Synthesis of Sugar Fatty Acid Esters using Lipase Immobilized in Supported Sol-Gels

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Nikhil Kumar

Abstract

Sugar fatty acid esters are of practical importance and have a variety of applications that include biodegradable detergents and emulsifiers in resin polymerization. Traditionally, they are synthesized chemically with low selectivity and different degrees of esterification. In this study, different sugar esters were enzymatically synthesized from a variety of sugars/sugar alcohols and fatty acids. The removal of water in an esterification reaction is critical in determining the reaction direction due to the reversibility of the reaction. Furthermore, if the water generated in the reaction is not removed continuously lower ester yields and a significant reduction in the activity and stability of immobilized enzymes can occur due to partial inhibition. The enzyme used was Lipozyme *Candida antartica* Lipase B (CAL-B) and the approach taken here was to investigate the feasibility of using Celite® supported sol-gel immobilized enzymes (CSSIE) as biocatalysts. The sol-gel consisted of PTMS (trimethoxypropylsilane) and TMOS (tetramethylorthosilicate) and was supported by three different types of Celite® – R632, R633 and R647. CSSIE was dried and reused a number of times without a significant loss of activity. The CSSIE were found to behave as highly porous adsorbents with a high capacity to absorb water by selective uptake of polar substances. Their strong affinity for water not only prevented significant co-adsorption problems but also offered the consistent performance required for industrial applications.

Maximum yield of product was obtained using CSSIE and results were compared to literature data using a variety of biocatalysts and moisture adsorbing media including unsupported sol-gels, Novozym, Celite® and molecular sieves. Although the moisture adsorbing capacity of the CSSIE decreased with reuse due to saturation, sol-gel aging marginally increased enzyme activity with a half life of 8 repeated uses with the highest conversion obtained when using the supported sol-gel 8-days after initial preparation. No appreciable side products were observed for any of the reaction combinations examined and the highest product yield (96.7%) was obtained for the product sorbitan monooleate. Experiments were also conducted to determine conversion and moisture content as a function of substrate molar ratio, quantity of enzyme, type of solvent, nature of the fatty acid and reaction temperature. The highest conversion was obtained by the use of acetone as the reaction solvent, longer chain fatty acids and a reaction temperature of 40°C. The maximum protein immobilization of 85% of the supplied protein was obtained using sol-gel supported by Celite®-R633. Sol-gel clusters on the surface of the Celite® were observed following repeated reuse of the CSSIE which may partially explain the decrease in conversion observed with reuse.

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List of Acronyms

HLB	Hydrophilic – Lipophilic Balance
CMC	Critical Micelle Concentration
CSSIE	Celite® Supported Sol-Gel Immobilized Enzymes
GC/MS	Gas Chromatography-Mass Spectrometry
IL	Ionic Liquids
FDA	U.S. Food and Drug Administration
CAL-B	*Candida antartica* Lipase B
PTMS	Trimethoxypropylsilane
TMOS	Tetramethylorthosilicate
HMDS	Hexamethyldisilazane
TMCS	Trimethylchlorosilane
BSTFA	N,O Bis(trimethylsilyl)trifluoroacetamide
KF	Karl Fischer
BCA	Bicinchoninic acid
HPLC	High Performance Liquid Chromatography
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
LSD	Least Significant Difference
SEM	Scanning Electron Microscopy
SS	Sum of Squares
MS	Mean Squares
Df	Degrees of freedom
ANOVA	Analysis of variance

1. Literature Review

Sugar fatty acid esters are widely used in the food, pharmaceutical, detergent, agricultural, fine chemical and personal care industries. They are tasteless, odorless, non-toxic, non-irritant, and biodegradable and have practical importance as emulsifiers, wetting agents, surfactants, detergents and lubricants (Colbert, 1974). They can be synthesized by an esterification reaction between sugars/sugar alcohols (e.g. sucrose, fructose, glucose, sorbitol, xylitol) and fatty acids (e.g. oleic acid, palmitic acid, myristic acid, stearic acid, caprylic acid).They are surface active in aqueous solutions and consist of a polar head (sugar) and a non-polar tail (fatty acid) (Colbert, 1974). Thus, they are amphiphillic and nonionic based on the absence of formally charged groups on the sugar head group. To overcome the problems of synthesizing sugar esters chemically as discussed in Section 1.4, this study explored an enzymatic approach using lipase immobilized in a sol-gel matrix supported on a Celite® carrier.

Celite® supported sol-gel immobilized enzymes (CSSIE) were prepared from sol-gels made of PTMS and TMOS and a variety of different Celite® types (R632, R633 and R647). The most common approach used to remove the water liberated during enzymatically-mediated esterification reactions is the addition of molecular sieves to the reaction media. This has been found to help in the absorption of the water produced as a by-product (Yoo, Park & Yoon, 2007). The yield of product for a variety of sugars and fatty acids when CSSIE was used was studied and is compared to the use of unsupported sol-gel enzymes, Celite®, Novozym and molecular sieves. A drying approach was explored to determine if the water could be removed from the CSSIE and allow reuse without a significant decrease in activity. Other key parameters were investigated like moisture content as a function of biocatalyst, reaction rate, fatty acid, reaction temperature and quantity of enzyme.

1.1 Chemical Synthesis Techniques

Two chemical techniques for performing the transesterification reaction between sugars and fatty acids have been described in Ota *et al.* (1972) and Farone & Serfass (1995). Both these approaches have been carried out chemically with different degrees of esterification at high temperatures and very low product yield. Ota *et al.* (1972) approach consisted of combining sugar with the fatty acid in a molar ratio 1:4.5 followed by heating at 230°C in a nitrogen gas stream for 2 hours with continuous stirring. Following this, the product was cooled to 80°C and acetic anhydride with sulfuric acid was added to the mixture and refluxed for 4 hours. The product was then washed with boiling water and dried under reduced pressure. Activated clay and activated carbon were added and the mixture was heated at 120°C for 1 hour for decolourisation following steam injection for deodorization. The final product was dissolved in twice the amount of ethanol and left undisturbed for 8 hours. The resulting product was a light brown solid with a melting point of 34.5°C, hydroxyl value of 23 and a saponification value of 243. This end product was fractionally crystallized and contained a mixture of sugar diesters (38%) with sugar fatty acid triesters as the main product.

Farone & Serfass (1995) have developed an approach where the sugar fatty acid ester transesterification is conducted in a series of steps as described below. The reaction between the fatty acid and methyl/ethyl alcohol was first carried out in the presence of a sulfuric acid catalyst to produce fatty acid esters and water.

$$\text{Fatty acid} + \text{Methyl / Ethyl Alcohol} \leftrightarrow \text{Fatty acid Ester} + \text{Water}$$

The sulfuric acid catalyst was then neutralized with a metal carbonate (potassium carbonate) to form a metal sulfate which was separated from the fatty acid ester, alcohol and water by filtration. The metal sulfate, alcohol and water were recovered from the fatty acid ester at the end of the reaction as by-products and reused in the process. The fatty acid ester was reacted with sugar dissolved in dimethyl sulfoxide in the presence of the metal carbonate catalyst to produce the sugar ester product and alcohol.

$$\text{Fatty acid Ester} + \text{Sugar} \leftrightarrow \text{Sugar Ester} + \text{Alcohol}$$

The reaction time was from 8 to 24 hours at a temperature of 105 – 125°C. The dimethyl sulfoxide was then separated from the reaction mixture by vacuum distillation. The sugar ester product and unreacted fatty acid ester were emulsified by the addition of water and the unreacted sugar and metal carbonate were dissolved in the water. The dimethyl sulfoxide was separated and reused in the process. The emulsion that contained the sugar ester product and unreacted fatty acid ester was separated from the water by filtration, decanting or centrifugation. The metal carbonate was recovered and used as a mineral supplement for molasses and animal feed. The final sugar ester product was purified by dissolving the unreacted fatty acid in ethyl acetate. The dimethyl sulfoxide, alcohol and ethyl acetate were separated for reuse by fractional distillation and drying of the non-aqueous solvents. All the unreacted sugar in a concentrated form was recovered.

In comparison, Ota *et al.* (1972) conducted the reaction using sorbitol and palmitic acid and obtained an end product of sorbitan tripalmitate ester monoacetate and 38% by weight of sorbitan dipalmitate ester monoacetate. Farone & Serfass (1995) obtained a 92% mixture of sucrose mono and di-stearate which is above the 80% commercial requirement. Farone & Serfass (1995) also proposed an integrated commercial facility that could be used to produce 800 tons of sucrose stearate per year.

1.2 Other Experimental Approaches

1.2.1. Supercritical Carbon Dioxide

Habulin, Sabeder & Knez (2008) synthesized sugar fatty acid esters using lipase with 2-methyl 2-butanol and supercritical carbon dioxide as the reaction solvent. Molecular sieves were used in the reaction. The inhibitory effect of sugar esters on the growth of microorganisms was tested and it was found that sucrose laurate inhibited the growth of *Bacillus cereus,* a food poisoning bacteria, at a concentration of 9.375 mg/ml. The highest palmitate acid conversions obtained for fructose palmitate synthesis in t-butanol and supercritical carbon dioxide were 65% and 61%, respectively.

1.2.2. Ionic Liquids and Ultrasound Radiation

Lee *et al.* (2008) synthesized sugar esters by mixing an aqueous sugar solution with an organic solvent and ionic liquid (IL). They found that this approach enabled high sugar concentrations in the IL which significantly improved the initial reaction rate and conversion of the lipase catalyzed reaction. The enzyme activity was greatly enhanced when ultrasound radiation was used in the IL and no significant effect on the stability of enzymes was observed. Xiao *et al.* (2005) investigated the synthesis of glucose esters using ultra-sound radiation and shaking in a non-aqueous media. They found that enzyme activity in ionic liquids increased 1.4 times when ultrasound radiation was applied.

1.2.3. Pervaporation Dehydration

Sakaki *et al.* (2006) applied pervaporation dehydration using a zeolite NaA membrane to remove the water formed in the lipase-catalyzed esterification reaction of sugar with palmitic acid in 2-methyl 2-butanol. The water content in the reaction mixture was reported to decrease ten-fold from 0.3 weight% to 0.03 weight% by this technique.

1.2.4. Molecular Sieves

Yoo, Park & Yoon (2007) performed the enzymatic synthesis of sugar esters in a pilot reactor removing the water by recycling the reaction media through a molecular sieve column outside the reactor. A yield of 98% xylitol monooleate was obtained under these conditions. The limitation of this approach however is that there is an increase in the reactor volume and mass transfer limitations could occur due to challenges associated with mixing. In addition, the price of molecular sieves is higher than other types of desiccant-based dehumidifiers and they have not yet been approved by the FDA for foodstuff and pharmaceutical applications.

1.2.5. CO_2-Acetone System

Tai & Brunner (2009) explored the production of fatty acid-sugar esterification in a CO_2-acetone system catalyzed using Novozym 435. Key process parameters such as pressure, temperature and concentrations of the substrate and enzyme were examined. The optimum temperature and pressure were found to be 50°C and 65 bar, respectively. Water was removed using a multi-phase distribution of acetone-CO_2-water-sugar system.

1.2.6. Packed-bed Reactor

Ye, Pyo & Hayes (2010) produced saccharide-fatty acid esters using immobilized *Rhizomucor miehei* lipase-catalyzed esterification in various solvent free systems at 65°C. The solvent-free suspensions consisted of molecular sieves and a packed-bed reactor operated under continuous recirculation. A fructose oleate yield of 88% with 92% monoester was obtained with high rates of reaction.

1.2.7. Recombinant DNA technology

Chang & Shaw (2010) discussed protein and metabolic engineering concepts to improve the catalytic efficiency, thermostability and pH stability of biocatalysts for the enzymatic synthesis of sugar esters. This review also focused on the advantages of using recombinant DNA technology for the large-scale production of enzymes and cloning of key enzyme genes for carbohydrate biosynthesis. This is an interesting approach as the production of large quantities of enzymes in a heterologous host would substantially lower the overall production cost. The importance of solvent engineering to improve sugar ester yields is also discussed in this review.

1.3 Challenges in Performing the Esterification Reaction

Some of the challenges faced in the synthesis of sugar esters include the following:

1. Choice of a solvent due to opposite polarities of substrates

Sugars are soluble only in hydrophilic solvents such as DMF, DMSO and pyridine which are toxic to sugars/fatty acids and unsuitable for commercial use. However it has been found that enzymes were quickly inactivated by these solvents and stability affected which has resulted in low reaction rates and conversions (Yoo, Park & Yoon, 2007). Due to the hydrophobicity of fatty acids, the use of water as a reaction solvent results in low product yields (Yoo, Park & Yoon, 2007). Some of the methods proposed to counteract this challenge is to use activated fatty acids (condensation of the compound molecule with a nucleotide) in polar solvents (Therisod & Klibanov, 1986), activated sugars in apolar solvents (Oguntimein, Erdmann & Schmid, 1993), achieve partial solubilization of both substances in intermediate polarity solvents (Coulon *et al.*, 1996) and the use of ionic liquids (organic salts that melt below 100°C) (Lee *et al.*, 2008) Regioselective modification of carbohydrate molecules has also been shown to be a challenge due to the presence of multiple hydroxyl groups that result in lower conversions (Chang & Shaw, 2010).

According to Yan *et al.* (2001), a solvent must be chosen that is non-toxic without affecting the stability and activity of different biocatalysts and be reasonably priced for large-scale use.

Furthermore, the solvent should dissolve enough substrate to allow the enzymatic reaction to occur. Acetone, t-butanol, dimethylformamide, dimethylsulfoxide and pyridine are the most widely used solvents in literature for sugar-fatty acid esterification reactions. Although sugars and sugar alcohols are highly soluble in hydrophilic solvents such as DMF, DMSO and pyridine, these are toxic and unsuitable for commercial use and t-butanol has been banned for use in the manufacture of food additives by FDA.

2. Formation of water as a by-product

Esterification is a reversible reaction and hence the water by-product must be continuously removed to reduce the rate of inhibition of the reverse reaction and to obtain higher yields of sugar ester product (Adachi & Kobayashi, 2005). Enzyme activity and stability has also been shown to be affected by a high concentration of water (Yoo, Park & Yoon, 2007). In addition, when the immobilized enzyme is covered by a layer of water this prevents the substrates from interacting with the enzyme (Chamouleau *et al.*, 2001) and reduces the reaction rate. Some methods that have been employed to remove the water formed during the reaction included evaporation under reduced pressure, azeotropic distillation (Yan *et al.*, 1999), microwave heating (Carillo-Munoz *et al.*, 1996), use of inorganic salts (Hertzberg *et al.*, 1992), pervaporation (Kwon *et al.*, 1995), evacuation in vacuum (Napier *et al.*, 1996), multiphase distribution of acetone-CO_2-water-glucose (Tai & Brunner, 2009), vapor permeation and membrane separation (Tsitsimpikou, Daflos & Kolisis, 1997), gas sparging (Won & Sun, 2001) and circulation of reaction media through an external column packed with molecular sieves (Yoo, Park & Yoon, 2007).

1.4 Advantages of an Enzymatic Process

The advantages of an enzyme-based approach in a chemical reaction has been compiled in Table 1 based on information reported by Fregapane *et al.* (1994) and Pauly, Engasser & Ghoul (1999). The enzymatic approach has the advantage of monomer formation and trace amounts of undesirable side products at mild reaction conditions (Yoo, Park & Yoon, 2007) unlike the chemical approach with different degrees of esterification at high temperatures and very low product yield (Ota *et al.*, 1972).

Table 1. Comparison of chemical-based and enzymatic approaches for sugar ester synthesis

Chemical Process	Enzymatic Process
Consumes high quantities of energy	Consumes low quantities of energy
Forms considerable amounts of undesirable side products	Forms trace amounts of undesirable side products
Produces a whole range of structural isomers due to the presence of multiple hydroxyl groups in carbohydrate substrates	Monomer formation
Forms very complex mixtures of various compounds due to the non-specific nature of the chemical condensation reaction between sugars/sugar alcohols and fatty acids	Specificity of enzyme usually results in the formation of simple product mixtures
High temperatures and pressures leads to parasitic reactions, polymerization and coloring of products	Mild reaction conditions; De-coloring of products
Low selectivity leads to formation of a mixture of sugar esters with different degrees of esterification	Enzymes are very selective and this led to monoester production
Requires toxic organic solvents	Does not require toxic organic solvents. Acetone could be used

1.5 Product Applications

a) Drug Delivery Applications

Sugar esters have different HLB (Hydrophilic – Lipophilic Balance) values that depend on the fatty acid chain length and have been used to modify the uptake and excretion of drugs. Sugar esters with large HLB values of 16 resulted in an increased uptake and excretion of drugs (Szuts *et al.,* 2010). They have also been used to improve the disintegration of drugs. For example sucrose palmitate, which has a large HLB value, improved the rate of dissolution of nifedipine (Ntawukulilyayo, Bouckaert & Remon, 1993); sucrose stearate [HLB = 16] improved the rate of dissolution of phenytoin (Otsuka & Matsuda, 1995) and glybuzole (Otsuka, Ofusa & Matsuda, 1998); three sugar esters of [HLB = 16] increased the rate of dissolution of spironolactone (Marton, Auner & Csoka, 2005) and the permeation rate through the skin and mucosa. When used as microemulsions they improved the diffusion of hydrocortisone through the stratum corneum (Lehmann, Keipert & Gloor, 2001) and increased the absorption of lidocaine hydrochloride by oral mucosal permeation (Ganem Quintanar *et al.,* 1998). The factors that led to an increase in the rate of transport of lidocaine included the high HLB value and formation of a microemulsion. Sugar esters have also been used as drug delivery agents for transdermal therapeutic agents (Csoka *et al.,* 2007); as tablet matrix formers with microcrystalline cellulose (Ntawukulilyayo, Demuynck & Remon, 1995) due to their ability to form hydrogen bonds between the sugar esters and cellulose in the final product; in the preparation of enteric coating emulsifying systems for insulin delivery (sucrose erucate) (Toorisaka *et al.*, 2005), and in solid-oil non-dispersions for transcutaneous protein delivery (sucrose erucate) (Tahara *et al.,* 2008). Sucrose stearates have also been used to develop the controlled release (excretion) of proniosome-based niosomes (Abd-Elbary, El-laithy & Tadros, 2008).

b) Cancer Applications

Sugar esters exhibited anti-cancer and anti-tumor properties (Ferrer *et al.*, 2005 and Benrezzak & Nigam, 1992). Maltose fatty acid esters have showed cytotoxicity against many tumor cells. Maltose tetrapalmitate has been used on rats as an immunoadjuvant against a weakly immuno transplanting mammary adenocarcinoma (Nishikawa *et al.*, 1981). Nishikawa *et al.* (1981) found that sugar fatty acids of stearates, palmitates and myristates were far more efficient anti-tumor agents than their derivatives with shorter fatty acid chains such as laurate and caprylate esters. Monoesters have also found to be in general, better anti-cancerous agents than higher substituted derivatives. Trehalosediesters of carbon chain lengths from 8 to 12 also inhibited tumor necrosis. However, the inhibitory effect of maltose and trihalose esters was greater than sucrose esters and monosaccharide fatty acid esters did not display any significant anti-cancer properties.

Ferrer*et al.* (2005) found that fatty acid esters of maltotriose had anti-tumor properties and inhibited cancer cell spreading. These esters were prepared by the transesterification reaction of vinyl laurate with maltotriose in the presence of an immobilized lipase. The solvents used were 2-methyl 2-butanol and dimethyl sulfoxide. Cytotoxic activity towards two human cancer cell types Hep-G2 and HeLa were observed. 6-O-palmitoyl maltotriose exhibited 50% inhibition values of 2.3 μM for Hep-G2 and 3.6 μM for HeLa. From this study, trisaccharides appeared to be more promising inhibitors than disaccharides and exhibited marginal cytotoxicity towards rat hepatocytes.

c) Hydrocarbon Emulsifiers

Hydrocarbons are organic compounds and a huge source of air and water pollution. Certain microorganisms degrade insoluble organic pollutants by producing emulsions (Kelkar, Kumar & Zinjarde, 2007; Zinjarde & Pant, 2002a; Chaillan *et al.,* 2004). These microbes showed increased degradation of hydrocarbons when emulsifiers were added externally (Lee *et al.,* 2006). Kelkar, Kumar & Zinjarde (2007) produced a sugar fatty acid ester hydrocarbon emulsifier (lauroyl glucose) which they examined for its potential to control environmental pollution. This emulsifier enhanced the disintegration of aliphatic, aromatic (benzene, toluene and xylene) hydrocarbons and crude oil. A linear relationship between the concentration of the sugar fatty acid ester (50 – 450 µg/L) and emulsification activity was observed under assay conditions. The microbial culture degraded 70% of the aliphatic portion of crude oil when the external sugar ester emulsifier was at a concentration of 200 mg/L.

d) Plant growth inhibition

Afach *et al.* (2006) enzymatically synthesized sugar fatty acid esters from D-allose (a rare sugar) using a lipase catalyzed transesterification approach with D-allose and vinyl octanoate. The growth inhibiting activity of the resulting 6-O-octanoyl-D-allose on lettuce seedlings was found to be six times greater than the effect of using D-allose. It was concluded that the introduction of the octanoyl group induced a 6-fold increase in its inhibitory activity.

1.6 Technical Specifications

1.6.1 Stability under Acidic and Basic Conditions

The stability of sucrose fatty acid esters under acidic and basic conditions was evaluated by Okumura *et al.* (2011). The glycoside and ester bond were preferentially hydrolyzed under acidic and basic conditions where the rate of hydrolysis was determined to be a function of the solution pH, product concentration and the position of the acyl sugar monoester. These monoester products had excellent stability at a pH from 5 to 7 and moderately stable at a pH of 7 but were unstable at pHs greater than 8.

