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Nair do Amaral Sampaio Neta

Sugar ester biosurfactants for food industry applications

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e da
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ABSTRACT

Sugar ester biosurfactants for food industry applications

Biosurfactants are compounds with surface activity constituting the major class of natural surfactants that present interesting features (e.g. emulsifying capacity) for the food industry. These compounds have several advantages over synthetic surfactants such as degradability, can be synthesized from renewable substrates as carbohydrates and fatty acids, and low toxicity. In this sense, the purpose of this thesis is to synthesize new biosurfactants, in particular fructose, sucrose and lactose esters, for potential use in the food industry. Several synthesis experiments were performed under different experimental conditions to maximize the esterification reaction yield, and consequently the production of biosurfactant. The sugar ester biosurfactants purity was the determinant factor for these studies. The synthesis assays were performed in shake flasks under controlled temperature, time and agitation, according to the optimal criteria for the lipases used, namely *Candida antarctica* type B (CALB) lipase and porcine pancreas lipase (PPL). An experimental design was conducted in order to optimize the yield of fructose esters production from fructose, oleic acid and ethanol using CALB. Temperature and reaction time were found to be the most significant parameters. The optimum conditions were 57.1 ºC, 100 rpm and 37.8 h and a maximum esterification yield of 88.4% was obtained. Afterwards, the lipases from CALB and PPL were used with different carbohydrates (fructose, sucrose and lactose), fatty acids (oleic and linoleic) and solvents (ethanol and ethyl acetate), in order to explore the synthesis of novel sugar esters with improved characteristics. The optimal conditions have been established for all set of experiments and the sugar ester biosurfactants have been characterized according to their surface activity and emulsification index (EI). In the experiments for which CALB was used, lactose ester synthesized using linoleic acid and ethanol presented the highest esterification yield (83.5%). However, the fructose esters obtained showed a better performance regarding their ability to reduce surface tension (35.8 mN/m) and to stabilize an emulsion (EI between 54.4 and 58.4%). Regarding the use of PPL, the highest esterification yields

(47.6%) were observed for sucrose esters, which presented the best reduction of surface tension (33.4 mN/m) and EI (between 58.1 and 58.4%). Finally, CALB was immobilized on chitosan and used to synthesize sugar ester biosurfactants. The enzyme immobilization on chitosan showed the highest yield in the lactose ester production (84.1%) compared with the results obtained with the lipase CALB immobilized on acrylic resin. Additionally, the production of fructose ester was found to be higher for CALB immobilized on acrylic resin (74.3%). Sugar ester biosurfactants were then added to samples of fresh coconut milk and characterized according to their surface activity, EI and particle size distribution. Results indicated the lactose ester as the best biosurfactant (surface tension reduction 38.0 mN/m, EI = 54.1%), although good results were also found for the other sugar esters. In summary, the results gathered in this thesis demonstrate the potential of sugar ester biosurfactants for food industry applications.

Keywords**:** *Candida antarctica* type B lipase, porcine pancreas lipase, esterification, sugar ester biosurfactants, surface and emulsification activity, food industry.

RESUMO

Ésteres de açúcar biosurfactantes para aplicações na indústria alimentar

Biosurfactantes são compostos com actividade de superfície que constituem a principal classe de surfactantes naturais e que apresentam características interessantes (por exemplo, capacidade emulsificante) para a indústria alimentar. Estes compostos têm várias vantagens sobre os surfactantes sintéticos, são biodegradáveis, podem ser sintetizados a partir de substratos renováveis tais como os carboidratos e ácidos graxos, e têm baixa toxicidade. Nesse sentido, o objectivo desta tese é sintetizar novos biosurfactantes, em particular os ésteres de frutose, sacarose e lactose, para potencial uso na indústria de alimentos. Vários experimentos de síntese foram realizados sob diferentes condições experimentais para maximizar o rendimento da reacção de esterificação e, consequentemente, a produção de biosurfactantes. A pureza dos ésteres de açúcar biosurfactantes foi o factor determinante para esses estudos. Os ensaios de síntese foram realizados em matraz sob condições controladas de temperatura, tempo e agitação, de acordo com os critérios óptimos para as lipases utilizadas, nomeadamente a lipase *Candida antarctica* tipo B (CALB) e lipase pâncreas de porco (PPL). Um delineamento experimental foi conduzido de forma a optimizar o rendimento da produção de ésteres de frutose a partir de frutose, ácido oléico e etanol utilizando CALB. A temperatura e o tempo de reacção foram considerados os parâmetros mais significativos. As condições óptimas foram 57.1 ºC, 100 rpm e 37.8 h e um rendimento máximo de esterificação de 88.4% foi obtido. Posteriormente, as lipases CALB e PPL foram utilizadas com diferentes carboidratos (frutose, sacarose e lactose), ácidos graxos (oléico e linoléico) e solventes (etanol e acetato de etila), a fim de explorar a síntese de novos ésteres de açúcar com características melhoradas. As condições óptimas foram estabelecidas para todo o conjunto de experimentos e os ésteres de açúcar biosurfactantes foram caracterizados de acordo com sua actividade superficial e índice de emulsificação (IE). Nos experimentos onde a lipase CALB foi utilizada, o éster de lactose sintetizado usando ácido linoléico e etanol

apresentou o maior rendimento de esterificação (83.5%). No entanto, os ésteres de frutose obtidos mostraram melhor desempenho quanto à capacidade de reduzir a tensão superficial (35.8 mN/m) e para estabilidade da emulsão (EI entre 54.4 e 58.4%). Quanto ao uso da PPL, o maior rendimento de esterificação (47.6%) foi observado nos ésteres de sacarose, que apresentaram a melhor redução de tensão superficial (33.4 mN/m) e EI (entre 58.1 e 58.4%). Finalmente, a lipase CALB foi imobilizada em quitosana e usada para sintetizar ésteres de açúcar biosurfactantes. A imobilização da enzima em quitosana apresentou o maior rendimento na produção de éster de lactose (84.1%) em comparação com os resultados obtidos com a lipase CALB imobilizada em resina acrílica. Além disso, a produção de éster de frutose foi maior com a lipase CALB imobilizada em resina acrílica (74.3%). Em seguida, ésteres de açúcar biosurfactantes foram adicionados às amostras de leite de coco fresco e caracterizadas de acordo com sua actividade superficial, EI e distribuição do tamanho das partículas. Os resultados indicaram o éster de lactose como o melhor biosurfactante (tensão superficial 38.0 mN/m, EI = 54.1%), embora bons resultados também foram encontrados para os outros ésteres de açúcar. Em resumo, os resultados obtidos nesta tese demonstraram o potencial dos ésteres de açúcar biosurfactantes para aplicações na indústria alimentar.

Palavras-chave: lipase *Candida antarctica* tipo B, lipase de pâncreas de porco, esterificação, ésteres de açúcar biosurfactantes, actividades de superfície e emulsificação, indústria alimentar.

PUBLICATIONS

This thesis is based on the following original research or review articles:

Chapter 1: Neta, N.A.S., Sancho, S.O., Gonçalves, L.R.B., Rodrigues, S., Teixeira, J.A. and Rodrigues, L.R. Sugar ester biosurfactants: their enzymatic synthesis and applications. (to be submitted)

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Chapter 5: Neta, N.A.S., Gonçalves, L.R.B., Rodrigues, S., Sancho, S.O., Rodrigues, L.R. and Teixeira, J.A. Enzymatic synthesis of sugar esters and their potential as surface-active stabilizers of coconut milk emulsions. *Food Hydrocolloids*. (submitted)

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ABBREVIATIONS

3D	Three dimensional
ANOVA	Analysis of variance test
a_w	Water activity
CALB	*Candida antarctica* type B lipase
CCRD	Central composite rotatable design
EC 3.1.1.3	Triacylglycerol acyl-hydrolase lipase
EI	Emulsification indexes
EtAc	Ethyl acetate
EtOH	Ethanol
FRU	Fructose
GOT	Glutamate oxaloacetate transaminase
GPT	Glutamate pyruvate transaminase
GRAS	Generally regarded as safe
He	Height of the emulsion
Ht	Total height of the liquid
IR	Infrared spectroscopy
IUBMB	International union of biochemistry and molecular biology
kDa	Molecular weight
LA	Linoleic acid
LAC	Lactose
Lipozyme®	Native soluble lipase b from *Candida antarctica*
Lipozyme™	Immobilized *Mucor miehei*
LMWE	Low molecular weight emulsifiers
MRLM	Multiple linear regression
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
Novozym 435	*Candida antartica* type B immobilized in acrylic resin
OLA	Oleic acid
PPL	Porcine pancreas lipase
Rf	Retention factor
rpm	Agitation - rotations per minute
RSM	Response surface methodology
SD	Standard deviation
SPSS	Statistical package for social sciences
SUC	Sucrose
TLC	Thin layer chromatography
VIF	Variance inflation factor
γ-GT	Gamma glutamyl transferase

General Introduction

"Só uma coisa torna um sonho impossível: o medo de fracassar. Nunca deixei que nenhum limite tirasse de mim a ambição da auto-superação. As pessoas que alcançam seu potencial pensam em aperfeiçoamento".

(Nair Sampaio)

SCOPE AND AIMS

Chemical catalysts contribute to the substantial decrease in energy and reduction of manufacturing costs. However, the chemical synthesis of sugar esters generates a final product that is often aggressive to the environment, and whose preparation requires high temperatures, making it a difficult process. Accordingly, these synthetic surfactants present some drawbacks, such as their costs, availability and environmental impact. Therefore, the enzymatic synthesis, using lipases as biocatalyst, appears to be an interesting green alternative for the production of sugar ester biosurfactants. Sugar ester biosurfactants produced by esterification reactions, are compounds with surfactant properties, i.e., capable of reducing the surface tension and of promoting the emulsification of immiscible liquids. In this sense, the general objective of this thesis was to develop an ecologically friendly process for the synthesis of sugar ester biosurfactants, by the esterification reaction of carbohydrates with fatty acids using lipases. In the reaction schemes, solvents that leave no toxic residues in the final product were used. Therefore, the specific aims of this thesis were:

- to produce sugar ester biosurfactants by esterification reactions of several combinations of carbohydrates (fructose, sucrose and lactose), fatty acids (oleic and linoleic acids), solvents (ethanol and ethyl acetate) and lipases (CALB and PPL);
- to characterize the esterification reaction products using several techniques (thin layer chromatography, emulsification index and surface tension);
- to increase the production yields of sugar ester biosurfactants using low-cost and biodegradable raw materials;
- to optimize the sugar ester biosurfactants production yields using experimental design statistic techniques;
- to compare CALB lipase performance for the synthesis of sugar ester biosurfactants when immobilized in two different supports;
- to determine the effect of sugar ester biosurfactants as a potential emulsifier in food formulations.

The produced and characterized sugar ester biosurfactants described in this thesis are intended to be applicable in the food industry in several products such as, coconut milk, ice cream, fruit juice, among others.

OUTLINE OF THE THESIS

This thesis is organized in six chapters that cover the research aims stated above. The thesis subjects are introduced in this chapter, while in Chapter 6 the main conclusions and perspectives extracted from the current work are given. In the other chapters, the research fields are covered as follows:

- In Chapter 1, an overview on the different approaches related with the production of sugar ester biosurfactants or sugar esters using chemical and enzymatic processes, and the importance of using lipases in organic reactions is given. The future perspectives regarding

the replacement of products obtained by chemical via by sugar ester biosurfactants in the food industry are discussed.

- In Chapter 2, the maximization of fructose ester production by response surface methodology using a compilation of mathematical and statistical techniques, is described. Synthesis was conducted by esterification of oleic acid with fructose using a lipase from *C. antarctica* type B (CALB) immobilized on acrylic resin.

- In Chapter 3, the production of sugar ester biosurfactants by enzymatic synthesis from CALB lipase, using different combinations of sugars (fructose, sucrose and lactose), fatty acids (oleic acid and linoleic acid) and solvents (ethanol and ethyl acetate), is discussed. The production of sugar esters is confirmed by TLC and esterification yields, surface activity and emulsification index are presented.

- The synthesis of fructose, sucrose and lactose esters catalyzed by porcine pancreas lipase (PPL) is described in Chapter 4. Different fatty acids (oleic and linoleic acid) and solvents (ethanol and ethyl acetate) are used. Furthermore, the sugar ester biosurfactants synthesized are characterized according to their surface activity and emulsification indexes.

- In Chapter 5, the use of CALB immobilized on acrylic resin (commercial) and chitosan for the production of sugar ester biosurfactants is evaluated. Additionally, the biosurfactants are characterized according to their ability to stabilize coconut milk.

Chapter 1

Sugar ester biosurfactants: their enzymatic synthesis and applications

"A alegria está na luta, na tentativa, no sofrimento envolvido. Não na vitória propriamente dita".

(Mahatma Gandhi)

Sugar ester biosurfactants can be synthesized either by chemical or enzymatic processes. Due to the high regiospecificity of enzymes, enzymatic synthesis is characterized by the production of sugar monoesters, whereas the chemical process usually leads to a mixture of sugar polyesters. Therefore, various sugar esters can now be prepared by a single reaction step employing lipase as biocatalyst. The immobilization of enzymes has immediate benefits (such as control stability/activity in unusual conditions of temperature, pressure and pH, or in non-conventional media, such as in organic solvents, and improve their efficiency and recovery) and the support type is generally considered as the most important component for a successful development and application of the immobilized biocatalyst in organic reactions. Sugar esters are non-ionic biosurfactants consisting of a hydrophilic (carbohydrate moiety) and a hydrophobic group (one or more fatty acids). By controlling the esterification degree, as well as the nature of fatty acid and sugar, a range of sugar esters can be synthesized. Being biosurfactants, these compounds present surface activity and a high emulsifying capacity, thus they can be used in a great variety of industries. The potential of sugar ester biosurfactants for applications in the food industry will be discussed in this chapter.

1.1 Introduction

Enzymes are considered natural catalysts (biocatalystics) (Hasan *et al.* 2006) that increase the speed of a reaction by lowering its activation energy if they occur. Without the use of enzymes, it is possible that reactions might not occur, thus, the reaction rate is very low under moderate conditions of pressure and temperature (Park *et al.* 2004).

Gutiérrez-Ayesta *et al.* (2007) conducted several experiments under different conditions to evaluate the ability of lipases to hydrolyse vegetable oils and phosphatides. Marked differences were observed in lipase hydrolytic activity in terms of source, degree of purity, state (free or immobilised), substrate, and reaction medium (solvent-free or biphasic). In view of the different structural characteristics of commercially available lipases, it is reasonable to assume that under fixed-reaction conditions, the catalytic activity is related with the enzyme structure.

Numerous advantages have been associated with the use of enzymes, as an example the production of purer products (monoesters) due to the high selectivity of the catalyst and the mild conditions of temperature, pressure and pH required for synthesis, in contrast to the extreme conditions associated with the chemical processes (Cameotra and Makkar 2004, Krajewska 2004).

Chemical catalysts contribute to the substantial decrease in energy and reduction of manufacturing costs, i.e., the chemical synthesis of sugar esters generates a final product that is often aggressive to the environment, and whose preparation requires high temperatures, making it a difficult process (Tsavas *et al.* 2002). In recent years, the most significant development in the field of synthetic chemistry has been the use of biological systems to replace chemical reactions. Reactions catalyzed by enzymes or enzyme systems display far greater specificities than more conventional forms of organic reactions (Ogawa and Shimizu 2002).

1.2 Lipases

Most lipases consist of approximately 300 amino acid residues and present a molecular mass between 25-75 kDa, are glycoproteins, bearing the glycosylated hydrophilic part around the active site, and are included in the hydrolase family group of enzymes (Caballero *et al.* 2009, Ciafardini *et al.* 2006, Hasan *et al.* 2006, Saxena *et al.* 2003). Moreover, lipases are water-soluble enzymes that play an important role in the metabolism of fats in the digestion process, evolving to deal with the biophysical properties of the interfacial microenvironment where their substrates are to be found. These enzymes are used several applications in food, dairy, detergent and pharmaceutical industries (Gupta *et al.* 2004, Reis *et al.* 2009). Joseph and collaborators (2008) reported that the hydrolases catalyze the hydrolysis of triglycerides to free fatty acids and glycerol, and also esterification reactions, interesterification, acidolysis, alcoholysis and aminolysis.

Novel biotechnological applications have been successfully established by using lipases for the synthesis of biopolymers, the production of enantiopure pharmaceuticals, agro-chemicals, and flavour compounds (Jaeger and Eggert 2002), as well as many other processes. Using lipases the operatinal costs, such as reaction time, energy expenditure and manpower, can be minimized due to the use of organic reactions (Hasan *et al.* 2006, Saxena *et al.* 2003).

1.2.1 Sources of lipases

There are several sources of lipases that have been extensively used in industrial processes, namely vegetal (*Asclepiadaceae, Euphorbiaceae* and *Caricaceae*), animal (pancreatic, hepatic and gastric) and microbial (bacteria and fungi). Microbial lipases can be produced by yeasts like *Candida* and *Torulopsis*, by filamentous fungi like *Rhizopus*, *Geotrichum* and *Humicola*, and by bacteria like *Pseudomonas* and *Staphylococcus*. The most common lipases from animal sources are the ones from porcine pancreas (PPL), while those from vegetal sources are extracted from soybean, barley and cotton (Gupta *et al.* 2004, Hasan *et al.* 2006). These enzymes have differences in their catalytic properties, and it is important to notice that most lipases currently used are produced by microorganisms (Cheng and Tsai 2006, Gupta *et al.* 2004, Ng and Tsai 2004, Ng and Tsai 2006, Villeneuve *et al.* 2000).

Microbial lipases are mostly extracellular, thus easier to isolate/recover, are generally more stable, and far more diverse as compared to other sources of lipases (Demirjian *et al.* 1999, Hasan *et al.* 2006, Leathers *et al.* 2010, Mahapatra *et al.* 2009).

The lipase obtained from *Candida antarctica* type B, also known as CALB, is one of the most frequently used lipases in organic reactions (McCabe and Taylor 2004). It consists of 317 amino acids and has a molecular mass of approximately 33 kDa. Furthermore, it is commercially available in the free and immobilized state (Ong *et al.* 2006).

CALB can be used for industrial processes, such as the synthesis of triglycerides and esterification of terpene alcohols. Its use has been reported for the synthesis of acyl hexose, oleate esters of fructose and glucose. Furthermore, this enzyme demonstrated a high potential for the synthesis of active compounds in the pharmaceutical industry, and in the manufacturing of pulp and paper (Liese *et al.* 2000, Yadav and Lathi 2003, Zhang *et al.* 2003).

Porcine pancreatic lipase (PPL) is one of the cheapest commercially available non-microbial enzyme, having a high thermostability and activity in anhydrous reaction media (Gogoi *et al.* 2008). This enzyme presents a molecular mass of approximately 50 kDa, it is the main enzyme involved in the digestion of triglycerides. Nevertheless, enzyme preparations are often impure, containing various hydrolases such as esterases, trypsin and other proteases, among others (Caro *et al.* 2008), which can impair the success of a given enzymatic reaction. The PPL commercially available has been used in several studies. It contains about 8 to 20% of enzyme, and according to Kazlauskas and Bornscheuer (1998) it also contains contaminant proteins, such as α-chymotrypsin, carboxypeptidase B, protease, phospholipase, and cholesterol esterase. Among the contaminants, only the α-chymotrypsin and cholesterol esterase have been considered potential inhibitors of the hydrolysis reaction of esters (Faber 1997). However, these limitations can be overcome by the addition of several substances, such as calcium and sodium ions (Verger 1997).

Finally, it is worth emphasizing the importance of fungi as enzyme producers. The most extensively studied lipase-producers are *Geotrichum candidum, Aspergillus niger, Aspergillus oryzae, Rhizopus delemar* and *Penicillium cyclopium.* Their enzymes are extracellular, theferore its recovery from fermentation broths is easier. Fungi lipases

constitute a source of new biocatalysts with special features and a high potential for organic reactions (Macedo *et al.* 2004).

In addition, lipases possess a wide substrate specificity, have the ability to recognize chirality and do not require labile cofactors (Jaeger and Eggert 2002, Yadav and Sivakumar 2004). As previously mentioned, these enzymes have been used for the synthesis of organic chemicals, mainly in aqueous media and in some cases non-aqueous media, since they are inexpensive, stable, and easy to recycle (Reetz 2002).

In non-aqueous media, lipases are used in esterification, transesterification, amidation, hydrolysis, hydrazinolysis, and epoxidation reactions. They are known to catalyze the synthesis of amides from non-activated esters n-octyl alkyl-amides (Chowdary and Prapulla 2002, Jaeger and Eggert 2002, Ng and Tsai 2006, Yadav *et al.* 2005, Yadav and Borkar 2006, Yadav and Borkar 2008, Yadav and Borkar 2009a, Yadav and Borkar 2009b, Yadav and Devi 2001).

Studies reported that the lipases specificity is controlled by the molecular properties of the enzyme, substrate structure and factors affecting the enzyme-substrate binding (Hou and Shimada 2009, Naik *et al.* 2010). According to this specificity, lipases are classified into different groups, namely random non-specific, regiospecific, stereospecific or fatty acid specific lipases. However, there are some lipases that do not have specificity to all fatty acids, regardless of the position on the glycerol, being hydrolyzed in equimolar concentrations (Shintre *et al.* 2002, Van de Velde *et al.* 2002).

