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Synthesis of lipase-catalyzed saccharide-fatty acid esters using a packed bed bioreactor system with continuous re-circulation of reaction medium: A continuation of batch-mode-related research

Oriana Okutu Obiri
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Oriana Okutu Obiri entitled "Synthesis of lipase-catalyzed saccharide-fatty acid esters using a packed bed bioreactor system with continuous re-circulation of reaction medium: A continuation of batch-mode-related research." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biosystems Engineering.

Douglas Hayes, Major Professor

We have read this thesis and recommend its acceptance:

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Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Vice Chancellor and
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SYNTHESIS OF LIPASE-CATALYZED SACCHARIDE-FATTY ACID ESTERS USING A PACKED BED BIOREACTOR SYSTEM WITH CONTINUOUS RE-CIRCULATION OF REACTION MEDIUM: A CONTINUATION OF BATCH-MODE-RELATED RESEARCH

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Oriana Okutu Obiri
December 2006

DEDICATION

This thesis is dedicated to my father, Mr. Richard Wesley Obiri who left me on April 11th, 2006 to go and be with the Lord. Thank you so much for being my inspiration and encouraging me to aim higher and higher. I wish you could be here to see the finish product but I know that you are in spirit. I love you!!

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I wish to thank all those who have helped me complete my Master of Science degree in Biosystems Engineering. My profound thanks to Dr. Douglas G. Hayes for first introducing me to this area of biological surfactants, and his constant guidance and advice during the course of this research. I would like to thank my committee members, Dr. Philip Ye and Dr. Svetlana Zivanovic for their counsel and time as they served on my committee. I would like to thank Hung Dung and Javier Gomez Del Rio for their answers to certain questions that had come up during my research.

I would like to thank the University of Tennessee Biosystems Engineering and Soil Science Department for their financial support. This research was sponsored by the United States Department of Agriculture Cooperative State Research, Education and Extension Service Grant 95-37500-2200. Finally, I would like to thank my family and friends for being there for me, your constant encouragement and reminder that I was doing this for all of you kept me going.

ABSTRACT

The purpose of this thesis study was to further study the batch synthesis of lipase-catalyzed saccharide-fatty acid esters and to introduce the fed-batch synthesis of saccharide-fatty acid esters as a preliminary design to the continuous synthesis of these esters in a packed bed bioreactor system. The batch reaction method is the conventional mode of synthesizing saccharide-fatty acid esters on a lab-scale basis and some conversion-related parameters of the reaction mode were investigated. These experiments included investigating the effect of water content on the equilibrium conversion of fructose oleates and exploring the activity retention of immobilized *Rhizomucor miehei* lipase (RML) in successive batch reactions. The batch synthesis of other saccharide-oleic acid esters was also a point of interest and was studied.

Results from the batch-related experiments revealed the following results: that the there is no loss of activity after successive use of the immobilized RML in batch reactions, that the presence of water reduces the thermodynamic equilibrium-controlled conversion of the reaction, and that the product conversion and the reaction rate of each type of saccharide are different.

Motivated by drawbacks associated with the batch mode of esterification, the fed-batch synthesis mode was proposed as a preliminary design towards the continuous synthesis mode, and was applied to the lipase-catalyzed esterification reaction between fructose and oleic acid in the lab. The study was approached by first designing and building a packed bed bioreactor system. Lipase-catalyzed synthesis of saccharide-fatty

acid esters was then conducted by continuous re-circulation of the reaction medium through the packed-bed reactor.

Success was achieved in designing and assembling the bioreactor system and it was employed for the fed-batch synthesis of saccharide-fatty acid esters. The results obtained demonstrated that the synthesis of saccharide-fatty acid esters in a packed bed bioreactor with continuous re-circulation of reaction medium did achieve a high product conversion without disadvantages such as the need for lipase recovery and replacement, large requirement for labor and frequent shutdown and start-up procedures. A kinetic mathematical model was created to predict the mass fraction of monoesters that were present in the re-circulation stream and that produced in the packed bed reactor. It was observed from the results that the derived kinetic model was reliable and correlated well with the experimental data.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

a_W	water activity
g	grams
hr	hours
kg	kilogram
min	minutes
ml	milliliter
mM	millimolar
^oC	degree Celsius
^oF	degree Fahrenheit
psi	pounds per square inch
rpm	revolutions per minute
wt%	weight percent

Abbreviations

$BaCl_2$	barium chloride
$CaSO_4$	calcium sulfate
CH_3COOK	potassium acetate
FFA	free fatty acid
HLB	hydrophilic lipophilic balance
HPLC	High Pressure Liquid Chromatography
K_2SO_4	potassium sulfate
LiCl	lithium chloride
ME	monoester
NaCl	sodium chloride
PBR	packed-bed reactor
RML	*Rhizomucor miehei* lipase
tBuOH	tertiary butyl alcohol
V_{MAX}	maximum velocity

CHAPTER 1

INTRODUCTION

1.1 - Overview

Surfactants, also known as emulsifiers, are amphiphilic compounds which have both hydrophobic and hydrophilic domains (Figure 1.1) and their amphiphilic nature enables them to exhibit different beneficial characteristics.

Characteristic properties include the ability of surfactants to form an ordered molecular film at the interface in order to reduce the interfacial tension between two phases, the capability to control the interfacial behavior and mass transfer of molecules across phases and also the capacity to alter foaming properties of aqueous solutions[1, 2]. These unique characteristics of surfactants make them highly applicable for a wide range of industrial processes such as their use as emulsifiers in several products. Other surfactant applications include their use as foaming agents, detergents, solubilizing agents, adhesives and wetting agents[3, 4]. Figure 1.2 illustrates the action of a surfactant as an emulsifier in an oil-in-water emulsion. In the diagram, the hydrophilic end of the surfactant molecules attach themselves to the water droplets in the oil phase and the hydrophobic end are exposed to the oil phase; this mechanism allows for stabilization of water droplets in the oil phase.

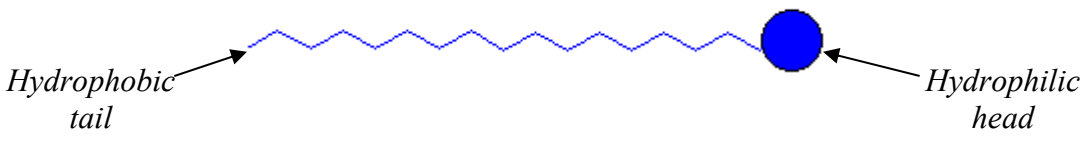

Figure 1.1: Structure of a surfactant

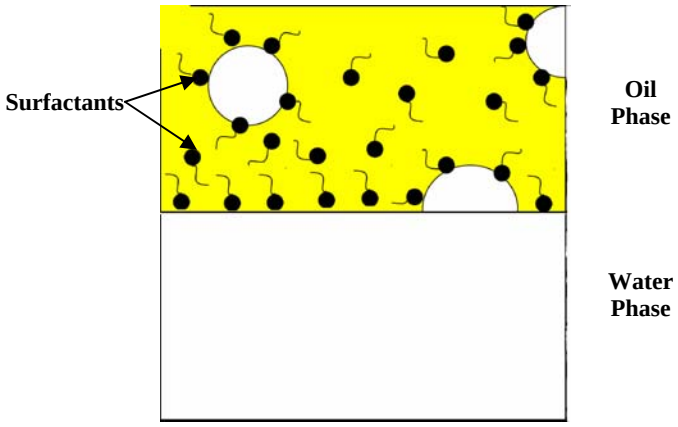

Figure 1.2: Illustration of emulsifier action in oil-in-water emulsion

Surfactants are molecules with distinct hydrophilic and hydrophobic moieties, and depending on the charges on the hydrophilic moiety, can either be classified as ionic or non-ionic.

The industrial market for surfactants has become diverse with a growing and constant demand over the last few years. In the early to mid 90's, the worldwide production of surfactants exceeded 3 million tons per annum which had an estimated value of US$4 billion. By the turn of the 21st century, surfactant production has risen to over 4 million tons[3].

Over the past decade, there has been an increase of environmental awareness with governmental environmental protection agencies implementing stricter rules and regulations for the biodegradability of industrial products. Furthermore, there has been increased consumer awareness of potentially adverse allergic effects caused by synthetic food emulsifiers and this has forced the food industry to search for more natural alternatives such as biological surfactants.

Biological surfactants, which are often referred to as biosurfactants, are identified as being biodegradable in the environment and biocompatible with human beings. They are either naturally produced by yeast or bacteria from various bio-based substrates such as sugars, fatty acids, amines and wastes, or chemically in the lab using similar substrates[5].

Biological surfactants have characteristics and properties similar to synthetic surfactants; most possess excellent heat resistance and pH resistance. Examples of biosurfactants are glycolipids, lipopeptides, phospholipids, saccharide fatty-acid esters, and amine-based fatty-acid esters[2, 5]. The focus of this research paper is on saccharide-fatty acid esters.

1.2 – Saccharide-Fatty Acid Esters

Saccharide-fatty acid esters are non-ionic biodegradable surfactants produced using materials from agricultural feedstock; saccharides which come from starches and celluloses or other naturally-occurring polysaccharides, and fatty acids derived from seed oils. They are the products of reactions such as esterification between hydroxyl(s) of the carbohydrate (sugar) group, and the carbonyl of a fatty acyl group, or an interestification reaction between a sugar hydroxyl group and an ester of a fatty acid. Sugars are included in the category of chemical compounds known as polyols which are defined as alcohols with multiple hydroxyl groups. Polyols act as acyl acceptors by taking on the carbonyl group of fatty acid molecules; examples of other polyols include glycerol and sugar alcohols.

Figure 1.3 illustrates an example of an esterification reaction in which a saccharide acyl acceptor covalently bonds to the carbonyl group of a fatty acid molecule in order to create room for the addition of a second alkyl group to synthesize an ester molecule. The reaction requires a catalyst, produces water as a co-product and is reversible.

The esters produced are typically a mixture of partially or completely acylated saccharides in which the position of the acylated hydroxyl is widely distributed. The product distribution is dependent on a number of factors such as the catalyst type, the inherent activity of the carboxyl and hydroxyl groups, and the relative ratio of acyl acceptor and acyl donor substrates. Saccharide-fatty acid monoesters are attracting attention because they are biodegradable compounds which make them environmentally-friendly and are cheap to produce since their raw materials are readily available, renewable and inexpensive.

Saccharide-fatty acid monoesters have a wide application in the food industry, serving as emulsifiers and additives in the candy and beverage industries, as a result of their biocompatibility; the most common esters being sucrose fatty acid esters. Saccharide-fatty acid esters have been found to demonstrate excellent functional properties which allows for their use both in food and non-food products, such as emulsification, emulsion stabilization and low-foaming properties. Some of the food products include diary products and baked goods and some of the non-food products include detergents, different types of cosmetics and oral care products[6, 7].

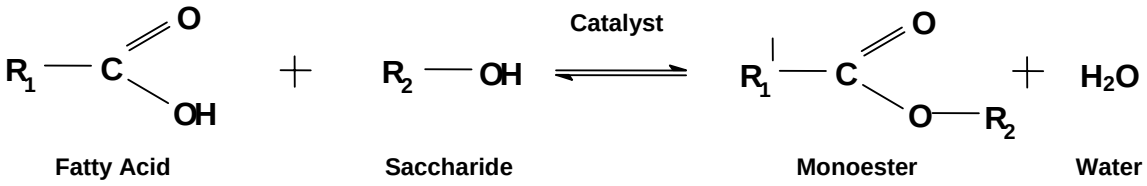

Figure 1.3: **General mechanism for a catalyzed esterification reaction. R_1 represents an alkyl group on the fatty acid and R_2 denotes the remainder of the saccharide molecule.**

1.3 – Proposed Work and Thesis Objectives

This thesis research study is a continuation of work performed by Xun Zhang and Hung Thai Dang in the research group of Dr. Douglas G. Hayes. The focus of this research was to experimentally study the batch synthesis of lipase-catalyzed saccharide-fatty acid esters and to investigate the fed-batch synthesis of saccharide-fatty acid esters as a preliminary design for future work on the continuous synthesis of saccharide-fatty acid esters. The synthesis of fructose oleates was chosen to serve as a reaction model and should be applicable to other saccharide oleate synthesis. Most of the research that has been conducted for the enzymatic synthesis of saccharide oleic acid esters has been done in batch mode and one of the aims of this research study was to design and optimize the fed-batch synthesis mode that will continuously re-circulate the reaction medium through a lipase-packed packed bed reactor.

Chapter 2 of this thesis presents background information into the conventional reaction scheme of producing saccharide oleic acid esters on a bench scale and then presents an alternative enzymatic reaction approach which with lipase as a biocatalyst. The chapter then introduces the large scale production of saccharide-fatty acid esters and

both the batch and continuous synthesis approach are explored. The chapter concludes with a literature review of analogous experiments that has been conducted involving the continuous synthesis of lipase-catalyzed polyol-fatty acid esters in different reactors.

Chapter 3 through to 5 focuses upon research performed by the author of this thesis. The objectives of this research study are: 1) To determine if immobilized lipase possesses high activity retention (or operational stability) during its use in successive batch experiments, 2) to investigate the effect of water activity on the equilibrium conversion of fructose oleates during batch synthesis, 3) to explore the batch synthesis of other saccharide-fatty acid esters, 4) to design and assemble a bioreactor system containing a saccharide adsorption column and packed-bed reactor that will employ continuous re-circulation of the reaction medium for the fed-batch synthesis of saccharide fatty acid esters, 5) to quantify the product conversion obtained in the packed bed bioreactor system and access the productivity, and 6) to develop a mathematical kinetic model that would predict the mass fraction of monoesters produced in the packed bed reactor.

Chapter 3 discusses different experiments conducted as a continuation of Zhang's and Dang's thesis work involving the batch synthesis of fructose-oleic acid with an introduction of each experiment, the methodology, a discussion of the results obtained leading up to the conclusions.

Chapter 4 is focuses upon the major objectives of this thesis study with a general overview of the synthesis using a bioreactor system with continuous re-circulation of the reaction medium. The thesis section was broken down into three phases listed in sequential order. Phase I delves into the design and building of the bioreactor system

based upon a packed bed reactor. Phase II discusses kinetic results obtained from the continuous synthesis of fructose-oleic acid esters. Phase III introduces a mathematical model developed to characterize the packed bed reactor and discusses its relevance to the Phase II results. The thesis culminates in Chapter 5 with a summary of the conclusions and recommendations for future work.

