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BIOREACTOR SYSTEM DESIGNS FOR LIPASE-CATALYZED SYNTHESIS OF SACCHARIDE- FATTY ACID ESTERS IN SOLVENT-FREE MEDIA

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To the Graduate Council:

I am submitting herewith a dissertation written by Ran Ye entitled "BIOREACTOR SYSTEM DESIGNS FOR LIPASE-CATALYZED SYNTHESIS OF SACCHARIDE- FATTY ACID ESTERS IN SOLVENT-FREE MEDIA." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biosystems Engineering.

Douglas G. Hayes, Major Professor

We have read this dissertation and recommend its acceptance:

X. Philip Ye, Svetlana Zivanovic, Qixin Zhong

Accepted for the Council:
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Vice Provost and Dean of the Graduate School

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

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A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Ran Ye

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DEDICATION

This dissertation is dedicated to my family.

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This dissertation could not have been completed alone and there are numerous people who have helped me to solve a tremendous amount of problems. Here, I am deeply grateful for everyone's help.

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Abstract

As nontoxic biobased surfactants derived from plant oils and cellulose or starch, saccharide-fatty acid esters are widely used in cosmetics, food, and pharmaceutical industries due to their biocompatibility, biodegradability as well as antimicrobial activity. Generally, saccharide-fatty acid esters are synthesized chemically under high pressure, temperature and the presence of alkaline or acid catalysts leading to low-quality products (chemo-degradation of double bonds and oxygenated moieties) and large amounts of byproducts. In contrast, biocatalytic synthesis enhances sustainability: near-ambient pressure and temperature, the absence of toxic, acids and bases catalysts, and improved selectivity of products. For lipase-catalyzed synthesis under nearly anhydrous conditions, the major hurdle to be overcome is the poor miscibility of the acyl donor and acceptor substrates, resulting in slow reaction rates. Although several approaches such as, the employments of organic solvents, complexation agents, and ionic liquids, have been reported in the literature, a robust solution is desperately needed. This study focused on employing immobilized lipases under completely solvent-free conditions to synthesize saccharide-fatty acid esters using the ester products to enhance miscibility. Experimentally, metastable saccharide particles with a diameter of 10-100 micron-sized suspensions of saccharide were formed in oleic acid-rich ester mixtures initially for synthesis of saccharide-fatty acid esters in packed bed bioreactor containing immobilized lipases. Water, a by-product that limits ester yield by promoting hydrolysis, was removed via free evaporation. In this dissertation, a bioreactor system was developed for the eco-friendly solvent-free, immobilized lipase-catalyzed synthesis of biobasaed surfactants utilizing suspensions as reaction medium with 88 wt% in 6 days; the performance of the bioreactor systems developed for Objective 1 was optimized through water concentration control and interval time with 91 wt% in 4.8 days; and to improve design of bioreactor system developed in Objective 1 by in-line filter and derive a mathematical model to

describe the production of esters by the bioreactor systems developed. Finally, 84 wt% ester content was achieved in 8.4 days.

Keywords: biocatalysis, lipase, saccharides-fatty acid esters, biobased surfactant, solvent-free, bioreactor, water control, fructose-oleic acid ester

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CHAPTER 1

INTRODUCTION & OBJECTIVES

1.1 Motivation of Enzymatic Synthesis of Biobased Surfactant

Surfactants are amphiphilic compounds employed for the decrease of surface and interfacial tensions and improvement of the solubility and mobility of hydrophobic or insoluble organic compounds by accumulating at the interface of immiscible fluids [1]. Specially, surfactants consist of polar and non-polar groups. The polar group or "head" interacts with hydrophilic molecules such as water. In contrast, the non-polar group or "tail" has affinity for lipophilic molecules, for instance, oil [1]. Generally, surfactants are classified into two major categories: ionic and non-ionic surfactants based on head group chemistry. More specially, an ionic surfactant dissociates in water releasing cation, anion or zwitterion. In contrast, a non-ionic surfactant with a polarization of electrostatic charges dissolves in water by formation of hydrogen bonds [1].

Due to their ability to co-solubilize water and oil, they are widely used in the laundry, personal care product, and dishwashing detergents industries. However, the majority of surfactants consumed in daily life are petroleum-based. With a high degree of price fluctuations for petroleum, scientists are making efforts on the development of a substitute from renewable and natural sources, such as seed oils and sugars from feedstocks and biomass. Although fats / seed oils are more expensive currently, this situation will change.

In this project, enzymatic synthesis of nontoxic, biodegradable and environmentally-friendly biobased surfactants is focused upon due to advantages compared with chemical synthesis. First, enzymatic methods provide lower energy consumption, for instance lower temperature and ambient pressure and lower amounts of reactants due to employment of near-stoichiometric ratios. Second, utilization of enzyme can offer

the safer and more environmentally-friendly operation conditions due to the absence of toxic metal or acidic/basic catalysts. Third, a narrower production distribution is often yielded by enzyme synthesis.

