ENZYMATIC PRODUCTION OF SUGAR FATTY ACID ESTERS

Von der Fakultät Chemie der Universität Stuttgart
Zur Erlangung der Würde eines Doktors der
Naturwissenschaften (Dr. rer. nat.) genehmigte Abhandlung

vorgelegte von

Dipl.-Ing. Youchun Yan
aus VR China

Hauptberichter:	Prof. Dr. Rolf D. Schmid
Mitberichter:	Prof. Dr. Dieter H. Wolf
Vorsitzender:	Prof. Dr. H. Bertagnolli

Tag der mündlichen Prüfung: 21. März 2001

Institut für Technische Biochemie der Universität Stuttgart

2001

LIST OF PUBLICATIONS

1. Yan, Y., Bornscheuer, U.T., Stadler, G., Lutz-Wahl, S., Otto, R.T., Reuss, R. and Schmid, R.D. (2001) Regioselective glucose synthesis on preparative scale. Submitted to *Eur. J. Lipid Sci. Technol.*.

2. Yan, Y., Bornscheuer, U.T. and Schmid, R.D. (2000) Efficient water removal in lipase catalyzed esterifications using a low-boiling-point azeotrope. submitted to *Biotechnol. Bioeng.* on 18/9/2000.

3. Yan, Y., Bornscheuer, U.T. and Schmid, R.D. (2000) Application of crystallization technique for the lipase-catalyzed solid phase synthesis of sugar fatty acid monoesters. In AOCS book "Crystallization and solidification properties of lipids" edited by N. Wildlak, R. Hartel and S. Narine. *in press.* AOCS Press. accepted on 20/9/2000.

4. Yan, Y., Bornscheuer, U.T., Stadler, G., Wahl, S.L., Reuss, R. and Schmid, R.D. (2001) Production of sugar fatty acid esters by enzymatic esterification in a stirred-tank membrane reactor: Optimization of Parameters by Response Surface Methodology. *J. Amer. Oil Chem. Soc.* **78**: 147-152.

5. Yan, Y., Bornscheuer, U.T., Stadler, G., Reuss, R. and Schmid, R.D. (2000) Enzymatic production of biosurfactant 6-*O*-glucose fatty acid monoesters. *J. Deterg. Cosmetics*. *in press*. accepted date: 12/5/2000.

6. Bornscheuer, U.T., Syldatk, C., Schmid, R.D., Yan, Y. and Otto, R.T. (2000) Procedure for selective esterifications of polyols. *German patent* DE 19924221 A1.

7. Yan, Y., Bornscheuer, U.T. and Schmid, R.D. (1999) Lipase-catalyzed synthesis of vitamin C esters. *Biotechnol. Letters*. **21**:1051-1054.

8. Yan, Y., Bornscheuer, U.T., Cao, L. and Schmid, R.D. (1999) Lipase-catalyzed solid-phase synthesis of sugar fatty acid esters: Removal of byproducts by azeotropic distillation. *Enzyme Microb. Technol*. **25**:725-728.

ACKNOWLEDGEMENTS

I would like to thank all those who contributed in one way or another to the research summarized in this thesis, first of all my supervisor, Professor Dr. Rolf D. Schmid for giving me the opportunity to do this work in his laboratory and also for his useful advise and encouragement. Furthermore, I would like to thank him very much for his support to my scholarship application as well as his numerous helps during my stay in Germany.

I would also like to thank Professor Dr. Uwe Bornscheuer, the former head of Biocatalysis group at the ITB, now at the Institute of Chemistry, Department of Technical Chemistry & Biotechnology, University of Greifswald, for his guidance, enthusiasm and numerous helpful discussions, corrections of my publications and the manuscript of this dissertation, even after his relocation to Greifswald.

I also wish to express my thanks to Professor Dr. Dieter H. Wolf for the review of this thesis and Professor Dr. Helmut Bertagnolli for the examination.

I am grateful to all my colleagues at ITB and especially the members of the Biocatalysis group, especially Dr. Sabine Lutz-Wahl, Dr. Markus Enzelberger, Dr. Ulrike Schmid, Dr. Sandra Pisch, Dr. Frank Zocher, Erik Henke, Markus Baumann, Sandra Vorlova and Marcus Kleinmaier, for their advice and assistance; to Prof. Dr.-Ing. Matthias Reuss, Prof. Dr. Christoph Syldatk, Dr. Ralf Otto, Gerhard Stadler at the Institute of Biochemical Engineering, University of Stuttgart for their cooperation. Particular thanks are to Dr. Lieming Ding for the correction of this thesis; to the former Chinese Ph.D. student Dr. Linqiu Cao for his help in the lab during my initial period.

I also appreciate the KAAD (Bonn, Germany) for their financial support and especially to Dr. Heinrich Geiger for his numerous helps during my stay in Germany. Thanks are to Henkel KGaA (Düsseldorf, Germany), Roche Diagnostics GmbH (Penzberg, Germany), Novo Nordisk A/S (Bagsveard, Denmark) and Sulzer Chemtech GmbH (Neunkirchen, Germany) for providing me with experimental materials; to Henkel KGaA also for supporting my patent application.

Last, my greatest debt is to my wife Wen Zhou for her love and dedication, and my son Qin Yan who has been at times neglected during the preparation of this work.

ABSTRACT

Sugar fatty acid esters (SFAE) are well known as bio-surfactants. Their excellent biodegradability as well as the fact that they are tasteless, odorless, nontoxic, non-irritant and non-ionic explains their increasing importance in numerous areas. On the other hand, for a long time, large scale production of SFAE remained mainly in the realms of organic chemistry and chemical processing. Chemical methods are mainly performed at high temperatures in the presence of alkaline catalysts. High energy consumption, coloring of products and low selectivity are major disadvantages of these methods. Moreover, some chemically synthesized SFAE are toxic and not readily biodegradable, thus causing their limited application in cosmetics, food industry and pharmaceutics. Enzymatic synthesis offers an alternative way.

In the present work, a novel and effective enzymatic method eliminating most difficulties was developed for the production of SFAE. Unprotected sugars and non-activated fatty acids were directly used as starting materials in order to decrease production cost. The selection of organic solvent is very crucial. It turned out that only ethyl methylketone (EMK) or a mixture of EMK and hexane are useful for the production of SFAE, because both organic solvents are not only easily eliminated and allowed for use in the manufacture of foods and/or food additives, but also can form an azeotrope with reaction water which is easily removed from the reaction medium by azeotropic distillation. For the application of the membrane pervaporation for the solvent regeneration, the selection of membrane material is quite important. It was found that Pervap® 2200 membrane is suitable to remove water from EMK.

In order to get high conversion, some important parameters such as reaction time (T_r), substrate ratio ($S_{r,}$ acyl donor to glucose), reaction temperature (R_t), solvent EMK or mixture of EMK and hexane amount ($S_{a,}$ based on excess of substrates) and enzyme load ($E_{l,}$ based on substrates) were investigated by response surface methodology in this work. In case of EMK, due to the various parameters to be considered, response surface methodology was used to identify best process conditions and up to 93 % yield of glucose stearate were achieved under optimized conditions (T_r = 58 h; S_r = 2.7; E_l = 8.9 % [w/w]; R_t = 78 °C; S_a = 1.9). In the case of a solvent mixture of EMK and hexane, 93% yield were also achieved at 59°C after 48 h using an equimolar ratio of glucose and stearic acid.

Since the price of the biocatalyst largely contributes to the overall process costs, the factors affecting its long-term stability have been investigated in detail. Systematic analyses using supports of different aquaphilicity were used to find an optimum in enzyme stabilization. For

the determination of the operation stability, the model production of glucose palmitate was studied for seven subsequent reaction cycles (each 48 h) at an enzyme concentration of 9% (w/w) using a 20:80 (v/v) ratio of hexane:EMK at 59°C. The reaction was terminated by addition of acetone, the enzyme separated by filtration and dried in *vacuo* immediately prior to re-use in the next cycle. It was shown that after 14 days, still about 86% synthetic activity can be achieved in the mixture of EMK and hexane. In contrast, CAL-B lost about 40% activity in reactions with pure EMK at 59°C and 80% activity if the ester synthesis is conducted at 75°C.

In this work it was proved that it is suitable a stirred-tank reactor in combination with a prevaporation membrane-module for the production of SFAE. The yield of 79.2% glucosestearate was achieved with 2L reactor in preparative scale, although a cheap substrate D-glucose was used instead of β-D(+)-glucose.

The process for the enzymatic synthesis of SFAE described herein demonstrates a useful solution to commercialize this technology. This process does not require prior modification of substrates: the renewable resources sugar and fatty acids. It offers also a number of advantages such as high productivity, high ratio of product to reaction mixture and reaction rate. More importantly, the solvents EMK and hexane are non-toxic, biocompatible and permitted for the use in food industry by German authorities. Furthermore, usage of only very small amounts of organic solvents and high reaction rates lead to high space-time yields, which is a very important factor for the industrialization. In addition, recovery of solvent by membrane vapour permeation/pervaporation, mild and ‘environmentally friendly’ reaction conditions result in low energy consumption and high enzyme stability. The reaction is performed with equimolar reactants, resulting in a more simple downstream processing. Therefore, this process is of great significance on a large scale.

ZUSAMMENFASSUNG

Enzymatische Herstellung von Zuckerfettsäureestern

Zuckerfettsäureester finden breite Anwendung als Detergentien und Emulgatoren. Als nichtionische, oberflächenaktive und bioabbaubare Substanzen wird darüber hinaus auch über ihren Einsatz als Pharmazeutika sowie in der Kosmetik- und Nahrungsmittelindustrie berichtet. Der größte Teil der im Handel befindlichen Zuckerfettsäureestern wird derzeit über chemische Verfahren in Ver- oder Umesterungsreaktionen unter Verwendung alkalischer Katalysatoren bei hohen Temperaturen hergestellt. Das Hauptproblem dieser Verfahren sind die hohen Energiekosten sowie die mangelnde Regioselektivität der Synthesereaktionen, wodurch die Bildung von Strukturisomeren verursacht wird, wenn an den verschiedenen Hydroxylgrupen der Zuckerkomponenten die Reaktion stattfindet. Außerdem ist die Entfernung der toxischen organischen Lösungsmittel (DMSO und DMF) sehr schwer. Dies beeinträchtigt zudem die Qualität des Produktes.

Im letzten Jahrzehnt wurden daher eine ganze Reihe von enzymatischen Methoden zur Synthese von Zuckerfettsäureestern vorgeschlagen. Eine von unserem Institut entwickelte Methode besteht darin, dass die Lipase-katalysierten Synthese von Zuckerfettsäureestern in einem System durchgeführt wird, welches hauptsächlich aus einer Festphase besteht. Diese beinhaltet den Zucker, die Fettsäure und das Produkt, wobei als Zuckerkomponente ungeschützte Monosaccharide (in diesem Fall Glucose) eingesetzt werden können. Hauptproblem bei dieser Methode ist jedoch, dass sie sehr schwer im großen Massstab durchzuführen ist. Ein anderes Problem beruht auf der Verwendung von Molekularsieb als Trocknungsmittel, da hierdurch aufwendige Wiedergewinnungsschritte notwendig sind.

In dieser Arbeit gelang es, ein neues effektives enzymatisches Verfahren zur Synthese von Zuckerfettsäureestern zu entwickeln. Es basiert auf der Umsetzung eines Zuckers (Glucose) mit einer Fettsäure (Kettenlänge C_6-C_{18}) mittels Lipase-Katalyse (optimal: Lipase aus *Candida antarctica* B immobilisiert an einem Träger aus Polypropylen) in Gegenwart geringer Mengen eines Lösungsmittels (optimal: Ethylmethylketon bzw. Mischung von Ethylmethylketon und Hexan). Lösungsmittel und Reaktionswasser bilden ein Azeotrop, welches aus dem Reaktionsmedium durch Distillation entfernt wird. Das Lösungsmittel wird durch Membran-Pervaporation wiedergewonnen, bevor es in das Reaktionsmedium zurückgeführt wird. Nach kurzer Reaktionszeit kommt es zur Verfestigung des Reaktionsgemisches, wobei die Reaktion jedoch durch die feine Verteilung von Lipase und Substraten fortschreitet und unter optimalen Bedingungen nach weniger als 48 Stunden

abgeschlossen ist. Durch NMR-spektroskopische Untersuchung des Produktes wurde nachgewiesen, dass die Veresterung regioselektiv an der primären Hydroxylgruppe des Zuckers erfolgt. Das Verfahren wurde hinsichtlich der Auswahl geeigneter Lösungsmittel, verschiedener Membranmaterialien, Substratverhältnis, Enzymmenge, Reaktionszeit, Temperatur und Lösungsmitteleinfluss optimiert. Dabei zeigte sich, dass insbesondere die Wahl und Konzentration des organischen Lösungsmittels einen erheblichen Einfluss auf Umsatz und Selektivität in der Reaktion hat. Als optimal erwies sich ein äquimolares Verhältnis von Zucker und Acyldonor. Im folgenden wird die Untersuchung und Optimierung verschiedener Reaktionsparameter ausführlicher geschildert:

Auswahl des Lösungsmittels: Die Löslichkeit der Zucker in für die Lebensmittelindustrie zugelassenen organischen Lösungsmittel wie Aceton, Ethylmethylketon, Hexan, Methanol wurde bestimmt. Bei Raumtemperatur liegt die Löslichkeit von Glucose in Ethylmethlketon bei nur 0,06 g/L. Trotz dieser geringen Löslichkeit ist jedoch die enzymatische Reaktion der geschwindigkeitsbestimmende Schritt, da die Lösungsgeschwindigkeit für Glucose in Ethylmethylketon bei 60°C ca. 25 mal höher als die Reaktionsgeschwindigkeit ist. Als Zusatz gelten organische Flüssigkeiten, die in kleinen Mengen zur Modifikation der Reaktionsphase eingesetzt werden, um die für den Stofftransport und damit für den Ablauf der Reaktion notwendige flüssige Phase zu erhalten. Die Verwendung der oben gennanten Lösungsmitteln hat einen weiteren Vorteil: Ethylmethylketon, Aceton und eine Mischung aus Ethylmethylketon und Hexan bilden mit dem Nebenprodukt Wasser oder Methanol ein Azeotrop, das vom Reaktionsmedium durch Distillation entfernt werden kann. Das optimale Verhältnis zwischen Reaktanden und Lösungsmittel lag in einem Bereich von 1:1,3 bis 1:1,6 (bezogen auf das Substratgewicht). Unter optimallen Bedingungen [EMK:Hexan 4:1 (v/v)], hohsten so 93% Glucosestearinsäureester dargestellt werden.

Einfluss des Wassergehaltes: Es konnte ein grosser Einfluss des Wassergehaltes in der Reaktionsmischung auf die Umsatz festgestellt werden. Dies beweist, dass die effektive Entfernung des in der Reaktion entstehenden Wassers eine wichtige Rolle spielt. Der Wassergehalt in der Reaktionmischung muss in einem Bereich von 0,02-0,4% liegen.

Auswahl des Membranmaterials: Nach der Produktinformation des Herstellers und den Ergebnisse dieser Arbeit, ist das geeignete Membranmaterial zur Wasserentfernung aus Ethylmethylketon die Pervap® 2200 Membran.

Acyldonoren: Bei Verwendung längerkettiger, gesättigter Fettsäuren (>C10) konnten Umsätze zwischen 70-100 % mit Glucose als Acylakzeptor erzielt werden. Im Gegensatz dazu lagen die Umsätze für kürzere Fettsäuren bei lediglich 40-60%. Die erhöhte Löslichkeit der Produkte führt hier zu einer schlechteren Kristallisation bzw. Verfestigung des Reaktionsgemisches, was sich ungünstig auf die Gleichgewichtslage auswirkt. Ferner kann auch eine Hemmung durch die höhere Konzentration des Produktes in der flüssigen Phase nicht ausgeschlossen werden.

Einfluss des Substratverhältnisses: Eine Variation des Verhältnisses zwischen Fettsäure und Zucker ergab, dass optimale Umsätze und Produktivitäten abhängig von der Reaktionstemperatur waren, bei 60°C erwies sich ein äquimolares Verhältnis als am Günstigsten.

Einfluss der Temperatur: Die Reaktionstemperatur konnte durch die Löslichkeit des Produktes beeinflusst werden. Der höchste Umsatz mit längerkettigen, gesättigten Fettsäuren wie Palmitinsäure und Stearinsäure wurde bei 60°C beobachtet, während sich die Bildung von Zuckerestern aus kürzerkettigen Fettsäuren wie Caprylsäure bei 25°C als optimal erwies.

Einfluss der Enzymmengen: Normalweise beschleunigt sich beim Einsatz grösserer Enzymmengen die Reaktionsgeschwindigkeit, dabei werden aber die Produktionskosten erhöht. Das optimale Verhältnis zwischen Reaktanden und Enzyme lag in einem Bereich von 8% bis 10% Enzym bezogen auf das Substratgewicht.

Enzymstabilität: Es konnte gezeigt werden, dass die immobilisierte Lipase CAL-B-EP 100 bei einer Reaktionstemperatur von 59°C nur geringfügig an Aktivität verlor und auch nach ihrem Einsatz in sieben Batchreaktionen (14 Tage) noch 86% synthetische Aktivität erhalten bleibt. Häufig wurde ein starker Einfluss von Temperaturen über 60°C bzw. organischer Lösungsmittel auf die Enzymsstabilität beschrieben. In dieser Arbeit wurde eine klare Korrelation zwischen der Polarität des organischen Lösungsmittels und der hydrolytischen Aktivität gefunden.

Design eines Bioreaktors: In dieser Arbeit erwies sich der traditionelle Rührkessel in Verbindung mit einem Pervaporation-Membranmodul als geeigneter Bioreaktor. Mit diesem

wurden Versuche im 200 g-Massstab durchgeführt und 79% Ausbeute on D-Glucoseestern der Stearinsäuren erzielt.

Ausserdem wurden ungewöhnliche Zuckerester wie z.B. Vitamin C-Fettsäureester, Salicin-Fettsäureester und Zimtsäure-Glucoseester, die für medizinische und pharmazeutische Anwendungen interessant sind, im beschriebenen System erfolgreich synthesiert.

Vorteile der Lipase-katalysierte Produktion von Zuckerfettsäureestern:
Zusammenfassend kann festgestellt werden, dass die Lipase-katalysierte Produktion von Zuckerfettsäureestern als neues Verfahren eine Reihe von Vorteilen gegenüber den bisher beschriebenen Methoden aufweist. Hervorzuheben sind hier die direkte Verwendung von Zuckern, einfacher Acyldonoren (freie Fettsäure), relativ hoher Substratkonzentrationen und die geringen Mengen an für die Lebensmittelindustrie durch die zuständige Behörde zugelassene Lösungsmittel sowie deren Regeneration durch Membranpervaporation. Das hier entwickelte Verfahren zeichnet sich durch hohe Raum-Zeit-Ausbeuten (0.05-0.12 g/ml*d) und Produktivitäten (0.56-1.3 mmol/g*h) aus. Die Immobilisierung der Lipase führt zu hohen Stabilitäten (noch 86% synthetische Aktivität nach 14 Tage) und erlaubt eine leichte Abtrennung vom Reaktionsgemisch.

ABBREVIATIONS

ACN	Acetonitrile
ACT	Acetone
ANOVA	Analysis of variance
a_w	Thermodynamic water activity
BHA	Butylated hydroxyanisol
BHT	Butylated hydroxytoluene
b.p.	Boiling point
C	Carrier
CAL-B	Lipase B from *Candida antarctica*
CCF	Central composite face
DMF	Dimethylformamide
DMP	Dimethylpyrrolidone
DMSO	Dimethylsulfoxide
DS	Degree of substitution
E, Enz	Enzyme
E_l	Enzyme load
EMK	Ethyl methylketone
EMR	Enzyme membrane reactor
EP 100	Polypropylene 100
Fa	Fatty acid
f.s.	Fast solidification
g	gram
G. F. T.	Gesellschaft für Trenntechnik
h	hour
Hex, HEX	Hexane
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
K_M	Michaelis-Menten constant
L-2	Chirazyme® L-2 lipase B from *Candida antarctica*
Lipozyme IM	Lipozyme, immobilized lipase from *Rhizomucor miehei*
M	Molecular mass
2-MBA	2-methyl-2-butanol
M_h	Molecular mass of hydrophilic fraction of surfactant
min	minute
n.a.	not available
n.d.	not determined
NMR	Nuclear Magnetic Resonance
n.s.	No solidification
P	Product
PBA	Phenylboronic acid complex
PEG	Polyethyleneglycol
P_l	Product in liquid phase
Pr.	Prediction
Q^2	Fraction of the variation of the response predicted by the model
p/r	Ratio of product to reaction mixture, w/w
R^2	Fraction of the variation of the response explained by the model,
RMS	Response surface methodology

R_t	Temperature
S	Substrate
S_a	Solvent to substrates
SFAE	Sugar fatty acid ester
S_l	Substrates in liquid phase
SP 435	Novozym SP435, lipase B from *Candida antarctica*
SP 525	Free lipase B from *Candida antarctica*
S_r	Acyl donor to glucose
s.s.	Slow solidification
b_0	Intercept
b_i	First order model coefficients
b_{ii}	Quadratic coefficients for the *i*th variable
b_{ij}	Interaction coefficients for the interaction of variables i and j
T, T_r	Reaction time
TBA	*tert*-Butanol
TDM	Tausend Deutsche Mark ~ 500 Euro
THF	Tetrahydrofuran
TLC	Thin layer Chromatography
U	Unit of lipase activity
V	Volume of reaction mixture
Vc	Vitamin C
v_s	Solubilisation rate
X_i	Independent variables
Y	Value of the response

Content

1. Introduction

1.1 General introduction

In the last decade due to raise of labor cost, shortage of available industrial land, and strong pressure on environmental protection the traditional manufacturing business are no longer as competitive as used to. Many of them are diminishing and some are being replaced by emerging technologies. Biotechnology, a potential star industry in the twenty-first century, has been identified as one of the strategic sciences and technologies by many countries in the world. High research priority and financial support have been shifted to that area, which has resulted in development of a new global industry. The sales of bio-products are around US$ 500 billion by the end of last century as compared to US$ 25 million in 1980 (Layman 1985), US$ 1.7 billion in 1992 and is increasing at a rate of 3-5% annually (Desai and Banat 1997) (Lin 1996).

The production of surfactants based on fats, oils and carbohydrates is a large industry. In 1995 the worldwide production exceeds 3 million tons per annum (at an estimated value of 4 billion US$) and was rising to over 4 million tons by the end of last century (Greek 1991) (Sarney and Vulfson 1995). The non-ionic surfactants occupy 24%. The consumption of surfactants as household/laundry detergents account for 54% of the total output, with only 32% destined for industrial use. On one hand, their excellent biodegradability as well as the fact that they are non-ionic explains their increasing importance in numerous areas. On the other hand, for a long time large scale production of sugar fatty acid esters (SFAE) remained mainly in the realms of organic chemistry and chemical processing. Chemical methods are mainly performed at high temperatures in the presence of alkaline catalysts. High energy consumption, coloring of products and low selectivity are major disadvantages of these methods. Moreover, some chemically synthesized SFAE are toxic and not readily biodegradable, thus causing their limited application in cosmetics, food industry and pharmaceutics where excellent toxicological properties are desired. They usually are not permitted to be used in the food industry. However, rapid advances in biotechnology over the past decade have led to considerable interest in the development of biological methods for manufacturing surfactants and other value-added compounds on the industrial scale (Ergan et al. 1988; Omar et al. 1988; McNeill et al. 1991; Mohamed et al. 1993; Carrillo-Munoz et al. 1996; Otero et al. 1996; Chevandier et al. 1997; From et al. 1997; Gandhi 1997; Okazaki et al. 1997; Polat et al. 1997; Tweddell et al. 1997; Soumanou et al. 1998; Torres et al. 2000). They

possess many advantages over the chemically manufactured process, including mild reaction conditions, high selectivity and low toxicity.

1.2 Biosurfactants

Biosurfactants - SFAE - have attracted the great attention of biotechnological researchers because they consist of two inexpensive, renewable and easily accessible starting agricultural materials – sugar and fat/oil (Adelhorst et al. 1990; Akoh 1994; Fujii 1996; Ogino 1996; Arcos et al. 1998). In recent years, growing consumer demand for 'green' products has focused attention on the utilization of carbohydrates as raw materials for specialty chemicals (Figure 1.2.1) (Hirota and Sadzuka 1996; Kokubo et al. 1996; Minamikawa and Hato 1996; Goto et al. 1997; Kim et al. 1997). Carbohydrates are abundantly available, renewable, resources and carbohydrate-based products are, non-toxic, biocompatible and biodegradable (Madsen et al. 1996; Baker et al. 2000; Baker et al. 2000; Baker et al. 2000). Moreover, carbohydrate-based products would qualify for the generally accepted 'natural' label, assuming that methods used for their production which are natural, i.e. enzymatic (Riva 1994). This is particularly important in food and personal-care applications (Fujiwara and Tamura 1996; Hayashi and Itagaki 1996; Bornscheuer 1999; Bornscheuer and Kazlauskas 1999; Hill and Rhode 1999; Bornscheuer 2000) (Kunieda and Hasegawa 1996) (Yahagi and Iwai 1996). Another advantage of enzymatic processes for carbohydrate conversions stems from the fact that carbohydrates are polyhydroxy compounds. Hence, the high regioselectivity of many enzymatic processes can provide an important advantage over less selective and more conventional chemical processes (Chopineau et al. 1988; Takeda 1996; Cameotra and Makkar 1998; Bousquet et al. 1999). Interestingly, these applications also constitute a convergence of the two major classes of renewable raw materials: carbohydrates and oleochemicals. SFAE possess distinct hydrophilic and hydrophobic moieties. For consistency in nomenclature it should be taken into consideration the number of hydroxyl groups or esterification sites available in a molecule prior to the synthesis of the carbohydrate.

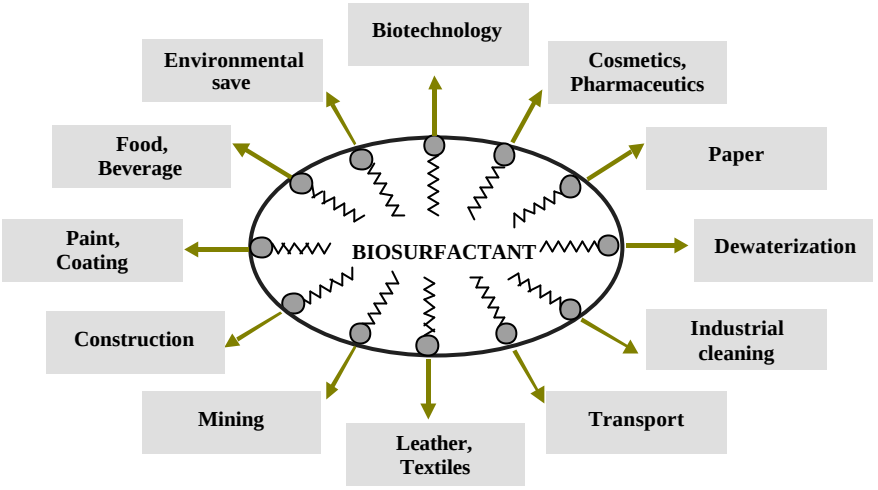


Figure 1.2.1 Applications of biosurfactants [adapted from (Haferburg et al. 1986)].

For example, free glucose has five hydroxyl groups available for substitution with long chain fatty acids of interest. The degree of substitution (DS) is therefore defined as the number of hydroxyl groups esterified with long chain fatty acids. SFAE with a degree of substitution 1-3 are highly hydrophilic, digestible, absorbable, and are usable as solubilizing, wetting, dispersing, emulsifying and stabilizing agents, and as antimicrobial and protective coatings for fruits. SFAE with a degree of substitution more than 4 are lipophilic, nondigestible, nonabsorbable fat-like molecules with physical and chemical properties of conventional fats and oils. These are often referred to as low-calorie fat substitutes (Zunft and Ragotzky 1997). It should be noted that enzymatic synthesis of sugar polyesters (DS > 4) is extremely difficult due to its steric hindrance and has not been successfully done so far.

1.3 Property parameter of surfactants--hydrophilic-lipophilic balance (HLB)

HLB is a measure of solubility of surfactants in water and this value often suggest their potential usefulness. Determination of HLB values for emulsifiers is generally relative to the emulsifying properties of surfactants with standard HLB values. The HLB of an emulsifier determines the type of emulsion that tends to form. In the ideal case, the HLB can be calculated according to the formula:

$$HLB=20*M_h/M$$

Where, M_h represents the molecular mass of the hydrophilic fraction in the molecule; M the total molecular mass of the surfactant.

The HLB value is an indicator of the behavioral characteristics and does not indicate emulsifying efficiency. The selection of a suitable emulsifier for an oil-in-water (o/w) or

water-in-oil (w/o) emulsion must be determined by experimentation. However, a low HLB value of 3-6 will promote or stabilize w/o emulsions, an intermediate value (8-13) will stabilize o/w emulsions, and a high value, such as 15-18, will act as a solubilizer.

1.4 Chemical synthesis of SFAE

The initial reports on the synthesis of SFAE (mono- and diesters) by transesterification involved the use of toxic solvents such as dimethylformamide (DMF), dimethylsulfoxide (DMSO), or dimethylpyrrolidone (DMP) as the mutual solvent for solubilizing sucrose and free fatty acids. This process, sometimes referred to as the Hass-Snell process, was first commercialized by Dai Nippon sugar manufacturing Co., Ltd. in Japan in 1959 to produce SFAE as food additives. SFAE manufactured by this technology were not permitted for use in food industry in most developed countries such as Germany and USA because of their odor and toxic materials present in the product.

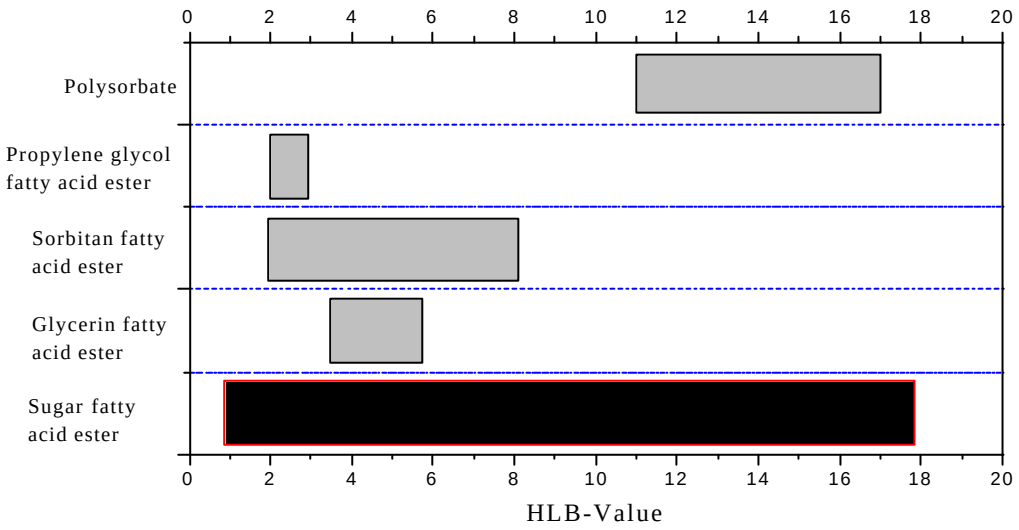


Figure 1.4.1 HLB values of food additive emulsifiers.

Osipow and coworkers first described the commercial process for the preparation of SFAE from methyl ester in DMF (Osipow and Rosenblatt 1967). A relatively safer process known as the Nebraska-Snell process was developed, which involved reaction of a microemulsion of sucrose with the fatty acid methyl ester in the presence of propylene glycol and potassium carbonate as solvent and catalyst. Feuge *et al.* developed a solvent-free interestification process, which involved the reaction between molten sucrose (mp 185°C) and fatty acid methyl ester in the presence of lithium, potassium and sodium soaps as solubilizers and catalysts at temperatures between 170-187°C (Feuge et al. 1970). The drawback to this

process is that molten sucrose is rapidly degraded to a black tarry mass at these temperatures. In both solvent and solvent-free processes, distillation is often required to remove the unreacted methyl esters, fatty acids, and alcoholic by-products. At present, mono- to octa-esters of sucrose and several different esters of sorbitan are commercially available. However, most of the emulsifiers manufactured by chemical methods using unprotected sugar moieties and fatty acids may not be used in food applications, because the complete removal of toxic organic solvents such as DMSO, THF and DMF used for the solubilization of sugar components is laborious.

The HLB value of SFAE as emulsifiers was reported (Fig. 1.4.1) to be dependent on: i) degree of substitution, ii) alkyl chain length in the ester group, and iii) the presence of acyl double or triple bonds, i.e., degree of unsaturation (Böge and Tietze 1999).

1.5 Enzymatic synthesis of SFAE

Enzymatic processes offer an alternative way to these surfactants. It is achieved by coupling of sugar and fatty acid with lipase as biocatalysts. The products are nonionic, digestible, absorbable and biodegradable. Enzymatic esterification of sucrose and of various monosaccharides with fatty acids in aqueous media has been carried out in the early 1980s, but the selectivity and the yield obtained were low (Ikeda and Klibanov 1993; Sarney et al. 1994; Redmann et al. 1997; Sarney et al. 1997; Kirk et al. 1998; Kitagawa and Tokiwa 1998; Sin et al. 1998; Degn et al. 1999; Gao et al. 1999; Garcia et al. 1999; Munoz et al. 1999; Tsuzuki et al. 1999; Park and Chang 2000). The use of enzymes enables the preparation of a wide range of monosaccaride fatty acid esters often as single regioisomers and with no requirement for laborious regioselective protection (Björkling et al. 1991; Mutua and Akoh 1993). Because of the different solubilities of sugars and fatty acids in organic solvents, it is not easy to mix sugars and fatty acids in the reaction medium. By complexation of sugar with boronic acid derivatives, Schlotterbeck *et al.* have found that phenyl boronic acid can inhibit lipase activities (Schlotterbeck et al. 1993).

Different systems based on non-aqueous enzymology have been developed for the synthesis of SFAE. Due to the low solubilities of sugars in organic solvents, different kinds of processesfor the enzymatic synthesis of SFAE have been explored: the use of bulky polar organic solvents such as pyridine or dimethylformamide (Chopineau et al. 1988; Riva et al. 1988; Janssen et al. 1990; Janssen et al. 1991), or the use of activated acyl donors (Therisod and Klibanov 1986; Pulido et al. 1992),

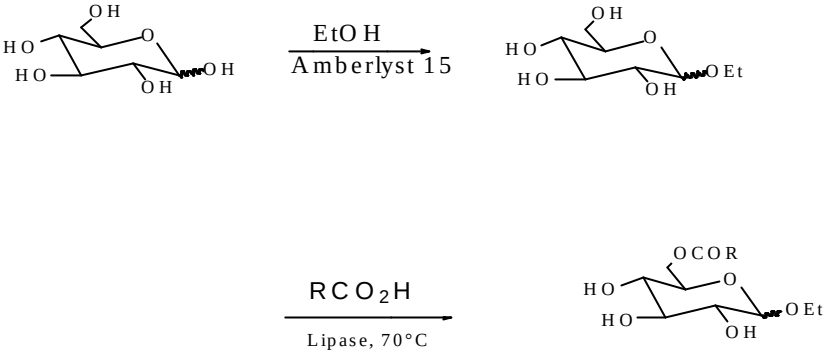


Figure 1.5.1 Lipase-catalyzed synthesis of SFAE using modified sugar (Adelhorst et al. 1990).

substrate immobilization (Sharma and Chattopadhyay 1993), or the use of sugar derivatives (alkyl or acetal) to increase the miscibility of the substrate (Fig. 1.5.1) (Adelhorst et al. 1990; Fregapane et al. 1991; Fregapane et al. 1994; Fregapane et al. 1994) or the use of solubilizing agents for sugars like organoboronic acids (Oguntimein et al. 1993; Schlotterbeck et al. 1993). Unfortunately, the solvents used in the enzymatic synthesis of SFAE are often deleterious to most lipases, resulting in partial or complete inactivation, and are too harmful for the final products to be used as food ingredients. Furthermore, synthesizing the sugar derivatives, protecting and deprotecting steps are laborious and may complicate product purification and increase process costs. The acylation of non-modified sugars was also studied in a solvent-free process using molten fatty acids as acyl donors (Adelhorst et al. 1990; Guillardeau et al. 1992). Because of the low solubility of sugar in the molten fatty acid, low reaction rate and poor regioselectivity has been achieved in this process. To overcome these problems, some processes for the synthesis of SFAE from the original sugar and long chain fatty acids using a large amount of solvent and catalyst have been reported (Ljunger et al. 1994; Ducret et al. 1995; Scheckermann et al. 1995; Ducret et al. 1996; Ducret et al. 1997).

