UNIVERSITÀ DEGLI STUDI DI MILANO
Department of Chemistry
Doctorate Degree in Industrial Chemistry
Cycle XXXII

Biocatalysis for Biomass Valorization: Protein Hydrolysates and Sugar Esters from Agri-Food Wastes

Ph.D. Thesis

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“ἐὰν μὴ ἔλπηται ἀνέλπιστον οὐκ ἐξευρήσει,

ἀνεξερεύνητον ἐὸν καὶ ἄπορον”

“Αν δεν ελπίζεις, δε θα βρεις το ανέλπιστο,
που είναι ανεξερεύνητο και απλησίαστο”

“He who does not expect will not find out the unexpected,
for it is trackless and unexplored”

Ηράκλειτος / Heraclitus

Abstract

During this doctorate work, two research topics have been studied within the aim of valorization of waste and by-products derived from the agri-food industry using a biotechnological approach for the production of high-value chemicals.

The first topic was the preparation and characterization of hydrolysates from rice bran protein. Rice bran (RB) is a waste derived from the milling process of the rice and is a rich source of highly nutritional proteins, lipids, carbohydrates, and a number of micronutrients (e.g. vitamins, minerals, antioxidants, and phytosterols). The sequential treatment of RB with carbohydrases and proteases was used to prepare mixtures of water-soluble peptides which were tested for their biological activity (ACE-inhibition) and as flavor enhancers. Carbohydrases, that catalyze the hydrolysis of the glycosidic linkages of rice bran polysaccharides, enhanced the extractability of the entrapped protein components. Then, proteases (Flavourzyme or/and Alcalase) allowed converting the protein fraction of rice bran into mixtures of more water-soluble peptides.

The prepared samples were submitted to ultrafiltration by using membranes with molecular weight cut-off of 10, 5 and 1 kDa and characterized by SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis), Gel Permeation Chromatography (GPC) and by sensory analysis. All samples with a molecular weight under 10 kDa exhibited ACE-inhibitory activity.

The highest activity was found for the samples P4'' (68.70%) with a molecular weight under 1 kDa and P2' (60.19 %) with a molecular weight under 5 kDa and the lowest activity for the sample "P5" (20.28 %) with a molecular weight under 5 kDa. It is noticeable that the choice of the enzyme for the first step treatment (carbohydrases) has a great effect on the ACE – inhibitory activity of the final hydrolysate. Interestingly, the sensory analysis revealed that the resulting protein hydrolysates exert only sweet and umami taste. It should be mentioned that the bitter taste was completely eliminated, which could be considered very promising for the application and utilization of the rice bran protein hydrolysates as food enhancers.

The second topic of this PhD work was the enzymatic synthesis of sugar-fatty acid esters that can be used as bio-surfactants.

Surfactants constitute an important class of chemicals widely used in almost every sector of industry. Environmental and health concerns about the effects of the conventional surfactants have increased the demand for surfactants from natural raw materials that possess good biodegradability and low toxicity, along with the desired functional performance.

Sugar fatty acid esters (SFAEs), usually called sugar esters, are fully biodegradable, non-ionic surfactants which are characterized by excellent emulsifying, stabilizing and detergency properties. Depending on carbon chain length and nature of the sugar head group, together with the many possibilities for linkage between the hydrophilic sugar and the hydrophobic alkyl chain, SFAEs cover a wide range of hydrophilic–lipophilic balance (HLB) values which result in tunable surfactant properties.

Chemical synthesis of SFAEs requires harsh reaction conditions which result, in most cases, in complex mixtures of isomers and by-products. Enzyme-based synthesis is an alternative strategy that can overcome the above-mentioned drawbacks. Sugar fatty acid esters can be prepared, indeed, through an esterification reaction between a sugar and a fatty acid catalyzed by a lipase.

SFAEs, including glucose monooleate (GluMO), monostearate (GluMS), monopalmitate (GluMP), monolaurate (GluML), and galactose monooleate (GalMO), monostearate (GalMS), monopalmitate (GalMP), monolaurate (GalML), were synthesized by enzymatic esterification of fatty acids and the corresponding sugar. After a screening of several lipases both in free and immobilized form, an immobilized lipase CALB (*Candida antarctica* lipase B) was selected as the biocatalyst to promote the ester bond formation. Reactions were carried out in organic solvent by using molecular sieves (4 Å) to scavenge the water by-product and thus shift the reaction toward sugar ester formation. Reaction yields and product characterization were assessed by NMR. Rational design of enzymatic reactions was carried out by using the synthesis of GluMP as the model reaction. Sugar: fatty acid ratio, temperature, and reaction time were selected as variables (response: product yield).

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Chapter 1: Food waste valorization

1.1 Scope of the thesis

The valorization of wastes and by-products derived from Agri-Food Industry is a hot topic nowadays not only because of the need to discharge the waste in a sustainable and more environmental-friendly way but also because of the continuous demand for starting materials. Food waste biomass is a source of high value chemicals that can provide the chemical industry plenty high added value products. Apart from the source itself, greener processes and methods should be also introduced in order to exploit food waste biomasses. The aim of the current thesis work was to exploit the protein and oil fraction of agri-food wastes (such as rice bran) for the production of high value chemicals by using a biotechnological approach. With respect to this aim, this thesis is divided into two main topics:

I. Preparation and characterization of hydrolysates from rice bran protein

II. Enzymatic synthesis of sugar-fatty acid esters (bio-surfactants)

1.2 Green Chemistry and Circular Economy

Green chemistry is defined, according to the Environmental Protection Agency (EPA), as a chemistry that designs chemical products and processes that are harmless to the environment, thus preventing the formation of pollution. To this purpose, chemicals should be designed in order to break down into harmless compounds once dispersed into the environment. Furthermore, efficient ways of synthesis which minimize or do not use harmful reagents and, if possible, make use of water instead of organic solvents, should be preferred over traditional ones [1, 2]. A very simple example of a process made Green is the replacement of mineral acids, which are very effective in hydrolysis reactions but are quite difficult to handle and dispose, with heterogenous or biocatalysts.

The first manual of Green Chemistry was published by P. Anastas and J.C. Warner in 1998 and in this book the twelve principles of Green Chemistry were introduced. They are considered the guiding framework for the design of new chemical products and processes and are applied to all aspects of the process lifecycle including the raw materials, the efficiency and safety of the transformation, as well as the toxicity and biodegradability of products and reagents used. The twelve principles of Green Chemistry are presented in the **Table 1.1**. Recently, also Poliakoff et al. [3] proposed a mnemonic, called **PRODUCTIVELY**, which conveniently captures the spirit of the twelve principles of green chemistry:

P – Prevent wastes
R – Renewable materials
O – Omit derivatization steps
D – Degradable chemical products
U – Use of safe synthetic methods
C – Catalytic reagents
T – Temperature, Pressure ambient
I – In-Process monitoring
V – Very few auxiliary substrates
E – E-factor, maximize feed in product
L – Low toxicity of chemical products
Y – Yes, it is safe

The last decades, the concept of green chemistry has been widely adopted both in industrial and academic field. As some authors suggest, sustainability is our ultimate common goal and green chemistry is the path to achieve it [4].

Table 1.1. The 12 principles of Green Chemistry [5, 6].

(1)	**Prevention**	It is better to prevent waste than to treat or clean up waste after it has been created.
(2)	**Atom economy**	Synthetic methods should be designed to maximize incorporation of all materials used in the process into the final product.
(3)	**Less Hazardous Chemical Syntheses**	Wherever practicable, synthetic methodologies should be designed to use and generate substances that possess little or no toxicity to human health and the environment.
(4)	**Designing Safer Chemicals**	Chemical products should be designed to preserve efficacy of function while reducing toxicity.
(5)	**Safer Solvents and Auxiliaries**	The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and, innocuous when used.
(6)	**Design for Energy Efficiency**	Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure.
(7)	**Use of Renewable Feedstocks**	A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable.
(8)	**Reduce Derivatives**	Unnecessary derivatization (use of blocking groups, protection/deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.
(9)	**Catalysis**	Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
(10)	**Design for Degradation**	Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.
(11)	**Real-time analysis for Pollution Prevention**	Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
(12)	**Inherently Safer Chemistry for Accident Prevention**	Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

The scenario of Circular Economy is a hot topic. Several authors and organizations described the concept of Circular Economy and different definitions appeared during the last years trying to give the interpretation of this concept [7, 8]. For instance, according to European Commission, the circular economy is an economy "where the value of products, materials and resources is maintained in the economy for as long as possible, and the generation of waste minimized" [9]. The transition to a more circular economy would make "an essential contribution to the EU's efforts to develop a sustainable, low-carbon, resource-efficient and competitive economy". Mitchell proposed in his report that "a circular economy is an alternative to a traditional linear economy (make, use, dispose of) in which we keep resources in use for as long as possible, extracting the maximum value from them while in use, then recovering and reusing products and materials" [10].

Circular economy mainly appears in the literature through three main "actions", which are also known as the 3R's Principles: Reduction, Reuse and Recycle.

The **Reduction principle** has as a goal the reduction of the primary energy, raw materials and waste by improving the efficiency in production and consumption processes. This can be achieved e.g. by introducing better technologies or more compact and lightweight products, simplified packaging, a simpler lifestyle, etc.

The **Reuse principle** aims to "any operation by which products or components that are not waste are used again for the same purpose for which they were conceived" [11]. The reuse of products is very beneficial for the environment as it requires less resources, energy, and labor respect to the manufacture of new products from virgin materials but also respect to recycling or disposal [12].

The **Recycle principle** aims to "any recovery operation by which waste materials are reprocessed into products, materials or substances whether for the original or other purposes. It includes the reprocessing of organic material but does not include energy recovery and the reprocessing into materials that are to be used as fuels or for backfilling operations" [11]. Waste recycling has the advantage of minimizing the quantity of waste that should be treated and/or disposed of, thus decreasing also its environmental impact. It is important though to notice, that if an industry or the society is capable to recycle all the waste produced, it may not be interested in reducing the amount of waste.

1.3 Valorization of biomass derived from food industry

The agri-food industry encloses various manufacturing processes that lead to the generation of accumulative quantities of several waste, especially organic residues. The definition of Food waste (FW) can be summarized as the "end products of various food processing industries that have not been recycled or used for other purposes. They are the non-product flows of raw materials whose economic value is less than the cost of collection and recovery for reuse; therefore, discarded as waste" [13]. Almost 90 million tons of FW are produced every year in the European Union, 38% of which is derived directly from the food manufacturing sector [14].

Food supply chain residues could be used as a raw material to produce a large variety of products (**Figure 1.1**). The advantages from their exploitation are numerous: reduction of carbon footprint and dependence on fossil fuels, reduction of the cost of the waste, renewable feedstock, cost and resource efficiency. The industrial development could become more sustainable by utilizing efficiently agricultural and forestry residues, aquatic biomass, and various waste streams through an integrated biorefining [15].

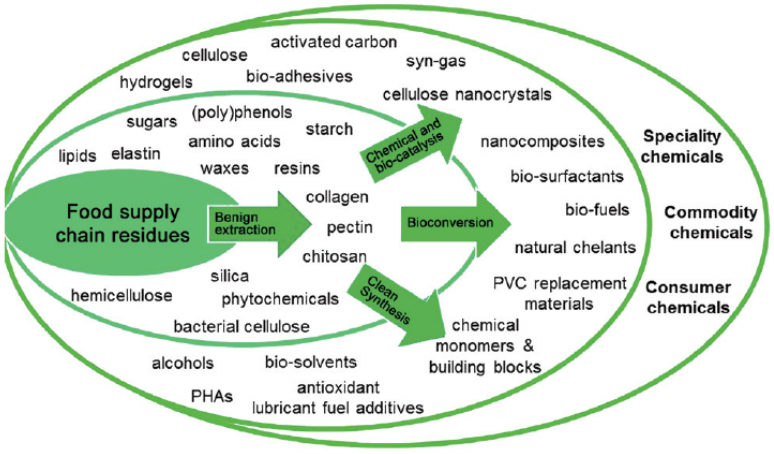

Figure 1.1. Food supply chain residues and the numerous possibilities for valorization [16].

Similar to oil refineries, where petroleum is transformed into classical fuels and building blocks commonly used nowadays in consumer products, bio-refineries convert organic waste and biomasses into a wide range of intermediates and products [17]. Following the waste bio-refinery concept, valorization routes of FW refer to both the extraction of high-value components already present in the substrates, using specifically tailored recovery procedures, and the subsequent conversion into chemicals, materials or biofuels by the utilization of classical or biological fermentative processes [18].

A proposed biorefinery concept for the valorization of waste exploiting the benefits of circular economy and green chemistry to produce biofuels and high value chemicals is presented in **Figure 1.2**. The "AgroCycle", for example, is a Horizon 2020 project (between 26 collaborative partners) that addresses the 'circular economy' by reducing waste all along the chain and by utilizing this waste as a feedstock for downstream industries such as pharmaceuticals and biochemicals [19].

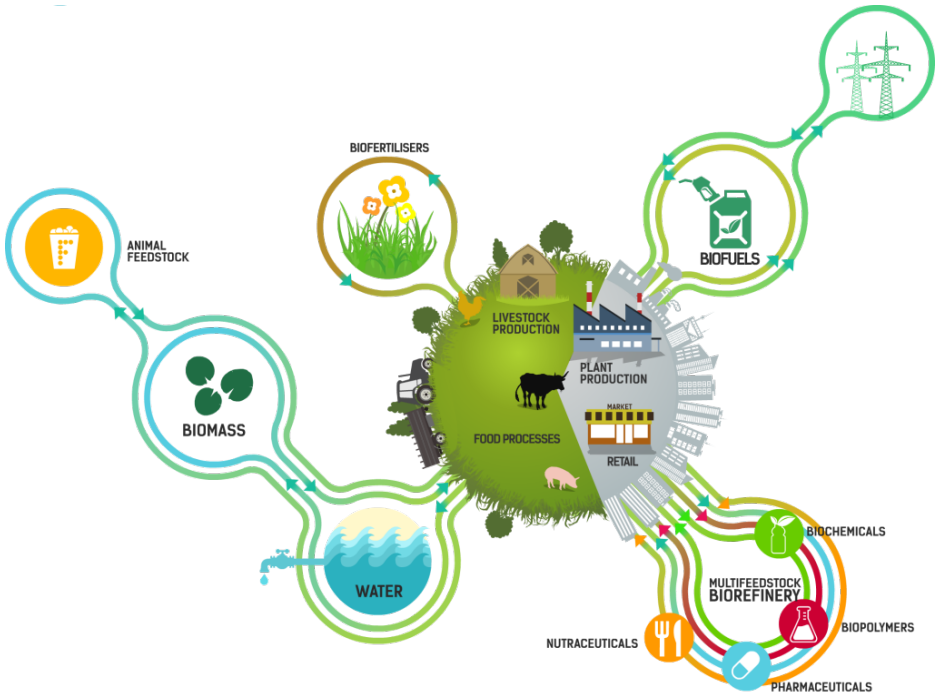

Figure 1.2. Agro-cycle innovations in the agricultural production chain [19].

The diversity of compounds found in food supply chain waste reflects also the variety of sectors in the chemical industry that could benefit from using this renewable feedstock, improving its green prospective. The various sectors of chemical industry where FW could be utilized as a starting material are presented in **Figure 1.3**.

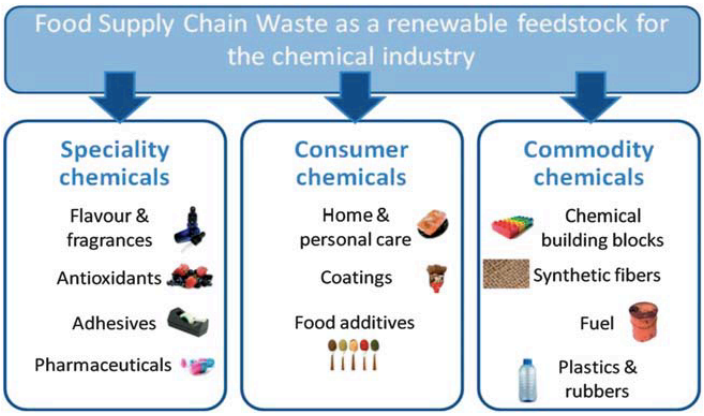

Figure 1.3. Sectors of the chemical industry that could benefit from the exploitation of food waste as feedstock [13].

In order to achieve sustainable and environmentally friendly production of compounds from food waste biomasses, it is fundamental to use also processes and catalytic materials that embrace the core of green chemistry. To this frame, bio-catalysis offers numerous advantages versus the conventional catalytic processes, gaining always more attention from the research community and industry.

Biocatalytic processes are used from humanity since antiquity for the production of food (bread, wine, cheese), even though the discovery of enzymes and their scientific background is very recent. Buchner first reported in 1897 that glucose was fermented by using yeast extracts, which did not contain any living yeast cells but only the catalytic components extracted them. These catalytic active components are called enzymes, coming from the Greek word "ἔνζυμον" meaning literally "in-yeast".

Enzymes are considered sustainable natural catalysts. These biocatalysts are derived from renewable sources and are non-hazardous, non-toxic with high biocompatibility and biodegradability. In addition, enzymes are advantageous compared to the use of precious metal catalysts that sometimes lead to traces of the noble metals requiring expensive removal processes to achieve acceptable ppm levels in end products. Reactions performed by using enzymes also require mild conditions (low temperatures and pressure, physiological pH) which make the overall bioprocess more energetically convenient. Furthermore, the usual protection and deprotection steps that are used in the conventional organic synthesis are not necessary with enzymes, as they already provide high selectivity and specificity. High selective products can be synthesized in a shorter time, less waste is generated, and the overall process is economically as well as environmentally more sustainable [20, 21].

The optimization of the synthetic protocols of the enzymes, dating back to the breakthroughs in DNA sequencing and in recombinant DNA technology, has driven down significantly their end cost and therefore, more enzymes are now commercially available at reasonable prices. The notion that enzymes are expensive catalysts is obsolete and can be applied at industrial level.

There are, however, some issues regarding the use of enzymes that have a severe impact on the biocatalytic process and should be taken into account. Firstly, enzymes can work also in organic media and not only in water. This is very important when either the reactants or the products are not soluble or stable in water. For instance, esterification and amidation reactions cannot be performed in water because are limited by the equilibrium and/or competing product hydrolysis. But the catalytic efficiencies of reactions using enzymes and organic solvents are generally much lower than those

performed in aqueous environment. This means, that a proper choice of the solvent or mixtures of solvents are critical to the reaction.
Secondly, enzymes, and especially natural ones, generally exhibit low reaction rates with non-natural substrates, compared to classical thermo-catalytic processes, as Nature conceive them to work in living organisms. Nonetheless, very good results can be obtained with enzymes in various processes, for example using a lipase, Pfizer developed an effective chemo-enzymatic process to manufacture pregabalin, the active pharmaceutical ingredient of the CNS drug Lyrica® [22].

In the current thesis, enzymatic catalysis is used for the valorization of protein and oil wastes derived from agri-food industry. Protein-rich food wastes (e.g. rice bran) can be used as an alternative source for the preparation of vegetable-derived bioactive peptides. These bioactive peptides are widely used in functional foods and nutraceuticals thanks to their unique functional, sensorial and biological characteristics (antioxidant, antihypertensive and anti-inflammatory properties) [23]. In addition, bio-derived surfactants from sugars, amino acids, fatty acids, hydroxy acids and lipids can all be derived from food supply chain waste including agricultural residues and food processing waste. Some commercialized surfactants (e.g. pentose sugars with fatty alcohols) are already derived from agricultural residues such as wheat bran and straw [24]. Sugar-based surfactants (e.g. alkyl polyglucosides) are also considered renewable surfactants with good detergent properties and low toxicity, compared to traditional fossil oil-based surfactants, and can be derived from starch and vegetable oils [25]. Both bioactive peptides (rice bran protein hydrolysates) and bio-derived surfactants (sugar fatty acid esters) are products that can be prepared from food waste by exploiting the benefits of enzymatic catalysis.

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Chapter 2: Preparation and characterization of hydrolysates from rice bran protein

2.1 Introduction

2.1.1 Rice bran: Definition and composition

The depletion of fossil fuels from one side and the environmental impact of their excessive utilization, in general, has created an urgent need for searching new sustainable ways and renewable sources to produce energy and chemicals. To this aim, the valorization of wastes and byproducts derived from agri-food industry for the production of high-added value chemicals is gaining always more scientific attention.

Rice is a primary staple food for more than half word's population and, according to Food and Agriculture Organization of the United Nations (FAOSTAT), its annual production (Rice paddy) in 2017 reached around 721,4 million metric tons (MMT). In **Figure 2.1**, are presented the top ten countries for the production of rice (paddy) in 2017 (FAOSTAT) worldwide [1]. Considering these numbers, it is understandable that the amount of waste produced during the production process of rice is also very high. For example, each tone of rice contains 70 kg of rice bran, a byproduct derived from the production chain of rice containing highly nutritional proteins. Rice bran, which forms 5% to 8% of the rice grain weight and is currently utilized as livestock feed, could be a sustainable source for the production of high-value products regarding also its great availability [2].

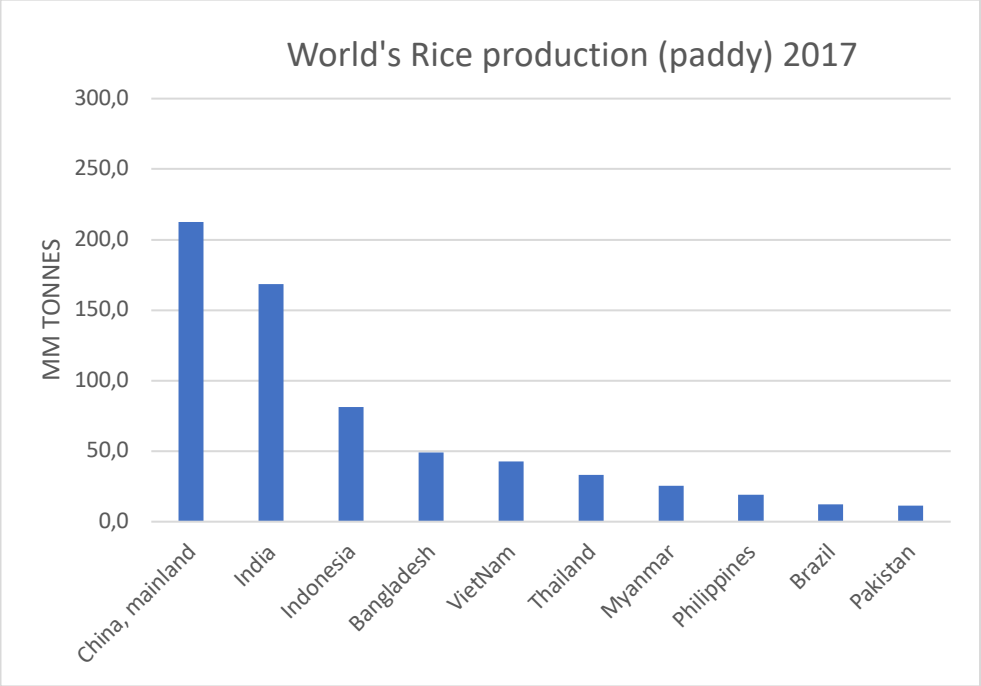

Figure 2.1. Top 10 countries of world's rice production in 2017 (MMT).

Rice undergoes several processing steps such as cleaning, hulling, milling, resulting in the end in undesired products such as husk, germ and bran. The proximate composition of rough rice as well as the composition of its milling fractions are presented in **Table 2.1**.

Table 2.1. Proximate composition of rough rice and its milling fractions at 14% moisture [3, 4, 5].

Rice fraction	**Crude protein** (gN x 5.95)	**Crude fat** (g)	**Crude fibre** (g)	**Crude ash** (g)	**Available carbohydrates** (g)	**Neutral detergent fibre** (g)	**Energy content** (kJ)	**Energy content** (hcal)	**Density** (g/ml)	**Bulk density** (g/ml)
Rough rice	5.8-7.7	1.5-2.3	7.2-10.4	2.9-5.2	64-73	16.4-19.2	1580	378	1.17-1.23	0.56-0.64
Brown rice	7.1-8.3	1.6-2.8	0.6-1.0	1.0-1.5	73-87	2.9-3.9	1520-1 610	363-385	1.31	0.68
Milled rice	6.3-7.1	0.3-0.5	0.2-0.5	0.3-0.8	77-89	0.7-2.3	1460-1 560	349-373	1.44-1.46	0.78-0.85
Rice bran	**11.3-14.9**	**15.0-19.7**	**7.0-11.4**	**6.6-9.9**	**34-62**	**24-29**	**670-1 990**	**399-476**	**1.16-1.29**	**0.20-0.40**
Rice hull	2.0-2.8	0.3-0.8	34.5-45.9	13.2-21.0	22-34	66-74	1110-1 390	265-332	0.67-0.74	0.10-0.16

Bran by definition is the outer covering of grain that is separated when making white flour. Bran coming from wheat or oat is usually added to other foods because of its high fibre content that is needed for a healthy body. In the case of rice, it is not feasible to use directly the bran as a food additive because apart from fibers it contains also lipids that are highly susceptible to oxidation.

In **Figure 2.2** the structure of the rice bran is presented. Rice bran (that appears as a red line) is called the layer between the white rice (*Oryza sativa L.*) and the hull (or husk), which is the external layer of the rice grain [6]. Rice bran is removed during the whitening process of the rice. Together with the husk and the germ, rice bran is considered a byproduct deriving from the rice production chain.

Figure 2.2. Rice bran structure.

Rice bran cannot be used for food consumption due to the fact that rancidity occurs when lipase present in bran is activated, during the milling process, hydrolyzing the glycerides into free fatty acids and glycerol. Stabilization of rice bran is necessary for possible applications in food industries by inactivating the lipase enzyme that makes the bran rancid and inedible.

Though rice bran has the highest protein content respect to the other milling fractions (**Table 2.1**), it is now underutilized as an animal feedstock. Its composition is very interesting from nutritional point of view, as it contains a large quantity of essential nutrients such as minerals, vitamins, fibre, amino acids and antioxidants [7, 8]. The composition of rice bran depends on rice variety, climate and rice processing methods. Rice bran has the potential to be used as a resource for the production of functional foods and feed ingredients. In **Table 2.2** are presented some of the important bioactive compounds that are present in rice bran.

Table 2.2. Bioactive compounds found in rice bran [9].

Steroidal compounds	**Anthocyanins and flavonoids**	**Polymeric carbohydrates**	**Phenolic and cinnamic acids**
Acetylated steryl glucosides	Anthocyanin monomers,	Arabinoxylans	Caffeic acid
Cycloartenol ferulate	dimmers and polymers	Glucans	Coumaric acid
Campesterol ferulate	Apigenin	Hemicellulose	Ferulic acid
24-methylenecycloartenol	Cyanidin glucoside		Gallic acid
ferulate	Cyanidin rutinoside		Catechins
γ-oryzanol	Epicatechins		Hydroxybenzoic acid
β-sitosterol ferulate	Eriodtyol		Methoxycinnamic acid
Tocopherols	Hermnetins		Sinapic acid
Tocotrienol	Hesperetin		Syringic acid
	Isohamnetins		Vanillic acid
	Luteolin		
	Peonidin glucoside		
	Tricin		

2.1.2 Rice bran oil

Rice bran oil (RBO) is an excellent source of commercially important bioactive phytochemicals with antioxidative and chemopreventive properties with various applications in pharmaceuticals, nutrition supplements and cosmetics, such as sterols, tocopherols, tocotrienols and γ-oryzanol, as well as other compounds at lower concentrations, such as lecithin and carotenoids. Also, some researchers reported that RBO has hypolipidemic, antiatherogenic, and antidiabetic properties [10].

The saponifiable lipids present in RBO include 68-71% triglycerides, 2-3% diglycerides, 5-6% monoglycerides, 2-3% free fatty acids (FFAs), 2-3% waxes, 5-7% glycolipids and 3-4%

phospholipids. RBO is composed by 47% monounsaturated, 33% polyunsaturated and 20% saturated fats. The most important unsaturated fatty acids in RBO are oleic acid and linoleic acid and the major saturated fatty acid is palmitic acid [11, 12]. Apart from the great nutritional properties, RBO features also other interesting properties such as good stability, appealing flavor and extending oil frying-life, thus it could be used as an alternative to bakery shortening.

2.1.3 Rice bran proteins

Because of their excellent functional properties, proteins of animal origin have been extensively studied and are commonly used in food systems. Their production though, has a significant environmental impact, as the estimated contribution of meat production to greenhouse gas emission ranges between 10% and 51% [13]. Moreover, the demand for meat products is on the rise. Indeed, according to the United Nations, the world population is expected to increase to over 9 billion by 2050, especially in 3rd world countries where meat consumption is on the rise. Consequently, there is a great interest in plant proteins and their functional characteristics as an alternative to animal protein isolates. Common plant products with high protein content, include soy protein isolates and commercial wheat gluten, a coproduct of commercial starch isolation [14].

Rice bran contains most of the protein of rice grain and is considered highly nutritional with unique functional properties that are comparable to other cereal proteins (e.g. starch). The protein content of rice bran varies from 10 to 16% depending on rice type and production process. Most of the rice bran proteins are storage proteins and can be grouped into four fractions according to their solubility by the Osborne fractionation method, known as albumin (30.9%), globulin (24.9%), prolamin (11.6%) and glutelin (32.5%) [15, 16].

Rice bran proteins have excellent quality: apart from their high nutritional value, they have also optimal digestibility and are rich in essential amino acids; last but not least, they are gluten-free and hypoallergenic. As a result, they have high potential as functional food ingredients, nutritional supplements and flavor enhancers. These proteins could be used in many applications regarding their nutritional value as well as their anti-inflammatory and hypoallergenic behavior. Numerous factors such as structural complexity, poor solubility, strong aggregation as well as the difficulty of separation from the other components of the vegetable material, make rice bran proteins hardly available, consequently limiting a possible industrial application.

In order to separate the proteins from the complex nature of rice bran and improve their biological and functional characteristics, it is necessary to hydrolyze them into smaller peptides. It is reported that protein hydrolysates of rice bran containing peptides with two to ten amino acid residues have

good antioxidative potential, as they were able to inhibit the oxidation of biological macromolecules by scavenging free radicals *in vivo* [17]. In addition, rice bran protein hydrolysates were found to have interesting sensorial characteristics and beneficial functions to health [18]. Consequently, they could be utilized as nutraceutical supplements in several foods such as sports nutrition, beverages etc.

2.1.4 Extraction methods – the enzymatic approach

Proteins in rice bran are usually aggregated or linked to other components like cell wall material or starch. Also, the high content of the rice bran in fibers (12%) and phytate (1.7%) make difficult the separation of these proteins from the other components present in rice bran [19, 20]. In fact, the poor solubility of rice bran proteins is caused by their strong aggregation and extensive disulfide bond cross-linking. In order to improve the functional properties of these proteins (such as emulsification, foaming capacity and gel forming ability), it is necessary to hydrolyze them into smaller peptides obtaining hydrolysates that have numerous applications as food additives and nutraceuticals. Several methods have been used in order to extract the rice bran proteins and consequently increase their solubility, such as alkaline extraction, colloid milling, microwave and ultrasonic-assisted extraction as well as enzymatic extraction (**Table 2.3**).

Protein hydrolysis consists of the cleavage of peptide bonds to give peptides of varying sizes and free amino acids. Such a process is performed industrially by the action of an acid or base, or by enzymatic processes [21, 22]. The conventional acid hydrolysis method is based on sample treatment in excessively acidic solutions accompanied by high temperatures. It is a low cost, quick and simple operation, which makes it applicable at the industrial level. However, some essential amino acids are destroyed, and asparagine and glutamine are converted into aspartic acid and glutamic acid, respectively. Additionally, obtained hydrolysates have poor functional properties due to the formation of salts after the neutralization process [23, 22].

Alkaline hydrolysis is a very simple process: the sample is solubilized by heating and mixed with alkaline solutions until reaching the desired degree of hydrolysis (DH), i.e. the desired percentage of cleaved peptide bonds, a parameter used for monitoring the reaction progress. The main drawback of alkaline hydrolysis is the production of hydrolysates with low amino acids content. [23, 22].

Alkaline extraction is a common method used to dissolve the soluble rice proteins in dilute alkaline solution. During this process, some of the hydrogen, amide, and disulfide bonds in rice glutelin are broken leading to the decreasing of the molecular size of protein and consequently improving the protein extraction [24]. However, alkaline conditions could have a negative effect on the nutritional properties of the derived products because under these conditions maillard reactions are promoted

that lead to dark color products and also the extraction of other components present in rice bran is increased (by co-precipitation with protein), lowering the quality of the final hydrolysate [20].

A system of using multiple solvents for the extraction of rice bran proteins has also been suggested in the literature. Proteins in rice bran are consist of water-soluble, salt-soluble, alcohol-soluble, and alkali-soluble proteins. Hamada and Abediyi et al. used sequential extraction of rice bran proteins with water, NaCl solution, 60% ethanol and 0.1M NaOH and both reported that the resulted protein yield was higher that 90% [25, 26]. However, the number of steps in a multiple-step process is crucial and should be minimized since the overall yield can be considerably reduced even if individual steps have high yields [27]. In industrial scale, the use of multiple steps for extraction could result in a loss of product per step, that consequently will lower the yield and the efficiency of the overall production process.

Colloid milling, sonication, high-speed blending and homogenization are some of the physical methods used to extract proteins. These physical processes are releasing proteins by promoting cell disruption and subsequently enhancing their extraction. Sonication is a promising technique for rice bran protein extraction, though the yields are relatively low even if a combination of this method with enzymatic treatment is used, as recently has been revealed by Tang et al. (15% rice bran protein) [28]. A longer period of sonication may be required to improve the yields [15].

Table 2.3. Protein extraction of rice bran by using different methods and conditions (adopted by Phongthai et al.) [16].

Methods/ Raw Materials	Conditions	Protein recovery/ yield %
Alkaline extraction		
Full fat rice bran	pH 9.0 at 24 °C	14-20
Defatted rice bran	pH 2.0-10.0 at extraction time of 32 to 58 min	1.4-12.2
Defatted rice bran	pH 8.0, extraction temperature of 63 °C for 340 min	31.3
Defatted rice bran	pH 9.0, stir at room temperature for 2 h	37.6-46.2
Rice bran	pH 9.5, shake at 50 °C for 2 h	32.9
Broken rice	0.1 % NaOH solution, stir at room temperature for 1 h and then left overnight	77.3
Rice bran	pH 11.0, solid/liquid ratio of 1:7.5 (w/v), stir for 1 h	25
Physical Extraction		
Defatted rice bran	Blending + alcalase (enzyme)	81
Defatted rice bran	Freezing for 16 h + thawing	12-56
Rice bran	Blending + amylase & protease	66
	High pressure + amylase & protease	67
	Colloid milling + homogenizing	37.8-67.5
Broken rice	Colloid milling	63.8
Enzymatic Extraction		
Defatted rice bran	Phytase & xylanase, stir at 55 °C for 2 h	34.0-74.6
Defatted rice bran	Amylase, ratio of rice bran to water 1: 17 at 50 °C	58.4
Defatted rice bran	0.1 % (w/w) papain & 5 % viscozyme, at 37 °C for 1 h	54.0-82.6
Rice bran	Cellulase & hemicellulase, pH 4 at 65 °C	35-46
Rice bran	0.5 % (w/w) a-amylase & viscozyme, pH 4.1-6.25, stir for 1h	30-35
Novel technology techniques		
Defatted rice bran	Ultrasonic at 100 W for 5 min, pH 11.0	~25.9/4.5
Defatted rice bran meal	Microwave at 800 W for 20-90 min, pH 8.0	67-70
Defatted wheat gem	Ultrasonic at 363 W for 24 min	37-57

Enzyme–aided valorization of plant waste (like rice bran) or residues from vegetable oil production represents an example of biorefinery of protein-rich biomass and fulfill the requirements of "circular economy" where waste is managed sustainably by turning it into a resource. Unlike chemical methods, enzymatic hydrolysis uses mild conditions and is easy to control. The process is usually performed in a reactor with temperature, pH, agitation and time controls, being the temperature and pH adjusted according to the ideal working conditions of the enzyme. Additionally, enzymes present substrate specificity, i.e. it is possible to predict which peptides will be obtained, thus allowing the process to be standardized; furthermore, there are no side reactions or decrease in the nutritional value of the protein source [29, 22].

