 % of polyunsaturated fatty acids. Therefore, the overexpression of *OLE1* and *FAD2* genes seems to lead to our best oil candidate for bioplasticizers production, which we verified by preparing rubber compounds tested for performances.

4.3.6. Physical properties: MICRO-OIL4

Physical properties of MICRO-OIL4 are shown in Table 4, in comparison with the other oils produced in this study (see Chapter 3 for more details about MICRO-OIL1 and 2).

	Glass transition temperature (°C)	Melting peak (°C)	Iodine value gI2/100g
MICRO-OIL 1	-88.7	10	65.3
MICRO-OIL2	-80.7	8	55.5
MICRO-OIL4	-74.4	8	56
MES	-60	-	-
AP-88	-97	-5	84.6

Table 4. Physical properties of the oils used in this study

As we widely discussed in Chapter 3, the physical properties of microbial oils are crucial for their influence on elastomer-oil compatibility, very important for tyre compound to maximise plasticisation effectiveness and to limit oil migration events [23].
Briefly, Table 4 shows that the glass transition temperature of MICRO-OIL4 is higher compared to the other microbial oils, especially in respect to MICRO-OIL1, probably due to the enriched MUFAs lower level of PUFAs in MICRO-OIL4 composition that determines a different fatty acid distribution. In addition, the iodine value is lower compared to MICRO-OIL1, and almost the same of MICRO-OIL2. Physical properties, although they are different compared to the other microbial oils, show values closer to MICRO-OIL2. Because of that, in the second part of this Chapter we will describe

the results obtained by compounding MICRO-OIL4, together with AP-88 and MES, with the same elastomer, SLR3402, used in the Chapter 3 of this thesis.

4.3.7. Styrene-butadiene-based NR-SBR formulations

4.3.7.1. Compounding

The NR-SBR compounds were prepared using the MICRO-OIL4, the vegetable (AP-88) and mineral oil (MES), as references. The final content of oils for each compound is 20 phr. The compounds were prepared in three steps. The temperature in the first step was set to 140 °C to create a chemical bond between the filler and silane coupling agent. During the second and third steps which was performed at low temperature, 90° and 80°, respectively, to avoid the pre-vulcanization of the rubber compounds, were added the vulcanizing agents, accelerators and antioxidants.

4.3.8. Characterization of the NR-SBR compounds

4.3.8.1. Static and dynamic properties of compounds

The results obtained in Chapter 3 on the compound obtained with MICRO-OIL2 and the elastomer SLR3402, we can claim that the obtained material has good static properties, especially modulus at different elongation, as well as interesting dynamic properties especially tanδ at 70°. Conversely, it showed a worse value of tensile strength compared to that obtained in compound containing the vegetable oils AP-88.

	NR-SBR MICRO-OIL4	NR-SBR AP-88®	NR-SBR MES
STATIC PROPERTIES			
M10 [MPa]	0.62	0.60	0.63
M50 [MPa]	1.61	1.42	1.70
M100 [MPa]	2.53	2.24	2.77
M300 [MPa]	8.77	8.55	10.53
Tensile strenght [MPa]	18.26	16.32	15.95
DYNAMIC PROPERTIES			
E' (23°C)	8.27	7.32	8.78
tanδ (23°C)	0.171	0.174	0.181
E' (70°C)	7.38	6.5	8.0
tanδ (70°C)	0.120	0.132	0.123

Table 5: Static and dynamical analysis were performed to determine compounds properties.

Here we show the static and dynamic properties obtained by using MICRO-OIL4, compared with the reference oils, MES and AP-88. As well as MICRO-OIL2, moduli at different percentages of elongation of the compound with MICRO-OIL4 are very similar to MES, and higher compared to AP-88. In addition, unlike MICRO-OIL2, tensile strength is significantly higher compared to both the reference oils.