CHAPTER 2

BACKGROUND

2.1 - The Conventional Reaction Scheme for Saccharide-Fatty Acid Ester Production

The conventional method of producing saccharide-fatty acid esters has been through chemical production, which involves the use of an alkali to catalyze the reaction at high temperature ($\geq 180^{o}C$)[3]. Though this method of production has been used in the past, it has many drawbacks associated with it. For instance, the conventional reaction exhibits low selectivity. The effectiveness of alkali catalysts in energizing the production of the desired sugar ester product is very low and as a result, undesirable by-products, such as dehydration products, form. Some of these products may have allergenic or carcinogenic properties, and others promote discoloration in the product[3, 8].

The uncontrollable production of structural isomers is also another result of the low selectivity drawback and this is due to the presence of numerous hydroxyl groups on the fructose molecule. Some of these isomers are not easily biodegradable, defeating the main purpose of producing biological saccharide-fatty acid esters[3]. The conventional method of fructose-oleic acid ester production also carries the burden of the need for high energy consumption.

2.2 - The Enzymatic Reaction Scheme for Saccharide-Fatty Acid Ester Production

Over the last decade, due to rapid advances in the biotechnology field, there has been considerable interest in developing a biological-based method of producing sugar-

based surfactants. The use of biological catalysts, particularly enzymes, to catalyze the esterification reaction between the sugar and fatty acid moieties is now being researched worldwide.

2.2.1 - The Enzyme Lipase

The advantage of enzymes over traditional catalysts is that they have high regioselectivity and enantioselectivity, in addition to the fact that they can function under mild conditions such as low operating temperatures. Moreover, they are more environmentally-friendly than many conventional catalysts. Currently, nearly 4000 enzymes are known and about 200 of these are used for commercial purposes[9].

Lipases (E.C. 3.1.1.3) are the most common enzymes employed to catalyze the esterification reaction between sugars and fatty acids. Lipases are commonly referred to as triacylglycerol hydrolases because they catalyze the hydrolysis of acylglycerols. However, as mentioned in the previous section, given the appropriate nearly-anhydrous organic medium conditions, lipases can catalyze and promote esterification reactions.

Most of the lipases used in industry are produced by microorganisms and are obtained through isolation and screening techniques; often, they are expressed recombinantly in *E. coli*. Microbial lipases are more readily available than lipases from plants and animals and they exhibit interesting properties such as the ability to differentiate between enantiomers and high thermostability[10]. These lipase-producing microorganisms include fungi, bacteria, yeasts and actinomyces. For example, *Candida*

antartica lipases are obtained from thermophilic yeast and *Staphylococcus aureus* lipases are obtained from Gram-positive bacteria[11].

Many lipases are differentiated from common esterases by their display of the "interfacial activation" phenomenon[10, 11]. Typically, the active sites of lipases are buried under a hydrophobic lid. In a reaction occurring at or near a lipid-water interface, the phenomenon of interfacial activation is exhibited through the change in orientation of the hydrophobic lid; the lid opens by penetrating into the lipid phase. The end result is that lipase' active site becomes free, making it accessible to the substrates in the reaction[10].

Due to new and improved biotechnological applications such as genetic engineering, there are large quantities of certain lipases now available at very low cost. Lipases are environmentally-friendly catalysts that can be used safely in different manufacturing industries. A list of industrial applications of lipases is provided in Table 1[8].

Lipases obtained from many non-thermophilic organisms will denature when temperatures exceed 40^{o}C in aqueous media. However, by immobilizing lipases, their thermal stability often improves. Enzyme immobilization is the physical localization of an enzyme into a specific micro-environment, whereby an enzyme is physically attached to a solid. The technique helps improve storage and thermal stability of the enzyme and facilitates enzyme recovery; the process also enhances enzyme dispersion[12, 13]. The advantage of an immobilized lipase is the possibility of reusing the lipase, or, alternatively using it in a continuous process involving packed-bed reactors[14].

The immobilization process can be achieved either by engineering the microenvironment of the enzyme or the macro-environment.

Table 1: Industrial applications of microbial lipases[8]

Industry applications of microbial lipases		
Industry	**Action**	**Product or application**
Detergents	Hydrolysis of fats	Removal of oil stains from fabrics
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavoring agents in milk, cheese, and butter
Bakery foods	Flavor improvement	Shelf-life prolongation
Beverages	Improved aroma	Beverages
Food dressings	Quality improvement	Mayonnaise, dressings, and whippings
Health foods	Transesterification	Health foods
Meat and fish	Flavor improvement	Meat and fish products; fat removal
Fats and oils	Transesterification, hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono-, and diglycerides
Chemicals	Enantioselectivity, synthesis	Chiral building blocks, chemicals
Pharmaceuticals	Transesterification, hydrolysis	Specialty lipids, digestive aids, resolution of enantiomeric drugs
Cosmetics	Synthesis	Emulsifiers, moisturizers
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with improved quality
Cleaning	Hydrolysis	Removal of fats

The former could involve the process of attachment using methods such as a) carrier-binding or adsorption in which the lipases are physically bound to water-insoluble carriers and b) cross-linking, a method which involves intermolecular binding of lipases by bi- or multi-functional reagents. An alternative process is immobilization through containment in a barrier and that can be achieved through methods such as a) entrapment which involves incorporating enzymes into the lattices of a semi-permeable gel and b) micro-encapsulation within lipid vesicles. Macro-environment-type immobilization techniques usually involve precipitation methods in an organic solvent. Overall, the most common immobilization technique for lipase is adsorption because it is least expensive and least detrimental for the sustained lipase activity and selectivity[12].

A lipase's active site features a "catalytic triad" which allows it to act as an effective catalyst. This catalytic triad functions through use of hydrogen bonds through three amino acid residues; aspartic acid (Asp), histidine (His), and serine (Ser) (Figure

2.1)[15, 16]. The -OH nucleophile on the serine molecule attacks the carbonyl group of a fatty acyl donor, forming an acyl-enzyme intermediate through an ester bond. When the acyl-enzyme intermediate is nucleophilically attacked by the hydroxyl group of a saccharide, the partially positively-charged carbonyl group of the intermediate is linked with the slightly negatively charged oxygen of the hydroxyl group of the sugar and this mode of action yields a sugar ester. This catalytic mechanism for lipase is represented pictorially in Figure 2.2[17].

Like many other enzymes, lipase has a high regioselectivity and can differentiate between identical functional groups in a single substrate producing regioselective products[18].

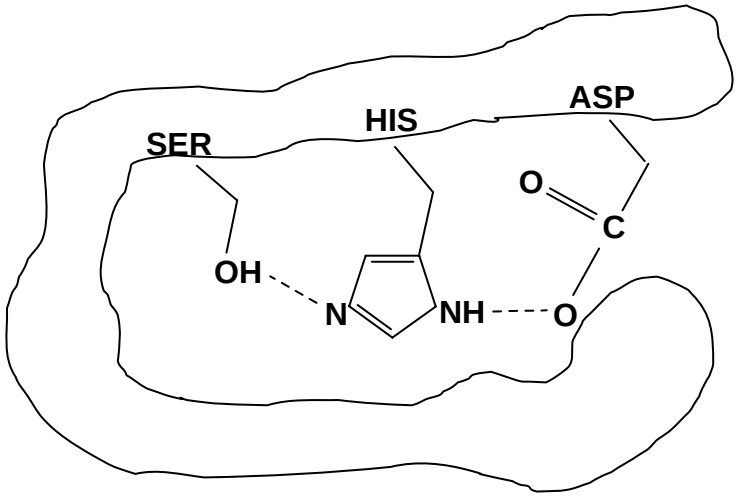

Figure 2.1: Structure of the catalytic triad of lipase[15].

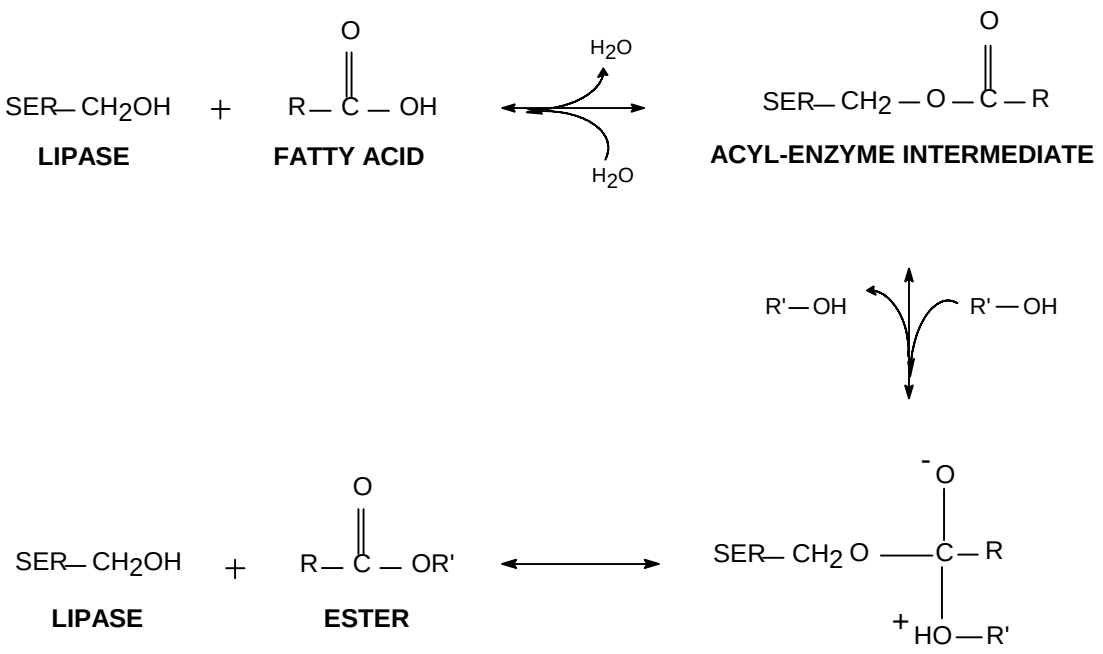

Figure 2.2: Catalytic mechanism of lipase for the formation of sugar esters. R represents an alkyl group on the fatty acid and R' is the remainder of the polyol molecule[17].

Hence, if used, there will be no need for extensive regioselective protection and subsequent de-protection in esterification reactions. The presence of enzymes allows the direct use of unmodified substrates, meaning fatty acids and sugars can be directly added to the reaction medium without need of protective group chemistry. Lipase catalysis does not demand high temperature conditions thereby making the production cost lower when compared to the chemical production method. A huge benefit of using lipase to catalyze the saccharide-fatty acid esterification reactions is the choice given to the manufacturer to control the regioselectivity of the reaction by properly selecting a lipase[19]. The saccharide ester products produced from the lipase biocatalysis are digestible, absorbable, non-ionic and most importantly biodegradable[5].

2.2.2 – Lipase Biocatalysis in Organic Media

Just like any other catalyst, lipases increase the rate of the reaction by reducing the activation energy over the time scale of the reaction without affecting the chemical equilibrium. Generally, lipases function in aqueous media; however, over the last 25 years, it has been discovered that they can function in the presence of many different organic solvents. Lipases are naturally designed to catalyze the hydrolysis of ester bonds in the presence of aqueous media as it is essential for there to be water in the lipase's microenvironment for it to function. On the other hand, when lipases are introduced into a non-aqueous or organic media, the reduction in water content in the environment brings about a reversal in the equilibrium conversion of the reaction. The synthesis of ester bonds is rather observed in a process known as esterification, as a result of the microaqueous environment[20]. The presence of the organic solvent also helps depress side reactions that might be water-dependent[21].

For the past decade, the employment of enzymes in non-aqueous media, in particular organic solvents, has become more common in the biotechnology sector. The advantages harnessed from using enzymes in organic media include a) enhanced solubility of hydrophobic substrates and products, b) reduction of enzymatic hydrolysis, c) alteration of enzymatic substrate regio- and stereoselectivity, d) enhanced thermostability, e) simplified post-reaction recovery of protein and products and f) reduction of microbial contamination[13].

2.2.3 – Water Control in Lipase-Catalyzed Esterification Reactions

Even though the presence of organic solvents shifts the thermodynamic equilibrium from a hydrolysis reaction to an esterification, the production of the water as one of the products of esterification can reverse the reaction's direction if it accumulates in the reaction media. Lower water content will highly favor an esterification reaction and increase the fatty acid conversion; hence, it is important to control the removal of water.

There have been reported techniques of controlling water activity in enzymatic esterification. Methods include solid salt hydrates[22], pervaporation[23] and evaporation[24]. In some experiments, the water has been removed from organic solvents using adsorbents such as alumina, silica gel, zeolites and ion exchange resins. Some of these techniques have been applied to esterification reactions involving saccharides and fatty acids. In previous research conducted in D.G. Hayes laboratory, *Zhang* and *Hayes* conducted esterification reactions involving fructose and oleic acids and they were faced with water accumulation issues due to the hygroscopicity of fructose[17]. The group attempted to control the water activity (a_W) of the reaction media by first reducing the a_W of fructose prior to its addition to reaction mixture; they accomplished this by storing the fructose over LiCl which is a low water activity agent. Unfortunately, they discovered that the water content of fructose ironically increased. *Dang* and *Hayes* also conducted similar esterification reactions but in their research; the group subjected the reaction media to vacuum pressure in an attempt to reduce the water content. The results showed that the water content was reduced from 1.5 to 1.0 wt. % leading to increase of about 1-2% in ester conversion[25, 26].

2.3 – Considerations for Large-Scale Production of Saccharide-Fatty Acid Esters

2.3.1 - Batch Synthesis Reaction of Lipase-Catalyzed Saccharide-Fatty Acid Esters

Most of the research that has been conducted for the enzymatic synthesis of saccharide-fatty acid esters has been done in batch reaction mode. Batch reactions involve the mixing of all the reactants required for a particular reaction together in a mixer-type reactor from the start of the reaction to the finish when the desired conversion is achieved. There is not any form of continuous input of the feed or withdrawal of product during the course of reaction. This method of reaction is beneficial if a company or a producer wants to make amounts of surfactants one batch at a time with no large demands of products in question.

Though the batch mode of reaction has indispensable advantages of its own, it is not an optimum option if the same product is to be made in large volumes. This is because the level of inconsistency in different batches is much higher in the batch mode than in the continuous mode; this may be due to changes sourcing from slightly different reaction conditions, equipment aging or contamination[27-29].

The different types of reactors used for the batch reaction mode require emptying and cleaning after every reaction batch is completed; this is done to prevent contamination. The constant repetitive emptying and cleaning tends to be highly inefficient in terms of time and income[27]. The requirement to centrifuge and filter the finished product of all the entire amount of enzymes used to catalyze the reaction, on a large scale increases the complexity, time and expense of the process[29].

Another disadvantage associated with the batch reaction mode is the loss of lipase when the enzyme is recovered from one batch, purified and re-used for another batch; over long term, this can prove to be costly. Other well-documented limitations of the batch mode include high labor costs and frequent start-up and shutdown procedures of the reactors[29].

