CONTENT

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INTRODUCTION

The miraculous properties of the milk thistle have been made popular by the herbalist Discorides since the time of ancient Greece. He wrote that the tea of milk thistle seeds could annihilate the poison from the venomous snake bite. Pliney The Elder promoted that a mixture of plant juice and honey helps the gallbladder. It was in 1534 that the milk thistle began to be given as treatment for liver deases by Otto Brunfels. In the late sixteenth century, Culpepper claimed that the plant infusions are excellent for obstructions of the liver and the spleen and also curative for jaundice and for breaking and expelling stones. Rademacher popularized the ethanol extract from the seeds for hepatosplenic disorders[1].

Over the past 25 years, the herbal industry of the U.S. exploded due to the increased interest of the population for herbal products and remedies. In 1994, the Congress of the Dietary Supplement Health and Education Act classified vitamins, minerals and herbs as dietary supplements rather than drugs thus leaving the Food and Drug Administration without too much power over their manufacturing protocols. Since then, the natural remedies market has registered huge profits[1].

Silymarin is a natural supplement and besides this, it has extraordinary medicinal properties, whereas it is included in many phytochemical products. Its functionalization with hydrocarbon radicals in the active hydroxyl positions could give compounds with much higher affinity for lipophilic media. On one hand, these compounds introduced into creams or cosmetics products may overcome the skin's barrier and may exhibit preventive effects on tissue degeneration. On the other hand, they may be included in capsules and pills intended for oral administration, in order to metabolize and to reach the affected target much more easily. This is also the direction of the study proposed in the following pages. In other words, an esterification reaction is desired between silybin, the main component of silymarin, and various fatty acids, the process being mediated by lipase as a cold-active enzyme.

CHAPTER 1. Substrate: silymarin and its constituents

1.1. Concept

Silybum marianum is a very effective natural remedy which has been used since the ancient times, known in the popular consciousness as the milk thistle. It originates from Southern and Southeastern Europe, but due to the multiple colonization of the past, the milk thistle was also spread in the warm areas of America, Asia and Australia along with its medicinal benefits and applications. 'Thistle' is the common name of a group of flowering plants characterised by spiked leaves with white veins from which the milk is extracted. *Silybum marianum* belongs to the aster family, *Asteraceae* or *Compositae*, as it could be found in daisies, artichokes and thistles. Reaching the maturity, the thistle plant blooms with a large, purple flower, while its spikes extend to the stem. In a heraldic direction, it is interesting to mention that the crowned thistle is the national emblem as well as a high chivalric order of Scotland since 1249 and also, the official symbol of the city of Nancy, the former capital of the Duchy of Lorraine [1].

From the biochemical point of view, *Silybum marianum* consists of a group of flavonolignans colectively known as the silymarin mixture. Highest concentrations of silymarin are found in the fruit, pericarp, seeds and leaves of the thistle plant with a range of 1.5%-3.5% flavonolignans in relation to the fruit weight [2].

In other words, silymarin comprises silydianin, silychristine and silybin, being noticed that the last compound shows more active biological properties. They fairly act as free radical scavengers and stabilizers of plasma membranes. Silymarin preparations for clinical use became officially adopted in 1969 [1], which has so far declined an avalanche of studies on its flavonolignan components that induce hepatoprotective effects, as well as antioxidative, antiinflammatory, hypolipidemic and neuroprotective effects [2].

1.2. Silybin and the other flavonolignans

Nowadays, the term silymarin refers to the extract of *S.marianum* from milk thistle which is rich in flavonolignans compounds. European Pharmacopeia claims that milk thistle extract contains 30% to 65% silymarin corresponding to 20-45% silydianin, 40-65% silybin A and B, 10-20% isosilybin A and B. From a chemical point of view, flavonolignans are natural polyphenols, biogenetically related to lignans due to their similar synthetic pathways [3]. They consist of two phenylpropanoid units linked to another complex structural part that ensures the binding of the C_6C_3 ring with that of the flavonoid nucleus in different positions. Due to their extended and complicated structure, these compounds show multiple chirality which leads to the existence in nature of several stereisomers. The first known source of flavonolignans was isolated 2000 years ago from milk thistle and as a consequence of multiple chirality, *S.marianum* contains 23 natural related components [4].

As previously noticed, silymarin is maily composed of individual flavonolignans and the flavonoid taxifolin. The chemical composition analyzed by HPLC-MS could be studied from the following table (Tabel 1)[5].

Tabel 1. Characterization of silymarin mixture by HPLC-MS[5].

Compound	Retention Time (min)	Content (%)
SB A	6,41	16,34+/-1,60
SB B	6,99	21,64+/-1,53
ISB A	8,15	5,73+/-1,16
ISB B	8,44	2,90+/-0,65
SC A	3,1	13,73+/-1,20
SC B	3,82	1,83+/-0,15
SD	3,68	4,55+/-0,62
DHSB	12,47	0,33+/-0,07
DHSC	8,02	0,56+/-0,09
TA	1,99	2,09+/-0,41

Silybin is the main component of silymarin and hence the most biologically active. Structurally, the chromone ring is responsible for the weak acidic properties and the antioxidant response is given by the phenolic hydroxyls from 3,4- and 4,5- suitable positions for the formation of complexes with

various metal ions. Very low water solubility (430 mg/L) restricts its therapeutic efficacy even if it is clinically safe at high doses (>1500 mg/day for humans) [6]. Silybin exists in two stereisomeric forms A (2R, 3R, 10R, 11R) and B (2R, 3R, 10S, 11S) having reduced solubility both in water and in lipid medium [7]. All silymarin components' structures are outlined in the figure below (Figure 1).

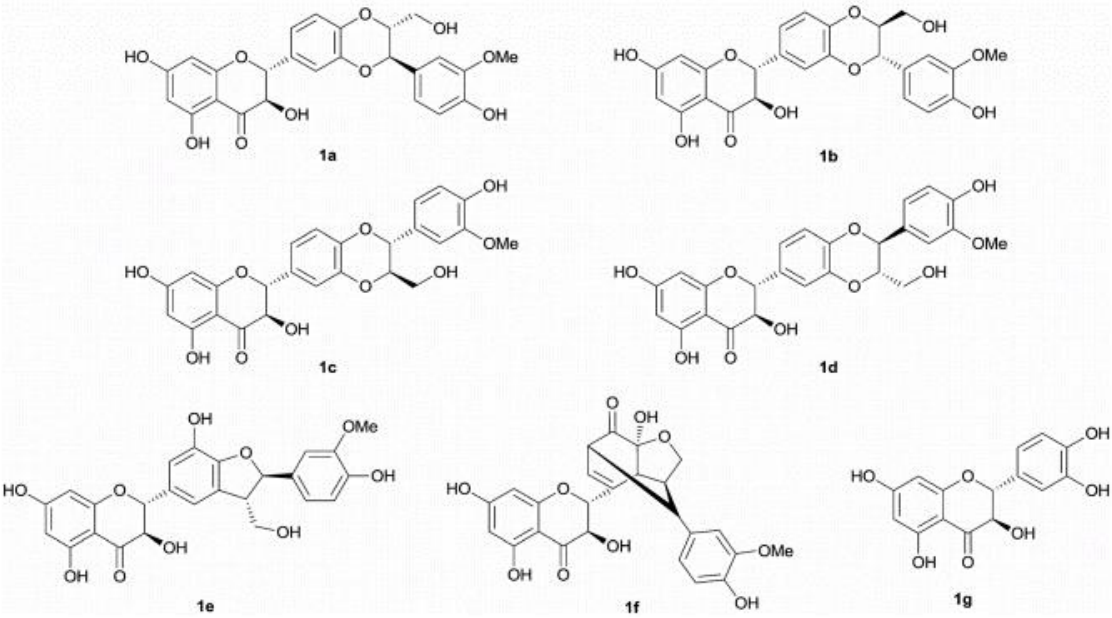

Figure 1. Structures of silymarin components: (1a)-silybin A, (1b)-silybin B, (1c)-isosilybin A, (1d)-isosilybin B, (1e)-silychristin A, (1f)-silydianin, (1g)-taxifolin [33].