Also for the dynamic properties, as already showed for MICRO-OIL2, the values corresponding to the storage modulus (E') and tanδ measured for MICRO-OIL4 at 23°C e 70°C are very similar to MES compounds, but it is interesting to highlight that the values of storage modulus (E') at both temperatures are higher than AP-88 compound. In addition, also the lower values of tanδ at 70°C compared to MES and especially AP-88, are confirmed.

These results suggest that MICRO-OIL4 used in NR-SBR compounding allows to obtain interesting characteristics: *i)* better tensile strength compared to MICRO-OIL2, MES and AP-88; *ii)* low hysteresis at high temperature, as well as high storage moduli (E'), both optimal conditions to get low rolling resistance and consequently reducing fuel consumption and CO_2 emissions. All these unpredictable and valuable properties of MICRO-OIL4, together with the engineering strain *L. starkeyi-OLE1-FAD2*, were taken into consideration for the patent application (Patent application number: 102020000006715).

4.4. Supplementary files

4.4.1. Molecular biology, methods and materials

Diagnostic PCRs were performed using Wonder Taq DNA Polymerase (Euroclone S.p.A). PCR amplification for cloning was performed with Q5® High-Fidelity DNA Polymerase (New England Biolab, Beverly, MA). Both the enzymes were used according to the manufacturer's instructions. Primers were purchased from Metabion (international AG/GmbH). DNA purification from PCR reaction mixture was done using NucleoSpin Gel and PCR clean-up (MACHEREY-NAGEL GmbH & Co. KG). Separation of DNA fragment was performed in 0,8% (w/v) agarose gel (XX) in TAE buffer (40 mM Tris-acetate pH 8.0 and 1 mM EDTA). DNA fragments were purified from gels using NucleoSpin Gel and PCR clean-up (MACHEREY-NAGEL GmbH & Co. KG). Plasmid assembly was done with the Gibson Assembly™ Master Mix (New England Biolab, Beverly, MA) according to manufacturer's protocol. Restriction endonucleases and DNA ligase (New England Biolab, Beverly, MA) were used accordingly to manufacturer's instructions.

4.4.2. *Lipomyces starkeyi* plasmids construction

4.4.2.1. Primers sequences used to plasmids construction

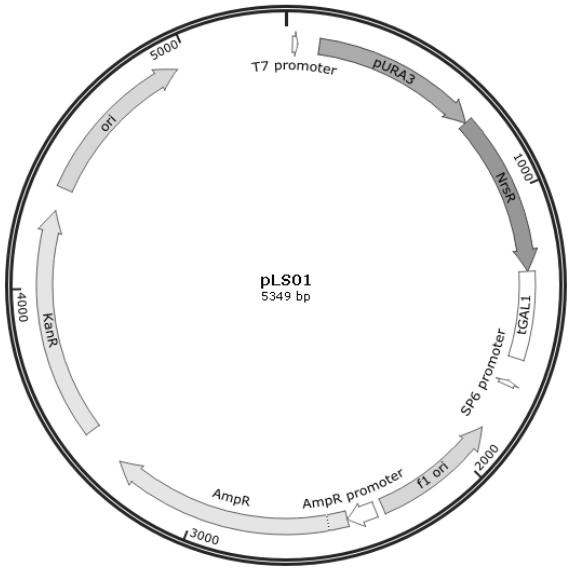
Name	Sequence
pURA3 FW	TCGATCCAGAATTCGTGAT**TCTAGACTCGAGATATC***ATGATCACTGAGCGATAGTTC*
pURA3 REV	**AGTTCAGGCTTTTTACCCAT***GTTGAATTTAGGGATATACTGTAGAAGAC*
tGAL1 FW	**GTCCGAGGGCAAAGGAATAA***AGTTTAGAGATGTACAAGGGGT*
tGAL1 REV	**GCTTGTCGACGAATTCAGAT***TGCCACGATAACTTTGTGC*
Nsr R FW	ATGGGTACCA CTCTTGACGA C
Nsr R REV	TTAGGGGCAG GGCATGCTCA
pTDH3 FW	CGTGAT**TCTAGACTCGA**GAT*TTAATTTGCTGAAGCGGTTTGC*
pTDH3 REV	**CGCTTAGCGATATCACTAGT*AGATCT***TGCGAATGTGGATTAGAGTAAGA
tPGK1 FW	***ACTAGTGATATCGCTAAGCGGCCGGCC****CTCCCGTTAATGTTGGGATTC*
tPGK1 REV	**TATCGCTCAGTGATCATGAT***CCTGTCAATTATGCTACCACTTG*
LsOLE1 FW	TCAGATACTA GTATGACTGC CAGTGCTGAG ACAACGTCC
LsOLE1 REV	AGATACACTA GTCTAAGCGG CACCAGCGCC CT
LsTDH3 FW	ATAATAATCCGAACTGCCGC
HygR FW	ATGGGTAAAA AGCCTGAACT CACC
HygR REV	TTATTCCTTT GCCCTCGGAC G
LsFAD2 FW	CTAGTAAACT AGTATGTCCA CAATAACATA CACAC
LsFAD2 REV	TATTCTACTA GTTTACTGAG CCCTTCTTCC