Industries which are considering the enzymatic synthesis of saccharide-fatty acid esters demand a reaction mode which will generate a continuous reaction mechanism and products that do not require excessive post production and purification steps. A continuous reaction mode is the most preferable method for the production of saccharide-fatty acid esters when it comes to large-scale production.

2.3.2 – Continuous Synthesis of Saccharide-Fatty Acid Esters

The continuous synthesis mode of reaction can be described as a method in which the feed and product streams are continuously fed and withdrawn from the reaction system, respectively, at steady state. This reaction method could involve the use of any reactor designed to account for the continuous feed injection and product collection. Continuous synthesis of polyol-fatty acid esters has recently received a lot of interest and attention from the biological surfactant industry due to some of the advantages demonstrated in other industries. One of the advantages is the yield of a very high biocatalyst-substrate ratio which leads to minimization of reaction times[27]; this is a major advantage over the batch mode as production rates and efficiencies are increased. The continuous reaction mode also provides better catalyst or enzyme protection and reduces

the potential for mechanical damage. The continuous synthesis process is mostly preferred because it generates large amounts of a product with minimum manual labor and the products typically tend to be of similar quality throughout the process, even when different batches are made.

One of the goals of this thesis is to build a packed bed reactor-based system for the synthesis of fructose-oleic acid esters implementing a technique of continuous re-circulation of the reaction medium; a synthesis design which will serve as a model for the synthesis of other saccharide-fatty acid esters. The continuous re-circulation of a reaction medium through a reactor is a characteristic feature of a fed-batch reactor design and this design was employed as a preliminary step for the proposed continuous synthesis mode work that will be conducted in the future.

An addendum to this goal was the task of mathematically modeling the kinetic reaction mode and this was accomplished by creating a mass balance of the packed bed reactor and determining the amount of esters produced in the reactor.

2.4 – Literature Review: Lipase-Catalyzed Synthesis of Saccharide-Fatty Acid Esters

2.4.1 – Continuous Synthesis of Polyol-Fatty Acid Esters in Different Reactors

Through diverse research that has been conducted, there are many different types of reactors that have been designed for the continuous synthesis of different types of lipase-catalyzed polyol-fatty acid esterification reactions; including packed bed (PBR), membrane, hollow-fiber and the like.

One of the most common reactor designs that have been used for continuous synthesis of polyol fatty acid esters is the PBR. *Arcos et al* conducted a series of esterification reactions involving glycerol and unsaturated fatty acids with an immobilized *Rhizomucor miehei* lipase (RML) as a catalyst using a PBR[30]. The dry immobilized enzyme was manually packed into a tygon tube and retained in the tubing with glass wool. The substrates, glycerol and unsaturated fatty acids such as conjugated linoleic acid and oleic acid, were transported individually using syringe pumps and were pre-mixed via a Y-connector prior to entering the packed bed reactor. Results from the experiment proved that the continuous synthesis mode was highly effective.

The group was able to determine that at a temperature of 50^{o}C, a fluid residence time of 1 hr, and a starting molar ratio of fatty acid to glycerol of 0.33 led to 90% product conversion. They also observed that the reactor types allowed for a high sustainability of immobilized enzyme activity which in turn permitted the continuous operation of the reactor for 12 days. The accumulation of the product water was minimized by using high amounts of the glycerol in proportion to the fatty acid. The excess glycerol phase performed as a desiccant by removing the water produced, thus reducing its activity in the fatty acid-rich phase[30].

Khaled et al designed a fixed bed reactor system consisting of a 50 cm long double jacket column which was used to contain the immobilized 1,3-selective *Rhizomucor miehei* lipase (Lipozyme IM®, Novozymes Inc., Franklinton, NC), a reactant reservoir, a magnetic stirrer, a peristaltic pump and a temperature-controlled pump[31]. The double-jacket fixed bed enzymatic reactor was packed with 200g of Lipozyme IM® which was used for the lipase-catalyzed esterification reaction between

fructose (99% pure) and oleic acid (technical grade) to produce fructose oleate. Using 2-methy-2-butanol as a solvent and temperature of approximately 55^{o}C, the group obtained yields of more than 80% after effluent recycling and drying provided that the fructose : oleic acid molar ratio was maintained at 2:1[31].

Membrane bioreactors are also commonly employed for continuous synthesis of esters. They are designed with the goal of integrating the actions of catalytic conversions, product separation and catalyst recovery, all in one single operation. Recently, membrane bioreactors have been used in two-phase bioconversion-type reactions due to their abilities to provide an interfacial contact area and to act as an interfacial catalyst together with the enzyme[29]. For example, in the esterification of polyol-fatty acid esters, there is the presence of two liquid phases, the polyol and the fatty acid, or a liquid-and-solid phase, if the polyol is a liquid or solid respectively.

The continuous synthesis of glycerides in a micro-porous membrane bioreactor using lipase to catalyze the reaction was conducted by *Hoq et al* in hopes of overcoming the drawbacks associated with batch-wise synthesis in an emulsion-based media[27]. In their experiments, they used a one-unit plate dialyzer as a micro-porous membrane bioreactor which constituted two frames covered with highly hydrophobic polypropylene membranes possessing efficient porosity. The main substrates for the reaction were prepared into two co-current feeds fed on separate sides of the membrane; one consisting of only oleic acid and the other, a mixture of glycerol, water and *Chromobacterium viscosum* var. *paralipolyticum* lipase. At the start of the reaction, the glycerol solution was fed into the bioreactor at a high flow rate, after which the oleic acid was fed. The recycle flow rate of the glycerol-water-enzyme solution was then adjusted to constant

value of 10ml/hr for the duration of the experiment. The substrates were reacted in the bioreactor and the effluent containing the glycerides was then collected.

One of the advantages observed from the use of the micro-porous membrane bioreactor over the emulsion system were the elimination of the need for stirring. It was also observed that the glyceride products were produced in pure state without any contamination by another phase. The group discovered from the experiments that the problem of auto-oxidation of the fatty acid was eliminated with the use of the bioreactor for continuous synthesis. This was because the bioreactor has no free space to hold air inside of it when it was filled with the substrates.

It was also observed that it was easier to control the produced water content in the continuous synthesis mode than in an emulsion system. A column packed with molecular sieves was used for continuous dehydration of the glycerol solution prior to being mixed with the fatty acid. Some of the challenges faced by the researchers involved the relationship between fatty acid flow rate and product conversion; it was discovered that product conversion was lower with an increased flow rate due to shorter residence times. The group also discovered that the catalytic rate of conversion was very low for the system and further research was recommended.

Watanabe et al conducted the enzyme-catalyzed continuous production of acylated mannoses using a tubular reactor packed with immobilized lipase[32]. The reactor system consisted of a column packed with the lipase and a delivery pump to transport the substrate through the reactor system. Mannose was dissolved with one of several different fatty acids tested (oleic acid, lauric acid, etc) using 2-methyl-2-propanol in a substrate reservoir connected to the system. In order to improve the miscibility of

mannose and the fatty acids, each of the substrates were dissolved in nearly-anhydrous acetronitrile before being fed to the substrate reservoir. All of these components were installed in a thermo-regulated chamber which was maintained at 50^{o}C and the accumulation of water was removed by rotary evaporation. They found that the continuous production of acyl mannoses using different types of fatty acids could be maintained for 3 weeks at a productivity of 350 $g\ L_{reactor}^{-1}\ day^{-1}$.

A similar tubular reactor was tested for use in the continuous synthesis of lauroyl and oleoyl erythritol using an immobilized lipase packed in a stainless steel column. In this particular research, the substrate reservoir was only filled with the fatty acids which in this case were different purity grades of oleic acid and lauric acid. The other substrate, erythritol powders, was packed into another stainless steel column which was connected in series and upstream to the packed column containing the lipase; the system was contained in a temperature-controlled oven. A pump was used to transport the fatty acid from the reservoir through the erythritol column, through the lipase-packed column/reactor for reaction and then into the effluent reservoir. In this research, the reactor system was continuously operated stably for a period of 2 weeks; a different fatty acid was used each week. It was concluded from the results, that in a 7-day continuous synthesis period, the productivity for monolauroyl and monooleoyl erythritol was approximately 1.25 $kg\ L_{reactor}^{-1}\ day^{-1}$and 1.36 $kg\ L_{reactor}^{-1}\ day^{-1}$, respectively[33].

Generally, there has been a number of investigations that has involved the design and assembling of different types of reactor systems for the continuous synthesis of different polyol-fatty acid esters. However, there is no documented research of a design

of a packed-bed bioreactor system that was used for the synthesis of saccharide-fatty acid esters using a continuous medium re-circulation approach and the absence of solvent.

2.5 – Summary of Batch Synthesis of Saccharide-Fatty Acid Esters

As mentioned earlier, this research study is a continuation of research work conducted by Xun Zhang and Hung Thai Dang. Zhang reported a 50 % conversion of oleic acid into esters starting out with a 2:1 oleic acid : fructose mole ratio of substrates. Dang attempted to attain a higher conversion using a 1:1 oleic acid : fructose mole ratio and obtained a 80% conversion of oleic acid.

A 1:1 stoichiometric mixture of oleic acid and fructose was prepared and reacted together in a reaction vial in the presence of tertiary butyl alcohol (tBuOH). Fructose was then added to the reaction medium at saturation levels using a stepwise-addition method throughout the entire course of the reaction. The mixture was stirred at 400 revolutions per minute (rpm) on a hot plate to attain a reaction media temperature of 65^{o}C. Lipozyme IM®, lipase from *Rhizomucor miehei* immobilized onto macroporous anionic resin beads were purchased from Novozymes, Inc. (Franklinton, NC) and was added to catalyze the esterification reaction. Aliquots were periodically taken out for high pressure liquid chromatography (HPLC) analysis and the reaction was carried out until the maximum product conversion was attained.

The presence of tBuOH at the start of the reaction acted as an aid to increase miscibility of the hydrophilic fructose in the hydrophobic oleic acid. As the reaction proceeded, the two primary hydroxyl groups of fructose at positions 1 and 6 of the

molecule were esterified, with a strong preference to the 6-hydroxyl group, yielding mono- and di-esters (ME and DE) and the presence of these esters improved the fructose solubility during the reaction[34]. tBuOH was completely removed after a few hours into the reaction via free evaporation and accumulated water produced by the reaction was also removed through free evaporation.

The research study conducted by Dang focused on optimizing the batch synthesis of lipase-catalyzed esterification of fructose-oleic acid and deriving a kinetic model. Fructose solubility was a crucial matter to address in the research study as this parameter affected the reaction rate and product conversion. It was discovered that the replacement of crystalline fructose with amorphous fructose yielded a higher conversion and reaction rate; however amorphous fructose was difficult to work with and opted for crystalline fructose. The group hence proceeded to compare the benefits of the method of incremental step-wise addition of crystalline D-fructose to the reaction media to that of the method of total sugar content addition at the start of the reaction. Hung discovered that the stepwise addition method yielded a higher product conversion and reaction rate.

A ternary phase diagram for the system consisting of D-fructose, oleic acid and fructose-oleic acid esters was created to aid in predicting fructose solubility at 60^{o}C. Figure 2.3 shows the complete phase diagram. It can be observed that the solubility of soluble fructose increases as the monoester (ME) and oleic acid (OA) mass ratio increases.

The effect of water content on product conversion was investigated by Dung. It was discovered that the removal of water from the reaction medium before reaction initiation did not increase the rate or the extent of reaction. Further tests were

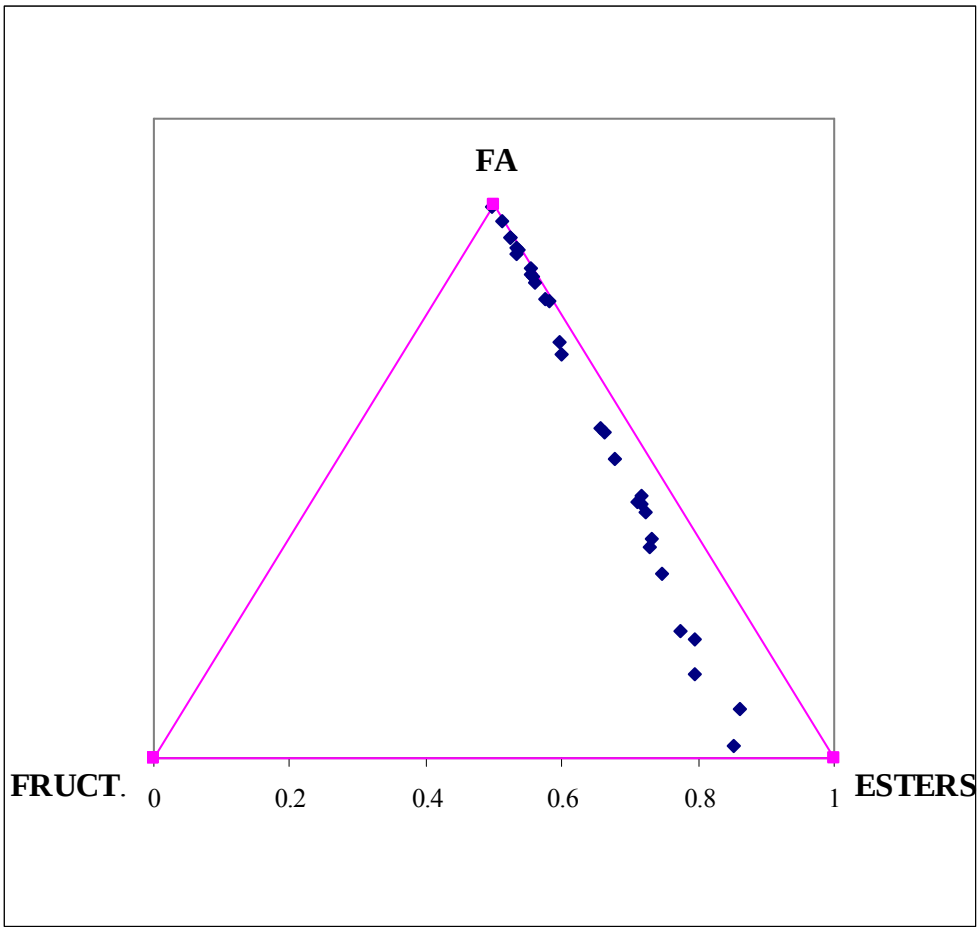

Figure 2.3: A complete triangular phase diagram for D-fructose, OA, and ME at 60°C[25]

conducted to determine if the removal of water from the media during the course of reaction would increase the 80% product conversion that had already been achieved. Through evaporation, the water content was decreased from 1.6 to 1.0 wt%; however, there was only a 2% increase in conversion. Hence, he concluded that the limitation for full product conversion was thermodynamic-equilibrium-related and it was essential to conduct further research into the effect of water content on equilibrium.