1.6.2 Effect of Different Biocatalysts

The influence of enzymes and different lipases on the enzymatic synthesis of sugar fatty acid esters has been studied by various researchers. The properties of sugar monoesters were shown to vary with the use of different biocatalysts as shown by Plou *et al.* (2002) in which different sources of enzymes such as lipase and protease were analyzed for the ester reaction using sucrose and maltose with vinyl laurate in 2-methyl 2-butanol and dimethyl sulfoxide. The lipase from *Thermomyces Lanuginosus* exhibited the highest activity in this study. Glucose esters were tested and hydroxyl groups at the 6 position were preferentially acylated. Monoesters were formed at a sugar:fatty acid molar ratios of 1:1. Lipases from different sources such as *Pseudomonas sp.* and *Rhizomucor meihei* were found to be regiospecific. Lipases from *Candida antartica* resulted in products of 6 and 6^1-monoesters in the study conducted by Woudenberg & Rantwijk (1996).

1.6.3 Influence of Acyl Donor

The influence of the acyl donor on the yield of product is well studied in the area of sugar ester synthesis. Kawase, Sonomoto & Tanaka (1992) detected the formation of an acyl-enzyme intermediate and Schmid & Verger (1998) found that the nature and type of the acyl donor had a notable effect on the reactivity with the observation that longer carbon chains led to higher conversions. It was also found that the rate of the ester synthesis with hydroxyl-containing vinyl ester compounds was about 20-100 times faster than with alkyl esters.

1.6.4 Effect of Support in Immobilized Enzymes

The advantage of using immobilized enzymes as biocatalysts has been widely recognized due to advantages such as increased mechanical strength, chemical and physical stability, enzyme loading capacity, hydrophobic and hydrophilic character and economic considerations (Mateo *et al.*, 2007). Most literature report on the enzymatic acylation of sugars that have been performed using immobilized commercial lipases. For example, Ferrer *et al.* (2002) used lipase from *Thermomyces langinosus* and studied the effect of using a polypropylene support which was found to increase the initial activity of the enzyme; however, a reduction in the yield of monoester noticed was attributed to the formation of excessive amounts of diesters. The use of different supports has been studied such as the covalent attachment to Eupergit C that has very low synthetic activity and silica granulation that exhibited high selectivity. There were also differences in reactivity and selectivity due to pore diameter and surface area of the support (Ferrer *et al.*, 2002).

1.6.5 Structural Dependence on Functional Properties

The effect of the sugar ester structure on the functional properties has also been studied by Ullrich, Metz & Mader (2008). Di and Tri-saccharide esters were more soluble in water than monoesters due to the increased hydrophilicity of sugar groups. Pharmaceutical excipients are substances other than the active drug that are contained in the finished product dosage. In this study, the application of gentle heat and shear stress led to nanosized pharmaceutical excipients without the use of any organic solvents that resulted in increased stability. This was beneficial as it led to the production of stable pharmaceutical excipients.

1.6.6 Hydophilic-Lipophilic Balance (HLB)

Surfactants have been widely used to modify the surface and interfacial interactions of immiscible substances such as oils and water. The polar ends of sugar ester surfactants are hydrophilic and the non-polar ends are lipophilic or hydrophobic. The non-polar regions vary in the number of chains, chain length, composition and chain saturation or branching while the head groups are polar, ionic or zwitter ionic (Colbert, 1974).

The hydrophilic-lipophilic balance (HLB) of a surfactant is the measure to which the sugar ester is hydrophilic or lipophilic determined by calculating the values for different regions of the molecule (Boyd, Parkinson & Sherman, 1972).

*HLB = (20 * Molecular mass of the hydrophilic portion of the molecule) / (Molecular mass of the whole molecule)*

A HLB value of 0 corresponds to a completely hydrophobic molecule and a value of 20 corresponds to a completely hydrophilic molecule. The HLB value of surfactants has been modified to predict the surfactant properties of a compound. Generally, a value of 0-3 corresponds to an antifoaming agent, 4-6 corresponded to a W/O emulsifier, 7-9 corresponds to a wetting agent, 8-18 indicates an O/W emulsifier, 13-15 indicates typical detergents and 10-18 indicates solubilisers or hydrotropes (Orafidiya & Oladimeji, 2002).

The effect of change in HLB with increased temperature was analyzed by Szuts *et al.* (2007). Sugar esters decomposed at temperatures below 220°C. Esters with high or medium HLB values were vitrified when melted. Hydrophilic and lipophilic sugar esters also softened and melted when subjected to heat treatment. An interesting phenomenon that was observed after melting and solidification was that the sugar esters had a partially amorphous structure which slowly crystallized in lime. The melting point of sucrose esters was from 40-79°C. When samples of sugar esters were heated to 100°C, the loss of mass that occurred was less than 1%. Crystal structures of sugar esters with a high or moderate HLB had a glass transition temperature instead of an amorphous melting temperature. HLB values which were characterized by these melting points disintegrated due to heating and did not recrystallize even when cooled. In general, recrystallization was faster for sugar esters with low HLB values. The original structure was not retained for any type of sugar ester irrespective of the HLB value after melting, solidification or heating.

A surfactant with a higher HLB value was water soluble and a lower HLB value was oil soluble. Hence, it was observed that high temperatures had a permanent effect on surfactant structure. This phenomenon was observed to be an important consideration in drug delivery where the modification in the structure of a sugar ester pharmaceutical product might alter the dissolution rate in the body which would result in mass transport limitations and other complications (Szuts *et al.,* 2007).

1.6.7. Critical Micelle Concentration (CMC)

Surfactants contain surface-active molecules and have the ability to self-assemble in aqueous solutions forming micelles, lamellae etc. Theoretically, as surfactants are added to a solution the surface concentration increases and surface tension decreases. The critical micelle concentration is the concentration of the surfactant beyond which the addition of surfactant solution results in the formation of more micelles but constant surface tension (De Vendittis *et al.,* 1981).

The CMC of sucrose fatty acid esters was analyzed by Molinier *et al.* (2005) and was found to be strongly affected by the composition of the mixture. Generally, micelle properties depend on the size of the alkyl chain and the head group. Micelles that were formed at surfactant concentrations just above the CMC were spherically shaped with a constant hydrodynamic radius up to a concentration at which the micelles became much larger in size and were not spherical with a variable diffusion coefficient. As expected, these micelles grew at higher surfactant concentrations and the concentration range for constant micelle radius was large. This condition, however, was not satisfied in the case of octanoate-derived esters. Sugar monoester micelles with a carbon chain length of 10 to 14 were oblate shaped with the exception of sucrose palmitate micelles that were prolate shaped and sucrose monooctanoates which were hexagonal with a radius of 13 Å. Micelles were also influenced by differences in the intramolecular hydrogen bonding of the sucrose head due to varying substitution.

1.6.8. Study of Interfacial and Foaming Properties

Interfacial and foaming properties of sugar ester surfactants have been of considerable interest to researchers in the recent past. Surface tension has often been a key concept used to characterize interfacial systems. Foaming has been defined by the foam volume that can be obtained from a unit volume of liquid and is a function of material, physicochemical conditions and test methods respectively (Koczo & Racz, 1991). The foam formation process has also been described as the formation of a new gas-liquid interface.

In particular, the foam property of a liquid has been characterized by its foam stability and is an important characteristic in surfactant solutions. Foaming and interfacial properties of sucrose laureates such as surface tension, thin film drainage, thickness and mobility were compared and studied by Husband *et al.* (1998). Dilaurates displayed poorer foaming properties and higher surface tension than monoesters. Crude sucrose monolaurate also exhibited high foam stability in comparison to the other sucrose laurates analyzed. Addition of diesters to monoesters in a molar ratio 1:4 improved the foaming properties to an optimum level which increased light scattering and reduction of the CMC. There were no appreciable differences in the surface tension and thin film properties between plain monoesters and the mixture of monoester and diester mixtures. Sucrose-based surfactants with a wide HLB range were produced by the esterification of the sucrose head group at a maximum of 8 sites and by variation in the length of the fatty acid. Molecular modelling experiments suggested that acyl chains of diester isomers were inclined at an angle greater than 120°. Diesters also had much higher light absorbance than monoesters which were constant with time. The foam stability increased with the concentration of the sugar ester. Foams corresponded to 40-100% of the total quantity of monoesters.

The reasons attributed to high foam stability of a mixture of monoesters and diesters were due to a higher surface activity than plain monoesters and the intermolecular interaction of the monoesters and diesters which was due to the solubilisation effect of monoesters in diesters and led to the formation of smaller aggregates and micelles. Thus, these interactions were attributed to mixed micelle formation and the bridging effect between monoester micelles and diesters. The lower surface activity of the diester indicated a more open structure with reduced efficiency of packing at the surface and formation of stable aggregates that affected the kinetics of adsorption. Coke *et al.* (1990) observed that interactions at interfaces increased interfacial viscoelasticity and simultaneously reduced drainage and increased foam stability.

Drainage of the foam lamellae was also not affected by crude diesters and hence it was concluded that the increased foamability was not an interfacial effect. In a mixture that contained monoesters and diesters, the presence of diesters reduced the CMC of the monoester but there was no effective change in the surface tension of the surfactant. This suggested that the effect of the diester was mainly in the bulk in the form of micelles rather than at the interface. The effect of presence of BSA protein on foam stability was also studied. Decrease in foam stability in the presence of a protein was attributed to competitive adsorption according to Clark, Wilde & Wilson (1991). Another interesting study that explained the formation of a viscoelastic layer due to the stabilization of the interface by proteins was conducted by Wilde & Clark (1996). According to this study, the protein was displaced from the interface by the surfactant but a high concentration of surfactant was not present to stabilize the interface. This resulted in the presence of both the protein and surfactant at the interface that led to the production of an unstable system.

In general, the foam stability increased with surfactant concentration. The binding of proteins with the fatty acid affected its stability according to Coke *et al.* (1990). Thus, the addition of sucrose diester to pure monoester enhanced its foaming properties due to the monoester-diester interaction and there was no observed effect on the surface tension and other thin film properties. Furthermore, these sucrose esters destabilized BSA protein containing foams. Liquid sugar esters also wetted the solid substrate and maintained contact due to intermolecular interactions and pair potentials. The degree of wetting or wettability was found to vary with the balance between the work of adhesion and the work of cohesion and depended on the solid surface properties.

1.7. Sol-gel Chemistry

The sol-gel process involves the chemical formation of an inorganic network in a solution at low temperatures (Schmidt, 1988). The solid (multiphase gel) is the final product formed from the liquid (colloidal solution) in the sol-gel process. The process starts with the hydrolysis of the precursor. The hydrolyzed precursor then undergoes condensation and gelation to form the gel. The properties of the gel change over time, with aging and drying. Aging of sol-gel is the expulsion of solution from the shrinking pores that contracts the gel-matrix due to polycondensation reactions. Structural reinforcement with additional cross links occurs due to sol-gel aging (Reetz, Zonta & Simpelkamp, 1995). Some common requirements of the precursors include solubility in the reaction media and reactivity to allow the gel-formation process to occur. They should also be able to form inorganic monomers for the sol-gel reaction. Schmidt (1988) described the applicability of finely divided silica that could be peptized to produce sols. Alkaloids are frequently used as precursors due to their solubility in common organic solvents and the ability to control hydrolysis.

Schmidt (1988) also described the potential advantages of the sol-gel process including the ease of purifying precursors, the use of chemistry to control reactions, formation of pre-organic network in the solution, introduction of permanent organic grouping in solutions and adjustment of appropriate viscosity for coatings. The gelation step involves the neutralization of surface charges, aggregation, further condensation by the reactive surface groups and the formation of the solid gel.

1.8. Enzyme Immobilization

Enzymes are catalysts that are selective and specific and can be used under mild experimental and environmental conditions. Immobilization has been found to be a powerful tool to improve enzyme properties such as stability, activity, specificity and selectivity leading to a reduction in inhibition and improved enzyme recovery. Recovery and reuse are important criteria that make enzymatic processes economically feasible. Immobilization also simplifies reactor design and can be used to control the reaction (Mateo *et al.,* 2007).

An ideal immobilization process should limit the use of toxic reagents and the immobilized enzyme should be highly stabilized. The enzyme support used for immobilization is vital for enzyme stabilization as undesirable support-enzyme interactions would decrease enzyme stability after immobilization and would not be suitable for reuse. Physical modification of immobilized enzymes generates a micro-environment that increases enzyme stability under distorting conditions. Chemical modification has also been found to improve enzyme performance after immobilization by increasing its surface reactivity. Also, chemical and genetic amination of enzyme surfaces e.g. glutaryl acylase / penicillin G acylase improves the covalent attachment and enzyme stability (Mateo *et al.,* 2007).

1.9. Sol-gels in Enzyme Immobilization

Reetz, Zonta and Simpelkamp (1995) carried out the lipase immobilization in sol-gels and found that the enzymatic activity was dramatically increased in an organic medium (88 fold when compared to the unimmobilized lipase). This was attributed to the high dispersion of lipase in the sol-gel matrix and the interaction between the lipophilic domains of the lipases and the hydrophobic regions of the sol-gel matrix. High enzyme activity of the heterogeneous biocatalyst was found to occur in water, which was 41% greater than in an aqueous homogeneous solution.

In this study carried out by Reetz, Zonta and Simpelkamp (1995), the lipase was entrapped in chemically inert hydrophobic silica gel that was prepared by the hydrolysis of alkyl substituted silanes in the presence of an enzyme. The gel precursors used were alkoxysilane derivatives like $RSi\text{-}(OMe)_3$ where R was an alkyl, aryl or alkoxy group. Gelatin and polyvinyl alcohols were used as aqueous solutions and PTMS & TMOS were used to prepare the sol-gel.

1.10. Celite® as an Enzyme Support Material

Celite®, also known as diatomaceous earth, is a naturally occurring soft, silicaeous, sedimentary rock that easily crumbles into a fine while to off-white powder with a typical chemical composition consists of 80 – 90% silica, 2 – 4% alumina and 0.5 – 2% iron oxide (De Martin et al., 1999). Common applications of Celite® include use as a filtration aid, liquid absorbents and reinforcing filler in plastics and rubber. Particle size and pore diameters for the Celite® usied in this study are provided in the Appendix (Section C). Wehtje, Adlercreutz & Mattiasson (2004) analyzed the use of Celite® as a support in enzyme immobilization and compared its activity to the use of controlled pore glass and glycerol-controlled pore glass as support materials. The enzyme immobilized by Celite® exhibited higher activities than the use of the other two support materials. Albumin was added during the immobilization of enzyme as an additive and this led to a stabilizing effect of the enzyme activity. Controlled pore glass supported enzyme required larger additions of albumin to provide the same activity exhibited by the Celite® supported enzyme. Without the addition of albumin, Celite® was the best support material and resulted in an activity of 0.25 mg / g Celite®. The enzyme used in this study was mandelonitrile lyase and a buffer solution of 1 ml / g support was used to prepare the enzyme solution.

The enzyme solution was added to the Celite® support material (1 – 2 ml solution / g support) and the water was removed by evaporation in a vacuum. The enzyme loading was also higher (20 mg / g Celite®) than the use of controlled pore glass. However, an activity loss occurred when the enzyme was added directly to the support without water removal, which was a critical phase during immobilization. The addition of polyethylene glycol as an additive made the properties of the support more favorable to retention of the enzyme activity and protected the enzyme from inactivation during the immobilization step.

1.11. Celite® Supported Sol-Gels

Meunier & Legge (2010) was the first group to exploit the effective use of CSSIE for the production of biodiesel in a transesterification reaction and investigated properties such as the activity, stability and reusability of the enzyme. Encouraging results were reported with the supported sol-gels showing good conversion, high initial enzyme activity and protein loading capacity. An average conversion of 60% was reported after a reaction time of 6 hours with the highest surface area coverage of the sol-gel supported on Celite®-R632.

2. Materials and Methods

2.1. General

Celite® - R632, R633, R647 samples were gifts from World Minerals (Santa Barbara, CA). Lipozyme, *Candida antartica* Lipase B (CAL-B) and Novozym 435 were gifted from Novozymes North America Inc. (Franklinton, NC). The biological source of Novozym was *Candida antartica* and its commercial activity was 10,000 PLU/g. Other materials used in this study were tetramethylorthosilicate (TMOS) (Aldrich), trimethoxypropylsilane (PTMS) (Sigma), oleic acid (90%) (Sigma Aldrich), palmitic acid (99%) (Sigma), stearic acid (98%) (Aldrich), lauric acid (98%) (Aldrich), myristic acid (98%) (Sigma), fructose, sucrose, sorbitol, xylitol (Aldrich), pyridine (99%) (Sigma), hexamethyldisilazane:trimethylchlorosilane (HMDS:TMCS 3:1) (Supelco), pentadecanoic acid (90%) (Sigma) (internal standard for fatty acid analysis), tetrahydroxyfuran (99%) (Sigma), BSTFA (Supelco) (derivatization agent for GC-MS analysis), dimethoxyformamide (99%) (Sigma), molecular sieves (4 Å, 1/16 inch pellet) (Supelco), Milli-Q water purification system (Millipore, Billerica, MA) and HYDRANAL Coulomat CG catholyte (coulometric KF titration) (Fluka). Acetone and 2-methyl-2-propanol (t-butanol) were used as the reaction solvents.

2.2. Preparation of CSSIE

Celite® supported sol-gel immobilized enzymes (CSSIE) were prepared following the immobilization of procedure described in Meunier & Legge (2010). The hydrolyzed precursor solution was prepared from PTMS (0.08 mol), TMOS (0.02 mol), ultrapure water (1 mol) and hydrochloric acid (200 µL of 0.1 M). Lipase and phosphate buffer (50 mM, pH 7) were added to the precursor (14 ml of protein concentration 4000 µg/ml) after sonicating (1 hr) and rotary evaporation (40°C for 30 min) to remove water and alcohol. This mixture was combined with the desired Celite® support (6 g) and the gel was air-dried in a Petri dish at 4°C for 24 hours and washed with 15 ml of phosphate buffer (50 mM, pH 7) to remove the protein that was unimmobilized. In the case of unsupported sol-gels, the gel was dried without the addition of Celite®.

2.3. Sugar Fatty Acid Esters Synthesis

The reaction mixture consisted of 3 mmol sugar, 3 mmol fatty acid, 5 g of CSSIE (or) 0.03 g of unsupported sol-gel (or) a combination of 0.03 g and 5 g of Novozym and molecular sieves / Celite® with 25 ml of solvent. Previous studies of Yoo, Park & Yoon (2007) indicate that a high solubility of the sugar in the solvent was vital for achieving high ester product yield, thus sugar was added to the acetone in a stoppered glass bottle and agitated at 250 rpm in a shaker at 25°C for 8 hours before introducing the fatty acid and enzyme into the reaction mixture. Once the sugar was dissolved in the solvent, esterification was initiated by adding the fatty acid with the enzyme and mixed using a magnetic stirrer at 125 rpm. The reaction was allowed to proceed by mixing for 12 hours at 25°C. The yield of product was calculated from the decrease in the concentration of reactants and increase in the concentration of the sugar monoester assayed using GC-MS.

2.4. Conversion as a Function of Reaction Temperature

An evaluation of temperature and type of sugar was conducted using oleic acid with sol-gel supported on Celite®-R633 using acetone as reaction solvent. The reactions were carried out using a shaking bath and constant temperatures of 25°C, 40°C, 50°C, 60°C. The reaction was conducted in a 10 ml sealed glass vial using a magnetic stirrer at 125 rpm.

2.5. Sol-Gel Aging

The procedure used to prepare and wash the CSSIE described in Section 2.2 was adopted. After washing, the excess solvent was allowed to evaporate from the gel at room temperature for 14 hours and the gels were stored in a sealed Petri dish at 4°C (Meunier & Legge, 2010). Throughout the duration of this experiment, the sample was aged and dried under these conditions for the reaction between fructose and oleic acid in acetone. For each experiment, a newly prepared reaction mixture and a small quantity of CSSIE were used and the remaining sol-gel was stored in a sealed Petri dish at 4°C.

2.6. Gas Chromatography-Mass Spectrometry (GC/MS) Analysis

2.6.1. Sample Derivatization

Sugar/Sugar Alcohols

The carbohydrates were derivatized (Sweeley *et al.*, 1963) by mixing 0.01 g of sugar/sugar alcohol with 1 ml of pyridine (99%) + 0.1 ml of HMDS and 0.1 ml of TMCS. The mixture was shaken vigorously and then heated at 60°C for 10 minutes. Sample concentrations ranging from 1 to 10 mmol/L were analyzed for calibration purposes.

Fatty Acids

The derivatization method used by Severson *et al.* (1977) was adapted to derivatize fatty acids in this study. The fatty acids were derivatized by mixing 0.05 g of fatty acid with 0.05 g of pentadecanoic acid (internal standard) + 26.25 ml of tetrahydrofuran and the BSTFA-based derivatization was carried out at 75°C for 45 minutes. Concentrations ranging from 1 to 10 mmol/L were analyzed for calibration purposes.

Sugar Fatty Acid Ester

The derivatization method used by Severson *et al.* (1977) was adapted to derivatize sugar fatty acid esters in this study. The purified product was reacted with BSTFA:DMF of ratio 1:1 and was heated at 75°C for 45 min.

2.6.2. Product Analysis

Reactant and product analysis was performed on a Varian GC-MS system (CP-3800 gas chromatograph / Saturn-2000 mass spectrometer / mass spectrometer). Trimethylsilyl derivatives (1µL) were prepared according to Sweeley *et al.* (1963) as described in Section 2.6.1 and injected into a Hewlett-Packard Ultra 2 (Agilent Inc.), 25 m * 0.22 mm fused-silica capillary column with a 5% phenylmethyl silicone phase of 0.33 µm film thickness. The carrier gas used was helium with a linear flow rate = 35 cm/s, split ratio = 25, electron impact ionization energy = 70 eV, scan range = 40-650 Da and scan rate = 0.88 scans/s. The injector and detector temperature were held at 285°C and the temperature program was: 100°C for 0.5 minutes, increased to 240°C at 8.3°C/min, increased to 300°C at 10°C/min and held for 10 minutes (based on Fregapane *et al.*, 1994). Quantitative data was obtained from peak areas and calibration plots. The yield of product was determined based on the decrease in the concentration of the reactants (conversion). Mass spectra fragmentation patterns were recorded and verified to identify sugar esters and fatty acids.