The overall goal of this research is to design an enzymatic bioreactor system for solvent-free lipase-catalyzed synthesis of saccharide-fatty acid esters, biobased surfactant. Attempts and efforts will be made toward solving two major problems that occur: slow reaction rate and control of water concentration. The poor miscibility of the lipophilic acyl donor and hydrophilic acyl acceptor is the primary reason resulting in the slow reaction rate. Some methods have been employed to overcome this problem, such as, employing polar organic solvents and ionic liquids (reviewed in Chapter 2). In this dissertation, metastable 10-100 micron-sized suspensions of saccharide were formed in oleic acid and ester mixtures (75/25 w/w) for synthesis of saccharide-fatty acid esters in packed bed bioreactor containing immobilized lipases (Objective 1). (described in Chapter 3). Water is a by-product that inhibits the ester yield by enhancing hydrolysis of ester. Therefore, water removal is required from bioreactor system via some approaches, for instance, free evaporation, molecular sieves, and vacuum pressure (described in Chapter 4). Additionally, optimization of the bioreactor system developed for Objective 1 was performed through the water control and reformation of suspensions media (described in Chapter 4). Different common acyl donors and acyl acceptors were used to examine the universality of the bioreactor system developed by Objective1and the relationship between concentrations of acyl acceptors and the initial rates was investigated in Chapter 5. Further, an on-line filter was developed and applied into the bioreactor system combining with the selected water removal method for the operation of a continuous process under the optimal condition (described in Chapter 6). In Chapter 7, some recommendations for future work were provided.

1.2 Objectives

The overall objective of research was to design and develop enzymatic bioreactors to produce saccharide fatty acid esters in solvent-free media. Currently, enhancing the reaction rate and conversion yield under solvent-free conditions is the first major hurdle to be cleared. Accordingly, achieving the overall objective requires fulfillment of three sub-objectives:

1. Investigate the optimal the conditions required to form metastable 10-500 micron-sized suspensions of saccharide crystals and their utilization in solvent-free bioreactor systems to produce saccharide-fatty acid esters
2. Optimize the water content and water removal approach in the bioreactor system of Objective1 to efficiently maximize the conversion of desirable products
3. Improve the design of the bioreactor system developed by Objectives 1 and 2 through incorporating of an on-line filter and derive a mathematical model to simulate the time course of reaction.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

As mentioned in Chapter 1, the overall objective of this proposal is to design an enzymatic bioreactor system for solvent-free lipase-catalyzed synthesis of saccharide-fatty acid ester biobased surfactant. To understand the current state-of-the-art, some relevant topics in the literature will be covered in this chapter, such as lipase-catalyzed reactions in nonaqueous media, chemical synthesis of saccharide-fatty acid esters, and application of saccharide-fatty acid esters. Also, kinetic model of bioreactors for lipase-catalyzed synthesis of saccharide-fatty acid esters will be described. For the section of enzymatic synthesis of saccharide-fatty acid esters in nonaqueous media, several sub-topics covered include organic solvent, supercritical carbon dioxide, ionic liquids, eutectic mixtures and solvent-free media.

2.2 Lipase-Catalyzed Reactions in Nonaqueous Media

Nonaqueous bioprocessing is attractive for several different reasons. Since the free energy of substrate dissolution relies on the physicochemical properties of solvents, the substrate specificity of an enzyme is solvent-dependent [1]. The employment of enzymes in nonaqueous media enhances their catalytic synthetic capability by allowing for a wider range of substrates that can be utilized [2]. In addition, low water content in nonaqueous media shifts ester or amide formation at equilibrium toward product synthesis. Moreover, organic solvents also reduce microbial contamination [3]. Furthermore, enzymes in nonaqueous media often possess improved thermostability due to the low water content in their microenvironment. Also, the downstream purification of product is simplified since solvents are often easily evaporated away [4-6].

Enzymes catalyze reactions in nonaqueous media under mild operational conditions and yield a narrow distribution of products and relatively high reaction rates compared to the absence of biocatalysts [7]. However, most benign solvents are relatively hydrophobic. Some enzymes will lose their inherent activity in the presence of organic solvent-based media. For instance, polar organic solvents can alter chemical and physical structure of an enzyme's active site of by removing its water molecules of hydration. The resultant conformational change will lead to the loss of enzyme activity. Secondly, since enzymes are typically insoluble suspensions in nonaqueous solvents, in contrast to aqueous media, this leads to diffusional mass transfer limitations between substrates and enzymes' active sites. Hence, a decrease of enzyme activity will occur. However, many organic solvents interact relatively benignly with many enzymes. There are small amount of water in such solvents allowing for the enzyme activity to remain activity since the solvents extract away minimal water used in formation of bonds with polar amino acids on the enzyme surface [2,3, 8,9].

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are enzymes which exhibit the ability of modifying of oils and fats by formation and breakage of ester bonds [10-11]. Many lipases possess a helical oligopeptide lid that protects the active site and interacts with a hydrophobic interface. The active site generally includes a catalytic triad composed of serine, histidine, aspartate; and, an α/β-hydrolase fold forms the catalytic features of lipase. Also, lipases require no cofactor or coenzymes. Furthermore, lipases are well studied, inexpensive and commercially available [10-12].