Kinetic modeling was another major aspect of the research study conducted by Dang. The Ping-Pong Bi-Bi model was applied to the lipase-catalyzed esterification

between fructose and oleic acid. By studying initial reaction involving a series of kinetic experiments performed in tBuOH-rich media, the kinetic parameters were determined and was applied to the batch synthesis of fructose-oleic acid esters.

With the success of Hung Dang's work, additional research studies were conducted by the author of this thesis to investigate different aspects of the lipase-catalyzed batch synthesis process. Specifically, the activity retention rate of the RML and the effect of water content on the equilibrium conversion of fructose-oleic acid esterification were investigated.

CHAPTER 3

BATCH SYNTHESIS OF SACCHARIDE-FATTY ACID ESTERS: ACTIVITY RETENTION, EFFECT OF WATER ON EQUILIBRIA, AND EFFECT OF ACYL ACCEPTOR TYPE

3.1 - Enzyme Activity Retention of the Immobilized Lipase during Successive Batch Runs of Lipase-Catalyzed Esterification of Fructose and Oleic Acid

One of the major characteristics of immobilized lipase is their activity retention capability. Lipase activity has been extensively studied and it has been found that lipases rarely lose their catalytic activity abilities during a time course of reaction under proper conditions. This factor contributes to the effective and profitable advantages of using immobilized lipases.

It was hypothesized that the activity of immobilized *Rhizomucor miehei* lipase (RML) in the batch synthesis of fructose-oleic acid esters would be retained during the time course of reaction. In order to investigate this hypothesis, a series of batch-mode esterification experiments were designed, which involved the re-use of the same enzyme used in the initial reaction medium. The maximum velocity of each of the successive reactions is then compared to the initial reaction in which the fresh lipase was used. If there was no change or an increase in the ratio of maximum velocities of the last two batch reaction to that of the first batch, then it can be concluded that there was no loss of activity.

3.1.1 – Method and Materials

The materials used for the lipase-catalyzed batch esterification of the fructose-oleic acid esters included the following from their respective sources. Lipozyme IM®, lipase from *Rhizomucor miehei* immobilized onto macroporous anionic resin beads, was purchased from Novozymes, Inc. (Franklinton, NC). D-fructose, > 99 % pure tBuOH, glacial acetic acid, and HPLC grade methanol, acetone, and acetronitrile of high purity were purchased from Fisher Scientific (Pittsburgh, PA) and used without further purification. Technical grade oleic acid (98% pure, determined by HPLC) and RML were purchased from Sigma Aldrich (St Louis, MO).

A reaction medium containing 70 wt% fructose oleate/30 wt% oleic acid mixture was prepared. The rationale for this choice of reaction medium was to ensure a high solubility of the fructose that was added. The reaction media was saturated with crystalline fructose by adding an amount that corresponded to the phase diagram (Figure 2.3). The reaction was carried out under normal atmospheric conditions over a hot plate and stirred at 400rpm to achieve thorough miscibility of the individual components. After the medium achieved a temperature of 65^{o}C, 0.5 g of immobilized RML was added. The medium was frequently re-saturated with fructose during the time course of reaction by decanting away the lipase-free medium, adding sugar to that medium to the point of saturation, removing any excess sugar by centrifugation, and re-adding the lipase into the medium. The reaction vial was left open in order to allow the evaporation of water that accumulated as a co-product. Aliquots of the reaction media was frequently retrieved to analyze its composition using HPLC analysis.

Upon achievement of approximately 90% conversion of the oleyl acyl groups, the RML was filtered out of medium, washed with acetone and dried in a temperature-regulated oven at 65^{o}C. The RML was the added to the successive batch medium prepared similarly to the initial batch medium and heated to a temperature of 65^{o}C. Three successive batch reactions were conducted. For the last two batches, small amounts of immobilized RML were lost during enzyme recovery. Even though a total amount of 0.5g was used in the initial reaction medium, only 0.35g and 0.3g was used for batch 2 and batch 3 respectively.

HPLC Analysis:

HPLC analysis used for identification of the different compounds in a product mixture in order to calculate product conversion was conducted using an analytical reverse phase C_{18}-stationary phase column from W.R. Grace (Deerfield, IL). The conditions for the HPLC system included a 1ml/min flow rate of the mobile phase (45:45:10 v/v/v acetone:acetronitrile:acetic acid), a column incubation temperature of 25^{o}C and the use of an evaporative light scattering detector (ELSD, model Mark III, from AllTech) operated at 84^{o}C drift tube temperature and 2.35 bar of nitrogen pressure. The HPLC system consists of an SD-200 dual-pump analytical system from Rainin Instruments (Woburn, MA). The Star Workstation Version 6.0 from Varian (Walnut Creek, CA) was used as a data acquisition system. Products were identified using standards. Response factor calibrations were determined for all the substrates and products and were used to calculate the concentration results obtained from the ELSD.

3.1.2 – Results and Discussion

Three successive batch reactions were conducted using the same immobilized RML in each stage. From HPLC analysis, the mass ratios of the esters produced were determined and plots of the product conversion were created for each batch (Figure 3.1). The maximum velocity (V_{MAX}) of each of the reaction was calculated and the V_{MAX} of the last two batch reactions were compared to that of the first batch reaction. It was discovered that the time course of the esterification reaction in three successive reactions was linear up to approximately 90% conversion (Figure 3.1). Table 2 lists the results that were obtained from the analysis.

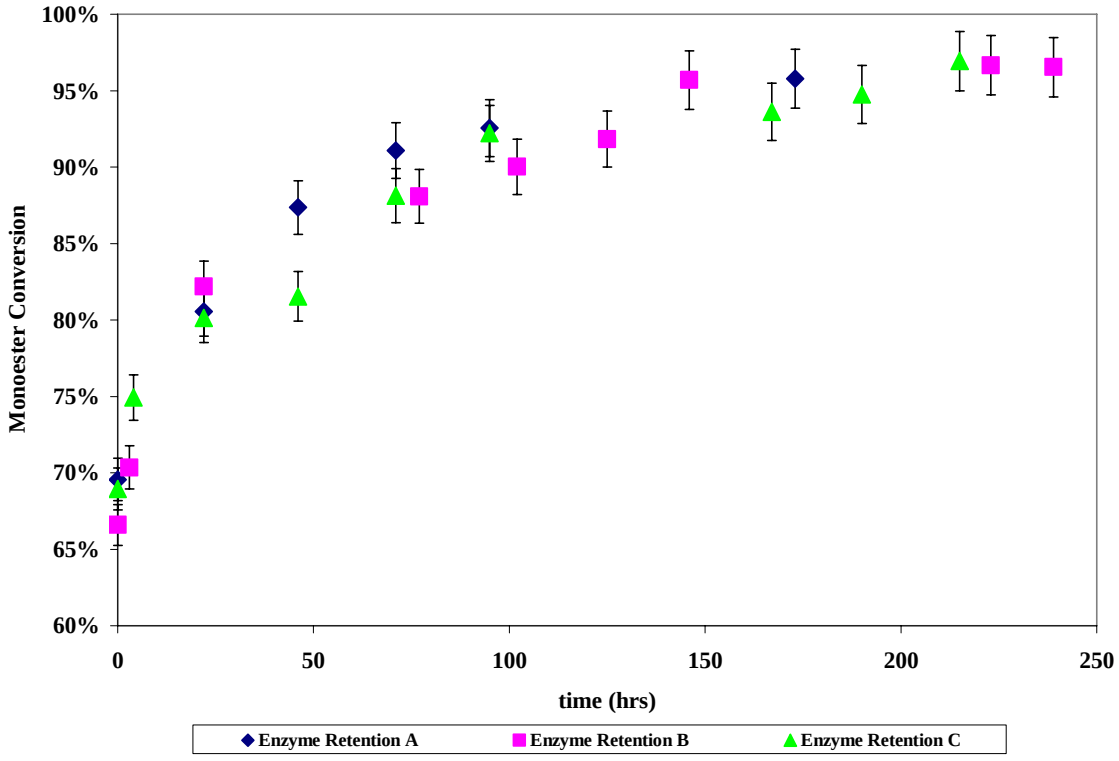

Figure 3.1 – Plot demonstrating the activity retention of immobilized *Rhizomucor miehei* lipase in three successive batch reactions, A, B and C.

Table 2: Enzyme activity retention results of immobilized RML

Batch Reaction	$V_{MAX,n}/V_{MAX,A}$*	Reaction Time, hours (days)
A	-	173 (7.2)
B	1.11 ± 0.25	239 (10.0)
C	1.21 ± 0.24	173 (7.2)
TOTAL		585 (24.4)

3.1.3 – Conclusions

From the results, it was determined that the immobilized RML did not lose activity after being subjected to approximately 24 days of reaction in three successive batch media. It was noticed that the activity increased slightly in batches *B* and *C* in comparison to batch *A* and it can speculated that immobilized RML is probably energized to its full activity level after being used in subsequent reactions. As mentioned earlier, during the recovery and reuse of RML between successive batches, small amounts of immobilized RML was lost; it can be argued that without the loss of these small amounts, a much higher relative activity could have been obtained for the two successive batches.

The excellent activity retention ability of lipases is one of the advantages that make lipases an efficient catalyst and this experiment is an additional proof to this well-documented characteristic.

3.2 - Effect of Water Content on the Equilibrium Conversion of Fructose-Oleic Acid Esterification

As mentioned previously, a 100 % conversion was never obtained in the esterification reaction between the sugar fructose and the oleic acid when a 1:1

* Error limits calculated based on 95% confidence interval using t-distribution

stoichiometric feed was used. It was initially proposed that the accumulation of water co-product in the reaction medium was the source of limitation for the full conversion.

Previous work demonstrated that the removal of water from the reaction medium before initiation of the reaction neither increased the rate nor the extent of reaction by any significant amount[25, 35, 36]. It was hypothesized that the limitation for the conversion at 80-90% was due to thermodynamic equilibrium and the effect of water content on equilibrium was further examined. The goal of this experiment was to investigate the effect of water accumulation on equilibrium-limited conversion. In order to address this issue, three different batch esterification of fructose oleates were run in parallel over different water activity-controlled environments.

Water activity is a fundamental principle derived from thermodynamics and physical chemistry; the term "water activity" describes the equilibrium amount of water available for the hydration of materials. The relationship between the amount of water and water activity is non-linear and can only be explained using isotherms. The water activity (a_W) value of pure water is 1.00 and this value decreases with the addition of salts to water[37]. Saturated salt solutions of NaCl, $BaCl_2$ and K_2SO_4 have high water activities close to $a_W = 1.00$, whilst others containing LiCl, CH_3COOK and $CaSO_4$ (which commonly used in dessicants), have low a_W values. Saturated salt solutions have the advantage of maintaining a constant humidity provided the amount of salt present is above the saturation level.

The experiments were designed to investigate and compare product conversion in three different environments: a low water activity-controlled environment, an environment in which the reaction was exposed to normal room temperature and open to

the atmosphere, and a high water activity-controlled environment. The water activity-controlled esterification reactions were operated within closed reaction systems exposed to different environments.

3.2.1 – Method and Materials

Three different conversion mixtures were prepared, each containing approximately 70wt% monoester – 30wt% oleic acid in separate reaction vials and each reaction medium was saturated with fructose using the ternary phase diagram (Figure 2.3) as a guide. The reaction media were stirred at 400 rpm and heated to a temperature of 65^{o}C after which, 0.3 grams of RML was added to each of the three prepared initial media. One of the reaction media-containing vials was placed in a desiccator and was surrounded with Drierite® anhydrous $CaSO_4$; a dessicant containing 97% $CaSO_4$ and 3% $CoCl_2$ manufactured by W.A. Hammond Company Ltd (Xenia, OH); the water activity of Drierite anhydrous $CaSO_4$ was measured and was recorded at $a_W < 0.022$. The second media was conducted in the open, under a normal room temperature of 24^{o}C and a room equilibrium relative humidity of 31.1%, to serve as a control for the experiment. The third reaction vial was placed in a desiccator and surrounded with a slurry of water and NaCl crystals, which has an $a_W = 0.752$, thereby creating a high water activity environment. The water activity of the Drierite® anhydrous $CaSO_4$ and the aqueous NaCl solution were measured using a Decagon AquaLab 3TE, a water activity measurement equipment purchased from EminTech Co. (Helsingborg, Sweden). The heating and stirring of each of the reaction media was continued during the time course of reaction.

The reaction media were run in parallel at a temperature of 60°C with the continuous addition of fructose in small batch increments to maintain the saturation level over total time course of 173 hrs. Aliquots of the reaction media were taken out separately from each of the three reactions to analyze the conversion of products via HPLC analysis. The values of the concentration-based equilibrium constants, K_c, were calculated using the following equation:

$$K_c = [ME]_{eq} * [H_2O]_{eq} * [S]_{eq}^{-1} * -[FA]_{eq}^{-1} \quad \textbf{(3.1)}$$

In order to determine the K_c, the concentrations of fructose, water, oleic acid and the monoesters produced in the reaction media were measured through HPLC analysis (Section 3.1.1). The water content was measured by the Karl Fischer titration technique using a Model 270 Multi-Mode Meter and Titration Controller from Denver Instruments (Denver, CO). Karl Fischer titration is a widely-used analytical method for quantifying water content in various non-aqueous solutions. The principle is based on the Bunsen Reaction between iodine and sulfur in an aqueous medium was applied to a non-aqueous system by adding methanol as a solvent and pyridine or primary amines as a buffering agent[38]. The water content measurement was accomplished by mixing aliquots of the reaction medium in methanol and injecting a 100µl sample into the Karl Fischer titrator. Error limits for K_c values were calculated based on a 95% confidence interval using t-distribution.

3.2.2 – Results and Discussion

The water activity for each created environment was measured and the product conversion and equilibrium constant for each reaction was calculated. The results are listed in Table 3. From the results obtained, it was observed that when the reaction took place in an environment with a low water activity, a higher product conversion (~ 93.1%) was achieved, whilst the product conversion for the reaction in the presence of a high water activity environment was only 84%. It was determined that a decrease in the water activity led to a slight increase in the percent product conversion.

3.2.3 – Conclusions

As discussed earlier in this thesis the extent of hydrolysis catalyzed by lipase is reduced when the microenvironment contains minimal amounts of water. Based on the hypothesis introduced for this particular experiment, the results prove that the presence of water limited the thermodynamic equilibrium-controlled conversion of the reaction. Usually in the lab, through the creation of a nearly-anhydrous environment, the batch synthesis of fructose oleates generates a conversion of approximately 90%.

