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Enzymatic Synthesis of Carbohydrate Fatty Acid Esters in a Highly Concentrated Aqueous System and in an Organic Solvent.

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A ti Sara

"…voltarei para buscar os instantes que não vivi junto do mar."

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Resumo

Ésteres gordos de hidratos de carbono são surfactantes largamente aplicados em indústria farmacêutica, alimentar e cosmética. A sua produção mediante catálise enzimática é hoje em dia investigada pelo seu potencial ambiental e económico. Neste âmbito, foi desenvolvido um sistema aquoso altamente concentrado para acilação de hidratos de carbono, em um passo, catalisada por lipase e usando ácido decanóico como dador acil. A extensão desta reacção foi analisada para um conjunto de hidratos de carbono com diferente tamanho e estrutura formados por unidades de glucose, verificando-se que a reacção de síntese é promovida por um excesso de fase sólida num sistema quase livre de solvente e também por interacções de natureza covalente, assim como não covalente com a água.

Ésteres de ácidos gordos com β-cyclodextrinas foram ainda sintetizados numa reacção catalisada por uma lipase em dimetilsulfido (DMSO) usando éster vinilíco como dador acil. Para esta reacção de transesterificação em solvente polar foram investigadas a lipase imobilizada de *Candida antarctica* (CALB) e a lipase livre, Amano de *Aspergillus niger.* Sendo que, esta ultima demonstrou catalisar em certa extensão a reacção enzimaticamente.

O método de purificação do produto e análise do grau de substituição nos hidratos de carbono modificados foi optimizado com vista a alcançar eficientemente reprodutibilidade dos resultados.

Palavras-chave: Síntese enzimática; Lipases; ésteres de hidratos de carbono com ácidos gordos; meio aquoso; DMSO; sistemas altamente concentrados.

Abstract

The application of enzymatic catalysis for the synthesis of carbohydrate fatty acid esters has been investigated as an alternative more environmental and economically attractive for large scale production of these compounds, which are largely used in pharmaceutical, food and cosmetic industry. In this way, a highly concentrated aqueous system was employed in a lipase catalysed acylation reaction of carbohydrates with decanoic acid as acyl donor. Reaction extents of several carbohydrates with different chain lengths containing glucose units were assessed. Parameters as carbohydrate quantity, nature of interactions with water and water content showed affect the reaction. Namely, synthesis reaction is successfully promoted by an excess of solid material in an almost solvent free aqueous system and by interactions with water not strictly of covalent nature.

Synthesis of fatty acid esters of β-cyclodextrins (CDs) was also investigated in a reaction lipase catalysed using vinyl-activated and acid decanoic as acyl donors in dimethylsulfoxide (DMSO). Immobilised lipase from *Candida antarctica* (CALB) and Amano lipase from *Aspergillus niger* were investigated for their catalytic properties regarding transesterification in solvents of increasing hydrophilicity. Amano lipase was proved to catalyse in some extent enzimaticaly the reaction.

The suitability of the method for the purification of the product and analysis of the degree of substitution (DS) of the modified carbohydrates was also investigated, leading to the most feasible and reproducible results.

Keywords: Enzymatic synthesis; Lipases; Carbohydrates fatty acid esters; aqueous medium; DMSO; high concentrated systems.

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List of abbreviations

ABS Absorbance
Asp Aspartic acid
Bp boiling point
CALB *Candida antarctica* Lipase B immobilized
CAC Critical Aggregation Concentration
CD Cyclodextrin
CMC Critical Micellar Concentration
DAME Decanoic acid methyl ester
DMSO Dymethil Sulfoxide
DMF Dimethylformamide
DS Degree of Substitution
Eq. Equation
FA Fatty acid
FAME Fatty acid methyl ester
FID Flame Ionization Detector
FTIR Fourier transfer infrared measurement
g Gram
GC Gas Chromatography
Glu Glutamic acid
GU Glucose Units
His Histidine
HLB Hydrophilic-Lipophilic Balance
HPLC High Performance Liquid Chromatography
HTS High Throughput Screening
log P Partition coefficient logarithm in a biphasic system octanal/ water
µL Microliter
mL Mililiter
mp Melting point
M_w Molecular weight
NMR Nuclear Magnetic Resonance
R&D Research and Development
SFAE Sugar Fatty Acid Esters
TLL *Thermomyces lunuginosas* Lipase
U Enzymatic Activity

Introduction

Nowadays, the synthesis of compounds from renewable resources has had a growing interest due to advantages with regard to performance, commercial potential and environmental compatibility. Namely due to their potential of replacing of the wide range of petroleum derived standard products. The same advantages are undoubtedly important for production industrial processes, where exists a constant search for more attractive environmental, economical and healthy alternatives.

In this way, biotechnology has had an important impact in diverse industrial fields. The potential of living cells and products derived from them has had an enlarged recognition. Biotechnology applications cover a wide range of areas, such as pre-treatment of raw material, processing operation and product modification, waste management, and energy recycling and conservation. Processes like fermentations and enzyme catalysis are a established technology, with decades of experience and refinement behind current practices, and advances are associated to new technologies such as metabolic and genetic engineering.

Great development has been carried out in terms of academic and industrial research in biocatalysis field, extending its application as sustainable industrial processes. Enzymatic catalysed reactions are an alternative to several reactions that are difficult to perform with chemical catalysts, with high levels of chemo-, regio- and stereoselectivity, cleanness and ease of disposal. According to Panke, 2002 is estimated that more than 130 processes of biocatalysis in industrial organic synthesis have been commercialised.

Surfactants constitute an important class of industrial chemicals widely used in almost every sector of modern industry, with their applications in oil, cosmetic, pharmaceutical, food, environmental bioremediation, textile, paper, polymer, and plastic, among others.

Namely, carbohydrate fatty acid esters are biosurfactants or bio based surfactants, which owing to their low toxicity, biodegradability, diversity and their possible production from renewable-resource of raw materials have shown to be a readily sustainable alternative for the typical surfactants from petrochemical sources. The development of new biosurfactants with new physicochemical properties enlarges their potential range of applications.

Therefore, there is a demand for efficient methods for the synthesis of carbohydrate fatty acid esters. The traditional regioselective acylation of a carbohydrate requires larges sequences of chemical protection/deprotection reactions, whilst the employment of hydrolytic enzymes allows selectivity in a direct way. However, depending on the different substrates used for an industrial application a study of the different reaction strategies involved is required.

In the present work methodologies for the acylation of different length carbohydrates with a fatty acid by lipases are developed, with emphasis on study of reaction systems conditions and accurate purification of the products and analysis of the extent of reaction.

Literature overview

Carbohydrates chemistry

Carbohydrates are the most abundant class of biomolecules that play important roles in biological systems such as the storage and transport of energy (starch, glycogen) and structural components (cellulose in plants, chitin in animals). Beyond their large range of applications as substitutes of petroleum products, they have a potential in the treatment of cancer, inflammatory diseases and infections. For this reason, carbohydrates have triggered a higher interest from the industry sector and lead to great developments in the carbohydrates field.

Carbohydrates contain carbon, hydrogen and oxygen in their composition with large quantities of hydroxyl groups. The presence of the hydroxyl groups allows carbohydrates to interact with the aqueous environment and to participate in hydrogen bonding, both within and between chains. Classification of carbohydrates is based on the size of the carbon chain, number of sugar units and location of the carbonyl groups. The simplest carbohydrates contain either an aldehyde moiety (aldoses) or a ketone moiety (ketoses). All carbohydrates can be classified as monosaccharide, oligosaccharides or polysaccharides. From two to ten monosaccharide units, linked by glycosidic bonds, make up an oligosaccharide and polysaccharides are much larger, containing hundreds of monosaccharide units. Derivatives of the carbohydrates can contain nitrogens, phosphates and sulphur compounds. Carbohydrates can also combine with lipid to form glycolipids or with protein to form glycoproteins.

Specifically, starch-based materials, because of the biocompatibility and degradability, are presently being used to prepare biodegradable materials with technological applications in a large number of areas such as medicine, pharmacy and biology (Wong, 1997; Marques, 2002).

Starch is the major form of stored carbohydrate in plants and a well-known material for industrial purposes. Only in a few plants the starch yield is in sufficient quantity to be economical, as corn, wheat, potato, rice, tapioca, and sago. Namely, corn starch is commonly used owing to its low cost and availability, although other starches are also employed.