Table S1. All the primers employed and developed in this study

Italics: sequence annealing to the genome. Bold: sequence annealing to the plasmid. Bold and italics: BglII, SpeI, EcoRV, BlpI, FseI restriction site.

4.4.2.2. The recombinant vector pLS01

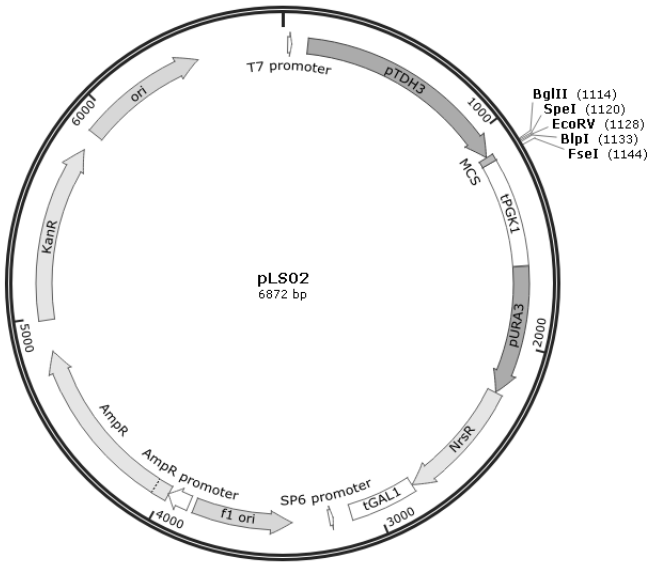
The sequences of the promoter *pURA3*, the terminator *tGAL1* have been amplified by PCR using as a template the genomic DNA of *L. starkeyi* and primers for *pURA3* and *tGAL1* (Table S1). The drug resistance Nsr^R cassette was obtained by amplification using the plasmid pZ_5 [24] as template and the primers for Nsr^R (Table S1).
All the PCR products have been recovered from 0,8% agarose and purified. The sequences *NrsR*, p*URA3* e t*GAL1*, have been subcloned into pStblue-1 vector using the cloning kit Gibson assembly (New England Biolab, NEB). Then, pLS01 has been amplified in *E. coli* and checked by analytical digestion using the enzymes HheI e SphI. The vector pLS01 is shown here as Fig.S1.



4.4.2.3. The recombinant vector pLS02

The EcoRV linearized vector pLS01 has been used as scaffold for the insertion of the constitutive and strong promoter *TDH3* and the terminator *PGK1,* both amplified by PCR using *L. starkeyi* genomic DNA as template and the specific primers described in Table S1, containing the restriction site for the enzymes BglII, SpeI, EcoRV, BlpI, FseI, that allow to create between promoter and terminator a multiple cloning site (MSC) using the cloning kit Gibson assembly (New England Biolab, NEB). The obtained vector pLS02 has been amplified in *E. coli* and checked by analytical digestion using the enzymes PstI e PmlI.