Table 3: Results for effect of water content on equilibrium product conversion

Agent used to control water activity (a_W)	a_W @ 24°C	% Monoester Conversion	Kc*
$CaSO_4$	<0.022	93.1 ± 6.7	4.2 ± 0.2
Control	0.311	89.9 ± 1.0	4.5 ± 0.2
NaCl	0.752	84.8 ± 4.8	6.1 ± 0.2

* Error limits calculated based on 95% confidence interval using t-distribution

It can also be observed that the K_c value for the there different experiments varied slightly, with the highest Kc vale occurring for the experiment that employed the highest a_W value (Table 3). The variation in the Kc value could be due to changes in changes in experimental conditions and parameters that were not accounted for; it is important to conduct further research to investigate the effect of these conditions. The values obtained are on order of magnitude higher than values reported for octyl glucoside octanoic acid ester synthesis in acetronitrile ($K_c = 0.55$) and glucose-lauric acid synthesis in 2-methyl-2-butanol ($K_c = 0.30$)[39].

It is concluded that the water content has an effect on the reaction's overall conversion, as predicted by thermodynamic equilibrium.

3.3 – Effect of Acyl Acceptor in Batch Synthesis of Other Lipase-Catalyzed Saccharide Oleic Acid Esters

The batch conversion of other types of saccharides and oleic acid into esters was a point of interest. Research was conducted involving sucrose, glucose and xylose as acyl acceptors. Sucrose is a disaccharide consisting of glucose and fructose monomeric units joined together by an a-1,2 glycosidic bond with the chemical formula $C_{12}H_{22}O_{11}$. Both glucose ($C_6H_{12}O_6$) and xylose ($C_5H_{10}O_5$) are monosaccharides just like fructose, with xylose differing from the others as it is a pentose sugar with the others being hexoses. Figures 3.2(a-d) illustrates the chemical structures of sucrose, fructose, xylose and glucose.

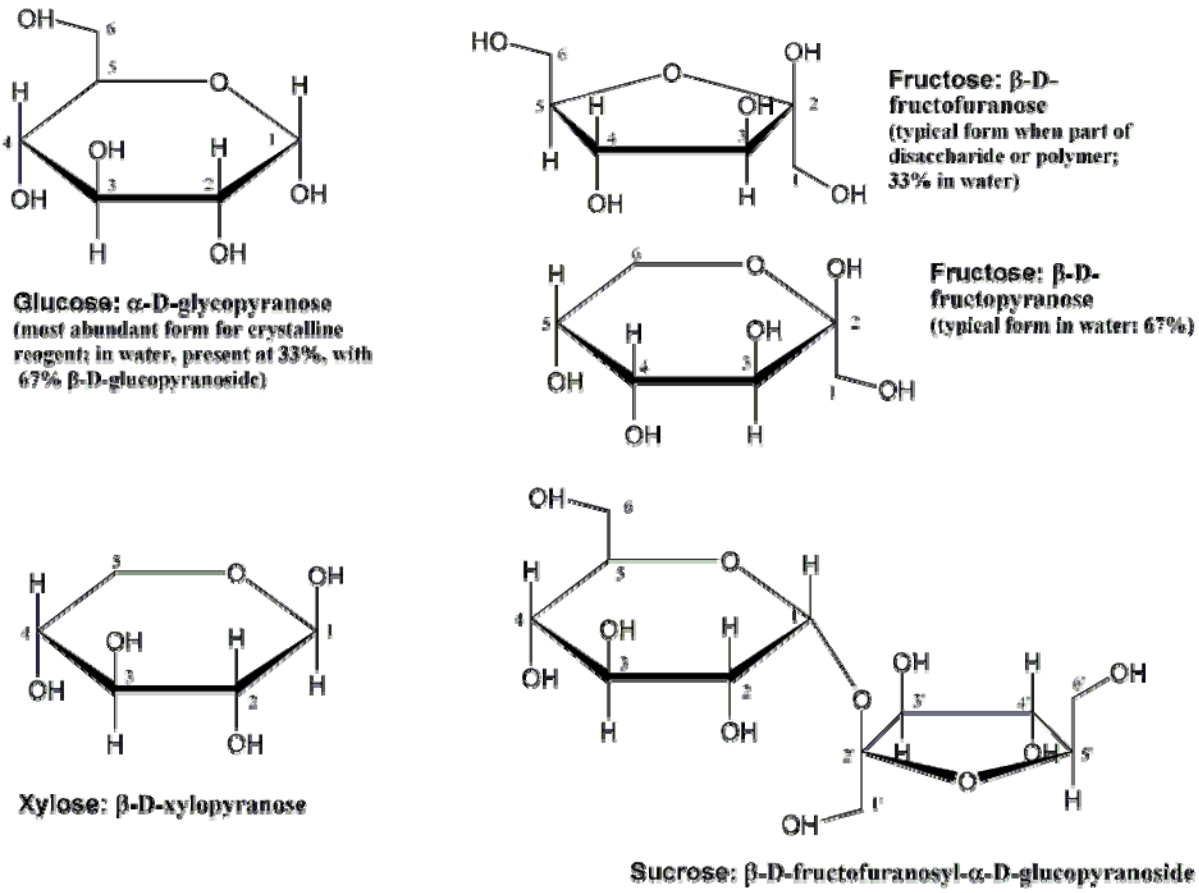

Figure 3.2: Chemical structures of sucrose, fructose, xylose and glucose

3.3.1 – Materials and Methods

Sucrose, D-xylose and D-glucose (≥ 99% pure) were purchased from Sigma-Aldrich (St. Louis, MO) and other materials used are described in Section 3.1.1. The reaction media for each of the batch synthesis of the saccharides-oleic acid esters were prepared similarly to that described for the batch synthesis of fructose-oleic acid esters in Section 3.1.1 of this thesis. The media was constantly saturated with the respective saccharide using the same procedure described in Section 3.1.1 and was continuously stirred at a rate of 400 rpm. Samples were collected for HPLC analysis (Section 3.1.1) to determine the conversion of oleic acid into the various saccharide oleic acid esters.

3.3.2 – Results and Discussion

The results demonstrate an 87% conversion was obtained for sucrose oleic acid monoesters or oleate, 78% conversion for glucose oleate and 72% conversion for xylose oleate according to the time courses of reaction plotted in Figure 3.3. For sucrose, the 6'-OH position of the molecules was esterified to generate 75wt% ME and the 6'-OH and 1'-OH position to produce 11% of DE. In the case of glucose, 71 wt% of ME and 7% of DE was achieved and for xylose, 62 wt% of ME and 8 wt% of DE. It was observed that the product conversion for the xylose and glucose were somewhat lower than the conversion obtained from fructose. Both xylose and glucose are monosaccharides just like fructose and it would seem that their product conversion would be similar to that of fructose (> 80%); however, this discrepancy may be related to the differences in the

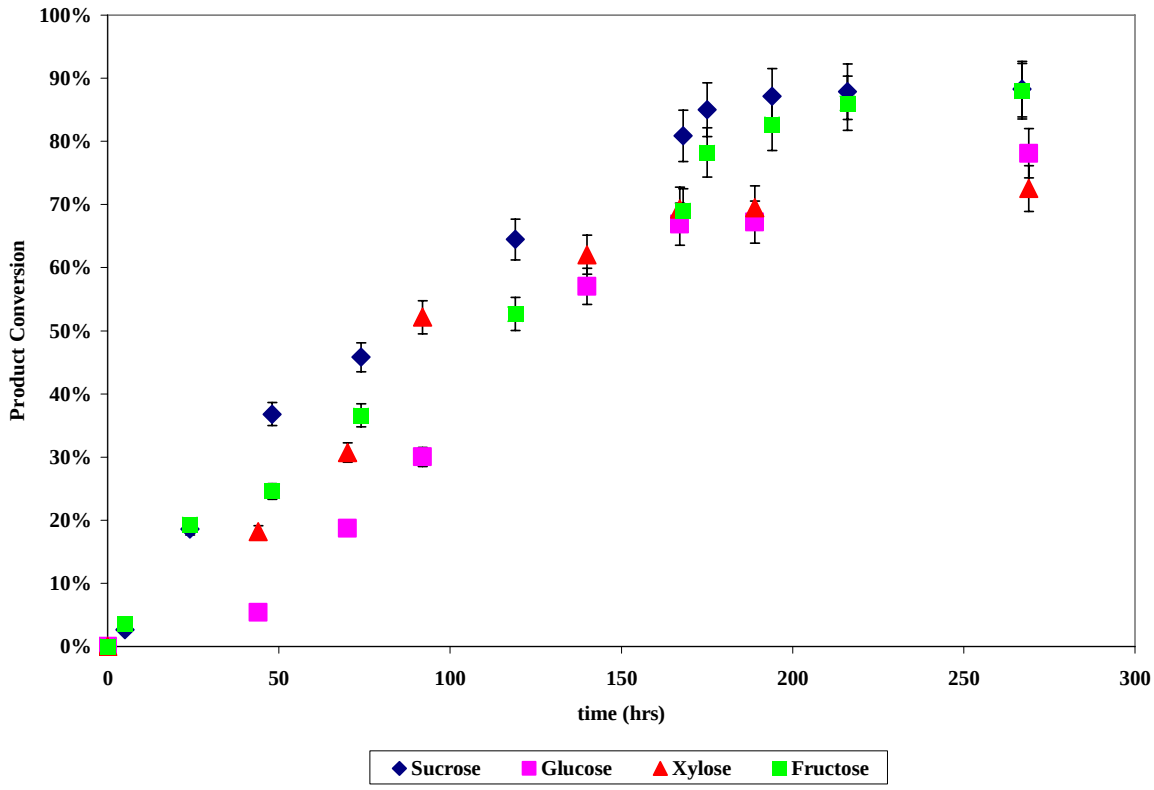

Figure 3.3 – Time course of reaction for the lipase-catalyzed synthesis of sugar oleates at 65°C

structure of the saccharide molecules and the respective solubility of each of the sugar molecules used. It may be possible that the availability and accessibility of the esterifiable –OH groups to the active site are limited for xylose and glucose as a result of their structure, compared to fructose.

Ikeda and Klibanov conducted experiments involving lipase-catalyzed acylation of different saccharides in hydrophobic solvents by complexation. The group reacted different sugars such as sucrose, lactose, xylose, fructose, glucose and mannose with phenylboronic acid and vinyl acrylate, catalyzing the reaction with lipase. In the results, they discovered that after 24 hrs of esterification, an 86% conversion was obtained for α-D-glucose, 10% for D-fructose, 0% for D-xylose and 31% for sucrose. The results led the group to conclude that the molecular size of the sugar was not a limiting factor in esterification of sugars. The group also concluded that some of the sugars such as fructose interacted slowly with the phenylboronic acid, hence the low conversion[40].

The dependence of esterification rate on similar sugars was also investigated by *Tsuzuki et al*. The group conducted different esterification reactions between sugars and palmitic acid, using n-hexane as a solvent at a temperature of 37^{o}C. Their results showed that after 72 hrs of incubation, the conversion of D-glucose was 92%, 44% for D-xylose, 89% for D-fructose and 81% for sucrose. They discovered that for glucose and sucrose, the 6'- OH group was the only primary hydroxyl group esterified whilst for xylose and fructose, the 1'-OH was the favorable choice[36].

Based on these results, it seems that D-xylose is not easily esterified. D-xylose is a 5-carbon ring monosaccharide that lacks a primary hydroxyl group when it exists in a pyranose (ringed) form[41], even though its secondary hydroxyl groups are available for

esterification. Immobilized RML is 1,3-selective and does not perform well in the catalyzed esterification of secondary –OH groups. When D-xylose is in linear form, the 5'-OH group is the primary hydroxyl group and usually in solution, there is the co-existence of both the linear and pyranose forms, although typically less than 1% of saccharides exist in their linear form. It is possible that the equilibrium position highly favors the existence of the linear form in the xylose oleate reaction medium and perhaps, the immobilized RML is active on the primary –OH groups present on the linear form, leading to a yield of 72% xylose monooleate conversion.

It was observed that glucose had a much lower conversion than the conversion for fructose oleates and this result does not agree with other published results such as the 86% conversion was obtained for α-D-glucose obtained by *Ikeda and Klibanov*[40]. This low yield may be related to the configuration of α- and β- anomers of the glucose molecule. *Halling et al* conducted experiments in order to determine the solubility of crystalline a-glucose and b-glucose in 2-methyl 2-butanol. Their results showed that the solubility of β-glucose was higher than that for α-glucose; the equilibrium ratio for crystalline β-glucose was 0.95 and that for crystalline α-glucose was 0.86[42]. Further experiments conducted by the group enabled to conclude that the equilibrium between α- and β- glucose anomers was highly complicated as dissolved α-glucose is known to mutarotate to its anomeric form β-glucose[42].

When drawn as a ring structure, a-D-glucose is has its 1'-OH group located below the plane of the ring, and *β*-D-glucose' is positioned above the ring; the presence of the OH group above the ring could block access to the primary 6'-OH group required for estrification. Hence, in regards to the relatively low conversion of glucose oleates, it is

possible that there was higher concentration of β-glucose as compared to α-glucose and may have contributed to limited conversion into glucose oleates. It is recommended that the experiment be repeated multiple times in order to rule out any experimental errors that might have occurred during this particular experiment.

Another reason for the incomplete conversion could be related to limited xylose and glucose solubilities in the oleic acid-ester reaction mixtures as compared to the solubility of fructose. There are no literature sources documenting the solubility of these saccharides in their own saccharide-oleate mixtures. It is important to conduct extensive research to explore the solubility of these sugars in their respective sugar-oleic acid ester mixtures and also in different organic solvents. It would also be advantageous to create ternary phase diagrams for each of the saccharides used and to further investigate the relationship between reaction rate and saccharide solubility.

The ~87% conversion obtained for sucrose oleic acid esters was acceptable, even though it can be argued that a much higher conversion was expected. This is because sucrose is a dissacharide with eight –OH groups with the 1'- and 6'- OH groups being the primary hydroxyl groups for esterification. As mentioned earlier from the results obtained by Tsuzuki et al, the 6'- position of sucrose was identified as the primary –OH group that was esterified in the esterification reaction with palmitic acid[36]. Hence it is possible that only one of the possible two primary hydroxyl group was esterified in the sucrose-oleic acid esterification reaction, thereby resulting in an 87% conversion. Another important contributing factor is that sucrose, being a dissacharide, perhaps may penetrate less effectively into the active site of lipases due to stearic hindrance, resulting in limited

conversion. The accumulation of water during the esterification of sucrose oleates could have also been a contributing factor to a less-than-perfect conversion.

3.3.3 – Conclusions

It was proved from the results that other saccharides can be reacted with oleic acid into sugar esters in a lipase-catalyzed esterification reaction. However, the product conversion and the reaction rate of each type of saccharide depend on the structure and solubility of the each sugar. It is important to develop saccharide oleic acid saccharide oleic acid ester tertiary phase diagrams for each of the saccharides.