In this direction there are many semisynthetic modifications to increase the bioavailability while retaining its biological activity. It is the case of Legalon®, a common drug intended for liver therapy, which contains bis-hemisuccinate derivated silybin with high affinity for aqueous environment. There are also other modifications in order to increase the solubility in water, such as phosphodiesterification and glyco-conjugation. At the opposite pole, silybin derived at 7-OH group with palmitate or at 23-OH with acyl residues tends towards hydrophobic media [7]. Otherwise the structural modifications are imperious because silybin undergoes intensive Phase II metabolism and is rapidly excreted in bile and urine, leading to low therapeutic efficacy. By all means, oral administration of the drug is preferred, although in the case of silymarin extracts only 9 ng/mL concentrations are absorbed into plasma. When an active agent is orally delivered, it must first dissolve in gastric and/or intestinal fluids in order to permeate the membranes of the gastrointestinal tract to reach systemic circulation [8].

1.3. Medicinal benefits of silymarin

In the last decades, many hypotheses and studies on the miraculous properties of silybin and silymarin have been stated. Silybin functions primarily as an antioxidant therefore this term must be correctly understood. Generally, antioxidants exert protective effects on the biomolecules and biocomponents which are subjected to oxidative stress. In the living organisms antioxidants associate themselves in complex biological systems, often as redox tandems in order to prevent and repair proteins, lipids and nucleotides from damage caused by reactive oxygen species (ROS, e.g., OH• , O_3 •, NO, ROO•, H_2O_2, $^1O_2$, HClO, etc). Being inductory of oxidative stress, ROS are a cause of autoimmune diseases through the interaction between the intact and the damaged proteins, involving the so-called cross-immune reactivity [9]. It has to be metioned that at larger concentrations, antioxidants could also become harmful oxidative stressors. The most important antioxidants are undoubtedly the vitamins (ascorbate, retinol and tocopherol), carotenoids and flavonoids. Recent studies revealed that antioxidants could also play the role of mediators for some nuclear receptors engaged in the production of intracellular enzymatic antioxidant systems. The combination of various antioxidants results in the formation of hybrids with a pronounced antioxidant effect. There are useful exemples described in the literature: tocopherol and procaine, flavonolignans and fatty acids, improved ascorbic acid, iron chelator deferiprone [10].

In the recent past, silymarin started to be introduced in dermatological and cosmetic preparations for its antioxidant effect and its capacity to withstand the UVB- and chemically- induced damages. There are various factors, both intrinsic (genetic) and extrinsic (environmental), leading to a perpetual process of functional and structural modifications in skin tissue. Solar radiation caused by UV (295-400nm) wavelengths reaches the earth surface and initiates skin reactions that result in reactive oxygen species formation (ROS). These free radicals drive to carbonylated proteins, peroxidated lipids and enzyme activation, thus causing the remodeling of the extracellular matrix. It is the first signal of collage and elastan fibers destruction, of lowering the skin hydration and increasing the inflammatory susceptibility. Collagenase and elastase hydrolyze the support and integration networks of skin tissue, breaking down collagen and elastic fibers. Moreover, hyaluronidases section the hyaluronic acid into smaller fragments, influencing the hydration capacity of the skin [5].

As a consequence of the antioxidative effect proposed by silybin, a number of other properties related to the antioxidative ones could be formulated. Reported activities include inhibition of lipid peroxidation of hepatocyte, microsomal and erythrocyte membranes in rats, and protection against genomic damages by suppression of hydrogen peroxide and super oxide anions and of lipoxygenase.

It is considered that silybin also influences hepatocyte synthesis by stimulating the activity of ribosomal RNA polymerase, as well as protecting against radiation-induced suppression of hepatic and splenic DNA and RNA synthesis [1]. Silymarin anti-oxidative, anti-fibrotic, antiinflammatory, membrane stabilizing, immunomodulatory and liver regenerating properties play an important role in experimental liver diseases. There are also significant responses of flavonolignans to mushroom (*Amanita sp.*) poisoning, hepatitis, cirrhosis and liver fibrosis [3]. Moreover, silymarin increases the activity of anti-oxidant enzymes like superoxide dimutase, catalase, gluthatione peroxidase, gluthatione reductase and gluthatione-S transferase. The molecular mechanism of the antioxidant activity is based on the functions of each hydroxyl groups, these offering the suitable positions for silybin derivatization without losing the biological activity of the resulting conjugates [8].

Silymarin inhibits all stages of carcinogenesis, acting as a prophylactic and therapeutic agent in the treatment of more advanced and agressive forms of cancer. It is highly recommended for effective control of chemotherapy and radiotherapy-induced toxicity by inhibiting the expression and the secretion of the growth factors that cause the formation of the tumor cell lines [8].

1.4. Silymarin extraction from natural sources

Freudenberg was the first who built the hypothesis of naturally biosinthetical processes formation of flavonolignans in *S. marianum* (purple flowering plant): starting from (+)-taxifolin and coniferyl alcohol in an oxidative environment provided for peroxidase enzyme catalytic activity. Firstly, there are formed neutral phenoxy and quinone methide radicals, followed by O-coupling. Finally, a thermodynamically controlled nucleophilic attack of the hydroxyl group on the quinone methide system through an intermediate affords the 2,3-trans-substituted 1,4-benzodioxane skeleton. The production of flavonolignans in the white-flowered variant is different, whereas instead of taxifolin, the 3-deoxy derivative eriodictyolmay is involved in the biosynthesis [3].

Synthetically, silymarin is extracted from *S. marianum* fruits with amphipolar solvents methanol, 80% methanol, ethanol and ethyl acetate by percolation or with the use of Soxhlet apparatus. The fatty materials from the fruit extract are removed by extraction with n-hexane or petroleum ether, beining obtained flavonolignan-containing extracts. Typically, the seeds of *S.marianum* are partially defatted by pressing, which lowers the fat content from approximately 25 to 8 %. Then the seeds are extracted with acetone (alternatively ethanol, methanol or ethyl acetate). Acetone extract is partially

evaporated and the remaining fat is removed by hexane extraction. Crude silymarin (complex) precipitates after further evaporation. Pure silybin is prepared by dissolving silymarin in absolute ethanol followed by addition of about 10 % water. Crude silybin, which precipitates could be further purified by recrystallization from ethanol [8].

At the industrial level, the extraction of silymarin is performed with ethyl acetate and acetone. Further, silymarin components are separated by solvent-solvent partition and different chromatographic techniques such as OCC, TLC, LPLC, RP- and NP-MPLC, RP- and NPHPLC. The separation of diastereoisomers of silybin, isosilybin and silychristin and regioisomers (−)-silandrin and (−)-isosilandrin was accomplished in the 2000s by RP-HPLC, MPLC and HPLC. Moreover, the isolation process of the extracted fractions was monitored by analytical TLC or HPLC [3].

Cold press methode is proposed by Derya Duranet al. as being a more economical technique compared to solvent extraction and hot press along with its simplicity and energy efficiency. It has been reported to be the best way to produce high-quality oil. The popularity of the method increased since no heat or chemical treatment is used during the cold press process and all beneficial nutritional properties of the raw material are transmitted to the oils. These oils consist of natural phytochemicals as tocopherols, fatty acids, sterols and antioxidant phenolic compounds. However, after the cold press method two fractions are obtained: the oil and the waste and silymarin is completely passed into the residue, so the problem arises in the light of the valorization of biomass waste [2].

A greener method of extracting the silymarin mixture from the milk thistle proposes the use of water that solubilizes natural compounds at high temperature and elavated pressure. Give the physical parameters: dielectric constant, surface tension and viscosity of the water, which all are absolutely dependent on the temperature, adjusting the temperature and the pressure could bring the water in a strong solvent stage with similar properties to organic solvents. Unlikely organic solvents, which require defatting of the milk thistle prior to extraction, water extraction did not require deffating. This procedure assumes that 0.5g of milk thistle seeds are milled to an average diameter of 0.4 mm and then introduce into the extraction cell along with 2 g of washed sea sand. The cell is installed in the GC oven and the water starts pumping into the cell with a constant flow. The pressure is maintained above the water pressure at the desired temperature of 140°C. After collectinng the fractions at well-defined time intervals, 1 mL of each sample is subjected to compressed nitrogen drying, then redissolved in methanol, filtered and analyzed by HPLC. The results could be observed in the next table (Tabel 2) [11].