2.7. Procedure for CSSIE reuse

At the end of the each reaction the CSSIE was separated by filtration using a 0.45 µm DB filter paper (Chromatographic Specialties Inc.). The CSSIE was then washed once with 5 ml of phosphate buffer (50 mM, pH 7.0) and excess buffer was evaporated from the gel at room temperature and the gel was stored in a sealed container for 24 hr before reuse. After 24 hr storage, the CSSIE was dried at 40°C for 1 hour before reuse.

2.8. Determination of Immobilized Mass of Protein in Sol-Gel

Protein analysis was determined according to Meunier & Legge (2010) using a Varian HPLC (High-Performance Liquid Chromatography) system (Varian Inc., Mississauga, ON) with an Agilent Zorbax Bio Series GF-250 column (Agilent Technologies, Mississauga, ON) and a Prostar 325 UV–Vis detector. The HPLC conditions employed a partial loopfill injection mode, absorbance of 280 nm and mobile phase flow rate of 1 ml/min. The mobile phase consisted of 200 mM of phosphate buffer at pH of 7. A quantity of 15 ml of Lipozyme CAL-B of protein concentration 2000 µg/ml was used. The retention time of the Lipozyme CAL-B was 16.7 minutes.

2.9. Moisture Content Analysis

The moisture content of the product was determined using a CA-06 Karl Fischer (KF) Moisture Titrator with an end point potential of 30 mv, VA-T=40, SENS=45, titration parameters = 1, 3, 5 minutes and end point waiting time of 30 sec. These were all parameters specific to the Moisture Titrator used in this study.

Product moisture content was calculated by the formula,

$$\frac{Moisture\ content\ of\ titration - \ Moisture\ content\ of\ blank\ solution}{(Sample\ weight + Tare\ weight) - \ Tare\ weight}$$

The product that contained a certain quantity of water was injected into the titration chamber that contained the reagent. Based on the amount of hydrogen iodide formed that was measured by the detection electrode, the water content of the sample was calculated. It was important that the stirrer speed was adjusted and the detection electrode & stirrer unit had to be connected properly. The desiccant tube (drying tube) was attached to remove any condensation that formed and the drain cock was closed. The titration flask and cathode chamber were filled with 100 ml of reagent and 1 vial of catholyte solution respectively.

At the end of the reaction, the enzyme was separated from the product mixture by filtration (0.45 µm filter paper) and the purified product was injected into the titration chamber that contained the reagent. The moisture content (%) was then measured.

2.10. CSSIE Reuse – Scanning Electron Microscopy (SEM)

A Hitachi S570 scanning electron microscope (Hitachi High-Technologies, Berkshire, England) was used to characterize the surface morphology and textural characteristics of the samples. All samples were coated with 24-carat gold prior to the analysis and electron beam energy of 15 kV was used for the analysis.

3. Results and Discussion

Meunier & Legge (2010) reported the development of Celite® supported sol-gel immobilized enzymes (CSSIE) for the production of biodiesel in a transesterification reaction and investigated properties such as the activity, stability and reusability of this enzyme preparation. Their results demonstrated that this approach can achieve good conversion with high initial enzyme activity and that they were able to achieve a high protein loading capacity. The objective of this study was to demonstrate the potential of this immobilized enzyme preparation for the production of sugar fatty acid esters.

The first section of the results and discussion presents the results obtained from experiments carried to select the support with highest lipase activity out using different Celite® carriers. This is followed by a series of experiments designed to optimize and characterize the selected support using different parameters with this system. The potential reusability of the enzyme preparation is then presented closing with experiments designed to explain the moisture absorbing properties of the preparation.

3.0. Selection of Celite® Support

3.0.1. Immobilized Mass of Protein in Sol-Gels

As a starting point, the suitability of the sol-gel and Celite® combination for protein immobilization was evaluated. A comparison was conducted using three different Celite® carriers that included: R632, R633 and R647. It had been shown in previous studies (Meunier

& Legge, 2010) that the greater the lipase immobilization the higher the enzyme activity, substrate conversion and product yield. The particle size and pore diameter for each type of Celite® carrier are given in the Appendix (Section C). As shown in Figure 1, the maximum protein immobilization occurred for the CSSIE supported with Celite®-R633 with 85% of the initial protein mass immobilized followed by the CSSIE supported with Celite®-R632 (77.5%), Celite®-R647 (58%) and unsupported sol-gel (55%). The determination of immobilization is shown in the Calculations (Section C). Meunier & Legge (2010) reported that R647 and R632 had significantly less percent sol-gel on the surface of the Celite® compared to R633. This was determined by measuring the increase in the surface area of the support after coating with lipase sol-gel. Celite®-R647 increased from 58.92 (uncoated) to 64.39 m^2/g; Celite®-R632 increased from 1.49 (uncoated) to 1.52 m^2/g; Celite®-R633 showed the largest increase from 0.9 (uncoated) to 1.37 m^2/g. This indicated that the lipase sol-gel did not adhere as well to the Celite®-R647 and Celite®-R632. Celite®-R633 was also reported to favor sol-gel adhesion over cohesion to the Celite® surface due to a smaller particle size (300-600 µm) than Celite®-R632 and Celite®-R647 (600-1400 µm). These are the characteristics of Celite®-R633 that might have resulted in higher immobilization in this study.

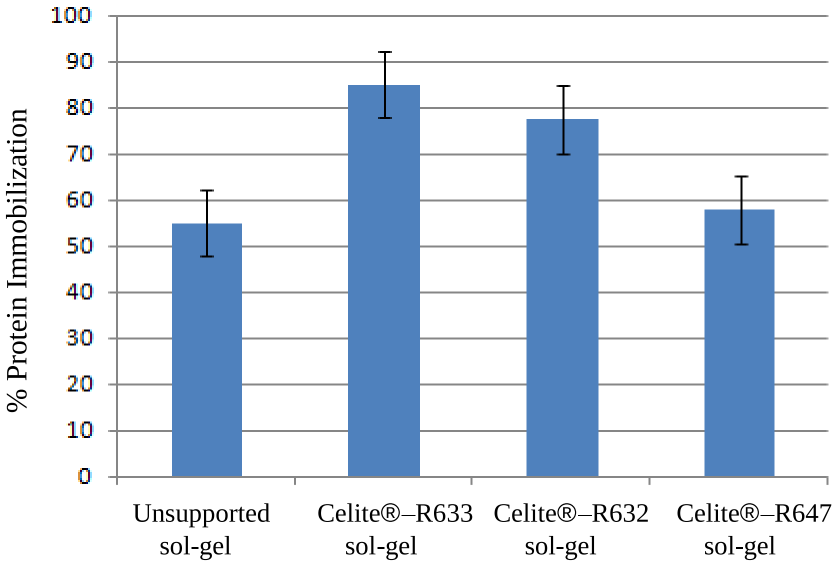


Figure 1. Percentage of immobilized protein for various sol-gel preparations. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.0.2. CSSIE Activity and the Effect of Sol-Gel Aging

This experiment was conducted to detect and evaluate enzyme activity using the sol-gels in the reaction between fructose and oleic acid in acetone. From Figure 2 it can be seen that fructose monooleate was produced without any evidence of side product formation based on GC-MS data. Stoichiometric and material balance calculations also confirmed the absence of any side products as shown in the Appendix (Section A) and Calculations (Section A) respectively. All the sol-gels used were active and the activity trend followed the sequence Celite®–R633 > Celite®–R632 > Celite®–R647 > Unsupported Sol-Gel based on oleic acid conversions.

Sol-gel aging as a phenomenon has been a topic of interest among researchers due to polycondensation reactions that complete gel formation over time. Aging causes the sol-gel structure to be reinforced with additional cross links which result in the contraction of the gel matrix and expulsion of solution from the shrinking pores (Jin & Brennan, 2002). Meunier & Legge (2010) reported that sol-gel aging occurred over time and that this resulted in increased enzyme activity. Jin & Brennan (2002) reported that this phenomenon is caused by significant structural and conformational changes in the entrapped proteins such as a result of changes in internal composition and protein-silica interactions. This might cause an increase in activity due to an increase in the protein's conformational flexibility over the aging time. Peltola *et al.* (2000) also reported similar aging phenomena in the conversion of atmospheric carbon dioxide to methanol due to confinement and matrix effects for the use of silica sol-gels where the precursor was TMOS. A study was conducted to determine if aging occurred for the transesterification reaction and it was found that during sol-gel aging the conversion of oleic acid initially gradually increased for the supported sol-gels. Maximum conversion of the oleic acid was obtained for a sol-gel aging time of 8 days (94.2%). After this, the conversion decreased for a sol-gel aging time of 16 days and 30 days as shown in Figure 2 for the Celite®-R633 supported sol-gel. Maximum conversion was obtained when the sol-gel supported when Celite®–R633 was used. Unsupported sol-gels showed a drop in conversion with for the first 8 days followed by an increase in conversion up to day 16. The aging times reported are based on the number of days after which the wash buffer had completely evaporated from the gel. From Figure 2, there appears to be less variability for the supported sol-gels over the aging period in comparison to the unsupported sol-gel. This might be due to greater structural and conformational stability of the protein due to additional cross link protein-silica interactions in the presence of the Celite® support over the aging period (Jim & Brennan, 2002). It is noted that the aging effect is in agreement with the increased activity with aging reported by Meunier & Legge (2010).

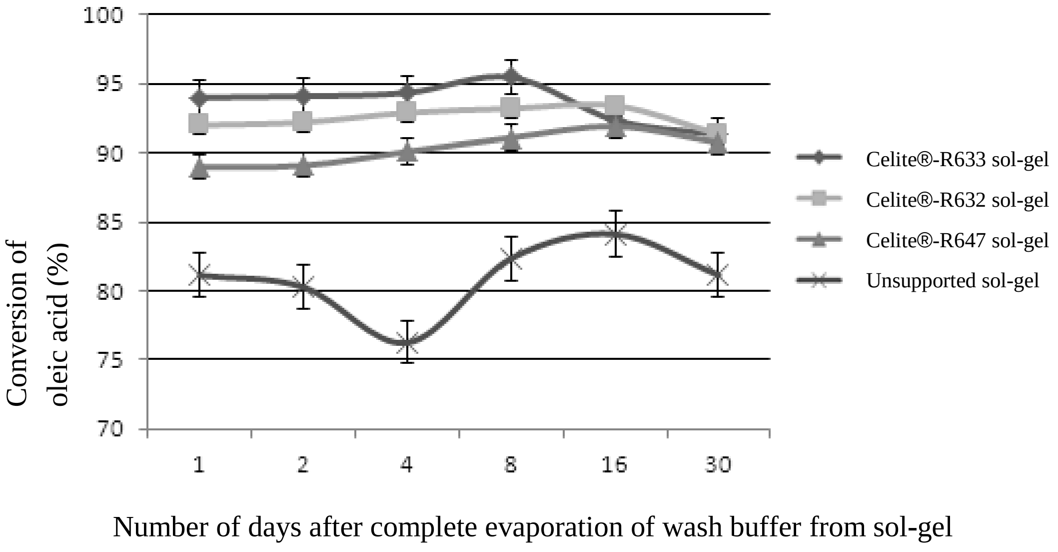


Figure 2. Conversion of oleic acid to fructose monooleate in acetone for various sol-gels. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.1. Oleic Acid Conversion using Selected Sol-Gel Supports

3.1.1. Conversion as a Function of Different Preparations

Yoo, Park & Yoon (2007) reported the highest conversion of oleic acid to sugar oleates when Novozym was combined with molecular sieves in the reaction media. A study was conducted to determine the effect of Novozym and molecular sieves and to compare these results to the use of Celite®-R633 supported sol-gels. From Figure 3 it is evident that the highest conversion of oleic acid was obtained when CSSIE was used followed by Novozym and molecular sieves with the lowest conversion with unsupported sol-gels. The difference was found to be statistically significant by randomized blocking and multiple comparison techniques using the least significant difference (LSD) approach and the Bonferroni inequality. The maximum conversion of oleic acid was 96.7% for sorbitan monooleate in acetone with a 1:1 molar ratio of sugar:oleic acid. The conversion was found to be partly dependent on the sugar substrates although the differences were not significant. When CSSIE, Novozym and molecular sieve and unsupported enzyme were used, the average conversions for different sugars were 92.23%, 79.66% and 77.45%, respectively. The lowest conversion for oleic acid was 70.32% when sucrose and unsupported sol-gel were used. The use of sugar alcohols (sorbitol and xylitol) as the substrates resulted in a higher oleic acid conversion than for sugars (sucrose and fructose). According to Yoo, Park & Yoon (2007), higher carbohydrate solubilities in the reaction solvent lead to higher conversions. The solubilities of the various sugars at 60°C in t-butanol are 17.5 g/L for xylitol, 10.6 g/L for sorbitol and 1.1 g/L for sucrose and in acetone the reported solubilities are 3.1 g/L for xylitol and 0.21 g/L for sucrose. The differences in solubility may be an important factor for the higher conversions observed using xylitol and sorbitol (sugar alcohols) compared to sucrose. Oleic acid conversions reported by Yoon, Park & Yoon (2007) for sugar fatty acid esters using Novozym and molecular sieves were also found to be similar to the results in this study.

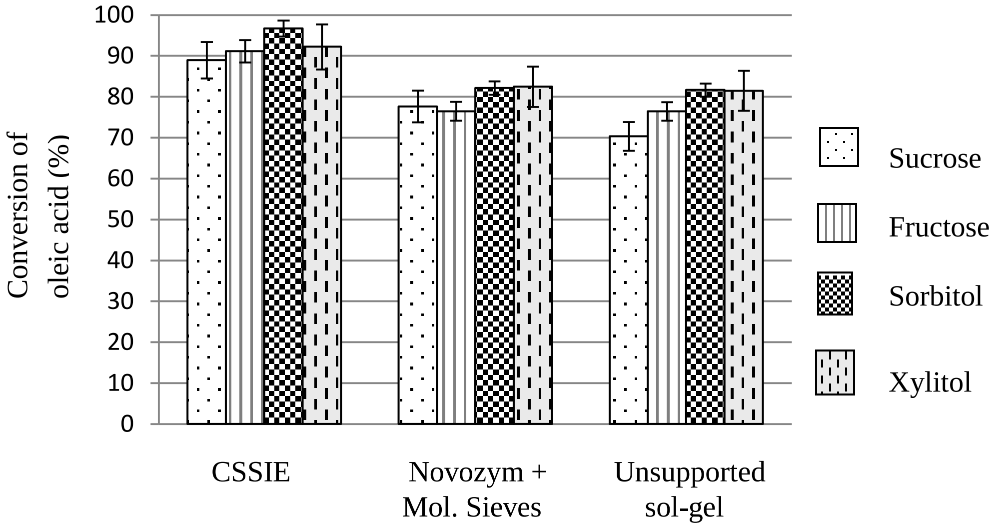


Figure 3. Conversion of oleic acid using CSSIE, Novozym and molecular sieves and unsupported sol-gel in the enzymatic esterification reaction between sugars/sugar alcohols and oleic acid in acetone. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.1.2. Effect of Sugar and Fatty Acid Molar Ratio on Conversion

The sugar ester yield for different molar ratios of sugar/oleic acid was studied for different sugars in acetone using Celite®-R633 supported sol-gel. As seen in Figure 4, when the molar ratio was increased from 1 to 3, there was an increase in the conversion of oleic acid for all sugars (~ 3% increase for sucrose and fructose). However, with further increases in molar ratio up to a maximum of 9, the conversion increased only marginally for the sucrose and fructose monoesters and was relatively constant for the xylitol and sorbitan monoesters. These results are in agreement with Yoo, Park & Yoon (2007) who reported similar conversions for different molar ratios and related the equilibrium constant of the esterification reaction to the substrate concentrations. Ferrer *et al.* (2002) reported that the pre-dissolution of the sugar in DMSO causes a notable acceleration of the reaction. It was also reported that a high initial amount of sugar inhibits sugar and fatty acid conversion so slight solubility of sugar in CO_2 saturated acetone was facilitated and maximum conversion was determined at a sugar:oleic acid molar ratio of 2:1. Tai & Brunner (2009) reported that a greater quantity of fatty acid (greater than a fatty acid: sugar molar ratio of 2:1) increased the viscosity of the reaction mixture which lowered the reaction rate. Mutua & Akoh (1993) reported a decrease in conversion at high molar values due to substrate inhibition or saturation at the enzyme active site.

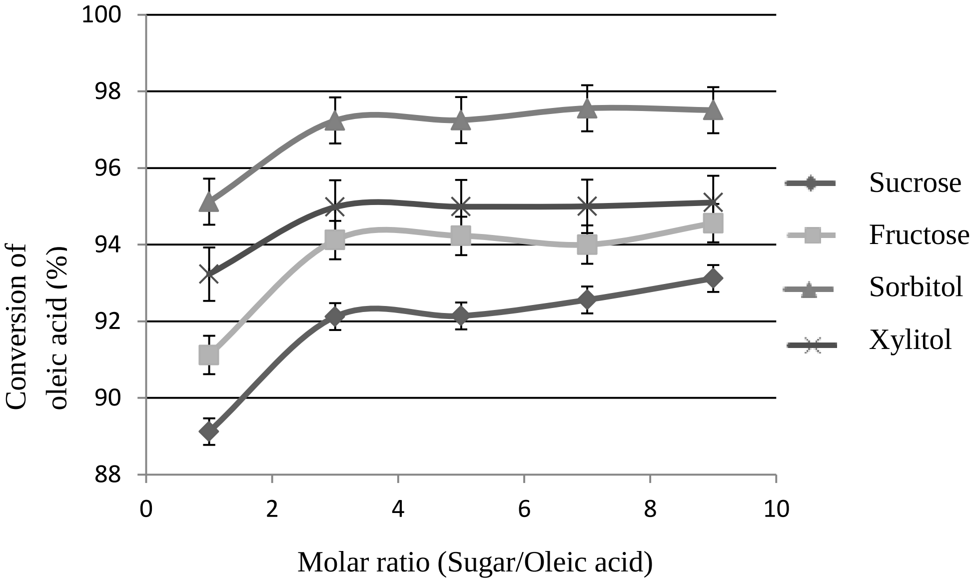


Figure 4. Conversion of oleic acid for different sugar-oleic acid molar ratios in the enzymatic esterification reaction between sugars/sugar alcohols and oleic acid in acetone. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.1.3. Time Course Analysis - Conversion for Various Loadings of CSSIE

Conversion of oleic acid as a function of the quantity of the CSSIE for sucrose monoester synthesis was investigated as a function of reaction time. The amount of CSSIE used for the reaction was from 1 g to 15 g for the reaction mixture along with the fatty acid. Once the enzyme was added, the conversion of oleic acid was determined every 2 hours for different CSSIE quantities as shown in Figure 5. The highest conversion of 92.1% was obtained when the largest quantity of enzyme (15 g) was used. A large difference in conversion was not observed between loadings of 5 g (89.96%) and 15 g (90.12%) of CSSIE. However, for enzyme loadings of 1 and 2.5 g, oleic acid conversion was observed to be lower than at higher loadings and which may be attributed to the accumulation of water (Yoo, Park & Yoon, 2007). The time taken for the conversion of oleic acid to level off was consistent for all quantities of enzyme (about 10 hours) but higher initial reaction rates were obtained with increasing CSSIE loading. The initial reaction rates were determined on the basis of the linear portion of the reaction time course and are provided in Table 2. The lowest conversion of oleic acid was obtained using 1 g of CSSIE (60%). Tai & Brunner (2009) reported similar results that with larger amounts of Novozym-435 the total conversion increased since more active sites were available for the reaction which resulted in higher initial reaction rates measured. However higher loadings of enzyme, greater than 50% Novozym-435 in relation to the % amount of dissolved palmitic acid, resulted in a slower reaction due to agglomeration of the enzyme particles. This loading would translate to 35 g CSSIE in terms of protein content which has not been evaluated in this study. Ferrer *et al.* (2002) also reported that the product yield increases with the lipase content over the range of 10 – 100 mg/ml solvent. Yoo, Park & Yoon (2007) reported that higher enzyme amounts increased the initial reaction rate with quantities similar to those reported in this study.

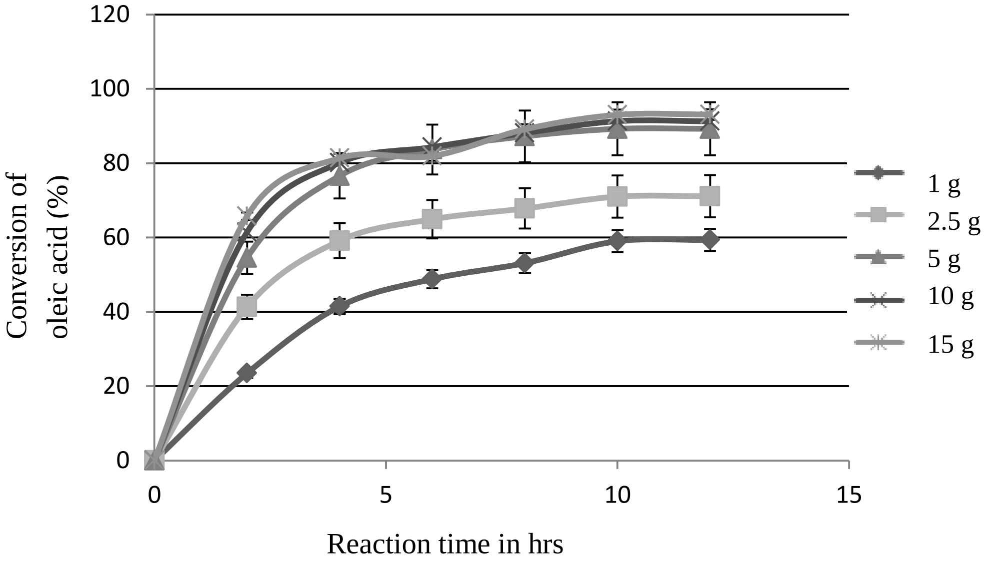


Figure 5. Conversion of oleic acid using different quantities of Celite®-R633 supported sol-gel in the enzymatic esterification reaction between sucrose and oleic acid in acetone. The error bars represent the 95% confidence interval of the sample mean based on n=3.

