To my family

PRODUCTION AND CHARACTERIZATION OF ENZYMATICALLY PRODUCED SURFACTANTS

A MASTER'S THESIS

in

Food Engineering

University of Gaziantep

by

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July 2000

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ABSTRACT

PRODUCTION AND CHARACTERIZATION OF ENZYMATICALLY PRODUCED SURFACTANTS

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July 2000, 66 Pages

Propyl laurate and lauric acid esters of fructose were produced by using immobilized lipase (Novozym 435). Molecular sieve was used to shift the reaction towards the synthesis. In the first part of the work, the optimum working parameters of the enzyme for the production of propyl laurate were investigated. The mono, -di and higher esters of lauric acid with fructose were obtained and analyzed qualitatively and quantitatively by HPLC. After the purification of products by column chromatography, the surfactant properties (HLB value, critical micelle concentration, surface tension and emulsion stabilization) were evaluated experimentally.

According to the results, maximum amount of propyl laurate was obtained when isopropyl alcohol was used. The product formation was followed by a linear increase up to lauric acid concentration of 5 mmole. The maximum activity was obtained at 60°C. The amount of product seemed to be linearly proportional to enzyme concentration up to 50 mg. There was a linear increase in the amount of propyl laurate up to 50 min of reaction time.

The amount of esters was observed to vary depending on the ratio of lauric acid to fructose and the reaction time. The HLB value of propyl laurate, fructose monolaurate and fructose dilaurate were calculated as 3.5, 8.95 and 5.3, their CMC values were calculated as 6.84×10^{-4} M, 7.20×10^{-5} M and 6.40×10^{-5} M,

respectively. The CMC values of esters obtained from conductivity curve and surface tension curve were similar to each other. It was found that when the concentration of surfactant increased, the stabilization of emulsion also increased. Fructose dilaurate showed better stability than the other esters.

Results showed that, these enzymatically prepared and purified substances have the properties of general surfactants. In addition, their use in food industry was found to be suitable depending on the advantages such as use of natural sources in their production, ease of purification and modification, absence of toxic impurity in the final product. They are also harmless to the environment because they are biodegradable.

Key words : *Candida antarctica* lipase, esterification, surfactants, emulsifiers, lauric acid, fructose

ÖZET

ENZİMATİK YOLLA YÜZEY AKTİF MADDELERİN ÜRETİMİ VE ÖZELLİKLERİNİN İNCELENMESİ

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Temmuz 2000, 66 Sayfa

Propil laurate ve laurik asidin früktoz esterleri, tutuklanmış lipaz enzimi (Novozym 435) kullanılarak üretildi. Reaksiyonu senteze yönlendirmek için, moleküller elek kullanıldı. Çalışmanın ilk bölümünde, propil laurate üretimi için, enzimin optimum çalışma parametreleri belirlendi. Laurik asit ve früktozdan mono, -di ve yüksek esterleri üretilerek ve kalitatif ve kantitatif olarak HPLC ile analiz edildi. Ürünlerin kolon kromatografisi kullanılarak saflaştırılmasından sonra, yüzey aktif maddesi özellikleri (HLB değeri, kritik miselle konsantrasyonu, yüzey gerilimi ve emülsiyon kararlılığı) deneysel olarak incelendi.

Sonuçlara göre, maksimum propil laurate miktarına isopropil alkol kullanılarak ulaşıldı. Ürün miktarı, 5 mmol laurik asit derişimine kadar lineer idi. Maksimum aktivite 60 ° C de elde edildi. Ürün oluşumu, enzim miktarı 50 mg oluncaya değin lineer artış gösterdi. Propil laurate miktarı 50 dakikaya kadar lineer olarak arttı.

Esterlerin miktarı laurik asit/früktoz oranına ve reaksiyon zamanına göre değişti. Propil laurate, früktoz monolaurate ve früktoz dilaurate ın HLB değerleri sırasıyla 3.5, 8.95 ve 5.3 dür. CMC değerleri ise 6.84×10^{-4} M, 7.20×10^{-5} M ve 6.40×10^{-5} M idi. İletkenlik ve yüzey gerilim grafiklerinden elde edilen CMC değerleri birbirine benzerlik

gösterdi. Yüzey aktif maddesinin konsantrasyonu arttığı zaman, emülsiyon kararlığının da arttığı gözlendi. Früktoz dilaurate diğer esterlerden daha iyi dayanıklılık sağladı.

Sonuçlar, enzimatik olarak hazırlanan ve saflaştırılan bu madddelerin, genel yüzey aktif maddelerinin özelliklerini taşıdıklarını göstermiştir. Gıda endüstrisinde kullanılmaları, üretimlerinde doğal kaynakların kullanımı, saflaştırılmalarının ve modifikasyonlarının kolay olması, son üründe toksik kalıntı içermemeleri gibi avantajlarının olmasından dolayı uygun bulundu. Biyolojik olarak parçalanabilme özelliği taşıdıklarından dolayı da çevreye zarar vermemektedirler.

Anahtar kelimeler: *Candida antarctica* lipaz, esterifikasyon, yüzey aktif maddeleri, emülgatörler, laurik asit, früktoz,

ACKNOWLEDGEMENT

I wish to express my appreciation to my supervisors, Assist. Prof. Dr. Sibel FADILOĞLU and Assist. Prof. Dr. Esra İBANOĞLU for their patient supervisions, continuos guidance, invaluable suggestions and continuos encouragement.

I wish to thank Assoc. Prof. Dr. Ahmet KAYA for his help and suggestions for the HPLC works.

I always feel indebted to my husband, Ahmet Seçkin, for his love and help to complete this work.

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LIST OF SYMBOLS

The following nomenclature defines the principal symbols used in the thesis.

Symbols	Description
HPLC	High Pressure Liquid Chromatography
HLB	Hydrophile - Lipophile - Balance Value
CMC	Critical Micelle Concentration
FDA	Food and Drug Administration
w/o	Water in oil
o/w	Oil in water
PIT	Phase Inversion Temperature
GC	Gas Chromatography
TLC	Thin-Layer Chromatography
C-NMR	Carbon-Nuclear Magnetic Resonance
PLU	Propyl Laurate Unit
THF	Tetrahydrofuran
$CHCl_3$	Chloroform
CH_3OH	Methanol
I	Current
mA	Miliamper

CHAPTER 1
INTRODUCTION

The word SurfActAnt is constructed from the phrase Surface Active Agent. It conveys the idea that surfactants are designed to concentrate at surfaces or interfaces. There are many other names used for surfactants; wetting agents, dispersants, emulsifiers, foaming agents, solubilizers and detergents [1].

The four major classifications of surfactants are; anionic, cationic, nonionic and amphoteric. Anionic surfactants have a negative charge and cationic surfactants have a positive charge in aqueous solution. Nonionic surfactants have no charge in aqueous solutions. Amphoteric surfactants develop a negative or positive charge depending on whether the solution is alkaline or acidic [2].

Nonionic surfactants are a class of synthetic surfactants. They are prepared by attaching ethylene oxide molecules to a water insoluble molecule. The ethylene oxide molecules, derived from petroleum, are water soluble polymers. Depending on the number of ethylene oxides and the number of carbon atoms, the synthetic surfactants can be classified as a wetting agent, a detergent, or an emulsifier.

The important aspects for the determination of surface activity are the Hydrophile Lipophile Balance value (HLB), Critical Micelle Concentration (CMC), surface tension reduction and emulsion stabilization [3]. CMC can be defined as the required minimum surfactant concentration to reach the lowest surface tension value [4]. This value can be calculated both by conductivity curve and surface tension curve of surfactant solution.

Surfactants are produced chemically, microbiologically and enzymatically. Compared with conventional chemical synthesis, enzymatically prepared surfactants have gained importance in last years. They have the advantage of low energy requirement, minimal thermal degradation, high biodegradability, being able to produced from natural substances [5].

The most important group of surfactants is the fatty acid derivatives. Fatty acid esters and fatty acid esters of sugars are the ones most widely used in the food

industry. The nature of enzyme, water activity, type of sugar and fatty acid, temperature, time and solvent are the important parameters for the production of sugar fatty acid esters by means of enzymatic reactions.

The enzymes used in the surfactant manufacture are lipases, proteases and glycosidases. Immobilized form of them have many advantages. Most researchers used immobilized form of C*andida antarctica* lipase for the production of surfactants. The main problem for the production of sugar fatty acid esters is the water produced during reaction. To shift the reaction towards synthesis, most researchers used molecular sieves [6].

The aim of this research is to synthesize surfactants from lauric acid and fructose by using immobilized lipase, to determine the optimum working parameters for the enzyme, to determine the effects of mole ratio of lauric acid to sugar and reaction time on product formation. The products were analyzed by using HPLC. After purification of esters by column chromatography, the surfactant properties, HLB value, CMC, surface tension and emulsion stability were investigated.

CHAPTER 2
LITERATURE REVIEW

Surfactant is a contraction of surface active agent and refers to a class of chemical compounds known technically as amphiphiles (from two Greek words meaning that they are not certain what they like). As shown in Figure 2.1, the molecules of such compounds consist of two regions of very different characteristics: one part is polar (either a dipole or a charged group) and the other is non-polar (usually a hydrocarbon or halocarbon chain) [7].

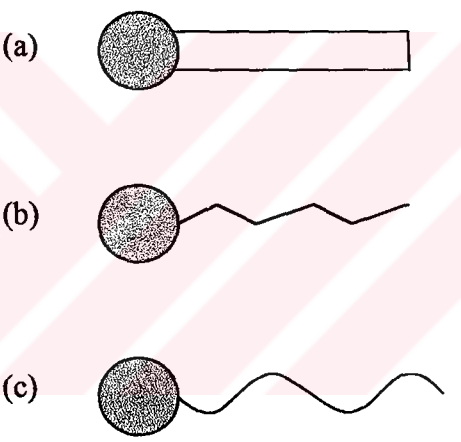


Figure 2. 1. Representation of a surfactant molecule. The round head group is polar and the tail is non polar. The representations here emphasize (a) space filling, (b) number of carbons in the chain, (c) flexibility.

Surfactants are designed to concentrate at surfaces or interfaces (Figure 2.2). The formation of such an ordered molecular film at the interface lowers the interfacial energy (tension) and is responsible for the unique properties of surfactant molecules. In addition to lowering the interfacial tension the molecular layer also dominates the interfacial rheological behavior and mass transfer.

Typical consumer products which use surfactants include bars of soaps, dishwashing liquids, laundry detergents, shampoos, mouthwashes and toothpaste. Most such surfactants must have FDA status as indirect Food Additives. Many

surfactants also have Direct Food Additives status. They can be an ingredient in food. Typical surfactants in this class include the molecules called "polysorbates"[8].

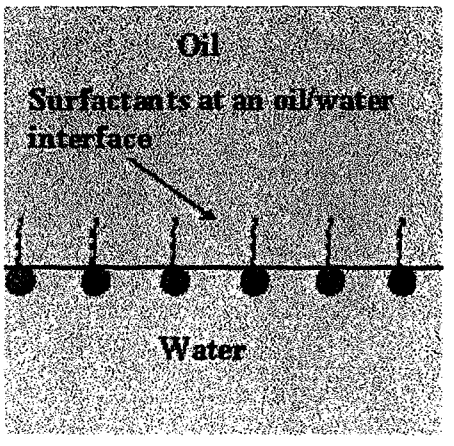


Figure 2. 2. Surfactants at an oil/water interface

2. 1. Other Names for Surfactants

Surfactants are found in almost every manufactured item in modern life. Because of these they are called by many different names. These words describe the many effects of surfactants on physical systems as well as their uses [9].

(a) Dispersants: They assist in the formation of stable dispersions by preventing aggregation of the solid particles in the system. This keeps the particles from sticking together and becoming too big to stay dispersed in the water (Figure 2.3).

(b) Fabric conditioners: As hair and fabric conditioners and softeners they lubricate and reduce the surface energy of fibers (Figure 2.4).

(c) Detergents: As shown in Figure 2.5, they remove contaminants from surfaces (soaps are detergents which are mild to human skin) [10].

(d) Solubilizers: They allow immiscible fluids to form isotropic (single phase) solutions (Figure 2.6).

(e) Emulsifiers: As emulsifiers, surfactants allow the stable dispersion of oil-in-water (o/w) by lowering interfacial tension and prevent droplet coalescence (Figure 2.7).

(f) Wetting agents: They reduce the free energy of the new interfaces (air/liquid and solid/liquid) being formed, thus promoting their formation and spreading of the liquid (Figure 2.8).

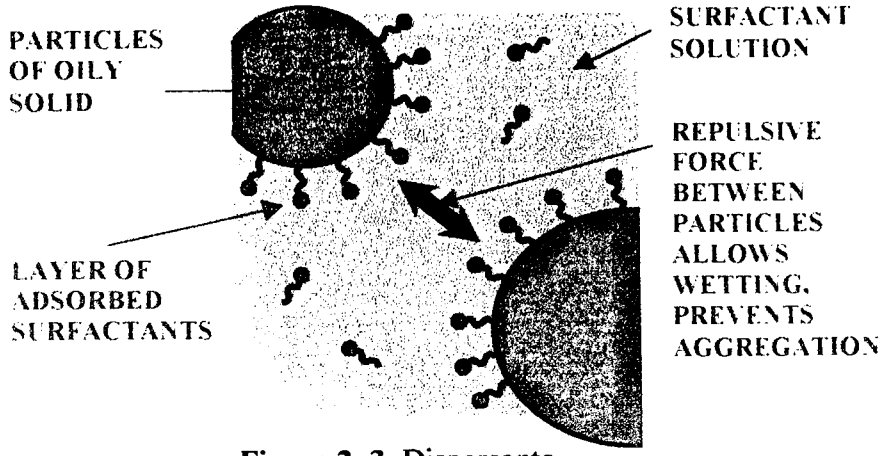


Figure 2. 3. Dispersants

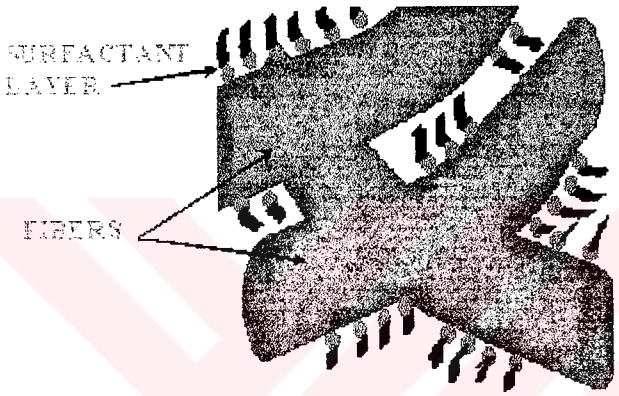


Figure 2. 4. Fabric conditioner

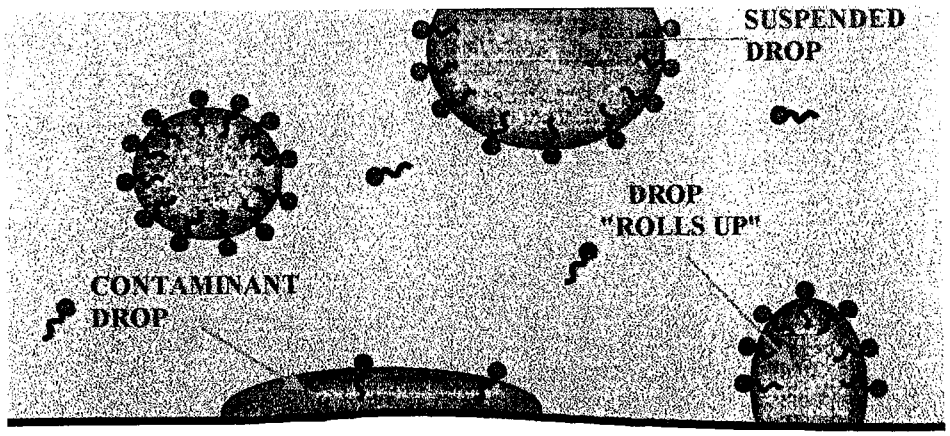


Figure 2. 5. Detergents

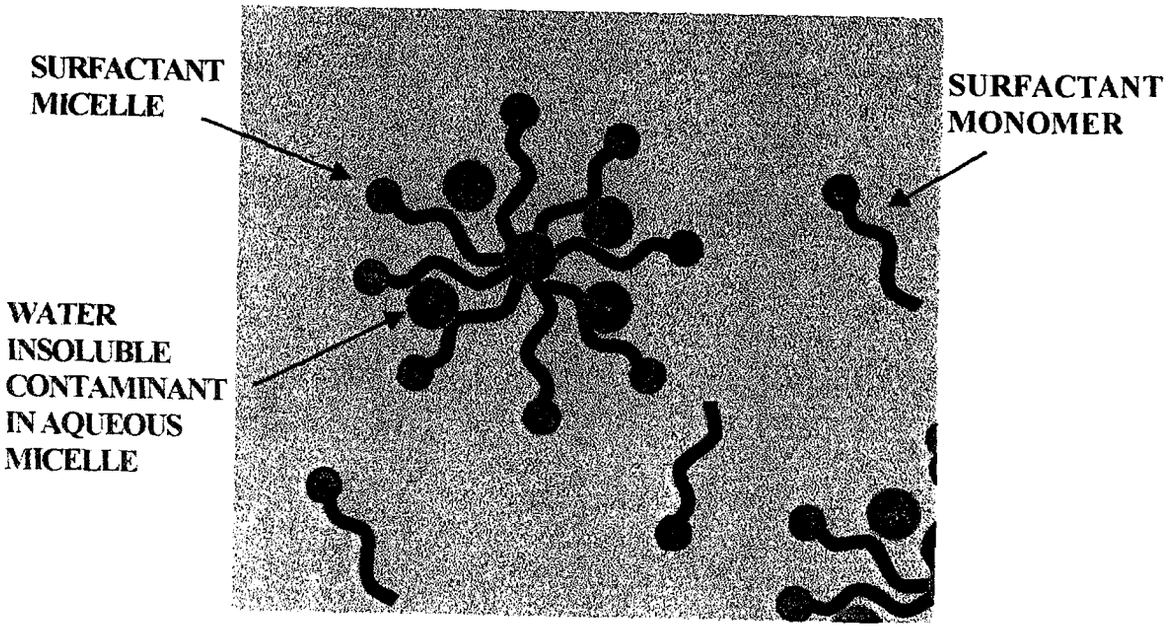


Figure 2. 6. Solubilizers

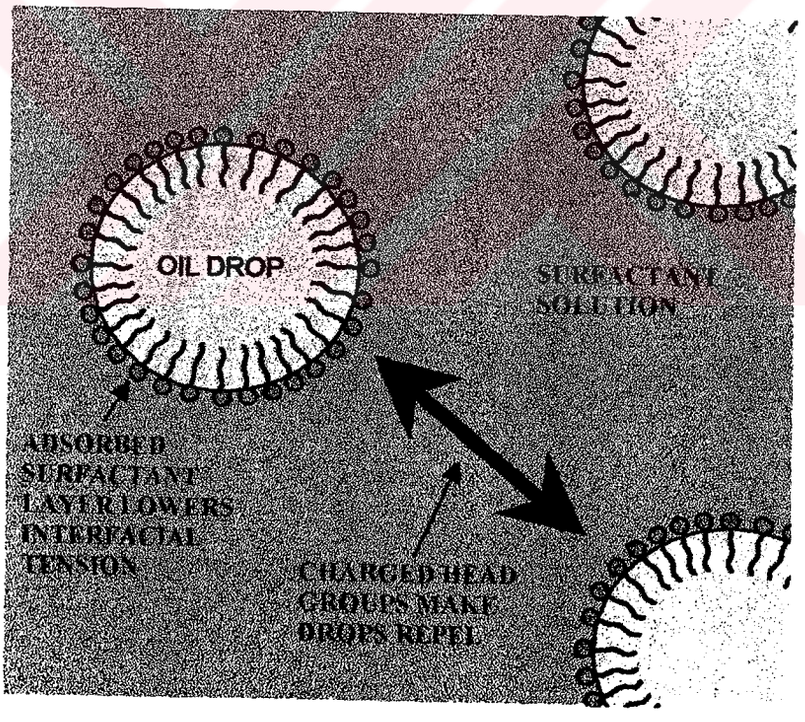


Figure 2. 7. Emulsifiers

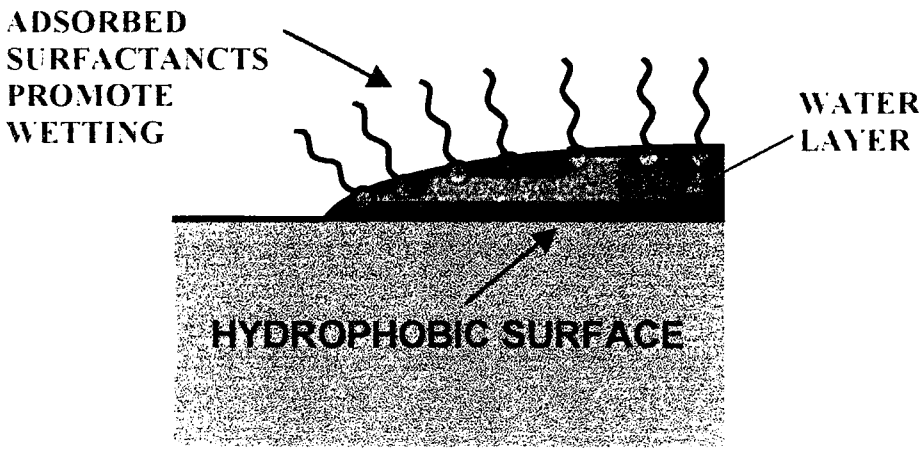


Figure 2. 8. Wetting agents

2. 2. Classification of Surfactants

There are many types of classification methods for surfactants.