Tabel 2. The efficiency of flavonolignan extraction by hot water procedure [11].

	Maximum yield (mg/g seed)			
Temperature(°C)	**Taxifolin**	**Silychristin**	**Silybinin A**	**Silybinin B**
100	0,6	2,3	1	1,6
120	0,7	2,5	1,2	2,2
140	0,5	2,4	1,2	2

1.5. Improvement of silymarin properties

It is undeniable that nowadays silymarin is a popular medicine due to its hepatoprotective, antioxidant, antiviral and antitumoral properties, but there are also some drawbacks concerning its water solubility, poor intestinal absorption as well as an elavated metabolism for each of its flavonolignan components [12]. In this direction studies have been carried out in order to increase the bioavailability of silymarin by developing new drug designs. These include drug modifications such as salts, esters and complexes with hidrophilic excipients, complexation reactions with cyclodextrines and phospholipid, formation of biocompatible polymer dispersions, lipid-based delivery systems, nanoemulsions and encapsulation in biodegradable nanomaterials [6].

In the main area of pharmacological interest stays the self-emulsifying drug delivery technique through which amphiphilic particles are formed, with the drug inside a surfactant molecules coat. The gastrointestinal absorption is greatly facilitated due to the surface interactions of these amphiphilic systems. The supersaturation of these systems could drive a faster absorption but thermodinamically, it is an unstable state that tends to return to the equilibrium by drug precipitation. This inconvinient can be overcome by precipitation inhibitors such as amorphous solid dispersions with PVP and PVA polymers [12]. The following figure outlines several methods by which the higher bioavailability of silymarine could be obtained (Figure 2).

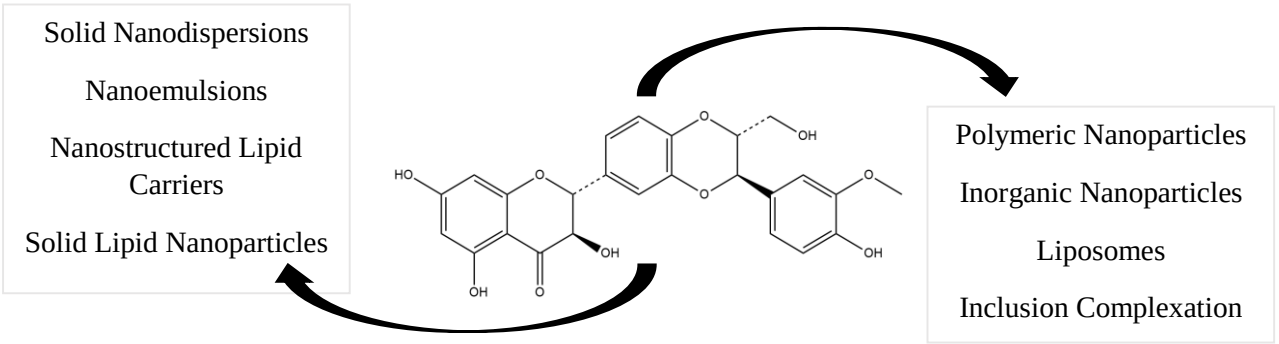

Figure 2. Silymarin modification methods.

A demonstrative study of the bioavailability improvement is given to the scientific community by S. Drouet et al. which achieved a selective acylation of silybin in 3-OH position to give 3-O-palmitoyl-silybin in the presence of Lewis acid $CeCl_3 \cdot 7H_2O$ as it could be oserved in the Figure 3. The yield reached was around 60%, with 85% of palmitate linked in position 3-OH and 15% in position 5-OH but with no negative change in antioxidant capacity of the derivative [7].

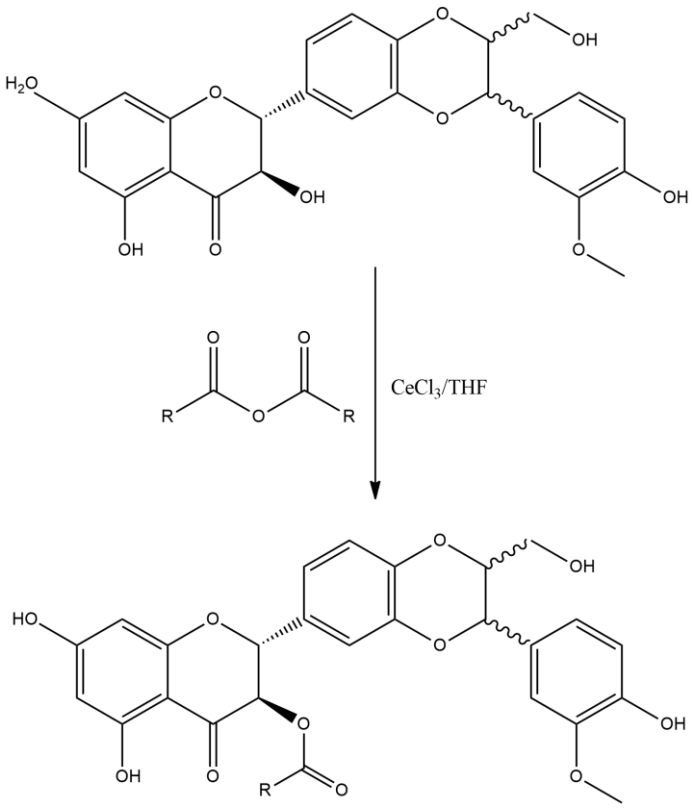

Figure 3. 3-O-palmitoyl-silybin synthesis

Two methods of radical scavenging were performed to demonstrate this: ferric reducing antioxidant power (FRAP) used for hydrophilic antioxidants, and copper reducing antioxidant capacity (CUPRAC) used for lipophilic and hydrophilic antioxidants. With FRAP assay the radical scavenging of silybin was 1.74 times more effective than give 3-O-palmitoyl-silybin while the derivative was 1.55 times more effective than silybin with the CUPRAC assay [7].

Besides the chemical modification methods, there are encapsulation variants of the compound of interest in matrices much better accepted by the organism. In the following are outlined some models. Silymarin could be included in natural β-cyclodextrines forming complexes often used because of their solubilization potential in the body barriers and of their preventive effect on metabolic degradation [6]. Other types of molecular complexes capable to enhance the bioavailability of the active constituents of silymarin are represented by phytophospholipid complexes known as phytosomes. The phytosome unit is a molecular complex between phospholipids and standardized polyphenolic constituents in a 1:1 or 2:1 molar ratios. The literature promotes the silybin-phytosome

system as having a more intense therapeutic activity compared to that of the unmodified compound [12]. Further, liposomes are hollow spherical nanoparticles with a closed shell of a lipid membrane (mono- or multi-layer), inside of which an aqueous solution can be encapsulated. These supramolecular aggregates owe their success as carriers of therapeutic drugs for many advantages including the capability to encapsulate both hydrophilic and lipophilic drugs, having targeting and controlled release properties, cell affinity, tissue compatibility, reduced drug toxicity and improved drug stability. Moreover, liposomal systems are known to find an immediate access to the reticulo-endothelial system (RES) rich sites like liver and spleen, and this self-targeted nature of liposomal carriers can be exploited well for drug distribution to hepatic site. Thus being said, flavonolignan components of silymarin could be integrated into liposomal systems in order to increase their properties [6].