Furthermore, lipases have been extensively studied with respect to their biochemical and physiological properties (Villeneuve *et al.* 2000). Most lipases have a great range of activity and stability between pH 6.0 and 8.0, and optimum temperature for maximum activity between 40 and 70 °C (Table 1.1) (Kazlauskas and Bornscheuer 1998).

Table 1.1 Biochemical properties of some lipases obtained from different sources

Properties	**Sources of lipases**			
	Candida rugosa	*Candida antarctica* (CALB)	Porcine pancreatic	*Geotrichum candidum*
Molecular weight (kDa)	65	33	50	54
Specificity	Non specific	1.3-specific	1.3 specific	Non specific
Temperature optimum (ºC)	37	57	45	40
pH optimum	7.0	7.0	8.0	6.3
Km value (µM)	0.17	0.19	0.30	0.71
Thermostability (ºC)	37	70	40	55

1.2.2 Use of lipases in esterification

The catalytic properties of enzymes have promoted the development of various products and also manufacturing processes where, the substrate specificity of lipases is known to be less rigorous compared to other enzymes (Gandhi *et al.* 2000, Kirk *et al.* 2002, van Beilen and Li 2002, Villeneuve *et al.* 2000). This results in the increase of enzymes with improved properties for established technical applications, and in the production of new enzymes tailor-made for entirely new areas of application where enzymes have not previously been used (Kirk *et al.* 2002, Villeneuve *et al.* 2000).

Lipases have been reported in several applications, such as in the synthesis of chiral compounds; carbohydrate ester synthesis; polyunsaturated fatty acid purification/enrichment; synthesis of biologically active compounds (e.g. alkaloids, antibiotics, terpenoids, pheromones); ester synthesis for perfumes and flavors (e.g. short-chain fatty acid esters synthesized with lipases are useful in making fruity flavors); synthesis of structured lipids and synthesis of organic carbonates (organic carbonates can be synthesized via lipase-catalyzed transesterification involving carbonates and alcohols in a water-restricted environment) (Fan and Qian 2010, Gandhi *et al.* 2000).

1.2.3 Immobilized lipases

Enzymes are naturally subjected to inactivation by chemical, physical or biological factors. Also, they are unstable, have a rapid loss of catalytic activity and are not regenerated (Kourkoutas *et al.* 2006). Therefore, new immobilization techniques have been developed to provide stability to the enzymes, and improve their efficiency and recovery (Kourkoutas *et al.* 2004, Roble *et al.* 2000). For example, the ester production using free lipases was found to be very low due to enzyme inhibition by short-chain acids (Kourkoutas *et al.* 2006).

Lipases have been successfully immobilized on a variety of matrices to be used in organic reactions (Hiol *et al.* 2000, Pahujani *et al.* 2008). Furthermore, the operational stability, especially in low-water media, and enzyme immobilization on various organic/inorganic supports also has been extensively studied aiming at an improved activity (Tzialla *et al.* 2010).

The use of immobilized lipases has been reported in several industrial areas, namely in the pharmaceutical, meat tenderization, clarification of beer, preparation of infant foods and dietary supplements, processing of fats and other lipids, as an additive in detergents, oleochemical industries, pulp and paper, the synthesis of drugs and fine chemicals, production of cosmetics, bioremediation and resolution of racemic mixtures and also in the bioenergetics in transesterification of oils and fats in the presence of solvents to produce biodiesel, as well as in medicine (Nitsawang *et al.* 2006, Sangeetha and Abraham 2006). Furthermore, these enzymes in an industrial context have been reported to be advantageous since their immobilization provided them a clear advantage to control their stability/activity in unusual conditions of temperature, pressure and pH, or in non-conventional media, such as in organic solvents (Bayramoglu *et al.* 2004, Caro *et al.* 2008, Singh *et al.* 2007).

The immobilization method and type of support to be used in a given process should be established empirically, while holding the choice of the binomial enzyme-support that has the better retention of activity. The choice of the method of restraint and the type of support will depend basically of the specific characteristics of the biological material. Given the variability of these factors, it can be stated that there is not a universal method suitable for any process (Corcoran 1985). Therefore, the lipase immobilization on

appropriate supports represents an important step. An ideal support would allow adsorbing the enzyme without affecting its activity and without interfering with the enzymatic reaction.

Lipases can be immobilized by a number of methods, including physical adsorption (active charcoal), covalent binding (cellulose, silica) and entrapment (cellulose) (Hasan *et al.* 2006, Wang *et al.* 2008). Physical adsorption and entrapment are the simplest methods of enzyme immobilization although they present some limitations, such as desorption and leakage of the enzyme. These limitations can lead to a low stability and catalytic activity. Alternatively, the covalent binding methods can be used, since a stronger binding between enzyme and solid support is obtained (Park *et al.* 2001, Park *et al.* 2002, Shim *et al.* 2007, Soares *et al.* 2003, Song *et al.* 2010). Nevertheless, the covalent binding methods result in a considerable decrease of the enzyme activity. It is assumed that this enzyme inactivation is caused by the damage of its active site and distortion of its native structure, due to the covalent bonds between the enzyme and the solid support (Giacomini *et al.* 1998). To overcome this problem, it is necessary to develop methods of pre-treatment or support types that prevent the loss of activity during the immobilization process (Lee *et al.* 2006, Song *et al.* 2010). Supports should be available, low cost, enable large scale operation, non-toxic, and have a high retention capacity and mechanical strength (Brígida *et al.* 2007, 2008).

Several materials have been reported as potential supports for enzymes immobilization. Alginate gels, carrageenan and polyacrylamide, alumina, ground kanuma, stalk of sugar cane, silica, acrylic resin and chitosan are some examples described in the literature (Biro *et al.* 2008, Bryjak and Trochimczuk 2006, Cruz *et al.* 2009, Emregul *et al.* 2006, Makas *et al.* 2010, Orrego *et al.* 2010, Tanvir *et al.* 2009, Wang *et al.* 2007).

Silica gel has been widely used as a support since it has a high mechanical strength, thermal and chemical stability, high resistance to microbial contamination and degradation, and a high surface area. Silica gel was used to immobilize lipase from *Candida cylindracea* which catalyzes the hydrolysis of triglycerides into free fatty acids (David *et al.* 2006).

Alumina has also been used to immobilize lipase from *C. antarctica* for the synthesis of butyl butyrate (Lozano *et al.* 2002). This support is highly resistant to high temperatures and pH's (Costa *et al.* 2001).

Furthermore, efforts have been made in order to develop immobilization supports commercially available, inexpensive and biodegradable. Brígida *et al.* (2007, 2008) used waste green coconut fiber for covalent immobilization of CALB. Besides being an interesting immobilization support, these wastes take up to seven years to decompose, contributing to the spread of tropical diseases, being responsible for leakage from landfills, if not properly disposed. Therefore, its use in added-value applications is also interesting from the environmental standpoint.

Among the potential supports that are abundant in nature, chitosan is the best example, being usually produced by alkaline hydrolysis of chitin, a process which results in N-deacetylation and depolymerization. Chitin poly (N-acetyl-glucosamine) is a polymer that can be obtained from the outer shell of crustaceans (shrimp and crab). Moreover, it is also naturally present in the cell walls of some microorganisms (Alsarra *et al.* 2002, Silva *et al.* 2006). Chitosan is a renewable and biodegradable low cost polymer (Alsarra *et al.* 2002).

Table 1.2 summarizes some types of supports used in the immobilization of lipases, as well as their microbial sources.

Table 1.2 Supports used for lipases immobilization

Support	**Microorganism**	**Method**	**Reference**
Chitosan	*Candida antarctica*	covalent	Rodrigues *et al.* (2008b)
Eupergit C	*Candida rugosa*	covalent	Knezevic *et al.* (2006)
Alumina	*Candida antarctica*	covalent	Lozano *et al.* (2002)
Silica gel and alumina	*Candida cylindracea*	covalent	Moreno and Sinisterra (1994)
Green coconut fiber	*Candida antarctica*	adsorption	Brígida *et al.* (2008)
Activated carbon	*Candida antarctica*	adsorption	Rodrigues *et al.* (2008a)
Polystyrene	*Candida rugosa*	covalent	Ye *et al.* (2009)

1.3 Esterification in organic solvents

The most diverse organic reactions such as hydrolysis, esterifications, interesterifications, alcoholysis, acidolysis, aminolysis and lactonizations can be conducted through synthesis routes using chemical or biochemical catalysts to achieve a given conversion under controlled conditions. The main advantages of these reactions are the enhanced solubility of nonpolar substrates and the possibility of shifting the equilibrium of the reaction towards the synthesis (Persson *et al.* 2002).

The synthesis of esters can be carried out either chemically or enzymatically. The chemical process occurs with a low selectivity and leads to a mixture of sugar esters with different degrees of esterification. It requires toxic organic solvents and is conducted at high temperatures, resulting in low quality of the final product (Yoo *et al.* 2007). To overcome this limitation biological catalysts and organic solvents that leave no toxic residues in the final product of the reaction, can be used (Pandey *et al.* 1999). Furthermore, the enzymatic method presents several advantages, namely the reduction of the number of reactional steps, the lack of racemization, and the minimal protection due to regiospecificity (Kim and Shin 2001).

Among several organic reactions, esterification is the most widely used in the organic process industry (Ali *et al.* 2007, Yadav and Thathagar 2002). The methyl or ethyl esters of fatty acids can be produced by esterification using biocatalysts (Arai *et al.* 2010, Fernandez-Lafuente 2010, Fukuda *et al.* 2009, Mbaraka and Shanks 2006, Sharma *et al.* 2001).

Nevertheless, the direct esterification of sugars with fatty acids catalyzed by lipase is a very complex procedure, due to the low solubility of sugars in organic media (Lortie 1997).

1.3.1 Influence of water

Water activity (a_w) has been described as an important parameter to optimize organic reactions in aqueous and non-aqueous systems and can be adjusted by a number of methods. In enzymatic reactions, a_w determines the equilibrium position of the hydrolase

reaction in low water systems (Adamczak and Bornscheuer 2009, Matsue and Miyawaki 2000).

Lipase-catalyzed esterification in organic solvents is a reaction in which water plays a crucial role (Giacometti *et al.* 2001). The nature of the organic solvents and the amount of water in the system influence the enzymatic reactions in non-aqueous reactions, thus a minimal amount of water is necessary to ensure the enzymes' optimal conformation and activity (Yaropolov *et al.* 2007). On the other hand, high water content reduces the reaction stability, the particles of enzyme present in the medium could be covered by a layer of water, thus preventing the contact of a lipophilic substrate (i.e. fatty acid) with the enzyme (Chamouleau *et al.* 2001). According to Adachi and Kobayashi (2005), the water should be removed in order to direct the reaction towards the product, so a maximum ester yield could be obtained, as illustrated in Figure 1.1.

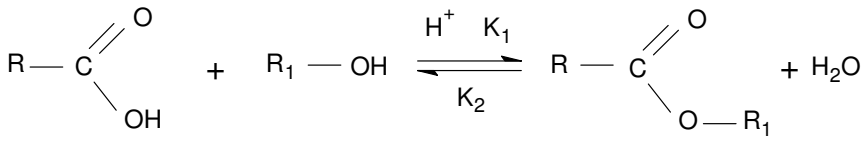

Figure 1.1 Schematic representation of the general esterification reaction with the production of ester and water where, the condition Keq>>1 or k1>>k2 must be fulfilled.

Several methods for removal of the water formed during the reaction have been reported, such as evaporation under reduced pressure, azeotropic distillation, use of molecular sieves and the use of sodium sulphate (Izák *et al.* 2005, Xuehui and Lefu 2001, Yan *et al.* 1999).

The influence of a_w is best predicted and analyzed in terms of its thermodynamic activity instead of water concentration (Chowdary and Prapulla 2002). According to Gandhi *et al.* (2000), this is readily observed from the effect of water content on the catalytic activity of lipase. The lipase presented a similar optimum at thermodynamic a_w of about 0.55 when used in solvents ranging from hexane to pentanone.

In contrast, Awang and co-workers (2000) reported that a_w ranging from 0.09 to 0.96 did not have a marked relevance on the yields of the synthesized ester in the esterification of dihydroxy stearic acid with octanol catalyzed by *Rhizomucor miehei* and Novozyme 435 lipases.

Studies conducted by Chowdary and Prapulla (2002), with the lipases from *Rhizopus oryzae, Mucor javanicus, Aspergillus niger* and *Penicillium roqueforti*, demonstrated that at higher levels of water activity (a_w 0.96), the enzymes promoted higher ester yields, probably due to protein aggregation, and the highest yield rates at lower levels of water activity (a_w 0.33).

Thus, the optimum a_w also depends on the type of support used for immobilization, as well as on the type of solvent used in the reaction, i.e., the optimum water requirement is dictated by the biological source of the enzyme, the organic solvent, and, possibly, the type of support used (Gandhi *et al.* 2000). For example, the effect of butanol, water and water-butanol ratio on the activity of immobilized *Mucor miehei* (Lipozyme™) was studied at various temperatures. The immobilized enzyme exhibited better stability than the free enzyme (Gandhi *et al.* 1997).

1.3.2 Influence of solvent system

Enzymatic reactions using organic solvents provide several advantages of industrial interest, such as increased solubility of non-polar substrates, reversibility of the thermodynamic equilibrium of the hydrolysis reactions, suppression of water-dependent side reactions, modification of substrate specificity and enantioselectivity, among others (Doukyu and Ogino 2010).

Depending on the miscibility of an organic solvent with water, and the relative proportion of solvent and water in the medium, there are three main types of organic solvent systems that can be used: water plus water-miscible organic solvent system (organic co-solvent system); water plus water-immiscible organic solvent system (two phase system or biphasic system); and nearly anhydrous organic solvent system (Davison *et al.* 1997, Ogino 2008).

The first organic solvent system is produced when water-miscible co-solvents are added to the medium to improve the solubility of compounds that are insoluble in aqueous systems and can reduce the mass-transfer limitations, leading to faster reaction rates for hydrophobic compounds. The increasing concentration of organic co-solvent would always lower the enzymatic activity, due to direct contact of the organic solvent with the enzyme. Moreover, the advantage of improving the solubility of the substrate is compensated by the achievement of a high enzymatic activity (Khmelnitsky *et al.* 1988, Ogino 2008).

The second organic solvent system consists of two phases, an aqueous phase containing a dissolved enzyme and another phase of an immiscible organic solvent (Khmelnitsky *et al.* 1988). The aqueous phase forms a separate layer in contact with the layer of the organic solvent. In this system, the enzymatic reaction occurs in the aqueous phase containing the enzyme. A hydrophobic substrate, such as steroids or fats, is mostly located in organic solvent layer and partitioned into the aqueous phase. The substrate is converted by the enzyme, and then the product is extracted into the organic solvent phase. This system is advantageous for the synthesis of esters due to the shift of the reaction towards synthesis (Ghatorae *et al.* 1994, Ogino 2008, Wu *et al.* 1993).

Finally, in the last organic solvent system, involving lyophilization, immobilization or modifications with amphipathic compounds, the enzymes are required for solubilisation purposes (Castro and Knubovets 2003, Klibanov 2001). Lyophilization causes a reversible damage in the enzyme structure (Lee and Dordick 2002). Co-lyophilization with additives such as carbohydrates, polymers, and salts prevents this damage and activates the lipases. The lipase lyophilized or precipitated from an aqueous solution at its optimum pH exhibits a high activity. In this system, lyophilized lipases often exhibit a high thermal stability, but show far lower catalytic activity than in water. The water content (water activity) in the system is essential to have sufficient lipase activity. It is generally known that hydrophobic solvents result in a higher lipase activity comparing to hydrophilic ones. Conformational mobility of lipases at such low water content is generally restricted. Therefore, the proteins are more rigid in this type of system than in water. This system has been demonstrated to be very useful in various enzymatic processes, such as in the synthesis and transesterification of esters, peptide synthesis, and transformation of various hydrophobic compounds (Serdakowski and Dordick 2008).

1.4 Sugar ester biosurfactants

Surfactants, biosurfactants, emulsifiers, bioemulsifiers, carbohydrate esters (sugar esters) or fatty acid esters are compounds that contain surface-activity and have a high emulsifying capacity. Among these, are named biosurfactants, the compounds that are obtained as metabolic products of bacteria, fungi or yeasts or produced through microbial enzymes (Banat *et al.* 2000, Nitschke and Pastore 2006, Reddy *et al.* 2009, Xie *et al.* 2007, Yin *et al.* 2009).

According to the literature, there are a large number of synthetic surfactants (Worakitkanchanakul *et al.* 2008). However, these synthetic surfactants present some drawbacks, such as their costs, availability and environmental impact. Therefore, the enzymatic synthesis, using lipases as biocatalyst, appears to be an interesting green alternative for the production of some sugar ester biosurfactants (Kiran *et al.* 2010). Table 1.3 shows some of the most common groups of surfactants of natural and synthetic origin.

Table 1.3 Natural and synthetic surfactants

Natural	**Synthetic**
Biosurfactants	Alkyl and aryl ether carboxylates
Fatty acid amides	Alkyl aryl sulfates
Fatty acid amines	Alkyl aryl ether sulfates
Derived protein	Alkyl phenol ethoxylate
Sucrose esters	Co-polymers of ethyl oxide/propylene
Sulfates of natural fatty alcohols	Ethoxylated fatty acids

In general, there are numerous advantages of the biosurfactants over their chemical counterparts, such as biodegradability (easily degradable in water and soil, which makes them suitable for applications such as bioremediation and wastewater treatment), low toxicity, selectivity, biocompatibility, ecological acceptability and effectiveness at extreme temperature and pH conditions (Batista *et al.* 2006, Chamouleau *et al.* 2001, Costa *et al.* 2006, Ghojavand *et al.* 2008, Luna-Velasco *et al.* 2007, Park *et al.* 2004, Thanomsub *et al.* 2006, Tsavas *et al.* 2002, Yin *et al.* 2009). Furthermore, the biosurfactants are odorless, biocompatible, and can be produced from renewable sources, thus at lower costs (Shin *et al.* 2009, Yutaka and Kitagawa 1998).

1.4.1 Properties

Biosurfactants possess several important physical and chemical properties, such as foaming, emulsifying and stabilizing capacities, low critical micellar concentration, detergent solubility, dispersion, power, among others. These properties are very important in evaluating its performance (Deleu and Paquot 2004, Lee *et al.* 2008).

The most important feature of any given biosurfactant is its ability to reduce the surface tension of a liquid medium (Ahimou *et al.* 2001, Bognolo 1999, Desai and Banat 1997, Ghojavand *et al.* 2008, Lee *et al.* 2008, Nguyen *et al.* 2008, Pletnev 2001). For example, a sugar ester biosurfactant, such as sophorolipid esters can effectively reduce the surface tension of water to values below 38.7 mN/m (Maier 2003, Mulligan 2005, Zhang *et al.* 2004). According to Busscher and co-workers (1994), a decrease larger than 8 mN/m of the surface tension is an indicative of biosurfactant production.

Besides, some biosurfactants are considered as food emulsifiers. Typically, the biosurfactants are molecules bearing a hydrophilic and a hydrophobic part. The hydrophobic part consists of fatty acid, whereas the hydrophilic part may consist of glycerol or one of its ester derivatives resulting from the reaction with organic acids such as lactic, citric, acetic, or tartaric acid (Figure 1.2). Food industries are extremely interested in these additives since they can safely be consumed by humans in quantities up to 125 mg kg^{-1} body weight per day. Additionally, they have useful properties that improve the production of some food products, such as bakery commodities (Suman *et al.* 2009).

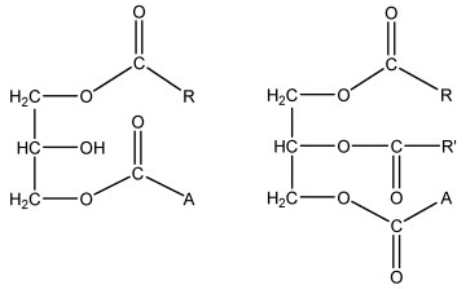

Figure 1.2 Chemical structures of mono- and diacylglycerol esters of fatty acids emulsifiers where typically R = lauric acid, myristic acid, palmitic acid, oleic acid, and stearic acid and A = diacetyl tartaric acid, acetic acid, lactic acid, and citric acid (adapted from Suman *et al.* 2009).

The potential of sugar ester biosurfactants in food industry is high although it is important that they accomplish specific acceptance criteria, present certified performances and comply with the existing restrictions (Makkar and Cameotra 2002, Suman *et al.* 2009).

1.4.2 Applications

Natural food emulsions exist long before food processing. A good example is milk, a natural emulsion/colloid in which fat is stabilized by a milk-fat-globule membrane. The development of technologies for processing oils, such as refining, bleaching, and hydrogenation, has led to the development of new food emulsifiers (Kinyanjui *et al.* 2003).

Manufacturers employ two types of emulsifiers or foaming agents in food, namely: LMWE (Low Molecular Weight Emulsifiers), such as mono-diglycerides, phospholipids, among others and macromolecules, such as proteins and hydrocolloids (Patino *et al.* 2008). The emulsifier film adsorbed at the oil-water or air-water interface is the source of many of the unique properties of food dispersions, particularly their stability and interactions, which translate into the shelf-life and textural properties so desired by manufacturers and appreciated by consumers (Sánchez *et al.* 2005). Therefore, there is a variety of segments of the food industry in which sugar ester biosurfactants may be used, namely in the production of aromas and maturation of cheeses, bakery products, cakes, biscuits, mayonnaise and sauces, instant products, sausages, among others (Liese *et al.* 2000, Pandey *et al.* 1999, Plou *et al.* 2002, Saxena *et al.* 1999, Sharma *et al.* 2001).