2. 2. 1. Classification Based on Raw Material

An early method of classifying surfactants based on the source of raw materials used in their manufacture, such as petroleum, fat or oil, coal or carbohydrate [1].

2. 2. 2. Classification Based on Ionic Charge

As shown in Figure 2.9, another classification based on the presence or absence of ionic charge. Although this type of classification is useful for the purpose of product formulation and application, it is not adequate for the purpose of either quantitative or qualitative analysis.

2. 2. 3. Classification Based on Structure

According to Kosaric (1992), surfactants may be classified in terms of their structure;

(a) hydroxylated and cross linked fatty acids

(b) polysaccharide-lipid complexes

(c) glycolipids

(d) lipoprotein-lipopeptides

(e) phospholipids

(f) complete cell surfaces

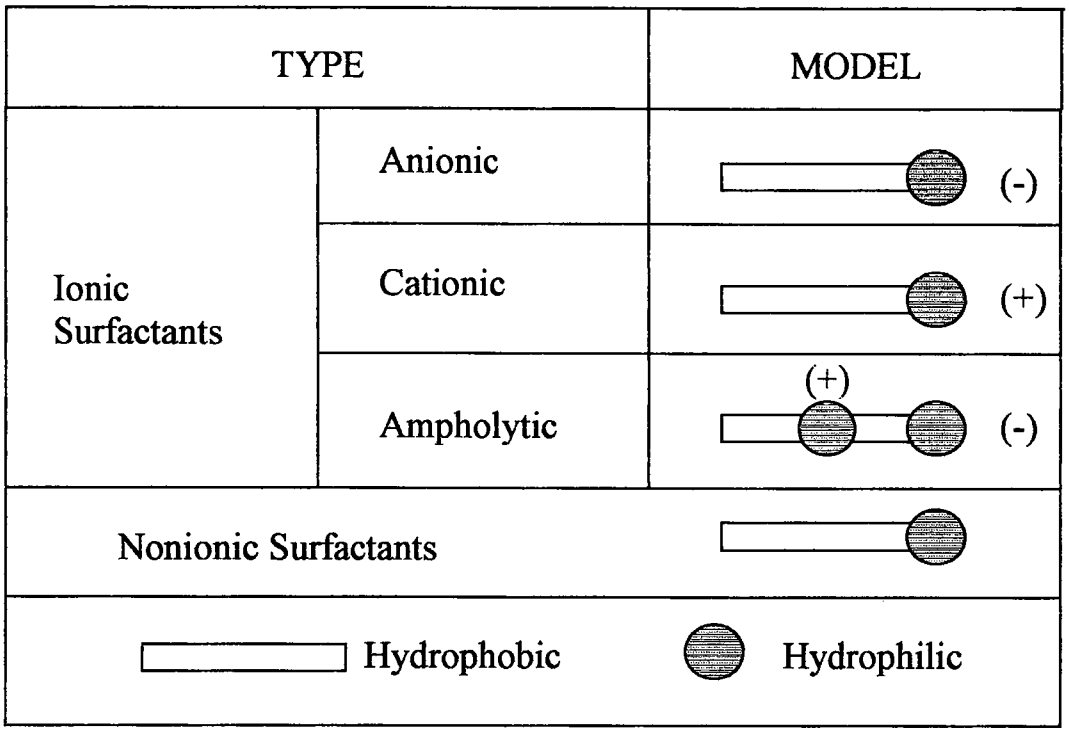


Figure 2. 9. Classification of surfactants depending on ionic charge

2. 2. 4. Classification Based on Elemental and Functional Group Analysis

An entirely different classification, based on elemental and functional group analysis in addition to charge type are designed to meet the particular need of analyst (Table 2.1).

2. 2. 5. Classification Based on Production Methods

Surfactants are produced chemically, microbiologically and enzymatically. The microbiologically and enzymatically prepared surfactants have gained importance in last years because they have many advantages [1]. These are discussed in later.

2. 3. Evaluation of Surfactant Activity

2. 3. 1. Critical Micelle Concentration

One of the most widely used indexes for evaluating surfactant activity is the critical micelle concentration (CMC). The CMC is the minimum surfactant concentration required for reaching the lowest interfacial or surface tension values.

Table 2. 1. Classification of surfactants by ionic type [11].

Anionics	**Cationics**	**Nonionics**	**Ampholytic**
Soaps	Primary alkyl ammonium salts	Esters of polyhydric alcohols	Betaines
Sulfated esters	Secondary alkyl ammonium salts	Alkolxylated amines	Aminoacids
Sulfated amides	Tertiary alkyl ammonium salts	Esters of polyoxy alkylene glycols	
Sulfated alcohol's	Quaternary alkyl ammonium salts	Ethers of polyoxy alkylene glycols	
Sulfated ethers	Acylated polyamines		
Sulfated carboxylic acid	Salts of heterocylic amines		
Petroleum sulfonates	Benzyl ammonium salt		
Sulfonated aromatic hydrocarbons			
Sulfonated esters			
Sulfonated amides			
Sulfonated ethers			
Acylated amino acids			

At concentrations above the CMC, amphiphilic molecules associate readily to form supramolecular structures such as micelles, bilayers and vesicles. The interfacial tension between phases changes very little above the critical micelle concentration because all additional surfactant molecules form micellar structures since the oil/water interface already has a monomolecular layer of amphiphiles. The forces that hold these structures together include hydrophobic, van der Waals, electrostatic and hydrogen bonding interactions. Since no covalent bonds are formed, these structures are fluid-like and are easily transformed from one state to another as conditions such as electrolyte concentration and temperature are changed [2].

Lipids can form micelles (spherical or cylindrical) or bilayers based mainly on the area of the hydrophilic head group and the chain length of the hydrophobic tail. Molecules with small chain lengths and large head groups generally form spherical micelles. Those with smaller head groups tend to associate into cylindrical micelles, while those with long hydrophobic chains form bilayers which, in turn, under certain conditions form vesicles. The formation of vesicles result in the solubilization of oil or water in the other phase, giving rise to microemulsion. The

unique properties of micelles are being explored for applications such as the extraction of proteins from fermentation broth and the removal of organics and metal ions from aqueous streams for environmental applications [12].

2. 3. 2. Hydrophile - Lipophile - Balance Value

For technological purposes, it is useful to be able to classify surfactants according to their stabilizing efficiency for a particular type of colloidal system. A well-established empirical procedure for doing this is the hydrophile-lipophile balance (HLB) method. It is based upon the idea that, for a given oil + water system, there is an optimum balance between molecular hydrophilic and lipophilic character which leads to maximum emulsification efficiency [8].

Surfactants with HLB numbers in the range 4-6 are suitable for preparing water in oil (w/o) emulsions, while those in the range 8-18 are suitable for oil in water (o/w) emulsions. A surfactant with an HLB number of 7 is neutral.

The original method of determining HLB numbers involved long and laborious experiments [3]. There are several alternative methods for calculating the HLB number of foods. For certain types of non-ionic surfactant, namely polyoxyethylene derivatives of fatty alcohols and polyhydric alcohol fatty acid esters, the HLB number may be calculated using the following formula.

$$HLB = 20 (1 - S / A) \quad (2.1)$$

where S is the saponification number of the ester and A is the acid number of the acid [13]. However for many fatty-acid esters, it is difficult to determine S accurately. In this case the following equations are used.

$$HLB = (E + P) / 5 \quad (2.2)$$

where E is the weight percentage of the oxyethylene content and P is the weight percentage of the polyhdric alcohol content. In surfactants where only ethylene oxide is used as the hydrophilic portion, the HLB is simply;

$$HLB = E / 5 \quad (2.3)$$

The required HLB number ranges for different systems are shown in Table 2.2 [13].

The use of HLB number in food systems is limited by the fact that many food emulsifiers interact strongly with starch or protein, this leads to a modification in the available lipophilic or hydrophilic groups on the emulsifier molecules. Using mixtures of polysorbates and sorbitan esters, in terms of the rate of droplet

Table 2. 2. HLB number ranges with their applications

HLB number range	Application
3 - 6	w/o Emulsifier
7 - 9	Wetting Agent
8 - 18	o/w Emulsifier
13 - 15	Detergent
15 - 18	Solubilizer

coalescence, the optimum HLB values for the stability of w/o and o/w emulsions are 3.5 and 12 respectively [7]. It was found that o/w emulsions prepared with blend of emulsifiers are more stable than those prepared with a single emulsifier of the same HLB number. The HLB values of some food emulsifiers are shown in Table 2.3.

Table 2. 3. HLB values of some food emulsifiers

Emulsifier	HLB value
Sorbitan tristearate (Span 65)	2.1
Glycerol monostearate	3.8
Sorbitan monooleate (Span 80)	4.3
Propylene glycol monolaurate	4.5
Succinic acid ester of monoglycerides	5.3
Sorbitan monopalmitate (Span 40)	6.7
Sorbitan monolaurate (Span 20)	8.6
Diacetyl tartaric acid ester of monoglycerides	9.2
Polyoxyethylene sorbitan monostearate (Tween 60)	14.9
Polyoxyethylene sorbitan monopalmitate (Tween 40)	15.6
Polyoxyethylene sorbitan monolaurate (Tween 20)	16.7
Sodium oleate	18.0
Sodium stearoyl-2-lactylate	21.0

2. 3. 3. Interfacial/Surface Tension Measurements

The most widely used methods for the detection of surfactants are interfacial/surface tension measurements. Surfactants have ability to reduce the

surface tension at the interface or surface. This property also helps to calculate CMC value of surfactant. Capillary rise, detachment methods can be used for the determination [14].

2. 3. 4. Phase Inversion Temperature (PIT)

PIT system, although not extensively employed in the food industry, is useful in relating the complex, temperature-dependent phase behavior of certain emulsifiers, for example, that of nonionic emulsifiers in water. As the temperature increases over a small temperature range, nonionic emulsifiers change from being preferentially water soluble to oil soluble [15].

2. 4. Production Methods of Surfactants

Surfactants constitute an important class of industrial chemicals widely used in almost every sector of modern industry. The manufacture of surfactant is on such a large scale that their production has always been considered to lie within the realms of organic chemistry and chemical engineering. However, rapid advances in biotechnology over the past decade have led to considerable interest in the development of biological methods for manufacturing on an industrial scale [16].

There are many disadvantages of chemically synthesized surfactants as compared to their microbial and enzymatic synthesized counterparts. Current chemical methods of their manufacture are typically based on high temperature esterification or transesterification carried out in the presence of an alkaline catalyst. The main drawbacks of these conventional methods are a high consumption of energy and the formation of undesirable side products. Additionally, a whole range of structural isomers are usually obtained due to the presence of multiple hydroxyl groups in the carbohydrate substrates [17].

2. 4. 1. Microbial Synthesis of Surfactants

Numerous strains of bacteria and fungi produce extracellular surface active lipids when grown in alkaline-rich media. This suggests that the physiological function of extracellular surfactants is to facilitate the utilization of alkanes by emulsification [18].

There are several reasons why the microbial production of surfactants have received so much attention in recent years. First is the diversity of biosurfactants, the

majority of which are unobtainable on a practical scale by organic synthesis because of their structural complexity. Finally, fermentation provide an inexpensive method on large scale biosurfactant manufacture [19].

One problem is the low final concentration of products in the fermentation broth. Together with difficulties associated with product recovery due to broth foaming, this has resulted in projected manufacturing costs beyond the acceptable level for many commercial applications. However, screening of microorganisms and optimization of the reaction conditions have led to substantial yield improvements [18].

2. 4. 2. Enzymatic Synthesis of Surfactants

Many isolated enzymes that catalyzed hydrolysis, alcoholysis, condensation, acylation or esterification reactions have been employed for the preparation of various surfactants, including monoglycerides, phospholipids, glycolipids and amino-acid based surfactants, from relatively inexpensive raw materials such as fats and plant oils. Compared with conventional chemical synthesis, these enzymatic methods have the advantages of low energy requirement, minimal thermal degradation, high biodegradability and therefore, have emerged as promising substitutes for conventional approaches for the production surfactants [2].

By their nature, surfactants are amphiphilic molecules containing a hydrophilic and hydrophobic moiety. One of the fundamental problem in applying isolated enzymes to the synthesis of surfactants is the efficient solubilization of the starting materials as it is by no means easy to find a solvent that would dissolve high concentrations of both hydrophobic and hydrophilic constituents in the reaction mixture at ambient temperature and that will not be deleterious for the enzyme activity [20].

Another problem is the choice of enzymes. In other words; the main question is how one can employ isolated enzymes for the preparation of surfactants. The use hydrolytic enzymes in non-aqueous media is suitable for this process. Indeed, many hydrolytic enzymes, such as lipases, proteases and glycosidases are available in large quantities. They are very robust and inexpensive, and do not require any cofactors to manifest their catalytic activity. Enzymes can not influence the equilibrium of a chemical reaction and therefore the removal of water from the reaction medium

forces them to work "in reverse", that is, to synthesize a chemical bond rather than to break it [20].

The major characteristics of microbial biosurfactants and enzyme synthesized surfactants are given in Table 2.4.

When we consider the application of enzymes to the synthesis of surfactants, the major group is the fatty acid derivatives.

Table 2. 4. Characteristics of Surfactants

Features	Microbial biosurfactants	Enzyme synthesized surfactants
Advantages	-Biodegradability -Diversity -Low production costs -In-situ applications	-Biodegradability -Ease of structural modification -Low recovery costs -Ease of purification
Disadvantages	-High recovery costs -High waste volume	-High enzyme costs -Low solubility of substrates
Key points for further development	-Strain improvement -Whole cell immobilization -Improved fermentation technology. -Metabolic engineering	-Enzyme immobilization -Enhanced enzyme stability and activity -Multiple-phase systems -Supercritical fluid technology

2. 5. Fatty Acid Derivatives

They are important surfactants for household, cosmetic and industrial purposes. Since the dramatic rises in oil prices, surfactants based on natural fats and oils and fatty acids obtained therefrom have gained importance [17]. Figure 2.10 shows an overview of the fatty acid derivatives and also a schematic classification into the fields of application.

Neutralization, esterification, amidation, ethoxylation or condensation of the fatty acids obtained by splitting fats and oils gives the corresponding surfactants or

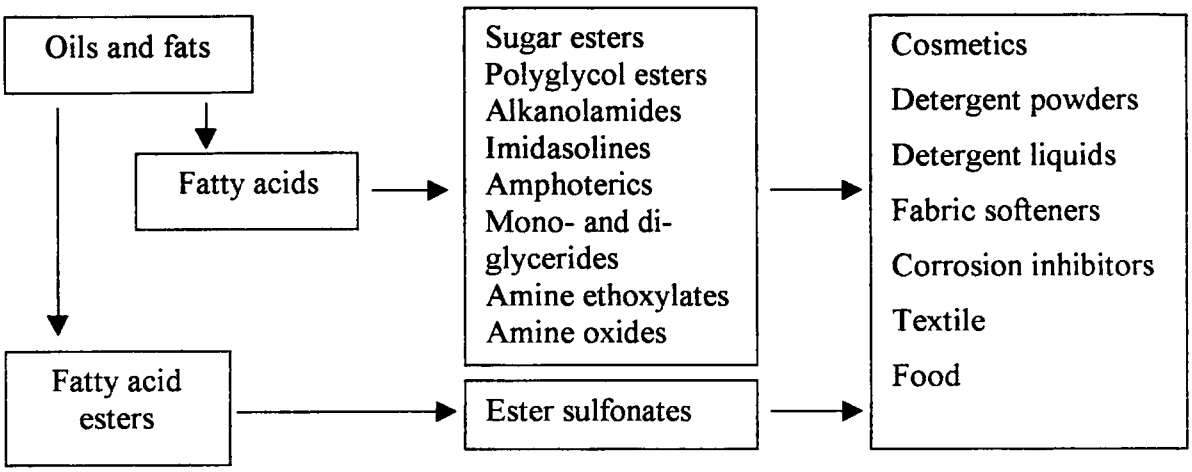


Figure 2.10. Fatty acid derivatives

emulsifiers, such as soaps, mono- and diglycerides, sugar esters, alkanolamides, polyglycol esters and imidazolines.

The essential fields of application of these fatty acid derivatives are detergent industry, cosmetics, fabric softeners, textile and food industry. These are discussed in later sections.

2. 5. 1. Fatty Acid Esters

Fatty acid esters include fatty acid monoglycerides and diglycerides which are obtained by glycerolysis of triglycerides. This reaction is run with an excess of glycerol at temperatures 200-250°C. The reaction is generally done under an inert gas atmosphere especially if saturated fats are used [17].