CHAPTER 4

SYNTHESIS OF SACCHARIDE-FATTY ACID ESTERS USING A PACKED BED BIOREACTOR SYSTEM WITH CONTINUOUS RE-CIRCULATION OF REACTION MEDIUM

4.1 – Overview

The design of the packed bed bioreactor system and its employment for the synthesis of fructose-oleic acid esters with continuous re-circulation of reaction medium were the steps taken to investigate the effectiveness of this mode of synthesis for application to other saccharide-fatty acid esters. The fed-batch synthesis design was employed as a preliminary design for the proposed continuous synthesis mode work that will be conducted in the future. As mentioned earlier, the research study was divided into three phase; Phase I entailed the design and building of the bioreactor system, Phase II involved the operation of the continuous synthesis of lipase-catalyzed fructose-oleic acid esterification and obtaining the time course of reaction. Phase III consisted of deriving a mathematical model to characterize the packed bed reactor.

4.2 – PHASE I – Designing and Assembling the Bioreactor System

4.2.1 – The Packed Bed Bioreactor System Design

The proposed packed bed bioreactor system to be used for the continuous synthesis of the enzyme-catalyzed fructose-oleic acid monoesters consisted of the following components.

- Peristaltic Pump
- Adsorption column packed with fructose crystals
- Packed bed bioreactor containing immobilized RML
- Oven
- Flexible and heat-resistant tubing
- Feed/Recycle reservoir to supply and collect the feed and recycle stream respectively

4.2.2 – Components and Materials

The Pump

A BioLogic LP Peristaltic Pump purchased from Bio-Rad Laboratories (Hercules, CA) was used to control the flow of the feed mixture through the bioreactor system. It is a two-channel, bi-directional, variable speed pump which delivers flow rates from 0.05 to 40 ml/min. The features of the pump consisted of control panel keys and status LEDs for monitoring and controlling the system, a power switch and plumping connections to which inlet and outlet lines can be attached to. A schematic drawing of the pump is attached in **Figure 4.1**.

Plumbing the Pump System

In plumbing the pump system, only tubing with wall thickness less than 1 mm was used in order to avoid damage to the pump. All plumping connections were made using Luer fittings (**Figure 4.2**) that were purchased from Bio-Rad.

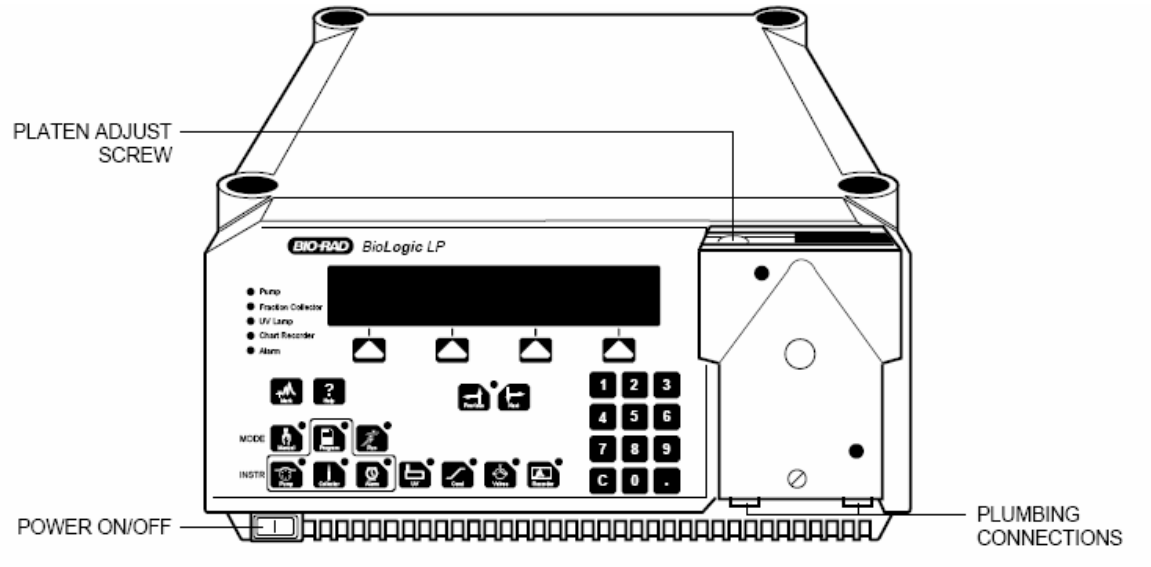

Figure 4.1: Schematic illustration of peristaltic pump[43]

	MALE LUER FOR SYSTEM TUBING CONNECTIONS		FEMALE LUER FOR PUMP TUBING
	MALE-TO-MALE CONNECTOR		FEMALE 'T' CONNECTOR

Figure 4.2: Examples of Luer fittings used for plumbing the pump[43]

Proper adjustment of the platen was an essential procedure that had to be completed before the peristaltic pump could be plumbed. In order to accomplish this task, the platen adjust screw was turned in the clockwise position until the end and then the screw was turned clockwise for 5 full turns for a tubing with an internal diameter size of 0.8 mm (1/32”).

The platen cam lever was then pulled away from the pump head to unlock the platen and to slide it away from the pump head frame. A lock-ring was slipped onto one end of the tubing and a barbed female Luer-fitting was inserted into the same end until the tubing reached the flange of the fitting. The Luer fitting was then clamped into place by sliding the lock-ring along the tubing over the barbed fitting. The procedure was repeated for the other end of the tubing. One end of the fittings was then inserted into one of the retaining brackets of the pump head and the tubing was pulled around the rollers in order to insert the other end fitting into the bracket at the other end. An illustration of the plumping procedure is shown in **Figure 4.3**.

The Columns

OmniFit® chromatography columns from Supelco, a subsidiary company of Sigma-Aldrich (St. Louis, MO), were used to contain the sugar and to hold the lipase, in the latter case, it acted as the packed bed reactor. The columns were made of borosilicate glass and consisted of two adjustable endpieces made of polytetrafluoroethylene (PTFE) fitted with 10μm frits. The unique design of the OmniFit® chromatography columns provided the following desirable properties: pressure-rating to 40 bar (600 psi), solvent

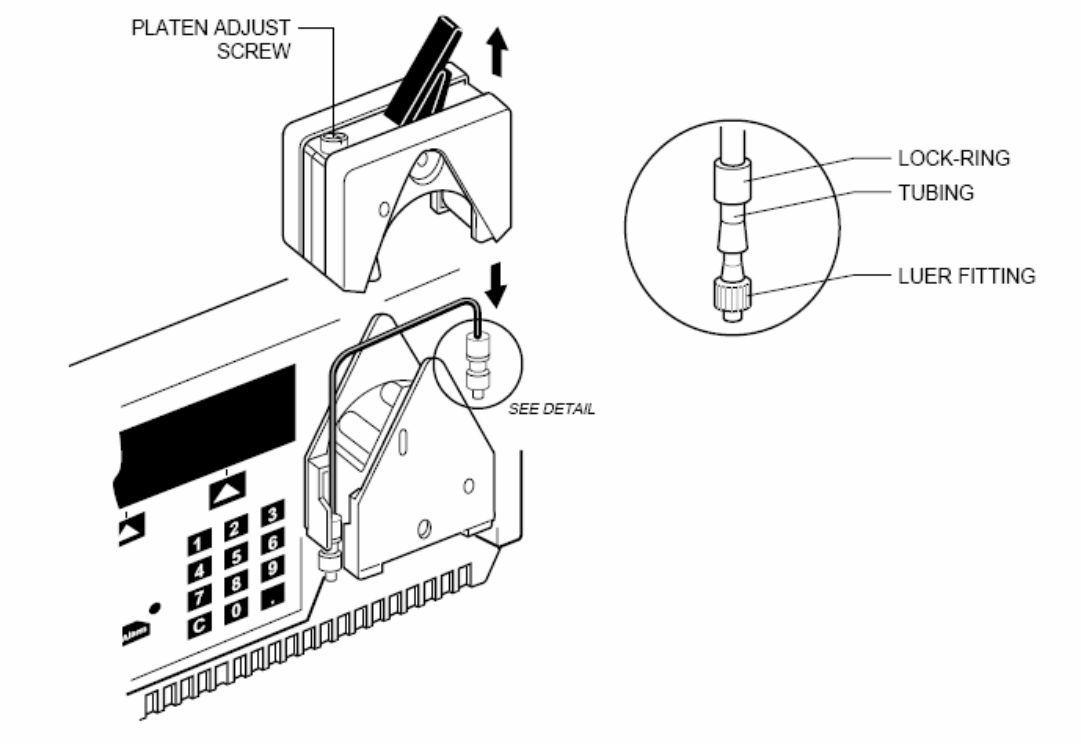

Figure 4.3: Plumbing the pump system illustration[43]

resistance, biocompatibility, height adjustment, robustness and ease of use. The dimensions of the columns used for the two different functions are contained in Table 4 and a schematic diagram of one of the columns is shown in **Figure 4.4**.

Packing the columns

The columns were first dismantled by unscrewing the removing one of the adjustable endpieces and pulling it gently out of the column. The 100mm x 10mm column and the 50mm x 10mm column were then with granulated fructose and immobilized RML, respectively. Each of the columns was filled to full volume capacity and the endpieces were replaced and screwed back onto the columns, making them ready for the next phase of the research. A photograph of a lipase-packed OmniFit® chromatography column is included in **Figure 4.5**.

Table 4: Dimensions of OmniFit® chromatography columns

Dimensions	Sugar Column	Lipase Column
Length (mm)	100	50
Inner Diameter (mm)	10	10
Volume (ml)	5.6	1.7

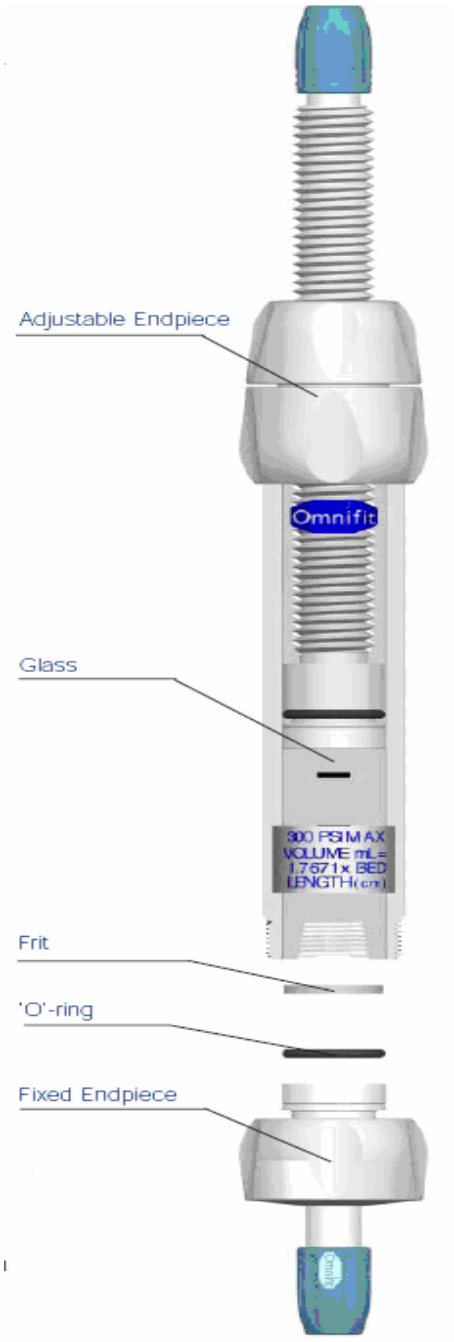

Figure 4.4: Schematic diagram of Omnifit® column[44]

Figure 4.5: Photograph of OmniFit® column packed with lipase

The Oven

The packed columns were enclosed inside a temperature–regulated oven and for this purpose; a medium Fisher Isotemp® Economy Lab Incubator was used from Fisher Scientific (Pittsburgh, PA). The incubator used gravity flow convection to generate uniform heating. Some of the unique specifications associated with the Fisher Isotemp® Economy Lab Incubator include a chamber volume capacity of 3.75 cubic feet, a temperature range of 30^{o} - 75^{o}C (86^{o} - 167^{o}F) with uniformity of $\pm 1^{o}$C at 37^{o}C and a resolution of 0.5^{o}C. A picture of the equipment is given in **Figure 4.6**.

Figure 4.6: Photograph of Fisher IsoTemp® Economy lab incubator

Tubing Specifications

A screen of the compatibility of tubing material with oleic acid was researched in order to determine the most suitable tubing material for feed mixtures containing high concentrations of oleic acid. C-FLEX®, found to have excellent compatibility with oleic acid, chosen based on additional advantages such as high flexibility, excellent heat-resistance, non-toxicity and lower gas permeability. C-FLEX® tubing is a thermoplastic elastomer composed of a styrene-ethylene-butylene modified block copolymer with silicone oil. Table 5 lists the physical characteristics.

Feed/Recycle Reservoir

A 250ml capacity Erlenmeyer flask was used for this purpose.

Table 5: Physical characteristics of C-FLEX® tubing[44]

Material	C-FLEX ®
Description	Clear, soft material
ID	1/32" (0.8 mm)
OD	1/12" (2.1 mm)
Wall thickness	1/32" (0.8 mm)
Temperature Range	-100 to 275°F (-73 to 135°C)
Max psi	51 psi at 70°F

4.2.3 – Method/Approach

An illustration of the packed bed reactor system is shown in Figure 4.7. The feed reservoir was connected to a peristaltic pump which transported the starting mixture into and through the packed bed bioreactor system. Connected to the peristaltic pump was first OmniFit® chromatography column which was packed with fructose sugar and this was in turn connected to the second OmniFit® chromatography column packed with immobilized RML. The sugar column, which in this case acts as an adsorption column, serves as a fructose sugar reservoir to constantly supply the feed stream with sugar to saturation level. The lipase column serves as the core component of the entire system: the packed bed reactor. The effluent from the packed bed reactor was then recycled back into the feed reservoir. The two OmniFit® columns were stored in the temperature-controlled Fisher Isotemp® Economy lab incubator set at a temperature of 70^{o}C the entire reaction time. All the components were connected together using clear, 0.8mm ID C-FLEX® tubing and 0.8mm ID Luer fittings.