Alkyl polyglucosides, derived from starch, are already produced on a commercial scale by Henkel for use in detergents (Böge and Tietze 1999). Björkling *et al.* showed that immobilized *Candida antarctica* lipase catalyzes the regioselective 6-*O*-acylation of ethyl glucose with fatty acids. A yield of over 90% was obtained by performing the reaction at 70°C in a solvent-free system if the water is removed continuously from the reaction by vacuum distillation. The process has been developed to pilot-plant scale by Novo Nordisk (Björkling et al. 1989; Björkling et al. 1991), which was also commercialized by Unichema International (Bosley 1997). The biodegradable surfactant based on ethyl glucoside esters can

be made using stirred-tank reactor and the reaction is driven to high conversion by removing water under vacuum. It has to be pointed out that production costs should always be considered in conjunction with actual product performance. Obviously, additional costs would be justified if the emulsifying properties of these biosurfactants were found to be superior in specific applications compared with currently used surfactants. The process is laborious with low yield in the first step and the HLB range of SFAE is also reduced. Unfortunately, acylation of sucrose is even more difficult than of glucose, due to its extreme low solubility in organic media and its large and hydrophilic structure, is generally not a suitable substrate for lipases.

1.5.1 Lipase-catalyzed solid phase synthesis of SFAE

In the group of Prof. Schmid, a method for the synthesis of SFAE based on a mainly solid-phase system was developed, where the acylation of a solid sugar with a fatty acid was performed *via* lipase-catalysis in the presence of a small amount of organic solvents [*tert*-butanol (TBA), acetone] serving mainly as adjuvant and molecular sieves for the removal of produced water (Cao et al. 1996; Cao et al. 1997; Cao et al. 1998; Cao et al. 1999). In spite of the fact that the sugar is nearly insoluble, various monosaccharide fatty acid esters were synthesized with high up to quantitative conversion, which is attributed to the high solubilization rate of solid sugar and the crystallization of the product from the reaction mixture (Yang and Robb 1994; Wolff et al. 1997). The principle of lipase-catalyzed solid phase synthesis of SFAE is represented in Figure 1.5.1.1. This method offers a number of advantages, in which both substrates (sugar and acyl donor) can be directly used without any modification. In comparison to other methods such as prior substrate modification methods (Adelhorst et al. 1990; Fregapane et al. 1991; Pelenc et al. 1993), this novel system is obviously more simple. In this system, high reaction rate and high productivity can be achieved within a few hours, and it is comparable to the use of modified sugars such as ethylglucoside, butylglucoside or acetalized sugars and the costs for the substrate modification can be saved. In addition, the enzyme in the solid phase system showed also high stability which may be related to the use of less organic solvent as adjuvant. Substrate or product inhibition can be avoided, the use of solid substrates and precipitation of the product in the reaction mixture and isolation of product by extraction and recrystallisation are also facilitated, resembling a simple downstream process. It should be emphasized that the regioselectivity is also adjustable even in the solid phase by selecting suitable lipase, substrates and adjuvants (Bornscheuer 1995).

Previous publications on enzymatic acylation of SFAE paid little attention to practical difficulties associated with the esterification process on a large scale. Such points as solvent recycling, separation and re-use of enzymes, bioreactor design, overall process modeling, and most critically removing water continuously during esterification must be addressed prior to assessing the commercial feasibility of this technology.

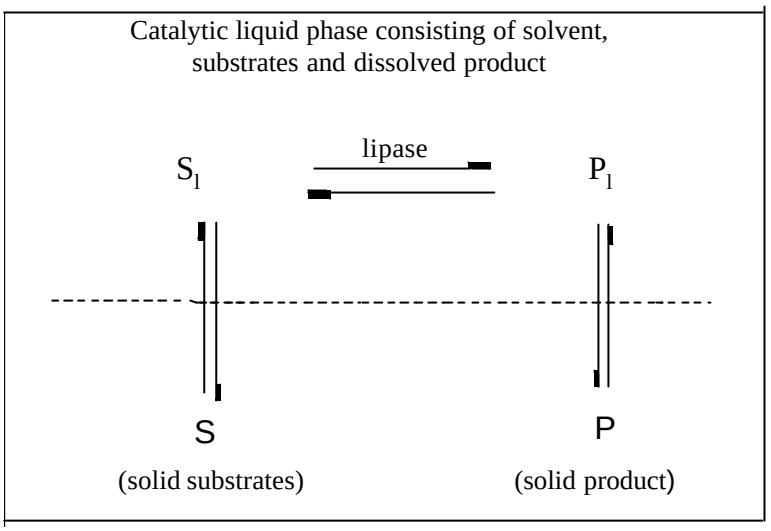


Figure 1.5.1.1 Solid phase consisting of solid substrates and solid product.

1.6 Target

The aim of this work is to develop a process to synthesize SFAE using unprotected sugar and non-activated fatty acids or fatty acid esters on a preparative scale. The reaction system should be performed in the absence of solvent or the presence of only little biocompatible organic solvent (mainly solid phase system) whilst removing the generated water continuously during esterification. The reaction rate, conversion, regioselectivity, productivity, and catalyst stability should be high. Furthermore, the products have to meet the requirements of the regulatory authorities to be used in food or food additives.

2. Lipase-catalyzed solid phase synthesis of SFAE using an azeotropic distillation to remove by-product generated during esterification or transesterification

2.1 Problems in the synthesis of SFAE

In previous work of Prof. Schmid's group, a method for the synthesis of SFAE was developed, based on a mainly solid phase system, where the acylation of a solid sugar with a fatty acid was performed *via* lipase-catalysis in the presence of a very small amount of organic solvents (TBA, acetone) which mainly serves as adjuvant. Water generated during the esterification was eliminated by direct addition of activated molecular sieves in the reaction system (Cao et al. 1996; Cao et al. 1997; Cao et al. 1998; Cao et al. 1999). Unfortunately, this is not practical on a large scale since:

- Molecular sieves occupy large space of the reactor, leading to a low space-time yields, when one takes into account that removal of 1 g water needs 17 g molecular sieves;
- Mass transfer limitations can occur due to difficult stirring;
- molecular sieves are broken due to the strong stirring which is necessary to keep mass transported in the solid phase system;
- The regeneration process of molecular sieves is very laborious and costly.

On the other hand, water removal is critical to achieve high yields in the synthesis of SFAE and enzyme activity and/or stability are negatively effected by higher concentrations of water (Dudal and Lortie 1995). Because the esterification is a reversible reaction, the presence of the reaction by-product water in the media lowers the maximum yield. Furthermore, the water liberated by the reaction, as a by-product, affects directly the activity of the enzymes which have their maximum activity within a strict hydration range. In addition, this method uses TBA as solvent, which is not permitted to be used in food industry.

2.2 Overview of water removal

To date, water removal in solvent-free systems has been achieved by performing reactions in open test tubes, evacuation *in vacuo*, pervaporation using special membranes and dry gas bubbling (Ergan et al. 1990; Padt et al. 1993; Fregapane et al. 1994; Kosugi and Azuma 1994; Napier et al. 1996; Kim et al. 1998). However, when organic solvents are present in the reaction medium, the selective removal of water is rather complicated if the boiling point of the solvent is lower than that of water. For instance, the vacuum evacuation method that was shown to be very effective in solvent-free systems can not be applied to the solvent-phase

reactions. Very few water removal methods have been reported for enzyme reactions that employ an organic solvent as a reaction medium.

Water removal from reactions can be carried out with adsorbents or by distillation methods. As discussed previously, water removal with addition of an adsorbent is not practical on a large scale. A close examination of the published work shows that some distillation methods could be chosen in order to remove the produced water from the reaction system:

1. Bulk solvents such as 2-methyl-2-butanol (2-MBA) have been used since the boiling point of 2-MBA is 100-103°C under atmospheric pressure so the generated water will be vaporized first by lowering the pressure (Ducret et al. 1995). Unfortunately, the solvents with boiling point above 100°C including 2-MBA are not allowed in the preparation of foods and/or food additives by regulatory authorities.
2. Jeong *et al.* developed a system in which the water was stripped by continuously sparging dry air through the reaction medium and was removed by a water trap packed with silica blue after condensing the effluent gas (Jeong and Lee 1997). The main problem of this method is the loss of the solvents during operation.
3. Bloomer *et al*. developed a method for synthesis of fatty acid esters such as fatty acid ethyl ester, using hexane or pentane as solvents. Water produced during the reaction was trapped by condensing the refluxing vapor phase and passing it over activated molecular sieves in a reflux trap (Bloomer et al. 1992). One major drawback of this method is that the reaction temperature should be kept around 100 °C and that still molecular sieves are necessary (Roure et al. 1997).
4. In recent years, water removal methods by circulating saturated salt solutions (Kvittingen et al. 1992) through microporous membranes (or gas phase equilibrated with saturated salt solutions) such as a silicone tube or a hollow fiber membrane have been developed (Rosell et al. 1996; Ujang et al. 1997). While these systems have several advantages over the conventional approaches for water removal, the use of these membranes may pose some difficulties for the solid phase enzymatic synthesis of SFAE because of 1) precipitation of substrates and product on the membrane surface; 2) difficult mass transfer in the solid reaction medium, thus leading to difficult release of the water molecules and; 3) abrasion on the membrane surface due to strong agitation of the solid substrates.

Thus, there are always some drawbacks in the literature methods to remove water from the organic solvent reaction media. It should be noted that most drawbacks are mainly attributed to the higher boiling point of the by-product water. In order to prevent evaporation of water generated during the esterification, it was necessary to raise the reaction temperature to above

100°C. On the other hand, lower temperatures allow for greater operational stability for enzymes, and lead to low energy consumption. The reaction temperature should be controlled from room temperature to 80 °C due to the enzyme stability. However, this would not be possible if the vapor phase consists of only water under atmospheric pressure.

2.3 Aim

The aim of this work is to develop a novel and effective enzymatic method for the synthesis of SFAE without any direct addition of molecular sieves in the reaction system. The development of the synthetic method involved the improvement of reaction rate, conversion, regioselectivity, productivity, safety and catalyst stability. All this is necessary for the products to be used in the food industry; On the other hand, the method must fulfil industrial purposes which means no modification of the substrates are needed to keep cost low (neither the sugars nor the acyl donors). Furthermore, the solvent used in the system should be recognized as safe in the food industry (Bundesministerium 1991).

2.4 Results

2.4.1 Synthesis of SFAE using fatty acid methyl ester by transesterification

In order to decrease the boiling point of the by-product, methyl palmitate was used instead of palmitic acid for synthesis of glucose palmitate by transesterification in the reaction medium consisting of TBA. Since the boiling point of methanol (b.p. 64.5°C) generated by transesterification is lower than that of TBA (b.p. 83°C), methanol can be removed from the reaction medium by selective distillation (Fig. 2.4.1.1).

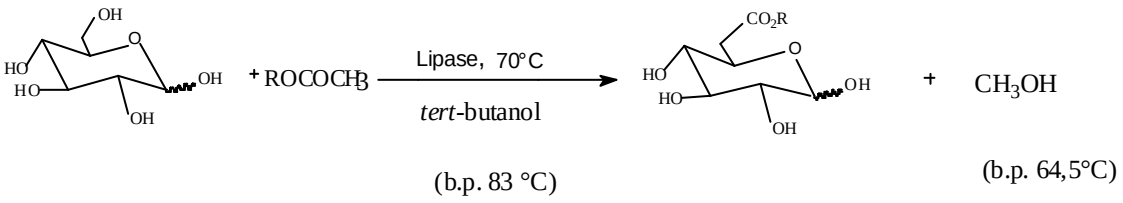


Figure 2.4.1.1 Fatty acid methyl esters as acyl donor in the synthesis of SFAE.

The reaction was carried out at 70°C for 48 h in a vessel which was connected to a condenser. The temperature in the condenser had to be adjusted to condense only the solvent TBA while eliminating the methanol which was kept under gaseous form. A yield of 70% was achieved

for glucose palmitate. The main disadvantage of this method is, that the products are not permitted to be used in the food industry by the regulatory authorities.

2.4.2 Selection of suitable organic solvents

To easily realize complete removal of the by-product water/methanol, initial experiments were performed using a solvent-free mixture of fatty acid/fatty acid methyl ester and glucose at 60°C using Novozyme® SP 435 as a catalyst *in vacuo*. It was found that the reaction kinetics under these solvent-free conditions were very poor, even when 5~10% glucose fatty acid ester was initially added to increase the contact interface of both substrates, apparently due to low miscibility of the reactants. In previous work it was found that the addition of TBA and acetone give rise to a notable acceleration for the lipase-catalyzed solid phase synthesis of SFAE. So the solvent can not be avoided in this system. A suitable organic solvent should dissolve enough substrate to carry out the reaction without affecting the enzyme activity or stability, and the product solubility should be low to favor an irreversible reaction by facilitating crystallization (Rubio et al. 1991; Kawashiro et al. 1997; Ke and Klibanov 1998; Koops et al. 1999).

Tab. 2.4.2.1 Organic solvents permitted for use in the food industry by German authorities.

Solvents for unlimited use		
CO_2	ethylacetate	propane
butylacetate	acetone	butane
N_2O	ethanol	
Solvents with limited use		
2-propanol	methylacetate	EMK
methanol	dichlormethane	hexane

Adapted from 'Verordnung über die Verwendung von Extraktionslösungsmitteln bei der Herstellung von Lebensmitteln vom 8.11.1991'(Bundesministerium 1991).

It is known that very few organic solvents are recognized to use in the food processing by the regulatory authorities. TBA is not accepted for use as solvent in the preparation of food or food additive. From all solvents which are permitted for the use in the manufacture of food products by the regulatory authorities (Tab. 2.4.2.1) butylacetate, ethylacetate, methylacetate, methanol, ethanol and 2-propanol can not be used as reaction medium for the synthesis of SFAE because they are competitive reactants. For the remaining solvents the solubility of glucose and glucose palmitate was determined (Tab. 2.4.2.2). Sugars are insoluble in

carbondioxide, butane, hexane, dinitrogenmonoxide and propane under the experimental conditions. It turned out, that only acetone and EMK can be used for the synthesis of SFAE since they can dissolve sufficient amounts of glucose and only limited amount of glucose palmitate. In addition, both solvents form suitable azeotropic mixture allowing the removal of the by-products generated during the enzymatic reaction.

Tab. 2.4.2.2 Solubility of β-D(+)-glucose and 6-*O*-β-D(+)-glucose palmitate in organic solvents at 25°C.

Solvents	**Solubility**	**[mg/ml]**
	Glucose	**Glucose palmitate**
Acetone	0.348*	4.60*
Hexane	n. d.**	n. d.**
EMK	0.079*	2.508*
Dichlormethan	< 0.002**	n. d.**

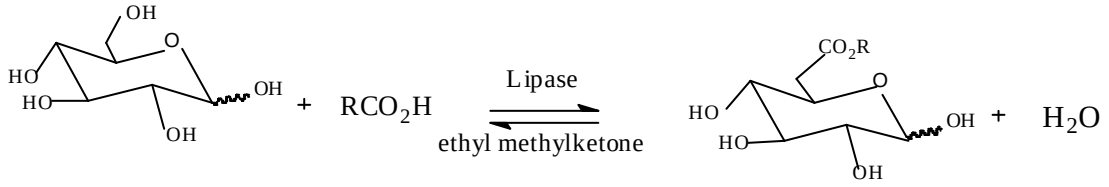
*: at 60°C; **: at 25°C; n. d.: not determined;

2.4.3 Development of new reaction systems

According to the above findings, the following two reaction systems were developed:

- esterification
- transesterification

For esterification, the reaction system consisted of glucose, fatty acid, lipase Novo SP 435 and EMK. Water generated during the reaction will form an azeotrope with EMK and can then be removed from the reaction medium by azeotropic distillation (Fig. 2.4.3.1). (Röck 1960).



(b.p. 78-80°C)

Azeotrope ⇒ 67% EMK and 33% water, b.p. 73.5°C

Figure 2.4.3.1 Lipase-catalyzed synthesis of SFAE in EMK.

For transesterification, the reaction system consisted of glucose, fatty acid methyl ester, lipase Novo SP 435 and acetone. Methanol generated during the reaction will form an azeotrope with acetone and can then be removed from the reaction medium by azeotropic distillation (Fig. 2.4.3.2).

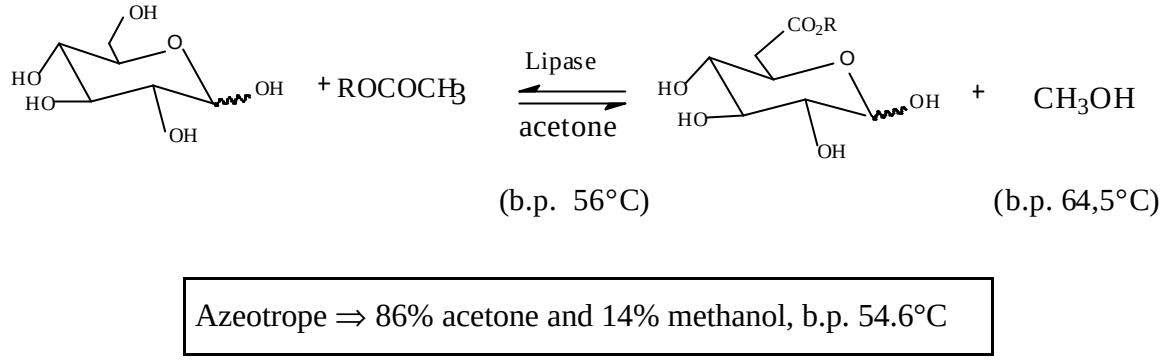


Figure 2.4.3.2 Lipase-catalyzed synthesis of SFAE in acetone.

2.4.4 Design of experimental apparatus on laboratory scale

An apparatus was designed for the synthesis of SFAE (Fig. 2.4.4.1). The reaction mixture which consisted of reactants and an organic solvent, was incubated in a two-necked round-bottom flask, placed in an oil bath, and stirred by a magnetic bar under reduced pressure. A Soxhlet extractor with molecular sieves was used to remove water or methanol produced from the refluxing mixture. This extractor was connected at the top to a condenser that was connected to a vacuum controller. The temperature of the reaction was maintained at 25-60°C and the pressure was progressively lowered so that the solvent and azeotrope in the reaction mixture were refluxed. The volatile components mixture was dried by passing through molecular sieves before returning to the reaction system. This provides constant removal of the water or methanol liberated during the enzymatic reaction, forcing the equilibrium towards synthesis.

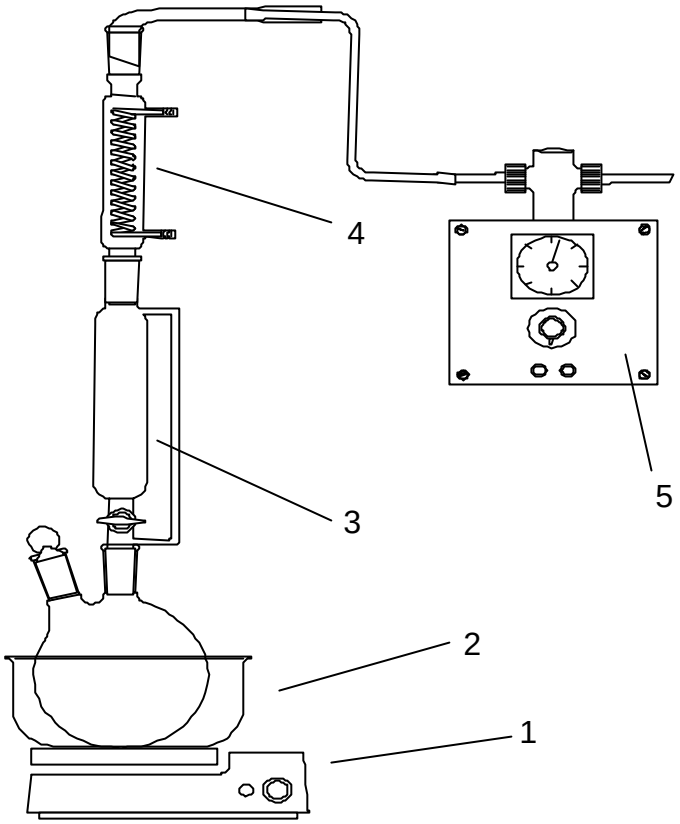


Fig. 2.4.4.1 Experimental apparatus for the synthesis of SFAE by removal of by-product using azeotropic distillation. 1. Heated magnetic stirrer; 2. Oil bath; 3. Soxhlet extractor; 4. Condenser; 5. Vacuum controller.

2.4.5 Influence of reaction temperature

2.4.5.1 Effect of temperature on solubilities of glucose and products

The solubilities of glucose, glucose palmitate and glucose stearate in acetone and EMK are shown in Figure 2.4.5.1.1. The solubility of glucose is not affected by temperature, but the solubilities of the glucose fatty acid esters significantly increased at temperatures above 50-60°C. Glucose palmitate has an approximately 18-fold higher solubility (4.6 mg/ml) in acetone at 60°C than at 25°C (0.25 mg/ml). In comparison with EMK, the solubility of glucose palmitate increases only 10-fold while the temperature increases from 25 ~ 60 °C. For the enzymatic synthesis of SFAE by esterification reaction in the range of 25-75°C, the solubilities of glucose, glucose caprylate, glucose palmitate and glucose stearate in acetone and EMK at 25, 40, 60 and 75°C were determined and are shown in Table 2.4.5.1.1. The solubility of glucose caprylate at 60°C in acetone is 15-fold higher than the solubility of glucose palmitate, thus explaining the lower yields obtained in the solid phase synthesis of SFAE reported previously. The solubilities of all products were slightly lower in EMK than in acetone.

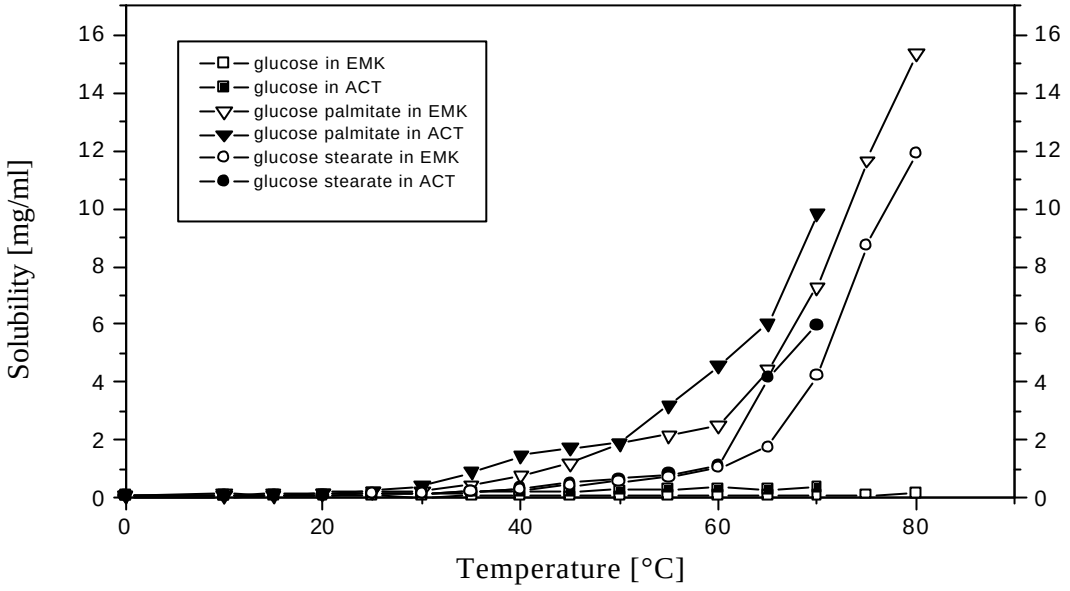


Figure 2.4.5.1.1 Influence of temperature on the solubilities of glucose, glucose palmitate and glucose stearate in acetone (ACT) and EMK.

Tab.2.4.5.1.1 Solubilities of glucose, glucose caprylate, glucose palmitate and glucose stearate at different temperatures.

Temp.	**Solubility [mg/ml]**							
	Glucose		**Glucose caprylate**		**Glucose palmitate**		**Glucose stearate**	
[°C]	ACT	EMK	ACT	EMK	ACT	EMK	ACT	EMK
25	0.14	0.06	2.98	1.66	0.25	0.23	0.15	0.13
40	0.21	0.07	17.26	5.59	1.47	0.77	0.36	0.31
60	0.35	0.08	69.30	16.60	4.60	2.51	1.10	1.03
75	n.d.	0.11	n.d.	51.23	n.d.	11.67	n.d.	8.74

n.d.-- not determined; ACT – acetone.

2.4.5.2 Effect of temperature on reaction rate

Reactions were carried out at three temperatures to evaluate the influence of temperature on the esterification. The lowest temperature studied was 25°C. Lower temperatures would result in slower initial reaction rates, since the reaction temperature has a great influence on the rate constant for esterification. The melting point of palmitic acid (62-63°C at atmospheric

pressure) also plays an important role. Higher temperatures accelerate reactions, but because of the increased solubility of SFAE in the reaction medium it might lead to an inhibition.
Figure 2.4.5.2.1 shows a plot of conversion *vs* time for three different temperatures. As expected, conversion was found to increase with increasing temperatures. After 50 h, an increase in product yield of 10.7 % was observed when the temperature was raised from 25°C to 40°C, whereas an increase from 40 to 60°C led to 14.1 % higher yields.

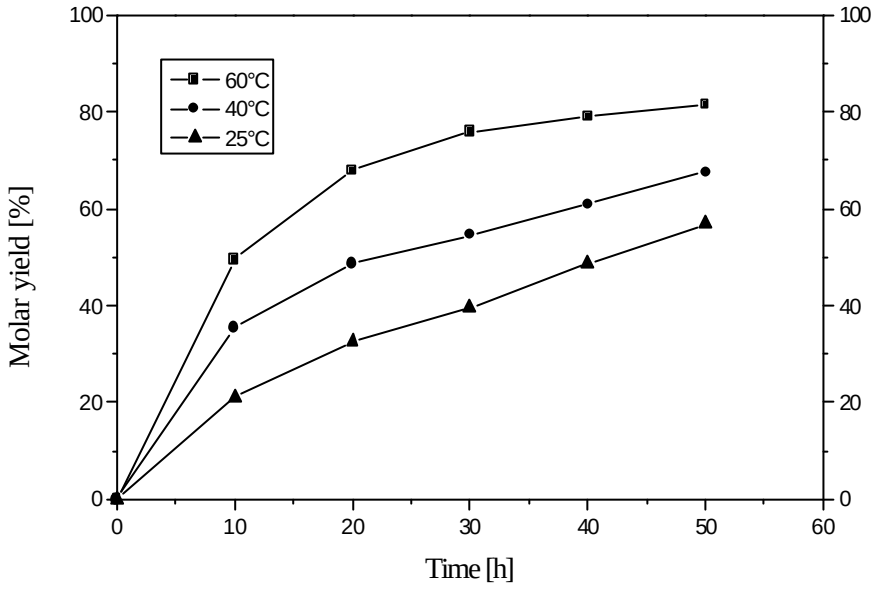


Figure 2.4.5.2.1 Influence of reaction temperature on glucose palmitate synthesis. Reaction conditions: 0.5 mmol glucose, 0.5 mmol palmitic acid, 50 mg CAL-B EP 100, 200 rpm in 50 ml EMK.

2.4.5.3 Effect of temperature on product inhibition

The solubilities of SFAE significantly increase with temperature. Indeed, it must be kept in mind, that the esterification is a reversible reaction. An important feature of the lipase-catalyzed solid phase synthesis of SFAE is that the SFAE formed during the reaction will crystallize from the liquid phase, if its saturation concentration, which is highly related to the temperature, is reached. If the reaction temperature is high, the SFAE concentration in the reaction liquid phase is also high thus, the product can not crystallize from the reaction medium, causing product inhibition. This had been clearly confirmed in the enzymatic reaction using caprylic acid as acyl donor because glucose caprylate had high solubility in acetone (69.3 mg/ml at 60°C) or EMK (51.2 mg/ml at 75°C) (Fig. 2.4.5.3.1). 87% conversion of glucose caprylate was achieved at 25°C, compared to 50% at 60°C. The solidification of the reaction mixture could be observed at 25°C.

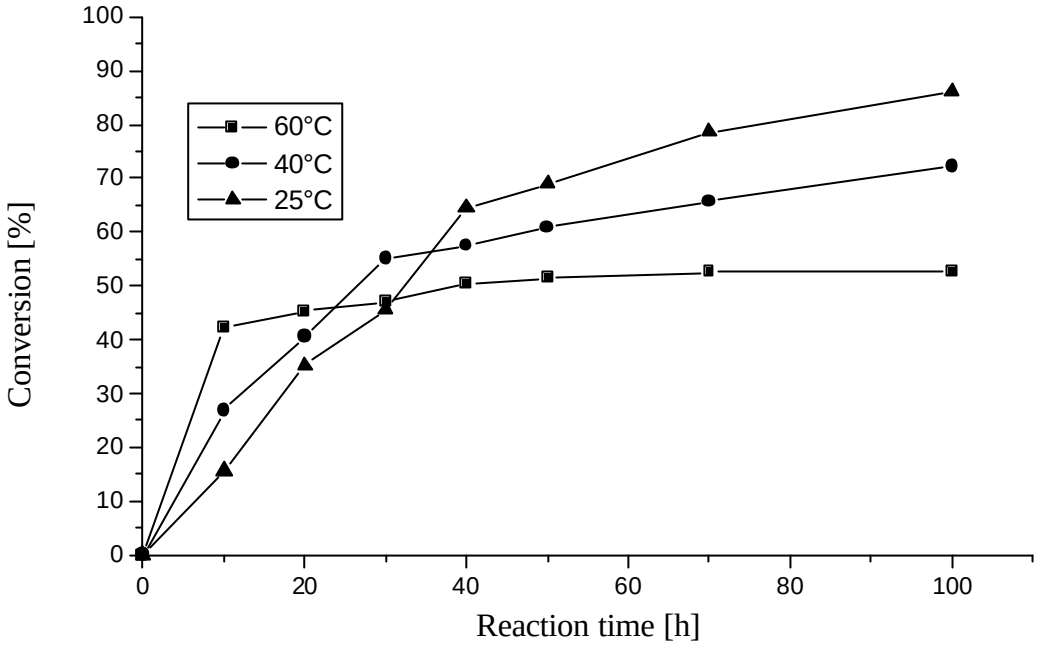


Fig. 2.4.5.3.1 Synthesis of 6-O-octanoyl-β-D(+)-glucose at different temperatures. Reaction conditions: 90 mg glucose, 72 mg caprylic acid, 45 mg SP 435, 5 ml EMK and 600 rpm.

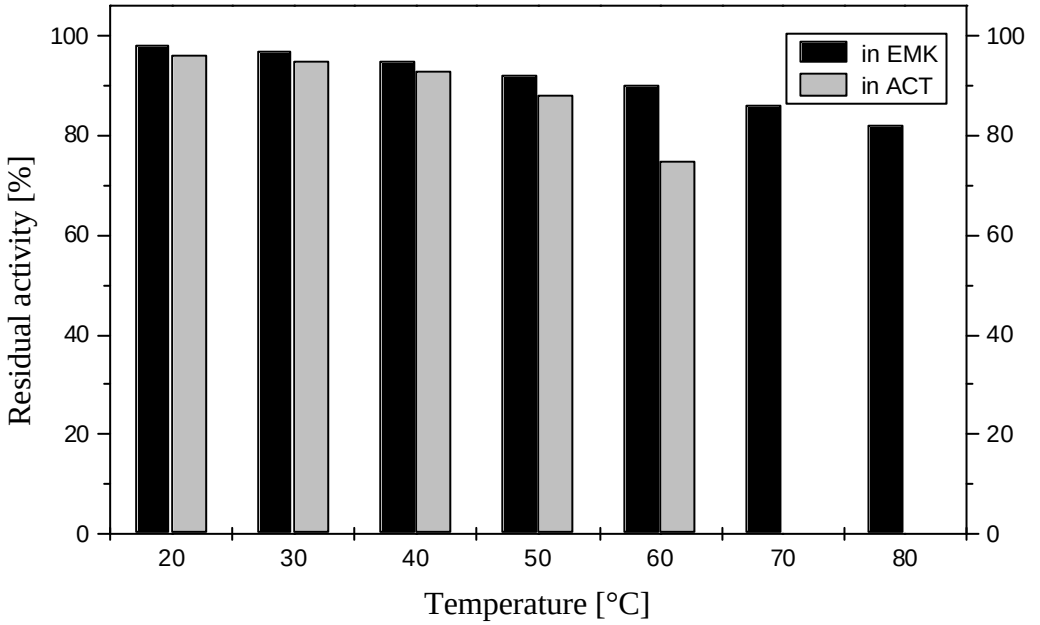


Fig. 2.4.5.4.1 Enzyme residual activity at different temperatures. Reaction conditions: 90 mg glucose, 128 mg palmitic acid or 135 mg methyl palmitate, 50 mg EP 100, 200 rpm, 50 ml EMK/acetone, reaction time 48h.

2.4.5.4 Effect of temperature on enzyme stability

Enzyme stability depends on the reaction conditions such as substrate, solvents, reaction temperature and reaction time. CAL-B is a very versatile lipase which is catalytically active even in polar solvents such as acetone or EMK. When immobilized, CAL-B is highly thermostable. As the price of the biocatalyst is often a significant part of the overall production cost, stability and re-use of lipases are very important for using this technology in industry. It was found that the activity loss of lipase immobilized EP 100 is about 60% after six times re-use in EMK at 60°C for 12 days. In comparison with acetone, the loss of activity of CAL-B appears slightly higher at the elevalted temperatures (Fig. 2.4.5.4.1).

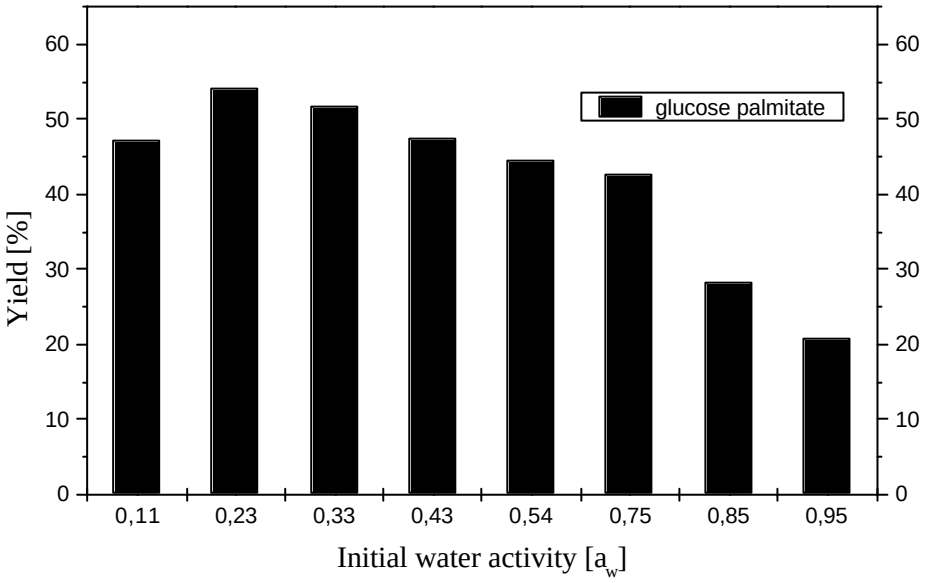


Figure 2.4.6.1 Influence of initial water activities of substrates and enzyme. Reaction conditions: 90 mg glucose, 128 mg palmitic acid, 50 mg EP 100, 200 rpm, 50 ml EMK, reaction time 48 h.

2.4.6 Effect of initial water activities

Starting from free fatty acid and glucose, water is one of the reaction products, which has to be removed by circulating the reaction solvent and the azeotrope through a column of molecular sieves. The water bound to the reactants and the enzyme were immediately adsorbed by the molecular sieves, if the influence of initial water activity was studied. The initial water activities of the substrates and enzyme were adjusted according to Goderis *et al.* (Goderis et al. 1986), to pre-equilibrate the desired saturated salt solution, catalyst and

reactants in a sealed container *via* the vapor phase before use. The effect of the initial water activities of the reactants and enzyme on the conversion were studied (Fig. 2.4.6.1). It was found that the highest yield was achieved at an initial water activity of 0.23. It is also indicated that esterification reactions are favored at lower initial water activities. Since a reaction at controlled humidity is impossible due to the use of molecular sieves, the water content of the reaction mixture was determined by Karl-Fischer titration during the reaction. It was found that the water content of the reaction mixture was stable between 0.05~0.2% (w/w).

2.4.7 Influence of reaction time

As indicated in previous sections, long reaction times normally favor the synthesis of SFAE and give also high conversions. Main focus was given on the influence of the reaction time on the reaction rate with different acyl donors (free fatty acid, a fatty acid methyl ester). It was found that the initial esterification reaction rate was very fast when free fatty acid was used as acyl donor, but then the reaction rate decreased progressively after a certain period of reaction time (Fig. 2.4.7.1). In contrast to the transesterification reaction in acetone, the change of the reaction rate was irregular under the experimental conditions used.