The reaction mixture contains 35-60 % of monoglycerides and 35-50 % of diglycerides, in addition to triglyceride, glycerol and fatty acids after a reaction time of 30-60 minutes. These mixtures are used as emulsifiers in food industry and in cosmetics [17]. Molecular distillation is suitable for further purification of such commercial products; the mixture from the reaction above can be separated by this method and monoglyceride concentrations of 90-95 % purity can be obtained [21].

Monoacylglycerols and their numerous derivatives such as ethoxylated monoglycerides, acetic, lactic, citric and diacetyl tartaric esters of monoglycerides are widely used as emulsifiers in the food industry. Drawbacks of this process include high energy consumption, and the products are often unusable as obtained, requiring redistillation to remove impurities and degradation products. In addition,

the highly unsaturated, heat sensitive oils can not be processed directly without prior hydrogenation. Thus, energy conservation and minimizing thermal degradation are probably the main advantages of introducing an enzyme-based technology [22].

Enzymatic glycerolysis of fats and oils has been performed in a nearly stoichiometric solvent-free mixture of substrates at ambient temperatures using 1,3-specific lipases. The equilibrium shift required for the reaction to proceed towards accumulation of the final product was achieved by decreasing the reaction temperature to below the melting point of the monoglycerides. Yields of up to 90 % were obtained with a variety of animal and plant lipids, including beef tallow, lard, rape seed, olive and palm oils [22].

Monoglycerides have also been prepared by enzymatic hydrolysis and alcoholysis of oils catalyzed by the 1,3-specific lipase. The first method is probably more suitable for the production of mono- and diglyceride mixtures with desirable emulsifying properties, whilst the second one yields very pure monoglyceride products. The alcoholysis of oils, although often carried out in batch, can be run in continuously in a packed-column reactor to facilitate product separation and recovery [22].

2. 5. 2. Sugar Fatty Acid Esters

Fatty acid esters of sugars or sugar alcohols constitute a very interesting group of nonionic surfactants, with potentially important applications in many industries, because of their surface active properties due to their composition [23].

They are tasteless, odorless and nontoxic, they are the best suited emulsifiers for foods. They are not irritant to the eyes and skin, they are suitable not only for foods but also for pharmaceuticals and cosmetic. They do not cause environmental pollution because they are biodegradable. They offer a full range of HLB values from 1 to 16, and in use all grades display exceptionally good surfactant properties. They give normal food products after human and animal digestion [4].

Sugar fatty acid esters are employed as industrial detergents and food emulsifies in numerous products and processes. They have been investigated extensively during the last years; they deserve particular consideration since they can be prepared from renewable resources and are physiologically, dermatologically and biologically acceptable surfactants. They can be synthesized either by chemical or enzymatic ways. Standard chemical esterification reactions involve high

temperatures which cause coloration of the final products and dehydration and cyclization in the case of sugar alcohols [17].

The use of a biological catalyst under mild conditions for the synthesis of esters of sugars or sugar alcohols can overcome this problem [24]. Figure 2.11 shows the key parameters in enzymatic synthesis of sugar esters [25].

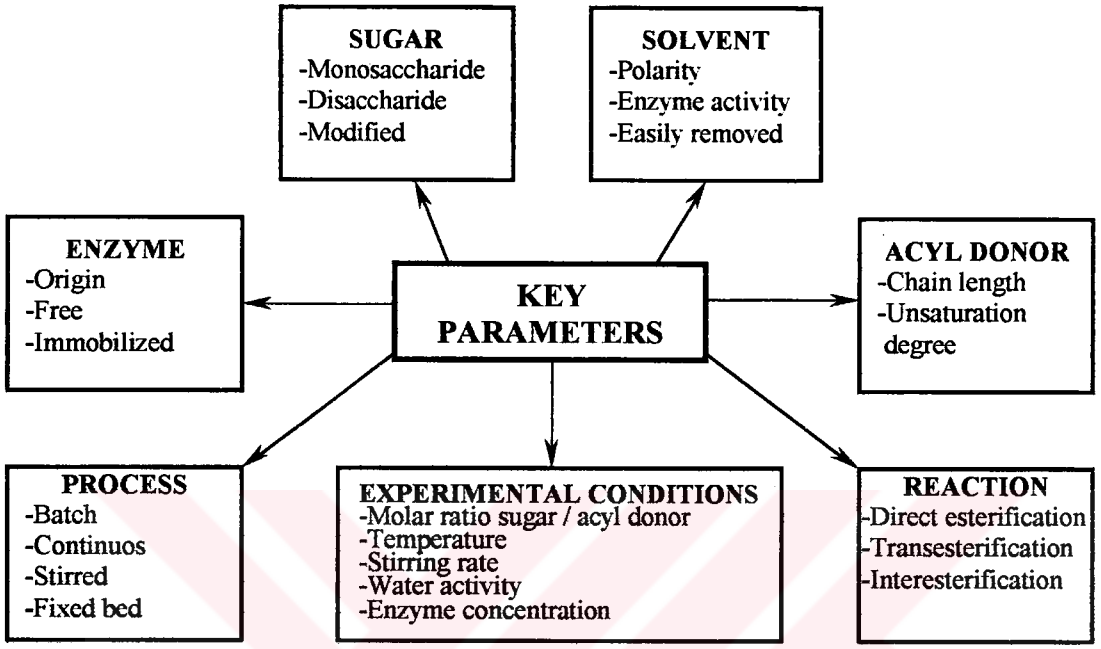


Figure 2. 11. Key parameters of enzymatic synthesis of sugar esters

-Effect of solvent : Different considerations have to be taken into account for selection of solvent. The best solvent has to be compatible with enzyme. The toxicity and processes to remove the solvent after synthesis have to be kept in mind.

In 1984, Seino *et al.*, described the first enzymatic method for the synthesis of these materials in aqueous solution [26]. Because the formation of ester linkages is thermodynamically favored in low water content media, alternative enzymatic procedures which employ such media have been developed. However, these process suffers from low miscibility of the two substrates with one another, and the necessity for use of toxic solvents such as pyridine [27]. Polar solvents of lower toxicity than pyridine have also been used to optimize the partial esterification of sugar alcohols in a one-step process in which there is no prior modification of the sugar. The main drawback of this alternative is the low solubility of the sugar in the reaction medium. Some researchers used 2-methyl-2-butanol as the solvent. Monosaccharides are more soluble in this solvent than in isopropyl alcohol and in acetone but acetone is easily

removed (BP.56.2°C) and also accepted by European Economic Community directives as an extraction solvent. So it is generally recognized as safe for use in the manufacture of foods and or food additives [28].

-Effect of water activity : Synthesis of fatty acid esters in organic solvents requires a medium characterized by a very limited hydration state. Initial water content and, water liberated during esterification reaction affected the amount of product and also the conversion of product [23].

It is, the fact that esterification is a reversible reaction, and the water liberated by the reaction affects directly the activity of the enzymes which have their maximum activity within a strict hydration state.

Reaction stoichiometries can be written as;

$$\text{sugar} + \text{acid} \leftrightarrow \text{monoester} + H_2O \qquad (2.4)$$

$$\text{monoester} + \text{acid} \leftrightarrow \text{diester} + H_2O \qquad (2.5)$$

To displace the equilibrium of the reaction, some researchers have performed the synthesis in a solvent-free process, under reduced pressure. In these cases, the water liberated by the reaction is vaporized and eliminated without inhibiting the esterase activity of the enzyme and thus favoring synthesis [29]. In such systems, continuous evaporation of water under reduced pressure requires the use of solvents with higher boiling points than water. Such solvents are often difficult to eliminate from the final product. Their use is based on the necessity for complete dissolution of the sugar for the production of fatty acid esters of sugars.

Some researchers used molecular sieves for detection of conversion and also selectivity of reaction for the fatty acid esters of sugars [28]. In these studies, it was found that reaction in the absence of molecular sieves yielded the highest percentage of monoester. However, accumulation of water effectively led to cessation of the reaction at shorter times. The higher molar ratio of diester to monoester obtained in the presence of molecular sieves can be attributed to the reduction of the water activity in the solution. The low activity of water favors the first esterification reaction to produce higher yields of the diester.

Because of the continuous water removal by molecular sieves, the hydrolytic processes (the reverse reactions) do not occur to a significant extend and they may be neglected. Acid concentration affects both reaction rates (v_1 and v_2) in similar fashion. Reaction 1 should occur faster than Reaction 2 because of higher reactivity

of the primary hydroxyl groups on fructose. Moreover, monoester precipitation because of its low solubility should also decrease v_2. Thus, the reactions explain the net accumulation of monoester during the initial stages of the reaction and the observed maxima in the concentration of this species. However, the monoester solubility in acetone was independent of acid concentration [29]. Possible adsorption of a relatively significant fraction of fatty acid on the desiccant or on the immobilized enzyme support was not the reason for the low amount of monoester produced when stoichiometric amounts of reactants (acid and fructose) were employed.

-Effect of solubility of sugar and esters : Monosaccharide solubility governs both the rate at which it can be converted to the corresponding esters and monoester selectivity. These factors, in turn, determine the potential viability of the process. When sugar solubility in acetone is low, the rate of sugar esterification is slow. Moreover, the small amount of monoester produced remains in solution, thereby rendering the initial product monoester susceptible either to further acylation to form the diester or to alcoholysis to regenerate the original sugar and diester. Under such conditions, sugar conversion will be low, and reaction selectivity will be poor. Determination of sugar solubility under reaction conditions should allow prediction of successful sugar monoacylation [24].

It was reported that for the selective monoacylation of sugar, monoester solubility in acetone is a key factor in achieving good yields of this species. When monoester solubility is low, its precipitation should lead to nearly quantitative yields using a small excess of acid. The temperature at which the reaction is initiated is selected on the basis of a compromise between the desire to enhance reaction rate by increasing temperature and the necessity of operating at a temperature which minimizes monoester solubility in order to prevent further reaction of this species to form the diester. By contrast, when the monoester has a significantly higher solubility under the initial reaction conditions, there are two strategies which can be employed to optimize the yield of the desired monoester: (i) favor monoester precipitation by decreasing reaction temperature and/or by using a longer-chain saturated fatty acid or (ii) favor the first esterification reaction [29].

-Effect of nature of the acyl donor : The choice of the acyl donor influences the acylation reaction by two manners: First, sugars can be acylated by direct esterification or transesterification reactions. The acyl donor used was a fatty

acid or a fatty acid methyl ester or a triacylglycerol. Direct esterification is characterized by water synthesis that favored hydrolysis reaction whereas transesterification reaction led to alcohol or acylglycerol productions when fatty acid ester or triacylglycerol are respectively used.

Second, the chain length and the degree of unsaturation affected the activity of the lipase. It was reported that when the number of double bond increased, the activity of lipase was found high [25]. This result is explained by the orientation change of the carbon chain due to double bonds.

-Effect of nature of the enzyme : Usually ester bonds synthesis are catalyzed by lipases (E.C.3.1.1.3). However, other hydrolases as proteases have also employed. Enzymes are used as free or immobilized on a support. Immobilization confers some interesting properties to the biocatalyst. Surface interactions between substrates and enzyme are increased by immobilization allowing a more homogenous substrate concentration near the enzyme. A simple filtration step allowed the recovery of immobilized lipases that can be reused several times [30-34].

2. 5. 3. Various Application of Fatty Acid Esters of Sugar

The fatty acid derivatives especially fatty acid esters, fatty acid esters of sugars or sugar alcohols offer a full range of HLB values from 1 to 16, and in use all grades display good surfactant functionality. The following is a discussion of several products in which fatty acid derivatives are employed and is a review of their effects.

Wheat products : Bread, frozen dough and noodle are some examples to this category. In bread, emulsifiers give strength to dough and increase the mechanical resistance at the time of kneading. They increase the volume after baking, soften the crumb and make uniform cavities. They improve quality when flour is mixed with sorghum or corn flour, or with bean or fish meal to add protein. In frozen dough, they prevent spoiling during refrigeration, improve the crumb after thawing and baking, and the make the bread voluminous and soft [15].

In noodle products, they are used for the purpose of preventing mixed dough from sticking to the machine and noodles to each other. They prevent retrogradation of boiled noodles during storage.

Confectioneries : The emulsifiers used in sponge cake shorten whipping time of cake batter by the all-in one method and promotes the cake-making process. They increase the volume of cake and improve the crumb, give uniformity to cake and

produce soft taste. They make stable emulsion of fatty materials and prevent stickiness to the machine in products like biscuit, cracker and cookie. They prevent blooming even in high-fat biscuit products. They are used to prevent the adherence to the teeth, machine or wrapping paper in products like caramel, candy and chewing gum.

They improve heat deformation of chocolate and reduces oil separation. They increase the water resistance and the sugar blooming in chocolate. They are also used effectively in products like rice cake, rice cracker, pudding and jellies [15].

Dairy products and substitutes : They are used to improve overrun by preventing excessive cohesion of fat during freezing due to stable emulsification and they provide smooth and melty taste in dairy products like ice-cream, ice-milk. The use of them affects the dryness of products. They prevent water separation in milk drink, whipping cream and condensed milk. They are also suitable to improve the quality of canned drinks like chocolate or coffee drink.

Processed fats and oils : They are very powerful for w/o or o/w emulsified fat. In products like margarine, they facilitate the improving creaminess, consistency and water holding capacity [15].

Other edible uses in food industry : In instant curry, they are useful in mixing of spices and wheat flour with oil easily. They improve operation efficiency and yield. They also give good emulsion and dispersion in cooking. They are effective in the prevention of precipitation of solid matter in thick sauce. They prevent caking of hygroscopic powdered foods and improve fluidity and solubility of spices in water.

In canned drinks; they are used for the purpose of preventing the precipitation caused by demulsification and denaturation of proteins during sterilization. They inhibit the growth of heat resistant spore forming bacteria and prevent denaturation of wheat at high temperature during distribution. They are also used for coating fruits to maintain freshness during prolonged storage life.

Miscellaneous uses : Detergents, drugs, cosmetics, plastics and textiles are the other industries that the fatty acid derivatives are used. They give softness and smoothness to skin, providing a thin film, thus, retaining adequate moisture. They are also used as soothing agent in skin and hair creams, lotions, shampoos, hair lotions, shaving creams and lipsticks. They used as a foaming agent in toothpaste [35].

They are used as additives and emulsifiers for polymerization of resins for food packaging films. They are also effective as antistatic agent for various kinds of textiles.

2. 5. 4. Synthesis and Modification of Phospholipids

Crude lecithin, recovered from plant oils, is a complex mixture of individual phospholipids, with the major components being phosphatidylcholine and phosphatidiylethanolamine. It is used for the production of a wide range of "special lecithin's", which are defined as products that have been processed to achieve the required surface-active properties. Special lecithin's have a wide variety of applications in the manufacture of paints and leather, in the food industry (for margarine, bakery goods, chocolate, and instant products). Derivatized phospholipids also have specific applications in personal care products and pharmaceuticals [36]. Due to the presence of numerous functional groups in phospholipid molecules, the use of enzymes offers superior control over the degree of modification necessary for obtaining a product with the desired properties [37].

Lysophospholipids, obtained by complete or partial hydrolysis of lecithin, another important class of industrially important surfactants. Phospholipase A_2-mediated hydrolysis is performed in 30 % phospholipid emulsion with aqueous buffer [36]. However, this batch process suffers from several complications. One such complication is the necessity to inactivate phospholipase A_2 after completion of the reaction, because it is practically impossible to recover and reuse the enzyme from the heterogeneous reaction mixture. Irreversible inactivation of phospholipase A_2 is achieved either by a combination of alkalization and heat treatment, or by digestion with protease(s). The proteases can subsequently be heat inactivated.

2. 5. 5. Synthesis of Pure Alkylglycosides

The major attraction of alkylglycosides as compared to sugar fatty acid esters is their much better stability under alkaline conditions. Pure alkylglycosides are also useful in biomedical and pharmaceutical applications. They are easily biodegradable. Their synthesis was carried out in an aqueous-organic two-phase system with immobilized glycosidases suspended in a concentrated solution of sugar (aqueous phase), with medium chain primary alcohols forming the organic phase [38]. Although a number of trans-glycosylation reactions took place under these

conditions, the oligosaccharide products were confined to the aqueous phase due to their insolubility in the organic solvent. However, pure alkyl glucoside is extracted in the organic phase [36].

2. 5. 6. Synthesis of Amino-Acid Based Surfactants

Amino acid esters and amides have been a subject of intensive investigations due to their excellent emulsifying properties, biocompatibility, and strong antimicrobial activity. These features have made them attractive for applications in cosmetic and personal care products, food, and pharmaceutical formulations. Additionally, there are also good reasons for considering amino acid based-surfactants as potential bulk detergents for industrial and household cleaning usages. Renewable and cheap raw materials are used in their production [39, 40]. Enzymatic acylation of the α-amino group of amino acid amides, using free fatty acids or their methyl esters has been reported in several publications [41].

2. 6. Systematic Analysis of Surfactants

There are a lot of methods for the analysis of surfactants depending on nature and type of surfactants. Table 2.5 shows the general procedures for the analysis of them [11]. First one is the recognition of surfactants in compositions. Certain physical properties that are characteristics of solutions of surfactants (such as foaming, lowered surface or interfacial tension, wetting power) often suffice to indicate that surfactants are present. Recognition of the charge type of surfactants can be accomplished by testing with certain dye-stuff whose colors or solubility characteristics are changed by the presence of surfactants of a given charge type.

Second step is the isolation of surfactants from compositions. Extraction methods are suitable under the proper conditions of pH, temperature, electrolyte concentration and temperature. Third one is the separation of mixtures of surfactants. Both instrumental and chemical methods are applied for the quantitative and structural analysis of surfactants

For the detection and quantification of surfactants GC and HPLC has been widely used. They have the advantages of high specificity and sensitivity and capability of handling large amounts of samples. Furthermore, the amount of sample required for analysis is small, without the need for tedious pre-purification. This assay was successfully used to monitor the surfactant production. TLC method can

also applied to analysis and purification of surfactants. It can provide reasonably good resolution and semi quantitative information provided with appropriate solvent systems. Carbon-Nuclear Magnetic Resonance (C-NMR) is applied for the structural and positional analysis of surfactants [31].

Table 2. 5. General diagram of systematic analysis of surfactants

1-Recognization	Certain physical properties are used; -testing with certain dye-stuff -precipitation reaction
2-Isolation from compositions	-extraction methods -selective adsorption and chromatographic methods (under proper conditions like pH, temperature, solvent, electrolyte concentration)
3-Seperation of mixtures of surfactants	-Chromatography (TLC, GC, paper and ion exchange) -liquid-liquid extraction
4-Structural analysis of surfactants	-Instrumental methods (NMR, infrared) -Physical properties (such as refractive index)
5-Quantitative analysis	-Both instrumental and chemical methods

There are different purification procedures for the surfactants. These are;

Sedimentation and centrifugation : Particles of radius greater than 1μm will settle down under gravity in a reasonable time. They will certainly sediment rapidly if the particles aggregated together to form flocs. The rapid settling of this floc is used in the purification of colloid. After that, centrifugation is necessary [9].

Dialysis : The colloid is retained on one side of a membrane (often in the form a bag) which is bathed in the wash solution. The pores of the membrane must be large enough to allow passage of the solvent and small molecules, but not the colloid. Dialysis is a diffusive process in which ions and other small molecules move through the membrane as a consequence of the concentration difference on either side. Good mixing of both regions, speeds the attainment of equilibrium [12].