The vector pLS02 is shown here as Fig.S2.



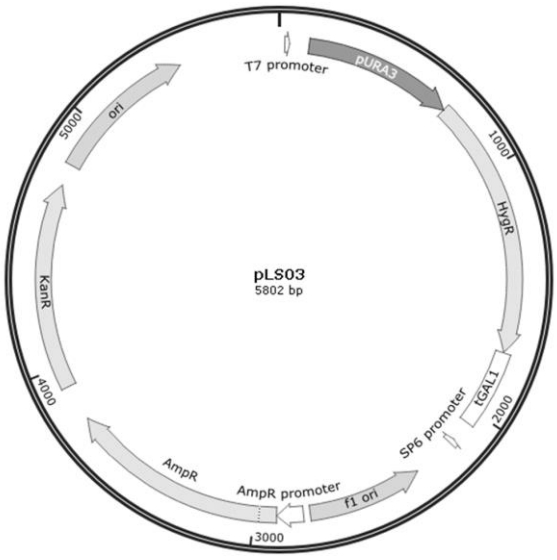
4.4.2.4. The recombinant vector pLS02-OLE1

The gene encoding for the Δ9 desaturase of *L. starkeyi*, *LsOLE1* was identified by matching the amino acid sequence of *S. cerevisiae* Δ9 desaturase against the putative amino acid sequences present in the genomic DNA sequence of *L. starkeyi* (www.genome.jgi.doe.gov/Lipst1_1/Lipst1_1.home.html), using the program BlastP(https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Protein). The sequence of *LsOLE1* was amplified by PCR using as a template the genomic DNA of *L. starkeyi* and the primers for *LsOLE1* (Table S1). Therefore, the vector pLS02 has been linearized by SpeI and then ligated to the *LsOLE*1 fragment. The vector pLS02-*LsOLE1* has been verified by sequencing using the specific oligonucleotides, LsTDH3 FW and tPGK1 REV, showed in the Table S1.

The vector pLS02-*LsOLE1* is shown here as Fig.S4.

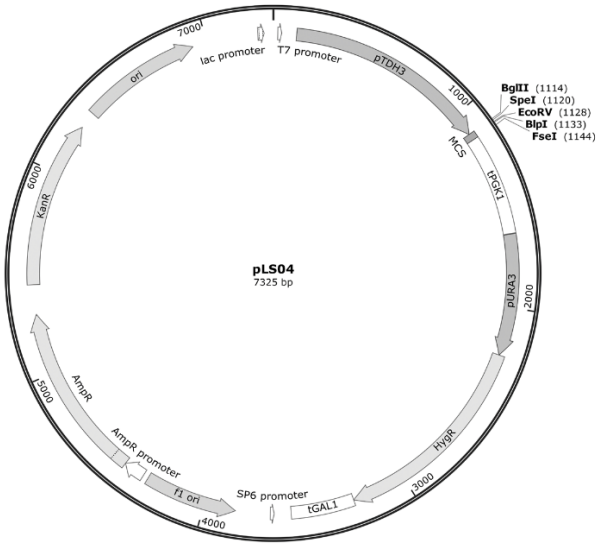
4.4.2.5. The recombinant vector pLS03

The drug resistance *HygR* was obtained by PCR amplification using the plasmid pZ_4 [25] as a template and the primers for *HygR* (Table S1).
The PCR product has been recovered from 0,8% agarose and purified. Then, *HygR* sequence has been integrated into pStblue-1 vector using the cloning kit Gibson assembly cloning (New England Biolab, NEB). The product has been amplified by *E. coli* and checked by analytical digestion using the enzymes HheI e HincII.
The vector pLS03, is shown in the Fig.S4.