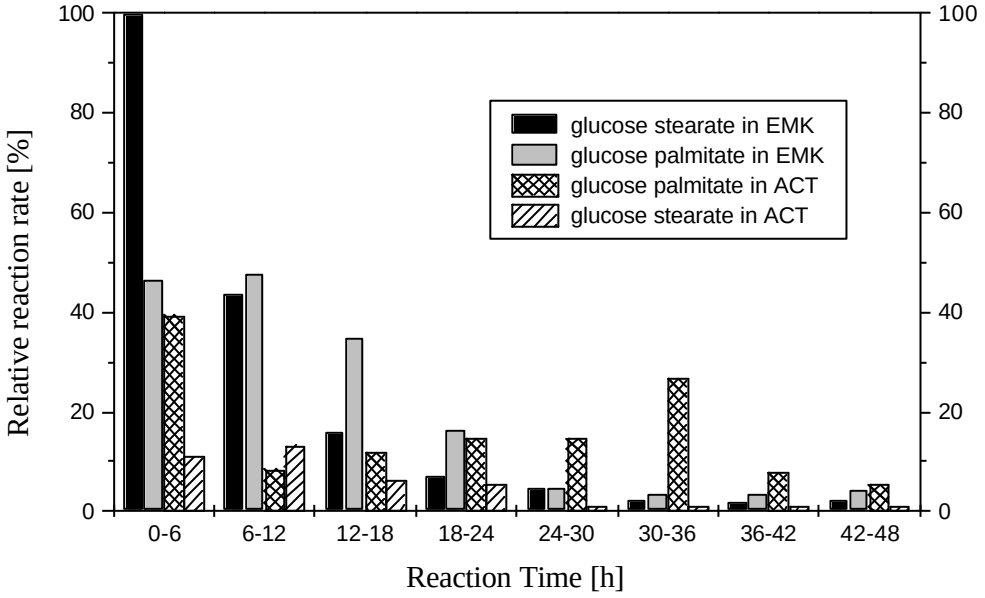


Figure 2.4.7.1 Influence of the reaction time on the reaction rate at 60°C.
Reaction conditions: 90 mg glucose, 128 mg palmitic acid or 142 mg stearic acid or 135 mg methyl palmitate, 50 mg EP 100, 200 rpm, 60°C, 50 ml EMK or acetone.

2.4.8 Influence of enzyme load

Lipase catalyzed synthesis of glucose stearate on different enzyme concentrations was investigated. If no mass transfer limitation is present, the relation between reaction rate and enzyme concentration should be linear. Due to the nature of the highly concentrated and mainly solid reaction mixtures, it is obvious that effective mixing of reactants and enzyme is important to provide good transport and contact of the reaction partners. The optimum enzyme concentration highly depends on the stirring status. It was found that the yield increased with increasing enzyme concentration until a maximum value was reached at 8-10% (w/w) enzyme load (see figure 2.4.8.1).

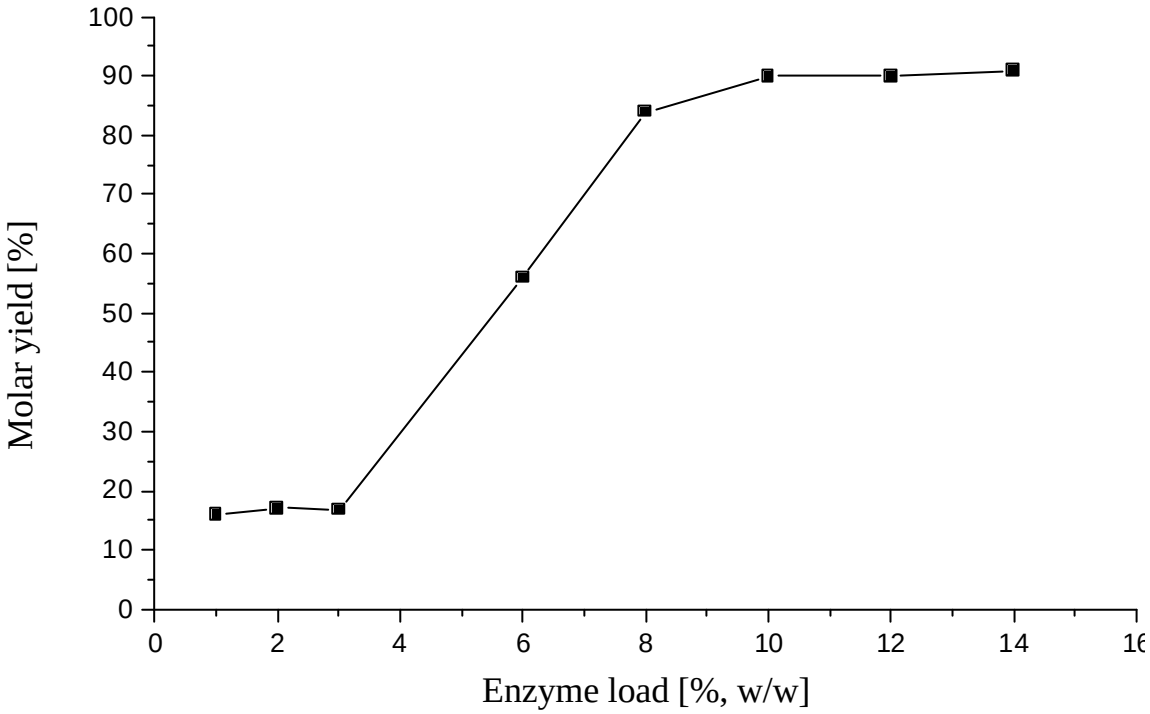


Figure 2.4.8.1 Effect of enzyme load on the product yield.
Reaction conditions: 90 mg glucose, 142 mg stearic acid, 200 rpm, 60°C and 50 ml EMK, 48 h.

2.4.9 Influence of substrate ratio

Reaction at different molar ratios of stearic acid to sugar were performed at 60°C (Tab. 2.4.9.1). In this series of experiments, the amount of glucose, EMK, SP 435 and the pressure were held constant. When the ratio of stearic acid to sugar was 5:1, the solubility of glucose in EMK at 60°C was sufficiently high to reach almost complete consumption of glucose in 24 h.

In the presence of a threefold excess of acid, 95% conversion of glucose was achieved after 48 h. When equimolar proportions are employed, a yield of 90% was achieved after 48 h reaction. With respect to the product purification, reactions at equimolar concentrations of fatty acid and sugar seems to be the best choice.

Tab. 2.4.9.1 Reaction between glucose and stearic acid at different molar ratios.

Stearic acid /Sugar	Reaction time [h]	Conversion [mol %]
5/1	8	42
	16	86
	24	100
	32	100
3/1	8	38
	16	58
	24	80
	48	95
1/1	8	38
	16	53
	24	76
	32	83
	48	90

Reaction conditions: 90 mg glucose, 50-100 mg SP 534, 200 rpm, 60°C and 50 ml EMK, 450 rpm.

2.4.10 Optimum reaction conditions

The following experiments indicated that two different sets of reaction conditions may be employed to produce high yields of SFAE: i) for middle chain length fatty acid such as caprylic acid, room temperature and long reaction time (100 h); ii) for long chain length fatty acids such as palmitic acid and stearic acid, moderate temperatures (around 60°C) and short times (48 h). Highest yields (81%) of SFAE were achieved with free palmitic acid, when an azeotropic distillation was employed to remove the water generated by esterification. Better results (90%) were found for the synthesis of 6-*O*-stearoyl-β-D(+)-glucose from stearic acid (Fig. 2.4.10.1). Considerably lower yields (66% for palmitate, 20% for stearate) were possible using the corresponding methyl ester, although the boiling point of the azeotrope using acetone/methanol is much lower than for EMK/water. In the case of caprylic acid, reactions

were also performed at lower temperatures, because the solubility of 6-*O*-caproyl-β-D(+)-glucose was very high at 60°C. It turned out that the highest yield 76% was achieved at 25°C, whereas a yield of 62 % was obtained at 40°C and a 40% yield at 60°C.

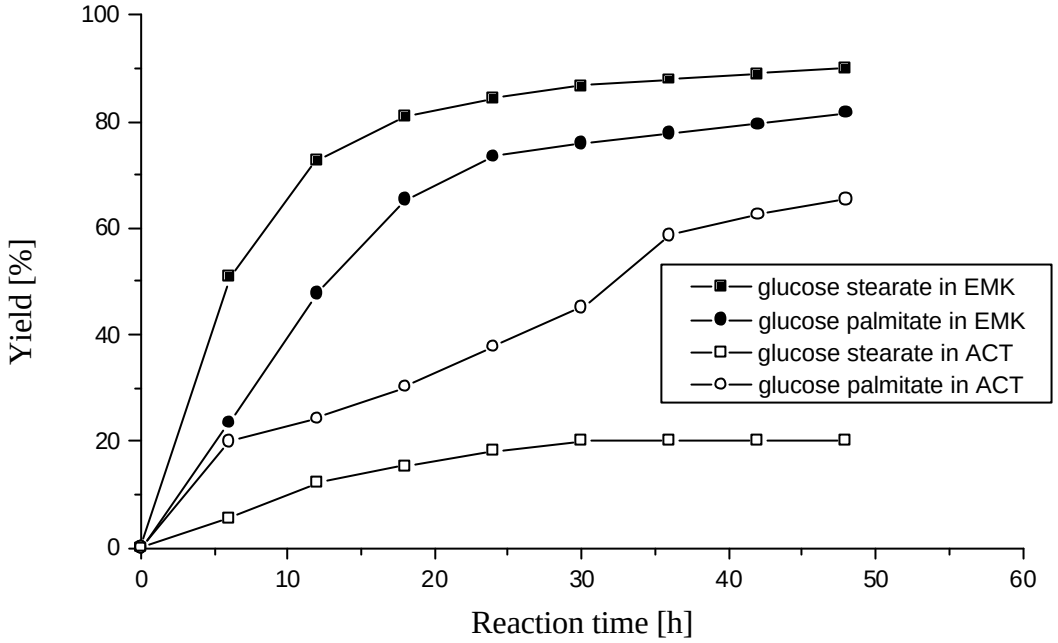


Fig. 2.4.10.1 Time course of the synthesis of 6-*O*-stearoyl-β-D(+)-glucose and 6-*O*-palmitoyl-β-D(+)-glucose.
Reaction conditions: 90 mg glucose, 128 mg palmitic acid or 142 mg stearic acid or 135 mg methyl palmitate or 157 mg methyl stearate, 50 mg EP 100, 200 rpm, 60°C and 50 ml EMK or acetone.

2.5 Discussion

2.5.1 Solubility and reaction temperature

The reaction temperature has a great influence on both, the rate constant of esterification and the solubility of the glucose fatty acid esters. The solubility of glucose in EMK increases only 0.05 mg/ml with increasing the temperature from 25 to 60°C. In contrast to the glucose fatty acid esters, their solubilities increase greatly with the temperature rising, for example, the solubility of the glucose caprylate in acetone increases 2.98 to 69.3 mg/ml with increasing temperature from 25 to 60°C. It lead to the saturation concentration of glucose fatty acid ester is too high under the reaction conditions, the product can not or difficulty crystallize from the reaction medium, shift the reaction equilibrium to undesired direction, namely a product inhibition. In addition, the high temperature leads to the denaturation of the enzyme. On the

other hand, the by-product existing in the reaction media significantly influences on the yield. To eliminate the by-product efficiently from reaction mixture, it is necessary to raise the reaction temperature above the boiling point of the azeotrope.

Optimum reaction temperatures for lipase catalyzed synthesis of SFAE should be adjusted depending on chain length of fatty acids. For synthesis of glucose fatty acid ester with a short chain length such as caprylic acid, lower temperature (25°C) was chosen to remain the low product concentration in the liquid phase where reaction occurred. Consequently, higher reaction temperature (60°C) was employed for the synthesis of long chain fatty acid glucose ester, to accelerate the reaction. Under these fixed reaction temperatures, the reaction pressure had to be decreased to evaporate the azeotrope and solvent since the kinetics of acylation and the reaction yield are critically dependent on the evaporation of methanol or water from the reaction medium.

2.5.2 Acyl donors -- free fatty acid *vs* fatty acid methyl ester

High yields have been achieved when azeotropic distillation was employed to remove the water generated by esterification for synthesis of 6-*O*-palmitoyl-β-D(+)-glucose and 6-*O*-stearoyl-β-D(+)-glucose from glucose and palmitic acid or stearic acid. The yield in the synthesis of glucose palmitate using palmitic acid methyl ester decreased to 67% compared to the 81% typically obtained using free palmitic acid. Similar results were achieved for the synthesis of 6-*O*-stearoyl-β-D(+)-glucose, where yield decreased from 90% to 20%. One explanation might be that the direct esterification of the free fatty acid is faster than transesterification using the methyl ester. The other explanation might be that transesterification reaction follows the Ping-Pong-Bi-Bi mechanism and the esterification reaction obeys an ordered Bi-Bi mechanism.

2.5.3 Substrate ratio

In the synthesis of SFAE, the excess of long chain fatty acid did not result in significant increase of conversion. In the case of synthesis of SFAE with short chain length, the excess of fatty acid seems to favor higher conversion. For example, caprylic acid is almost completely dissolved in EMK and the solubility of glucose caprylate in EMK at 60°C is 16.60 mg/ml, the excess of caprylic acid can drastically change the composition of the liquid phase in which the reaction occurs.

2.5.4 Stability of enzyme (temperature and solvent)

CAL-B is a 33 kDa protein. Like most carboxylic esterases of known structure, it consists of the common structural framework such as the α/β-hydrolyze fold and the catalytic triad in the active site. The immobilized lipase CAL-B was reported to be a very highly thermostable enzyme and it can be used in continuous operation at 60-80°C without any significant loss in activity (Arroyo and Sinisterra 1994). Here, it was found that CAL-B is more stable in EMK than in acetone. This might be attributed to the fact that acetone is more water-soluble (∞ g/100g water) than EMK (26 g/100g water), thus acetone might dissolve water bound to the enzyme (Klibanov 1997). Studies on the stability of the biocatalyst showed that the immobilized lipase CAL-B was highly stable (12 days, 60% residual activity) in the reaction medium under the reaction conditions investigated.

3. Production of SFAE by enzymatic esterification in a stirred-tank membrane reactor

3.1 Possible technologies for solvent regeneration

A close examination of the published work shows that five techniques can be used in the solvent regeneration, e.g. absorption, distillation, selective extraction, reverse osmosis and membrane pervaporation. The absorption process is not practical on a large scale due to its complicate and high-cost regeneration process. Reverse osmosis operation is currently applied only in the laboratory scale. Distillation and extraction are the traditional methods for the recovery of solvents in industry. In the case of the azeotrope mixture of EMK and water, EMK can be regenerated. In the case of an azeotrope mixture of acetone and methanol, CH_2Cl_2 was added to the azeotrope and extractive distillation was used to break the mixture of methanol and acetone since methanol can form a new azeotrope (Röck 1960) (methanol:CH_2Cl_2, 92:8, v:v, b.p. 39.2°C). However, more investment and higher process costs are needed in distillation than in membrane pervaporation (Tab. 3.1.1).

Tab. 3.1.1 Economic comparison of methods for solvent recovery.

	Investment costs [TDM]*		**Energy costs**
	10 m^3	30 m^3	**[TDM/year]****
Distillation	1800	3500	700
Membrane	1500	3000	150

*Investment costs based on manufacturer's prices for skid mounted units, including piping, process control, cooling units and start up; ** Energy costs based on heating vapor DM 50/metric ton, electricity DM 0.20/kWh (Adapted from the internal data of Langbein Engelbracht GmbH).

3.2 Membrane pervaporation

3.2.1 Introduction

In recent years, there has been increasing interest in the use of pervaporation membrane separation technique for the selective separation of organic solvent mixtures due to its high separation efficiency and flux rates coupled with potential savings in energy costs (Padt et al. 1993; Napier et al. 1996; Ujang et al. 1997). This technique depends on the fact that 'non-porous, water permeable membranes' permit easier passage of water than solvent, thus

changing the composition of the mixture (but which does not contain pores extending through the membrane) (Fig. 3.2.1). A lot of research had clearly demonstrated that pervaporation could be applied to fractionating mixtures of close boiling-temperature liquids, or mixture leading to azeotropes (Binning 1961; Neel et al. 1987). In the beginning of the 80ies this technology was first commercialized by the German company G. F. T. (Gesellschaft für Trenntechnik, Homburg/Saar). After that, about 50 plants with larger productive capacities (ranging from 2 000 to 15 000 liters/day) were installed by G. F. T. in western Europe and in USA. In 1988 the first large scale industrial pervaporation plant was operated in the sugar refinery of Bethéniville, Marne, France. This plant is used for the dehydration of alcohol and designed by G. F. T. to produce 150 000 liters of refined ethanol per day. The pervaporators are fed with a 94% alcoholic mixture issued from a predistillation column and the residual water-content in the dehydrated product is less than 2000 p.p.m. (Rapin 1988).

In order to adapt this technique on the production of SFAE, the key problem was to separate methanol from the azeotrope mixture of acetone and methanol or water from the azeotrope mixture of EMK and water, before acetone or EMK are returned to the reaction medium.

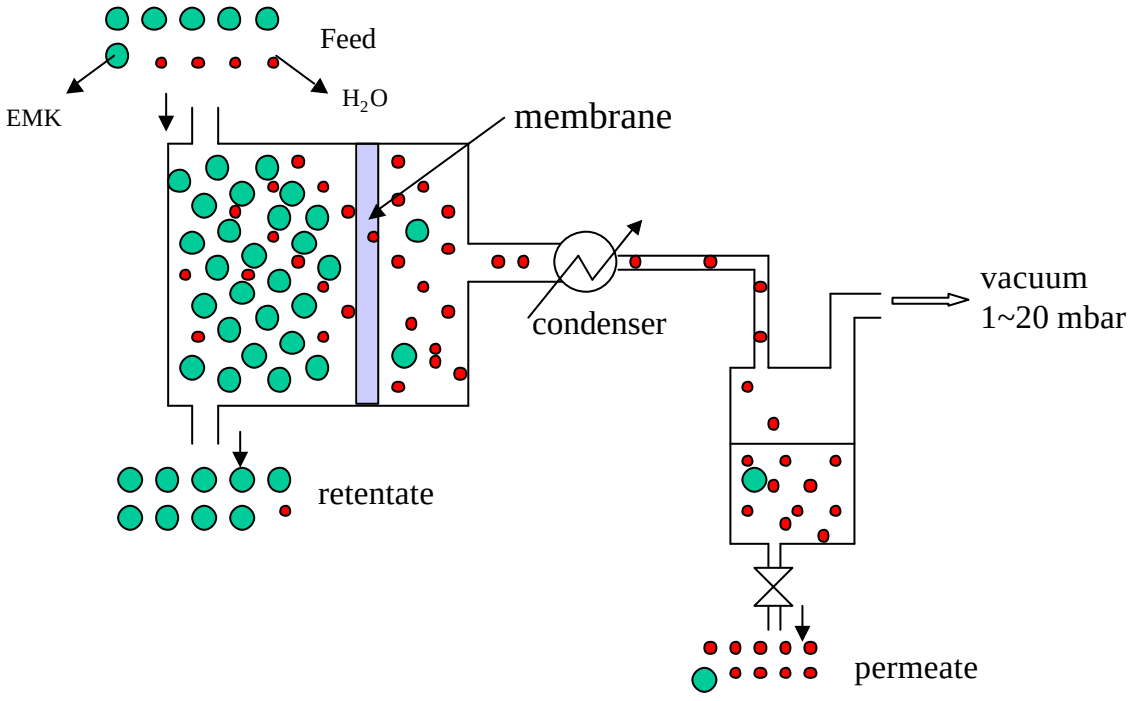


Figure 3.2.1 Continuous-flow vacuum pervaporation.

3.2.2 Design of a stirred-tank membrane reactor

In pervaporation, the liquid mixture feed is in contact with one side of a dense non-porous membrane and is removed from the downstream side in the vapor phase under vacuum after

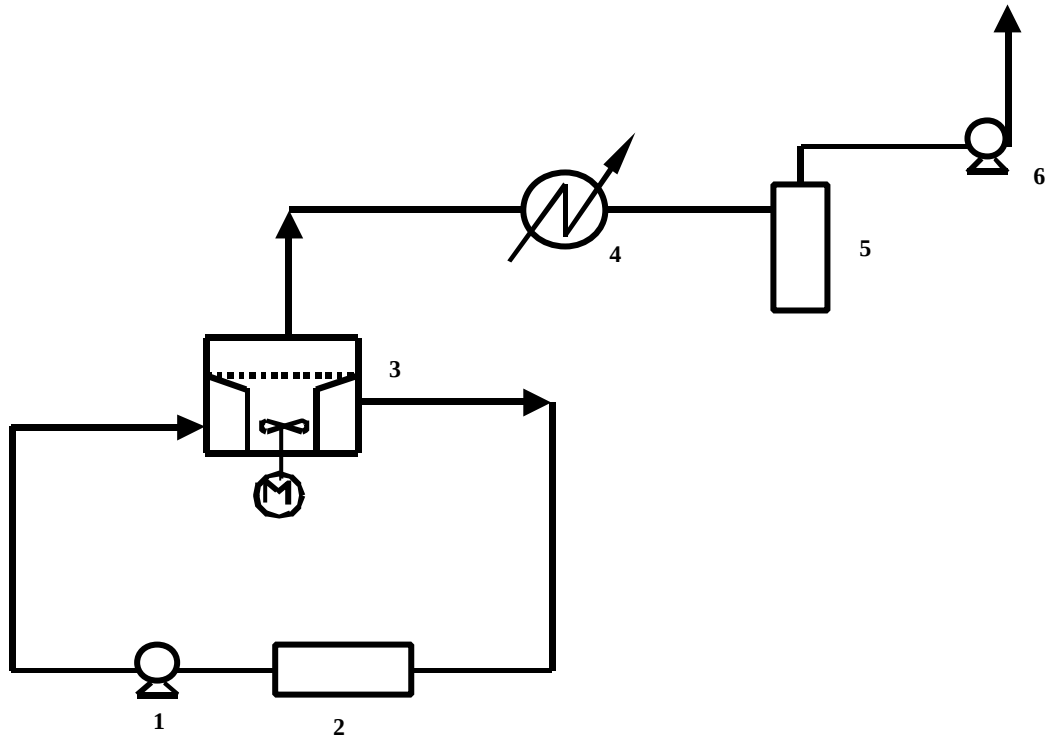


Fig. 3.3.2.1. Process diagram of lipase-catalyzed synthesis of SFAE in a stirred-tank membrane reactor. 1 pump, 2 water bath, 3 membrane reactor, 4 condenser, 5 permeate container, 6 vacuum pump.

diffusing through the membrane and condensation. This technique depends on the fact that the given membrane permits easier passage of one liquid than the other, thus changing the composition of the mixture through passing the permselective membrane. In vapor permeation, the whole feed mixture is in the vapor phase, so that the separating component just has to permeate through a permselective, non-porous membrane (Huang 1991). Recently, Gemert and coworkers reported an enzyme catalyzed process in which reaction water formed was continuously removed through a non-porous, water permeable membrane and the most interesting is that the enzyme was immobilized on the membrane (Gemert and Cuperus 1998). However, in the process of solid phase synthesis of SFAE, using a membrane reactor in which the membrane was equipped at the bottom side is not suitable because of 1) the risk of precipitation of substrates and product on the membrane surface; 2) difficult transfer of water molecules from the solid reaction medium, which can result in very low conversions (data not shown) and; 3) the abrasion of the membrane surface due to strong agitation of the solid substrates. As shown in Chapter 2, water generated during esterification can be removed from the reaction medium by forming an azeotrope with EMK. Thus a membrane reactor was designed with a membrane located at the top to avoid the problems mentioned above (Figure

3.3.2.1). In order to obtain good permeation rates and a high degree of separation for a liquid mixture, it is essential to choose a right membrane as well as best operating conditions.

3.3.3 Membrane selection

For dehydration of an organic solvent, hydrophilic polymers appears to be the most suitable because the high polarity of water makes hydrophilic membranes more selective towards water (Jirage and Martin 1999). The main characteristies of the membrane are selectivity and permeability. Here, a membrane is needed that can separate EMK from water or acetone from methanol (Figure 3.3.3.1). Unfortunately, a membrane that can be applied to separate the latter could not be identified. Our previous work showed, that the water content of the reaction mixture should be in the range of 0.02-0.2%, and that the amount of water generated during esterification must be reduced to a maximum water content in EMK of 2-4%. It turned out that a Pervap® 2200 membrane is best as based on the manufacturer's information. This membrane is highly selective for separating water from an organic solvent even at elevated solvent concentrations. This makes its application especially attractive for the dehydration of an azeotropic mixture, which cannot be separated by simple distillation.

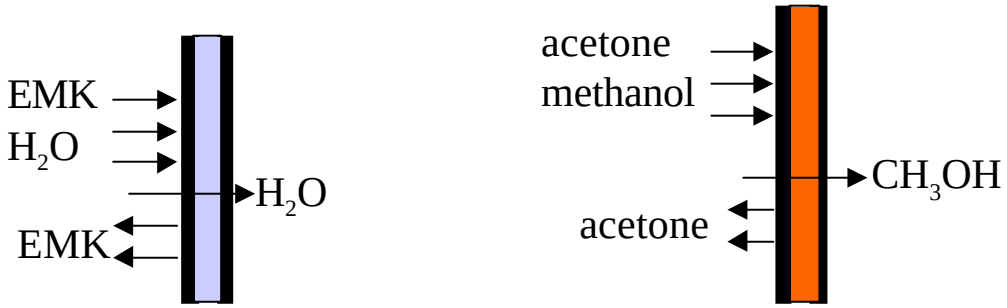


Figure 3.3.3.1 Membrane selectivity required for synthesis of SFAE.

3.3.4 Parameters influencing water removal

3.3.4.1 General consideration

The dehydration process can be divided into three steps: i) water sorption into the membrane at the upstream side; ii) diffusion through the membrane; and iii) water desorption into a vapor phase at the downstream side. The driving force - the difference of partial pressures of the permeating components between the feed and permeate sides of the membrane - were determined by feed concentration, feed flow rate, feed pressure, temperature and permeate pressure. In the present operating system the feed was not liquid but a mixed vapor and the feed concentration, feed flow rate and feed pressure depend on the process of esterification. It

is well known that pervaporation selectivity is not significantly altered by increasing downstream pressure, as long as the permeate pressure does not exceed the saturated vapor pressure of the pervaporate evolved. Beyond this limit, the transport selectivity either increases or decreases, depending on the respective volatility of the two feed components. In the case of the azeotrope mixture of EMK and water, the faster migrant water is the less volatile component and an increase of downstream pressure will diminish selectivity. Therefore, the permeate pressure should be as low as possible to favor removal of water. Thus the maximum vacuum value of the employed vacuum pump at 10 mbar was chosen.

3.3.4.2 Temperature

Temperature has a great influence on both the permeate flux and the synthesis of SFAE (Huang 1991; Yan et al. 1999). In general, the permeate flux can be increased by raising the temperature without any reduction of selectivity because of the higher water vapor partial pressure of the upstream as well as the simultaneous increase of the diffusion velocity of the permeate through the membrane. According to results in Chapter 2, 25, 40, 50, 60 and 75°C were used as test temperatures. The influence of the operating temperature on the water permeate flux through the membrane is shown in Figure 3.3.4.2.1. The water permeate amount significantly increases above 1.0% at temperatures above 60°C on the feed water content in EMK. In the application of the vapor permeation, the temperature was set as high as possible to achieve maximum permeation rates and minimize the required membrane area and plant costs. On the other hand, high temperatures lead to low monoester formation due to higher solubility of the SFAE in the reaction solvent. It is essential to keep the reaction temperature above the boiling point of the azeotropic mixture of EMK and water (b.p. 73.5°C) to vaporize the water generated during esterification. However, at 75°C the product solubilities are too high to ensure crystallization of medium chain fatty acid glucose monoesters like glucose caprylate, thus avoiding high conversion. In this case, the reaction and dehydration units should be separated, each operated at different temperatures.

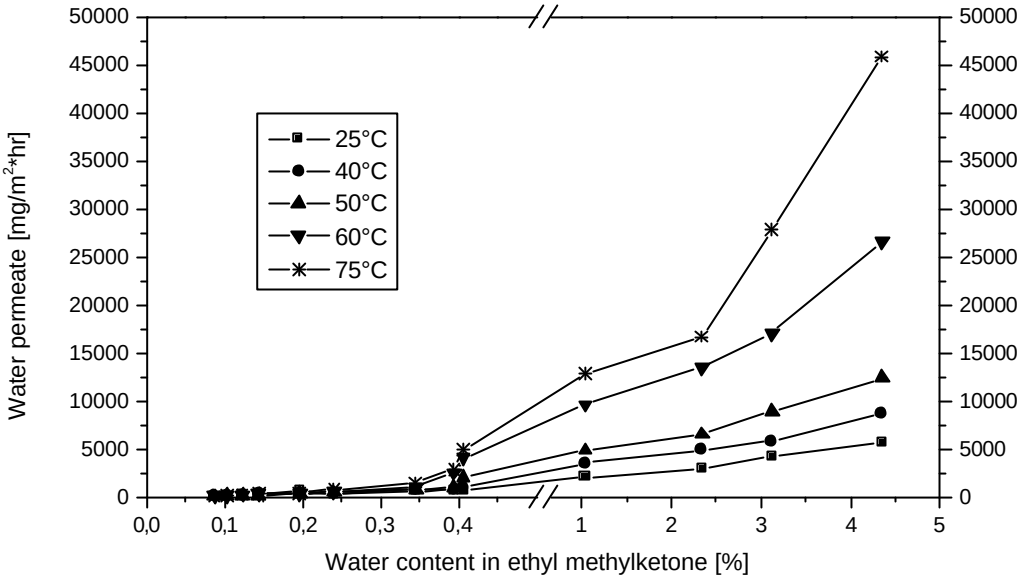


Fig. 3.3.4.2.1 Relationship between water content in EMK and water permeate at different temperature. Test conditions: 20 ml EMK with 4.3-4.6% water, membrane reactor (Φ 58mm x 8mm, membrane area 23cm^2), vacuum 8-12 mbar (downstream), collecting container –8°C, operating temperature (upstream) 25, 40, 50, 60°C, membrane at bottom of reactor; 75°C, membrane at top of reactor.

3.4 Production of SFAE with continuous dehydration of solvent by membrane vapor permeation

3.4.1 Influence of fatty acid chain length

From previous studies on the lipase-catalyzed synthesis of SFAE (Cao et al. 1996; Cao et al. 1997; Cao et al. 1998; Cao et al. 1999; Yan et al. 1999) in a solid phase system, it was already known that the fatty acid chain length had a considerable influence on product formation. This was mainly due to the higher solubility of medium chain fatty acids in the reaction system, thus suppressing the crystallization of product, which is required to achieve high conversions (Flores et al. 2000). A related pattern was also observed in this process using fatty acids ranging from C_8-C_{18} (Figure 3.4.1.1). Highest yields of monoester 73% were achieved using stearic acid at a fatty acid : glucose ratio of 1:1, whereas conversion of monoester was only 18% in case of caprylic acid.

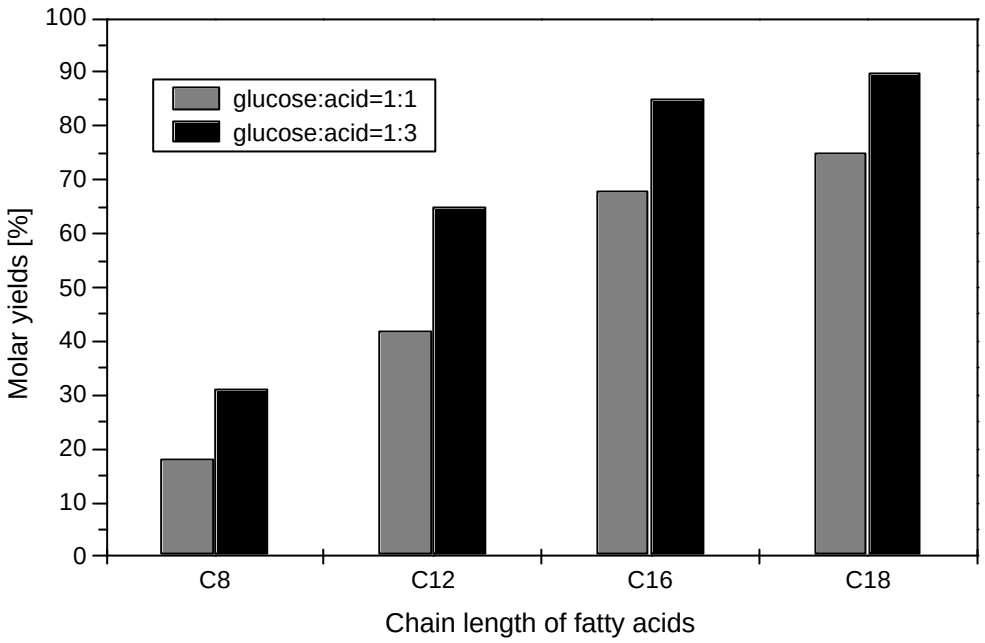


Figure 3.4.1.1 Acylation of SFAE with different chain long fatty acids. Reaction conditions: 0.5 mmol glucose, 0.5-1.5 mmol fatty acids, 50 mg L-2 lipase, 0.4-1 ml EMK, 75°C, 650 rpm, 48 h.

3.4.2 Influence of substrate ratio

In our previous works, an equimolar ratio of sugar to fatty acid was found is sufficient at 60°C, as an excess of acyl donor is not required to achieve a high conversion. In the present system, however, a considerably higher conversion was observed when the ratio of fatty acid to glucose was increased from 1:1 to 6:1. Thus the synthesis of 6-*O*-octanoyl-glucose monoester increased from 18 to 64% (Figure 3.4.2.1). A possible explanation might be that at a higher reaction temperature, the equilibrium is shiftted toward the undesired direction because high product concentration lower precipitation. By increasing the substrate ratio, it was possible to increase the concentration of substrate in the reaction medium and improve the glucose transformation rate, shifting the equilibrium to the ester synthesis.

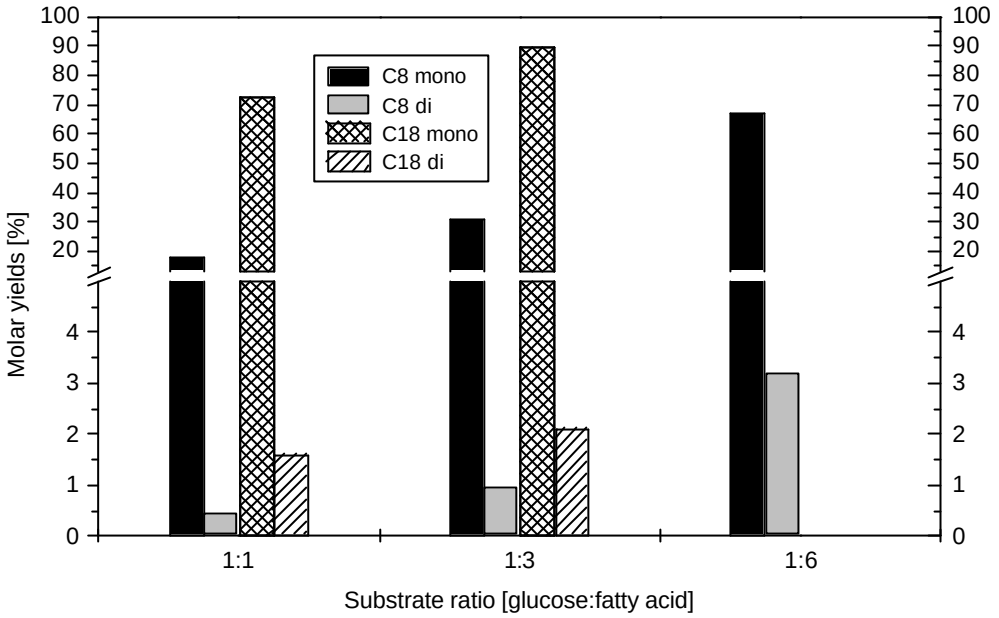


Fig. 3.4.2.1 Synthesis of SFAE at different substrate ratios.
Reaction conditions: 0.5 mmol glucose, 0.5-3 mmol fatty acids, 50 mg L-2 lipase, 0.4-1 ml EMK, 75°C, 650 rpm, 48 h.

3.4.3 Influence on regioselectivity

A series of glucose fatty acid esters were produced in the stirred-tank membrane reactor at 75°C. The results are shown in Figures 3.4.2.1 and 3.4.3.1. At a ratio of 3:1 (stearic acid:glucose), yields of 90% of stearic acid glucose monoester were achieved. That means, a two fold excess of stearic acid increased the monoester formation by about 17%. At the same time, diester formation increased only about 0.5%. The esterification of glucose and fatty acid produces at first monoester. If the fatty acid is in excess in the reaction system, the monoester can be further esterified to produce the diester. This can explain why the diester content increased from 0.45 to 3.21% (Fig. 3.4.3.1) when the glucose:acid ratio is increased from 1:1 to 1:6 as shown capric acid. The rate of diester production increased with increasing concentrations of fatty acid and the concentration of monoester in reaction media, but with decreasing chain length of the fatty acid. The fact that different diester formation was observed for stearic, palmitic and capric acids at same molar ratio of glucose to acid, indicates that the amount of diester can not be attributed to only acid concentration in the solvent, because the acid was totally soluble under the experimental conditions. According to Arcos *et al.* (Arcos et al. 1998; Arcos et al. 1998; Arcos et al. 2000), another reaction that simultaneously produces the diester and whose reaction rate does not depend on acid concentration can occur. They claim that interesterification between two mols of monoester

produces one mol diester and one mol glucose. This reaction rate depends only on the monoester concentration in the solvent. The solubility of glucose caprylate of 51.23 mg/ml at 75°C in EMK is about six times higher than the solubility of glucose stearate (8.74 mg/ml). This explains the results shown in Figure 3.4.3.1. The solubility of monoester in the reaction solvent plays the key role to achieve a high yield of monoester. If the solvent amount is fixed, an increase of the reaction temperature leads to significant increases in the solubility of the monoester. In contrast, the production of monoester does not always increase with the reaction temperature because to dissolve sufficiently fatty acid in EMK needs a certain temperature.