The numerous applications of lipase-catalyzed reactions in industry and research are related to the several different reactions it can catalyze, including hydrolysis, esterification, alcoholysis, acidolysis and transesterification (**Fig. 2.1**) [13]. Fatty acids, employed in soap production, are produced by lipase-catalyzed hydrolysis of triacylglycerols [14] or they could be obtained through environmental sewage disposal treatment from digestion of oil-rich waste liquids [15-17]. The ability of lipases to

hydrolyze lipids has led to their use in laundry detergents [18-22]. Lipases are important for the food industry [23-24] For example, the enhancement of flavors in cheese products and acceleration of cheese ripening require lipases [25]. Lipases assist in the formation of fruit-enhancing flavors such as apple and strawberry for beverage production [26]. Also, lipases can modify animal fat as an alternative to butter. Akoh and co-workers utilized *Rhizomucor miehei* lipase to generate a inexpensive replacement for cocoa butter through acidolysis of beef tallow by stearic acid due to an increase in cost of coca butter, producing a "structured lipid" which is generally defined as a lipid structurally modified and reconstructed to obtain a desired nutritional, physical, and chemical function [27]. This approach potentially reduced production costs due to the use of an inexpensive animal by-product (tallow) [27]. Lipases also are employed in the chocolate industry as coffee whiteners [27-29]. In the pharmaceutical industry, lipases are used in the preparation of single-isomer chiral drugs by hydrolysis and transesterification reactions conducted in organic solvents [30-31]. Both asymmetric synthesis of racemic alcohols, acids, esters or amines [32] and the desymmetrization of prochiral compounds [33] depend on lipases as a highly stereoselective biocatalyst to accomplish. In addition, lipase-catalyzed synthesis is employed to produce esters as cosmetic ingredients that retain moisture [34]. Recently, hexyl laurate, an ingredient applied widely in cosmetics, was synthesized by esterification in a solvent-free system in the presence of immobilized *R. miehei* lipase [35]. Also recently, fatty chlorogenate esters, which possess high antioxidant capacity, were obtained at 93% yield through employment of *Candida antarctica* B lipase [36].

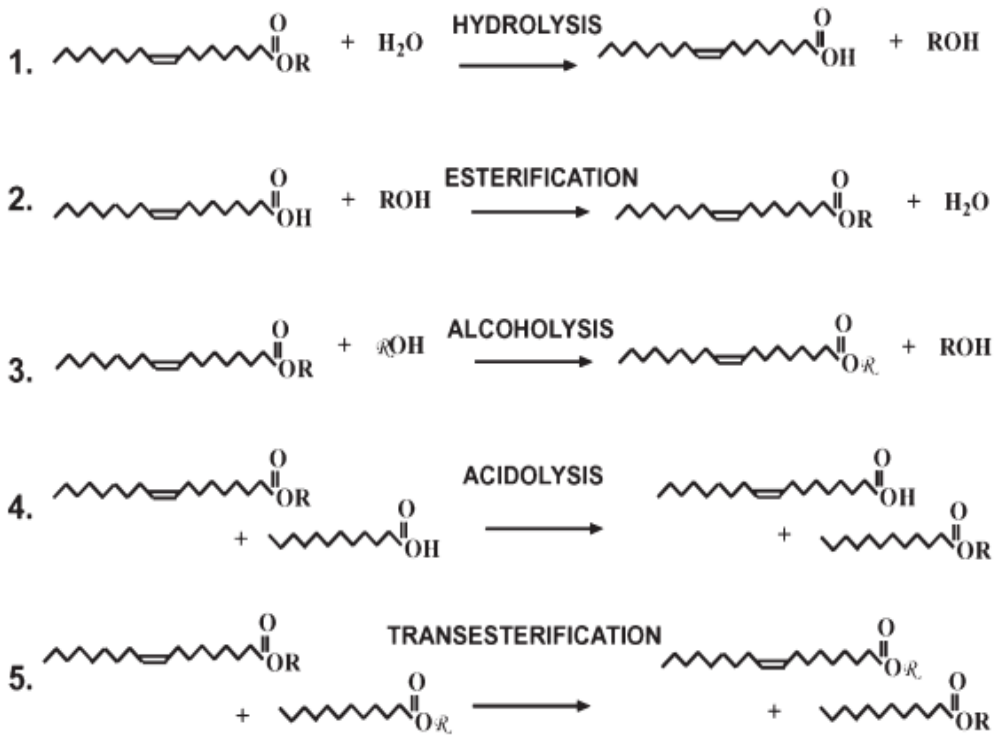


Fig. 2.1. Lipase-catalyzed reactions [13].

2.2 Chemical Production of Saccharide-Fatty Acid Esters

In general, saccharide-fatty acid esters are synthesized by chemical methods under extreme conditions, such as high temperature and pressure, and/or the presence of alkaline or acid catalyst [37, 38]. For example, the synthesis of sorbitan-fatty acid esters involves a two-stage process that consists of dehydration of sorbitol in the presence of acid (e.g. NaH_2PO_3) at 150-200°C, followed by alkali (e.g., $Na_2CO_3^-$)-catalyzed esterification with fatty acids at 200-250°C [39]. In addition, sucrose esters are synthesized by base- ($K_2CO_3^-$) catalyzed transesterification with fatty acid methyl ester (R'COOMe) serving as acyl donor in dimethyl formamide (DMF) at 90 °C [39, 40].

