Synthesis of sucrose fatty acid esters as catalyzed by alkaline protease AL 89 and Candida antarctica lipase B in hydrophilic solvents.

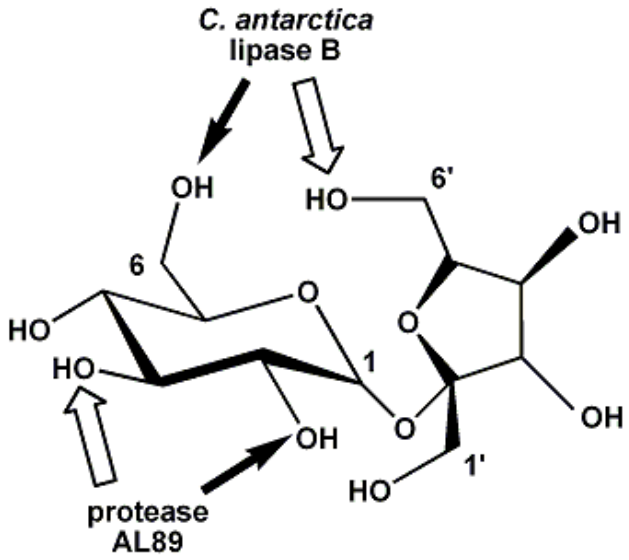
Ritthitham, Sinthuwat

Publication date:
2009

Document Version
Publisher's PDF, also known as Version of record

AALBORG UNIVERSITY

Synthesis of sucrose fatty acid esters as catalyzed by alkaline protease AL 89 and *Candida antarctica* lipase B in hydrophilic solvents



Sinthuwat Ritthitham **Ph.D. thesis 2009**

Section of Biotechnology
Department of Biotechnology, Chemistry and Environmental Engineering
Aalborg University

Preface

The experimental work presented in this thesis was conducted in the laboratory of the Bioprocess group at the Section of Biotechnology, Aalborg University during 2004-2009. The PhD. was financially supported by a grant from the Royal Thai government.

The thesis is divided into two sections of which section one consists of six chapters addressing the chemistry and biocatalysis related to the properties and synthesis of sucrose fatty acid esters. Section two covers the research results in the form of two published papers, one published book chapter and one paper prepared for submission.

Acknowledgements

I would like to express my deepest gratitude to whom I am indebted for their guidance and support me for the completion of this thesis:

First and foremost, I am especially grateful to thank my supervisor, Associate Professor Lars Haastrup Pedersen for providing me an opportunity to do scientific research in Denmark, for making possible for me to work in this fascinating world of enzyme technology and for guiding me as a very patient leading supervisor. His overview, knowledge of details, valuable experiences, hospitality, generosity, and improvisation has been essential for my scientific journey. On the process of manuscript preparation for the scientific paper publications and thesis submission, he intensively and patiently helped, guided, and taught me with good scientific writing and even seriously took the trouble of proof reading. Moreover, he provided me the documents strongly supported for my scholarship application and extension as well as his numerous helps for my visa application during I stayed in Denmark. Additionally, I would like to thank his family for inviting me many great dinner times.

I would like to thank Associate Professor Reinhard Wimmer and Associate Professor Allan Stensballe for carrying out the NMR and MS analysis.

Thank you to Gunnar Andersen who has fixed countless small but important laboratory instruments for me. Thank you to Tanja Bergmann, a very kind laboratory technician, for providing me with experimental materials and thanks to the members in Bioprocess group for an enjoyable time, for laughs, and for good talks.

Thanks to Wantana Khamme Christiansen, for her kindness preparing the original taste of Thai food every week, which made my life in Denmark more pleasant.

Finally, I would like to thank my parents and all my relatives in Thailand for their encouragement and unconditional support throughout my study and stay in Denmark.

Aalborg, December 2009

Summary

This thesis aimed to investigate enzyme catalyzed synthesis of *O*-acyl sucrose in organic hydrophilic solvents. The synthetic activity, regio-selectivity, and enzyme stability of alkaline protease AL 89 in hydrophilic, aprotic solvents was investigated using activated vinyl fatty acid esters as acyl donors. The regio-selectivity of the alkaline protease was shown to be towards the 2-OH of sucrose catalyzing the formation of 2-*O*-acyl sucrose which again served as a substrate for the protease resulting in the synthesis of 3-*O*-acyl sucrose. The optimal reaction conditions (substrate molar ratio, amount of water, temperature) were found at 70 °C with the acyl acceptor to donor ratio of 1:1.5 and a water content of 0-2% (v/v). The fatty acid chain length of acyl donor did not significantly affect the formation rate.

6-*O*- and 6'-*O*-stearoyl sucrose were enzymatically synthesized by *Candida antarctica* lipase B in *tertiary*-alcohols with polar solvents (either pyridine or DMSO) as co-solvents. The highest initial formation rate of 6-*O*- and 6'-*O*-stearoyl sucrose were obtained when performing the reaction in a solvent system of 45% pyridine in 2-methyl-2-butanol in which solid sucrose was continuously dissolved and consumed for the synthesis of esters. The sucrose solubility increased in the solvent system with hydrophilic co-solvents in the following order: no-co solvent< 45%Pyridine < 20%DMSO < 55%Pyridine while the regio-selectivity towards the formation of 6-*O*-stearoyl sucrose decreased in the order of no-co solvent>45% pyridine> 55% pyridine> 20%DMSO. The regio-isomeric distribution defined as the final concentration of 6-*O*-stearoyl sucrose to 6'-*O*-stearoyl sucrose in the reaction system with no-co solvent was 2:1 whereas in the reaction system with 20% DMSO it was 1:1.

A procedure for separation of mono-*O*-caproyl sucrose regio-isomers was developed by chromatography. The stability of the purified 2-*O*-caproyl sucrose and 3'-*O*-caproyl sucrose was observed to be strongly dependent on the drying temperature during the solidification step. In the presence of water at 60 °C, the acyl group of the purified 2-*O*-caproyl sucrose migrated to form 3-*O*- and 6-*O*-

caproyl sucrose. At the same conditions, the stability of the purified 3'-*O*-caproyl sucrose was higher than 2-*O*-caproyl sucrose as impurities of 6-*O*-caproyl sucrose were not detected. Performing solidification by lyophilization, absolute purity of 3'-*O*-caproyl sucrose and 96% purity of 2-*O*-caproyl sucrose with 4% of 3-*O*-caproyl sucrose was obtained.

The synthesis, analysis, purification and characterization of mono-*O*-acyl sucrose presented in this work could be applied for the preparation of pure and well-defined mono-*O*-acyl sucrose regio-isomers for the investigation of structure-function properties in relation to their applications as emulsifiers and surface active compounds.

This thesis is based on the following publications referred to by Roman numerals and presented in section II.

I. Pedersen L.H., **Ritthitham S.,** and Kristensen M., (2009). Activity and stability of proteases in hydrophilic solvents, In *Modern Biocatalysis: Stereoselective and environmentally friendly reactions*, Wolf-Dieter Fessner and Thorleif Anthonsen, Editors., Wiley-VCH: Weinheim. p.55-66 (ISBN 978-3-527-32071-4)

II. **Ritthitham S.,** Wimmer R., Stensballe A., and Pedersen L.H., (2009). Selectivity and stability of alkaline protease AL-89 in hydrophilic solvents. *J. Mol. Catal. B: Enzym*. 59. 266-273.

III. **Ritthitham S.,** Wimmer R., Stensballe A., and Pedersen L.H., (2009). Analysis and purification of *O*-decanoyl sucrose regio-isomers by reversed phase high pressure liquid chromatography with evaporative light scattering detection. *J. Chromatogr. A*. 1216. 4963-4967.

IV. **Ritthitham S.,** Wimmer R., and Pedersen L.H., (2009). Controlling the regio-selectivity in lipase catalyzed synthesis of sucrose stearate by polar co-solvents in *tertiary* alcohols. *In preparation.*

Abbreviations

Asp	Aspartic acid
CALB	*Candida antarcitca* lipase B
COSY	Correlation Spectroscopy
CMC	Critical Micellar Concentration
DABCO	1,4 diazabicyclo [2.2.2] octane
DBU	1,8-diazabicyclo[5.4.0] undec-7-ene
DIAD	Diisopropylazodicarboxylate
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
ELSD	Evaporative Light Scattering Detection
FAB MS	Fast Atom Bombardment Mass Spectroscopy
ESI MS	Electron Spray Ionization Mass Spectroscopy
His	Histidine
HLB	Hydrophilic-Lipophilic Balance
HPLC	High Pressure Liquid Chromatography
NMR	Nuclear Magnetic Resonance
Ser	Serine
Ph_3P	Triphenylphosphine
THF	Tetrahydrofuran
TOCSY	Total Correlation Spectroscopy
TLC	Thin Layer Chromatography
Å	Ångstrøm (0.1 nm)

Section I

Contents of section I

1.
Introduction

Carbohydrates are the most abundant organic compounds on the planet. They are renewable, sustainable resources which are at the same time relatively inexpensive sources of carbon and hydrogen from which a wealth of bulk and fine chemicals can be produced. Their chemical structures are polyhydroxyl aldehydes or ketones which can be reduced to give sugar alcohols, oxidized to give sugar acids, derivatized or substituted at one or more of the hydroxyl groups resulting in many different functional properties. Different positions and the degree of substitution result in different physicochemical properties, which in turn will be vital to industrial applications (Khan, 1995).

Sugar fatty acid esters constitute an interesting group of non-ionic surfactants with potentially important applications in a range of industries. The surface active properties of this type of amphiphilic molecules have very good emulsifying, stabilizing and conditioning effects. In addition, they are not harmful to the environment as they are completely biodegradable under both aerobic and anaerobic conditions.

Biocatalysis in organic solvents developed over the last three decades of the 20th century into well-established processes for the synthesis of chemical compounds (Koeller and Wang, 2001). The use of biocatalysts in ester synthesis offers advantages especially regarding substrate specificity and regio-selectivity that are difficult to obtain by conventional chemical catalysis. Moreover, enzymes are very versatile, active, selective, and environmentally friendly catalysts that generally work under mild conditions. Hydrolytic enzymes, including lipases and proteases, can catalyze condensation reactions provided that the thermodynamic equilibrium is shifted. This can be obtained by performing the reaction in organic solvents instead of water (Iyer and Ananthanarayan, 2008). Enzymes show selectivity at three levels: chemo-, regio- and stereo-selectivity. Chemo-selectivity is the ability of the enzyme to direct the catalytic reaction to a specific functional group in the molecule such as a hydroxyl or an amino group. Regio-selectivity concerns the

ability of enzyme to distinguish between several functional groups of the same kind and direct the reaction towards one particular position of the substrate, as known from hydroxyl groups of carbohydrates. The regio-selectivity is an important issue as the regio-isomers of a particular compound may differ in their toxicity, smell, taste, and biodegradability. The stereo-chemical properties of enzymes are extremely attractive in organic synthesis. Enzymes may be used for production of enantiopure chiral molecules either by enantioselective asymmetric synthesis or by de-racemization (resolution) of racemic mixtures.

1.1
Aim of the research

The research presented in this thesis concerns the synthesis, analysis, purification and characterization of *O*-acyl sucrose from esterification reactions using vinyl activated fatty acid esters as acyl donors. The synthetic methods were investigated and developed by using enzymatic biocatalysis with focus on the alkaline protease from *Bacilllus pseudofirmus* strain AL 89 and *Candida antarctica* lipase B (CALB). The hydrolytic and synthetic activity, stability, and the regio-selectivity of the alkaline protease AL 89 was investigated in a solvent system of hydrophilic aprotic solvents. The parameters affecting the rate of *O*-acyl sucrose synthesis with different fatty acid chain length of acyl donors were included in this work. The separation and purification of mono-*O*-caproyl sucrose regio-isomers was established based on low and high pressure chromatography.

2.
Carbohydrate based surfactants

Surfactants are surface active molecules containing a water-soluble and a fat soluble part and can be classified into 4 groups: anionic, cationic, amphoteric, and nonionic. The carbohydrate-based surfactants are typically nonionic surfactants primarily used in products such as detergents, cosmetics, pharmaceuticals and foods as well as in many industrial processes. They are physiologically, dermatologically and biologically acceptable, odorless, tasteless, and biodegradable. They are stable at pH 4 to 8 and at temperatures up to 180 °C. The overall performances such as emulsification, detergency, foam power, and wettability, are comparable with other surfactants and for some applications even superior. The uses of surfactants are determined by the particular functionality with respect to their solubility in oil and water which can be expressed and quantified as their hydrophilic-lipophilic balance or HLB value.

Sugar fatty acid esters offer a wide range of HLB values (see Table 1), ranging from zero to twenty, depending on (i) the alkyl chain length of acyl group, (ii) the degree of substitution or the number of ester groups per molecule, (iii) the degree of unsaturation of the acyl chain (Gupta, 1983). The behavior of sugar fatty acid esters is caused by the presence of the hydrophilic free hydroxyl groups and the hydrophobic alkyl chains.

Table 1 Hydrophilic-Lipophilic Balance (HLB) of some sugar fatty acid esters (herbaria, 2009)

Compounds	HLB value*
Sorbitan trioleate	1.8
Sorbitan tristearate	2.1
Sorbitan monoleate	4.3
Sorbitan monostearate	4.7
Sorbitan monopalmitate	6.7
Sorbitan monolaurate	8.6
Maltose monostearate	11.2
Maltose monopalmitate	11.8
Maltose monomyristate	12.4
Sucrose monostearate	11.2
Sucrose monopalmitate	11.8
Sucrose monomyristate	12.4

*HLB = (L/T) x 20 (L and T referred to the molecular weight of the hydrophilic part of the molecule and the total molecular weight, respectively)

2.1
Sucrose based emulsifiers

Sucrose fatty acid esters classified as nonionic surfactants are widely used in foods, cosmetics and pharmaceuticals as emulsifiers (Allen and Tao, 1999). Sucrose esters were approved in 1959 for use as food additives in Japan (Polat and Linhardt, 2001) and subsequently found worldwide approval for a wide range of applications including personal care products, cosmetic applications and food emulsifiers (Hill and Rhode, 1999; Garti *et al.*, 2000). The Acceptable Daily Intake (ADI) of sucrose esters is 0-2.5 mg/kg body/day (Lauridsen, 1976). The properties of sucrose esters can range from water soluble surfactants (high HLB, most hydrophilic) to oil soluble surfactants (low HLB, most hydrophobic) (see Table 2).

Table 2 Approximation of HLB values of surfactants as a function of their solubility in water (Sartomer, 2009)

Solubility in water	HLB value*	Description
Insoluble	4-5	Water in oil emulsifier
Poorly dispersible (milky appearance)	6-9	Wetting agent
Translucent to clear	10-12	Detergent
Very soluble	13-18	Oil in water emulsifier

*HLB = (L/T) x 20 (L and T referred to the molecular weight of the hydrophilic part of the molecule and the total molecular weight, respectively)

2.2
Synthesis of sucrose fatty acid esters

Chemical catalysts are generally used for the synthesis of sucrose esters for example, sodium methoxide, or alkaline catalysts. However, the alkaline catalyzed transesterification reaction normally proceeds faster than the acid catalyzed counterpart (Freedman e*t al.*, 1986). With potassium carbonate as catalyst, the synthesis of sucrose fatty acid esters by tranesterification with methy fatty acid esters has been carried out at 90 °C at a pressure of 80-100 mmHg in DMF for 9 to 12 hrs obtaining a mixture of sucrose fatty acid esters ranging from mono-, to pentaesters. As sucrose fatty acid esters containing one to three fatty acids are approved for a range of industrial applications, several methods have been developed to achieve reactions with a higher selectivity to provide a high amount of monoesters. Using potassium carbonate as the catalyst and propylene glycol as a solvent, 85% yield of sucrose monoesters and 15% yield of for sucrose diesters were obtained. In this reaction, the product itself formed an emulsion to conduct the reaction in a developing micro-emulsion system (Osipow and Rosenblatt, 1967). Recently, Cruces *et al* (2001) reported 72% yield of 2-*O*-lauroyl sucrose obtained using Na_2HPO_4 as catalyst at 40 °C for 5 hrs in DMF with vinyl laurate as acyl donor.

Alternatively biocatalysts, mainly proteases and lipases, can be used for the synthesis of sucrose fatty acid esters in the organic solvents with the main advantages of their selectivity, especially for the synthesis of mono-*O*-acyl sucrose (see Table 3).

Table 3 Enzyme catalyzed acylation of sucrose in organic solvents

Enzyme	Solvent	Acylating agent (Acyl chain length)	Acylation position on sucrose molecule	Reference
Protease N	DMF	Vinyl ester (C8)	1’	Carrea *et al.*, 1989
Protease N	DMF	Methyl ester (C8-C12)	1’	Potier *et al.*, 2001
Subtilisin	DMF	2,2,2-trichloroethyl butyrate (C4)	1’	Riva *et al.*, 1988
Subtilisin BPN’	Pyridine	Vinyl ester (C2-C10)	1’, 6	Rich *et al.*, 1995
Subtilisin Carlsberg	Pyridine	Vinyl ester (C2-C10)	1’,6	Rich *et al.*, 1995
Subtilisin	Pyridine	Vinyl ester (C12-C18)	1’	Polat *et al.*, 1997
Thermolysin	DMSO	Vinyl laurate (C12)	2	Pedersen *et al.*, 2002
Alkaline protease AL 89	DMF:DMSO (1:1 v/v)	Vinyl ester (C10-C18)	2	Ritthitham *et al.*, 2009
Candida antarctica lipase B	*t*-butanol	Ethyl butyrate (C4)	6, 6’	Woudenberg *et al.*, 1996
Candida antarctica lipase B	*t*-pentanol-Pyridine (11:9 v/v)	Vinyl stearate (C18)	6, 6’	Ritthitham *et al.*, (Paper IV)

2.3
Applications of sucrose fatty acid esters

Sucrose fatty acid esters have been commercially and extensively used in foods where they improve the emulsion stability and the textural properties (Farooq and Haque, 1992). Phosphorylated sucrose stearate prepared by dry-heating sucrose stearate with metaphosphoric acid showed a higher solubility and better emulsifying properties than sucrose stearate and improved the thermal behavior

of potato starch by increasing the gelatinization temperature, decreasing the viscosity and inhibiting retrogradation (Yamagishi *et al.*, 2004).

Mono-*O*-lauroyl sucrose was proved to affect cancer cell growth as the antitumor activity was shown both *in vivo* and *in vitro* (Kato *et al.*, 1971) and it could inactivate the food pathogenic bacteria *Escherichia. coli* O157:H7. Moreover, a synergistic inhibitory effect with heat in the presence of EDTA was observed (Hathcox and Beuchat, 1996). 6-*O*-lauroyl sucrose at 1 g/l completely inhibited the growth of *Streptococcus sobrinus* (Devulapalle *et al.*, 2004).

In pharmaceutical applications, sucrose stearate and sucrose palmitate were used as a tablet matrix forming agent in order to control the dissolution rate of drug release (Ntawukulilyayo *et al.*, 1995).

Olestra, a sucrose-based fat substitute (non caloric fat) containing six or more fatty acids per sucrose molecule, has been developed by Proctor and Gambel in the early 1970s and marketed under the brand name Olean (Stauffer, 1999). The nutritional properties are similar to triglycerides, but it is not digestible by lipolytic enzymes.

3.
Biocatalyst in organic solvents

In the present work, two different serine hydrolases were used as biocatalysts for the synthesis of *O*-acyl sucrose, the alkaline protease from *Bacillus pseudofirmus* AL 89, classified as a member of the subtilisin-like protease family and the commercial lipase from *Candida antarctica* lipase B: Novozym 435.

3.1
Subtilisin (EC 3.4.21.14)

Subtilisins belong to the clan (or superfamily) of subtilisin-like serine proteases and have been classified into the following six families based on their amino acid sequences: subtilisin, thermitase, proteinase K, lantibiotic peptidase, kexin (pro-protein convertases), and pyrolysin. Subtilisins are produced only by microorganisms, mainly from *Bacillu*s strains and their molecular weight ranges from 15 to 30 KDa with few exceptions, like a 90 KDa subtilisin from *Bacillus subtilis* (natto) (Kato *et al.*, 1992). Based on sequence alignment of the catalytic domain (Siezen and Leunissen, 1997), subtilisin family proteases are sub-grouped into three subfamilies: true subtilisin (> 64% sequence identity within catalytic domain), high-alkaline protease (> 55% sequence identity within catalytic domain), and intracellular protease (> 37% sequence identity within catalytic domain). The subfamily of true subtilisin and alkaline proteases are industrially important biocatalysts utilized in laundry detergents and as biocatalysts for the synthesis of carbohydrate esters in organic solvents.

The secondary and tertiary structure of subtilisin has been determined by X-ray crystallography, showing a globular protein with α-helixes and a large β-sheet. Generally, the fold of subtilisins consists of 8 α-helixes and 9-11 β-strands with the calcium ion binding sites (Nonaka *et al.*, 2004). The N-terminal amino acid sequences of the subtilisins (see Fig 1) start with alanine (Yamagata *et al.*, 1995).