CHAPTER 2. The cold-active enzymes

2.1. Concept

The biotechnology industry is highly promoted because it is environmentally friendly and has a valuable potential to take the place of the chemical industry based on the petrochemical refineries, which use harsh reaction conditions. With the interest of the scientific world focused on renewable resources and Green Chemistry, the bio-catalytic approach becomes highly appreciated in the new generation of synthetic processes, where chemicals of industrial value are obtained under mild conditions from renewable sources of biomass, in most cases [13]. Chemical syntheses based on enzymes used as biocatalysts are topical because of the reduction of the number of synthesis stages, high efficiency of atoms, by avoiding the protection steps [14], to a good regio- / enantio-type selectivity, which could be achieved under mild process conditions, in other words, green conditions. New bio-catalytic concepts have been launched using enzymes, either solubilized in the reaction medium (homogeneous bio-catalysis) or immobilized (heterogeneous bio-catalysis) [15].

This study brings in the spotlight the enzymes from organisms that live in extremely harsh environmental conditions, which to some extent simulate the conditions available in industrial processes. Extremophile microorganisms that live in cold environments represent a particularly interesting source of living material adapted to abnormal conditions or considered extreme in comparison to those confortable for human beings [16]. In contrast, organisms that live in moderate environmental conditions may be termed mesophiles or neutrophiles. As previously mentioned, microrganisms that adapted and colonized cold places on Earth may be divided into psychrophilic or psychrotolerant depending on the optimal temperature of development. Psychrophilic organisms grow onto significant low temperature range between -20 and 10°C being although unable to develop at temperatures higher than 15°C. Meanwhile, psychrotolerants optimally grow between 20-25°C and even register high metabolic activiy at values below 0°C. This behavior could be explained by the fact that psychrophiles are usually found in marine ecosystems while psychrotolerants thrive in terrestrial cold environments.These cold-adapted microorganisms mostly include bacteria, yeasts, fungi and algae. Cold and frozen areas of the terrestrial biosphere include polar circles (Arctic and

Antarctic), deep water and frozen altitudes of mountains along with glaciers, ice sheets and permafrost [17]. As a general information, the temperature recorded annually at the poles is always below zero degrees, and during the winter, the thermometers reach -80°C [16]. The polar regions represent 15% and the permafrost 20% of the total surface of the Earth, while the microorganisms able to maintain their life throughout the geological evolution of these platforms are of psychrotolerant type. The aquatic surface of the Earth represents 75% with an average temperature of 3°C, but it is extraordinary to be notified that at the bottom of the seas and oceans the micro-organisms of psychrophilic type coexist and develop in the absence of light, at high pressures and without valid food. At the level of glaciers, they develop in the wires and films of liquid that spread over the mineral grains in order to ensure their nutrient needs [16].

2.2. Diversity of cold-active enzymes

Microorganisms inhabiting low-temperature environments have been adapted in order to survive by the production of cold-active enzymes to ensure the minimum rate of chemical and metabolic reactions and of ice-binding proteins, designed to control the growth of ice crystals thus combating frost and membrane rigidity. Usually, the optimum temperatures for the activity of the cold-adapted enzymes are in the range of 20-30°C, which can be considered close to that of the thermophiles. The ability of these enzymes to incorporate a significant fraction of their activity at low temperatures is based on their structural flexibility. It is worth mentioning that an increase in the flexibility of the enzyme is not necessarily proportional to the decrease in their thermo-stability. There are two strengths related to the active enzymes operating, one of them is for the manipulation of thermo-labile and sensitive substrates at low temperatures and the other facilitates the inactivation of the enzyme as the temperature increases [17].

One aspect that may be less well known is that microorganisms that live in very cold areas, somehow avoid frost, rather than fight it. This contribution is due exclusively to proteins that mediate the binding capacity of ice crystals. The ice-binding proteins are adsorbed on the ice surface leading simultaneously to the decrease of the freezing point and to the slight increase of the melting point of water. This dependence could be clearly observed following the analysis of thermal hysteresis, whose width correlates with the concentration and functions of the protein [18].

Moreover, the adsorption of proteins on the ice crystals leads to their stabilization and inhibition of the recrystallization process. Indeed, ice recrystallization inhibition is of utmost relevance in all freezing processes involving living cells and food products, since large ice crystals damage cell membranes and impair cell viability and food quality [18].

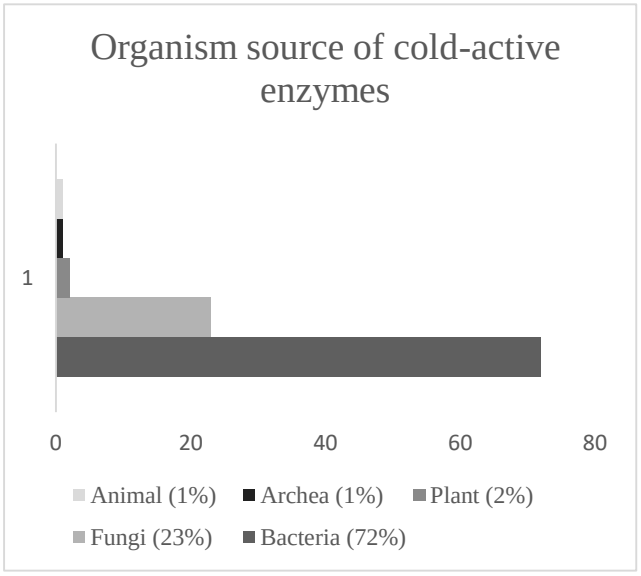

Figure 4.

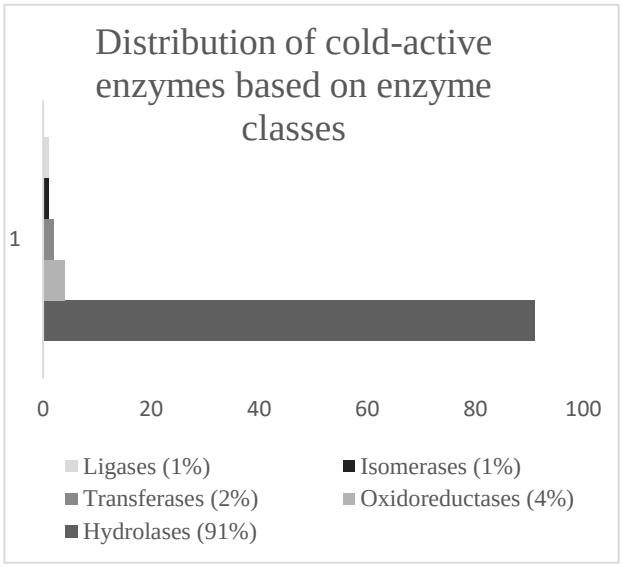

Figure 5.

The majority of cold-adapted enzymes have been expressed in heterologous hosts being obtained from psychrophilic and psychrotolerant microorganisms, bacteria or fungi as it could be observed in Figure 4. Distribution of cold-active enzymes based on enzyme classes could be analyzed in Figure 5. To perform catalysis at low temperature demands high flexibility for proteins that adversely alters the stability of the enzyme. However, there are very interesting examples of enzymes with high thermostability, as is the case of the superoxide dismutase, DaSOD, isolated from a psychrophilic organism, *Deschampsia antarctica,* whose optimum temperature is 20°C but it records a catalytic response even at -20°C. At 0 degrees it retains up to 80% of its catalytic activity [16].

Most of the time, the catalytic properties of mesophilic organisms at reduced temperatures are unusual, as is the case of *Candida albicans* lipase, with an optimal temperature of 15°C or *Arabidopsi* β-amylase 3 with great residual activity at low temperatures. More surprising is to discover a thermophilic enzyme with high activity at low temperatures. For instance, β-galactosidase isolated from *Pyrococcus furiosus* has optimal activity at 90°C, but retaining 8% of its activity at 0 degrees. Comparatively, the lactase activity of *P. furiosus* at 0°C was still 40% of the optimal activity from the main β-galactosidase use in the food industry (28 U/mg at 50°C and pH 7.0) from *K. marxianus*. In addition, the lactase activity of *P. furiosus* at 0°C was 31% of the optimal activity of a cold-active β-galactosidase from *Arthrobacter psychrolactophilus* strain F2 (33 U/mg at 10◦C and pH 8.0) [16].