2.3 Applications of Saccharide-Fatty Acid Esters

Due to their amphiphilicity, nontoxic and biodegradability, saccharide-fatty acid esters are widely utilized biobased nonionic surfactants or emulsifiers in foods, pharmaceuticals and cosmetics [41]. These esters are being increasingly employed since they are harmless to the environment, compatible with eyes and skin, and utilize low-cost renewable feedstocks [42-43]. Typical applications include baked goods, fruit coatings, and confectionery foods [44-46]. Sucrose-fatty acid esters also are applied in detergents industry [47]. Emulsifiers are useful functional additives widely employed in food processing involving multiphase systems. They enable two distinct phases to form a stable quasi-homogeneous material that remains stable for a significantly long time. Also, emulsifiers modify and improve the physical property of the continuous phase in a food product. Similar to other emulsifiers, sucrose-fatty acid esters possess both lipophilic and hydrophilic functional groups. The relative proportion of hydrophilic and lipophilic behavior for surfactants and emulsifiers is often expressed as the hydrophilic-lipophilic balance, or HLB. The HLB value occurs

within the range of 0 to 20, with low numbers (<9) indicative of more lipophilic behavior and high numbers (>11) reflecting more hydrophilic preference. Saccharide-fatty acid esters can be tailored through the number of fatty acyl groups per molecule and the length of the fatty acyl chain to cover almost the entire HLB range.

Utilization of sugar esters as additives for drug formulations has been investigated. Some studies indicated sucrose-fatty esters significantly enhance the percutaneous permeation of ionized and unionized drug species by *in vitro* experiments using excised hairless mouse skin, demonstrating their potential application in transdermal drug delivery [48]. Sugar-fatty acid esters also exhibited some antitumoral properties. 6, 6′-Diestertrehaloses of fatty acids (C8, C10, and C12) and n-dodecyl-β-D-maltoside have been identified as a new class of anti-cancer agents due to their inhibitory effects on tumor necrosis factor gene expression [49].

Sugar-fatty acid esters are potentially useful as food preservation agents to prevent food spoilage since they have antimicrobial activity, especially toward Gram-positive bacteria (spore-formers). In addition, yeasts and moulds also suffer growth retardation through sucrose-fatty acids ester treatments [50]. 6-O-lauroyl sucrose and maltose when present at 0.8 mg/ml and 4 mg/ml, respectively, are capable of inhibiting the growth of *Bacillus* sp. and *Lactobacillus plantarum*, respectively. However, sucrose dilaurates and 6-O-lauroylglucose did not show antimicrobial activity because of their low aqueous solubility [51]. In addition, commercial sucrose esters are widely used in canned beverages for inhibitory function on the germination of spore-forming bacterium, especially in Japan (Mitsubishi-Kagaku Foods Corporation, Japan). *Bacillus* may lead to food poisoning once the concentration of *B. cereus* reaches 10^6 g/g in foods. It produces two types of food-borne intoxications in humans: the emetic form (causing vomiting) and the diarrheal form. The minimal inhibitory concentration (MIC) of sucrose laurate against *B. cereus* food poisoning bacteria is 9.375 mg/ml [52]. Maltose and maltotriose esters inhibited the growth of

Streptococcus sobrinus and galactose laurate, fructose laurate and 6-*O*-lauroylmannose showed the highest inhibitory effect against *S. mutans*. Both bacteria have been intensively implicated as causative organisms of dental cavities caries. Hence, the esters are potentially useful oral-hygiene product additive [53-54].

Since sugar-fatty acid esters are fully biodegradable and totally nontoxic to humans, higher animals, and crops, they appear to be good candidate for insecticides. Some related researches have been reported. Twelve synthetic sucrose ester (SE) products with C7-C10 chain length and various degrees of esterification were examined for their capability to inhibiy seed germination of prosomillet and velvetleaf. Most of the sucrose esters tested resulted in virtually total inhibition of germination at low concentration (100 ppm). Among them, sucrose esters with seven and eight carbon acyl groups were the most active; and, nonanoyl and decanoyl esters were not effective. Di-, tri-, and tetraacyl esters were also highly effective [55]. Another research group found a sucrose octanoate mixture containing a large fraction of monoester yielded the highest activity against arthropod pests, with additional esters tested consisting of sorbitol octanoate, sorbitol decanoate, sorbitol caproate, xylitol octanoate, xylitol decanoate and xylitol dodecanoate in the range from 1200 to 2400 ppm [56]. Moreover, sucrose octanoate (4,000 ppm) exhibited high pesticide activity against nymph and adult whiteflies [57].

The combination of antimicrobial activity with other functional properties such as emulsification and stabilization emphasizes the potential value of sucrose esters in the manufacture of foods, cosmetics and pharmaceuticals. But, care must be taken that other ingredients in a formulation do not impair their activity [50].

2.4 Lipase Catalyzed Synthesis of Saccharide-Fatty Acid Esters in Organic Solvent

Solvents are usually employed for the lipase catalyzed synthesis of saccharide-fatty acid esters on a laboratory scale to help co-solubilize saccharide and acyl donor. The solvent type has various effects on the lipase-catalyzed production of sugar-fatty acid esters. **Table 2.1** lists common organic solvents employed for the lipase-catalyzed synthesis of saccharide-fatty acid esters. Many literature examples also compare different types of solvents and sources of lipases to optimize reaction rate and yield. Recently, different lipases (from *Pseudomonas cepacia*, *R. miehei, Candida antarctica* and *Thermomyces lanuginosus*) and solvents (2-methyl-2-butanol, acetone, and methyl ethyl ketone, or MEK) were compared in lipase-catalyzed synthesis of lactose monolaurate and sucrose monolaurate using response surface methodology [58]. The optimal synthesis conditions occurred at 61 °C using in 2-methyl-2-butanol, *R. miehei* lipase (Lipozyme IM, Novozymes, Inc, Franklinton, NC USA, abbreviated "RML" in this dissertation), and vinyl laurate and lactose at molar ratio of 3.8:1 initially, yielding 99.3% conversion.