This polysaccharide is composed of a mixture of two forms of α-D-Glucose polymers, amylose and amylopectin. With the exception of so-called waxy (corn) or glutinous (rice) starches, most starches found in nature are composed of a mixture of amylopectin (80-90%) and amylose (10-20%). Amylose is essentially a linear polysaccharide linked by α-(1-4) linkages, where molecules coil into a helical structure and form a colloidal dispersion in hot water (which helps to thicken gravies). Amylopectin is completely insoluble and a highly branched polysaccharide linked by α-(1-6) bonds, which provides starches with different properties (Parker and Ring; 2001).

Many commercial products from starches are obtained by hydrolysis reaction which can be accomplished by the use of heat, acid or enzymes, as catalysts or a combination of two of

them. Hydrolysis is a chemical reaction in which water is used to break long polysaccharide chains into smaller chains which have different properties and molecular compositions depending on the starch and how it is digested into simple carbohydrates that are in general more enable and soluble. Properties include hygroscopicity, fermentability, viscosity, sweetness, stability, gelation, solubility and bioavailability. Namely, the usually starch processing by heating in the presence of water disrupts the native crystalline structure, a phenomenon known as gelatinisation. In an excess of water (>90% w/w), above a characteristic temperature known as the gelatinisation temperature, the natural starch granule loses its crystalline order and swells irreversibly to many times its original size and ruptures. At the same time the polysaccharide amylose (if present) is preferentially solubilised.

In general, amylose is substantially hydrolyzed by beta-amylase efficiently which hydrolyzes α-(1→4) linkages resulting in maltose and glucose. Whereas amylopectin is hydrolyzed into maltose and higher molecular weight dextrins, because the beta-amylase enzyme is unable to hydrolyze past the α–(1→6) branch point in the starch molecule.

Dextrins and starch have the same general formula, $-[C_x(H2O)_y)]_n-$ ($y = x - 1$), but dextrins are smaller and less complex molecules than starch. Unlike starch, dextrins are soluble in water and are precipitated by alcohol. The severity of the heat and acid treatment determines the degree of solubility, which is the basis for classifying dextrins. Chemical properties of dextrins rely on the extent of the starch from which they are derived. Dextrin forms a strongly adherent paste when mixed with water and have found widespread used as bodying agents, coatings, spray-drying aids, fat replacers, adsorbents, adhesives, film formers, freeze-control agents, crystallization inhibitors, nutritive supplements, and flavour carriers (Pizzi, A. and Mittal, K.L.; 1994).

Macrocyclic structures of glucose, called cyclodextrins (CDs), containing six, seven, or eight glucose monomers are produced from amylose by a highly selective enzymatic cyclization by the action of cyclodextrin glucosyltransferases (CGTases). CDs possess homogenous toroidal structures (Figure 1) with an internal hydrophobic cavity and an external hydrophilic side that confer the ability of CDs to form complexes with a variety of organic compounds, which in turn helps to alter the apparent solubility of the molecule, to increase stability in the presence of light, heat and oxidizing conditions, and to decrease the volatility of compounds. CDs can also be used as processing aids to isolate compounds from natural sources and to remove undesired compounds such as cholesterol from food products (Cserháti et al., 2003). In the pharmaceutical industry, CDs have mainly been used as complexing agents to increase the aqueous solubility of poorly water-soluble drugs, and to increase their bioavailability and stability. In addition, they can be used to reduce or prevent gastro-intestinal or ocular irritation, reduce or eliminate unpleasant smells or tastes, prevent

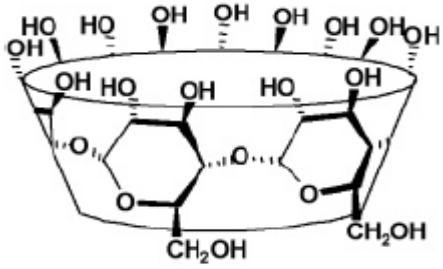
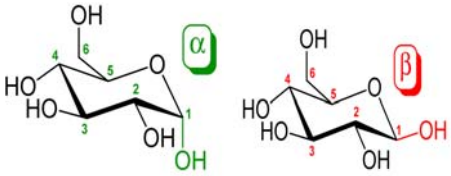
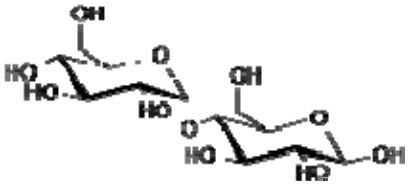
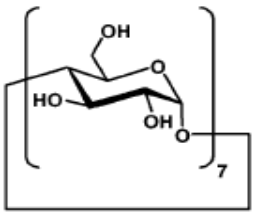
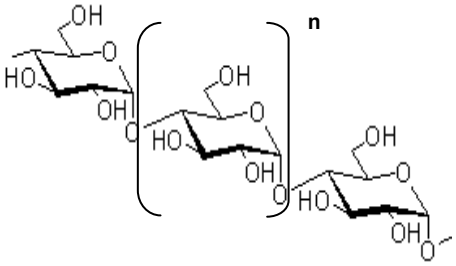


Figure 1 - β-Cyclodextrin structure. Adapted from Villalonga, R. et al, 2007.

drug–drug or drug–additive interactions, or even to convert oils and liquid drugs into microcrystalline or amorphous powders (Villalonga, R. et al, 2007). Examples of starch-derived carbohydrates used in the present study are presented in Table 1.

Table 1- Example of some starch derivates and their properties and general applications.

Name	Structure	Number glucose units	$M_{W\,(GU)}$ (g/mol)	Comments
D- Glucose		1	180,15	Soluble in water; Relative Sweet :74,3; Mixture anomeric; Glucose syrup and crystalline; digestible; Pharmaceutical, food and beverages industry
Maltose		2	171,1	Soluble in water; Relative sweet:32,5; Mono-hydrated powder; Digestible, Essentially food industry
β-cyclodextrin		7	162,14	Cyclic olygossacharide, with a hydrophobic cavity; Less soluble of cyclodextrins; crystalline powder; Pharmaceutical industry (drug delivery)
Yellow dextrin		-	162,14	Lowest molecular weight. Very soluble in water; Low viscosity; good stability. Adhesives and gels to food, cosmetic, paper, industry and textiles.
White dextrin		-	162,14	Intermediate molecular weight dextrin; 1-95% soluble in water (depend reaction conditions). Prone to retrogradation. High molecular weight. Application as adhesives essentially.
Amylose		200 to 20000	162,14	Linear polysaccharide; weakly soluble in water. Undigestible. Pastes, gels, food industry essentially.

Carbohydrate Fatty Acid Esters

Carbohydrate fatty acid esters are composed of mono-, oligo- or polysaccharide esterified with fatty acids of various chain lengths. Carbohydrate fatty acid esters with different physicochemical properties are formed by: i) variation of the modification degree, ii) position of the carbohydrates modified hydroxyl groups, and modification iii) carbohydrate, and iv) fatty acid nature (Garofalakis et al., 2000).

Sugar fatty acid esters with degrees of substitution of 1 to 3 are nonionic, digestible, absorbable, and biodegradable detergents of low toxicity. Carbohydrate fatty acid polyesters (DS 4-14) are lipophilic, nondigestible, nonabsorbable molecules (Akoh, 1994).

There has been a growing interest due to their several physical and biological properties (Allen et al., 1999) and also, in part, of their biologic product character. Namely, a selective modification of carbohydrates might lead to synthesis of novel polymers and, sugar fatty acid esters with new and specific properties, and consequently numerous applications can be achieved.

Carbohydrates-based compounds

In the 1960s, the chemical industry started to develop synthetic ways to produce surfactants from petroleum. Nevertheless, before the chemical surfactants became widespread natural surfactants (biosurfactants) produced from animal or plant materials were already used, such as soap, lecithin and saponin, already consumed for home and industrial purposes.

The rapid advance in biotechnology has lead to an increasing interest in biosurfactants manufactured on an industrial scale. One of the most important factors for biosurfactant development has been the increase of the environmental awareness among consumers, since the biodegradability of household and industrial detergents has become almost as important as their functional performance. On the other hand, consumer attentiveness for adverse allergic effects caused by artificial food additives, such as food emulsifiers, but also new legislation, are also forcing the food industry to find natural alternatives (Sarnay et al, 1995).

However, production of strictly natural surfactants has been of undersized feasibility, given that they are found in small amounts and their separation and purification processes are associated to high costs. Although in the production by microorganisms biologic processes had been achieved high yields, the manufacture of the synthetic analogues has been more attractive economically (Holmberg et al., 2001). Nowadays, the term “natural surfactant” or “biosurfactant” means surfactants synthesised from natural materials and processes, such as surfactants derived from carbohydrates (Dembitsky et al., 2004).