Enzymatic extraction is an alternative and environmental-friendly method for the extraction of rice bran protein, as it requires milder process conditions at neutral or slightly basic pH. In addition, procedures involving enzymes are very appealing because they provide easier control of the reaction parameters and minimize the formation of secondary products [30]. Also, the nutritional value of the derived protein hydrolysates is not affected negatively by the enzymatic process, but, on the contrary, their physicochemical and functional characteristics are improved.

Proteolytic modification is an efficient approach to improve the functional properties of proteins such as solubility and viscosity, as well as to reduce their allergenic potential. Enzymatic hydrolysis is the preferred method, as acid and alkaline hydrolysis methods tend to be difficult to control and can yield variable products with reduced protein quality and biological value [31]. On this ground, preparation of protein hydrolysates through enzyme-catalyzed hydrolysis using defatted rice bran as starting material is a convenient way for their valorization.

Carbohydrases are a class of hydrolytic enzymes that catalyze the hydrolysis of carbohydrates. Particularly, carbohydrases named as cellulase (EC 3.2.1.4) and xylanase (EC 3.2.1.8) degrade the cell walls of plant biomass substrates that consist mainly of cellulose and xylans. Some recent studies suggest that the use of these carbohydrases may increase the protein yield of rice bran as they releasing more protein from the polysaccharide matrix of rice bran [15, 24]. Other enzymes, such as α-amylase (EC 3.2.1.1) and phytase (EC 3.1.3) could also enhance the protein extraction by attacking and breaking the interaction of proteins with starch and phytate respectively, in the rice bran structure. Specifically, carbohydrases catalyze the hydrolysis of glycosidic bonds. For example, endo-1,4-xylanases hydrolyze internal β-1,4 bonds of xylans to give xylose monomers, endo-1,3(4)-β-glucanases catalyze the hydrolysis of internal 1,3 and 1,4 bonds of β-glucans (including cellulose) and a-amylases split the internal α-1,4-glucosidic bonds and are therefore aimed at the degradation of starches. In addition, cellulases are specialized in the hydrolysis of α-1,4-glucosidic bonds of cellulose and hemicellulases are able to hydrolyze hemicelluloses, which are polymers consisting of different monomer units (**Figure 2.3**).

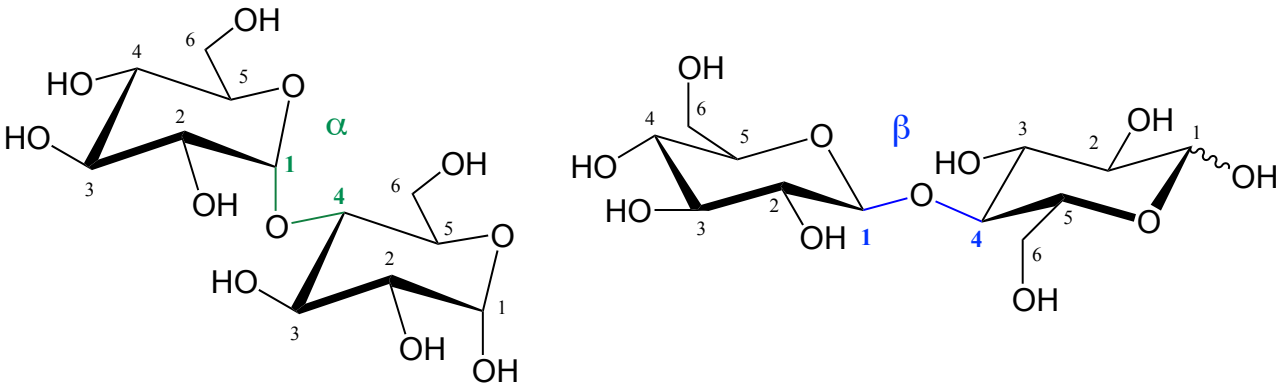

Figure 2.3. Examples of α-1,4-glucosidic (mannose) and β-1,4-glucosidic bonds (cellobiose).

For the hydrolysis of protein fraction of rice bran and the preparation of hydrolysates with low molecular weight peptides, proteolytic enzymes are required. The proteases are the class of hydrolytic enzymes that catalyze the hydrolysis of the peptide bond. They are divided into endopeptidases and exopeptidases based on the position of the hydrolyzed peptide bond. Endopeptidases are digesting

internal peptide bonds whereas exopeptidases liberate amino acids by hydrolyzing N-terminal peptide bonds.

2.1.5 ACE inhibitory peptides

Biologically active peptides are defined as specific protein fragments that have positive effects on body functions or conditions and may influence human health [32, 33]. Food-derived bioactive peptides correspond to cryptic sequences from parent protein (also known as "cryptides" [34]), which render them inactive and need to be released by hydrolytic reactions in order to perform their specific roles. Depending on their amino acid sequence, these peptides can accomplish wide-range of activities (**Figure 2.4**), including antioxidant, cholesterol lowering capacity, antimicrobial, antithrombotic, antiobesity and antidiabetics, blood pressure-lowerings etc. [35]. Protein hydrolysates are mixtures of polypeptides, oligopeptides and amino acids manufactured from protein sources using partial hydrolysis. There has been growing interest in these preparations over the last two decades, with novel bioactive peptides continually being discovered [32].

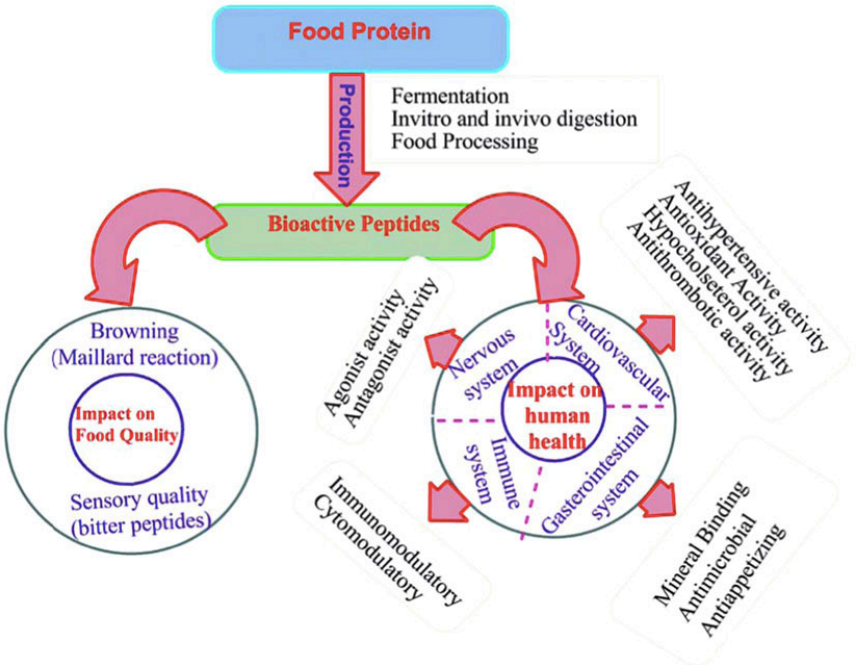

Figure 2.4. Production and wide range of activities exhibited by bioactive peptides (adopted by Admassu et al.) [35].

During the last years, many studies suggested that several peptides derived from plant and animal proteins exert antihypertensive properties. Hypertension is a chronic disease that is usually stated as excessive high blood pressure (systolic and diastolic values ≥ 140 mm Hg and ≥ 90 mm Hg, respectively), which is characterized by insufficient relaxation of blood vessels and reduced blood flow.

The elevated blood pressure is also a high-risk factor for stroke, arteriosclerosis and myocardial infarction and it can be treated by ACE inhibitors [36]. ACE stands for "Angiotensin Converting Enzyme", an enzyme that catalyzes the reaction of Angiotensin I to Angiotensin II. ACE is a dipeptidyl carboxypeptidase (EC. 3.4.15.1) and was originally isolated from horse blood [37]. Angiotensin II is a compound responsible for the enhancement of the blood pressure caused by narrowing the blood vessels [18]. ACE-inhibitory peptides bind tightly to the ACE active site, competing with angiotensin I for occupancy, and inactivate ACE, preventing eventually blood pressure enhancement (**Figure 2.5**).

Captopril, enalapril and lisinopril are some of the known commercially synthesized ACE inhibitors used to treat hypertension. These synthetic drugs, however, are accompanied with several adverse effects such as coughing, taste disturbances, and skin rashes [38]. Peptides derived from food proteins are known to exhibit ACE inhibitory activity and are considered to be milder and safer as compared to synthetic drugs, with minimal side effects and therefore safer to use [39, 19]. Moreover, these peptides are multifunctional and can easily absorbed in the gastrointestinal tract [40, 41]. Recently, Wang et. al. prepared hydrolyzed proteins of rice bran with molecular weight <4kDa by using Trypsin and demonstrated that these hydrolysates exhibited good ACE-inhibitory activity with a relatively low IC_{50} value (300 mg/mL) [42].

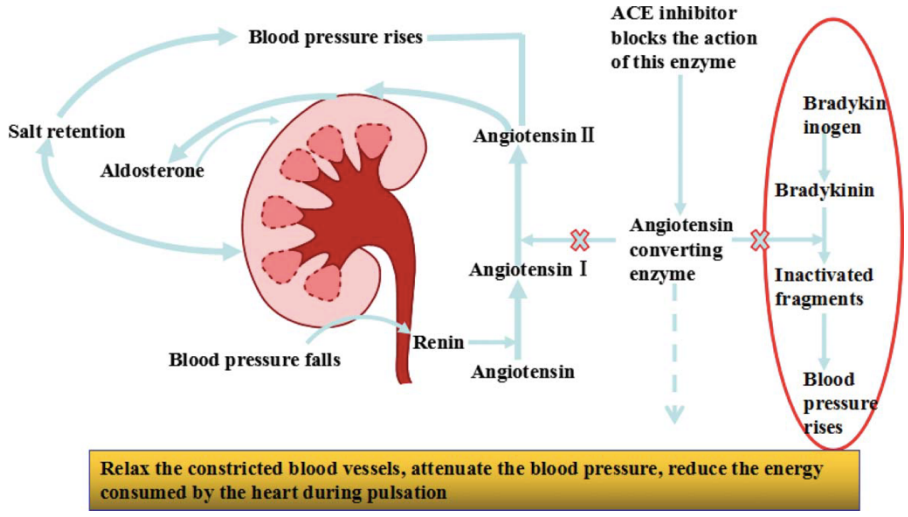

Figure 2.5. Proposed anti-hypertensive properties of peptides from rice bran protein [18].

2.1.6 Taste active peptides

Umami taste has long been apprehended in many traditional foods including soy sauce, tomatoes, fish and fish sauces, hydrolyzed vegetable protein, cheeses and fermented Asian foods, although this taste quality was officially recognized only some time ago.

The word “umami” came from a Japanese word (うま味) which means a “pleasant savory taste”, “mouthfulness” or “delicious”. In 2002, umami was recognized as the fifth basic taste (together with salty, sweet, sour and bitter) to describe a pleasant savory or MSG (monosodium glutamate)-like taste [43]. Synergism and interactions with other tastes (e.g. bitterness suppression), are two important characteristics of umami. Umami substances are very important for food seasoning and healthy eating. In addition to MSG, various substances were found to be responsible for umami taste including some free L-amino acids, bi-functional acids, peptides and their derivatives [44].

Purine 5’-ribonucleotides such as guanosine 5’- monophosphate (GMP), inosine 5’-monophosphate (IMP), and adenosine 5’-monophosphate (AMP) were found to induce an umami taste sensation, with recognition thresholds of 30 and 5 $mmol \cdot kg^{-1}$ for GMP and IMP, respectively. Apart from l-glutamate and ribonucleotides, additional umami-tasting molecules have been identified in various natural sources. Analysis of the peptides from vegetable protein hydrolysates revealed pyroglutamyl peptides such as pGlu-Pro-Ser, pGlu-Pro, pGlu-Pro-Glu, and pGlu-Pro-Gln as important umami-like molecules [45].

In the last years, fifty-two peptides have been reported to show umami taste, including twenty-four dipeptides, sixteen tripeptides, five octapeptides, two pentapeptides, two hexapeptides, one tetrapeptide, one heptapeptide, and one undecapeptide [46]. Particularly, certain peptides and some derivatives are essential for achieving superior products, thus the knowledge of the structural and physicochemical characteristics of these tastants is important for their utilization in food industry [44].

Enzymatic hydrolysis is an efficient process to extract proteins from food waste (e.g. meat by-products, residues from vegetable oil production, etc.) and obtain high value-added ingredients, e.g. protein hydrolysates. However, a major challenge that may affect the sensorial characteristics of protein hydrolysate-based products is the bitter and unpalatable taste. Consequently, it is mandatory to limit the bitter taste of protein hydrolysates for further application as protein ingredients [47].

Bitterness increases as the length of the peptides are elongated, because the extended peptide chain length may increase interactions with bitter receptors [48]. The degree of hydrolysis (DH) influences also bitterness. DH is defined as the percentage of peptide bonds cleaved during enzymatic hydrolysis, which is a predictor of reduced average molecular weight (MW) of peptides. The intensity of bitterness is higher when DH values are very low. As proteolysis proceeds, more hydrophobic amino acids are exposed, leading to increased bitterness. With extensive hydrolysis (high DH values), bitter peptides can be further degraded into smaller peptides or free amino acids, resulting in reduced bitterness. Proteolytic enzymes used to prepare hydrolysates can also influence bitterness due to different peptide profiles caused by their different specificities.

A promising method to remove bitterness from protein hydrolysates was found to be the enzymatic hydrolysis by using exopeptidases, including amino- and carboxy-peptidases. Exopeptidases can selectively break peptide bonds at the N- or C-terminals of bitter peptides, releasing free hydrophobic amino acids and further reducing bitter taste [49]. Sequential hydrolysis by endo- and exo-peptidase has been shown to reduce the bitterness of wheat gluten hydrolysates. Cheung et al. also reported recently that exopeptidase treatment of whey protein hydrolysates decreased bitterness and increased the umami taste [50].

2.2 Experimental

2.2.1 Materials and Methods

The Defatted rice bran (DRB) used for the experiments was provided by SCITEC-CNR. The lipids were removed from the rice bran by Soxhlet extraction in hexane for 4 h. The enzymes α-Amylase (derived from *Aspergillus oryzae*, activity 50 U/mg) and amyloglucosidase (derived from *Aspergillus oryzae*, activity 70 U/mg) were purchased from Sigma-Aldrich (Milano, Italy). All the other enzymes were kindly provided by Novozymes® (Bagsværd, Denmark). Specifically, the following carbohydrases were used: Viscozyme® (endo-β-glucanases derived from *Aspergillus aculeatus*, activity 100 FBG/g; FBG= Fungal Beta-Glucanase), Ultraflo® (endo-β-glucanases derived from *Humicola insolens*, activity 45 FBG/g), Celluclast® (cellulase derived from *Trichoderma reesei*, activity 700 EGU/g; EGU= Endo-Glucanase Unit) and Ceremix Plus® (mixture of enzymes produced by submerged fermentation of several organisms: *Humicola insolens*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*). Also, proteases used were Alcalase® (derived from *Bacillus licheniformis*) and Flavourzyme® (derived from *Aspergillus oryzae*). All the other reagents have been purchased by Sigma-Aldrich.

Thin Layer Chromatography (TLC) analysis was performed on silica-gel F_{254} precoated aluminum sheets (0.2 mm layer, Merck, Darmstadt, Germany).

^{1}H and ^{13}C spectra were recorded at 400.13 and 100.61 Hz, respectively, on a Bruker AVANCE 400 spectrometer equipped with a TOPSPIN software package (Bruker, Karlsruhe, Germany) at 300 K. ^{1}H and ^{13}C chemical shifts (δ) are given in parts per million. The ^{13}C NMR signal multiplicities were based on APT (attached proton test) spectra. The ^{13}C NMR signals were assigned with the aid of ^{1}H-^{13}C correlation experiments (heteronuclear multiple quantum correlation spectroscopy, HMQC, and heteronuclear multiple bond correlation spectroscopy, HMBC).

Electrospray ionization mass spectra (ESI-MS) were recorded on a ThermoFinnigan LCQ Advantage spectrometer (Hemel Hempstead, Hertfordshire, United Kingdom). Matrix Assisted Laser Desorption Ionization Time-of-Flight mass spectra (MALDI-Tof) were recorded on an OmniFLEX (Bruker Daltonics, Germany).

A Lyophilizer Lio 5P was used for freeze drying.

2.2.2 Pretreatment of DRB

A pretreatment of DRB was used for a set of experiments (P3 and P4) in order to remove the water-soluble polysaccharides and enhance the accessibility of carbohydrases to the cell walls of rice bran. As a "pretreatment" procedure is considered the suspension in water of the rice bran at a fixed temperature and for a specific time. This step allows the extraction of the water-soluble components from the DRB without the use of enzymes but only due to hydrothermal treatment. The procedure is as follows: 30 g of DRB are suspended in 300 mL of distilled H_2O and heated at 80 °C in an oil bath (Silicon oil) for 24 h under vigorous magnetic stirring (500 rpm). The derived mixture is separated by centrifugation (15 min, 4000 rpm). The solid fraction (PT-DRB) is dried at 60 °C for 10 h, while the liquid fraction is lyophilized and preserved in the freezer for analysis (^{1}H NMR) in order to determine the derived water-soluble sugars.

Most of the experiments (P1, P2, P5, P6, and HC1 – HC12) were carried out without any pretreatment.

2.2.3 Composition of polysaccharides of DRB

With the aim to study the composition of the polysaccharides present in DRB, the next experimental procedure was followed. At the first place, DRB was treated with the mixture of two enzymes, α-amylase and amyloglucosidase in order to remove starch present in rice bran as an alternative method to carbohydrase-aided protein extraction. Typically, 10 g of DRB were suspended in deionized water (10% wt. DRB/H_2O) and the resulting slurry was kept under magnetic stirring at 45 °C. The pH was adjusted to 4.5 by using 1 M solution of citric acid. The reaction was started by adding α-amylase and amyloglucosidase (0.5% w/w DRB) in the reaction mixture. After 24 h, the reaction was terminated by inactivating the enzymes at 100 °C for 15 min. Then, the insoluble residue was isolated by centrifugation (8000 rpm, 10 min) followed by a filtration step on a Buchner funnel equipped with a filter paper (Whatman® quantitative, ashless, grade 41, Merck). The solid residue was washed thoroughly with abundant deionized water, filtered under reduced pressure and then dried in the oven at 65-68 °C, until a constant weight was reached and milled to a fine powder. The supernatant (I) and the washings were collected, concentrated under reduced pressure, freeze dried and analyzed by ^1H NMR (D_2O, 400 MHz) and mass spectrometry.

The obtained de-starched solid residue (2 g) was suspended in deionized water (10% wt. DRB/H_2O) and the slurry was kept under magnetic stirring at 80-85 °C for 24 h and worked-up as previously described (2.2.2. pretreatment of DRB). The supernatant (II) was recovered, freeze-dried and analyzed by ^1H NMR (D_2O, 400 MHz). The analytical data confirmed the presence of a mixture of complex carbohydrates.

Finally, a sample from the supernatant (II) was submitted to chemical hydrolysis in order to characterize the sugar composition. 20 mg of the lyophilized supernatant (II) was placed in a sealed test tube and treated with 2 M trifluoroacetic acid (4 mL, 1:20 w/v). The reaction was heated at 120-125 °C for 2 h and then cooled to room temperature. The crude mixture was transferred into a round-bottom flask and dried under reduced pressure. The residue was solubilized in deionized water, freeze dried and analyzed by TLC, ^1H NMR and ^{13}C NMR (D_2O, 400 MHz). TLC was revealed by using an ethanolic solution of 1,3-dihydroxynaphthalene (0.2 g/100 mL) containing H_2SO_4 (20% v/v) and heating the TLC at 110 °C.

2.2.4 Study of several Carbohydrases for the hydrolysis of DRB Carbohydrates

A series of different carbohydrases and combinations of them (**Table 2.4**) were studied for the hydrolysis of the rice bran polysaccharides. In a typical experiment, 3g of DRB was suspended in 30 ml distilled H_2O in a 2-neck glass reactor equipped with a mercury thermometer and placed in an oil-bath. The enzymatic reactions were adjusted at the suggested optimal pH for each enzyme using 0.1 M HCl and was maintained at the optimal temperature under magnetic stirring. The hydrolysis reaction was started up by adding the enzyme to the temperature-equilibrated mixture. After 4 h, the reaction was terminated by inactivating the enzymes at 100 °C for 15 min. The final mixture was then centrifuged in order to separate the soluble carbohydrates (liquid fraction) from the solid fraction. The DRB so enriched in proteins (DRBP) was dried at 130 °C for 30 min before the next usage.

Table 2.4. Temperature and pH for each enzyme used in enzymatic hydrolysis of DRB carbohydrates (should be in the same order as the graph)

Entry	Enzyme mixtures	T (°C)	pH
HC-1	Ceremix	50	6
HC-2	Viscozyme	50	5
HC-3	Ultraflo	60	4.5
HC-4	Celluclast	45	5
HC-5	Ceremix + Celluclast	47	5.5
HC-6	Ceremix + Viscozyme	50	6
HC-7	Ceremix + Ultraflo	55	5.5
HC-8	Ceremix + Viscozyme + Celluclast	50	5.5
HC-9	Viscozyme + Celluclast	47	5
HC-10	Viscozyme + Ultraflo	55	4.5
HC-11	Celluclast + Ultraflo	55	5
HC-12	Ceremix + Celluclast + solid fitase*	47	5.5

2.2.5 Carbohydrate hydrolysis of DRB

As a first step, DRB has been treated with carbohydrases in order to hydrolyze the rice bran carbohydrates and enrich the remaining DRB solid in proteins. Deriving from the previous study of different carbohydrases, it was chosen the combination of two enzymes, specifically Ceremix and Celluclast, as the mixture of enzymes for the first step of DRB carbohydrate hydrolysis. In addition, for comparison some samples were prepared by using the enzyme Viscozyme for this step. It should be mentioned that RB has been used after the pretreatment with water or directly treated with the carbohydrases in order to study the influence of the water-soluble compounds to the final products and also to investigate what kind of compounds can be removed only by using heat and water. It is also important here the fact that this pretreatment step it is expected to help the carbohydrases by making more accessible the structure of RB.

The enzymatic reactions are performed in glass reactor, where 30 g of DRB are added together with deionized water (10% wt. DRB/H_2O). The glass reactor is heated in an oil bath (Silica oil, AP 100, Aldrich) and the temperature is controlled by mercury thermometer. The temperature is set at 47-50^oC and pH is adjusted by hydrochloric acid (HCl) solution 0.1M (pH 5.5-6). The enzymes studied for the carbohydrate hydrolysis are Viscozyme, used in 5% w/w loading with respect to DRB and the combination of 2 other carbohydrases, named Ceremix and Celluclast, used in 5% w/w loading with respect to DRB. The enzymes are charged to the reactor and the reaction is left for 4 hours. In order to inactivate the enzymes, the temperature is increased at 80-100^oC for 15-20 min. After the reaction, the liquid fraction that contains the soluble RB carbohydrates is separated by centrifugation and lyophilized. The remaining solid that is now enriched in proteins (DRBP) is dried at 60^oC for 4 h before the next usage.

2.2.6 Protein hydrolysis of DRB protein-enriched residue

The remaining dried biomass of rice bran from the previous step is now called Defatted rice bran enriched in proteins (DRBP) because it is the solid fraction separated by filtration after the treatment with carbohydrases, enzymes that hydrolyzed the rice bran sugars and resulted consequently in the enrichment of rice bran in proteins. In this step, the scope is to solubilize the rice bran proteins and prepare protein hydrolysates that have low molecular weight. In order to achieve that, two proteolytic enzymes were chosen, namely Flavourzyme and Alcalase. In order to study the influence of the proteolytic enzymes to the final product properties two different paths were chosen by using: (a) only Flavourzyme or (b) the combination of Alcalase and Flavourzyme.

The solid fractions derived from the previous step of carbohydrate hydrolysis of DRB with Viscozyme (DRBP-V) and the combination of Ceremix and Celluclast (DRBP-CC) were used for the preparation of protein hydrolysates. The reactions were carried out in a 2-neck glass reactor equipped with a mercury thermometer. Firstly, 2-40 g of DRBP-V or DRBP-CC were suspended in distilled H_2O (10 wt.% DRBP/H_2O), and the mixture was heated at 50 °C under magnetic stirring. The hydrolysis reactions were started up by adding the first enzyme (Alcalase at pH = 8-9 or Flavourzyme at pH = 6-7) to the temperature-equilibrated mixture at 1 wt.% loading enzyme/DRBP. The next enzyme (Flavourzyme at pH = 6-7) was added after 2 h (1 wt.% loading enzyme/DRBP), and the reaction were carried out for a total of 24 h (**Figure 2.6**). The enzymatic reactions were adjusted at the suggested optimal pH for each enzyme using 0.1 M HCl or 0.1M NaOH. The reactions were terminated by inactivating the enzymes at 100 °C for 15 min. The final mixtures were then centrifuged at 4000 rpm for 15-30 min in order to separate the protein hydrolysates (liquid fraction). The so-derived liquid fractions were lyophilized obtaining light-brown crystals. The solid fractions (remaining DRBP) were dried at 60 °C for 10 h to calculate the weight-loss percentage.

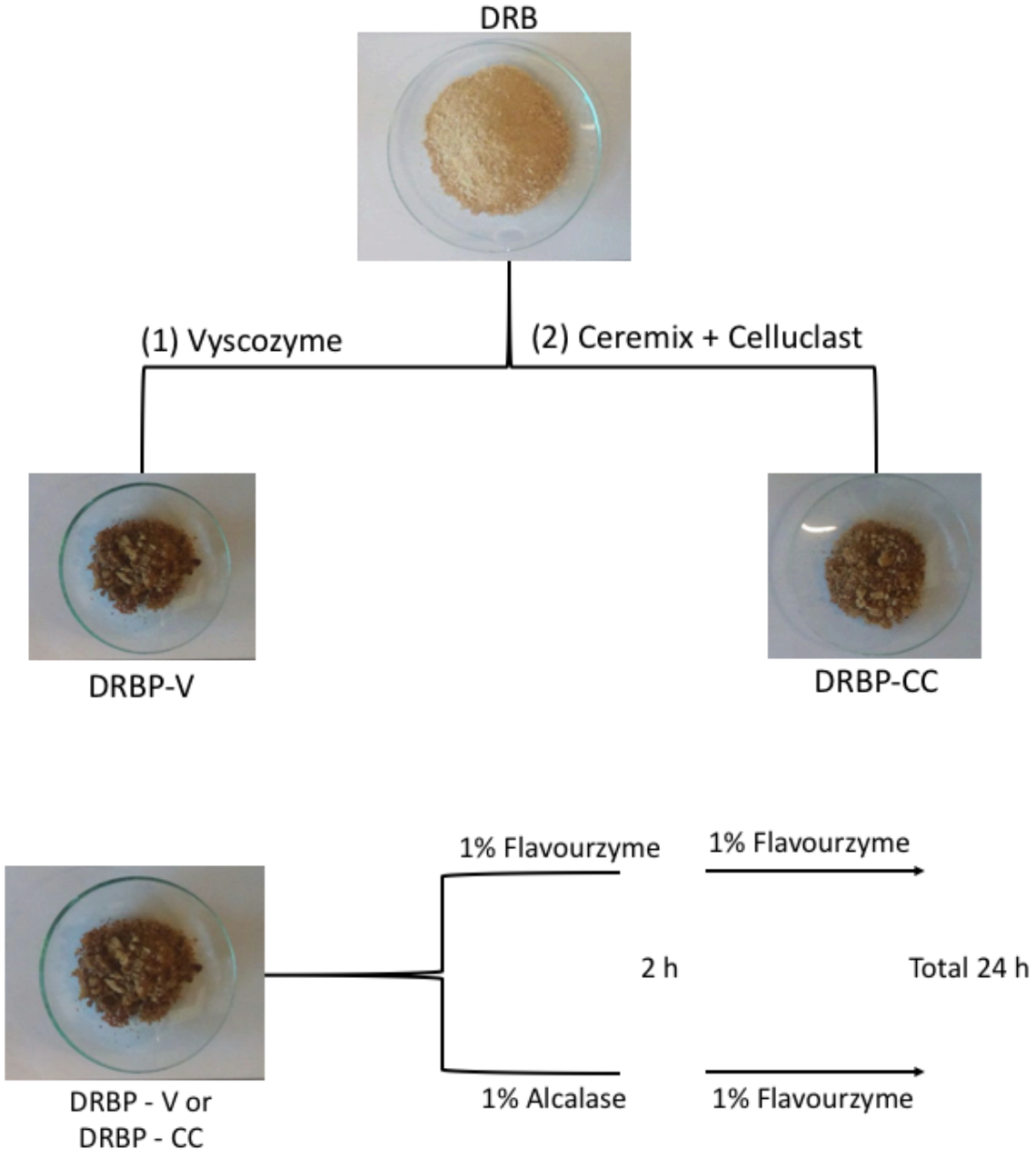

Figure 2.6. Schematic representation of the 2 different reaction paths followed for hydrolysis of DRBP using: (a) 2% Flavourzyme (b) 1% Alcalase and 1% Flavourzyme.

2.2.7 Ultrafiltration

The protein hydrolysates have been separated according to their molecular weight by using the ultrafiltration technique. Ultrafiltration is a kind of separation technique, a filtration method, where membranes are used instead of filters. This separation was necessary for preparing samples with low molecular weight that are easier characterized and analyzed.

Ultrafiltration was performed by using stirred ultrafiltration cell (model 8400 of 400 mL capacity and model 8050 of 50 mL capacity) purchased by the Merck Millipore Corporation. Ultrafiltration membranes were made of regenerated cellulose with molecular weight cut-off (MWCO) of 10 and 5 kDa. Each sample (P1, P2, P3, P4, P5, P6, P7, P8) was solubilized in milli-Q H_2O before transferred to the ultrafiltration unit. Typically, 3 g of samples were solubilized in 300 ml milli-Q H_2O using an Ultrasonic bath (UV power: 80 %, 25^oC) for approximately 15 min. Ultrafiltration was carried out at room temperature under nitrogen (N_2) gas pressure (max. operating pressure 75 psi) and magnetic stirring (200 rpm). The derived ultra-filtrated samples under the MWCO of the membrane were lyophilized and preserved for analysis. Hydrolysates above the MWCO of the membrane were also recovered from the cell and lyophilized.

2.2.8 Characterization techniques

I. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE)

The Electrophoresis system used in this work was a XCell SureLock™ MiniCell purchased by Invitrogen (Thermo Fisher). Also, Novex 16% Tricine Gels and Novex Tricine SDS Running Buffer and Sample buffer were purchased by the same company. For the Gel staining, a Silver Staining Kit (containing Developer, Stopper, Sensitizer, Developer Enhancer and Stainer), provided by SilverQuest™, was used. As a fixative solution 5 vol.% glutaraldehyde and 50 vol.% methanol in MilliQ water was prepared.

Sample preparation: Each sample was dissolved in MilliQ water. The final concentration of the samples is presented in the following table (**Table 2.5**). The samples for electrophoresis were prepared as follows: 20 μL of sample and 20 μL of Novex Tricine SDS Sample Buffer (2x) are added to Eppendorfs and the samples are heated at 80 ºC for 5 min in a water bath.

Table 2.5. Samples prepared for SDS-PAGE and their final concentration.

Sample	**Sample mass**	**MilliQ H_2O volume**	**Final concentration**
*P1 < 5 kDa**	21 mg	500 μL	42 mg/mL
*P1 < 10 kDa**	26 mg	500 μL	52 mg/mL
P2 < 5 kDa	23 mg	500 μL	46 mg/mL
P2 < 10 kDa	21 mg	500 μL	42 mg/mL
P4 < 5 kDa	30 mg	1 mL	30 mg/mL
P4 < 10 kDa	25 mg	500 μL	50 mg/mL
P3 < 5 kDa	22 mg	500 μL	44 mg/mL
P3 < 10 kDa	21 mg	500 μL	42 mg/mL

1L of running buffer solution was prepared using 900 mL of MilliQ water and 100 mL Novex Tricine SDS Running Buffer (10x). After inserting the gel in the chamber of SDS-PAGE unit, all the parts of the chamber are filled with the running buffer. 20 μL of each sample were loaded in the gel according to **Table 2.6**. The electrophoresis run was performed at 120 V for 2 hours, then the gel was removed and stained.

Table 2.6. SDS-PAGE sample loading

Gel well	Sample	Loaded mass	Sample Information
1	P1 < 5 kDa	420 µg	No PT – Flav
2	Empty	520 µg	-
3	P1 < 10 kDa	460 µg	No PT – Flav
4	P2 < 5 kDa	420 µg	No PT – Alc + Flav
5	P2 < 10 kDa	300 µg	No PT – Alc + Flav
6	MARKER	500 µg	(molecular weights are expressed in kDa)
7	Empty	440 µg	-
8	P4 < 5 kDa	420 µg	PT – Alc + Flav
9	P4 < 10 kDa	420 µg	PT – Alc + Flav
10	Empty	520 µg	-
11	P3 < 5 kDa	460 µg	PT – Flav
12	P3 < 10 kDa	420 µg	PT – Flav

Gel Staining procedure: The gel was stained using Silver staining technique. It was left in the Fixative solution for one hour in agitation. During this time, silver staining solutions were prepared following the SilverQuest™ Silver Staining Kit instruction:

- **Sensitizing solution**: 30 mL EtOH + 10 mL Sensitizer + 60 mL MilliQ H_2O
- **Staining solution**: 99 mL MilliQ H_2O + 1 mL Stainer
- **Developing solution**: 90 mL MilliQ H_2O + 10 mL Developer + 1 drop Developer Enhancer

After the fixing, the gel was soaked in 30% EtOH for 10 minutes. EtOH was removed and the Sensitizing solution was added for 10 minutes. The Sensitizing solution was removed and EtOH was added for 10 minutes. The EtOH was removed and MilliQ water was added for 10 minutes. The MilliQ water was removed and the Staining solution was added for 15 minutes. The gel was washed using MilliQ water for less than a minute. MilliQ water was removed and developing solution was added until the bands were clearly visible. In the end, 10 mL of Stopper was added directly on the top of the gel to stop the development reaction.

II. Gel Permeation Chromatography

Gel permeation chromatography (GPC), sometimes referred to as gel filtration chromatography (GFC) or size exclusion chromatography (SEC), implies the chromatographic fractionation of macromolecules according to their molecular size.

To perform the GPC analysis, an AKTA FPLC (Fast Protein Liquid Chromatography) system (Amersham Pharmacia Biotech) was used. The FPLC was connected with a UV-900 detector and an AKTA INV-907 Valve for the injection of the samples that were monitored by the UNICORN® control platform. The OHpak SB-802,5 HQ column (length 300 mm) has been used for the analysis, purchased by Shodex™, with size exclusion limit of 10,000 Da. As mobile phase NaCl 0.1 M solution was chosen prepared by HPLC water. Samples were diluted in HPLC water (samples concentrations of 5, 10 or 20 mg/ml) and 20 μL of sample were injected each time to the column. The flow rate was set at 0.8 mL/min. As a reference, a HPLC standard peptide mixture (STD) has been used bought from Sigma-Aldrich, which contained peptides with known molecular weights. The composition of the standard mixture, the corresponding weights as well as the retention on the column (expressed in mL) are shown in **Table 2.7**.

Table 2.7. Composition of the HPLC standard mixture and their corresponding weights.