4.4.2.6. The recombinant vector pLS04

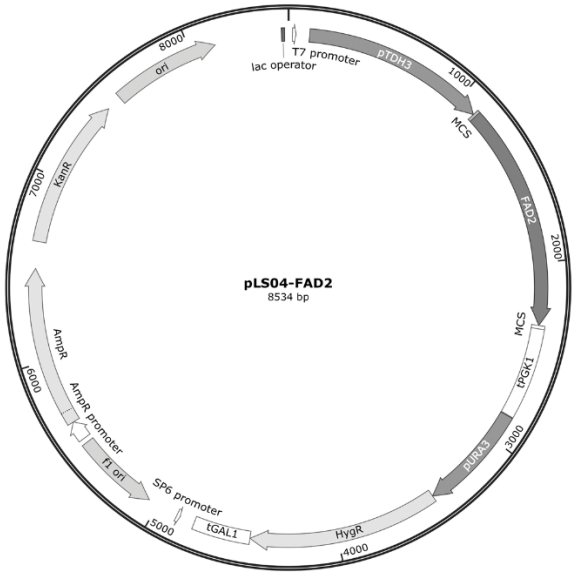
The recombinant vector pLS02 (see paragraph 1.2.2.) has been digested by NheI enzyme for linearization and *NrsR* removal. The same enzyme has been used to cut the pLS03 vector and recover *HyrR* gene. After that a cut and sew has been made to obtain the vector pLS03 checked by analytical digestion using the enzymes SacII.
In the Fig. S5 is shown the vector pLS04.



4.4.2.7. The recombinant vector pLS04-FAD2

The Δ12 desaturases of *L. starkeyi*, termed *LsFAD* sequence was obtained from [21]. The *L. starkeyi FAD2* gene sequence was amplified by PCR using as a template the genomic DNA from *L. starkeyi* and the primers for *LsFAD2* (Table S1). Therefore, the vector pLS04 has been linearized by EcoRV-HF and then ligated to the *LsFAD2* fragment. The vector pLS04-*LsFAD2* has been verified by sequencing using the specific oligonucleotides, *LsTDH3* FW and *tPGK1* REV, showed in the Table S1.

The Fig.S6 showed the vector pLS04-*LsFAD2.*



4.4.3. RTq PCR on genomic DNA*: OLE1* and *FAD2* gene copy number quantification

To verify the presence of supernumerary genes in the strain overexpressing *LsOLE1* and *LsFAD2*, we performed real-time PCR on genomic DNA of *L. starkeyi-OLE1-FAD2.* The results confirmed that cells contained two copies of both the genes, *LsOLE1* (Fig.S7A) and *LsFAD2* (Fig.S7B) in their genomes.

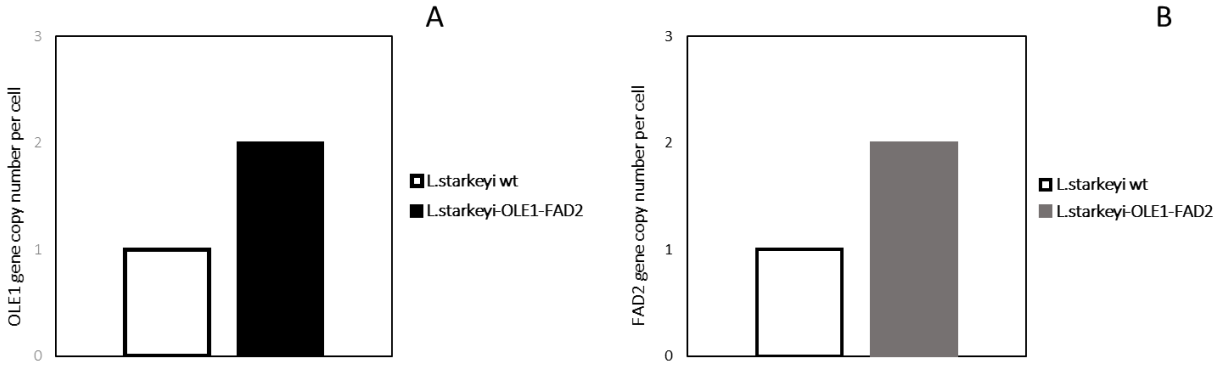


Fig.S7. Gene copy number of *LsOLE1* and *LsFAD2*. A) *LsOLE1* gene copy number in control cells (empty bars) and cells overexpressing *LsOLE1* (black bars). B) *LsFAD2* gene copy number in control cells (empty bar) and cell overexpressing *LsFAD2* (shaded bar) analysed during exponential phase.