Table 2. Reaction rates for various amounts of CSSIE

CSSIE Loading (g)	Initial Reaction Rate (mmol/L·hr)
1	12.6
2.5	24.6
5	31.2
10	37.2
15	40.8

3.1.4. Reaction Rate as a Function of Solvent

The rate of the reaction was evaluated as the conversion of oleic acid at varying times and for acetone and t-butanol. Each reaction mixture consisted of sugar/sugar alcohol:oleic acid with a molar ratio 3:1 and CSSIE (Celite®-R633 supported) in the solvent. Based on Figures 6 and 7 acetone resulted in a higher sugar ester yield in comparison to t-butanol for all the reaction combinations considered. The sugar ester yield was highest with sorbitol (97.1%) and lowest with sucrose (91.4%) with acetone as the reaction solvent. The sugar ester yields with sorbitol and sucrose in t-butanol were 89.16 and 79.31%, respectively. The average product yield for acetone and t-butanol was 93.1 and 85.2%, respectively. Therefore the use of acetone as the reaction solvent for the synthesis of sugar ester was found to be a more favorable alternative to t-butanol based on these results. The time taken for the conversion of oleic acid to level off was consistent for both types of reaction solvents (about 10 hours). The initial reaction rates of all sugars/sugar alcohols in acetone were also higher than t-butanol as seen in Table 3. For example, the reaction rate of sorbitol monooleate in acetone (43.8 mmol/L·hr) was higher than in t-butanol (31.2 mmol/L·hr) as shown in Table 3. These results are in agreement with data reported by Tai & Brunner (2009) who selected acetone because it is an accepted extraction solvent by the FDA for the manufacture of food products and additives. The reaction rates for the sorbitol were also higher than for the sugars in both solvents. According to Yoo, Park & Yoon (2007), higher solubilities in the reaction solvent lead to higher reaction rates. The differences in solubility may be an important factor for the higher reaction rates observed using sorbitol compared to sucrose and fructose. High initial rates of reaction (900 μmol/h/g enzyme) were reported by Yoo, Park & Yoon (2007) who attributed this to the high activity of the enzyme in acetone.

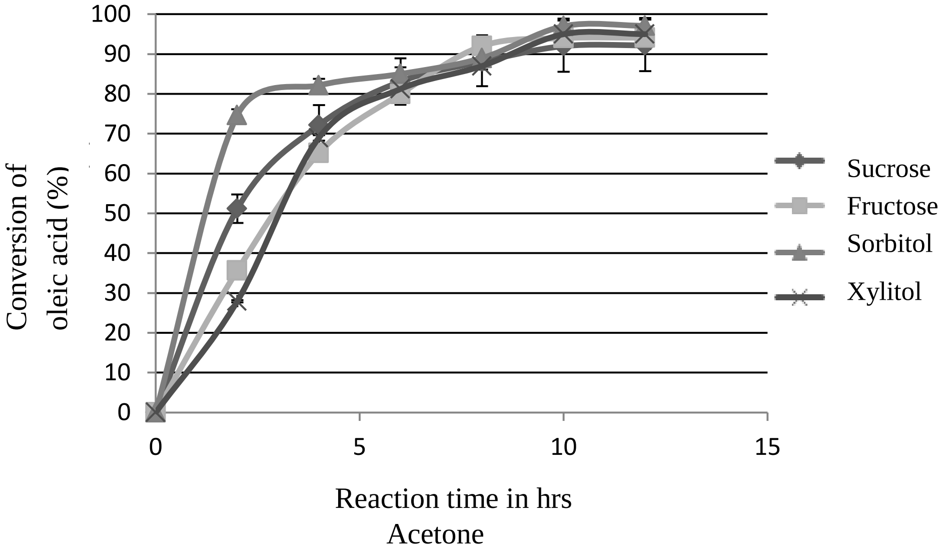


Figure 6. Time course for the conversion of oleic acid in the enzymatic esterification reaction between sugars/sugar alcohols and oleic acid in acetone. The error bars represent the 95% confidence interval of the sample mean based on n=3.

Table 3. Reaction rates of sugars/sugar alcohols in solvent

Sugar/sugar alcohol	**Initial Reaction Rate (mmol/L·hr)**	
	Acetone	**t-Butanol**
Sucrose	30.6	21.0
Fructose	21.1	15.6
Sorbitol	43.8	31.2
Xylitol	17.4	16.8

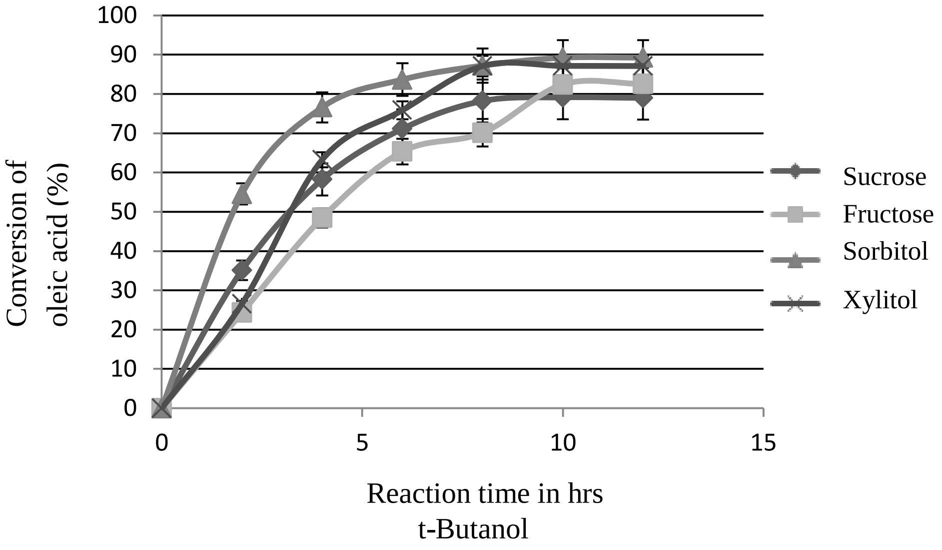


Figure 7. Time course for the conversion of oleic acid in the enzymatic esterification reaction between sugars/sugar alcohols and oleic acid in t-butanol. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.1.5. Conversion as a Function of Fatty Acid

This study was conducted to determine the conversion of oleic acid using different fatty acids for a variety of sugars in acetone using the sol-gel supported by Celite®-R633 (Figure 8). The acyl donors used in this study were palmitic, stearic, myristic and lauric acid. The procedure used is provided in Section 2.3 and was carried out at room temperature. The highest conversions were obtained when stearic acid (18 carbon chain) was used followed by palmitic acid (16 carbon chain), myristic acid (14 carbon chain) and lauric acid (12 carbon chain). Shorter fatty acid chains resulted in a decrease in product yield in comparison to the use of longer fatty acid chains. This is consistent with the results reported by Tai & Brunner (2009) which could be a result of the nature of the Lipozyme CAL-B and the formation of an acyl-enzyme intermediate (Kawase *et al.,* 1992). The conversion was highly repeatable as demonstrated by the small error bars. The yield of product when stearic acid was used was statistically analyzed by multiple comparison techniques using the least significant difference (LSD) approach and was comparable to the use of oleic acid. The lowest conversion of 62% was obtained for fructose monolaurate production where lauric acid was the acyl donor. Plou *et al*. (2002) studied the influence of the acyl donor and fatty acid chain length (from 12 – 18 carbon atoms) on conversion to sugar esters and reported similar results in that longer carbon chains resulted in higher conversion. Kawase *et al.* (1992) and Schmid & Verger (1998) also reported that the nature of the acyl donor had a notable reactivity.

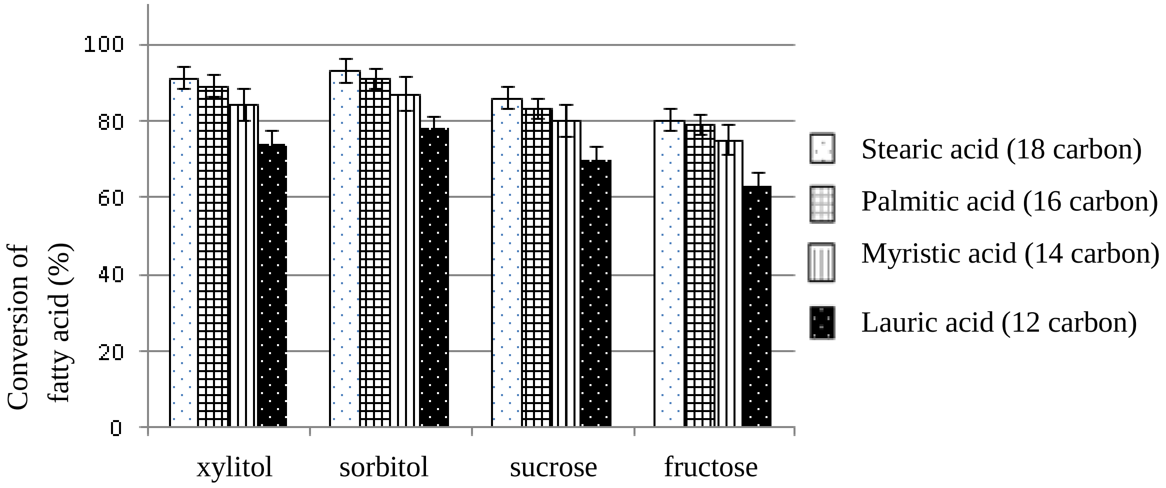
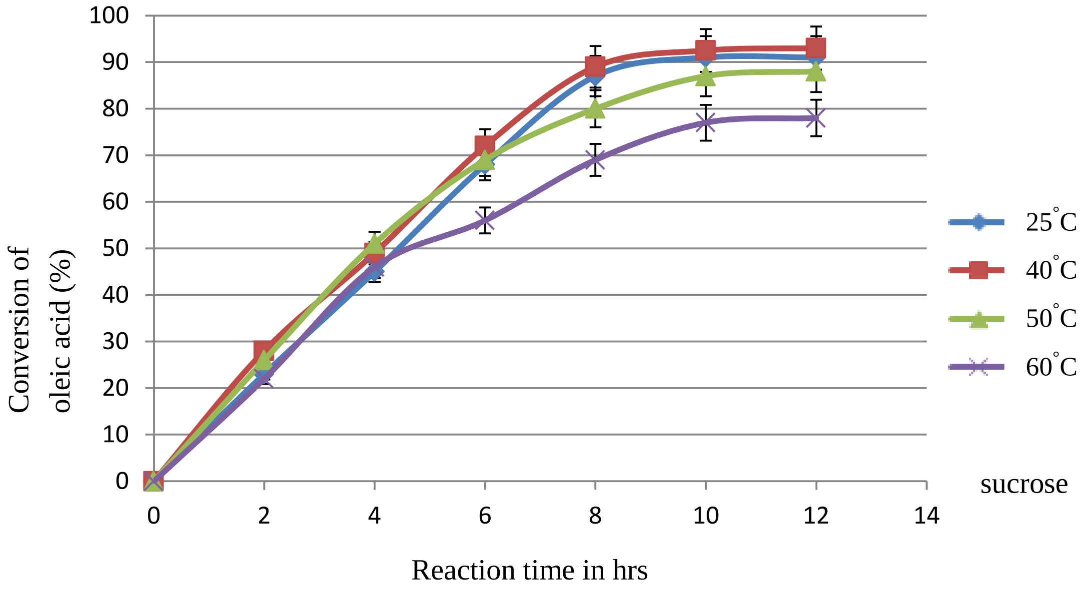


Figure 8. Conversion of fatty acid using Celite®-R633 sol-gel in the enzymatic esterification reaction between sugars/sugar alcohols and fatty acids in acetone. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.1.6. Time Course Analysis – Conversion as a Function of Reaction Temperature

Oleic acid conversion was measured at different reaction temperatures for a variety of sugars using sol-gel supported by Celite®-R633 as shown in Figure 9. Based on these results the optimum temperature for maximum conversion with the highest initial reaction rate was obtained at 40°C. The conversion of oleic acid steadily decreased at higher temperatures of 50°C and 60°C. This was similar to results reported by Tai & Brunner (2009) who found that the stability of the enzyme was adversely affected at higher temperatures (50 & 60ºC) and resulted in decreased reaction rates and yield. The initial reaction rates for all sugars followed a similar trend as shown in Table 4. The maximum conversion of oleic acid was 96.5% for sorbitan monooleate with no side product formation. The lowest conversion was 64% for the production of sucrose monooleate at 60°C. These results are similar to those obtained by Ferrer *et al.* (2002) who reported that maximum fatty acid conversion was observed at a temperature of 40°C. The enzyme used in their study was granulated lipase and Accurel-supported lipase. They also observed significant reductions in enzyme activity at temperatures greater than 40°C. Tai & Brunner (2009) attributed these high levels of conversion to a temperature effect on the configuration of the active site of the enzyme. Similar temperature effects on sugar fatty acid production have also been reported by Mutua & Akoh (1993) and Ward (1997) using immobilized lipase from *Candida antartica*.


100
90
80
70
60
50
40
30
20
10
0
Conversion of oleic acid (%)
0
2
4
6
8
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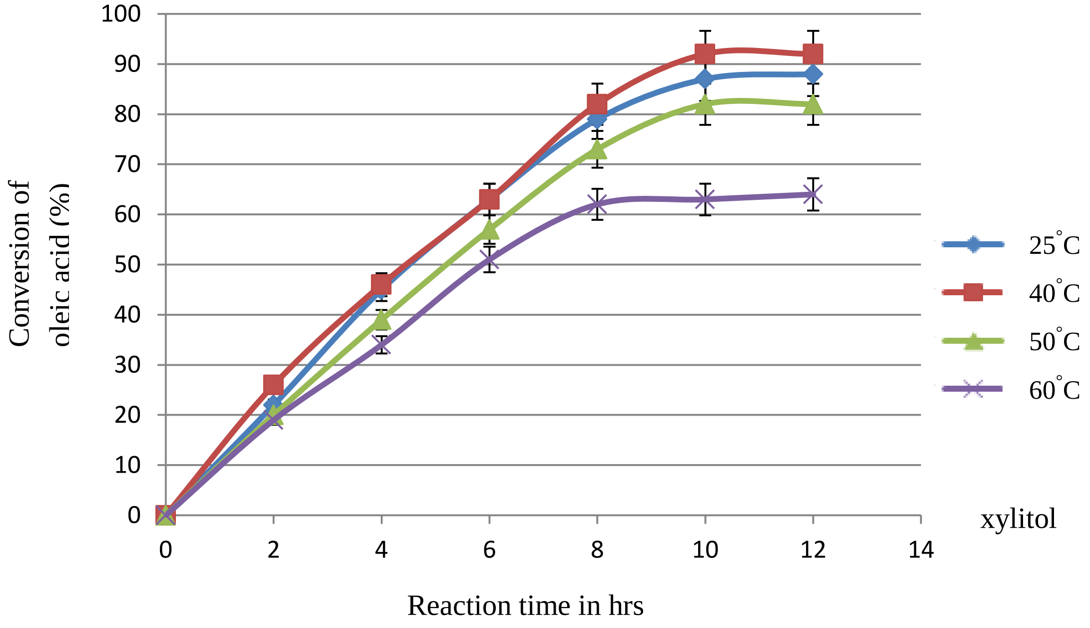
Reaction time in hrs
25°C
40°C
50°C
60°C
sucrose


100
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Conversion of oleic acid (%)
0
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Reaction time in hrs
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50°C
60°C
xylitol

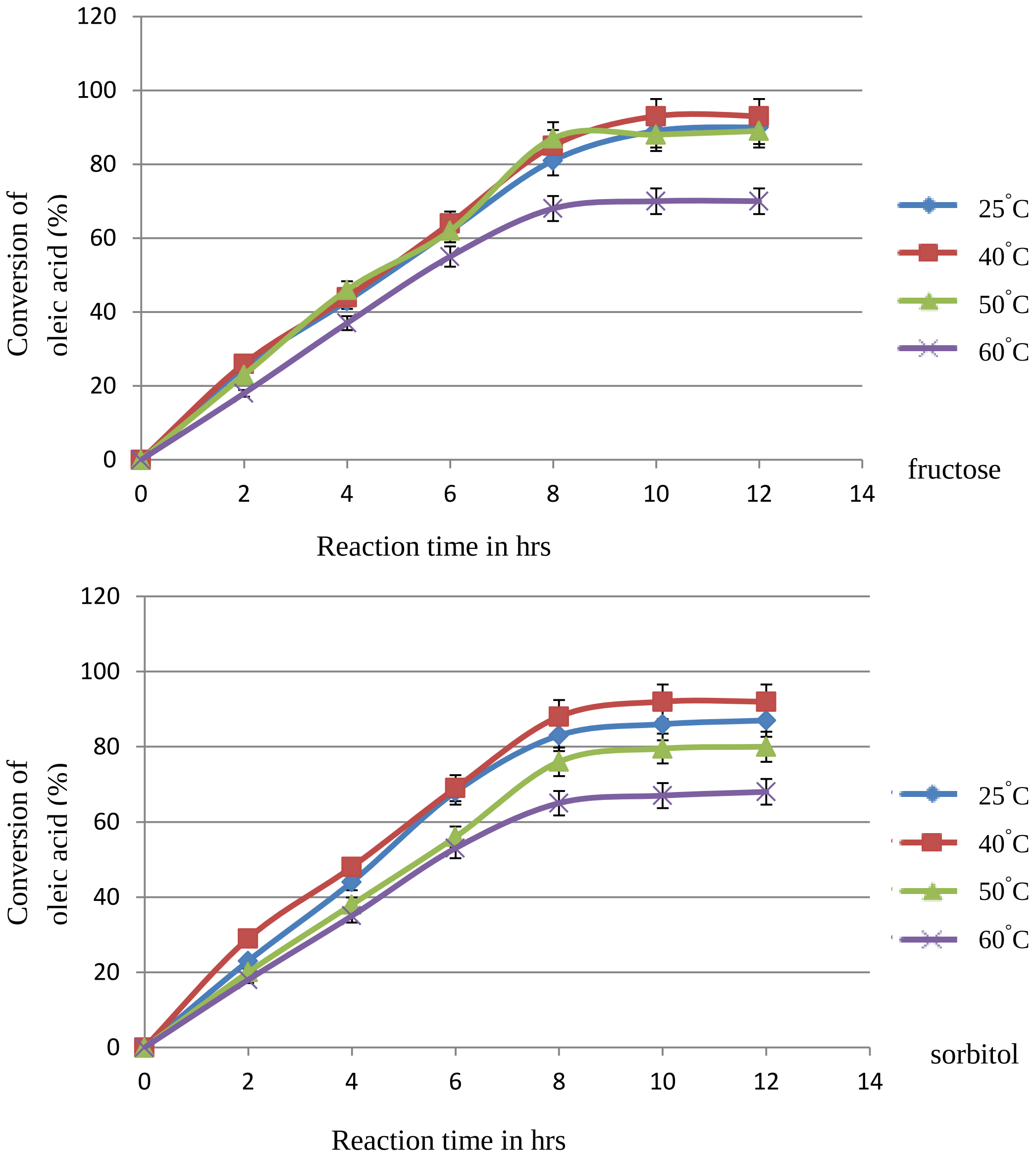


Figure 9. Time course for the conversion of oleic acid as a function of reaction temperature in the enzymatic esterification reaction between sugars/sugar alcohols and oleic acid in acetone. The error bars represent the 95% confidence interval of the sample mean based on n=3.