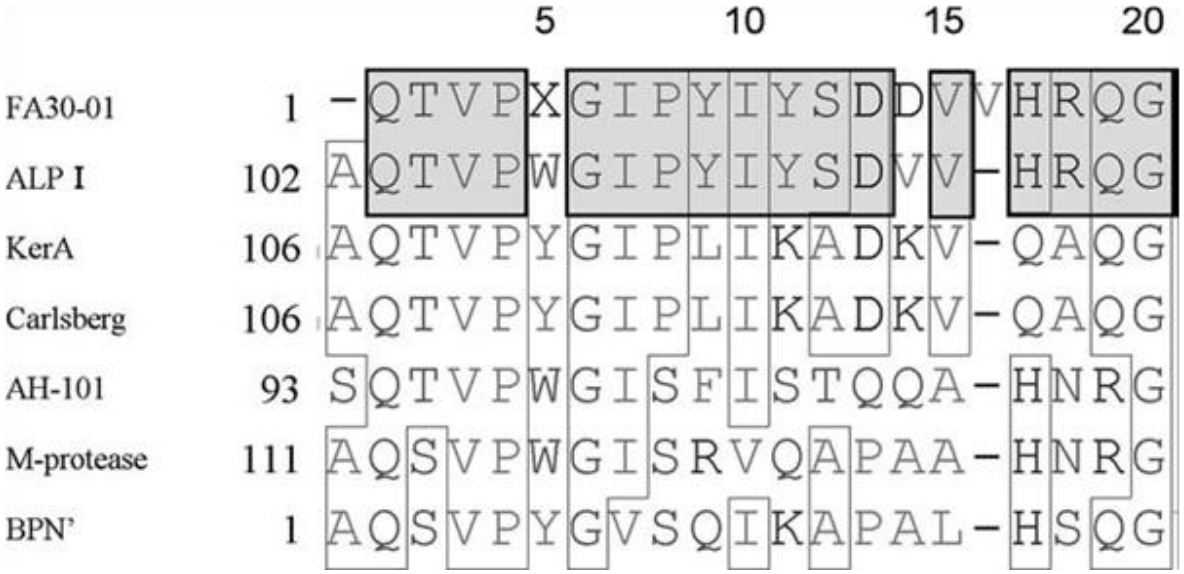


Fig 1 **Alignment of the N-terminal sequence of subtilisin ALP (I), Subtilisin Carlsberg (Carlsberg), alkaline protease (AH101), alkaline protease (M-protease) and subtilisin BPN' (BPN') (Kojima *et al.*, 2006)**

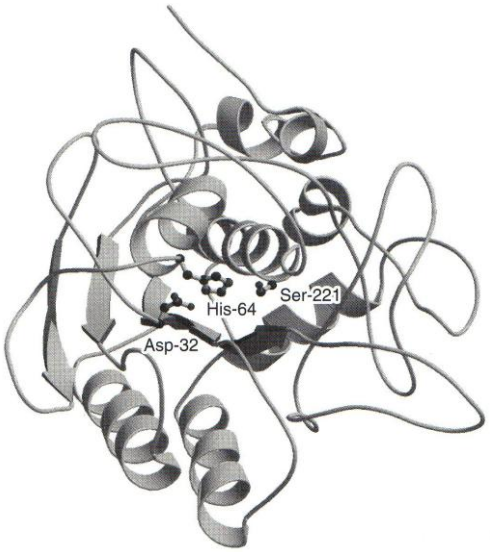


Fig 2 **3-D structure of subtilisin showing the residues of the catalytic triad, Ser 221, His 64, Asp 32 (Arnorsdottir *et al.*, 2005)**

The active site region of the mature protease is well conserved in all subtilisins and contains the catalytic triad with three essential amino acids: Asp32, His 64 and Ser 221 (see Fig 2). The catalytic mechanism involving the serine residue is composed of two steps: acylation and deacylation (see Fig 3). In the acylation step, serine reacts with the substrate and subsequently forms an ester bond leading to the acyl-enzyme-intermediate and the formation of free alcohol. In the deacylation step, an alcohol attacks the intermediate. The ester product is released and the enzyme turns to its original state (Jaeger and Rietz, 1998). The histidine residue has a dual role: first, it accepts a proton from serine to facilitate formation of the ester bond, and secondly, it stabilizes the negatively charged transition state. By stabilizing the positive charge of histidine, the aspartic acid residue contributes to a rate enhancement in the order of 10^4 (Branden and Jooze, 1999).

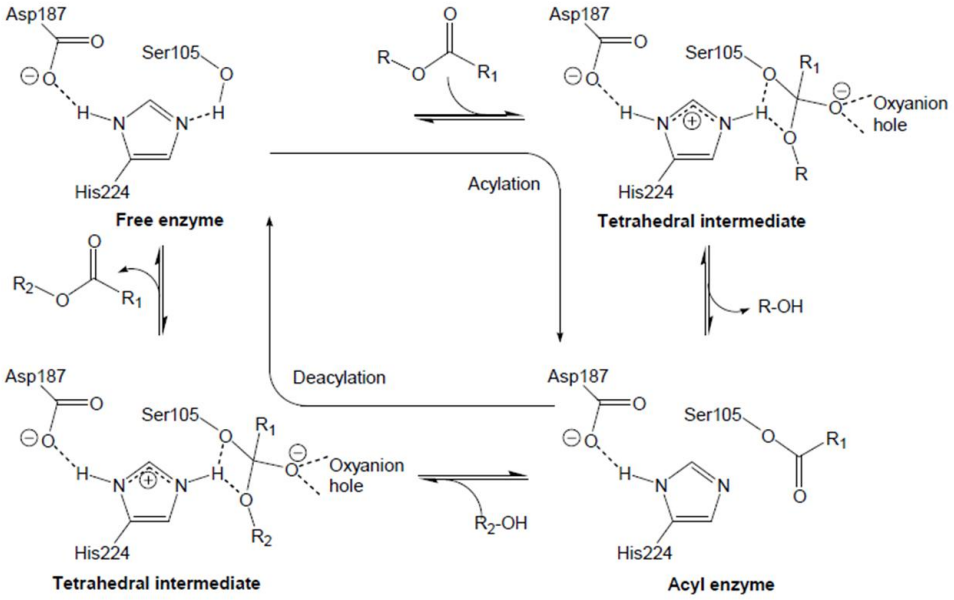


Fig 3 Catalytic mechanism of serine hydrolases involving the catalytic triad (Ottosson, 2001)

Subtilisins have been successfully used for the regio-selective acylation of carbohydrates in anhydrous media. The transesterification of sucrose with fatty acid esters using subtilisin as a catalyst showed a high regio-selectivity toward formation of 1'-*O*-acyl-sucrose (experimentally 80-90% of the product). Formation

of an ester bond by transesterification of sucrose with vinyl laurate involving the catalytic triad of the subtilisin active site (see Fig 4) demonstrated that the 1'-OH was preferred to adducts formed at the other OH-groups. Thus, the 1'-OH adduct was the least sterically constrained when exposed to the reaction media and the energy driving the reaction was proved to depend on entropic factors (Fuentes *et al.*, 2002).

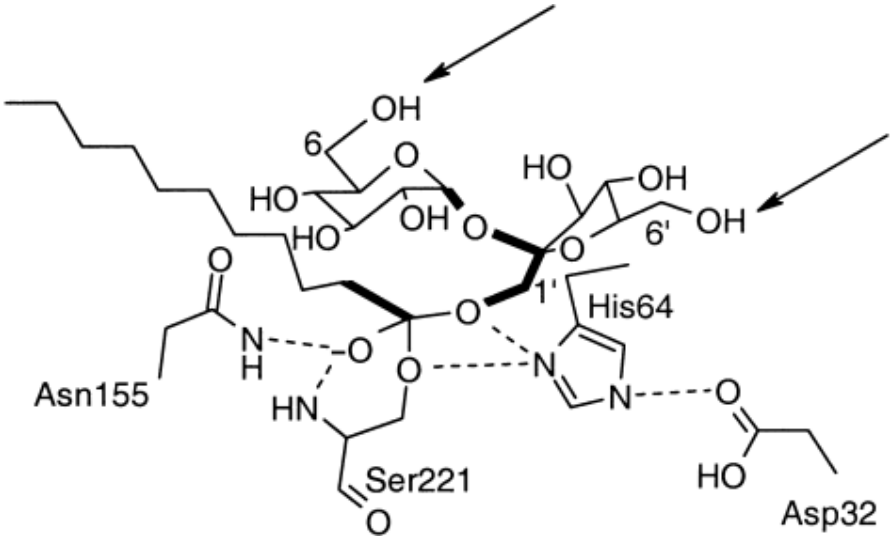


Fig 4 Active site of subtilisin (catalytic triad and oxyanion hole) and its interaction with sucrose (6-OH, 6'-OH and 1'-OH) in the process of transesterification of sucrose with vinyl laurate (Fuentes *et al.*, 2002).

3.2

Bacillus pseudofirmus AL 89 alkaline protease

The alkaline protease AL 89 was the biocatalyst used for the investigation of *O*-acyl sucrose synthesis in hydrophilic organic solvents. The enzyme was produced by *Bacillus pseudofirmus* strain AL 89 which was isolated from soil samples at a Soda Lake in Ethiopia. With respect to proteolytic activity in aqueous solution, the enzyme proved to be highly active at pH 11.0 and 60 $^{\circ}$C (Gessesse *et al.*, 2003). At temperatures above 60 $^{\circ}$C, Ca^{2+} was shown to improve its stability. The enzyme was classified as a member of subtilisin family with a

molecular weight of 24 kDa. Alignment of the purified protease AL-89 protein sequence compared to the subtilisin ALP I sequence showed the 87% homology to subtilisin ALP I pre-pro sequence (see Fig 5). Ser 50 and Asp 185 of subtilisin ALP I sequence was substituted with Pro 50 and Ser 185 on the protease AL 89 sequence, respectively (Fallesen, 2009).

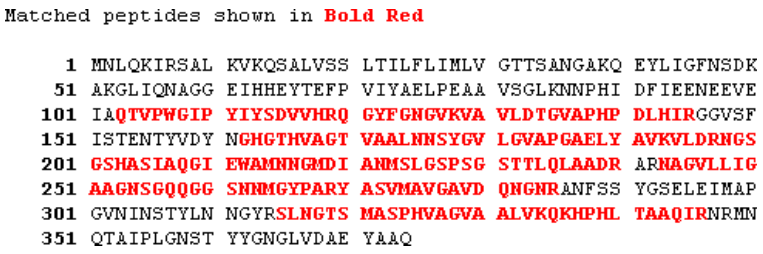


Fig 5 Alignment sequence of alkaline protease AL 89. The bold red represented the peptide matched with subtilisin ALP I (Fallesen, 2009)

The alkaline protease AL-89 proved to be catalyzing for the synthesis of 2-*O*-lauroyl sucrose in hydrophilic organic solvents. The synthetic activity of alkaline protease AL 89 depended on the pH in the purification of the enzyme preparation. After the concentrated enzyme was dialyzed against milliQ water, the pH of the enzyme solution was adjusted with NaOH from pH 6.0 to pH 10.0. The pH-adjusted enzyme solution was then solidified by lyophilization. The lyophilized enzyme preparation was employed for the synthesis of sucrose laurate by performing the synthetic reaction in different hydrophilic solvents and the results showed that lyophilized enzyme at pH 6.0 could not catalyze the ester synthesis reaction while a high concentration of 2-*O*-lauroyl sucrose was obtained with the lyophilized-enzyme at pH 10.0 in the reaction system of DMF: DMSO (1:1 v/v) (Pedersen *et al.*, 2003). This supported the findings of Schulze and Klibanov (1991) that the enzymes working in organic solvents were profoundly affected by pH of the aqueous solution from which they were solidified. This phenomenon has been referred as “pH memory”, indicating that enzymes maintain the conformation induced by the pH at which they were lyophilized.

In this work, the alkaline protease AL 89 was prepared by dialyzing the concentrated enzyme solution against 10 mM sodium carbonate buffer pH 10.0 and subsequently lyophilized for 3 days. The residual proteolytic activity assayed in aqueous conditions after dissolving the enzyme in hydrophilic organic solvents was investigated for the estimation of inactivating rate and the half-life ($t_{d½}$).

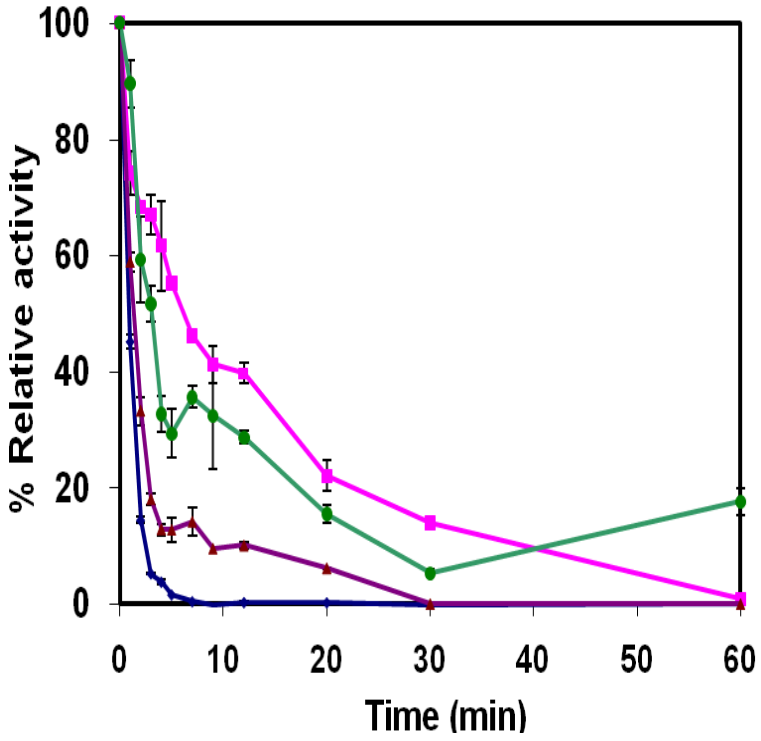


Fig 6 **Proteolytic activity assayed in 10 mM Tris-HCl buffer pH 10.0 with azocasein as a substrate at 60 °C. Protease AL 89 (10 g/l) was dissolved in different organic solvents and subsequently transferred to aqueous buffer solution at time interval for the activity assay.**

Symbols: DMF-DMSO (1:1 v/v) solvent mixture (-▲-), DMF (-■-), MilliQ water (-●-), DMSO (-♦-)

The operational stability of enzymes in organic media is an important factor. With regard to the use of biocatalysts in organic synthesis; therefore, the enzymes should be sufficiently stable during the reaction process. The results presented in Fig 6 demonstrated that the enzyme stability in DMF was higher than in water with the half life of $(t_{d½})_{DMF}$: 10 min and $(t_{d½})_{water}$: 4 min, while the highest inactivating rate was observed in DMSO with the half life of $(t_{d½})_{DMSO}$: 1 min.

Enzyme-water interactions contribute to the efficiency of catalysis in organic solvents and there are two distinct forms of water in a lyophilized enzyme preparation: tightly and loosely bound. Tightly bound water does not exchange with other water molecules in the enzyme or in the bulk organic solvent and is necessary to maintain the enzyme conformation and catalytic activity. The displacement of tightly bound water by organic solvent results in a dramatic change of protein structure resulting in denaturation. Loosely bound water is necessary for enhancing the enzyme activity by increasing enzyme flexibility and active site polarity (Clark, 2004). Thus, adding the water into the reaction could improve the enzyme activity. The activity and stability of lyophilized subtilisin BPN' dissolved in the polar solvent THF was improved 4 times when addition of 0.2% water (Wangikar *et al.*, 1997), while the stability of subtilisin Carlsberg was improved in *tert*-amyl alcohol containing 2% of water (Schulze and Klibanov, 1991).

3.3
Candida antarctica Lipase B (EC. 3.1.1.3)

Lipases (triacylglycerol acylhydrolases) are part of the hydrolase family that act on fatty acid carboxylic ester bonds hydrolyzing long-chain triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol (see Fig 7). The characteristic folding pattern of lipases is known as the α/β hydrolase fold (Ollis *et al.*, 1992) and composed of a central β-sheet up to eight different strands (β1-β8) connected by up to six α-helices (Jaeger and Reetz, 1998).

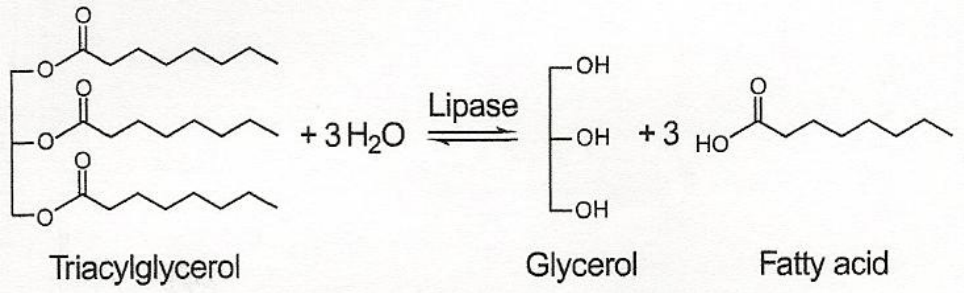
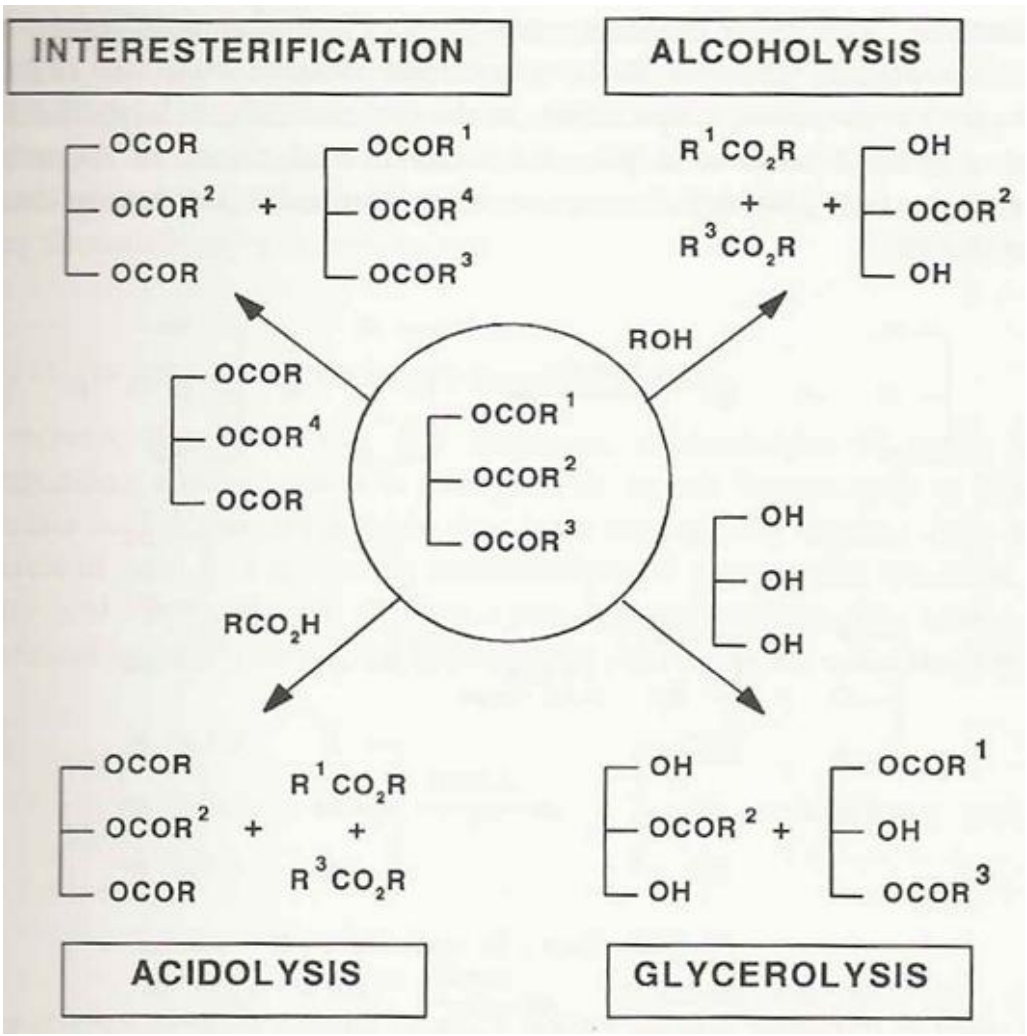


Fig 7 Catalytic action of lipases hydrolyzing a triglyceride to glycerol and free fatty acids and the reverse reaction (condensation) to form the triglyceride (Jaeger and Reetz, 1998)

By controlling the activity of water and reactants in the process, esterification, interesterification, acidolysis, alcoholysis and aminolysis can be the dominating reaction (see Fig 8). Thus, lipases are versatile biocatalysts and industrially important in the synthesis of value-added products as they usually proceed the reaction with high regio-and/or enatioselectivity which can not be obtained by chemical methods (Houde *et al.*, 2004).



.