2.3. Gene cloning and protein engineering

The most convenient method to discover, even create new enzymes refers to the expression of protein chains in different cell and microbial cultures. Many studies have revealed the heterologous hosts used for the expression of cold-active enzymes as it could be observed in Figure 6. From the existence of microbial diversity, only a minor fraction can be reproduced through laboratory experiments in human handling conditions. Extremophile microorganisms depend upon drastic conditions to grow and reproduce, nonetheless, cloning techniques suppress the obstacle by various methods [17]. Metagenomics is the main culture-independent approach involving DNA extraction from the environmental sample, isolation of targeted genes and cloning the material further to create a genomic library. Novel enzymes could also be assembled by computational genomics using available information in genome databases without the need of a natural sample. The discovery of novel cold-adapted enzymes is a real challenge, because only a few genomes of psychrophiles have been deposited in public databases [19].

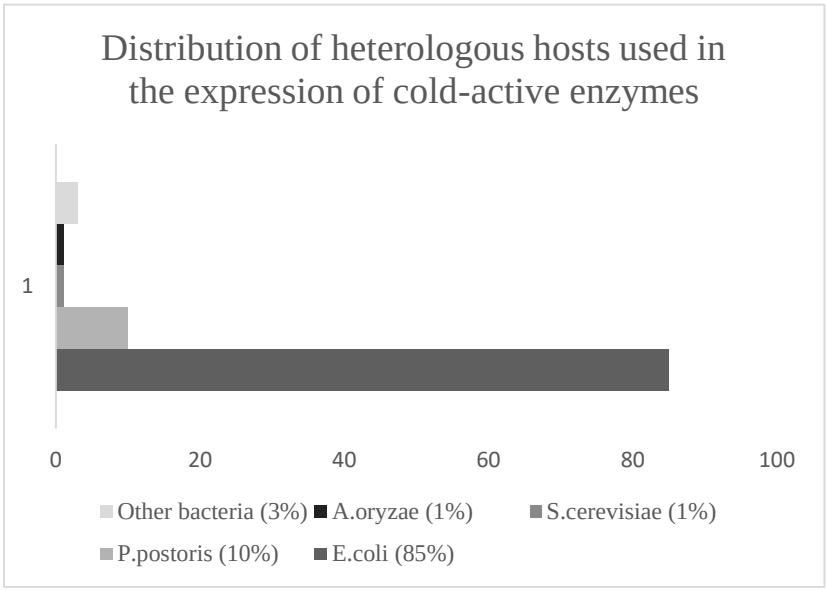

Figure 6.

The first step is the isolation of the adapted microorganism, which stands out with good enzymatic activity. The main cloning method is based on the creation of specific primers for gene amplification that uses a DNA strand as a template [19]. This method is possible if the genome of the species is completely sequenced and stored in the gene bank, so the microorganism can be cultivated. There are several valid hosts for gene expression, but *E. coli* is the most popular in this case. Various genotypes have been used but the preferred strain is BL21. Nevertheless, other expression hosts have been researched, such as *Halobacterium* sp. for the expression of a cold-adapted hydrolase, and *Pichia pastoris*, used as the expression host for 9 proteins including various fungal enzymes. Half of the

adapted genes were cloned into pET vectors and only 5 were integrated into pCold plasmids. The enzyme purification process often calls for His tag fusion while optimal temperature is an important parameter of enzyme characterization as a result of their complete or partial adaptation to cold [16].
By far the most preferred cold-active enzymes are from the hydrolase class, more specifically lipases and esterases. This concentrates all the research efforts on solving their three-dimensional protein structure. Several strategies have been suggested to promote proper expression and folding of cold-active enzymes expressed in heterologous host, increasing their solubility, activity, and yield [19].

2.4. Applications of cold-active enzymes

Introduction of cold-adapted enzymes to traditional production schemes actually diminishes the panorama of the energy consumed at each step of the process, which is explainable because no thermal energy is needed, the reaction yields are appreciable, the enzymes retain their stereo-specificity at low temperatures, which excludes secondary reactions. Moreover, their thermal lability at higher values rapidly leads to the inactivation of enzymes, hanging up the process [20].
The ability to heat-inactivate cold-active enzymes has particular relevance to the food industry where it is important to prevent any modification of the original heat-sensitive substrates and products. This is also of benefit in sequential processes (e.g. molecular biology) where the action of an enzyme needs to be terminated before the next process is undertaken, easily by heat-inactivation [20]. Examples of biotechnological applications of enzymes are provided further and the application classes are listed in Figure 8.

Nonetheless, the industrial applications of cold-active enzymes are still in the early stages. As far as concerns the food sector, cold-active amylases (EC 3.2.1.1) are of great interest for baking and brewing products, since they could be easily inactivated during cooking. A patent developed from *Bacillus licheniformis* with Novozymes improved the catalytic activity in a decreased temperature range from 10 to 60°C. Another patent developed with ColdZYMES ApS was used to heterologously express *Clostridium* α-amylase at lower temperatures than 10°C. The cold-active variants are frequently exploited for milk processing or refrigerated storage as is the case of β-D-Galactosidase usage. Among proteases (EC 3.4.) from psychrophilic sources, that from *Pseudoalteromonas* strain SM9913 might be used in tenderizing collagen-rich meat, while that from *Flavobacterium balustinum* promises wider applications, due to its optimal temperature of 40°C and high thermolability (full inactivation at 50 °C in ca. 10 min) [18].

Cold-active enzymes could be used in chemical manufacturing for organic compounds that are highly volatile and can only be modified at low temperature. In other cases, low temperature may make separations of the product easier and less expensive. Cold-active enzymes could be added to detergents for low-temperature washes or to other solutions for cleaners. Some enzymes might replace chemical preservatives in foods by depleting metabolites required by other organisms, disrupting microbial cells, or degrading other enzymes. Psychrophilic microorganisms and their enzymes are already crucial to nutrient cycling and biomass degradation and production. We can take advantage of the natural role of psychrophiles and use ones producing useful enzymes in waste-water treatment, biopulping and bioremediation in cold climates. Psychrophilic methanogens would be useful in anaerobic digestors to increase methane production in Northern regions. In research, reactions could be performed at low temperature and then the mixture heated to readily inactivate the enzyme before proceeding to the next step. Other cold-active enzymes could substitute for currently used enzymes that require higher temperatures than the cells or substrates require[17].

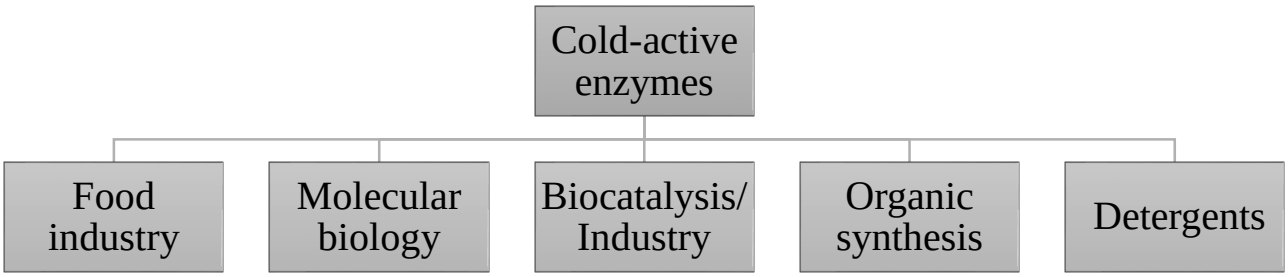

Figure 8. Application classes of cold-active enzymes.

In the field of molecular biology, the enzymes are the main actors. Several *in vitro* reactions necessite low temperature, a condition that could be fulfilled and enhanced by the cold-active enzymes. Ligases (EC 6.5.1.1) catalyse the formation of phosphodiester bonds, joining DNA fragments with protruding or blunt ends. For DNA with protruding ends, the optimal ligation temperature is a compromise between the ligase Topt and the optimal temperature for annealing short DNA protruding ends, usually very low. For this reason, the reaction efficiency can be increased by low temperatures (4–8°C) and by extending the incubation time over several hours. Proteases (EC 3.4.) are used to remove protein contaminants from nucleic acid preparations. The most popular is Proteinase K, which retains relatively high activity at 20°C and is stable up to 95°C [18].