Table 4. Reaction rates as a function of reaction temperature and type of sugar

Temperature (ºC)	**Initial Reaction rate (mmol/L·hr)**			
	Sucrose	**Fructose**	**Sorbitol**	**Xylitol**
25°C	30.6	21.0	43.8	17.4
40°C	39.78	26.53	58.4	20.85
50°C	40.12	22.10	47.45	17.66
60°C	27.28	19.89	42.12	15.94

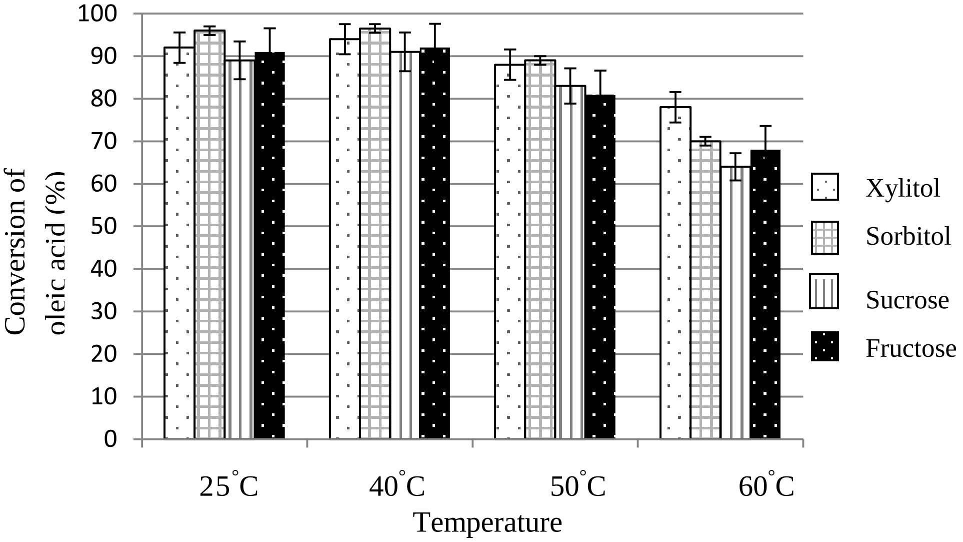


Figure 10. Conversion of oleic acid using Celite®-R633 supported sol-gel at different reaction temperatures in the enzymatic esterification reaction between sugars/sugar alcohols and oleic acid in acetone. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.2. Moisture Absorbing Capacity of the Supported Sol-Gel

3.2.1. Moisture Content as a Function of Biocatalyst

The presence of water in the reaction medium is disadvantageous due to the reversibility of the esterification reaction and reduction in substrate-enzyme interaction due to the formation of a layer of water around the immobilized enzyme. Some methods that have been employed to remove the water formed during the reaction include evaporation under reduced pressure, azeotropic distillation (Yan *et al.*, 1999), microwave heating (Carillo-Munoz *et al.*, 1996), use of inorganic salts (Hertzberg *et al.,* 1992), pervaporation (Kwon *et al.*, 1995), evacuation in vacuum (Napier *et al.,* 1996), multiphase distribution of acetone-CO_2-water-glucose (Tai & Brunner, 2009), vapor permeation and membrane separation (Tsitsimpikou, Daflos & Kolisis, 1997), gas sparging (Won & Sun, 2001) and circulation of reaction media through an external column packed with molecular sieves (Yoo, Park & Yoon, 2007). In this study the water content for different biocatalysts was measured to determine the potential for absorption of water. As a starting point, sugar esters were synthesized using a variety of sugars and oleic acid in acetone. The moisture content at the end of the reaction was determined by calculating the difference in the moisture content of the product and the blank solution described in Section 2.9. As seen in Figure 11, the lowest product moisture content was obtained for sol-gel supported with Celite®-R633 with fructose. For all types of CSSIE the final moisture content was found to be significantly less (< 0.1%) than the Novozym - molecular sieve combination. When Novozym or unsupported sol gel was used the water content was found to be in the range of 0.3 - 0.4%, which was close to the theoretical quantity of water formed for specified quantity of sugars and oleic acid from mass balance and stoichiometric relationship. The average moisture content with Novozym and molecular sieves was 0.15%. The moisture content for Novozym and molecular sieves was similar to the results obtained by Yoo, Park & Yoon (2007). These results can be partly explained based on some of the physical characteristics of Celite®. The manufacturer reports a much higher water adsorption capacity (240% mass of water to mass of Celite®) compared to 84% for R632 and 163% for R647. Similar results were obtained by Sabeder, Habulin & Knez (2005) for sugar ester production using Novozym and molecular sieves. They reported that at molecular sieve concentrations (weight of molecular sieve/weight of reaction mixture) greater than 12.1% there was a decrease in yield of product due to the excessive removal of essential water from the vicinity of the enzyme and interference in the mass transfer phenomena. Tarahomjoo & Alemzadeh (2003) who analyzed the influence of quantity of molecular sieve to glucose palmitate production in hexane made similar observations.

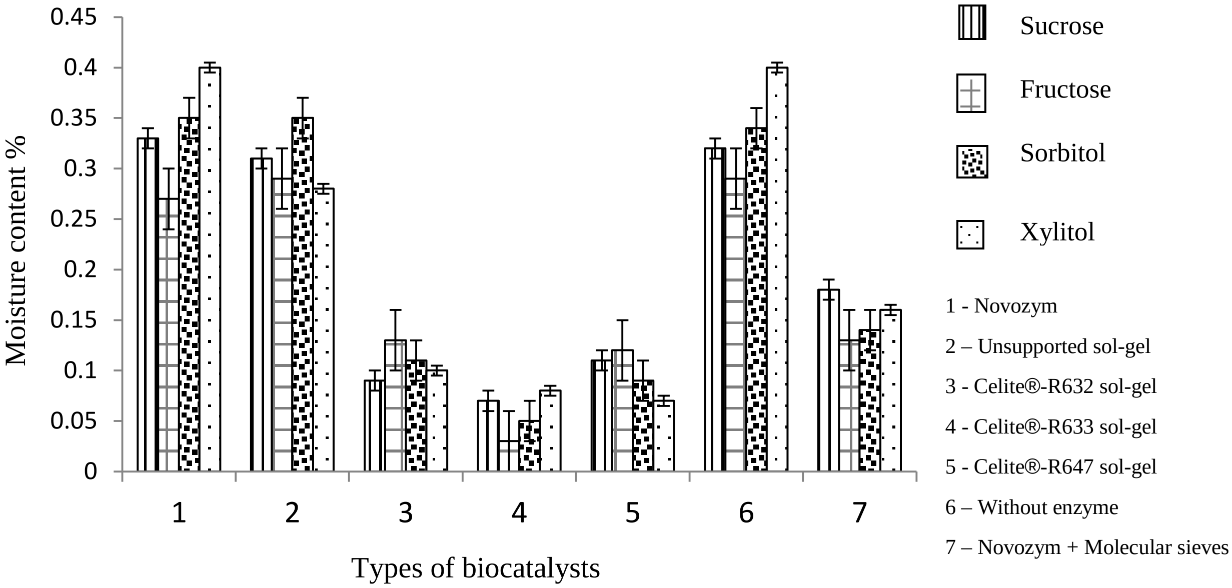


Figure 11. Moisture content of the reaction medium with various sugars and oleic acid in acetone for different biocatalysts combinations and a reaction time of 12 hours. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.2.2. Moisture Content as a Function of CSSIE amount

An experiment was conducted to determine the effect of different loadings of CSSIE and the effect on the resulting moisture content in acetone for fructose monooleate production. This study is similar to that reported by Yoo, Park & Yoon (2007) and Sabeder, Habulin & Knez (2005) who examined the effects of different molecular sieve loading. They observed that when large amounts of molecular sieve (15 g) were added, conversion was greater than 90%. They also reported that the final water content was 0.3% without molecular sieves which is consistent with the results reported here using unsupported sol-gel.

The moisture content was determined for different quantities of CSSIE when producing fructose monooleate. From Figure 12 it is observed that the addition of large amounts of CSSIE decreased water content during the reaction and at the end of the reaction. Samples were taken at 2 hour intervals and moisture content analyzed. In all cases the moisture content leveled off after 8 hours regardless of CSSIE.

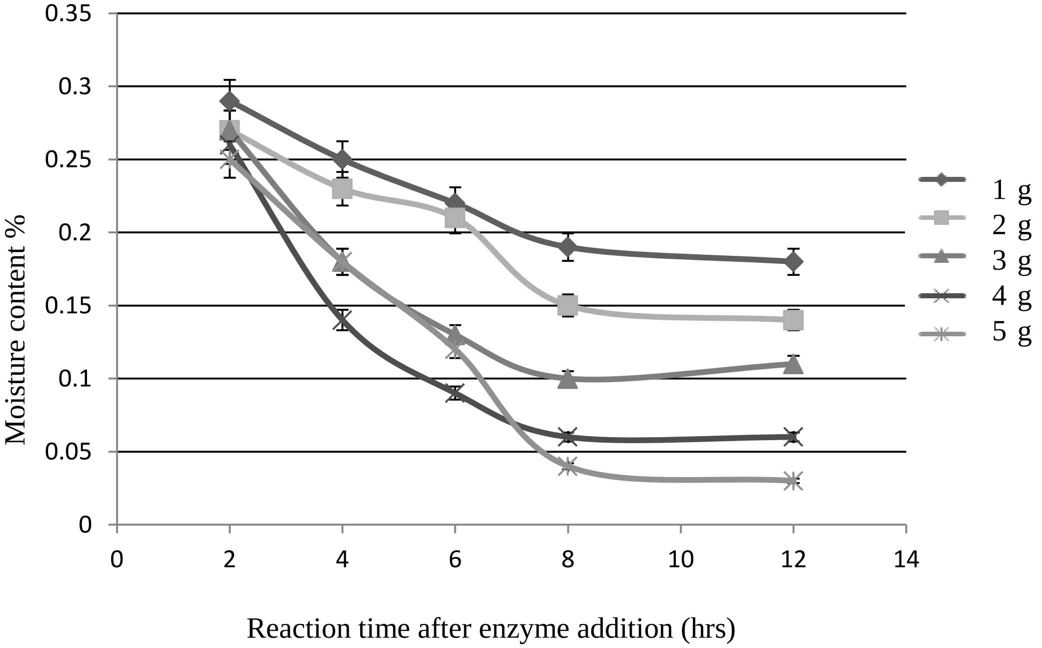


Figure 12. Moisture content in reaction medium for fructose with oleic acid in acetone for different quantities of Celite®-R633 supported sol-gel. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.2.3. Moisture Content as a Function of Reaction Temperature

Experiments were conducted to determine the effect of reaction temperature on the moisture content of the reaction medium for different temperatures and sugar/sugar alcohols with oleic acid and Celite®-R633 supported sol-gel. From Figure 13 it is observed that the maximum moisture content occurred at 40°C for sorbitol. This could be attributed to the fact that this was the combination for which the highest enzyme activity was observed. The moisture content was found to be the highest consistently for sorbitol which agree with the previous finding that conversion was the highest for sorbitol. The lowest moisture content at 60°C was the lowest for which can be related to the low conversions observed at this temperature. The lowest moisture content was observed for sucrose and fructose which can be related to the lower yield of products compared to the sugar alcohols.

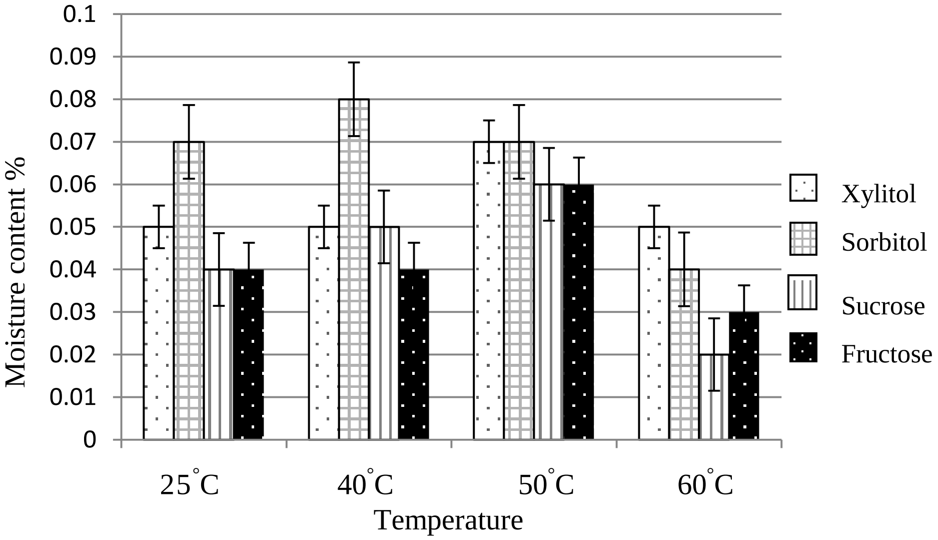


Figure 13. Moisture content at different temperatures in the enzymatic esterification reaction between sugars/sugar alcohols and oleic acid in acetone and a reaction time of 12 hours. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.2.4. Moisture Content as a Function of Fatty Acid

This study was conducted to determine if there was a relationship between moisture content and the different fatty acids using Celite®-R633 supported sol-gel in acetone (Figure 14). A similar pattern for moisture content was observed to that for the conversion data for the different fatty acids. This is reasonable as the greater the conversion the more water produced as a product. From Figure 14 it is observed that the fatty acids with a shorter chain length resulted in decreased water contents due to reduced substrate conversion. The highest moisture content occurred for xylitol monostearate at 0.12% and the lowest for fructose monolaurate at 0.03%.

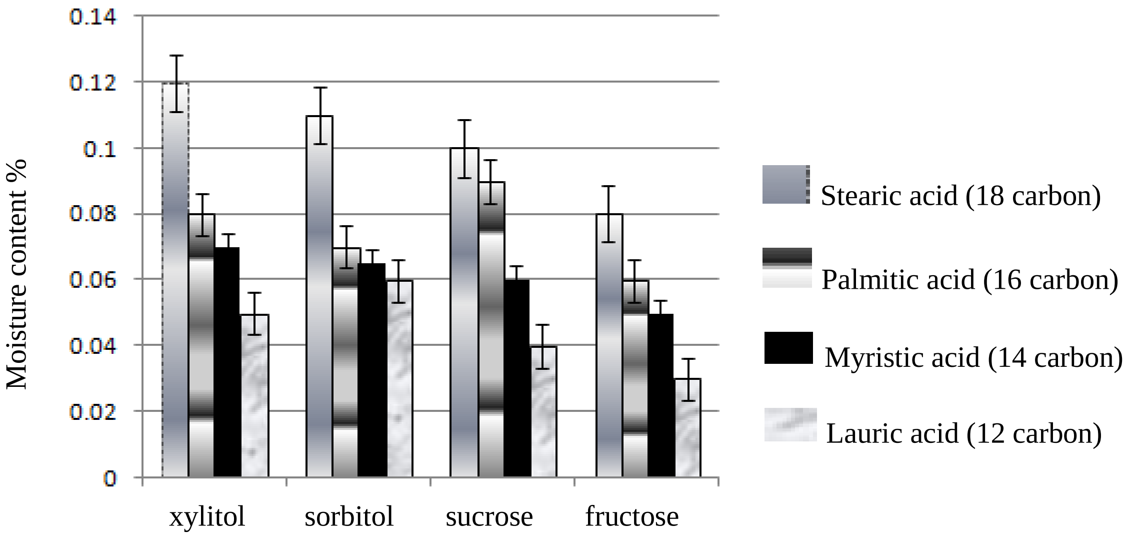


Figure 14. Moisture content as a function of fatty acid in the enzymatic esterification reaction between sugars/sugar alcohols and fatty acids in acetone. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.2.5. Moisture Content as a Function of Celite®+ Novozym

The moisture absorbing capacity of Celite® was evaluated in comparison to CSSIE for the synthesis of fructose monooleate. A combination of Novozym-Celite® was used instead of

CSSIE alone and the moisture content was determined. From Figure 15 it is seen that the maximum water adsorption was observed for Celite®-R632 followed by Celite®-R633 then Celite®-R647. From Section 3.2.1 the maximum water adsorption was obtained Celite®-R633 sol-gel. Adlercreutz (2005) discussed the potential of water absorption for enzymes supported with Celite®. He reported that Celite® (30-80 mesh) could absorb 2 mg of water per gram of Celite® and act as a suitable support for chymotrypsin with the highest activity in comparison to other supports like Accurel PA6 (polyamide) and controlled pore glass. The reaction was conducted between N-acetyl-L-phenyl alanine ethyl ester with 1-butanol with the enzyme horse liver alcohol dehydrogenase. It was observed that the enzymes bound varying quantities of water depending on the thermodynamic water activity and that the polyamide support Accurel PA6 adsorbed more water than Celite®. De Martin *et al.* (1999) also examined the water absorbing properties of Celite® and reported the adsorption and release of water for Celite®-R640 rods. It was found that the rods could be used to maintain a constant water activity within defined ranges of water concentration and adsorbed water in excess of 90% of the Celite's® weight. Adsorption isotherms were presented and it was observed that water penetrated into the inner zones of the rods and that water exchange with the external phase was very slow. The rods were also ground into a powder and it was found that the adsorption properties changed dramatically. It is apparent that the macroscopic 3-dimensional organization underwent partial destruction although the pores were unbroken. It was concluded that the ability of Celite®-R640 to buffer the water activity was mainly related to its porosity (0.8 cm^3/g).

One of the reasons that Celite®-R633 (pore diameter 6.5 μm) could absorb more water than Celite®-R647 (0.07 μm) as observed in this study may be due to differences in the pore diameter. The Celite® - sol-gel - lipase combination that had the greatest water adsorbing effect for Celite®-R633 supported sol-gels could be due to the thermodynamic water activity and increased water penetration into the inner zones of sol-gel. This could increase the water binding capacity of the Celite®-R633 supported sol-gel. It is also evident that Celite® alone did not facilitate maximum water adsorption although a certain quantity of water was absorbed by the Celite®. In terms of % moisture content the effect of 5 g of Celite®-R632 was comparable to the effect of 5 g of molecular sieve and the addition of larger amounts of Celite® lowered the moisture content.

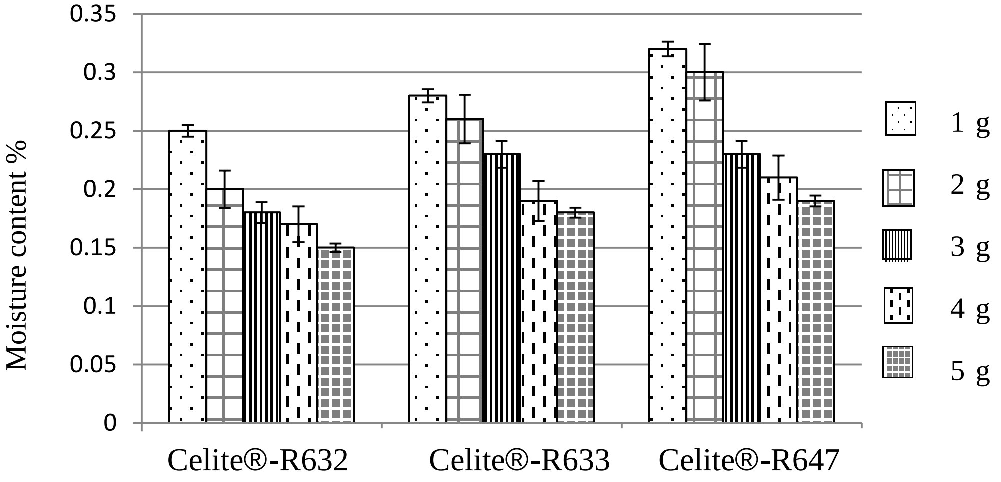


Figure 15. Moisture content in the enzymatic esterification of fructose with oleic acid in acetone using Celite® and Novozym and a reaction time of 12 hours. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.3. Reusability of the Immobilized Lipase

A gram of CSSIE was found to adsorb 0.00846 g of water for the first use as shown in the Calculations (Section B). To preserve this water absorption capacity of the sol-gel so that the high substrate conversions could be achieved, a drying approach was developed to remove the water absorbed so that the sol-gel could be reused. The approach was to dry the Celite®-R633 supported sol-gel at 40°C. To establish a time frame for drying, the weight of CSSIE was determined at ten minute intervals during the drying process and results are presented in Figure 16. From Figure 16 it is observed that a constant weight was reached after 40 minutes of drying at 40°C. For subsequent re-use experiments, the CSSIE was dried for 40 minutes.

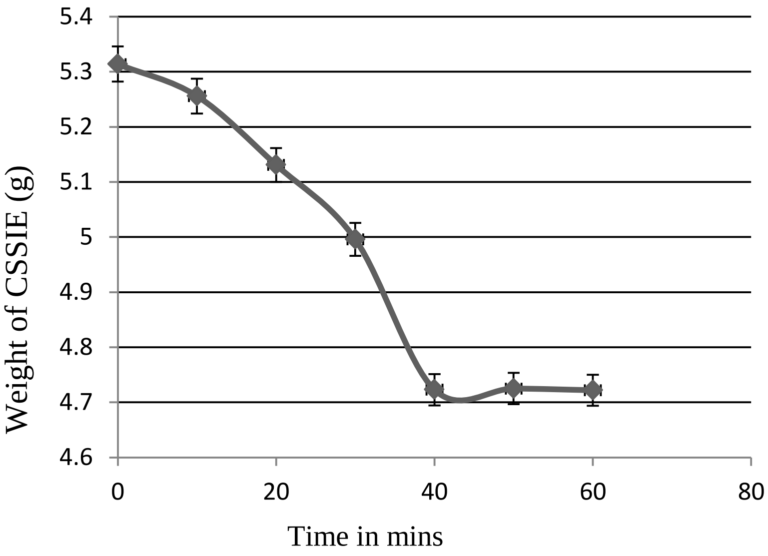


Figure 16. Celite®-R633 supported sol-gel mass as a function of time at 40°C. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.3.1. Moisture Content as a Function of CSSIE Reuse

Moisture content of the reaction medium was determined following reuse of supported sol-gels during the production of fructose monooleate without drying (Fig. 17). Moisture content was found to increase with the number of reuses for all three forms of Celite®. This is likely due to the reduction in water adsorbing capacity of the CSSIE as it becomes saturated with water. The increase in moisture content could also be partly attributed to changes in the nature of the supported sol-gels due to aging and reuse. It was observed that CSSIE facilitated water adsorption was experienced until the 3rd reuse. The moisture content obtained with the 4th reuse of CSSIE (0.3%) was equal to the theoretical quantity of water that would be predicted based on the amount of product formed. The maximum water adsorption was observed for Celite®–R633 for each reuse compared to Celite®–R632 and R647. A gram of CSSIE was found to adsorb 0.00592 g of water for the first reuse without drying as shown in the Calculations (Section B).

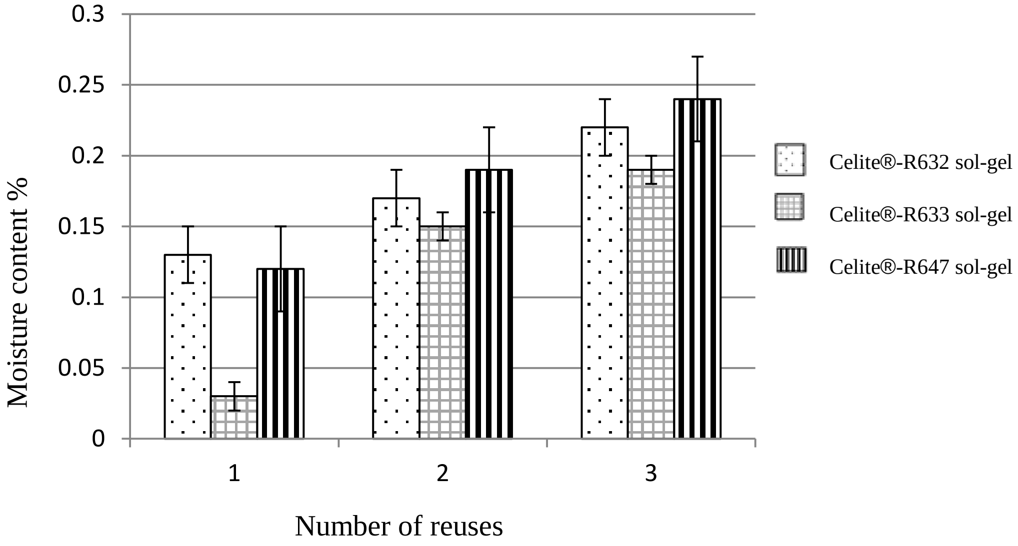


Figure 17. Moisture content of the reaction medium for the production of fructose monooleate following reuse CSSIE without drying. The error bars represent the 95% confidence interval of the sample mean based on n=3.