Carbohydrate-based fat replacers, such as processed starches and fibers, mimic fat by binding water, providing lubricity, body and a pleasing mouthfeel. They add bulk, viscosity, structure, slip, and texture to products. Their energy values are lower than fat and only small

amounts are required to bind water and fill the void left by the fat removed. For these reasons, they have a high interest considering the health and wellness trend that is taking the food industry (Michaelides, J. et al.; 2004).

Properties of carbohydrate fatty acid esters

Surfactants are defined as chemical structures responsible for the decreasing of the surface tension of liquid-solid (wetting properties), liquid-liquid and liquid-gas interfaces (alteration of foaming properties) and the dispersion and/or the solubilisation of otherwise insoluble substances. Surfactant compounds are also denominated by amphiphilic compounds, in reference to their chemical structure characterised by a lipophilic and a hydrophilic moiety. Their physical properties, such as the capacity to reduce the surface tension of water, to stabilize emulsions, form foams and detergent capacity are determined by the concrete molecular structure of the surfactant and its charge (cationic, nionic, zwitterionic and non- ionic) (Silbey et al., 2004).

Thus, carbohydrate esters consist of amphiphilic molecules with a carbohydrate moiety acting as the hydrophilic group (or heads) and one or more fatty acids (linked by ester bonds, SFAE) or alcohols (linked by ether bonds, glycosidic) as lipophilic component (or tales) (Plou et al, 2002).

Carbohydrate derivatives are distinguished by their simultaneus high lipophilicity and hydrophilicity, and also by their physiochemical properties, namely a high thermostability. This resistance results from the strength of the hydrogen bonds between the hydroxyl groups of carbohydrates and water, preventing any meaningful dehydration of the polar part (Soderman et al., 2000; Stuberaunch, 2001). Their feasibility results from the fact they are tasteless, odourless, not irritant to eyes and skin. And, depending of their degree of composition not toxic and highly biodegradable (Baker et al., 2000).

The classification of the surfactant properties can be done by a *Hydrophilic-Lipophilic Balance,* HLB that permits to predict the behaviour and consequently proceed with an adequate selection of their applications. A lower HLB means a higher lipophilicity and with a higher HLB, the hydrophilicity will be higher. The HLB of non-ionic surfactants can be calculated from its molecular structure by using an empirical formula. In the case of the SFAE this classification will be, therefore modulated by the kind of acyl group (simple or branched chain), the degree of substitution (mono-, di-, triesters, etc.) and by the degree of polymerization of the carbohydrate (monosaccharide, disaccharide, etc.) (Coulon et al., 1998).

Physico-chemical properties

SFAEs have the capacity to stabilize emulsions, working as emulsifiers. An emulsifier forms a surface film in the interface between the colloid drops and the dispersion medium, reducing in this way the interfacial tension and prevents the coagulation (Levine, 1996). Soultani, 2003 investigated diverse physical properties of sucrose commercial esters and mono-

and di- esters of fructose, namely the effect of the chain length of the fatty acid on the capacity to stabilize emulsions of these sugar esters. Another property common among the SFAE is the formation of stable foams as was studied by Drummond (1998) for monoesters of lactose and lactitol of diverse long chain fatty acids.

SFAE molecules, like other surfactants, can self assemble when dissolved in water leading to formation of supramolecular aggregates denominated micelles. In the classic model of the spherical micelle, the hydrophobic moieties tend to be placed inside, with the polar heads on the surface in contact with the water. The formation of micelles only occurs above a limit of surfactant concentration designated by critical micellar concentration (CMC). A higher trend to form micelles will correspond to a lower CMC. In the case of non ionic surfactants, such as the SFAE, the formation of aggregates increases with the length of the carbohydrate chain. The way the surfactant interacts with the different surfaces is likewise important for its characterisation and subsequently application. In general the SFAEs have low CMC (Polat, 2001) and high surface activity with gradual absorption and continuing activity. Specific conclusions about these properties are reached by respective analysis methods, having been done to date several studies (Zhang et al., 1997; Ferrer et al., 2002; Soderman et al., 2000).

Polymeric biosurfactants are being considered a new class of amphiphilic materials that exhibit valuable properties for aqueous formulations. Hydrophobically modified polymers (HMP) have attracted much attention due to their biocompatible properties and their good surface tension lowering effects, being used on emulsion stabilization, emulsion polymerisation and preparation of solid particles with controlled surface properties (Akkara et al., 1999). Several of polysaccharides are able to form hydrogels that are three-dimensional, hydrophilic and polymeric networks capable of absorbing large amounts of water or biological fluids (Ferreira L. et al, 2002).In addition, they have a strong tendency to self-associate and/or to associate with surfactants, forming spontaneously hydrophobic cores, where intra- or inter-molecular associations of HMP mainly result from hydrophobic interactions within the same polymer chains or between different chains (Bai G. et al., 2008; Villalonga, 2007).

Biologic properties

Besides, some compounds of this class are associated to antitumoral and antimicrobial biologic activities. The mechanism of action of carbohydrate esters in the antimicrobial activity is still unclear. Nevertheless, the effect of the SFAE of several microorganisms involved in food spoilage and poisoning (*Pseudomonas fluorescens*, *Bacillus* sp.) and in a diversity of diseases such as *Staphylococcus aureus*, *Escherichia coli* or the higher eukaryotic microorganism, *Pichia jadinii* have been studied (Devulapalle et al., 2004; Watanabe et al., 2000; Ferrer et al., 2005). SFAE seems to cause changes in cellular morphology (altering its permeability) and induces autolysis processes that result in cell death. It has been postulated that sugar esters reorganize the cellular membrane altering its permeability, which causes a loss of important metabolites (Ferrer et al., 2005; Cho et al., 1990). Another potential of the SFAE is the antitumoral activity

related to its effect in some tumoral factors and cells (Okabe et al., 1999; Ferrer et al., 2005) or by the carbohydrates role in the cellular signalling (Matsumoto et al., 2000; Ueoka et al., 2002).

Applications and commercialization

Sugar fatty acid esters are used as additives in pharmaceutical, cosmetic, petroleum and food industries due to their good detergent and emulsifying properties. There is an emergent interest for their use as fine chemicals in oral-care products and medical supplies because of their special biologic activities, such as antimicrobial, plant growth inhibitors and antitumoral actions that are well reported and might open new markets (Table 2). Sucrose esters, by far the most developed derivative of the carbohydrate esters, are being produced at about 25 Ton/year (Hill and Rhode, 1999).

Polysaccharides are also an interesting starting material to produce biopolymer esters that can be used in injection molding operations, biodegradable emulsifiers, compatibilizers, detergents and which exhibit a great potential in drug/gene delivery research and in other biomedical applications (Akkara et al., 1999; Marques, A.P. et al, 2002). In addition, modified sugars and dextrins (tapioca, corn, potato, and rice derivatives) are traditional ingredients to provide enhanced functionality in reduced-fat systems. The degree of digestible carbohydrates determines their ability to lower the overall caloric content as well as fat content (Michaelides, 2004).

Table 2- Examples of applications or/and activities of several carbohydrates fatty acid esters.

Carbohydrate	Activity or Application	Reference
Sucrose	Food emulsifiers	Nakamura, 1999; Watanabe T., 1999.
	Antimicrobial properties	Marshall DL,1994; Cho, 1990; Thomas LV, 1998; Watanabe, 2000; Hathcox, 1996.
	Insecticide activity	Chortyk et al., 1996; Peterson JK, 1997.
Maltose	Antimicrobial	Devulapalle, 2004; Ferrer, 2004.
	Antitumoral	Okabe, 1999; Ferrer, 2005.
	Cosmetic additive	Philippe, M., 1996.
β-cyclodextrin	Liposome-like properties; specific affinity lectin proteins and DNA	Ravoo, 2000; Mazzaglia, 2004; Cryan, 2004.
	Drug-delivery cores. pharmaceutical	Uekama et al., 1998; Hirayama et al, 1995.
Dextrin	Emulsifier, adhesive and dispersive in cosmetics, printing ink, coatings.	Suzuki, T., 1998.
Amylose	Material for food of slow digestive absorption	Kameyama, 1993.
	Coating food	Cole, M., 1969.

Production of carbohydrate fatty acid esters

Nowadays, the R&D of efficient synthetic methods to produce carbohydrate fatty acid esters is a relatively dynamic field. These compounds constitute an alternative significantly advantageous in a world aware of environmental issues. Moreover from inexpensive organic raw materials, as low molecular weight carbohydrates and several polysaccharides it is possible to produce value-added products.

Carbohydrate esters can be synthesised using either chemical and/or enzymatic processes. Methods can be more selective in terms of the degree of substitution (mono-, di-, triesters, etc.) and regioselectivity and, also more feasible for an industrial scale or limited to the laboratory scale. Besides the specific synthesis of the product its purification and analysis is as well an important challenge in the elaboration of a method.