The main emulsifiers used by the food industry are monoglycerides and esters of lactic acids (Kamel 1997). Monoglycerides are widely used as anti-staling agents and account for approximately one third of the emulsifiers used in the baking industry. These compounds can also act as mild dough conditioners, leading to improved dough machining properties, enhanced slicing performance and superior bread quality (Sawa *et al.* 2009, Stauffer 2000).

Esters of lactic acid are part of the aroma array of cheeses made from goats' and ewes' milk (e.g., Feta, Manchego, Serra da Estrela and Roncal), but the number and type of esters found vary between cheese varieties (Mukdsi *et al.* 2009). Among the esters identified in these cheeses, the ethyl esters of the straight-chain fatty acids of C2-C10 are most frequently found (Liu *et al.* 2004). These esters, which are potent flavour compounds

at less than 5 ppm, are important for development of the characteristic "fruity" type flavours such as ethyl butanoate and ethyl hexanoate (Moio and Addeo 1998).

Several studies have been reported on the use of sugar ester biosurfactants in bakery products. In the last years, the bakery industry became more demanding regarding the increase of products shelf-life and consistent quality through the use of food additives. These additives, including emulsifiers, enzymes, soy flour, oxidants and reductants, are essential for improving dough machinability, reducing resting time, and improving baked goods' shelf-life. Furthermore, these additives increase the volume and aeration thus reducing the stickiness, improve texture and shelf-life of starch-containing products, crumb whiteness, aroma and flavour (Haros *et al.* 2002, Leon and De Barber 2002, Makkar and Cameotra 2002, Moayedallaie *et al.* 2010, Nitschke and Costa 2007, Suman *et al.* 2009).

Sugar ester biosurfactants are used to assist blending and emulsification of ingredients, to control the agglomeration of fat globules, stabilize aerated systems, modify rheological properties of wheat dough, improve consistency, and to interact with the components of the flour and other ingredients in the mix for softer crumb improving the palatability. According to their chemical structure, emulsifiers can interact and form complexes with starch, protein, shortening, and water. Interaction of an emulsifier with the protein can improve the strength and allow better retention of carbon dioxide (Colla *et al.* 2010, Demirkesen *et al.* 2010, Makkar and Cameotra 2002, Nitschke and Costa 2007, Suman *et al.* 2009). An improvement of dough stability, texture, volume and preservation of bakery products was obtained by the addition of rhamnolipid surfactants (Haesendonck *et al.* 2004).

According to Sawa and co-workers (2009), mixing properties, bread quality and crumb firmness during bread storage were strongly influenced by the type and level of monoglyceride added to flour during bread processing. Polyunsaturated monoglycerides showed the greatest strengthening effects on dough properties.

Additionally, emulsifiers can be added to cereal flours in order to produce specific desired characteristics, such as soft structures. In fact, during extrusion, emulsifiers form complexes with amylose that affect texture, cell distribution, and density of the extruded products. Furthermore, emulsifiers act as lubricants for the melted dough reducing the specific mechanical energy (De Pilli *et al.* 2007). In bakery and ice cream formulations, the

sugar ester biosurfactants act by controlling consistency, delaying staling and solubilizing flavor oils; they are also utilized as fat stabilizers and anti-spattering agents during cooking of oil and fats (Kosaric 2001).

1.5 Conclusions

The synthesis of sugar ester biosurfactants can be accomplished chemically or enzymatically. The chemical process occurs with low selectivity and leads to a mixture of sugar esters with different degrees of esterification require toxic organic solvents and is performed at high temperatures. Also, the use of these solvents is being gradually restricted in many industrial applications. This issue can be overcome using a biological catalyst, such as immobilized lipases, and organic solvents that do not leave toxic residues in the final reaction products. Enzyme immobilization is advantageous, namely due to the possibility of reusing the catalyst the use in continuous processes; ease of handling; higher thermal and chemical stability of the enzyme; regeneration and ease of recovery; and reduction of operational costs.

Sugar ester biosurfactants can be synthesized from sugars and fatty acids using lipases. These compounds present numerous advantages as compared to their chemical counterparts, such as biodegradability, low toxicity, selectivity, biocompatibility, environmental acceptability and effectiveness under extreme conditions of temperature and pH. Therefore, sugar ester biosurfactants have a variety of potential applications, including pharmaceuticals, detergents, cosmetics and food industry. In the food industry, these compounds can be used in the production of aromas and maturation of cheeses, bakery products, cakes, cookies, mayonnaise and sauces, instant products, sausages, among others.

Sugar ester biosurfactants are expected to gain a significant market share due to increasing knowledge; metabolic, systems and synthetic biology efforts to increase production yields and product diversity; and improved downstream technologies that facilitate product recovery.

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Chapter 2

Maximization of fructose esters synthesis by response surface methodology

"O sucesso é a soma de pequenos esforços – repetidos dia sim, e no outro dia também".

(Robert Collier)

In this section, enzymatic synthesis of fructose fatty acid ester was performed in organic solvent media, using a purified lipase from *Candida antartica* B immobilized in acrylic resin. Response surface methodology with a central composite rotatable design based on five levels was implemented to optimize three experimental operating conditions (temperature, agitation and reaction time). A statistical significant cubic model was established. Temperature and reaction time were found to be the most significant parameters. The optimum operational conditions for maximizing the synthesis of fructose esters were 57.1 ºC, 100 rpm and 37.8 h. The model was validated in the identified optimal conditions to check its adequacy and accuracy, and an experimental esterification percentage of 88.4% (±0.3%) was obtained. These results showed that an improvement of the enzymatic synthesis of fructose esters was obtained under the optimized conditions.

2.1 Introduction

Sugar esters are non-ionic biosurfactants that consist of a carbohydrate moiety as hydrophilic group and one or more fatty acids as lipophilic component. By controlling the esterification degree and the nature of fatty acid and sugar, it is possible to synthesize sugar esters within a wide range of properties.

An increasing interest in the production of sugar esters has been reported, since they can be used as surface-active components in many industrial fields, as cosmetics, health-care, pharmaceuticals and food industries (Nakamura 1997, Watanabe 1999). Furthermore, these compounds have certain advantages over synthetic surfactants, such as being prepared from renewable sources; tasteless, odorless, stable over a broad pH range and non-irritant. In food industry, fructose esters can be used in the production of aromas and maturation of cheeses, bakery products, cakes and biscuits, mayonnaise and sauces, instant products and sausages, among others (Tarahomjoo and Alemzadeh 2003). In addition, sugar esters properties as antibiotics (Marshall and Bullerman 1994), antitumorals (Okabe *et al.* 1999) and insecticides (Chortyk *et al.* 1996) are well reported and might open new markets. For the past few years, several researchers have investigated the lipase-catalyzed synthesis of sugar containing acrylic esters for their biomedical applicability (Chang and Shaw 2009, Park and Chang 2000, Staples *et al.* 2000). Moreover, these compounds are biodegradable, biocompatible and essentially non-toxic (Naoe *et al.* 2001, Torres and Otero 2001).

Sugar esters can be synthesized either by chemical or enzymatic processes. Chemical production of sucrose esters is usually base-catalyzed at high temperatures, has a low selectivity, forming colored derivatives as side-products (Nakamura 1997). Enzymes have been successfully applied to the regioselective transformations of mono- and oligosaccharides, including acylation, deacylation and oxidation reactions. The enzyme-catalyzed synthesis of sugar esters provides regio- and stereoselective products (Cruces *et al.* 1992, Riva *et al.* 1998, Soedjak and Spradlin 1994). Previously, sugar esters were synthesized mostly by esterification in aqueous media causing hydrolytic side reactions. To prevent these side reactions, solvents such as pyridine and dimethylformamide were used as reaction media (Ferrer *et al.* 1999). However, the solubility of sugars and the activity of enzyme were decreased due to the increased hydrophobicity introduced by these organic

solvents in the reaction system. In addition, the use of sugar esters as food additives and pharmaceuticals was incompatible with the use of these toxic solvents. Due to the high regiospecificity of enzymes, enzymatic synthesis is characterized by the production of a more defined product sugar monoester, whereas chemical process usually leads to a mixture of sugar polyesters (Maugard *et al.* 1997). Therefore, various sugar esters (e.g. fructose or sucrose esters) can now be prepared by a single reaction step employing enzymes - lipase - as a biocatalyst (Roy and Chawla 2001, Sabeder *et al.* 2006, Tarahomjoo and Alemzadeh 2003).

Enzymatic synthesis in organic medium is based on the ability of lipases to catalyze reverse hydrolysis, i.e., the formation of ester bonds. These reactions take place in a medium presenting a low water activity and allow much higher conversions in a shorter time. In these conditions, the thermodynamic equilibrium of the reaction is shifted towards synthesis reaction instead of hydrolysis. The enzymatic process yields up to 80% conversion within 8 h of incubation, and synthesis can be performed in a batch reactor at a temperature as low as 64 ºC in presence of microbial lipase like *Candida antartica*. Enzymatic synthesis of fructose fatty acid esters is showed in Figure 2.1.

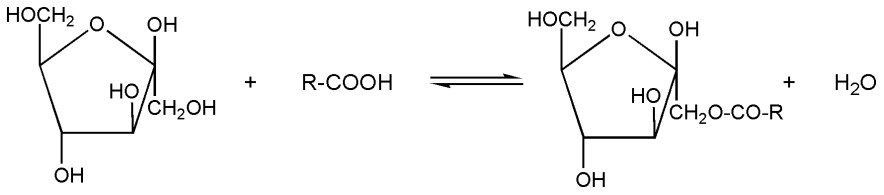

Figure 2.1 Lipase-catalyzed syntheses of fructose fatty acid esters (Sabeder *et al.* 2006).

The enzymatic esterification of sugar esters is gaining importance due to mild reaction conditions and excellent selectivity associated with lipase-catalyzed reactions. Therefore, the optimization of sugar ester synthesis is very important for its economical manufacturing (Adnani *et al.* 2010).

Nevertheless, several factors can affect both the conversion yield and the rate of esterification. These factors include the reaction solvent, reaction temperature, reaction time, the type and concentration of the acyl donor, enzyme content and initial substrate concentration. Thus, it is difficult to search for the major factors and to optimize them as several parameters are involved (Lundstedt *et al.* 1998).

The classical method of optimization involves changing one variable at a time, keeping the others at fixed levels. Being single dimensional, this laborious and time consuming method often does not guarantee determination of optimal conditions, neither takes into account possible interactions among various operational factors. Experimental design and optimization are tools that enable building models and evaluating the significance of the different factors considered, as well as their interactions. Furthermore, with these models, a small number of experimental trials are used to search the optimal factor levels that conduct to the desired response (Adnani *et al.* 2010, Montgomery 1997).

The aim of this work is to optimize, using a response surface method (RSM), the operational conditions (temperature, agitation and reaction time) that maximize the synthesis of fructose esters. Therefore, a 2^k full-factorial central composite design, based on a preliminary design that used five factors (temperature, agitation, reaction time, fructose concentration and enzyme concentration) (*data not shown*) was conducted. Fructose esters are synthesized by esterification of oleic acid with fructose using a lipase from *C. antarctica* type B (CALB) immobilized in acrylic resin.

2.2 Material and Methods

2.2.1 Materials

All chemicals used were analytical grade. The commercial triacylglycerol lipase from *Candida antartica* B immobilized in acrylic resin (CALB) (Novozym 435) was purchased from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO).

2.2.2 Methods

2.2.2.1 Synthesis of fructose esters

The fructose esters synthesis experiments (esterification reactions) were conducted in flasks by adding oleic acid (0.5 mmol), fructose (0.6 mmol), CALB (12.5 mg), sodium sulfate anhydrous (0.1 g) and ethanol 99% (0.6 mL). The flasks were incubated for different reaction times under controlled agitation and temperature. The synthesis procedure has been previously described by Sabeder and collaborators (2006).

2.2.2.2 Experimental design & data analysis

The optimal temperature, agitation and reaction time levels for maximizing the synthesis of fructose esters were studied using a 2^k full-factorial design with 3 factors and 3 replicates of the central point. Based on previous experiments (*data not shown*) and studies, the amounts of the reactants showed no significant effect on the esterification percentage.

The initial experimental design was augmented using an additional central composite design allowing the optimization of those experimental conditions of operation by means of a response surface methodology. Since the new runs were made after a period of 15-days from the initial ones, to control day-to-day variation, blocking technique was considered. Therefore, a new block of experimental data was included, consisting in nine other experimental points, being three of them replicates of the initial central point. The other six new points, also called star points, were introduced to make the central composite design rotatable, setting the distance from the central design equal to ±1.682. The parameters range was chosen according to previous experiments and reported knowledge on the optimum conditions for enzymatic synthesis of fructose esters (Sabeder *et al.* 2006, Shieh *et al.* 1996, Yan *et al.* 2001, Zaks *et al.* 1985). In total, the 3 independent factors (temperature, agitation and reaction time corresponding to the actual factors x_1, x_2 and x_3, respectively) were studied at 5 levels (±1.682; ±1 and 0) and a set of 20 experiments were carried out randomly (Table 2.1).

Table 2.1 Experimental range and levels of the factors tested in the 2^3 full factorial central composite rotatable design

Variable	Symbol	**Coded (X_i) variable level**				
		-1.682	-1	0	+1	+1.682
Temperature (ºC)	x_1	46.6	50	55	60	63.4
Agitation (rpm)	x_2	58	75	100	125	142
Reaction time (h)	x_3	3.8	12	24	36	44.2

For the statistical treatment, the actual factors were coded according to the following equation (1):

$$X_i = \frac{x_i - x_0}{\Delta x_i}, \quad i = 1, 2, 3 \tag{1}$$

where X_i is the coded value of the independent factor, x_i is the real value of the independent factor, x_0 is the real value of the independent factor at the central point and Δx_i is the step change value.

It is expected that the behavior of the system could be explained by a quadratic or cubic equation (2), which is used for predicting the optimal esterification percentage point (Y), based on the coded values of the independent factors (X_i):

$$Y = \beta_0 + \sum_{i=1}^{3}\left(\beta_i X_i + \beta_{ii} X_i^2 + \beta_{iii} X_i^3\right) + \sum_{i<j}^{3}\left(\beta_{ij} X_i X_j + \beta_{iij} X_i^2 X_j + \beta_{ijj} X_i X_j^2\right) + \beta_{123} X_1 X_2 X_3 + \varepsilon \tag{2}$$

where Y is the predicted response, which takes into account the block correction (α_{block} is equal to the value of the block 1 or block 2 correction for the experimental data obtained at the first or second block of essays, respectively); the β's are first, second and third order parameters whose values are to be determined using multiple linear regression model (MRLM) and the statistically significant ones selected using a stepwise method. The first order parameters are related with the screening process, the second order with the model

curvature and the third order parameters due to asymmetry issues. Furthermore, to ensure that the model is hierarchical parameters could be included in the final model regardless their statistical significance. X_1 X_2 and X_3 are the coded independent factors and ε is a random error term of the regression model.

Design-Expert 6.0.6., Trial version and Statistical Package for Social Sciences (SPSS), version 14, were used for the experimental design and regression analysis of the experimental data. The significance of the regression model was evaluated using analysis of variance (ANOVA). The quality of the fit obtained using the regression model equation was statistically checked by means of two diagnostic residuals: the multiple or adjusted coefficient of determination (R^2 or R^2_{adj}, respectively) and the predicted coefficient of determination (Q^2). The R^2-values describe the goodness of fit, giving an idea of how well current runs can be reproduced by the mathematical model. The Q^2-value describes the goodness of prediction, showing how well new experiments can be predicted using the mathematical model. R^2 and Q^2 values higher than 0.75 and 0.60 indicate that the model is good, and Q^2 values lower than 0.25 indicate that the model is useless (Mandenius and Brundin 2008).

The discrimination ability of the model was also inferred by calculating the adequate precision value, which compares the range of the predicted values at the design points to the average prediction error. A value greater than 4 is envisaged to assure adequate model discrimination. The significance of the regression coefficients was tested using a *t*-test. Also, the required non multi-collinearity condition between the independent variables was evaluated using the variance inflation factor (VIF). Values lower than 10 should be obtained to ensure that the independent variables are not collinear. Finally, the contour plots obtained from the fitted quadratic or cubic model were also used to infer about the optimal experimental conditions keeping the independent factors within the experimental range studied. To validate the optimal operation condition levels that maximize the synthesis of fructose esters, 3 additional experimental trials were carried out at the optimal operating conditions to confirm the predicted esterification value obtained by the analysis of the response surface.

2.2.3 Characterization of the product

After the esterification reactions procedure described above, the product obtained was characterized according to the techniques described below.

2.2.3.1 Quantification of fructose esters

The ester content was calculated taking into account the residual fatty acid amount in the reaction mixture, which was determined by the volumetric method. Briefly, 0.1 g of a sample from the reaction mixture was diluted in 20 mL of 0.1 wt% phenolphthalein solution in absolute ethanol, and then titrated with a sodium hydroxide solution (0.1 mol/L) (Leitgeb and Knez 1990).

2.2.3.2 Fructose ester purification and characterization

At the end of the esterification reaction, the lipase, together with the sodium sulfate anhydrous (non reactive species), were removed by filtration using filter paper with a pore-size of 60-µm (Macherey-Nagel Inc.). Afterwards, the ethanol was evaporated from the reaction media using a rotoevaporator. The remaining product (fructose ester) was then analyzed and identified by thin layer chromatography (TLC), using a chloroform/hexane (1:1, v/v) mixture for elution. Subsequently, the fructose ester spot was identified with iodine according to Ducret and collaborators' work (1995).

Additionally, the purified reaction product was characterized by infrared spectroscopy (IR). The solid product was crushed with a mulling agent, nujol. Subsequently, a thin film of the mull was applied on the surface of a NaCl cell and measured. Infrared absorption spectra were recorded on a Bio-Rad model FTS 165 spectrophotometer with a spectral band between 450 and 4000 cm^{-1}.

2.3 Results and Discussion

Sugar esters have been attracting a considerable interest in several fields, such as food industry, mainly due to their advantages as compared to synthetic surfactants (Nakamura 1997, Tarahomjoo and Alemzadeh 2003, Watanabe 1999). Sugar esters can be synthesized either by chemical or enzymatic processes, although enzymatic esterification of sugar esters is gaining importance due to mild reaction conditions and excellent selectivity associated with lipase-catalyzed reactions (Sabeder *et al.* 2006).

The rate of esterification, as well as the conversion yield, is affected by a number of factors such as the solvent, temperature, time, type and concentration of the acyl donor, enzyme content and initial substrate concentration (Polat and Linhardt 2001, Roy and Chawla 2001, Sarney *et al.* 1996, Shieh *et al.* 1996, Yan *et al.* 2001). Consequently, as several parameters are involved, the optimization of the esterification rate and conversion yield can be very laborious if no alternative approaches, such as experimental design and optimization tools, are used (Lundstedt *et al.* 1998). Even using an experimental design to build models and study interactions among different factors, when a great number of factors is used the optimization may be difficult to assess.

Based on this discussion, a preliminary design was conducted with five factors (temperature, agitation, reaction time, fructose concentration and enzyme concentration) (*data not shown*) and, although the ratio substrate/enzyme has been reported as an important factor (Sabeder *et al.* 2006), the significance of substrate and enzyme concentrations were found to be not significant, as well as their interactions. Also, it is well known that water concentration is a critical factor in ester synthesis reactions catalyzed by lipases, since an excess of water would favor the reverse reaction, i.e., the hydrolysis of ester bonds. Nevertheless, in the current work this factor was excluded from the design since a desiccant (sodium sulfate anhydrous) was added to the reaction medium in order to prevent the reverse reaction. Therefore, a response surface methodology (RSM) with a three-factor-five-level central composite rotatable design (CCRD) was employed for modeling and optimization of the enzymatic esterification of fructose esters.

The influence of three operating variables, namely temperature, agitation and reaction time, on the esterification process was evaluated by means of a 2^3-full factorial rotatable central composite design. In total 20 runs were carried out, being six of them at

the central point. The ranges of values of the variables used in the augmented experimental design were 46.6-63.4 ºC, 58-142 rpm and 3.8-44.2 h, respectively (Table 2.1).

A statistically significant cubic polynomial model (P<0.0001) was fitted to the experimental data (Table 2.2) with an R^2-value and an R^2adj-value of 0.9995 and 0.9981, respectively.

Table 2.2 Experimental design and results (experimental and model prediction) obtained using the 2^3 full factorial central composite rotatable design used for the optimization of the synthesis of fructose esters

Run	Block	Temperature (ºC)	Agitation (rpm)	Time (h)	Esterification (%)	
					Experimental	Model Prediction*
1	1	60	75	12	67.24	67.15
2	1	50	125	12	64.79	64.70
3	1	50	75	36	79.62	79.53
4	1	55	100	24	84.84	84.92
5	1	60	125	12	75.77	75.68
6	1	50	75	12	85.91	85.82
7	1	55	100	24	84.35	84.92
8	1	60	75	36	87.45	87.36
9	1	60	125	36	86.16	86.07
10	1	55	100	24	84.81	84.92
11	1	50	125	36	79.77	79.68
12	2	55	100	24	83.48	83.10
13	2	55	100	24	83.25	83.10
14	2	55	100	24	83.37	83.10
15	2	55	100	44.2	84.34	84.47
16	2	55	142	24	85.42	85.55
17	2	55	100	3.8	78.19	78.32
18	2	55	58	24	85.32	85.45
19	2	63.4	100	24	54.84	54.97
20	2	46.6	100	24	71.96	72.09

(*) Model predicted values include block corrections.