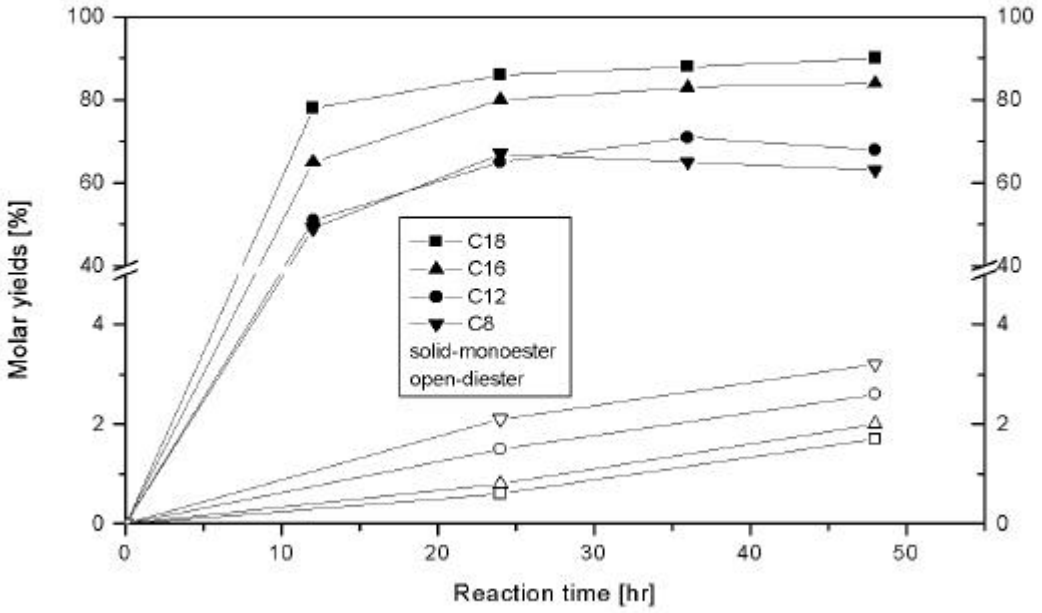


Fig. 3.4.3.1 Synthesis of SFAE with various fatty acids.
Reaction conditions: 0.5 mmol glucose, 1.5-3 mmol fatty acids, 50 mg L-2 lipase, 0.4-1 ml EMK, 75°C, 650 rpm, 48 h.

3.5 Optimization of enzymatic esterification by response surface methodology

3.5.1 Introduction

Since a temperature around 75°C is a pre-condition for a successful azeotropic distillation, higher yields can only be obtained, if all other reaction parameters (i.e., reaction time, substrate ratio, amount of EMK and enzyme load) are optimized. Because of the complicated interaction of these parameters, response surface methodology (RMS) was used to identify best reaction conditions.

RSM is a useful statistical technique for the investigation and optimization of complex processes. It enables the evaluation of multiple parameters, alone or in combination, on response variables. The main advantages of RSM are the reduced number of experiments needed to provide sufficient data for statistically acceptable results. RSM has been successfully applied in many areas such as food process (Vainionpää 1991), enzymatic synthesis (Ismail et al. 1998) and enzymatic modification of fats and oils (Huang and Akoh 1996; Xu et al. 1998; Xu et al. 1998; Xu et al. 1998; Xu et al. 1999), the production of SFAE by lipase-catalyzed esterification has not yet been optimized, in particular for solid phase synthesis systems.

3.5.2 Experimental design

The experiments consisted of a total of 29 runs with the sets of five factors, which includes reaction time (T_r , h), substrate ratio (S_r , acyl donor to glucose, mol/mol), reaction temperature (R_t , °C), EMK amount (S_a , wt-% based on fold substrates) and enzyme load (E_l, wt-% based on substrates). These factors had been determined important as shown in chapter 2 and the set ranges of the factors were also based on these findings with each used in three levels according to the central composite face (CCF) design (Montagomery 1997; Umetrics 1997). The actual variables and their levels are listed in Table 3.5.2.1.

3.5.3 Statistical analysis

Modde 4.0 (*Umetri*, Sweden) was used to analyze the experimental data. Second-order coefficients were generated by regression analysis with backward elimination. Responses were first fitted for the factors by multiple regression. The fit of the model was evaluated by the coefficients of determination (R^2 and Q^2) and analysis of variance (ANOVA). The

insignificant coefficients were eliminated after examining coefficients, and the model was finally refined. The polynomial equation to be fitted to the measured, dependent variables Y was of the form:

$$Y = b_0 + \sum_{i=1}^{5} b_i X_i + \sum_{i=1}^{5} b_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} b_{ij} X_i X_j \qquad [3.5.3.1]$$

Where Y is the value of the response, X_i (i=1-4) is the independent variables, b_0 is the intercept, b_i is the first order model coefficients, b_{ii} is the quadratic coefficients for the ith variable and b_{ij} is the interaction coefficients for the interaction of variables i and j.

The performance of the enzymatic synthesis of SFAE was evaluated by analyzing responses to the conversion. Optimization of the reaction parameters was calculated using the predictive models from RSM. The synthesis of SFAE was carried out at the predicted optimal conditions. The observed responses obtained at these conditions were analyzed and compared to the predicted values.

3.5.4 Model fitting

Using the normalized results as the responses for the fitting of the RSM models, the quadratic relationships required for modeling were set up. The analysis of variance for the five process variables indicated that the SFAE yields can be well described by polynomial model. The second-order polynomials fitted to the measured data were computed by stepwise regression analysis using the standardized variables X_i. Standardized values were used to attain proper preference between the variables in the calculation of the models. The equations tabulated in Tab. 3.5.5.1 are, however, computed with those actual variables which also occur in the standardized models. The coefficient of determination (R^2) of the model for the response is 0.996 (Q^2=0.899). According to the ANOVA, there was no lack of fit and there was a satisfactory coefficient of determination. Prediction results were close to the observed, and all absolute errors of the predictions were less than 1.0. This indicates that the model represents the real relationships between reaction parameters well.

Tab. 3.5.2.1 Set factor levels, measured responses, and the comparison between the calculated net incorporation and the predicted results by the RSM-fitted model.

No.	Run No.	Factors					Conversion* [%]	Pr.
		T_r	S_r	E_l	R_t	S_a		
1	5	24	1	6	74.5	2.5	52.3	52.3
2	26	60	1	6	74.5	0.5	64	63.7
3	3	24	3	6	74.5	0.5	52.8	52.9
4	27	60	3	6	74.5	2.5	82.3	82.2
5	21	24	1	12	74.5	0.5	57.8	58
6	23	60	1	12	74.5	2.5	73.4	73.4
7	4	24	3	12	74.5	2.5	69.2	69.6
8	25	60	3	12	74.5	0.5	83.2	83.2
9	24	24	1	6	78.5	0.5	46.3	46.1
10	14	60	1	6	78.5	2.5	83.3	83
11	16	24	3	6	78.5	2.5	67.6	67.7
12	2	60	3	6	78.5	0.5	75.8	75.5
13	17	24	1	12	78.5	2.5	63.5	63.7
14	18	60	1	12	78.5	0.5	76	75.8
15	29	24	3	12	78.5	0.5	63.9	64.1
16	19	60	3	12	78.5	2.5	85	85
17	22	24	2	9	76.5	1.5	72.9	72
18	20	60	2	9	76.5	1.5	89.5	90.5
19	13	42	1	9	76.5	1.5	80.3	80.8
20	10	42	3	9	76.5	1.5	89.2	88.8
21	6	42	2	6	76.5	1.5	78.6	79.4
22	9	42	2	12	76.5	1.5	86.4	85.6
23	1	42	2	9	74.5	1.5	84.6	84.2
24	8	42	2	9	78.5	1.5	87	87.4
25	15	42	2	9	76.5	0.5	76.8	77.2
26	7	42	2	9	76.5	2.5	84.7	84.4
27	28	42	2	9	76.5	1.5	87	86.7
28	11	42	2	9	76.5	1.5	86.5	86.7
29	12	42	2	9	76.5	1.5	86.8	86.7

T_r --reaction time (24-60 h), S_r --acyl donor to glucose molar ratio (1-3), E_l --enzyme load (6-12 wt-% of substrates), R_t --reaction temperature (74.5-78.5°C), S_a --Solvent amount (0.5-2.5 fold of substrates), Pr.-prediction, conversion*--of glucose.

3.5.5 Main effects of parameters on response

The influence and effects of parameters on esterification of sugar and fatty acid on the product yield have widely been investigated for the past twenty years. However, only a few reports have been found that the reaction performed in a mainly solid phase. As expected, the results obtained from the present work are similar to our previous work (Yan et al. 1999; Yan et al. 1999). The yield of glucose fatty acid monoester was increased by longer reaction time, high enzyme load and higher substrate ratio. Reaction temperature was kept in a narrow range between 74.5°C to 78.5°C in order to evaporate the azeotrope and to change the gaseous EMK to liquid phase when the azeotrope was broken. The different effects of the changing parameters were showed in Fig. 3.5.6.1. It can be observed that all five

Tab. 3.5.5.1 Regression coefficients and significance values of the second-order polynomials after backward elimination.

Variable	Coefficient	Significance
constant	86.6668	5.94293e-018
Tr	9.23333	2.46359e-011
Sr	4.00555	1.87283e-008
El	3.07777	1.48073e-007
Rt	1.6	2.15308e-005
Sa	3.59445	4.39183e-008
Tr*Tr	-5.42929	4.01997e-006
Sr*Sr	-1.87928	0.00506487
El*El	-4.12929	3.06534e-005
Rt*Rt	-0.829282	0.130047
Sa*Sa	-5.87929	2.19689e-006
Tr*Sr	-0.249995	0.230139
Tr*El	-1.45	6.70746e-005
Tr*Rt	0.500001	0.0317162
Tr*Sa	-0.425002	0.0582344
Sr*El	-0.125002	0.53421
Sr*Rt	-1.05	0.000604544
Sr*Sa	1.67867e-006	1
El*Rt	-1.05	0.000604539
El*Sa	-2.27501	2.40397e-006
Rt*Sa	1.125	0.000384746

parameters had positive effects. The reaction time had the most significant effects on conversion for the first-order factors, which indicated that longer reaction times were favored. For the second-order factors, solvent amount, reaction time and enzyme load had negative effects on conversion. No significantly effective interactions in the model were observed, that means the production of SFAE can be optimized by one-factor design.

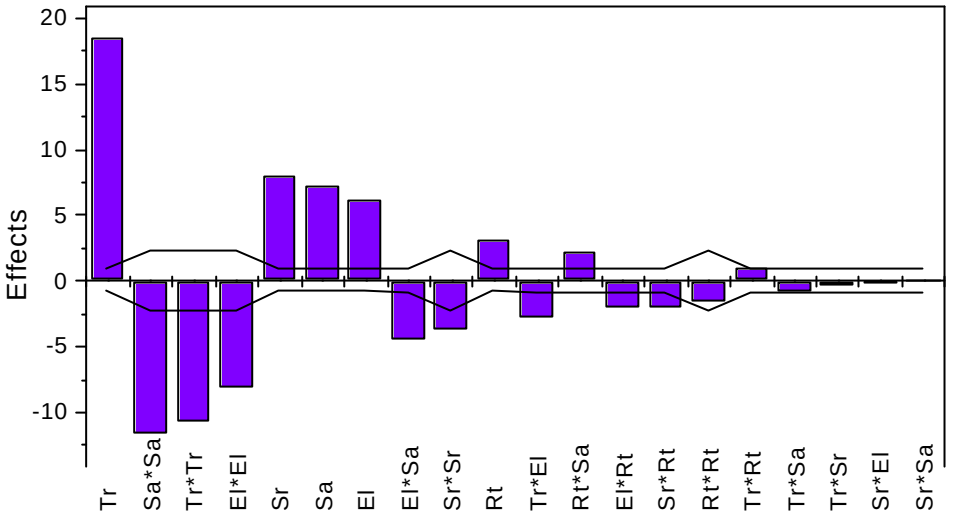


Figure 3.5.5.1 Main effects of the factors and their significance.

3.5.6 Optimization

The merits of using RMS are to evaluate the relationships between each parameter and predict the result and behavior under given reaction conditions. Moreover, optimal parameters can be obtained by iterative calculation with more than one response and targets. Optimization of the yield of SFAE was performed by taking into account reaction time, temperature, glucose/stearic acid molar ratio, solvent amount and enzyme load. However, it should be noticed that the range of each parameter has to be pre-decided before optimization, e.g. a shorter reaction time always resulting in lower product conversion. But the shorter reaction time with a sufficient monoester yield can achieve a higher space-time yield which is very important factor to realize this technology in the industry, thus needs to be pre-decided. To evaluate the relationships and interactions of parameters, surface plot give the good prescriptions. The behavior of the product conversion under the above process conditions is presented in Figures. 3.5.7.1-3.5.7.10. Generally, higher substrate ratio, higher enzyme load and longer reaction time contribute positively to monoester conversion. The effect of temperature is not significant to conversion since the given range of temperature here is very

narrow. The Influence of solvent amount seems to be complex. In the range of 0.4-1.6 wt-%, the conversion increased greatly, and increasing solvent leads to decreasing monoester yield. The optimization with the response and five variables can not just be calculated mathematically through equation 3.5.3.1 as more than one optimum condition may exist. As a result, possible optimum conditions, however, can be iteratively calculated in the set ranges and targeted response of conversion (Tab. 3.5.7.1). It shows that optimum conditions are different and a conversion up to 93% can be achieved under the given reaction conditions.

Tab. 3.5.6.1 Optimum conditions generated by Modde 4.0 in the set parameter ranges and targets through iterative calculation*.

No	Tr	Sr	El	Rt	Sa	conversion	iteration
1	57.18	2.96	9.38	77.53	1.81	93.41	226
2	57.35	2.80	9.02	78.18	1.86	93.58	136
3	60.00	2.12	8.01	78.50	2.18	92.11	171
4	58.02	2.72	8.89	78.27	1.86	93.57	129
5	52.80	2.16	9.18	76.62	1.56	91.21	11
6	60.00	2.40	9.00	76.50	1.50	91.67	0
7	49.20	3.00	9.00	76.50	1.50	91.52	0
8	49.20	2.00	9.00	78.50	1.70	91.14	7

*Set parameter ranges: Tr, 24-60; S_r, 1-3; E_l, 6-12%; R_t, 74.5-78.5°C; S_a, 0.5-2.5. Set targets: conversion 92.7% (minimum 88.2%). Abbreviations see Tab. 3.5.2.1.

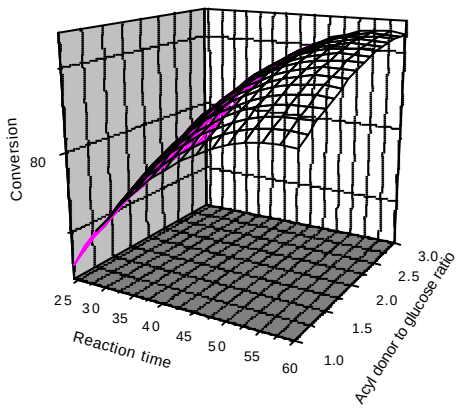


Fig. 3.5.7.1

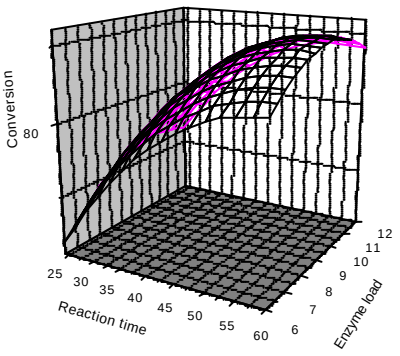


Fig. 3.5.7.2

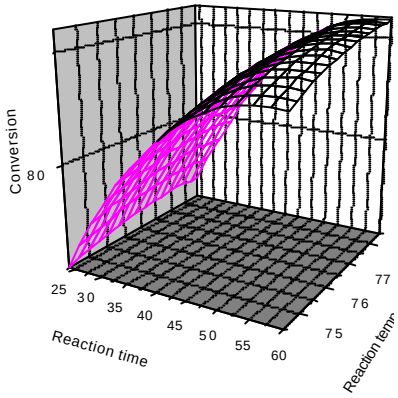


Fig. 3.5.7.3

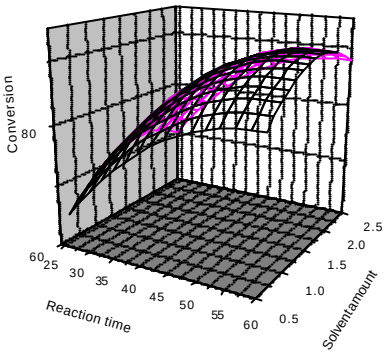


Fig. 3.5.7.4

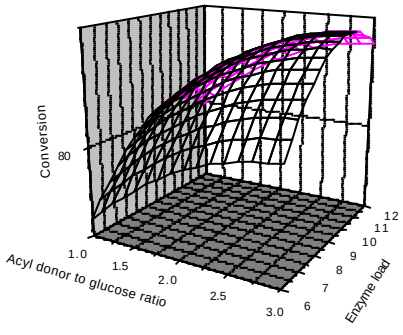


Fig. 3.5.7.5

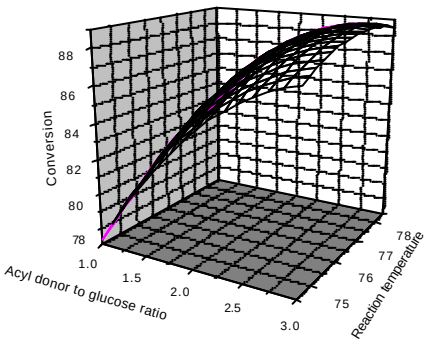


Fig. 3.5.7.6

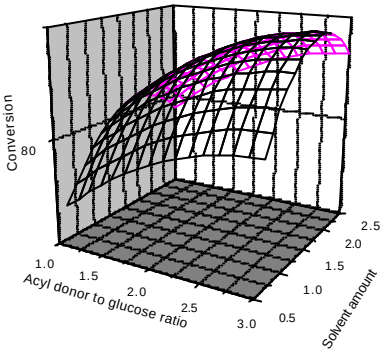


Fig. 3.5.7.7

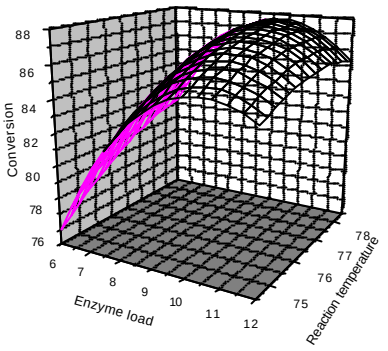


Fig. 3.5.7.8

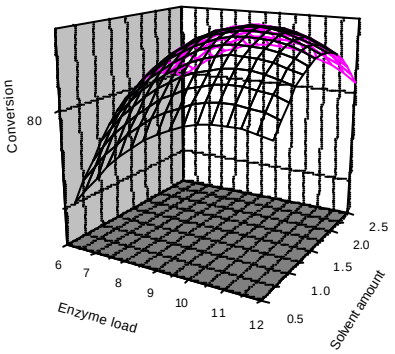


Fig. 3.5.7.9

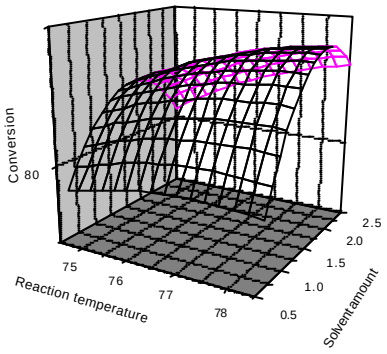


Figure 3.5.7.10

Figure 3.5.7.1-10 Surface plots for the evaluation of effects of parameters.

4. Enzymatic synthesis of SFAE in a stirred-tank membrane reactor: Removal of reaction water using a low-boiling-point azeotrope

4.1 Result and discussion

4.1.2 Effect of addition amount of hexane to EMK

After a carefully study of literature, a ternary azeotrope with EMK and hexane was found (Bludworth et al. 1945; Gmehling et al. 1994) (e.g., 56°C b.p. at 26:55:19 EMK:hexane:water) which can realize the removal of reaction water from the reaction media by azeotropic distillation at 59°C is arround the optimum temperature (60°C) of immobilized lipase B from *Candida antarctica*. First, the effect of the percentage of hexane in the reaction mixture was investigated. Figure 4.1.2.1 illustrated the formation of SFAE with different percentage of hexane in EMK. At percentages of hexane of 60% or higher, the reaction is extraordinarily slow, this is probably due to a bad solubilization of sugar in the solvent medium. If the percentage of hexane increased to 100%, the esterification was not performed since no glucose was solubilized in hexane. In contrast, at no hexane (pure EMK), the initial acylation proceeded very fast, but the maximum conversion of monoester did not exceed 52%, and it was reached only after 8 h of reaction. This phenomenon may be related to the fact that the equilibrium of the esterification is rapidly reached because there is accumulation of the by-product water in the reaction media under these conditions (no azeotrope was evaporated from reaction medium). At 20% hexane, although the reaction is initially not so fast, the formation of monoester is almost the best after 48 h reaction. Thus, a medium containing 20% hexane was chosen for further study. At same time, addition of 20% hexane can lower the boiling temperature of the solvent mixture by as much as 15°C. This would have important significance for the synthesis of SFAE on a large-scale operation since low-temperature evaporation conditions is highly advantageous in term of energy consumption and enzyme stability. It is also easier to dry the evaporated solvents *via* a membrane pervaperator before the solvent was returned to the reaction vessel as mentioned in Chapter 3.

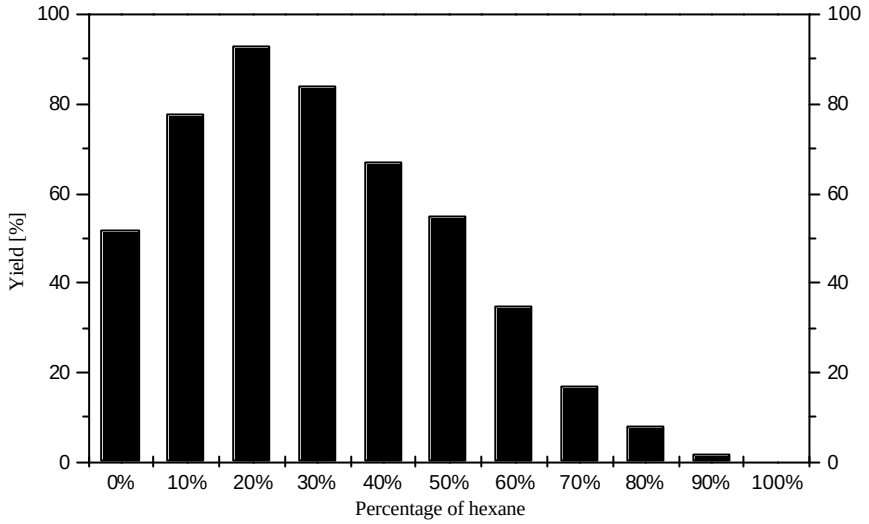


Figure 4.1.2.1 Relationship between product yields and the percentage of hexane in EMK. Reaction conditions: 0.5 mmol glucose, 0.5 mmol fatty acids, 50 mg magnet EP 100 lipase, 0.4 ml EMK +/ hexane, 59°C, 650 rpm, 48 h.

4.1.3 Synthesis of SFAE with different fatty acid chain length in the ternary solvent system

It was observed in this process using fatty acids ranging from C_8-C_{18} (Figure 4.2.3.1). At an equimolar of fatty acid to glucose, highest yields of monoester (93%) were achieved using stearic acid, whereas for caprylic acid the highest conversion of monoester was only 61%. Compared of the date presented in Fig. 4.1.3.1 indicated that all conversions of monoesters using mixtures of EMK and hexane were better than those using EMK only, especially in the case of caprylic acid. One explanation might be that the solubilities of SFAE are lower in the mixture of EMK and hexane compared to the pure EMK.

Our previous investigation showed the low solubility of the product in reaction medium can favor the lipase-catalyzed solid phase synthesis of SFAE. Moreover, the solubility of SFAE in the liquid reaction phase mainly depends on the solvent used. It is well known that crystallization can be facilitated by lowering the temperature (decreasing solubility with decreasing temperature). Alternatively, the "cold crystallization technique" can also be used for this purpose, in which two or more organic solvents can be combined to facilitate the crystallization of the substance without lowering the temperature. This technique was also implemented for the solid phase synthesis. Two organic solvents -- EMK and hexane were

mixed together as reaction solvents. One organic solvent EMK offers a little higher product solubility, the other hexane allows near to non-solubility. A binary mixture of the two solvents has a low product solubility (0.78 mg/ml 6-*O*-stearoyl-glucose compared to 0.98 mg/ml in pure EMK) and thus allows high conversion by solidification of the reaction mixture. It is notable that the highest conversions (99%) to 6-*O*-stearoyl-β-D-glucose in 48 h was achieved with this binary solvent mixture.

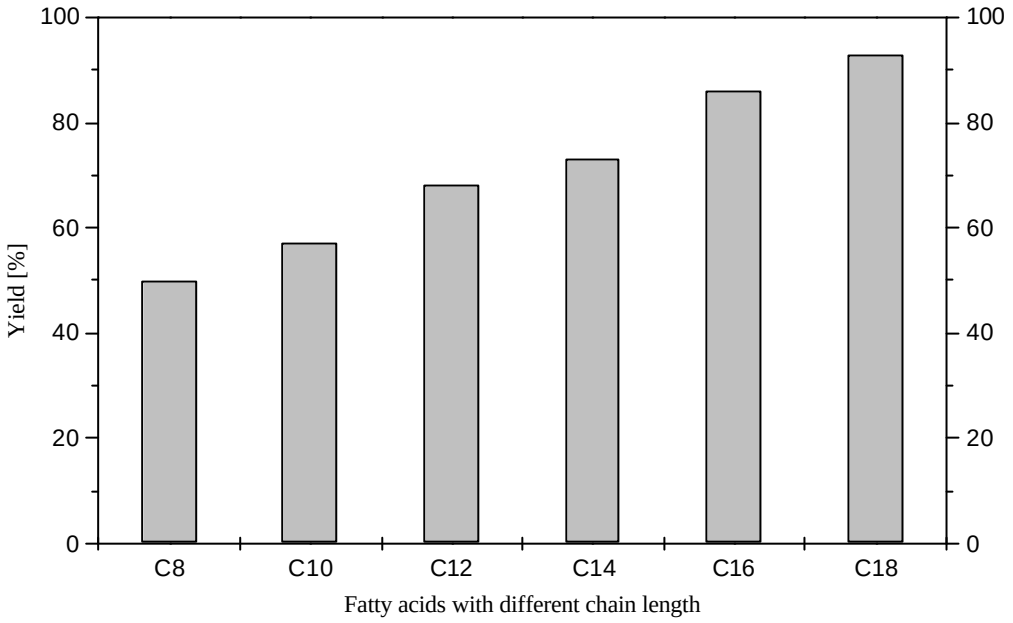


Fig. 4.1.3.1 Acylation of SFAE with different chain long fatty acids.
Reaction conditions: 0.5 mmol glucose, 0.5 mmol fatty acids, 50 mg magnet EP 100 lipase, 0.4 ml mixture of EMK and hexane (4:1, v:v), 59°C, 650 rpm, 48 h.

4.1.4 Enzyme stability

In non-aqueous media such as organic solvent, enzymes retain a high conformational rigidity from stronger non-covalent interactions than in water (Klibanov 1989). However, the solvent can cause inhibition or inactivation by directly interacting with the enzyme. As the cost of biocatalyst is a significant part (85%) of the overall production cost, the stability and reuse of lipase are very important for using this technology in the industry. Since lipase specificities are often determined in hydrolysis reactions and the results are frequently directly applied to biotransformation involving hydrolysis or enzymatic esterification, then the hydrolytic activity of lipase immobilized EP 100 in different solvent system at different operating temperature was first investigated. Tab. 4.1.4.1 shows that there is apparent correlation between the polarity of the solvent, represented as log P, and the hydrolytic activity of lipase,

but no relationship between log P and conversion. No product can be detected in hexane (log P = 3.50), 84% conversion in EMK (log P = 0.29), 83% conversion in acetone (log P = -0.23), 86% conversion in mixture of 20 % hexane in EMK (log P = 0.93). It was found that the enzyme hydrolytic activity lost greatly about 42% after first reaction in the solid phase synthesis of glucose palmitate at 59°C using acetone (EMK) as reaction solvent, whereas for hexane the enzyme activity lost 14% only. It was revealed the enzyme hydrolytic activity generally decreased with increasing polarity of the solvents and is in good agreement with earlier work, in which has been reported enzyme activity and/or stability correlated with a number of solvent properties of which the most important and most popular one is log P (Laane et al. 1987) and either the electron pair acceptance index or the polarizability (Valivety et al. 1991; Valivety et al. 1992; Valivety et al. 1993). The data in Tab. 4.1.4.1 suggested that both reaction temperature and reaction solvent have greater influence to enzyme stability, thus resulting in the loss of hydrolytic activity of enzyme. Indeed, in the case of the synthesis of SFAE, the loss of synthetic activity of lipase is more important than that of hydrolytic activity.

Tab. 4.1.4.1 Enzyme hydrolytic activity in solid phase synthesis of SFAE.

Solvents	**Log P value**	**Conversion [%]**	**Enzyme hydrolytic activity (U/g)**							
			Cycle number							
			0	1	2	3	4	5	6	7
EMK, 59 °C	0.29	84%	125	72	42	41	43	28	27	26
Acetone, 60°C	-0.23	83%	125	63	40	29	24	21	18	18
Hexane, 59°C	3.50	0%	125	108	104	103	103	101	102	100
EMK, 75°C	0.29	68%	125	60	39	30	26	23	23	22
EMK+ 20% Hexane, 59°C	0.93	86%	125	84	56	45	49	38	30	27

Reaction conditions: 0.5 mmol glucose, 0.5 mmol palmitic acid, 50 mg EP 100 lipase, 0.4 ml solvent, 48 h, 650 rpm, at different temperatures.

Then the operational stability of the lipase was also investigated (Fig. 4.1.4.1). As indicated in Fig. 4.1.4.1, the temperature greatly influences the residual synthetic activity of enzyme. In contrast to hydrolytic activity, no drastic loss of synthetic activity was observed at 59°C reaction temperature, but at 75°C the enzyme residual synthetic activity drastically decreased after every run. At an enzyme concentration of 9% (w/w) using 20% hexane in EMK as reaction solvent at 59°C. More than 90% of substrates were converted within 48 h in the first run. Acetone was then added into the reaction medium. The enzyme was separated by

magnetic filtration and dried in vacuum immediately. It was reused for another synthesis. After seven runs (336 h), the percentage of substrates converted into esters was over 77%, and the residual synthetic activity of enzyme was kept to 86%, which demonstrated the excellent operational stability of lipase B from *Candida antarctica* immobilized on the magnetic EP 100 under the experimental reaction conditions. The immobilized lipase *Candida antarctica* B used here has high activity even in a very dry organic solvent (water content 0.002%). It might be that commercial lipase Chirazyme® L-2, Lyo., BM used here is impure. The high activity of the crude lipase relative to the purified enzyme preparation may be due to the presence of significant amounts of stabilizers, such as carbohydrates or sugar alcohols in the crude preparation. These hydrophilic compounds may hold water tightly even during lyophilization. This provides the enzyme with a suitably hydrated environment associated with enzyme molecules, stabilizes enzyme structure, and consequently enhances higher catalytic activity.

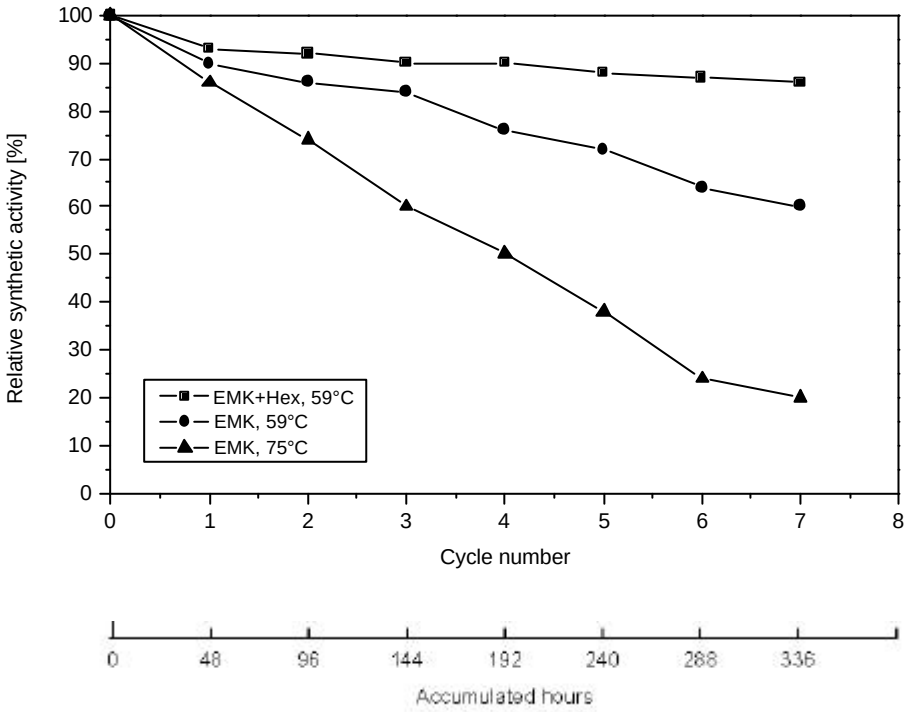


Fig. 4.1.4.1. Enzyme operational stability in solid phase synthesis of SFAE. Reaction conditions: 0.5 mmol glucose, 0.5 mmol palmitic acid, 50 mg L-2 lipase, 0.4 ml EMK or mixture of EMK and hexane (4:1, v:v), 59 or 75°C, 650 rpm.

5. Production of SFAE on preparative scale

5.1 Introduction

Given the increasing demand for SFAE, the goal of large-scale manufacturing is to produce the excellent biodegradable, non-ionic SFAE that can be performed by azeotropic distillation combining with membrane pervaporation for dehydration of EMK as shown in the previous chapters. To achieve this goal, the properties of enzyme and the solubilities of substrates in EMK should be taken into consideration to augment traditional concepts in engineering design. Production and processing of SFAE is a relative simple process that can be manipulated at three main levels of process engineering: reactor, solvent recovery and down stream process (Malcata 1990; Berovic 1999). It is important that critical issues at all of these levels be addressed simultaneously to achieve maximum productivity and optimal quality of the final SFAE.

5.2 Bioreactor design

The design of a bioreactor for the production of SFAE must cover three important factors: the type of reactor, the mode of operation (batch or continuous), and conditions of operation.

5.2.1 Type of bioreactor

The design of a bioreactor for the production of SFAE should support the transformation of water molecule in a high concentration suspension system and be compatible with the physicochemical properties of immobilized enzyme abrasiveness, surface, particle size and sensitivity to inhibitor (Nienow et al. 1996; Wang and Zhong 1996; Wang and Zhong 1996; Oh et al. 1997; Garcia et al. 2000; Giorno and Drioli 2000). Stirred-tank bioreactor and fixed-bed bioreactor are the possible candidates for the production of SFAE. Fixed-enzyme-bed bioreactor have been successfully used in a continuous manner for the enzymatic modification of fats and oils (Xu et al. 1998; Xu et al. 1999). However, it may present considerable disadvantages for the synthesis of SFAE in suspension. The possibility of clogging and the longer residence times due to flow through the enzyme bed will restrict the transport of solid substrate and product, resulting in lower product yield. These problems may be overcome by the use of other reactors, such as ceramic matrix reactor, that provide straight channels through which the particles are converted and provide a narrow residence time distribution that could be adjusted to minimize the effects of by-product water for the reversible reaction. Alternatively, conventional stirred-tank bioreactor can be used for the production of SFAE in

a mainly solid phase system. The product at a high yield due to efficient removal of reaction water by azeotropic distillation, the low investment and the ease of scale-up make this technology attractive.

5.2.2 Mode of bioreactor operation

At the laboratory scale, the usual mode of operation is batch production. Since the market demands small amount of various kinds of SFAE (the total market size for SFAE is estimated to be approximately 4 000 t/a), batch operation can provide a high product flexibility and simple downstream process due to the high productivity. However, it is labor intensive compared with a continuous manner.