A Slovenian research group [59] investigated the influence of organic solvents (2-methyl 2-butanol, *ert*-butanol, acetone and MEK) on the synthesis of fructose palmitate. A conversion of 82% was obtained for MEK in 72 h at 40 °C using 10 % (w/w) molecular sieves for removal of the reaction product, water. In addition, *Candida antarctica* B immobilized lipase products (SP 435 and SP 382 from Novozymes, abbreviated "CALB" in this proposal) successfully catalyzed fructose-palmitic acid esterification to obtain 53% and 44% fructose palmitate in 2-methyl 2-butanol at 40°C under stirring at 600 rpm with molecular sieves. Under the same condition, 30% final yield was achieved by RML-catalyzed esterification. The initial

rate of esterification achieved using CALB was higher than achieved using RML for a common concentration of biocatalyst [59].

Table.2.1. Lipase-catalyzed synthesis of saccharide-fatty acid esters in different organic solvent systems

Solvents	Acyl acceptor	Lipase	Product	Yield (%)
Diisopropylether	Arabinose	PPL [a]	5-OAcetylarabinofuranoside	68 [61]
	Glucose		6-O-Acetylglucopyranoside	62 [61]
	Fructose		1-O-Acetylfructoside	70 [61]
Acetone	Xylitol	CALB [b]	Xylitoyl laurate	70 [62]
			Xylitoyl myristate	70 [62]
	Glucose	CALB [b]	Xylitoyl palmitate	98 [63]
	Methyl-α-D-glucoside	CALB [b] and RML[c]	6-lauroyl glucose ester	41 [64]
			methyl-A-D-glucopyranoside	
	α-D(+)-glucose	CALB[b]	D(+)-glucose palmitate	86 [65]
Dioxane	D(+)-glucose	CALB[b]	D(+)-glucose stearic	95 [65]
2-methyl-2-butanol	Sucrose	*Pseudomonas cepacia* lipase [d]	Sucrose monolaurate	34.2 ± 1.66 [58]

Table.2.1. Continued

Solvents	Acyl acceptor	Lipase	Product	Yield (%)
Ethyl methylketone	Lactose	RML[c]	Lactose monolaurate	52.4 ± 1.88 [58]
	D-fructose		Fructose oleate	83 [66]
	fructose		fructose palmitate	73.4 [67]
	α-Butylglucoside	CALB [b]	α-Butylglucoside Oleate	95 [68]
	Gulcose	CALB [b]	glucose stearate	93 [70]
	Galactopyranose	CALB [b]	galactopyranose sophorolipid	60 [71]
Tetrahydrofuran	D-psicose	CALB [b]	D-psicose dilaurate	90 [72]
Dimethylformamide	D-psicose 1,2-O -isopropylidene-D -xylofuranose	RML [c]	D-psicose dicaprate	86 [72]
	D-Glucose		xylose 5-arachidonate	85 [73]
			1- or 6-O-stearate glucose	83 [73]
Heptane	n-Butanol	CALB [b]	n-Butyl acetoacetate	24 [74]

Table.2.1. Continued

Solvents	Acyl acceptor	Lipase	Product	Yield (%)
Toluene	Glucose	CALB [b]	6-O-Vinylacetyl glucose	25 [75]
Acetonitrile	Mannose	RML [c]	6-O-Lauroyl mannose	15 [75]
		Thermomyces lanuginosus lipase [e]		74 [76]
		CALB [b]		60[77]
	D(+)-Mannose	CALB [b]	acyl mannoses	70 [78]
2-Methyl-2-propanol				

[a] PPL= porcine pancreatic lipase
Immobilized onto Accurel nylon beads, Novozymes, Inc., Franklinton, NC, USA
[b] Lipase B from CALB (lipase B, *Candida antarctica* lipase immobilized on Accurel EP-100) Novozymes, Inc., Franklinton, NC, USA
[c] Lipozyme IM (*Mucor miehei* lipase immobilized on an macroporous anion exchange resin) Novo Nordisk A/S, Bagsvaerd, Denmark
[d] Amano Lipase PS-C I (from *P. cepacia* (Lot# 07703EE)) Sigma–Aldrich ,St. Louis, MO, USA
[e] Lipozyme TL IM from *Thermomyces lanuginosus* immobilized on silica., Novo Nordisk A/S, Bagsvaerd, Denmark