Two data blocks were considered for establishing the model allowing controlling day-to-day variation between the first 11 experimental runs and the last 9, corresponding to the initial design and to the augmented design, respectively. The model had no lack of fit (P=0.0227), an adequate model discrimination (adequate precision value of 96.4) and a Q^2-value of 0.7056, showing a very satisfactory predictive performance of the model.

The main effects, quadratic and cubic effects and interactions of the three operating variables on the esterification percentage were evaluated. Globally, all effects evaluated were statistically significant ($P \leq 0.0020$) except agitation ($P=0.8625$). However, since second and third order interaction parameters involving agitation were statistically significant, agitation was included in the final form of the cubic model to ensure a hierarchical model. The parameters of the final cubic model estimated using the response surface methodology and their standard errors are shown in Table 2.3.

Table 2.3 Regression parameters of the optimal cubic model selected using a stepwise method, for the 2^3 full factorial central composite design

Factor	β's coefficient (coded factors)	Standard errors	*P*-values
Intercept	84.0	0.2	<0.0001
X_1	4.1	0.2	<0.0001
X_2	0.03	0.2	0.8625*
X_3	6.6	0.2	<0.0001
X_1^2	-6.9	0.1	<0.0001
X_2^2	0.9	0.1	0.0004
X_3^2	–0.6	0.1	0.0020
$X_1 X_2$	3.5	0.1	<0.0001
$X_1 X_3$	2.7	0.1	0.0001
$X_2 X_3$	1.4	0.1	<0.0001
X_1^3	–3.2	0.1	<0.0001
X_3^3	–1.7	0.1	<0.0001
$X_1^2 X_2$	–1.8	0.2	0.0004
$X_1 X_2 X_3$	–3.9	0.14	<0.0001

(*) Parameter with no statistical significance included in order to keep a hierarchical model.

Considering the results obtained Eq. (2) takes the simpler form of:

$$Y = \beta_0 + \sum_{i=1}^{3}\left(\beta_i X_i + \beta_{ii} X_i^2\right) + + \beta_{111} X_1^3 + \beta_{333} X_3^3 + \sum_{i<j}^{3}\left(\beta_{ij} X_i X_j\right) + \beta_{112} X_1^2 X_2 + \beta_{123} X_1 X_2 X_3 + \varepsilon \qquad (3)$$

Also, no statistical evidences of multi-collinearity were found since the variance inflation factor (VIF) values calculated for all the terms included in the model (linear, quadratic and cubic terms) were lower than 5. The predicted values were in good

agreement with the experimental values (Table 2.2), showing that the cubic model could be used to predict and optimize the esterification percentage by determining the optimal operating conditions (temperature, agitation and reaction time).

The optimization process was carried out based on the contour plots and the 3-D response surface (Figure 2.2).

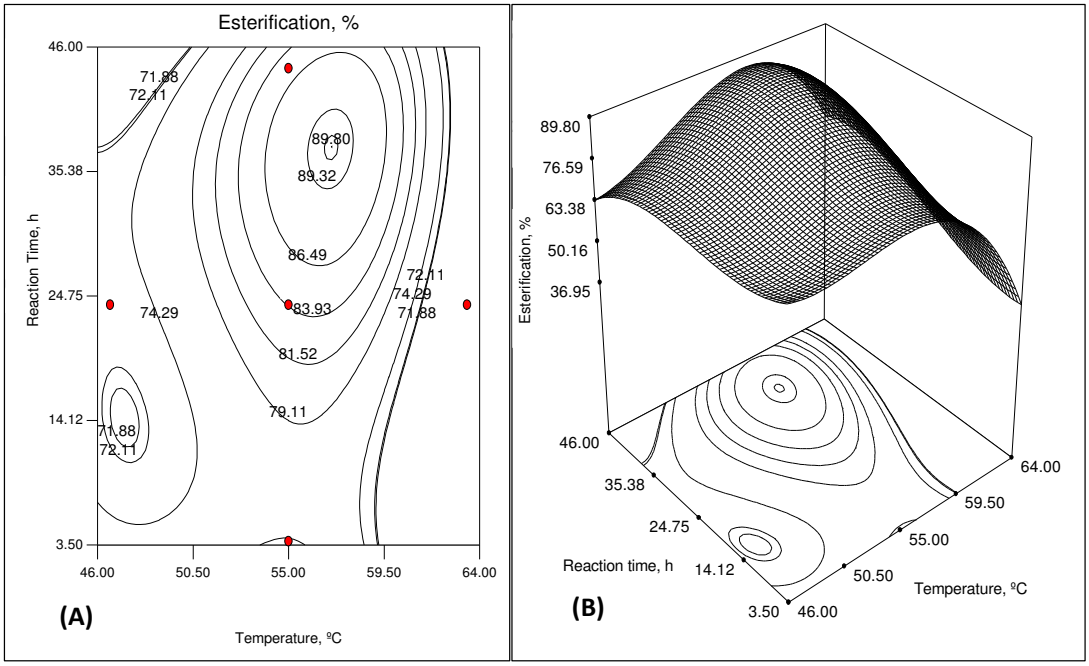

Figure 2.2 Response surface described by the model for an agitation set equal to 100 rpm (central point), in the region explored experimentally: (A) contour plots showing the predicted esterification percentage; (B) 3-D surface.

For optimization purposes agitation was set equal to 100 rpm (equal to the value of the initial design central point), since this effect was not statistically significant. Under this agitation, the fitted surface showed a possible maximum point, based on the contour plots analysis.

The predicted maximum esterification percentage was equal to 89.8% (±0.4%) for a temperature of 57.1 ºC and a reaction time of 37.8 h. Three model validation experiments

were carried out at those optimal operating conditions and showed good correspondence between experimental (88.4% ±0.3%) and predicted maximum esterification percentage.

Furthermore, according to Figure 2.2 and model predictions, the conversion of fatty acid into ester after 3.5 h, 57.1 ºC and 100 rpm is about 78%. However, after 37.8 h under the same temperature and agitation conditions, conversion reaches 89.8%. Thus, from a practical point of view it would be beneficial to stop the reaction after 3.5 hours instead of waiting for the optimum reaction time to be reached (37.8 h) because it would cause a pronounced decrease in productivity (moles of ester per hour). It is important to notice that, before the optimization of the operational conditions for the enzymatic synthesis of fructose ester, the maximum esterification percentage obtained was 74.3%±0.2%, for 72 h, 40 ºC and 250 rpm. Also, enzymatic process yields up to 80% conversion have been reported for the synthesis of sugar esters using a lipase from *C. antartica* (Roy and Chawla 2001, Sabeder *et al.* 2006, Tarahomjoo and Alemzadeh 2003).

Afterwards, the esterification product obtained at the validated maximum point was analyzed by TLC and IR spectroscopy in order to confirm the synthesis of the fructose ester according to the reaction scheme presented in Figure 2.1, since ethyl oleate is a possible by-product of the reaction that occurs in the presence of ethanol and oleic acid (Bousquet *et al.* 1999, De *et al.* 1999, Foresti and Ferreira 2005, Habulin *et al.* 1996, Hazarika *et al.* 2002).

The TLC plate is illustrated in Figure 2.3, where a spot with a retention factor (Rf) (distance traveled by the compound divided by the distance traveled by the solvent) of 0.5 was found to correspond to the fructose ester. The fructose ester Rf value was found to be in accordance with previous reports (Khaled *et al.* 1991, Pyo and Hayes 2008).

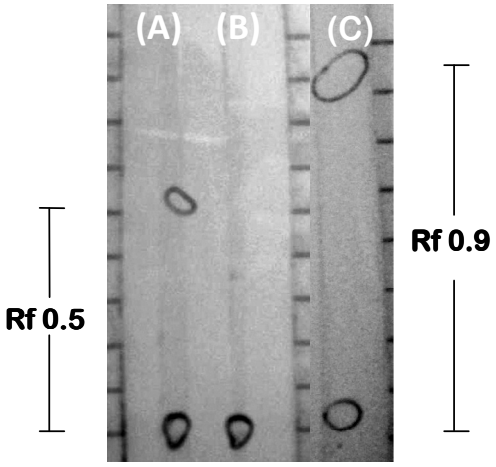

Figure 2.3 Thin-layer chromatography of the fructose ester. Lane (A) corresponds to the product synthesized at the validated maximum point of the design, namely the fructose ester. Lane (B) corresponds to a control experiment where no enzyme was used, thus no fructose ester was formed. Lane (C) corresponds to a standard of ethyl oleate.

Several authors reported higher Rf values (Rf~0.9) for ethyl oleate ester (Bousquet *et al.* 1999, De *et al.* 1999, Khaled *et al.* 1991, Seino *et al.* 1984), as confirmed also by TLC in the current work (Figure 2.3 – lane C). Furthermore, the fructose ester was analyzed by infrared spectroscopy (Figure 2.4) and the band peaks obtained confirmed the presence of an ester: 1741 cm^{-1} (C=O, ester); 2923 cm^{-1} (CH); 1463 cm^{-1} (CH_2); 1178 cm^{-1} (C=C) (Seino *et al.* 1984). Therefore, it was possible to conclude that the product synthesized at the maximum point of the experimental design corresponds to the expected fructose ester.

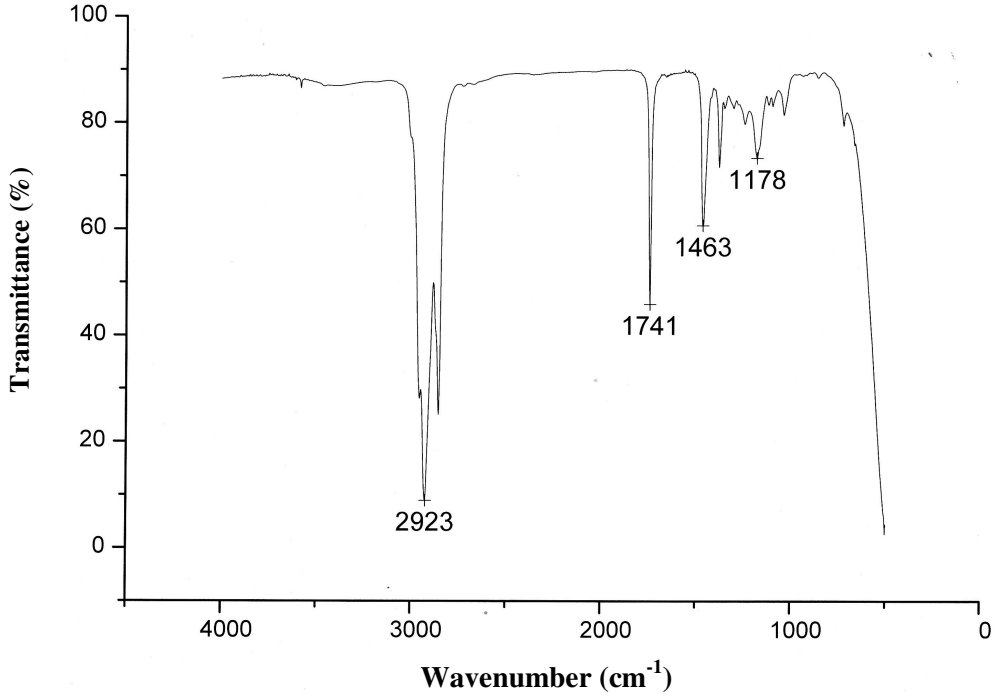

Figure 2.4 Infrared absorption spectra of the fructose ester obtained in the optimized conditions for enzymatic synthesis.

Despite the enzymatic synthesis of highly biodegradable surfactants from renewable resources (sugar and fatty acids) has been widely investigated (Polat and Linhardt 2001, Sabeder *et al.* 2006), statistical design of experiments and RSM have been only applied in a few studies (Shieh *et al.* 1996, Yan *et al.* 2001). Therefore, the current work can be regarded as a useful input for the development of more efficient processes for the enzymatic synthesis of fructose esters. However, it is important to refer that the results of such optimization are more limited in application than those which come from a more mechanistic standpoint, and therefore the optimum conditions found are only valid under the same experimental domain and reaction system used.

2.4 Conclusions

The modeling and optimization of immobilized *C. antartica* B catalyzed esterification reaction to synthesize fructose ester was successfully performed using a response surface methodology based on a central composite rotatable design (R^2 and Q^2 equal to 0.9995 and 0.7056, respectively). Furthermore, fructose ester was confirmed to be the product of the esterification process by TLC and IR spectroscopy. The effects of three main reaction operating parameters (temperature, agitation and reaction time) and of their interactions were evaluated over the given ranges. The results obtained showed that the established cubic model can be used to predict the esterification percentage under any given conditions within the experimental range. Moreover, under the optimized operating conditions an effective enhancement of the synthesis of fructose esters was achieved. An 88.4% (±0.3%) esterification percentage was obtained in a 37.8 h experiment conducted at 57.1 ºC and 100 rpm, which corresponds to an improvement of about 15% comparing to the values previously reported in the literature. Finally, this study can be seen as an effective contribution to the development of more efficient bioprocesses for industrial synthesis of fructose esters.

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Chapter 3

Enzymatic synthesis of sugar ester biosurfactants catalyzed by *Candida antarctica* type B

"Quem não sonha não realiza. Quem não ousa não conhece seus limites".

(Arquimedes Bastos)

Sugar ester biosurfactants are compounds that exhibit surface activity and have high emulsifying capacity; therefore they are widely used in various industrial fields, especially in the food industry. This work consists on the production of sugar esters by enzymatic synthesis using fructose, sucrose or lactose, fatty acids (oleic or linoleic acids), and as solvents, ethanol or ethyl acetate. The esterification reactions catalyzed by a lipase from *Candida antarctica* type B were conducted for 72 h at 40 °C and 250 rpm. Parameters including esterification yield, surface tension and emulsification index were determined. The synthesis of sugar esters was confirmed by thin layer chromatography. Lactose ester synthesized using linoleic acid and ethanol presented the highest esterification yield (83.5%) compared to the other sugar esters and experimental conditions used. However, the fructose esters obtained showed a higher performance regarding the ability to reduce surface tension (35.8 mN/m) and to stabilize an emulsion (emulsification index for 2 minutes (58.4%), 24 hours (56.2%) and 48 hours (54.4%)). Although further work is required in order to improve fructose esters synthesis yields, these results are promising and suggest an opportunity for its use in the food industry.

3.1 Introduction

Synthesis of sugar esters can be carried out either chemically or enzymatically. The chemical process occurs with a low selectivity and leads to a mixture of sugar esters with different degrees of esterification; it requires toxic organic solvents and is conducted at high temperatures, which causes coloration of the final products (Ferrer *et al.* 2005, Yoo *et al.* 2007).

Enzymatic synthesis of these sugar esters constitutes an interesting alternative, since enzymes present a greater specificity than more conventional forms of organic reactions, thus more differentiated sugar esters can be obtained (Ogawa and Shimizu 2002). The enzymatic method is also usually conducted at low temperatures, leading to a reduction of the required number of steps in the reaction, lack of racemization and minimal protection due to regio-specificity (Kim and Shin 2001). Furthermore, enzymatic reactions provide several advantages of industrial interest, such as increased solubility of hydrophobic substrates, suppression of water-dependent side reactions, alteration of substrate-, regio-, and stereo-specificity, recovery and reusability of enzyme, often enhanced thermostability in nearly anhydrous organic solvent system, elimination of microbial contamination, potential for enzymes to be used directly in a chemical process, among others (Doukyu and Ogino 2010).

Nevertheless, some problems have been reported regarding the enzymatic synthesis of sugar esters. The choice of solvents for enzymatic esterifications of underivatized sugars with fatty acids is very difficult, because one reactant (sugar) is polar and the other (fatty acid) is non polar. In addition, most enzymes are quickly inactivated under hydrophilic organic solvents (e.g., pyridine, dimethyl sulfoxide, and dimethyl formamide) which are able to dissolve high concentrations of both sugars and fatty acids (Degn and Zimmermann 2001, Ganske and Bornscheuer 2005, Lee *et al.* 2008). Also, the use of these solvents is being progressively restricted for many industrial applications, namely for food applications (Rojas-Melgarejo *et al.* 2006, Sabeder *et al.* 2005). To overcome this limitation, organic solvents that leave no toxic residues in the final product of the reaction have been proposed (Ferrer *et al.* 2005).

Several lipases have been used for the enzymatic synthesis of sugar esters, namely the ones from *Acinetobacter* sp., *Arthrobacter* sp., *Aspergillus* sp., *Aspergillus niger*,

Aspergillus oryzae, *Bacillus laterosporus* and *Candida antarctica* (Ferrer *et al.* 2005, Pandey *et al.* 1999).

Sugar esters are non-ionic biosurfactants that consist of a carbohydrate moiety as hydrophilic group, and one or more fatty acids as lipophilic component. By controlling the esterification degree and the nature of the fatty acid and sugar, it is possible to synthesize sugar esters within a wide range of properties. Biosurfactants are compounds that present surface-activity and have high emulsifying capacity, thus are widely used in various industrial areas, including in pharmaceutical, cosmetic, detergent and food industry. Moreover, they are tasteless, odorless and non-toxic, therefore present a high potential for food applications, such as in the production of aromas, maturation of cheeses, bakery products and sauces (Liese *et al.* 2000, Pandey *et al.* 1999, Plou *et al.* 2002, Saxena *et al.* 1999, Sharma *et al.* 2001, Szuts *et al.* 2007). Sugar esters have been used as sweetening agents and surfactants, as well as in the delivery of physiologically active agents (Somashekar and Divakar 2007). Recently, some sugar esters have been found to possess antitumor and antibiotic activities (Villo *et al.* 2010).

Aiming at its application in the food industry, this work reports the enzymatic synthesis of sugar ester biosurfactants by *C. antarctica* type B (CALB) lipase, using different combinations of sugars (fructose, sucrose and lactose), fatty acids (oleic acid and linoleic acid) and solvents (ethanol and ethyl acetate). The esterification yields were determined and sugar esters synthesis was confirmed by thin layer chromatography (TLC). Furthermore, all sugar esters were characterized regarding their surface activity and emulsification index.

3.2 Material and Methods

3.2.1 Materials

All chemicals used were analytical grade. Triacylglycerol lipase purified from *C. antartica* B immobilized in acrylic resin (CALB) (Novozym 435) was purchased from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO).

3.2.2 Methods

3.2.2.1 Esterification reactions

The synthesis experiments were conducted in flasks (100 mL) by adding 0.5 mmol fructose, sucrose or lactose; 0.5 mmol oleic acid or linoleic acid; 22.5 mg CALB; and 0.6 mL ethanol or ethyl acetate (99%), according to Table 3.1.

Table 3.1 Summary of the reaction schemes studied in the current work

Samples	Solvents	Fatty acids	Carbohydrates
A	Ethanol	Oleic acid	Fructose
B			Sucrose
C			Lactose
D		Linoleic acid	Fructose
E			Sucrose
F			Lactose
G	Ethyl acetate	Oleic acid	Fructose
H			Sucrose
I			Lactose
J		Linoleic acid	Fructose
K			Sucrose
L			Lactose

Sodium sulfate anhydrous (0.1 g) was added to all reactions in order to prevent the occurrence of hydrolysis of the ester bonds. For the reaction to occur, the flasks were incubated at 40 ºC and 250 rpm for 72 h. The synthesis procedure has been previously described by Sabeder and collaborators (2006). At the end of the esterification reaction, the lipase, together with the desiccant sodium sulfate anhydrous (non reactive species), were removed by filtration using filter paper with a pore-size of 60-µm (Macherey-Nagel Inc.). Afterwards, the solvent was evaporated from the reaction media using a rotoevaporator. Three independent reactions were conducted for each experimental condition above described.

3.2.2.2 Thin Layer Chromatography (TLC)

The obtained products (sugar esters) were identified by TLC, using commercial plates (Merck) coated with a 0.25 mm layer of silica gel (Khaled *et al.* 1991). A mixture of chloroform/hexane (1:1, v/v) was used for elution. Subsequently, the sugar esters spots were identified with iodine according to Ducret and collaborators' work (1995).

3.2.2.3 Esterification yields

The sugar esters content was quantified by calculating the residual fatty acid amount in the reaction mixture, which was determined by the volumetric method described elsewhere (Leitgeb and Knez 1990). Briefly, 0.1 g of sample from the reaction mixture were diluted in 20 mL of 0.1 wt% phenolphthalein solution in absolute ethanol, and then titrated with a standardized 0.1 mol/L aqueous sodium hydroxide solution. Measurements were done in triplicate.

3.2.3 Characterization of the product

After the esterification reactions, the sugar esters obtained were characterized according to the techniques described below.

3.2.3.1 Surface tension

The surface tension was determined at room temperature (25±1 ºC) using the Ring method as described elsewhere (Rodrigues *et al.* 2006). A KRUSS Tensiometer (Kruss model K10) equipped with a 1.9 cm Du Nouy platinum ring was used. Measurements were done in quintuplicate and results represent the means ± standard deviation. The concentration of final product used in these measurements was 30% (v/v).

3.2.3.2 Emulsification index (EI)

Emulsification index was determined for all the sugar esters obtained. Briefly, 2 ml sample and 1 ml of n-hexadecane were homogenized using a vortex for 2 minutes at 25 °C.