5.2.3 Conditions of bioreactor operation

As indicated in Chapter 2, in the case of the production of SFAE in a solid phase system, high by-product water content in reaction medium may limit productivity. In addition, accumulation of product in the liquid catalyzed phase may reduce the selectivity and productivity due to the product inhibition. A key part to consider in the design of a batch stirred-tank bioreactor for the production of SFAE is the stirrer. The stirring rate affects the rate of reaction, through the supply of the dissolved sugar and the removal of by-product water. Furthermore, stirrer affects the enzyme distribution and can cause mechanical damage, which is of great significance in terms of manufacture cost, if it was taken into account that the enzyme cost is approx. 80% of total material costs. The process design parameters such as temperature, solvent amount, enzyme load, substrate ratio and reaction time should be evaluated in term of the long-term ability to the immobilized enzyme to produce SFAE. Process parameters that maximize the long-term productivity will increase the volumes of production and reduce the operation costs of manufacturing.

5.3 Solvent regeneration

For the separation of the water from EMK, different processes such as water-adsorbing materials, reverse osmosis and pervaporation could be used. For the methods of addition of water-adsorbing materials, it is not practical due to its complicated and high-costly regeneration process. The reverse osmosis has some disadvantages such as time-consuming, high-energy consumption and difficulty to scale up. In contrast, membrane pervaporation separation techniques for the recovery of organic solvent has been greatly developed recently because of its high separation efficiency and flux rates coupled with potential savings in energy costs as mentioned in Chapter 3 (Schroen and Woodley 1997). In order to obtain a

suitable pervaporation module, some considerations should be followed: i) requirement of a low hydraulic resistance on the permeate side of the membrane; ii) optimal energy input; iii). module costs per membrane area installed. According to the ways of establishing the driving force for pervaporations, the pervaporations can be divided into vacuum pervaporation, sweep gas pervaporation and thermopervaporation. A comparison of these three operational modes, thermopervaporation is favored due to easy operation and extremely simple permeate transport out of the module. However, it requires more investment since the packing density has to be kept low and many cooling walls have to be dispersed between the membranes. In the case of vacuum pervaporation, the same results can be achieved with the additional expense of a vacuum pump. The condenser can be placed out side of the module because the specific downstream transport resistance is lowered at the low pressure. Sweep gas pervaporation required the cooling of a large gas stream to low temperature, significantly enlarged condenser area for reasons of decreased condensing efficiency due to the high concentration of inert gas, and much lower condensation temperatures due to the decreased pressure of the vapors. Thus it can not be used efficiently in direct organic solvent recovery. Therefore, the plate-and-frame type of pervaporator under reduced pressure was chosen for drying EMK for the production of SFAE.

5.4 Downstream processing

Optimal bioreactor design should result in the production of a high proportion of SFAE in reaction mixture. However, in the case of a solid phase system, the solid sugar and crystallized SFAE may attach or coprecipitate on the surface of the immobilized enzyme in the reaction mixture. The main goals of downstream processing of SFAE are: i) collection and purification of enzyme from the reaction system for re-use of enzyme, ii) elimination of unreacted substrates to achieve high purity of SFAE.

5.4.1 Collection and purification

On a laboratory scale, processing of collection of enzyme is relatively straightforward. Following extraction of SFAE with addition of more than 30 times acetone [v/v], the enzyme and unreacted solid sugar are separated from the product solution by filtration through filtration paper for qualitative analysis. The enzyme and unreacted sugar were dried in vacuum for the re-use. However, this method is time-consuming, difficult to scale-up and expensive since the concentrated product surfactant solution, which form a protected layer on the surface of filtration paper, result in the production of a solution difficult to pass through the filtration material. Some of the criteria for the selection of the appropriate methods for

enzyme collection and purification include: i) capability of processing a large amount of SFAE; ii) preservation of enzyme activity; iii) easy scale-up; iv) low cost of operation and; v) final product SFAE that meets the standards as food grade. On the basis of the above criteria and preliminary results, the separation technology of magnetic filtration and selective extraction seems to be promising candidates for use at the industrial scale. Using this magnetic carrier EP 100 not only can highly catalyze the esterification reaction, but also can very easily separate the immobilized enzyme from the reaction medium by a magnetic filter.

5.4.2 Elimination of unreacted sugar

Unreacted sugar can be separated from the reaction mixture by selective extraction which relies on the different solubilities of sugar and SFAE in acetone at mild temperatures (40~50°C). The separation of solid sugar particles and product solution can be accomplished in two ways: by settling under gravity or by using centrifugal separators. Separation under gravity can be carried out in any scale and the process can be handled easily with low specific energy consumption. The separation velocity and the phase purity of a particle-free system were investigated. It was found the separation is sufficient under gravity, but the separation time needed to obtain rather pure phase is quite long (3~5 h). This long process results from the high viscosity of the detergent-rich liquid phase as well as from the low interfacial tensions of this detergent-based extraction system. The procedure by sedimentation is time-consuming, but is not labor-intensive and requires little investment. On the other hand, a centrifugal separator could be also employed to separate extraction solution containing solid sugar particles. There are obvious advantages such as continuous processing, large capacity and high efficiency for separation, however, at expense of high investment.

5.5 Production of SFAE on preparative scale

In this research, SFAE were produced on preparative scale ~200g in a stirred-tank reactor in combinatoin with solvent recovery by membrane pervaporation in a batch manner (Fig. 5.5.1).

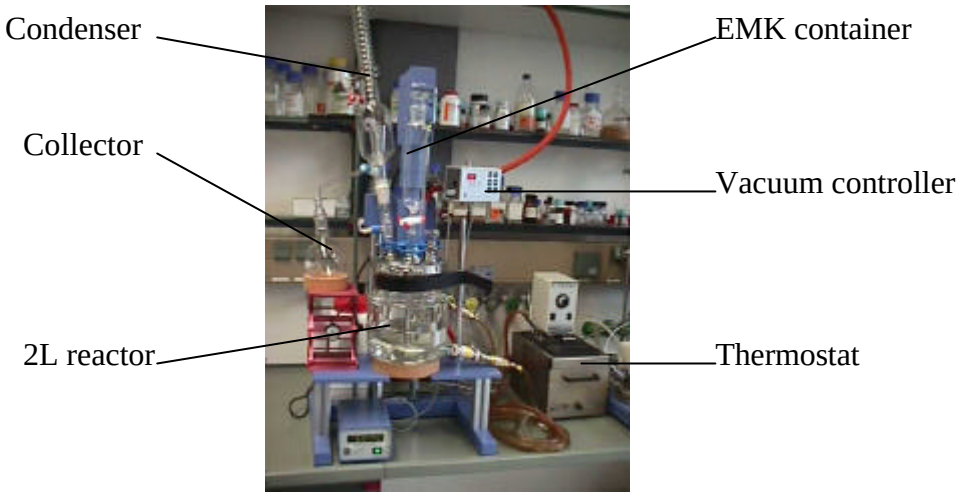


Figure 5.5.1 Experimental apparatus for preparative synthesis of SFAE.

Table 5.5.1. Reaction conditions and yields in the production of SFAE on preparative scale.

D-glucose [g]	**Fatty acids [g]**	**Lipase L-2 [g]**	**EMK Flow rate [ml/min]**	**Temp. [°C]**	**Time [h]**	**Yield [%]**
90	C_{18} 142	20	0.7	60	48	79.2
180	C_{18} 284	40	1.1	60	48	74.2
90	C_{16} 128	20	0.7	60	48	61.8
90	C_{16} 128	20	0.7	60	48	62.5
180	C_8 116	40	1.0	60	48	24.4
180	C_8 116	40	1.0	50	48	31.8

From previous studies on the synthesis of SFAE (Cao et al. 1997; Otto et al. 1998; Yan et al. 1999; Yan et al. 1999; Yan et al. 2000) in a solid phase system, it was already known that the fatty acid chain length had a considerable influence on product formation. Here, a related pattern was also observed on preparative synthesis of SFAE using fatty acids with a chain length of C_8, C_{16}, C_{18} (Table 5.5.1). At an equimolar of fatty acid to glucose, highest yields of monoester (79%) were achieved using stearic acid, whereas for caprylic acid the highest yield of monoester was only ~32%.

6. Lipase-catalyzed synthesis of uncommon SFAE

6. 1 Lipase-catalyzed synthesis of vitamin C fatty acid esters

6.1.1 Introduction

Antioxidants are widely used in foods and cosmetics to prevent oxidation of lipids. Synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), are widely used as food additives, but their application has been reassessed because of possible toxicity or carcinogenic components formed during their degradation (Kaitaranta 1992). Much research has been done to develop safe and natural antioxidants in the last decade due to the tendency of consumer to prefer “natural” products (Bashir et al. 1995; Guyot et al. 1997; Hubbs 1997; Buisman et al. 1998; Bülter and Elling 2000).

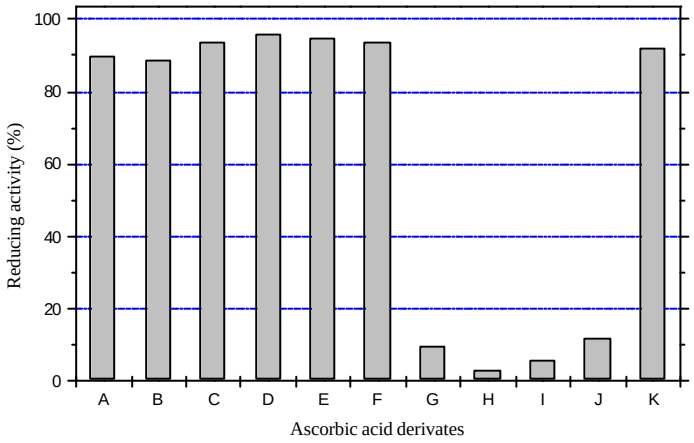


Figure 6.1.1.1 Reducing activity of vitamin C and its deviates [adapted from (Nostro 1997)].

A: ascorbic acid, B: 6-*O*-octanoyl-ascorbic acid, C: 6-*O*-dodecanoyl-ascorbic acid, D: 6-*O*-tetradecanoyl-ascorbic acid, E: 6-*O*-octadecanoyl-ascorbic acid, F: 6-*O*-dodecanedioyl-ascorbic acid, G: 6-*O*-docosanedioyl-ascorbic acid, H: 6-*O*-thapsoyl-ascorbic acid, I: 6-*O*-suberoyl-ascorbic acid, J: 6-*O*-adipoyl-ascorbic acid, K: 5,6-*O*-octylidene-ascorbic acid.

L-ascorbic acid (Vitamin C), one of the most potent dietary antioxidants, provides optimal nutritional support to all physiological functions, including notably, vascular and capillary integrity of the circulatory system. Vitamin C has a very efficient antioxidant activity in aqueous media. It has been recognized to be safe and used as additives in food and food manufacture for centuries. However, its highly hydrophilic behavior prevents its application in cosmetics or other products which contain fats and oils. However, this can be circumvented by using fatty acid esters of vitamin C, which, due to an amphiphilic structure, not has good

solubility and miscibility in a hydrophobic environment, but also seems to enhance the radical scavenging performances compared to its free counterpart (Fig. 6.1.1.1) (Liu et al. 1996). Recent studies indicate that lipophilic vitamin C esters are much more effective in the prevention of low density lipoprotein peroxidation (Liu et al. 1992; Liu et al. 1996). At present, 6-*O*-palmitoyl ascorbic acid is produced commercially by chemical means. This is encountered with a number of disadvantages, which reside in the use of less biocompatible chemicals and solvents, the formation of by-products due to the instability of vitamin C and, hence, low yields (Chen et al. 1994).

Enzymatic processes offer an alternative way to these antioxidants. Although the lipase-catalyzed synthesis of vitamin C esters has been already described (Humeau et al. 1995; Humeau et al. 1998; Grüning and Hills 1999), it is so far hampered by long reaction times or the use of solvents, which are not permitted for the manufacture of food related products. For instance, Humeau *et al.* (Humeau et al. 1998; Humeau et al. 1998) achieved approximately 40% conversion of vitamin C to 6-*O*-ascorbyl palmitate after 5 h when using a 9-fold excess of palmitic acid methyl ester in TBA. No significant increase in conversion was observed after prolonged reaction times and no attempt for production isolation was reported.

In this work, vitamin C fatty acid esters are synthesized in a mainly solid phase system, in the presence of small amounts of organic solvent (acetone or TBA), catalyzed by immobilized lipase B from *Candida antarctica*, followed by a mild downstream processing to reduce the formation of undesired by-products. To increase the reaction rate and shift the equilibrium toward product synthesis, activated esters – i.e. fatty acid vinyl esters - are routinely employed (Fig. 6.1.1.2). This takes advantages of the tautomerization of the vinyl alcohol generated during the transesterification to the highly volatile acetaldehyde. Thus, the undesired back reaction is suppressed (Lobell and Schneider 1994; Bornscheuer and Yamane 1995; Shibatani et al. 1997). As vitamin C and its esters are very sensitive to oxidation, a mild extraction method for the isolation of reaction products was also developed.

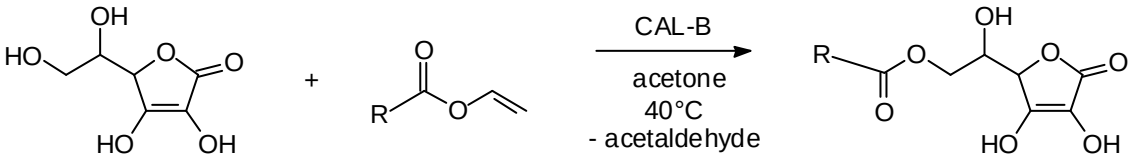


Figure 6.1.1.2 Reaction scheme for the lipase-catalyzed synthesis of vitamin C fatty acid esters (R = C_7-C_{15})

6.1.2 Results and discussion

6.1.2.1 Influence of fatty acid chain length

From previous studies on the lipase-catalyzed synthesis of SFAE in a solid phase system, it was already known that the fatty acid chain length had a considerable influence on product formation. This was mainly due to the higher solubility of medium chain fatty acids in the reaction system, thus suppressing the precipitation/crystallization of product, which is required to achieve high conversions. A related pattern was observed in the synthesis of vitamin C esters using fatty acid vinyl esters ranging from C_8-C_{16} (Figure 6.1.2.1.1). At a fatty acid: vitamin C ratio of 2:1, highest yields (91%) of isolated product were achieved using palmitic acid vinyl esters, whereas for caprylic acid vinyl esters the highest yield was 63%. It is also obvious from Figure 6.1.2.1.1 that in TBA always slightly higher yields can be achieved compared to acetone.

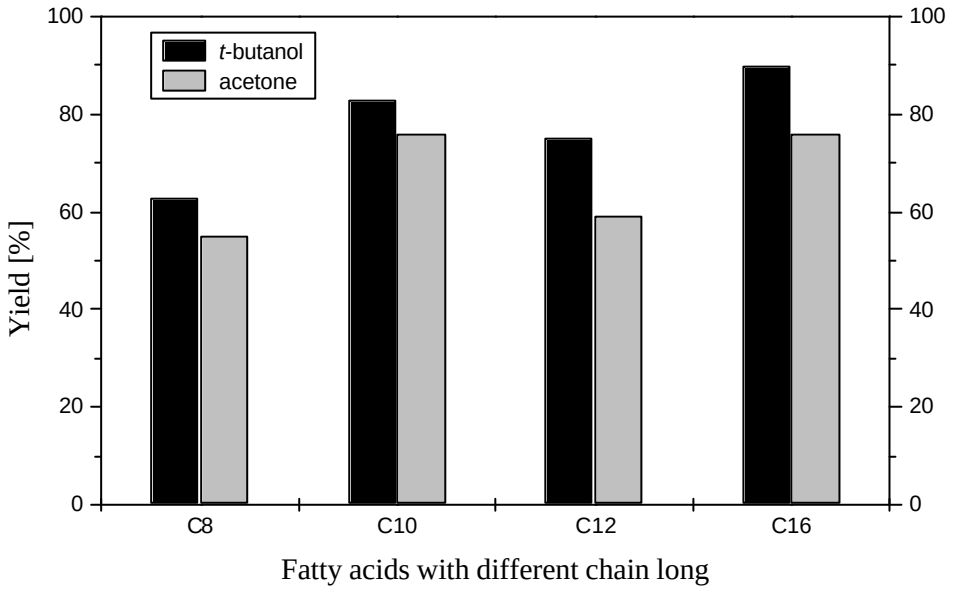


Figure 6.1.2.1.1 Effect of fatty acid chain length and solvent on product yield.

6.1.2.2 Effect of substrate ratio

An equimolar ratio of vitamin C to fatty acid vinyl ester would be sufficient and no excess of the acyl donor is required to achieve a high conversion. However, a considerably higher yield was observed when the ratio of vinyl ester to vitamin C was increased from 1:1 to 3:1 (Figure 6.1.2.2.1~ 4). As to be seen in Figure 6.1.2.2.1~4 the yield of 6-*O*-palmitoyl-L-ascorbic acid, 6-*O*-lauroyl-L-ascorbic acid, 6-*O*-decanoyl-L-ascorbic acid and 6-*O*-octanoyl-L-ascorbic acid

in acetone increased from 60 to 86%, 38 to 62%, 40 to 61%, 39 to 60% respectively. It was also observed from Figure 6.1.2.2.1 to 6.1.2.2.4, that slightly higher product yields in TBA can be obtained compared to acetone.

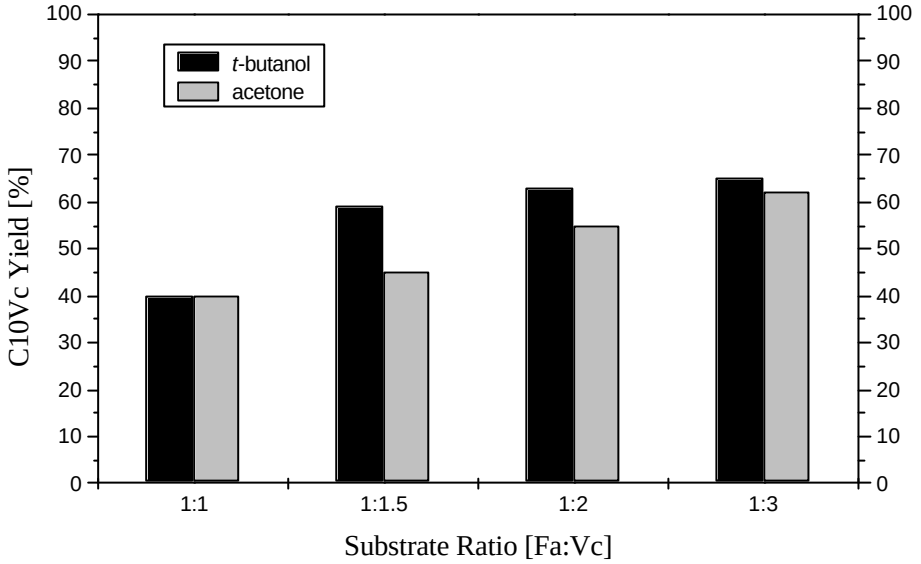


Figure 6.1.2.2.1 Influence of substrate ratio and solvent on 6-*O*-octanoyl-L-ascorbic acid yield.

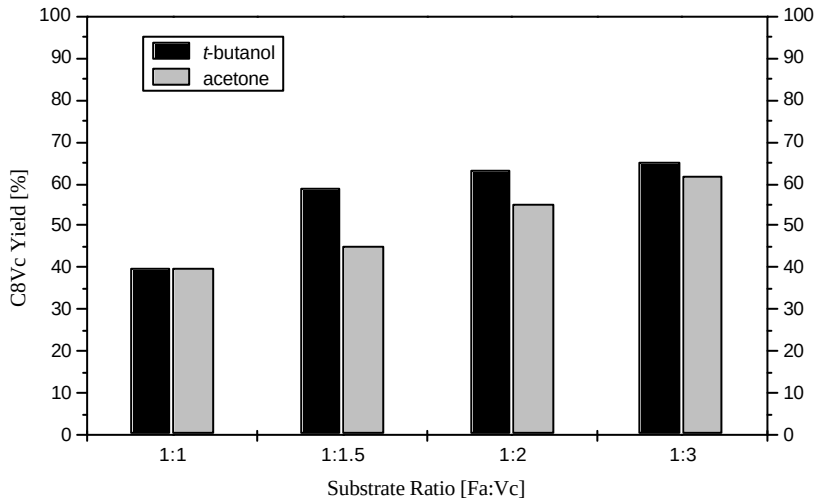


Figure 6.1.2.2.2 Influence of substrate ratio and solvent on 6-*O*-decanoyl-L-ascorbic acid yield.

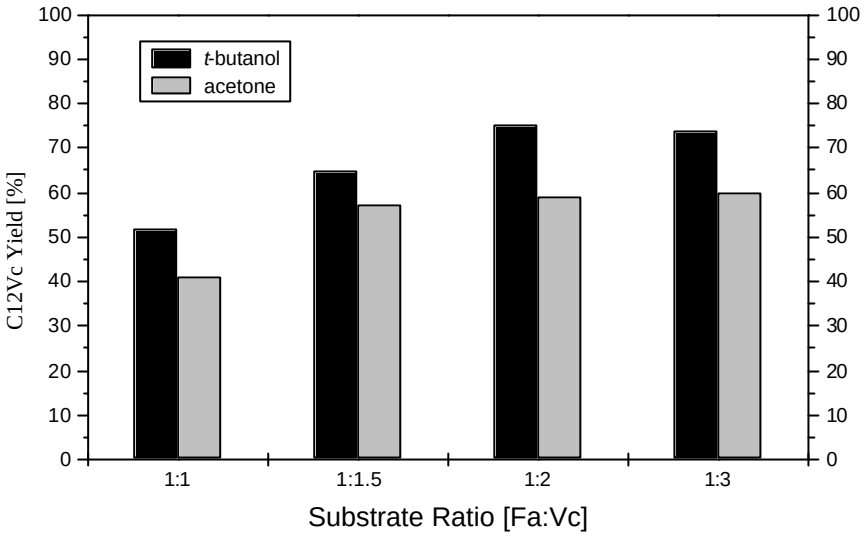


Figure 6.1.2.2.3 Influence of substrate ratio and solvent on 6-*O*-lauroyl-L-ascorbic acid yield.

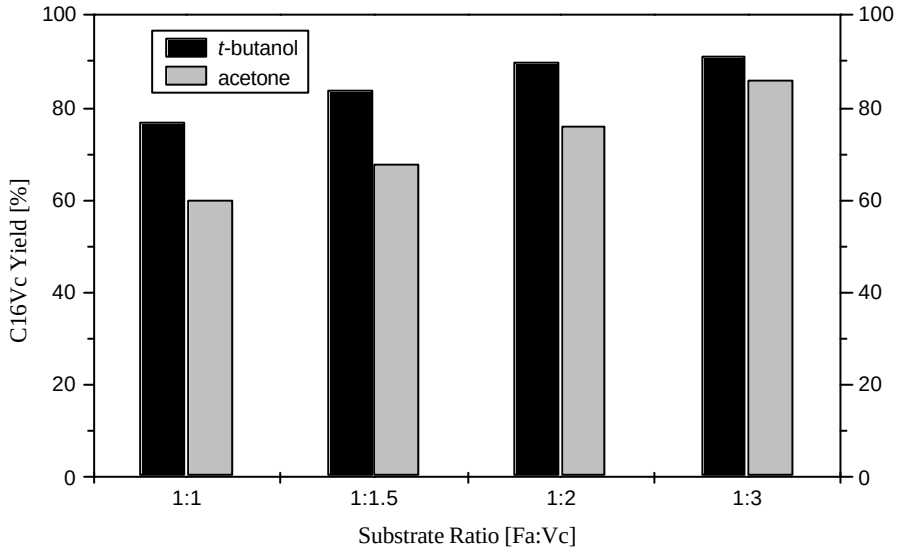


Figure 6.1.2.2.4 Influence of substrate ratio and solvent on 6-*O*-palmitoyl-L-ascorbic acid yield.

6.1.2.3 Product isolation

Vitamin C is very sensitive and easily undergoes oxidation, degradation and rearrangements. Even its esterified counterparts have to be handled carefully to avoid formation of undesired by-products. Initially, it was attempted to purify the vitamin C esters from the crude reaction mixtures by common laboratory-scale methods like silica gel chromatography. However, it turned out that considerable losses of product occurred (data not shown). It was therefore focussed on a milder downstream procedure for the separation of vitamin C esters from unreacted substrates and which should also be applicable on a large scale (Scheme 6.1.2.3.1). In addition, only those solvents should be used, which are biocompatible with a later application of the vitamin C esters in food related products or cosmetics.

Best results were achieved, when the crude reaction mixtures was extracted with water, which not only removes unreacted vitamin C, but also degrades excess vinyl esters to the free fatty acids. Extraction with *n*-hexane then allowed separation of free fatty acids from product, which remained insoluble in this solvent. This procedure enabled the isolation of all vitamin C esters in excellent purity (>98%) and high yields (Figure 6.1.2.2.1~ 4). NMR spectroscopy revealed that acylation occurred exclusively at the primary hydroxyl group as shown in Figure 6.1.1.2. It should be emphasized, that the separation of vitamin C esters from remaining fatty acid methyl esters (Humeau et al. 1995; Humeau et al. 1998; Humeau et al. 1998) is not practical by simple extraction or separation and would require more laborious procedures, which might also affect the stability of the vitamin C esters.

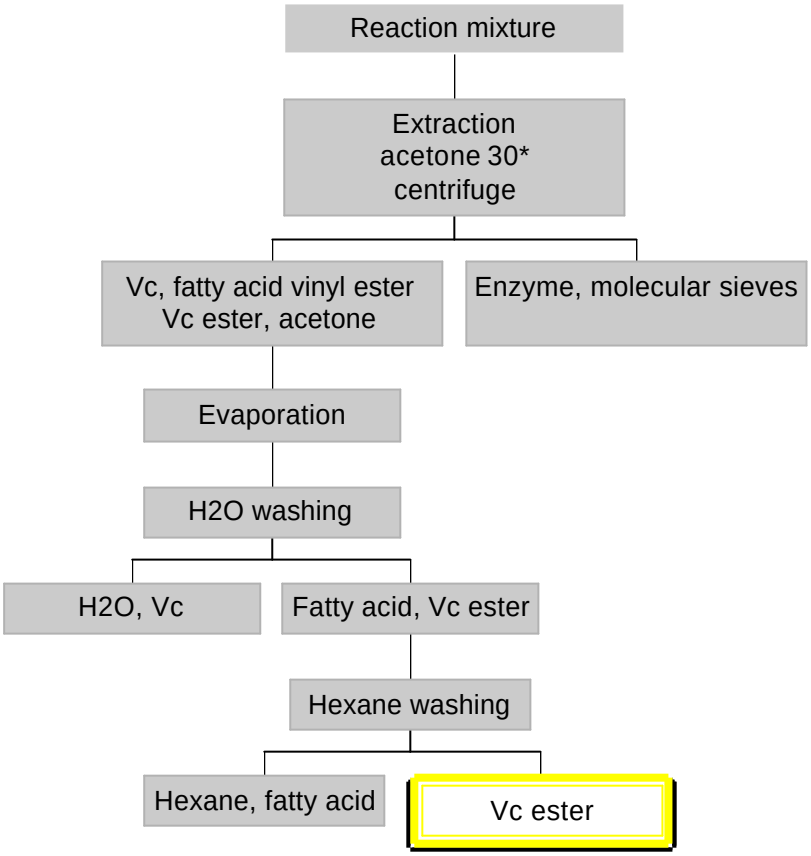
Although this method employs the more expensive (commercially available) vinyl esters compared to the use of fatty acid methyl esters, it is clearly superior to already published procedures for several reasons: i) the vitamin C esters are isolated in high yield and purity after reasonable reaction times; ii) equimolar amounts (or only a moderate excess of vinyl esters) of substrates can be used; iii) a mild product isolation is feasible and; iv) the procedure employs acetone which is easy to be eliminated and recognized as safe for use in the manufacture of food and food additives by European Community directives.

6. 2 Lipase-catalyzed synthesis of arylaliphatic glycolipids

6.2.1 Introduction

Aromatic glycolipids are of great interest in medical and pharmaceutical field. The glucose esters of unsaturated aryaliphatic acids like cinnamic acid or its hydroxylated derivatives were applied in tumor treatment (Kimura et al. 1987; Liu et al. 1992). Furthermore, alkyl and phenolic glucosides (e.g. salicin) are known for their antimicrobial as well as antiviral and

antiinflammatory activities (Rekker and Kort 1979; Nonaka et al. 1981; Nonaka and Nishioka 1983; Nihro et al. 1992). Combining both aromatic



Scheme 6.1.2.3.1 General purification scheme of vitamin C fatty acid esters.

glucose esters and phenolic glucosides led to the formation of novel aromatic arylglucoside esters possessing putative pharmaceutical effects. These kinds of glycolipids were usually obtained by solvent extraction from plants like certain *Prunus* spec., *Rheum*. Spec. and from the finally *Salicaceae*. Some of these compounds can also be obtained by biotransformations using plant cell suspension cultures of *Glycyrrhza echinata* and *Aconitum japonicum* (Kashiwada et al. 1984; Ushiyama et al. 1989) as well as by co-factor-dependent enzymatic synthesis (Corner *et al.*, 1965). The latter appears to be low yields with high undesired side-reactions and difficult product isolation. Recently, Prof. Schmid's group has reported a method for the synthesis of simple esters derived from glucose and aliphatic fatty acids (Cao et al. 1996), in which lipase B from *Candida antarctica* (CAL-B) was used as biocatalyst. CAL-B is a very versatile lipase which is catalytically active even in polar solvents such as TBA or acetone and allowed access to a wide range of aromatic glycolipids with widespread

properties. In this work, a process for the synthesis of SFAE based on phenolic glucosides or unsaturated arylaliphatic acids, which are known to display anticancer activity, was described.

6.2.2 Lipase-catalyzed synthesis of salicin fatty acid esters

Salicin fatty acid esters were synthesized by lipase B from *Candida antarctica* in a mainly solid phase system (Figure 6.2.2.1), where the acylation of a solid sugar derivative-- natural occurring drug salicin-- with fatty acids was performed in the presence of a very small amount of organic solvents (EMK). Water generated during the esterification formed an azeotrope with EMK and was eliminated from the reaction medium by azeotropic distillation. D-(-) salicin and non-activated fatty acids with different chain length (C_8, C_{10}, C_{14}, C_{16}, C_{18}) were used as the reactants.

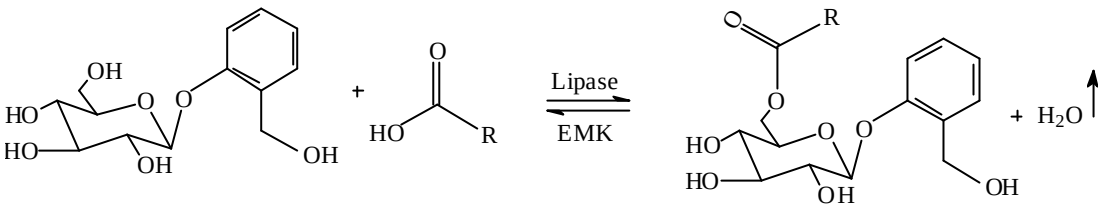


Figure 6.2.2.1 Reaction scheme for the lipase-catalyzed synthesis of salicin fatty acid esters (R= C_7, C_9, C_{13}, C_{15}, C_{17})

6.2.2.1 Results and discussion

6.2.2.1.1 Influence of fatty acid chain length on monoester yields

As indicated in the previous chapters, long chain fatty acids as acyl donors normally favored the solidification of the reaction mixture and gave also high conversion (Cao et al. 1996; Otto et al. 1998; Otto et al. 1998; Otto et al. 1998; Otto et al. 1999) (Yan et al. 1999; Yan et al. 1999; Yan et al. 2000; Yan et al. 2000; Yan et al. 2000; Yan et al. 2000). The influence of the fatty acids on the synthesis of salicin esters was investigated (Figure 6.2.2.1.1.1). As acyl donor, fatty acids with different chain length were used in the presence of EMK at a molar ratio of fatty acid:salicin of 1:1. The highest yield of 6-*O*-stearoyl-D(-) salicin (67%) was achieved by the use of long chain fatty acid--stearic acid. Using palmitic acid the salicin monoester yield of 53% after 48 h reaction time at 40°C was obtained. No salicin diester was detected and solidification was observed after 24 h by the use of long chain fatty acids such as stearic acid and palmitic acid as acyl donors. With myristic acid the mixture solidified after about 36 h. In contrast, with short chain fatty acids such as caprylic acid and capric acid no solidification of the reaction mixtures was observed. A rational explanation for this phenomenon is as follows: the different solubilities of the salicin esters of fatty acids with

different chain lengths in reaction medium. The salicin fatty acid ester formed during the reaction will crystallize from the liquid phase, if its saturation concentration is reached. Through the crystallization, product inhibition can be avoided and thereby the equilibrium of the solid phase synthesis is favored. Therefore, higher yields of the salicin esters of long chain fatty acids were achieved.

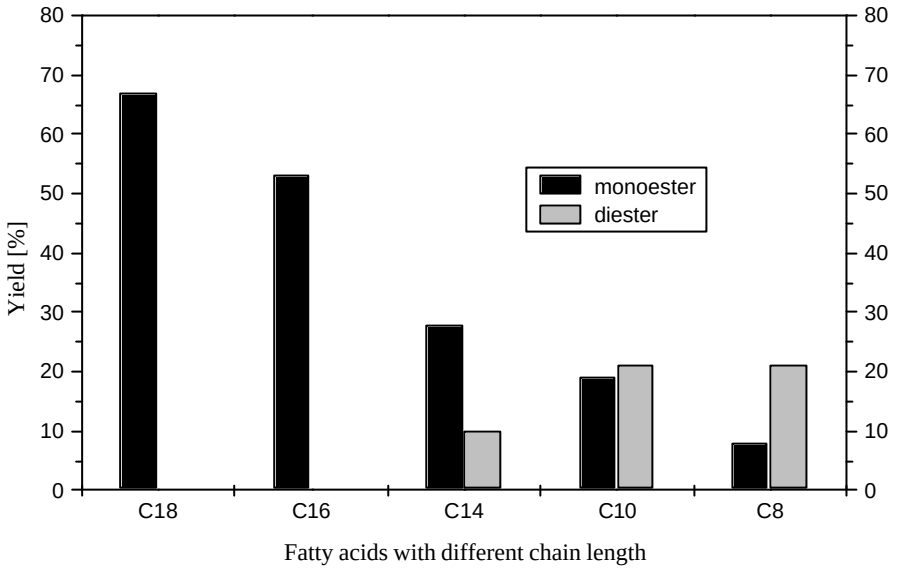


Fig. 6.2.2.1.1.1 Influence of fatty acids chain length on product yield.
Reaction condition: 0.5 mmol salicin, 0.5~1.0 mmol fatty acids, 50 mg CAL-B, 200 rpm, 50 ml EMK, temperature 25-40°C, time 24-48 h.

6.2.2.1.2 Regioselectivity of salicin by using different acyl donors

As shown in Figure 6.2.2.1.1.1, a drastic change of regioselectivity of salicin was found when going from stearic acid to caprylic acid. The yields of diester from 0 increased to 21%. It is known that the solubility of salicin fatty acid esters depends mainly on the chain length of the acyl moiety. Generally, the solubility of salicin fatty acid esters decreased with increasing chain length and thus for short chain fatty acids the possibility of diacylation increases. Furthermore, the salicin monoesters of fatty acids with long chains crystallized easily from the reaction mixture, thus diacylation was not favored. According to the model of Kazlaukas & Bornscheuer, in a lipase-catalyzed acylation of sugar or sugar derivatives, the primary hydroxyl groups reacts first, followed by the secondary hydroxyl group (Bornscheuer and Kazlauskas 1999). Indeed, ^{1}H-NMR and ^{13}C-NMR spectra gave evidence that the acylation

proceeded first at the primary hydroxyl group of glucoside and then at the primary hydroxyl group at the aromatic ring of D-(-)-salicin.

6.2.3 Lipase-catalyzed synthesis of cinnamic acid glucose ester

CAL-B consists of the common structural framework such as α/β-hydrolyze fold and the catalytic triad in the active site (Ollis et al. 1992). It allows access to a wide range of aromatic glycolipids (Otto et al. 1998; Otto et al. 1998; Otto et al. 1998; Otto et al. 1999). Since the cinnamic acid glucose has antiinflammatory and anticancer activities it was expected to synthesize it by CAL-B catalyzed esterification (Figure 6.2.3.1), which was based on a solid phase system.

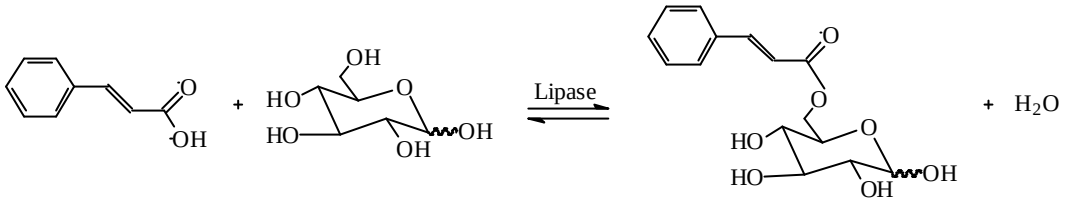


Fig. 6.2.3.1 Reaction scheme for the lipase-catalyzed synthesis of glucose cinnamic acid ester.