	Components of standard mixture (STD)	**MW (Da)**	**Retention (ml)**
1	Angiotensin II Acetate (ASP-ARG-VAL-TYR-ILE-HIS-PRO-PHE)	1046.2	12.55
2	Methionine Enkephalin Acetate (TYR-GLY-GLY-PHE-MET)	573.7	14.36
3	Leucine Enkephalin (TYR-GLY-GLY-PHE-LEU)	555.6	19.05
4	VAL-TYR-VAL	379.5	30.73
5	GLY-TYR	238.2	32.82

III. Hydrolysis degree

The Degree of hydrolysis (DH) is defined as the percentage of the total number of peptide bonds in a protein which have been cleaved during hydrolysis (Adler-Nissen, 1986). In this work, DH has been calculated by using the TNBS method [51]. This method is based on the reaction of primary amino groups with TNBSA reagent (**Figure 2.7**).

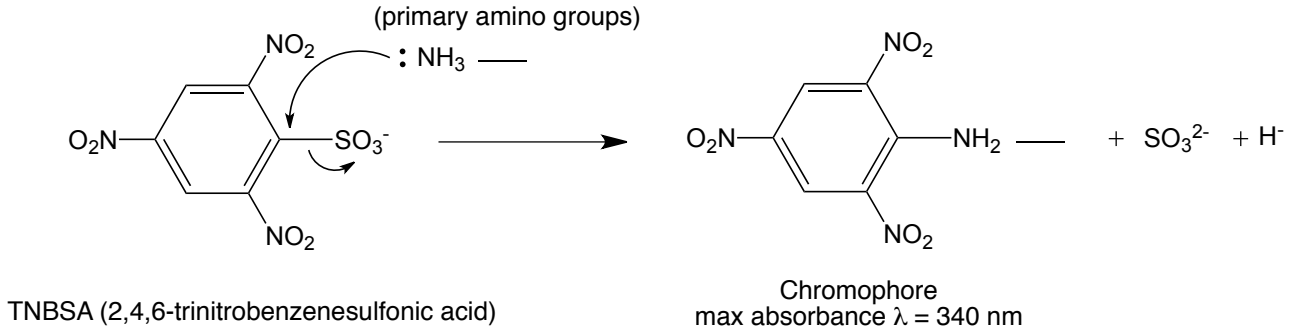

Figure 2.7. The reaction of TNBSA with primary amino groups leads to the formation of a chromogenic derivative that has a maximum absorbance at 340 nm.

Experimental procedure: For the construction of the calibration curve, five standard solutions of L-leucine were prepared with increased concentrations (0.4, 0.8, 1.2, 1.6 and 2.0 mM) in 1% w/v sodium dodecyl sulfate (SDS/H_2O). 0.1% w/v aqueous solution of TNBSA was freshly prepared before analysis by diluting the initially prepared solution of 1% w/v TNBSA in MeOH. As a buffer 0.1 M $NaHCO_3$ (pH 8) was prepared. For the preparation of the standard L-leucine samples, 0.25 mL of standard solutions were transferred in test tubes containing 2 mL buffer solution (NaHCO3 0.1 M) and then 2 mL of 0.1% TNBSA were added and mixed carefully. The tubes were incubated in water bath at 37 ° C for 1 h. Finally, 4 mL of 0.1 M HCl were added to each tube to stop the reaction. The absorbance of the standard solutions is measured at 340 nm, subtracting from all the values obtained the value of the blank solution ("zero"). Tests were performed in triplicate, obtaining the average of the three values.

To calculate the HD of RBP hydrolysates, solutions of samples were prepared at concentrations of 0.1 mg/mL in 1% w/v SDS/H_2O. 0.25 mL of each solubilized sample were transferred to test tube with 2 mL buffer solution (NaHCO3 0.1 M) and then 2 mL of 0.1% TNBSA were added and mixed carefully. The procedure was similar to the standards, as previously described.

To confirm the reliability of the method, an industrial hydrolysate named "KS" with known degree of hydrolysis (DH = 30%) was also characterized. A solution of 1.5 mg/mL of KS in 1% w/v SDS

was prepared. The KS hydrolysate is composed by 15% proteins and 85% H_2O, consequently the solution is prepared at a higher concentration. The results are presented in **Table 2.8**.

The DH is calculated by the following equation:

$$\mathbf{DH\%} = \left(\frac{\mathbf{AN_2 - AN_1}}{\mathbf{N_{pb}}}\right) \times \mathbf{100}$$

Where AN_1 is the amino nitrogen content of the protein substrate before hydrolysis (mg/ g protein), AN_2 the amino nitrogen content of the protein substrate after hydrolysis (obtained by comparison with calibration curve) the and N_{pb} the nitrogen content of the peptide bonds in the protein substrate, that here is considered 123.3 mg/ g protein [51].

The concentration of free amino nitrogen was calculated by the following relation:

$$\frac{\text{mol}_{\text{Leu}}}{\text{L}_{\text{sample}}} = \frac{\text{mol}_{\text{amino N}}}{\text{L}_{\text{sample}}} \xrightarrow{\times 14 \frac{\text{g}}{\text{mol}}} \frac{\text{mg}_{\text{amino N}}}{\text{L}_{\text{sample}}}$$

Table 2.8. Calculations for the evaluation of the Degree of Hydrolysis (DH).

Sample	Abs	Abs_0	mg N_{amino}/ L solution	AN2 (mg N_{amino} / g protein)	DH %
P1	0.6266	0.1423	3.56	35.61	**28.9**
P2	0.6572	0.1729	4.51	45.11	**36.6**
P3	0.6035	0.1192	2.84	28.43	**23.1**
P4	0.5648	0.0805	1.64	16.41	**13.3**
KS	0.6845	0.2002	5.35	35.72	**29.0**

IV. Elemental Analysis

In order to evaluate the protein content of the rice bran, samples were submitted to elemental analysis. The evaluation of the protein content was performed by adopting the Association of Official Analytical Chemists method (AOAC). This method involves firstly the determination of the percentage of the nitrogen content of the sample by weight (e.g. by using elemental analysis). Then, the protein content is calculated by multiplying the value obtained with a statistically determined factor, that is equal to 6.25, assuming that all the nitrogen in the sample is attributed to the protein fraction.

V. ACE inhibitory activity assays

Samples with molecular weight below 10 and 5 kDa were prepared and submitted to ACE-inhibition activity assays. RBP hydrolysates were tested for their ACE-inhibitory activity with a method described in several previous studies [52, 53, 54], evaluating hippuric acid (HA) formation from hippuryl-histidyl-leucine (HHL), a mimic substrate for angiotensin I [55].

To perform the assays, 100 µL of 2.5 mM HHL in 100 mM tris-formic acid (tris-HCOOH), 300 mM NaCl pH 8.3 (buffer 1) was mixed with 30 µL of RBP hydrolysate solution in buffer 1. The test was carried out on six different concentrations obtained by diluting the mother solution (1 mg RBP hydrolysate/ml). Samples were preincubated at 37 °C for 15 min, then 15 µL of ACE solution, in 100 mM tris-HCOOH, 300 nM NaCl, 10 µM ZnCl2, pH 8.3, were added. Samples were incubated for 60 min at 37 °C, then the reaction was stopped with 125 µL of 0.1 M HCl. The aqueous solution was extracted twice with 600 µL of ethyl acetate; the solvent was evaporated, the residue was dissolved in 500 µL of buffer 1 and then analyzed by HPLC, in order to determine HA. HPLC analyses were performed with a HPLC 1200 Series (Agilent Technologies, Santa Clara, US) equipped with an autosampler using the following conditions: column, Lichrospher 100 C18 (4.6 Å~ 250 mm, 5 µm; Grace, Italy); flow rate, 0.5 mL/min; detector, λ 228 nm; mobile phase, water and MeCN, gradient elution from 5 to 60% MeCN in 10 min and 60% MeCN for 2 min, then back to 5% MeCN in 3 min; injection volume, 10 µL; Rt (HA), 4.2 min.

The evaluation of the inhibition of ACE activity was based on the comparison between the concentrations of HA in the presence or absence of the inhibitor (Inhibitor Blank). The phenomenon of autolysis of HHL to give HA was evaluated by a reaction blank, i.e. a sample with the higher inhibitor concentration but without the enzyme. The percentage of ACE inhibition was calculated considering the area of the HA peak with the following formula:

$$\textbf{ACE inhibition}\ (\%) = \frac{\mathbf{A_{IB} - A_N}}{\mathbf{A_{IB} - A_{RB}}} \times \mathbf{100}$$

where A_{IB} is the area of HA in the Inhibitor Blank (IB) sample (i.e., sample with enzyme but without inhibitor), A_N is the area of HA in the samples containing different inhibitor amounts, and A_{RB} is the area of HA in the Reaction Blank (RB) sample (i.e., sample without enzyme and with inhibitor at the highest concentration).

IC_{50} was also calculated, that is the inhibitor concentration needed to observe a 50% inhibition of the ACE activity. Statistical analyses were performed with Statgraphics Plus (version 2.1 for Windows). The experimental data were evaluated by using one-way analysis of variance followed by Fisher's least significant difference procedure; values with different letters were significantly different for $p < 0.05$.

VI. Sensory Analysis

The samples were submitted to sensory analysis in the laboratory of LABCAM S.R.L. (Albenga, Italy). According to the experimental procedure, the samples were diluted in two different concentrations, 0.1 g/ 100 mL and 0.5g/100mL in water. The samples analyzed for their sensorial characteristics were prepared according to the following method: At the first step, 1% Viscozyme is used for hydrolyzing the carbohydrate fraction of DRB and increase at the same time the protein content of the remaining solid residue (DRBP). At the second step, the hydrolysis of the DRB protein is achieved by using 2 different paths as before: (1) 2% of Flavourzyme and (2) 1% Alcalase + 1% Flavourzyme.

2.3 Results and discussion

2.3.1 Study of different carbohydrases for the enrichment of the defatted rice bran (DRB)

As mentioned above, the heterogeneous and complex nature of rice bran proteins (RBPs) are limiting factors in their utilization as food ingredients. With the aim of preparing high-protein products, a fully enzymatic approach was pursued.

To enhance the extractability of the entrapped proteins through the hydrolysis of the carbohydrate fraction of DRB, four commercial enzyme preparations individually and in combination were screened for their ability to solubilize proteins. Specifically, the following commercial products were used:

Table 2.9. Commercial enzyme mixtures.

Product name	**Containing enzymes**	**Enzyme activities**
Ceremix	Endo-1,4-Xylanase Endo-α-amylase Metallo endoprotease (neutral) Endo-1,3(4)-β-glucanase	130 FXU/g 115 KNU-B/g 0.3 AU-N/g 380 BGU/g
Viscozyme	Endo-1,3(4)-β-glucanase (Xylanase, Cellulase, Hemicellulase)	100 FBG/g
Ultraflo	Endo-1,3(4)-β-glucanase (Cellulase, Xylanase)	45 FBG/g
Celluclast	Cellulase	700 EGU/g

These preparations are industrial enzymes, kindly supplied by Novozymes and mostly used in the brewing industry. As the manufacturer never discloses the exact composition of the carbohydrase mixtures, standardization of the experimental procedures was not trivial. On an empirical base, we decided to use 5 % w/w of enzyme with respect to DRB. When in combination, equal quantity of each product was used up to the final amount of 5 % w/w.

Temperature and pH value were selected on the basis of either the activity profiles reported in the technical information sheets or data from the literature. 4 h was setup as the reaction time. Reactions were carried out in water (10% w/v DRB/H_2O), adjusting pH for each enzyme with HCl. At the end of the reaction, enzymes were inactivated by heating at 100^{o}C for 15 min.

The protein content of the solid residue obtained after centrifugation was calculated using the method of AOAC (Association of Official Analytical Chemists) [56], where a statistical factor of 6.25 is used to convert the results from elemental analysis (Nitrogen content, N%) into the percentage of protein (P%) in the analyzed sample of biomass. According to this method, the protein content of the Defatted Rice Bran (DRB) used as starting material was 19.5%.

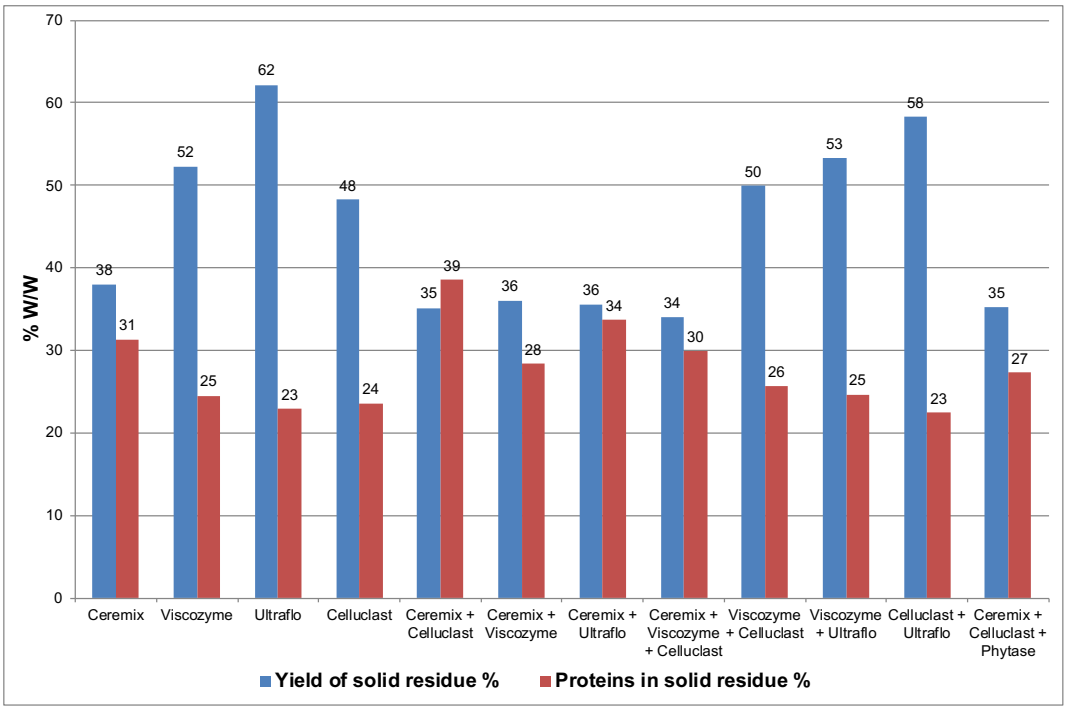

Figure 2.8. Solid residue yield and protein content (%) of the solid residue after rice bran carbohydrate hydrolysis with different enzyme preparations.

Results of the experiments, reported in **Figure 2.8**, clearly indicate that, the selected enzyme mixtures or combination of them successively hydrolyzed the carbohydrates present in DRB with remarkable differences: the higher the value of solid residue yield, the lower the hydrolysis of the DRB carbohydrates and therefore the lower the enrichment of the DRB residue in proteins. The combination of Ceremix and Celluclast shows the best performance as it hydrolyses most of the carbohydrates present in DRB (35% yield of remaining solid residue displaying the highest percentage of proteins (39%), which is used in the successive step of DRB protein hydrolysis). With respect to the combination of Ceremix and Celluclast, the use of Viscozyme demonstrates a lower removal capability of carbohydrates of DRB featuring 52% solid residue yield. For comparison purposes, Defatted Rice Bran Protein Hydrolysates have been prepared by using either Viscozyme or the combination of Ceremix and Celluclast for the step of the carbohydrates' hydrolysis of DRB.

It has been reported that the interaction with phytate molecules can cause a modification in the protein structure leading to the formation of an insoluble protein-phytate complex [15]. Thus, the use of phytase an enzyme that catalyzes the stepwise removal of phosphate groups in phytate can help increasing the solubility of RB proteins. In our hands, addition of phytase to a combination of carbohydrases (Ceremix and Celluclast) did not show advantages on extractability of proteins bound to cellular components and/or phytate as non-significant enhancement of the protein content was observed (35% of solid residue, 27% proteins).

The solid residue of DRB after the treatment with carbohydrases (Ceremix and Celluclast or Viscozyme), was submitted to treatment with proteases (Flavourzyme or both Alcalase and Flavoyrzyme). Flavourzyme® and Alcalase®, are two different commercially prepared mixtures that contain proteolytic enzymes of microbial nature, widely used for the production of bioactive peptides [57]. According to Merz et al., that studied extensively the composition of Flavourzyme®, identified and characterized eight different enzymes that include two aminopeptidases, two dipeptidyl peptidases, three endopeptidases, and one α-amylase from the *Aspergillus oryzae* strain [58]. In Alcalase® the key enzymatic activity is provided by a serine endopeptidase.

Defatted rice bran was used with or without pretreatment in water at 80ºC for 24h, before the use of carbohydrases. The reactions of protein hydrolysis were carried out in water (10% w/w DRB/H_2O) for 24 h at 50 ºC. 2 % w/w of enzyme with respect to DRB was used (Flavourzyme). When in combination (Flavourzyme and Alcalase), equal quantity of each product was used up to the final amount of 2 % w/w. Specifically, 1% of Flavourzyme was transferred to the reaction mixture and after 2h another 1% of Viscozyme or Alcalase was added. The reaction left under stirring for 24 h overall time. At the end of the reaction, enzymes were inactivated by heating at 100ºC for 15 min and the reaction mixture was centrifuged to separate the hydrolysates from the solid residue. In **Table 2.10** are presented the final hydrolysates obtained after lyophilizing the supernatants. The % yields of the prepared hydrolysates refer to the rice bran obtained after the hydrolysis of the carbohydrates.

Table 2.10. Prepared defatted rice bran hydrolysates.

Sample	**PT with H_2O**	**1st step: treatment with carbohydrases**	**2nd step: treatment with proteases**	**Yield %**
P1	no	ceremix-celluclast	flavourzyme	**23.4**
P2	no	ceremix-celluclast	alcalase+flavourzyme	**37.6**
P3	yes	ceremix-celluclast	flavourzyme	**19.6**
P4	yes	ceremix-celluclast	alcalase+flavourzyme	**22.7**
P5	no	viscozyme	flavourzyme	**29.9**
P6	no	viscozyme	alcalase+flavourzyme	**36.2**

PT: pretreatment of defatted rice bran with water at 80ºC for 24h.

All the hydrolysates were then ultrafiltrated by using membranes with molecular weight cut-off of 10 and 5 kDa. The ultrafiltration yields were calculated by weight (**Table 2.11**, **Figure 2.9**). The pretreatment in hot water and the use of Ceremix and Celluclast led to the maximum amount of smaller peptides under 5kDa (96.2% and 96.6% of samples P3 and P4, respectively). In addition, the use of both Flavourzyme and Alcalase relatively increased the amount of smaller peptides as samples prepared by Flavourzyme contained more peptides with molecular weight above 10 kDa (P1 vs P2 and P5 vs P6). Interestingly, the choice of carbohydrases at the first treatment affects the size of the final hydrolysate: Viscozyme provided samples with more amount of larger peptides compared to samples prepared by using Ceremix and Celluclast (24.1% of P5 and 8.8% of P6 are above 10 kDa).

Table 2.11. Ultrafiltration yields of rice bran protein hydrolysates.

Sample	Sample description	%[P<5kDa]	%[5kDa<P<10KDa]	%[P>10kDa]
P1	no PT – ceremix+celluclast – flavourzyme	87.23	4.26	8.51
P2	no PT – ceremix+celluclast – flavourzyme+alcalase	90.70	4.65	4.65
P3	PT – ceremix+celluclast – flavourzyme	96.23	2.51	1.26
P4	PT – ceremix+celluclast –flavourzyme+alcalase	96.65	2.23	1.12
P5	no PT – viscozyme – flavourzyme	70.44	5.46	24.10
P6	no PT – viscozyme – flavourzyme+alcalase	85.36	5.84	8.80

PT: pretreatment of defatted rice bran with water at 80°C for 24h.

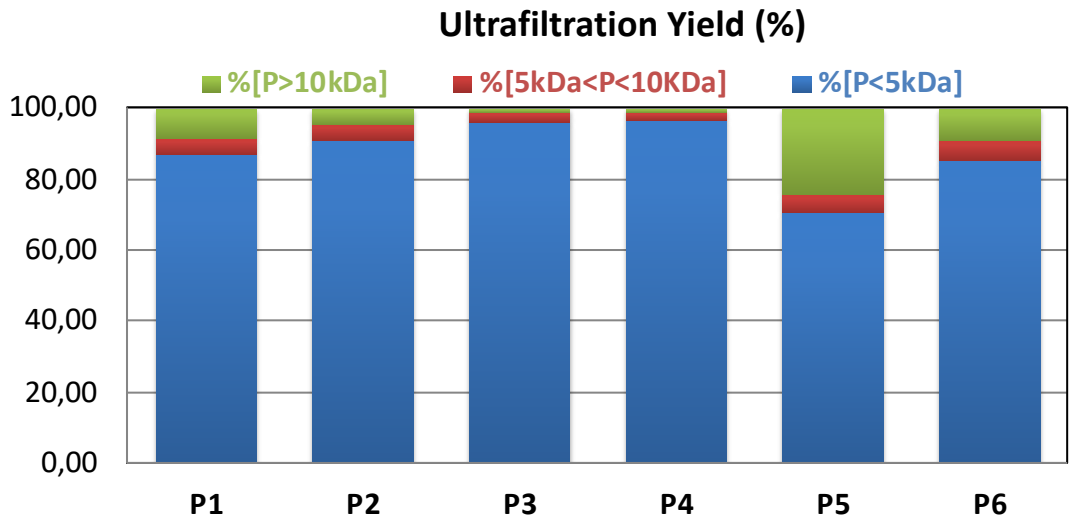

Figure 2.9. Ultrafiltration yields of rice bran protein hydrolysates. Samples obtained under 5 kDa, between 5 and 10 kDa and above 10kDa.

2.3.2 Hydrolysis Degree measurements

Degree of hydrolysis (DH) is defined as the proportion of cleaved peptide bonds in a protein hydrolysate. [59, 60]. There are several methods for the determination of DH including the pH-stat methods, 2,4,6-trinitrobenzenesulfonic acid (TNBSA), o-phthaldialdehyde (OPA), trichloroacetic acid soluble nitrogen (SN-TCA), and formol titration methods [61, 60]. All the reported methods are based on the assumption that a free amino group and a free carboxyl group are released when a peptide bond is broken. To quantify the increasing in the concentration of such groups is a way to evaluate the progress of protein hydrolysis. DH affects the size and hence the amino acid composition of the peptides, thus affecting the taste as well as the functional and biological properties of protein hydrolysate preparations [40].

To determine DH of DRB hydrolysates we used the TNBSA method, based on the reaction of 2,4,6-trinitrobenzenesulfonic acid (TNBSA) with amino acids that give a yellow product whose absorbance is measured at 340 nm.

Standard solutions of L-leucine were used to prepare the calibration curve for quantification (**Figure 2.10**). The measured degree of hydrolysis (DH) of the samples P1-P4 is reported in **Table 2.12**. To validate our procedure, a commercial sample of soy protein hydrolysate (KS) was used as reference standard: KS was found to have a DH of 29%, a value corresponding to the one provided by the supplier (30%).

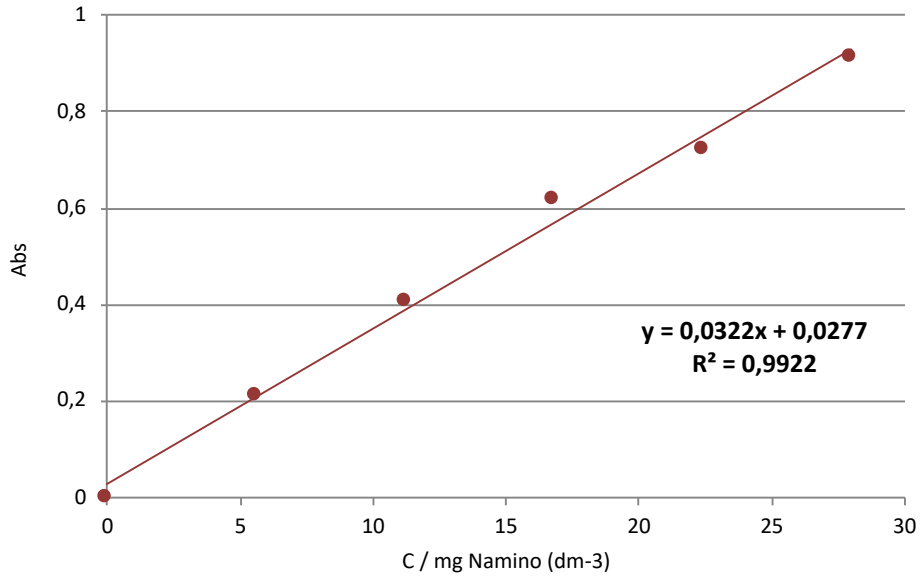

Figure 2.10. Calibration curve for the evaluation of DH of RBP hydrolysates. L-leucine standard solutions have been prepared.

According to the values obtained, protein hydrolysates prepared without pretreatment with water at 80 °C, have higher DH (P1 and P2). In addition, the combined use of the enzyme mixtures Alcalase

and Flavourzyme led to a slightly increased DH value (P2, 36.6 %) with respect to the use of only Flavourzyme (P1, 28.9 %). On the contrary, the hydrolysates prepared with pretreatment (P3 and P4) had a completely different behavior: the sample prepared by using only Flavourzyme has higher DH value (P3, 23.1 %) with respect to the one prepared by the use of both enzyme mixtures (P4, 13.3 %).

Table 2.12. Degree of Hydrolysis (DH%) of RBP hydrolysates.

Sample	**PT with H_2O**	**1st step: treatment with carbohydrases**	**2nd step: treatment with proteases**	DH %
P1	no	ceremix-celluclast	flavourzyme	**28.9**
P2	no	ceremix-celluclast	alcalase+flavourzyme	**36.6**
P3	yes	ceremix-celluclast	flavourzyme	**23.1**
P4	yes	ceremix-celluclast	alcalase+flavourzyme	**13.3**
KS	Soy protein hydrolysate (commercial sample)			**29.0**

PT: pretreatment of defatted rice bran with water at 80°C for 24h.

2.3.3 SDS-PAGE and GPC characterization

According to the SDS-PAGE characterization (**Figure 2.11**), RBP hydrolysates contain very short peptides that are difficult to detect well with this method. On the "gel well" with number 3, 5 and 9 bands at ~7 kDa are clearly visible. In addition, a pale shadow is visible under 1 kDa for the wells with number 4, 5, 8 and 9, probably due to very short peptides. Finally, at 1, 11 and 12 nothing is visible.

No	Sample	Sample information
1	P1 < 5 KDa	No PT – Flav
2	Empty	-
3	P1< 10 KDa	No PT – Flav
4	P2 < 5 KDa	No PT – Alc + Flav
5	P2 < 10 KDa	No PT – Alc + Flav
6	MARKER	(MW are expressed in KDa)
7	Empty	-
8	P4 < 5 KDa	PT – Alc + Flav
9	P4 < 10 KDa	PT – Alc + Flav
10	Empty	-
11	P3 < 5 KDa	PT – Flav
12	P3 < 10 KDa	PT – Flav

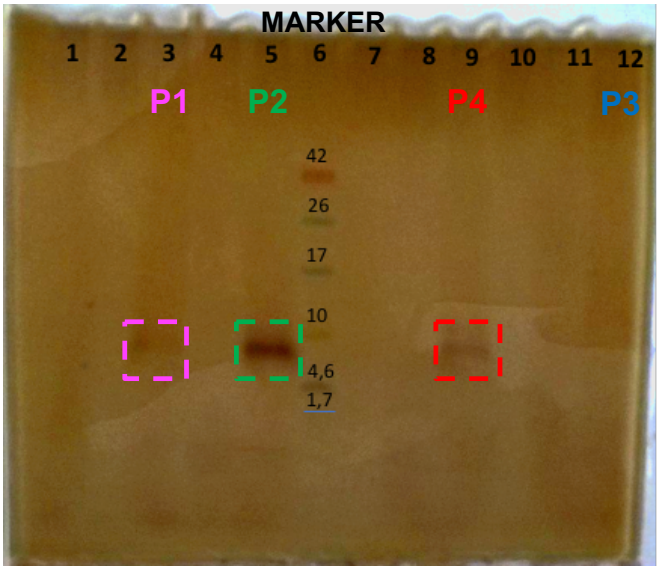

Figure 2.11. SDS-PAGE gel of the samples P1, P2, P3 and P4 under 10 and 5 kDa, where PT stands for pretreatment of DRB in water at 80ºC for 24h.

Samples P1 and P3 prepared by using only Flavourzyme, have less intense band at ~7kDa compared to the samples P2 and P4 prepared by using the combination of Alcalase and Flavourzyme. In addition, the pretreatment in water at 80 ºC for 24 h seems to have an important affection on the activity of the proteolytic enzymes, as it can be seen by comparing the samples P2 (5^{th} well) and P4 (9^{th} well), prepared without and with pretreatment, respectively. The pretreatment probably allows these enzymes to work better by liberating the rice bran structure from the water-soluble components (carbohydrates).

The molecular weight distribution of rice protein hydrolysates was also determined by gel filtration chromatography (GPC) using a Shodex SB-802,5 HQ column (exclusion limit 10.000 Da) (eluent: 0.1 M NaCl in 0.1 M phosphate buffer pH 6.8).

In **Figure 2.12** are shown the peaks of the five components and their corresponding molecular weights (1046, 574, 556, 380 and 238) contained in the standard peptide mixture (STD) used to set up the separation conditions.

In the following figures are presented the spectra from GPC analysis. Specifically, in **Figure 2.12** are shown the peaks of the five components of the standard peptide mixture (STD) and their corresponding molecular weights (1046, 574, 556, 380 and 238). The chromatogram shows how much material exited the column at any one time, with the higher molecular weight peptides eluting first, followed by successively lower molecular weight (and therefore smaller) peptides emerging later. The primary separation is according to elution volume.

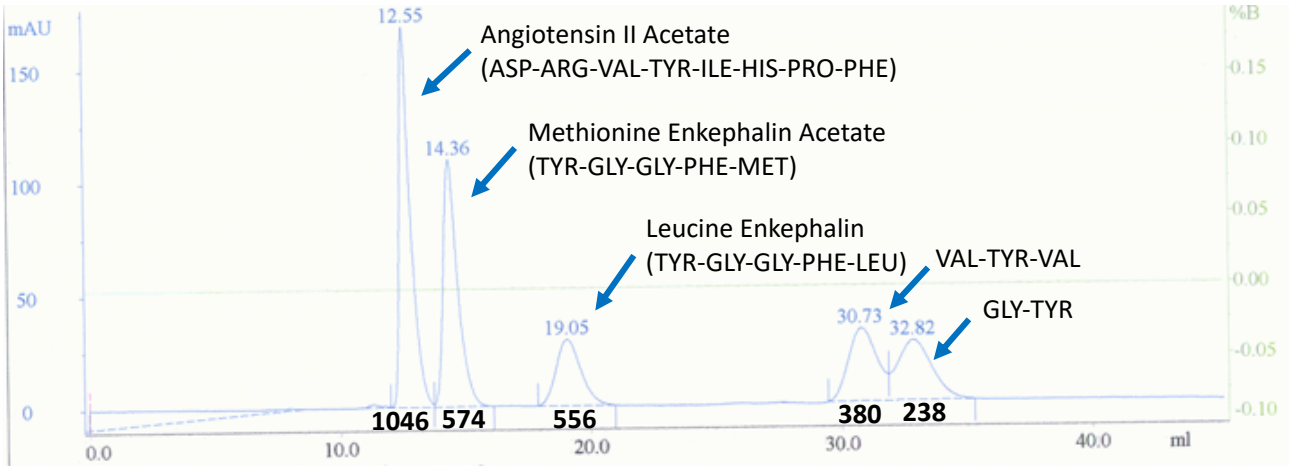

Figure 2.12. GPC analysis of the standard peptide mixture (STD) and the corresponding weights of the peptides contained in this mixture.

The chromatograms of the samples P1, P2, P3 and P4 with molecular weight under 10 kDa (A) and under 5 kDa (B) compared to the standard peptide mixture, are presented in **Figure 2.13**. Very intense peaks appeared in the range between 1046 and 576 Da for the samples P1 and P3 and less intense for sample P4. These peaks are clearly visible at MW under 5 and 10 kDa. Moreover, these samples have another peak that corresponds to peptides with MW between 556 and 380 Da. Some peptides with MW over 1050 kDa are also appeared and specifically the intense peak of sample P2 prepared under 10 kDa (Figure 2.16 (A)) corresponds to peptides with MW 7kDa, confirmed also by the previous SDS-PAGE characterization, where this sample had a very intense band at this weight.

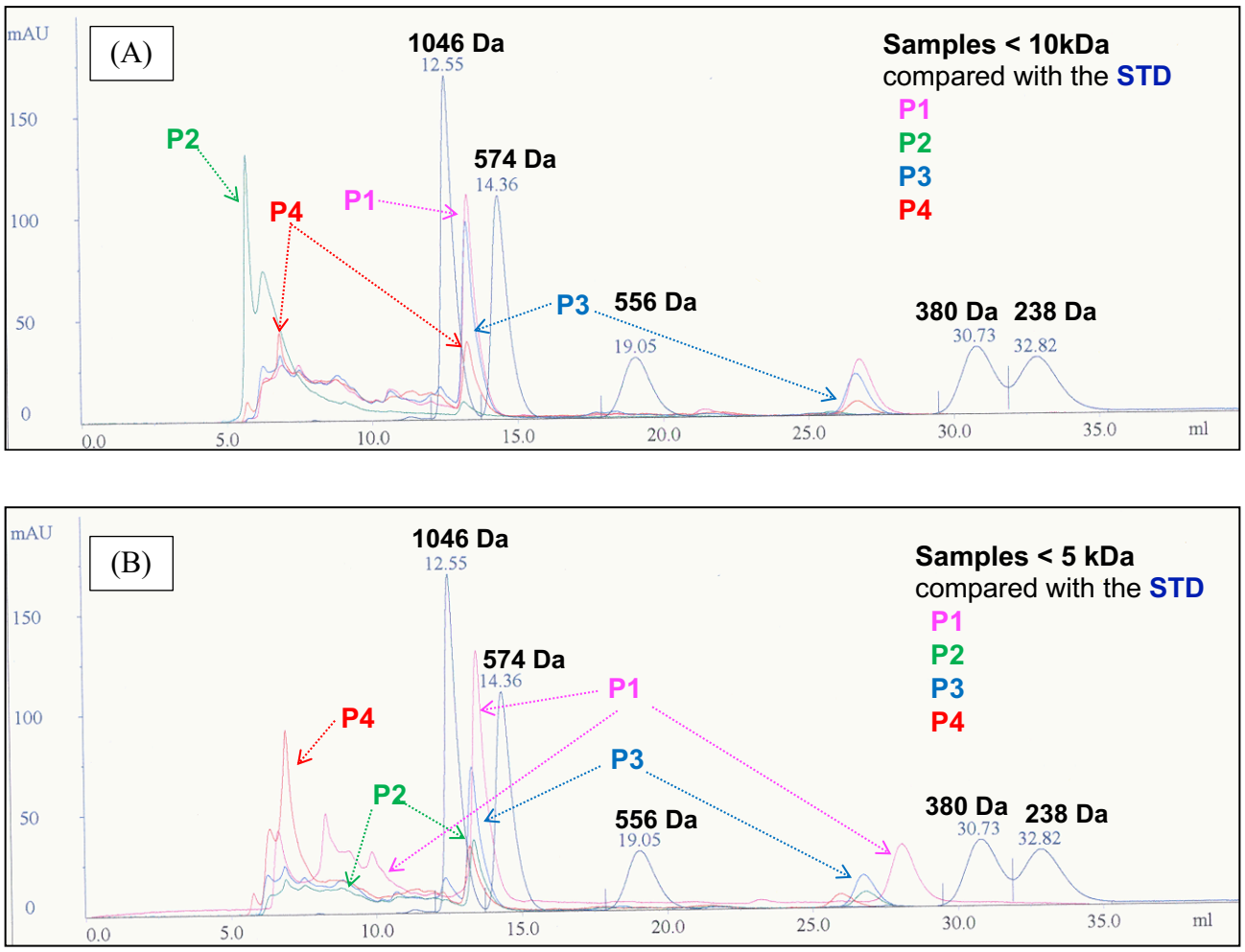

Figure 2.13. GPC analysis of samples P1, P2, P3 and P4 with MW < 10 kDa (A) and MW < 5kDa (B) compared with the standard peptide mixture (STD).