Next, the emulsion was left to settle for 2 minutes and the height of the emulsion was measured (T2). The index was calculated using the equation (1).

$$\text{Emulsion index (\%)} = He/Ht \times 100 \quad (1)$$

where He is the height of the emulsion and Ht is the total height of the liquid.

To evaluate the stability of the emulsion, these were left to settle for 24 (T24) and 48 hours (T48). All determinations were performed in triplicate and results represent the means ± standard deviation (Cooper and Goldenberg 1987).

3.2.3.3 Statistical analysis

The data were statically evaluated using ANOVA and Tukey-test at 95% of confidence level ($\alpha = 0.05$). Origin Pro 7.5 software (OriginLab) was used for data processing.

3.3 Results and Discussion

3.3.1 Thin Layer Chromatography (TLC)

According to the TLC results, it was possible to confirm the presence of sugar esters (fructose, sucrose and lactose esters) by measuring the retention factor (Rf). All sugar esters obtained in this study presented Rf values of 0.5 as shown in the Figure 3.1.

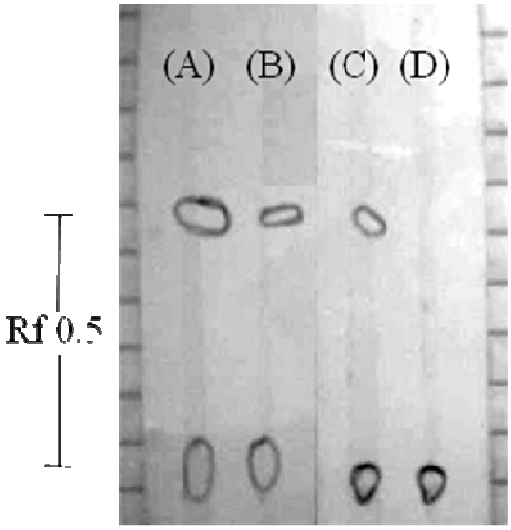

Figure 3.1 Thin-layer chromatography of the sugar esters obtained from the different reaction schemes. Lane (A) corresponds to fructose ester, lane (B) to sucrose ester and lane (C) to lactose ester. Lane (D) corresponds to a control experiment where no enzyme was used, so no sugar ester was formed.

The results obtained are in accordance with the study by Khaled *et al.* (1991), that reported an Rf = 0.5 for sucrose esters using an elution mixture of 80:10:8:2 chloroform:methanol:acetic acid:water. Also, Tortorello and Delwiche (1983) reported an Rf value of 0.51 for fatty acid esters. Moreover, Pyo and Hayes (2008), reported an Rf = 0.58 for fructose esters using the same TLC conditions as the ones used in the current work. It is important to notice that oleic acid could also be used to produce ethyl oleate, however its Rf value has been reported by several authors using the same solvent system as being higher (Rf~0.9) (Bousquet *et al.*1999, De *et al.* 1999, Khaled *et al.* 1991, Seino *et al.* 1984), thus excluding this possibility.

3.3.2 Esterification yields

The yields of fructose, sucrose and lactose esters obtained by enzymatic synthesis with CALB using two fatty acids (oleic and linoleic) and two solvents (ethanol and ethyl acetate) are presented in Figure 3.2. Results represent the average of three independent assays ± standard deviation. The experimental conditions of each sample are given in section 2.2.1.

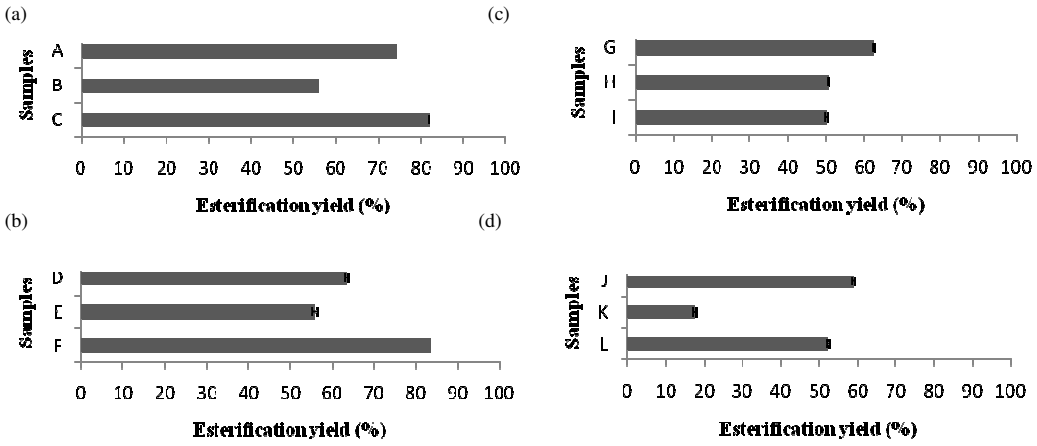

Figure 3.2 Sugar esters yields obtained by enzymatic synthesis with CALB using: ethanol, oleic acid and fructose, sucrose and lactose as carbohydrates (samples A, B, C) (a); ethanol, linoleic acid and fructose, sucrose and lactose as carbohydrates (samples D, E, F) (b); ethyl acetate, oleic acid and fructose, sucrose and lactose as carbohydrates (samples G.H, I) (c); and ethyl acetate, linoleic acid and fructose, sucrose and lactose as carbohydrates (samples J, K, L) (d).

In general, ethanol was found to be a better solvent than ethyl acetate for the envisaged esterification reactions producing higher yields independently of the fatty acid and carbohydrate used in all the samples. From Figure 3.2a, it was found that oleic acid possesses a better performance in the synthesis of fructose (74.3%) and lactose (81.8%) esters, samples A and C, respectively. Furthermore, Figure 3.2b shows that, using linoleic acid, the highest esterification yield (83.5%) was obtained for sample F consisting of a lactose ester. Also, sample D (fructose ester) presented a good esterification yield (63.5%). In addition, the differences observed between samples C and F (Figures 3.2a and 3.2b) indicate that, linoleic acid is slightly better than oleic acid for the synthesis of lactose esters. Overall, it was found that the fatty acids (oleic and linoleic) promoted good yields in the synthesis of esters for all carbohydrates tested. Such results can be credited to the specificity of the enzyme selected.

Concerning Figure 3.2c, it was found that using oleic acid, a higher yield for fructose ester (62.3%) (sample G) was obtained. For samples H and I (sucrose and lactose esters), similar esterification yields were obtained. Moreover, from Figure 3.2d, a significant

reduction in the synthesis of sucrose ester (17.4%) (sample K) was found when using linoleic acid and ethyl acetate as solvent. However, samples J and L (fructose and lactose esters, respectively) showed higher esterification yields, 58.8% and 52.2%, using the same conditions. The difference between these samples J, K and L is the type of carbohydrate used in the synthesis reaction.

Regarding the synthesis of fructose esters the best results were found for sample A (74.3%), of sucrose esters for the sample B (56.0%) and of lactose esters for sample F (83.5%). For all these samples, oleic acid showed a better performance in the synthesis of the fructose and sucrose esters, except for lactose esters for which the linoleic acid performed slightly better. As for the best solvent, the use of ethanol provided better yields than ethyl acetate.

It is important to notice that the same experimental conditions (amounts of reactants, temperature, time and agitation) were used for all the synthesis reactions conducted in this work. Therefore, the differences observed in the esterification yields can be explained by the different amounts of *cis* double bonds (unsaturated) present in the chemical structures of the fatty acids used. Oleic acid possesses one *cis* bound, while linoleic acid has two. Studies by Selmi *et al.* (1998) clearly showed that linolenic and linoleic acids are esterified more slowly than oleic acid. Also, the authors reported that the higher the number of unsaturated bonds, the lower the rate of synthesis, and consequently the lower the final esterification yield. Hence, the different yields obtained in the current work when comparing reactions conducted with one or the other fatty acid are in perfect agreement with the fact that lower unsaturated fatty acids (e.g. oleic acid) are esterified faster than higher unsaturated fatty acids (e.g. linoleic acid).

Sabeder and collaborators (2006) used SP 435 and SP 382 lipases from CALB to synthesize fructose esters. The authors obtained 53% and 44% esterification yields, respectively, which are lower than the ones obtained in the current work. Both samples were catalyzed for esterification using fructose, palmitic acid and 2-methyl-2-butanol. Using the same solvent, 2-methyl-2-butanol, Ducret *et al.* (1996) obtained a lower yield (58.1%) of fructose monooleate using similar experimental conditions as the ones used in the current study, namely fructose and oleic acid esterification by CALB for 24 h, i.e., the higher values reported in this study are justified by the presence of a specific solvent

(ethanol and ethyl acetate). Furthermore, Patil *et al.* (2010) obtained even lower esterification and transesterification yields, 12% and 21% respectively, for the production of fructose esters. These authors used fructose, oleic acid, sorbitol and ascorbic acid, and CALB lipase. Coulon *et al.* (1995) studied the kinetic of acylation of fructose by transesterification or direct esterification. Even if both reactions have a similar initial rate, the conversion yield of the transesterification reaction was found to be 1.5 fold higher than the direct esterification reaction. When oleic acid methyl ester was used, 65% of fructose was converted to fructose ester, against 46% in the presence of oleic acid.

Walsh *et al.* (2009) studied the synthesis of sucrose esters using 2-methyl-2-butanol as solvent. The authors reported lower sucrose ester yields (20.9%) than the ones found in the current study (56.0%), thus ethanol appears to be a better solvent for conducting the esterification reaction. According to Habulin *et al.* (2009), lower yields were also found in the synthesis of sucrose palmitate and sucrose laurate in supercritical CO_2 conditions (52% and 47%, respectively). These lower sucrose ester yields were probably due to a low solubility of sucrose in tertiary alcohols.

Other researchers studied the synthesis of lactose esters, and all reported yields lower than the ones obtained in the current study. Wu *et al.* (2004) using for the enzymatic synthesis, lactose, divinyl butanedioate, pyridine (as solvent) and a protease from *B. subtilis* (as biocatalyst), reported a esterification yield of 62%. Wang *et al.* (2005) submitted lactose to transesterification with divinyl hexanedioate in anhydrous pyridine catalyzed by an alkaline protease from *B. subtilis* and reported a yield of 77%. Walsh *et al.* (2009) using CALB in 2-methyl-2-butanol obtained 21.8% of lactose monolaureate. Based on these studies, it is possible to conclude that the results obtained in the current work are superior regarding the esterification yields.

3.3.3 Surface tension

The surface tension values measured for the sugar esters synthesized are presented in Figure 3.3. The concentration of final product used in these measurements was 30% (v/v) and the results represent the average of five independent assays ± standard deviation. The experimental conditions of each sample are given in section 2.2.1.

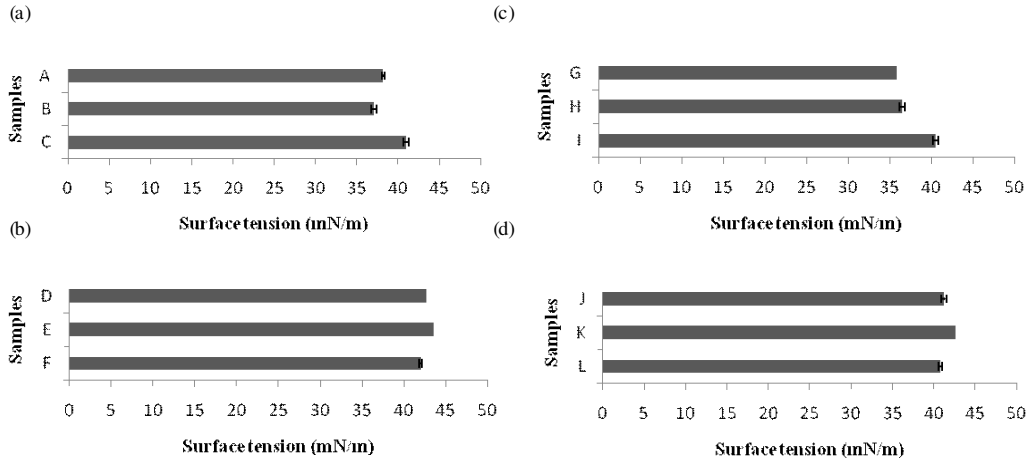

Figure 3.3 Surface tension values for the sugar esters obtained by enzymatic synthesis using CALB in the different experimental conditions studied, namely ethanol, oleic acid and fructose, sucrose and lactose as carbohydrates (samples A, B, C) (a); ethanol, linoleic acid and fructose, sucrose and lactose (samples D, E, F) (b); ethyl acetate, oleic acid and fructose, sucrose and lactose (samples G, H, I) (c); and ethyl acetate, linoleic acid and fructose, sucrose and lactose (samples J, K, L) (d). Surface tension of water was measured as a control (71.6 mN/m).

According to Figure 3.3a, using ethanol and oleic acid in the synthesis reaction, the most significant surface tension reduction comparing with the control (34.5 mN/m) was obtained for the sucrose ester (sample B). Moreover, the fructose ester (sample A) also showed a significant reduction of surface tension (33.4 mN/m). Becerra *et al.* (2008) obtained a surface tension value of 43.1 mN/m for sucrose monoesters synthesized using a lauric acid derivative. Thus, the surface activity of the sucrose esters synthesized in the current study proved to be superior. Rahman and Herawan (2000) reported a 38.3 mN/m surface tension for fructose esters, which is similar to the value obtained in this work. Surface activity is a very important feature when food applications are envisaged, since the greater the ability of a compound (sugar ester biosurfactants) to reduce surface tension, the greater the stability of the emulsion formed.

Comparing the esters obtained using different solvents (ethanol or ethyl acetate) (Figure 3.3b and 3.3d, respectively), it was found that in the presence of linoleic acid, the surface tension values for both solvents were higher than the 40 mN/m. However, switching the fatty acid to oleic acid and using ethyl acetate as solvent (Figure 3.3c)

resulted in the best results regarding surface activity. The fructose ester (sample G) was found to possess the lowest surface tension (35.8 mN/m) among all the sugar esters synthesized, which was not expected from the previous results (Figure 3.2) since ethyl acetate produced the lowest esterification yields, and the reduction of surface tension is usually related with the presence of sugar esters. Furthermore, sucrose ester (sample H) also presented a reduced surface tension value (36.5 mN/m) in comparison to the others samples. Thus, the sucrose esters were found to be potent surface active compounds when compared to the other sugar esters synthesized in this work.

Lactose ester synthesis was found to be the one that gave the highest esterification yields (Figure 3.2), however this was not followed by a great decrease in the surface tension (values above 40 mN/m). Nevertheless, the results gathered in Figure 3.3 clearly show a decrease in the surface tension as compared to water, thus confirming the presence of sugar ester biosurfactants. Busscher *et al.* (1994) suggested that a decrease larger than 8 mN/m of the surface tension in relation to water is an indicative of biosurfactant activity. Accordingly, all the sugar esters produced in this work presented surface activity, thus these esters could potentially be used in the food industry. It is important to notice that apart from their obvious role as agents that decrease surface and interfacial tensions, hence promoting the formation and stabilization of emulsions, biosurfactants can have several other functions in food. For example, to control the agglomeration of fat globules, stabilize aerated systems, improve texture and shelf-life of starch-containing products, modify rheological properties of wheat dough and improve consistency and texture of fat-based products (Nitschke and Costa 2007).

The results (Figure 3.3) clearly showed that ethyl acetate was the solvent that promoted the synthesis of sugar esters with lower surface tension values. The same behavior was observed for the esters synthesized using oleic instead of linoleic acid. Again, a possible explanation is the fact that oleic acid possesses one *cis* double bond, while linoleic acid possesses two *cis* double bonds, as previously discussed. According to Leshem *et al.* (1988), for a fixed monolayer area in a completely expanded state, an increase in the number of *cis* double bonds causes a concomitant increase in the surface tension. Table 3.2 summarizes the best results obtained for the surface tension values of the samples studied.

Table 3.2 Best results obtained for the surface tension values of the samples studied. The Tukey-test was used at 95% of confidence level to evaluate the existence of statistical significant differences between the different samples

Samples[#]	**Surface tension Mean (mN/m)**±SD*
A	38.2±0.2[a]
B	37.1±0.4[b,c]
G	35.8±0.0[b,d]
H	36.5±0.3[b]

SD = Standard Deviation; [#]The experimental conditions of each sample are shown in section 2.2.1; *Values with different letters present statistically significant differences ($p<0.05$).

A statistical significant difference at 5% level of probability could be observed between the samples A and B (both synthesized with ethanol), and G and H (both synthesized with ethyl acetate). These compounds correspond to fructose esters (samples A and G) and sucrose esters (samples B and H), all synthesized using oleic acid as fatty acid. For these samples it was found that the low values of surface tension obtained were due to the use of the oleic acid, thus resulting in more active fructose and sucrose esters.

3.3.4 Emulsification index (EI)

The emulsification indexes were determined for all the sugar esters synthesized and the results are presented in Table 3.3. Emulsion stability was studied in different time points, namely 2 minutes, 24 hours and 48 hours. The experimental conditions of each sample are given in section 2.2.1 and the results represent the average of three independent assays ± standard deviation. Tukey-test was used at 95% of confidence level to evaluate the existence of statistical significant differences between the emulsification indexes obtained for the different time points in each sample.

Table 3.3 Emulsification indexes determined for the fructose, sucrose and lactose esters obtained for the different experimental conditions studied: ethanol, oleic acid and fructose, sucrose and lactose as carbohydrates (samples A, B, C) (a); ethanol, linoleic acid and fructose, sucrose and lactose (samples D, E, F) (b); ethyl acetate, oleic acid and fructose, sucrose and lactose (samples G, H, I) (c); and ethyl acetate, linoleic acid and fructose, sucrose and lactose (samples J, K, L) (d). The Tukey-test was used at 95% of confidence level to evaluate the existence of statistical significant differences between the different time points in each sample

Group	Samples[#]	Emulsification index Mean (%)±SD*		
		2 min	24 hours	48 hours
a	A	37.8±0.2[a]	33.9±0.1[b]	29.5±0.0[c]
	B	30.0±0.3[a]	30.0±0.2[a]	30.0±0.2[a]
	C	30.2±0.3[a]	25.1±0.0[b]	25.0±0.4[b]
b	D	30.0±0.1[a]	29.5±0.4[a]	29.2±0.4[a]
	E	29.0±0.2[a]	29.0±0.1[a]	29.0±0.2[a]
	F	35.1±0.1[a]	30.0±0.2[b]	30.0±0.3[b]
c	G	58.4±0.2[a]	56.2±0.0[b]	54.4±0.2[c]
	H	33.1±0.0[a]	27.5±0.1[b]	27.3±0.3[b]
	I	35.1±0.3[a]	35.1±0.4[a]	30.0±0.3[b]
d	J	28.4±0.1[a]	28.4±0.5[a]	28.1±0.1[a]
	K	30.4±0.0[a]	30.3±0.1[a]	30.1±0.2[a]
	L	31.0±0.3[a]	30.5±0.1[a]	30.0±0.0[a]

SD = Standard Deviation; [#]The experimental conditions of each sample are shown in Table 3.1; *Values with different lowercase in the same line present statistically significant differences ($p<0.05$) for each sample in all time points.

Two important parameters are used to evaluate the power of a given emulsifier, namely the emulsification index (EI) and the emulsion stability (Abu-Ruwaida *et al.* 1991, Cooper and Goldenberg 1987, Singh *et al.* 2007). According to Table 3.3a, it could be observed that the sample with the highest emulsification index was sample A (fructose ester), with an EI of 37.8% for 2 minutes. Sucrose and lactose esters (samples B and C, respectively) presented similar values (30.0%) for all the time points studied. Furthermore, from Table 3.3b, similar values (~29.0%) for fructose and sucrose esters (samples D and E,

respectively) were found for all the time points. Lactose ester (sample F) presented the best EI value (Table 3.3b) for 2 minutes (35.1%) compared to samples D and E.

Comparing all the results obtained for the emulsification index test, sample G was the one that presented the highest percentage: 58.4% (2 min), 56.2% (24 h) and 54.4% (48 h). Sample G corresponds to the fructose ester synthesized using oleic acid and ethyl acetate. The EI values obtained for this sample are consistent with its surface activity previously discussed (Figure 3.3). Samples H and I (sucrose and lactose esters) showed values close to 30.0% for all time points. From Table 3.3d, all the samples (fructose, sucrose and lactose esters) presented similar EI values for all time points (30.0%).

In general, the esters obtained from oleic acid showed a better performance regarding the emulsification index as compared to the ones obtained from linoleic acid. Furthermore, except for sample G, all sugar esters presented similar emulsification index values ranging from 25.0 to 37.8% for all time points. Moreover, the solvent that showed better results was ethyl acetate, which is also in agreement with the previous results of surface tension activity (Figure 3.3).

The results obtained for each sample at different time points (Table 3.3) showed variability at 5% level probability. The emulsions' stability for samples B, D, E, J, K and L showed no statistically significant differences among the different time points. For these samples, the emulsions were found to be stable for all the time points studied, thus no changes in the emulsification indexes could be observed.

It is important to notice that these emulsions were found to be stable for several weeks (*data not shown*), which means that these sugar esters provide stability to the emulsions over a long time. Although some studies on the use of sugar ester biosurfactants have been reported (Nitschke and Costa 2007), to our knowledge this is the first report on the emulsification index values for fructose, sucrose and lactose esters under the experimental conditions used. The stability of the emulsions conferred by the presence of these particular sugar esters is very promising, since they can compete with others obtained by chemical via that have been used in the field of food additives (Muller *et al.* 2002, Singh *et al.* 2007).