Monoesters of mono and oligosaccharides, due to their higher solubility in water, are products that have been more under focus in the research works in the last decades. However, regioselective acylation of carbohydrates is an arduous task due to their multifunctionality (different hydroxyl groups can have similar reactivity) (Descotes, G. et al., 1999). And hence, the development of regioselective SFAE synthesis methods constitutes *per se* a valuable contribution for organic chemistry as a strategy of selective protection of polyhydroxyl compounds.

On the other hand, since the pioneering work of Landoll (1982), to prepare copolymers by grafting hydrophobic hydrocarbon groups onto a hydrophilic polymer (cellulose in that case), several families of polymeric biosurfactants based on polysaccharides with well-defined structures have been developed (Akkara, J. et al., 1999). Attempts have been made to prepare polysaccharide-based biosurfactants by microbial synthesis but this led only to poorly controlled structures in terms of number of hydrophobic groups, nature of the groups, distribution along the polysaccharide backbone, etc. For these reasons it is also difficult to understand and control the emulsifying properties of these compounds.

Chemical synthesis

Currently chemical production of sucrose esters is carried out by a transesterification reaction base-catalysed between a methyl or an ethyl ester of a fatty acid and the disaccharide at high temperatures and in a polar aprotic solvent. In function of the quantity of the acyl donor and the time reaction are obtained distinct degrees of substitution. This reaction produces a mixture of regioisomers (low selectivity) and a medium yield of product. Moreover, coloured derivatives are formed as side-products, there are many intermediate stages, and yet numerous

purification steps are needed to remove toxic solvents used (Nakamura et al., 1999; Chaiyaso *et al.*, 2006).

A selective chemical acylation of the carbohydrates can be achieved often by complex protecting-groups methodologies. Notwithstanding, there are some direct chemical acylation methods with some selectivity using unprotected sugar moieties and fatty acids. These methods use extreme reaction conditions or may not be used in food applications, because toxic organic solvents such as tetrahydrofuran or dimethylformamide are required for solubilisation and removal of the product from the reaction mixture (Tsavas *et al.*, 2002).

On the whole, the chemical synthesis of carbohydrate fatty acid esters is hampered by less environmental friendly solutions, such as the severe reaction conditions resulting in high energy consumption, formation of unwanted side products due to low selectivity and the use of toxic solvents.

Enzymatic synthesis

Enzymatic catalysis has been applied for several decades to the synthesis of biosurfactants based on carbohydrates and its application has been a promising way to overcome the issues connected to the chemical synthesis. The advantages of biocatalysis are mainly related to chemical selectivity of the reaction and the mild conditions required, making synthesis processes more efficient, environmental and economically attractive.

Some lipases and proteases are found to react with different substrates besides their natural substrates and to catalyze esterification or transesterification reactions (Gubitz et al., 2003; Kaewprapan et al., 2007).

Nowadays, enzymatic catalysis has an important role in the synthesis of the carbohydrate fatty acid esters, by contributing towards their characterization as natural biosurfactants and also by adding enzymes in the field of carbohydrates chemistry.

Biocatalysis in organic synthesis

Enzyme biocatalysts are currently applied in the production of fine chemicals, pharmaceuticals, and agricultural chemicals. It is widely acknowledged that there is a growing need for more environmentally acceptable processes in the chemical industry. By Sheldon, R. A. 2005, a ‘Green Chemistry’ or ‘Sustainable Technology’ approach efficiently utilises preferably renewable raw materials, eliminates waste and avoids the use of toxic and/or hazardous reagents and solvents in the manufacture and application of chemical products.

In fact, biocatalysis reactions proceed under mild reaction conditions (physiological pH and at near room temperature and pressure). Enzymes are an environmentally compatible catalyst because they are biodegradable and nontoxic proteins and they often use water as solvent, which is combined with high activities and chemo-, regio- and stereoselectivity in

multifunctional molecules. Furthermore, the use of enzymes generally circumvents the need for functional group activation and avoids the protection and deprotection steps required in traditional organic syntheses. Enzymatic catalysed reactions can achieve acceleration 10^{12} times higher, and each molecule of catalyst can turn over up to one million molecules of product (Faber, 2004). However, there are resistances towards shifting traditional chemical processes that are associated with high efficiency yields. Several intrinsic difficulties encountered in enzyme biocatalysis include product and substrate inhibition, low solubility of reactants in the reaction media, slower reaction rates, and the fact enzymes are prone to be inactivated under various operational conditions.

Nowadays, different approaches in the enzymes and biotransformations field have overcome several limitations. Advances in recombinant DNA techniques have made, in principle, possible to produce virtually any enzyme at a commercially acceptable price. Advances in protein engineering techniques, such as site directed mutagenesis and in vitro evolution, have permitted to manipulate enzymes such that they exhibit the desired substrate specificity, activity, stability, pH profile, temperature resistance etc. Furthermore, the development of effective immobilisation techniques has opened the way for optimising the performance, recovery and recycling of enzymes, and also varying of the reactions conditions by medium engineering have promoted more efficient biotransformations (Bornscheur, 2005).

Lipases as biocatalysts in organic synthesis

Hydrolases, especially lipases, are an important group of biotechnologic relevant enzymes and the most used enzymes in organic synthesis. Lipases (E.C. 3.1.1.3) comprise a group of enzymes which catalyze, *in vivo,* the hydrolysis of triacylglycerols into free fatty acids and glycerol. Likewise, lipases are readily attractive for the organic chemistry for synthesis of chiral and novel compounds, and as well industrial biocatalysts.

First, these enzymes have a wide range of substrates, being able to act as synthesis intermediates with high regio- and stereoselectivity. They can catalyze in a sensitive way a large number of reactions including ester bond hydrolysis in an aqueous conventional medium and its reverse ester synthesis such as condensation (esterification) and alcoholysis (transesterification) reactions in non-conventional media. In addition lipases are largely produced from microorganisms, playing a vital role in commercial ventures with vast applications in fat, oil, paper, food and detergent industries. Most of lipases can act in a wide range of pH and temperature and their employment does not require cofactors. Lastly, lipases might be stable in organic solvents where they are insoluble and some of them exhibit selectivity (Klibanov, 2001; Schmid et al., 2001; Gupta et al., 2004).

Namely, lipases catalyse reverse hydrolysis reactions with high regio- and enantioselectivity in alcohols racemic mixtures and polyhydroxyl compounds, such as carbohydrates (Schmid et al., 1998). Thus, these enzymes have been a useful tool in the development of processes to enzimaticaly synthesise carbohydrate fatty acid esters and used to modify regioselectively mono- or even polysaccharides in one step reactions.

Availability, production and activity analysis

Lipases are universal enzymes that can be found in animal (Carrière et al., 1994), plant (Huang, 1984; Mukheriee, 1994) and microorganism (Jaeger et al., 1994; Gilbert, 1993) and also in viruses (Canaan et al., 2004). In the biotechnology field efforts have been made especially employing lipases from microorganisms, which segregate lipases in the growing medium what is advantageous from a technical point of view compared to intracellular enymes. The advances in genetic engineering have promoted an increase of the lipases production in a commercial scale by recombinant bacteria and yeasts.

For instance, *Thermomyces lanuginosus* lipase (also known as *Humicola lanuginosa*) employed in detergents is largely produced (hundred of tones per year) by fermentation of a strain of *Arpegillus orizae* by cloning of the gene that encodes this lipase. More recently, there is an increasing search of lipases from extremophile microorganisms to find out if their enzymes are more stable at high temperatures.

However, one of the main reasons argued by organic chemists against the use of biocatalysis for organic synthesis is the lack of reproducibility obtained in lipase catalyzed reactions when commercial lipases from different batches of the same supplier are used, in spite of the same number of lipase units indicated by the supplier. This problem is very important, namely, when the lipase from the same microorganism gives different enantiopreference (Mária et al., 1999). Hereby, most of the lipases are produced in two different isoforms by the microorganisms, denominated A and B. Both isoenzymes are very similar showing the same enantiomeric preference, but slight structural differences can lead to different enantioselectivities. Almost all lipase preparations contain both isoenzymes, the unique reasonable exception is the lipase from *Candida antarctica* (CAL), for which both of the isoenzymes are also available due to genetic engineering procedures (Faber, 2004).

The purification protocols of lipases are usually rather tedious, employing no specific techniques such as the precipitation, hydrophobic interaction chromatography, by size exclusion or ionic exchange. Alternatively, affinity chromatography can reduce the individual steps of the purification as well as reverse micellar and aqueous two-phase systems (Gupta et al., 2004).