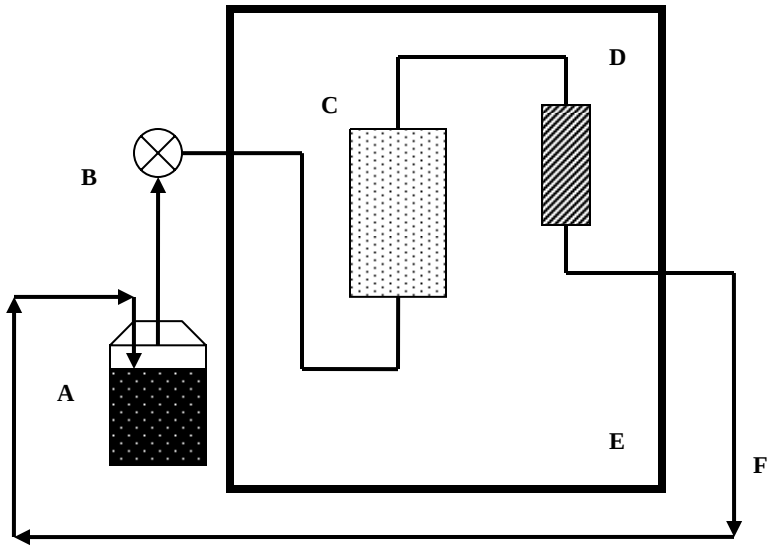

Figure 4.7: **Schematic Diagram of packed bed bioreactor for fed-batch synthesis of saccharide-fatty acid esters. (A) Feed Reservoir with oleic acid-monoester conversion mixture, (B) peristaltic pump, (C) glass column containing substrate sugar, (D) glass column containing immobilized RML, (E) temperature-controlled oven and (F) recycle stream of effluent.**

4.3 – PHASE II – Synthesis of Fructose-Oleic Acid Esters with Continuous Re-circulation of Reaction Medium

4.3.1 – Method and Materials

In order to determine if the fed-batch synthesis mode of fructose-oleic acid esters production was effective in converting oleic acid into esters, the packed bed bioreactor system was tested with a conversion mixture of monoester and oleic acid. A batch of fructose oleates was previously prepared using the batch esterification scheme (Section 3.1.1) and it was filtered to remove the lipase. A feed mixture of monoester and oleic acid (30/70 w/w) was then prepared in a 250 ml Erlenmeyer flask. This reaction mixture served as the feed that was continuously re-circulated through the packed bed reactor and the sugar adsorption column at a flow rate of 0.1 ml/min. The Erlenmeyer flask containing the reaction mixture was set over a hot plate and was heated to a temperature range between 60-65^{o}C in order to ensure that there was no loss in temperature as the feed went through the different column located in the IsoTemp® Incubator, which was set at 70^{o}C.

The feed mixture first went through the adsorption column, by way of the C-FLEX® tubing, where the reaction media was saturated with fructose. The adsorption column was packed with fructose crystals with a total mass of 4.44g filling a total bed volume of 5.5ml. The fructose-saturated reaction was then pumped into the lipase-packed column where biocatalysis of the esterification reaction occurred, producing fructose-oleic acid esters. The packed bed reactor was packed with lipase with a total mass of 16.44g filling a volume of 1.57ml. The reaction media was then recycled back into the

feed reservoir for a continuous cycle of the reaction media through the bioreactor system. The recycle system was run for a total of 423.5 hours (~ 17.6 days). During this time course, aliquots of the reaction media were frequently taken out for HPLC analysis to determine the conversion of oleic acid into esters. The aliquots taken were very minute and were less than 1% of the total weight content of the feed/recycle reservoir. The reactor was shut down after all the fructose crystals were depleted from the sugar column and dissolved in the reaction stream.

4.3.2 – Quantification Results and Discussion

The equation used to determine the conversion of fatty acyl group into product was based on weight percent:

$$\textbf{\%Conversion of fatty acyl groups} = \frac{[ME]+[DE]}{[FFA]+[ME]+[DE]} \quad \textbf{(4.1)}$$

Using this equation, the results shown in Table 6 were obtained.

TABLE 6: Conversion results obtained from fed-batch synthesis of fructose-oleic acid esters in a packed bed bioreactor system

	Wt. %FFA	Wt. %ME	Wt. %DE	CONVERSION
START OF CONTINUOUS SYNTHESIS (Day 1)	70.71	21.74	7.55	29.29 wt%
END OF CONTINUOUS SYNTHESIS (Day 17)	18.79	52.88	28.34	81.21 wt%

The initial reaction mixture contained 29.29wt% of batch-synthesized fructose oleates that had been purified from the immobilized RML used to catalyze it, and 70.71wt% of technical grade OA. From the results obtained, it can be observed that a product conversion of 81.21% was obtained after 17 days of continuous synthesis reaction and the original oleic acid percent composition in the reaction was depleted to only 18.79wt%. The time course of conversion is plotted in Figure 4.8.

In that original mixture, it was determined that the composition of monoesters was 21.74wt% and that for diesters was 7.55wt% of the entire mixture; hence the ratio of ME:DE was 2.9:1w/w. After 17 days of continuous esterification of the reactants into products in a recycle stream, it was discovered that the ME: DE ratio had decreased to 1.9:1. It seems that the production of diesters was much higher than the production of monoesters. This could be attributed to the fact that there was the presence of a large proportion of monoesters originally in the reaction mixture and since it is the monoesters that are further esterified into diesters, this resulted in more diesters being synthesized. The concept of the reaction favoring the diester production can be observed in Figure 4.9; a plot which compares the conversion of monoesters to that of diesters.

After the bioreactor system was shut down, the immobilized RML packed into the packed bed reactor was found to be intact. The lipase was cleaned of any fructose oleate or oleic acid remnants using acetone, dried and re-weighed. The weight recorded was 16.25g indicating a minimum 0.19-gram loss of enzyme, which could have resulted from transfer of product onto the analytical microbalance for measurement.

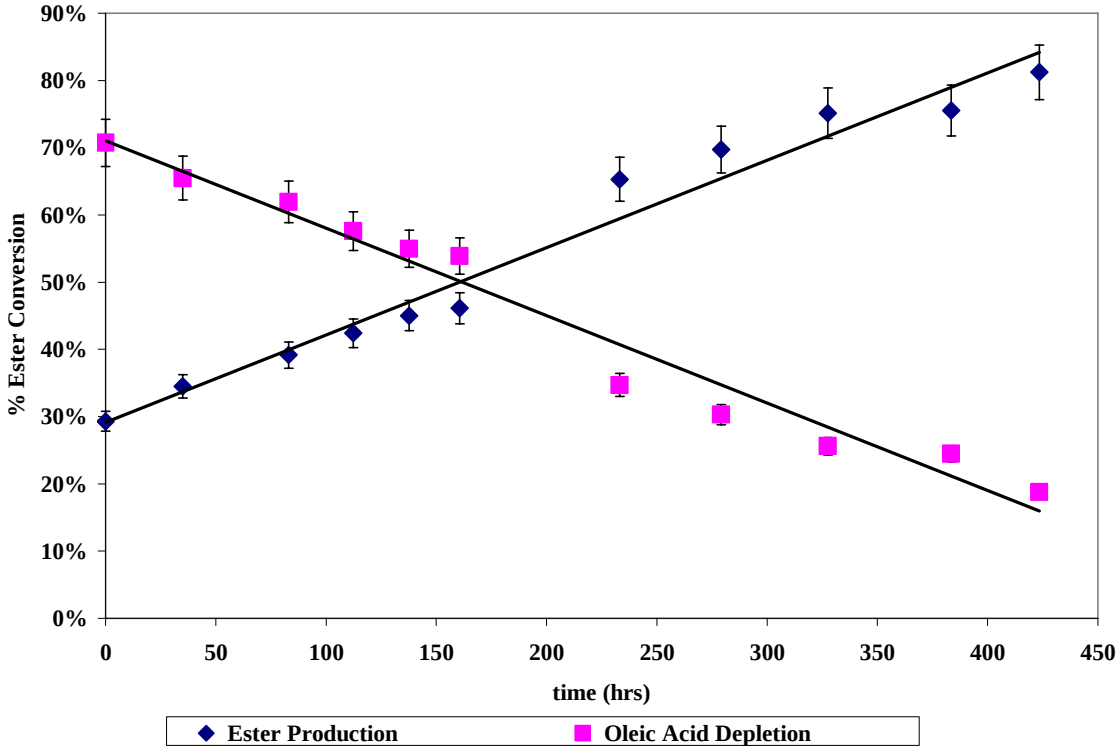

Figure 4.8: Time course of product conversion for fructose-oleic acid esters in a packed bed bioreactor system operated with continuous re-circulation of the reaction medium

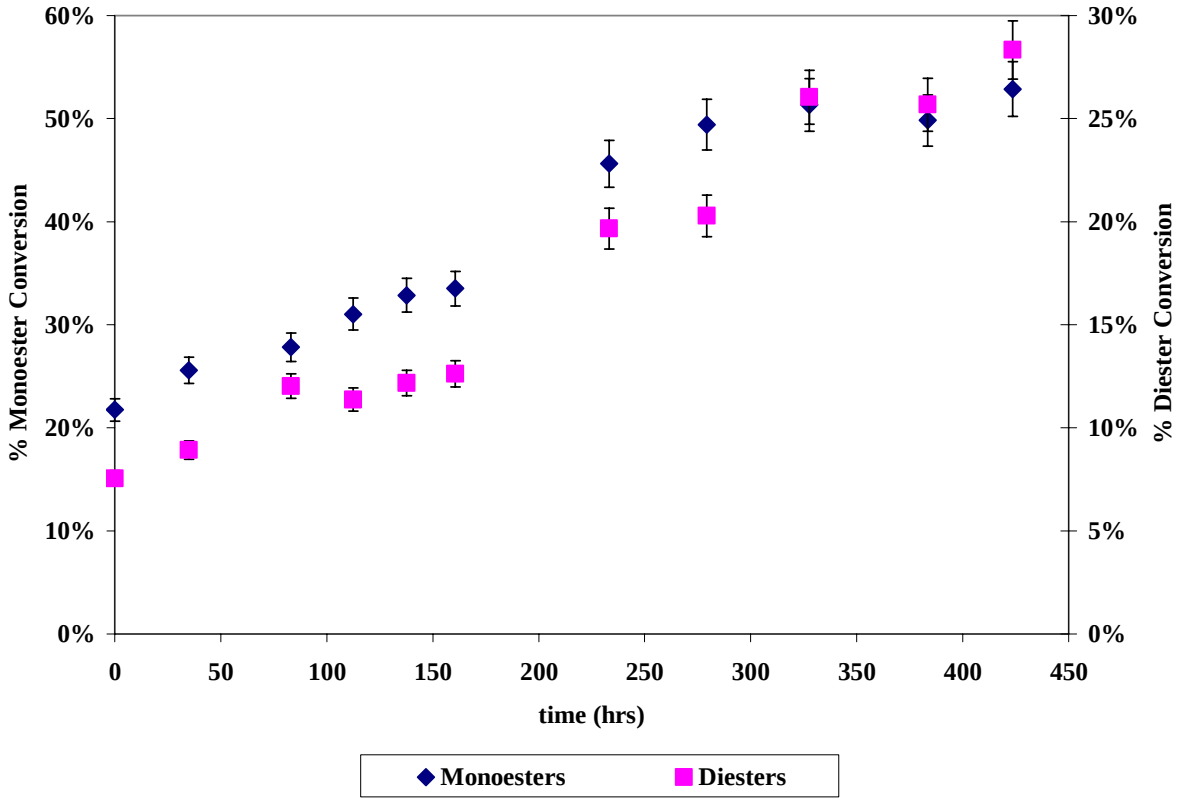

Figure 4.9: Time course of fructose-oleic acid monoester and diester conversion in a packed bed bioreactor system operated with continuous re-circulation of the reaction medium

The results obtained show that it took the bioreactor system a total of 423.5hrs to transform a product conversion of 30% into 81%. As compared to the batch synthesis mode, which usually takes a total of less than 300 hours to obtain a product conversion of approximately 80%-90% starting with 0%, the reaction time was very slow. The productivity of the synthesis of fructose-oleic acid esters with continuous re-circulation of reaction medium was compared that of batch synthesis. The productivity was calculated according to the cumulative amount of the product per unit volume of the reactor will be determined using the equation shown below (Equation 4.2). The equation to calculate the productivity per mass unit of enzyme per unit time, P (kg/g_{enzyme}-hr) is:

$$P = \frac{M_P Q C_P}{m} \qquad \textbf{(4.2)}$$

where:

M_P is the molecular mass of the product (kg/mol)

Q is the volumetric flow rate (L/hr)

C_P is the concentration of the sugar-oleic acid ester in the effluent (mol/L)

m is the mass of enzyme (g)

The results of productivity are tabulated in Table 7.

TABLE 7: Comparing productivity of the fed-batch synthesis to batch synthesis

	REACTION TIME	**PRODUCTIVITY**
SYNTHESIS WTH CONTINUOUS RE-CIRCULATION	**279 hrs**	**0.096 kg/g_{enzyme}-hr**
	423 hrs	**0.110 kg/g_{enzyme}-hr**
BATCH SYNTHESIS	**267 hrs**	**0.423 kg/g_{enzyme}-hr**

The results shown here are based on different batches of lipase that were used for the individual reactions.

4.3.3 – Conclusions

The fed-batch bioreactor system designed to serve as a preliminary design of the proposed continuous synthesis conferred most of the advantages that were predicted earlier in this study when the batch mode was compared to the continuous mode of ester synthesis (Section 2.3). The column provided excellent protection for the lipase; the immobilized RML used in the bioreactor system was found to be intact with no unit loss observed after 423.5 hrs of continuous operation and could be re-used in another phase of fed-batch synthesis of fructose oleates without having to recover it from the reactor. The time course of the reaction was linear which can be compared to the linear trend obtained in the lipase-catalyzed batch reactions (Section 3.1). For those batch reactions, the activity retention of the immobilized RML was measured and found to be excellent; a characteristic which can be said of the lipase used in the packed bed bioreactor system. However it is recommended that activity retention experiments similar to those designed for the batch reactions (Section 3.1) should be conducted in order to build a stronger scientific conclusion.

The conversion of esters from an approximately 30% composition to an 81% constitution demonstrated that the fed-batch synthesis mode of reaction in a packed bed reactor is an applicable and effective method of synthesizing saccharide-fatty acid esters. One of the expectations predicted was not achieved and that involved the advantage of a

minimization of reaction times as a result of the yield of a high biocatalyst-substrate ratio. The reaction for the fed-batch synthesis of fructose-oleic acid esters was catalyzed using a batch of immobilized RML different from that used in the batch synthesis of fructose oleates (Chapter 3); hence it would not be fair to perform a direct comparison of the reaction times in the two mode of reaction. It is possible that the reaction time was actually minimized in the fed-batch synthesis with respect to the particular batch of immobilized RML used.

As mentioned before, the results obtained show that the reaction times for the bioreactor system were slow. One of the reasons for the limitation in ester conversion may be the result of the incidence of sugar caramelization. Caramelization is a complex reaction that occurs when sugar is heated at high temperatures for long periods of time and involves a series of reaction listed as follows: 1) equilibration of anomeric and ring forms, 2) condensation, 3) intramolecular bonding, 4) isomerization of aldoses to ketoses, 5) dehydration reactions, 6) fragmentation reactions, and finally 7) unsaturated polymer formation and color change[45]. It is possible that the condensation stage could have produced a high percentage of accumulated water in the flow stream if the reaction medium and resulted in a limited conversion of reactants into esters.