The logarithm of a solvent's water/ octanol partition coefficient (*log P*), which is generally seen as a critical measure of solvent polarity, is also a significant parameter for enzyme activity and stability associated with lipase catalyzed synthesis of sugar-fatty acid esters in nonaqueous media [42,79]. *Log P* values for several common solvents are given in **Table 2.2**. Higher enzyme activities and stabilities are achieved using nonpolar solvents possessing *log P* values > 3 [80-82], which are also suitable for the lipase catalyzed interesterification of oils and fats [79]. However, the solubility of sugar in organic solvents with high *log P* values is very poor. **Table. 2.2** provides the activity and stability of lipase and glucose solubility in common organic solvents for the esterification of glucose and myristic acid. Although organic solvents have benefited many lipase-catalyzed reactions in laboratory-scale experiments as described in the literature, there are a limited number of organic solvents listed in **Table. 2.2** which performed adequately for lipase catalyzed synthesis of sugar-fatty acid esters, all of which possessed *log P* values between 0 and 1.5: *tert*-butanol, *tert*-pentanol, acetone, and tetrahydrofuran, or THF. More polar solvents with *Log P* values < 0 in general performed poorly, despite their ability to solubilize glucose. Polar solvents usually lead to the loss of enzyme activity by their ability to remove water molecules of hydration from the enzyme's microenvironment and by promoting the hydrolysis of ester products resulting in the decrease of product yield and formation of by-product [13, 60, 64]. In addition to improving substrate solubilization in the absence of enzyme inactivation, an ideal organic medium will be environmentally-friendly and easily recovered and recycled after completion of the reaction [9, 41, 60]. Of the moderately polar solvents listed above that performed best for lipase-catalyzed saccharide-fatty acid esterification, acetone, a solvent accepted by European economic community directives (88-344-CEE) as an extraction solvent in the manufacture of food products and additives [83], ranks highest in its achievement of the ideal solvent conditions .

Solvent-systems that combine two or more organic solvents have been useful. One binary solvent system (2-methyl-2-butanol: dimethyl sulfoxide, or DMSO, 4:1 v/v) was applied for the esterification of maltose, sucrose, and glucose by lipases from *T. lanuginosus* and CALB by combining a small fraction of solvent which greatly enhances saccharide solubilization at the expense of enzyme stability (DMSO) with a larger fraction of a solvent the allows for high enzyme activity but poorly solubilizes acyl acceptor (2-methyl-2-butanol). Using this approach, the conversion of sucrose monolaurate was > 80% using *T. lanuginosus* lipase [51]. Myristic acid esters of fructose, α-D-methylglucopyranoside and maltose was also synthesized successfully in a mixture of tert-butanol: pyridine 55/45 v/v) using immobilized CALB, achieving productivity values of 22.3 μmol min^{-1} g^{-1}, 26.9 μmol min^{-1} g^{-1}, and 1.9 μmol min^{-1} g^{-1}, respectively, even though neat pyridine denatures many lipases [84].

Although the utilization of organic solvents in enzymatic synthesis has some benefits, there are several substantial disadvantages for their employment for large-scale synthesis, such as the loss of enzyme activity they often cause and the concern for their impact on environmental and personal safety. Therefore, other alternative approaches are desirable.

Table 2.2. Activity of Lipase (Immobilized CALB lipase, Novozymes, Inc, Franklinton, NC USA), Glucose Solubility, and Enzyme Stability for the Esterification of Glucose and Myristic Acid as a Function of Solvent Hydrophobicity [66]

Solvents	Solvent hydrophobicity[a] (*log P*)	Enzyme activity[b] (imol/min g)	Glucose Solubility[c] (mM)	Residual activity[d] (%)
Dimethylsulfoxide	-1.3	0	29	0
Dioxane	-1.1	1.1	7.5	53
Dimethylformamide	-1.0	0	12	0
Acetonitrile	-0.33	0	1.1	27
Acetone	0.23	3.0	2.6	46
Tetrahydrofuran	0.49	1.6	2.1	46
Pyridine	0.69	0	134	0
tert-Butanol	0.80	3.7	12	75
tert-Pentanol	1.4	3.6	10	71
Toluene	2.5	0	0.6	54
Hexane	3.5	0	0	80

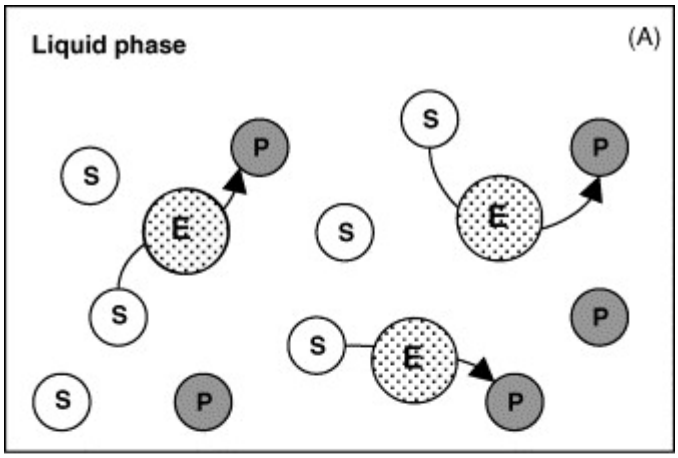
[a] After decanting the solvent, the immobilized enzyme preparation and the molecular sieves were washed 4 times with warm (45°C) *tert*-butanol followed by drying *in vacuo* for 2h. Additional molecular sieve (500 mg) was added before reuse.
from [79]
[b] The enzymes were recovered after 24 h of reaction to determine their residual activity.
[c] Determined after the initial 24 h of incubation at 45°C, before enzyme addition.
[d] Measured by reacting 150 mg glucose x $1H_2O$ and 750 mg myristic acid in 5 ml of solvent in the presence of 35 mg lipase and 0.5g molecular sieves at 45°C and rotary shaking (250 rpm) for 24 h. The reaction was carried out at 45°C (250 rpm).