2.5. Cold-active lipase

Extreme organisms develop in a reduced temperature range of 25 and 45°C, due to their adaptation to the inhabiting environment by producing enzymes, along with other biomolecules, which possess specific bio-catalytic activity at low temperatures [21].

Lipases (E.C. 3.1.1.3) are triacylglycerol acyl hydrolases, one of the most important classes of enzymes together with carbohydrases and proteases. Their specialty is both hydrolysis and synthesis of fatty acid esters in aqueous or non-aqueous media [21]. The first reported three-dimensional structures of lipases were claimed by Brandy et al. and Wrinkle et al. They had investigated lipases from *Mucor miehei* and human pancreas, respectively using X-ray crystallography. Various sources of lipases have been researched since then (including *Geotrichum candidum*, *Candida rugosa*, *Chromobacterium viscosum*) [22]. The context of our study is based on the cold-active lipases, which offer bio-catalytic response in the temperature range of 0-30°C. Their cold-adaptation provides such flexibility around the active sites, along with low enthalpy and affinity towards substrates, but high specific activity at low temperatures. Most of lipases investigated so far have been isolated from psychrophilic and psychrotolerant microorganisms of polar areas, deep water or chilled food samples. However, these enzymes are slightly thermostable because they have developed in the context of very low temperatures, so the improvement of their thermal stability is obtained by immobilization, directed evolution, protein engineering, chemical treatments. An extremely interesting observation is that the cold-resistant lipases extracted from organisms of tropical areas show very good thermo-stability compared to the analogues of the alpine areas [21].

Like all other lipases, the cold-resistant possess the canonical α/β hydrolase fold (central, hydrophobic β-sheet that is covered by α-helices from both sides); the active site contains the catalytic triad, Ser105(nucleophile)-His224(basic residue)-Asp/Glu187(acidic residue) and an oxyanion hole. In most cases, the active site is covered by a lid which opens in the presence of an interface to facilitate contact with the substrate [22].

Naturally, the conformational structure of the lipases is adapted to accommodate the substrate at low temperatures. Their behavior is compared with those of mesophilic and thermophilic enzymes by crystallographic studies or site-directed mutagenesis [21]. For instance is the case of *Pseudomonas immobilis* and *Pseudomonas fragi* IFO 3458 lipases which compared with their counterparts reveals that they have a very low content of arginine residues in comparison with the lisine residues, weak hydrophobic core, very few salt bridges and few aromatic-aromatic interactions. In addition, the arginine residues are distributed differently from mesophilic enzymes. Some of them are at the level

of salt bridges, but most cover the surface in the idea of increasing the conformational flexibility. The high content and aggregation of glycine residues depict local mobility. Another feature is associated with the production of trehalose and exopolysaccharides, acting like cryoprotectants for protein precipitation and denaturation. If glycine is substituted by proline, the shift in the acyl chain length enhances the thermo-stability of the enzyme [21].

Cold-active enzymes, in general, inherit heat instability, which undergoes rapid inactivation of the enzymes at moderate temperatures. For industrial applications, the needs of thermostability are crucial. The psychriphilic yeast, *Candida antarctica* expresses two lipases, namely *C. antarctica* lipase A and *C. antarctica* lipase B, with different physicochemical behavior. CAL-A is considered to be the most thermostable enzyme (>90°C), while CAL-B is smaller in size and less thermostable. The factors commonly considered to increase thermal stability are the hydrophobicity, number of hydrogen bonds, amino acid composition, amino acid distribution and interactions in the protein. Directed evolution with random mutagenesis based on error-prone PCR (epPCR) and iterative saturation mutagenesis guided by rational design are more frequently employed nowadays to combat the thermolability of these enzymes [23].

Lipase A from *Bacillus subtilis* after ep-PCR shows an increase of 15°C of the melting point and of 20°C at optimal temperature, comparing with wild-type lipase. From *P. fragi*, a variant is obtained after two rounds of evolution which displays a shift of 10 degrees in the optimal temperature. The protein engineering strategy was adapted to enhance the thermostability where the disulphide and other bonds are modified to decrease the entropy of the unfolded form of proteins or to decrease the unfolding rate of irreversibly denatured proteins. CAL-B and *Geobacillus zalihae* T1 lipase were successfully engineered by mutating five amino acid pairs to cysteine and by introducing an ion-pair in the inter-loop [21].

As stated, lipases are structurally modified to accommodate the substrate at reduced temperatures. The greatest advantage of cold-active enzymes is the consumption of a small amount of energy associated with the high flexibility of their activity under low water conditions. They are cost-advantageous, of wide variety, stable to organic solvents and specific in mild reaction conditions [21].

CHAPTER 3. The aim: cold-active enzyme-based esterification of silybin with fatty acids

3.1. Contribution of fatty acids

Fatty acids are the main monomeric components of lipid derivatives such as triglycerides, phospholipids, cholesteryl esters, which undoubtedly participate in the proper physiological functioning of cells, tissues and therefore of the body. Their classification depends on several aspects, either structural or functional; in the following some of them will be listed [24]. There are four different fatty acids characterized by the same number of eight carbons, but by a different number of double bonds in particular positions and conformations. When there is one carbon-to-carbon double bond, the molecule is monounsaturated, whereas polyunsaturated fatty acids have more than one double bond [25]. Saturated fatty acids are mainly involved in the production and storage of energy, being the first line of bio-components that by consuming 1 gram of lipids generates 9.3 kcal. Saturated fatty acids are also engaged in lipid transport and phospholipid and sphingolipid synthesis ensuring the structural integrity of cellular membranes [24]. Stearic acid, the 18-carbon chain, is the most popular saturated fatty acid found in foods, but palmitic acid, the 16-carbon saturated chain, is the most prevalent in most nutrients, as well as in the body [25].

Monounsaturated fatty acids are key components due to their unsaturation, which confers a slight fluidity in the membrane lipid bilayer. Thus, they accompany monounsaturated fatty acids, displaying more structural properties [24]. Oleic acid is a major monounsaturated fatty acid, with the cis-configuration of the double bond. It is normally found in olive oil, palm oil and canola, which are promoted as monounsaturated oils. As mentioned earlier, polyunsaturated fatty acids contain two or more double bonds. Because of the cis-configuration, the hydrocarbons chain aims a slightly bending, property with a special meaning in the physiological responses of fatty acids. The cis unsaturated fatty acids with their bent chains would behave in a more disorderly fashion, while the saturated and trans unsaturated fatty acids could stack together tightly exposing structural rigidity [25].

The term ‘essential’ for saturated and monounsaturated fatty acids are not commonly exploited, as this term is intended for polyunsaturated bio-components introduced through food intake that fulfill particular physiological functions [24].

Essential fatty acids are important constituents of cell membranes because the multitude of double bonds ensuring fluidity and affinity at the binding sites of enzymes and membrane receptors. They cannot be synthesized by the human or animal body, for which they are purchased from the diet. There are a series of essential fatty acids omega-6 derived from cis-linoleic acid and omega-3 derived from α-linolenic acid. For instance, the linoleic acid is polyunsaturated, having two double bonds, which extends the tendency to bend the chain. The common linoleic acid is an omega-6 mostly encountered in vegetable oils. There is also α-linoleic acid, a less widespread omega-3, found in flaxseed oil, canola, soy and wheat germ oil. All the omega-3 polyunsaturated fatty acids are generally considered to be essential [25]. The omega-9 series derived from oleic acid is considered not to be part of the essential fatty acids category. However, all these series of polyunsaturated acids are metabolized similarly due to the large hydrocarbon chains, which are degraded by the same enzymes. These long-chain metabolites are of particular physiological importance in the brain, retina, liver, kidneys, gonads, adrenal glands [26].