Gel-filtration : The mostly used purification procedure for the sugar esters is the gel filtration method. The column materials are selected as a silica gel and Sephadex depending on nature of surfactant. After the column is packed, it is equilibrated with proper solvent. Then the surfactant solution is fed to the column and the surfactant is purified by applying selected elution program [28].

Ultrafiltration : It is the extend of the filtration process to very small particles. This necessarily requires the use of membranes with a very small pore radius, and is usually assisted by applying pressure.

Ion-exchange : The surface of most colloid is electrically charged. These charges are neutralized by the adsorption of ions of opposite sign on the external faces of the colloid crystals [12].

CHAPTER 3
MATERIALS AND METHODS

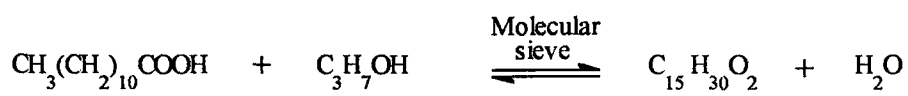
3. 1. Materials

Lipase from *Candida antarctica* (designated "Novozym 435",had an activity of 7000 PLU/gr) was kindly provided by Novo Nordisk A/S (Bagsvaerd, Denmark). Anhydrous fructose, glucose and D-sorbitol were purchased from Sigma Chemical Co (St. Louis, MO) [42]. HPLC grade acetone, acetonitrile, tetrahydrofuran, chloroform, methanol, acetic acid (glacial), hexane, 2methyl-2-propanol, isopropyl alcohol were purchased from Merck (Darmstadt, Germany). The molecular sieves (an effective pore diameter of 4°A) and silica gel (15 – 40 μm, average pore diameter of 60°A) were supplied by Sigma Chemical Co. Potassium hydroxide and phenolphthalein were purchased from Riedel de Haen (Hannover, Germany). In the preparation of solutions distilled water was used. HPLC analyses was performed using triple distilled water.

3. 2. Methods

3. 2. 1. Esterification Reaction of Lauric Acid

The esterification was carried out in 100 ml conical plastic-stoppered flask at 60°C with shaking at 120 strokes/min on a thermoconstanter shaker water bath (NÜVE; Sanayi ve Malzemeleri İmalat ve Ticaret A.Ş., İstanbul, Türkiye) for 15 min. The reaction was initiated by adding 100 mg of lauric acid, 50 mg of Novozym 435 and 4 ml of chosen solvent as shown below. To enhance the reaction towards synthesis, 200 mg of molecular sieves were used [29]. The effects of lauric acid and Novozym 435 concentration, type of solvent, temperature and time were investigated.



3. 2. 1. 1. Assay of Enzyme Activity

Reaction was terminated by removing enzyme and molecular sieves by centrifugation. The ester formation was calculated on the basis of the acid values of the reaction mixture before and after the incubation time [42]. The acid values were determined by titration with 0.05 N KOH using phenolphthalein (1%) as an indicator. The reaction mixture operated without the enzyme was titrated in the same way and used as a blank.

Enzyme was immediately introduced in a new mixture for repetitive assays after centrifugation. All reactions were performed triplicate.

3. 2. 1. 2. Stability of Enzyme

Novozym 435 (50 mg) was incubated in a mixture of 4 ml isopropyl alcohol and 100 mg lauric acid in a thermoconstanter water bath shaker at 40°C. After 6 days of incubation period, the enzyme activity was calculated [6].

3. 2. 2. Esterification Reaction Between Lauric acid and Fructose

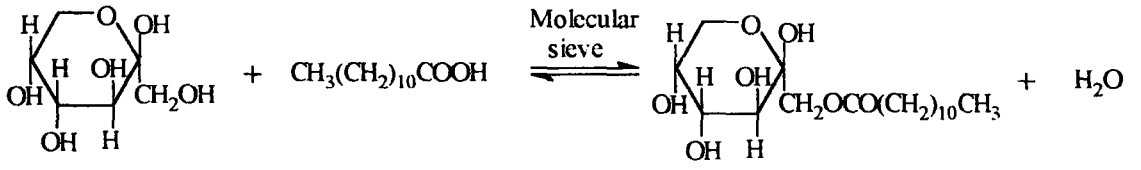
3. 2. 2. 1. Solubilities of Sugars

The solubilities of fructose, glucose, D-sorbitol in acetone and isopropyl alcohol at 5, 28, 40°C were determined according to Arcos *et. al.*,(1998). At the temperature of interest, 50 ml of solvent (acetone or isopropyl alcohol) was saturated with chosen sugar overnight. Then the solution was filtered to remove the undissolved sugar. A 20 ml filtrate was taken and solvent was evaporated using vacuum oven at 50°C. The solid sugar was dissolved in 2 ml of water and subjected to quantification by HPLC [29].

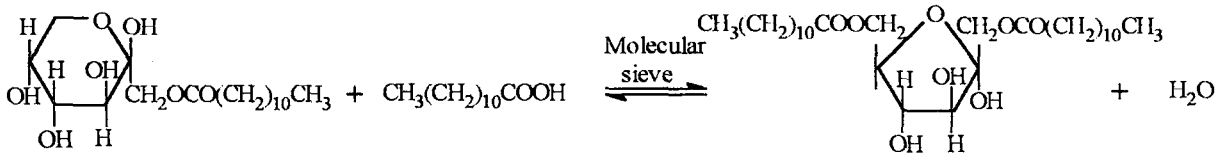
An HPLC system consisted of HP series 1100 wavelength detector, an Jasco PU-980 model isocratic pump, Supelcosil LC-NH_2 (25 x 4.6 mm) column, a software program Borwin version 1.21 were used to determine the solubilities of sugars. Mobile phase used was triple distilled water. Flow rate was 2.0 ml/min and wavelength was 254 nm [43].

3. 2. 2. 2. Reactions

Reactions given below were performed by mixing the 300 mg of lauric acid, 60 mg of fructose, 200 mg of molecular sieve, 100 mg of Novozym 435 in a



Fructose Lauric acid Fructose monolaurate Water



Fructose monolaurate Lauric acid Fructose dilaurate Water

stoppered glass erlenmeyer. After addition of 2 ml of acetone, reaction was allowed to occur in a thermoconstanter shaker water bath for 96 hour at 40°C [23]. The effects of mole ratio of acid to sugar were investigated using 0.5/1, 1/1, 3/1, 5/1 mole ratio of lauric acid to fructose. For determination of amount of products, a sample was taken in one-hour intervals and analyzed by HPLC [44].

3. 2. 2. 3. Analysis of Reaction Mixtures by HPLC

After separation of immobilized enzyme and molecular sieves by centrifugation, the composition of the remaining solution was analyzed using an HPLC apparatus consisting of a Jasco PU-980 model isocratic pump and Nova-Pak C-18 (3.9 x 150 mm) column, an HP 1100 series variable wavelength detector and a software program of Borwin version 1.21 was used to identify the product. Mobile phase was a mixture of acetonitrile, water, THF, acetic acid (70.0:25.0:5.0:0.1) (v/v). Flow rate was 1.0 ml/min and wavelength was 214 nm [23].

For determining the conversion of lauric acid to esters, calibration curves of lauric acid and esters were prepared by HPLC.

3. 2. 2. 4. Purification of Esters

The immobilized enzyme and molecular sieves were removed from the reaction mixtures by centrifugation after incubation. Then the solvent was removed

by evaporation in vacuum oven for 3 hours at 40°C. The products were purified by column chromatography .

A column (1.6 cm in diameter, 50 cm in height) was packed with silica gel (25 gr silica gel in 100 ml of chloroform). The flow rate was adjusted 1.3 ml/min using a peristaltic pump (EYELA, Micro tube pump, M-3, Tokyo, Rikakikai Co., Ltd.). After that, column was equilibrated with chloroform by washing three times of the bed volume. A reaction mixture of 350 mg was dissolved in 1.5 ml of chloroform and was loaded to the column.

The flow rate was adjusted as 1 ml/min for the elution. Firstly the excess acid and higher esters were eluted with 100 ml of chloroform. The diester was then eluted using a 99.0:1.0 (v/v) mixture of 100 ml of $CHCl_3$ and CH_3OH. Elution of monoester was done with a mixture of 100 ml of $CHCl_3$ and CH_3OH (97.0:3.0) (v/v) [29].

Ten milliliter fractions were collected and fractions were assayed by HPLC [11]. These purified esters were used to investigate the surfactant properties of esters and used as standards for HPLC analysis.

3. 2. 3. Statistical Analysis

One-way analysis of variance (ANOVA) and multiple range test (LSD) were carried out using Statgraph package program (Statistical Graphics Corporation, V 5.0).

3. 2. 4. Surfactant Properties of Esters

3. 2. 4. 1. Hydrophilic Lipophilic Balance Value

HLB value of product was obtained using the Griffin equation [3].

According to this equation;

$$HLB = 20 \times [1 - (S/A)] \qquad (3.1)$$

where S = Saponification index of the ester group,

A = Acidity index of the fatty acid.

3.2.4.2. Critical Micelle Concentration

CMC of surfactant solutions were calculated by conductivity method [11]. The apparatus used produces a pulsed, bipolar voltage having a frequency of 1 kHz and a peak to peak amplitude of about 0.5 V. This waveform was generated from an integrated circuit powered by a 9-V power supply (Leader-Lag.1265-Audio Signal

generator). This alternating voltage waveform is used to avoid polarizing effects that would occur if a dc voltage were applied to the electrodes [45].

The electrodes consist of two 0.5 cm diameter carbon rods which were coated with paraffin except for the bottom tips. This wax deposit (which acts as an insulator) ensures that a constant surface area is exposed to the solution; otherwise, the conductivity would increase as the total volume of liquid increased, covering a larger surface area of the electrodes. A digital ampermeter (ITT MX 20 type avometer) is placed in series between one electrode and the pulsed voltage supply. This instrument is sensitive ac mA setting, allowing for 0.01 mA sensitivity.

The currents (I) were recorded at different concentrations (C) of surfactant solution. Then the plot of current/concentration (I/C) vs. $C^{1/2}$ was obtained. Data points of the exponential curve were connected by straight lines and the intersection point of two lines gave the value [45].

3. 2. 4. 3. Determination of Surface Tension

Surface tension of surfactant solutions were calculated by using capillary rise method [11]. For a surface having a radius of curvature R the pressure difference, ΔP, is a function of the surface tension and can be expressed as;

$$\Delta P = 2\gamma / R \qquad (3.2)$$

$$2\gamma / r = \rho \times g \times h \qquad (3.3)$$

where ΔP = pressure difference, γ = surface tension, ρ = density, g = acceleration due to gravity, r = radius of the capillary, h = height of the liquid in capillary.

The principle was that, if we know the capillary radius, we can make an absolute determination of γ for a liquid [45]. The apparatus was consisted of a capillary tube with an attached graduated scale that is marked from 0 to 10 cm in 1 mm increments.

At different concentrations of surfactant solution the height of the liquid in the capillary tube was marked and this value vs. concentration was plotted. The inflexion point of this curve gives the CMC value of surfactant.

3. 2. 4. 4. Determination of Emulsion Stability

The ability of surfactants to stabilize emulsions, water in oil was assessed as follows: 3 volumes of surfactant solutions in xylene (as the oil phase) were mixed

with 2 volumes of the water with a homogenizator (Art-Miccra D-8 type, Müllheim, Germany) at a speed of 33000 min^{-1} for 5 min. Each emulsion contained 0.25 and 0.50 % (w/v) surfactant as specified. The emulsion formed was transferred to a graduated cylinder and kept at 30°C, and the separation of the phases was measured as a function of time. The height of the clear xylene layer was used as a monitored parameter for 30 hours. The curve of separation of phases vs. time were plotted [46].

CHAPTER 4
RESULTS AND DISCUSSION

4. 1. Esterification Reaction Between Lauric Acid and Isopropyl Alcohol

4. 1. 1. Effect of Different Solvents

Propyl laurate was produced by using five different solvents at 60°C for 15 minutes in order to investigate the effect of solvent in the reaction medium. The results are shown in Table 4.1.

Maximum amount of product was obtained when isopropyl alcohol was used. So further studies were carried out in isopropyl alcohol, also considering that its high boiling point allow to study at high temperatures. In addition, the use of isopropanol in the preparation of food additives is permitted by the regulatory authorities [28].

Table 4.1. Product formation depending on type of solvent

Solvent	Product (mmole)
Isopropyl alcohol	0.23
Acetone	0.19
2-methyl-2-propanol	0.13
Hexane	0.04
Acetonitrile	0.02

4. 1. 2. Effect of Accumulation of Water during Reaction

The effect of water produced in the reaction was studied. Two reaction mixtures were prepared in the presence and absence of molecular sieves. The molecular sieves absorb the water formed during the reaction, thereby reducing its

activity in solution. The other reaction conditions were kept constant. The quantity of molecular sieves employed (200 mg) was more than that necessary to completely absorb the amount of water which would be produced by complete esterification of the acid used in this experiment [6].

Figure 4.1 indicates the result of this comparative study. The reaction in the presence of molecular sieves yielded slightly ($P < 0.05$) higher amount of propyl laurate than that in the absence of molecular sieves. The difference between them was small, because the amount of lauric acid used in reaction was small. If we consider the long reaction time and production of higher esters than monoester, the difference will be large.

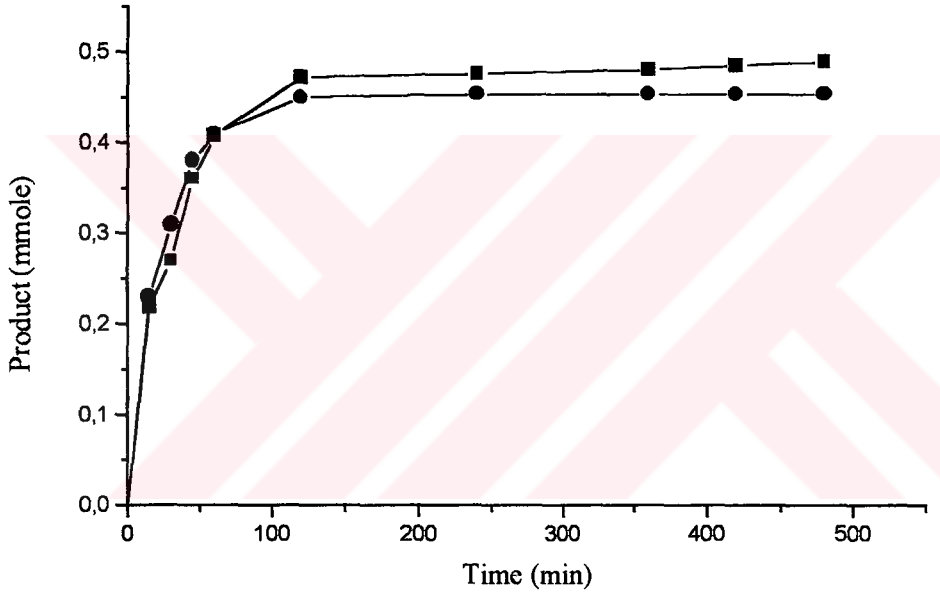


Figure 4.1. Effect of accumulation of water during esterification reaction. Reaction conditions : 100 mg of lauric acid, 50 mg of Novozym 435 and 4 ml of isopropyl alcohol, reaction temperature 60°C. Reaction with molecular sieve (■), without molecular sieve (●).

4. 1. 3. Effect of Lauric Acid Concentration

In order to see the effect of substrate concentration on the product formation, different amounts of lauric acid (0.125 mmole to 17.5 mmole) were used in the reaction medium while keeping the other reaction parameters as constant.

As seen in the Figure 4.2, the amount of propyl laurate was increased linearly up to lauric acid concentration of approximately 5.0 mmole. Then the product

formation was slowed down and it was reached to 1.80 mmole of product at a lauric acid concentration of 12.5 mmole. There were no significant (P>0.05) change observed in the product formation after that substrate concentration.

This result was consisted with the previous studies [47], it was observed that the reaction is essentially first order at very low substrate concentrations. At high substrate concentrations, the product formation was essentially independent of the substrate concentration.

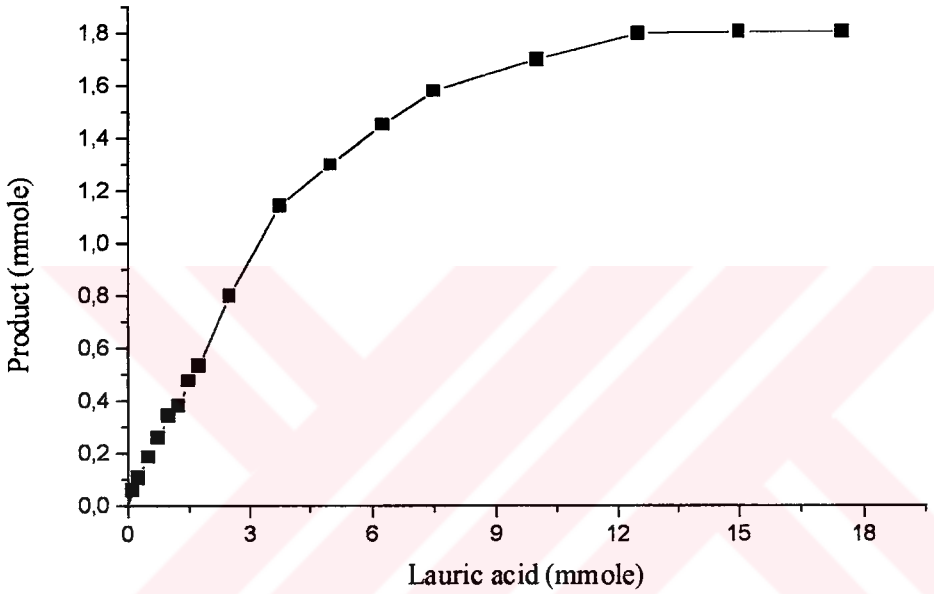


Figure 4.2. Effect of lauric acid concentration on product formation.
Reaction conditions : Lauric acid, 50 mg of Novozym 435, 200 mg of molecular sieve and 4 ml of isopropyl alcohol, reaction temperature 60°C, reaction time 15 min.

4. 1. 4. Effect of Enzyme Concentration

Enzyme concentrations of 10 mg to 300 mg were used in the esterification reaction for the conversion of lauric acid into propyl laurate. Figure 4.3 shows the effect of enzyme concentration on the product formation.

The amount of propyl laurate produced seemed to be linearly proportional to the enzyme concentration up to 50 mg showing that the system was in steady state. After this point linearity deviates and the product formation was slowed down. The amount of propyl laurate reached 0.46 mmole at an enzyme concentration of 300 mg.