2.3.4 ACE-inhibition activity of the Rice Bran Protein Hydrolysates (RBPHs)

Although a number of biologically active peptides with antihypertensive properties have been obtained from different plant proteins, there is poor information about the ACE-inhibitory activity of rice bran protein and its hydrolysates.

Only recently, Wang et al. investigated enzymatic hydrolysis of rice bran protein using trypsin. The authors succeeded to isolate and characterize an ACE inhibitory tripeptide (Tyr-Ser-Lys, MW: 395.0 Da) from trypsin hydrolysate with molecular weight under 4 kDa [42].

In **Table 2.13** the values of maximum ACE-inhibition percentage of the different samples of DRB hydrolysates, obtained by sequential filtration through ultrafiltration membranes with MW cut-off of 10 and 5 kDa, at the same concentration are reported. If the percentage of ACE inhibition is greater than 50%, it is possible to calculate the IC50 value which is the sample concentration that serves to inhibit the enzyme activity by 50%. Thus, samples with lower IC50 values are more active than those with higher IC_{50}.

Table 2.13. Results of ACE inhibitory assays.

Sample	PT with H_2O	1st step: treatment with carbohydrases	2nd step: treatment with proteases	MW	ACE-INHIBITION (%)	IC_{50} (µg/ml)
P1	no	ceremix -celluclast	flavourzyme	<10 kDa	30.22 ± 0.38	/
P1’	no	ceremix -celluclast	flavourzyme	<5 kDa	57.96 ± 1.57	780.65 ± 15.13
P2	no	ceremix -celluclast	alcalase+flavourzyme	<10 kDa	49.50 ± 1.55	/
P2’	no	ceremix -celluclast	alcalase+flavourzyme	<5 kDa	60.19 ± 1.17	842.58 ± 16.65
P3	yes	ceremix -celluclast	flavourzyme	<10 kDa	46.17 ± 1.24	
P3’	yes	ceremix -celluclast	flavourzyme	<5 kDa	49.44 ± 0.66	/
P4	yes	ceremix -celluclast	alcalase+flavourzyme	<10 kDa	49.43 ± 1.83	
P4’	yes	ceremix -celluclast	alcalase+flavourzyme	<5 kDa	56.81 ± 1.08	761.91 ± 25.86
P5’	no	viscozyme	flavourzyme	<5 kDa	20.28 ± 0.72	/
P6’	no	viscozyme	alcalase+flavourzyme	<5 kDa	32.16 ± 1.55	/

PT: pretreatment of defatted rice bran with water at 80°C for 24h.

In **Figure 2.14** the graphical comparison among the samples is shown. Each ACE-inhibitory activity value is the average ± of three different assays performed by using 1.0 mg/ml as maximum concentration (see Experimental for details).

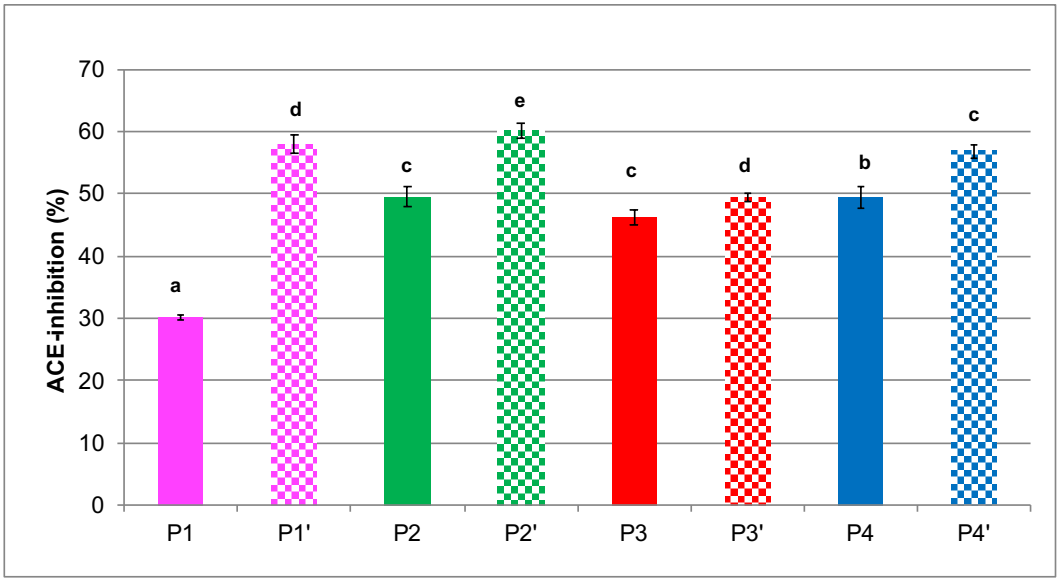

Figure 2.14. ACE-inhibition activity of the samples under 10 kDa (P1, P2, P3 and P4) and 5 kDa (P1', P2', P3'and P4'). Different letters on the bars mean significant difference ($p < 0.05$).

A comparison of ACE-inhibitory activity of samples obtained with the same sequential steps of hydrolysis with carbohydrases and proteases but using ultrafiltration membranes having different MW cut-off indicated that fractions with peptide sizes <5 kDa were always more active than the corresponding fractions with MW <10 kDa. In one case (P1 vs P1') the antihypertensive efficacy is as much as doubled. In addition, the double digestion with Alcalase and Flavourzyme is always more effective than Flavourzyme digestion even if, in some cases, differences are not significant (e.g. P1' vs P2'and P3 vs P4).

Regarding the effects of pretreatment step on the ACE inhibition percentage, results are contradictory. In the case of samples with MW<10 kDa prepared with Flavourzyme, the pretreatment step provides more active samples (cf. P1 vs P3) whereas for samples with MW<5 kDa the opposite is true: hydrolysates obtained without pretreatment are more effective (sample P1' is more active than P3' i.e. 57.96 vs 49.44% ACE inhibition, for P1' and P3', respectively). These contradictory results need further investigation. On the basis of the present data a possible explanation could be the presence of a higher amount of peptides with molecular weight above 10 kDa in the sample P1 (8.51%) compared with the sample P3 (1.26%), according to ultrafiltration yields presented in **Figure 2.9** and **Table 2.11**.

As for hydrolysates obtained with Alcalase and Flavourzyme, both fractions with peptide sizes <10 kDa and <5 kDa have almost the same activity (49.55 vs 49.43% ACE inhibition, for P2 and P4, and 60.19 vs 56.81% ACE inhibition, for P2' and P4', respectively, being the sample without pretreatment the most effective).

Data of **Table 2.13** clearly indicate also that the ACE-inhibition activity is affected mostly by the choice of the enzyme mixture at the first step treatment when carbohydrases are used to solubilize carbohydrates and to enrich the rice bran residue in proteins. Specifically, samples prepared by Ceremix and Celluclast showed up to the double activity with respect to the samples prepared by Viscozyme (P1' vs P5' and P2' vs P6') **(Figures 2.15** and **2.16)**. The use of Ceremix and Celluclast led also to the highest enrichment in proteins of rice bran residue (cf. **Figure 2.8**), as previously shown, thus allowing the proteolytic enzymes to produce a larger amount of bioactive peptides.

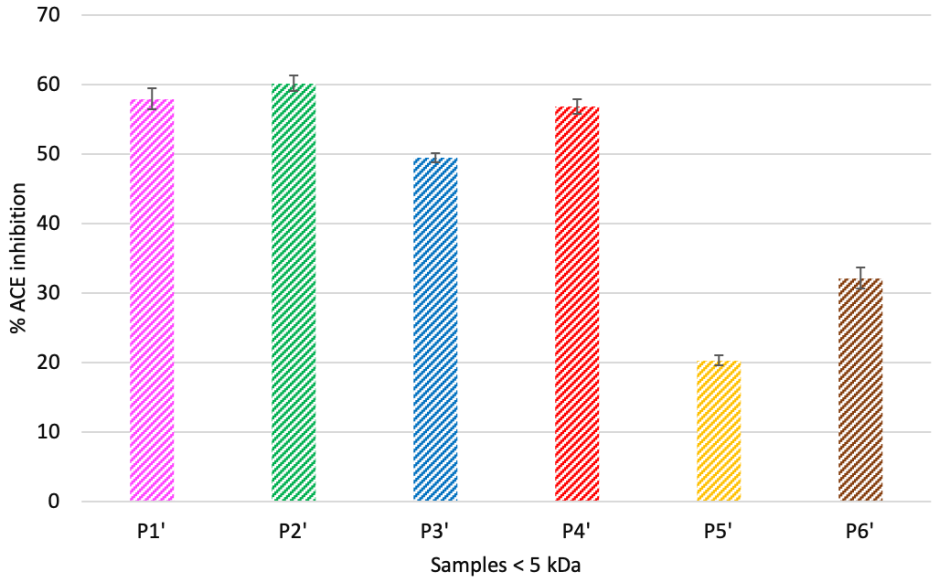

Figure 2.15. ACE inhibition activity at maximum concentration of samples with MW< 5kDa.

The curves obtained plotting ACE-inhibition percentage *vs* hydrolysates concentration are reported in **Figure 2.16**.

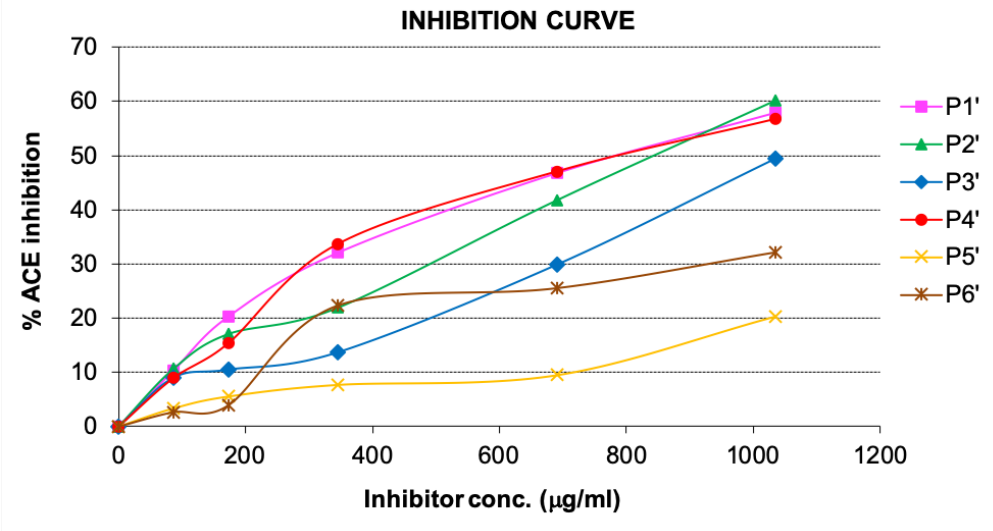

Figure 2.16. ACE inhibition activity of the samples in six different concentrations.

As the most promising mixture of peptides was obtained with a double digestion performed with Alcalase and subsequently Flavourzyme and then purifying by ultrafiltration with 5 kDa membranes, two of the previous samples (P2'and P4') as well as the sample showing the highest ACE-inhibitory activity among those prepared with Flavourzyme (P1') were selected and further ultrafiltrated by using a membrane of MW cut-off of 1kDa. The obtained samples with MW < 1kDa (P1'', P2'', and P4'') were in turn submitted to assays for evaluating ACE-inhibitory activity. Results of these assays (highest ACE inhibition percentage and IC50 values) in comparison with those of fractions with peptide size < 10 kDa and < 5 kDa (P1, P2, P4 and P1', P2', P4', respectively) are reported in **Table 2.14**.

Table 2.14. ACE-inhibitory activity of < 10 kDa, < 5 kDa and < 1 kDa fractions from RBPHs.

Sample	PT with H_2O	1st step: treatment with carbohydrases	2nd step: treatment with proteases	MW	ACE-INHIBITION (%)	IC_{50} (µg/ml)
P1	no	ceremix -celluclast	flavourzyme	<10 kDa	30.22 ± 0.38	/
P1'	no	ceremix -celluclast	flavourzyme	<5 kDa	57.96 ± 1.57	780.65 ± 15.13
P1''	no	ceremix -celluclast	flavourzyme	<1 kDa	35.94 ± 1.39	/
P2	no	ceremix -celluclast	alcalase+flavourzyme	<10 kDa	49.50 ± 1.55	/
P2'	no	ceremix -celluclast	alcalase+flavourzyme	<5 kDa	60.19 ± 1.17	842.58 ± 16.65
P2''	no	ceremix -celluclast	alcalase+flavourzyme	<1 kDa	51.32 ± 1.92	925.43 ± 15.80
P4	yes	ceremix -celluclast	alcalase+flavourzyme	<10 kDa	49.43 ± 1.83	/
P4'	yes	ceremix -celluclast	alcalase+flavourzyme	<5 kDa	56.81 ± 1.08	761.91 ± 25.86
P4''	yes	ceremix -celluclast	alcalase+flavourzyme	<1 kDa	68,70 ± 2.94	552.73 ± 13.20

It is known that the bioactivity of peptides is related to their chain length, amino acid composition, and sequence. According to the literature, the most effective ACE-inhibitory peptides identified until now contain 2−20 amino acids and have a good hydrophobicity/hydrophilicity balance and some particular structural characteristic related to sequence [62, 63]. In general, smaller peptides showed more inhibitory activity than larger counterparts.

On this ground, the ACE inhibitory activity is expected to increase going from fractions with peptide sizes under 10kDa to those with peptide sizes under 1kDa. Indeed, only the hydrolysates P4, P4' and P4'' exhibited such behavior (**Figure 2.19**).

By contrast, in both the second hydrolysates prepared with Alcalase and Flavourzyme, i.e. P2, P2' and P2" and the sample obtained using only one protease (Flavourzyme) i.e. P1, P1' and P1", the most active fraction is that obtained by ultrafiltration through a 5 kDa cut-off membrane.

In addition, the three fractions P2, P2' and P2" showed ACE inhibition activities not significantly different from each other (**Figure 2.18**). On the contrary, the fraction P1" having peptide size under 1 kDa, shown an ACE inhibitory activity similar to that of < 10 kDa fraction and extremely lower than that of P1' fraction (**Figure 2.17**), thus suggesting that the bioactive peptides liberated from the native protein with Flavourzyme have a MW in the range 1-5 kDa. These results are worth of a more in-depth analysis.

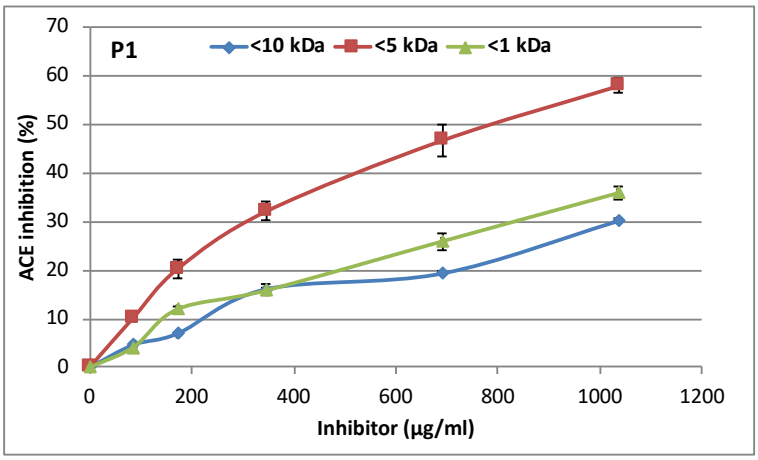

Figure 2.17. ACE inhibition vs concentration of P1, P1' and P1" fractions (MW< 10, 5 and 1 kDa, respectively).

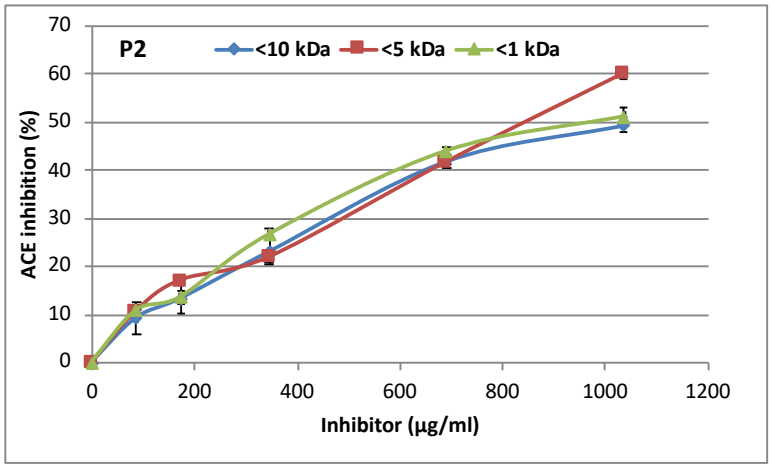

Figure 2.18. ACE inhibition vs concentration of P2, P2' and P2" fractions (MW< 10, 5 and 1 kDa, respectively).

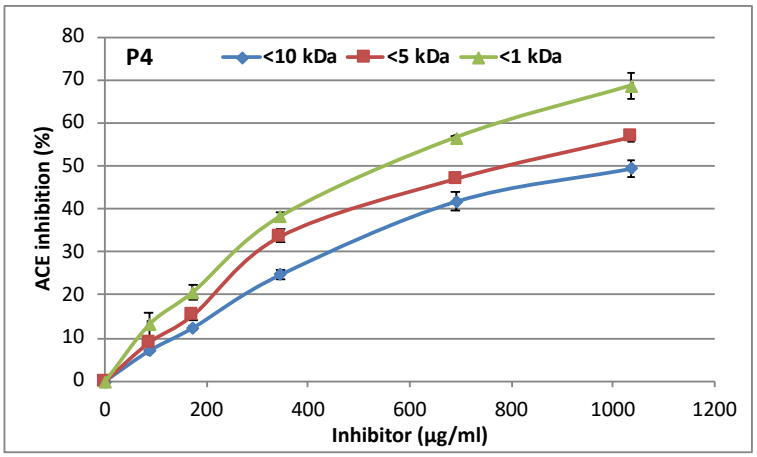

Figure 2.19. ACE inhibition vs concentration of P4, P4' and P4" fractions (MW< 10, 5 and 1 kDa, respectively).

2.3.5 Sensorial Characteristics of the prepared RBPHs

To evaluate a possible application of Rice Bran Protein in food and pharmaceutical industry, a sensory profile of two of the above-mentioned hydrolysates, i.e. P5' and P6' was carried out. The two samples were prepared by hydrolyzing carbohydrates with Viscozyme and using either Flavourzyme or the combination of Flavourzyme and Alcalase for the protein hydrolysis of DRB followed by ultrafiltration through a membrane with MW cut-off of 5 kDa.

The sensory profiling took place at the Sensory Laboratory of at Special Company for Professional Training and Technological and Commercial Promotion of the Chamber of Commerce of Savona (Albenga, Italy) by panelists trained to evaluate the five basic tastes (sweet, bitter, sour, salty and umami). Each sample was tasted at two different concentrations: 0.1 g/ 100 mL and 0.5 g/100 mL. Results of sensory analysis are reported in the **Figures 2.20** and **2.21**.

For the DRBP hydrolysate prepared only by using the enzyme Flavourzyme, P5, sweetness and savoriness (that is Umami taste) are recognized for both concentrations. At 0,5 g/100 mL the Umami taste is five times more intense with respect to the diluted sample 0,1 g/100 mL, though the sweet taste remains in the same levels for both concentrations, so that umami is the most intense attribute of such sample.

By contrast, P6 sample at low concentration of 1g/100 mL reveals only sweet taste. By increasing the concentration to 0,5 g / 100 mL, the assessors recognized both sweet and umami taste, with sweet prevailing over umami.

Figure 2.20. Taste Profile of the DRBP hydrolysate (P5) prepared by using the enzyme Flavourzyme.

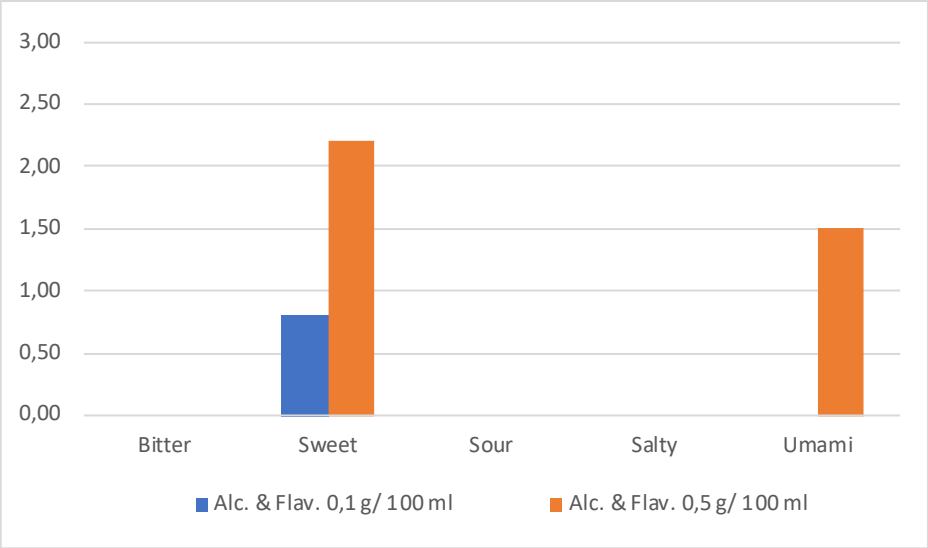

Figure 2.21. Taste profile of the DRBP hydrolysate (P6) prepared by using the combination of Alcalase and Flavourzyme.

The assays have given statistically accepted results, which is confirmed by the factor CVr% that should be less than 20% to consider "good" a result. Interestingly, sensory analysis revealed that the tasted protein hydrolysates elicit only sweet and umami taste. In both samples the salty, acid and bitter taste were completely absent, increasing the potential use of these hydrolysates as flavor enhancers. Moreover, a taste of chicken broth and toasted cereals has been distinguished by some panelists for the sample Flavourzyme at 0.5% concentration.

It is worth to mention that our research group has already developed a protocol for producing peptide mixtures with flavor-enhancing properties from rice middlings [30]. According to this protocol, rice middling proteins are extracted and hydrolyzed at the same time in ammonium bicarbonate buffer, with the aim to obtain peptides with a high number of polar residues. In fact, it is known that polar peptides, unlike hydrophobic peptides, are more rarely associated with the bitter taste. Enzymatic hydrolysis was carried out with two different commercial food-grade protease/peptidase complexes, Umamizyme and Flavourzyme, that were selected on the basis of their capacity to enhance flavor and to debitter bitter protein hydrolysates [30]. Sensory evaluation revealed that although umami taste was the most intense attribute, both hydrolysates were also characterized by a moderate bitter taste as well as a sour taste when Umamizyme was used.

On the contrary, the protein hydrolysates prepared now elicit only sweet and umami taste. It is worth noting that the use of carbohydrases, the different hydrolysis conditions and the ultrafiltration methodology allowed to eliminate completely the bitterness from the mixture of peptides prepared from rice bran protein. The suitable taste profile makes these hydrolysates potential ingredients for the food industry.

2.3.6 Sugar composition of DRB

The pretreatment with water was useful to investigate the sugar composition of defatted rice bran (DRB). When DRB was submitted to a treatment with hot water (85 °C for 24 h) up to 30% solubilization of the starting material was achieved depending on the nature of the rice bran. NMR and MS analysis (**Figures 2.22**, **2.23** and **2.24**) indicated that the aqueous extract, after precipitation of complex-carbohydrates with 2-propanol, is mainly composed by saccharose, which represents 10% of the whole starting material, and 12.8% of proteins.

Figure 2.22. TLC of the supernatant from the pretreatment of DRB in hot water compared with a standard sample of saccharose (Sacch). Eluent: *n*-propanol/ethyl acetate/water (8:1:1). For TLC visualization reagent: see experimental.

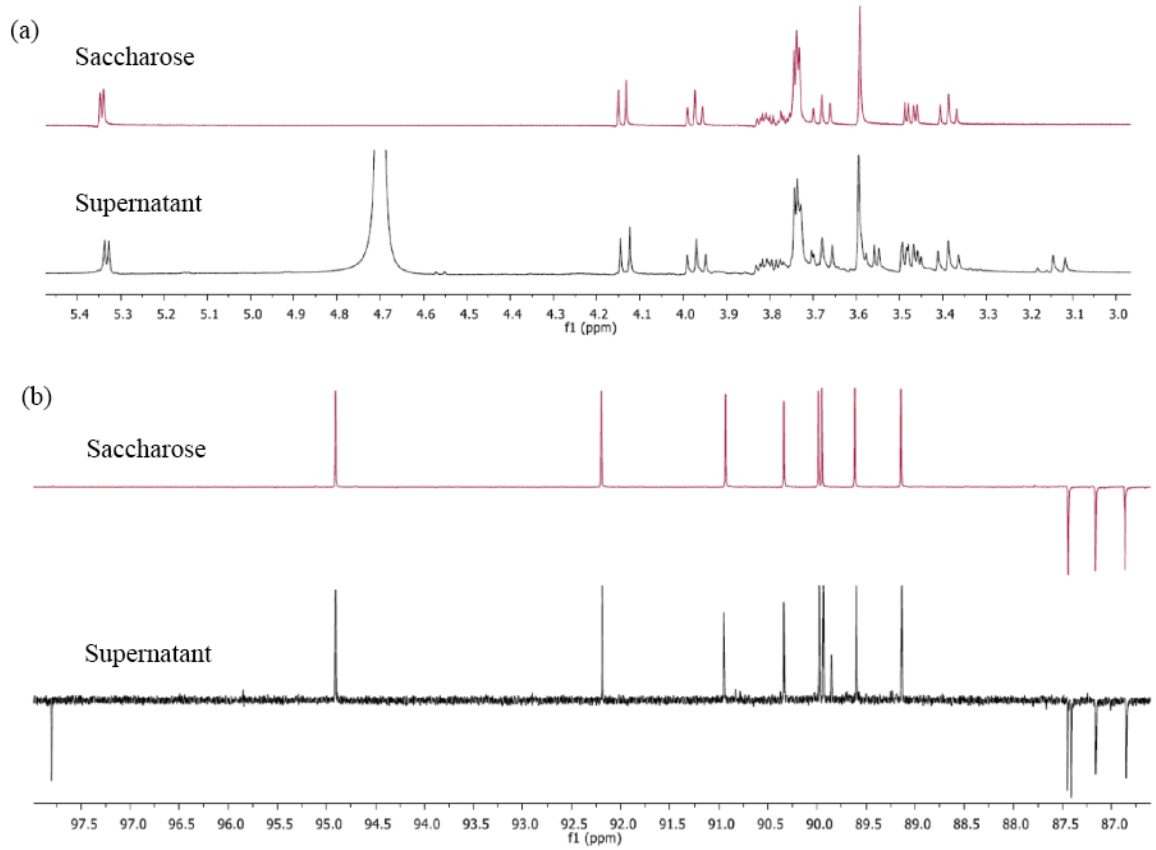

Figure 2.23. ^{1}H and ^{13}C NMR spectra (a and b, respectively) of the supernatant derived from the pretreatment in hot water.

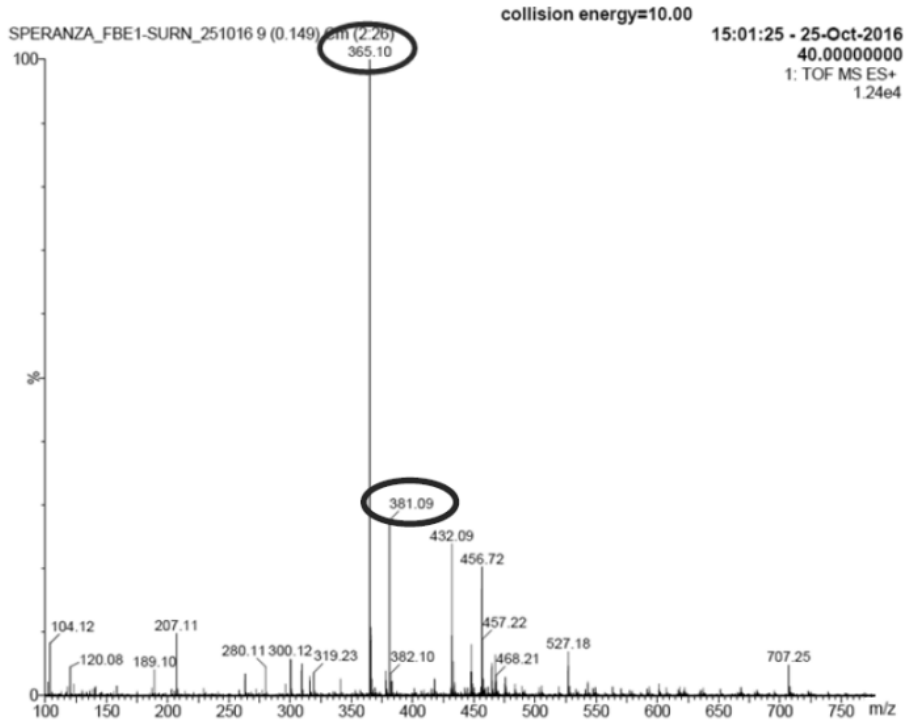

Figure 2.24. Q-Tof mass spectrum of the supernatant. Mass values in the circle correspond to the molecular mass of saccharose (m/z: 365 $[M + Na]^{+}$, 381 $[M + K]^{+}$).

In order to study the composition of the polysaccharides present in rice bran, DRB (10 g) was treated with a mixture of α- amylase and amyloglucosidase in order to hydrolyze the starch always present in commercial sample of rice bran. The resulting extract (ca. 40% w/w) was found to be composed mainly by glucose, according to ^{1}H NMR analysis (**Figure 2.25**) and very poor in protein (only 5.6%).

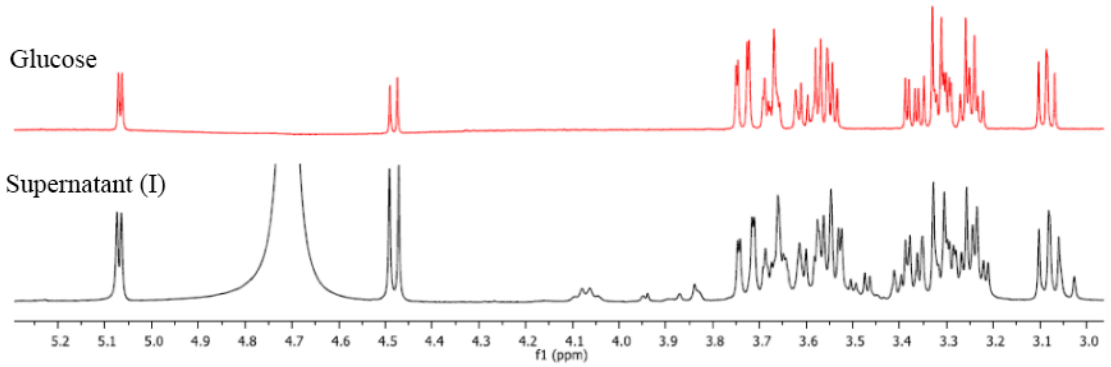

Figure 2.25. ^{1}H NMR (D_2O, 400 MHz) of the supernatant (I) derived from the enzymatic hydrolysis with α-amylase and amyloglucosidase.

Re-solubilization of the de-starched solid residue with hot water (80-85°C for 24 h) led to an extract containing the water-soluble carbohydrates of DRB. Composition of the polysaccharides fraction of this extract (**Supernatant II**) was investigated by submitting it, after lyophilization, to chemical hydrolysis with 2 M TFA (120°c, 2h). TLC and ^{1}H and ^{13}C NMR analysis of the hydrolyzed sample (**Figure 2.26** and **2.27**) revealed the presence of four monosaccharides: arabinose, glucose, xylose and galactose in order of abundance.

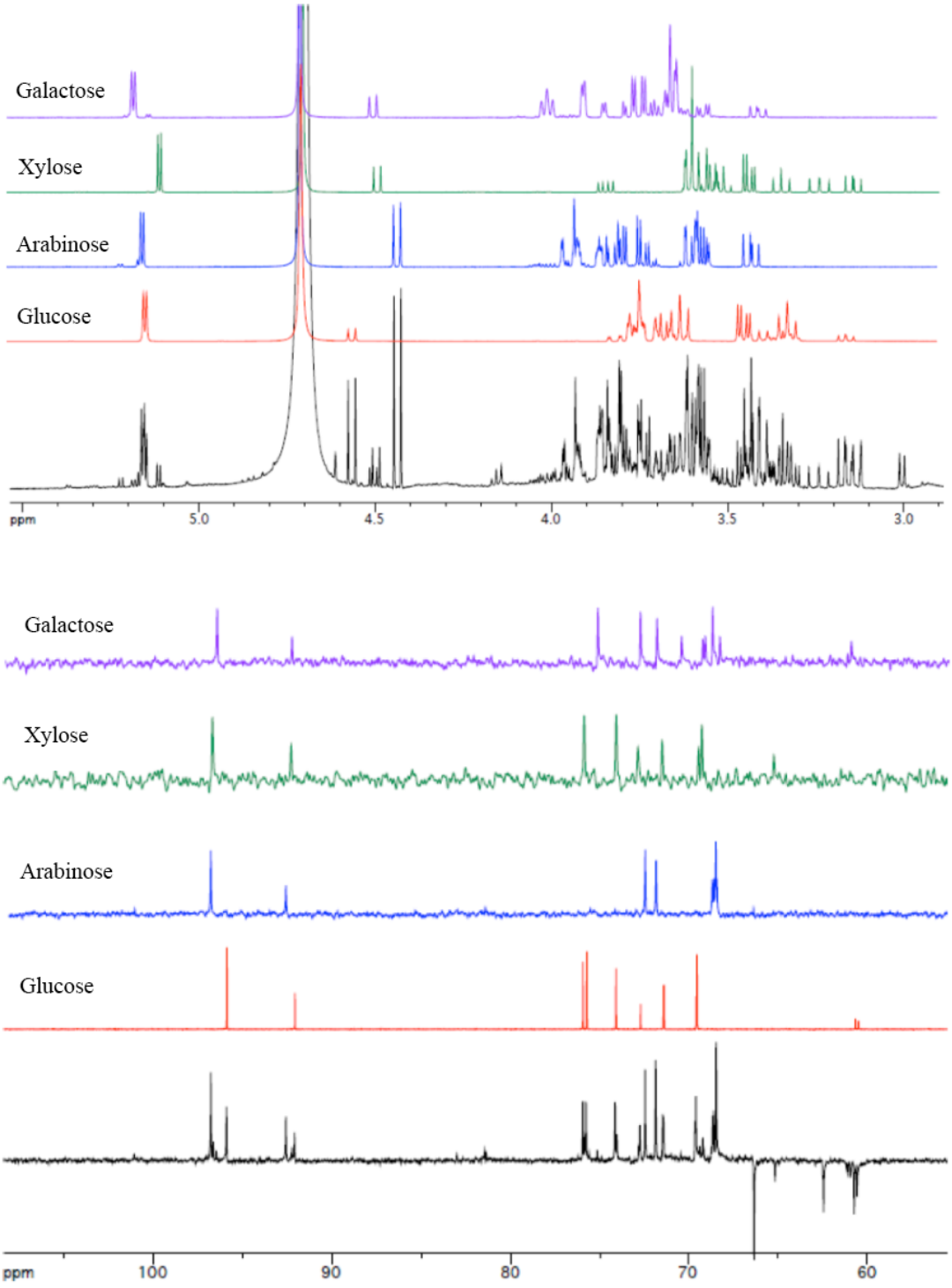

Figure 2.26. ^{1}H NMR and ^{13}C NMR spectra (D_2O, 400 MHz) of the product from the chemical hydrolysis (TFA) of the supernatant (II).