The analysis of the lipase activity can be carried out by diverse approaches that are based in measurement of natural hydrolytic activity in aqueous medium. These are very important in biotechnology as a tool to detect lipolytic microorganisms, in screening methods, in direct evolution of these enzymes and to determine the activity in purified and raw lipase preparations. Each method used has their inherent advantages and limitations. Some are more adequate to multiple assays, as high throughput screening (HTS) and at the same time less exact (Beisson et al., 2000). The chromatography analysis (GC and HPLC) is the most extended method. A faster method for analysis of the synthesis activity of the lipase in transesterification reactions is the fluorometric method (Konarzcycka-Bessler et al., 2003)

Structure and action mechanism

Lipases in their natural aqueous medium act as hydrolytic enzymes. The general action mechanism in the case of hydrolysis with their natural substrates consists in a nucleophilic attack of the enzyme to the carbonyl group of the ester, forming an acyl-enzyme intermediate (since, the exit of an alcohol occurs), which is attacked by an external nucleophile thus placing an acyl group to an alcohol. More concretely, when the acyl-enzyme intermediate is formed by the action of this in the ester, a transesterification occurs which distinguishes from the esterification by the direct action of the enzyme in the carboxylic acid and therefore, water is released (Figure 2).

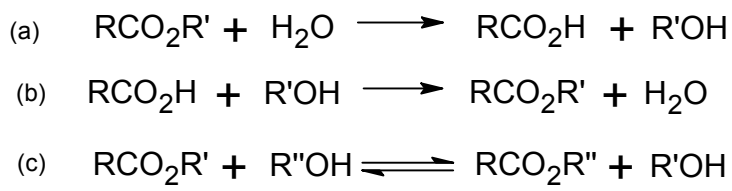


Figure 2 - Reactions lipase catalyzed: (a) Hydrolysis; (b) Esterification; (c) Transesterification.

Lipases in the aqueous medium do not present activity only when the substrate is dispersed in a monomeric state. When the substrate concentration in the reaction medium is above its limit of solubility, a second lipophilic phase is formed and there is a significant increase of the lipase activity. In fact, lipases do not hydrolyse their substrates in concentration under a critical concentration, denominated critical aggregation concentration (CAC), showing higher activity above it, which is designated by “interfacial activation” (Pandey et al., 1999). In fact, a unique property of lipases that distinguishes them from esterase hydrolases is their enhanced activity at or near the lipid/water interfaces and do not hydrolyze dissolved substrates in the bulk fluid (Berg et al., 2001).

The mode of action of an interfacial enzyme can be reached depending on the experimental system. For instance, unimolecular film systems consist of only two phases, the bulk and the film, and the enzyme is distributed between the two and it can be optimized in terms of lipid packing, chain length specificity, and product solubilisation. This is not the case with the other dispersed systems available, such as liposomes, micelles, and emulsions. Several structure/function studies have led to better characterisation aspects of interfacial enzymology. The pH dependent activity, substrate specificity and inhibition of lipases can result from both “classical” interactions between the substrate or inhibitor and the active site, as well as from the adsorption of the enzyme at the surface of the aggregated lipid. For this reason, the size of the interfacial area was already reported to be the rate limiting step for lipase-catalyzed reactions (Carriére et al., 1997; Oloulou et al., 2006).

Enzyme appropriate form of employment can be chosen to promote a considerable interfacial area depending on the specific purpose (Moniruzzama et al., 2006). Enzymes have been employed in aqueous and nonaqueous systems in various states such as native enzymes, suspended enzyme powder, solid enzyme adsorbed on support, polyethylene glycol-modified

enzymes soluble in organic solvents, enzyme entrapped within a gel, microemulsion or reversed micelle and immobilised enzyme. No general guidelines are available yet for choosing the best form to perform it (Krishna, 2002).

During the nineties first lipases structures by X-ray crystallography were discovered and their three-dimensional structure suggests the interfacial activation can result from the presence of a peptidic loop that covers the active site of the enzyme in solution, as a lid. From structures of lipases crystallized with substrate analogues, there is evidence that from the contact with the aqueous/lipidic interface the lid suffers a conformational reorganization that makes the substrate accessible to the active site (Moniruzzama et al., 2006). However, it still is a matter of debate whether the kinetic property of interfacial activation is associated with the conformational changes occurring in lipases (i.e., the opening of the lid giving access to the active site).

Lipases which structures were discovered show a common architecture composed by a nucleus of β sheets more or less parallel, surrounded by a specific sequence of α–helices. The hydrolysis of esters occurs owing to a catalytic triad situated in the active site composed by a serine nucleophile residue activated through hydrogen links associated with histidine (His) and aspartic acid (Asp) or glutamic acid (Glu) (Petkar et al., 2006). The serine proteases follow essentially the same mechanism (Silverman, 2002). Hitherto, lipases investigated have demonstrated structural and functional similarity, independent of organism and even in the case of low homology of the aminoacid sequences (Schmid, R. et al., 1998). Nevertheless, despite these similarities, slight variations in the active site structure might affect the catalytic properties and the lipase stability in determined reactions conditions.

All the lipases accept fatty acid esters of middle size chain (four carbon atoms, C_4) to large (sixteen carbon atoms, C_{16}), some are even able to hydrolyse fatty acid esters with very large size chain (C_{22}). The substrate specificity of lipases, referred to the acyl group, is determinated by the form of the active site which imposes a specific chain length ideal presented by each lipase.

In a non-aqueous medium, with an external nucleophile different from water, the enzyme can act in a synthetic way promoting acylation reactions (Faber, 2004). The direct esterification between an acid and an alcohol, even enzimaticaly catalysed, is in general slow and reversible with an equilibrium constant of around one (Borsncheuer et al., 2005). In order to achieve a high ester yield it is needed as little water as possible in the mixture. Several means to removal water are by high vacuum evaporation (Napier et al.,1996), azeotropic distillation (Gubicza et al., 2003) or molecular sieves (Kim, et al., 1998) as drying agents, which are employed when cheap substrates are used and, hence losses have less importance or when it is predetermined to scale industrially.

In the fine chemical field, where in general the substrates are expensive and the added value of the products is higher, laboratorial biocatalysed acylation reaction has been preferably done by transesterification reactions. In these reactions there is no releasing of water and therefore, the water activity is more susceptible to control, being constant and it should be adjusted only in the beginning of the reaction. Conversely to the hydrolytic reactions, where the

external nucleophile (water) is always in excess, the concentration of alcohol to acylate in the transesterification reactions can be limited.

Transesterification reactions, where common esters (with methyl, ethyl groups) are employed as acyl donors, are reversible. This is due to the nucleophilicity of the external nucleophile and the leaving group (methanol, ethanol respectively) are very similar, and for that reason both compete for the acyl-enzyme intermediate in both ways of the reaction. Thus, the reaction rate is slow which can lead to losses of selectivity for these kinetic reasons.

A shift in the equilibrium of the reaction in a desired way can be achieved by three different approaches, use of excess of acylating agent, evaporation of the alcohol produced by high vacuum (not applied when solvents of low boiling point are used since they will vaporize as well) or by activated acyl donors favouring an increase or a decrease in the reaction irreversibility. Namely, the use of activated acyl donors can be considered an irreversible process because the equilibrium position can be shifted to the product formation. The enol esters are considered the most useful activated acyl donors (Bornscheuer et al., 2005). The alcohol produced, as a leaving group, tautomerizes to the correspondent carbonylic compound, which is not a nucleophile, shifting the equilibrium to the desired side. Specifically, by use of vinyl esters the product of tautomerization (acetaldehyde) is volatile, converting to a process virtually irreversible where isolation of the reaction product is facilitated (Figure 3).

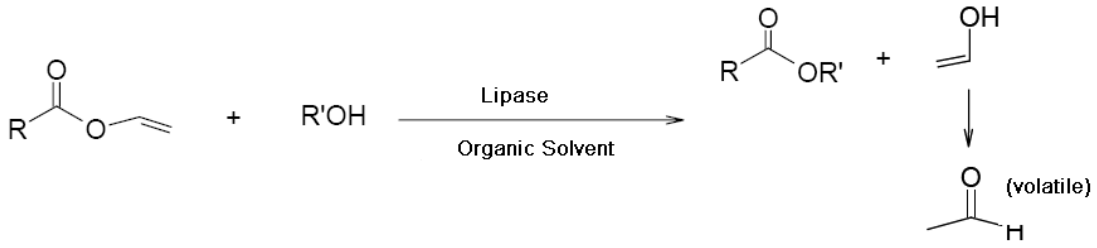


Figure 3- Scheme of irreversible transesterification catalysed by a lipase employing a vinyl ester, as acyl donor with tautomerization of the vinyl enol produced.