Fig 8 Synthetic reactions catalyzed by lipase in low water condition (Koskinen and Klibanov, 1996)

Lipases act at the interface between an insoluble substrate phase and an aqueous phase in which the enzyme is dissolved (oil-water interface). According to their conformations, the lipases are classified into two different forms, namely open (active) and closed (inactive) (see Fig 9). The active site of lipases is covered by a lid-like structure resulting in inaccessibility of the substrate to the active site (closed form). The active site becomes fully accessible above the critical micellar concentration (CMC) of the substrate (Reis *et al.*, 2009; Geraldine *et al.*, 2008). In the presence of substrate (opened form), the lipase is bound to an interface and the interfacial activation takes place by the movement of a lid. The hydrophobic pocket of the lid thus expose to the hydrophobic phase (lipid phase), which enhances hydrophobic interactions between the enzyme and the lipid surface. The interfacial activation is unique to the class of lipases and responsible for the synthetic reactions they catalyze. Moreover, the amino acid sequences of the lid also affect the lipase activity and enantioselectivity in organic solvents (Secundo *et al.*, 2006).

The yeast *Candida antarctica* isolated from a sample from Antarctica produced two lipases designated A and B, which exhibit different characteristics as presented in Table 4 (Kirk and Christensen, 2002). The primary structure of *Candida antarctica* lipase B (CALB) consists of a single polypeptide chain of 317 amino acid residues with a molecular weight of 33 kDa, which is a fairly small protein compared to other lipase (Uppenberg *et al.*, 1994). The catalytic triad results from Ser105, His224, Asp187 with the mechanism of serine hydrolases; however, the sequence of the catalytic triad residues is arranged as the mirror image of the catalytic triad of the serine protease (see Fig 10).

Table 4 Characteristics of *Candida antarctica* lipase A (CALA) and *Candida antarctica* lipase B (CALB) (Kirk and Christensen, 2002)

	Candida antarctica lipase A	*Candida antarctica* lipase B
Molecular weight (kD)	45	33
Isoelectric point (pI)	7.5	6.0
pH optimum	7.0	7.0
pH stability	6-9	7-10
Interfacial activation	yes (but low)	No
Positional specificity toward triglycerides	*Sn*-2	*Sn*-3

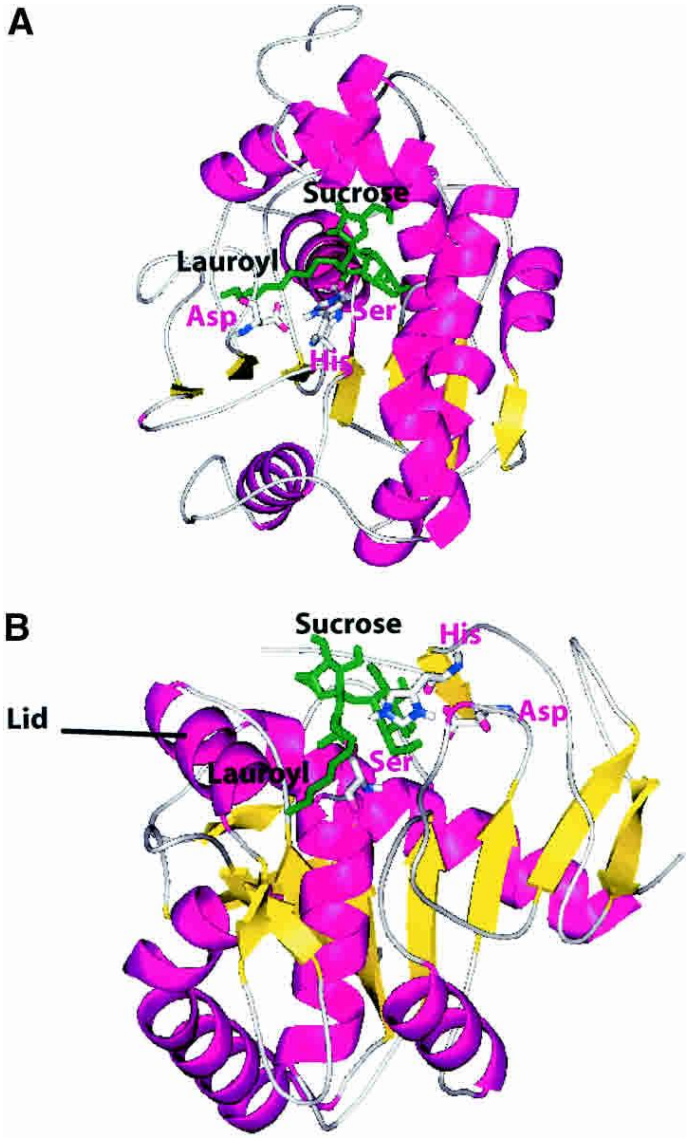


Fig 9 Three-demensional (3D) structures of lipase in the transesterification reaction of sucrose with vinyl laurate (Fuentes *et al.*, 2004)

A) *Candida antarctica* lipase B (CALB), showing the opened form

B) *Thermomyces (Humicola) lanuginosus* lipase (TIL), showing the closed form with lid

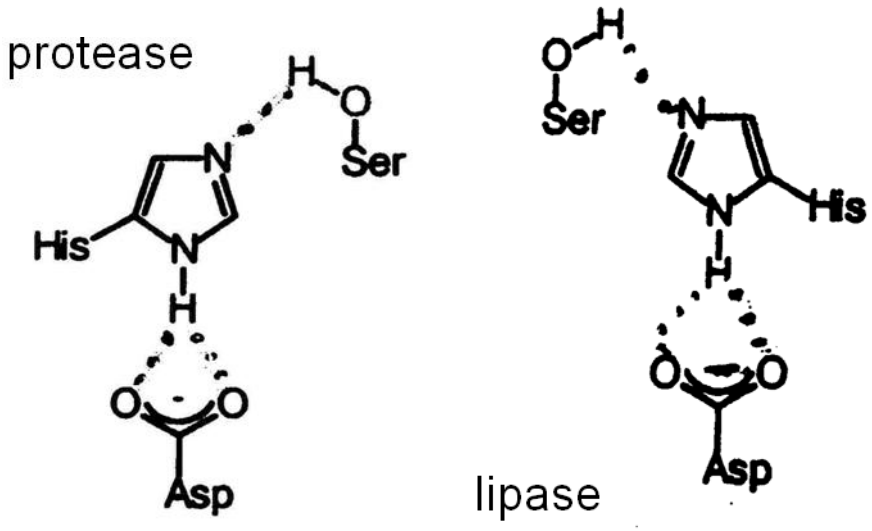


Fig 10 Mirror image of the catalytic triad of serine protease (Rantwijk, 2005)

Contrary to most other lipases, CALB lacks the lid that regulates the access to the active site, thus it does not display the interfacial activation, or the increasing of the activity caused by exposure to a water-lipid interphase (Martinelle *et al.*, 1995). Moreover, CALB does not contain the consensus amino sequence (GXSXG) around the nucleophilic active site serine located at the C-terminal end of β5strand. It has a threonine at the first conserved glycine and the substitution of threonine with valine by site-directed mutagenesis resulted in the loss of activity (Bornscheuer *et al.*, 2002).

CALB has proven to be a particularly useful biocatalyst in organic chemistry in the preparation of regio-isomeric and enantio-isomeric products as it provides a good stability and activity catalyzing a diverse range of reactions (Anderson *et al.*, 1998; Berglund, 2001). The regioselective synthesis of *O*-acyl sucrose by a CALB-catalyzed process in organic media leads to a mixture of 6-*O*-acyl sucrose and 6'-*O*-acyl sucrose (Wongdenberg *et al.,* 1996; Paper IV Ritththam *et al.*, 2009).

4.
Detection and characterization of *O*-acyl sucrose

The methods used for qualitative and quantitative determination, and preparation of *O*-acyl sucrose for structural characterization are presented in this chapter.

4.1
Thin layer chromatography (TLC)

Thin layer chromatography (TLC) has been widely used as a preliminary qualitative technique for the analysis of carbohydrates and their derivatives to give an impression of the reaction. This technique is considered as a rapid, advantageous and simple method for the preliminary investigation of sucrose fatty acid ester synthesis. The samples are directly applied to the chromatographic plate without any pretreatments and run in a closed chamber often for a short period of time. The resolution depends on solvent systems adaptable to the sorbents used (Ghebregzabher *et al.*, 1976).

The separation of acetyl and benzoyl derivatives of sugars on TLC glass plate has been described by Deferrari *et al.,* (1962). The TLC plates coated with a mixture of silicic acid with 10% starch as binder were developed by the ascending method using a methanol-benzene solvent system as a mobile phase. After the evaporation of mobile phase, the dried plate was sprayed with silver nitrate-ammonia sodium-methylate reagent and heated at 110 °C for 10 min to develop the brown spots which were detectable by UV absorption.

In the reactions of *O*-stearoyl sucrose synthesis as catalyzed by alkaline protease AL 89 and *Candida antarctica* lipase B, TLC was used for monitoring the synthesis and purification process. Samples of reaction mixture (5 µl) containing sucrose, sucrose fatty acid esters and biocatalyst were applied directly to a commercial aluminum TLC plate coated with silica gel (Merck, Germany) and separated according to the mobility in the mobile phases

consisting of chloroform and methanol (5:1, v/v). To localize the sucrose fatty acid ester and calculate the R_f values, the TLC plate was sprayed with sulphuric acid (conc) and methanol (1:1, v/v) and subsequently heated for 5 min at 150 °C. Sucrose and sucrose fatty acid esters obtained from enzymatic synthesis showed up as carbon black spots (see Fig 11 and Fig 12).

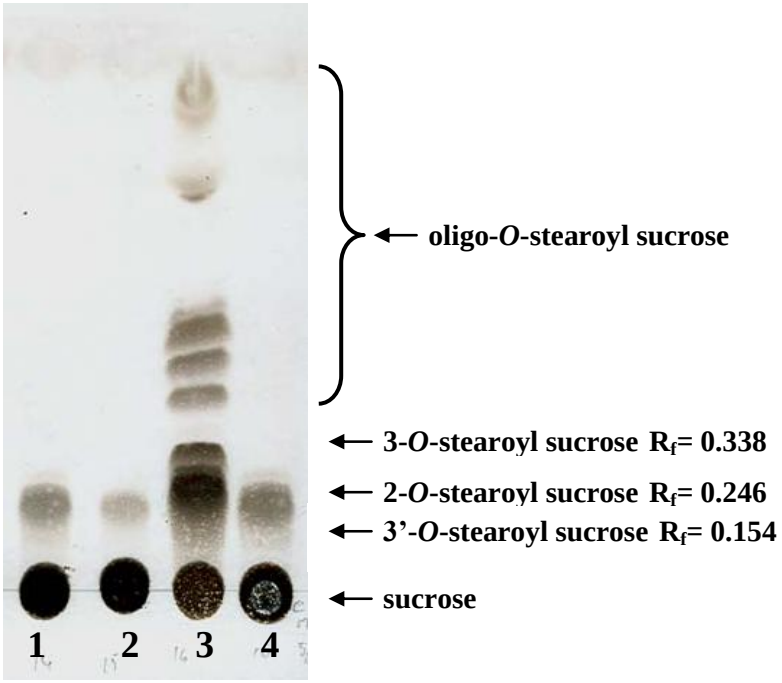


Fig 11 Analytical TLC plate of the reaction mixture for sucrose fatty acid ester synthesis as catalyzed by alkaline protease AL-89 in DMF-DMSO (1:1 v/v). The reaction mixture (0.2 M sucrose, 0.2 M vinyl sterate, 10 g/l lyophilized enzyme) was incubated at 45 °C, 250 rpm for 24 hr. Mobile phase was chloroform : methanol (5:1 v/v)

Lane 1 Control (only substrate)

Lane 2 Control with 10 g/L Casein (instead of enzyme) treated with 10 mM Sodium carbonate buffer pH 10.0

Lane 3 Reaction with 10 g/L alkaline protease AL89 dialyzed against 10 mM Sodium carbonate buffer pH 10.0

Lane 4 Control with 10 g/L Celite (instead of enzyme) treated with 10 mM Sodium carbonate buffer pH 10.0

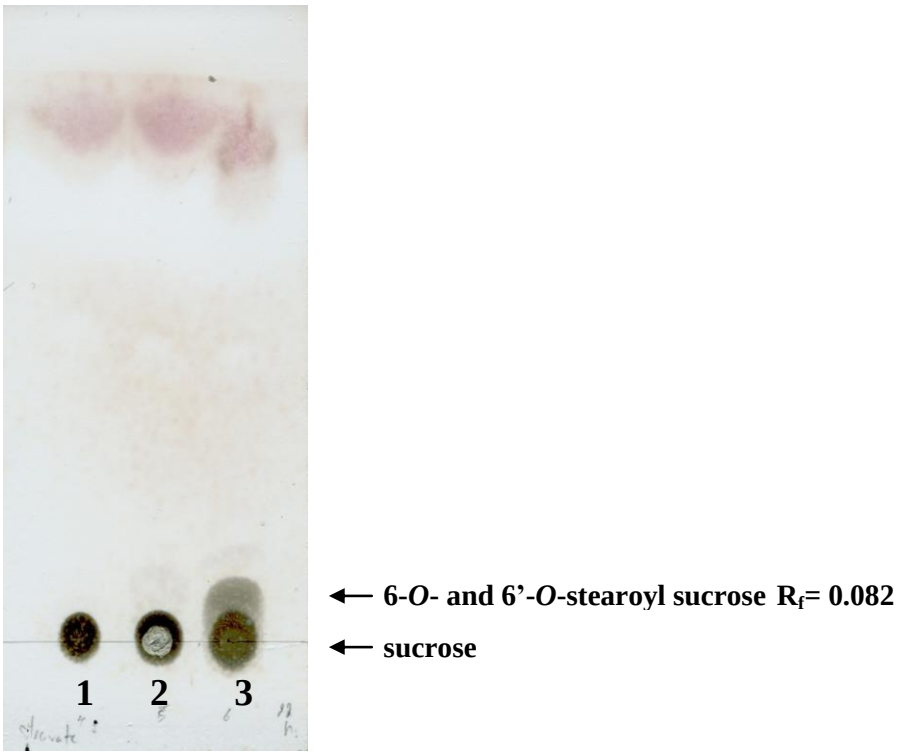


Fig 12 Analytical TLC plate of the reaction mixture for sucrose fatty acid ester synthesis as catalyzed by *Candida antarctica* lipase B in 45%Pyridine in 2-methyl-2-propanol (v/v). The reaction mixture consisting of 0.03 M sucrose 0.1M vinyl state, 20 g/L CALB, 20 g/L molecular sieve was incubated at 50 °C, 250 rpm for 48 hr. Mobile phase was chloroform : methanol (5:1 v/v)

Lane 1 Control (substrate incubated at the same condition)
Lane 2 Reaction with celite (instead of enzyme) treated with 10 mM Sodium carbonate buffer pH 10.0 (100 g/l)
Lane 3 Reaction with *Candida antarcitca* lipase B

4.2
Preparative thin layer chromatography

For milligram scale preparation of *O*-stearoyl sucrose for further structural analysis, preparative TLC was performed by applying the reaction mixture directly to the TLC glass plate (20 x 20 cm, 1500 microns thickness) with a loading volume of 500 µl and the mobile phase consisting of chloroform : methanol (5:1 v/v). To visualize the location of *O*-stearoyl sucrose on the TLC plate, a small strip of the TLC plate was developed.

The part of the stationary phase containing the purified esters was scrapped off and ground into fine powder. The esters were then extracted by chloroform - methanol (1:1 v/v). The purity of the extracted esters was qualitatively determined by analytical TLC (see Fig 13). The crude reaction mixture was separated by preparative TLC into seven fractions. Mono-*O*-stearoyl sucrose was distributed into 4 fractions (F1 to F4) while the oligo-*O*-stearoyl sucrose fraction were detected from F 5 to F 7.

The disadvantage to purify sucrose fatty acid esters by this technique is that it is tedious and time consuming. Furthermore, it is difficult to obtain a high purity if the bands are very close in relative mobility (R_f value).

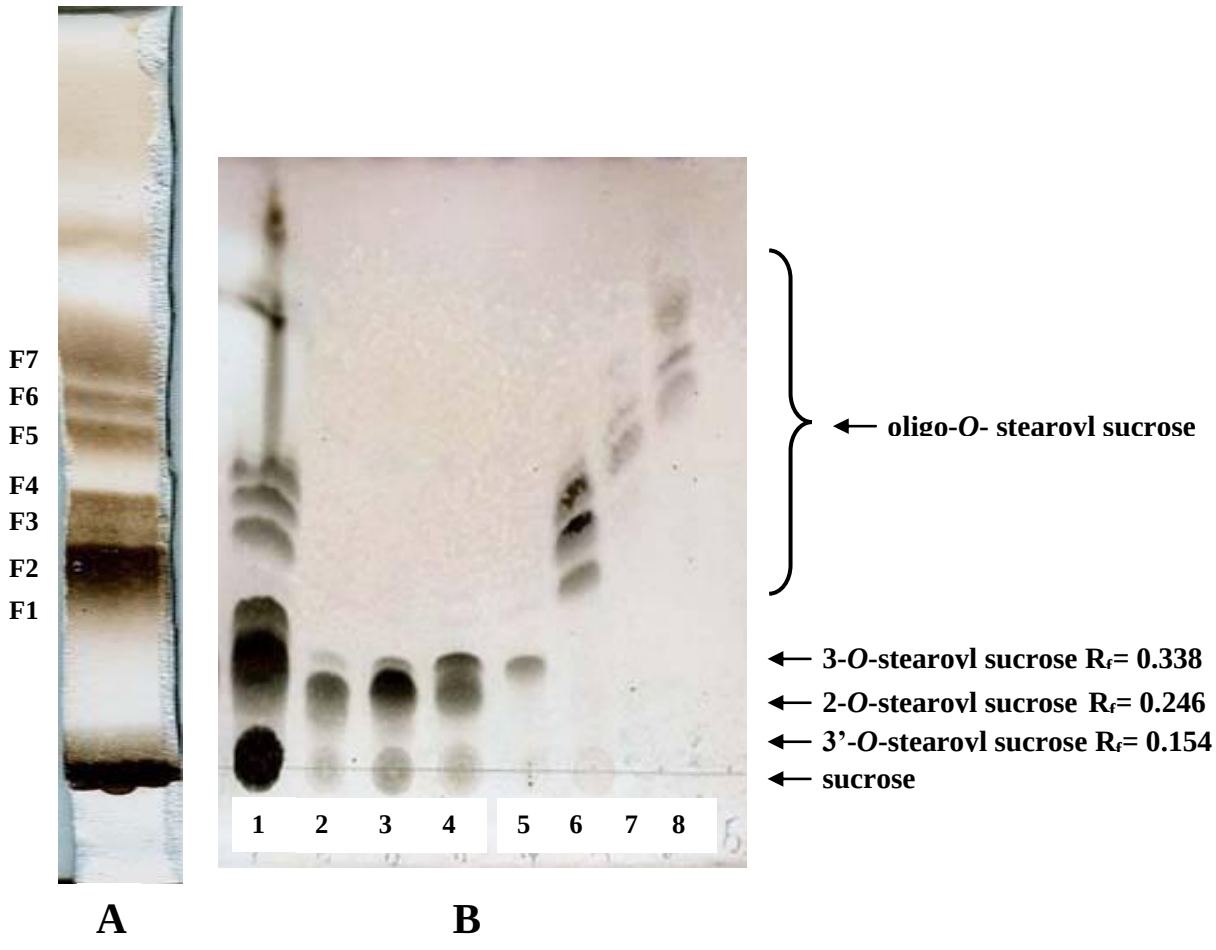


Fig 13 Separation of *O*-stearoyl sucrose on Preparative TLC (A) and the purity of purified fractions as determined by analytical TLC (B). Mobile phase was chloroform : methanol (5:1 v/v).

Lane 1 Crude reaction mixture

Lane 2-8 Purified fraction 1 to fraction 7

4.3

High Pressure Liquid Chromatography (HPLC)

HPLC is an efficient tool for analyzing and quantifying sucrose fatty acid esters in a reaction mixture. This technique is more convenient than gas chromatography as it does not require previous derivatization of the sample (Karrer and Herberg, 1992). Many procedures for sucrose fatty acid ester separation with HPLC were reported with the different eluent systems (Cormier *et al.*, 1978; Kaufman and Garti, 1981; Jasper *et al.*, 1987). In this work, a reversed phase C-18 Chromolith column packed with monoliths rod particles of highly porous silica was connected to the Evaporative Light Scattering Detector (ELSD). The mobile phase of acetonitrile in water flowed at the rate of 2 ml/min with a gradient system developed according to the acyl fatty acid chain length (see Table 5).

Table 5 Gradient of acetonitrile in water (%) for the analysis of *O*-acyl sucrose with RP-HPLC

Time (min)	Acyl fatty acid chain length C10-C12	Acyl fatty acid chain length C14-C18
0	20	50
1	20	50
7	55	80
8.5	100	100
12	100	100

HPLC analysis of the reaction mixture revealed mono-*O*-acyl sucrose product peaks of 3'-*O*-, 2-*O*-, and 3-*O*-acyl sucrose as identified by NMR (see Fig 14). Oligo-*O*-acyl sucrose was detected in the crude reaction mixture with the acyl chain lengths of 10 (vinyl caprate) and 12 (vinyl laurate).