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5

Conclusions and future perspectives

The research presented in this thesis deals with the development of sustainable plasticizers oils, to replace the oils derived from petroleum distillation traditionally used in tyre industry. As we discussed, the potential of vegetable oils as bioplasticizers is high, but at the same time there are concerns related to the sustainability of their production.
Here we propose an integrated second generation biorefinery process based on oleaginous yeasts able to accumulate microbial oils to use as plasticizers, starting from waste industrial feedstock (second generation biomasses), fully reflecting the concept of circular economy.
The major appeal of the proposed process lies in the high similarity between the composition of microbial and vegetable oils, therefore offering a promising alternative in terms of performances, but at the same time in terms of sustainability. It was surprisingly observed that, at least in specific compounds, microbial oils led to improved final mechanical properties of the cured rubber compounds as compared to the chosen vegetable oils.

The microbial oils have been produced during this study by microbial fermentation, in particular we selected: *i)* two promising oleaginous yeasts, *R. toruloides and L. starkeyi*, as producers; *ii)* we selected crude glycerol as residual feedstock for yeasts cultivation; *iii)* we optimized fermentation conditions and oil profile; *iv)* we tested the most promising oil (MICRO-OIL1, 2, 4), together with reference oils, as plasticizers for tyre production, evaluating the performances of the obtained materials.

Among the microbial oils, their plasticizing capability was initially investigated by the preparation of Natural Rubber-Butadiene-based rubber compounds (NR-BR), and by comparing the data with the ones obtained by the use of MES and reference vegetable oils (CASTOR OIL, AP-75 and AP-88).
The characterization of the prepared compounds showed an improvement of processability directly related to plasticizing capability.
The tensile mechanical studies of the compounds revealed that by using MICRO-OIL1 (obtained from the wild type yeast *R. toruloides*) and MICRO-OIL2 (obtained from the wild type yeast *L. starkeyi*) it is possible to obtain static properties similar to those obtained with the mineral oil MES and ameliorated if compared to the tested vegetable oils. The dynamic analysis confirmed the similarity between microbial oils and MES in terms of storage modulus (E'), as well as a better performance compared to the tested vegetable oils. In addition, MICRO-OIL1 and 2 allowed to obtain compounds with the lower value of tanδ at 70°, predictive of a lower rolling resistance of tyre. The good performance conferred to NR-BR compounds by these microbial oils was the basis for developing a Patent Application (number: IT 102020000006688).

The natural presence in MICRO-OIL1 of carotenoids, known for their antioxidant properties, led us to investigate their possible protective action against thermal aging. Therefore, we compared compounds prepared with MICRO-OIL1 with or without the addition of the antioxidant-antiozonant 6PPD. Tensile mechanical analysis after thermal aging revealed similar static properties in the obtained compounds. Based on these data, we can conclude that the composition of MICRO-OIL1 might have a protective action against thermal aging.

As already discussed in the introduction, the oleaginous yeasts selected for this work showed the capability to grow on a wide range of second generation biomasses. But it is important to consider the fermentation strategy to improve SCO production: different fed-batch technique have been tested, to easily control nutrient addition during cellular growth and lipid accumulation [1-4]. In the context of raw substrates, a fed-batch strategy allows a cell adaptation and growth during the initial batch phase performed with pure glycerol, and oil accumulation during the feeding with crude glycerol. The cells with this strategy have been able to: *i)* metabolize the crude glycerol into oil ($Y_{o/g} \geq 0.20$); *ii)* accumulate oils in a percentage higher than 45% of their CDW, resulting a valuable strategy for SCOs production.