A second study was conducted to determine the effect of drying after each conversion cycle. CSSIE was dried after every use at 40°C for 40 minutes with the objective of removing the water adsorbed from the previous reaction and to increase water adsorption capacity for the subsequent use. Figure 18 shows that the moisture adsorbing capacity of CSSIE decreased with reuse as the water content of the medium increased. This was observed until the 5th reuse which compared to the 3rd reuse without drying. The moisture content obtained with the 6th reuse of CSSIE (0.3%)

was equal to the theoretical quantity of water that would be predicted based on the amount of product formed. This illustrates that the drying approach could be used to extend the usefulness of the CSSIE adsorption capacity and extend the number of possible reuses. A gram of CSSIE was found to adsorb 0.0073 g of water for the first reuse with drying in comparison to 0.00592 g of water without drying as shown in the Calculations (Section B). Claon & Akoh (1994) utilized this enzyme drying method for a reaction between geraniol and acetic acid using *Candida Anatartica* lipase SP382 40 BLU/g immobilized on acrylic resin. In their study, the immobilized enzyme was removed by passing the reaction mixture through an anhydrous sodium sulfate column and was rinsed with hexane and dried in a desiccator. This was found to increase the stability of the enzyme and allow reuse. Adlercreutz (2005) also reported that the drying of Celite® supported enzymes increased the water absorbing capacity of the enzyme.

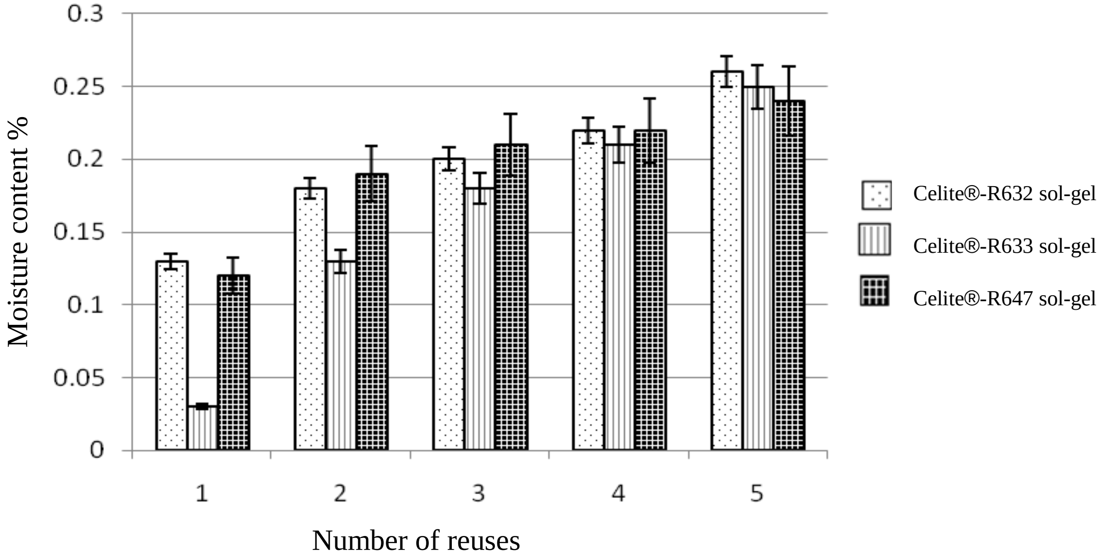


Figure 18. Moisture content in the synthesis of fructose monooleate with reuse of the CSSIE following drying at 40°C for 40 min after each use. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.3.2. Enzyme Half Life

Enzyme half-life is an important consideration in the engineering of immobilized enzymes. A study was conducted to compare the half-life of CSSIE to that of Novozym which has a reported half-life in the way of reuses of 9 repeated uses as reported by Yoo, Park & Yoon (2007). Claon & Akoh (1994) reported a half life of 7 repeated uses for a reaction between geraniol and acetic acid using *Candida antartica* lipase immobilized on acrylic resin.

The oleic acid conversion was determined for Celite®-R633 supported sol-gel with various sugar/sugar alcohols in acetone. After the CSSIE was separated from the reaction mixture it was dried and then reused. The half-life of the CSSIE was defined as the number of repeated uses until the conversion was one-half of the conversion obtained with the first CSSIE use. Figure 19 shows that the activity of the CSSIE increased marginally (1%) until the 4th reuse during which increased oleic acid conversion was observed. Conversion with the 5th reuse was equal to the conversion obtained for the initial CSSIE cycle. Conversion decreased substantially after the 5th reuse and an enzyme half-life of 7 reuses was observed. The half-life was found to be the same for all sugar esters based on oleic acid conversion. The relatively constant conversion for up to 4 reuses could be due to the sol-gel aging effect discussed in Section 3.0.2 and the subsequent conversion drop off could be due to decreased enzyme activity and water absorption capacity of the supported sol-gel in spite of drying after every use.

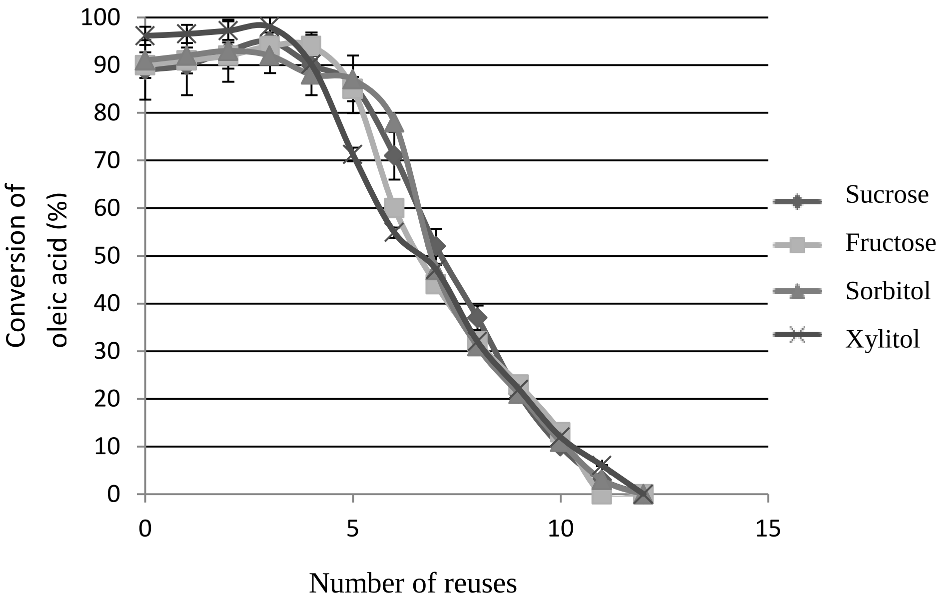
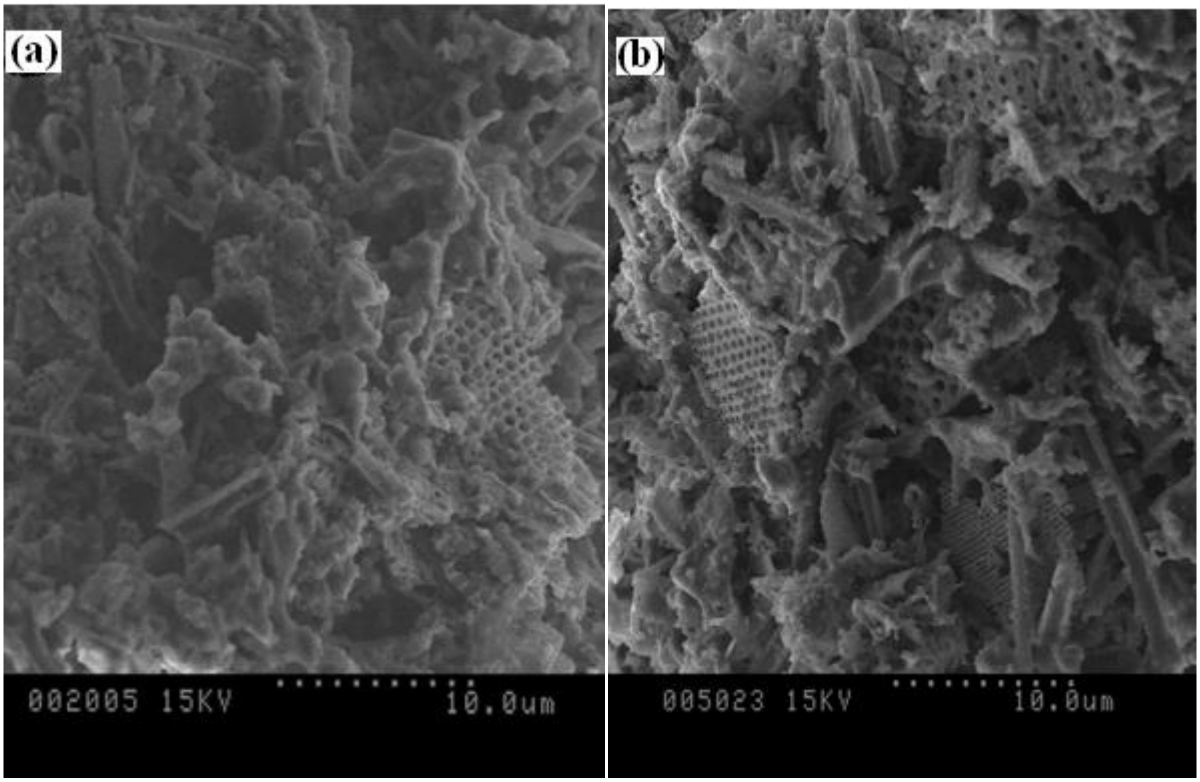
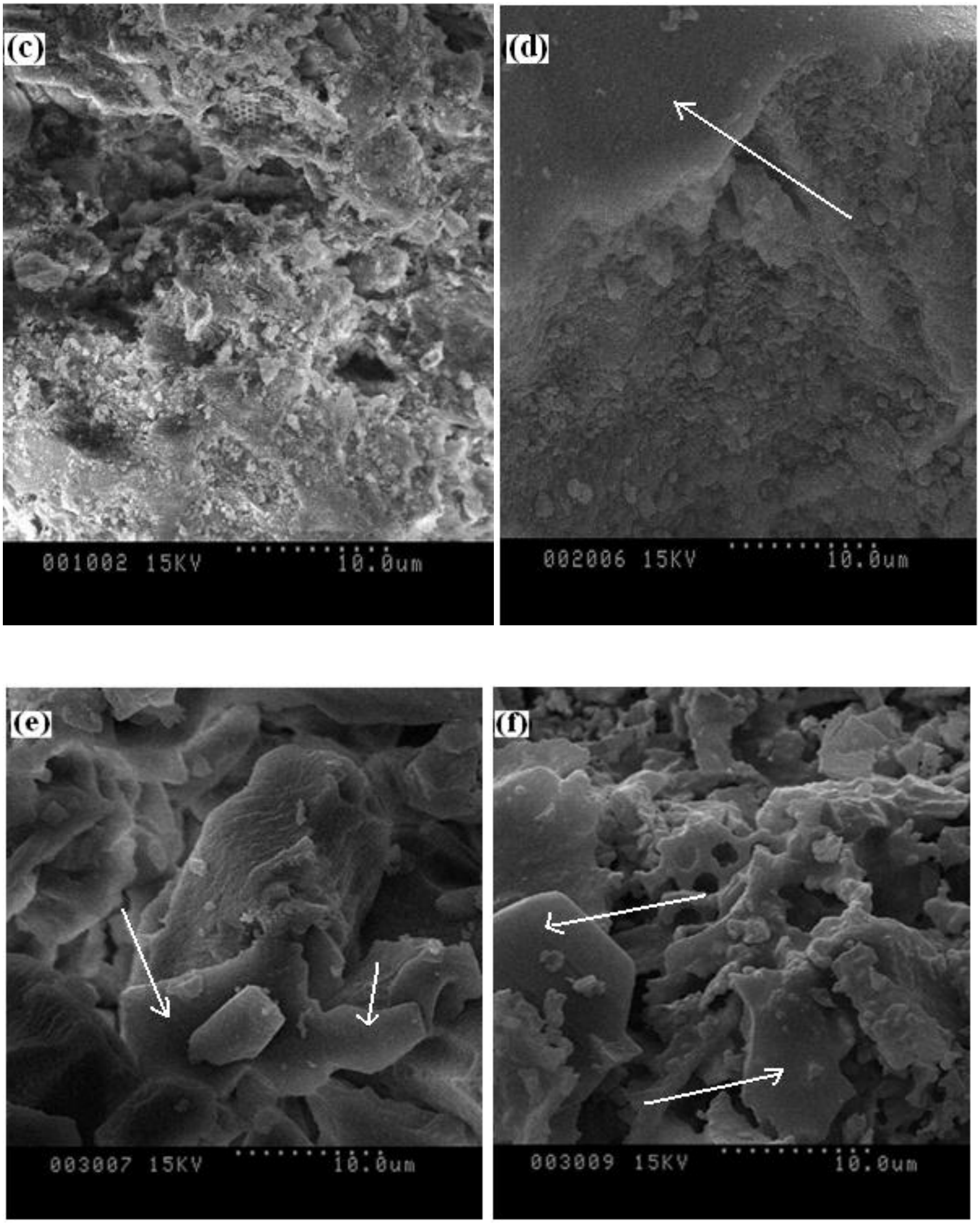


Figure 19. Conversion of oleic acid with CSSIE (Celite®-R633) reuse in the enzymatic esterification reaction between sugars/sugar alcohols and oleic acid in acetone. The error bars represent the 95% confidence interval of the sample mean based on n=3.

3.4. Scanning Electron Microscopy (SEM) Images

In order to determine what features might be changing as a function of reuse, SEM images of the supported sol-gel were obtained for various samples as a function of reuse. Figure 20 shows SEM images that were collected for a variety of uncoated Celite® samples and sol-gels including (a) Celite®-R633 (b) Celite®-R632 (c) Celite®-R647 (d) Unsupported lipase sol-gel (e) Celite® R633 supported sol-gel after 1st use (f) Celite®-R633 supported sol-gel after 2nd use (g) Celite®-R633 supported sol-gel after 3rd use. The particle sizes and the pore diameters of the uncoated Celite®-R633, R632 and R647 are provided in Appendix (Section C). Definite clusters of sol-gel on the surface of the Celite® were observed in the SEM images of the supported sol-gel. These sol-gel clusters were visible even after drying and reuse. The enzyme activity and moisture absorbing capacity could be attributed to the preservation of sol-gel clusters and the porosity of the Celite® with repeated use. Meunier & Legge (2010) conducted SEM analysis of CSSIE and determined that R633 had 38 sol-gel clusters in 477,000 µm^2 of surface area imaged with an average cluster size of 1270 µm^2. R633 and R632 had a comparable number of sol-gel clusters; however, R647 had very few clusters. It was suggested that R633 and R632 favored adhesion of sol-gel as a thinner layer on the surface rather than cohesion of thicker sol-gel clusters as with R647.

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002006 15KV 10.0um
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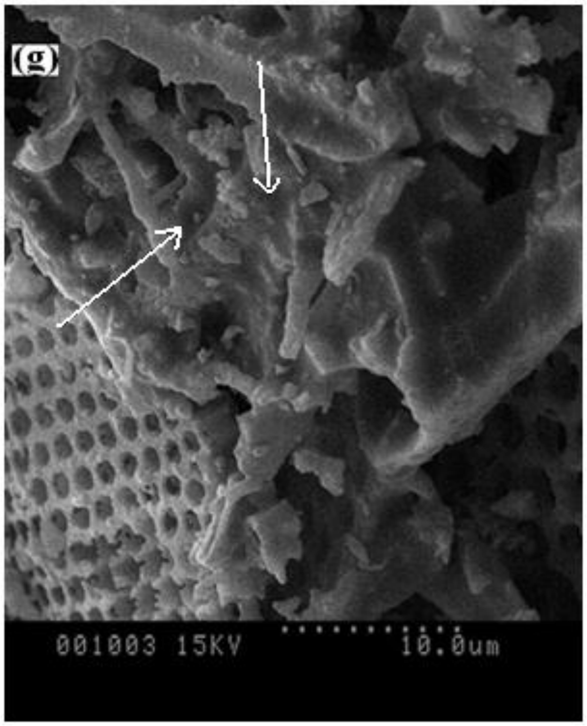


Figure 20. SEM Images at 3000 x magnification (a) Celite®-R633 (b) Celite®-R632 (c) Celite®-R647 (d) Unsupported lipase sol-gel (e) Celite®-R633 supported sol-gel after 1stuse (f) Celite®-R633 supported sol-gel after 2nd use (g) Celite®-R633 supported sol-gel after 3rd use. Arrows indicate sol-gel clusters on the surface of the Celite®.

4. Research Highlights

1. CSSIE was made up of sol-gel that consisted of PTMS and TMOS and supported by three different types of Celite®–R632, R633 and R647. They were found to be effective as biocatalysts in the esterification reaction between sugars/sugar alcohols and fatty acids and adsorbed quantities in excess of 90% of the water by-product which resulted in high sugar fatty acid ester product yields. About 20 hours of total reaction time was required before oleic acid conversion leveled off. The highest yield of 96.7% was obtained for the production of sorbitan monooleate with no appreciable quantities of side products formed for any of the reactions reported based on stoichiometric calculations and GC-MS data.
2. Maximum product yield and water adsorption was achieved by Celite®-R633 supported sol-gel. Highest oleic acid conversions were obtained with the use of sorbitol as the acyl acceptor. Sugar alcohols (sorbitol and xylitol) resulted in higher oleic acid conversions than sugars (sucrose and fructose). Acetone was a more favorable reaction solvent than t-butanol based on the higher oleic acid conversions obtained.
3. A CSSIE drying and reuse approach was developed which involved heating for 40 minutes at 40^0C before reuse. This appeared to activate the adsorption sites by removing the water from the previous reaction. A half-life of 8 repeated uses was observed. The moisture adsorbing capacity of the sol-gel supported by Celite®-R633 was 20% more than Novozym and molecular sieve. Celite® partially adsorbed water but did not facilitate maximum water adsorption. Supported sol-gels experienced an aging effect with highest conversions observed with a sol-gel aging time of 8 days.
4. Excess of dissolved sugar favored the conversion of fatty acid to product. Quantities of sugar greater than a sugar:fatty acid molar ratio of 3:1 did not significantly increase oleic

acid conversions. Large quantities of enzyme also favored higher conversion of reactants. Concentrations of enzyme in excess of 1.66 g/mmol fatty acid did not significantly increase oleic acid conversion.

5. Highest oleic acid conversions were obtained with the use of longer chain fatty acids and a reaction temperature of 40°C. Maximum immobilization of 85% of initial protein mass was obtained by using the sol-gel supported by Celite®-R633.

5. Future Research Prospects and Recommendations

Accurel supported sol-gel immobilized enzymes

Adlercreutz (2005) reported that the polyamide support Accurel PA6 adsorbed more water than Celite®. It might be useful to explore the use of Accurel supported sol-gel immobilized enzymes for fatty acid sugar ester synthesis.

Membrane Separation

Although membrane separation techniques such as pervaporation dehydration (Sakaki *et al.*, 2006) have been explored as an approach to remove water its potential in sugar ester purification would be interesting. Membranes composed of cellulose and synthetic polymers or ceramic materials could also be modified to be highly selective towards polar molecules and have the advantage of low cost, high durability and ease of manufacture. Reverse osmosis, electrodialysis and freeze drying are also practical options that could be possible alternatives to the approach utilized here.

Zeolites

The use of zeolites as an adsorbing agent has not been used in sugar fatty acid ester synthesis. They facilitated selective adsorption of polar molecules due to their uniform pore size and high adsorption capacity (Grace Division, 2006). This approach may be an effective water adsorbent in esterification reactions. The preferential uptake of water by zeolites was carried out even at low concentrations due to its negatively charged framework which increases the ion exchange due to intermolecular attractive forces with the positive ions of polar molecules. An advantage of this technique would be the reversibility of the adsorption process and the lack of any structural change of the zeolite (Grace Division, 2006).

6. Conclusions

From the results of this study the use of sol-gels supported with Celite®–R633 as a biocatalyst is a feasible approach for the production of sugar fatty acid esters due to the water adsorption characteristics of the Celite® support. The selective uptake of polar molecules by CSSIE may provide consistent performance which would be required in large scale industrial application of these enzymes in esterification reactions.

Although it might be too early to conclude that CSSIE is the most favorable biocatalyst for the enzymatic synthesis of sugar fatty acid esters the results of this study suggest that Celite®-R633 should be considered as a promising support for lipase immobilized sol-gels. The CSSIE seems to be an effective alternative for the removal of water and are comparable to approaches like molecular sieves in combinations with Novozym.

References

Abd-Elbary, A., El-laithy, H.M. & Tadros, M.I., (2008). Sucrose stearate-based proniosome-derived niosomes for the nebulisable delivery of cromolyn sodium. Int. J. Pharm. 357, 189–198.

Adachi, S., & Kobayashi, T. (2005). Synthesis of esters by immobilized-lipase-catalyzed condensation reaction of sugars and fatty acids in water-miscible organic solvent. Journal of Bioscience and Bioengineering, 99(2), 87-94.

Adlercreutz, P. (2005). On the importance of the support material for enzymatic synthesis in organic media. European Journal of Biochemistry, 199, 609-614.

Afach, G., Kawanami, Y., Kato-Noguchi, H., & Izumori, K. (2006). Practical production of 6-O-octanoyl-D-allose and its biological activity on plant growth. Bioscience, Biotechnology and Biochemistry, 70(8), 2010-2012.