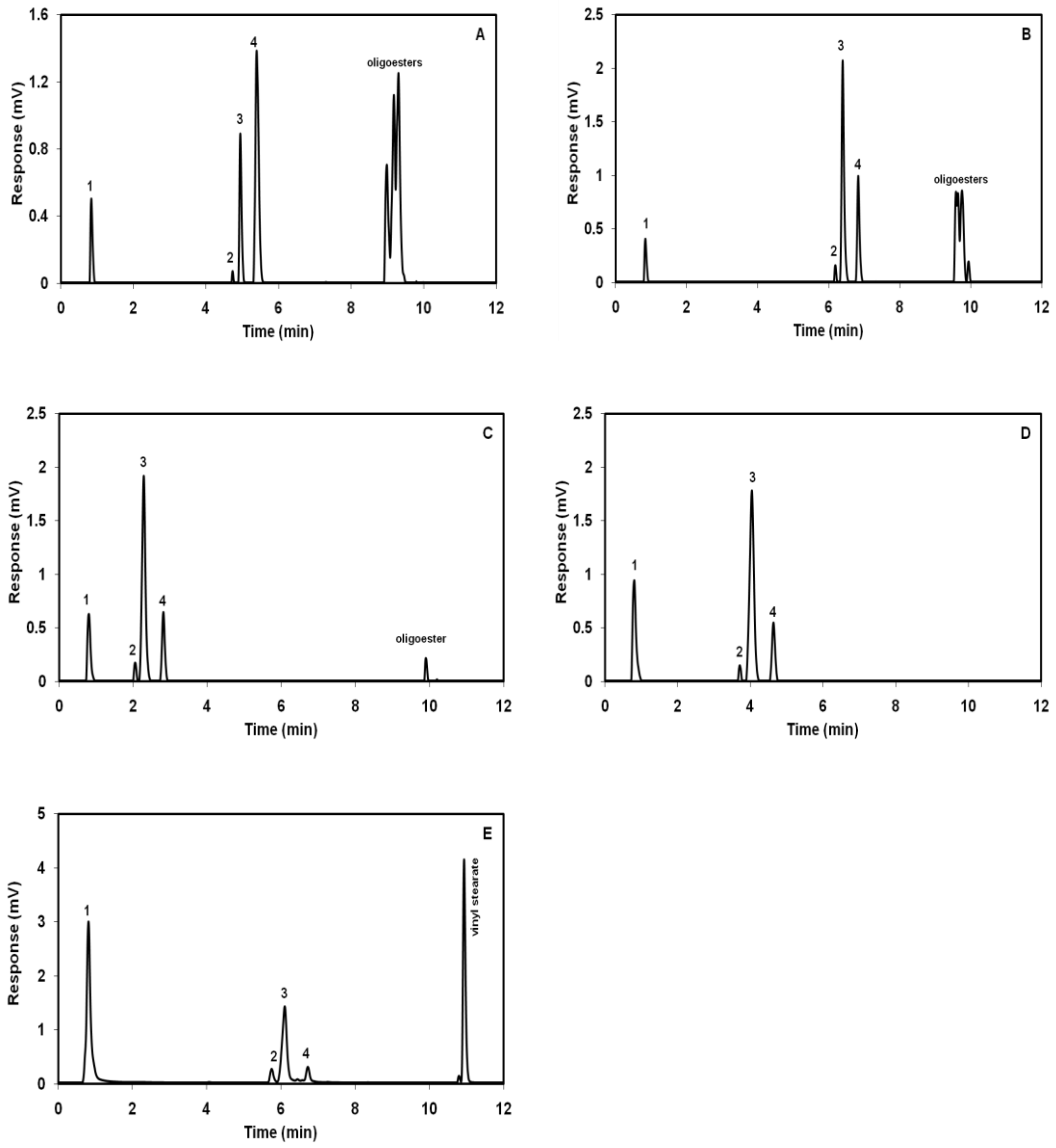


Fig 14 HPLC chromatogram of the crude reaction mixture from the synthetic reaction as catalyzed by alkaline protease AL-89 in DMF and DMSO (1:1 v/v) with the acyl donor of vinyl caprate (A), vinyl laurate (B), vinyl myristate (C), vinyl palmitate (D) and vinyl stearate (E). Chromatographic conditions: See text and Table 5

Peak identification: **1: sucrose**
2: 3'-*O*-acyl sucrose
3: 2-*O*-acyl sucrose
4: 3-*O*-acyl sucrose

Commercial sucrose fatty acid esters from Sisterna (see Table 6) were analyzed and compared with the sucrose fatty acid esters synthesized in this work. The samples analyzed in this work were Sisterna SP 70C (sucrose stearate; 70% monoester), Sisterna SP 30C (sucrose distearate; 30% monoester), Sisterna L70C (sucrose laurate; 70% monoester). The samples, without any further purification, were dissolved and appropriately diluted with methanol prior to injection for HPLC analysis. The HPLC chromatogram of Sisterna L70 C (see Fig 15, black line) compared with the sucrose laurate synthesized by protease AL 89 (Fig15, red line) showed the differences in the regio-isomeric distribution of *O*-lauroyl sucrose. In the sample of Sisterna L70 C, the 3-*O*-lauroyl sucrose was detected in a high content while the 2-*O*-lauroyl sucrose was the main ester product obtained from the enzyme catalyzed reaction. The HPLC chromatogram of Sisterna SP 70C (sucrose stearate; 70% monoester) and Sisterna SP 30C (sucrose distearate: 30% monoester), showed the mixture of *O*-palmitoyl sucrose and *O*-stearoyl sucrose (see Fig 16).

Table 6 Commercial sucrose fatty acid esters produced from Sisterna (Sisterna, 2009)

Product name	Fatty acids	INCI-name*	HLB	Physical Form	% Mono Ester
Sisterna PS750-C	**Palmitate/Stearate**	**Sucrose Palmitate**	**16**	**Powder**	**75**
Sisterna L70-C	**Laurate**	**Aqua (and) Sucrose Laurate (and) Alcohol**	**15**	**Liquid** (40% Sol.)	**70**
Sisterna SP70-C	**Stearate/Palmitate**	**Sucrose Stearate**	**15**	**Powder**	**70**
Sisterna SP50-C	**Stearate/Palmitate**	**Sucrose Stearate**	**11**	**Powder**	**50**
Sisterna SP30-C	**Stearate/Palmitate**	**Sucrose Distearate**	**6**	**Powder**	**30**
Sisterna SP10-C	**Stearate/Palmitate**	**Sucrose Polystearate**	**2**	**Powder**	**10**
Sisterna SP01-C	**Stearate/Palmitate**	**Sucrose Polystearate**	**1**	**Powder**	**0**

*International nomenclature cosmetic ingredient name

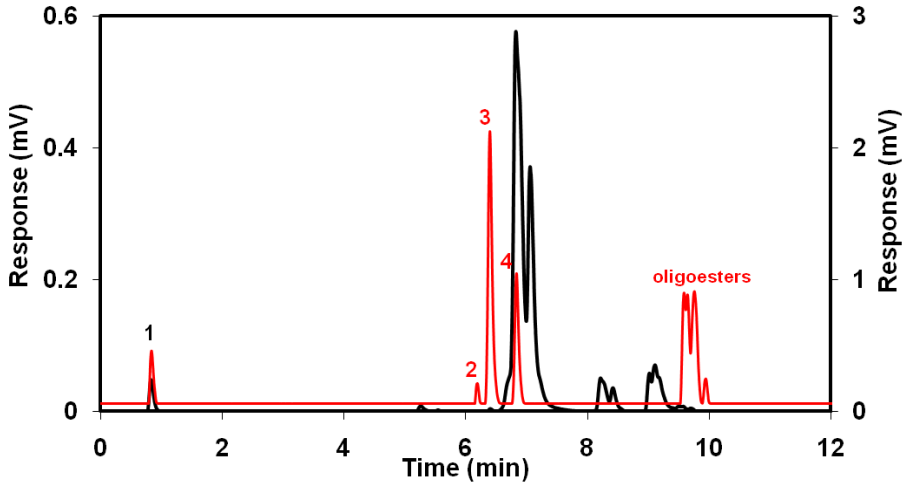


Fig 15 **HPLC chromatogram of Sisterna L70C (sucrose laurate solution, black line) compared with *O*-lauroyl sucrose synthesis as catalyzed by protease AL 89 in a solvent mixture of DMF-DMSO (1:1 v/v) (red line). Chromatographic conditions: See text and Table 5.**

Peak identification: **1: sucrose**
2: 3'-*O*-lauroyl sucrose (Rt= 6.27 min)
3: 2-*O*- lauroyl sucrose (Rt= 6.47 min)
4: 3-*O*- lauroyl sucrose (Rt= 6.91 min

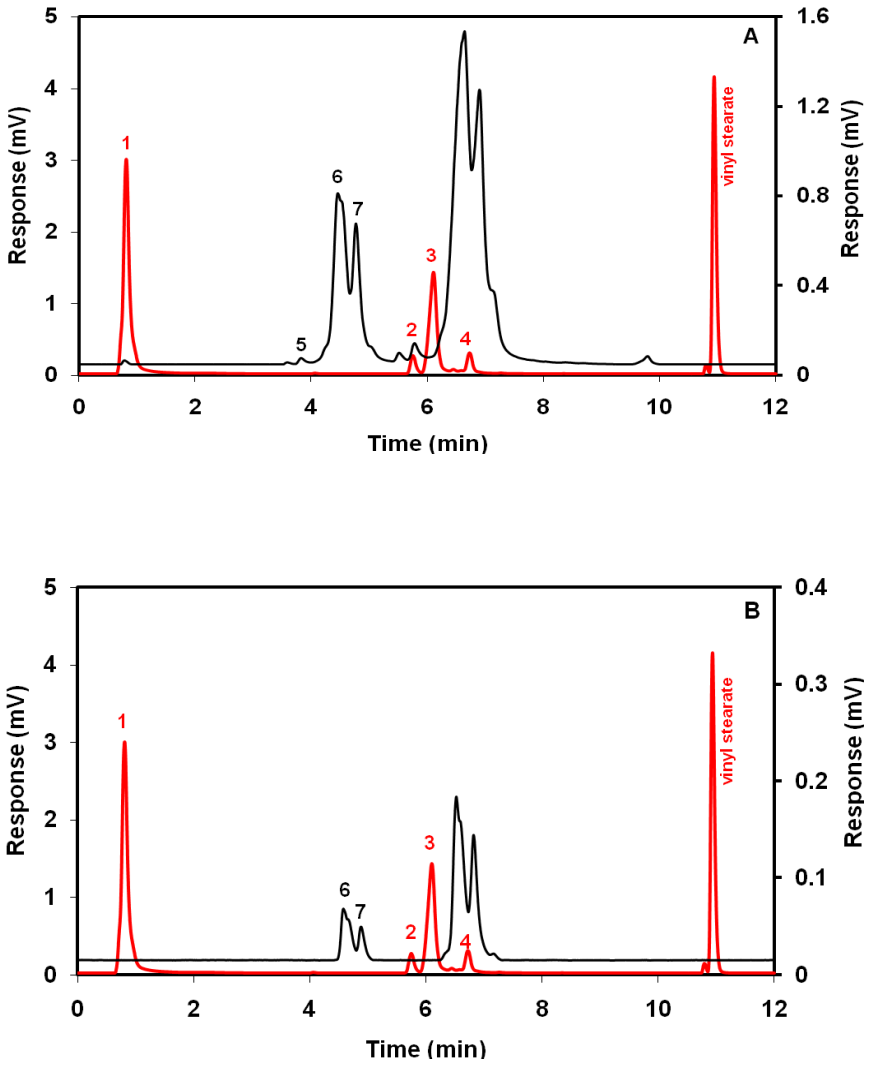


Fig 16 **HPLC chromatogram of a) Sisterna SP70C (sucrose stearate); b) Sisterna SP30C (sucrose distearate) compared with *O*-stearoyl sucrose synthesized by protease AL 89 (red line) in DMF-DMSO solvent mixture (1:1 v/v). Chromatographic conditions: See text and Table 5**

Peak identification: **1: sucrose**

2: 3'-*O*-stearoyl sucrose (Rt= 5.67 min)

3: 2-*O*-stearoyl sucrose (Rt= 6.02 min)

4: 3-*O*-stearoyl sucrose (Rt= 6.63 min)

5, 6, 7: *O*-palmitoyl sucrose (Rt= 3.75 min, 4.44 min, 4.76 min)

4.4
Mass Spectroscopy

Mass spectrometry is a technique used for analyzing the mass of atoms, molecules or fragments of molecules. The gaseous molecules are ionized and the ions are accelerated in an electric field. The mass spectrum represents the separation of ions according to their mass to charge ratio (m/z). In this work, the mass spectrometer with the electron spray ionization probe operating in the positive ionization mode (ESI MS) was used for determining the molecular mass of mono-*O*-acyl sucrose as sodium adducts (see Table 7). In principle, the sodium salts, even though having not been added to the sample, are usually detected at low concentrations originating from glassware and storage bottles or present as impurities even in analytical grade solvents (Cech and Enke, 2001).

Table 7 Molecular mass of *O*-acyl sucrose synthesized by protease AL 89 as analyzed by ESI MS

Acy donor	Molecular formula	Molecular mass Sodium Adduct
Vinyl caprate (C10)	$C_{22}H_{39}O_{12}$	514.2785
Vinyl laurate (C12)	$C_{24}H_{43}O_{12}$	547.2661
Vinyl myristate (C14)	$C_{26}H_{47}O_{12}$	575.2946
Vinyl palmitate (C16)	$C_{28}H_{51}O_{12}$	603.3303
Vinyl stearate (C18)	$C_{30}H_{55}O_{12}$	631.3794

The mass spectrum of the purified 2-*O*-stearoyl sucrose is depicted in Fig 17 with the intensity peak representing the molecular mass of 631.379 [M+Na] in the positive ionization mode. Pseudomolecular ions and very few fragments were also produced under the ESI analysis conditions since the ESI MS is a very soft ionization method (Pierez-Victoria *et al.*, 2007). The ion at m/z 365.1 was from the molecular ion [M+Na] of a sucrose moiety released from a fragmented molecule due to ester bond cleavage, which was also observed by Moh *et al.,* (2000). The sucrose spectrum and other ions involving the cleavage of the

glycosidic bond between the fructose and glucose moiety were not found in this work. Fast Atom Bombardment mass spectroscopy (FAB-MS) analysis of commercial mono-*O*-caproyl sucrose and mono-*O*-lauroyl sucrose showed the spectrum of these fragments due to glycosidic bond cleavage (de Koster *et al.*, 1993) The mass spectrum of purified 3-*O*-caproyl sucrose with the formation of a dimer [2M+Na] with m/z 1015.5 was shown in Fig 18.

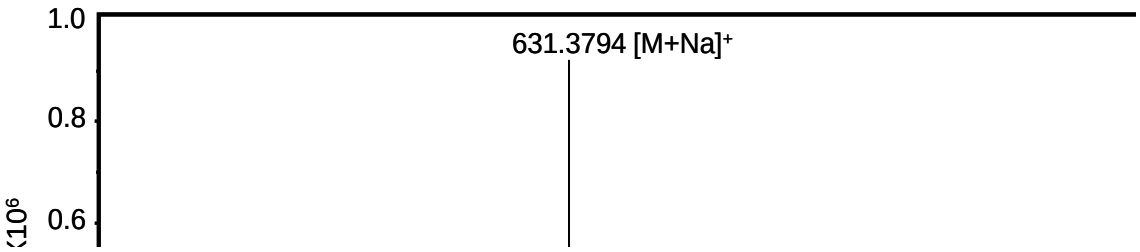


Fig 17 Mass spectrum of purified 2-*O*-stearoyl sucrose analyzed by ESI MS. The samples was dissolved in methanol and diluted in 50% methanol in acetonitrile containing 1% formic acid

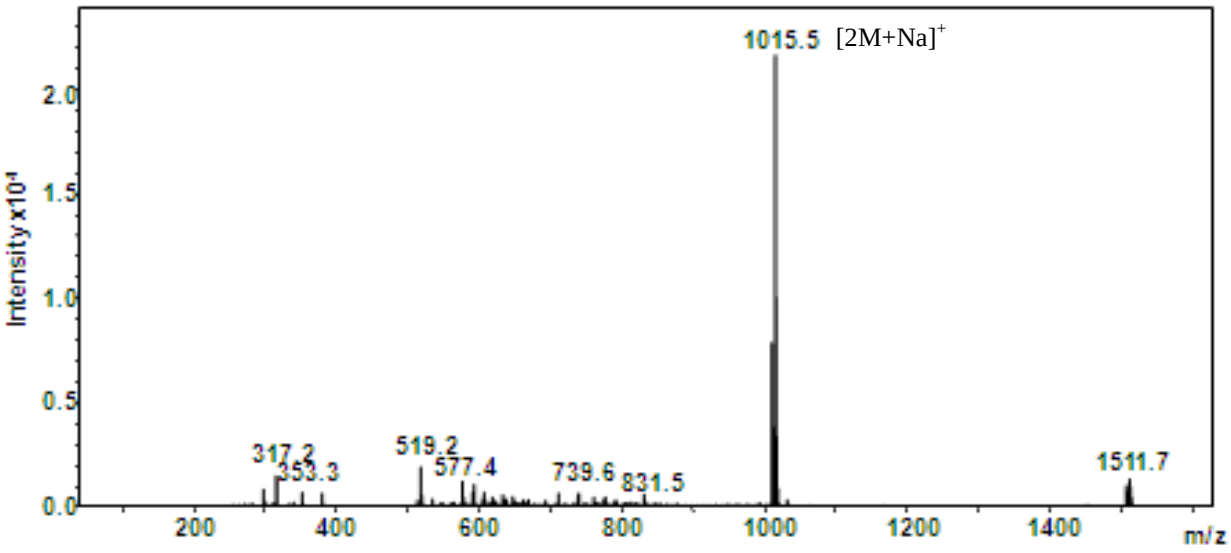


Fig 18 Mass spectrum of purified 3-*O*-caproyl sucrose analyzed by ESI MS. The sample was dissolved in acetonitrile and diluted with 50% methanol in acetonitrile containing 2.5% formic acid

4.5
NMR Spectroscopy

The ^{1}H and ^{13}C-NMR spectroscopy has been routinely applied to elucidate the structure of the synthesized sucrose fatty acid esters. The NMR spectrum of sucrose and mono-*O*-acyl sucrose was assigned by two dimensional COSY (Correlation Spectroscopy) and TOCSY (Total Correlation Spectroscopy) analysis. The chemical shifts of each hydrogen and carbon atom of sucrose compared to the corresponding sucrose fatty acid esters could identify the acylation position (see Fig 19). The chemical shifts of *O*-acyl sucrose synthesized in this work are summarized in Table 8.

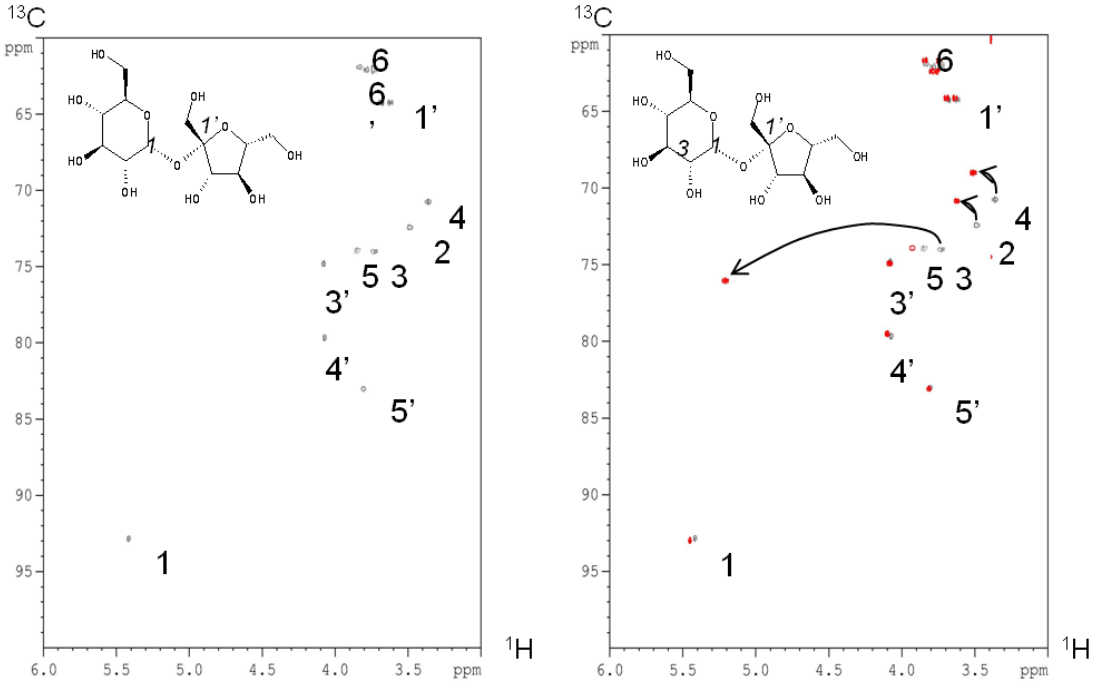


Fig 19 Chemical shift of 3-*O*-acyl sucrose as recorded by ^{1}H ^{13}C HSQC at 298 °K on a 600 MHz spectrometer equipped with a triple-gradient TXI (H/C/N) probe

Table 8 Chemical shifts **(δ, ppm)** of sucrose and its esters in chloroform-methanol 1:1 (v/v)

atom	sucrose	2-*O*-acyl sucrose	3-*O*-acyl sucrose	4-*O*-acyl sucrose	6-*O*-acyl sucrose	3'-*O*-acyl sucrose	4'-*O*-acyl sucrose	6'-*O*-acyl sucrose
C1	92.77	90.12	92.91	92.50	93.54	92.79	93.00	92.4
C2	72.33	73.50	70.72	72.50	73.07	72.33	72.27	72.4
C3	73.94	71.24	75.94	71.70	74.54	74.03	73.86	74.1
C4	70.67	70.95	68.93	71.50	71.34	70.43	70.62	70.9
C5	73.87	73.67	73.82	71.90	72.20	73.87	73.77	73.5
C6	61.84	61.85	61.61	61.70	64.49	61.84	61.78	62.3
H1	5.41	5.55	5.45	5.46	5.27	5.41	5.46	5.37
H2	3.48	4.65	3.62	3.57	3.35	3.48	3.53	3.48
H3	3.72	3.89	5.20	3.89	3.60	3.65	3.76	3.73
H4	3.36	3.43	3.51	4.78	3.21	3.40	3.40	3.34
H5	3.84	3.89	3.92	4.03	3.87	3.84	3.92	3.82
H6a	3.73	3.73	3.74	3.52	4.13	3.73	3.77	3.86
H6b	3.83	3.86	3.84	3.61	4.24	3.83	3.90	3.74
C1'	64.14	63.05	64.04	64.30	64.89	64.97	63.56	64.00
C2'[a]	-	-	-	-	-	-	-	-
C3'	79.64	77.29	79.43	79.50	80.31	79.54	77.58	79.4
C4'	74.69	74.22	74.80	74.80	75.75	72.70	77.94	76.3
C5'	82.97	82.64	83.04	83.00	83.65	83.31	82.22	80.00
C6'	61.99	62.13	62.30	62.20	63.26	62.00	62.69	66.5
H1'a	3.68	3.54	3.69	3.62	3.48	3.66	3.75	3.67
H1'b	3.61	3.41	3.63	3.69	3.52	3.59	3.63	3.63
H3'	4.07	4.18	4.10	4.08	3.94	5.29	4.36	4.04
H4'	4.08	4.05	4.08	4.08	3.92	4.30	5.22	3.99
H5'	3.80	3.76	3.81	3.80	3.69	3.92	4.00	3.98
H6'a	3.79	3.80	3.79	3.74	3.58	3.79	3.79	4.44
H6'b	3.73	3.73	3.75	3.79	3.63	3.73	3.79	4.25

[a] On the inverse-detected carbon spectra measured, the C2' carbon atom was not showing up because it is not bearing a hydrogen substituent.