This transesterification methodology was firstly applied using lipases and then extending to proteases. This is one of the main enzymatic techniques of protection of functional groups in organic synthesis due to its chemoselectivity, being useful in cases such as aminoalcohols, and essentially at the level of regioselectivity achieved by the biocatalysis that presents a high value in the case of polyhydroxyl compounds (Faber, K., 2004). In case of the polyhydroxyl compounds, such as carbohydrates, lipases and serine proteases permit a regioselective acylation of determinated hydroxyl groups, acylating preferably the primary alcohols, since these are less stringent stereochemically (Plou et al., 2002).

Lipase from *Thermocmyces lanuginosus*

Thermocmyces lanuginosus (previously known as *Humicola lanuginosa*) is a thermopile filamentous fungus that grows in an optimum way between 48-52ºC, although it can be cultivated at temperatures between 30-55ºC. This extremophile fungus can tolerate an

exposition during some minutes at higher temperatures such as 68ºC and its spores can survive for five minutes in boiling water. (https://fungalgenomis.concordia.ca/fungi)

This fungus, amongst several interesting degradative enzymes, segregates a lipase (TLL) which is a unique chain polypeptide with a molecular weight of 27500 Daltons and its optimum hydrolytic activity occurs at pH 8.0, being stable in a range of pH 4 to 7. The major activity occurs at 60ºC of temperature, being considerable until 65ºC and deactivated when is kept during 20 minutes at 80ºC. The structure of this enzyme is very similar to the *R. miehei* lipase. The active site (Ser146, His258, and Asp201) is situated under a short branch of the helices that acts as lid. In regard to the hydrolysis of the triglycerides this lipase does not present specificity of position (Figure 4).

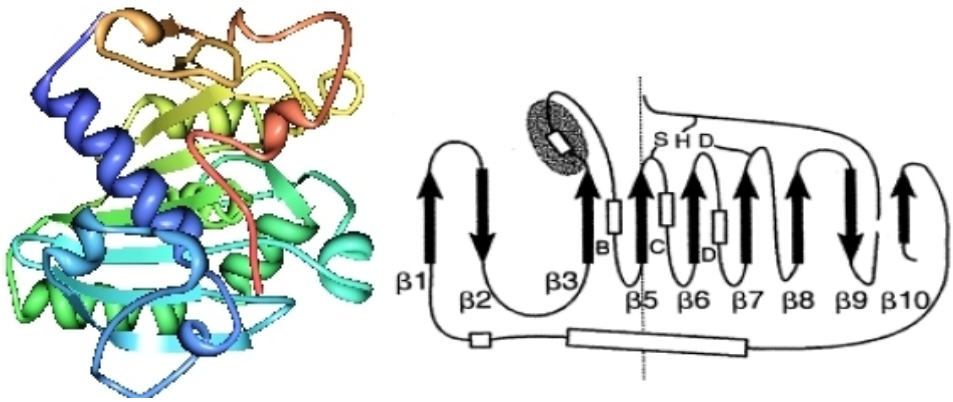


Figure 4- Three dimensional strutucture (left) of the lipase *Thermomyces lanuginosus* by X-rays crystallography with lid area before extreme of the red line from 1DTEA pdb file (Brozozowski et al., 2000). On the right is presented the topological localization in the same lipase, indicating the zone of the lid at grey (Adaptaded from Muralidhar et al, 2002).

Lipase B from *Candida antarctica*

Candida antarctica is a yeast whose enzymes are able to act in extreme conditions and are largely used in the detergent formulation. The lipase from *C. Antarctica* (CAL) is one of the most versatile lipases used in biotransformations. Similarly to other microorganisms, this yeast produces two isoenzymes A an B. The isoenzyme A needs a calcium cation and is more termostable, whereas the B isoenzyme is less termostable (even so more than major of the lipases) and is not dependent of metallic cations. The isoenzyme A is highly active in a non specific way with triglycerides while the isoenzyme B presents more specificity but is less active in a wide range of substrates.

CAL-B is a lipase that has shown a high resistance to deactivation by addition of organic solvents (Salis et al., 2003). In contrast, as several lipases like *T. lanuginosus* lipase, this enzyme seems to be very rigid and does not show a pronounced effect of interfacial activation, which is consistent with the absence of structural changes observed in the X-ray crystalline structures with or without an inhibitor. It is possible that this enzyme does not have the lid region for the active site control and for this reason, this lipase shows similar activity in monomeric substrates in solution and in aggregated substrates adsorbed in an interface (figure

5 Figure 5) (Muradihar et al., 2002). The conformation seems to be always “opened” with a restricted entry to the active site (catalytic triad: Ser105, Asp187, His224) (McCabe et al., 2004).

Kinetic studies of transesterification reaction in organic solvents catalyzed by CALB demonstrated that the lipase follows a bi-bi ping-pong mechanism with competitive inhibition for the substrate (alcohols used the group acyl acceptors) (Martinelle et al., 1995). This lipase owing to its versatility has been the target of numerous projects based in direct mutagenesis and evolution to extend and modulate the catalytic and physical properties of CALB (Luntz, 2004; Magnusson et al., 2005).

CAL-B immobilised in an acrylic macroporous resin is commercialised as Novozym® 435 and is one of the most used enzymes in biocatalysis being employed in industrial processes as a catalyst for synthesis of simple esters, amides, alcohols, amines and carboxylic acids optically active (Anderson et al., 1998).

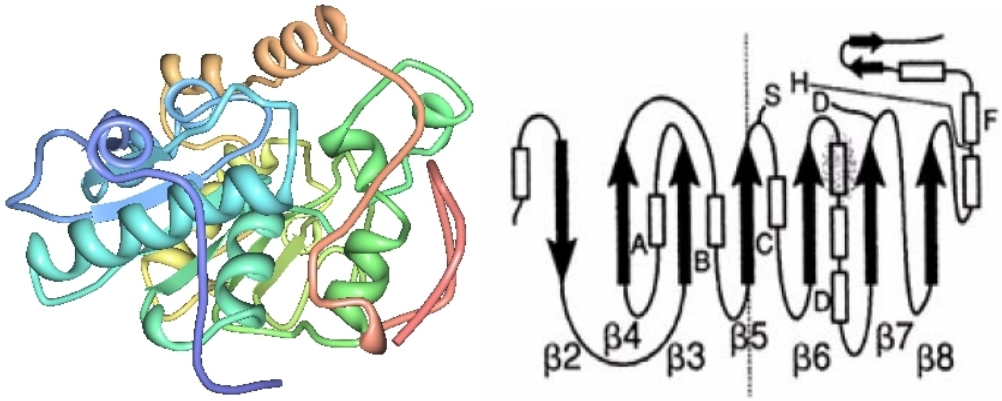


Figure 5- Three dimensional estructural (left) of the lipase B of *Candida antarctica*. File from pdb (1TCA) of the structure by x-Ray crystallography (Uppenberg et al., 1994). On the right it is presented the topological localization of the lid region (light grey region) that is roughly inexistent (Adapted from Muralidhar et al., 2002).

Reactions systems - Trends and state of art

The discovery of hydrolases, such as lipases and serine proteases, capable of functioning in the reverse synthetic direction in non-conventional media has found widespread applications in ester synthesis. A number of successful examples have been reported on utilization of hydrolases for acylation of carbohydrates such as mono- and oligosaccharides (Cruces et al., 1992; Sharma, 1993, Koskinen et al., 1996; Degn et al., 2001), but relatively few on polysaccharides (Donnelly, 1998; Akkara et al., 1999).

The yield of reversed hydrolysis reactions is strongly affected by the type of solvent used, water content of the reaction mixture, enzyme stability in the solvent, and the substrates solubility. Nevertheless, other different factors also affect enzymes activity, stability and diverse types of selectivity in a given solvent, depending of system physico-chemical properties.

In the enzymatic acylation of carbohydrates the choice of the solvent is a very important task. Namely, one reactant is polar (carbohydrate), the other has a nonpolar character (i.e. fatty acid vinyl ester or fatty acid or acid anhydride etc.) and the product is amphiphilic (carbohydrate ester). And yet, because of the hydrophobic and hydrophilic nature of substrates and consequent heterogeneous immiscible mixtures, which hinder enzymatic reactions it is particularly hard to find optimal reaction conditions. Therefore, a compromise between enzyme activity and substrate solubility required has to be achieved in a medium engineering approach.