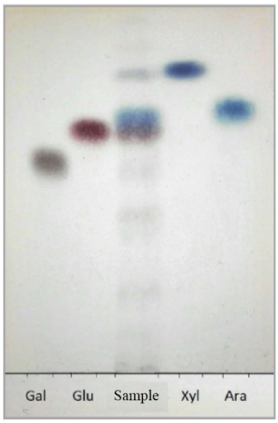

Figure 2.27. TLC of the product from the chemical hydrolysis (TFA) of supernatant (II) in comparison with a standard sample of galactose (Gal), glucose (Glu), xylose (Xyl), arabinose (Ara). Eluent: *n*-propanol/ethyl acetate/water (8:1:1). For TLC visualization reagent: see experimental.

2.4 Conclusions and perspectives

Aim of this part of the thesis was to assess the antihypertensive properties of rice bran protein hydrolysates and their peptide size-based fractions prepared by membrane ultrafiltration using the in *vitro* inhibition of angiotensin I-converting enzyme (ACE) activity.

In order to enhance the extractability of the entrapped protein, rice bran was treated firstly with carbohydrases, enzymes hydrolyzing glycosides, oligo- and polysaccharides. After a screening of four commercial enzyme preparations (Viscozyme, Ceremix, Celluclast, Ultraflo), it was found that a combination of Ceremix and Celluclast led to the highest enrichment of rice bran residue in protein. Two different proteases (Flavourzyme and/or Alcalase) were used to produce hydrolysates which were separated by membrane ultrafiltration into peptide fractions with different sizes (<10 kDa, <5kDa and <1kDa) and investigated for their antihypertensive efficacy.

The highest ACE-inhibitory activity was found in both <1kDa and <5kDa fractions depending on the mode of preparation of the peptide mixtures. It is worth noting that the choice of the carbohydrases on the first step treatment affects significantly the activity of the final hydrolysate. In fact, the samples treated with Ceremix and Celluclast had almost double activity with respect to the samples treated with Viscozyme.

Two of the fractions with peptide sized < 5kDa prepared by using either Flavourzyme or a combination of Alcalase and Flavourzyme, were submitted to sensory analysis that described umami and sweet as the only taste attributes for both samples. The complete lack of the bitter and sour tastes is very positive from the point of utilizing these mixtures as flavor enhancers.

In conclusion, this study suggests that the protein components of rice bran possess peptide amino acid sequences that can be exploited as sources of antihypertensive agents.

The potential of RBP to be used as an attractive raw material for the preparation of peptides with ACE Inhibitory activity by Flavourzyme and Alcalase/Flavourzyme digestion depends on several factors including the processing conditions for extraction of RB protein (selection of carbohydrases) and hydrolysis conditions (selection of proteases). These issues need to be further investigated.

Unless synergistic antihypertensive effect, in most cases total hydrolysates have been found to possess lower activity than single peptides. For this reason, purification will be carried out in order to obtain a single/a few peptide(s) with specific amino acid sequence. Specifically, the hydrolysates will be purified by chromatographic techniques such as gel filtration, reverse phase HPLC followed by identification using spectroscopic methods (NMR and LC-MS). The isolated peptide(s) will be synthesized and its (their) activity, stability and bioavailability will be investigated. Its (their) mechanism of action will be studied by kinetics of enzyme inhibition and molecular docking simulations.

2.5 References

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Chapter 3: Chemo-enzymatic synthesis of sugar fatty acid esters

3.1 Introduction

3.1.1 Surfactant: Definition and categories

The word "**Surfactant**" has been created by the contraction of the three words "**Surface-Active Agents**". These materials can be defined as the molecules that lower the surface or interfacial tension between two or more immiscible substances (either liquids or liquids and solids) and may act as wetting agents, emulsifiers, foaming agents and dispersants.

Surfactants are amphiphilic organic molecules, which means that consist of hydrophobic moieties (known as tails) and hydrophilic moieties (known as heads). When a sufficient amount of surfactant molecules is added in a solution, these molecules tend to combine together forming bigger structures called "micelles". Typically, in an aqueous solution, micelles form an aggregate where the hydrophilic heads are in contact with water and the hydrophobic tails are grouped together in the center of the micelle protected from water (**Figure 3.1**).

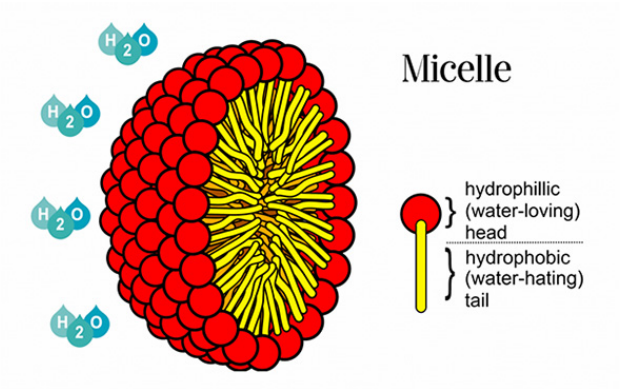


Figure 3.1. Micelle structure in aqueous solutions [1].

According to the polarity of the head group, surfactants can be classified in four categories: anionic, cationic, non-ionic and amphoteric (**Figure 3.2**). Anionic surfactants have a negatively charged hydrophilic end that help them lift and suspend soils in micelles. Therefore, they are typically used in soaps and detergents. Most common anionic surfactants are sulfates, sulfonates phosphates, and gluconates. Cationic surfactants, on the other head, have a positively charged hydrophilic end and can be used as antistatic products (fabric softeners) and antimicrobial agents (disinfectants). For instance, alkyl ammonium chlorides are commonly used cationic surfactants. Amphoteric surfactants, known also as zwitterionic, have both anionic and cationic centers and are used widely in personal care products (shampoos, cosmetics). Amino oxides and betaines are some examples of zwitterionic surfactants. Finally, nonionic surfactants have polar head groups that are not electrically charged and

contain oxygen covalently bonded with the hydrophobic tail. Nonionic surfactants together with anionic ones have numerous applications as wetting, emulsifying, spreading and foaming agents [2]. Some examples of widely used nonionic surfactants are alkyl, fluorinated and silicone-based polyethylene oxide (PEO) surfactants.

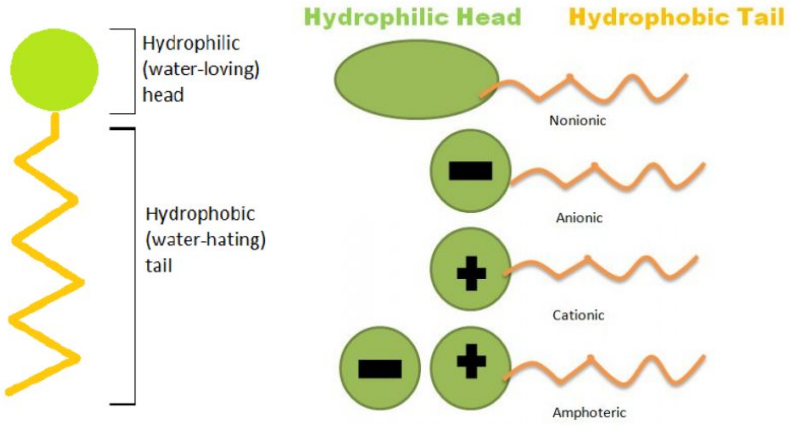


Figure 3.2. Hydrophilic head and hydrophobic tail of surfactants and their classification [3].

The global surfactants market was estimated at $43,655 million in 2017, according to the report published recently by Allied Market Research on 2018, and it is projected to reach $64,408 million by 2025, registering a compound annual growth rate of 5.4% from 2018 to 2025 [4].

The surfactant market is expected to growth in the next years, because of the rising urban population, the increasing home healthcare industry and growing household consumption expenditure, that are triggered by the accelerating economic conditions and improving consumer confidence. However, the surfactant industry growth would be challenged by stringent regulations, volatility in the raw material prices and intense competition. A few notable trends in the last years include increasing preference for sugar surfactants, growing demand of surfactants by pharmaceutical companies, and expanding bio-based surfactants market [5].

3.1.2 Sugar Fatty Acid Esters (SFAEs)

Sugar Fatty Acid Esters (SFAEs) are non-ionic surfactants, characterized as biodegradable, nontoxic and nonirritant. They are also tasteless and odorless with high antimicrobial activity. These properties are making them very attractive compounds for food, pharma and cosmetic industry. In addition, SFAEs cover a wide range of hydrophilic-lipophilic balance (HLB) values, extending their applications as food additives, low calorific sweeteners, emulsifiers, and antimicrobial agents in foods and pharmaceuticals. As food additives, SFAEs were already approved fifty years ago with their initially use being foaming agents for cakes and then, subsequently used as emulsifiers of oil and fat and processed milk products (e.g. whipped cream, coffee whiteners) [6].

Nowadays, the only sugar esters available on the market that are used as emulsifiers, are sorbitan esters and ethoxylated sorbitan esters (known under the trade names Span® and Tween®) and sucrose esters [7]. Sucrose esters were approved for use as food additives in Japan in 1959 and then found worldwide approval for application as nonionic surfactants and emulsifiers in food products [8, 9]. They are usually produced by alkaline-catalyzed transesterification (e.g. K_2CO_3) of a fatty acid methyl ester with sucrose using as solvent dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) at around 90-100 ^oC and/or reduced pressure [10].

3.1.3 Properties of SFAEs

SFAEs are characterized by unique physicochemical properties thanks to the many possibilities for linkage between the hydrophilic sugar head group and the hydrophobic alkyl chain as well as the carbon chain length and the nature of sugar head group. Sugar Esters can be solids, liquids or waxy materials depending on their composition and have special thermal properties [11]. Also, they can exhibit different surface-active properties, as they are able to decrease the surface tension of water, such as HLB values, critical micelle concentration (CMC), emulsifying stability, and foaming ability [12].

Hydrophilic **L**ipophilic **B**alance (**HLB**) is defined the balance of the size and strength of the hydrophilic and lipophilic moieties of a surfactant molecule. The HLB scale ranges from 0 to 20. In the range of 3.5 to 6.0, surfactants are more suitable for use in water in oil (W/O) emulsions. Surfactants with HLB values in the 8 to 18 range are most commonly used in oil in water O/W emulsions [13]. The longer the fatty acid chains in the SFAEs and the higher the degree of esterification, the lower the HLB value (**Figure 3.3**).

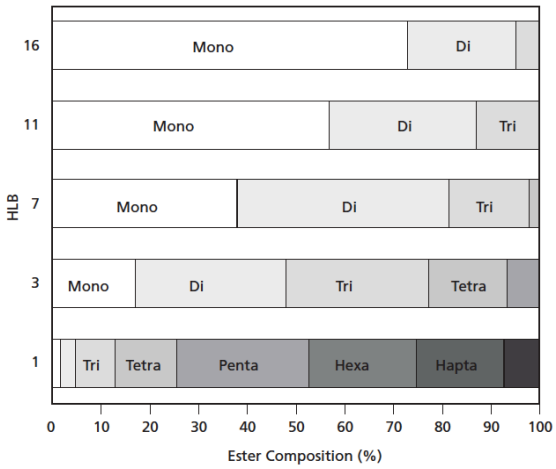


Figure 3.3. HLB values of SFAEs and their composition.

As previously referred, surfactants, and so also sugar esters, tend to form thermodynamic stable aggregates, known as micelles, when they are in aqueous solutions. Critical Micelle Concentration (CMC) is the concentration above which micelles start to form, depending on the surface structure and on the experimental conditions. Below the CMC, the surfactants are solubilized as monomers in the solution. When the CMC is reached, all additional surfactants introduced to the system are promoting the formation of new micelles or the growth of aggregates [14]. CMC is affected by the chain length of the hydrophobic group and, specifically, when the alkyl chain length increases the CMC decreases. In addition, temperature plays an important role in micelles formation and surface activity. By increasing the temperature, larger micelle sizes can be obtained and lower CMC.

Emulsification is one of the most important functions of sugar esters thanks to the original hydrophilic group of sugar/sugar alcohol and the original lipophilic group of fatty acids. By varying the degree of substitution or the fatty acid chain lengths, wide ranges of functionality can be obtained. SFAEs that have three or less fatty acids reduce the surface tension of water.

For instance, Neta et al. [15] studied the biocatalytic esterification of fructose, glucose and lactose with oleic acid in ethanol using CALB (*Candida antarctica* lipase B), immobilized either on acrylic resin or on chitosan, and found out that the derived esters were able to stabilize emulsions of fresh coconut milk. Particularly, lactose ester was the most efficient as it provided the highest decrease in surface tension of the mixture.

3.1.4 Enzymatic versus Chemical synthesis of SFAEs

Sugar esters of glucose, fructose and sucrose are industrially synthesized by trans-esterification of the methyl esters of the corresponding fatty acid by using basic or metallic catalysts. The reactions are normally carried out at high temperatures, above 150 ºC, and reduced pressure. These harsh conditions imply high energy costs for the overall production process. The esters produced under high-temperature processes contain also undesired byproducts, as heterogeneous mixtures of products with different esterification degrees are obtained in different positions of acylation. This may happen because of the intermolecular migration of the acyl group occurring during the reaction process and could be prevented by using complicated protection and deprotection steps for the synthesis of sugar esters. In contrast, the enzymatic synthesis of sugar esters is a one-step process that does not require protection and deprotection of the sugar hydroxyl groups, simplifying the overall synthetic procedure. Also, the use of enzymes requires milder reaction conditions (e.g. temperatures at 40-60 ºC), reducing the energy costs and making the whole process an eco-friendly alternative to the chemical synthesis. Enzymes also provide high degree of product selectivity (chemo-, regio-, enantio- and diastereoselectivity). In case of sugar ester synthesis, the final product is usually a monoester with traces of diesters. As a result, obtaining relatively simple product mixtures, the purification process of the desired sugar ester is also simplified [16]. In **Figure 3.4**, the process steps of the conventional esterification (left) in comparison to the enzymatic one (right), for the production of SFAEs is presented.

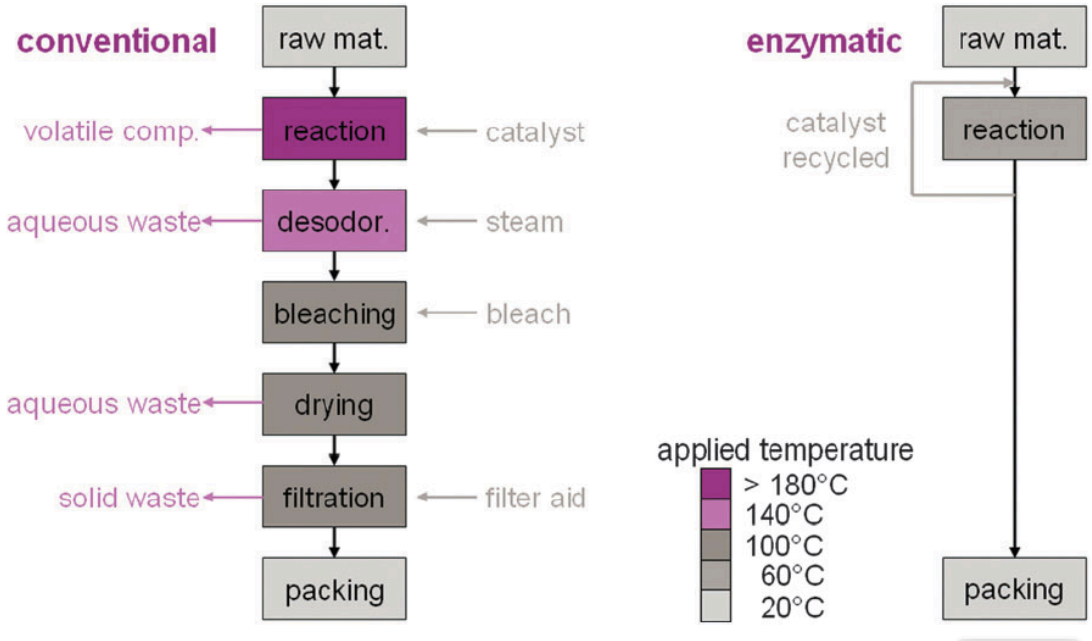


Figure 3.4. Process steps of conventional (left) and enzymatic (right) esterification for production of cosmetic fatty acid esters [17].

3.1.5 Enzymatic approach for the synthesis of SFAEs

In the last years, many scientists are shifting their attention on the utilization of enzymes as biocatalysts for the production of SFAEs, as the demand for greener processes and products is always growing. The enzymatic approach is advantageous regarding the high specificity and regioselectivity of the reaction provided at lower temperatures as well as because of the easier downstream processes. Among the different kind of enzymes, Lipases (triacyl glycerol hydrolases, EC 3.1.1.3) are the most appropriate for the esterification reactions. Lipases naturally catalyze the hydrolysis of triglycerides into di-, or monoglycerides, fatty acids and glycerol in aqueous media. When nonaqueous solvents are used, lipases are able to catalyze the synthesis of esters as they shift the reaction equilibrium, though the presence of a sparing amount of water is essential.

The use of lipases as biocatalysts in organic solvents, apart from inverting the thermodynamic equilibrium that favors synthesis and not hydrolysis, is also advantageous because it allows the suppression of side reactions depended on water, the elimination of microbial contamination, the solubility enhancement of non-polar substrates and the modification of substrate and stereo specificity [18]. On the other hand, enzymatic activity in organic solvents can be very challenging, as many factors contribute to the activity reduction and deactivation of lipases in non-aqueous environment, such as loss of critical water from the surface of the enzyme, deformation of active side geometry, direct denaturation by solvent intrusion into protein structure etc. [18].

According to the mechanism of interfacial activation on triglyceride substrates, the activity of lipases increases upon binding to lipid surfaces [19]. This docking causes a conformational change of the enzyme that involves the opening of the lid, a mobile amphipathic structure. The length and complexity of the lid depend on the enzyme and normally covers the pocket of the catalytic active site of most lipases [20, 21]. The inner walls of this pocket are covered with hydrophobic amino acids and their steric hinderance determines the substrate-selectivity of lipases. Instead, the activity of lipases is provided by the three hydrophilic amino acid residues situated in the bottom of the pocket, in the vicinity of the active site serine residue. The catalytic mechanism is based on the ability of the Ser-His-Asp triad to stabilize the tetrahedral intermediate formed during the hydrolysis of triglycerides in the presence of water. In **Figure 3.5** is presented the catalytic mechanism of lipase *Candida antarctica* B (CALB), based on Ser-His-Asp triad. Histidine and Aspartate residues are required to stabilize the deprotonation of Serine. Then, the nucleophilicity of the hydroxyl residue of serine is enhanced and attacks the carbonyl group of the substrate, forming an acyl-enzyme intermediate. The de-acylation step is strictly related to the type of molecules populating the interface. In the next two steps, the alcohol moiety of the starting ester is released and hosted in the alcohol channel of the catalytic site, while a nucleophile (a water, a monoglyceride or a simple alcohol)

attacks the acylated enzyme, leading to the release of the product and regeneration of the catalytic site [20, 22].

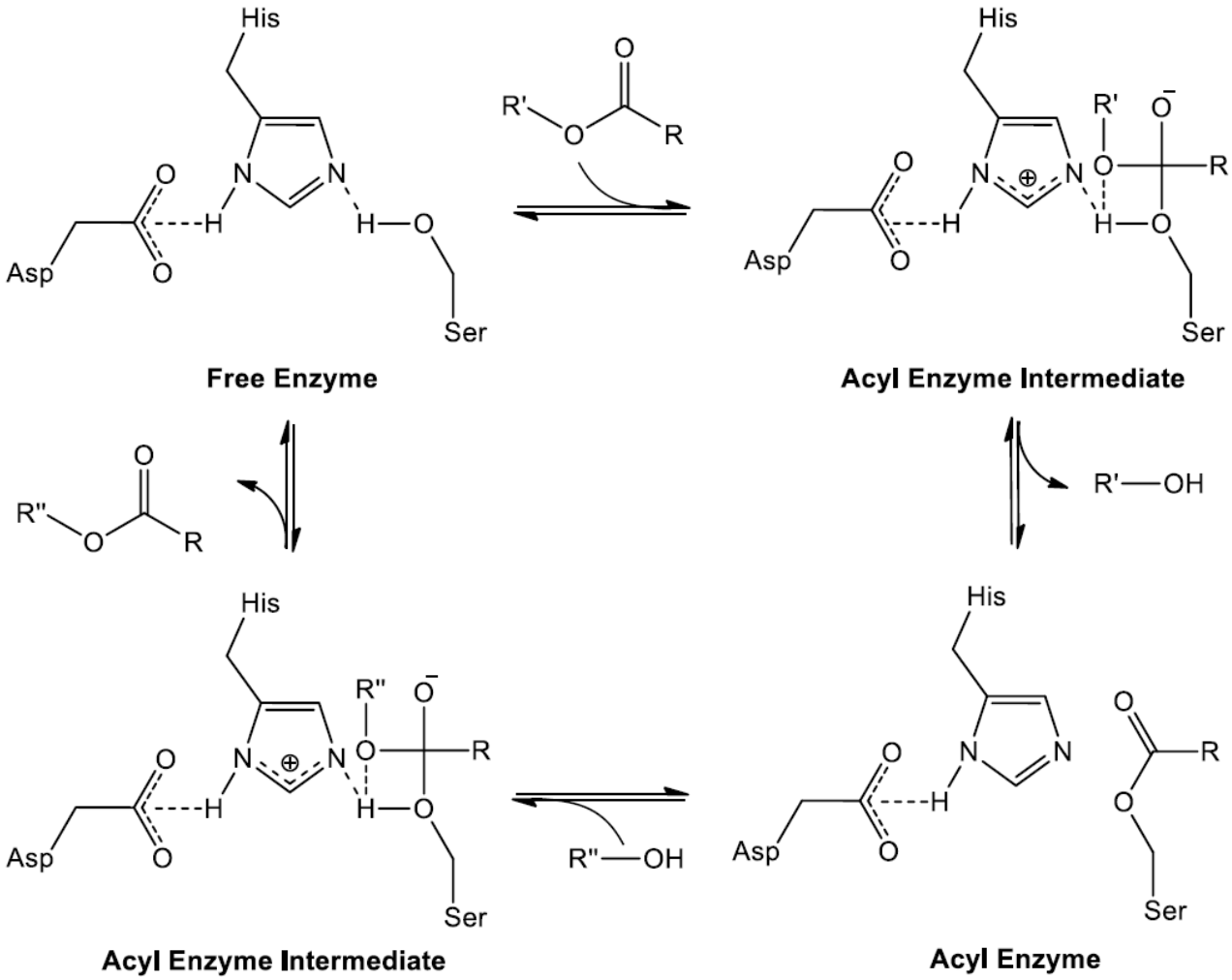


Figure 3.5. The catalytic mechanism of lipase *Candida antarctica* B based on a Ser-His-Asp triad [22].

The lipase Candida antarctica B (CALB) immobilized on acrylic resin is known with the trade name Novozym 435, commercially prepared by Novozymes. In Novozym 435, the immobilization of CALB is succeeded by adsorption on a macroporous acrylic resin named Lewatit VP OC 1600 *via* interfacial activation. This commercial lipase is the most widely used lipase both in research and industry, regarding its successful performance in a variety of reactions (esterification, transesterification, polymer preparation, hydrolysis, amidation etc.) [23].

3.1.6 Parameters that influence the enzymatic synthesis of SFAEs

There are several parameters that affect the enzymatic esterification of sugars and fatty acids that should be taken into account, including the solvents, the substrates, temperature and the presence of water [16, 24, 25].

The choice of the solvent is very essential for the SFAEs synthesis because of the different solubilities of sugars and fatty acids in organic solvents. Ideally, the solubility of the formed ester in the solvent should be low in order to allow its continuously removal by precipitation, driving the reaction equilibrium towards the product. To find a suitable organic solvent for the enzymatic esterification, it is required to consider not only the solubility of the two substrates but also the solvent toxicity on the biocatalyst. Immobilization of lipases seems to improve their resistance in organic solvents [16].

The characteristics of the two reactants (sugars and fatty acids) play important role in the efficiency of a specific lipase to catalyze the esterification process. Lipases derived from different sources exert different catalytic efficiency towards different substrates. Some lipases are highly selective towards long and medium fatty acid chain lengths and some others have a high selectivity for short and branched chain fatty acids. The selectivity and activity of lipases is also affected by ionic and steric effects of the two substrates, such as substitution, unsaturation, branching and carbon chain length [26].

The temperature affects many parameters of the enzymatic esterification reaction including the solubilities of both the reactants and products, the reaction rate, the equilibrium position and the stability of the enzyme. The immobilization of the enzyme is reported to improve their stability at high temperatures. For example, the immobilized *Candida antarctica* lipase B (CALB) is thermally stable at temperatures between 60 and 80 ^{o}C without showing any significant loss of catalytic activity [27, 28].

The activity of water is very crucial for the production of sugar esters in non-aqueous solvents. Even though the ester formation does not require the presence of water, a small amount of it is necessary for achieving the hydration of the enzyme and maintain it stable and active [29]. During the esterification process, water is also produced and, if not removed, can be accumulated forcing the reaction equilibrium towards the hydrolysis of the previously formed ester. Consequently, the amount of water should be controlled and kept extremely low to allow the ester synthesis, but not completely absent. Chamouleau et al. studied the effect of activity and water content on the enzymatic esterification of fructose with palmitic acid using molecular sieves and observed that even though the use of a drying agent increased the performance of the reaction, it caused also a loss of enzymatic selectivity. This loss was due to the fact that molecular sieves remove also water in the

microenvironment of the enzyme, increasing the hydrophobicity and consequently decreasing fructose solubility [30].

An extensive study on lipase synthesis of sugar fatty acid esters have been performed by Šabeder et al [31]. According to this work, fructose esters were synthesized enzymatically by using CALB and many parameters that influence the esterification reaction were studied deeply. Specifically, comparison was made by studying different organic solvents, biocatalyst concentration, molecular, molecular sieves concentration, temperature, stirring rate and different fatty acids as acyl donors (palmitic and lauric acid). The optimum conditions for the synthesis of fructose palmitate in 2-methyl-2-butanol (2M2B) were determined: 10 % (w/w of substrates) of CALB and 12.1% of molecular sieves at 60 ^{o}C for 72h at stirring rate of 600 rpm.

3.1.7 Enzyme immobilization methods

Lipases can be used for the esterification reaction either in free form or immobilized on a solid surface. Immobilized enzymes are defined as the ones "that are physically attached to specific solid supports and consequently confined and can be used repeatedly and continuously while maintaining their catalytic activities" [32]. Immobilized enzymes are generally preferable in the industrial sector as immobilization technology provides numerous advantages, such as better stability and activity, easier control of the reaction, easy separation of the enzyme from the derived products, reusability and improved productivity and economic efficiency [33]. Some widely used supports for immobilization of enzymes are cellulose, sepharose, agarose beads, zeolites, porous glass, epoxy resins like sepabeads, offering large areas for enzyme–support interactions [34]. There are several techniques that could be used for the immobilization of the enzymes. Most common ones are the absorption, covalent bonding, entrapment and cross-linking (**Figure 3.6**).

Adsorption on a solid substrate is considered a straightforward immobilization technique, based on weak bonds such as Van der Waal's forces, electrostatic and hydrophobic interactions. This method involves the contact of the solid support with a solution of the enzyme for a certain period of time under suitable conditions in order to maintain the enzymatic activity. The non-adsorbed enzyme molecules are removed from the surface by washing with buffer [33]. This method leaves the enzyme structure intact, allowing enzymes to retain their activity and also promoting the transport of the substrates to the enzyme's active center. A disadvantage of this method is the low stability of the derived immobilized enzymes, that may lead to a fast washing out of the enzyme from the carrier [35]. Adsorption method includes also the following subcategories: physical adsorption, electrostatic binding and hydrophobic adsorption.

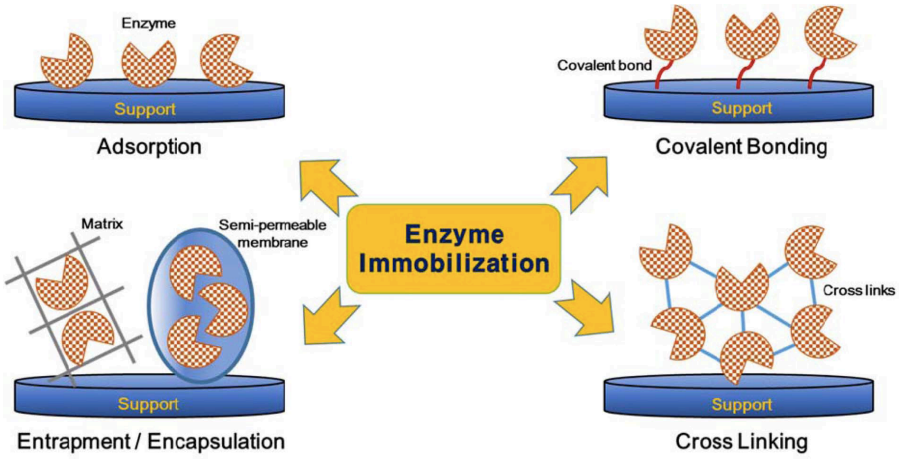


Figure 3.6. Enzyme immobilization techniques.

Covalent binding is another immobilization technique where stable complexes are formed through covalent bonds between the enzyme functional groups and a support matrix. This method is carried out by firstly activating the surface using linker molecules, such as glutaraldehyde or carbodiimide, and then by covalent coupling of the desired enzyme to the activated support. These linkers are acting as the bridge between surface and enzyme via covalent bonding. This technique provides strong bindings between enzymes and support matrix, minimizing the enzyme leakage from the support. Disadvantages of covalent linkage include reduced activity of proteins, by forming linkage on active sites, use of toxic reagents and reduced activity if the covalent bond is formed on the active site of the enzyme [36].

During the immobilization by entrapment, the enzyme is not directly attached to the support surface but entrapped within a polymeric network. This network allows only the pass of substrate and products but retains the enzyme, thus constraining the diffusion of the enzyme. This method is carried out by mixing the enzyme into a monomer solution and then polymerization is followed by a chemical reaction or by changing experimental conditions. Some advantages of this technique include better stability, less enzyme leaching and ease optimization of pH, polarity or amphiphilicity of the enzyme by modifying the encapsulating material [37]. However, this method has some limitations: low enzyme capacity and possible leakage of the entrapped enzymes caused by the large pore size of support matrix.

Cross-linking immobilization is an irreversible method performed by the formation of intermolecular cross linkages between the enzyme molecules by covalent bonds. The process is carried out by using a multifunctional reagent that acts as linkers, connecting enzyme molecules into three-dimensional cross-linked aggregates. It is a simple method based on the strong chemical binding of enzyme biomolecules, providing minimal enzyme leakage, possibility to adjust enzyme microenvironment by using suitable stabilizing agents and subsequently increase stability. This method, however, requires the use of glutaraldehyde, which could result in severe enzyme modifications, leading to enzyme conformational changes and loss of activity.

3.1.8 Scope of the work

The second part of the present PhD thesis is the enzymatic synthesis of Sugar Fatty Acid Esters (SFAEs). SFAEs are non-ionic surfactants usually called sugar esters, which are characterized by excellent emulsifying, stabilizing and detergency properties. SFAEs are widely used in many market sectors (i.e. food, detergent, cosmetic and pharmaceutical industry); depending on carbon chain length and nature of the sugar head group, SFAEs cover a wide range of hydrophilic-lipophilic balance (HLB). They have many advantages over petrochemical-derived surfactants as they are neither harmful to the environment nor skin irritants, are fully biodegradable and non-toxic. More interestingly, they can be produced from renewable resources [38]. The aim of this work was to set up a chemoenzymatic synthesis of SFAEs as an eco-friendly alternative to the chemical methods [39, 40, 41] that could allow in a future the utilization of vegetable oils and sugar rich biomasses as starting materials. As a first approach to this topic, it was decided to study the esterification reaction of monosaccharides (specifically glucose and galactose) with free fatty acids unsaturated (oleic acid) or saturated (palmitic acid, lauric acid, stearic acid) via enzymatic approach.

3.2 Experimental

3.2.1. Materials and Methods

All reagents (glucose, galactose, oleic acid, palmitic acid, stearic acid, lauric acid) and solvents (Dimethylsulfoxide, tert-butanol, 2-methyl-2butanol, 2-butanone, hexane, heptane, tert-butyl methyl ether, dichloromethane, methanol, ethanol) used in this work were purchased from Sigma-Aldrich, VWR International, Fluorochem, Fluka, Herk-Schuchardt and Merk and were used without further purification. All reagents and solvents were of commercial quality from freshly opened containers.

Commercial immobilized lipase from *Candida antarctica* B (CALB, Novozym® 435) was kindly provided by Novozymes (Bagsvard, Denmark). The following commercial lipases used in the screening process were purchased by Amano Enzymes (Japan): *Porcine pancreas lipase* (PPL), *Pseudomonas fluorescens lipase, Rhizopus niveus lipase, Aspergillus niger lipasem, Phospholipase D, Thermomyces lanuginosus lipase* (TLL), *Candida rugosa lipase* (CRL), *Pseudomonas cepacia lipase* (PCL), *Rhizomucor miehei lipase* (RML).

Analytical and Column Chromatography

Flash column chromatography (CC) purifications were carried out using silica gel 60 with particle size 40–63 μm (Merck, Darmstadt Germany). Reactions were monitored by thin layer chromatography (TLC), which was performed on commercial silica gel on aluminum sheets (Merck, Silica gel 60 F254). TLC plates were afterwards dipped into a staining solution that contains ammonium molybdate $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (21 g) and Ce(IV)sulphate (1g) and sulphuric acid (98%, 31 ml in total 500 ml aq. solution); upon heating, organic substances were oxidized giving blue spots on a white to light blue background.

NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded at 400.13 and 100.61 Hz, respectively, on a *Bruker AVANCE 400* spectrometer equipped with *TOPSPIN* software package (Bruker, Karlsruhe, Germany) at 300 K, unless stated otherwise. ^1H and ^{13}C chemical shifts (δ) are given in parts per million and were referenced to the solvent signals (δ_H 7.26 - δ_C 77.16, δ_H 3.31 - δ_C 49.00, δ_H 2.50 - δ_C 39.52 and δ_H 4.79 ppm from tetramethylsilane (TMS) for CD_3OD and DMSO-d_6 respectively).

^{1}H NMR signals were assigned with the aid of ^{1}H-^{1}H correlation spectroscopy (^{1}H-^{1}H COSY). ^{13}C NMR signal multiplicities were based on APT (Attached proton test) spectra. ^{13}C NMR signals were assigned with the aid of ^{1}H-^{13}C correlation experiments (Heteronuclear multiple quantum correlation spectroscopy, HSQC, and Heteronuclear multiple bond correlation spectroscopy, HMBC).

Mass spectroscopy

Electrospray ionization mass spectra (ESI-MS or ESI-Q-Tof-MS) were recorded on a Thermo Finnigan LCQ Advantage spectrometer (Hemel Hempstead, Hertfordshire, U.K.) and Micromass Q-Tof micro mass spectrometer (Waters, Milford, Massachussets, U.S.A.), respectively.

3.2.2. Enzyme screening

A series of several lipases have been used both in free and immobilized form, to study the esterification of sugars with fatty acids. Experiments were performed firstly by using glucose as an acyl acceptor and oleic acid and as an acyl donor, according to the following procedure:

The reactions were carried out in a glass reactor heated in an oil bath at 40 or 65 ºC for 24 to 72 h. Glucose (1 mmol) and oleic acid (1 mmol or 2 mmols) were transferred in the reactor and were mixed under magnetic stirring (200 rpm) in a solution of dimethyl sulfoxide (DMSO) and tert-butanol (t-BuOH) 1:9 (5 ml). After 15 min, molecular sieves (90 mg; 12.1% w/w) and the selected enzyme (10-12% w/$w_{reactants}$) were transferred to the reaction mixture and the reaction was left under stirring at the desired temperature. Molecular sieves were added for the removal of the water produced from the esterification in order to avoid the hydrolysis of the formed ester(s).