Barkay, T., Nayon-Venezia, S., Ron, E.Z., & Rosenberg, E., (1999). Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan. Appl. Environ. Microbiol. 65, 2697–2702.

Benrezzak, O. & Nigam, V. N. (1992). Modulation of protein kinase C by palmitoyl esters of maltose. Cancer Biochem.Biophys. 13, 13-22.

Boyd, J., Parkinson, C., & Sherman, P. (1972). Factors affecting emulsion stability, and the HLB concept. Journal of Colloid and Interface Science, 41(2), 359-370.

Carrillo-Munoz, J., Bouvet, D., Guibe-Jampel, E., & Loupy, A. (1996). Microwave-promoted lipase-catalyzed reactions resolution of (n)-1-phenylethanol. Journal of Organic Chemistry, 61(22), 7746-7746.

Chaillan, F., Fleche, A., Bury, E., Phantavong, Y.H., Grimont, P., Saliot, A., & Oudot, J., (2004). Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. Res. Microbiol. 155, 587–595.

Chamouleau, F., Coulon, D., Girardin, M., & Ghoul, M. (2001). Influence of water activity and water content on sugar esters lipase-catalyzed synthesis in organic media. Journal of Molecular Catalysis B: Enzymatic, 11(4-6), 949-954.

Chang, S. W., & Shaw, J. F. (2010). Biocatalysis for the production of carbohydrate esters. New Biotechnology, 26(3-4), 109-116.

Claon, A. P., Akoh, C. C. (1994). Enzymatic synthesis of geranyl acetate in n-hexane with *Candida antartica* lipases. Journal of Oil Chemists Society, 71, 575-578.

Clark, D. C., Wilde, P. J., & Wilson, D. R. (1991). Destabilization of α-lactalbumin foams by competitive adsorption of the surfactant Tween 20. Colloids and Surfaces, 59, 209-223.

Coke, M., Wilde, P. J., Russell, E. J., & Clark, D. C. (1990). The influence of surface composition and molecular diffusion on the stability of foams formed from protein/surfactant mixtures. Journal of Colloid and Interface Science, 138(2), 489-504.

Colbert, J. C. (1974). Sugar Esters-Preparation and Applications, Noyes Data Corporation. New Jersey.

Coulon, D., Ismail, A., Girardin, M., & Ghoul, M. (1996). Enzymatic synthesis of fructose monooleate in a reduced pressure pilot scale reactor using various pilot scale donors. Trends Biotechnol., 51, 115.

Csoka, G., Marton, S., Zelko, R., Otomo, N., & Antal, I. (2007). Application of sucrose fatty acid esters in transdermal therapeutic systems. Eur. J. Pharm. Biopharm. 65, 233–237.

De Martin, L., Ebert, C., Garau, G., Gardossi, L., & Linda, P. (1999). Penicillin G amidase in low-water media: Immobilisation and control of water activity by means of Celite® rods. Journal of Molecular Catalysis B: Enzymatic, 6(4), 437-445.

De Vendittis, E., Palumbo, G., Parlato, G., & Bocchini, V. (1981). A fluorimetric method for the estimation of the critical micelle concentration of surfactants. Analytical Biochemistry, 115(2), 278-286.

Farone, W.A., & Serfass, R.W., Kimball Chase Tech. Ltd. (1995). Sugar-Ester Manufacturing Process. U.S. Pat. 5,756,716.

Ferrer, M., Perez, G., Plou, F. J., Castell, J. V., & Ballesteros, A. (2005). Antitumour activity of fatty acid maltotriose esters obtained by enzymatic synthesis. Biotechnology and Applied Biochemistry, 42(1), 35-39.

Ferrer, M., Plou, F. J., Fuentes, G., Cruces, M. A., Andersen, L., & Kirk, O. (2002). Effect of the immobilization method of lipase from *Thermomyces lanuginosus* on sucrose acylation. Biocatal. Biotrans., 20(1), 63-71.

Fregapane, G., Sarney, D. B., Greenberg, S. G., Knight, D. J., & Vulfson, E. N. (1994). Enzymatic synthesis of monosaccharide fatty acid esters and their comparison with conventional products. JAOCS, 71(1), 87-91.

GanemQuintanar, A., Quintanar-Guerrero, D., Falson-Rieg, F., & Buri, P., (1998). Ex vivo oral mucosal permeation of lidocaine hydrochloride with sucrose fatty acid esters as absorption enhancers. Int. J. Pharm. 173, 203–210.

Grace Davison. (2006). Zeolite Molecular Sieve. Retrieved from http://lartpc-docdb.fnal.gov/0000/000084/001/zeolite_molecular_sieve.pdf (25 September, 2011)

Habulin, M., Sabeder, S., & Knez, Z. (2008). Enzymatic synthesis of sugar fatty acid esters in organic solvent and in supercritical carbon dioxide and their antimicrobial activity. Journal of Supercritical Fluids, 45(3), 338-345.

Halling, P. J. (1992). Salt hydrates for water activity control with biocatalysts in organic media. Biotechnology Techniques, 6(3), 271-276.

Hertzberg, S., Kvittingen, L., Anthonsen, T., & Skjak-Braek, G. (1992). Alginate as immobilization matrix and stabilizing agent in a two-phase liquid system: Application in lipase-catalyzed reactions. Enzyme and Microbial Technology, 14(1), 42-47.

Husband, F. A., Sarney, D. B., Barnard, M. J., & Wilde, P. J. (1998). Comparison of foaming and interfacial properties of pure sucrose monolaurates, dilaurate and commercial preparations. Food Hydrocolloids, 12(2), 237-244.

Jin, W., & Brennan, J. D. (2002). Properties and applications of proteins encapsulated within sol–gel derived materials. Analytica Chimica Acta, 461(1), 1-36.

Kawase, M., Sonomoto, K., & Tanaka, A. (1992). Inspection of an acyl-enzyme intermediate in a lipase reaction by gas chromatography-mass spectrometry and modelling of the reaction mechanism. Biocatal Biotransformation, 6(1), 43-50.

Kelkar, D. S., Kumar, A. R., & Zinjarde, S. S. (2007). Hydrocarbon emulsification and enhanced crude oil degradation by lauroyl glucose ester. Bioresource Technology, 98(7), 1505-1508.

Koczo, K., & Racz, G. (1991). Foaming properties of surfactant solutions. Colloids and Surfaces, 56, 59.

Kwon, S.J., Song, K. M., Hong, W.H., & Rhee, J.S. (1995). Removal of water produced from lipase-catalyzed esterification in organic solvent by pervaporation. Biotechnology and Bioengineering, 46(4), 393-395.

Lee, M., Kim, M.K., Singleton, I., Goodfellow, M., & Lee, S.T., (2006). Enhanced biodegradation of diesel oil by a newly identified Rhodococcus baikonurensis EN3 in the presence of mycolic acid. J. Appl. Microbiol. 100, 325–333.

Lee, S. H., Nguyen, H. M., Koo, Y., & Ha, S. H. (2008). Ultrasound-enhanced lipase activity in the synthesis of sugar ester using ionic liquids. Process Biochemistry, 43(9), 1009-1012.

Lehmann, L., Keipert, S., & Gloor, M., (2001). Effects of microemulsions on the stratum corneum and hydrocortisone penetration. Eur. J. Pharm. Biopharm. 52, 129–136.

Lobnik, A., & Wolfbeis, O. S. (2001). Probing the polarity of sol-gels and ormosils via the absorption of nile red. Journal of Sol-Gel Science and Technology, 20(3), 303-311.

Marton, S., Auner, A., & Csoka, G., (2005). Increased solubility applying solid-in-solid solutions. Eur. J. Pharm. Sci. 25, 155–157.

Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., & Fernandez-Lafuente, R. (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme and Microbial Technology, 40(6), 1451-1463.

Meunier, S. M., & Legge, R. L. (2010). Evaluation of diatomaceous earth as a support for sol-gel immobilized lipase for transesterification. Journal of Molecular Catalysis B: Enzymatic, 62(1), 53-57.

Molinier, V., Fenet, B., Fitremann, J., Bouchu, A., & Queneau, Y. (2005). PFGSE-NMR study of the self-diffusion of sucrose fatty acid monoesters in water. Journal of Colloid and Interface Science, 286(1), 360-368.

Montgomery, D. C., & Runger, G. C. Applied statistics and probability for engineers. Hoboken, NJ: Wiley, 2011.

Mutua, L.N. & Akoh, C.C. (1993). Synthesis of alkyl glycoside fatty acid esters in non-aqueous media by Candida sp. Lipase. J. Am. Oil Chem. Soc. 70, 43–46.

Napier, P. E., Lacerda, H. M., Rosell, C. M., Valivety, R. H., Vaidya, A. M., & Halling, P. J. (1996). Enhanced organic-phase enzymatic esterification with continuous water removal in a controlled air-bleed evacuated-headspace reactor. Biotechnology Progress, 12(1), 47-50.

Nishikawa, Y., Yoshimoto, K., Nishijima, M., Fukuoka, F., & Ikekawa, T. (1981). Chemical and biochemical studies on carbohydrate esters. V. Anti Ehrlich ascites tumor effect and chromatographic behaviors of fatty acyl monoesters of sucrose and trehalose. Chem. Pharm. Bull. 29, 505-513.

Ntawukulilyayo, J. D., Bouckaert, S., & Remon, J. P. (1993). Enhancement of dissolution rate of nifedipine using sucrose ester coprecipitates. Int. J. Pharm. 93, 209–214.

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

Oguntimein, O. B., Erdmann, H., & Schmid, R. D. (1993). Lipase Catalyzed Synthesis of Sugar Esters in Organic Solvents. Biotechnol.Lett.,15, 175.

Okumura, H., Kitazawa, N., Wada, S., & Hotta, H. (2011). Stability of sucrose fatty acid esters under acidic and basic conditions. Journal of Oleo Science, 60(6), 313-320.

Orafidiya, L. O., & Oladimeji, F. A. (2002). Determination of the required HLB values of some essential oils. International Journal of Pharmaceutics, 237(1–2), 241-249.

Ota, M., Osaka-Shi, Seisheiro, Y., & Yamato-Takada-Shi, (1972). Mixed Sugar Alcohol Esters of Higher and Lower Fatty acids, U.S. Pat 3,649,647.

Otsuka, M., & Matsuda, Y., (1995). Effect of cogrinding with various kinds of surfactants on the dissolution behavior of phenytoin. J. Pharm. Sci. 84, 1434–1437.

Otsuka, M., Ofusa, T., & Matsuda, Y., (1998). Dissolution improvement of water-insoluble glybuzole by co-grinding and co-melting with surfactants and their physicochemical properties. Colloid Surf. B 10, 217–226.

Pauly, G., Engasser, J.M., & Ghoul, M., Laboratoires Serobiologiques (Societe Anonyme) (1999). Method for Enzymatic Synthesis of Sucrose Esters, U.S. Pat. 6,355,455 B1.

Peltola, T., Jokinen, M., Rahiala, H., Pätsi, M., Heikkilä, J., Kangasniemi, I., & Yli–Urpo, A., (2000). Effect of aging time of sol on structure and in vitro calcium phosphate formation of sol-gel-derived titania films. J. Biomed. Mater. Res., 51 (2), 200-208.

Plou, F. J., Cruces, M. A., Ferrer, M., Fuentes, G., Pastor, E., & Bernabe, M., (2002). Enzymatic acylation of di- and trisaccharides with fatty acids: Choosing the appropriate enzyme, support and solvent. Journal of Biotech., 96(1) 55-66.

Reetz, M. T., Zonta, A., & Simpelkamp, J. (1996). Efficient immobilization of lipases by entrapment in hydrophobic sol-gel materials. Biotechnology and Bioengineering, 49(5), 527-534.

Šabeder, S., Habulin, M., & Knez, Ž. (2006). Lipase-catalyzed synthesis of fatty acid fructose esters. Journal of Food Engineering, 77(4), 880-886.

Sakaki, K., Aoyama, A., Nakane, T., Ikegami, T., Negishi, H., Watanabe, K. (2006). Enzymatic synthesis of sugar esters in organic solvent coupled with pervaporation. Desalination, 193(1-3), 260-266.

Schmid, R. D., & Verger, R. (1998). Lipases: Interfacial enzymes with attractive applications. Angewandte Chemie International Edition, 37(12), 1608-1633.

Schmidt, H. (1988). Chemistry of material preparation by the sol-gel process. Journal of Non Crystalline Solids, 100(1–3), 51-64.

Severson, R. F., Ellington, J. J., Schlotzhauer, P. F., Arrendale, R. F., & Schepartz, A. I. (1977). Gas chromatographic method for the determination of free and total solanesol in tobacco. Journal of Chromatography A, 139(2), 269-282.

Sweeley, C.C., Bentley, R., Marita, M. & Wells, W.W (1963). Gas-liquid Chromatography of trimethylsilyl derivatives of sugars and related substances. J. Am. Chem. Soc. 85:2497.

Szűts, A., Pallagi, E., Regdon, J., Géza, Aigner, Z., & Szabó-Révész, P. (2007). Study of thermal behavior of sugar esters. International Journal of Pharmaceutics, 336(2), 199-207.

Szuts, A., Szucs, M.B., Eros, I., Otomo, N., & Révésza, P. (2010). Study of gel-forming properties of sucrose esters for thermosensitive drug delivery systems. International Journal of Pharmaceutics, 383, 132-137.

Tahara, Y., Honda, S., Kamiya, N., Piao, H., Hirata, A., Hayakawa, E., Fujii, T., Goto, M., (2008). A solid-in-oil nanodispersion for transcutaneous protein delivery. J. Control. Release.131, 14–18.

Tai, H. P., & Brunner, G. (2009). Sugar fatty acid ester synthesis in high-pressure acetone-CO2 system. Journal of Supercritical Fluids, 48(1), 36-40.

Tarahomjoo, S., & Alemzadeh, I. (2003). Surfactant production by an enzymatic method. Enzyme and Microbial Technology, 33(1), 33-37.

Therisod, M., & Klibanov, A. M. (1986). Facile Enzymatic Preparation of Monoacylated Sugars in Pyridine. J. Am. Chem. Soc., 108, 5638.

Toorisaka, E., Hashida, M., Kamiya, N., Ono, H., Kokazu, Y., & Goto, M. (2005). An enteric coated dry emulsion formulation for oral insulin delivery. J. Control. Release. 107, 91–96.

Tsitsimpikou, C., Daflos, H., & Kolisis, F. N. (1997). Comparative studies on the sugar esters synthesis catalyzed by *Candida Antarctica* and *Candida Rugosa* lipases in hexane. Journal of Molecular Catalysis – B: Enzymatic, 3(1-4), 189-192.

Ullrich, S., Metz, H., & Mäder, K. (2008). Sucrose ester nanodispersions: Microviscosity and viscoelastic properties. European Journal of Pharmaceutics and Biopharmaceutics, 70(2), 550-555.

Ward, O. P., Fang, J., & Li, Z. (1997). Lipase-catalyzed synthesis of a sugar ester containing arachidonic acid. Enzyme and Microbial Technology, 20(1), 52-56.

Wehtje, E., Adlercreutz, P., & Mattiasson, B. (1993). Improved activity retention of enzymes deposited on solid supports. Biotechnology and Bioengineering, 41(2), 171-178.

Wilde, P.J., & Clark, D.C. (1996). Methods of testing protein functionality. G.M. Hall, Editor, Blackie Academic and Professional, London, 110–152.

Won, K., & Sun, B. L. (2001). Computer-aided control of water activity for lipase-catalyzed esterification in solvent-free systems. Biotechnology Progress, 17(2), 258-264.

Woudenberg, M., & Rantwijk, F. (1996). Regioselective acylation of disaccharides in tert-butyl alcohol catalyzed by *Candida Antarctica* lipase. Biotechnol. Bioeng., 49, 328.

Xiao, Y., Wu, Q., Cai, Y., & Lin, X. (2005). Ultrasound-accelerated enzymatic synthesis of sugar esters in nonaqueous solvents. Carbohydrate Research, 340(13), 2097-2103.

Yan, Y., Bornscheuer, U.T., Cao, L., & Schmid, R.D. (1999). Lipase-catalyzed solid-phase synthesis of sugar fatty acid esters: Removal of byproducts by azeotropic distillation. Enzyme and Microbial Technology, 25(8-9), 725-728.

Yan, Y., Bornscheuer, U.T., Stadler, G., Lutz-Wahl, S., Reuss, M. & Schmid, R.D. (2001). Production of sugar fatty acid esters by enzymatic esterification in a stirred membrane tank reactor: Optimization of parameters by response surface methodology. J. Am. Oil Chem. Soc., 78(2), 147–152.

Ye, R., Pyo, S., & Hayes, D. G. (2010). Lipase-catalyzed synthesis of saccharide-fatty acid esters using suspensions of saccharide crystals in solvent-free media. JAOCS, Journal of the American Oil Chemists' Society, 87(3), 281-293.

Yoo, I. S., Park. S. J., & Yoon, H. H. (2007). Enzymatic synthesis of sugar fatty acid esters. J. Ind. Eng. Chem, 13(1), 1-6.

Zinjarde, S.S., & Pant, A., (2002a). Hydrocarbon degraders from a tropical marine environment. Mar. Poll. Bull. 44, 118–121.

Appendix

A. Example Chromatograph for GC-MS Analysis

Samples containing a variety of sugars with oleic acid and CSSIE were derivatized and then analyzed by the GC-MS. A typical chromatogram for a full complement of reactants and products is shown in Figure 21. This chromatogram reflects good separation of all esters produced, unconverted sugars and oleic acid. The molecular formula and weights of the derivatized products are provided in Table 5. Quantitative data was obtained from peak areas and calibration plots. The yield of product was evaluated from the conversion of oleic acid and stoichiometric and mass balance calculations in the absence of any evidence of side products which could be attributed to the regioselectivity of the enzyme. Mass spectra fragmentation patterns were recorded and used to identify of the sugar esters and fatty acids. The retention times and mass spectral data for each peak matched the retention times and mass spectral data obtained for derivatized samples of the individual compounds.

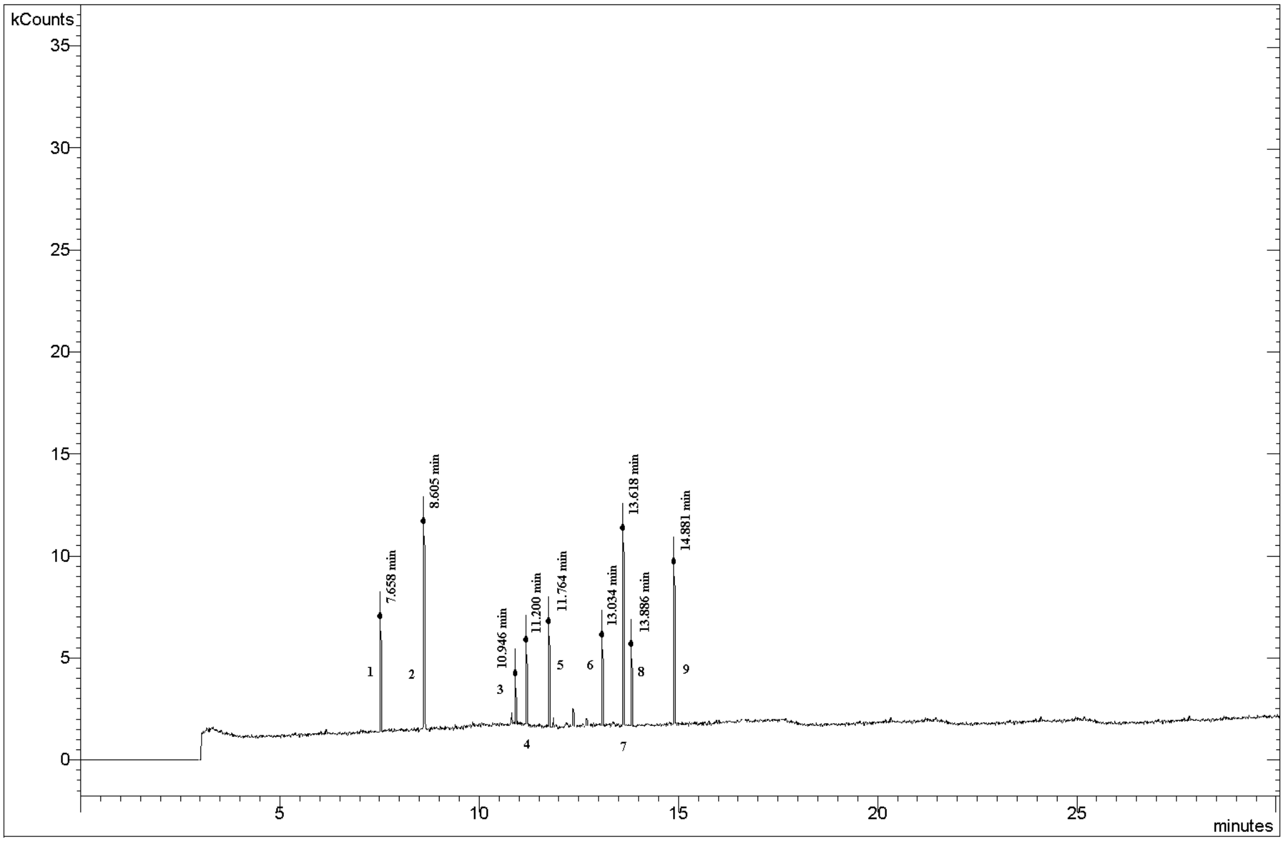


Figure 21. Example chromatogram of a sample containing unconverted reactants and products

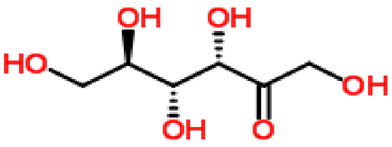
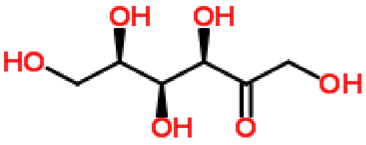
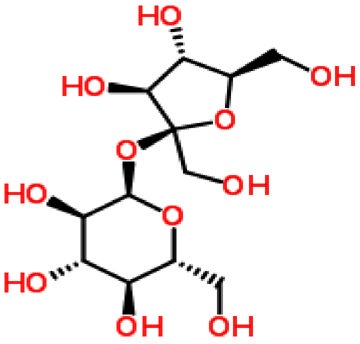
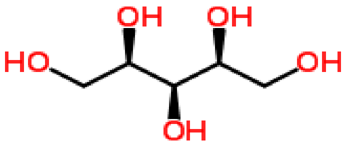
Table 5. Assignment of retention times for compounds analyzed by GC-MS.