5.

Regio-selective synthesis of *O*-acyl sucrose as catalyzed by alkaline protease AL 89 in hydrophilic solvents

This chapter focuses on the synthesis of *O*-acyl sucrose catalyzed by alkaline protease AL 89 in hydrophilic aprotic solvents in terms of regio-selectivity, formation rate and production yield. The protease catalyzed acylation of sucrose was studied using vinyl fatty acid esters as acyl donors. The process conditions were optimized with respect to substrate molar ratio, temperature and the water content. During the synthesis and the purification the regio-isomeric distribution and acyl migration were studied.

5.1
Conformation of sucrose in hydrophilic solvents

Sucrose ($C_{12}H_{22}O_{11}$), with the IUPAC systematic name α-D-glucopyraonosyl β-D-fructofuranoside, is a naturally occurring carbohydrate found in sugar cane, sugar beet and many other plants including maple trees. Sucrose is a non reducing sugar as the α-glucose and fructose molecules are connected at their anomeric carbon atoms and; therefore, does not contain a free hemi-acetal linkage. The total eight hydroxyl groups on the sucrose molecule are classified into primary hydroxyl (at carbon atom 6, 1' and 6') and the secondary hydroxyl groups (2, 3, 4, 3', and 4').

The conformation of sucrose dissolved in aprotic solvents investigated by NMR (Lichtenthaler and Immel, 1995; Bernet and Vasella, 2000) showed the two inter residue H-bonds between fructose and glucose molecule of C(1F)OH$^{\cdots\cdots}$O (2G) and C(6F)OH$^{\cdots\cdots}$O (5G) with the distances of 1.85 and 1.89 Å, respectively (see Fig 20).

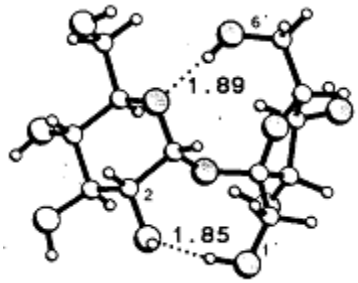


Fig 20 Distances of two inter residue H-bonds between the fructose and glucose moieties of sucrose dissolved in hydrophilic aprotic solvents (Lichtenthaler *et al.*, 1995)

The two sucrose conformers named S1 and S2 in hydrophilic aprotic solvents investigated by Lichtenthaler *et al.,* (1995) are presented in Fig 21. As determined by ^{1}H NMR, the S1 and S2 conformers in equilibrium are distributed with the ratio of 2:1 and it is clearly evident that the highest positive electrostatic potential is found in the area of the 2-OH group on the glucose moiety, leading to enhanced acidity of this hydroxyl group over the others, and as such it de-protonates first at alkaline conditions.

With the sucrose conformer ratio of S1 to S2 at 2:1, the benzylation of sucrose with benzyl bromide catalyzed by sodium hydride (NaH) in DMF resulted in the formation of 2-*O*-,1'-*O*- and 3'-*O*- benzyl sucrose in the ratio of 11:2:1 (Lichtenthaer *et al.*, 1995). The three primary hydroxyl groups are preferentially alkylated, acylated, oxidized and displaced by halogen in the order 6-*O* ≈ 6'-*O* >> 1'-*O* (James *et al.*, 1989)

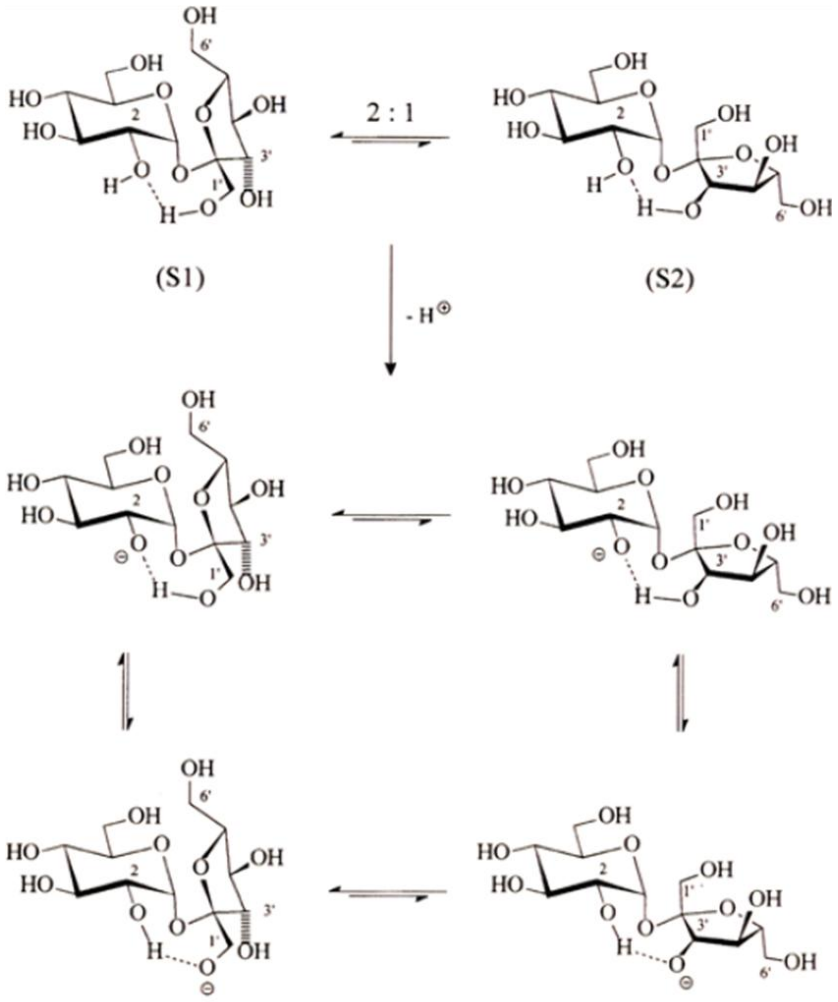


Fig 21 Conformation of sucrose in an aprotic hydrophilic solvent, showing a 2:1 equilibrium distribution of S1 and S2 conformer (Lichtenthaler *et al.*, 1995)

5.2

Synthesis of *O*-acyl sucrose

By a non enzymatic approach, Chauvin *et al* (1993) reported the synthesis of 2-*O*- and 3-*O*-lauroyl sucrose catalyzed by sodium hydride in anhydrous pyridine using 3-lauroylthiazolidine-2-thiones as acyl donor with the yield of 70% and 2%, respectively. The ester products were not observed in the reaction without sodium hydride indicating that the catalyst activated the 2-OH, leading to the formation of the stabilized nucleophilic 2-oxyanion which then reacts with the esterifying agent to form 2-*O*-acyl sucrose. The disodium hydrogen phosphate (Na_2HPO_4) was also

reported as an effective catalyst for the synthesis of 2-*O*-acyl sucrose in DMSO with vinyl fatty acid esters as acyl donors at 40 °C with production yields of 2-*O*-acyl sucrose higher than 50 % (Cruces *et al.*, 2001). Vinyl fatty acid esters are commonly used as acylating agents for the synthesis of sugar fatty acid esters because the vinyl group is a good leaving group. During transesterification, vinyl alcohol is released and immediately tautomerized to volatile acetaldehyde. The conversion of vinyl alcohol to acetaldehyde is practically complete, making the process irreversible and simple for product isolation (Degueil-Castaing *et al.*, 1987; Yang *et al.*, 1999).

Celite, a diatomaceous earth which is used as a filtering agent or a carrier for enzyme immobilization, treated with 10 mM phosphate buffer (pH 8.0) was previously reported by Plou *et al.,* (1999) as a potential catalyst for the synthesis of 2-*O*-lauroyl sucrose in DMSO. However, the initial formation rate of 2-*O*-stearoyl sucrose catalyzed by alkaline protease AL 89 in a DMF - DMSO solvent mixture (1:1 v/v) was 16.2 mM/hr or 23 fold higher than the Celite catalyzed process (100 g/L) under the same conditions (paper II: Ritthitham *et al.*, 2009).

The regioselectivity, is extremely important in the synthesis of carbohydrate esters and was shown to be strongly dependent on the type and source of the biocatalyst, the solvent medium, and the type of the acylating agents used (Gandhi *et al.*, 2000). With a subtilisin as biocatalysts, sucrose was shown to be regioselectively acylated at the position C-1’ (Soedjak and Spardlin, 1994; Polat *et al.*, 1997) and with the metalloprotease thermolysin and the alkaline protease AL 89, substitution at position C-2 was obtained (Pedersen *et al.*, 2002; Pedersen *et al.*, 2003).

5.3

The effect of substrate molar ratio, water content, and temperature on the synthesis of *O*-stearoyl sucrose

The substrate molar ratio, water content and temperature affected the initial rate of 2-*O*-, 3-*O*-, and 3'-*O*-stearoyl sucrose synthesis as catalyzed by alkaline protease AL 89 in a DMF-DMSO solvent mixture (1:1 v/v). The highest initial synthetic rate was obtained with the molar ratio of sucrose to vinyl stearate 1: 1.5 at 70 °C and a water content ranging from 0 to 2.5% (v/v). As discussed in Chapter 2, a small amount of water plays an essential role in maintaining the enzyme flexibility necessary for the catalytic activity in organic solvents (Halling, 1990; Goldberg *et al.*, 1990; Gubicza and Szakacs-Schmidt, 1994; Klibanov, 1997). In reactions at high water contents; however, water causes the hydrolysis of the acyl-enzyme intermediate, leading to a decrease in the synthetic activity (Valivety *et al.*, 1992(a); Valivety *et al.*, 1993). Control of the water content, and consequently of the water activity (a_w), in a reaction could be performed by different ways such as pervaporation (Keurentjes *et al.*, 1994; Kwon *et al.*, 1995), the use of saturated salts solutions (Valivety *et al.*, 1993; Wehtje *et al.*, 1993; Rosell *et al.,* 1996), salt pairs (Halling, 1992; Kvittingen *et al.*, 1992; Robb *et al.*, 1994; Kim *et al.*, 1998) or vacuum (Napier *et al.*, 1996). The initial water content influenced both the production yield and the enatioselectivity of the lipase catalyzed 2-methyl-1-pentanoic synthesis in hexane as reported by Zarevucka *et al.,* (1997).

Proteases from different microbial sources showed widely varying needs of water to maintain the enzyme activity in anhydrous organic solvents. Kitagawa *et al.,* (2002) investigated two different proteases from *Streptomyces sp.* and *Bacillus sp.* for their requirements of water necessary for the synthesis of glucose fatty acid ester in DMF. The transesterification activity of the *Streptomyces* protease decreased with addition of water while that of *Bacillus* protease increased at high water content (20% v/v). The variation in optimal water activity depends on the amount of water tightly bound to the enzyme and the solvent used. For example, an enzyme working in hydrophilic organic solvents need more water than that in hydrophobic organic solvents (Gandhi *et al.*, 2000)

5.4
Synthesis of *O*-acyl sucrose with different acyl chain lengths

A number of investigations on protease-catalyzed transesterification showed a decreasing initial reaction rate with the increasing acyl donor fatty acid chain length as well as the size of the acyl acceptor (Therisod and Klibonov, 1986; Carrea *et al*, 1989; Riva *et al*, 1988; Kitagawa *et al*, 1998; Tai, *et al*, 2001). The same tendency was shown in a lipase-catalyzed esterification of maltose and sucrose, respectively (Pedersen *et al*, 2002). However, the synthesis of 2-*O*-acyl-sucrose catalyzed by alkaline protease AL-89 in DMF-DMSO solvent mixture was not affected by the fatty acid acyl chain length (from C_8- C_{18}) as demonstrated in Table 9. From the results, the highest total production yield (0.85 mol product/mol sucrose) was obtained with vinyl laurate. The reaction with vinyl caprate showed the lowest monoester production yield of 0.31 mole product/ mole sucrose. The explanation could be that sucrose oligoesters were formed in the latter process as detected by HPLC (see Fig 14 (A), chapter 4 page 24).

Table 9 Initial formation rate and production yield of *O*-acyl-sucrose synthesis as catalyzed by alkaline protease AL 89 in DMF-DMSO (1:1 v/v) solvent mixture. Reaction conditions: 0.2 M sucrose, 0.15 M vinyl fatty acid ester, 10 g/L lyophilized alkaline protease AL 89, 60 °C, 250 rpm.

Acy donor (fatty acid chain length)	2-*O*-acyl-sucrose		3-*O*-acyl-sucrose		3'-*O*-acyl sucrose		Total Yield (mol mol^{-1})
	Initial rate (mM min^{-1})	Yield (mol mol^{-1})	Initial rate (mM min^{-1})	Yield (mol mol^{-1})	Initial rate (mM min^{-1})	Yield (mol mol^{-1})	
Vinyl caprate (C10)	5.18±0.54	0.18	1.80±0.40	0.12	0.40±0.11	0.01	0.31
Vinyl laurate (C12)	7.02±1.21	0.60	1.70±0.37	0.21	0.93±0.20	0.04	0.85
Vinyl myristate (C14)	5.43±1.33	0.40	1.71±0.62	0.29	0.50±0.18	0.01	0.70
Vinyl palmitate (C16)	5.93±0.18	0.39	1.42±0.01	0.22	0.45±0.01	0.02	0.63
Vinyl stearate (C18)	5.30±0.20	0.45	1.80±0.13	0.25	0.31±0.01	0.02	0.72

5.5
Acyl migration

During the synthesis of *O*-caproyl sucrose as catalyzed by alkaline protease AL 89 in DMF-DMSO solvent mixture (1:1 v/v), it was observed that the concentration of 2-*O*-caproyl-sucrose decreased corresponding to an increasing 3-*O*-caproyl-sucrose concentration (see Fig 22). The interchange between two neighboring regio-isomers is often a result of acyl migration which occurs spontaneously or could be activated by acid or alkaline (Bornemann *et al.*, 1992), small amounts of water (Thevenet *et al.*, 1999), temperature (Paper III, Ritththam *et al.*, 2009) or even taking place on an HPLC column during the analysis as observed with a NH_2 spherisorb column (Molinier *et al.*, 2003).

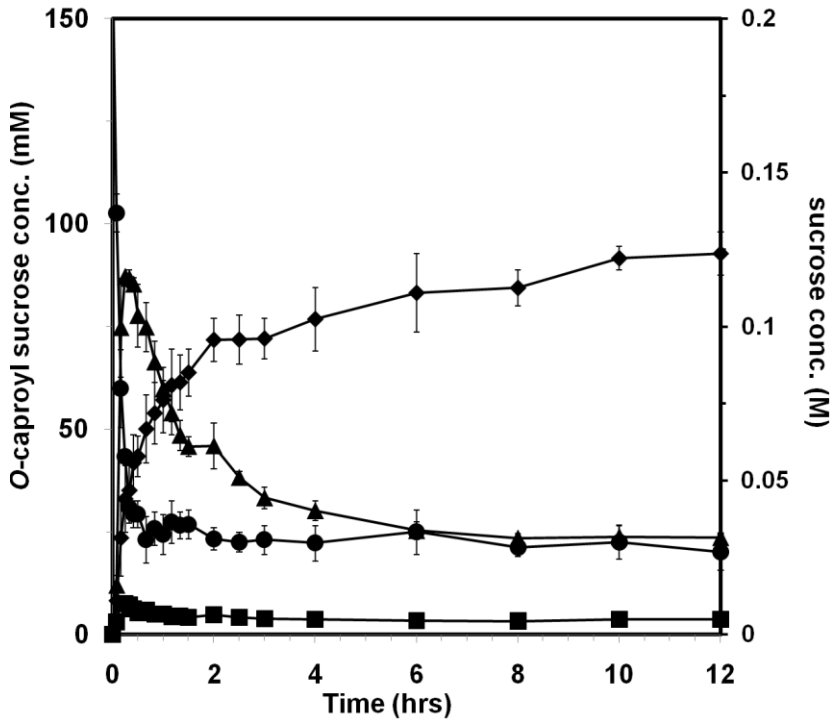


Fig 22 Progression profile of *O*-caproyl sucrose synthesis as catalyzed by alkaline protease AL-89 in DMF-DMSO solvent mixture (1:1 v/v) showing the inter conversion between two regio-isomeres
Symbols: 2-*O*-caproyl sucrose (-▲-), 3-*O*-caproyl sucrose (-♦-), 3'-*O*-caproyl sucrose (-■-), sucrose (-●-)

The acyl migration taking place on the glucose moiety from the C-2 to the C-3 position was confirmed by many investigations including Tsuda and Yoshimoto (1981) and Yoshimoto and Tsuda (1983). From the report of Molinier *et al.,* (2003) using N-acylthiozolidinethion as an acyl donor in the DMF with butyl lithium as a catalyst, the acyl group easily migrated from 2-*O*-acyl sucrose to form 3-*O*-acyl sucrose both in aqueous and organic medium. From 3-*O*-acyl sucrose the acyl group could migrate to form 6-*O*-acyl sucrose in the presence of water on analytical NH_2 spherisorb HPLC column.

The acyl migration from 2-*O*- to 3-*O*-acyl-sucrose was also observed in the reaction catalyzed by 100 g/L Na_2HPO_4 in DMSO at 40 °C (Cruces *et al.*, 2001). In the present work, the migration of 2-*O*- to 3-*O*-caproyl sucrose in DMF-DMSO solvent mixture (1:1 v/v) was confirmed to be catalyzed by alkaline protease AL 89 (Paper II: Ritthitham *et al.*, 2009).

During the purification of mono-*O*-caproyl sucrose regio-isomers by low pressure liquid chromatography using an Accubond SPE ODS (C18) pre-packed column followed by high pressure liquid chromatography with reversed-phase C-18 preparative column, the dried fractions of purified 2-*O*-caproyl sucrose and 3'-*O*-caproyl sucrose showed impurities when analyzed by HPLC. The distribution of 2-*O*-caproyl sucrose to 3-*O*-, 6-*O*-caproyl sucrose resulting from intramolecular acyl migration was observed during the drying process at 60 °C, atmospheric pressure. The intermolecular acyl migration from 3'-*O*-caproyl sucrose to 3-*O*-caproyl sucrose was also activated during these drying conditions. However, performing the drying process by lyophilization improved the stability of 2-*O*-caproyl sucrose and 3'-*O*-caproyl sucrose, respectively. As a result, 8.5% of 3-*O*-caproyl sucrose was detected as an impurity in the purified 2-*O*-caproyl sucrose fraction while no migration products were observed in the purified 3'-*O*-caproyl sucrose fraction (Paper III: Ritthitham *et al.*, 2009).