The reaction was monitored by TLC (CH_2Cl_2/MeOH; 10:1) and samples were analyzed after 24, 48 and 72 h. Two samples were taken from the reaction mixture each time: One was diluted in MeOH and the other one in a biphasic system of hexane: H_2O in order to check the presence of any surfactant that could lead to the formation of an emulsion between the two non-mixed solvents after vigorous shaking. Only the organic phase was deposited on the TLC plate.

The following lipases tested have been tested:

- CALB (*Candida antarctica B,* Novozym 435)
- *Rhizopus niveus*
- *Candida rugosa*
- *Pseudomonas cepacia*
- *Porcine pancreas*
- *Pseudomonas fluorescens*
- *Thermomyces lanuginosus*
- *Phospholipase D*
- *Aspergillus niger*

3.2.3. Enzyme immobilization and activity assays

The enzyme immobilization was performed at the Department of Drug Sciences of the University in Pavia and aimed at the preparation of stable and active enzymes under the selected reaction conditions (DMSO:t-BuOH 1:9, 65 °C). Immobilization was achieved either by adsorption or covalent method. The work was divided into four parts, that included:

1. Enzyme activity assay without the reaction conditions (Free enzyme).
2. Enzyme immobilization.
3. Enzyme activity assay after immobilization without reaction conditions
4. Enzyme stability assay under reaction conditions.

Four candidate lipases were tested:

I. *Candida rugosa lipase* (CRL)
II. *Pseudomonas cepacia lipase* (PCL)
III. *Thermomyces lanuginosus lipase* (TLL)
IV. *Rhizomucor miehei lipase* (RML)

A weighed amount of enzyme is added to a phosphate buffer (25mM pH 7 for the adsorption immobilization and 1M pH 8 for the covalent immobilization) in a plastic container and the mixture is left on mechanical rollers for 1 hour for mixing. As supports are used the octyl-Sepharose and Sepabeads, depending on the immobilization method: adsorption or covalent immobilization, respectively. The support is firstly washed with water on a Buchner filter (1g) and then is added to the enzyme – buffer mixture. The ratio between the volume of the support ($V_{support}$) and the total volume (V_{total}) is 1:10 (support density= 0.7 g/mL). The mixture is left on the rollers under gentle agitation for 24 hours. The immobilized enzymes ("derivates") were recovered by filtration under vacuum, washed with water, dried and stored at 4 ^{o}C.

The enzymes, both in free and immobilized form, were tested for their retained activity according to the following procedure [42]. In a plastic baker with 18.4 ml of Tris HCl buffer (2.5 mM, pH 7) and NaCl (100 mM), are added 0.6 ml of acetonitrile (CH_3CN) and 1ml tripropionin (substrate) under mechanical agitation. Then, 10 mg of enzyme are added to the above mixture that catalyze the hydrolysis of tripropionin to dipropionin and propionic acid (**Figure 3.7**).

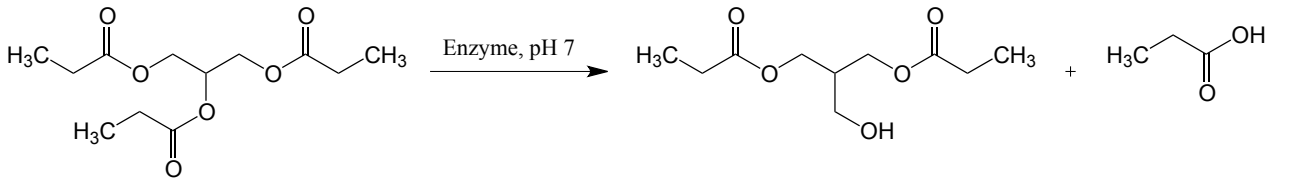


Figure 3.7. Enzymatic hydrolysis of tripropionin to propionic acid.

The hydrolytic activity of the enzyme was quantified by titration at 25 ºC and pH 7 using a pH-Stat (718 STAT Titrino, Metrohm, Herisau, Switzerland). The enzyme was added, and the pH was maintained at 7.0 using 0.1M NaOH as titrant. Experiments were done in triplicate; thus, the average activity was calculated. The hydrolytic activity was evaluated by measuring the initial hydrolysis rate calculated from the NaOH (100 mM) consumption (mmol NaOH min^{-1}/mL of enzyme preparation). One International Unit (UI) of lipase activity was defined as the amount of enzyme that hydrolyzes 1μmol of tripropionin per min at pH 7.0 and 25°C to give propionic acid [43]. The activity values were calculated using the following equation:

$$\text{Activity [UI/g]} = \frac{V_{NaOH/min} \times C_{NaOH}}{\text{W}_{\text{enzyme}}}$$

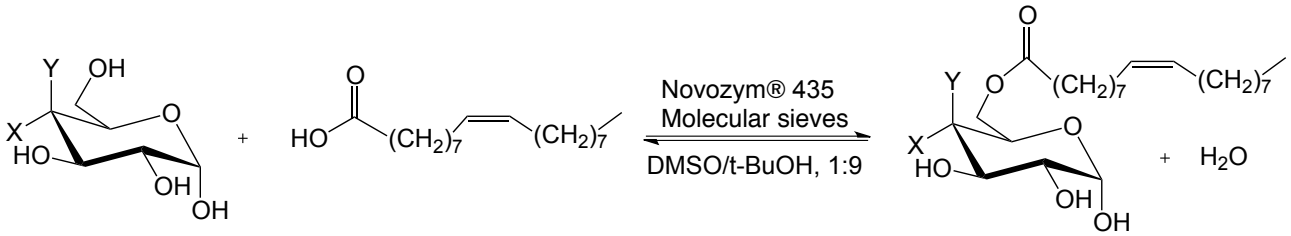
where $V_{NaOH/min}$ are the ml of titrant (NaOH) per min, C_{NaOH} the concentration of titrant equal to 100mM and W_{enzyme} the weight of enzyme in g. Also, the percentage of the retained activity was calculated as follows:

$$\%\ \textit{R}\text{etained Activity} = \frac{\text{Activity}_{\text{immobilized}}}{\text{Activity}_{\text{free}}} \times 100$$

The enzyme stability assays are actually activity assays performed to the enzymes used under the final reaction conditions (solvent mixture t-BuOH: DMSO, 1:9, 65 ºC) in definite time intervals. The experiments were performed in small scale in Eppendorf tubes with total reaction volume of 1 ml (solvents) and 40 mg of enzyme. The tubes were heated at 65 ºC in water bath. Finally, the enzyme was recovered by simple filtration.

3.2.4. Synthesis of Glucose and Galactose monooleate

$C_{24}H_{44}O_7$; MW: 444.60



X: OH; Y: H for glucose
X:H; Y:OH for galactose

Oleic Acid

GluMO or GalMO

Glucose monooleate and Galactose monooleate were prepared according to the following experimental procedure:

The reactions were carried out in a glass reactor heated in an oil bath at 65 °C for 48 h. Sugar (Glu or Gal, 900 mg; 5 mmol) and oleic acid (2825 mg; 10 mmol) were transferred in the reactor and were mixed under magnetic stirring (200 rpm) in a solution of DMSO and t-BuOH 1:9 (25 ml). After 15 min, molecular sieves (450 mg; 12.1% w/w) and the enzyme Novozym® 435 (375 mg; 10% $w/w_{reactants}$) were transferred to the reaction mixture and the reaction was left under stirring for 48 h at the desired temperature. Molecular sieves were added for the removal of the water produced from the esterification in order to avoid the hydrolysis of the formed ester(s).

The reaction was monitored by TLC (CH_2Cl_2/MeOH; 10:1) and samples were analyzed after 24 and 48 h. Two samples were taken from the reaction mixture each time: One was diluted in MeOH and the other one in a biphasic system of hexane: H_2O in order to check the presence of any surfactant that could lead to the formation of an emulsion between the two non-mixed solvents after vigorous shaking. Only the organic phase was deposited on the TLC plate.

After 48 h, the reaction mixture was filtered in order to remove the molecular sieves and immobilized CALB, and washed with t-BuOH, which was removed under vacuum conditions. The enzyme was recovered after washings with H_2O (50 ml) and dried in the oven at 55 °C overnight. The molecular sieves were also washed and reactivated for reuse.

Glucose Monooleate

Due to the difficulties encountered in removing the oleic acid, the product purification was carried out by two successive flash columns chromatography (CH_2Cl_2/MeOH; 10:1; 12:1), and Glucose Monooleate (184 mg; 0.41 mmol) was obtained as a white powder.

The reaction was carried out also with lower amounts of starting reagents, obtaining similar yields.

Yield: **8**%.

Rf (Eluent CH_2Cl_2/MeOH; 10:1): 0.28

^{1}H NMR (DMSO-*d*$_6$ + D$_2$O (1 drop), 400 MHz): δ (ppm) 5.32 (m, 2H, C**H**=C**H**), 4.89 (d, J = 3.6 Hz, 1H, H^1), 4.25 (dd, J = 11.7, 1.9 Hz, 1H, H^{6a}), 3.99 (dd, J = 11.7, 6.1 Hz, 1H, H^{6b}), 3.76 (ddd, J = 9.8, 6.0, 1.8 Hz, 1H, H^5), 3.43 (t, J = 9.2 Hz, 1H, H^3), 3.12 (dd, J = 9.6, 3.7 Hz, 1H, H^2), 3.04 (dd, J = 9.9, 8.9 Hz, 1H, H^4), 2.26 (t, J = 7.4 Hz, 2H, OOCC**H$_2$**), 1.97 (m, 4H, C**H$_2$**CH=CHC**H$_2$**), 1.50 (m, 2H, OOCCH$_2$C**H$_2$**), 1.23 (s, 20H, (C**H$_2$**)$_4$CH$_2$CH=CHCH$_2$(C**H$_2$**)$_6$), 0.85 (t, J = 6.8 Hz, 3H, CH$_2$C**H$_3$**).

^{13}C NMR (DMSO-*d*$_6$ + D$_2$O (1 drop), 100 MHz): δ (ppm) 176.33 (OO**C**), 132.97 (**C**H=**C**H), 95.46 (C^1), 75.96 (C^3), 75.29 (C^2), 73.66 (C^4), 72.40 (C^5), 67.09 (C^6), 36.75 (OOC**C**H$_2$), 34.57 (**C**H$_2$CH$_2$CH$_3$), 32.72 - 31.36 (**C**H$_2$)$_4$CH$_2$CH=CHCH$_2$(**C**H$_2$)$_4$), 29.86 (**C**H$_2$CH=CH**C**H$_2$), 27.75 (OOCCH$_2$**C**H$_2$), 25.38 (**C**H$_2$CH$_3$), 17.23 (**C**H$_3$).

MS (ESI$^+$): *m/z* theoretically calculated for $[C_{24}H_{44}O_7]^+$: 444.60; experimentally obtained: 467.97 $[M+Na]^+$.

<u>Galactose monooleate</u>

Due to the difficulties encountered in removing the oleic acid, the product purification was carried out by flash column chromatography, conducted in non-isocratic conditions (CH_2Cl_2/MeOH; 5 column volumes 20:1 → 10:1), and Galactose Monooleate (22 mg; 0.05 mmol) was obtained as a white powder.

The reaction was carried out also with lower amounts of starting reagents, without being able in obtaining pure product.

Yield: **1**%.

Rf (Eluent CH_2Cl_2/MeOH; 10:1): 0.27

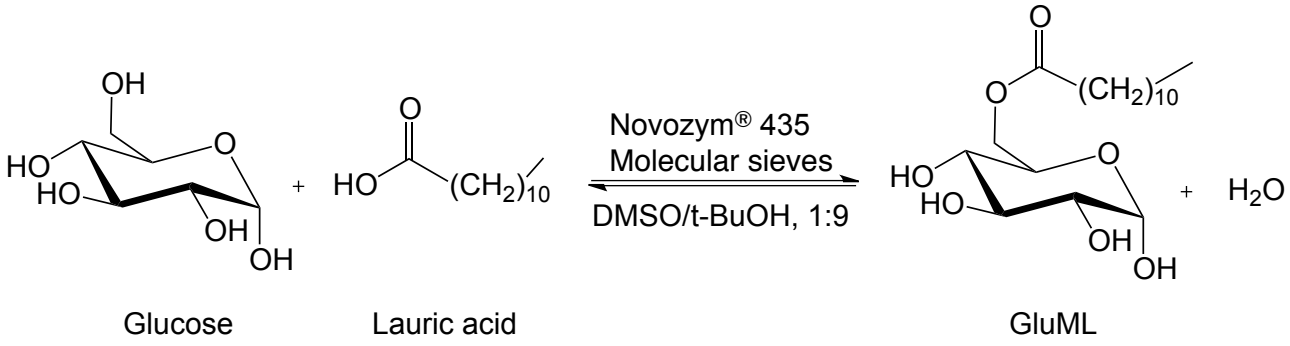
^{1}H NMR (DMSO-*d*$_6$ + D$_2$O (1 drop), 400 MHz): δ (ppm) 5.30 (m, 2H, **C**H=**C**H), 4.93 (d, *J* = 3.3 Hz, 1H, H^1), 4.04 (m, 2H, H^{6a}, H^{6b}), 3.98 (dd, *J* = 12.0, 6.3 Hz, 1H, H^5), 3.65 (d, *J* = 1.6 Hz, 1H, H^4), 3.55 (dd, *J* = 10.0, 2.7 Hz, 1H, H^3), 3.50 (dd, *J* = 10.0, 3.4 Hz, 1H, H^2), 2.25 (t, *J* = 7.3 Hz, 2H, OOCC**H$_2$**), 1.95 (d, *J* = 5.4 Hz, 4H, C**H$_2$**CH=CHC**H$_2$**), 1.48 (br s, 2H, OOCCH$_2$C**H$_2$**), 1.22 (s, 20H, (C**H$_2$**)$_4$CH$_2$CH=CHCH$_2$(C**H$_2$**)$_6$), 0.83 (t, *J* = 6.5 Hz, 3H, C**H$_3$**).

^{13}C NMR (DMSO-*d*$_6$ + D$_2$O (1 drop), 100 MHz): δ (ppm) 173.63 (OO**C**), 130.12 (**C**H=**C**H), 92.85 (C^1), 69.51 (C^5), 69.22 (C^2), 68.67 (C^3), 68.02 (C^4), 64.33 (C^6), 33.82 (OOC**C**H$_2$), 31.66 (**C**H$_2$CH$_2$CH$_3$), 29.63 - 28.51 (**C**H$_2$)$_4$CH$_2$CH=CHCH$_2$(**C**H$_2$)$_4$), 26.93 (**C**H$_2$CH=CH**C**H$_2$), 24.78 (OOCCH$_2$**C**H$_2$), 22.48 (**C**H$_2$CH$_3$), 14.34 (**C**H$_3$).

MS (ESI$^+$): *m/z* theoretically calculated for $[C_{24}H_{44}O_7]^+$: 444.60; experimentally obtained: 467.55 $[M+Na]^+$, 481.51 $[M+K]^+$, 499.3 $[M+Na+MeOH-H]^+$, 411.05 $[2M+Na]^+$.

3.2.5. Synthesis of Glucose monolaurate

$C_{18}H_{34}O_7$; MW: 362.46.



The reactions were carried out in a glass reactor heated in an oil bath at different temperatures (65 or 75 °C) for 24 or 48 h, according to **Table 3.1**. Glucose and lauric acid at different molar ratios (1:1, 1:2 or 1:3) were transferred in the reactor and were mixed under magnetic stirring (200 rpm) in a solution of DMSO and t-BuOH 1:9. After 15 min, molecular sieves (450 mg; 12.1% w/w) and the enzyme Novozym® 435 (375 mg; 10% $w/w_{reactants}$) were transferred to the reaction mixture and the reaction was left under stirring at the desired temperature and time. Molecular sieves were added for the removal of the water produced from the esterification in order to avoid the hydrolysis of the formed ester(s).

The reaction was monitored by TLC (CH_2Cl_2/MeOH; 10:1) and samples were analyzed after 24 and 48 h. Two samples were taken from the reaction mixture each time: One was diluted in MeOH and the other one in a biphasic system of hexane: H_2O in order to check the presence of any surfactant that could lead to the formation of an emulsion between the two non-mixed solvents after vigorous shaking. Only the organic phase was deposited on the TLC plate.

The separation of the produced sugar fatty ester succeeded according to the following procedure: After the end of the reaction, the reaction mixture was filtered in order to remove the molecular sieves and immobilized CALB, and was washed with t-BuOH, which was removed under vacuum conditions. The enzyme was recovered after washings with H_2O (50 ml) and dried in the oven at 55 °C overnight. The molecular sieves were also washed and reactivated for reuse. The so-obtained filtrate contains now DMSO, unreacted glucose, fatty acid and the formed ester.

In order to selectively remove the DMSO and the unreacted glucose from this mixture, H_2O (20 vol/vol_{DMSO}) was added and the so-formed white slurry (fatty acid and sugar-fatty ester) was then filtrate. The white precipitate obtained from the filtration was dried overnight at room temperature. The product purification was carried out by selective dissolution of the free fatty acid in hexane (0.05 $vol/w_{Lauric\ acid}$). A fluffy suspension of the product was obtained, which was centrifuged to separate the supernatant.

Table 3.1. Experimental conditions of glucose monolaurate synthesis.

CODE	Sugar/Fatty Acid Ratio	Reaction Temperature [°C]	Reaction Time [h]	NMR Yield%	Yield %
Glu-LA 1	1:2	65	48	20 ± 2	13
Glu-LA 2	1:2	65	48	22 ± 2	16
Glu-LA 3	1:2	65	24	18 ± 2	15
Glu-LA 4	1:3	75	24	25 ± 2	21
Glu-LA 5	10:30	75	24	23 ± 2	14

Glucose Monolaurate (**90% Purity**) was obtained as a white fluffy powder and its purity was determined by ^{1}H NMR test, comparing the area ratio of the protons of the α-carbons (-C**H$_2$**COOH) of bonded and free fatty acid.

Rf (Eluent CH_2Cl_2/MeOH; 10:1): 0.28

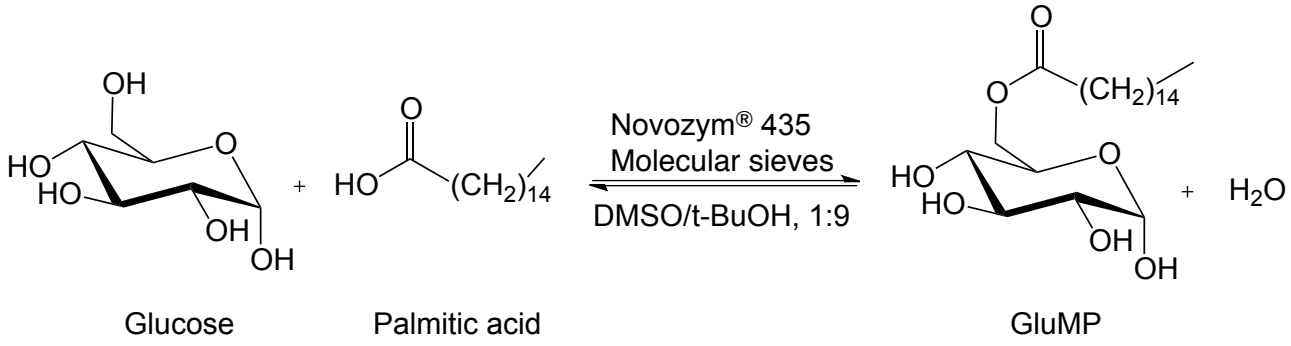
^{1}H NMR (DMSO-*d$_6$* + D$_2$O (1 drop), 400 MHz): δ (ppm) 4.89 (d, J = 3.3 Hz, 1H, H^{1}), 4.25 (d, J = 11.5 Hz, 1H, H^{6a}), 3.99 (dd, J = 11.6, 6.1 Hz, 1H, H^{6b}), 3.75 (m, 1H, H^{5}), 3.43 (t, J = 9.1 Hz, 1H, H^{3}), 3.12 (dd, J = 9.4, 3.3 Hz, 1H, H^{2}), 3.04 (t, J = 9.3 Hz, 1H, H^{4}), 2.26 (t, J = 7.1 Hz, 2H, OOCC**H$_2$**), 1.50 (m, 2H, OOCCH$_2$C**H$_2$**), 1.23 (s, 16H, (C**H$_2$**)$_8$), 0.84 (t, J = 6.2 Hz, 3H, C**H$_3$**).

^{13}C NMR (DMSO-*d$_6$* + D$_2$O (1 drop), 100 MHz): δ (ppm) 176.36 (OO**C**), 95.47 (C^{1}), 75.99 (C^{3}), 75.31 (C^{2}), 73.68 (C^{4}), 72.41 (C^{5}), 67.09 (C^{6}), 36.77 (OOC**C**H$_2$), 34.59 (**C**H$_2$CH$_2$CH$_3$), 43.64 - 41.68 ((**C**H$_2$)$_6$), 27.76 (OOCCH$_2$**C**H$_2$), 25.39 (**C**H$_2$CH$_3$), 17.25 (**C**H$_3$).

MS (ESI$^+$): *m/z* theoretically calculated for $[C_{18}H_{34}O_7]^+$: 362.46; experimentally obtained: 385.41 $[M+Na]^+$, 747.29 $[2M+Na]^+$.

3.2.6. Synthesis of Glucose monopalmitate

$C_{22}H_{42}O_7$; MW: 418.56.



The reactions were carried out in a glass reactor heated in an oil bath at different temperatures (55, 65 or 75 °C) for 24 or 48 h, according to **Table 3.2**. Glucose and palmitic acid at different molar ratios (1:1, 1:2 etc.) were transferred in the reactor and were mixed under magnetic stirring (200 rpm) in different solvents (**Table 3.2**). After 15 min, molecular sieves (450 mg; 12.1% w/w) and the enzyme Novozym® 435 (375 mg; 10% $w/w_{reactants}$) were transferred to the reaction mixture and the reaction was left under stirring at the desired temperature and time. Molecular sieves were added for the removal of the water produced from the esterification in order to avoid the hydrolysis of the formed ester(s).

The reaction was monitored by TLC (CH_2Cl_2/MeOH; 10:1) and samples were analyzed after 24 and 48 h. Two samples were taken from the reaction mixture each time: One was diluted in MeOH and the other one in a biphasic system of hexane: H_2O in order to check the presence of any surfactant that could lead to the formation of an emulsion between the two non-mixed solvents after vigorous shaking. Only the organic phase was deposited on the TLC plate.

After the end of the reaction, the reaction mixture was filtered in order to remove the molecular sieves and immobilized CALB, and was washed with t-BuOH, which was removed under vacuum conditions. The enzyme was recovered after washings with H_2O (50 ml) and dried in the oven at 55 °C overnight. The molecular sieves were also washed and reactivated for reuse. The so-obtained filtrate contains now DMSO, unreacted glucose, fatty acid and the formed ester. In order to selectively remove the DMSO and the unreacted glucose from this mixture, H_2O (40 vol/vol $_{DMSO}$) was added and the so-formed white slurry (fatty acid and sugar-fatty ester) was then filtrate. The white precipitate obtained from the filtration was dried overnight at room temperature. The product purification was carried out by selective dissolution of the free fatty acid in hot heptane (0.1 $vol/w_{Palmitic\ acid}$).

A suspension of the product **Glucose Monopalmitate** (**89% Purity**) was obtained as a white powder and its purity was determined by ^{1}H NMR test, comparing the area ratio of the protons of the α-carbons (-C**H₂**COOH) of bonded and free fatty acid.

Rf (Eluent CH_2Cl_2/MeOH; 10:1): 0.28

Table 3.2. Experimental conditions of glucose monopalmitate synthesis.

CODE	Sugar/Fatty Acid Ratio	Solvents	Reaction Temp. [°C]	Reaction Time [h]	NMR Yield%	Yield %
Glu-PA 1	1:2	DMSO/t-BuOH 1:9	65	48	25 ± 2	19
Glu-PA 2	1:2	DMSO/t-BuOH 1:9	65	48	21 ± 2	18
Glu-PA 3	1:2	DMSO/t-BuOH 1:9	65	24	20 ± 2	13
STD 1	1:1	DMSO/t-BuOH 1:9	55	24	11 ± 2	2
STD 2	1:3	DMSO/t-BuOH 1:9	55	24	19 ± 2	10
STD 3	1:1	DMSO/t-BuOH 1:9	55	48	11 ± 2	7
STD 4	1:3	DMSO/t-BuOH 1:9	55	48	15 ± 2	9
STD 5	1:1	DMSO/t-BuOH 1:9	75	24	18 ± 2	17
STD 6	1:3	DMSO/t-BuOH 1:9	75	24	24 ± 2	10
STD 7	1:1	DMSO/t-BuOH 1:9	75	48	17 ± 2	10
STD 8	1:3	DMSO/t-BuOH 1:9	75	48	25 ± 2	19
Glu-PA 4	10:30	DMSO/t-BuOH 1:9	75	24	24 ± 2	17
Glu-PA 5	2:6	DMSO/t-BuOH 1:9	75	24	25 ± 2	17
Glu-PA 6	1.5:4.5	DMSO/t-BuOH 1:9	75	24	24 ± 2	15
Glu-PA 7	1:3	DMSO/t-BuOH 1:9	75	24	23 ± 2	12
Glu-PA 8	1:3	t-BuOH	75	24	22 ± 2	15
Glu-PA 9	1:3	2M2B	75	24	14 ± 2	5
Glu-PA 10	1:3	DMSO	75	24	0	0

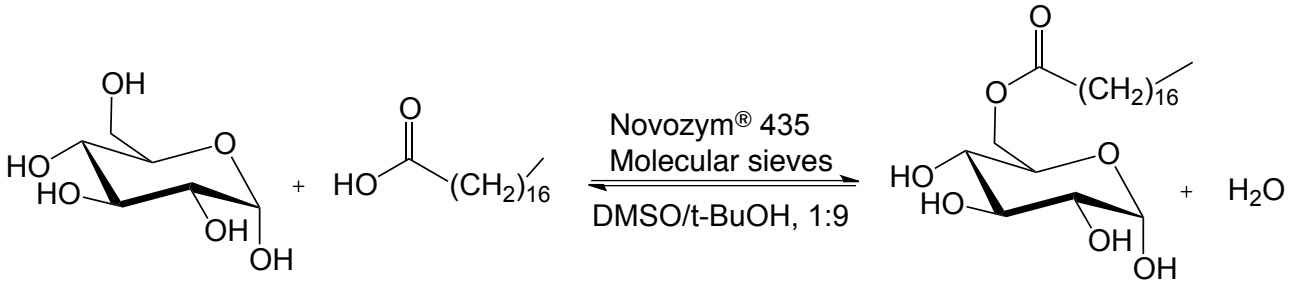
^{1}H NMR (DMSO-d_6 + D_2O (1 drop), 400 MHz): δ (ppm) 4.89 (d, J = 3.6 Hz, 1H, H^{1}), 4.24 (dd, J = 11.6, 6.0 Hz, 1H, H^{6a}), 3.99 (dd, J = 11.7, 6.0 Hz, 1H, H^{6b}), 3.75 (ddd, J = 1.7, 5.9, 9.6 Hz 1H, H^{5}), 3.43 (t, J = 9.2 Hz, 1H, H^{3}), 3.12 (dd, J = 9.6, 3.7 Hz, 1H, H^{2}), 3.04 (t, J = 9.4 Hz, 1H, H^{4}), 2.25 (t, J = 7.3 Hz, 2H, OOCC**H_2**), 1.49 (m, 2H, $OOCCH_2C$**H_2**), 1.23 (s, 24H, (C**H_2**)$_{12}$), 0.84 (t, J = 6.8 Hz, 3H, C**H_3**).

^{13}C NMR (DMSO-d_6 + D_2O (1 drop), 100 MHz): δ (ppm) 173.56 (OO**C**), 92.56 (C^{1}), 73.07 (C^{3}), 72.38 (C^{2}), 70.73 (C^{4}), 69.50 (C^{5}), 64.14 (C^{6}), 33.88 (OOC**C**H_2), 31.70 (**C**$H_2CH_2CH_3$), 29.83 - 28.49 ((**C**H_2)$_{10}$), 24.86 ($OOCCH_2$**C**H_2), 22.50 (**C**H_2CH_3), 14.35 (**C**H_3).

MS (ESI^{+}): *m/z* theoretically calculated for $[C_{22}H_{42}O_7]^{+}$: 418.56; experimentally obtained: 442.20 $[M+Na]^{+}$, 451.26 $[M+MeOH]^{+}$.

3.2.7. Synthesis of Glucose monostearate

$C_{24}H_{46}O_7$; MW: 446.62.



Glucose Stearic acid GluMS

The reactions were carried out in a glass reactor heated in an oil bath at different temperatures (65 or 75 °C) for 24 or 48 h, according to **Table 3.3**. Glucose and stearic acid at different molar ratios (1:2 or 1:3) were transferred in the reactor and were mixed under magnetic stirring (200 rpm) in a solution of DMSO and t-BuOH 1:9. After 15 min, molecular sieves (450 mg; 12.1% w/w) and the enzyme Novozym® 435 (375 mg; 10% $w/w_{reactants}$) were transferred to the reaction mixture and the reaction was left under stirring at the desired temperature and time. Molecular sieves were added for the removal of the water produced from the esterification in order to avoid the hydrolysis of the formed ester(s).

The reaction was monitored by TLC (CH_2Cl_2/MeOH; 10:1) and samples were analyzed after 24 and 48 h. Two samples were taken from the reaction mixture each time: One was diluted in MeOH and the other one in a biphasic system of hexane: H_2O in order to check the presence of any surfactant that could lead to the formation of an emulsion between the two non-mixed solvents after vigorous shaking. Only the organic phase was deposited on the TLC plate.

After the end of the reaction, the reaction mixture was filtered in order to remove the molecular sieves and immobilized CALB, and was washed with t-BuOH, which was removed under vacuum conditions. The enzyme was recovered after washings with H_2O (50 ml) and dried in the oven at 55 °C overnight. The molecular sieves were also washed and reactivated for reuse. The so-obtained filtrate contains now DMSO, unreacted glucose, fatty acid and the formed ester. In order to selectively remove the DMSO and the unreacted glucose from this mixture, H_2O (40 vol/vol $_{DMSO}$) was added and the so-formed white slurry (fatty acid and sugar-fatty ester) was then filtrate. The white precipitate obtained from the filtration was dried overnight at room temperature. The product purification was carried out by selective dissolution of the free fatty acid in hot heptane (0.1 $vol/w_{Stearic\ acid}$).

A suspension of the product **Glucose Monostearate** (**87% Purity**) was obtained as a white powder and its purity was determined by ^{1}H NMR test, comparing the area ratio of the protons of the α-carbons (-C**H$_2$**COOH) of bonded and free fatty acid.

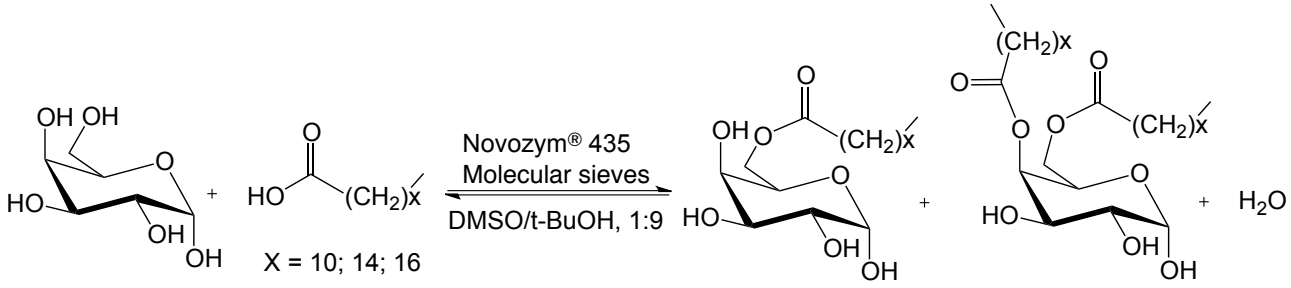
Rf (Eluent CH_2Cl_2/MeOH; 10:1): 0.29

Table 3.3. Experimental conditions of glucose monostearate synthesis.

CODE	Sugar/Fatty Acid Ratio	Reaction Temperature [°C]	Reaction Time [h]	NMR Yield%	Yield %
Glu-SA 1	1:2	65	48	19 ± 2	14
Glu-SA 2	1:2	65	24	17 ± 2	12
Glu-SA 3	1:3	75	24	24 ± 2	17
Glu-SA 4	10:30	75	24	23 ± 2	21
Glu-SA 5	10:30	75	24	24 ± 2	23

^{1}H NMR (DMSO-*d*$_6$ + D_2O (1 drop), 400 MHz, 313 K): δ (ppm) 4.90 (d, J = 3.6 Hz, 1H, H^1), 4.26 (dd, J = 11.7, 1.7 Hz, 1H, H^{6a}), 4.02 (dd, J = 11.7, 5.9 Hz, 1H, H^{6b}), 3.76 (m, 1H, H^5), 3.45 (t, J = 9.2 Hz, 1H, H^3), 3.15 (dd, J = 9.6, 3.6 Hz, 1H, H^2), 3.07 (t, J = 9.4 Hz, 1H, H^4), 2.25 (t, J = 7.3 Hz, 2H, OOCC**H$_2$**), 1.50 (m, 2H, $OOCCH_2C$**H$_2$**), 1.23 (s, 36H, (C**H$_2$)$_{14}$**), 0.84 (t, J = 6.6 Hz, 3H, CH_2C**H$_3$**).
^{13}C NMR (DMSO-*d*$_6$ + D_2O (1 drop), 100 MHz, 313 K): δ (ppm) 176.40 (OO**C**), 95.45 (C^1), 76.08 (C^3), 75.34 (C^2), 73.70 (C^4), 72.45 (C^5), 67.01 (C^6), 36.79 (OOC**C**H_2), 34.50 (**C**$H_2CH_2CH_3$), 32.68 - 31.37 ((**C**H_2)$_{12}$), 27.70 ($OOCCH_2$**C**H_2), 25.28 (**C**H_2CH_3), 17.11 (**C**H_3).
MS (ESI$^+$): *m/z* theoretically calculated for $[C_{24}H_{46}O_7]^+$: 446.62; experimentally obtained: 469.62 $[M+Na]^+$.

3.2.8. Synthesis of Galactose saturated fatty acid mono and diesters



The reactions were carried out in a glass reactor heated in an oil bath at different temperatures (65 or 75 °C) for 24 or 48 h, according to **Table 3.4**. Galactose and lauric, palmitic or stearic acid at different molar ratios (1:2 or 1:3) were transferred in the reactor and were mixed under magnetic stirring (200 rpm) in a solution of DMSO and t-BuOH 1:9. After 15 min, molecular sieves (450 mg; 12.1% w/w) and the enzyme Novozym® 435 (375 mg; 10% w/$w_{reactants}$) were transferred to the reaction mixture and the reaction was left under stirring at the desired temperature and time. Molecular sieves were added for the removal of the water produced from the esterification in order to avoid the hydrolysis of the formed ester(s).

The reaction was monitored by TLC (CH_2Cl_2/MeOH; 10:1) and samples were analyzed after 24 and 48 h. Two samples were taken from the reaction mixture each time: One was diluted in MeOH and the other one in a biphasic system of hexane: H_2O in order to check the presence of any surfactant that could lead to the formation of an emulsion between the two non-mixed solvents after vigorous shaking. Only the organic phase was deposited on the TLC plate.