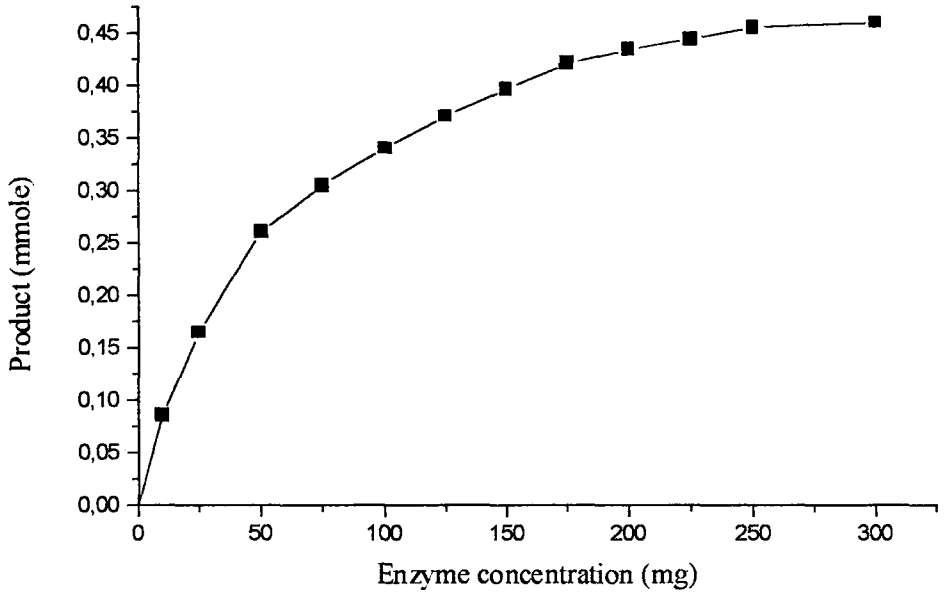


Figure 4.3. Effect of enzyme concentration on product formation
Reaction conditions : Novozym 435, 100 mg of lauric acid, 200 mg of molecular sieve and 4 ml of isopropyl alcohol, reaction temperature 60° C, reaction time 15 minutes.

The progress curve of the lipase catalyzed esterification reaction was same with the general esterification reactions [47]. According to Mutua and Akoh (1993), any further increase in the enzyme concentration did not result in an increase in the amount of desired product, because the substrate concentration became a limiting factor in the face of an excess of active enzyme sites [48].

4. 1. 5. Effect of Temperature

To evaluate the effect of temperature on product formation, temperature was changed from 40°C to 80°C. As seen from the Figure 4.4, the enzyme exhibited its maximum activity at 60°C. Under the given reaction conditions, the same optimum temperature for *Candida antarctica* lipase has been reported by the Novo Nordisk A/S which is the manufacturing firm of the Novozym 435 [42]. This high optimum temperature for the *Candida antarctica* lipase permits to study at high temperatures.

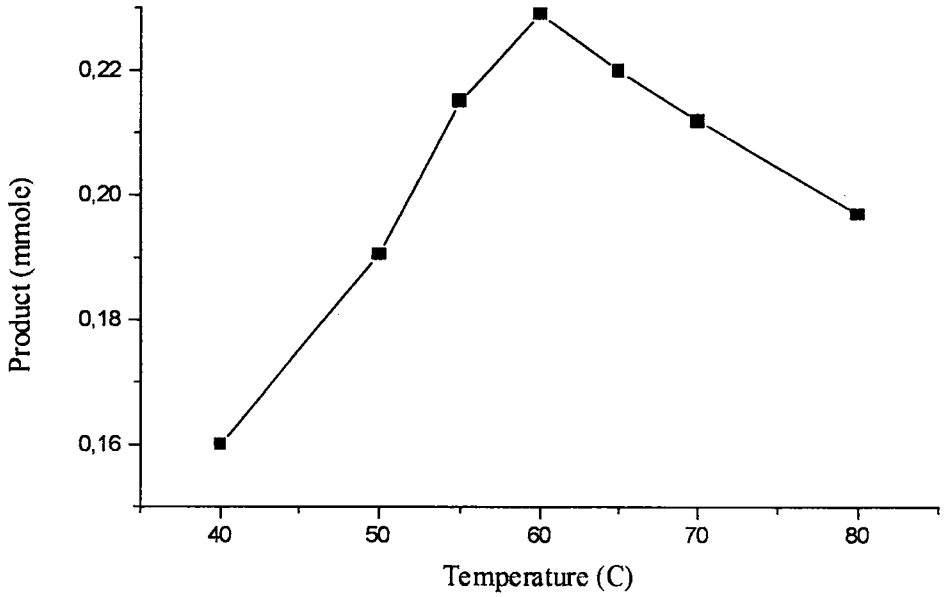


Figure 4.4. Effect of temperature on product formation.

Reaction conditions : 100 mg of lauric acid, 50 mg of Novozym 435, 200 mg of molecular sieve and 4 ml of isopropyl alcohol, reaction time 15 min.

4. 1. 6. Effect of Incubation Time

The effect of incubation time on the propyl laurate formation was studied from 5 min to 520 min (Figure 4.5).

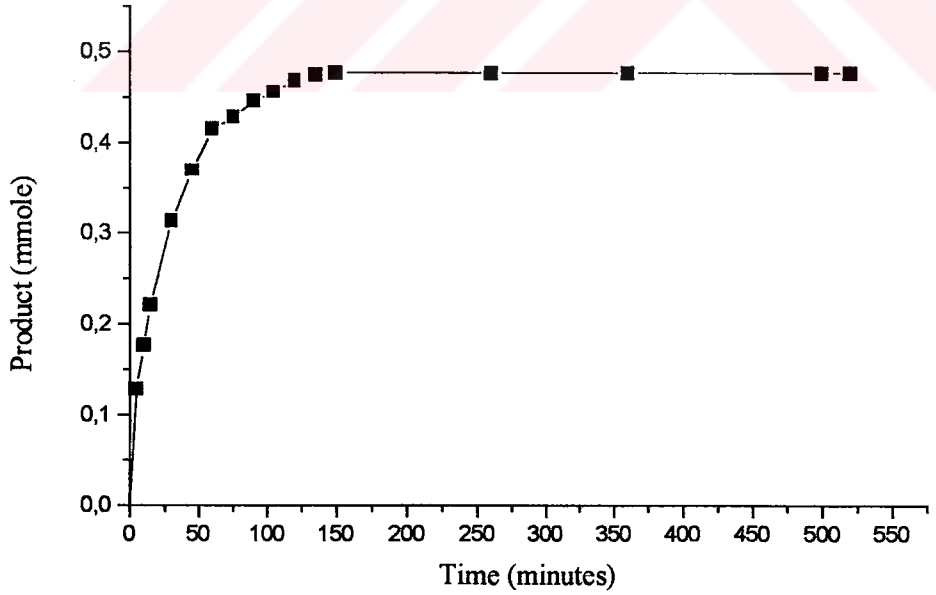


Figure 4.5. Effect of incubation time on product formation.

Reaction conditions : 100 mg of lauric acid, 50 mg of Novozym 435, 200 mg of molecular sieve and 4 ml of isopropyl alcohol, reaction temperature 60°C.

There was a linear increase in the amount of propyl laurate up to 50 min. The amount of product reached 0.4 mmole at that time value. After that, the amount of product formation slowed down up to 100 min and it reached 0.48 mmole of propyl laurate. No significant ($P>0.05$) change was observed in the amount of product after 150 min.

4. 1. 7. Stability of Enzyme

After 6 days of use at 40°C in isopropyl alcohol containing lauric acid, the activity of Novozym 435 at 60°C was reduced to 70 % of its initial value. Thus, the stability of the enzyme used in this work is high in the reaction medium considering the reaction time required. The same results were reported by other researchers [28,29]. So, the enzyme has very high activity for long time at high temperatures.

4. 1. 8. Repetitive Use of Enzyme

The results of repeated use of immobilized enzyme in the system are shown in Table 4.2. After the third use of the same enzyme at 60°C, for 15 min, the retained activity was 59 %. It seemed that this offers the benefit of applying the enzyme economically several times.

Table 4.2. Repetitive use of Novozym 435

Number of use	μmole of propyl laurate produced	Retained activity (%)
1	179	100
2	122	68
3	106	59

4. 2. Esterification Reaction Between Lauric Acid and Fructose

The esterification reaction between lauric acid and fructose were done and analyzed by HPLC (Appendix B). The products were the fructose monolaurate, fructose dilaurate and higher esters.

4. 2. 1. Solubility of Sugars

Solubility studies of two monosaccharide (glucose and fructose) and one sugar alcohol (D-sorbitol) in acetone and isopropyl alcohol were tabulated in Table 4.3. The solubilities were recorded at three different temperatures (5, 28 and 40°C). As seen from the Table 4.3, the solubilities of fructose and glucose were similar to each other within same conditions. The solubilities of sugar was increased with increasing temperature. High temperatures are expected to increase the reaction rate and the complete consumption of the limiting reagent (sugar) can be obtained in a few hours [29]. The solubility of D-sorbitol was less than that of sugars.

Table 4.3. Solubility of sugars

Temperature (°C)	Solubility (mg/ml)					
	FRUCTOSE		GLUCOSE		SORBITOL	
	Acetone	Isopropyl alcohol	Acetone	Isopropyl alcohol	Acetone	Isopropyl alcohol
5	0.23	0.58	0.24	0.61	0.21	0.16
28	0.37	1.73	0.42	1.84	0.27	0.31
40	0.72	3.60	0.81	3.90	0.49	0.58

The solubilities of sugars in isopropyl alcohol were higher than acetone. Even though the use of isopropanol in the preparation of food additives is permitted by the regulatory authorities, most researchers have discontinued its use and adopted acetone as a solvent. So, further experiments in this study were carried out in acetone. Because this solvent is inexpensive, volatile (easy to evaporate, from the final product, B.P. 56.2°C) and permitted for use in the manufacture of food products by EEC (directive 88 – 344 – CEE) [6]. The toxic effect of the solvent can be reduced by minimizing the necessary solvent/sugar ratio.

4. 2. 2. Effect of Lauric Acid / Fructose Mole Ratio

Reactions at different molar ratios of lauric acid to fructose (0.5/1, 1/1, 3/1, 5/1) were performed at 40°C. Figures 4.6, 4.7, 4.8 and 4.9 indicate the effect of reactants on the product formation.

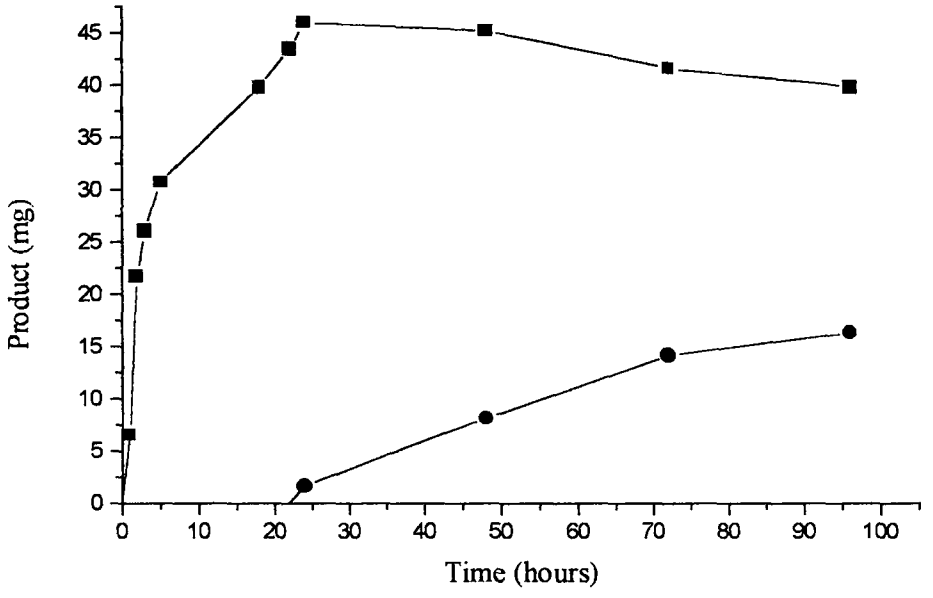


Figure 4.6. Product distributions for the esterification of lauric acid with fructose. Lauric acid/fructose = 0.5/1, at 40°C. Fructose monolaurate (■), fructose dilaurate (●).

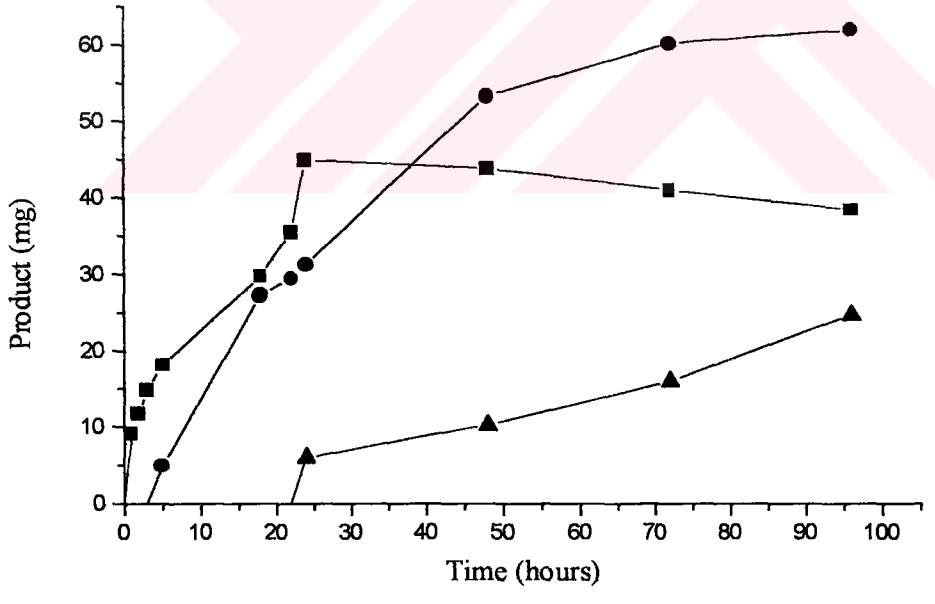


Figure 4.7. Product distributions for the esterification of lauric acid with fructose. Lauric acid/fructose = 1/1, at 40°C. Fructose monolaurate(■), fructose dilaurate (●), higher ester and fatty acid mixture (▲).

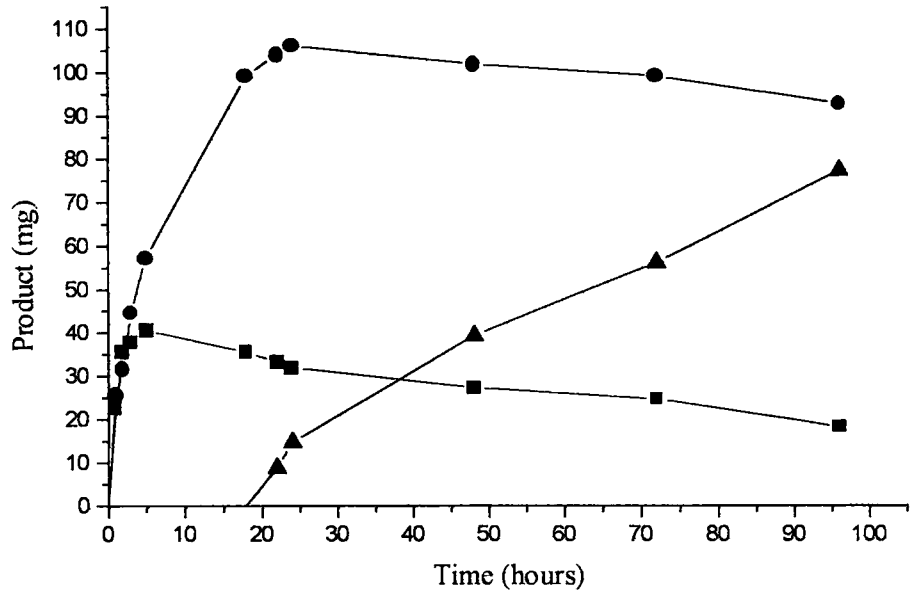


Figure 4.8. Product distributions for the esterification of lauric acid with fructose. Lauric acid/fructose = 3/1, at 40°C. Fructose monolaurate(■), fructose dilaurate (●), higher ester and fatty acid mixture (▲).

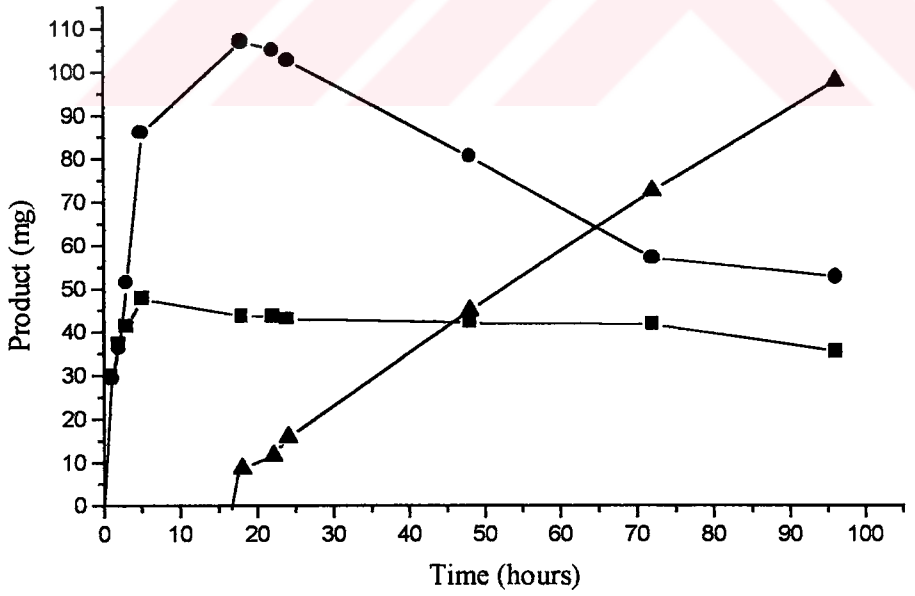


Figure 4.9. Product distributions for the esterification of lauric acid with fructose. Lauric acid/fructose = 5/1, at 40°C. Fructose monolaurate(■), fructose dilaurate (●), higher ester and fatty acid mixture (▲).

Although monoester and diester were obtained, higher esters were not detected for 96 hours of reaction time in the presence of 0.5/1 mole ratio of lauric acid to fructose. The product was only monoester up to a reaction time of 24 hours. The amount of fructose monolaurate reached to a maximum value of 46 mg in 24 hours. After that point, while the amount of monoester began to decrease, the diesters were produced. The formation of them continued up to 96 hours, but the amount of them did not reach the amount of monoester.

In the presence of equal molar of lauric acid and fructose, all fractions were obtained. At the beginning of reactions, both the monoester and diester were produced. The amount of monoester increased to 44 mg in 24 hours. Then, the amount of it began to decrease. The higher esters were detectable at that point. The fructose dilaurate continued to increase up to 72 hours. After that its formation slowed down . The amount of diester was higher than other fractions.

Both fructose monolaurate and dilaurate showed a fast increase up to 5 hours in the presence of 3/1 mole ratio of lauric acid to fructose. While the diester continued to increase, the amount of monoester slowed down after that point. The higher esters became detectable and diester started to decrease in 24 hours. For 96 hours of reaction period, the amount of monoester, diester, and higher esters were 18 mg, 93 mg and 77 mg, respectively.

In the presence of 5/1 mole ratio of lauric acid to fructose, higher esters again were not detectable up to18 hours reaction time. The amount of fructose monolaurate reached to 47 mg in 5 hours and then decreased. The amount of fructose dilaurate increased up to 18 hours and then slowed down. At that point higher esters were detectable and the amount of them reached to 98 mg after 96 hours reaction time.