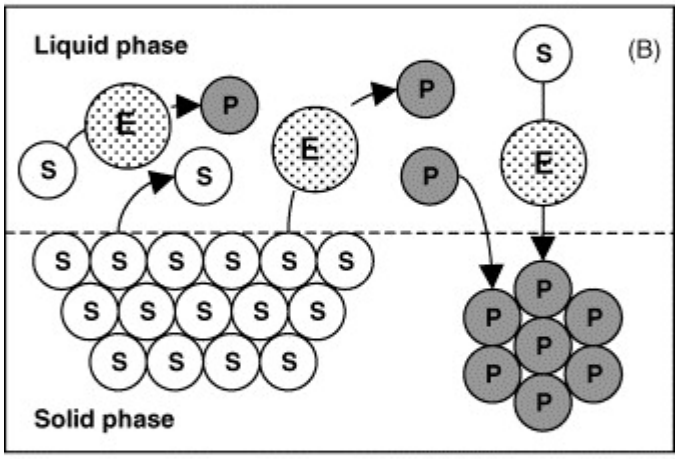
2.5 Lipase Catalyzed Synthesis of Saccharide-Fatty Acid Ester Reactions Using Eutectic Mixtures

Another approach invented for lipase-catalyzed synthesis of saccharide fatty acid is to employ a mainly solid-phase system composed of saccharide, fatty acid and product in the presence of a small amount of organic solvent (e.g. *tert*-butanol or acetone) acting as adjuvants to create small catalytic liquid phases at near-ambient conditon [85]. Compared with reactions in organic solvent media, the initial reaction rate and overall productivity of reaction in "solid-phase" systems are often improved several-fold [65]. As shown in **Fig. 2.2 A&B**, the success of this approach can be attributed to the solid-phase substrate acting as a "reservoir" to replenish liquid-phase substrate converted to product. Also, the precipitation of product simplifies downstream product purification and can enhance product selectivity. For instance, saccharide-fatty acid monoester product formed in "solid-phase media" readily precipitates, lessening the ability of lipase to catalyze diester formation. In **Fig. 2.2.C**, the eutectic mixture consisting of solid and liquid phase provides a method which decreases the melting point of a mixture, even lower than the melting point of each pure compound in the mixture [86]. An organic solvent usually decreases the melting point resulting in a eutectic mixture at room temperature or below [86]. The eutectic mixture contains mostly substrate molecules and a small amount (5–30%, gram-solvent per gram-total substrate mixtures) of organic solvent in **Fig. 2.2.C.** Eutectic media displays a stable biocatalytic liquid phase with extremely high concentration of substrates. The lowest melting point, called the eutectic point, can be identified at a specific composition of the mixture. Due to the utilization of small amounts of organic solvents in eutectic media, they can be considered to be more biocompatible and environment friendly for enzymatic reactions than in pure organic phase media [87-88]. Although acyl acceptors are almost insoluble in the catalytic liquid phase of

system, final products in high yield composed of most monoesters were obtained due to crystallization of monoesters in the catalytic liquid phase. The final conversion of β-D(+)-glucose with palmitic acid was up to 86% at 48 h [65]. The reaction rate for the esterification of β -D(+)-glucose with stearic acid was equal to 0.4 mmol saccharide fatty acid ester per gram CALB lipase per hour [89]. The acylation of β-D (+)-glucose with palmitic acid in *tert*-butanol by CALB was synthesized in a solid-phase system with highest selectivity (98% monoacylation). The selectivity in the acylation of fructose has the significantly relationship with fatty acid chain length and the type of organic solvent. Monoacylation was created in the presence of less hydrophobic solvents (e.g. *tert*-butanol) [90]. In the solid-phase system, the polarity of the biocatalytic phase has essential relationship with the fatty acid. In contrast, the organic solvent is insignificant for the polarity of the reaction phase. The influence of the saccharide or saccharide-fatty acid ester (desirable products) on lipase stability can be neglected due to their low solubility in the system [91].

Although the synthesis of fatty acid ester in the solid phase system exhibits high reaction rate, quantitative saccharide-fatty acid ester yield and high selectivity, they are organic solvent based reactions leading to the increase of the cost of process and its inherently batch nature has a negative effect on continuity of the process [60].


Liquid phase
(A)
S
P
E


Liquid phase
(B)
S
P
E
Solid phase

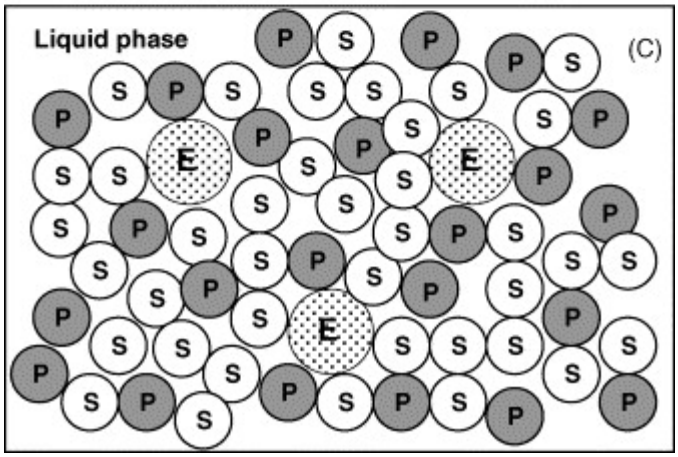

Liquid phase
(C)
S
P
E

Fig.2.2. Schemes of substrate mixtures in organic solvent media (A), solid phase media (B), and eutectic media (C); S: substrate; P: product; E: enzyme [85]

2.6 Synthesis of Saccharide Fatty Acid Ester in Two-Phase Supercritical Carbon Dioxide Systems

The supercritical carbon dioxide, as an interesting alternative medium for synthesis of saccharide-fatty acid esters, exhibits several advantages over organic solvents as reaction media due to its nontoxicity, low flammability, low cost operating condition (temperature > 31°C; pressure > 7.3 MPa) compared with traditional chemical synthesis approaches. The solvent power of supercritical CO_2 is controlled through adjusting temperature and pressure. The simple recovery process for products and enzyme particles from reaction media through a series of depressurization steps is an additional benefit [93]. Experiments demonstrate the stability of enzymes in supercritical carbon dioxide is satisfactory and comparable with enzyme stability in organic solvents [94].