6.
Separation and purification of *O*-acyl sucrose regio-isomers

The synthesis of sucrose fatty acid esters can be catalyzed by biocatalysts or non-enzymatically by chemical catalysts. In all cases, complex mixtures of regio-isomeric esters can be obtained ranging from mono- to octa-esters with a maximum of 255 possible isomers. However, the narrow product profiles can be obtained due to the regio-selectivity of enzymes. In this chapter, the separation of seven mono-*O*-caproyl sucrose regio-isomers using low pressure column chromatography followed by high pressure column chromatography is presented.

6.1
Separation of *O*-stearoyl sucrose by solvent extraction

Flow chart of the protocol developed for the separation of mono-*O*-stearoyl sucrose is shown in Fig 23. From the initial reaction mixture, the unreacted vinyl stearate and sucrose was extracted by hexane and 70% methanol in water, respectively. The extraction of the mono-*O*-stearoyl sucrose mixture was achieved by chloroform-methanol-water with the volumetric ratio of 5:1:1.

Analytical TLC was employed to estimate the purity of the extraction process (see Fig 24) and the concentration of 2-*O*-, 3-*O*- and 3'-*O*-stearoyl sucrose was determined by HPLC as described in Chapter 4.

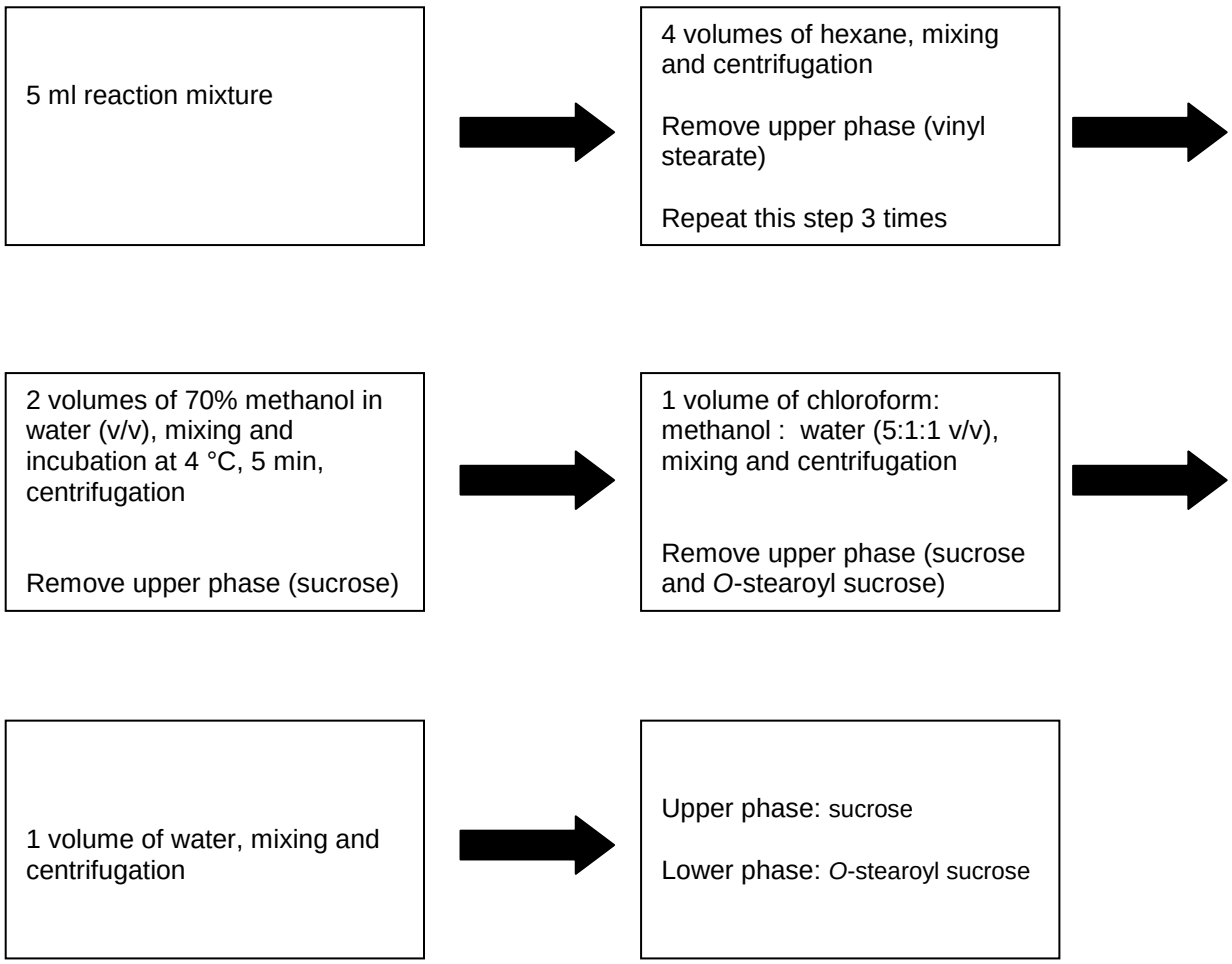


Fig 23 Extraction protocol for the separation of *O*-stearoyl sucrose

As the polarity of the different regio-isomers in the reaction mixture are relatively close, the separation of 2-*O*-, 3-*O*-, and 3'-*O*-stearoyl sucrose in the extraction solvent system is difficult to obtain. Thus, the complete separation of each mono-*O*-stearoyl sucrose regio-isomer could not be achieved (see Fig 24). The extraction yield ($g_{extracted}/g_{crude\ reaction\ mixture}$) of 2-*O*-, 3-*O*-, and 3'-*O*-stearoyl sucrose quantified by HPLC was 12.5%, 7.7% and 5% respectively, which was not a satisfactory yield.

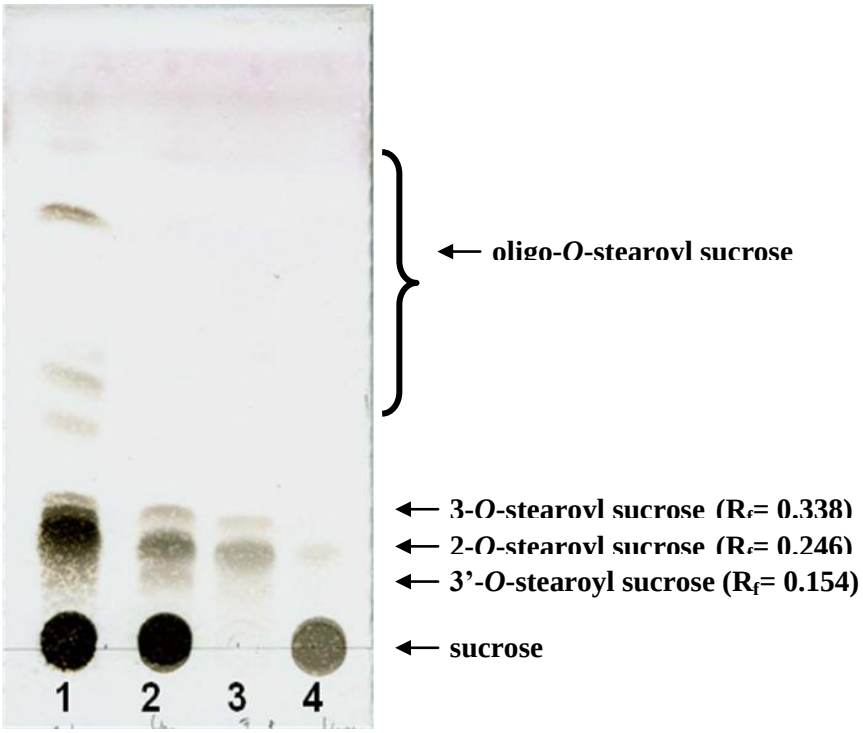


Fig 24 Analytical TLC of the purified fractions obtained by solvent extraction.
Mobile phase: chloroform : methanol (5:1 v/v)
Lane 1 Fraction of hexane
Lane 2 Fraction of 70% methanol in water
Lane 3 Fraction of chloroform-methanol-water (lower phase)
Lane 4 Fraction of chloroform-methanol-water (upper phase)

6.2

Separation of *O*-caproyl sucrose regio-isomers by low and high pressure liquid chromatography

A chromatographic procedure was developed for the separation of mono-*O*-caproyl sucrose regio-isomers. The crude reaction mixture containing unreacted sucrose, mono-*O*-caproyl sucrose, oligo-*O*-caproyl sucrose was absorbed on the Accoubond column (300 mm x 60 mm) and the mono-*O*-caproyl sucrose fraction was eluted by 80% methanol in water. The mono-*O*-caproyl sucrose was then separated into 7 different regio-isomers by preparative C-18 HPLC column (Lichrosorb RP 18 stainless steel: 250mm x10 mm, 7 µm particle size) by an acetonitrile gradient in water. The position of substitution of the regio-isomers was characterized by NMR.

Using the gradient of acetonitrile in water from 40 to 45% and 40 to 55% for 1 hr, four *O*-caproyl sucrose regio-isomers were separated with the retention time of 12.78, 13.66, 16.14 and 16.65 min (see Fig 25 A and B). However, starting the gradient by a step down in acetonitrile concentration from 50% to 40% followed by an increase to 55% acetonitrile improved the separation process obtaining the separation of seven regio-isomers within 25 min in a single run. In particular the separation of 3'-*O*-caproyl sucrose and 2-*O*-caproyl sucrose was well resolved (see Fig 25 C).

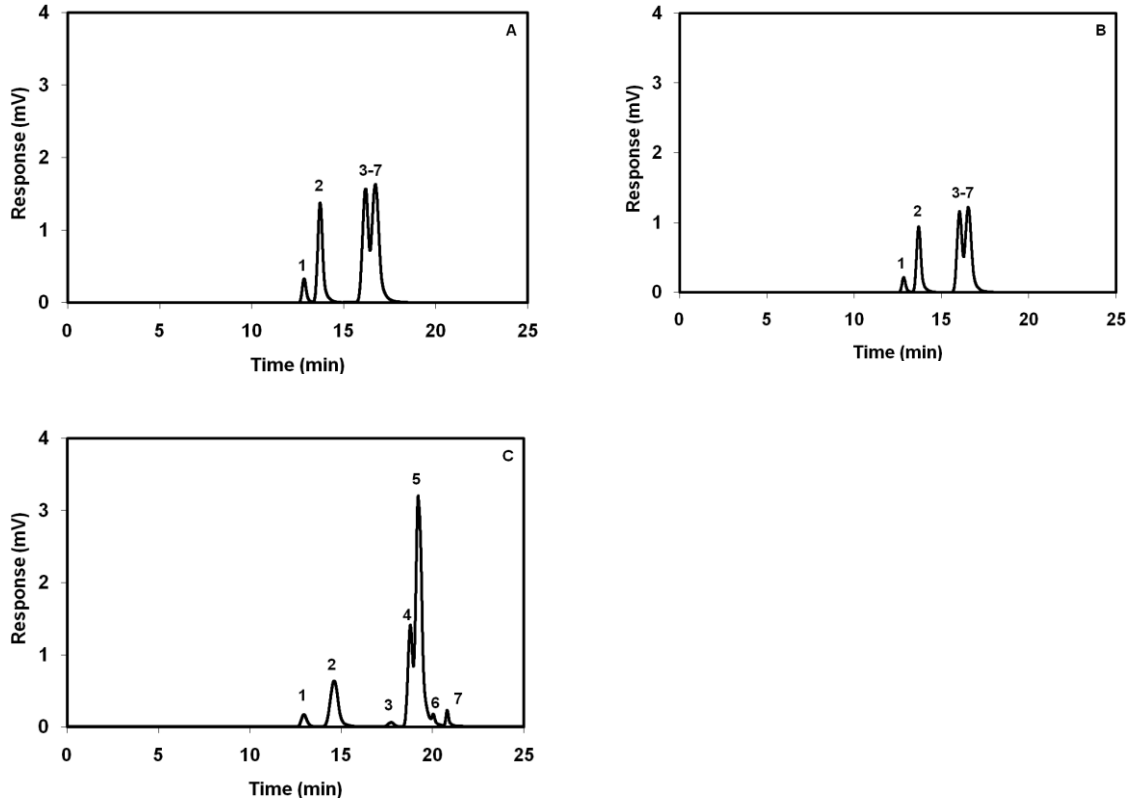


Fig 25 Effect of gradient condition on the separation of regio-isomers prepared by Lichrosorb RP18 Preparative HPLC column

A: Acetonitrile-water gradient from 40% to 45% Acetonitrile for 60 min

B: Acetonitrile-water gradient from 40% to 55% Acetonitrile for 60 min

C: Acetonitrile-water gradient under the condition as described in paper III (Ritthitham *et al*, 2009)

Peak identifications: **(1) 3'-*O*-caproyl sucrose** **(2) 2-*O*-caproyl sucrose** **(3) 4-*O*-caproyl sucrose** **(4) 6-*O*-caproyl sucrose** **(5) 3-*O*-caproyl sucrose** **(6) 6'-*O*-caproyl sucrose** **(7) 4'-*O*-caproyl sucrose**

The separation of synthetic and commercial sucrose fatty acid esters was investigated by many research groups with different HPLC methods (see Table 10). However, the procedures reported by those methods were in analytical scale.

Table 10 HPLC procedures for the separation of synthetic and commercial sucrose fatty acid esters

Sucrose fatty acid esters	**Column (length x diameter, particle size)**	**Mobile phase**	**Flow rate (ml/min)**	**Total elution time (min)**	**Regio-isomer identification**	**Reference**
Commercial sucrose fatty acid esters F160, F 50 from Ryoto Co, Ltd, Japan	Lichrosorb RP 18 (250x4.6 mm, 10 µm)	Isocratic methanol:water (95: 5 v/v)	1	40	Not Reported	Kaufman and Garti., 1981
Commercial sucrose fatty acid esters F160 from Ryoto Co, Ltd, Japan	Bondapak C18 (150x3.9 mm, 10 µm) and Nova-Pak C18 (150x3.9 mm, 4 µm)	Isocratic acetone:water (70:30 v/v)	0.5	30	Not Reported	Torres *et al.*, 1990
Commercial sucrose fatty acid esters F160, F 140 from Ryoto Co, Ltd, Japan	RP C18- ODSA* (150x 4.6 mm, 5 µm)	Isocratic methanol:water (75: 25 v/v)	1.2	120	Not Reported	Moh *et al.*, 2000
Synthesized mono, di, tri, tetra, penta *O*-octanoyl sucrose	RP C18- ODSA* (150x 4.6 mm, 5 µm)	Gradient methanol in water	1	80	Not Reported	Wang *et al.*, 2006

Sucrose fatty acid esters	**Column (length x diameter, particle size)**	**Mobile phase**	**Flow rate (ml/min)**	**Total elution time(min)**	**Regio-isomer identification**	**Reference**
Commercial sucrose fatty acid esters S1670, S 1170 from Ryoto Co, Ltd, Japan	RP C18- ODSA* (150x 4.6 mm, 5 µm)	Gradient methanol containing 10%THF (v/v) in water	1	80	6-*O*-acyl sucrose 6,3'-di-*O*-acyl sucrose	Wang *et al.*, 2007
Commercial sucrose monolaurate (Fluka)	Spherisorb ODS2 (250 mm x 4.6 mm, 3 µm)	Isocratic acetonitrile:water (35:65 v/v)	0.3	40	6-*O*-lauroyl sucrose 2-*O*-lauroyl sucrose 1'-*O*-lauroyl sucrose	Perez-Victoria *et al.*, 2007

*: Octadecyl silanized silica gel

7.

Synthesis of 6-*O*- and 6'-*O*-stearoyl sucrose as catalyzed by *Candida antarctica* lipase B: the effect of hydrophilic solvents on the sucrose solubility, initial reaction rate and the regio-selectivity

The synthesis of 6-*O*- and 6'-*O*-acyl sucrose can be performed by enzymatic or non-enzymatic (chemical) methods. The following methods are normally used and eventually combined to obtain a high yield of the two regio-isomers.

i) Esterification with free fatty acids using chemical catalysis:
The chemical process named the Mitsunobu reaction was catalyzed by diisopropylazodicarboxylate (DIAD) at room temperature. The solution of sucrose in anhydrous DMF is added to triphenylphosphine the carboxylic acid and DIAD. The mixture of monoesters (6-*O*-, 6'-*O*-acyl sucrose) and diester (6,6'-di-*O*-acyl sucrose) was obtained in proportions depending on the stoichiometry of the reactants. Recently, Wang *et al.,* (2007) reported the synthesis of 6-*O*-acyl sucrose with a high yield by the process called the stannylene acetal method; however, the by product, 6,3-di-*O*-acyl sucrose was obtained simultaneously via dibutylstannylene acetal intermediates.

ii) Regioselective esterification obtaining 2-*O*-acyl-sucrose followed by controlled the migration toward 6-*O*-acyl sucrose:
The selective esterification of 2-*O*-acyl sucrose was achieved using acylating agent such as N-acylthiazolidinethione in DMF and this product can be transformed into 6-*O*-acyl sucrose when 1,8-diazabicyclo [5.4.0] undec-7-ene (DBU) is added.

iii) Chemoenzymatic synthesis:

A two step procedure for the synthesis of 6'-*O*-acyl sucrose was reported by Chauvin and Plusquellec (1991). Sucrose was first selectively acylated by an acylating agent (3-acyl-5-methyl-1,3,4 thiadiazole-2(3H)-thiones) and with the 1,4 diazabicyclo [2.2.2] octane (DABCO) as catalyst in DMF. As a result, the crude mixture of 6-*O*-acyl sucrose, 6'-*O*-acyl sucrose and 1'-*O*-acyl sucrose, was obtained. The 6-*O*-acyl sucrose and 1'-*O*-acyl sucrose were then eliminated by hydrolysis using *Candida cylindracea* lipase (CCL).

iv) Enzymatic synthesis:

The synthesis of 6-*O*- and 6'-*O*-acyl sucrose has been catalyzed by lipases via esterification or transesterification reactions. Many microbial lipases proved to catalyze regioselective acylation of sucrose. *Candida antarctica* Lipase B (Novozyme 435) showing selectivity toward synthesis of 6-*O*- and 6'-*O*-acyl sucrose while the lipase from *Thermomyces lanuginosus* (Ferrer *et al.*, 2005) *Pseudomonas sp* (Rich *et al.*, 1995) or *Mucor miehei* (Kim *et al.*, 1998) catalyzed regio-selective synthesis of 6-*O*-acyl sucrose. In paper IV (Ritthitham *et al*., 2009) the synthesis of 6-*O*- and 6'-*O*-stearoyl sucrose as catalyzed by *Candida antarctica* Lipase B in different organic solvents was investigated with the results showing that increasing the solvent hydrophilicity decreases the overall enzyme selectivity.

7.1
Synthesis of 6-*O*- and 6'-*O*-stearoyl sucrose

6-*O*-stearoyl sucrose and 6'-*O*-stearoyl sucrose were synthesized by *Candida antarctica* lipase B via transesterification of sucrose using vinyl stearate as an acyl donor at 50 °C in *tertiary*-alcohols with and without hydrophilic co-solvents. With hydrophilic co-solvent (DMF, DMSO, DMA, pyridine), the solubility of sucrose improved, leading to an increase of the initial synthetic rate, production

yield, product distribution, and degree of conversion (Reyes-Duarte *et al.*, 2005). In this work, the correlation between hydrophilicity of the reaction media and the initial formation rate was not found. However, most lipases are inactivated by a hydrophilic co-solvents employed but exhibit a relatively high stability in *tert*-alcohol (2-methyl-2-propanol, 2-methy-2-butanol) (Degn and Zimmermann, 2001). 2-methyl-2-butanol was used as a reaction medium in the enzymatic acylation of underivatized carbohydrates because this solvent is considered as a nontoxic and slightly polar solvent. At the same time it does not serve as a substrate for lipase due to sterical hindrance in the active site (Moreau *et al.*, 2007).

In this work, the lipase-catalyzed 6-*O*- and 6'-*O*-stearoyl sucrose formation was performed in *tertiary* alcohols 2-methyl-2-propanol (*tert*-butanol) and 2-methyl-2-butanol (*tert*-pentanol), respectively in the presence or absence of pyridine and DMSO as co-solvents. The progress of the reactions was monitored by HPLC from which a chromatogram was presented in Fig 26. 6-*O*- and 6'-*O*-stearoyl sucrose were detected at retention time of 6.41 and 6.87 min, respectively.