3.4 Conclusions

In the current work, synthesis of sugar ester biosurfactants catalyzed by CALB, using three types of carbohydrates, two types of fatty acids and two organic solvents, was studied. According to the results, the highest esterification yield (83.5%) was obtained for the synthesis of lactose ester from lactose and linoleic acid in the presence of ethanol, in comparison with the other sugar esters and conditions studied. However, the fructose esters showed a better performance regarding their surface activity and the ability to stabilize emulsions, suggesting that further studies should be conducted in order to maximize their synthesis yield.

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Chapter 4

Production of sugar ester biosurfactants by enzymatic synthesis using porcine pancreas lipase

"O planejamento é uma ferramenta administrativa, que possibilita perceber a realidade, avaliar os caminhos, construir um referencial futuro, estruturando o trâmite adequado e reavaliar todo o processo a que o planejamento se destina".

(Autor Desconhecido)

Sugar ester biosurfactants and their corresponding emulsification properties result from the presence of both hydrophilic and hydrophobic regions on the same molecule. This work aimed the study of the effect of several variables (carbohydrates, fatty acids and solvents) on the enzymatic synthesis of sugar esters, using porcine pancreas lipase as biocatalyst. According to the results obtained, the highest esterification yields (47.6%) were observed for sucrose esters, which presented the best performance regarding surface activity (33.4 mN/m), and formation and stability of emulsions given by their emulsification indexes (2 min: 58.4%; 24 h: 58.2% and 48 h: 58.1%). Fructose esters also presented good esterification yields (41.4%), surface activity (36 mN/m) and emulsification index (above 50% in some experimental points). In general, the reagents that yielded the best results regarding sugar esters formation were oleic acid and ethanol. Several applications of sucrose and fructose ester biosurfactants have been suggested for the food industry due to their ability to reduce surface tension and form stable emulsions.

4.1 Introduction

Lipases are biocatalysts of great importance in different areas, which may catalyze reactions in both aqueous and organic medium. This phenomenon is primarily due to its ability to use a wide range of substrates, stability at a large temperature, pH and organic solvents ranges, and chemo-regio and enantioselectivity (Borkar *et al.* 2009, Kim and Shin 2001, Krajewska 2004, Saxena *et al.* 2003, Song *et al.* 2008).

The most common lipases of animal origin are obtained from porcine pancreas (Gupta *et al.* 2004, Hasan *et al.* 2006). Porcine pancreas lipase (PPL) has hydrophilic amino acid residues inside its core and contains up to 32 water molecules hydrogen bonded to several amino acid residues. The surface amino acid residues are more hydrophobic, thus conferring stability to the enzyme when in a non-polar medium (Kiran *et al.* 2001). It is important to notice that PPL is one of the cheapest commercially available non-microbial enzyme (Gogoi *et al.* 2008). PPL act as biocatalyst in the enzymatic synthesis of sugar ester biosurfactants, produced from carbohydrates (Mulligan 2005).

The sugar ester biosurfactants produced by PPL can be synthesized from renewable substrates and present a great diversity. Moreover, these compounds possess structural characteristics and physical properties that make them comparable or superior in terms of efficiency to their chemical counterparts (Rashedi *et al.* 2005). They are used in several applications based on their ability to reduce surface tension, increase solubility (Mulligan 2005), and form stable emulsions of oil in water (Kiran and Divakar 2001, Rashedi *et al.* 2005).

Sugar ester biosurfactants present a range of different biological functions/properties, thus enabling their potential use in the food industry. Several studies reported their use in the manufacture of cereal products (e.g. yeast raised bread, cakes, donuts, pastries and desserts), dairy products (e.g. ice cream, cream coffee, liquid and dry whipped toppings) and of coconut milk (Benincasa *et al.* 2004, Kiran and Divakar 2001, Mukherjee *et al* 2009, Nayak *et al.* 2009, Salihu *et al.* 2009, Tangsuphoom and Coupland 2009, Tugrul and Cansunar 2005).

In this study, it was investigated the synthesis of fructose, sucrose and lactose esters catalyzed by PPL. Different fatty acids (oleic and linoleic acid) and solvents (ethanol and ethyl acetate) were studied. Additionally, the sugar esters synthesized were evaluated for

their surface activity and ability to form and stabilize emulsions. The sugar ester biosurfactants synthesized in the current work are aimed at applications in the food industry.

4.2 Material and Methods

4.2.1 Materials

All chemicals used were analytical grade. Commercial porcine pancreatic lipase (Type II) was purchased from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO).

4.2.2 Methods

4.2.2.1 Synthesis of sugar esters

Sugar ester biosurfactants were synthesized through esterification between a carbohydrate (fructose, sucrose or lactose) and a fatty acid (oleic or linoleic acids) catalyzed by PPL using ethanol or ethyl acetate as solvents. The different reagent combinations used are described in the Table 4.1.

Table 4.1 Combinations of carbohydrates, fatty acids and solvents used to synthesize sugar esters by porcine pancreas lipase

Samples	Solvents	Fatty acids	Carbohydrates	Sugar esters formed
1	Ethanol	Oleic acid	Fructose	Fruest
2			Sucrose	Sucest
3			Lactose	Lacest
4		Linoleic acid	Fructose	Fruest
5			Sucrose	Sucest
6			Lactose	Lacest
7	Ethyl acetate	Oleic acid	Fructose	Fruest
8			Sucrose	Sucest
9			Lactose	Lacest
10		Linoleic acid	Fructose	Fruest
11			Sucrose	Sucest
12			Lactose	Lacest

The synthesis were conducted in flasks containing 0.5 mmol fructose, sucrose or lactose; 0.5 mmol oleic acid or linoleic acid; 22.5 mg porcine pancreas lipase and 0.6 mL ethanol or ethyl acetate (99%). Sodium sulfate anhydrous (0.1 g) was added to all reactions. The flasks were incubated at 40 ºC and 250 rpm for 72 h, according to methodology described by Sabeder and collaborators (2006).

After the esterification reaction, the lipase together with the sodium sulfate anhydrous (non reactive species) were removed by filtration using filter paper with a pore-size of 60-µm (Macherey-Nagel Inc.). Afterwards, the solvent was evaporated from the reaction media using a rotoevaporator. All the synthesis experiments were carried out in triplicate.

4.2.2.2 Thin Layer Chromatography (TLC)

Thin-layer chromatography (TLC) was performed on commercial silica gel plates (Merck Co. Inc, Damstadt, Germany) pre-coated with a 0.25 mm layer (Khaled *et al.* 1991). TLC plates were spotted with the samples from the reaction mixture dissolved in ethanol and developed using as mobile phase: chloroform/hexane, 1:1 (v/v). Sugar esters spots were visualized with iodine according to Ducret and collaborators' work (1995).

4.2.2.3 Esterification reactions yields

The sugar ester biosurfactants content was quantified by calculating the residual fatty acid amount in the reaction mixture, which was determined by the volumetric method described elsewhere (Leitgeb and Knez 1990). Briefly, 0.1 g of sample from the reaction mixture were diluted in 20 mL of 0.1% (w/v) phenolphthalein solution in absolute ethanol, and then titrated with standardized sodium hydroxide solution of 0.1 mol/L. Measurements were done in triplicate.

4.2.3 Characterization of the sugar ester biosurfactants

Sugar ester biosurfactants synthesized were further characterized according to the following techniques.

4.2.3.1 Surface tension

The surface tension was determined at room temperature (25±1 ºC) using the Ring method as described elsewhere (Rodrigues *et al.* 2006). A KRUSS Tensiometer (Kruss model K10) equipped with a 1.9 cm Du Nouy platinum ring was used. The results represent the average of five independent measurements ± standard deviation. The concentration of final product used in these measurements was 30% (v/v).

4.2.3.2 Emulsions formation and stability

The sugar esters ability to form and stabilize emulsions was evaluated through the determination of the emulsification indexes (EI). Briefly, 2 ml sample and 1 ml of n-hexadecane were homogenized using a vortex for 2 minutes at 25 °C. Next, the emulsion was left to settle for 2 minutes and the height of the emulsion was measured (T2). The index was calculated using the equation (1).

$$EI\ (\%) = He/Ht \times 100 \quad (1)$$

where He is the height of the emulsion and Ht is the total height of the liquid. To check the stability of the emulsions, the samples were left to settle for 24 (T24) and 48 hours (T48). All determinations were performed in triplicate and results represent the mean ± standard deviation (Cooper and Goldenberg 1987).

4.2.3.3 Statistical analysis and data analysis

Statistical analysis of the data was conducted using Origin Pro 7.5 (OriginLab) software. The data were analyzed and compared through one-way analysis of variance (ANOVA) and Tukey-test at 95% of confidence level (p = 0.05).

4.3 Results and Discussion

4.3.1 Thin Layer Chromatography (TLC)

According to the TLC results, it was possible to confirm the presence of sugar esters (fructose, sucrose and lactose esters) by measuring the retention factor (Rf). All sugar esters obtained in this study presented Rf values of 0.5. The results are in accordance with the studies of Khaled *et al.* (1991) and Pyo and Hayes (2008), that reported Rf values of 0.5 and 0.58 for sucrose and fructose esters, respectively. Moreover, Tortorello and Delwiche (1983) reported an Rf value of 0.51 for fatty acid esters.

4.3.2 Esterification reactions yields

Figure 4.1 illustrates the sugar esters yields obtained by enzymatic synthesis with PPL using different fatty acids, carbohydrates and solvents combinations. The experimental conditions used for each sample are given in Table 4.1 and the results represent the average of three independent assays ± standard deviation.

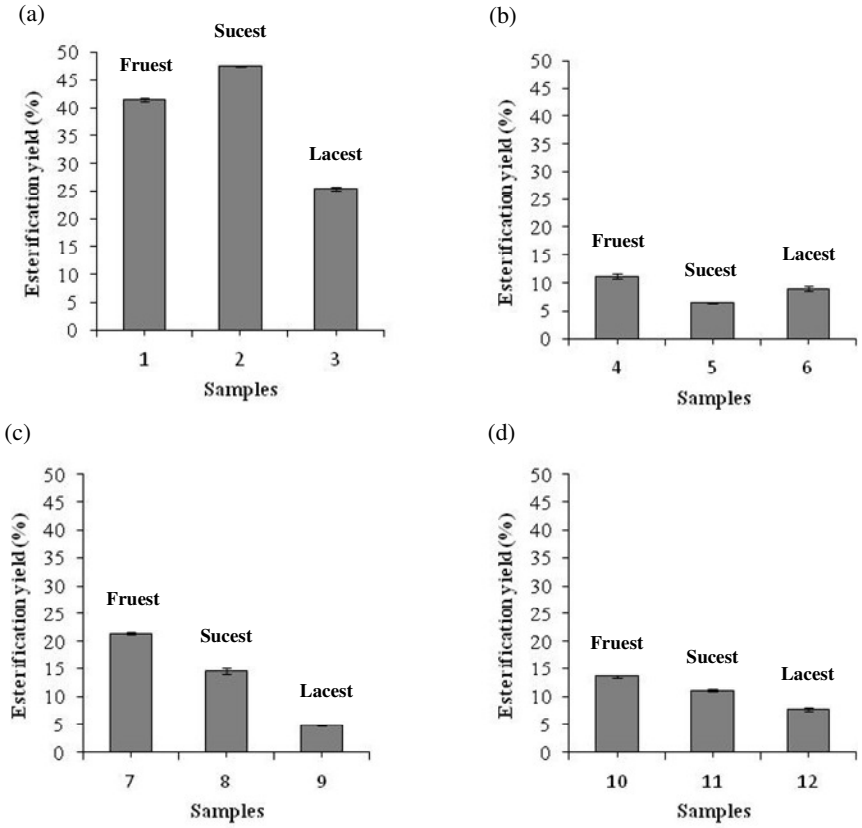

Figure 4.1 Yields of the sugar esters obtained using porcine pancreas lipase in the following conditions: ethanol, oleic acid, fructose, sucrose or lactose (a); ethanol, linoleic acid, fructose, sucrose or lactose (b); ethyl acetate, oleic acid, fructose, sucrose or lactose (c); ethyl acetate, linoleic acid, fructose, sucrose or lactose (d).

Figure 4.1a (esterification using ethanol and oleic acid) corresponds to the best results obtained for the synthesis of sugar esters (samples 1, 2 and 3, respectively). Fructose ester showed a yield of 41.4%, sucrose ester of 47.6% and lactose ester of 25.4%.

Figure 4.1b corresponds to the esterification reaction using ethanol and linoleic acid. Sample 4 (fructose ester) presented the highest yield (11.2%) followed by sample 6 (lactose ester) with a yield of 9.0% and sample 5 (sucrose ester) with a yield of 6.4%. The yields obtained for samples 7, 8 and 9, synthesized with ethyl acetate and oleic acid are illustrated in Figure 4.1c. For these samples, yields of 21.4%, 14.5% and 4.8% were obtained for fructose, sucrose and lactose esters, respectively. Figure 4.1d presents the results obtained using ethyl acetate and linoleic acid for the synthesis reactions. The

highest yield (13.6%) was again obtained for fructose ester (sample 10). The sucrose ester (sample 11) presented a yield of 11.1%, and lactose ester a yield of 7.7%. From these results, except for the system with ethanol and oleic acid, fructose ester was the product with the highest yield obtained under all the tested conditions

It is well known that an esterification reaction yield of 100% cannot be achieved since only part of the alcohol and fatty acid present will react, resulting in a equilibrium between alcohol, fatty acid, ester and water. Therefore, in the present study, the highest esterification yield was obtained for sample 2 using sucrose as carbohydrate (47.6%). Moreover, sample 1 obtained from fructose also presented a high esterification yield (41.4%). The differences observed in the esterification yields of both samples (1 and 2) were found to be statistically significant as shown by the Tukey test at 5% level of probability. It is also important to notice that for all results presented in Figure 4.1a, the samples only differed on the carbohydrate used, therefore the results suggest that the use of oleic acid and ethanol (as solvent) is the condition that provides the best esterification yields for PPL.

Plou *et al.* (2002) suggested that the longer the carbon chain of the fatty acid used in the enzymatic esterification reaction, the higher the yield of sugar ester obtained. Ku and Hang (1995) reported a fructose ester yield of 24.4% obtained from oleic acid in the presence of tertiary butyl alcohol by *Byssochlamys fulva* NTG9, being this value lower than the fructose ester yields obtained in the current study. Moreover, Bagi and Simon (1999) conducted a direct esterification for 5 days using PPL immobilized on Sorsilen to synthesize fructose butyrate, which yielded 10.6%. The reaction mixture contained fructose, acyl donor (butyric acid in esterification, or tributyrin in transesterification) and acetonitrile. Although comparisons are hampered by the different experimental conditions used in the above mentioned studies, the yields obtained in the present study for fructose esters were found to be superior. Additionally, Somashekar and Divakar (2007) used PPL as biocatalyst to enzymatically produce sucrose and glucose esters. A maximum yield of 18% and 8% for glucose and sucrose esters was achieved, respectively. This result was also lower comparing the sucrose esters produced this study (samples 2, 8 and 11).

Kiran and Divakar (2001) reported maximum esterification yields of lactic acid with stearic acid (24.4%) and with palmitic acid (22.2%) by PPL, both using ethylmethyl ketone

as solvent. Similar lactose ester yields were obtained in the current study using oleic acid, lactose and ethanol (25.4% - sample 3). However, the yields obtained for the synthesis of lactose esters using ethyl acetate were very low (4.8% - sample 9; 7.7% - sample 12). These results are slightly higher than the ones reported by Ku and Hang (1995). These authors performed an enzymatic esterification of lactose and linoleic acid in tertiary butyl alcohol with lipase from *B. fulva* NTG9, and found that no lactose ester was synthesized (0%). Thus, the lactose ester yield obtained with oleic acid and ethanol in the present study (25.4%) was higher than the results obtained by Ku and Hang (1995). This yield is comparable to the one reported by Kiran and Divakar (2001), however, the solvent used herein (ethanol) can be obtained by renewable sources, such as sugar cane, and is less toxic and polluent than ethylmethyl ketone.

Selmi and collaborators (1998) reported that linoleic acid is esterified more slowly than oleic acid because the higher the number of unsatured bonds, the lower the synthesis rate and yields obtained. These observations can explain the differences observed for the two fatty acids in the present study because, in general, oleic acid showed a better performance than linoleic acid.

4.3.3 Surface tension

Figure 4.2 presents the surface tension values determined for the sugar ester samples produced according to Table 4.1. The results represent the average of five independent assays ± standard deviation.

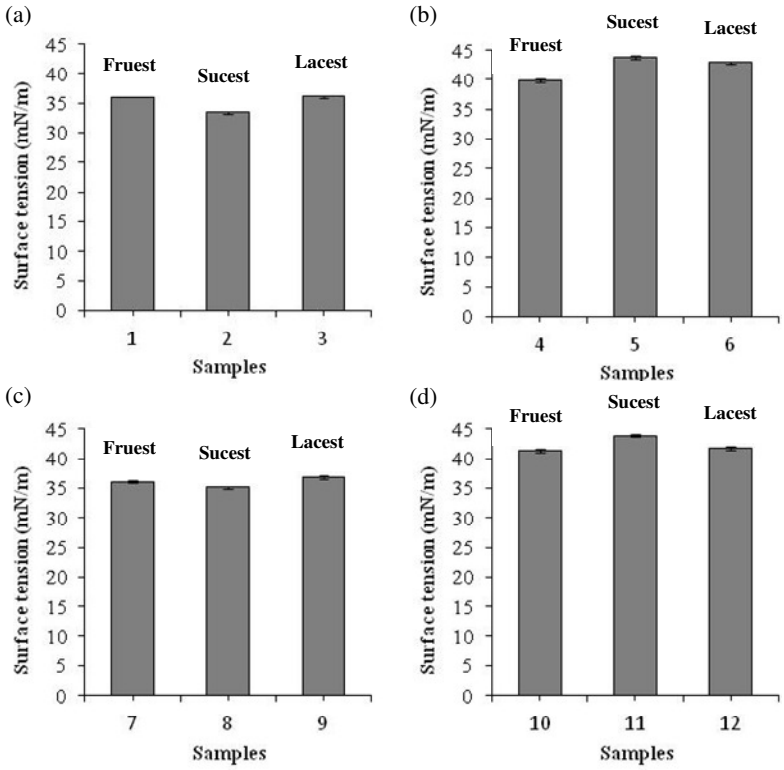

Figure 4.2 Surface tension values for the sugar esters obtained by enzymatic synthesis using porcine pancreas lipase in the different experimental conditions studied: ethanol, oleic acid, fructose, sucrose or lactose (a); ethanol, linoleic acid, fructose, sucrose or lactose (b); ethyl acetate, oleic acid, fructose, sucrose or lactose (c); ethyl acetate, linoleic acid, fructose, sucrose or lactose (d).

According to Figure 4.2a, the samples 1, 2 and 3, synthesized in the presence of ethanol and oleic acid, presented a surface tension of 36.0 mN/m (sample 1, fructose ester), 33.4 mN/m (sample 2, sucrose ester) and 36.1 mN/m (sample 3, lactose ester). Also, similar results are presented in the Figure 4.2c for the esters synthesized with ethyl acetate and oleic acid, where the samples 7, 8 and 9 showed surface tension values of 36.1 mN/m (fructose ester), 35.1 mN/m (sucrose ester) and 36.8 mN/m (lactose ester), respectively.

The surface tension values obtained for the experimental conditions represented by the Figures 4.2b and 4.2d were slightly higher than the ones shown in Figures 4.2a and 4.2c (between 39.8 and 43.9 mN/m). The results suggest that under the same conditions the

use of ethanol generally leads to lower surface tension values as compared to ethyl acetate. Additionally, the experiments conducted with oleic acid showed lower surface tension values than linoleic acid independently of the carbohydrate and solvent used. These results are possibly related with the amount of *cis* double bonds present in the chemical structure of the oleic (one bond) and linoleic acid (two bonds). Leshem *et al.* (1988) reported that, an increase in the number of these bonds causes a concomitant increase in the surface tension.

Table 4.2 summarizes the best results for surface tension obtained herein. Data were analyzed by the Tukey-test at 5% level of probability.

Table 4.2 Summary of the best results obtained for the surface tension values of the samples presented in Figures 4.2a and 4.2c

Group	Samples[#]	Surface tension (mN/m)±SD*
a	1	36.0 ± 0.0^{a}
	2	$33.4\pm0.2^{b,c}$
	3	$36.1\pm0.2^{a,d}$
c	7	$36.1\pm0.2^{a,d,e}$
	8	$35.1\pm0.2^{b,f}$
	9	36.8 ± 0.3^{b}

SD = Standard Deviation; [#]The experimental conditions of each sample as shown in Table 4.1; *Values with different letters present statistically significant differences ($p<0.05$).

According to Table 4.2, no statistical significant differences were observed among samples 1, 3 and 7. These samples were synthesized in the presence of oleic acid and ethanol (samples 1 and 3, fructose and lactose esters, respectively) or ethyl acetate (sample 7, fructose ester). Therefore, for these samples the surface tension was found to be independent of the type of carbohydrate and solvent used. A surface tension of 38.3 mN/m for fructose esters obtained with distillated palm fatty acid, *Mucor miehei* lipase and tert-butyl alcohol has been reported (Rahman and Herawan 2000).

From Figure 4.2 it was possible to observe that the lowest surface tensions correspond to sucrose esters synthesized with oleic acid, regardless of solvent used (Figures 4.2a and 4.2b).