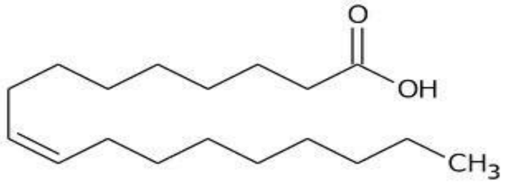
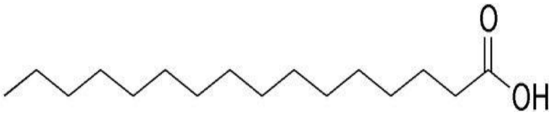
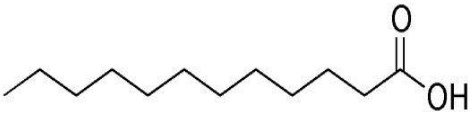
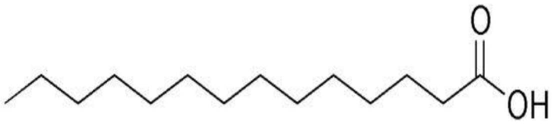
Peak Number	Sample	Molecular weight (g/mol)	Molecular formula	Retention Time (minutes)
1	Xylitol	224	$C_8H_{20}O_5Si$	7.658
2	Fructose	252	$C_9H_{20}O_6Si$	8.605
3	Sorbitol	254	$C_9H_{22}O_6Si$	10.946
4	Oleic acid	354	$C_{21}H_{42}O_2Si$	11.200
5	Sucrose	414	$C_{15}H_{30}O_{11}Si$	12.616
6	Xylitol monooleate	488	$C_{26}H_{52}O_6Si$	13.034
7	Fructose monooleate	516	$C_{27}H_{52}O_7Si$	13.618
8	Sorbitan monooleate	518	$C_{27}H_{54}O_7Si$	13.886
9	Sucrose monooleate	678	$C_{33}H_{62}O_{12}Si$	14.881

B. Chemical Structures of Compounds used in this Study

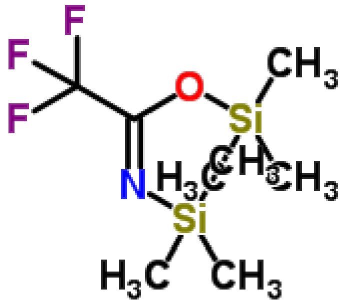
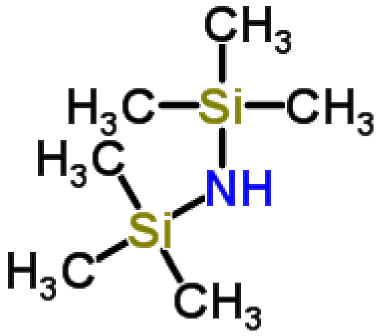
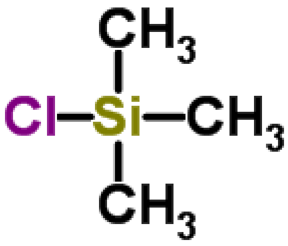
The structures of the compounds used in this study are provided in Table 6.

Table 6. Structures of sugars, fatty acids and of the derivatization agents

 Fructose Systematic Name - D-Fructose Molecular formula - $C_6H_{12}O_6$ Molecular weight - 180.1559	 Sorbitol Systematic name - D-Sorbitol Molecular formula - $C_6H_{12}O_6$ Molecular weight - 180.1559
 Sucrose Systematic Name - β-D-fructofuranosyl α-D-glucopyranoside Molecular formula - $C_{12}H_{22}O_{11}$ Molecular weight - 342.11	 Xylitol Systematic Name - D-Xylitol Molecular formula - $C_5H_{12}O_5$ Molecular weight - 152.0685

 Oleic acid Systematic Name - (9Z)-octadec-9-enoic acid Molecular formula - $C_{18}H_{34}O_2$ Molecular weight - 282.256	 Palmitic acid Systematic Name - (9Z)-octadec-9-enoic acid Molecular formula - $C_{16}H_{32}O_2$ Molecular weight - 256.24
 Lauric acid Systematic Name - Dodecanoic acid Molecular formula - $C_{12}H_{24}O_2$ Molecular weight - 200.177	 Myristic acid Systematic Name - Tetradecanoic acid Molecular formula - $C_{14}H_{28}O_2$ Molecular weight - 228.209

In the process of derivatization of the substrates/products with BSTFA, HMDS or TMCS, the active hydrogen was replaced by –Si$(CH_3)_3$ (trimethylsilyl group) in the –OH, –COOH, =NH, –NH_2 and –SH groups.

 N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) Molecular formula - $C_{18}H_{18}F_3NOSi_2$ Molecular weight - 257.087	 Hexamethyldisilazane Molecular formula - $C_6H_{19}NSi_2$ Molecular weight - 161.105
 Chlorotrimethylsilane Molecular formula - C_3H_9ClSi Molecular weight - 108.016	 Stearic acid Molecular formula - $C_{18}H_{36}O_2$ Molecular weight - 284.48

C. Celite® Particle Size and Pore Diameter

Table 7. Particle size and pore diameters for the Celite® used in this study

(Based on manufacturer's specifications provided by Meunier & Legge, 2010)

Celite®	Particle Size (µm)	Pore Diameter (µm)
R633	300 - 600	6.5
R632	600 - 1400	7
R647	600 - 1400	0.07

Calculations

A. Mass Balance

The mass balance for sucrose monooleate synthesis using Celite®-R633 supported sol-gel was conducted as follows.

When 3mmol of sucrose reacts with 3 mmol of oleic acid, 3 mmol of sucrose monooleate and 3 mmol of water were formed. Thus on a mass basis 1.0269 g of sucrose would react with 0.847 g of oleic acid and form 1.82 g of sucrose monooleate ester and 0.0565 g of water. This translates into the formation of 0.3 g of water for 100 g of sugar monooleate. This is in agreement to moisture content % from Karl Fischer data.

The final water content in the reaction medium was 0.03 g for every 100 g of product formed using Celite®-R633 supported sol-gel which translates into to 90% water of the water being adsorbed.

B. Calculation of Water Adsorbing Capacity of CSSIE

Consider for example the production of sucrose monooleate in acetone using Celite®-R633 supported sol-gel.

Quantity of water adsorbed by supported sol-gel after its first use

Initial weight of CSSIE used in the reaction mixture = 5 g

From mass balance calculations 1.0269 g of sucrose reacted with 0.847 g of oleic acid and produced 1.82 g of sucrose ester and 0.0565 g of water.

From the GC-MS data an oleic acid conversion of 95.13% was observed thus 0.0537 g of water was formed with no side products formed.

From KF data 0.07% water was present in the final product using the supported sol-gel. By the use of the unsupported sol-gel 0.33% water was present in the final product. Therefore, 78.79% of the total water formed in the reaction (0.0537 g) was absorbed by 5 g of CSSIE for the first use = 0.0423 g

Quantity of water absorbed per g of CSSIE = 0.00846 g

Quantity of water adsorbed by supported sol-gel after its first reuse without drying

Initial weight of CSSIE = 5.314 g

From mass balance calculations 1.0269 g of sucrose reacted with 0.847 g of oleic acid and formed 1.82 g of sucrose ester and 0.0565 g of water.

From the GC-MS data an oleic acid conversion of 96.12% was observed, thus 0.0543 g of water was formed with no side product formation observed.

From KF data 0.15% water was present in the final product. The use of the unsupported sol-gel resulted in 0.33% water in the final product.

Therefore 54.54% of the total water formed in the reaction (0.0543 g) was absorbed by 5 g of CSSIE = 0.0296 g

Quantity of water absorbed per g of CSSIE while reusing it for the first time without adopting the drying approach = 0.00592 g

Quantity of water adsorbed by supported sol-gel after its first reuse with drying

Initial weight of CSSIE = 4.722 g

From mass balance calculations 1.0269 g of sucrose reacted with 0.847 g of oleic acid and formed 1.82 g of sucrose ester and 0.0565 g of water.

From the GC-MS data an oleic acid conversion of 96.12% was determined thus 0.0543 g of water was formed.

From KF data 0.12% water was present in the final product. By the use of the unsupported sol-gel 0.33% water was present in the final product.

Therefore 63.64% of the total water formed in the reaction (0.0543 g) was absorbed by 5 g of CSSIE = 0.0345 g

Quantity of water absorbed per g of CSSIE while reusing it for the first time with drying = 0.0073 g

Thus the CSSIE drying approach increased the water absorption capacity of the biological catalyst.

C. Determination of Protein Immobilized in Lipase Analysis by HPLC

Mass Balance of Protein

$$
\begin{aligned}
&Mass\ of\ protein\ immobilized\ within\ the\ sol-gel \\
&\qquad = \{Total\ mass\ of\ protein\ initially\ used\ to\ prepare\ the\ sol-gel \\
&\qquad -\ Mass\ of\ protein\ from\ buffer\ wash\ 1 \\
&\qquad - Mass\ of\ protein\ from\ buffer\ wash\ 2\ \}
\end{aligned}
$$

Mass of protein from each buffer wash was determined from the calibration plot in Figure 22.

Initially 15 ml of enzyme with a protein concentration of 2000 µg/ml was used and the total initial mass of protein was 30,000 µg.

Quantity of buffer used for each wash = 15 ml

Mass of immobilized of protein in sol-gel supported by Celite®-R633

= 30,000-3,000-1,500 µg = 25,500 µg

= 85% of initial protein mass immobilized

Immobilized mass of protein in sol-gel supported by Celite®-R632

= 30,000-3,750-3,000 µg = 23,250 µg

= 77.5% of initial protein mass immobilized

Immobilized mass of protein in sol-gel supported by Celite®-R647

= 30,000-7,350-5,250 µg = 17,400 µg

= 58% of initial protein mass immobilized

Immobilized mass of protein in unsupported sol-gel

= 30,000-9,750-3,750 µg = 16,500 µg

= 55% of initial protein mass immobilized

The retention time of the lipase was 16.7 minutes assuming that the largest peak on the chromatogram was lipase. The calibration plot provided in Figure 22 is for Lipozyme CAL-B lipase.

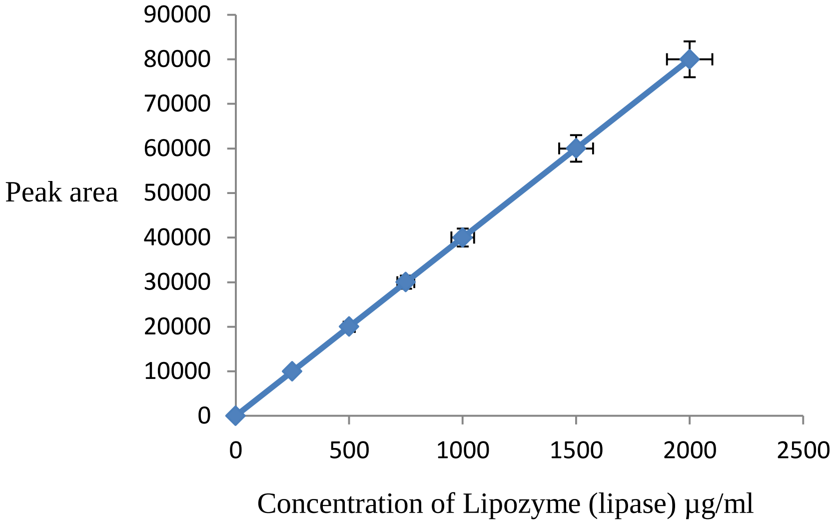


Figure 22. Calibration plot for determining protein loading in sol-gels. The error bars represent the 95% confidence interval of the sample mean based on n=3.

Resp. Fact. RSD = 21.85%

Regression coefficient (R^2) = 0.999548

Curve Type = Quadratic

$y = 3.350398e - 3x^2 + 5.8215e1x + 1.178849e + 2$

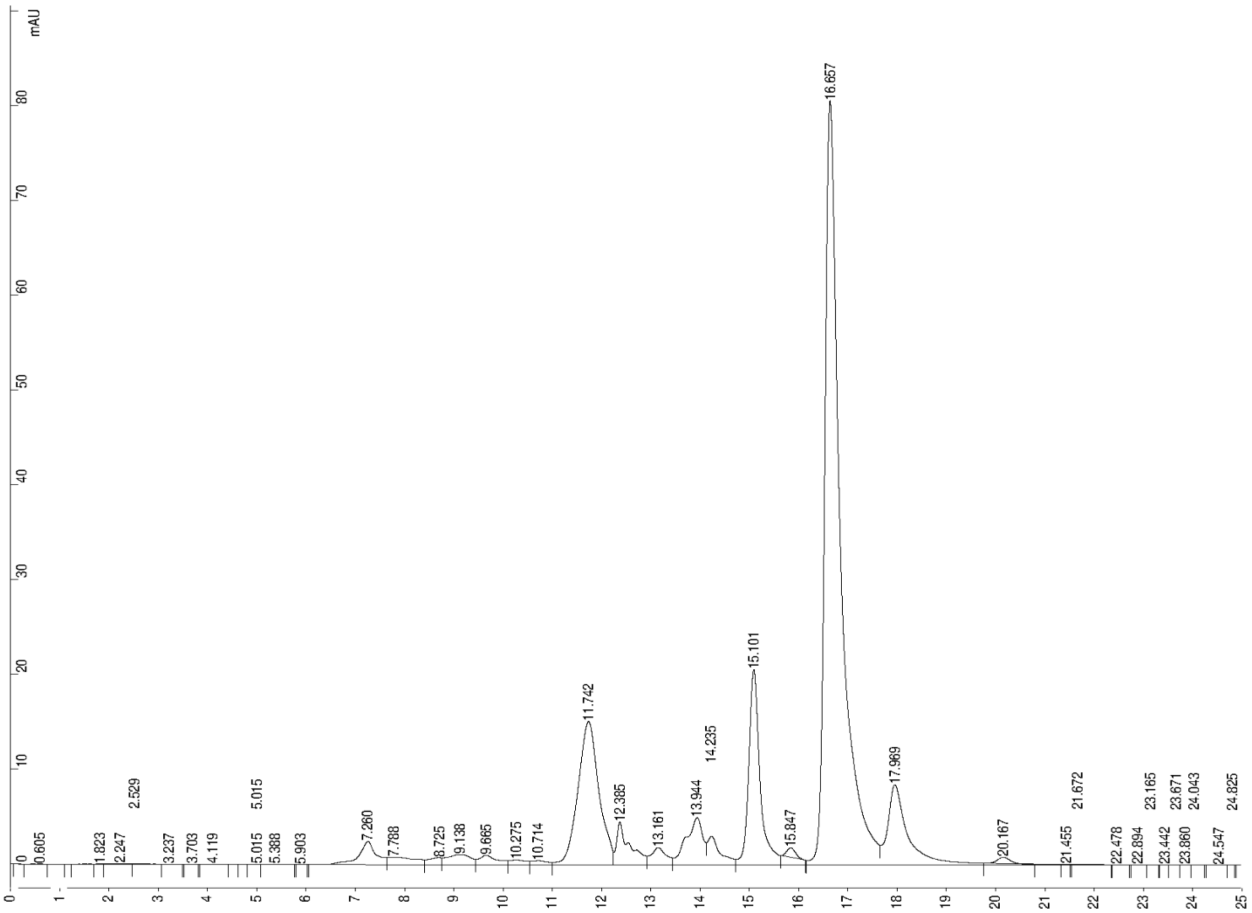


Figure 23. Chromatogram for a sample of 1000 µg/ml total protein based on manufacturers specifications.

Statistical Data Analysis

The effect of the Karl-Fischer (KF) titration reagent on moisture content was statistically analyzed using different preparations. The statistical calculations were based on randomized block designs and least significant difference (LSD) methods by Montgomery & Runger (2011).

Experimental Details

Blocked Variable – Number of times 100 ml of KF reagent was used for titration

Factor – Different types of enzymes with (or) without molecular sieves

Response Variable – Moisture Content

Number of replicates – 3

The amount of Karl Fischer reagent used for each replicate was always 100 ml. It was suspected that there was an error in the response variable along with the instrumental error and hence a blocked variable was chosen to be the number of times the same reagent was used. To neglect the sol-gel aging effect fresh sol-gel was prepared two days before each analysis.

Product moisture content was calculated by the formula,

$$\frac{Moisture\ content\ of\ titration - \ Moisture\ content\ of\ blank\ solution}{(Sample\ weight + Tare\ weight) - Tare\ weight}$$

Karl Fischer Titration

$I_2 + SO_2 + H_2O \rightarrow 2HI + SO_3$

The KF reagent contained iodine and sulphur dioxide. Based on the amount of hydrogen iodide formed measured by the detection electrode the water content of the sample was calculated. The stirrer speed was adjusted and the detection electrode and stirrer unit were connected directly before each analysis. The desiccant tube was attached to remove any condensation that formed and the drain cock was closed. The titration flask and cathode chamber were filled with 100 ml of fresh reagent and 1 vial of catholyte solution. The same reagent was reused for 3 titration replicates. Three types of samples were compared for the production of fructose monooleate in acetone.

Treatment 1 – Novozym (0.03 g)

Treatment 2 – Novozym (0.03 g) + Molecular sieves (5 g)

Treatment 3 – Sol-gel supported by Celite®-633 (5 g)

At the end of the reaction the treatments were separated from the product mixture by filtration with a 0.45 µm DB filter paper (Chromatographic Specialties Inc.) and the purified product was injected into the titration chamber that contained the KF reagent. The percentage moisture content was determined and analyzed statistically.

Statistics and Data Analysis

Number of times same reagent was used (Block)	**% Moisture Content for Different Samples**		
	Novozym	**Novozym + molecular sieve**	**Supported sol-gel**
1	0.33	0.13	0.06
2	0.35	0.15	0.03
3	0.3	0.16	0.05

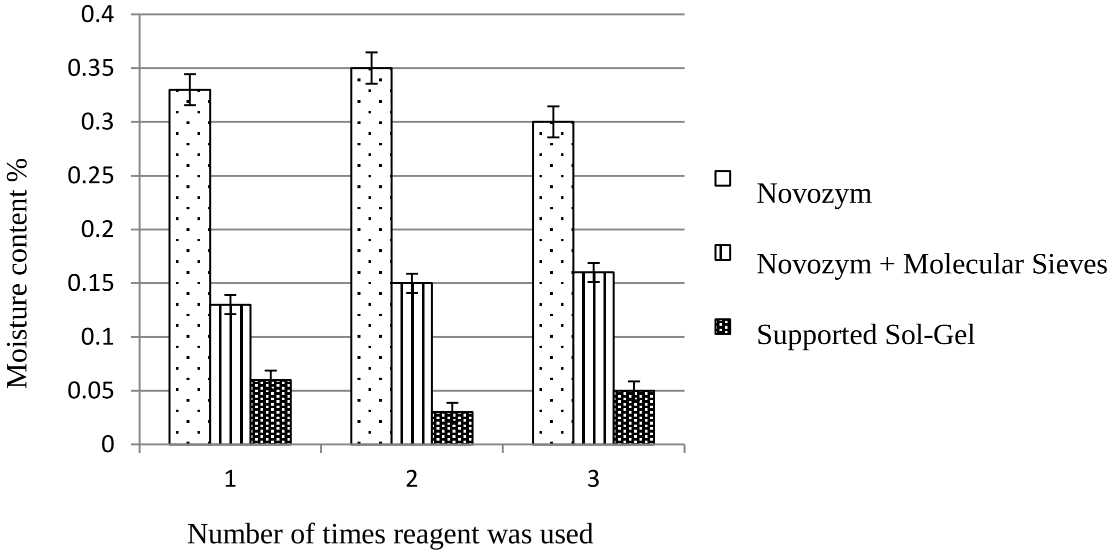


Figure 24. Moisture content in fructose monooleate production for different preparations. The error bars represent the 95% confidence interval of the sample mean based on n=3.

The statistical techniques used were based on the approach by Montgomery & Runger (2011) and the following methods were adopted to determine the Analysis of Variance (ANOVA) table.

1. Randomized Block Design by Single factor experiment
2. Multiple comparisons using the Least Significant difference (LSD)

Table 8. Randomized block design by single factor experiment

Number of times same reagent was used (Block)	**Percentage moisture content of product for different treatments**			
	Novozym	**Novozym + molecular sieve**	**Supported sol-gel**	**Block totals**
1	0.33	0.13	0.06	0.52
2	0.35	0.15	0.03	0.53
3	0.3	0.16	0.05	0.51
Treatment totals	0.98	0.44	0.14	1.56

Table 9. ANOVA table of effect of KF reagent on moisture content

Source	SS	Df	MS	F_{obs}
Factor	0.1208	2	0.0604	111.851
Block	0.0000667	2	0.00003335	0.0632
Error	0.00213	4	0.00053	
Total	0.123	8		

Summary of results

1. Variation of moisture content for different treatments was significant.
2. The number of uses of KF reagent did not influence the product moisture content for three replications.
3. Blocking accounted for 3% variation which was insignificant.

Discussion of analysis and conclusions

- The LSD approach confirmed that the use of different treatments significantly affected the moisture content and that supported sol-gels adsorbed more water than Novozym and molecular sieves.
- The use of the same KF reagent did not significantly affect the moisture content. However if the number of replications were increased it would possibly change statistical data and interpretations. Prior to this analysis KF reagent was changed after every use.
- The effect of blocking was not vitally important since only 3% of variation not associated with enzymes was explained by blocks.