Organic solvent reaction systems

Although enzymes natural medium is aqueous, in order to promote reverse hydrolysis processes, enzyme reactions in organic media have been applied with success. Consequently, a number of potential applications of enzymes that are either impossible or marginal in water become quite feasible and commercially attractive in other solvents.

Role of the water and enzyme activity

It is well known that most of enzymes present highest catalytic activity in water. Water helps enzyme to maintain its catalytically active conformation in the majority of noncovalent interactions increasing its flexibility, but also participates in most of the denaturation reactions. Resulting from this, there is the possibility to use different media instead of water, which at the same time can cause difficulties in the reaction proceeding.

In general, the majority of the organic compounds is insoluble in water and can promote undesirable side-reactions reactions (racemisations, polymerization and decomposition). Besides that, the thermodynamic equilibrium of several organic chemical processes, such as the reverse hydrolysis in aqueous solution, is unfavourable in water and this account at least partially for a low degree of conversion. The water activity has to be controlled and kept sufficiently low in this case. However, water removal is tedious and expensive, due to its high boiling point and high vaporization heat and in principle, this is overcome by switching from water to organic solvents (Faber, 2004).

Thus, with the use of non-conventional reaction media, rather than water has provided new possibilities for producing useful chemicals (emulsifiers, surfactants, wax esters, chiral drug molecules, biopolymers, peptides and proteins, modified fats and oils, structured lipids and flavour esters). Altogether, the use of an organic reaction medium can offer some interesting advantages as enhancement of the thermal stability of the enzyme, easy separation of the suspended enzyme from the reaction medium for recycling, increased solubility of the substrate, favourable equilibrium shift to synthesis over hydrolysis, elimination of undesired reactions caused by water and the generation of a *de novo* selectivity of the enzyme (Bell et al,. 1995; Klibanov, A.; 2001). Even though enzymes exhibit considerable activity in nonaqueous media,

the activity is low compared to that in water, presenting slower reaction rates, since enzymes are prone to be inactivated under various operation conditions.

Several strategies have been explored to overcome the lower activity of enzymes in organic solvents making them more appealing to organic chemists. These include the methods of enzyme preparation (immobilization, control of the pH value, lyophilisation with lyoprotectants and salts, addition of water-mimicking compounds like formamide, glycol or dimethylformamide (DMF), imprinting with substrates and substrate analogues, cross-link crystallization and adding certain additives with beneficial influence on the microenvironment of the enzymes), extractive biocatalysis (via integration of reaction and separation), protein engineering (chemical modification of the enzyme), genetic engineering, and novel enzyme screening (Klibanov, A, 2001; Ghanem, A., 2003; Paiva, 1997; Tsuzuki et al., 1999; Bornscheuer et al., 2001; Krishna, 2002; Reetz, 2002; Verma et al, 2008).

Reverse hydrolysis reactions

Although the hydrolytic reactions are usually dominant with the hydrolases in dilute aqueous solutions, as was already mentioned, it is possible to shift the equilibrium position in favour of synthesis by altering the reaction conditions, regardless of the presence of bulk water. For example, increasing the concentrations of starting materials and using activated esters such as vinyl esters as acyl donors are typical ways to shift the equilibrium toward esterification via two different, strategies thermodynamic and kinetic control, respectively (Bornscheuer, U. T; 1999). Nonetheless, there are a few successful cases of synthetic reactions catalyzed by hydrolases in aqueous solutions, in literature (Yang et al., 2003; Chang, R.C. et al., 2001; Patel, R.N., 2000) relative to reports of reactions in nonaqueous media.

It is worth mentioning that basic catalysis is an aspect to take in account, namely in the transesterification reactions commonly developed for the enzymatic synthesis of carbohydrate fatty acid esters. Ferrer et al., 1999 demonstrated that the support employed in the enzymes immobilization can interfere in the reaction catalyzing *per se* the acylation. The buffer employed in the lyophilisation (Ferro et al., 1994) or immobilization (Pedersen et al., 2003) of the enzyme can be responsible further the catalysis of acylation process of the carbohydrate, namely when are used inorganic buffers.

Effect of the solvent nature

Activity and specificity of hydrolases in organic solvents is highly influenced by the nature of the solvent. The parameters investigated have included the dielectric constant, the dipole moment, the water solubility and miscibility, hydrophobicity and partition coefficient logarithm in a biphasic system octanal/ water (log P).

Namely, consistent correlations have been obtained with the solvent hydrophobicity and it has been shown that enzymes as lipases generally have higher activities at high log P values (Pedersen, 2002). However, a hydrophobic solvent is not necessarily a good choice for

achieving optimal rates and degrees of conversion, since limit the solubility of hydrophilic substrates at the same time (Degn, 2001). For this reason, lipases application in the acylation of carbohydrates is practically limited to mono and disaccharides, since many polyhydroxyl compounds are insoluble in organic media and hence, standard nonaqueous enzymology is considered unable to sustain their catalytic transformations.

Two main strategies have been developed to overcome this particular limitation. The first is based on the use of organic solvents suitable for the solubilisation of both the carbohydrate and the acylating agent. The second is based on the hydrophobization of the sugar moiety by different methods of derivatisation: complexation with phenylboronic acids, formation of acetals or chemical acetylation, followed by solvent-free esterification with fatty acids. In this last case, the esterification takes place mainly in a solid phase system containing a small amount of solvent, called adjuvant, which is necessary to create a catalytic liquid phase in which the reaction occurs. Solvent free processes constitutes attractive reaction methods for achieving sufficiently high conversion levels, at the same time the product can be used with a minimum of purification (Cau et al.,1999). However, single-step acylations in organic solvents are more probable to attract the interest of industries than multi-step processes (Plou, F. et al., 2002).

Polar solvents like pyridine, dimethylsulfoxide (DMSO), dimethylformamide (DMF) and dimethylacetamide (DMA) have been previously employed as solvents capable of dissolving carbohydrates namely in protease catalysed reactions. Nevertheless these solvents may inactivate, lipases in particular (this has a different meaning to “especially lipases”), and they are not compatible with applications in the food and pharmaceutical industry. In order to avoid the use of these solvents, and at the same time to exploit the use of lipases in these reactions, several processes have been recently reported using more benign solvents such as tertiary alcohols or ketones (*tert*-butyl alcohol, 2-methyl-2-butanol or acetone) that dissolve the carbohydrate only partially (Table 3). Herein, the reaction is performed in a heterogeneous system where most of the substrate is suspended in the reaction mixture. For instance, the use of acetone as organic solvent for the synthesis of carbohydrate esters has the advantage that it can be easily removed from the reaction system for product recovery owing to its volatility.

However, the solubility of disaccharides in these solvents is rather lower comparing with monosaccharides, being difficult to attain notable yields of acylation reaction. Woudenberg-van Oosterom et al., 1996 demonstrated that the rate of the reaction vary directly with the solubility of the disaccharide.

In addition, lipase catalysed processes have also been developed using a medium constituted of two miscible solvents. More specifically, the sugar was dissolved in a low amount of a hydrophilic solvent (basically DMSO), and then was added to a tertiary alcohol, namely 2-methyl-2-butanol, where the carbohydrate solubility is rather poor. This procedure increases substantially the solubility of the carbohydrate and thus, allows the acylation to proceed (Degn et al., 2001).

Table 3- Examples of organic solvents used in the enzimatic modication of carbohydrate with fatty acids.

Carbohydrate	Solvent	Acyl donor	Biocatalyst	References
Glucose	*tert-butyl alcohol*	C2-C20	CALB and Mucor miehei immobilised	Degn, 1999
Glucose	*acetone or t-butanol (adjuvant)*	C12	CALB immobilised	Cao et al., 1999;
Glucose (derivatizated)	*tert*-butyl alcohol	C6-C18	CALB immobilised	Moreau et al., 2004
Glucose	acetone	C12	*C. antárctica* lipase	Arcos, 2001
Sucrose	pyridine	C12-C18	Subtilisin	Polat et al.,1997
Sucrose; maltose, Palatinose, Leucrose Trehalose,	*tert*-butyl alcohol	C4-C12	C.antartica lipase	Woudenberg et al., 1995
Maltose; Leucrose, maltriose;	2-methyl-2-But/DMSO mixture	C8-C18 Vynil esters	T. lanuginosus (immobilised in celite); CALB	Ferrer et al; 2000,2004
Maltose	*tert*-butyl alcohol	C18	*B. fuNa* NTG 9 lipase	Ku and Hang ,1995
Glucose (pervaporation)	2-methyl 2-butanol	C16	CALB and Mucor miehei immobilised	Sakaki et al., 2006
Maltose	Acetone	C12	CALB (Novzyme 435)	Liu, Q. et al.; 2008
Glucose	2-methyl 2-butanol	C12	*CalB (Novozyme 435)*	Flores and Halling; 2002 Salis et al.; 2004
α-cyclodextrin (methyl and hydroxypropyl derivatives)	n-heptane	carboxylic acids, C1-C8	*Rhizomucor miehei* lipase	Pattekan, H. H; 2002
β-cyclodextrin	DMSO	Vynil decanoate	Thermolysin and subtisilin (immobilysed)	Pedersen, N. R; 2005
β-cyclodextrin	DMF	Divynil esters	Subtilisin	Xiao, Y; 2004
Amylose Cyclodextrin (zinc-selenide slides)	Isooctane	Fatty acid ester	Subtilisin	Akkara, JA; 1999

Alternative reaction media

Conversion, yield, selectivity, and catalyst consumption are among the key targets of chemical reaction development in general and are particularly important for industrial application of reactions since they are the measure of overall reaction efficiency.