After the end of the reaction, the reaction mixture was filtered in order to remove the molecular sieves and immobilized CALB, and was washed with t-BuOH, which was removed under vacuum conditions. The enzyme was recovered after washings with H_2O (50 ml) and dried in the oven at 55 °C overnight. The molecular sieves were also washed and reactivated for reuse. The so-obtained filtrate contains now DMSO, unreacted glucose, fatty acid and the formed ester. In order to selectively remove the DMSO and the unreacted glucose from this mixture, H_2O (20 or 40 vol/vol $_{DMSO}$) was added and the so-formed white slurry (fatty acid and sugar-fatty ester) was then filtrate. The white precipitate obtained from the filtration was dried overnight at room temperature. Dissolution of the majority of the free fatty acids in hexane (0.05 vol/w_{LA}) or in hot heptane (0.1 vol/$w_{PA\ or\ SA}$) were performed for the purification of the formed esters. The so-obtained suspensions were either centrifuged (Gal-LA) to separate the supernatant or filtrated (Gal-PA or Gal-SA).

The white powders obtained were submitted to ^{1}H NMR and MS (ESI^+) tests. From the interpretations of the spectra, it was hypothesized that the samples were 1:1 mixtures of mono- and diesters in which part of the unreacted free fatty acid was still present.

Rf (Eluent CH_2Cl_2/MeOH; 10:1): 0.26; 0.63; 0.85

Table 3.4. Experimental conditions of the synthesis of galactose saturated fatty acid mono- and diesters.

CODE	Fatty Acid	Sugar/Fatty Acid Ratio	Reaction Temp. [°C]	Reaction Time [h]	Yield of Mixtures %
Gal-LA 1	Lauric Acid	5:15	75	24	4
Gal-PA 1	Palmitic Acid	5:15	75	24	5
Gal-SA 1	Stearic Acid	5:15	75	24	3
Gal-LA 2	Lauric Acid	1:3	65	24	11
Gal-PA 2	Palmitic Acid	1:3	65	24	2
Gal-SA 2	Stearic Acid	1:3	65	24	3

MS (ESI$^+$) GAL-LA 2: *m/z* theoretically calculated for $[C_{18}H_{34}O_7]^+$: 362.46; experimentally obtained: 385.22 $[M+Na]^+$, 567.22 [Diester M+Na]$^+$.

MS (ESI$^+$) GAL-PA 2: *m/z* theoretically calculated for $[C_{22}H_{42}O_7]^+$: 418.56; experimentally obtained: 441.25 $[M+Na]^+$, 679.25 [Diester M+Na]$^+$.

MS (ESI$^+$) GAL-SA 2: *m/z* theoretically calculated for $[C_{24}H_{46}O_7]^+$: 446.62; experimentally obtained: 469.58 $[M+Na]^+$, 735.52 [Diester M+Na]$^+$.

3.2.9. Evaluation of the Yield

I) NMR Method

The partial yields have been calculated on samples composed only by the formed sugar fatty acid ester and the unreacted fatty acid. These samples were obtained from the reaction mixture after the separation steps of immobilized enzyme and molecular sieves and after the H_2O filtration that lead to the binary mixture of formed ester and unreacted fatty acid. The content of sugar fatty acid esters is quantified by calculating the area ratios between discriminating protons of the product and the residual fatty acid, which was determined by ^{1}H NMR test.

Samples (10 mg) were dissolved in DMSO-d_6 (0,6 ml) and ^{1}H NMR analyses were conducted, by setting a relaxation delay (t_R) of 3 s. The *Area Ratio* is calculated between the signal of the proton related to the anomeric carbon of the sugar moiety in the formed ester (**H^1**) and the signal of the two protons related to the α-carbon of the unreacted fatty acid (-C**H$_2$**COOH), thus the value obtained through the signal integration is divided by two.

It is possible to calculate the mmoles of the formed ester in the analyzed samples ($n_{ester(sample)}$), and therefore the mmoles in the initial binary mixtures ($n_{ester(mixture)}$), as the amount of the two compounds is related to the Area ratios determined by ^{1}H NMR, according to the following equation:

$$Area\ ratio = \frac{n_{fatty\ acid}}{n_{ester}} \Rightarrow$$

$$n_{fatty\ acid} = Area\ ratio \times n_{ester} \quad (1)$$

where $n_{\text{fatty acid}}$ and n_{ester} the mmoles of the unreacted free fatty acid and produced ester in the sample respectively.

It is considered that every analyzed sample is composed by the formed ester and the unreacted fatty acid, so by resolving the following equation the amount of the formed ester can be calculated:

$$W_{sample} = W_{ester} + W_{fatty\ acid}$$

$$W_{sample} = (n_{ester(sample)} \times MW_{ester}) + (n_{fatty\ acid(sample)} \times MW_{fatty\ acid})$$

and substituting $n_{fatty\ acid(sample)}$ with equation (1):

$$W_{sample} = n_{ester(sample)} \times MW_{ester} + (Area\ ratio \times n_{ester(sample)} \times MW_{fatty\ acid})$$

$$n_{ester\ (sample)} = \frac{W_{sample}}{MW_{ester} + (Area\ ratio \times MW_{fatty\ acid})}$$

where W_{sample} is the weight of the analyzed sample and MW_{ester} and $MW_{fatty\ acid}$ are the molecular weights of the formed ester and free fatty acid, respectively.

Then, the corresponding amount of the formed ester in the initial binary mixture can be calculated according to the following relation:

$$\frac{n_{ester\ (sample)}}{W_{sample}} = \frac{n_{ester\ (mixture)}}{W_{mixture}}$$

$$n_{ester\ (mixture)} = \frac{n_{ester\ (sample)} \times W_{mixture}}{W_{sample}} = \frac{\cancel{W_{sample}}}{MW_{ester} + (Area\ ratio\ \times MW_{fatty\ acid})} \frac{W_{mixture}}{\cancel{W_{sample}}}$$

And so:

$$n_{ester\ (mixture)} = \frac{W_{mixture}}{MW_{ester} + (Area\ ratio\ \times MW_{fatty\ acid})}$$

where $W_{mixture}$ is the total weight if the binary mixture.

Finally, the yield of the non-isolated products *(% NMR Yield*) is obtained by the mmoles of the formed ester in the mixture ($n_{ester(mixture)}$) over the mmoles of the limiting agent, which is the sugar (n_{sugar}):

$$\%\ \boldsymbol{NMR\ Yield} = \frac{n_{ester(mixture)}}{n_{sugar}} \times 100$$

$$\%\ \boldsymbol{NMR\ Yield} = \frac{W_{mixture}}{n_{sugar} \times [MW_{ester} + (Area\ ratio\ \times MW_{fatty\ acid})]} \times 100$$

II) Titration method

The sugar fatty acid ester content was quantified by calculating the amount of the residual fatty acid in the reaction mixture obtained after the precipitation in H_2O and composed only by the product and the non-reacted fatty acid, which was determined by a titration volumetric method [44].

Samples (0.03 g) were diluted in a solution of diethyl ether/absolute ethanol 1:2 (30 ml) and phenolphthalein (2 drops) was used as pH indicator. Then the diluted samples were titrated with standardized sodium hydroxide solution 0.1 M, according to the *NGDC10-1976* method. Titration measurements were performed in triplicate to obtain the standard deviation and the percentage of the residual fatty acid was calculated using the equation:

$$\% FA = \frac{V_{NaOH} \times M_{NaOH} \times MW_{FA}}{W_{sample} \times 10} \times 100$$

where $\% FA$ is the percentage of unreacted fatty acid, V_{NaOH} is the volume of NaOH consumed during titration, M_{NaOH} is the molarity of the standardized NaOH solution, MW_{FA} is the molecular weight of the free fatty acid and W_{Sample} is the weight of the analyzed sample.

Then, the yield of the non-isolated products by titration (*% T Yield*) over the mmoles of the limiting agent (n_{sugar}) is obtained by using the following equations:

$$\%ester = 100 - \%FA$$

$$n_{ester} = \frac{\%ester \times W_{mixture}}{MW_{ester}}$$

$$\% \boldsymbol{T\ Yield} = \frac{n_{ester}}{n_{sugar}} \times 100$$

where n_{ester} are the mmoles of the product obtained, $W_{mixture}$ is the total weight of the reaction mixture and MW_{ester} is the molecular weight of the product. The *% ester* is calculated as the difference of the %FA from the total amount (100) by assuming that in the mixture there is only the formed ester and the unreacted free fatty acid.

3.2.10. Contact Angle Measurements

Pellets of each of the glucose saturated fatty acid ester were prepared with powdered pure ester (300 mg).

Contact angle measurements were performed on a Krüss Easy instrument. A drop of 5 μL was produced and gently placed on the surface of each pellet. Side view pictures were taken immediately after the water droplet left the syringe tip, using a high-resolution camera.

The drop profile was extrapolated using an appropriate fitting function. Measurements were repeated several times to obtain a statistically relevant population, since substrates surfaces were really variables even in the near spots.

3.3 Results and Discussion

3.3.1 Enzyme screening for glucose and oleic acid esterification

The lipases from *Rhizopus niveus, Candida rugosa, Pseudomonas cepacia, Porcine pancreas, Pseudomonas fluorescens, Pseudomonas fluorescens, Thermomyces lanuginosus, Aspergillus niger and Phospholipase D* have been screened in the esterification reaction of glucose and oleic acid. All the experiments were performed at 65 ºC for 48 h, with the exception of the reaction with *Porcine pancreas* lipase that was carried out at 45 ºC. Glucose and oleic acid have been used in 1:1 molar ratio using DMSO and t-BuOH (1:9) as solvents. In all the biotransformations no product was detected. This result could be ascribed to concurrent factors. A poor stability/inactivation of the assayed enzymes at high temperature (45 °C or 65 °C) and/or in the presence of DMSO and *t*-BuOH is plausible if one considers that the enzymes were used in their native state (i.e. as "free" enzymes). Moreover, the presence of traces of water in the reaction mixture might have hampered the reaction. Concerning substrate specificity, it can be expected that all the lipases considered in the screening can accept oleic acid as the substrate, as supported by literature data [16, 45, 46]. In absence of experimental data regarding the stability of the free enzymes under the operational conditions applied to the esterification reaction, it is hard to draw conclusions on the results of this screening; however, taking into account literature data [47], the lack of stability of the biocatalysts appears to be a major driver.

On the contrary, the use of the commercial immobilized lipase B from *Candida antarctica* (CALB) led to the formation of the desired ester (glucose monoleate). Immobilized enzymes have usually a higher stability compared to the free enzymes, especially under unusual conditions (pH, temperature and pressure) and in non-conventional environments as is the case of organic solvents [23, 38].

In order to further study some of the above enzymes, immobilized enzymes were prepared by two different methods: adsorption on octyl-Sepharose and covalent binding on Sepabeads.

3.3.2 Activity of immobilized lipases

The following lipases were immobilized and characterized for their activity, before and after immobilization, and their stability was assayed under the esterification reaction conditions:

- *Candida rugosa lipase* (CRL)
- *Pseudomonas cepacia lipase* (PCL)
- *Thermomyces lanuginosus lipase* (TLL)
- *Rhizomucor miehei lipase* (RML)

The hydrolytic activities of the immobilized enzymes were evaluated titrimetrically using a pH-stat, by measuring the initial hydrolysis rate of tripropionin to proprionic acid, calculated from the NaOH consumption (mmol NaOH min^{-1}/mL of enzyme preparation). Briefly, the reaction was carried out in a mixture containing 18.4 ml of Tris HCl buffer (2.5 mM) and NaCl (100 mM), 0.6 ml CH_3CN and 1 ml tripropionin. 10 mg of enzyme were added, and the mixture was titrated with NaOH (100 mM) maintaining the pH at 7. One International Unit (UI) of lipase activity was defined as the amount of enzyme that hydrolyzes 1μmol of tripropionin per min at pH 7.0 and 25°C.

According to the results of the activity assays, for the enzymes both in free and immobilized form (**Table 3.5**), the adsorption method was more effective compare to covalent immobilization. In fact, the Average Activity of immobilized enzymes is much higher when octyl-Sepharose is used as carrier with respect to Sepabeads for all the enzymes. PCL enzyme immobilized on octyl-Sepharose has the highest activity (1632.04 UI/g), followed by RML enzyme (1430.36 UI/g). On the contrary, when covalent immobilization was performed on Sepabeads, PCL did not display any activity and the other enzymes shown a significant loss in activity with respect to the free and immobilized form on octyl-Sepharose.

The percentage of the retained activity of the enzymes prepared by both immobilization techniques has been calculated by the following equation and the results are presented in **Table 3.5**:

$$\%\ Retained\ Activity = \frac{\text{Activity}_{\text{immobilized}}}{\text{Activity}_{\text{free}}} \times 100$$

Table 3.5. Average activity of free and immobilized enzymes on two different carriers.

	Average Activity (UI/g)			**Retained Activity (%)**	
Enzymes	free form	immob. on octyl-Sepharose	immob. on Sepabeads	immob. on octyl-Sepharose	immob. on Sepabeads
CRL	2435.00	1217.812	371.95	50.01	15.28
TLL	574.54	403.773	44.05	70.28	7.67
PCL	5734.64	1632.036	0.00	28.46	0.00
RML	4406.67	1430.357	213.19	32.46	4.84

All the enzymes have a noticeable loss of activity after the immobilization with respect to the free form. The immobilized enzymes on octyl-Sepharose still retain significant amount of activity with respect to the ones immobilized on Sepabeads and can be tested under the reaction conditions. In case of immobilization by adsorption on octyl-Sepharose, desorption and leakage of enzyme may occur leading to a loss of protein in the reaction mixture. On the other hand, although covalent binding provides a stronger binding between the enzyme and the carrier, it resulted in a considerable decrease of the enzyme activity due to a possible modification of the enzyme active site and distortion of its natural structure [47].

In order to obtain more amount of immobilized enzymes, successive immobilizations have been performed for the enzymes PCL and RML by adsorption on octyl-Sepharose, where PCL1, PCL2 and PCL3 refer to the first, second and third immobilization, respectively, and RML1 and RML2 to the first and second immobilization, similarly. In **Table 3.6** are presented the results of the activity assays of those samples.

Table 3.6. Average Activity of immobilized enzymes.

Immobilized enzymes	**Average Activity (Ui/g)**		**Retained Activity (%)**
	free form	immob. on octyl-Sepharose	
PCL1	5734.64	1632.036	28.46
PCL2	>>	1035.549	18.06
PCL3	>>	712.299	12.42
RML1	4406.67	1430.357	32.46
RML2	>>	486.768	11.05

The stability of the immobilized enzymes was tested under reaction conditions (DMSO: t-BuOH 1:9, 65°C) and the residual activity of the enzymes recovered after the reaction were measured. In **Figure 3.8** are presented the curves of the residual activity of TLL, PCL and RML immobilized on octyl-Sepharose. The curves are based on constant monitoring of the activity of the enzymes after definite intervals of time. The enzymatic activity for these enzymes is decreasing dramatically, reaching deactivation after 48 h. PCL was the only immobilized lipase that was relatively stable until 24 h.

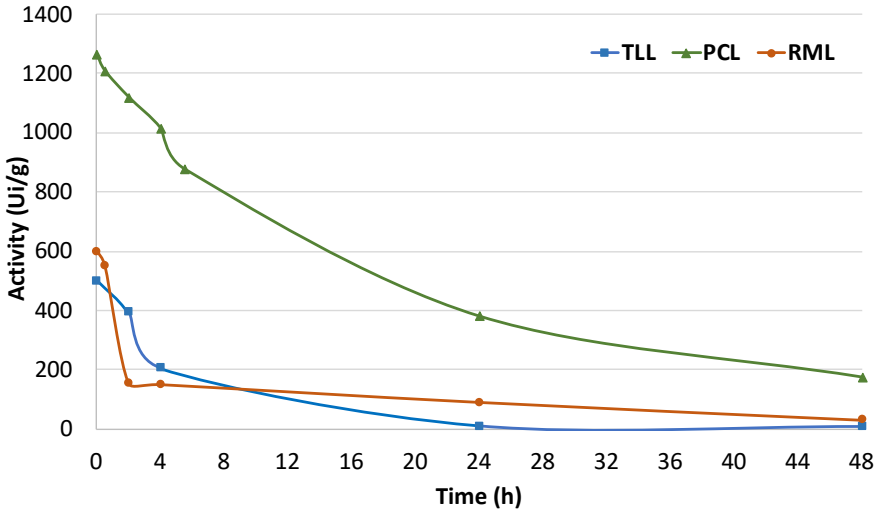


Figure 3.8. Stability of the immobilized lipases TLL, PCL and RML on octyl-sheparose under the reaction conditions ((DMSO:t-BuOH 1:9, 65°C).

Experiments for the esterification of glucose with oleic acid were performed by using the derivative immobilized enzymes, according to the conditions presented in **Table 3.7**. Unfortunately, all the attempts did not lead to the formation of the desired product. A further study is needed for the preparation of more stable immobilized enzymes under the esterification conditions, by using different substrates for immobilization and/or different immobilization methods.

It was decided to continue the experiments by using the commercial immobilized lipase from *Candida antarctica* B (CALB) and study further the reaction conditions.

Table 3.7. Experimental conditions tested for the immobilized enzymes.

Enzyme	Activity	Sugar	Fatty Acid	Reactants Molar ratio	Solvent	Temperature [ºC]
PCL1	1600	Glucose	Oleic Acid	1:2	DMSO:t-BuOH	65
RML1	1430	Glucose	Oleic Acid	1:2	DMSO:t-BuOH	60
PCL1	1600	Glucose	Oleic Acid	1:2	DMSO:t-BuOH	65
PCL3	730	Glucose	Oleic Acid	1:2	DMSO	Room Temp.
RML1	1430	Glucose	Lauric Acid	1:1	t-BuOH	Room Temp.
CRL1	1218	Glucose	Oleic Acid	1:1	t-BuOH	Room Temp.
PCL2	1035	Galactose	Oleic Acid	1:2	DMSO:t-BuOH	60
RML2	487	Galactose	Oleic Acid	1:2	DMSO:t-BuOH	60
PCL3	730	Galactoe	Oleic Acid	1:2	DMSO	Room Temp.
PCL1	1600	Lactose	Oleic Acid	1:4	2M2B	65
PCL3	730	Lactose	Lauric Acid	1:2	2M2B	65
RML2	487	Lactose	Oleic Acid	1:2	2M2B	65

3.3.3 Synthesis of glucose and galactose monoolates

Glucose monoolate (GluMO) and galactose monoolate (GalMO) were successfully synthesized by using the lipase Novozym 435 at 65 ºC for 48 h. In order to increase the conversion, an excess of fatty acid over sugar has been used (2:1 molar ratio). In **Figure 3.9** the reaction scheme for the synthesis of GluMO and GalMO is presented.

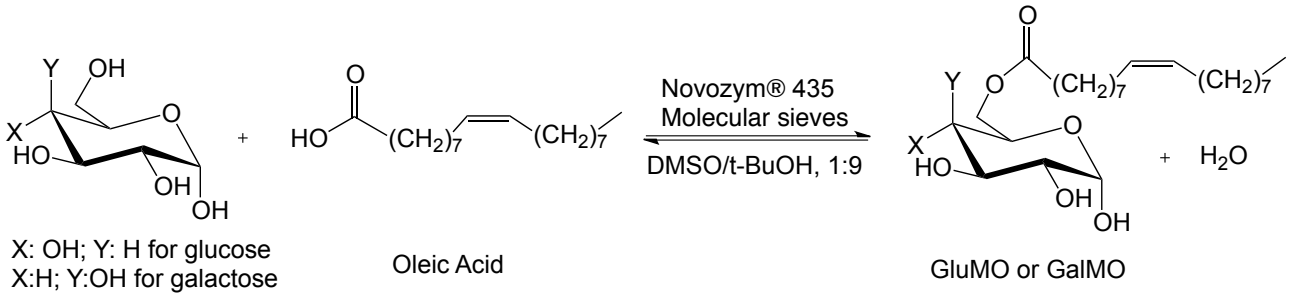


Figure 3.9. Reaction scheme for the synthesis of glucose monooleate and galactose monooleate.

Both products were successfully characterized by ESI-MS analysis and ^{1}H and ^{13}C NMR spectroscopy. ^{1}H and ^{13}C NMR signals were assigned with the aid of ^{1}H-^{1}H and ^{1}H-^{13}C correlation experiments (COSY, HSQC and HMBC).

The NMR analysis has been initially performed by using as a solvent deuterated methanol (CD_3OD). The high complexity of the obtained spectra though, due to the presence of both α- and β-anomeric species of glucose moiety, did not allow the interpretation of all the signals. In addition, the low solubility of both products in this solvent leaded to their precipitation during the analysis, making the final spectra even less clear.

In order to overcome this problem, it was decided to use as a solvent DMSO-d_6 with one drop of D_2O in order to make the exchangeable -OH protons disappear. In this solvent-system, both glucose and galactose monooleates were completely solubilized and, according to the spectra recorded shortly after the sample preparation, only a series of signals related to the α-anomer of the sugars were visible, making the interpretation of the spectrum much easier (**Figure 3.10**). Reinvestigation of the same samples after one week showed the appearance of the other β-anomeric species.

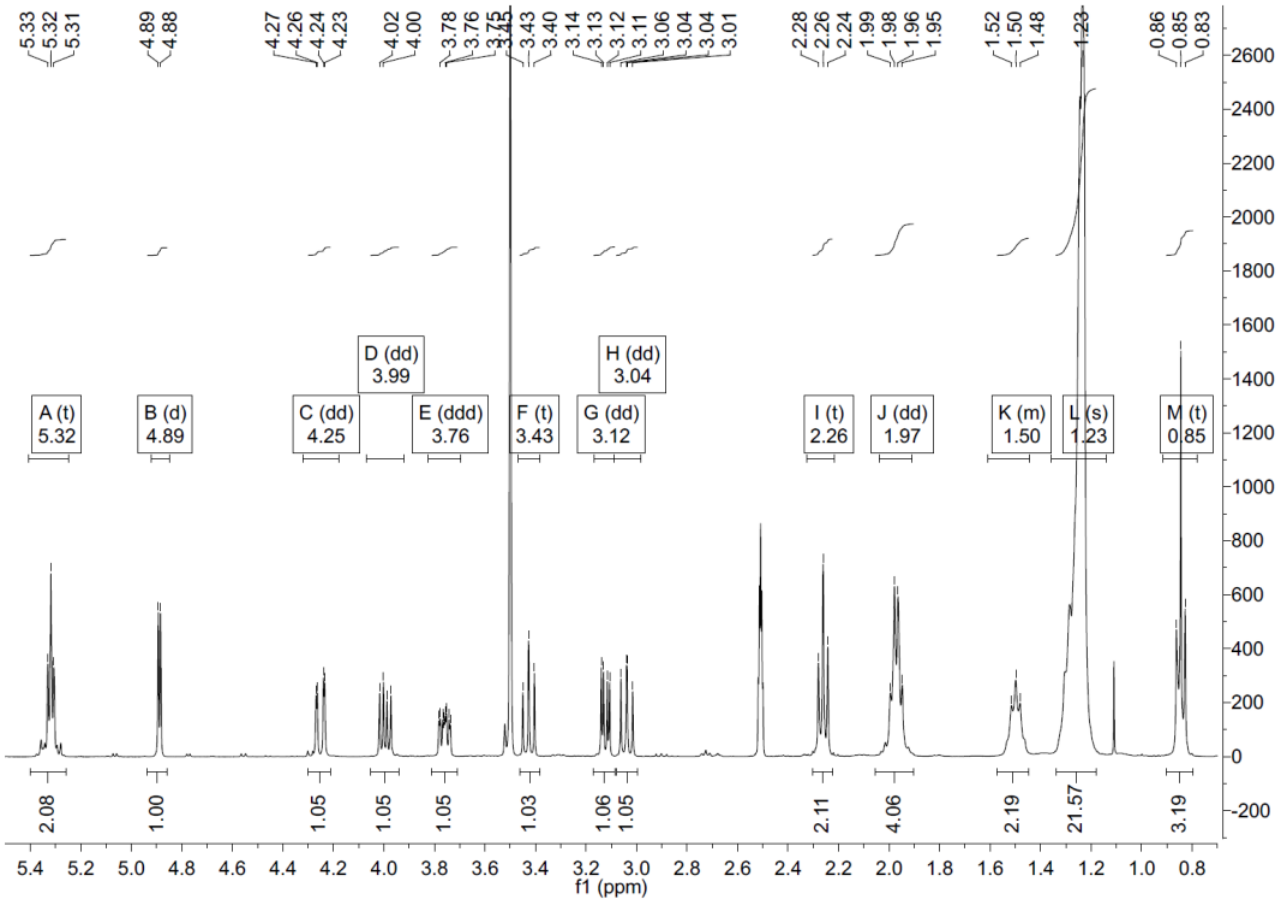


Figure 3.10. ^{1}H NMR spectrum of glucose monooleate (GluMO) analyzed in DMSO-d_6 with one drop of D_2O.

The position of the ester bond was confirmed by the observed downfield shift of the two protons in the six position (H^6) of the glucose moiety compared to free glucose (**Figure 3.11)** [48], and also from their interaction with the carbonyl carbon observed in the ^{1}H-^{13}C heteronuclear multiple bond correlation spectroscopy (HMBC) (**Figure 3.12**).

Both products were purified through two successive flash columns chromatography (CH_2Cl_2/MeOH; 10:1; 12:1), or with one long flash column chromatography conducted in non-isocratic conditions ((CH_2Cl_2/MeOH; 5 column volumes from 20:1 to 10:1). However, despite the distance of the spots between product and fatty acid on TLC, it was difficult to remove the oleic acid, as it was continuously eluted from the column, probably due to its acidic and oily nature, leading to rather poor yields: 8% for glucose monooleate and only 1% for galactose monooleate.

Since the subsequent purification attempts, using precipitation methods in H_2O or different eluents (Hexane/ethyl acetate, 1:2; or ethyl acetate/methanol 2:1), did not lead to an increase in yield, it was decided to use saturated fatted acids as acyl donors for the next experiments.

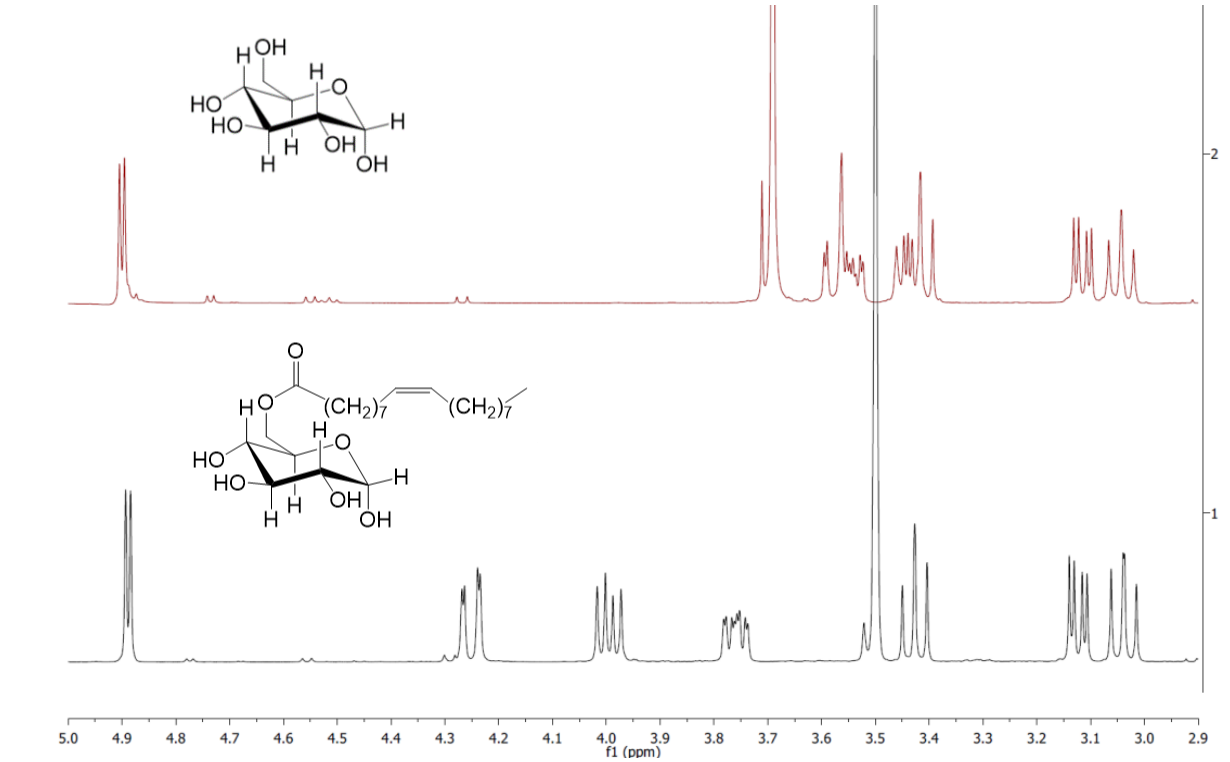


Figure 3.11. Comparison of ^{1}H NMR spectra of glucose and glucose monooleate, both recorded in DMSO-d_6 with one drop of D_2O.

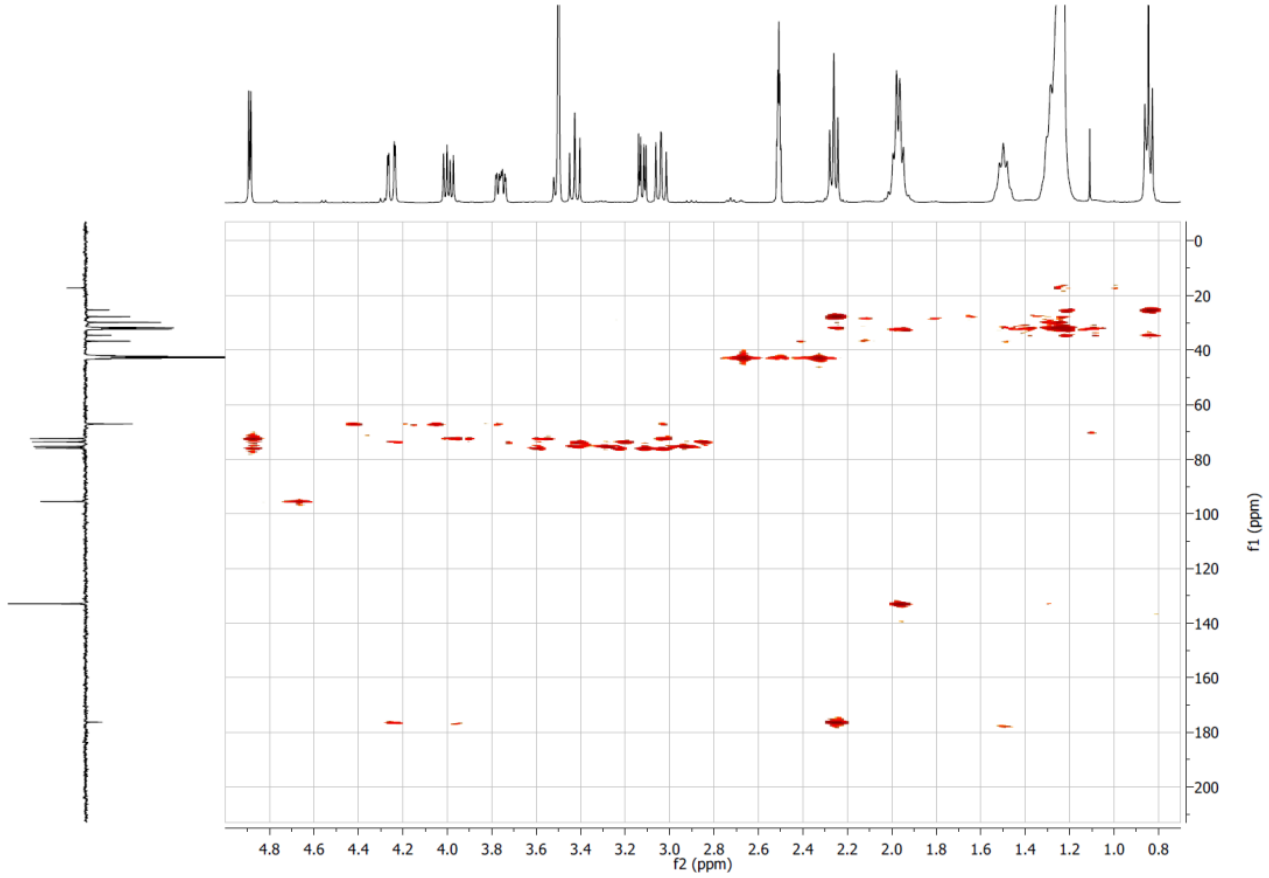


Figure 3.12. HMBC of glucose oleate in which the horizontal and vertical axis indicate the ^{1}H and ^{13}C chemical shift (ppm), respectively.

3.3.4 Synthesis of glucose esters using saturated fatty acids

In order to better set up the experimental conditions, the esterification of glucose with saturated fatty acids was studied. The selected saturated fatty acids were the following: lauric acid, commonly used in the synthesis of chemical surfactants; palmitic acid, that is the main component of palm oil and its world production is always increasing; and stearic acid, due to its equal chain length with oleic acid. The experiments were carried out at the previous conditions as well as in shorter reaction times (**Table 3.8**), leading to the formation of the products: glucose monolaurate, glucose monopalmitate and glucose monostearate (**Figure 3.13**).

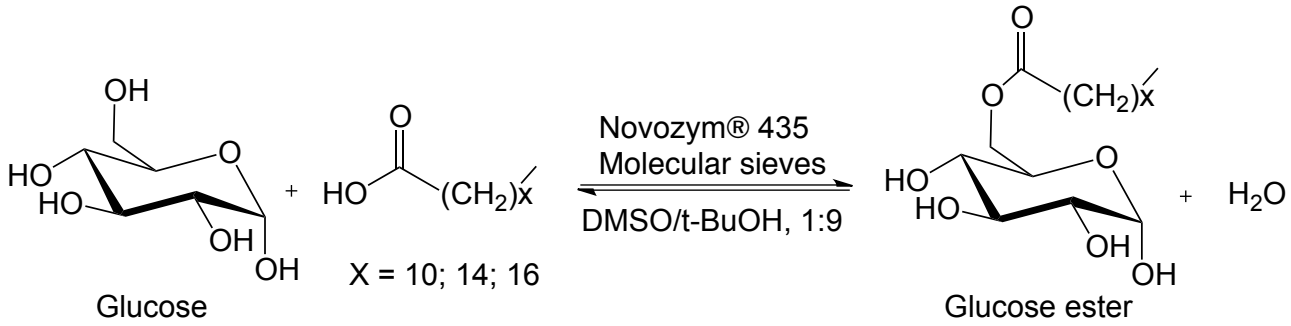


Figure 3.13. Reaction scheme for the synthesis of glucose fatty acid esters, where X = 10, 14, and 16 correspond to lauric acid, palmitic acid and stearic acid, respectively.

Table 3.8. Experimental conditions for glucose esterification with saturated fatty acids.

CODE	Fatty Acid	Sugar/Fatty Acid Ratio	Reaction Temp. [°C]	Reaction Time [h]	NMR Yield %	Yield %
Glu-LA 1/2	Lauric Acid	1:2	65	48	21 ± 2	15 ± 2
Glu-PA 1/2	Palmitic Acid	1:2	65	48	23 ± 2	18 ± 1
Glu-SA 1	Stearic Acid	1:2	65	48	19 ± 2	14
Glu-LA 3	Lauric Acid	1:2	65	24	18 ± 2	15
Glu-PA 3	Palmitic Acid	1:2	65	24	20 ± 2	13
Glu-SA 2	Stearic Acid	1:2	65	24	17 ± 2	12

The highest NMR yield has been found for the esterification of glucose with palmitic acid performing the reaction for 48 hours. Therefore, assuming a similar trend, the study of the reaction conditions was performed using only palmitic acid in the subsequent experiments.

I. Quantification of synthesized esters

The determination of the conversion was not feasible directly from the reaction mixtures due to the high complexity of the reaction system, e.g. different solubility of the reactants and low solubility of the formed esters. In order to determine the glucose fatty acid esters content, it was necessary to carry out purification steps in order to obtain simpler mixtures. First, the reaction mixture was filtrated in order to remove the immobilized enzyme and the molecular sieves. Then, t-BuOH was removed under reduced pressure and the products with the unreacted fatty acids were precipitated in H_2O. The successful partial purification was confirmed by the presence of only two spots in TLC plates.