6.2.3.1 Results and discussion

6.2.3.1.1 Solvent choice

Enzyme-catalyzed reactions in organic solvents rather than in aqueous media have made it possible to alter the enzyme properties. It has been shown that organic solvent may have a profound effect on the solubilization of substrates and products as well as the activity of enzymes (Rubio et al. 1991; Yang and Robb 1994). In the solid phase synthesis system, the solvent selection is crucial to achieve a high product yield. For this reason, several organic solvents were first examined for the synthesis of glucose cinnamic acid ester. It was found that only very few solvents could be used in the esterification of the glucose with cinnamic acid (Table 6.2.3.1.1.1).

In these experiments, it was found that a relatively high conversion (43%) could be achieved in acetone and no solidification was observed, while a relatively low conversion (24%) was obtained in cyclohexane and fast solidification occurred. This phenomenon let us investigate the possibility for the synthesis of glucose cinnamic acid ester in two-solvents mixture of acetone and cyclohexane. A series of experiments for the synthesis of glucose cinnamic acid ester at different ratio of acetone to cyclehexane were carried out, in order to maximize conversion and productivity (Tab. 6.2.3.1.2). Best result (70% conversion) was achieved at

ratio of acetone to cyclohexane of 2:1 [v/v], substrate ratio of 1:2 [glucose:cinnamic acid, mol/mol] at 55°C for 7 days.

Tab. 6.2.3.1.1 Screening of solvents for the synthesis of cinnamic acid glucose ester.

Solvents	Product*	Solvents	Product*
dioxane	+	trichlormethane	-
acetonitrile	-	diethyl ether	+
acetone	+++++	*n*-hexane	+
2,2,4-trimethylpentane / *iso*-octane	-	cyclohexane	+++
tetrahydrofuran	+	hexane	+
dichloromethane	-	*iso*-hexane	+
TBA	++++	hetpane	+
EMK	+++	n-pentane	-

Reaction conditions: 0.5 mmol cinnamic acid, 0.5 mmol glucose, 50 mg L-2, 100 mg molecular sieves, 0.6 ml solvents, reaction temperature 55°C, at 500 rpm and reaction time 7 days. *Product detected by TLC.

Tab. 6.2.3.1.2 Relationship between ratio of cyclohexane to acetone and conversion.

Ratio of cyclohexane to acetone [v/v]	Conversion* [%]
4:1	26
3:1	34
2:1	43
1:1	55
1:2	70
1:3	62
1:4	50

Reaction conditions: 0.5 mmol cinnamic acid, 0.25 mmol glucose, 50 mg L-2, 100 mg molecular sieves, 0.6 ml solvents, reaction temperature 55°C, at 500 rpm and reaction time 7 days. *conversion of glucose.

6.3 Lipase-catalyzed synthesis of amino-sugar derivatives

6.3.1 Introduction

Esters of alkyl glycosylamines are already known as a new generation of nonionic surfactants with interesting technological and economical properties. They have excellent detergent and lathering properties and are easy to be rinsed, and not irritating. They even diminish the irritation of the skin and the mucous membrane of the eye caused by other surfactants used in combination with them. In addition, they have a low environmental impact because they are biodegradable.

At present, fatty acid glucamide are manufactured by chemical method at high temperature employing inorganic alkaline catalyses. Besides high energy consume and low productivity, the formation of chloride salts by neutralization is the major disadvantage of these chemical approach since they may lead to production of nitrosamine and cause some concern of the safety of their use in human consumption. Enzymatic processes offer an alternative way to these surfactants. In fact, the enzymes which are highly regioselective and enantioselective may be employed for selective acylation of amino-sugar molecules (Zaks and Klibanov 1985; Oosterom et al. 1996; Valivety et al. 1997; Clapés et al. 1999). Because of the different solubilities of amino-sugars and fatty acids in organic solvents, it is not easy to mix sugars and fatty acids in the reaction medium. In the previous work a method for the synthesis of SFAE was reported, which was based on a mainly solid phase system. The acylation of a solid sugar (glucose) with a fatty acid was performed *via* lipase-catalysis in the presence of a small amount of organic solvents (Cao et al. 1996) (Yan et al. 1999; Yan et al. 1999; Yan et al. 2000; Yan et al. 2000; Yan et al. 2000; Yan et al. 2000), high yields and regioselectivities were obtained. Here, it was expected to report that lipases not only accept glucose as acyl acceptor, but also allow the efficient synthesis of amino-sugar fatty acid esters.

6.3.2 Result and discussion

N-methyl-glucamine contains the amine and five hydroxyl functions (one primary hydroxyl group) that can react with fatty acid. Because of the high activity of lipase for primary hydroxyl groups and amines, these two groups can be acylated to form either the amide compound or the N-methyl-glucamine monoester. First the lipase-catalyzed esterification with equimolar N-methyl-glucamine to palmitic acid in the presence of a small amount of TBA at 40°C was investigated. Under these conditions, only 45% palmitic acid was consumed after

72 h to yield a product mixture containing 65% palmitoyl-N-methyl-glucamide and 35% 6-*O*-palmitoyl-N-methyl-glucamine. In order to selectively synthesize the targeted N-acylation of N-methyl-glucamine, it is necessary to optimize the reaction condition such as substrate ratio, reaction temperature and reaction time.

6.3.2.1 Influence of the substrate ratio

Investigating different ratios of palmitic acid and N-methyl-glucamine, it was found that an increase in the fatty acid concentration led to a considerable increase in productivity of 6-*O*-palmitoyl-N-methyl-glucamine and amine conversion (Tab. 6.3.2.1.1). In contrary, amidification was preponderant when the molar ratio of palmitic acid to amine was lower than 1. In order to improve the efficiency of synthesis, the reaction was carried out at 60°C, with a molar ratio of palmitic acid to amine of 1:4. The highest conversion of palmitic acid (95%) was achieved and only palmitoyl-N-methyl-glucamide was detected after 48 h.

6.3.2.2 Influence of fatty acids chain length

N-methyl-glucamine was acylated with various fatty acids under the optimal conditions. Results are described in Table 6.3.2.2.1. All other fatty acids had a lower reactivity than palmitic acid. In the case of caprylic acid, a new product was obtained in 7% yield, which was confirmed as diacylated amide-ester 6-*O*, N-dioctanoyl-N-methyl-glucamide.

Table 6.3.2.1.1 Influence of substrate ratio on selective acylation.

Palmitic acid/amine [mol/mol]	**Amine conversion [%]**	**Amidification [%]**
4:1	100	5
2:1	70	25
1:1	45	65
1:2	30	88
1:4	22	100

Reaction conditions: 0.25~1 mmol N-methyl-glucamine, 0.25~1 mmol palmitic acid, 80 mg L-2, 200 mg molecular sieves, 0.6 ml TBA, 40°C, at 350 rpm and reaction time 72 h.

Table 6.3.2.2.1 Acylation of N-methyl-glucamine with various fatty acids.

Acyl donor	Conversion* [%]	Compounds
Stearic acid	93	Stearoyl-N-methyl-glucamide
Palmitic acid	95	Palmitoyl-N-methyl-glucamide
Lauric acid	54	Lauroyl-N-methyl-glucamide
Caprylic acid	46	Octanoyl-N-methyl-glucamide, 6-*O*, N-dioctanoyl-N-methyl-glucamide

Reaction conditions: 1 mmol N-methyl glucamin, 0.25 mmol fatty acids, 2 ml TBA, 200 mg lipase from *Candida antarctica* B on EP 100, 1 g molecular sieves, 60 °C, 250 rpm, 24 h.
* conversion of fatty acid.

7. Materials and methods

7.1 Chemicals

7.1.1 Sugars and sugar derivatives

Table 7.1.1 Characteristic parameters of sugars and sugar derivatives used.

Sugar/sugar derivatives	Melting point (°C)	Supplier
b -D(+)-Glucose	150	Sigma
D(+)-Glucose	146	Fluka
D(+)-Sucrose	169-170	Fluka
L-ascorbic acid (vitamin C)	160-165	Fluka
D(+)-Trehalose	not available	Fluka
D(+)-Lactose*H_2O	not available	Fluka

7.1.2 Fatty acids and other acyl donors

Table 7.1.2 Acyl donors used.

Fatty acid or acyl donor	Supplier
Oleic acid	Sigma
Stearic acid	Sigma
Palmitic acid	Fluka, Henkel
Myristic acid	Sigma, Henkel
Lauric acid	Sigma
Capric acid	Fluka, Henkel
Caprylic acid	Fluka, Henkel
Caproic acid	Henkel
Stearic acid methyl ester	Fluka
Palmitic acid methyl ester	Fluka
Palmitic acid vinyl ester	Tokyo Kasei Kogyo Co. Ltd.
Lauric acid vinyl ester	Tokyo Kasei Kogyo Co. Ltd.
Capric acid vinyl ester	Tokyo Kasei Kogyo Co. Ltd.
Caprylic acid vinyl ester	Tokyo Kasei Kogyo Co. Ltd.
Tripalmitin	Fluka

7.1.3 Organic solvents

The organic solvent used in enzymatic acylation of sugar were dried over activated molecular sieve. The solvents used for HPLC were filtered over a nitrocellulose filter and then sonicated in a water bath for 15 minutes.

Table 7.1.3 Characteristic parameters of the organic solvents used in this work.

Organic solvent	**Log P**	**Supplier**
Ethyl methylketone	0.29	Fluka
Dimethylformamide	1.0	Fluka
DMSO	-1.3.	Fluka
Dioxane	-1.1	Fluka
Acetonitrile	-0.36	Riedel-de Haën
Acetone	-0.30	Riedel-de Haën
γ-Butyrolactone	not available	Fluka
Tetrahydrofuran	0.49	Riedel-de Haën
Dichloromethane	0.64	Riedel-de Haën
t-Butanol	0.83	Fluka
t-Butylmethylether	1.6	Fluka
t-Amylalcohol	1.4	Fluka
3-Methyl-3-pentanol	1.5	Fluka
Ethylacetate	0.68	Riedel-de Haën
Triethylene glycol dimethyl ether	-1.74	Fluka
Ethylene glycol	-1.93	Fluka
Methanol	-0.76	Riedel-de Haën
Chloroform	2.0	Riedel-de Haën
Acetic acid	-2.3.	Fluka
Water	not available	laboratory
1-Butanol	0.8	Fluka
Diethylether	0.85	Riedel-de Haën
Hexane	3.5	Riedel-de Haën

7.1.4 Miscellaneous chemicals

Table 7.1.4 Other chemicals used in this work.

Substance	**Supplier**
LiCl	Fluka
$KC_2H_3O_2$	Fluka
$MgCb_2$*$6H_2O$	Fluka
$FeSO_4$	Fluka
Tris (2-amino-2-hydroxymethyl-1,3-propandiol)	Fluka
H_2SO_4	Fluka
HCl	Fluka
K_2CO_3	Fluka
NaOH	Fluka
$Mg(NO_3)_2$*$6H_2O$	Fluka
Triton X-100	Fluka
NaCl	Fluka
KCl	Fluka
K_2SO_4	Fluka
Span 60	Fluka
PEG 2000 monomethyl ether	Fluka
Molecular sieve	Fluka
Duolite 568	Novo
Polypropylene EP 100	Akzo Nobel
Ambelite XAD-7	Sigma
Celite®545	Fluka
Hyflo Super Cel®	Fluka
TLC aluminium sheet silica gel 60 F_{254}	Merk
Silica gel 60 (70-230 mesh)	Fluka

7.2 Equipment

Hydrolytic activity

Dosimat 665,	Metrohm, Switzerland
Impulsomat 614,	Metrohm, Switzerland
pH-Meter 632	Metrohm, Switzerland
Stirrer	Metrohm, Switzerland
Recorder	Linseis, Germany
Incubator	Metrohm, Switzerland
Thermostat chamber	Lauda, Germany

Karl-Fisher-Titration

Dosimat 665	Metrohm, Switzerland
KF-Titrino 701	Metrohm, Switzerland
Stirrer Tri Stand 703	Metrohm, Switzerland

Thin-layer chromatography (TLC)

Silica gel plate 60 F_{254}	Merk, Germany
Development chamber	Migge GmbH, Germany

High performance liquid chromatography (HPLC)

HPLC-equipment	Sykam, Switzerland
Column (Nucleosil C_{18} 5µm, 250 x 4 mm)	Sykam, Switzerland
RI detector (ERC-7512)	
HPLC controller S 2000	Sykam, Switzerland
Low pressure gradient mixer S 8110	Sykam, Switzerland
Integrator C-R5A Chromatopac	Shimazu Europa GmbH, Germany
Solvent delivery system S 1100	Sykam, Switzerland

Thermomixer	Eppendorf GmbH/Germany
Tensiometer	Lauda Tensiometer TD1, Lauda-Königshofen, Germany

Rotation vapor	Rotavapor R-134, Büchi, Switzerland
Melting point	
Equipment	Schmelzpunktbestimmungs-apparat, Glasapparatfabrik Hawel, Switzerland
Sonication	
Sonorex super RK 514H	Bandelin Electronic, Berlin/Germany
Dessicator	Migge GmbH, Leonberg/Germany
Flash liquid column chromatography	
Silica gel 60 (70-230 mesh)	Fluka, Buchs/Switzerland
Column	Migge GmbH, Leonberg/Germany
Experimental apparatus	
Soxhlet extractor 30 ml	Migge GmbH, Leonberg/Germany
Membrane reactor with area 23 cm^2	Jülich Forschung GmbH/Germany
Membrane Pervap® 2200	Sulzer Chemtech GmbH/Germany

7.3 Screening of lipases

Screening of immobilized lipases was performed according to the following procedure: 1 mmol palmitic acid and 1 mmol *b*-D(+)-glucose were mixed together in the presence of 0.7 ml t-butanol. The reaction was started by addition of 40 mg immobilized lipases as listed in Table 7.3.1.1. To remove the water produced in the reaction, 200 mg molecular sieve was added. The reaction mixture was stirred with a magnetic bar and thermostated to 60°C. After 24 hours the reactions were stopped and the mixture was extracted with 5 ml tetrahydrofuran at 60°C. 0.1 ml THF solution was sucked into a round bottom flask and the organic solvent was evaporated under vacuum. The residue was dissolved in 1 ml organic solvent (methanol:acetonitrile = 70:25) and analyzed by HPLC.

Table 7.3.1.1 Use of different preparation of immobilized lipases from *Candida antarctica*.

Trade name	**Support**
SP 435	Acrylic resin
CAL-B-SP525	EP 100
CAL-B	EP 100
Chirazyme® L-2, Lyo., BM	Magnetic PEG
Chirazyme® L-2	
CAL-B	Activated silica gel
CAL-B	Activated PEG
CAL-B	Celite
CAL-B	Magnetic PEG
CAL-B	EP 100

*PEG modified CAL-B

7.3.1 Determination of hydrolytic activity of lipases

Enzyme activity (lipase hydrolytic activity) was determined with a pH-stat (718 STAT Titrino, Metrohm, Schwitzerland). A certain amount of immobilized lipase (10 mg) was added to 20 ml aqueous solution (thermostated at 60°C) containing 2% (w/w) gum arabicum 5% (w/w) olive oil and 470 µl 22% (w/w) $CaCl_2$. This solution was automatically titrated with NaOH solution (0.1 M) to the desired pH before lipase was added. The consumption of NaOH was recorded on a recorder as a function of time. The lipase activity is then expressed in Units (U). One unit corresponds to the liberation of 1 µmol fatty acid per minute.

7.3.2 Determination of synthetic activity of immobilized lipases

20 mg immobilized lipase was added to 2 ml ethyl methylketone or a mixture of ethyl methylketone and hexane, containing 45 mg *b*-D(+)-glucose, 64 mg palmitic acid and 50 mg molecular sieve. The mixture was shaken (12000 rpm) and incubated on a thermoblock at 75°C or 59°C for 2 h. Samples were taken at intervals (20 minutes), centrifuged (14000 rpm, 4 min.) and the supernatants were analyzed by HPLC. The initial reaction rate was taken as the synthetic activity of immobilized lipases.

7.4 Determination of solubility, solubilisation rate and reaction rate

7.4.1 Determination of solubility of sugars

10 mg solid sugar was added to 1 ml organic solvent in a 2 ml Eppendorf tube until saturated solutions were obtained. After incubation at a given temperature for 24 hours on a thermomixer and centrifugation (14000 rpm, 4 min.), the concentration of the sugar was analyzed from the supernatant by HPLC.

7.4.2 Determination of solubility of SFAE

Pure solid SFAE was slowly added to 1 ml organic solvent and incubated on a thermomixer at a specified temperature until a suspension was obtained. After centrifugation (14000 rpm, 4 min.), the concentration of sugar fatty acid esters in the supernatant was determined by HPLC-analysis.

7.4.3 Determination of solubilisation rate

1 g solid sugar powder was added to 200 ml organic solvent in a capped glass flask. The suspension was incubated in an oil bath, thermostated at a specified temperature. 1 ml of the suspension was taken at intervals, then centrifuged at 14000 rpm for 30 seconds and the supernatant was analyzed by HPLC.

7.4.4 Determination of reaction rate in a solution saturated with glucose

In order to ensure that the reaction takes place at a saturated glucose concentration, 5 mg glucose, was added to a mixture consisting of 1 mmol palmitic acid and 0.7 ml ethyl methylketone. The reaction was started by addition of 10 mg immobilized lipase CAL-B-EP 100 and 50 mg molecular sieve (3Å, 8 mesh), and stopped by separating the enzyme from the reaction mixture by centrifugation (14000 rpm, 4 min.). The supernatant was analyzed by HPLC. The same reaction was repeated several times under variation of the reaction times.

7.5 Determination of saturation concentrations of SFAE

100 mg 6-*O*-palmitoyl-glucose was added to 500 µl solvent (in a 2 ml Eppendorf). and mixed with a thermomixer at 25 °C for 2 hours. After centrifugation (14000 rpm, 10 minutes), the supernatant was discarded. The residue was weighted. The adsorbed adjuvant was estimated by controlling the weight. The adjuvant concentration was expressed as a percentage of adjuvant adsorbed.

7.6 Screening of water binding materials

400 mg water binding material such as $CuSO_4*5H_2O$/ $CuSO_4*3H_2O$, Lactose, Na_2SO_4, and molecular sieve were added to a reaction mixture containing 2 mmol β-D(+)-glucose, 2 mmol octanoic acid, 100 mg CAL-B-SP435 and 1.6 ml TBA. The reaction was stopped after 24 h reaction times. The reaction mixture was extracted with 4 ml THF at 60°C. The conversion was determined by HPLC.

7.7 Enzymatic reactions

7.7.1 General procedures for enzymatic reactions

The reaction mixture consisted of equimolar free glucose and fatty acid (typically 0.5 mmol) and an organic solvent (1-3 weight equivalents of substratees), which was incubated in a two-necked round-bottom flask, placed in an oil bath, thermostated at 25-60°C (depending on the fatty acid chain length) and stirred by a magnetic bar (250 rpm) under the reduced pressure. For small laboratory-scale synthesis, a Soxhlet extractor with molecular sieves (4Å or 5Å 10 mesh, activated at 300°C, 8 h) was used to remove product water or methanol. This extractor was connected at the top to a condenser that was connected to a vacuum controller. The reaction was started by addition of the lipase. The temperature of the reaction was maintained at 25-60°C and the pressure was progressively lowered so that the solvent and azeotrope in the reaction mixture were refluxed. The condensed solvent was dried by passing through molecular sieves before returning to the reaction system. This provides constant removal of the water generated in the reaction, forcing the equilibrium towards synthesis. Samples from the suspension were taken at intervals, centrifuged and the supernatants analyzed by thin layer chromatography (TLC) and HPLC to determine enzymatic conversions. Productivities were calculated from the conversion and expressed as mmol product formed per gram enzyme per hour (mmol/g*h).

7.7.2 Procedure for lipase-catalyzed esterification in a stirred-tank membrane reactor

The reaction mixture consisted of equimolar sugar and fatty acid (usually 0.5 mmol) in a solvent EMK or a mixture of EMK and hexane (1~2 weight equivalents of substrates) as adjuvants. The reaction mixture was incubated in a membrane reactor, placed in a magnetic stirrer and agitated by a magnetic bar (650 rpm). The reaction temperature was kept at 75 or 59°C. At this temperature the azeotrope -- the mixture of EMK and water (b.p. 73.5°C) or the mixture of EMK, hexane and water (b.p. 56°C) produced during esterification -- was vaporized. The gaseous mixture was contacted with the membrane Pervap® 2200. Water

vapor passed through the membrane, leading to break the azeotrope. EMK and hexane vapor were changed back into a liquid phase and returned to the reaction medium. This provides constant removal of water generated in the reaction and drives the equilibrium towards SFAE synthesis. The permeate container equipped with a condenser (temperature kept at –8°C), was connected to a vacuum pump (maintained on 10 mbar). The reaction was started by addition of lipase immobilized CAL-B on EP 100 (0.2 weight equivalents of substrates). Samples from the reaction mixture were periodically collected and analyzed by thin layer chromatography (TLC) and HPLC as described previously (Cao et al. 1997).

7.7.3 General procedures for preparative synthesis of SFAE

The reaction mixture consisted of equimolar (typically 1 mol) free carbohydrate (solid particles), fatty acid and organic solvent (normally 1.0 ml/min EMK). The product was synthesized using lipase B from *Candida antarctica* (Chirazyme® L-2) in a mainly solid phase system in a stirred-tank reactor under reduced pressure (2 L). Esterification was performed in the presence of a small amount of EMK maintaining a catalytic liquid phase as well as forming a suitable azeotropic mixture with the water generated during esterification. The reaction water was removed from the reaction medium by azeotropic distillation and solvent was dried by membrane pervaporation before returning to the reaction medium in a batch manner. The reaction was controlled by HPLC, until the desired conversion was achieved. In case of glucose fatty acid esters (C_{12}-C_{18}), the reaction mixture solidified, if high conversion was achieved. The SFAE were purified by the methods described in chapter 7.

7.7.4 General procedure for synthesis of vitamin C fatty acid esters

The reaction mixture consisted of vitamin C, lipase, molecular sieves (3 Å, activated by heating overnight to 250°C under reduced pressure) and an organic solvent (TBA or acetone). The reaction mixture was incubated in a uncapped vial, placed in an oil bath, thermostated at 40°C and stirred by a magnetic bar (450 rpm) for 6 h to dry the reaction medium. The reaction was started by addition of the fatty acid vinyl ester. The temperature of the reaction was maintained at 40°C, so that the by-product acetaldehyde was evaporated during the transesterification. This provides constant removal of the by-product, forcing the equilibrium towards synthesis. Samples from the suspension were taken at intervals, centrifuged and the supernatants analyzed by TLC. The products were isolated to determine enzymatic yields.

7.7.5 General procedure for synthesis of amino-SFAE

The reaction mixture consisted of equimolar amounts of amino-sugar and fatty acid in an organic solvent [usually TBA (one weight equivalents of substrates) as adjuvant, and activated molecular sieves 3 Å, 10 mesh, 0.6 weight of substrates] for the adsorption of water generated during esterification. The reaction mixture was incubated in a capped vial, placed in an oil bath thermostated to 60°C and stirred by a magnetic bar (450 rpm). The reaction was started by addition of the immobilized lipase (0.2 weight equivalents of substrates). Samples from the reaction mixture were analyzed by TLC. At the end of the reaction, the mixture was extracted with 20 ml THF by stirring at room temperature for 30 min. The immobilized enzyme was separated from the reaction mixture by flotation allowing easy recovery of the biocatalyst. Organic solvent was removed in *vacuo* by rotary evaporation and the crude product was purified by silica gel chromatography [methanol:ethyl acetate=3:7].

7.7.6 General procedure for synthesis of salicin fatty acid esters

The reaction mixture (5 mmol scale) consisted of free fatty acids and salicin and an organic solvents (EMK or acetone). The reaction mixture was incubated in a two-necked round-bottom flask equipped with a Soxhlet extractor on top of a condenser, which was connected to a vacuum pump. Activated molecular sieves was placed in the Soxhlet extractor for the removal of by-products (water or methanol). The condensed solvent was dried by passing through the activated molecular sieves before returning to the reaction system. The apparatus was placed in an oil bath, thermostated to 40-60 °C and stirred by a magnetic bar under reduced pressure. The reaction was started by addition of lipase. Samples from the suspension were taken at intervals, centrifuged and the supernatants were analyzed by thin layer chromatography. At the end of the reaction, the mixture was extracted with dichloromethane and filtered to remove lipase and unreacted salicin. The salicin fatty acid esters were purified by column chromatography.

7.8 Investigation of enzyme stability

7.8.1 Enzyme stability by using different fatty acids

1 mmol different fatty acid was added to a mixture consisting of 1 mmol *b*-D(+)-glucose, 0.7 ml TBA and 200 mg activated molecular sieve (3Å, 10 mesh). The mixture was stirred with a magnetic bar and incubated in an oil bath thermostated to 60°C. The reaction was started by addition of 40 mg immobilized lipase CAL-B-EP 100. After 24 h, the reaction was stopped. To the reaction mixture 40 ml THF was added while stirring and the enzyme and sugar(not

reacted) were separated by filtration. The residue containing sugar and immobilized enzyme was dried under vacuum for two hours. The conversion was analyzed by HPLC. The isolated enzyme was reused for the same reaction under identical conditions. The conversion after the second reuse was also analyzed by HPLC.

7.8.2 Enzyme stability by using different solvents.

To a mixture containing 2 mmol *b*-D(+)-glucose, 2 mmol octanoic acid, 100 mg CAL-B on EP 100 and 500 mg molecular sieves, 1.4 ml solvent was added. The reaction mixture was stirred by a magnetic bar (200 rpm) and incubated at 60°C in an oil bath. After 24 h, the reaction was stopped. The immobilized enzyme was isolated and reused. The conversion was determined by HPLC.

7.8.3 Enzyme stability at different reaction temperatures

The reaction mixture containing 2 mmol *b*-D(+)-glucose, 2 mmol octanoic acid, 1.4 ml TBA, 100 mg CAL-B EP 100 and 500 mg molecular sieve was incubated at different reaction temperatures (30-70°C) and, stirred by a magnetic bar (200 rpm). After 24 h, the immobilized enzyme was isolated and reused. The conversion was determined by HPLC.

7.8.4 Operational stability of immobilized lipase

20 mg immobilized lipase was added to 2 ml EMK or the mixture of EMK and hexane, containing 45 mg *b*-D(+)-glucose, 64 mg palmitic acid and 50 mg molecular sieve. The mixture was shaken (12000 rpm) and incubated on a thermoblock at 75°C or 59°C for 24 hours. Samples were taken at intervals (20 minutes), centrifuged (14000 rpm, 4 min) and the supernatants were analyzed by HPLC. The initial reaction rate was taken as the synthetic activity of immobilized lipases. The reaction mixture was solubilised in acetone. The immobilized enzyme was isolated by filtration. After drying the immobilized enzyme in a desiccator (under reduced pressure), the used lipase was added to a new reaction mixture and carried out under identical condition. This process was repeated several times. The conversion was determined by HPLC. The synthetic activity of immobilized lipase was expressed by the conversion.

7.9 TLC-Analysis

7.9.1 TLC-analysis for glucose fatty acid esters

TLC analysis was performed on activated silica gel plates $60F_{240}$ (Merck) using chloroform, methanol, acetic acid and water (70:20:8:2) or ethylacetate, methanol and water (80:20:5) as developing system. The plates were sprayed with 50% sulfuric acid and heated at 110°C on a heating block for 5 minutes to develop the sugar and sugar ester spots.

7.9.2 TLC-analysis for vitamin C fatty acid esters

TLC analysis was performed on non-activated silica gel plates $60F_{240}$ (Merck) using ethylacetate:methanol:water (80:20:5, by vol.) as developing system. Spots were visualized by dipping into Cer-reagents (25 g molybdophosphoric acid and 10 g Cer(IV)-sulfate are dissolved in a mixture containing 300 ml water and 80 ml concentrated H_2SO_4 with water then added to a final volume of 1 L) and heated at 110°C on a heating block for 5 min to develop the vitamin C, vitamin C ester, fatty acid vinyl esters and/or fatty acids spots.

7.9.3 TLC-analysis for salicin fatty acid esters

TLC analysis was performed on activated silica plates $60F_{240}$ (Merck) using chloroform, methanol and water (65:15:2) as developing system. The plates were sprayed with anisaldehyde solution (acetic acid:sulfuric acid:anisaldehyde = 100:2:1) and heated at 110°C on a heating block for 3 minutes to develop the fatty acid, salicin fatty acid monoester, salicin fatty acid diester and salicin.

7.10 HPLC-Analysis

All solutions and reaction mixtures were analyzed by a HPLC system (Sykam, Gilching, Germany) equipped with a Nucleosil 120-5C_{18} column. For the analysis of 6-*O*-palmitoyl glucose ester a mobile phase consisting of methanol:acetonitrile:water (70:25:5) was used; monoester and fatty acid were detected with a refractive index detector. For the analysis of all other carbohydrates the mobile phase consisted of the following solvent system: A: methanol:water:acetic acid (49.5:50:0.5) and B: methanol. For the analysis of 6-*O*-octanoyl-glucose, a mobile phase of methanol:acetonitrile:water (50:30:20) was used. Analysis was performed at a flow rate of 1.0 ml/min at 39 °C with a light scattering detector at 2.5 bar nitrogen pressure.

7.11 NMR-Spectra

^{1}H- and ^{13}C-NMR-spectra were recorded on a Bruker AC250F, Bruker CXP 300 spectrometer (^{1}H at 200 MHz, ^{13}C at 100.6 MHz) in DMSO-d_6.

7.12 General procedure for the purification of SFAE

The SFAE synthesized in this work were purified according to Scheme 7.12. For the purification of a specific SFAE, this procedure must be modified according to the properties of SFAE, e.g. solubility, chain length of acyl moiety. Generally, the SFAE of long chain fatty acid can be purified by recrystallization in acetone or methanol. In contrast, the SFAE of short chain fatty acid should be purified by column chromatography.

7.12.1 Glucose fatty acid esters

In the case of glucose fatty acid esters, the procedure for column chromatography is generally not necessary. Highly pure glucose fatty acid esters can be obtained by recrystallisation in acetone. Glucose fatty acid esters were solubilised in acetone at 50°C. The insoluble sugar was separated by filtration. The SFAE solution in acetone was cooled to -10°C and white crystals formed, which were collected by filtration. The purity of the glucose fatty acid esters achieved with this procedure was higher than 99% as conformed by HPLC determination.

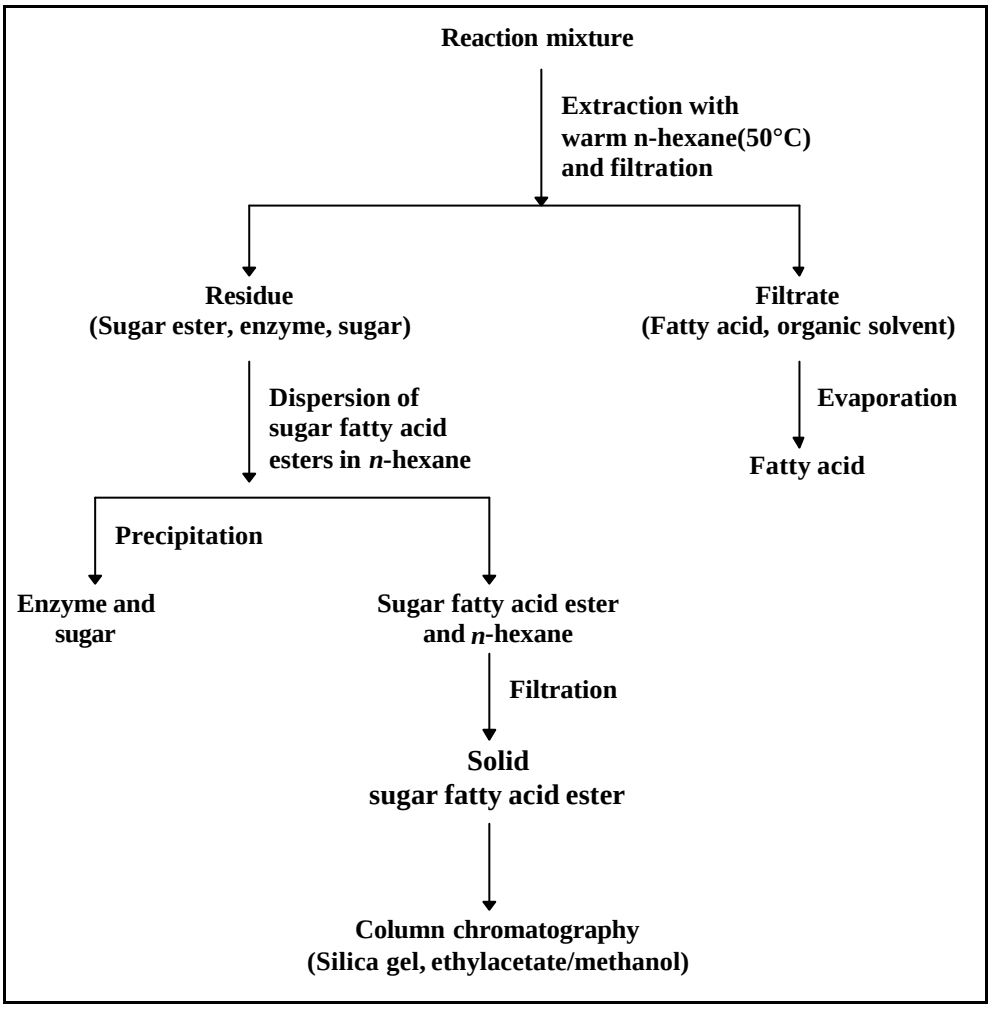
7.12.2 Vitamin C fatty acid esters

At the end of the reaction, the mixture was extracted three times with 20 ml mixture of acetone at 50°C, filtered to remove enzyme and molecular sieves. The fatty acid ascorbyl ester solution was heated to evaporate acetone in a rotary evaporator. For the caprylic acid ascorbyl ester, after dissolving the concentrate in 15 ml EMK, 5 ml pure water was added to hydrolyse unreacted fatty acid vinyl ester and to dissolve the unreacted vitamin C. The organic phase was collected by separation and then the solvent evaporated. For the other fatty acid ascorbyl esters, 5 ml (three times) was directly used to wash the concentrate. Then, the crude product was washed three times with hexane to remove fatty acids. A powdered, white solid was obtained. The purity of the fatty acid ascorbyl esters achieved with this procedure was higher than 99%.

7.12.3 Salicin fatty acid esters

At the end of the reaction, the mixture was extracted three times with 20 ml dichloromethane by stirring each at room temperature for 20 min. Hereby, the immobilized enzyme was separated by flotation from the reaction mixture allowing an easy recovering of the enzyme

preparation. Organic solvent from the supernatant was removed *in vacuo* and the crude product was purified by silica gel chromatography. For separating the mono- and diesters, it was achieved by removal of salicin myristic diester and myristic acid using chloroform:methanol (30:1) followed by elution with ethylacetate:methanol (30:1) to isolate the monoester. Thus, first the chloroform and methanol (30:1) was used to separate the salicin myristic diester and myristic acid and, then ethyl acetate and methanol (30:1) were added to separate the salicin myristic diester and salicin myristic monoester.



Scheme 7.12 General purification scheme of SFAE.

7.13 Immobilization of lipases

Immobilization of lipases was performed as described in literature (Wisdom 1984; Gray et al. 1990; Bosley and Peilow 1991; Montero et al. 1993; Huang and Ju 1994; Agrawal and Burns 1996; Bosley and Peilow 1997; Castillo et al. 1997; Lee and Akoh 1997).

7.13.1 Deposition of lipases on Celite

200 mg CAL-B was dissolved in 10 ml of phosphate buffer (20 mM, pH 7.0). The lipase solution was cooled in an ice bath. Then 2 g Celite powder was added while stirring. The mixture was stirred at 10°C for 2 h, then 10 ml chilled acetone was added to the solution. The immobilized lipase was collected by filtration and washed several times with chilled acetone and dried under vacuum for 2 h.

7.13.2 Adsorption of lipase on EP 100 and Amberlite XAD-7

1 gram of lipase powder was dissolved in 100 ml phosphate buffer (20 mM, pH 6.0). The solution was added to 5 gram EP100, pre-wet with 15 ml ethanol or acetone and incubated in an ice bath. The mixture was stirred at room temperature overnight. The immobilized lipase was collected by filtration and washed with distilled water for several times, then with sodium phosphate buffer (20 mM, pH 7.0) and dried in vacuum for 2 h and stored at 4°C prior to use.

7.13.3 Immobilization of lipase on activated silica gel

Silanisation was performed as follows: to 250 ml *p*-aminopropyl-triethoxysilane in acetone (1%) 10 g silica (80-800 mesh) was added. After evaporation of acetone, the mixture was heated to 110°C overnight. To 4 g activated silica gel, 40 ml 2.5% glutaraldehyde was added. The reaction mixture was stirred at room temperature for 1 h. After thorough washing with water, 40 ml 1% (w/v) lipase solution (dissolved in sodium phosphate buffer, 50 mM, pH 7.0) was added and stirred at room temperature for 2 h. The immobilized lipase was collected by filtration and washed several times with distilled water and buffer, (20 mM, pH 7.0). The immobilized enzyme was collected by filtration, dried overnight under vacuum and stored in the refrigerator.