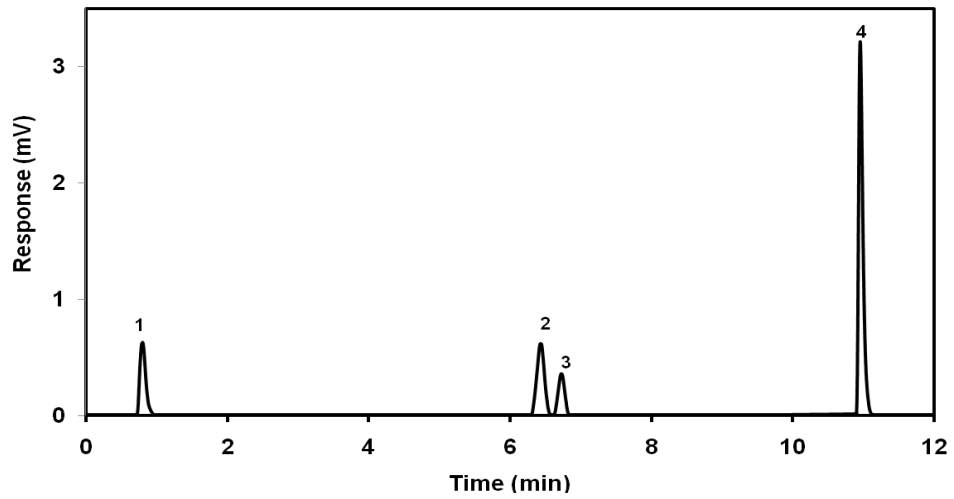


Fig 26 HPLC chromatogram of transesterification reaction mixture of 6-*O*-and 6'-*O*-stearoyl sucrose synthesis as catalyzed by *Candida antarctica* lipase B
Reaction condition: 0.03 M sucrose, 0.1 M vinyl stearate, 20 g/l *Candida antarctica* lipase B, 20 g/l molecular sieve, 250 rpm, 50 °C
Peak identification: 1: sucrose (R_t 0.81 min)
2: 6-*O*-steroyl sucrose (R_t 6.41 min)
3: 6'-*O*-steroyl sucrose (R_t 6.87 min)
4: vinyl stearate (R_t 10.94 min)

The solubility of sucrose in the reaction system containing 2-methyl-2-propanol was higher than that with 2-methyl-2-butanol. In the reaction system with pyridine and DMSO as co-solvent, the solubility of sucrose increased in the order of no-co solvent < 45%Pyridine < 20%DMSO < 55%Pyridine (see Table 11).

Table 11 Solubility of sucrose (mM) in different reaction system at 50 °C

Co solvent	Solvent	Sucrose solubility (mM)
None	2-methyl-2-propanol	0.98
	2-methyl-2-butanol	0.27
45%Pyridine	2-methyl-2-propanol	8.66
	2-methyl-2-butanol	4.79
55% Pyridine	2-methyl-2-propanol	18.64
	2-methyl-2-butanol	14.47
20%DMSO	2-methyl-2-propanol	13.14
	2-methyl-2-butanol	6.63

A reaction system constituted by two miscible solvents, 2-methyl-2-butanol containing 20% DMSO, was reported for the synthesis of mono-*O*-acyl sucrose catalyzed by *Humicola lanuginosa* lipase. A sucrose conversion of 70% and 80% was achieved for the synthesis of 6-*O*-lauroyl sucrose and 6-*O*-palmitoyl sucrose, respectively (Ferrer *et al.*, 1999). The highest productivity of mono-*O*-palmitoyl sucrose as catalyzed by *Candida antarctica* lipase B at 60 °C was obtained (45 g/l) in a reaction system of 2-methyl-2-butanol with 15% DMSO co-solvent (Reyes-Duarte *et al.*, 2005). In this work; however, the highest initial synthetic rate of 6-*O*- and 6'-*O*-stearoyl sucrose as catalyzed by *Candida antarctica* lipase B at 50 °C was found in a reaction system of 2-methyl-2-butanol containing 45% pyridine with the value of 2.55 µM/min and 1.63 µM/min, respectively. In the reaction system, solid sucrose was dissolved at a maximum solubility of 4.79 mM and continuously consumed for 6-*O*- and 6'-*O*-steroyl sucrose synthesis. After 4 days the sucrose concentration started to decline (see Fig 27 and Table 11).

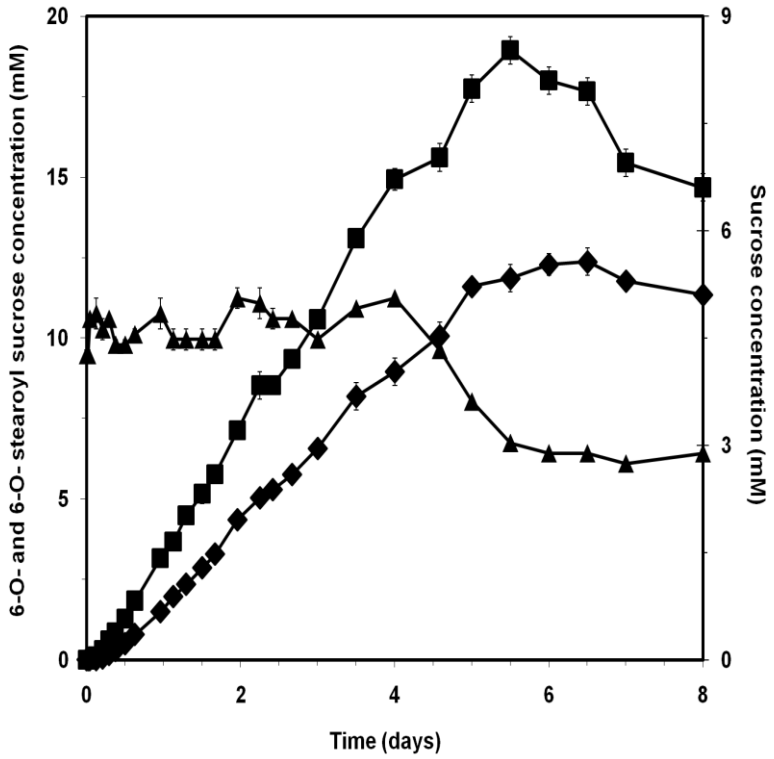


Fig 27 Production of 6-*O*- and 6'-*O*-stearoyl sucrose as catalyzed by *Candida antarctica* lipase B at 50 °C in 2-methyl-2-butanol containing 45% Pyridine.
Reaction condition: 0.03 M sucrose; 0.1 M vinyl stearate; 20 g/l CALB; 20 g/L molecular sieve; 250 rpm, 50 °C
Symbols: 6-*O*-stearoyl sucrose (-■-), 6'-*O*-stearoyl sucrose (-♦-), sucrose (-▲-)

7.2

Effect of hydrophilic co-solvents on regio-selectivity

In general, the reaction system influences the enzyme activity and specificity (Ryu and Dordick., 1991; Wescott and Klibanov., 1993), enantioselectivity (Parida and Dordick., 1991; Tawaki and Klibanov., 1992; Berglund., 2001) chemoselectivity (Tawaki and Klibanov., 1993) and regioselectivity (Rubio *et al.*, 1991). By changing the ratio of hydrophobic to hydrophilic solvent, the regioselectivity of *Candida antarctica* lipase B was changed (Paper 4, Ritthitham *et al.*, 2009). The effect of DMSO as a co solvent in 2-methyl-2-butanol on the synthesis of 6-*O*-lauroyl sucrose as catalyzed by *Thermomyces lanuginosus* lipase was reported by Ferrer *et al.,*(1999) and Ferrer *et al.*, (2002a). A high content of 6-*O*-lauroyl sucrose

was obtained in a reaction system with DMSO concentration higher than 15% while the DMSO concentration lower than 10% increased the synthesis of 6,6'-di-*O*-lauroyl sucrose and 6,1'-di-*O*-lauroyl sucrose. In this work, the decreasing of the overall regio-selectivity of *Candida antarctica* lipase B for the synthesis of 6-*O*-stearoyl sucrose was in the order of no co-solvent> 45% pyridine> 55% pyridine>20%DMSO (see Table 12). A distribution ratio (defined as molar ratio of 6-*O* to 6'-*O*-stearoyl sucrose at steady state) was in a range of 1.1-1.5 in a reaction system with hydrophilic co-solvents and the ratio was changed from 2:1 in the absence of co-solvent to 1:1 in the presence of DMSO.

Table 12 Effect of hydrophilic co solvents on the regio-isomeric and distribution ratio

Co-solvent	2-methyl-2-alcohol	Regio-isomeric ratio[a]	Distribution ratio[b]
None	Propanol	2.20	2.31
	Butanol	2.12	1.73
45% pyridine	Propanol	1.47	1.46
	Butanol	1.53	1.29
55% pyridine	Propanol	1.42	1.31
	Butanol	1.54	1.37
20% DMSO	Propanol	1.14	1.17
	Butanol	1.23	1.15

[a] :initial synthetic rate of 6-*O*-stearoyl sucrose/ initial synthetic rate of 6'-*O*-stearoyl sucrose
[b] :6-*O*-stearoyl sucrose (M)/ 6'-*O*-stearoyl sucrose (M)

By controlling the hydrophilicity of the reaction system, the synthesis of 1'-*O*-butoyl sucrose as catalyzed by subtilisin BPN' and subtilisin Carlsberg was preferentially over 6-*O*-butoyl sucrose (Rich *et al.*, 1995). In the explanation, the hydrophilic solvent is capable to solubilize and stabilize the sucrose molecule. The increasing of hydrophobic solvent greatly reduced the degree of salvation of the glucose moiety in the medium; therefore, the reactivity of 1'-OH relative to 6-OH was reduced.

8.
Conclusion and outlook

In this work, the synthesis of *O*-acyl sucrose in hydrophilic solvents was studied using alkaline protease from *Bacillus pseudofirmus* AL 89 and *Candida antarctica* lipase B as biocatalysts. The stability of the protease was investigated by dissolving lyophilized enzyme in different reaction media and determination of the proteolytic activity in aqueous buffered solution. The stability of alkaline protease was highest in DMF with a half life ($t_{d(1/2)}$) of 10 min and the stability decreased in the order of $t_{d(½)DMF} > t_{d(½)DMF\text{-}DMSO} = t_{d(½)water} > t_{d(½)DMSO}$.

In DMF-DMSO (1:1 v/v) solvent mixture alkaline protease AL 89 regioselectively catalyzed the synthesis of 2-*O*-acyl sucrose (see Fig 27, black arrow) and the transformation of 2-*O*-acyl sucrose to 3-*O*-acyl sucrose (see Fig 27, white arrow). The optimal reaction conditions for *O*-acyl sucrose synthesis were found at 0-2% (v/v) water and 70 °C with a substrate molar ratio of sucrose and vinyl fatty acid 1:1.5. Varying the vinyl fatty acid chain length from C_{10} to C_{18} did not significantly affect the transesterification rate.

With *Candida antarctica* lipase B as biocatalyst in organic solvents, sucrose was acylated at 6-OH and 6'-OH. Adding of hydrophilic co-solvents to the reaction system of tertiary alcohols affected sucrose solubility, initial synthetic rate and the enzyme regio-selectivity. The highest initial synthetic rate of 6-*O*- and 6'-*O*-stearoyl sucrose was found in the reaction system consisting of 45% pyridine in 2-methyl-2-butanol with a value of 25.68 and 16.78 n moles/min, respectively. Solid sucrose was continuously solubilized and converted into the corresponding esters as the reaction proceeded. As an effect of co-solvent, the regiomeric distribution ratio of 6-*O*-stearoyl sucrose to 6'-*O*-stearoyl sucrose varied from 2:1 to 1:1 in the reaction system with no-co solvent and co solvent with 20% DMSO respectively. The control of enzyme selectivity for the synthesis of 6-*O*-stearoyl sucrose by incorporated hydrophilic solvents decreased in the order of no-co solvent > 45% pyridine > 55% pyridine >20%DMSO. Thus the preference for 6-O-acyl sucrose

synthesis as catalyzed by *Candida antarctica* lipase B decreased, especially when the DMSO was used as co solvent (see Fig 28, black arrow).

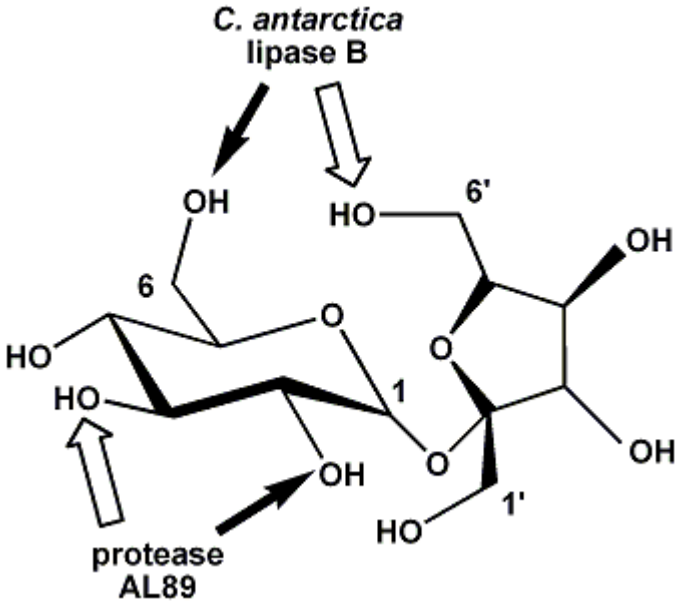


Fig 28 Regioselectivity of alkaline protease AL 89 and *Candida antarctica* lipase B for the synthesis of *O*-acyl sucrose in organic solvents

The separation of mono-*O*-caproyl sucrose regio-isomers was performed by column chromatography. The first column with AccuBOND C18 stationary phase was used for the separation of mono-*O*-caproyl sucrose from oligo-*O*-caproyl sucrose and unreacted substrates. The mono-*O*-caproyl sucrose regio-isomers absorbed onto the stationary phase could be stepwise eluted from the column at 80% (v/v) methanol in water. The mono-*O*-caproyl sucrose fraction containing seven regio-isomers was further separated by Lichrosorb RP 18 preparative HPLC column using a gradient of acetonitrile in water. The structural analysis of the purified regio-isomers was performed by NMR and MS with the substitution identified at the C-2, C-3, C-4, C-6, C-3, C-4', and C-6' position, respectively.

During the drying process, a high content of 3-*O*-caproyl sucrose in the purified 2-*O*-caproyl sucrose fraction was observed as the effect of acyl migration. In the presence of water at 60 °C, the formation of 3-*O*- and 6-*O*-caproyl sucrose from the purified 2-*O*-caproyl sucrose was detected as impurities. However, the migration was minimized when performing the drying process by lyophilization as 96% of purified 2-*O*-caproyl sucrose was obtained with the impurity of 3-*O*-caproyl sucrose. The purified 3'-*O*-caproyl sucrose was more stable than the purified 2-*O*-caproyl sucrose under lyophilization as no impurity was detected by HPLC. Thus, the results clearly demonstrated that the temperature is one of the key parameters controlling the acyl migration and thereby the purity of 2-*O*- and 3'-*O*-caproyl sucrose. The separation and purification of mono-*O*-caproyl sucrose regio-isomers presented in this work facilitates further characterization and comparison of the specific properties of the respective regio-isomers.

9.

References

Allen, D.K., and Tao, B.Y., (1999). Carbohydrate-alkyl ester derivatives as biosurfactants. *J. Surfactants. Deterg*. 2. 383-390.

Anderson, E.M., Larsson, K.M. and Kirk O., (1998). One biocatalyst-many applications: the use of *Candida antarctica* B-lipase in organic synthesis. *Biocatal. Biotransform*.16 (3). 181-204.

Arnorsdottir J., Kristjansson M.M., and Ficner R., (2005). Crystal structure of a subtilisin-like proteinase from a psychrotrophic *Vibrio s*pecies reveals structural aspects of cold adaptation. *FEBS Lett*. 272. 832-845.

Baczko K., Cauvin C N., Banoub J., Thibault P., and Plusquellec D., (1995). A new synthesis of 6-*O*-acyl sucrose and of mixed 6,6'-di-*O*-acyl sucrose. *Carbohydr. Res*. 267. 79-88.

Ball P., Ziemelis K., and Allen L., (2001). Synthesis methods that exploit enzymatic activities. *Nature*. 409. 225-235.

Berglund P. (2001). Controlling lipase enantioselectivity for organic synthesis. *Biomol. Eng*. 18. 13-22.

Bernet B., and Vasella A., (2000). Intra -and intermolecular H-bonds of alcohols in DMSO 1H-NMR analysis of inter-residue H-bonds in selected oligosaccharides: cellobiose, lactose, N,N''-diacetylchitobiose, maltose, sucrose, agarose and hyaluronates. *Chimica Acta* 83. 2005-2071.

Bornemann S., Cassells J.M., Dordick S., and Hacking A.J., (1992). The use of enzymes to regioselectively deacylate sucrose esters. *Biocatal. Biotransform*. 7. 1-12.

Bornscheuer , U.T., Bessler, C., Srinivas R. and Krishna, S.H., (2002). Optimizing lipases and related enzymes for efficient application. *Trends Biotechnol*. 20(10). 433-436.

Branden C. and Jooze J., (1999). Introduction to protein structure. 2nd edition. ISBN 0815323050 Page 137-148.

Cao L., Fischer A., Bornscheuer U.T., and Schmid R.D., (1997). Lipase-catalyzed solid phase synthesis of sugar fatty acid esters. *Biocatal. Biotransform*. 14. 269-273.

Carrea G., Riva S., and Secundo F., (1989). Enzymatic synthesis of various 1'-*O*-sucrose and 1-*O*-fructose esters. *J. Chem. Soc., Perkin*. 12. 1057-1061.

Cech N.B., and Enke C.G., (2001). Practical implications of some recent studies in electrospray ionization fundamentals. *Mass Spectrum. Rev*. 20. 362-387.

Chauvin C., and Plusquellec D., (1991). A new chemoenzymatic synthesis of 6'-*O*-acyl sucrose. *Tetrahedron Lett*. 32 (29). 3495-3498.

Chauvin, C., Baczko. K, and Plusquellec D., (1993). New highly regioselective reactions of unprotected sucrose: Synthesis of 2-*O*-acylsucrose and 2-*O*-(N- alkyl carbamoyl) sucrose. *J. Org. Chem*. 58. 2291-2295.

Clark D.S., (2004). Characteristics of nearly dry enzymes in organic solvents: implications for biocatalysis in the absence of water. *Philos. Trans. R. Soc. London, Ser B*. 359. 1299-1307.

Cormier R., Mai L.H., and Pommez P., (1978). Analysis of fatty acid esters of sucrose by means of HPLC. *Proceedings Technical Session Cane Sugar Refining Research*. 35-45.

Cruces M.A., Plou F., Ferrer M., Bernabe M., and Ballesteros A., (2001). Improved synthesis of sucrose fatty acid monoesters. *J. Am. Oil Chem. Soc*. 78 (5). 541-546.

Davies D.B., and Chirstofides J.C., (1987). Comparison of intramolecular hydrogen bonding conformations of sucrose containing oligosaccharides in solution and the solid states. *Carbohydr. Res*. 163. 269-274.

de Koster G.G., Pajarron A.M., Heerma W.,and Haverkamp J., (1993). Fast atom bombardment mass spectrometry of sucrose monocaprate and sucrose monolaurate. *Biol. Mass. Spectrom*. 22. 277-284.

Deferrari J. O., Muchnik de Lederkremer R., Matsuhiro B., and Sproviero J.F., (1962). Thin layer chromatography of acyl derivatives of sugars. *J. Chromatogr*. 9. 283-290.

Degn P., and Zimmermann W., (2001). Optimization of carbohydrate fatty acid ester synthesis in organic media by a lipase from *Candida antarctica*. *Biotechnol. Bioeng*. 74(6). 483-491.

Degueil-Castaing M., De Jeso B., Drouillard S., and Maillard B., (1987). Enzymic reactions in organic synthesis: ester interchange of vinyl esters. *Tetrahedron Lett*. 28(9). 953-954.

Devulapalle K.S., de Segura A.G., Ferrer M., Alcalde M., Mooser G., and Plou F.J., (2004). Effect of carbohydrate fatty acid esters on *Streptococcus sorbrinus* and glucosyltransferase activity. *Carbohydr. Res*. 339. 1029-1034.

Fallesen, 2009. Master thesis. Recombinant production of a protease from *Bacillus pseudofirmus* AL 89. Aalborg University. Denmark.

Faroq K., and Haque Z.U., (1992). Effect of sugar esters on the textural properties of nonfat low calorie yogurt. *J. Dairy Sci*. 75 (10). 2676-2680.

Feber K., (2000). Biotransformations in organic chemistry. Springer Verlag

Ferrer M., Cruces M.A., Bernabe M., Ballesteros A., and Plou F.J., (1999). Lipase-catalyzed regioselective acylation of sucrose in two solvent mixtures. *Biotechnol. Bioeng*. 65 (1). 10-16.

Ferrer M., Plou F.J., Pastor E., Fuentes G., Cruces M.A., Andersen L., Kirk O., Christensen M., and Ballesteros A.,(2002a). Effect of the immobilization method of lipase from *Thermomyces lanuginosus* on sucrose acylation. *Biocatal. Biotransform*. 20. 63-71.

Ferrer M., Soliveri J., Plou F.J., Lopez-Cortes N., Reyes-Duarte D., Christensen M., Copa-Patino JL., and Balllesteros A., (2005). Synthesis of sugar esters in solvent mixture by lipase from *Thermomyces lanuginosus* and *Candida antarctica* B, and their antimicrobial properties. *Enzyme Microb. Technol*. 36. 391-398.

Freedman B., Butterfield R.O., and Pryde E.H. (1986). Transesterification kinetics of soybean oil. *J. Am. Oil Chem. Soc*. 63 (10). 1375-1380.

Fuentes G., Ballesteros A., and Verma C., (2004). Specificity in lipase: a computational study of transesterification of sucrose. *Protein Sci*. 13. 3092-3103.

Fuentes G., Ballesteros A., and Verma C., (2007) Enthalpic and entropic contributions in the transesterification of sucrose: computational study of lipase and subtilisin. *J Biomol. Struct. Dyn*. 25(2). 145-155.