Different surface tension ranges have been reported for sugar esters synthesized from different experimental conditions. For example, in the synthesis of sucrose esters with fatty acids C12-C16, the range of surface tension values measured was between 31.5 mN/m and 35.2 mN/m, respectively. For lactose esters, using fatty acids C14-C16, the surface tension values were found to be within 38.6 mN/m and 39.5 mN/m (Plou *et al.* 2002). In summary, the sucrose and lactose esters obtained in this study presented high surface activity, thus representing promising alternative surfactants to be used in many industrial applications. This is very important because the greater the ability of a sugar ester biosurfactant to reduce surface tension, the greater the stability of the emulsion formed (Ahimou *et al.* 2001, Ghojavand *et al.* 2008, Lee *et al.* 2008, Nguyen *et al.* 2008).

According to Mulligan (2005), a good surfactant can lower surface tension of water from 72 to 35 mN/m. Yanke *et al.* (2004) found a surface tension value equal of 34.0 mN/m for sucrose palmitate obtained using methyl palmitate, anhydrous potassium carbonate and potassium oleate. Becerra *et al.* (2008) reported a surface tension value of 43.1 mN/m for sucrose monoesters using lauric acid derivative. Both works showed higher surface tension values than those found in the present study.

Nitschke and Costa (2007) reported that a decrease in the surface tension promotes the formation and stabilization of emulsions, and several other functions in food, such as: control of the agglomeration of fat globules, stabilization of aerated systems; improvement of texture and shelf-life of starch-containing products, modification of rheological properties of wheat dough and improvement of consistency and texture of fat-based products. Van Haesendonck and Vanzeveren (2004) suggested the use of sugar ester biosurfactants to improve properties of butter cream, croissants and frozen confectionery products. Being good surfactants, the sucrose and fructose esters obtained in the present study could be further explored for this type of food applications.

4.3.4 Emulsification index (EI)

The emulsification index (EI) was determined for all sugar esters synthesized according to Table 4.1 and the results are gathered in Table 4.3. The results represent the average of three independent assays ± standard deviation.

Table 4.3 Emulsification indexes determined for the sugar esters obtained for the different experimental conditions studied: ethanol, oleic acid and fructose, sucrose and lactose as carbohydrates (a); ethanol, linoleic acid and fructose, sucrose and lactose as carbohydrates (b); ethyl acetate, oleic acid and fructose, sucrose and lactose as carbohydrates (c); ethyl acetate, linoleic acid and fructose, sucrose and lactose as carbohydrates (d)

Group	Samples[#]	Emulsification index Mean (%)±SD*		
		2 min	**24 hours**	**48 hours**
a	1	55.9±0.0[a]	53.2±0.2[b]	50.2±0.3[c]
	2	58.4±0.1[a]	58.2±0.0[a]	58.1±0.4[a]
	3	38.0±0.2[a]	35.4±0.1[b]	33.2±0.0[c]
b	4	55.7±0.0[a]	55.6±0.4[a]	54.6±0.0[b]
	5	56.2±0.1[a]	56.1±0.0[a]	56.0±0.4[a]
	6	33.1±0.0[a]	32.2±0.3[b]	30.1±0.4[c]
c	7	53.2±0.4[a]	50.1±0.1[b]	48.2±0.0[c]
	8	56.2±0.0[a]	55.1±0.4[b]	52.1±0.2[c]
	9	33.2±0.3[a]	31.1±0.0[b]	30.1±0.4[c]
d	10	50.1±0.0[a]	50.1±0.1[a]	49.8±0.0[a]
	11	52.1±0.4[a]	50.0±0.1[b]	48.3±0.2[c]
	12	31.6±0.0[a]	31.3±0.1[a]	31.0±0.4[a]

SD = Standard Deviation; [#]The experimental conditions of each sample are shown in Table 4.1; *Values with different lowercase in the same line present statistically significant differences ($p<0.05$) for each sample in all times.

An important parameter for evaluating the power of an emulsifier is the EI (Abu-Ruwaida *et al.* 1991, Cooper and Goldenberg 1987, Singh *et al.* 2007). According to Table 4.3, the EI values obtained for samples 1 (55.9%) and 2 (58.4%) were the highest among the sugar esters obtained using oleic acid and ethanol. Samples 1 and 2 correspond to fructose and sucrose ester, respectively. However, EI significantly ($p<0.05$) decreased along the time for fructose ester (sample 1). Similar EI values were obtained for samples 4 (55.7%) and 5 (56.2%), which were synthesized using ethanol and linoleic acid, corresponding to fructose and sucrose esters, respectively. Sample 8, a sucrose sugar ester obtained using ethyl acetate and oleic acid, also showed similar EI compared to the above mentioned samples. The other samples presented lower EI for the 2 minutes assay.

Comparing the EI values obtained for samples 2, 5, 10 and 12 in the different time points (2 min, 24 h and 48 h), no statistical significant differences could be observed, indicating that the referred samples showed good emulsion stability.

The sucrose ester obtained from sucrose with oleic acid and ethanol (sample 2) presented the highest and most stable EI, the highest yield (Figure 4.1a) and the lowest surface tension (Table 4.2). Being sucrose a world-wide commodity relatively cheap (cheaper than lactose and fructose), these results open new possibilities for the production of sugar ester biosurfactants. Furthermore, correlating the surface tension results with the emulsification indexes, it was found that fructose (samples 1 and 7) and sucrose esters (samples 2 and 8) synthesized from oleic acid showed the best performances.

In summary, according to emulsification index test (Table 4.3) it is clear that the oleic acid and ethanol showed the best performance for surfactant production compared to linoleic acid and ethyl acetate. Moreover, compounds with EI values above 50% are considered promising emulsifiers, and in the present work all fructose and sucrose esters showed EI values above 50% even for the 48 h assay. Although some studies on the use of sugar ester biosurfactants have been reported, to our knowledge this is the first report on the emulsification index for fructose, sucrose and lactose esters for the experimental conditions used.

Finally, these emulsions were found to be stable for several weeks (*data not shown*), i.e. these sugar esters provide stability to the emulsions over time. Several studies have reported the potential of sugar ester biosurfactants for food applications, namely as emulsifiers; solubilizers; demulsifiers; wetting agents; foaming or de-foaming agents; thickeners; lubricating agents; and as functional ingredients due to their interaction with lipids, proteins and carbohydrates (Singh *et al.* 2007). Other segments of food industry in which sugar ester biosurfactants can be used are: the production of aromas and maturation of cheeses, bakery products, cakes, biscuits, mayonnaise and sauces, instant products, sausages, among others (Liese *et al.* 2000, Pandey *et al.* 1999, Plou *et al.* 2002, Saxena *et al.* 1999, Sharma *et al.* 2001).

4.4 Conclusions

In this study, sugar esters were enzymatically synthesized from three types of carbohydrates, two types of fatty acids and two types of solvents using PPL as biocatalyst.

The sucrose esters presented the highest esterification yield (47.6%), and superior surface activity (33.4 mN/m), besides their ability to form stable emulsions (EI above 58% for all assays). Additionally, fructose esters also showed good results. In general, the best sucrose and fructose esters were synthesized from oleic acid using ethanol as solvent, and represent promising products for application in the food industry. The use of sucrose and ethanol is interesting from the economical and environmental point of view. Sucrose is the cheapest carbohydrate among the ones studied herein, and ethanol is non pollutant and non toxic, and easily obtained from renewable sources.

4.5 References

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Chapter 5

Enzymatic synthesis of sugar ester biosurfactants and their potential as surface-active stabilizers of coconut milk emulsions

"Tentar e falhar é, pelo menos, aprender. Não chegar a tentar é sofrer a inestimável perda do que poderia ter sido. Uma longa viagem começa com um único passo".

(Albino Teixeira e Lao Tsé)

Sugar esters were produced by esterification reactions. They are compounds with surfactant properties (biosurfactants), *i.e.* capable of reducing the surface tension and promote the emulsification of immiscible liquids. As with all emulsions, coconut milk is not physically stable and prone to phase separation. The aim of this work was to evaluate the synthesis of fructose, sucrose and lactose esters from the corresponding sugars using *Candida antarctica* type B lipase immobilized in two different supports, namely acrylic resin and chitosan. The enzyme immobilized on chitosan showed the highest yield of lactose ester production (84.1%). Additionally, the production of fructose ester was found to be higher for the acrylic resin support (74.3%) as compared with the chitosan (70.1%). The same trend was observed for the sucrose ester, although with lower percentage yields. Sugar esters were then added to samples of fresh coconut milk and characterized according to their surface tension, emulsification index and particle size distribution. Although the microscopic analysis showed similar results for all sugar esters, results indicated lactose ester as the best biosurfactant, with a surface tension of 38.0 mN/m and an emulsification index of 54.1%, when used in a ratio of 1:10 (biosurfactant: coconut milk, v/v) for 48 hours experiments.

5.1 Introduction

Enzymes possess high catalytic activity, unique specificity of action and the ability to function under mild operating conditions. Numerous advantages have been associated to the use of enzymes, such as for example the production of purer products (monoesters) due to the high selectivity of the catalyst and the mild conditions of temperature, pressure and pH required for the synthesis, in contrast to the extreme conditions associated with the chemical processes. Hence, since the 1970's there has been considerable interest in enzyme technology and much of this is due to the potential of immobilized enzymes (Choudhary *et al.* 2004, Krajewska 2004, Lamb and Stuckey 1999, Rezaei *et al.* 2007).

Lipases are an important group of biocatalysts, which not only modify oils and fats, but are also used for organic chemistry (Bruno *et al.* 2005). There are several industrial lipase applications in food and flavor production, pharmaceuticals, synthesis of carbohydrate esters (or sugar esters), amines, amides, biodetergents, cosmetics and perfumery (Hung *et al.* 2003).

However, the current market price of lipases is about one order of magnitude higher than the energy costs associated with standard processes. Hence, in order to reduce the overall process costs, efficient methods for lipase immobilization are required since immobilization allows enzyme to be reused, extending its useful active life (Bruno *et al.* 2005, Soares *et al.* 2002, Villeneuve *et al.* 2000). The immobilized lipase B from *Candida antarctica* (CALB) is an interesting lipase with potential application in a number of industrial processes such as: detergents, fungicide, foaming agents, demulsifying, solubilizing, emulsifiers, dispersants or in the synthesis of sugar esters used in the flavor industry. CALB has been mostly used in the immobilized form commercially available from Novo Nordisk (Novozyme 435) (Chang *et al.* 2008, Foresti and Ferreira 2005, Kose *et al.* 2002, Rodrigues *et al.* 2008).

Several immobilization supports have been pursued in order to improve the use of enzymes in industrial applications. Chitosan is a natural polymer, with a low cost, renewable and biodegradable, highly economic and with an environmental relevance, having a high potential as a source of polysaccharides. It has been used as a matrix for immobilization of lipase, and it is a good support for enzyme immobilization in the food industry since it is non-toxic (food grade). Chitosan is obtained by partial N-deacetylation

of chitin, a by-product of the fishing industry, which is the main component of the shells of crab, shrimp, and krill. It is available in different forms (powder, gel, fibers and membranes) it has high protein affinity and allows an easy derivatization (Alsarra *et al.* 2002, Chiou and Wu 2004, Hirano 1999, Saboktakin *et al.* 2010a,b, Spagna *et al.* 2001, Wu *et al.* 2010).

Orrego and collaborators (2010) developed and tested formulations of chitosan membranes, verifying that they are efficient as support for the immobilization of lipases from *Candida rugosa* and *C. antarctica*. Furthermore, according to Hung *et al.* (2003), the immobilization of *C. rugosa* enzyme using chitosan as support is a promising technique for large-scale preparation of immobilized lipases for industrial applications.

Biosurfactants are compounds with surfactant properties, *i.e.* capable of reducing the surface tension and promote the emulsification of immiscible liquids (Amézcua-Vega *et al.* 2007, Banat 2000, Banat *et al.* 2000, Daoshan *et al.* 2004, Rodrigues *et al.* 2006a). Moreover, these compounds could replace products already in use in the food industry due to their biodegradability (Chamouleau *et al.* 2001, Park *et al.* 2004, Tsavas *et al.* 2002).

An oil-in-water emulsion formed from the aqueous extract of coconut (*Cocos nucifera,* LINNAEUS) solid endosperm called coconut milk is used as an ingredient in cooking in the many tropical countries (Tangsuphoom and Coupland 2009a). As with all emulsions, coconut milk is not physically stable and is prone to phase separation. The emulsion is relatively unstable because of the large droplet size and the poor emulsifying properties of coconut proteins adsorbed at the oil-water interface. To make more stable products, emulsifiers are usually added during manufacturing and the stabilized coconut milk is preserved by chilling, freezing, pasteurization, or sterilization (Tangsuphoom and Coupland 2005, 2009a,b).

This work reports the use of acrylic resin (commercial) and chitosan as supports for immobilization of CALB for the production of sugar ester biosurfactants. Additionally, the sugar ester biosurfactants were characterized according to their ability to stabilize coconut milk.

5.2 Material and Methods

5.2.1 Materials

All chemicals used were analytical grade. Commercial coconut milk was purchased from a local market (Fortaleza-CE, Brazil); the triacylglycerol lipase purified from *C. antartica* B immobilized in acrylic resin (CALB) (Novozym 435) was purchased from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO); powdered chitosan, 85.2% deacetylation degree was purchased from Polymar Ind Ltd. (Fortaleza-CE, Brazil); and native soluble lipase B from *C. antarctica* B (Lipozyme® CALB L) was kindly provided by Novozymes Latin America Ltd. (Araucária, Brazil).

5.2.1.1 Sample preparation of fresh coconut milk

Coconuts with 12-14 months' maturity from 'ordinary tall' coconut cultivars were used for the extraction of coconut milk. The fresh coconut pulp with added water in proportion 3:1 (w/v), respectively, was crushed by cutter (Arno, France) for 5 minutes. The viscous exudates resulting from the cutter extraction was squeezed through cheesecloth to obtain fresh coconut milk, according to methodology described by Seneviratne *et al.* (2009). Immediately after the fresh coconut milk extraction, surface tension, emulsification index and microscopic analysis were performed.

5.2.2 Methods

5.2.2.1 Preparation and activation of the immobilization support

In this work, chitosan was used as a solid support for lipase (lipase from *C. antarctica* type B) immobilization, based on the methodology described by Rodrigues *et al.* (2008). Briefly, 4.0 g of powdered chitosan was added to 96.0 mL of a 5% acetic acid solution. The obtained solution was dropped into a gently stirred 1M NaOH solution for 24 h, at room temperature. Afterwards, the mixture was washed with an excess of distilled water.

The support was activated with glycidol, ethylenediamine (EDA) and glutaraldehyde. Activation of chitosan with glycidol was carried out by etherification and further oxidation with sodium periodate of the resulting support - glyceril (Guisán 1988), 10 g of Chitosan - Glyoxyl gel was reacted with 40 mL of a 2M ethylenediamine solution, pH 10.0 (Cardias *et al.* 1999). Finally, 9 mL of sodium bicarbonate buffer, pH 10.0, containing 5% (v/v) of glutaraldehyde, was added to 1 g of chitosan- Glyoxyl - EDA. The mixture was kept under agitation for 60 min at 25 ºC for 18 h. After this time, supports were washed with distilled water to remove the excess of activating agent.

5.2.2.2 Immobilization of CALB

CALB was immobilized on chitosan gels, after activation with glycidol, ethylenediamine and glutaraldehyde. The immobilization was carried out in 100 mM bicarbonate buffer, pH 10.05, at 25 ºC and 5 h, under gentle stirring. In the immobilization assays, 109 U per gram of support was used.

5.2.2.3 Lipase activity

The hydrolytic activity of soluble and immobilized CALB was measured spectrophotometrically (410 nm) using p-nitrophenyl butirate (pNPB) as substrate, in 2-propanol, at pH 7.0 and 25 ºC, according with methodology described in literature (Bhatnagar *et al.* 2005).

5.2.2.4 Sugar ester biosurfactants production

Sugar ester biosurfactants were produced by esterification reactions. The synthesis experiments were conducted in flasks by adding oleic acid (0.5 mmol); fructose, sucrose or lactose (0.5 mmol); immobilized lipase (22.5 mg); sodium sulfate anhydrous (0.1 g); ethanol 99% (0.6 mL) and incubating the mixture at 40 ºC, 250 rpm for 72 hours. This procedure has been previously described by Sabeder and co-workers (2006). At the end of the esterification reaction, the lipase, together with the sodium sulfate anhydrous (non reactive species), were removed by filtration using filter paper with a pore-size of 60-µm

(Macherey-Nagel Inc.). Afterwards, the ethanol was evaporated from the reaction media using a rotoevaporator.

5.2.2.5 Thin Layer Chromatography (TLC)

The obtained products (sugar esters) were identified by thin layer chromatography (TLC), using commercial plates (Merck) coated with a 0.25 mm layer of silica gel (Khaled *et al.* 1991). A mixture of chloroform/hexane (1:1, v/v) was used for elution. Subsequently, the sugar esters spots were identified with iodine according to Ducret and collaborators' work (1995).

5.2.2.6 Quantification of the sugar esters

The sugar esters content was quantified by calculating the residual fatty acid amount in the reaction mixture, which was determined by the volumetric method described elsewhere (Leitgeb and Knez 1990). Briefly, 0.1 g of sample from the reaction mixture were diluted in 20 mL of 0.1 wt% phenolphthalein solution in absolute ethanol, and then titrated with standardized sodium hydroxide solution of 0.1 mol/L in water. Measurements were done in triplicate.

5.2.3 Characterization of the product

After the synthesis of the sugar ester biosurfactants, these compounds were characterized according to the techniques described below.

5.2.3.1 Surface tension

Surface tension was determined in the commercial coconut milk, the fresh coconut milk and the fresh coconut milk with added sugar ester biosurfactants (produced according to section 5.2.2.4) at different ratios, such as 1:1, 1:5, 1:10 and 1:100 (sugar ester: coconut milk, v/v). The surface tension was determined at room temperature (25±1 ºC) by the Ring method as described elsewhere (Rodrigues *et al.* 2006b). A KRUSS Tensiometer (Kruss model K10) equipped with a 1.9 cm Du Nouy platinum ring was used. Measurements were

done in quintuplicate and results represent the means ± standard deviation. The concentrations of sugar ester biosurfactants used to prepare the different ratios of ester: fresh coconut milk was 30% (v/v) and the samples were prepared at 25 °C.

5.2.3.2 Emulsification index (EI)

EI was determined for the commercial coconut milk, the fresh coconut milk and the fresh coconut milk with added sugar ester biosurfactants at different ratios, such as 1:1, 1:5, 1:10 and 1:100 (sugar ester: coconut milk, v/v). To determine the EI, a 2 mL sample and 1 mL of n-hexadecane were homogenized using a vortex for 2 minutes at 25 °C. Next, the emulsion was left to settle for 2 minutes and the height of the emulsion was measured (T2). The index was calculated using the equation (1).

$$\text{Emulsion index (\%)} = He/Ht \times 100 \quad (1)$$

where He is the height of the emulsion and Ht is the total height of the liquid. To check the stability of the emulsions, these were left to settle also for 24 (T24) and 48 hours (T48) (Cooper and Goldenberg 1987). All determinations were performed in triplicate and results represent the means ± standard deviation.

5.2.3.3 Determination of particle size distribution of coconut milk samples

Particle size distribution of commercial coconut milk, fresh coconut milk and fresh coconut milk with added sugar ester biosurfactants at different ratios, such as 1:1, 1:5 and 1:10 (biosurfactant: coconut milk, v/v), were determined by optical imaging and drop size analysis using an optical microscope. One drop of the sample was transferred to the slide and a cover slip was placed over the sample. The samples were analyzed and identified using an Olympus model CH31 optical microscope (Olympus, BX51M) with an attached camera and objective lens of 50 X (for the fresh coconut milk) and 200 X (for all other samples) with 1024x720 of resolution. The Image Pro Plus 6.0 software from Media Cybernetics was used to obtain the distribution and size of the particles in the samples.

Furthermore, photographs were taken from typical fields to compare the changeability of the fat globules (Peamprasart and Chiewchan 2006).

5.2.4 Statistical analysis

The data were statically evaluated using ANOVA and Tukey test at 95% of confidence level ($\alpha = 0.05$). Origin Pro 7.5 software (OriginLab) was used for processing data.

5.3 Results and Discussion

5.3.1 Thin Layer Chromatography (TLC)

From the TLC analysis, it was possible to verify the presence of the desired sugar esters (fructose, sucrose and lactose esters) by measuring the retention factors (Rf). Figure 5.1 illustrates an example of the TLCs obtained for the sugar esters synthesized.

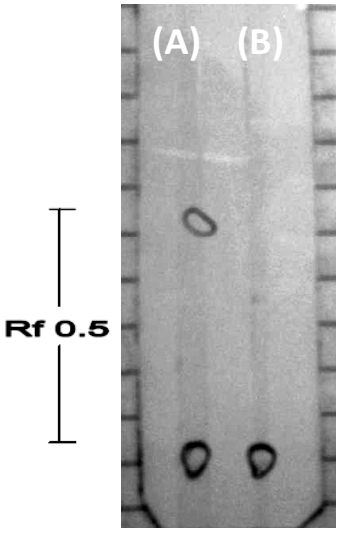


Figure 5.1 Thin-layer chromatography of sugar esters. Lane (A) corresponds to the product synthesized at the given experimental conditions. Lane (B) corresponds to a control experiment where no enzyme was used, thus no sugar ester was formed. This Figure shows the result of lactose ester.