Further it will be presented some aspects concerning the applications and implications of polyunsaturated fatty acids in the human body. The fluidity of the cell membrane is characterized by the composition of constituent lipids. Incorporation of saturated acids and cholesterol molecules increases rigidity. On the contrary, an advanced intake of unsaturated acids contributes to the formation of the fluid mosaic of the membrane, as well as to the improved response of the receptors to analogous hormones, growth factors or membrane proteins. A valuable example is insulin resistance due to the rigid membrane unable to bind insulin receptors. Thus the therapeutic implication of the lipid composition of the cell membranes on diabetes mellitus is outlined. Polyunsaturated fatty acids are shown to inactivate encapsulated viruses. Since neutrophils, macrophages and T cells stimulate the release of unsaturated fatty acids, it could be concluded that they are involved in the body mechanism of defense. Furthermore, their beneficial actions also sum up anti-fungal, anti-inflammatory, anti-viral and anti-bacterial responses. Studies have shown that acids have a guaranteed impact on atherosclerosis by modulating the expression of uncoupled proteins in vascular tissue [26].

3.2. Esterification mediated by cold-active lipases

The lipase-mediated esterification reaction has come to prominence in recent decades, due to the interest in organic esters for biotechnology and the chemical industry. The main difference between lipases and esterases consists of the occuerence of non-polar residues clustered around the active site at high accesibility values [27].

The mechanism of the lipase esterification reaction is similar to the one proposed for the serine-based protease-mediated reaction, involving two tetrahedral intermediates. The serine residue in the triad attacks as the nucleophile of the reaction the acid, losing a water molecule. An acyl-enzyme complex is formed. The alcohol molecule interacts through nucleophilic attack with the first tetrahedral complex to form the second intermediate. Thus, finally the enzyme releases the ester and regains its native form. The mechanism of the reaction could be analyzed in Figure 9. In nature, lipases are found to be active at oil-water interface. In vitro, they are found to be active in aqueous as well as anhydrous organic solvents. The interfacial activation was hypothesized to be due to a conformational change resulting from the adsorption of the lipases onto a hydrophobic interface, where a significant role is also held by the lid [22].

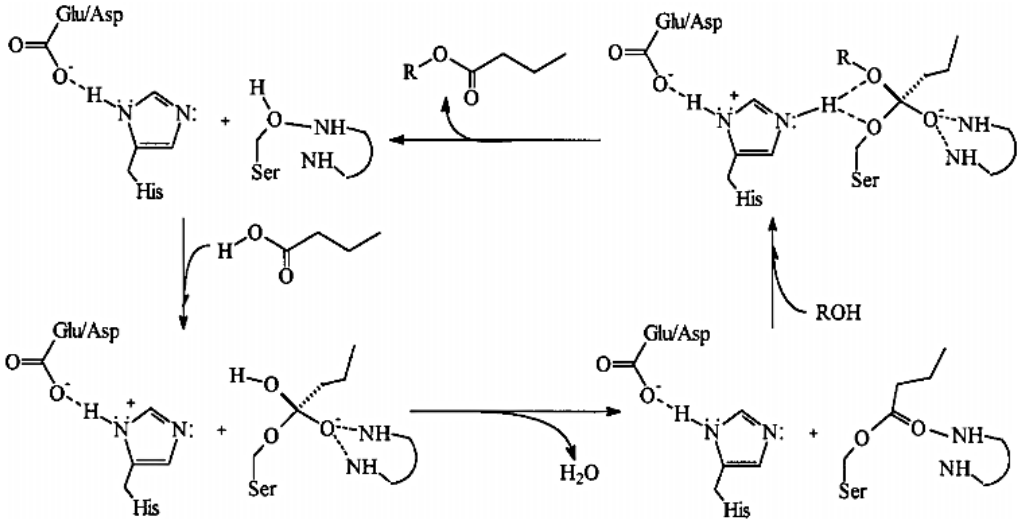

Figure 9. Mechanism of esterification by lipase catalyst.

During the esterification reaction, lipase could be deactivated due to several factors regarding temperature, exposure to interfaces, chemical denaturants, shear stress. The esterification reaction is always performed in non-aqueous solvents, in which the stability of the enzyme depends on the properties of the solvent. As Nakano et al have argued, lipase stability in organic solvent could be enhanced by random mutagenesis. Thermal deactivation of enzymes is greatly reduced by

immobilization. Arroyo et al. and Moreno et al. have promulgated studies on the temperature deactivation of immobilized enzymes, having as reference lipase B from *Candida antarctica* and lipase from *Candida rugosa*. The deactivation in the case of the immobilized enzyme was much slower. Studies on deactivation of lipids in *Aspergillus oryzae* and *Candida cylindricea* under shear stress have shown that deactivation occurs due to energy consumption per unit volume and gas hold-up [22].

3.3. The design of the biocatalyst

In the following, the focus will be on the enzyme immobilization methods highly recommended for the industrial applications. The immobilization technology was born in the 60s, aiming to improve the stability, reusability, activity, specificity and manipulation of the catalyst. Conventionally, there are 4 major immobilization techniques: adsorption, covalent binding, cross-linking and entrapment, for each one knowing both the advantages and the disadvantages [28]. In Figure 10, there are outlined the immobilization possibilities, excepting the physical adhesion.

By adsorption on a solid support, a physical process itself, the enzyme retains its catalytic activity unchanged, but weak interactions between the enzyme and support can easily lead to the release of the biocatalyst. However, this method of adsorption immobilization is the most preferred [29]. A relatively small number of cold-adapted enzymes have been immobilized on solid supports such as diethyl-amino-ethyl-Sepharose or agarose-coated polyethylene-imine. For instance, cold-adapted β-galactosidase from *Pseudoalteromonas sp.* is responsible for lactose degradation during storage of milk at low temperatures, was immobilized on DEAE-Sepharose. In this situation, the storage stability at 4°C lasted one week, but no extensive measurements have been made at higher temperatures [28].

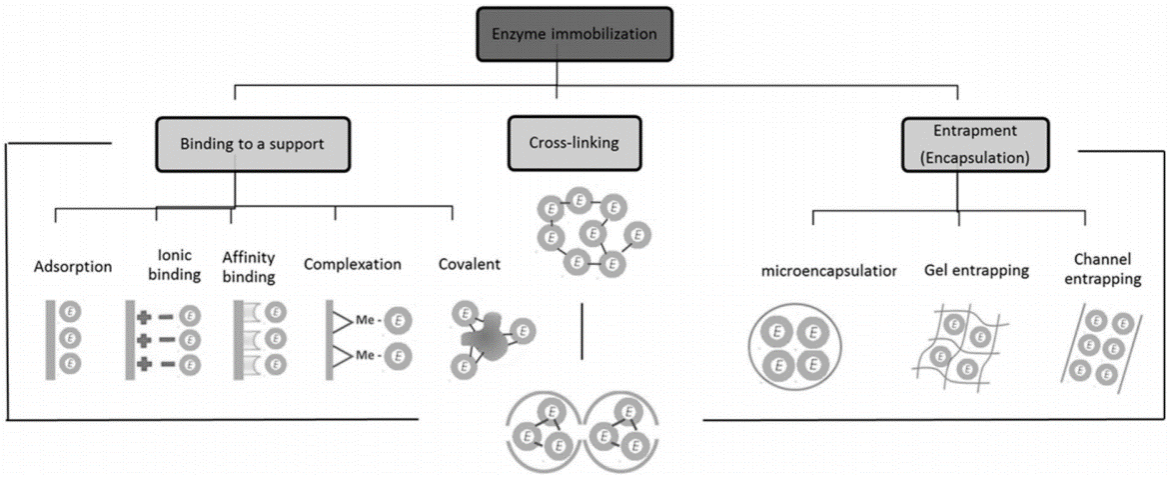

Figure 10. Enzyme immobilization techniques.

A more modern approach refers to enzyme immobilization via covalent attachment to stimulus-responsive or smart polymer and magnetic particles. The enzyme could be easily removed from the reaction mix by applying a magnetic field for decantation or magnetically stabilized fluidized bed reactors [29]. The disadvantage in this case is that the nanoparticles oxidize in air [28]. The particular case of lipase immobilization on magnetic nanoparticles, initially follows an activation step with carbodiimide, the resulting suspension being sonicated and held at a constant low temperature of 4°C [30].