Fuentes G., Cruces M.A., Plou F.J., Ballesteros A., and Verma C., (2002). Computational studies of subtilisin catalyzed transesterification of sucrose: importance of entropic effects. *Chem.Bio.Chem*. 9. 907-910.

Gandhi N N., Patil N.S., Sawat S.B., Joshi J.B., Wangikar P.P., and Mukesh D., (2000). Lipase-catalyzed esterification. *Catal. Rev.- Sci. Eng*. 42(4). 439-480.

Garti N., Clement V., Fanun M.,and Leser M.E., (2000). Some characteristics of sugar ester nonionic microemulsions in view of possible food applications. *J. Agric. Food. Chem*. 48. 3945-3956.

Geraldine J., Mala S., and Takeuchi S., (2008). Understanding structural features of microbial lipases: an overview. *Anal. Chem. Insights*. 3. 9-19.

Gessesse A., Hatti-Kaul R., Gashe B.A., and Mattiasson B. (2003). Novel alkaline protease from alkaliphilic bacteria grown on chicken feather. *Enzyme Microb. Technol*. 32. 519-524.

Ghebregzabher M., Rufini S., Momaldi B and Lato.M., (1976). Thin layer chromatography of carbohydrates. *J. Chromatogr*. 127. 133-162.

Goldberg M., Thomas D., and Legoy M.D., (1990). The control of lipase-catalysed transesterification and esterification reaction rates: Effects of substrate polarity, water activity and water molecules on enzyme activity. *Eur. J. Biochem*. 190. 603-609.

Gubicza L.,and Szakacs-Schmidt A., (1994). Effects of the reaction conditions on the enantioselective esterification using *Candida cylindracea* lipase. *Biocatal. Biotransform.* 9. 131-143.

Gupta, R.K., (1983). Sucrose esters and sucrose ester/glyceride blends as emulsifiers. *J. Am. Oil Chem. Soc*. 60. 862-869.

Halling P.J., (1990). High-affinity binding of water by proteins is similar in air and in organic solvents. *Biochim. Biophys. Acta*. 1040. 225-228.

Halling P.J., (1992). Salts hydrates for water activity control with biocatalysts in organic media. *Biotechnol. Tech*. 6. 271-276.

Hathcox A.K. and Beuchat L.R., (1996). Inhibitory effects of sucrose fatty acid esters, alone and in combination with ethylenediaminetetraacetic acid and other organic acids, on viability of *Escherichia coli* O157:H7. *Food Microbiol*. 13. 213-225.

Hill K., and Rhode O., (1999). Sugar-based surfactants for consumer products and technical applications. *Fett-Lipid*. 101. 25-33.

Houde A., Kademi A., and Leblance D., (2004). Lipases and their industrial applications.*Appl. Biochem. Biotechnol*. 118. 155-170.

Iyer P. V., and Ananthanarayan L., (2008). Enzyme stability and stabilization-aqueous and non aqueous environment. *Process Biochem*. 43. 1019-1032.

Jaeger K.E., and Reetz. M.T., (1998). Microbial lipase form versatile tools for biotechnology. *Trends Biotechnol*. 16. 396-403.

James C.E., Hough L., and Khan R., (1989). Sucrose and its derivatives. *Prog. Chem. Org. Nat. Prod*. 55. 117–184

Jaspers M.E.A.P., Van Leeuwen F.F., Nieuwenhuis H.J.W., and Vianen G.M., (1987). High performance liquid chromatographic separation of sucrose fatty acid esters. *J. Am. Oil Chem. Soc*. 64(7) 1020-1025.

Karrer, R., and Herberg, H. J., (1992). Analysis of sucrose ester fatty acid esters by high temperature gas chromatography. J*. High. Resolut. Chromatogr*. 15. 585-589.

Kato A., Ando K., Jamura G., and Arima K., (1971). Effects of some fatty acid esters on the viability and transplantability of Ehrlich ascites tumor cells. *Cancer Research*. 31. 501-504.

Kato T., Yamagata Y., Arai T., and Ichishima E., (1999). Purification of a new extracellular 90 KDa Serine proteinase with isoelectric point of 3.9 from *Bacillus*

subtilis (natto) and elucidation of its distinct mode of action. *Biosci. Biotechnol. Biochem*. 56. 1166-1168.

Kaufman V.R., and Garti N., (1981). Analysis of sucrose fatty acid esters composition by HPLC. *J. Liq. Chromatogr.* 4 (7). 1195-1205.

Keurentjes J.T.F., Janssen G.H.R., and Gorissen J.J., (1994). The esterification of tartaric acid with ethanol: kinetics and shifting the equilibrium by means of pervaporation. *Chem. Eng. Sci*. 49. 4681-4689.

Khan R. (1995) Sucrose properties and applications. M. Mathlouthi, P. Reiser (Eds).,Chapman & Hall, London, 264-278.

Kim J.E., Han J.J., Yoon J.H., and Rhee J.S., (1998). Effect of salt hydrate pair on lipase-catalyzed regioselective monacylation of sucrose. *Biotechnol. Bioeng*. 57. 121-125.

Kirk O., and Christensen M.W., (2002). Lipase from *Candida antarctica*: unique biocatalysts from a unique origin. *Org. Process Res. Dev*. 6. 446-451.

Kitagawa M., and Tokiwa Y., (1998). Synthesis of a polymerizable glucose ester in hydrophilic organic solvents catalyzed by alkaline protease from *Streptomyces sp*. *J. Carbohydr. Chem*. 17. 893-899.

Kitagawa M., Raku T., Shimakawa H., Fan H., and Tokiwa Y., (2002). Effect of water on the enzymatic synthesis of vinyl sugar ester in hydrophilic organic solvent. *Macromol. Biosci*. 2. 233-237.

Klibanov A.M., (1997). Why are enzymes less active in organic solvents than in water?. *Trends Biotechnol*. 15 (3). 97-101.

Koeller K.M. and Wong C.H., (2001). Enzymes for chemical synthesis. *Nature*. 409. 232-240.

Kojima M., Kani M., Tominaga M., Kitazume S., Inoue A., and Horikoshi K., (2006). Isolation and characterization of a feather-degrading enzyme from *Bacillus pseudofirmus* FA 30-01. *Extremophiles*. 10 (3). 229-235.

Koskinen A.M.P., and Klibanov A.M., (1996) Enzymatic reactions in organic media. 1st edition. ISBN 0 7514 0259 1.

Kvittingen L., Sjursnes B., Anthonsen T., and Halling P.J., (1992). Use of salt hydrates to buffer optimal water level during lipase catalysed synthesis in organic media: A practical procedure for organic chemists. *Tetrahedron*. 48. 2793-2802.

Kwon S.J., Song K.M., Hong W.H., and Rhee J.S., (1995). Removal of water produced from lipase catalyzed esterification in organic solvent by pervaporation. *Biotechnol. Bioeng*. 46. 393-395.

Lauridsen J.B., (1976). Food emulsifier: surface activity, edibility, manufacture, composition and application. *J. Am. Oil Chem. Soc*. 53. 400-408.

Lichtenthaler F.W., and Immel S., (1995). Computer simulation of chemical and biological properties of sucrose, the cylclodextrins and amylase. *Int. Sugar J*. 97. 1153. 12-22.

Lichtenthaler F.W., Immel S., and Pokinskyj P., (1995). Selective 2-*O*-benzylation of sucrose: A faciale entry to its 2-deoxy-and 2-keto-derivaties and to sucrosamine. *Molecular Modelling of Saccharides*. 8. 1939-1947.

Lichtenthaler F.W., and Peters S., (2004). Carbohydrates as green raw materials for the chemical industry. *Chimie*. 7. 65-90.

Ma M., DiLollo A., Mercuri R., Lee T., Bundang M., and Kwong E., (2002). HPLC and LC-MS studies of the transesterification reaction of methylparaben with twelve 3- to 6-carbon sugar alcohols and propylene glycol and the isomerization of the reaction products by acyl migration. *J. Chromatogr. Sci*. 40. 170-178.

Martinelle M., Holmquist M., and Hult K., (1995). On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginose* lipase. *Biochim. Biophys. Acta*. 1258. 272-276.

Moh M.H., Tanga T.S., and Tan G.H., (2000). Improved separation of sucrose ester isomers using gradient high performance liquid chromatography with evaporative light scattering detection. *Food Chem*. 69. 105-110.

Molinier V., Wisniewski K. Bouchu A., Fitremann J, and Y.Queneau., (2003). Transesterification of sucrose in organic medium: study of acyl group migrations. *J. Carbohydr. Chem*. 22(7):657-669.

Moreau B., Lognay G., Blecker C., Destain J., Gerbaux P., Chery F., Rollin P., Paquot M., and Marlier M., (2007). Chromatographic, spectrometric and NMR characterization of a new set of glucuronic acid esters synthesized by lipase. *Biotechnol. Agron. Soc. Environ*. 11. 9-17.

Napier E.P., Lacerda H.M., Rossel C.M. Valivety R.H., Vaidya A.M., and Halling P.J. (1996). Enhanced organic-phase enzymatic esterification with continuous water removal in a controlled air-bleed evaculated headspace reactor. *Biotechnol. Progr*. 12. 47-50.

Nonaka T., Fujihashi M., Kita A.,Saeki K., Ito S., Horikoshi K., and Miki K., (2004). The crystal structure of an oxidatively stable subtilisin-like alkaline serine protease, KP-43, with a C-terminal β-barrel domain. *J. Biol. Chem*. 279 (45). 47334-47351.

Ntawukulilyayo, J.D., Demuynck C., and Remon J.P., (1995). Microcrystalline cellulose-sucrose esters as tablet matrix forming agents. *Int. J. Pharm*. 121. 205-210.

Ollis D.L., Cheah E., and Cygler M., (1992). The α/β hydrolase fold. *Protein Eng*. 5(3). 197-221.

Osipow L., and Rosenblatt W., (1967). Micro-emulsion process for the preparation of sucrose ester. *J. Am. Oil Chem. Soc*. 44(5). 307-309.

Ottosson J., (2001). Ph.D Thesis. Enthalpy and entropy in enzyme catalysis- A study of lipase enantioselectivy. Kungl Teknishka Hogskolen Stockholm. Sweden.

Parida S., and Dordick J.S., (1991). Substrate structure and solvent hydrophobicity control lipase catalysis and enantioselectivity in organic media. *J. Am. Chem. Soc*. 113. 2253-2258.

Park O.J., Kim D.Y., and Dordick J.S., (2000). Enzyme catalyzed synthesis of sugar containing monomers and linear polymers. *Biotechnol. Bioeng*. 70. 208-216.

Pedersen N.R., Halling P.J., Pedersen L.H., Wimmer R., Matthiesen R., and Veltman O.R., (2002). Efficient transesterification of sucrose catalyzed by the metalloprotease thermolysin in dimethylsulfoxide. *FEBS Lett*. 519. 181-184.

Pedersen N.R., Wimmer R., Emmersen J., Degn P., and Pedersen L.H., (2002). Effect of fatty acid chain length on initial reaction rates and regioselectivity of lipase catalyzed esterification o disaccharides. *Carbohydr. Res*. 337. 1179-1184.

Pedersen N.R., Wimmer R., Matthiesen R., Pedersen L.H., and Gessesse A., (2003). Synthesis of sucrose laurate using a new alkaline protease. *Tetrahedron: Asymmetry*. 14. 667-673.

Perez-Victoria I., Zafra A., and Morales J.C., (2007). Determination of regioisomeric distribution in carbohydrate fatty acid monoesters by LC-ESI-MS. *Carbohydr. Res*. 342. 236-242.

Plou F., Cruces M.A., Ferrer M., Fuentes G., Pastor E., Bernabe M., Christensen M., Comelles F., Parra J.L., and Ballesteros A., (2002). Enzymatic acylation of di- and trisaccharides with fatty acids: choosing the appropriate enzyme, support and solvent. *J. Biotechnol*. 96. 55-66.

Plou F.J., Cruces M.A., Pasto E., Ferrer M., Bernabe M., and Ballesterose A. (1999). Acylation of sucrose with vinyl esters using immobilized hydrolase: demonstration that chemical catalysis may interfere with enzymatic catalysis. *Biotechnol Lett*. 21. 635-639.

Polat T., Bazin HG., and Linhardt RJ., (1997). Enzyme catalyzed regio-selective synthesis of sucrose fatty acid surfactant. *J. Carbohydr. Chem*. 16. 1319-1325.

Polat T., and Linhardt R.J., (2001). Syntheses and applications of sucrose based esters. *J. Surfactants. Deterg*. 4(4). 407-414.

Potier P., Bouchu A., Gagnaire J., and Queneau Y., (2001). Proteinase N-catalyzed regioselective esterification of sucrose and other mono-and disaccharides. *Tetrahedron: Asymmetry*. 12. 2409-2419.

Queneau Y., Fitremann J., and Trombotto S., (2004). The chemistry of unprotected sucrose: the selectivity issue. *Chimie*. 7. 177-188.

Reis P., Holmberg K., Watzke H., Leser M.E., and Miller R., (2009). Lipases at interfaces: a review. *Adv. Colloid Interface Sci*. 147-148. 237-250.

Reyes-Duarte D., Lopez-Cortes N., Ferrer M., Plou F.J., and Ballesteros A., (2005). Parameters affecting productivity in the lipase-catalyzed synthesis of sucrose palmitate. *Biocatal. Biotransform*. 23. 19-27.

Rantwijk Van F., (2005). Reaction of serine and metallo hydrolases: hydrolysis, esterification, transesterification, aminolysis, perhydrolysis. In *Adv. Course on Biocatalysis*, Delf University of Technology, The Netherlands, April 2005.

Rich J.O., Bedell B.A., and Dordick J.S., (1995). Controlling enzyme-catalyzed regioselectivity in sugar ester synthesis. *Biotechnol. Bioeng*. 45. 426-434.

Riva S., Chopineau J., Kieboom A.P.G., and Klibanov A.M., (1988). Protease-catalyzed regioselective esterification of sugars and related compounds in anhydrous dimethylformamide. *J. Am. Chem. Soc*. 110. 584-589.

Robb D.A., Zhen Y., and Halling P.J., (1994). The use of salt hydrates as water buffers to control enzyme activity in organic solvents. *Biocatal. Biotransform*. 9. 277.283.

Rosel C.M., Vaidya A.M., and Halling P.J., (1996). Continuous in situ water activity control for organic phase biocatalysis in a packed-bed hollow fiber reactor. *Biotechnol. Bioeng*. 49. 284-289.

Rubio, E., Fernandez-Meyorales A., and Klibanov A.M., (1991). Effect of the solvent on enzyme regioselectivity. *J. Am. Chem. Soc*. 113. 695-696.

Ryu K., and Dordick J.S., (1992). How do organic solvents affect peroxidase structure and function?. *Biochemistry*. 31. 2588-2598.

Samia S., Engasser J.M., and Ghoul M., (2001). Effect of acyl donor chain length and sugar/acyl donor molar ratio on enzymatic synthesis of fatty acid fructose esters. *J. Mol. Catal B: Enzym*. 11. 725-731.

Schulze B., and Klibanov A.M., (1991). Inactivation and stabilization of subtilisins in neat organic solvents. *Biotechnol. Bioeng*. 38. 1001-1006.

Secundo F., Carrea G., and Tarabiono C., (2006). The lid is a structural and functional determinant of lipase activity and selectivity. *J. Mol. Cat. B: Enzym*. 39. 166-170.

Siezen RJ., and Leunissen J.A.M. (1997). Subtilase: the superfamily of subtilisin-like serine protease. *Protein Sci*. 6. 501-523.

Soedjak HS., and Spardlin J.E., (1994). Enzymatic transesterification of sugars in anhydrous pyridine. *Biocatal. Biotransform*. 11. 241-248.

Stauffer C.E., (1990). Eagan Press Handbook Series: Emulsifiers. ISBN 1891127020

Tai, A., Okazaki. S., Tsubosuka N., and Yamamoto I., (2001). Protease- catalyzed monoacylation of 2-*O*-α-D-glucopyranosyl-L-ascorbic acid in pyridine. *Chem. Pharm. Bull*. 49(8): 1047-1049.

Tawaki S., and Klibanov A.M., (1992). Inversion of enzyme enantioselectivity mediated by the solvent. *J. Am. Chem. Soc*. 114. 1882-1884.

Tawaki S., and Klibanov A.M., (1993). Chemoselectivity of enzymes in anhydrous media is strongly solvent dependent. *Biocatal. Biotransform*. 8. 3-19.

Therisod M., and Klibanov A.M., (1986). Facile enzymatic preparation of monoacylated sugars in pyridine. *J. Am. Chem. Soc*. 108. 5638-5640.

Thevemet S,.Wernicke A., Belniak S., Descotes G., Bouchu A., and Queneau Y. (1999). Esterification of unprotected sucrose with acid chlorides in aqueous medium: kinetic reactivity vs acyl-or alkyloxycarbonyl group migration. *Carbohydr. Res*. 318. 52-66.

Torres M.C., Dean M.A., and Wagner F.W. (1990). Chromatographic separations of sucrose monostearate structural isomers. *J. Chromatog*. 522. 245-253.

Tsavas P., Polydorous S., Voutsas E.C., Magoulas K.G., Naragi K., and Halling P.J, (2002). Sucrose solubility in mixtures of water, alcohol, ester and acid. *J. Chem. Eng. Data*. 47(3). 513-517.

Tsuda Y., and Yoshimoto K., (1981). General path of *O*-acyl migration in D-glucose derivatives. *Carbohydr. Res*. 87. C1-C4.

Uppenberg J., Hansen M.T., Patkar S., and Jones A., (1994). The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida antarctica*. *Structure*. 2 (4). 453-454.

Valivety R.H., Halling P.J., and Macrae A.R., (1992a). Reaction rate with suspended lipase catalyst shows similar dependence on water activity in different organic solvents. *Biochim. Biophys. Acta*. 1118(3). 218-222.

Valivety R.H., Halling P.J., and Macrae A.R., (1993). Water as a competitive inhibitor of lipase catalyzed esterification in organic media. *Biotechnol Lett*. 15. 1133-1138.

Wang Q., Zhang S., Zhang P., Zhu J., and Yang J., (2006). Separation and quantitation of sucrose esters using HPLC with evaporative light scattering detection. *J. Liq. Chromatogr. Related Technol*, 29: 2399-2412.

Wang Q., Zhang S., and Yang J., (2007). HPLC analysis of sucrose ester analogs using evaporative light scattering detection. *J. Liq. Chromatogr. Related Technol*. 30. 2395-2407.

Wang Q., Zhang S., and Yang J., (2007). Regioselective formation of 6-O-acyl sucroses and 6,3-di-O-acylsucrose via the stannylene acetal method. *Carbohydr. Res*. 342. 2657-2663.

Wangikar P.P., Michels P.C., Clark D.S., and Dordick J.S., (1997). Structure and function of sutilisin BPN' solubilized in organic solvents. *J. Am. Chem. Soc*. 119. 70-76.

Wehtje E., Svensson I., Aldercreutz P., and Mattiason B., (1993). Continuous control of water activity during biocatalysis in organic media. *Biotechnol.Tech*. 12. 873-878.

Wescott C.R., and Klibanov A.M. (1993). Solvent variation invers substrate specificity of an enzyme. *J. Am. Chem. Soc*. 115. 1629-1631.

Woudenberg M., Rantwijk V., and Sheldon R.A., (1996). Regioselective acylation of disaccharides in *tert*-butyl alcohol catalyzed by *Candida antarctica* lipase. *Biotechol. Bioeng*. 49. 328-333.

Yamagata Y., Sato T., Hanzawa S., and Ichishima E., (1995). The structure of subtilsin ALP I from alkalophilic *Bacillus sp.* NKS-21. *Curr. Microbiol*. 30. 201-209.

Yamagishi Y., Hattori M., Yoshida T., and Takahashi K., (2004). Improvement of the functional properties of sucrose stearate by phosphorylation. *J. Agri. Food Chem*. 52. 8039-8045.

Yang H., Henke E., and Bornscheuer U.T., (1999). The use of vinyl esters significantly enhanced enantioselectivities and reaction rates in lipase catalyzed resolution of arylaliphatic carboxylic acids. *J. Org. Chem*. 64. 1709-1712.

Yoshimoto K., and Tsuda Y., (1983). General path of *O*-acyl migration in D-glucose derivatives: acyl migration of methyl mono-*O*-myristoy-α-and -β- D-glucopyranosides and mono-*O*-myristoyl-D-glucopyranoses. *Chem. Pharm. Bull*. 31. 4324-4334.

Zarevucka M., Rejzek M., Hoskovec M., Svatos A., Wimmer Z., Koutek B., and Legoy M.D., (1997). Initial water content and lipase-mediated ester formation in hexane. *Biotechol. Lett*. 19. 745-750.

http://www.theherbarie.com/ visited 01 06 09

http://www.sartomer.com/ visited 01 06 09

http://www.sisterna.com/ visited 01 06 09

Section II

Contents of section II

Chapter 1

Activity and stability of proteases in hydrophilic solvents

Pedersen L.H., **Ritthitham S.,** and Kristensen M., (2009).

In *Modern Biocatalysis: Stereoselective and environmentally friendly reactions*,
Wolf-Dieter Fessner and Thorleif Anthonsen, Editors.
Wiley-VCH: Weinheim. p. 55-66
(ISBN 978-3-527-32071-4)