The promising behaviour of microbial oils in NR-BR compounds led us to go forward with the research of other elastomers for tyre formulations. A compatibility test based on the evaluation of thermal properties of elastomers and oils revealed a good score between one of the microbial oils, MICRO-OIL2, and one of the selected elastomers, the styrene-butadiene rubber SLR3402, confirmed also after compounding it. In particular, the compound based on styrene-butadiene and natural rubber (NR-SBR), prepared with MICRO-OIL2 showed performances comparable to those obtained with MES, also confirmed by static properties and by the storage modulus obtained from dynamic analysis. Interestingly, as described above, even with this elastomer, the MICRO-OIL2 allowed to obtain lower values for tanδ, compared to MES and AP-88, suggesting a lower rolling resistance. On the other hand, AP-88, having lower moduli at different percentage of elongation and dynamic properties, showed higher values of tensile strength.

In the last part of this work, with the aim of fully demonstrating the potential of oleaginous yeasts as platform for the production of plasticizer oils, we proposed a metabolic engineering approach to modify the fatty acids profile of microbial oils. We decided to overexpress, in the yeast *L. starkeyi*, two genes encoding for enzymes involved in fatty acids desaturation, the Δ9 and the Δ12 desaturases. These enzymes are involved in the first two steps of fatty acids desaturation: the Δ9 desaturase provides the substrate for Δ12 desaturase, involved in the production of the first

precursor, linoleic acid, of PUFAs. The engineering led to the production of MICRO-OIL4, that was surprisingly enriched in MUFAs, instead of PUFA, as predictable from the description but never tested in this biological context. The findings are novel and inventive, and therefore they resulted in the submission of the Patent Application (number: IT 102020000006715).

In order to investigate the plasticizing properties of MICRO-OIL4, we prepared rubber compounds based on the elastomer mentioned before, SLR3402, comparing the obtained compound with those prepared with MES and AP-88, as reference. The static properties of the compound prepared with MICRO-OIL4 showed a good elasticity proven by the moduli at different percentage of elongation similar to MES, and also to MICRO-OIL2, greater reinforcement compared to all the reference oils considered. In addition, the value corresponding to storage modulus (E') and tanδ also confirmed a similar behaviour of MES compound, then again, a slightly lower hysteresis, contrary to what observed for AP-88. Then, the application of MICRO-OIL4 within the styrene-butadiene compound demonstrated the potential for a higher reinforcement and lower rolling resistance when compared to all the reference oils used in that study. These promising outcomes earned interest for the industrial application evidenced by the submission of the Patent Application (number: IT 102020000006715).

Overall, the second generation biorefinery model proposed in this work based on a renewable and residual biomass (crude glycerol) and oleaginous microorganisms to obtain bioplasticizer fully aligns with the bio-based concept, aimed to the production of bio-based material, defined as material wholly or partly derived from biomass [5], producing zero waste, minimizing environmental impacts producing at the same time economical value.

In this work the capability of microbial oils to work in model rubber tyre compounds as bioplasticizers was demonstrated. At the same time, we also highlighted that the composition and physical properties of microbial oils can play a pivotal role in elastomer-oil affinity. In fact, the unique and attractive perspective to modify the composition of microbial oils by metabolic engineering allowed to obtain unexpected cured rubber properties.

The work here presented can be considered at Technology Readiness Level (TRL) 4, defined as "– technology validated in lab". From here on, considering the promising results obtained, many aspects related to the production of these oils, fermentation time and downstream processing (cell lysis and SCOs extraction), renewable biomass selection and costs, shall be considered and explored in order to go up to TRL 5/6. At that moment, an economical evaluation will be possible, in order to make this

biobased and sustainable process able to compete with the existing production chains [6].

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