Cold-active enzymes have been covalently immobilized on several carriers, including agarose, chitosan, Sepharose, silica and graphene oxide. By covalent modifications generally the catalytic activity is diminished. Immobilization leads to the improvement of the thermal stability of the enzymes, which are markedly adapted to the temperatures considered critical, at which the enzymes begin to disorganize and destabilize. A psychrophilic pullulanase (*Exiguobacterium* sp.) starts losing its activity from 50°C, pointing no activity at 60°C. The immobilized enzymed on epoxy-functionalized silica maintains its thermal stability up to 90°C for one hour of incubation [28, 29].

Cross-linking technique aims to covalently link the enzymes together by creating aggregates. Firtly the enzymes are precipitated, then cross-linked using agents such as glutaratealdehyde or aldehyde dextran.. Enzymes could also be encapsulated into a polymeric matrix. Nonetheless, few attempts have been published on this topic. For instance, a cold-adapted cellulase from *Pseudoalteromonas* sp. was covalently immobilized in sodium alginate gel beads for the ethanol fermentation of kelp cellulose. After 7 cycles, the enzymes still show 58% activity [28, 29].

Another topic of particular importance is the use of organic solvent in enzymatic environment. This intention is industrially supported for the ease of solubilization of water-insoluble substrates, but the enzyme can be negatively denatured. In particular, cold-adapted enzymes are more sensitive to changes in temperature, outside the optimum range, and solvent. After the first identification of organic solvent-tolerant lipase Lip9 from *Pseudomonas aeruginosa* LST-03 with increased activity in n-decane, n-octane and DMSO, many enzymes were cloned, and the enzyme tolerance to an organic solvent was improved by directed evolution. Recently, the polar organic solvents, methanol and DMSO were shown to increase the conformational flexibility of the cold-adapted and organic solvent-tolerant lipases PML (*Proteus mirabilis*) and LipS (*P. mandelii*). The armistice path for best results could treat enzymes with organic solvent if they are properly immobilized on a solid support, to maintain both their structure conformation and thermo-stability [28].

3.4. Monitoring techniques of the reaction

The purpose of this thesis is the creation of a bio-catalytic system that performs the regio- / enantio-selective acylation of the silybinins from the silymarin mixture with omega-6 and 9-type fatty acids, in order to improve the affinity of flavonolignans to lipophilic media. Silybin has a considerable proportion of hydroxyl groups that cannot attract the molecule to aqueous environments because the aromatic hydrocarbon rings are opposing. We consider that by fatty acid acylation, the large hydrocarbon chain of the acid will improve the lipophilicity of the molecule, thus giving it a nonpolar character. For HPLC analysis, a non-polar type C18 column with polar mobile phase will be probably used. Further, there are presented some of the literature date from which we could assume the analysis parameters in terms of separation between silymarin components.

Silymarin is a complex mixture containing mainly $C_{25}H_{22}O_{10}$ compounds biosynthesised by various couplings of the flavonoid taxifolin and the lignan precursor coniferyl alcohol. The silybins were the first flavonolignans separated from the mixture. The analytical separation of the silybins and isosilybins by reversed-phase HPLC is relatively straight forward, but the complete separation of the less retained silychristin and silydianine is more difficult. W. Smith at al. used preparative-HPLC with a range of mobile phases to isolate the seven main compounds in commercial silymarin: silybin A, silybin B, isosilybins A and B, silychristins A and B and silydianin. The prepared sample was injected into the HPLC with a mobile phase of 50:50 MeOH:water, giving the fraction 1 (silychristin/silydianin) in between 2.4 and 5.4 minutes, the fraction 2 (silynins) between 8.4 and 11.1 minutes and the third fraction of isosilybins at 13.3 to 15.4 minutes [31].

A. Kuki et al. proposed a separation method of 11 silymarin constituents by HPLC-MS. Deffated dried powder was disolved in MeOH giving a stock solution of 1 mg/mL for injection. The mobile phase A was water containing 0.1% formic acid and the mobile phase B consists of methanol. Gradient elution at a flow rate of 1 mL/min was applied, while the stationary phase was C18. The module was equipped with a thermostable autosample using a precission of 8 times injections. The properties of the silymarin components derived from MS/MS spectra as further listed in the Table 3 [32].

A preparative HPLC separation can only be used to obtain minimum amounts of pure silybin diastereomers and such procedures are extremely laborious. Li et al. used reverse-phase silica with MeOH/water/HCOOH 45 : 55 : 0.1 and a column load of just 0.6 mg per injection, the authors were able to isolate roughly 20 mg of each silybin diastereomer (97% purity) in 80 injections. A similar approach was employed by Graf et al. 43 In a rather complex procedure with precipitation (MeOH/water) as a key step and 154 HPLC injections, the authors prepared ca. 5 g of each silybin

diastereomer in purity over 98%. Preparative HPLC was also used in a number of other studies to prepare pure silybin diastereomers [33].

Table 3. Silymarin components separated by HPLC-MS [32].

Peak number	Component	Retention time (min.)	Most intense peaks (m/z)	Relative intens.(%) m/z=301	Intensity ratio m/z=463/453
1.	Unknown	31,08	179,463	2	4,77
2.	Silychristin A	37,31	125,179	1	1,33
3.	Silydianin	40,22	151,179	10	0,2
4.	Silychristin B	43,84	125,179	1	1,23
5.	Silybin A	63,7	125,301	74	0,6
6.	Silybin B	67,4	125,301	78	0,58
7.	Silybin isomer	68,06	125,301	76	0,59
8.	Silybin isomer	68,65	125,301	77	0,56
9.	Isosilybin A	77,91	125,453	18	0,15
10.	Isosilybin B	80,25	125,453	18	0,15
11.	Isosilybin isomer	81,51	125,453	19	0,17

Diastereomeric discrimination of silybin A and silybin B was observed during combination of enzymatic acylation and alcoholysis using lipases. In this procedure, unresolved silybin was O-acetylated at position C-23 by Novozym435 in acetone using vinyl acetate as an acetyl donor. Following alcoholysis using the same enzyme as the catalyst in methyl t-butyl ether with n-butanol produced silybin B and 23-O-acetylsilybin A. The products were then separated by chromatography on silica gel and silybin B was subjected to the same procedure again. Novozym435 can be used for preparation of silybin diastereomers in high yields and purity (silybin A: 42% yield, >95%; silybin B: 67% yield, 98%). To avoid silybin isomerization the acetylation is performed also by the catalysis of Novozym435 (instead of acid-catalyzed esterification). This method is robust and scalable and allows the routine production of multigram amounts of pure silybins and it has been used in other studies [33].

CONCLUSIONS

In the context of the residues recovery from industry by catalytic methods environmental friendly, we propose a bio-catalytic solution for improvement of silymarin uptake. Silymarin extracted from milk thistle is well known for its antioxidant, anti-inflammatory and antiviral effects, being one of the most effective non-invasive drugs in the therapy of degenerative liver disease, and recent studies are debating its effect on inhibiting tumor cell evolution. Its medicinal benefits are unquestionable, which is why it falls into the category of dietary supplements, not requiring specialized prescriptions and being safe even in high doses.

The problem discussed in the literature refers to its composition non-availability, as a complex of several flavolignans, it did not exert good solubility neither in water nor in hydrophobic environment, therefore consisting the drawback of silymarin. In this research direction, we want to improve the biovalability of silymarin by selective acylation of its most biologically active compounds, silybin A and B, with common or omega-6/-9 fatty acids. Thus, by introducing a hydrocarbon chain with substantial length, the lipophilic character of the silybins should be accentuated, and thus may more easily overcome the cellular phospholipid barriers in the skin tissue.

The targeted reaction is esterification mediated by cold-adapted lipase type biocatalysts and in order to increase the stability of the enzyme at different temperature ranges or solvent concentrations, it will be performed the immobilization on magnetic particles.

The reaction products obtained from the enzymatic acylation process will be analyzed by high performance chromatographic analysis.

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