Yield calculation by NMR analysis

In **Figure 3.14** is presented the ^{1}H NMR spectrum of the sample received from the experiment "Glu-PA 3" after the purification step, as an example for the calculation of the yield. 10,8 mg of the sample "Glu-PA 3" were dissolved in DMSO-d_6 (0,6 ml). The signals highlighted in red belong to glucose palmitate and to non-reacted palmitic acid, thus their integration is related to the ratio of the two compounds in the mixture.

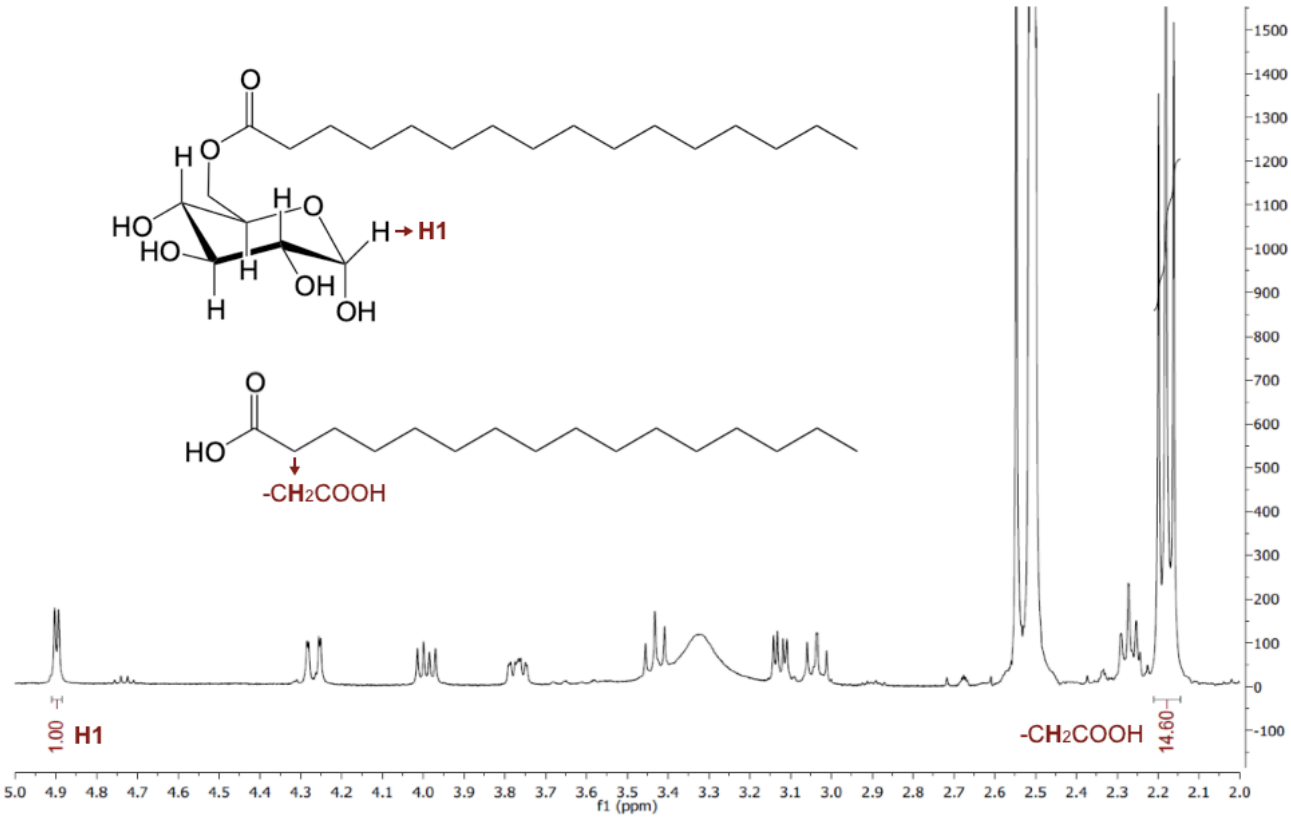


Figure 3.14. ^{1}H NMR spectrum of the sample recieved from the experiment "Glu-PA 3", dissolved in DMSO-d_6.

The Area Ratio is calculated between the signal of the proton related to the anomeric carbon of the sugar moiety in glucose monopalmitate (**H**[1]) and the signal of the two protons related to the α-carbon of the palmitic acid (-C**H**$_2$COOH), thus the value obtained through the signal integration is divided by 2. For the sample "Glu-PA 3" the Area ratio was found:

Area Ratio = 7.3

As this value is related to the ratio of the amount of the two compounds in the mixture, it is possible to calculate the moles of the product in the sample [$n_{GluMP\ (sample)}$] and consequently in the binary mixture [$n_{GluMP\ (mixture)}$], using the following equations:

$$n_{GluMP\ (sample)} = \frac{W_{sample}}{MW_{ester} + (Area\ ratio\ \times MW_{fatty\ acid})} = \frac{10.8}{418.56 + (7.3 \times 256.43)}$$
$$= 0.0047\ mmol$$

where MW_{GluMP} = 418.56 g/mol and MW_{PA} = 256.43 g/mol.

$$n_{GluMP\ (mixture)} = \frac{n_{ester\ (sample)} \times W_{mixture}}{W_{sample}} = \frac{0.0047 \times 458.4}{10.8} = 0.2001\ mmol$$

where $W_{mixture}$ = 458.4 mg.

Finally, the yield was calculated from the following equation:

$$\%\ \boldsymbol{NMR\ Yield} = \frac{n_{ester(mixture)}}{n_{sugar}} \times 100 = \frac{0.2001}{181.2/180.16} \times 100 = \mathbf{20\% \pm 2}$$

where W_{Glu} = 181.2 mg and MW_{Glu}= 180.16 g/mol.

The standard deviation of the final value was calculated from the difference between the sample taken from this reaction and was assumed as the error of the method.

Yield calculation by titration method

For some experiments, the yield has been calculation using the titration method NGDC10-1976 [44] in order to confirm also the reliability of the NMR method.

The formed product content (glucose monopalmitate) was quantified by calculating the amount of the non-reacted free fatty acid in the sample of the binary mixture. 0.03 g of sample were diluted in 30 ml of solvent system diethyl ether/ ethanol (1:2) and titrated with standardized sodium hydroxide solution 0.1 M. Two drops of Phenolphthalein were added to the solution as pH indicator. Titration measurements were performed in triplicate to obtain the standard deviation. Here are presented, as an example, the calculations of the yield for the sample "Glu-PA 3". The percentage of the residual fatty acid (%PA) was calculated using the following equation:

$$\%\ \mathrm{PA}\ (1) = \frac{V_{\mathrm{NaOH}\ (1)} \times M_{\mathrm{NaOH}} \times MW_{\mathrm{PA}}}{W_{\mathrm{sample}\ (1)} \times 10} = \frac{0.97 \times 0.1 \times 256.43}{0.03 \times 10} = 82.9\%$$

where MW_{PA}=256.43 g/mol and M_{NaOH}=0.1 mol/L. Similarly, the calculation for the other measuments of sample "Glu-PA 3" are presented in the following table:

Table 3.9. Yield by titration of sample Glu-PA 3 repeated three times.

Sample	W_{sample} [g]	V_{NaOH} [ml]	% PA
Glu-PA 3 (1)	0.0300	0.97	82.9
Glu-PA 3 (2)	0.0306	0.98	82.1
Glu-PA 3 (3)	0.0307	0.94	78.5

So, the average %PA = **81.2%**

The percentage of the formed ester (% GluMP) and consequently its mmoles were:

$$\%\ \mathrm{GluMP} = 100 - \%\mathrm{PA} = 100 - 81.2 = 18.8\%$$

$$n_{\mathrm{GluMP}} = \frac{\%\mathrm{GluMP} \times W_{\mathrm{mixture}}}{MW_{\mathrm{GluMP}}} = \frac{18.8 \times 10^{-2} \times 458.4}{418.56} = 0.2059\ \mathrm{mmol}$$

where $W_{mixture}$ = 458.4 mg and MW_{GluMP} = 418.56 g/mol.

Finally, the non-isolated product yield by titration method (T Yield) was calculated:

$$\% \, \mathbf{T\ Yield} = \frac{n_{GluMP}}{n_{Glu}} \times 100 = \frac{0.2059}{181.2/180.16} \times 100 = \mathbf{20.5}\%$$

where W_{Glu} =181.2 mg and MW_{Glu}=180.16 g/mol.

The values obtained with the two methods were similar and it was decided to carry out the measurements only with the NMR method that was more straightforward and also because the titration method required more amount of samples.

II. Design of Experiments – study of the reaction conditions

In order to perform a systematic study of the reaction conditions through design of experiments, it is necessary to evaluate the interactions of the three critical variables for the yield response, that in this work, were the moles of the fatty acid, the reaction time and the reaction temperature.

The design of experiments (DoE) is a fundamental tool of the Quality by Design (QbD), which has gained a significant attention in pharmaceutical industry, as an efficient method to design and control processes included in production of therapeutical compounds. QbD is used to evaluate the manufacturing performance by making the most suitable design choices for process parameters [49]. DoE is a systematic method, alternative to the One-Variable-At-a-Time (OVAT) methodology, that is used to determine cause-and-effect relationships between the critical factors affecting a process and the output of the that process. It is a very useful tool for managing the process inputs and optimizing the output [50].

DoE is a promising instrument for improving also enzymatic reactions. Recently, Carteret C. et al. have used a full factorial DoE to optimize the transesterification of rapeseed oil with methanol catalyzed by the immobilized *Mucor miehei* lipase on mesoporous silica [51].

As a model reaction to perform the DoE, the synthesis of glucose monopalmitate by CALB has be chosen. For the DoE experiments and the generation of the related matrix (**Table 3.10**), the amount of palmitic acid (1-3 mmol), the reaction time (24-48 h) and the reaction temperature (55-75 °C) were chosen as the three critical parameters. The NMR yield (%) was chosen as the answer (Y), while all the other reaction conditions and purification procedures remained constant in order to minimize variation sources.

From the elaboration of the experimental responses, the following equation was generated, whose coefficients were reported in a histogram (**Figure 3.15**):

$$\mathbf{Y = 17{,}67 + 3{,}34X_1 - 0{,}5475X_2 + 3{,}605X_3 - 0{,}2325X_1X_2 + 0{,}085X_1X_3 + 0{,}5325X_2X_3 + 0{,}8375X_1X_2X_3}$$

Table 3.10. Full factorial design (2^3) matrix.

STD	X_1 PA (mmol)	X_2 Time (h)	X_3 Temp. (°C)	X_1X_2	X_1X_3	X_2X_3	$X_1X_2X_3$	Y NMR Yield (%)
1	1 (-1)	24 (-1)	55 (-1)	1	1	1	-1	11 ± 2
2	3 (1)	24 (-1)	55 (-1)	-1	-1	1	1	19 ± 2
3	1 (-1)	48 (1)	55 (-1)	-1	1	-1	1	11 ± 2
4	3 (1)	48 (1)	55 (-1)	1	-1	-1	-1	15 ± 2
5	1 (-1)	24 (-1)	75 (1)	1	-1	-1	1	18 ± 2
6	3 (1)	24 (-1)	75 (1)	-1	1	-1	-1	24 ± 2
7	1 (-1)	48 (1)	75 (1)	-1	-1	1	-1	17 ± 2
8	3 (1)	48 (1)	75 (1)	1	1	1	1	25 ± 2

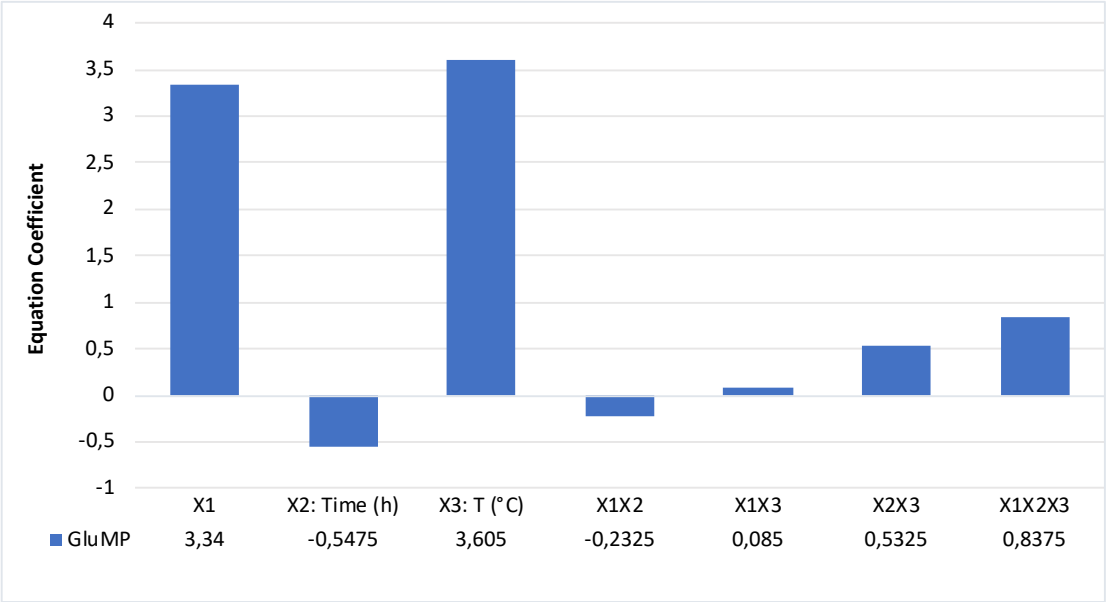


Figure 3.15. Histogram of the coefficients related to the elaboration of equation of the full factorial design.

From the preliminary analysis of the results, it is possible to observe that the three critical parameters are independent from each other. The interaction between the pairs of factors is minimal, particularly the one between the amount of palmitic acid and temperature. This means that by increasing both of these two factors, the yield is influenced in a positive but non-additive way.

Another interlocutory data is represented by the positive interaction of temperature and reaction time, which is contradictory to the trend of single data that have opposite sign. It is possible that the value obtained by **STD 4** experiment may represent an outliner due to the generation of water not absorbed by molecular sieves, which caused the reversion of the equilibrium. Taking into account this fact, time can be considered as an irrelevant variable, at least at 75 °C. Consequently, in order to save time

and to avoid the possible hydrolysis of the formed esters, it was decided to carry out the subsequent experiments for 24 hours.

Further experiments have to be performed to get a better knowledge of these reaction conditions and to understand if the model has predictive validity. However, from this preliminary study, it was possible to obtain the highest NMR yield, by using a ratio of 1:3 of glucose/palmitic acid, at 75 °C for 24 h. These optimized reaction conditions were used for the next experiments to study the esterification of palmitic acid with glucose in different solvents and also to reuse the biocatalyst.

III. Recycling of the biocatalyst

In order to study the stability of the biocatalyst during the esterification, three successive reactions were performed (**Table 3.11**) by reusing the enzyme CALB (Novozym® 435). The conditions were selected according to the results obtained from DoE study. All the experiments were performed by using glucose and palmitic acid at molar ratio 1:3 respectively, at 75 ºC for 24 h in the solvent mixture DMSO/t-BuOH 1:9. The enzyme was recovered after each cycle, washed with water and dried in the oven at 55 °C overnight before the next use.

Table 3.11. Experimental conditions of the three successive reactions for the enzyme stability tests.

CODE	Sugar/Fatty Acid Ratio	Molecular Sieves (mg)	Novozym® 435 (mg)	NMR Yield (%)	Yield (%)
Glu-PA 5	2:6	230	190	**25 ± 2**	**17**
Glu-PA 6	1.5:4.5	170	140	**24 ± 2**	**15**
Glu-PA 7	1:3	115	95	**23 ± 2**	**12**

In the selected experimental conditions, it is possible to reuse the biocatalyst up to three times obtaining similar NMR yields (25-23%) (**Figure 3.16**). This is also an evidence for the stability of the enzyme in the selected organic solvents and under these conditions.

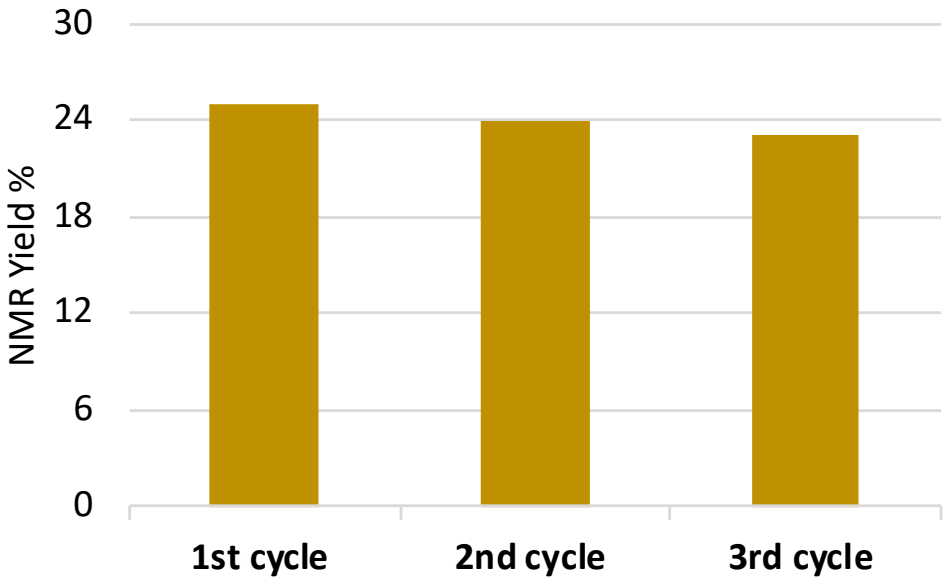


Figure 3.16. NMR yield reusing the biocatalyst up to 3 times.

The Hydrolytic activity of the biocatalyst after each cycle was evaluated by the method previously described. According to the results presented in **Figure 3.17**, the lipase CALB lost some activity after each cycle compared to the fresh enzyme. However, this lost in activity did not affect significantly the formation of the desired ester, as the obtained NMR yields were similar after each cycle.

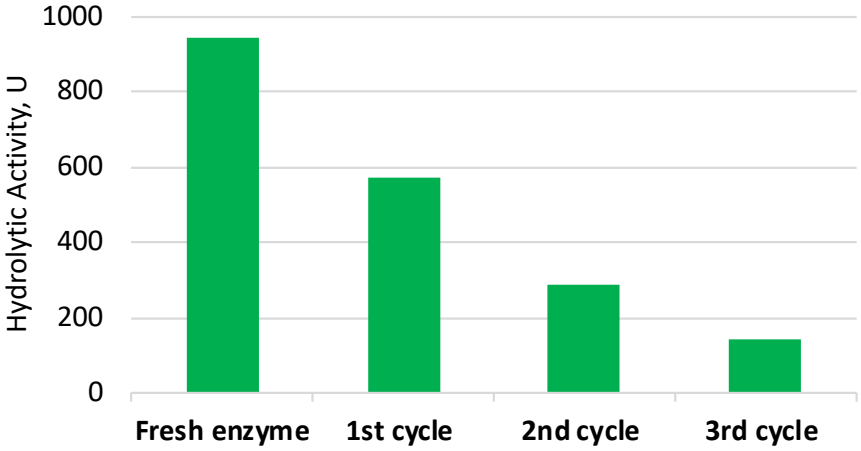


Figure 3.17. Hydrolytic Activity of the biocatalyst after each recycling compare to the fresh enzyme.

This could be explained due to the fact that the evaluation of enzymatic activity is performed in aqueous environment, by studying the enzymatic hydrolysis of tripropionin to propionic acid and measuring finally the activity by titrating the derived propionic acid. On the contrary, the synthesis of glucose monopalmitate is performed in organic solvents, so the behavior of the biocatalyst differentiates.

IV. Study of different green solvents

In the selected optimized conditions received from DoE study, different solvents were chosen to perform the esterification of glucose with palmitic acid. Particularly, t-butanol (t-BuOH), 2-methyl-2-butanol (2M2B) and dimethyl sulfoxide (DMSO) were used and compared to the previous solvent mixture of 10% DMSO:t-BuOH. All the reactions were carried out at 75°C for 24 h in molar ratio 1:3 of glucose: palmitic acid (**Table 3.12**).

Table 3.12. Experimental conditions for glucose monopalmitate synthesis with different solvents.

CODE	Sugar/Fatty Acid Ratio	Solvents	Reaction Temp. / °C	Reaction Time / h	NMR Yield %	Yield %
Glu-PA 5	1:3	10% DMSO:t-BuOH	75	24	**25 ± 2**	**17**
Glu-PA 8	1:3	t-BuOH	75	24	**22 ± 2**	**15**
Glu-PA 9	1:3	2M2B	75	24	**14 ± 2**	**5**
Glu-PA 10	1:3	DMSO	75	24	**0**	**0**

It is feasible to obtain the desired product (glucose monopalmitate) by using as a solvent either only t-BuOH or 2M2B. On the contrary, by using DMSO the reaction did not lead to the formation of the desired product (**Figure 3.18**). By comparing the NMR yields of the formed ester in different solvents, it is clear that the presence of DMSO in small amount of 10% in t-BuOH plays a fundamental role, enhancing the solubility of both reactants (glucose and palmitic acid) and leading finally to the highest NMR yield (25%), even though it has a negative effect in the enzyme activity.

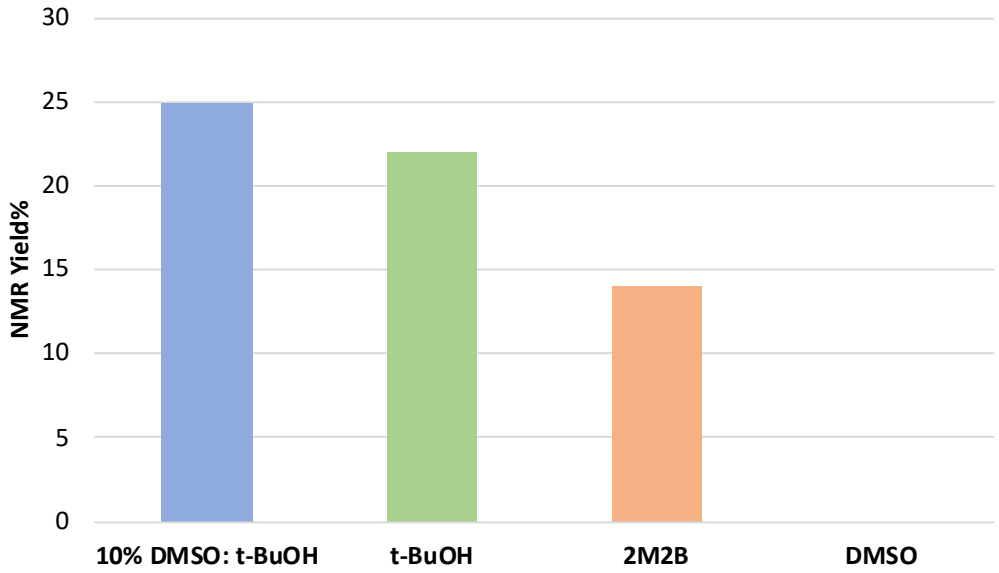


Figure 3.18. NMR yields of the esterification of glucose with palmitic acid in different solvents.

In recent study Ren and Ramsal [41] successfully synthesized glucose esters with palmitic, lauric and hexanoic acid by using the same lipase CALB in a solvent system of 80% DMSO and 20% 2M2B obtaining high conversion. Even though they obtained better results compared to this work, they used a large amount of dimethyl sulfoxide as a solvent, which makes the overall reaction less Green. With the aim of using a synthetic approach, as green as possible, in this work it was possible to obtain glucose esters of palmitic, lauric and stearic by using much lower percentage of this solvent, specifically 10% DMSO and 90% t-BuOH. However, a further study is needed to improve the performance of the biocatalyst in greener solvents, such as t-BuOH and 2M2B, and eventually increase the yields.

V. Characterization of the formed esters

The surfactant properties of the synthesized glucose saturated fatty acid esters (GluML, GluMP and GluMS) were studied. The Hydrophilic-Lipophilic Balance (HLB) of the three esters was calculated according to the Griffin method. Since all the three products showed very low water solubility it was difficult to evaluate their Critical Micelle Concentration (CMC) and surface tension. Also, their oil solubility, both in edible and industrial waste oils (rapeseed oil, rice oil and tall oil) was poor to allow interfacial tension studies.

In order to assess the surfactants wettability, contact angle measurements on the product surface were carried out on (**Figure 3.19**, **Table 3.13**). Specifically, two solvents were used to evaluate their hydrophilicity (water) and lipophilicity (diiodomethane) behavior.

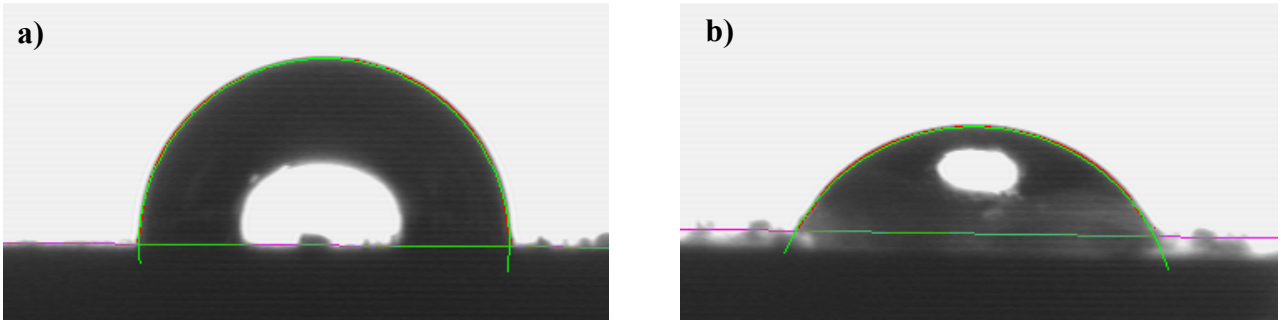


Figure 3.19. Side view pictures obtained from the high-resolution camera of Krüss Easy instrument taken immediately after the syringe tip left a 5 μL: **a)** drop of water; **b)** drop of diiodomethane.

The contact angle values were similar to those expected from the literature, in accordance to the esters HLB index. Indeed, the higher are the HLB values, the lower is the hydrophobicity and the greater the lipophobicity [41].

Table 3.13. HLB and contact angle values of synthesized glucose saturated fatty acid esters.

Sample	HLB	Water	Diiodomethane
		<θ>* / °	<θ>* / °
GluML	9.89	88 ± 2	61 ± 2
GluMP	8.56	89 ± 2	61 ± 2
GluMS	8.02	91 ± 1	57 ± 3

*averaged between three values.

By comparing the data between the three compounds, it is possible to infer a quite comparable degree of either hydrophobicity (very similar contact angles were detected, all around 90°) or lipophilicity (about 60°). However, the differences among the contact angles are very small and not so appreciable to certainly explain the HLB / wettability correlation. Further studies are required to have a deep explanation of the surface / wetting properties of the adopted compounds.

3.3.5 Synthesis of Galactose esters using saturated fatty acids

In the previous optimized reaction conditions, the esterification of galactose with saturated fatty acids has been studied. Again, lauric acid, palmitic acid and stearic acid have been used performing the reaction at 75ºC and 65ºC for 24h in the solvent mixture of 10% DMSO: t-BuOH (**Table 3.14**). Galactose esters were successfully formed, obtaining though mixture of mono- and di-esterified products (**Figure 3.20**).

Table 3.14. Experimental conditions for galactose esterification with saturated fatty acids.

CODE	Fatty Acid	Sugar/Fatty Acid Ratio	Reaction Temp. [°C]	Reaction Time [h]	Yield of Mixtures %
Gal-LA 1	Lauric Acid	1:3	75	24	4
Gal-PA 1	Palmitic Acid	1:3	75	24	5
Gal-SA 1	Stearic Acid	1:3	75	24	3
Gal-LA 2	Lauric Acid	1:3	65	24	11
Gal-PA 2	Palmitic Acid	1:3	65	24	2
Gal-SA 2	Stearic Acid	1:3	65	24	3

Regarding the fact that the products were a mixture of mono and diesters it was not feasible to calculate the yield by NMR. The presence of three and more products was confirmed by TLC analysis. Particularly, for the experiments performed at 75 ºC, high number of products were detected, confirming that acyl migration from the C six position (C6) of galactose moiety to other hydroxyl groups at high temperatures. Indeed, a further esterification on the primary alcohol group may occur, catalyzed by the enzyme CALB, leading to complex mixtures of diesters in different positions.
However, because of the similar behavior of these compounds with the starting saturated fatty acid, most of them were dissolved during the purification steps. The purification of these products was not possible also by flash column chromatography or by other purification methods, so they were not identified and characterized.

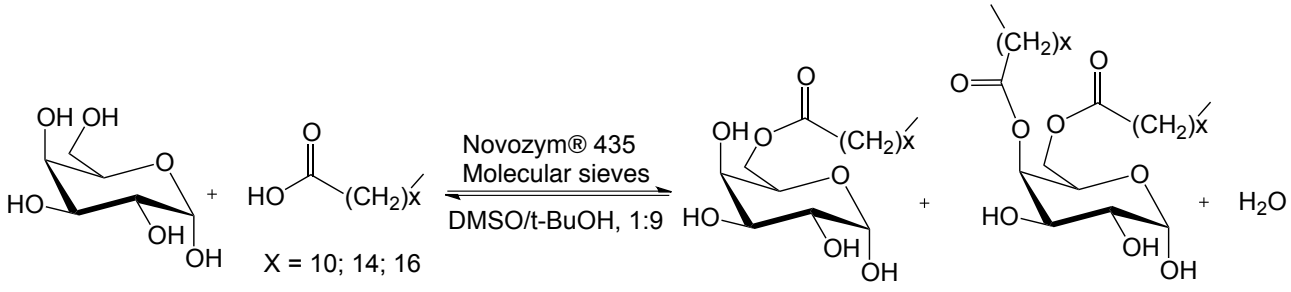


Figure 3.20. Reaction scheme of galactose with saturated fatty acids, where X = 10, 14, and 16 correspond to lauric acid, palmitic acid and stearic acid, respectively. The formation of mixture of mono- and diesters occurred at these conditions.

In order to investigate further the mixture of the formed products, experiments performed also by decreasing the temperature from 75 to 65 ºC, leading finally to the formation of one diester, as it was confirmed by TLC analysis.

The purification procedure used for glucose saturated esters was adopted also here and the obtained final mixtures, after the precipitation in water, were subjected to ESI-MS and ^{1}H and ^{13}C NMR analysis. The presence of monoester and the derivative bearing two acyl groups was confirmed. Though, it was not feasible to determine the position of the second acyl group, due to the overlapping of signals in NMR spectra. Possibly, the acyl migration occurred in the C four position (C4) of galactose moiety.

The yields of these reactions are lower than those obtained from glucose. This could be explained by the poorer solubility of galactose in the selected conditions compared to glucose. In addition, most of the formed diesters were dissolved during the removal of the non-reacted fatty acids by washings with hexane/heptane, as their behavior is similar to that of the starting acid. Yields could be improved by monitoring and studying further the reaction conditions and also by optimizing the purification steps for galactose esters.

3.4 Conclusions and Perspectives

The aim of this part of the thesis was the enzymatic synthesis of sugar fatty acid esters (SFAEs), that can be used as bio-surfactants.

As a first approach, an enzymatic screening of several lipases, both in free and immobilized form, for the esterification of glucose with oleic acid was performed. The commercial immobilized lipase from *Candida antarctica* B (CALB) was selected among the tested lipases as the biocatalyst that promoted the ester bond formation. The reactions were carried out at 65ºC for 48h in 10% DMSO: t-BuOH solvent mixture. Molecular sieves were added to the reaction mixture to remove the water by-product and thus shift the reaction toward sugar ester formation. Glucose monoolate (GluMO) and Galactose monoolate (GalMO) were synthesized and characterized, obtaining though low yields regarding the great difficulty in the purification of these esters from the non-reacted oleic acid.

Saturated fatty acids were also used for the esterification of glucose and galactose catalyzed by CALB. Particularly, Glucose monolaurate (GluML), glucose monopalmitate (GluMP) and monostearate (GluMS) were successfully synthesized. The reaction of galactose with saturated fatty acids led to the formation of a mixture of mono- and diesters.

An alternative purification procedure was developed, providing an easy and fast method for the recovery of the formed saturated esters. Briefly, the crude reaction was filtrated to remove molecular sieves and the immobilized CALB and t-butanol was evaporated. Then, in order to remove DMSO, water was added to the filtrate leading to the precipitation of the formed ester and non-reacted fatty acid. Finally, the precipitate was suspended to hexane or heptane for the selective dissolution the non-reacted fatty acid and the pure esters were recovered by filtration.

A valid method for the evaluation of the yields of the non-isolated products has been developed by using ^{1}H NMR analysis of the mixture obtained after the precipitation in water that contained the formed ester and the non-reacted fatty acid.

Rational design of enzymatic reactions (DoE study) was carried out by using the synthesis of GluMP as the model reaction. Sugar: fatty acid ratio, temperature, and reaction time were selected as variables (response: product yield). From the elaboration of the data obtained, the highest NMR yield was achieved by using a ratio glucose/palmitic acid of 1:3, at 75 °C for 24 h.

In the selected experimental conditions (glucose: palmitic acid 1:3, 75ºC, 24h) it was succeeded to recycle the catalyst up to three times obtaining similar yields with respect to the first cycle, although there was some loss of activity of the biocatalyst.

Different solvents were also studied for the esterification of glucose with palmitic acid in the previously selected conditions. In particular, t-butanol (t-BuOH), 2-methyl-2-butanol (2M2B) and dimethyl sulfoxide (DMSO) were used and compared to the previous solvent mixture of 10% DMSO:

t-BuOH. Glucose monopalmitate was obtained both in t-BuOH and 2M2B, two solvents that are considered green, in lower yields with respect to the mixture. DMSO has a fundamental role in enhancing the solubility of both species, thus increasing the product yield despite its negative effect on the enzyme activity.

A further study is needed on the enzymatic esterification of sugars and fatty acids in order to increase the reaction yields. Also, there is a need to search for other enzymes that could promote the sugar fatty acid ester synthesis, preferably in immobilized form. The choice of a proper immobilization technique is also crucial, as the stability and activity of the derived immobilized enzyme should be taken into consideration. The utilization of oil wastes with high free fatty acid content (such as rice bran oil) and disaccharides (lactose) for the synthesis of sugar esters *via* enzymatic approach is another interesting topic that needs to be explored.

3.5 References

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Acknowledgments

The doctorate thesis work has been carried out at the Laboratory of Organic Chemistry at the Department of Chemistry of the University of Milan.

I would like to thank my supervisor, Professor Giovanna Speranza, for the assignment and supervision of my PhD work. I would also like to thank her for the scientific guidance, the moral support and the confidence throughout the course of this work. In addition, I would like to thank my co-tutor Dr. Nicoletta Ravasio from CNR – SCITEC for her support and advice during my studies.

Moreover, I am grateful to Professor Daniela Ubiali and Dr. Teodora Bavaro from the department of Drug Sciences of University in Pavia, for the excellent collaboration and their valuable help for the enzymatic activity assays and SDS-PAGE characterization. Many thanks also for the fruitful collaboration go to Dr. Giovanna Boschin from the department of Pharmaceutical Sciences of the University of Milan for the ACE-inhibitory activity assays, and to Prof. Giuseppe Cappelletti of our department for the characterization of the surfactant properties of the synthesized sugar fatty acid esters. I would like to thank also Dr. Carlo Morelli from my group, for his support and useful advice during the practical work in the laboratory.

Especially, I would like to thank the postgraduate students Luca Vitali, Giuseppe Mazzola, Davide Rosa and Sara Sangiorgio that have collaborated with me during these three years, for their assistance and excellent cooperation in conducting the experiments. Also, I would like to thank all the people in my group for moral support and great understanding they have shown during the last three years.

Last but not least, I would like to thank warmly my husband, Filippo, for his tremendous support and love and my beloved parents, Valerios and Maria, that are always by my side.