In all mole ratios, monoester was obtained within 1 hour. The amount of fructose monolaurate was lower than others in all ratios except 0.5/1 mole ratio. Maximum amount of higher esters (98 mg) were obtained when the lauric acid/fructose mole ratio was 5/1.

When we examined all mole ratios, it was observed that, when the ratio of acid to sugar was increased, the formation of esters were increased subsequently and higher amounts of esters were obtained in shorter times. Although an increase in the excess of acid enhances the maximum yield of the diester, it also favors the production of triesters. The use of excess amount of acid also reduces the time required to reach a given extend of conversion.

There are several production methods based on selective production of monoesters and diesters from fatty acid and sugars. The affecting reaction parameters were found as the acid/sugar mole ratio, temperature and solubilities of esters and sugars in these studies [29, 49].

At high temperatures, the maximum percentage of monoester was high due to the increased solubility of sugar. The decrease in temperature reduced the solubility of the carbohydrate, the rate of the first esterification reaction decreased more rapidly than that of the second [6]. So, accumulation of the monoester was reduced and higher yields of the diester were favored. In addition, because of the high activation energies associated with esterification of secondary hydroxyl groups, formation of triesters and higher esters become less favorable as the temperature decreased. Consequently, higher charges of reactants reduce the reaction time, increase the process selectivity, reduce the amount of acetone in the reaction mixture and the toxicity, the cost of the process and the size of the reactor, a factor essential for industrial scale operations [28].

4. 2. 3. Amount of Lauric Acid Used in the Reaction

Figure 4.10 shows the relation between the amount of lauric acid used and reaction time. In all mole ratios of lauric acid to sugar, the reaction followed the same trend. Up to 20 hours, there were a rapid increase in the amount of lauric acid used. Then the rate of conversion of lauric acid was almost constant. In mole ratio of 5/1, the amount of lauric acid used was higher than the other. The amount of lauric acid used was increased, when the mole ratio was increased.

4. 3. Purification Results

4. 3. 1. Purification of Propyl Laurate

Propyl laurate produced for 6 hours at 60°C was purified by column chromatography. At the end of the reaction period, the amount of product was calculated as 0.48 mmole. The results of column chromatography after the HPLC are given Table 4.4.

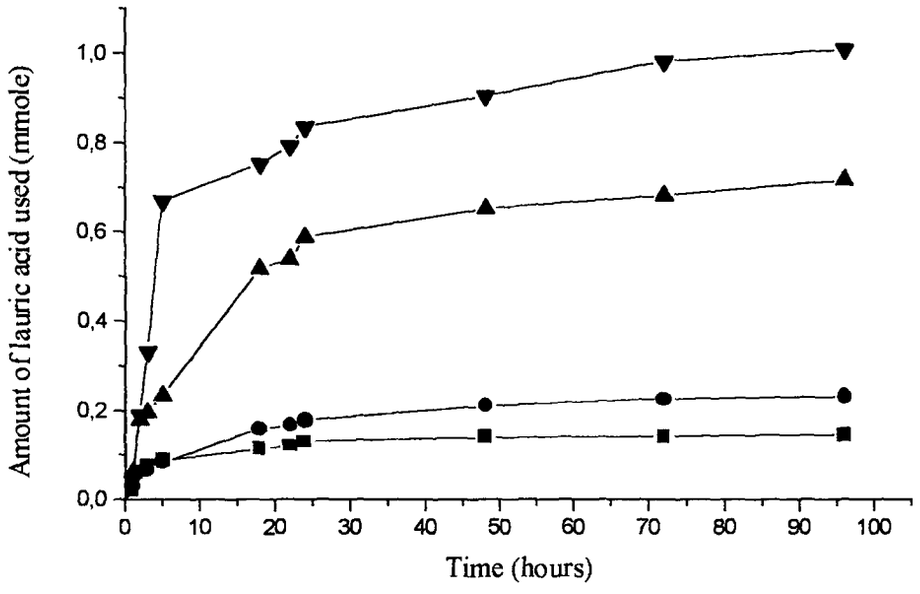


Figure 4.10. Amount of lauric acid used in reaction.

Reaction conditions : 60 mg of fructose, 100 mg of Novozym 435, 200 mg molecular sieve, 2 ml acetone, 40°C, lauric acid / fructose mole ratio 0.5/1 (■), 1/1 (●), 3/1 (▲), 5/1 (▼).

4. 3. 2. Purification of Lauric Acid Esters of Fructose

The product obtained by the reaction of lauric acid with fructose in molar ratio of 5/1 and incubation period of 18 hours was purified. At that time, all types of esters were present in the reaction mixture in high amounts.

At the end of this reaction period, the amount of monoester, diester and higher esters were found as 43.8, 107.17 and 8.71 mg, respectively. The amount of unreacted lauric acid was 149.50 mg. Table 4.4 shows the results of this purification procedure.

These purified esters were used as standards for the HPLC analyses (Appendix A) and also for the determination of surface active properties.

Table 4.4. Column chromatography results of esters

For the propyl laurate	
Tube number	Fraction
1 - 3	Unreacted lauric acid
5 - 8	Monoester (propyl laurate)
For the lauric acid esters of fructose	
Tube number	Fraction
1 - 4	Higher esters and unreacted lauric acid
6 - 9	Monoester (Fructose monolaurate)
12 - 15	Diester (Fructose dilaurate)

4. 4. Surface Active Properties of Esters

The surfactant properties of purified esters and the mixture of higher esters and lauric acid mixture were estimated. The determined parameters were the HLB value, CMC, surface tension and emulsion stability.

4. 4. 1. HLB value

The calculated HLB values of the esters given in Table 4.5. The HLB values show similarities to ones in the literature. Thus, they are emulsifiers with functional properties similar to those of compounds which constitute the largest class of emulsifiers employed in the food industry.

Table 4.5. HLB value of products

Emulsifier	HLB
Propyl laurate	3.50
Fructose monolaurate	8.95
Fructose dilaurate	5.30

According to Griffin classification, the emulsifier having HLB value in between 4 to 6 is suitable for the w/o emulsion, the emulsifier having that in between 8 to 18 is suitable for o/w emulsion and the surfactant with an HLB number of 7 is neutral, it can be used in either emulsion type [3].

The HLB value of propyl laurate and fructose dilaurate were in the first region, so they were suitable for w/o emulsions. The HLB value of fructose monolaurate was near to neutral, so it helps to stabilize the oil/water emulsions and water/oil emulsions [9].

4. 4. 2. Critical Micelle Concentration

CMC of propyl laurate, fructose monolaurate, fructose dilaurate and the mixture of higher esters and lauric acid were obtained by conductivity method. Figure 4.11, 4.12, and 4.13 illustrate the results of conductivity measurements for products. The calculated CMC values were given in Table 4.6.

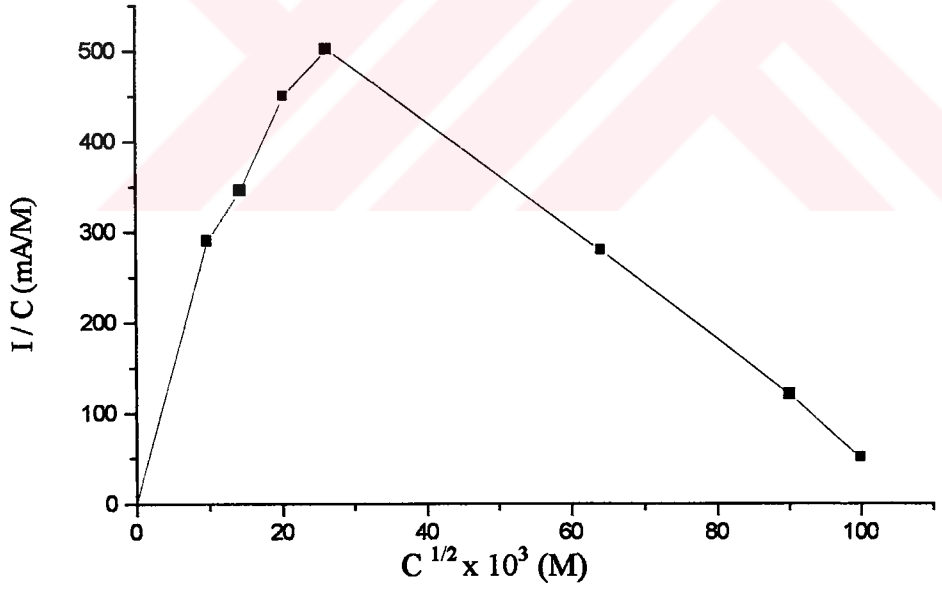


Figure 4.11. CMC curve of propyl laurate

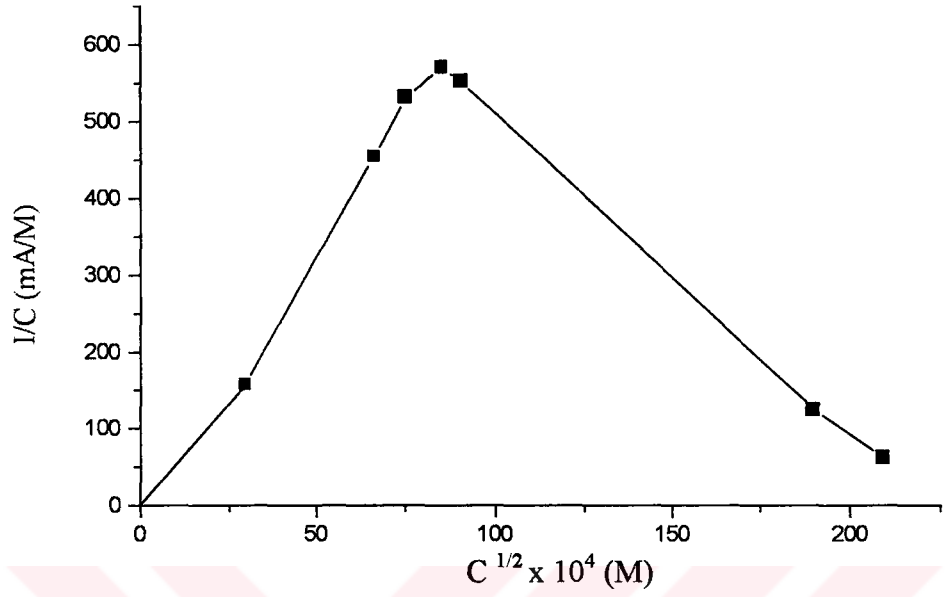


Figure 4.12. CMC curve of fructose monolaurate

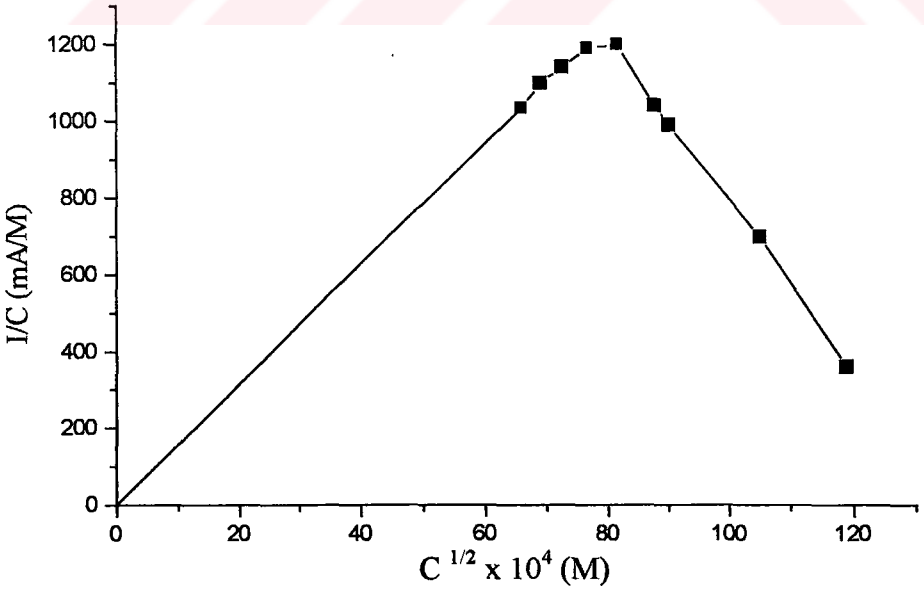


Figure 4.13. CMC curve of fructose dilaurate

According to these results, propyl laurate have the largest CMC value. In general, the CMC value of a surfactant in an aqueous medium decreases, as the number of carbon atoms in the hydrophobic group increases to about 16. Above 18 carbons, CMC remains unchanged with an increase of the chain length [1].

4. 4. 3. Surface Tension

The determination of surface tension of purified substances were obtained using capillary rise methods. The height of surfactant solutions in capillary is directly proportional to the surface tension. Height vs. concentration curves of aqueous solutions are given in Figures 4.14, 4.15 and 4.16, for propyl laurate, fructose monolaurate, fructose dilaurate, respectively.

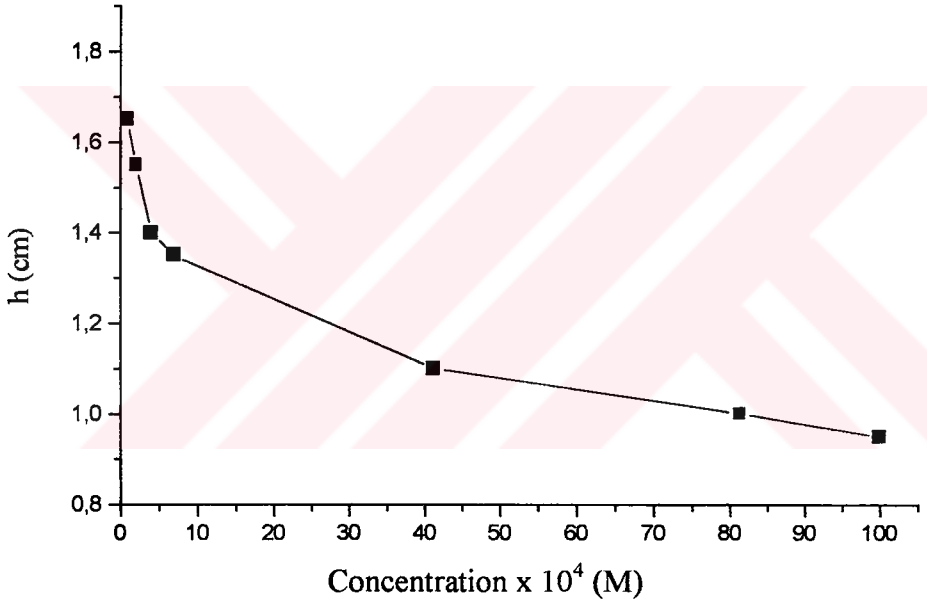


Figure 4.14. The curve of reduction of surface tension of propyl laurate.

The presence of small quantities of any synthesized esters considerably reduces the surface tension of water. When the concentration of surfactants increased, the height in the column was decreased. The height of pure water was 1.8 cm in the capillary under the same conditions. Fructose dilaurate decreased the height in the capillary more than the others at the same concentrations. The ability for decreasing the height in the capillary was found to be the lowest with propyl laurate.

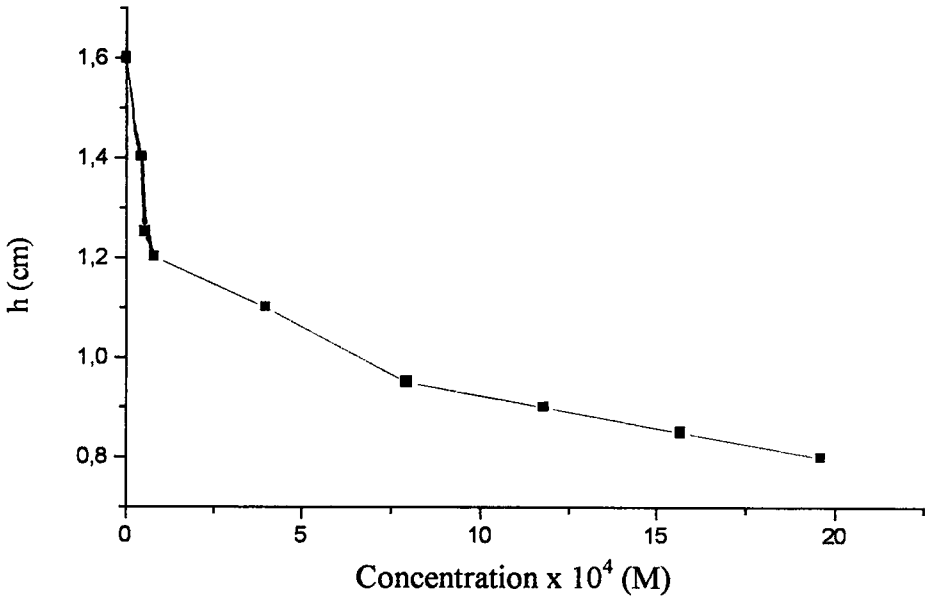


Figure 4.15 The curve of reduction of surface tension of fructose monolaurate.

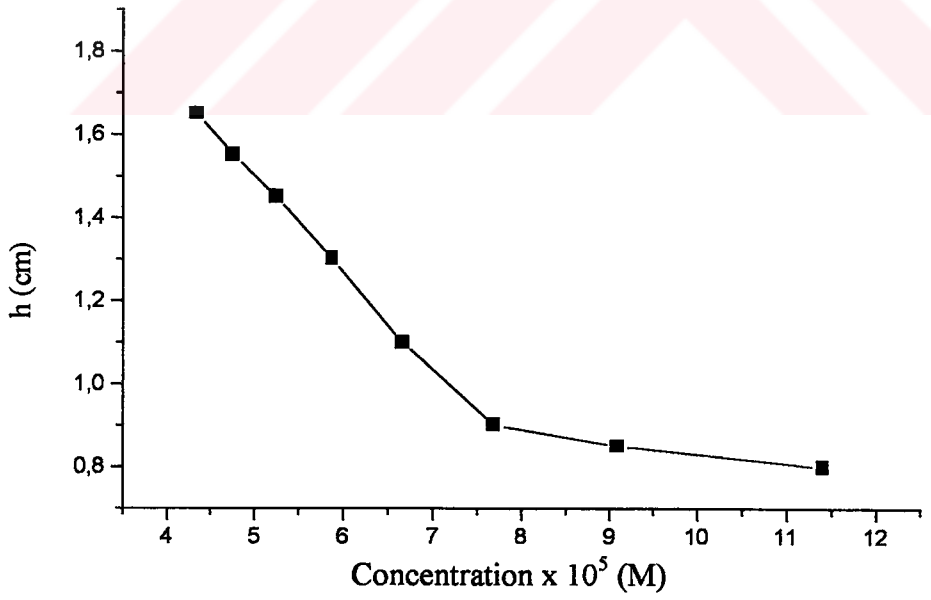


Figure 4.16. The curve of reduction of surface tension of fructose dilaurate.

At the same time CMC values of surfactants were calculated from the surface tension curve . CMC can be determined from the inflexion of the surface tension vs. concentration curve. The CMC values obtained from these plots are seen in Table 4.6.

Table 4.6. CMC value of emulsifiers.