The use of enzymes in organic solvents has some drawbacks like it is already illustrated decreased of catalytic activities, which are generally several orders of magnitude lower than in aqueous solution. Organic solvents toxicity can also affect the compliance with safety and solvent disposal legislation of the industry processes. And on the other hand, organic solvent media forms a homogeneous liquid phase with a small quantity of carbohydrates due to their low solubility. This limits the applicability of these systems for industrial uses even though high conversion yields of almost 100% can be attained (Kim, 2005). Therefore, with the necessity of using aqueous reaction media dismissed, one can explore enzymes not only in relatively simple organic solvents and their mixtures, but also in a variety of other environments, including, highly

concentrated substrate suspensions (sometimes referred to as eutectic mixtures) and solid phase reactions (Bell et al., 1995; Kimblov et al., 2001).

Biphasic systems are commonly applied in the chemical industry, especially in catalytic reactions. Several examples of the systems proposed are supercritical fluids (Ghaziaskar, HS, 2006; Habulin, M, 2008), gas phase, liquid crystals (Knez, Z. et al., 2002), melts and low-vapour-pressure ionic liquids that are not dealt within the present work. Because of the number of physically important parameters and their interdependencies, it is difficult to predict thermodynamic conversion and yield for biphasic systems. Analytical solutions for both conversion and yield as a function of substrate ratio, phase volume ratio, equilibrium constant, and partition coefficients have been studied and discussed theoretically and experimentally (Peters, M et al., 2007; Sheldon, 2005).

Solid and highly concentrated systems

A synthetic strategy in enzymatic synthesis consists of reaction mixtures with mainly undissolved substrates and/or products in which the compounds are present mostly as pure solids. It retains the main advantages of conventional enzymatic synthesis and the reaction yields are improved and the necessity to use organic solvents to shift the thermodynamic equilibrium toward synthesis is reduced to by-product, which makes the synthesis favourable even in water (Ulijn and Halling, 2004).

This type of reaction mixture is mostly applied in peptide synthesis and it is presented as an economical alternative for industrial and large scale applications. Namely, its reaction system can achieve high yields similar to those in conventional enzymatic reactions combined with a high ratio of product to reactor volume what leads to more efficient operation costs. On the other hand, systems without organic solvents are more environmentally friendly, economic and besides this the contamination of the product is lower.

The ‘solid phase media’ or ‘solid-to-solid system’ (Erbeldinger et al., 1998), which is a type of solid–liquid two-phase system, has a feature of liquid phase similar to that of organic solvent media. The solid phase media comprise a small volume of liquid phase and mostly solid substrate. However, since the substrate molecules that exist in liquid phase can be only engaged in enzymatic reactions, reactants in liquid phase cannot greatly contribute to enhancement of overall productivity. According to a report by Cao et al. (1997), the initial reaction rate and overall productivity of a reaction in solid phase media were enhanced approximately 10 times compared to those in organic solvent media. However, the substrate concentration in the liquid phase was only approximately 0.75M (Cao et al., 1999) or lower (Yan et al., 1999). Reactions do not always proceed well in the solid-to-solid system, whose switch-like behaviour was carefully investigated and reviewed in several referenced studies on kinetics, enzyme concentration, pH/temperature effects, mixing and solvent selection. These have opened new perspectives for the understanding, modelling, optimisation and the possible large scale application of the strategy. Different factors can be responsible for limiting the reactions. Namely, there may be direct effects on the enzyme molecules (substrate/product

inhibition), availability of essential water, diffusion and viscosity of reactants (Ulijn et al., 2001, 2003; Ulijn and Halling, 2004).

An esterification catalysed by lipase of *Mucor miehei* of sucrose and maltose was carried out in the absent of solvent at a temperature of 50ºC, mixing the disaccharide, the enzyme and the fatty acid in the presence of salt pairs that control the water activity. Notwithstanding, the low yield and the reactions conditions in general (reactants amounts, enzyme, etc.) do not make this reaction attractive to production of these carbohydrate esters (Kim et al., 1998).

Eutectic mixture formation is a well-known melting technique that lowers the melting point of a mixture below the melting point of each pure compound in the mixture (Gill and Vulfson, 1994). An adjuvant usually helps to decrease the melting point and the molten mixture can be maintained at room temperature or below. The eutectic mixture, which exists in a state of homogeneous liquid solution, consists of mostly substrate molecules and a small quantity (5–30%, gram-solvent per gram-total substrate mixtures) of organic solvent. As the molar ratio of a binary mixture is changed, the lowest melting point can be found at a specific mole fraction. This point is called the eutectic point and the corresponding mole fraction is the eutectic composition. Often, the term 'heterogeneous eutectic mixture' is used (Gill and Vulfson, 1994; Erbeldinger et al., 1998). The term, eutectic media, can also be used for eutectic substrate mixtures comparative to organic media. Eutectic media exhibit a transparent and stable liquid phase with extremely high substrate concentrations, corresponding to approximately 100 times higher than in the organic media. Since small amounts of organic solvents are used in eutectic media, they can be considered to be more biocompatible for enzymatic reactions than with organic phase media. There are a few reports (Gill and Vulfson, 1993; López-Fandiño et al., 1994) that extremely high concentrations of eutectic substrate mixtures were used in biocatalytic reactions. There has also been report of results for enzymatic dipeptide synthesis using eutectic media (Kim et al., 2005; Shin et al., 2003). Based on the computer-aided molecular dynamics simulations, it was confirmed that a eutectic mixture can be formed by the enhancement of overall molecular kinetic energy. However, this eutectic system has not been thoroughly investigated for industrial application to enzymatic reactions (Kim et al., 2005).

Ionic Liquids Systems

Ionic liquids (ILs) are organic salts those that melt below 100 ºC have potential interest as 'green solvents'. Specifically, their non-volatile character and thermal stability make them attractive alternatives for volatile organic solvents. Moreover, due to their associated synthetic flexibility, ILs are referred to as 'designer solvents'. Several groups have reported that ILs can be used as an alternative reaction media for biocatalysis and it was observed that their use enhanced the reactivity, selectivity, and stability of some enzymes. Namely, ILs have been studied as possibly good solvents for the esterifications of glucose. Ganske and Bornscheuer, 2005 carried out a two-phase system containing ILs and t-BuOH using vinyl ester as acyl donor. Even though ILs can present a lower solubility in comparison to some organic solvents and in

this case, large quantities of organic solvent were even so used. Sang Lee, 2007 prepared a high concentration of sugars in ILs, having developed a new procedure referred to as 'water-mediated supersaturation' that entails mixing an aqueous sugar solution into ILs followed by removal of the water from the solution. Recently Zhao, H., 2008 presented ILs able to dissolve carbohydrates without deactivation of the enzymes, namely immobilised CAL-B.

Chemicals and Materials

Phosphate buffers solution

General method

Dean-Stark distilation

Analytical Method

General approach

Calculations

Total Carbohydrate Analysis

General method

Analytical method development

Product recovery

Effect of the drying time in the purification of product

Dean-Stark distilation

Rotary Evaporation

Gas Chromatography

Total carbohydrate analysis

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Reaction conditions

Reaction with different carbohydrates

Effect of the amount of water in the acylation reaction system

Effect of chain length of the carbohydrate

Effect of solubility of the carbohydrate and appearance of the reaction system

Effect of the quantity of carbohydrate

Maltose time course

Enzymatic acylation of β-cyclodextrin in DMSO

Reaction conditions

Synthesis of β-cyclodextrin esters

Product recovery and reaction study approach

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Appendix I
Analytical method development

Appendix II
Enzymatic acylation in a highly concentrated aqueous system

Appendix III
Enzymatic acylation of β̃–cyclodextrin in DMSO