7.13.4 Modification of lipase B with activated PEG

To 200 ml *p*-aminopropyl-triethoxysilane in acetone (1%) 10 g PEG 2000 monomethyl ether was added. After the evaporation of acetone, the mixture was heated to 110°C for two hours. The reaction mixture was dissolved in 50 ml acetone, then petrol ether was slowly added in order to precipitate the PEG. The activated PEG was collected by filtration and washed with petrol ether. To 4 g activated PEG, 40 ml 2.5% glutaraldehyde was added. The reaction mixture was stirred at room temperature for 1 h. The activated PEG was extracted with chloroform. After evaporation of chloroform, 40 ml 1% (w/v) lipase solution (dissolved in sodium phosphate buffer, 50 mM, pH 7.0) was added and stirred at room temperature for 2 h. To this mixture, 40 ml chilled acetone was slowly added while stirring and non-modified

lipase was precipitated and filtrated. After evaporation of excess solvent under vacuum, the immobilized lipase was dissolved in acetone and precipitated with petrol ether. Immobilized lipase was collected by filtration, dried overnight under vacuum and stored at 4°C prior to use.

7.13.5 Immobilization of lipase on magnetic polypropylene

The magnetic support was prepared according to Figure 7.13.5: 4 g EP was pre-wetted with 15 ml ethanol, then 60 ml distilled water and 20 ml $FeSO_4$ (1.5%) were added under bubbling with nitrogen gas. Then 4 ml hydrogen peroxide (0.06%) was slowly added to the solution under stirring at 50°C. During the process, the pH was kept to 10.0 by adding 3 N sodium hydroxide. When the color of the mixture became dark, the EP was collected by filtration, thoroughly washed with distilled water and dried under vacuum for use.

2 g of this support was dissolved in 6 ml ethanol, then 40 ml CAL-B solution (1%, w/v) solubilized in sodium phosphate buffer (20 mM, pH 6.0) was slowly added to the mixture and stirred at room temperature overnight. The immobilized lipase was collected by filtration and washed with distilled water, buffer (50 mM, pH 7.0) and dried under vacuum see above.

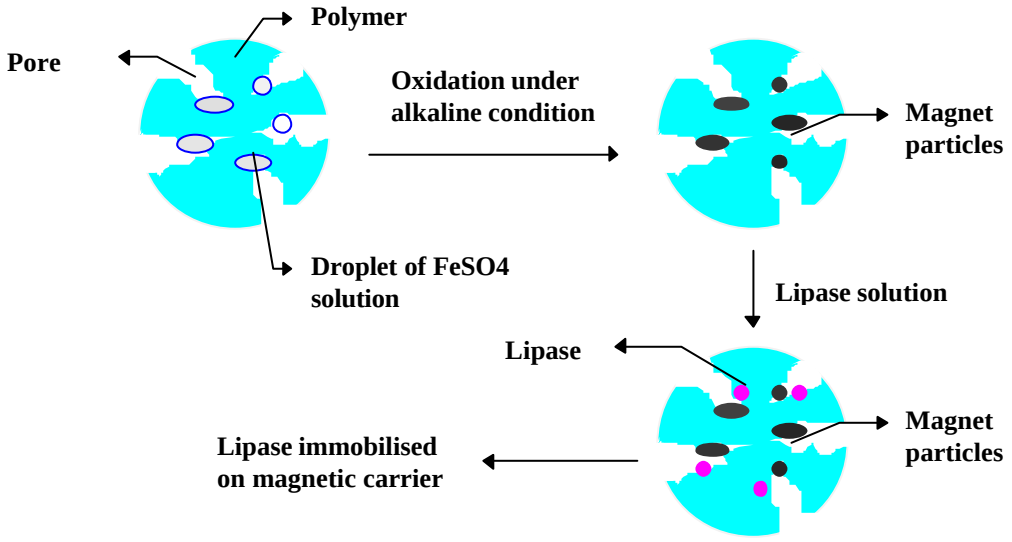


Figure 7.13.5 Preparation of magnetic EP 100

7.14 Adjustment of water activity

The immobilized enzyme, solvent as well as reactants were equilibrated with a series of saturated salt solutions (for water activities between 0.11-0.95) at room temperature for 3

days. The relationship between the water activity and salt solutions is shown in Table 7.14.1 (Goderis et al. 1986).

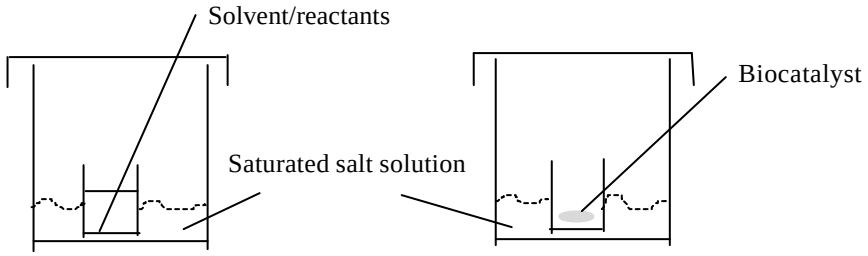


Figure 7.14.1 Pre-equilibration of a substrate solution and biocatalyst to a desired water activity.

Table 7.14.1 Water activity of different salt solutions (Goderis et al. 1986).

Saturated salt solution used	Water activity [a_w]
K_2SO_4	0.95
KCl	0.85
NaCl	0.75
$Mg(NO_3)_2*6H_2O$	0.54
K_2CO_3	0.43
$MgCl_2*6H_2O$	0.33
$KC_2H_3O_2$	0.23
LiCl	0.11

7.15 Determination of water content

The water content of free enzyme or immobilized enzymes was determined using a Karl-Fischer titrator (701 KF Titrino, Metrohm, Switzerland). The enzyme sample was added to 20 ml dry methanol, the methanol containing enzyme was automatically titrated with a standard solution. The water content was automatically calculated by the consumption of an organic base. The principle is represented in equation 7.15.

$$SO_2 + H_2O + I_2 \longrightarrow H_2SO_4 + 2\,HI \qquad (7.15)$$

7.16 Determination of aquaphilicity

The aquaphilicity was measured according to the method described by Reslow *et al.* (Reslow et al. 1992) with some modification (Figure 7.16): 100 mg dry support was added to 2 ml diethylether saturated with water in a capped flask (2.5 ml). The mixture was shaken overnight to reach the equilibrium. Then 1 ml diethylether solution was transfered to an Eppendorf tube (2 ml) containing 200 mg activated molecular sieve. The water remaining in diethylether was adsorbed by molecular sieve. The increase of the weight of molecular sieve corresponds to the amount of water dissolved in diethylether. The water adsorbed by the support will be estimated by comparing the water dissolved in diethylether and water saturated concentration of diethylether. Aquaphilicity was defined as the ratio of water concentration of the support and water concentration in the solvent.

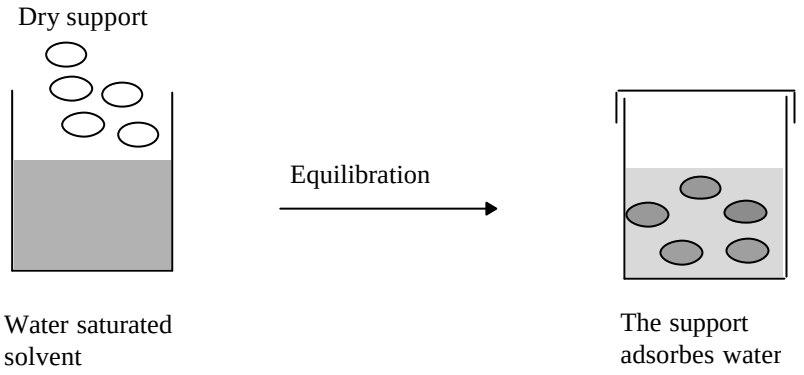


Fig. 7.16 Scheme of aquaphilicity determination (Reslow et al. 1992)

7.17 Water removal measurement

The water removal was measured at steady state using a membrane reactor. The solvent with a certain water content was incubated in the membrane reactor at different temperatures. Samples from the organic solvent were periodically collected and analyzed. The water content in the organic solvent phase was measured by Karl-Fischer titration. The water amount passing the membrane was calculated from the mass balance at the organic solvent side.

7.18 Determination of surface tension and critical micellar concentrations

Surface tension was measured in aqueous solutions with a tensiomat using a series of aqueous solutions at various concentration of biosurfactants at room temperature (25°C). The solutions were equilibrated overnight at room temperature on a shaking apparatus. Measurement was

repeated five times, and the mean value was taken. Critical micellar concentration was estimated by plotting the concentration vs. surface tension. The concentration at the inflexion point was taken as the CMC of a biosurfactant.

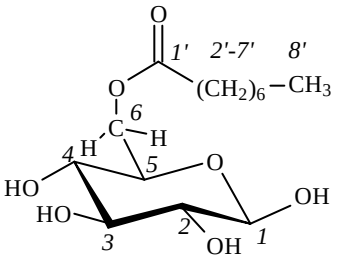
7.19 Determination of melting points

The melting points of sugar fatty acid esters were determined using the Bücher melting point determination apparatus (Hawel, Switzerland).

7.20 Characterization of SFAE

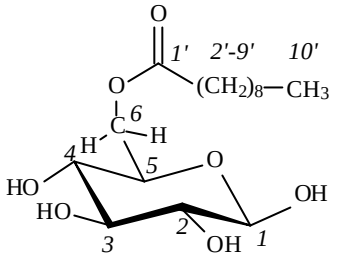
7.20.1 Glucose fatty acid esters

6-O-octanoyl-b-D-*glucose*



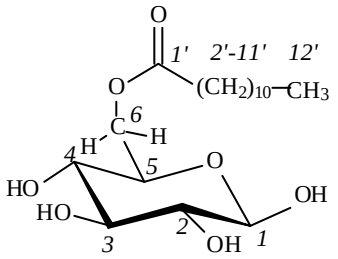
6-O-octanoyl-b-D-*glucose*: crystallized as white crystals from acetone, mp 126-127°C; solubility in acetone: 75.2 mg/ml at 60°C; insoluble in *n*-hexane. ^{1}H NMR (DMSO-d_6/TMS) δ (ppm), 0.99 (t, 3H, H-8'), 1.39 (m, 8H, H-4'-7'), 1.64 (m, 2H, H-3'), 2.41 (t, 2H-2'), 3.17 (m, 1H, H-4), 3.25 (m, 1H, H-2), 3.56 (m, 1H, H-3), 3.90 (m, 1H-5), 4.12 (dd, 1H, J = 1.65, 11.60, H-6a), 4.39 (dd, 1H, J = 6.2, 11.64, H-6b), 4.66 (d, J = 6.67, 1H, OH-3 or 2), 4.88 (d, 1H, J = 4.87, OH-4), 5.03 (dd, 1H, J = 4.10, 4.10, H-1), 5.16 (d, 1H, J = 5.68, OH-2 or 3), 6.47 (d, 1H, J = 4.65, OH-1). The shifts and coupling of H-6 protons were found. This result was in accordance to those in the literature (Adelhorst *et al*., 1990; Fregapane, 1994). ^{13}C-NMR (DMSO-d_6) δ (ppm): octanoyl moiety, 12.94 (C-8', CH_3), 21.05 (C-7', CH_2), , 23.49 (C-3', CH_2), 27.40 (C-4'-C-5', CH_2), 30.14 (C-6', CH_2), 32.46 (C-2', CH_2), 171.93 (C-1', C=O); sugar moiety, 62.85 (C-6, CH_2), 68.14 (C-4, CH), 69.55 (C-5, CH), 71.19 (C-2, CH), 71.87 (C-3, CH), 91.29 (C-1, CH).

6-O-decanoyl-b-D-*glucose*



6-O-decanoyl-b-D-*glucose*: crystallized as white crystals from acetone. mp 128-129°C; solubility in acetone 48.1 mg/ml at 60°C; insoluble in *n*-hexane. ^{1}H NMR (DMSO-d_6/TMS) δ (ppm): 0.86 (t, 3H, H-10'), 1.24 (m, 12H, H-4'-9'), 1.50 (m, 2H, H-3'), 2.27 (t, 2H-2'), 3.03 (m, 1H, H-4), 3.12 (m, 1H, H-2), 3.42 (m, 1H, H-3), 3.76 (m, 1H-5), 3.99 (dd, 1H, J = 6.21, 11.58, H-6a), 4.26 (d, 1H, J = 1.29, H-6b), 4.53 (d, J = 6.67, 1H, OH-3 or 2), 4.76 (d, J = 4.68, 1H, OH-4), 4.89 (dd, 1H, J = 3.73, 3.71, H-1), 5.04 (d, 1H, J = 5.61, OH-2 or 3), 6.34 (d, 1H, J = 4.47, OH-1). The shifts and coupling of H-6 protons were found. This result was in accordance to those in the literature (Adelhorst *et al*., 1990). ^{13}C-NMR (DMSO-d_6) δ (ppm): decanoyl moiety, 13.88 (C-10', CH_3), 22.03 (C-9', CH_2), 24.41 (C-3', CH_2), 28.37 (C-4', CH_2),28.60 (C-5', CH_2),28.68 (C-6', CH_2),28.81 (C-7', CH_2), 31.21 (C-8', CH_2), 33.38 (C-2', CH_2), 171.93 (C-1', C=O); sugar moiety, 63.78 (C-6, CH_2), 69.05 (C-4, CH), 70.47 (C-5, CH), 72.11 (C-2, CH), 72.78 (C-3, CH), 92.21 (C-1, CH).

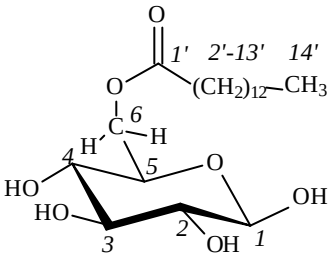
6-O-lauroyl-b-D-*glucose*



6-O-lauroyl-b-D-*glucose*: crystallized as white crystals from acetone. mp 130-131°C; solubility in acetone 20.1 mg/ml at 60°C; insoluble in *n*-hexane. ^{1}H NMR (DMSO-d_6/TMS) δ (ppm): 0.85 (t, 3H, H-12'), 1.26 (m, 12H, H-4'-11'), 1.49 (m, 2H, H-3'), 2.27 (t, 2H-2'), 3.03 (m, 1H, H-4), 3.12 (m, 1H, H-2), 3.42 (m, 1H, H-3), 3.76 (m, 1H-5), 3.98 (dd, 1H, J = 6.22, 11.65, H-6a), 4.26 (dd, 1H, J = 1.59, 11.57, H-6b), 4.53 (d, J = 6.64, 1H, OH-3 or 2), 4.76 (d,

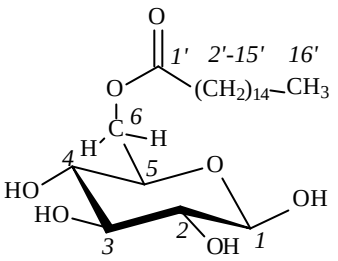
J = 4.76, 1H, OH-4), 4.89 (dd, 1H, J = 4.12, 4.11, H-1), 5.04 (d, 1H, J = 5.71, OH-2 or 3), 6.34 (d, 1H, J = 4.66, OH-1). The shifts and coupling of H-6 protons were found. This result was in accordance to those in the literature (Adelhorst *et al.*, 1990). ^{13}C-NMR (DMSO-d_6) δ (ppm): lauroyl moiety, 13.87 (C-12', CH_3), 22 01 (C-11', CH_2), 24.38 (C-3', CH_2), 28.35 (C-4', CH_2), 28.63 (C-5', C6', CH_2), 28.83 (C-7', CH_2), 28.92 (C-8', C-9', CH_2), 31.21 (C-10', CH_2), 33.38 (C-2', CH_2), 172.83 (C-1', C=O); sugar moiety, 63.78 (C-6, CH_2), 69.03 (C-4, CH), 70.45 (C-5, CH), 72.09 (C-2, CH), 72.76 (C-3, CH), 92.19 (C-1, CH).

6-O-myristoyl-b-D *glucose*



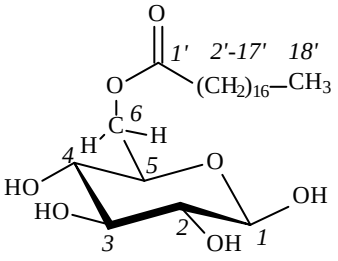
6-O-myristoyl-b-D-*glucose*: crystallized as white crystals from acetone. mp 131-133°C; solubility in acetone: 13.2 mg/ml at 60°C; insoluble in *n*-hexane. ^{1}H NMR (DMSO-d_6/TMS) δ (ppm): 0.85 (t, 3H, H-14'), 1.26 (m, 20H, H-4'-13'), 1.50 (m, 2H, H-3'), 2.27 (t, 2H-2'), 3.02 (m, 1H, H-4), 3.12 (m, 1H, H-2), 3.42 (m, 1H, H-3), 3.76 (m, 1H-5), 3.98 (dd, 1H, J = 6.22, 11.60, H-6a), 4.26 (d, 1H, J = 11.60, H-6b), 4.53 (d, J = 6.74, 1H, OH-3 or 2), 4.76 (d, J = 4.73, 1H, OH-4), 4.89 (dd, 1H, J = 4.23, 3.82, H-1), 5.04 (d, 1H, J = 5.68, OH-2 or 3), 6.34 (d, 1H, J = 4.58, OH-1). This result was in accordance to those in the literature (Adelhorst *et al.*, 1990). ^{13}C-NMR(DMSO-d_6) δ (ppm): myristoyl moiety, 13.87 (C-14', CH_3), 22 02 (C-13', CH_2), 24.39 (C-3', CH_2), 28.36 (C-4', CH_2), 28.65 (C-5', C6', CH_2), 28.84 (C-7', CH_2), 28.95 (C-8', C-9',C-10', CH_2), 31.22 (C-12', CH_2), 33.36 (C-2', CH_2), 172.83 (C-1', C=O); sugar moiety, 63.78 (C-6, CH_2), 69.03 (C-4, CH), 70.45 (C-5, CH), 72.09 (C-2, CH), 72.76 (C-3, CH), 92.19 (C-1, CH).

6-O-palmitoyl-b-D-*glucose*



6-O-palmitoyl-b-D-*glucose*: crystallized as white crystals from acetone. mp 135-136°C; solubility in acetone 4.6 mg/ml at 60°C; insoluble in *n*-hexane. ^{1}H NMR (DMSO-d_6/TMS), δ (ppm): 1.03 (t, 3H, H-16'), 1.44 (m, 24H, H-4'-15'), 1.69 (m, 2H, H-3'), 2.45 (t, 2H-2'), 3.21 (m, 1H, H-4), 3.31 (m, 1H, H-2), 3.60 (m, 1H, H-3), 3.95 (m, 1H-5), 4.18 (dd, 1H, J = 6.23, 11.64, H-6a), 4.44 (d, 1H, J = 11.46, H-6b), 4.71 (d, J = 6.75, 1H, OH-3 or 2), 4.94 (d, 1H, J = 4.82, OH-4), 5.08 (dd, 1H, J = 4.10, 3.97, H-1), 5.22 (d, 1H, J =5.67, OH-2 or 3), 6.53 (d, 1H, J = 4.61, OH-1). The shifts and coupling of H-6 protons were found. This result was in accordance to those in the literature (Adelhorst *et al*., 1990). ^{13}C-NMR(DMSO-d_6) δ (ppm): palmitoyl moiety, 13.11 (C-16', CH_3), 21.27 (C-15', CH_2), 23.64 (C-3', CH_2), 27.62 (C-4', CH_2),27.89 (C-5'), 27.91 (C6', CH_2), 28.10 (C-7', CH_2), 28.19 (C-8', C-9', CH_2), 28.23 (C-10',11', 12',13' CH_2), 30.47 (C-14', CH_2), 32.60 (C-2', CH_2), 172.06 (C-1', C=O); sugar moiety, 63.04 (C-6, CH_2), 68.29 (C-4, CH), 69.72 (C-5, CH), 71.35 (C-2, CH), 72.02 (C-3, CH), 91.45 (C-1, CH).

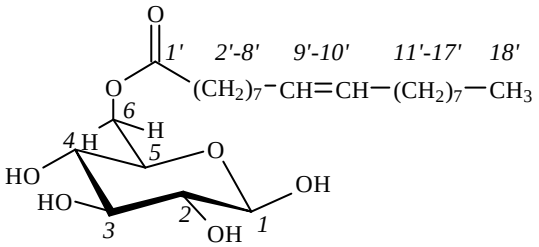
6-O-stearoyl-b-D-*glucose*



6-O-stearoyl-b-D-*glucose*: crystallized as white crystals from acetone. mp 134-136°C; solubility in acetone: 3.9 mg/ml at 60°C; insoluble in *n*-hexane. ^{1}H NMR (DMSO-d_6/TMS), δ (ppm): 0.85 (t, 3H, H-18'), 1.25 (m, 28H, H-4'-17'), 1.50 (m, 2H, H-3'), 2.27 (t, 2H-2'), 3.02 (m, 1H, H-4), 3.12 (m, 1H, H-2), 3.42 (m, 1H, H-3), 3.76 (m, 1H-5), 3.98 (dd, 1H, J = 6.26, 11.58, H-6a), 4.26 (d, 1H, J = 11.45, H-6b), 4.53 (d, J = 6.66, 1H, OH-3 or 2), 4.76 (d, 1H, J = 4.72, OH-4), 4.89 (dd, 1H, J = 3.86, 3.86, H-1), 5.04 (d, 1H, J = 5.64, OH-2 or 3), 6.34 (d, 1H,

J = 4.56, OH-1). The shifts and coupling of H-6 protons were found. This result was in accordance to those in the literature (Adelhorst *et al.*, 1990). ^{13}C-NMR (DMSO-d_6) δ (ppm): stearoyl moiety, 13.87 (C-18', CH_3), 22.05 (C-17', CH_2), 24.42 (C-3', CH_2), 28.41 (C-4', CH_2), 28.68 (C-5'), 28.72 (C6', CH_2), 28.89 (C-7', CH_2),29.01 (C-8', C-9', 10', 11', 12', 13', 14', 15'; CH_2), 31.26 (C-16', CH_2), 33.37 (C-2', CH_2), 172.06 (C-1', C=O); sugar moiety, 63.82 (C-6, CH_2), 69.05 (C-4, CH), 70.49 (C-5, CH), 72.11 (C-2, CH), 72.78 (C-3, CH), 92.21 (C-1, CH).

6-O-leoyl-b-D-*glucose*

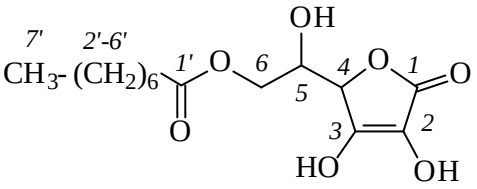


6-O-leoylb-D-*glucose*: crystallized as white crystals from acetone. mp 111-112°C; solubility in acetone: 77.6 at 60°C; insoluble in *n*-hexane. ^{1}H NMR (DMSO-d_6/TMS) δ (ppm): 85 (t, 3H, H-18'), 1.25 (m, 28H, H-4'-17'), 1.50 (m, 2H, H-3'), 1.98 (m, 4H, H-8' and H-11'), 2.27 (t, 2H-2'), 3.02 (m, 1H, H-4), 3.12 (m, 1H, H-2), 3.43 (m, 1H, H-3), 3.76 (m, 1H-5), 3.98 (dd, 1H, J = 6.29, 11.65, H-6a), 4.26 (dd, 1H, J = 11.43, 1.21, H-6b), 4.53 (d, J = 6.67, 1H, OH-3 or 2), 4.76 (d, 1H, J = 4.82, OH-4), 4.89 (dd, 1H, J = 4.18, 4.01, H-1), 5.04 (d, 1H, J = 5.69, OH-2 or 3), 5.33 (m, 2H, H-9'-10'), 6.34 (d, 1H, J = 4.63, OH-1). The shifts and coupling of H-6 protons were found. This result was in accordance to those in the literature (Adelhorst *et al.*, 1990). ^{13}C-NMR(DMSO-d_6) δ(ppm): oleoyl moiety, 13.87 (C-18', CH_3), 22.03 (C-17', CH_2), 26.50 (C-3', CH_2), 26.52(C-4', CH_2), 28.38 (C-5'), 28.45 (C6', CH_2), 28.54 (C-7', C-12', CH_2), 28.63 (C-13'), 28.78 (C-14', CH_2), 29.04 (C-8', C-11', CH_2), 31.22 (C-16', CH_2), 33.35 (C-2', CH_2), 129.54 (C-9'-C10'), 172.80 (C-1', C=O); sugar moiety, 63.82 (C-6, CH_2), 69.05 (C-4, CH), 70.49 (C-5, CH), 72.11 (C-2, CH), 72.78 (C-3, CH), 92.21 (C-1, CH).

7.20.2 Vitamin C fatty acid esters

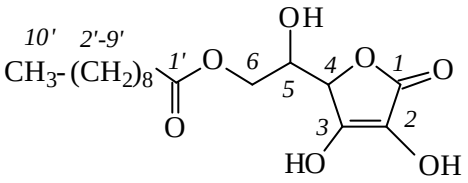
Vitamin C fatty acid monoesters were confirmed by NMR spectroscopy on a Bruker AM 400. ^{13}C NMR spectra at 100.6 MHz, in $CDCl_3$, with an internal reference of Tetramethylsilane.

6-O-octanoyl-L-asorbic acid



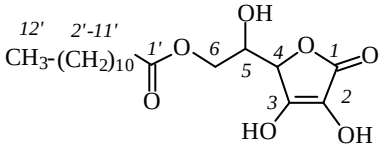
1 mmol of ascorbic acid and 3 mmol of caprylic acid vinyl ester were esterified and product was isolated as described in chapter 7.12.2: yield 196 mg (65%), white solid. R_f 0.70 [ethyl acetate:methanol:water(80:20:5)]; ^{13}C NMR (DMSO): δ (ppm) = 172.6 (C-1=O), 170.2 (C-1'=O), 152.0 (C-2), 118.1 (C-3), 74.9 (C-4), 65.4 (C-5), 64.3 (C-6), 33.2 (C-2'), 31.0 (C-6'), 28.3 (C-4'), 28.2 (C-5'), 24.2 (C-3'), 21.9 (C-7'), 13.8 (C-8').

6-O-decanoyl-L-asorbic acid



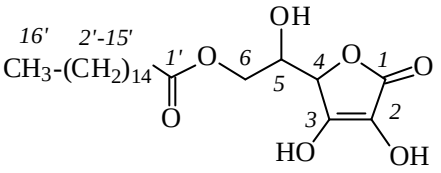
1 mmol of ascorbic acid and 1.5 mmol of capric acid vinyl ester were esterified and product was isolated as described in chapter 7.12.2: yield 288 mg (87 %), white solid. R_f 0.72 [ethyl acetate:methanol:water(80:20:5)]; ^{13}C NMR (DMSO): δ (ppm) = 172.6 (C-1=O), 170.2 (C-1'=O), 152.0 (C-2), 118.1 (C-3), 74.9 (C-4), 65.4 (C-5), 64.3 (C-6), 33.2 (C-2'), 31.2 (C-8'), 28.7 (C-6'), 28.6 (C-5'), 28.5 (C-7'), 28.3 (C-4'), 24.2 (C-3'), 21.9 (C-9'), 13.8 (C-10').

6-O-lauroyl-L-asorbic acid



1 mmol of ascorbic acid and 3 mmol of lauric acid vinyl ester were esterified and product was isolated as described in chapter 7.12.2: yield 265 mg (74 %), white solid. R_f 0.75 [ethyl acetate:methanol:water(80:20:5)]; ^{13}C NMR (DMSO): δ (ppm) = 172.6 (C-1=O), 170.2 (C-1'=O), 152.0 (C-2), 118.1 (C-3), 74.9 (C-4), 65.4 (C-5), 64.3 (C-6), 33.2 (C-2'), 31.2 (C-10'), 28.9 (C-6', 7'), 28.8 (C-5', 8'), 28.6 (C-9'), 28.3 (C-4'), 24.2 (C-3'), 21.9 (C-11'), 13.8 (C-12').

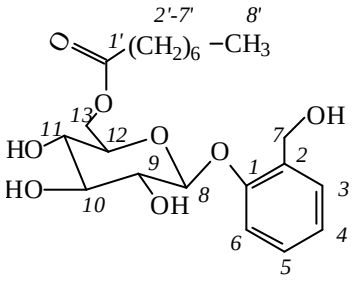
6-O-palmitoyl-L-asorbic acid



6-*O*-palmitoyl-L-asorbic acid: 1 mmol of ascorbic acid and 3 mmol of palmitic acid vinyl ester were esterified and product was isolated as described in chapter 7.12.2: yield 380 mg (91%), white solid. R_f 0.78 [ethyl acetate:methanol:water(80:20:5)]; ^{13}C NMR ($CDCl_3$): δ (ppm) = 172.6 (C-1=O), 170.2 (C-1'=O), 152.0 (C-2), 118.1 (C-3), 74.9 (C-4), 65.4 (C-5), 64.3 (C-6), 33.2 (C-2'), 31.2 (C-14'), 28.9 (C-8', 9', 10'), 28.8 (C-7', 11'), 28.6 (C-6', 12'), 28.5 (C-13'), 28.4 (C-5'), 28.3 (C-4'), 24.2 (C-3'), 21.9 (C-15'), 13.8 (C-16').

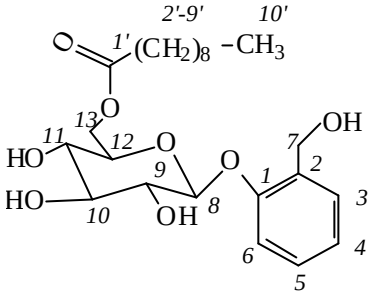
7.20.3 Salicin fatty acid esters

6-O- octanoyl –D-salicin



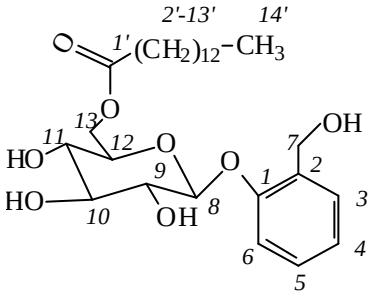
6-O- octanoyl –D-salicin: 0.5 mmol of salicin and 0.5 mmol of caprylic acid were esterified and product was isolated as described in chapter 7.12.3: yield 36 mg (9%), white solid. R_f 0.47 [chloroform:methanol:water(65:15:2)]; ^{13}C NMR (CD_3OD): δ (ppm) = 175.9 (C-1'=O), 157.0 (C-1=O), 129.8-132.4 (C-2, C-3, C-5), 123.9 (C-4), 117.1 (C-6), 103.2 (C-8), 77.8 (C-10), 75.5 (C-12), 75.0 (C-9), 71.6 (C-11), 64.6 (C-13), 61.0 (C-7), 35.0 (C-2'), 32.8 (C-6'), 30.1 (C-4', C-5'), 26.0 (C-3'), 23.7 (C-7'), 14.4 (C-8').

6-O- decanoyl –D-salicin



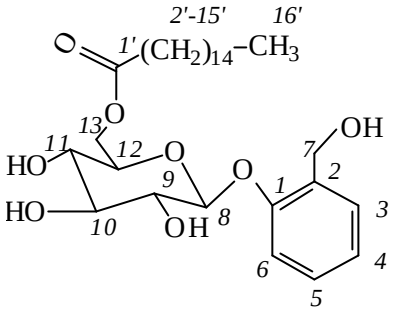
6-O- decanoyl –D-salicin: 0.5 mmol of salicin and 0.5 mmol of capric acid were esterified and product was isolated as described in chapter 7.12.3: yield 82 mg (19%), white solid. R_f 0.48 [chloroform:methanol:water(65:15:2)]; ^{13}C NMR (CD_3OD): δ (ppm) = 175.9 (C-1'=O), 157.0 (C-1=O), 129.8-132.4 (C-2, C-3, C-5), 123.9 (C-4), 117.1 (C-6), 103.2 (C-8), 77.8 (C-10), 75.5 (C-12), 75.0 (C-9), 71.6 (C-11), 64.6 (C-13), 61.0 (C-7), 35.0 (C-2'), 32.8 (C-6'), 31.21 (C-7', C-8'), 30.1 (C-4', C-5'), 26.0 (C-3'), 23.7 (C-9'), 14.4 (C-10').

6-O- myristoyl –D-salicin



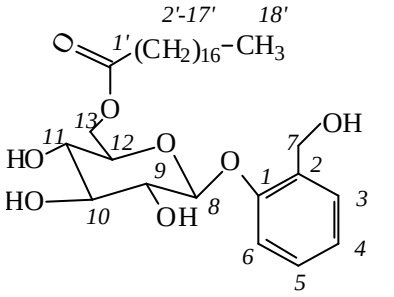
6-O- myristoyl –D-salicin: 0.5 mmol of salicin and 1.0 mmol of myristic acid were esterified and product was isolated as described in chapter 7.12.3: yield 137 mg (28%), white solid. R_f 0.54 [chloroform:methanol:water(65:15:2)]; ^{13}C NMR (CD_3OD): δ (ppm) = 175.9 (C-1'=O), 157.0 (C-1=O), 129.8-132.4 (C-2, C-3, C-5), 123.9 (C-4), 117.1 (C-6), 103.2 (C-8), 77.8 (C-10), 75.5 (C-12), 75.0 (C-9), 71.6 (C-11), 64.6 (C-13), 61.0 (C-7), 35.0 (C-2'), 32.9 (C-12'), 30.1-30.8 (C-4'- C-11'), 26.0 (C-3'), 23.7 (C-13'), 14.4 (C-14').

6-O-palmitoyl –D-salicin



6-O -palmitoyl –D-salicin: 0.5 mmol of salicin and 1.0 mmol of palmitic acid were esterified and product was isolated as described in chapter 7.12.3: yield 278 mg (53%), white solid. R_f 0.57 [chloroform:methanol:water(65:15:2)]; ^{13}C NMR (CD_3OD): δ (ppm) = 175.2 (C-1'=O), 157.0 (C-1=O), 129.8-132.4 (C-2, C-3, C-5), 123.8 (C-4), 117.0 (C-6), 103.2 (C-8), 77.8 (C-10), 75.5 (C-12), 75.0 (C-9), 71.6 (C-11), 64.6 (C-13), 61.0 (C-7), 35.0 (C-2'), 33.1 (C-14'), 30.2-30.8 (C-4'-C-13'), 26.0 (C-3'), 23.7 (C-15'), 14.5 (C-16').

6-O- stearoyl –D-salicin



6-O- stearoyl –D-salicin: 0.5 mmol of salicin and 0.5 mmol of stearic acid were esterified and product was isolated as described in chapter 7.12.3: yield 370 mg (67%), white solid. R_f 0.59 [chloroform:methanol:water(65:15:2)]; ^{13}C NMR (CD_3OD): δ (ppm) = 175.9 (C-1'=O), 157.0 (C-1=O), 129.8-132.4 (C-2, C-3, C-5), 123.9 (C-4), 117.1 (C-6), 103.2 (C-8), 77.8 (C-10), 75.5 (C-12), 75.0 (C-9), 71.6 (C-11), 64.6 (C-13), 61.0 (C-7), 35.0 (C-2'), 33.1 (C-16'), 30.1 – 30.8 (C-4'- C-15'), 26.0 (C-3'), 23.7 (C-17'), 14.5 (C-18').

8. References

Adelhorst, K., F. Björkling, S. E. Godtfredsen and O. Kirk (1990). "Enzyme catalysed preparation of 6-O-acylglucopyranosides." Synthesis **2**: 112-115.

Agrawal, A. and M. A. Burns (1996). "Selective extraction using preferential transport through adsorptive membrane." Biotechnol. Bioeng. **52**: 539-548.

Akoh, C. C., Ed. (1994). Carbohydrate polyesters as fat substitutes. New York, Dekker.

Arcos, J. A., M. Bernabe and C. Otero (1998). "Quantitative enzymatic production of 6-O-acylglucose esters." Biotechnol. Bioeng. **57**: 505-9.

Arcos, J. A., M. Bernabé and C. Otero (1998). "Different strategies for selective monoacylation of hexoaldoses in acetone." J. Surfactants Detergents **1**: 345-352.

Arcos, J. A., H. S. Garcia and C. G. Hill, Jr. (2000). "Continuous enzymatic esterification of glycerol with (poly)unsaturated fatty acids in a packed-bed reactor." Biotechnol. Bioeng. **68**: 563-570.

Arroyo, M. and J. V. Sinisterra (1994). "High enantioselective of 2-arylpropionic acids catalyzed by immobilized lipase from Candida antarctica: A mechanistic approach." J. Org. Chem. **59**: 4410-4417.

Baker, I. J. A., D. N. Furlong, F. Grieser and C. J. Drummond (2000). "Sugar fatty acid ester surfactants: Base-catalyzed hydrolysis." J. Surf. Det. **3**: 29-32.