Emulsifier	CMC value obtained from conductivity curve	CMC value obtained from surface tension curve
Propyl laurate	6.84×10^{-4} M	6.87×10^{-4} M
Fructose monolaurate	7.20×10^{-5} M	8.15×10^{-5} M
Fructose dilaurate	6.40×10^{-5} M	7.69×10^{-5} M

The fructose monolaurate and fructose dilaurate have approximately closer CMC values. The CMC value of propyl laurate was higher than that of them. The mixture of higher esters and lauric acid also decreased the surface tension and have a CMC value approximately 1%. This value was expressed on percentage basis, because they were obtained as a mixture from column chromatography.

The CMC values of esters obtained from conductivity curve and surface tension curve were similar to each other. Results showed that surfactants have ability to reduce the surface tension greatly.

These calculated CMC values were the same with the literature [50]. According to Ducret et al.(1996), the CMC value obtained for the monooleate esters of glucose, fructose and sorbitol, which have similar hydrophilic character remained around 8×10^{-5} M, while xylitol monooleate, which has a smaller hydrophilic group leads to a lower CMC value. As expected, the effect of hydrophobicity of the alkyl chain is clear from that determination.

4. 4. 4. Emulsion Stability

Figures 4.17 and 4.18 indicate the stabilization of w/o type emulsions with 0.25 % (w/v) and 0.50 % (w/v) surfactant respectively. There was also a control curve corresponding to the phase separation of the emulsion in the absence of a surfactant. After just a few minutes the aqueous and organic layers are completely separated in the absence of surfactant but in their presence, phase separation was significantly retarded.

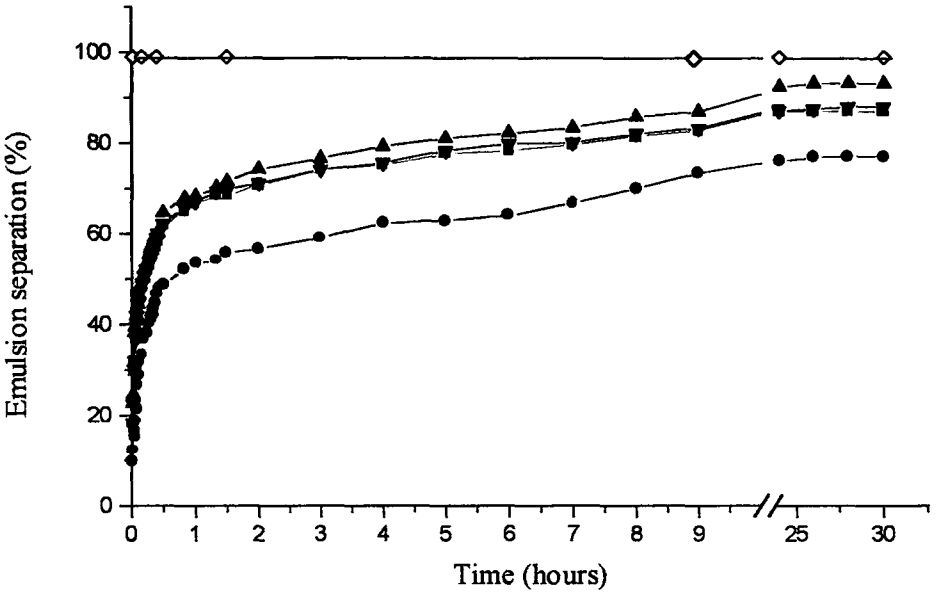


Figure 4.17. Stabilization of emulsion at 30°C by using 0.25 % (w/v) of surfactant solution. Fructose monolaurate(■), Fructose dilaurate (●), higher esters and lauric acid mixture (▲), propyl laurate (▼) and control (◊).

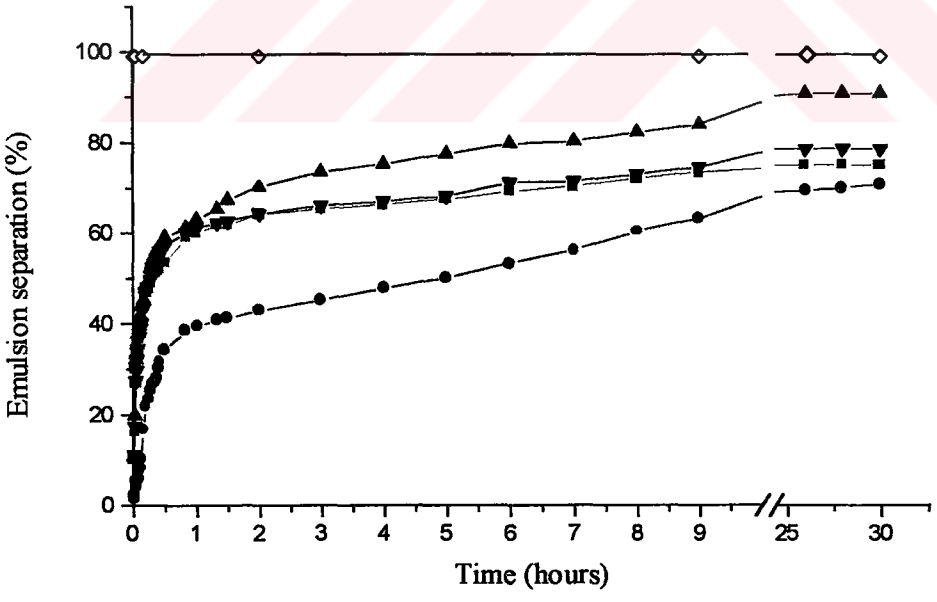


Figure 4.18. Stabilization of emulsion at 30°C by using 0.50 % (w/v) of surfactant solution. Fructose monolaurate(■), Fructose dilaurate (●), higher esters and lauric acid mixture (▲), propyl laurate (▼) and control (◊).

When the concentration of surfactant was increased, the stabilization of emulsion was also increased. It was seen from the graph that, there was a fast linear increase in the separation of phases up to 1 hour in each condition. Then the rate of separation of phases was gradually decreased up to 9 hours. After that time, no change was observed in the separation of phases.

While the most stable emulsion was the one prepared with fructose dilaurate in both concentrations, the stability of higher esters and lauric acid mixture were the weakest. For the emulsion prepared with 0.50 % (w/v) fructose dilaurate, the separation of phases was reached to 70 % in 30 hours. Under the same conditions, separation was 75 % for the emulsion prepared with fructose monolaurate, 78 % for the propyl laurate and 91 % for higher esters and fatty acid mixture.

For the emulsion prepared with 0.25 % (w/v) fructose dilaurate, the separation of phases was reached to 77 % in 30 hours. Under the same conditions, separation was 87 % for the emulsion prepared with fructose monolaurate, 88 % propyl laurate and 93 % for higher ester and lauric acid mixture.

It is known that, the type of emulsion formed by water and oils depends primarily on the nature of surfactant and, to a minor extend, on the process used for preparing the emulsion and the relative proportion of oil and water present.

In general, o/w emulsions are produced by emulsifying agents that are more soluble in the water than in the oil phase, whereas w/o emulsions are produced by emulsifying agents that are more soluble in the oil than in the water phase [4].

These results showed that, these enzymatically synthesized products have surfactant properties. The emulsifying properties of the propyl laurate and lauric acid esters of fructose are superior to those of surfactants produced current industrial processes which employ conditions under which dehydration of the sugar takes place. In addition their use in food industry was found suitable depending on advantages such as use of natural sources in their production, ease of purification and modification, absence of toxic impurity in the final product and biodegradability.

The esters of lauric acid and fructose have desirable functional properties which would render them of interest not only to the food industry, but also to the cosmetic, pharmaceutical and detergent industries.

CHAPTER 5
CONCLUSION

1. Propyl laurate and lauric acid fructose esters (fructose monolaurate, fructose dilaurate and higher esters) were produced by using immobilized *Candida antarctica* lipase (Novozym 435). The surfactant properties of these esters were also investigated after purification of them.

2. The optimum parameter of enzyme were obtained by producing propyl laurate. Maximum amount of product was obtained using isopropyl alcohol. The product formation was followed a linear increase up to lauric acid concentration of 5 mmole. There were no significant change observed in the amount of product after substrate concentration of 12.5 mmole. The amount of product seemed to be linearly proportional to enzyme concentration of 50 mg. Maximum activity was obtained when temperature 60°C. There was a linear increase in the amount of propyl laurate up to 50 min.

3. After 6 days of use at 40°C in isopropyl alcohol containing lauric acid, the activity of Novozym 435 at 60°C was reduced to 70 % of its initial value. The retained activity of enzyme was high so it offers the benefit of applying economically.

4. Four different mole ratio were compared the see the effect of acid /sugar mole ratio on the esterification reaction of lauric acid with fructose. In the presence of equal amount of lauric acid and fructose, the amount of fructose monolaurate increased to maximum value of 48.51mg at a time of 48 hours. In the presence of 0.5/1 mole ratio of lauric acid to fructose, higher esters were not detected. Maximum amount of higher esters (98 mg) were obtained when the mole ratio was 5/1.

5. When the ratio of acid to sugar was increased, the formation of esters were increased subsequently and more amount of esters were obtained in a short time. Although an increase in the excess of acid enhances the maximum yield of the diester, it also favors the production of triesters.

6. The HLB values of propyl laurate, fructose monolaurate and fructose dilaurate were found as 3.50, 8.95 and 5.3, respectively. The calculated HLB values of esters were found similar to those of compound, which constitute the largest class of emulsifiers employed in the food industry.

7. The CMC of esters were calculated by conductivity method. The values for propyl laurate, fructose monolaurate, fructose dilaurate were 6.84 x 10^{-4} M, 7.20 x 10^{-5} M and 6.40 x 10^{-5} M, respectively.

8. The emulsion stability of these esters were tested preparing water-in-oil emulsions containing 0.5 % (w/v) and 0.25 % (w/v) surfactant solution at 30°C. The phase separation was found to be high when the concentration was low. In both cases, fructose dilaurate showed better stability than the others. The separation of phases was reached to 70 % for the emulsion prepared with 0.5 % (w/v) fructose dilaurate, 75 % for the fructose monolaurate, 78 % for the propyl laurate and 91 % for higher esters and fatty acid mixture in 30 hours. For the emulsion prepared with 0.25 % (w/v) fructose dilaurate, the separation of phases was reached 77 % in 30 hours. Under the same conditions separation was 87 % for the emulsion prepared with fructose monolaurate, 88 % for propyl laurate and 93 % for higher ester and lauric acid mixture.

9. The surfactant properties of propyl laurate and lauric acid esters of fructose obtained by enzymatic synthesis are found superior to those of surfactants produced by current industrial processes.