As mentioned earlier, the fed-batch synthesis mode was employed as a preliminary design to facilitate a continuous bioreactor system design. The future design should reduce the residence time sugar is exposed to high temperatures during the reaction. This technique might probably aid in the product conversion. Most importantly, it is essential to conduct further test runs of the fed-batch bioreactor system, which would

involve exploring different reaction conditions in an attempt to achieve maximum conversion into fructose-oleic acid esters.

4.4 – PHASE III – Mathematical Modeling of Packed Bed Reactor

4.4.1 – Development of the Mathematical Model

An overall mass balance of the monoesters being produced on the entire bioreactor system was created based on the diagram shown in Figure 4.10. A mass balance applied to the feed/recycle reservoir was performed using the following formula.

Mass in – Mass Out + Generation = Accumulation

The "GENERATION" term is not applicable, thereby generating the following equation.

$$X_{ME,R}R - X_{ME,RES}R = \frac{\partial}{\partial t}\left(X_{ME,RES}M_{RES}\right) \quad \textbf{(4.3)}$$

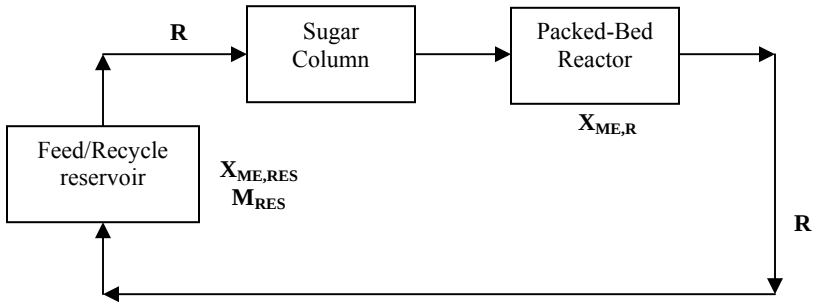

Figure 4.10: Block diagram of the bioreactor system showing major parameters employed in the mathematical model derivation.

where:

$\mathbf{X_{ME,RES}}$ is the mass fraction of the monoester in the feed/recycle reservoir

$\mathbf{X_{ME,R}}$ is the mass fraction of the monoester produced in the packed reactor

$\mathbf{M_{RES}}$ is the mass of reaction medium in the feed/recycle reservoir (g)

R is the mass flow rate of the reaction medium from the packed bed reactor (g/hr)

This equation assumes that the net increase in the mass flow rate of the recycle stream due to adsorption of fructose in the "saccharide column" is negligible. If one assumes the overall mass in the reservoir remains constant, i.e., that the mass of water leaving the reservoir via "free" evaporation is negligible, the following equation is derived:

$$\frac{\partial X_{ME,RES}}{dt} = \frac{R}{M_{RES}} X_{ME,R} - \frac{R}{M_{RES}} X_{ME,RES} \quad \textbf{(4.4)}$$

A monoester component mass balance on the packed bed reactor was then performed:

$$W = F_{FAO} \int_{X_{E,RES}}^{X_{E.R}} \frac{\partial X_{ME}}{r'_{FA}} \quad \textbf{(4.5)}$$

where:

$\boldsymbol{F_{FA0}}$ is the initial molar flow rate of the fatty acid entering the PBR (mol/hr)

$\boldsymbol{r'_{FA}}$ is the rate of reaction for fatty acid (mol g_{Enz}^{-1} hr^{-1})

$\boldsymbol{W}$ is the weight of catalyst in the PBR (g_{Enz})

The Ping-Pong Bi Bi kinetic mechanism[46] is a model that is frequently used to describe lipase-catalyzed reactions involving two substrates, including polyol-fatty acid esterification[25, 46] (and references therein):

$$r'_{FA} = \frac{V_{MAX}[FA][S]}{K_S[FA] + K_F[S] + [FA][S]} \quad \textbf{(4.6)}$$

where:.

V_{MAX} if the maximum velocity of the enzymatic reaction (mol g_{Enz}^{-1} hr^{-1})

K_{FA} is the Michaelis constant for fatty acid (mol L^{-1})

K_S is the Michaelis constant for saccharide (mol L^{-1})

[S] is the concentration of saccharide (mol L^{-1})

[FA] is the concentration of fatty acid (mol L^{-1})

It is assumed that K_{FA} and K_S are negligible because the values obtained by Dang[25, 26] for the same reaction under similar operating conditions but in batch mode (K_S = 401mM and K_{FA} = 387mM) were quite small; hence, Equation 4.6 simplifies to:

$$r'_{FA} = V_{MAX} \quad \textbf{(4.7)}$$

Equation 4.7 was then inserted in Equation 4.5; the resultant equation was integrated and algebraically manipulated, resulting in the equation given below.

$$X_{ME,R} = X_{ME,RES} + \frac{V_{MAX} W}{F_{FAO}} \quad \textbf{(4.8)}$$

Equation 4.8 was then inserted into Equation 4.4 and the following model equation was obtained:

$$\left[X_{ME,RES} - X_{ME,RES,0}\right] = \frac{V_{MAX} WR}{M_{RES} F_{FAO}} t \quad \textbf{(4.9)}$$

where:

$X_{ME,RES,0}$ is the mass fraction of monoesters in the feed/recycle reservoir at the start of the reaction.

Equation 4.9 was then used to solve for the mass fraction of the esters present in the feed/recycle reservoir, $X_{ME,RES}$. Subsequently, the value of $X_{ME,RES}$ was inserted into Equation 4.8 to obtain the mole fraction of ester produced in the packed bed reactor, $X_{ME,R}$. In summary, the following assumptions were employed in obtaining Eq. 4.9:

1. Changes in density due to adsorption of fructose in the saccharide column and to conversion of oleic acid into monoester were neglected
2. The loss of mass in the reservoir from the evaporation of water was neglected.
3. The increase of mass for the recycle stream due to adsorption of saccharide in the saccharide column was neglected.
4. The production of diesters from monoester was neglected.

4.4.2 – Results and Discussion

The value of the kinetic parameter, V_{MAX} was obtained from a prior experiment conducted by Dang et al[25], with a value of 4.6 mmol hr^{-1} $g_{catalyst}^{-1}$. The mathematical

model equation obtained was then applied and used to predict the mass fraction of esters that should be present in the feed/recycle reservoir and the PBR as a function of time. Figure 4.11 compares calculated and measured values of the mass fraction of monoesters present in the feed/recycle reservoir during the time course of the reaction. Figure 4.11 also includes the mass fraction of monoester in the PBR obtained from the kinetic model. It can be observed from the graph that at after a reaction time of 160 hrs, the mass fraction of monoesters that is predicted by the kinetic model is 0.52, experimentally a mass fraction of 0.46 was obtained. At the end of the time course of reaction (423.5 hrs), it is predicted that a mass fraction of 0.90 monoesters should be present in the feed/recycle reservoir whilst experimentally, a mass fraction of 0.81. These results show that kinetic model predicts an approximate 9% difference in the monoester mass fraction obtained experimentally and this discrepancy may be caused by differences in the reaction rate effective during the experiment and that used in the mathematical model.

It can be hypothesized that the mass fraction of monoesters in the feed/recycle reservoir ($X_{ME,RES}$) at any given time is less than the mass fraction of monoesters exiting from the packed bed reactor ($X_{ME,R}$); this is because the feed/recycle reservoir has other reactants (oleic and fructose) present that are still involved in the reaction. The results obtained show that at a time of 160 hrs, the mass fraction of monoesters in the feed/recycle reservoir is 0.52 whilst that exiting from the packed bed reactor is 0.54; results that provide evidence that the hypothesis is true. The model predicts a linear relationship between $X_{ME,R}$ and $X_{ME,RES}$.

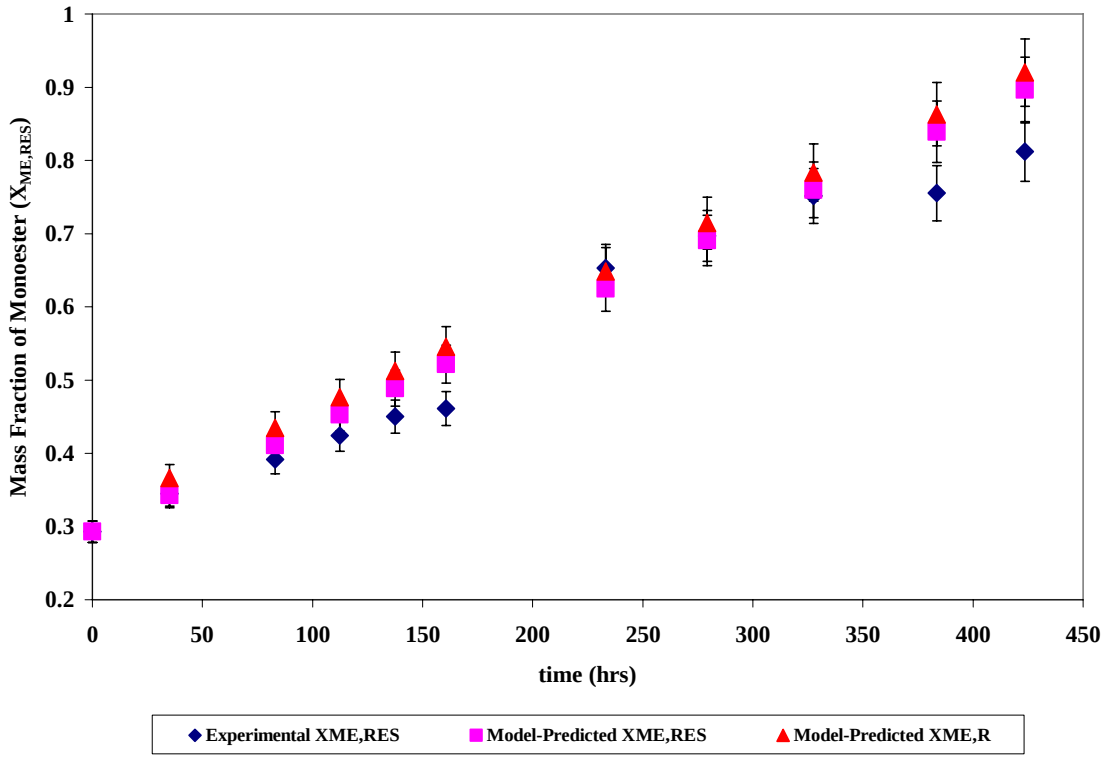

Figure 4.11: Time course of fructose-oleic acid esterification in the bioreactor system. Plotted are the experimental and calculated values of $X_{ME,RES}$, the latter obtained from Equation 4.9, and $X_{ME,R}$, obtained from Equation 4.8.

4.4.3 – Conclusions

It can be seen from the results that the derived kinetic model is well correlated with the experimental data and thus proves its reliability; also the trend of mass fraction of esters being produced experimentally and that predicted by the model are both found to linear. The model was based on a kinetic parameter that was derived from a previous experiment. Although the experiment was successful, it is was based off of a number of assumptions and hence serves as just an initial stage toward acquiring a full reversible kinetics model for the fed-batch synthesis of fructose-oleic acid esters in a packed bed

bioreactor system with continuous re-circulation of reaction medium. It is essential for further experiments to be conducted to develop a full kinetic model which include the effect of factors such as initial water concentration, diester production and polarity of the reaction mixture.

CHAPTER 5

CONCLUSIONS & RECOMMENDATIONS

The goals of this research focused upon both the batch and fed-batch synthesis mode of saccharide-fatty acid esters. The objectives set for the batch-related experiments included a) investigating the activity retention of immobilized RML b) determining the effect of water content on the equilibrium-based conversion of fructose-oleic acid esters and c) determining if the procedure employed previously by Zhang and Hayes and Dang and Hayes[17, 25] for fructose-monooleate synthesis can be applied successfully to the conversion of other saccharide and oleic acid into esters. The results from the three successive batch experiments with the use of the same unit of RML in a series of batch reactions led to the conclusion that there was no loss of activity for a total of 585 hrs of reaction. Immobilized RML can thus be re-used frequently after proper recovery and purification step in multiple reactions. It was deduced from the results of the effect of water content experiments that the presence of water reduces the reaction's thermodynamic equilibrium ability to promote full conversion of reactants into esters. The batch synthesis of sucrose oleates, xylose oleates and glucose oleates was a success in terms of conversion; however, the product conversion for the latter two were lower than that obtained for the synthesis of fructose oleates in previous batch reactions. It was concluded that the rate and extent of product conversion of each type of saccharide oleate was dependent on the molecular configuration of each saccharide, the availability of primary hydroxyl groups and the degree of saccharide solubilization. It is recommended that further research be conducted to explore the solubility of sucrose, glucose and

glucose in fatty acid/saccharide mixtures, moreover, to create tertiary phase diagrams for each system.

The choice of the feedstock source of sugar used in the synthesis is an important concept to consider when the industrial large-scale production of fructose-oleic acid esters is in question. It is recommended that fructose syrup serve as an alternative to anhydrous fructose crystals; this will be a cost-effective measure due to the lower cost for the former. However, the presence of water as part of the syrup composition would intensify the problem of limited product conversion. The issue of water content accumulation for fructose syrup would be one that has been addressed in lab-scale research and can be extended to large-scale production.

The objectives laid out for the synthesis of fructose-oleic acid esters through a packed-bed bioreactor with continuous re-circulation of reaction medium involved a) to design and assemble a packed bed bioreactor system, b) to synthesis fructose oleates in the bioreactor system and to quantify the product conversion, and c) to derive a mathematical model to predict the performance of the bioreactor system. The design and assembly of the packed bed bioreactor system was achieved successfully and results from the synthesis reaction experiments demonstrated that this method of synthesis was effective in producing a high conversion of fructose and oleic acid reactants into fructose-oleic acid esters that a conversion of reactants into products was achieved. An 81wt% ester conversion was obtained from a start-up reaction medium containing only 30wt%. It was observed that some of the predicted advantages expected of the continuous synthesis mode were achieved in the fed-batch design, such as reduced start-up and shutdown times and excellent lipase protection. Further test runs of the packed bed bioreactor system are

highly recommended to examine the effect of temperature and re-circulation flow rate. Redesign of the saccharide adsorption column using silica gel as a support is recommended as silica gel provides better sugar distribution in the column and might help reduce heat intensity on the sugar that leads to caramelization. With respect to the developed kinetic model, it is recommended that further experiments be conducted to develop a full kinetic model which would include the effect of factors such as initial water concentration, diester production and polarity of the reaction mixture.

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