Baker, I. J. A., B. Matthews, H. Suares, I. Krodkiewska, D. N. Furlong, F. Grieser and C. J. Drummond (2000). "Sugar fatty acid ester surfactants: Structure and Ultimate aerobic biodegradability." J. Surf. Det. **3**: 1-11.

Baker, I. J. A., R. I. Willing, D. N. Furlong, F. Grieser and C. J. Drummond (2000). "Sugar fatty acid ester surfactants: Biodegradation pathways." J. Surf. Det. **3**: 13-27.

Bashir, N. B., S. J. Phythian, A. J. Reason and S. M. Roberts (1995). "Enzymatic esterification and de-esterification of carbohydrates: synthesis of a naturally occurring rhamnopyranoside of p-hydroxybenzaldehyde and a systematic investigation of lipase-catalysed acylation of selected arylpyranosides." J. Chem. Soc. Perkin Trans. **1**: 2203-2222.

Berovic, M. (1999). "Scale-up of citric acid fermentation by redox potential control." Biotechnol. Bioeng. **64**: 552-557.

Binning (1961). Separation of mixtures. US 2981680.

Björkling, F., S. E. Godtfredsen and O. Kirk (1989). "A highly selective enzyme-catalysed esterification of simple glucosides." J. Chem. Soc., Chem. Commun.: 934-935.

Björkling, F., S. E. Godtfredsen and O. Kirk (1991). "The future impact of industrial lipases." Trends Biotech. **9**: 360-363.

Bloomer, S., P. Adlercreutz and B. Mattiasson (1992). "Kilogram-scale ester synthesis of acyl donor and use in lipase-catalyzed interesterifications." J. Am. Oil Chem. Soc. **69**(10): 966-973.

Bludworth, J. E., G. Flower and M. D. Cumberland (1945). Separation of organic compounds. US 2381032.

Böge, K. and L. F. Tietze (1999). "Synthesis of alkyl polyglycosides." Henkel-Referate **53**: 15-20.

Bornscheuer, U. T. (1995). "Lipase-catalyzed syntheses of monoacylglycerols." Enzyme Microb. Technol. **17**: 578-586.

Bornscheuer, U. T. (1999). "Recent advances in the lipase-catalyzed biotransformation of fats and oils." Recent Res. Devel. Oil Chem. **3**: 93-106.

Bornscheuer, U. T. (2000 Ed). Enzymes in lipid modification. Weinheim, Wiley-VCH.

Bornscheuer, U. T. and R. J. Kazlauskas (1999). Hydrolases in organic synthesis - regio- and stereoselective biotransformations. Weinheim, Wiley-VCH.

Bornscheuer, U. T. and T. Yamane (1995). “Fatty acid vinyl esters as acylating agents: A new method for the enzymatic synthesis of monoacylglycerols.” J. Am. Oil Chem. Soc. **72**: 193-197.

Bosley, J. (1997). “Turning lipases into industrial biocatalysts.” Biochem. Soc. Transact. **25**: 174-178.

Bosley, J. A. and A. D. Peilow (1991). Supported Enzyme. EP 424130.

Bosley, J. A. and A. D. Peilow (1997). “Immobilization of lipases on porous polypropylene reduction in esterification efficiency at low loading.” J. Am. Oil Chem. Soc. **74**(2): 107-111.

Bousquet, M.-P., R.-M. Willemot, P. Monsan and E. Boures (1999). “Enzymatic synthesis of unsaturated fatty acid glucoside esters for dermo-cosmetic applications.” Biotechnol. Bioeng. **63**: 730-736.

Buisman, G. J. H., C. T. W. Helteren, G. F. H. Kramer, J. W. Veldsink, J. T. P. Derksen and F. P. Cuperus (1998). “Enzymatic esterifications of functionalized phenols for the synthesis of lipophilic antioxidants.” Biotechnol. Lett. **20**: 131-136.

Bülter, T. and L. Elling (2000). “Enzymatic synthesis of UDP-galactose on a gram scale.” J. Mol. Catal. B: Enzymatic **8**: 281-284.

Bundesministerium (1991). Verordnung über die Verwendung von Extraktionslösungsmitteln und anderen technischen Hilfsstoffen bei der Herstellung von Lebensmitteln (Technische Hilfsstoff-Verordnung - THV) vom 08.11.1991 (BGB1. I S.2100). Bonn, Bundesdruckerei.

Cameotra, S. S. and R. S. Makkar (1998). “Synthesis of biosurfactants in extreme conditions.” Appl. Microbiol. Biotechnol. **50**: 520-529.

Cao, L., U. T. Bornscheuer and R. D. Schmid (1996). “Lipase-catalyzed solid phase synthesis of sugar esters.” Fat Sci. Technol. **98**: 332-335.

Cao, L., U. T. Bornscheuer and R. D. Schmid (1998). “Lipase-catalyzed solid phase synthesis of sugar esters, IV: Selectivity of lipases towards primary and secondary hydroxyl groups in carbohydrates.” Biocatal. Biotransform. **16**: 249-257.

Cao, L., U. T. Bornscheuer and R. D. Schmid (1999). “Lipase-catalyzed solid-phase synthesis of sugar esters. Influence of immobilization on productivity and stability of the enzyme.” J. Mol. Catal. B: Enzymatic **6**: 279-285.

Cao, L., A. Fischer, U. T. Bornscheuer and R. D. Schmid (1997). “Lipase-catalyzed solid phase synthesis of sugar fatty acid esters.” Biocatal. Biotransform. **14**: 269-283.

Carrillo-Munoz, J. R., D. Bouvet, E. Guibé-Jampel, A. Loupy and A. Petit (1996). “Microwave-promoted lipase-catalyzed reactions: Resolution of (±)-1-phenylethanol.” J. Org. Chem. **61**: 7746-7749.

Castillo, E., V. Dossat, A. Marty, J. S. Condoret and D. Combes (1997). “The role of silica gel in lipase-catalyzed esterification reactions of high-polar substrates.” J. Am. Oil Chem. Soc. **74**: 77-85.

Chen, S.-T., S.-Y. Chen, S.-J. Chen and K.-T. Wang (1994). “Vinyl carboxylate, an acylating reagent for selective acylation of amines and diols.” Tetrahedron Lett. **35**: 3583-3584.

Chevandier, F., E. Dubreucq and P. Galzy (1997). “Lipase catalysed synthesis of propanediol monoesters in biphasic aqueous medium.” Biotechnol. Lett. **19**: 913-917.

Chopineau, J., F. D. McCafferty, M. Therisod and A. M. Klibanov (1988). “Production of biosurfactants from sugar alcohols and vegetable oils catalyzed by lipases in a nonaqueous medium.” Biotechnol. Bioeng. **31**: 208-214.

Clapés, P., C. Morán and M. R. Infante (1999). “Enzymatic synthesis of arginine-based cationic surfactants.” Biotechnol. Bioeng. **63**: 333-343.

Degn, P., L. H. Pedersen, J. Duus and W. Zimmermann (1999). “Lipase-catalysed synthesis of glucose fatty acid esters in *tert*-butanol.” Biotechnol. Lett. **21**: 275-280.

Desai, J. D. and I. M. Banat (1997). "Microbial production of surfactants and their commercial applications." Microbiol. Mol. Rev. **61**: 47-64.

Ducret, A., A. Giroux, M. Trani and R. Lortie (1995). "Enzymatic preparation of biosurfactants from sugars and sugar alcohols of fatty acids in organic media under reduced pressure." Biotechnol. Bioeng. **48**: 214-221.

Ducret, A., R. Lortie and M. Trani (1997). Sugar/sugar alcohol esters. US 5635614.

Ducret, A., P. Pepin, M. Trani and R. Lortie (1996). "Lipase-catalyzed selective esterification of ibuprofen." Ann. N. Y. Acad. Sci. **799**: 747-751.

Dudal, Y. and R. Lortie (1995). "Influence of water activity on the synthesis of triolein catalyzed by immobilized Mucor Mielei lipase." Biotechnol. Bioeng. **45**: 129-134.

Ergan, F., M. Trani and G. Andre (1990). "Production of glycerides from glycerol and fatty acid by immobilized lipases in non-aqueous media." Biotechnol. Bioeng. **35**: 195-200.

Ergan, F., M. Trani and G. André (1988). "Solvent free triglyceride synthesis using lipozyme IM-20." Biotechnol. Lett. **10**: 629-634.

Feuge, R. O., J. Zeringue, T. J. Weiss and M. Brown (1970). "Preparation of sucrose esters by interesterification." J. Am. Oil Chem. Soc. **47**: 56.

Flores, M. V., J. J. W. Sewalt, A. E. M. Janssen and A. Padt (2000). "The Nature of fatty acid modifies the equilibrium position in the esterification catalyzed by lipase." Biotechnol. Bioeng. **67**: 364-371.

Fregapane, G., D. B. Sarney, S. G. Greenberg, D. J. Knight and E. N. Vulfson (1994). "Enzymatic synthesis of monosaccharide fatty acid esters and their comparison with conventional products." J. Am. Oil Chem. Soc. **71**: 87-91.

Fregapane, G., D. B. Sarney and E. N. Vulfson (1991). "Enzymatic solvent-free synthesis of sugar acetal fatty acid esters." Enzyme Microb. Technol. **13**: 796-800.

Fregapane, G., D. B. Sarney and E. N. Vulfson (1994). "Facile chemo-enzymatic synthesis of monosaccharide fatty acid esters." Biocatalysis **11**: 9-18.

From, M., P. Adlercreutz and B. Mattiasson (1997). "Lipase catalyzed esterification of lactic acid." Biotechnol. Lett. **19**: 315-317.

Fujii, T. (1996). "Biosurfactants." J. Jpn. Oil Chem. Soc. **45**: 1013-1022.

Fujiwara, M. and T. Tamura (1996). "Recent knowledge on physicochemical properties of surfactant solutions and their application in the detergent field." J. Jpn. Oil Chem. Soc. **45**: 1145-1156.

Gandhi, N. N. (1997). "Applications of lipase." J. Am. Oil Chem. Soc. **74**: 621-634.

Gao, C., M. J. Whitcombe and E. N. Vulfson (1999). "Enzymatic synthesis of dimeric and trimeric sugar-fatty acid esters." Enzyme Microb. Technol. **25**: 264-270.

Garcia, H. S., K. J. Keough, J. A. Arcos and C. G. Hill, Jr. (2000). "Interesterification (acidolysis) of butterfat with conjugated linoleic acid in a batch reactor." J. Dairy Sci. **83**: 371-7.

Garcia, T., N. Sanchez, M. Martinez and J. Aracil (1999). "Enzymatic synthesis of fatty esters Part II. Optimization studies." Enzyme Microb. Technol. **25**: 591-597.

Gemert, R. W. and F. P. Cuperus (1998). Enzymatically catalysed process. WO 98/36058.

Giorno, L. and E. Drioli (2000). "Biocatalytic membrane reactors: applications and perspectives." Trends Biotechnol. **18**: 339-349.

Gmehling, J., M. Jochen, F. Kai and K. Jörg (1994). Azeotropic data. Weinheim, Wiley-VCH.

Goderis, H. L., B. L. Fouwe, S. M. Cauwenbergh and P. P. Tobback (1986). "Measurement and control of water content of organic solvents." Anal. Chem. **58**: 1561-1563.

Goto, M., T. Ono, F. Nakashio and T. A. Hatton (1997). "Design of surfactants suitable for protein extraction by reversed micelles." Biotechnol. Bioeng. **54**: 26-32.

Gray, C. J., J. S. Narang and S. A. Barker (1990). "Immobilization of lipase from *Candida cylindraceae* and its use in the synthesis of menthol esters by transesterification." Enzyme Microb. Technol. **12**: 800-807.

Greek, B. F. (1991). "Sales of detergents growing despite recession." Chem. Eng. News **69**: 25-52.

Grüning, B. and G. Hills (1999). Enzymatische Herstellung von regioselektiven Fettsäureestern der Ascorbinsäure. EP 0965645A2.

Guillardeau, L., D. Montet, N. Khaled, M. Pina and J. Graille (1992). "Fructose caprylate biosynthesis in a solvent free medium." Tenside Surf. Det. **29**: 342-344.

Guyot, B., B. Bosquette, M. Pina and J. Graille (1997). "Esterification of phenolic acids from green coffee with an immobilized lipase from Candida antarctica in solvent-free medium." Biotechnol. Lett. **19**: 529-532.

Haferburg, D., R. Hommel, R. Claus and H. P. Kleber (1986). "Extracellular microbial lipids as biosurfactants." Adv. Biochem. Eng. / Biotechnol. **33**: 53-93.

Hayashi, T. and H. Itagaki (1996). "Alternative to animal testing for safety assessment of cosmetic surfactants." J. Jpn. Oil Chem. Soc. **45**: 1179-1188.

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

Hirota, S. and Y. Sadzuka (1996). "Surface modifications of liposomes." J. Jpn. Oil Chem. Soc. **45**: 1125-1132.

Huang, F.-C. and Y.-H. Ju (1994). "Improved activity of lipase by vacuum drying on hydrophobic microporous support." Biotechnol. Tech. **8**: 827-830.

Huang, K.-H. and C. C. Akoh (1996). "Optimization and scale-up of enzymatic synthesis of structured lipids using RMS." J. Food Sci. **61**: 137-141.

Huang, R. Y. M. (1991). Pervaporation Membrane Separation Processes. Amsterdam, Elsevier.

Hubbs, J. (1997). Enzymatic process for the manufacture of ascorbic acid, 2-keto-L-gulonic acid and esters of 2-keto-L-gulonic acid. WO 97/43433.

Humeau, C., M. Girardin, D. Coulon and A. Miclo (1995). "Synthesis of 6-O-palmitoyl L-ascorbic acid catalyzed by Candida antartica lipase." Biotechnol. Lett. **17**: 1091-1094.

Humeau, C., M. Girardin, B. Rovel and A. Miclo (1998). "Effect of the thermodynamic water activity and the reaction medium hydrophobicity on the enzymatic synthesis of ascorbyl palmitate." J. Biotechnol. **63**: 1-8.

Humeau, C., M. Girardin, B. Rovel and A. Miclo (1998). "Enzymatic synthesis of fatty acid ascorbyl esters." J. Mol. Catal. B: Enzymatic **5**: 19-23.

Ikeda, I. and A. M. Klibanov (1993). "Lipase-catalyzed acylation of sugars solubilized in hydrophobic solvents by complexation." Biotechnol. Bioeng. **42**: 788-791.

Ismail, A., S. Soultani and M. Ghoul (1998). "Optimization of the enzymatic synthesis of butyl glucoside using response surface methodology." Biotechnol. Prog. **14**: 874-878.

Janssen, A. E. M., C. Klabbers, M. C. R. Franssen and K. V. Reit (1991). "Enzymatic synthesis of carbohydrate esters in 2-pyrrolidone." Enzyme Microb. Technol. **13**: 565-571.

Janssen, A. E. M., A. G. Lefferts and K. Riet (1990). "Enzymatic synthesis of carbohydrate esters in aqueous media." Biotechnol. Lett. **12**: 711-716.

Jeong, J. C. and S. B. Lee (1997). "Enzymatic esterification reaction in organic media with continuous water stripping: Effect of water content on reactor performance and enzyme agglomeration." Biotechnol. Techn. **11**: 853-858.

Jirage, K. B. and C. R. Martin (1999). "New developments in membrane-based separations." Trends Biotech. **17**: 197-200.

Kaitaranta, K. J. (1992). "Control of lipid oxidation in fish oil with various antioxidative compounds." J. Am. Oil Chem. Soc. **69**: 810-813.

Kashiwada, Y., G.-I. Nonaka and I. Nishioka (1984). "Tannins and related compounds. XXIII. Rhubarb (4): Isolation and structures of new classes of gallotannins." Chem. Pharm. Bull. **32**: 3461-3470.

Kawashiro, K., H. Sugahara, S. Sugiyama and H. Hayashi (1997). "Effect of organic solvents on enantioselectivity of protease catalysis." Biotechnol. Bioeng. **53**: 26-31.

Ke, T. and A. M. Klibanov (1998). "On enzymatic activity in organic solvents as a function of enzyme history." Biotechnol. Bioeng. **57**: 746-750.

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

Kim, P., D.-K. Oh, S.-Y. Kim and J.-H. Kim (1997). "Relationship between emulsifying activity and carbohydrate backbone structure of emulsan from Acinetobacter calcoaceticus RAG-1." Biotechnol. Lett. **19**: 457-459.

Kimura, Y., H. Okuda, S. Nishibe and S. Arichi (1987). "Effects of caffeoylglycosides on arachidonate metabolism in leukocytes." Planta Medica **53**: 148-153.

Kirk, O., F. D. Pedersen and C. C. Fuglsang (1998). "Preparation and properties of a new type of carbohydrate-based cationic surfactant." J. Surfactants Detergents **1**: 37-40.

Kitagawa, M. and Y. Tokiwa (1998). "Synthesis of polymerizable sugar ester possessing long spacer catalyzed by lipase from *Alcaligenes sp.* and its chemical polymerization." Biotechnol. Lett. **20**: 627-630.

Klibanov, A. M. (1989). "Enzymatic catalysis in anhydrous organic solvents." Trends Biochem. Sci. **14**: 141-144.

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

Kokubo, S., K. Matsuda and T. Katsuragi (1996). "Control of interface in various food systems." J. Jpn. Oil Chem. Soc. **45**: 1157-1168.

Koops, B. C., H. M. Verheij, A. J. Slotboom and M. R. Egmond (1999). "Effect of chemical modification on the activity of lipases in organic solvents." Enzyme Microb. Technol. **25**: 622-631.

Kosugi, Y. and N. Azuma (1994). "Continuous and consecutive conversion of free fatty acid in rice bran oil to triacylglycerol using immobilized lipase." Appl. Microbiol. Biotechnol. **41**: 407-412.

Kunieda, H. and Y. Hasegawa (1996). "Controlling phase behavior in nonionic surfactant system." J. Jpn. Oil Chem. Soc. **45**: 1053-1062.

Kvittingen, L., B. Sjursnes and T. Anthonsen (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.

Laane, C., S. Boeren, K. Vos and C. Veeger (1987). "Rules for optimization of biocatalysis in organic solvents." Biotechnol. Bioeng. **30**: 81-87.

Layman, P. L. (1985). "Industrial surfactants set for strong growth." Chem. Eng. News **23**: 23-48.

Lee, K.-T. and C. C. Akoh (1997). "Effects of selected substrate forms on the synthesis of structured lipids by two immobilized lipases." J. Am. Oil Chem. Soc. **74**: 579-584.

Lin, S. C. (1996). "Biosurfactants: recent advances." Chem. Technol. Biotechnol. **66**: 109-120.

Liu, G.-T., T.-M. Zhang, B.-E. Wang and Y.-W. Wang (1992). "Protective action of seven natural phenolic compounds against peroxidative damage to biomembranes." Biochem. Pharmcol. **43**: 147-152.

Liu, X. Y., F. L. Guo, Y. C. Liu and Z. L. Liu (1996). "Remarkable enhancement of antioxidant activity of vitamin C in an artificial bilayer by making it lipo-soluble." Chem. Phys. Lipids **83**: 39-43.

Liu, Z. Q., L. P. Ma and Z. L. Liu (1996). "Making vitamin C lipophilic enhances its protective effect against free radical induced peroxidation of low density lipoprotein." Chem. Phys. Lipids **95**: 49-57.

Ljunger, G., P. Adlercreutz and B. Mattiasson (1994). "Lipase catalysed acylation of glucose." Biotechnol. Lett. **16**: 1167-1172.

Lobell, M. and M. P. Schneider (1994). "Synthesis of hydroxycarboxylic acid vinyl esters." Synthesis: 375-377.

Madsen, T., G. Petersen, C. Seirero and J. Torslov (1996). "Biodegradability and aquatic toxicity of glycoside surfactants and a nonionic alcohol ethoxylate." J. Am. Oil Chem. Soc. **73**: 929-933.

Malcata, F. X. (1990). "Immobilized lipase reactors for modification of fats and oil." J. Am. Oil Chem. Soc. **67**: 890-910.

McNeill, G. P., S. Shimizu and T. Yamane (1991). "High-yield enzymic glycerolysis of fats and oils." J. Am. Oil Chem. Soc. **68**: 1-5.

Minamikawa, H. and M. Hato (1996). "Physical properties of synthetic glycolipid/water systems --- oligasaccharide stereochemistry and molecular self-assembly." J. Jpn. Oil Chem. Soc. **45**: 1001-1012.

Mohamed, H. M. A., S. Bloomer and K. Hammadi (1993). "Modification of fats by lipase interesterification I: Changes in glyceride structure." Fat Sci. Technol. **95**: 428-431.

Montagomery, D. C. (1997). Design and analysis of experiments. New York, John Wiley & Sons Inc.

Montero, S., A. Blanco, M. D. Virto, L. C. Landeta, I. Agud, R. Solozabal, J. M. LAscaray, M. Renobales, M. J. LIama and J. L. Serra (1993). "Immobilization of *Candida rugosa* lipase and some properties of the immobilized enzyme." Enzyme Microb. Technol. **15**: 239-247.

Munoz, F. G., A. P. Oseguera, J. Cassani, M. J. Estrada, R. V. Duhalt and A. L. Munguia (1999). "Enzymatic synthesis of fructoseyl glycerol." J. Carbohyd. Chem. **18**: 275-283.

Mutua, L. N. and C. C. Akoh (1993). "Synthesis of alky glycoside fatty acid esters in non-aqueous media by *Candida sp.* lipase." J. Am. Oil Chem. Soc. **70**: 43-46.

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

Neel, J., G. J. Nguyen, R. Clement and R. Francois (1987). Separation of water-organic liquid mixtures by pervaporation. San Antonio, Bakish Mat. Corp. 126-140.

Nienow, A. W., G. Hunt and B. C. Buckland (1996). "A fluid dynamic study using a simulated viscous, shear thinning broth of the retrofitting of large agitated bioreactors." Biotechnol. Bioeng. **49**: 15-19.

Nihro, Y., S. Sogawa, A. Izumi, A. Sasamori, T. Sudo, T. Miki, H. Matsumoto and T. Satoh (1992). "3-O-alkyascorbic acids as free radical quenchers. 3. protective effect on coronary occlusion-reperfusion induced arrhythmias in anesthetized rats." J. Med. Chem. **35**: 1618-1623.

Nonaka, G.-I. and I. Nishioka (1983). "Tannins and related compounds. X. Rhubarb (2): Isolation and structures of a glycerol gallate, gallic acid glucoside gallates, galloylglucoses and isolindleyin." Chem. Pharm. Bull. **31**: 1652-1658.

Nonaka, G.-I., I. Nishioka, T. Nagasawa and H. Oura (1981). "Tannins and related compounds. I. Rhubarb (1)." Chem. Pharm. Bull. **29**: 2862-2870.

Nostro, P. L. (1997). "Supramolecular aggregates from vitamin C derivatives: Structure and properties." Internet J. Sci. Biol. Chem. **4**.

Ogino, K. (1996). "Research and development of novel surfactants." J. Jpn. Oil Chem. Soc. **45**: 921-932.

Oguntimein, G. B., H. Erdmann and R. D. Schmid (1993). "Lipase catalyzed synthesis of sugar ester in organic solvents." Biotechnol. Lett. **15**: 175-180.

Oh, D.-K., J.-H. Kim and T. Yoshida (1997). "Production of a high viscosity polysaccharide, methylan, in a novel bioreactor." Biotechnol. Bioeng. **54**: 115-121.

Okazaki, S. Y., N. Kamiya, M. Goto and F. Nakashio (1997). "Enantioselective esterification of glycidol by surfactant-lipase complexes in organic media." Biotechnol. Lett. **19**: 541-543.

Ollis, D. L., E. Cheah, M. Cygler, B. Dijkstra, F. Frolow and M. Franken (1992). Protein Eng. **5**: 197-203.

Omar, I. C., H. Saeki, N. Nishio and S. Nagai (1988). "Hydrolysis of triglycerides by immobilized thermostable lipase from Humicola lanuginosa." Agric. Biol. Chem. **52**: 99-105.

Oosterom, M. W., F. Rantwijk and R. A. Sheldon (1996). "Chemoenzymatic synthesis of 6-O-alkanoyl-N-alkylgluconamides." Fett/Lipid **98**: 390-393.

Osipow, L. I. and W. Rosenblatt (1967). "Micro-emulsion process for the preparation of sucrose esters." J. Am. Oil Chem. Soc. **44**: 307.

Otero, C., I. Del-Val, L. Robledo, C. Torres, J. A. Arcos and J. Perez-Gil (1996). "Conformational changes of different isolipases from Candida rugosa in liquid interfaces and after their contact with low-water-content media." Ann. N. Y. Acad. Sci. **799**: 324-327.

Otto, R., U. T. Bornscheuer, C. Syldatk and R. D. Schmid (1998). "Lipase-catalyzed synthesis of arylaliphatic esters of b-D(+)-glucose, alkyl- and arylglucosides and characterization of their surfactant properties." J. Biotechnol. **64**: 231-237.

Otto, R., H. Scheib, U. T. Bornscheuer, J. Pleiss, C. Syldatk and R. D. Schmid (1999). "Substrate specificity of lipase B from *Candida antarctica* in the synthesis of arlyaliphatic glycolipids." J. Mol. Catal. B: Enzymatic **8**: 210-211.

Otto, R. T., U. T. Bornscheuer, H. Scheib, J. Pleiss, C. Syldatk and R. D. Schmid (1998). "Lipase catalyzed esterification of unusual substrates: Synthesis of glucuronic acid and ascorbic acid (vitamin C) esters." Biotechnol. Lett. **20**: 1091-1094.

Otto, R. T., U. T. Bornscheuer, C. Syldatk and R. D. Schmid (1998). "Synthesis of aromatic *n*-alkyl-glucoside esters in a coupled ß-glucosidase and lipase reaction." Biotechnol. Lett. **20**: 437-440.

Padt, A. V., J. J. W. Sewalt and K. V. Riet (1993). "On-line water removal during enzymatic triacylglycerol synthesis by means of pervaporation." J. Membrane Sci. **80**: 199-208.

Park, H. G. and H. N. Chang (2000). "Enzymatic regioselective synthesis of sucrose acrylate esters." Biotechnol. Lett. **22**: 39-42.

Pelenc, V. P., F. M. B. Paul and P. F. Monsan (1993). Method for the enzymatic production of a-glucosides and esters of a-glucosides, and utilization of products thus obtained. WO 9304185.

Polat, T., H. G. BAzin and R. J. Linhardt (1997). "Enzyme catalyzed regioselective synthesis of sucrose fatty acid ester surfactants." J. Carbohyd. Chem. **16**: 1319-1325.

Pulido, R., F. O. López and V. Gotor (1992). "Enzymatic regioselective acylation of hexoses and pentoses using oxime esters." J. Chem. Soc. Perkin Trans. **1**: 2981-2988.

Rapin, J. L. (1988). The BETHENIVILLE pervaporation unit. The first large-scale productive plant for the dehydration of ethanol. Third intern. confer. on pervaporation processes in the chem. industry, Nancy, France.

Redmann, I., M. Pina, B. Guyot, P. Blaise, M. Farines and J. Graille (1997). "Chemoenzymatic synthesis of glucose fatty acid esters." Carbohyd. Res. **300**: 103-108.

Rekker, R. F. and H. M. Kort (1979). "The hydrophobic fragmental constant; an extension to a 1000 data point set." Eur. J. Med. Chem. **14**: 479-488.

Reslow, M., P. Adlercreutz and B. Mattiasson (1992). "Modification of the microenvironment of enzymes in organic solvents. Substitution of water by polar solvents." Biocatalysis **6**: 307-318.

Riva, S. (1994). Enzymatic synthesis of carbohydrate esters. in Carbohydrate polyesters as fat substitutes. C. C. Akoh Ed. New York, Dekker**:** 37-64.

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

Röck, H. (1960). Destillation im Laboratorium: Extraktive und Azeotrope Destillation. Darmstadt, Dr. Dietrich Steinkopff Verlag.

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

Roure, F., A. Ducret, M. Trani and R. Lortie (1997). "Enantioselective esterification of racemic ibuprofen in solvent media under reduced pressure." J. Chem. Tech. Biotechnol. **69**: 266-270.

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

Sarney, D. B., M. J. Barnard, M. Virto and E. N. Vulfson (1997). "Enzymatic synthesis of sorbitan esters using a low-boiling-point azeotrope as a reaction solvent." Biotechnol. Bioeng. **54**: 351-356.

Sarney, D. B., H. Kapeller, G. Fregapane and E. N. Vulfson (1994). "Chemo-enzymatic synthesis of disaccharide fatty acid esters." J. Am. Oil Chem. Soc. **71**: 711-714.

Sarney, D. B. and E. N. Vulfson (1995). "Application of enzymes to the synthesis of surfactants." *Trends Biotechnol.* **13**: 164-172.

Scheckermann, C., A. Schlotterbeck, M. Schmidt, V. Wray and S. Lang (1995). "Enzymatic monoacylation of fructose by two procedures." Enzyme Microb. Technol. **17**: 157-162.

Schlotterbeck, A., S. Lang, V. Wray and F. Wagner (1993). "Lipase-catalyzed monoacylation of fructose." Biotechnol. Lett. **15**: 61-64.

Schroen, C. G. P. H. and J. M. Woodley (1997). "Membrane separation for downstream processing of aqueous-organic bioconversions." Biotechnol. Prog. **13**: 276-283.

Sharma, A. and S. Chattopadhyay (1993). "Lipase catalysed acylation of carbohydrates." Biotechnol. Lett. **15**: 1145-1193.

Shibatani, S., M. Kitagawa and Y. Tokiwa (1997). "Enzymatic synthesis of vinyl sugar ester in dimethylformamide." Biotechnol. Lett. **19**: 511-514.

Sin, Y. M., K. W. Cho and T. H. Lee (1998). "Synthesis of fructose esters by Pseudomonas sp. lipase in anhydrous pyridine." Biotechnol. Lett. **20**: 91-94.

Soumanou, M. M., U. T. Bornscheuer and R. D. Schmid (1998). "Two-step enzymatic reaction for the synthesis of pure structured triglycerides." J. Am. Oil Chem. Soc. **75**: 703-710.

Takeda, T. (1996). "Synthesis and properties of chemically degradable surfactants." J. Jpn. Oil Chem. Soc. **45**: 975-990.

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

Torres, C., M. Bernabé and C. Otero (2000). "Enzymatic synthesis of lactic acid derivatives with emulsifying properties." Biotechnol. Lett. **22**: 331-334.

Tsuzuki, W., Y. Kitamura, T. Suzuki and S. Kobayashi (1999). "Synthesis of sugar fatty acid esters by modified lipase." Biotechnol. Bioeng. **64**: 267-271.

Tweddell, R. J., S. Kermasha, D. Combes and A. Marty (1997). "Optimization of the reaction medium for esterification catalyzed by a lipase from Pseudomonas fluorescens." Biotechnol. Lett. **19**: 939-942.

Ujang, Z., N. A. Sharbati and A. M. Vaidya (1997). "Organic-phase enzymatic esterification in a hollow fiber membrane reactor with in situ gas-phase water activity control." Biotechnol. Prog. **13**: 39-42.

Umetrics, A. (1997). *MODDE for Windows: Statistical modeling and experimental design, Graphical Software for Statistical Experimental Design (User's Guide)*. Umea.

Ushiyama, M., S. Kumagai and T. Furuya (1989). "Biotransformation of phenylcarboxylic acids by plant cell cultures." Phytochemistry **28**: 3335-3339.

Vainionpää, J. (1991). "Modelling of extrusion cooking of cereals using response surface methodology." J. Food Eng. **13**: 1-26.

Valivety, R., P. Jauregi, I. Gill and E. Vulfson (1997). "Chemo-enzymatic synthesis of amino acid-based surfactants." J. Am. Oil chem. Soc. **74**: 879-886.

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

Valivety, R. H., P. J. Halling, A. D. Peilow and A. R. Macrae (1992). "Lipases from different sources vary widely in dependence of catalytic activity on water activity." Biochem. Biophys. Acta **1122**: 143-146.

Valivety, R. H., G. A. Johnston, C. J. Suckling and P. J. Halling (1991). "Solvent effects on biocatalysis in organic systems: Equilibrium position and rates of lipase catalyzed esterification." Biotechnol. Bioeng. **38**: 1137-1143.

Wang, S.-J. and J.-J. Zhong (1996). "A novel centrifugal impeller bioreactor. I. Fluid circulation, mixing, and liquid velocity profiles." Biotechnol. Bioeng. **51**: 511-519.

Wang, S.-J. and J.-J. Zhong (1996). "A novel centrifugal impeller bioreactor. II. Oxygen transfer and power consumption." Biotechnol. Bioeng. **51**: 520-527.

Wisdom, R. A. (1984). "Enzyme esterification of fats: Factors influencing the choice of support for immobilized lipase." Enzyme Microb. Technol. **6**: 443-446.

Wolff, A., L. Zhu, V. Kielland, A. J. J. Straathof, J. A. Jongejan and J. J. Heijnen (1997). "Simple dissolution-reaction for enzymatic conversion of suspension of solid substrate." Biotechnol. Bioeng. **56**: 433-440.

Xu, X., H. Mu, C.-E. Hoy and J. Adler-Nissen (1999). "Production of specific-structured lipids by enzymatic interesterification in a pilot enzyme bed reactor: Process optimization by reponse surface methodology." Fett/Lipid **101**: 207-214.

Xu, X., A. R. Skands, J. Adler-Nissen and C.-E. Hoy (1998). "Production of specific-structured lipids by enzymatic interesterification in a pilot enzyme bed reactor: Optimization of the reaction by reponse surface design." Fett/Lipid **100**: 463-471.

Xu, X., A. R. H. Skands, J. Adler-Nissen and C.-E. Hoy (1998). "Production of specific structurned lipids by enzymatic interesterification: optimization of the reaction by response surface design." Fett/Lipid **100**: 463-471.

Xu, X., A. R. H. Skands, C.-E. Hoy, H. Mu, S. Balchen and J. Adler-Nissen (1998). "Production of specific-structured lipids by enzymatic interesterification: Elucidation of acyl migration by reponse surface design." J. Am. Oil Chem. Soc. **75**: 1179-1186.

Yahagi, K. and H. Iwai (1996). "Application of surfactants in personal care products." J. Jpn. Oil Chem. Soc. **45**: 1133-1144.

Yan, Y., U. T. Bornscheuer, L. Cao and R. D. Schmid (1999). "Lipase-catalyzed solid phase synthesis of sugar fatty acid esters: Removal of by-products by azeotropic distillation." Enzyme Microb. Technol. **25**: 725-728.

Yan, Y., U. T. Bornscheuer and R. D. Schmid (1999). "Lipase-catalyzed synthesis of Vitamin C fatty acid esters." Biotechnol. Lett. **21**: 1051-1054.

Yan, Y., U. T. Bornscheuer and R. D. Schmid (2000). “Efficient water removal in lipase-catalyzed esterifications using a low-boiling-point azeotrope.” Biotechnol. Bioeng. **submitted**.

Yan, Y., U. T. Bornscheuer, G. Stadler, S. Lutz-Wahl, M. Reuss and R. D. Schmid (2001). “Production of sugar fatty acid esters by enzymatic esterification in a stirred-tank membrane reactor: Optimization of parameters by response surface methodology.” J. Am. Oil Chem. Soc.: **78**: 147-152.

Yan, Y., U. T. Bornscheuer, G. Stadler, R. Reuss and R. D. Schmid (2000). “Enzymatic production of biosurfactant 6-*O*-glucose fatty acid monoesters.” Journal of Detergent & Cosmetics **in press**.

Yan, Y., R. T. Otto, U. T. Bornscheuer, C. Syldatk and R. D. Schmid (2000). Procedure for selective esterifications of polyols. DE 19924221A1.

Yang, Z. and D. A. Robb (1994). “Partition of coefficients of substrates and products and solvent selection for biocatalysis under nearly anhydrous conditions.” Biotechnol. Bioeng. **43**: 365-370.

Zaks, A. and A. M. Klibanov (1985). “Enzyme-catalysed processes in organic solvents.” Proc. Natl. Acad. Sci. **82**: 3192-3196.

Zunft, H.-J. F. and K. Ragotzky (1997). “Strategien zur Fettsubstitution in Lebensmitteln.” Fett/Lipid **99**: 204-213.

Curriculum vitae

Name:	Yan, Youchun
Date of Birth:	May 10, 1966
Place of Birth:	Zhejiang, P.R. China
Citizenship:	Chinese
Civil Status:	Married, one son

University Education

May 1997---February 2001 (Ph.D.)	Institute of Technical Biochemistry, University Stuttgart, Germany
September 1987---January 1990 (M.Sc.)	Department of Food Engineering, Zhengzhou Grain College, Zhengzhou, China
September 1980---July 1984 (B.Sc.)	Department of Food Engineering, Zhengzhou Grain College, Zhengzhou, China

School Education

September 1978---July 1980	High school, Jinhua, Zhejiang, China
September 1976---July 1978	Middle School, Lanxi, Zhejiang, China
February 1971---July 1976	Primary school, Lanxi, Zhejiang, China

Work Experience

February 1990-October 1993	Research engineer, Grain Research Institute of Zhejiang province, Hangzhou, China
August 1984-August 1987	Assistant engineer, Shengxian Oleochemicals Co., Zhejiang, China