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APPENDICES

APPENDIX A

Calibration Curves

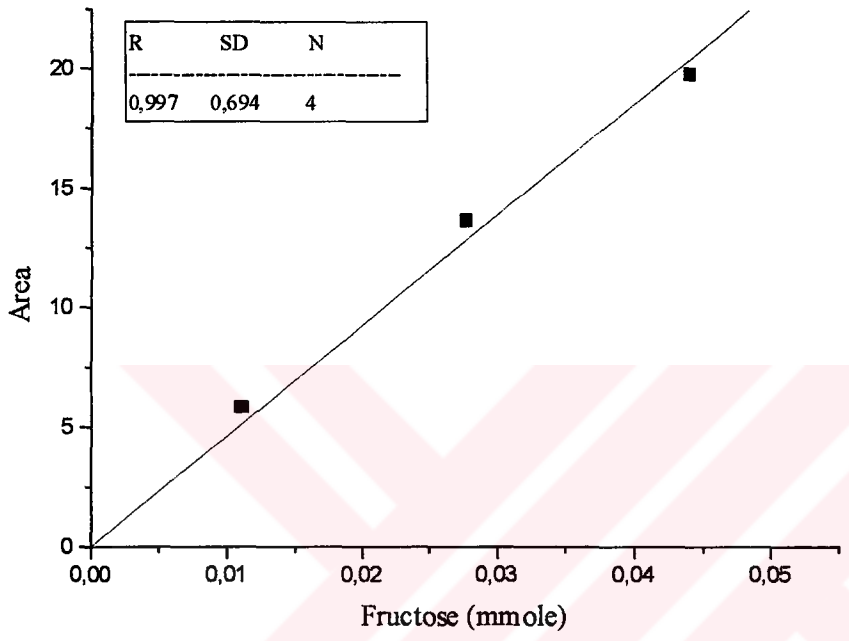

Figure A.1. Calibration curve of fructose

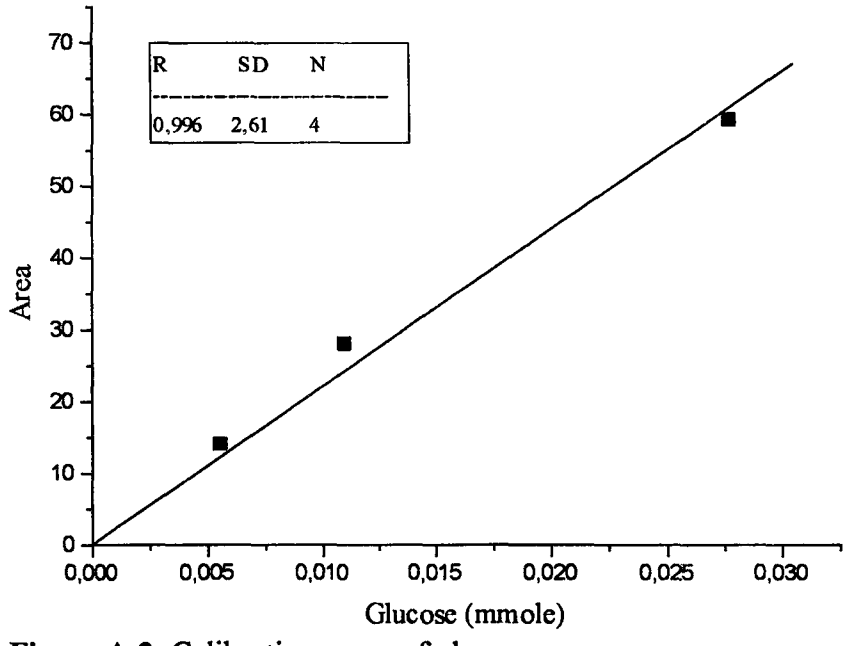

Figure A.2. Calibration curve of glucose

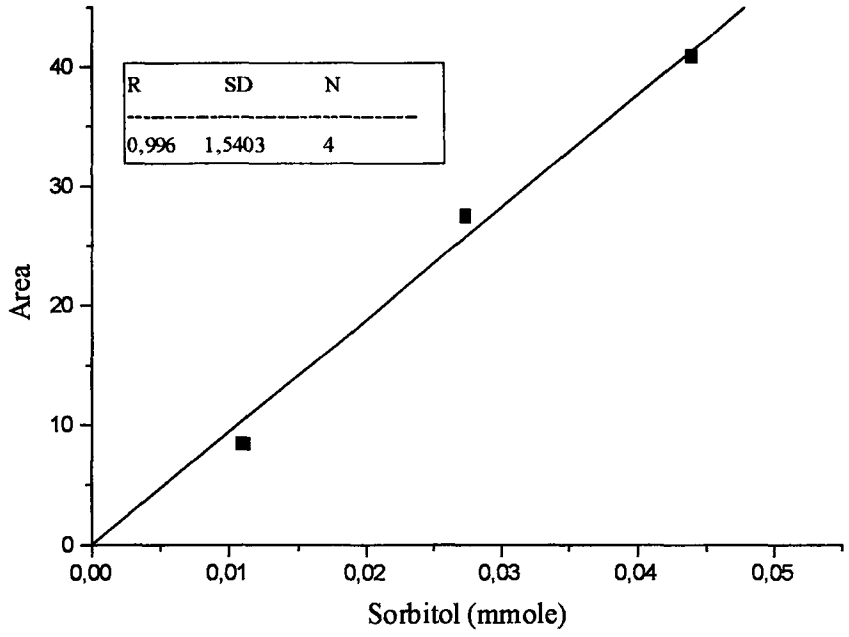

Figure A.3 Calibration curve of sorbitol

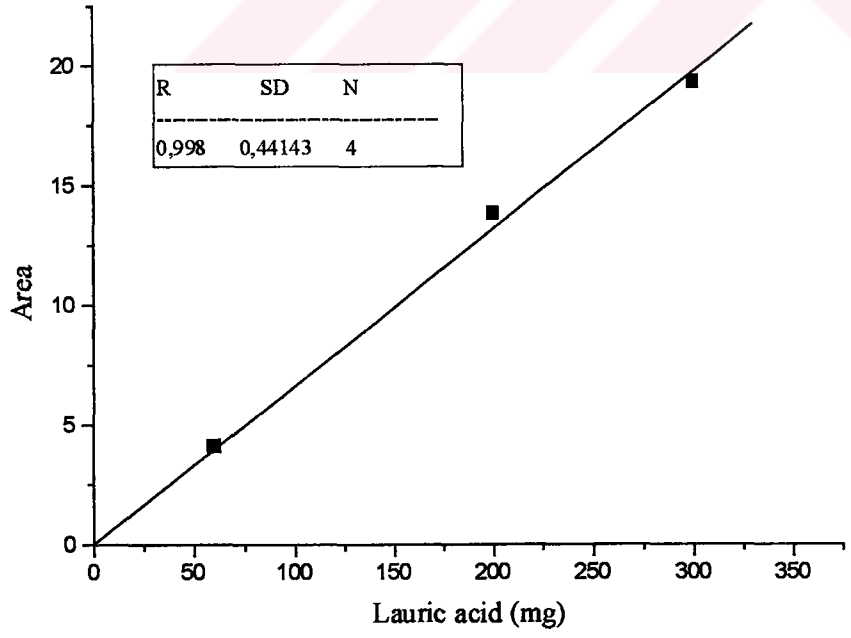

Figure A.4. Calibration curve of lauric acid

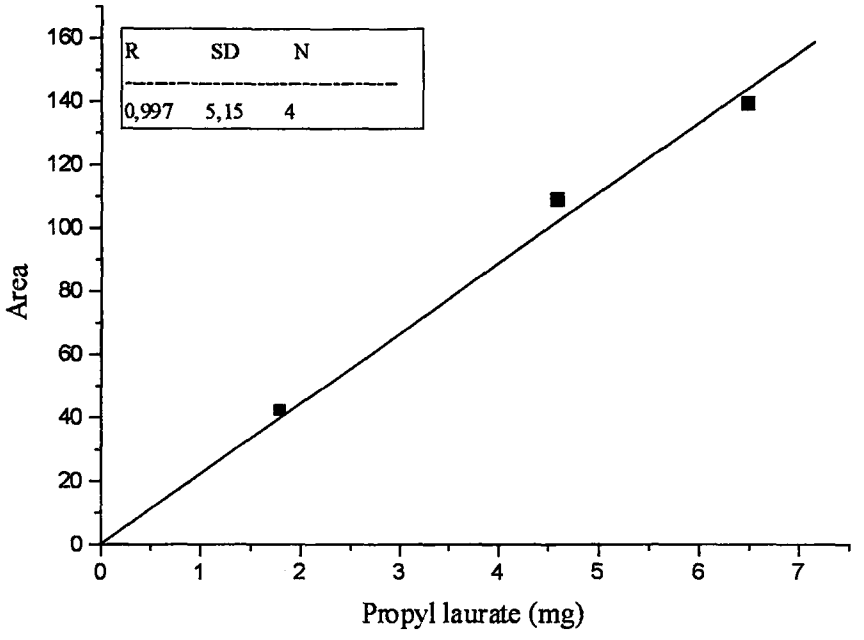

Figure A.5. Calibration curve of propyl laurate

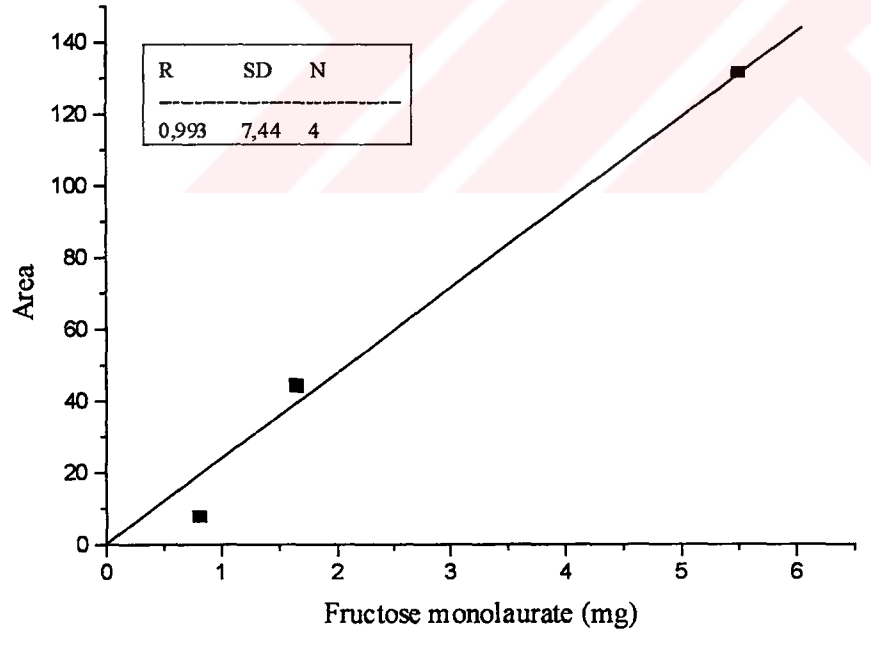

Figure A.6. Calibration curve of fructose monolaurate

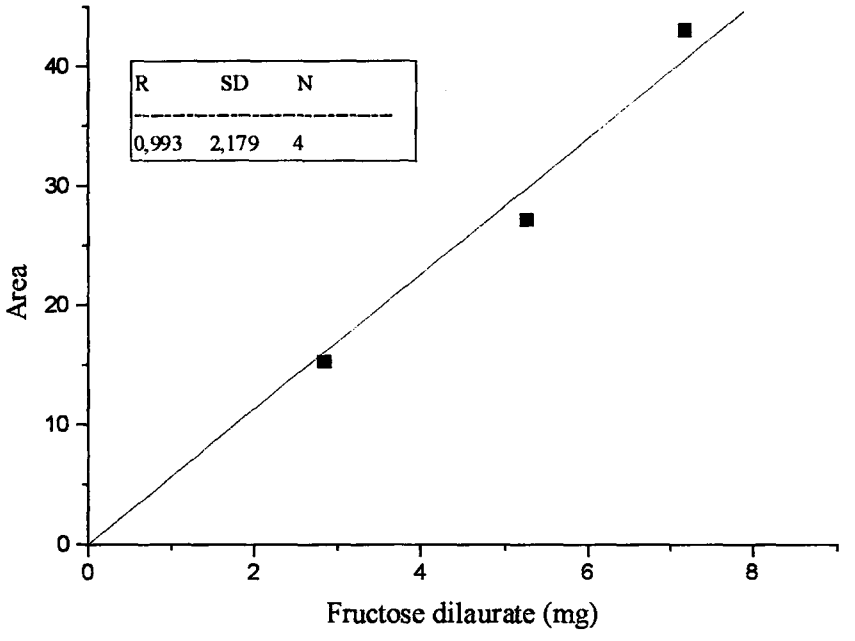

Figure A.7. Calibration curve of fructose dilaurate

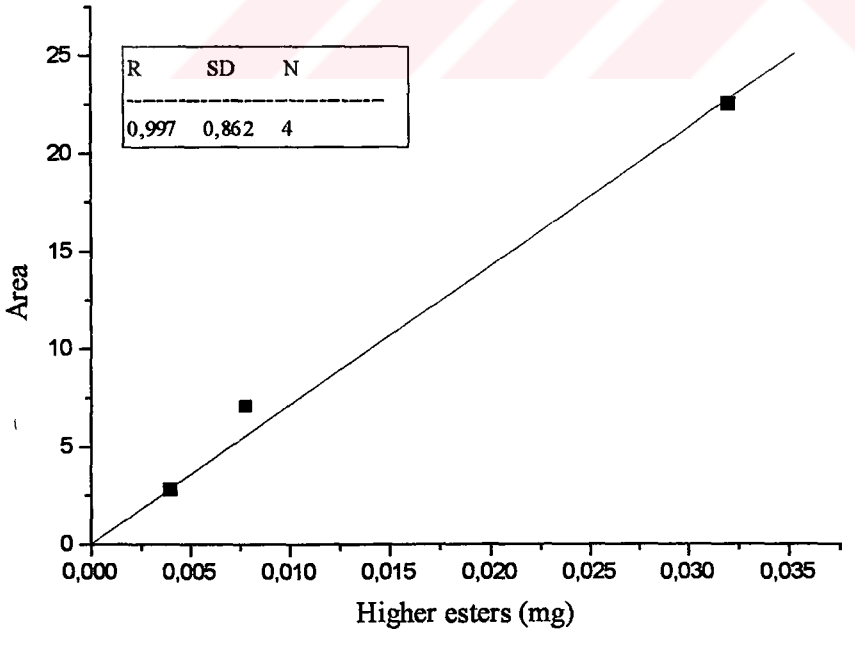

Figure A.8. Calibration curve of higher esters

APPENDIX B
HPLC Chromatograms

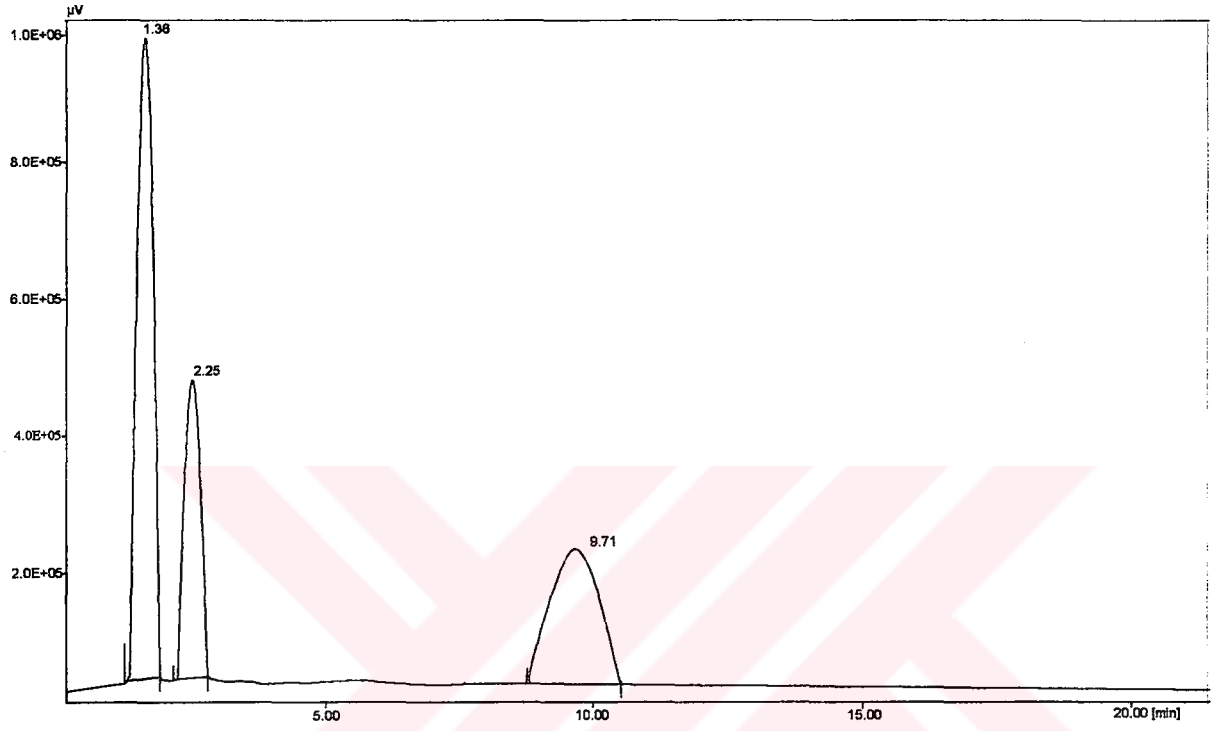

Figure B.1. HPLC chromatogram of propyl laurate

File Name : PROP_127.CH1

Info : 100 mg Lauric acid, 50 mg Novozym 435, 4 ml isopropyl alcohol,

200 mg molecular sieve, 60°C, 4 hour

Control Method

#	Name	Retention time
1	Isoproyl Alcohol	1.36
2	Lauric acid	2.25
3	Propyl laurate	9.71

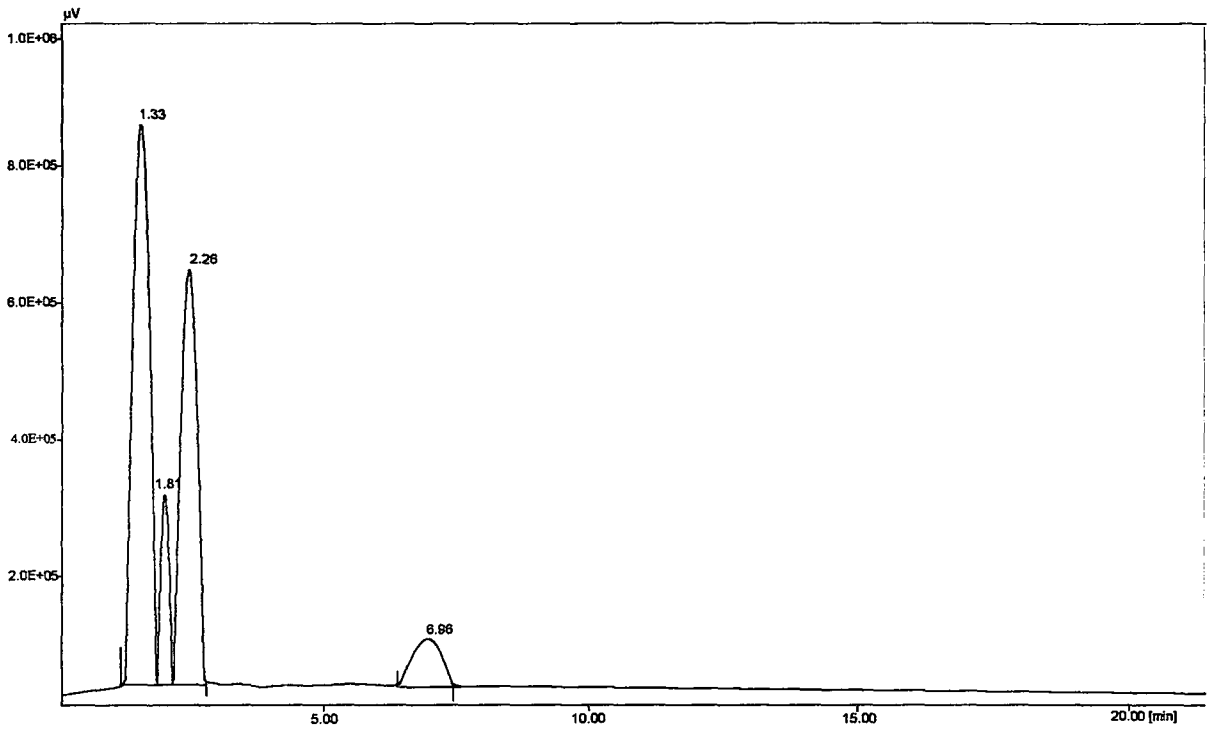

Figure B.2. HPLC chromatogram of lauric acid esters of fructose

File Name : EST_214.CH1

Info : Lauric acid/fructose mole ratio 5/1, 100 mg Novozym 435, 2 ml acetone, 200 mg molecular sieve, 40°C, 5 hour

Control Method

#	Name	Retention time
1	Acetone	1.33
2	Fructose monolaurate	1.81
3	Lauric acid	2.26
4	Fructose dilaurate	6.96

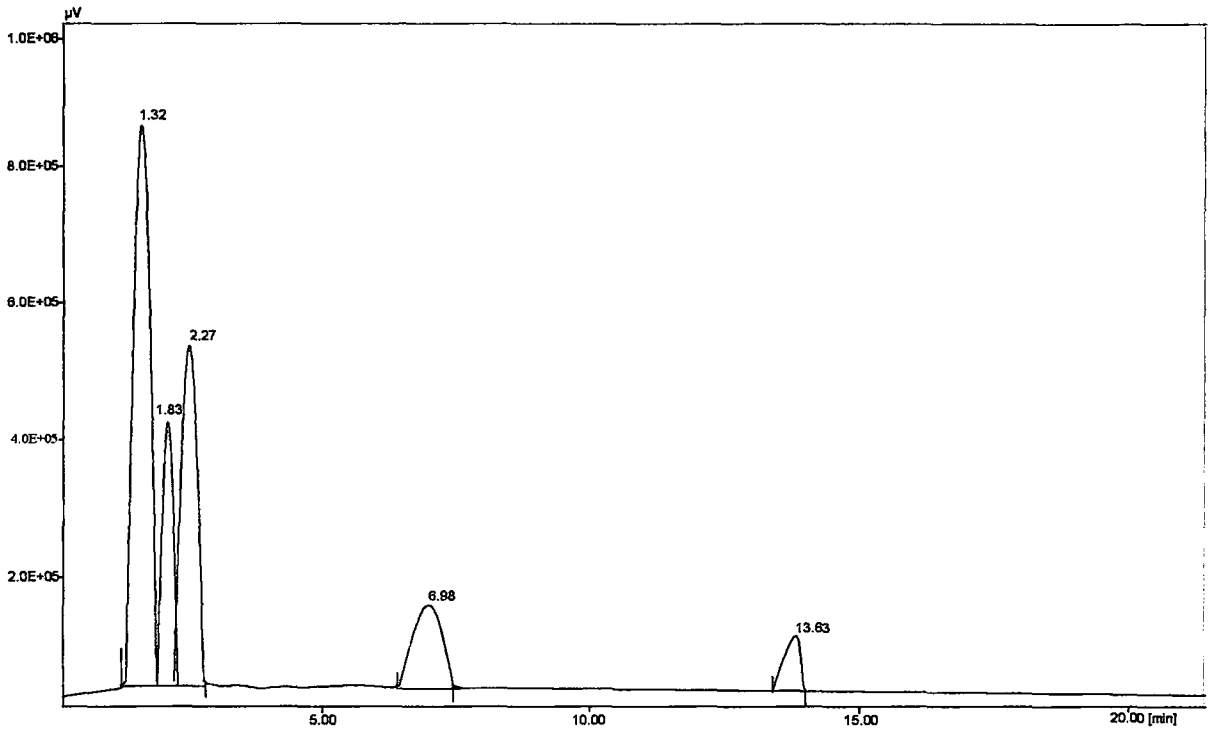

Figure B.3. HPLC chromatogram of lauric acid esters of fructose

File Name : EST_258.CH1

Info : Lauric acid/fructose mole ratio 5/1, 100 mg Novozym 435, 2 ml acetone, 200 mg molecular sieve, 40°C, 24 hour

Control Method

#	Name	Retention time
1	Acetone	1.32
2	Fructose monolaurate	1.83
3	Lauric acid	2.27
4	Fructose dilaurate	6.98
5	Higher ester	13.63

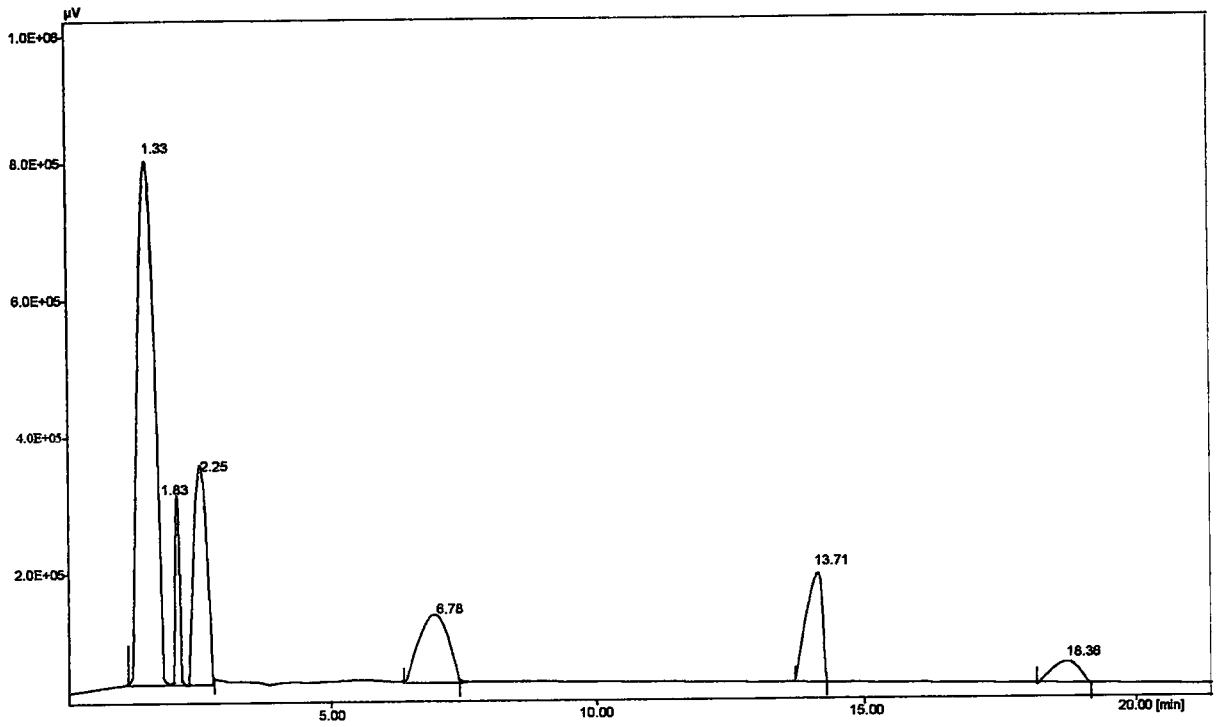

Figure B.4. HPLC chromatogram of lauric acid esters of fructose

File Name : EST_271.CH1

Info : Lauric acid/fructose mole ratio 5/1, 100 mg Novozym 435, 2 ml acetone, 200 mg molecular sieve, 40°C, 96 hour

Control Method

#	Name	Retention time
1	Acetone	1.33
2	Fructose monolaurate	1.83
3	Lauric acid	2.25
4	Fructose dilaurate	6.78
5	Higher ester	13.71
6	Higher ester	18.36