The Rf values obtained for all sugar esters were found to be 0.5 which is in accordance with previous reports. Khaled *et al.* (1991) and Tortorello and Delwiche (1983) reported an Rf value of 0.51 for fatty acid esters. Therefore, it was possible to conclude that the desired sugar esters were obtained.

5.3.2 Quantification of sugar esters

Table 5.1 presents the yields of sugar esters obtained from fructose, sucrose and lactose, by enzymatic synthesis using lipase from *C. antarctica* immobilized on the chosen supports, namely acrylic resin and chitosan.

Table 5.1 Sugar esters yields obtained by enzymatic synthesis with immobilized lipase from *C. antarctica* type B (CALB)

CALB	Sugar esters	Yield (%)±SD*
Acrylic resin	**fructose ester**	**74.3±**0.3[a]
Chitosan		**70.1±**0.0[b]
Acrylic resin	**sucrose ester**	**56.1±**0.2[c]
Chitosan		**55.0±**0.3[d]
Acrylic resin	**lactose ester**	**83.6±**0.3[e]
Chitosan		**84.1±**0.3[f]

SD = Standard Deviation; *Values with different letters in the same column present statistically significant differences ($p<0.05$).

According to Table 5.1, it was possible to observe statistically significant differences at 5% level of probability between the two types of supports for all the sugar esters produced. The lowest yield was obtained for the sucrose esters. *C. antarctica* lipase showed a better performance for the synthesis of lactose ester using both supports. Moreover, the lactose ester yield obtained for the immobilization with acrylic resin (83.6%) was found to be much higher than the values that have been reported using other methods by Walsh *et al.* 2009 (21.8%), Wang *et al.* 2005 (77%) and Wu *et al.* 2004 (62%). Furthermore, the immobilization with chitosan proved also to be an interesting and efficient support for the production of lactose ester (84.1%). According to the literature, chitosan is a good candidate for the immobilization of lipase when compared to other

polysaccharides, such as alginate and agarose (Betigeri 1999). Lipase from *C. antarctica* has been widely reported for the synthesis of sugar esters, being Habulin *et al.* (2008), Iwamoto *et al.* (2008), Perez-Victoria and Morales (2007), some of the most recent examples.

The yield of fructose ester was found to be higher for the immobilization with acrylic resin as compared to the immobilization with chitosan. The same trend was observed for the sucrose ester, although with lower yields. Immobilization with acrylic resin yielded 74.3% of fructose ester, while immobilization with chitosan yielded 70.1%. Although being similar, these yields were found to be statistically different and higher than the results reported by Sabeder *et al.* (2006). These authors reported yields of fructose ester of 53% when using immobilized lipase SP 435 from *C. antarctica* B, and a yield of 44% when using immobilized lipase SP 382 also from *C. antarctica* B. Moreover, the yields of sucrose ester (56.1% in acrylic resin and 55.0% in chitosan) obtained are higher than those found by Walsh *et al.* 2009 (20.9%) using the same biocatalyst.

The sugar ester yields obtained in the current study were found to be higher than the ones that have been previously reported in the literature. Also, immobilization of lipase proved to be advantageous due to the ease of recovery of the sugar esters, as well as to the possibility of biocatalyst reuse. Further studies on the reusability of the immobilized lipase are required in order to confirm the supports' potential for industrial enzymatic production of sugar ester biosurfactants (Kanwar *et al.* 2007, Nasratun *et al.* 2009, Pahujani *et al.* 2008, Song *et al.* 2010, Zhang *et al.* 2010).

5.3.3 Surface tension

Table 5.2 presents the surface tension values determined for the coconut milk with added sugar ester biosurfactants. The Tukey test was used at 95% probability to evaluate if there was a statistical significant difference between the surface tensions obtained for the different samples.

Table 5.2 Surface tension values measured for the fresh coconut milk, the commercial coconut milk and the fresh coconut milk with added sugar ester biosurfactants.

Groups	Products	Samples	Sugar ester: coconut milk (v/v)	Surface tension Mean (mN/m)±SD*#◊
1	Fresh coconut milk with Fructose ester	A	1:1	**39.7±**0.3[a]
		B	1:5	**40.0±**0.0[a, A, 1]
		C	1:10	**41.1±**0.2[b, C, 3]
		D	1:100	**54.3±**0.3[c]
2	Fresh coconut milk with Sucrose ester	E	1:1	**40.2±**0.3[a]
		F	1:5	**40.0±**0.0[a, A, 1]
		G	1:10	**41.9±**0.2[b, D, 4]
		H	1:100	**54.9±**0.2[c]
3	Fresh coconut milk with Lactose ester	I	1:1	**38.0±**0.0[a]
		J	1:5	**39.2±**0.3[b, B, 2]
		K	1:10	**40.0±**0.0[c, E, 1, 5]
		L	1:100	**52.1±**0.2[d]
4	Fresh coconut milk	M	-	**52±**0.3[a]
	Commercial coconut milk	N	-	**46±**0.2[b]

SD = Standard Deviation; *Values in each groups with letters different in the same column present significant difference ($p<0.05$); #Capital letters in the same column indicate comparison between the ratios 1:5 and 1:10, in each group; ◊Subscripts numbers indicate comparison in the ratios 1:5 and 1:10, between all groups.

According to Table 5.2, for the fresh coconut milk with fructose ester in the ratios 1:1 and 1:5 (samples A and B), and for the fresh coconut milk with sucrose ester in the same ratios (samples E and F), no statistically significant differences were observed, suggesting that similar results could be obtained using a higher dilution, thus with less costs if a commercial application is envisaged. For the fresh coconut milk with lactose ester, this was not observed since statistical significant differences were found for all the ratios studied.

Comparing the results obtained for all the sugar esters used, it was possible to verify that both fructose and sucrose ester at a ratio of 1:5 resulted in the same surface tension (40.0 mN/m). Using lactose ester, this surface tension was obtained for a higher dilution (1:10), thus meaning that this ester was more efficient in lowering the surface tension of the mixture.

From the results of the current study, it was found that the addition of each of the sugar ester biosurfactants lead to a significant reduction in surface tension of the fresh coconut milk. This is very important because the greater the ability of a sugar ester biosurfactant to reduce surface tension, the greater the emulsion stability formed in coconut milk. Results obtained with fresh coconut milk were better than with commercial coconut milk. Therefore, these results open an interesting perspective of the potential application of sugar ester biosurfactants in the food industry. To our knowledge this is the first report on the use of sugar ester biosurfactants to stabilize fresh milk coconut emulsions.

5.3.4 Emulsification index (EI)

Emulsification indexes (EI) determined for the same samples used for the surface tension measurements at different time points are gathered in Table 5.3.

Table 5.3 Emulsification index (EI) determined for fresh coconut milk, commercial coconut milk and fresh coconut milk with added sugar esters

Groups	Products	Samples	Sugar esters: coconut milk (v/v)	After 2 min	After 24 h	After 48 h
				%**EI**±SD*	%**EI**±SD*	%**EI**±SD*
1	**Fresh coconut milk with Fructose ester**	A	1:1	**58.9**±0.0^{a}	**54.6**±0.4^{a}	**52.4**±0.3^{a}
		B	1:5	**58.1**±0.2^{a}	**53.2**±0.9^{a}	**50.2**±0.2^{b}
		C	1:10	**58.0**±0.0^{a}	**52.2**±0.3^{a}	**49.1**±0.0^{c}
		D	1:100	**31.4**±0.5^{b}	**28.1**±0.1^{b}	**26.6**±0.2^{d}
2	**Fresh coconut milk with Sucrose ester**	E	1:1	**58.2**±0.2^{a}	**56.3**±0.3^{a}	**52.2**±0.3^{a}
		F	1:5	**58.2**±0.2^{a}	**55.4**±0.1^{b}	**52.3**±0.1^{a}
		G	1:10	**56.2**±0.3^{b}	**54.2**±0.3^{c}	**51.1**±0.0^{b}
		H	1:100	**38.6**±0.5^{c}	**35.2**±0.9^{d}	**34.6**±0.2^{c}
3	**Fresh coconut milk with Lactose ester**	I	1:1	**60.5**±0.9^{a}	**58.3**±0.9^{a}	**56.4**±0.3^{a}
		J	1:5	**59.2**±0.9^{b}	**58.0**±0.0^{a}	**56.2**±0.2^{a}
		K	1:10	**58.2**±0.2^{c}	**56.3**±0.3^{b}	**54.1**±0.0^{b}
		L	1:100	**36.7**±0.3^{d}	**34.4**±0.3^{c}	**31.6**±0.3^{c}
4	**Fresh coconut milk**	M	-	**37.2**±0.5^{a}	**33.4**±0.0^{a}	**29.6**±0.3^{a}
	Commercial coconut milk	N	-	**54.0**±0.0^{b}	**51.1**±0.5^{b}	**47.6**±0.0^{b}

SD = Standard Deviation; %EI = percentage of emulsification index (mean); *Values in each groups with letters different in the same column present significant difference ($p<0.05$).

An important parameter for evaluating the power of an emulsifier is the EI and the emulsion stability (Abu-Ruwaida *et al.* 1991, Cooper and Goldenberg 1987, Singh *et al.* 2007). All emulsions studied showed high EI values, being the EI value above 52% for all the ratios sugar ester: coconut milk used (except for 1:100), as well as for all the time points evaluated (2 minutes, 24 and 48 hours).

Regarding the emulsion of fresh coconut milk with fructose ester, no statistical significant differences were observed for 48 hours. Therefore, comparing the results obtained for the different sugar ester: coconut milk ratios, the best condition studied was 1:10 for 24 hours with an EI of 52.2% (sample C), since it provides the highest EI at a lower concentration ratio.

Furthermore, for the emulsions with lactose and sucrose esters no statistical significant differences were observed for 24 and 48 hours, meaning that these esters provide a higher stability to the emulsion comparing with fructose esters. Emulsification indexes of 52.3% and 56.2% were obtained at a concentration ratio of 1:5 (the highest dilution for which the differences observed have statistic significance) and 48 hours for sucrose and lactose esters, respectively.

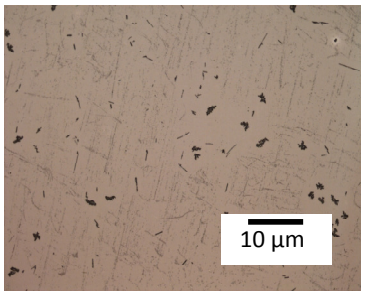
For the food industry it is important and necessary to use emulsifiers that comprise low-cost and high stability of the emulsion obtained (Shin *et al.* 2009, Yutaka and Kitagawa 1998). According to the results of the current work, the emulsifier that meets these characteristics is the lactose ester. In addition to achieving stability for a longer time (48 hours), it has a high EI (54.1%) at a low concentration ratio (1:10) (sample K).

Moreover, even for concentration ratios of 1:10 of all sugar ester biosurfactants added to the fresh coconut milk, the results are superior to those found for samples using commercial coconut milk. To our knowledge this is the first report on emulsification indexes for mixtures of coconut milk with sugar esters.

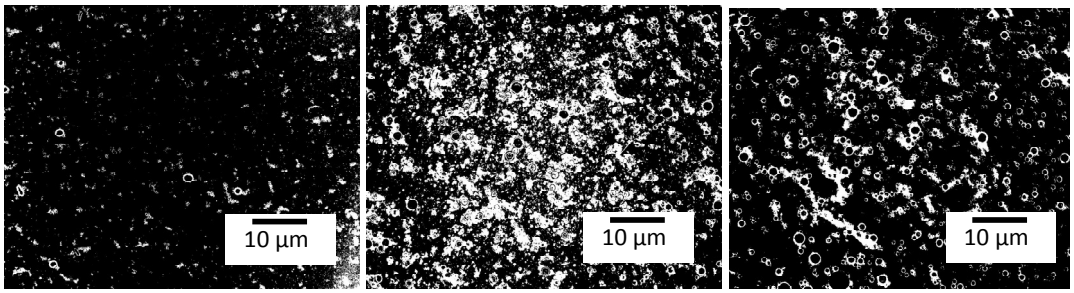
5.3.5 Particle size distribution of coconut milk samples

The microstructures of the coconut milk samples from different experimental conditions were examined using an optical microscope. Figure 5.2 illustrates the structure

of the fresh coconut milk and the fresh coconut milk containing lactose ester in different concentration ratios.



(a)



(b) (c) (d)

Figure 5.2 Micrographs (200 X magnification) of the fresh coconut milk (a) and the fresh coconut milk with lactose ester in different concentration ratios (fresh coconut milk: lactose ester): 1:1 (b), 1:5 (c) and 1:10 (d).

The micrographs taken from the emulsions obtained with fresh coconut milk with fructose and sucrose esters showed similar structures (*data not shown*). Based on the results illustrated on Figure 5.2, it was possible to determine the number of particles and their size distribution (Figure 5.3).

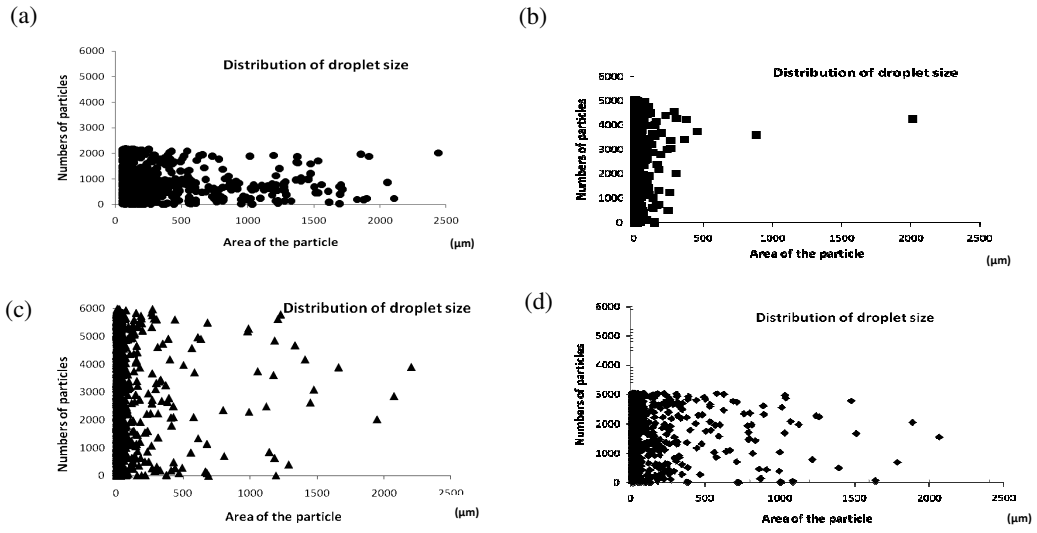


Figure 5.3 Particle size distribution of the fresh coconut milk (a), the fresh coconut milk with lactose ester in different concentration ratios: 1:1 (b), 1:5 (c) and 1:10 (d).

From Figures 5.2a and 5.3a, it was found that the coconut fibers possess a variable size and are non-uniformly dispersed, showing some aggregates. These results are in accordance with the ones reported by Jirapeangtong *et al.* (2008).

Figure 5.3b (ratio 1:1 of fresh coconut milk and lactose ester) showed that most of the particles were sized between 0 and 10 μm, which explains the stability of the emulsion formed. Within this range of sizes, the probability of occurring coalescence of the particles is very low, since they are very small and uniform regarding the low volume of the dispersed phase. Although this behavior has been observed for the higher concentration ratio studied (1:1), it does not preclude its potential use for stabilizing coconut milk. Figures 5.3c and 5.3d, showed a highest dispersion of the sugar ester biosurfactants globules in the aqueous phase, indicating a lower stability of the product. Other researchers reported the use of chemical surfactants to stabilize coconut milk, namely SDS and Tween. Tangsuphoom and Coupland (2009a) studied the effect of mixing chemical surfactants (SDS and Tween 20) with coconut milk under heat. Coconut milk emulsified with SDS was found to be stable in all heating treatments studied, meaning that no change in droplet size and no phase separation occurred. According to Seow and Gwee (1997), polysorbates,

such as Tween 20 and Tween 60, are widely used to improve the stability of sterilized coconut milk products, although typically at somewhat lower levels (around 0.3 wt%) and in combination with gums.

Although some studies on the use of surfactants for the stabilization of coconut milk have been reported, to our knowledge this is the first report on the use of sugar ester biosurfactants for that purpose.

5.4 Conclusions

The enzymatic synthesis of fructose, sucrose and lactose esters was conducted using two different types of supports (acrylic resin and chitosan) for enzyme immobilization of CALB. The enzyme immobilized on chitosan presented higher yields (84.1%) in the production of lactose ester as compared to the acrylic resin support. Furthermore, the lactose ester showed a better performance regarding the decrease of fresh coconut milk surface tension and the stabilization of the emulsion as given by the emulsification index. The use of CALB immobilized on chitosan presents a promising alternative for the enzymatic production of sugar esters due to the possibility of enzyme reuse and economic viability.

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Chapter 6

Conclusions and future perspectives

"Superar o fácil não tem mérito, é obrigação;
vencer o difícil é glorificante; ultrapassar o
outrora impossível é esplendoroso".

(Alexandre Fonteles)

This chapter presents the major conclusions extracted from this thesis. More detailed conclusions can be found at the end of each individual chapter. Furthermore, some perspectives for future research in this field are discussed.

6.1 Conclusions

The main objective of this thesis was the production of sugar ester biosurfactants using non-toxic reagents, namely: (1) lipases - *Candida antarctica* type B and porcine pancreas; (2) sugars - fructose, sucrose and lactose; (3) fatty acids - oleic acid and linoleic acid; and (4) solvents - ethanol and ethyl acetate. In this thesis it was sought to define the optimal production conditions and to characterize the synthesis products aiming at its subsequent incorporation in coconut milk. To achieve these goals, many issues have been studied and various strategies have been implemented successfully.

The main conclusions drawn from this work are presented below:

- The synthesis of fructose, sucrose and lactose esters was confirmed by TLC and IR spectroscopy;
- The modeling and optimization of the esterification reaction to synthesize fructose ester by immobilized CALB was successfully performed using a response surface methodology based on a central composite rotatable design (R^2 and Q^2 equal to 0.9995 and 0.7056, respectively). A cubic model was established that can be used to predict the esterification percentage under any given conditions within the experimental range studied;
- Under the optimized operating conditions an effective enhancement of the synthesis of fructose esters was achieved (yield = 88.4%);
- Synthesis of sugar ester biosurfactants catalyzed by CALB, using three types of carbohydrates, two types of fatty acids and two organic solvents, was studied. The highest esterification yield was obtained for the synthesis of lactose ester (yield = 83.5%) from lactose and linoleic acid in the presence of ethanol, in comparison with the other sugar esters and conditions studied. However, the fructose esters (yield = 74.3%) showed a better performance regarding their surface activity and ability to stabilize emulsions.
- Sugar esters were enzymatically synthesized from three types of carbohydrates, two types of fatty acids and two types of solvents using porcine pancreas lipase as biocatalyst. The sucrose esters presented the highest esterification yield (yield =

47.6%) and superior surface activity reduction, besides the ability to form stable emulsions. Fructose esters also showed good results.

- In general, the best sucrose and fructose esters were synthesized from oleic acid using ethanol as solvent, and represent promising products for application in the food industry. The use of sucrose and ethanol is interesting from the economical and environmental point of view. Sucrose is the cheapest carbohydrate among the ones studied herein, and ethanol is a non pollutant and non toxic solvent that can be easily obtained from renewable sources.
- The enzymatic synthesis of fructose, sucrose and lactose esters was studied using CALB immobilized on two different types of supports (acrylic resin and chitosan). The enzyme immobilized on chitosan presented higher yields of lactose ester synthesis (yield = 84.1%) as compared to the acrylic resin support (yield = 83.6%). Furthermore, the lactose ester showed a better performance regarding the decrease of fresh coconut milk surface tension and the stabilization of the emulsion as given by the emulsification index.
- The use of CALB immobilized on chitosan presents a promising alternative for the enzymatic production of sugar ester biosurfactants due to the possibility of enzyme reuse and economic viability.
- To obtain a maximum yield of lactose ester it is more advantageous to use CALB immobilized on chitosan. On contrary, CALB immobilized on acrylic resin is a better option to increase the yield of fructose ester synthesis.
- In general, CALB showed better results than the PPL lipase in the synthesis reactions, surface tension and emulsification index.
- Finally, this study can be seen as a contribution to the development of more efficient bioprocesses for industrial synthesis of sugar ester biosurfactants.

6.2 Future perspectives

The results obtained in this thesis provided some interesting perspectives on the use of sugar ester biosurfactants in the food industry. Their application in food emulsions, such as coconut milk, opened opportunities that worth further exploration. Furthermore, the development of new solutions that enable the decrease of the operational costs associated with sugar esters production will broaden their potential applications. Some suggestions for future work are presented below:

- Study of other lipases in the synthesis reactions to evaluate their impact on the product yield;
- Immobilize lipase using alternative cheap supports such as alginate gels, carrageenan and polyacrylamide, alumina, ground kanuma, stalk of sugar cane, among others;
- Characterize the kinetics of the enzymatic reactions;
- Incorporate the sugar ester biosurfactants in other food matrices, such as ice cream, fruit juice, cake, bread, among others.

The results gathered in this thesis are promising and have led to new questions that warrant further research.