A supercritical carbon dioxide batch-stirred-tank device (**Fig. 2.3**) was created to catalyze fructose-palmitic acid esterification in the presence of lipase from CALB (Novozym 435, Novozymes, Inc.). Sixty percent conversion was obtained at 60 °C and 10 MPa after 24 h [95]. Since only palmitic acid is soluble in the supercritical phase, the downstream isolation of the fructose palmitate was easily achieved [95]. Furthermore, silica gel used as saccharide solid support material was introduced into lipase-catalyzed reaction with 50-60% yield and high selectivity of final product (most monoester) [93].

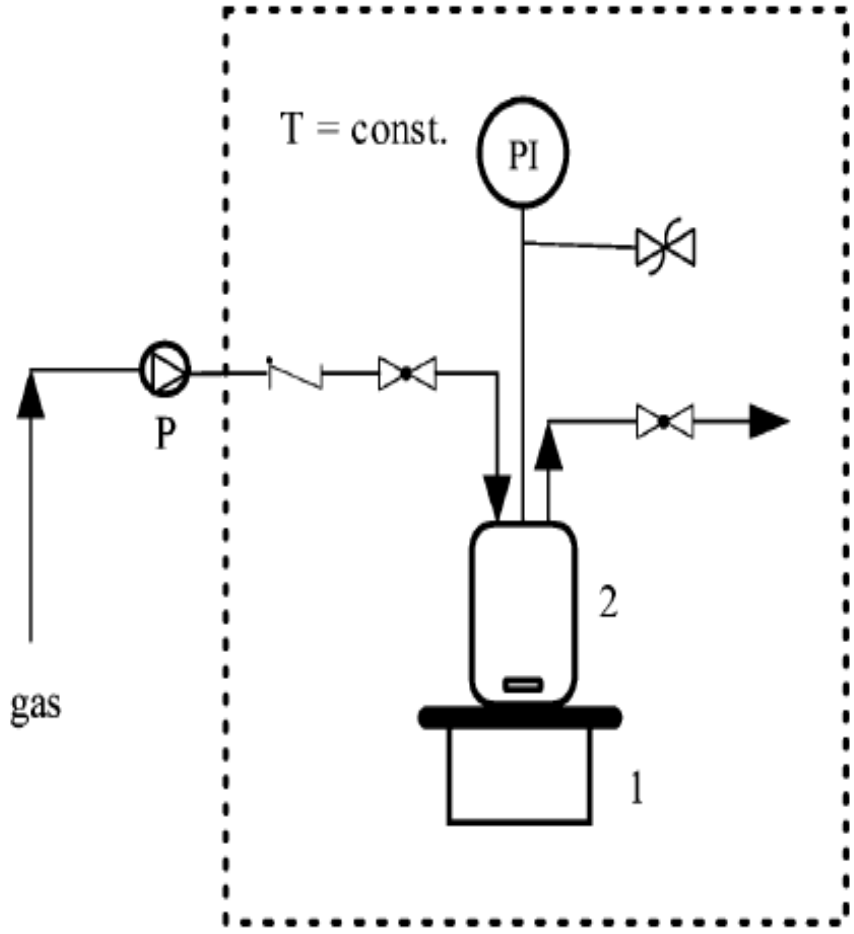


Fig. 2.3. Schematic diagram of the supercritical-CO2 bioreactor apparatus employed for lipase-catalyzed synthesis of saccharide-fatty acid esters: (1) magnetic stirrer and heater; (2) bioreactor; (P) high-pressure pump; (PI) pressure indicator [95] .

A high-pressure acetone–CO_2 supercritical phase system has been employed as a medium for glucose palmitate ester synthesis catalyzed by CALB (at 65 bar and 50 °C using 3% (v/v) acetone [92]. The reaction mechanism for the esterification of palmitic acid and glucose in a high-pressure acetone–CO_2 system, shown in **Fig. 2.4,** consists of glucose particles suspended in the reaction mixture [92]. A pseudo- dynamic equilibrium was assumed between glucose, palmitic acid, glucose palmitate, acetone, CO_2, and H_2O, as depicted in **Fig. 2.4.** The top or "light" phase consisted mostly of acetone and CO_2, with substrates and products present at small concentration, served as the reaction phase utilized by lipase. Solid glucose in the "heavy" phase served as a reservoir, with glucose molecules diffusing to the light phase to replace light-phase glucose molecules converted to ester. Glucose palmitate and water precipitate as a solid and liquid, respectively, into the heavy phase which contains only small amounts of acetone and CO_2. Excess water as a by-product from esterification can be removed without applying water removal methods since water is transferred to the heavy phase when its concentration increases beyond the solvent capacity of the light phase. The apparatus for this experimental approach, shown in **Fig. 2.5** is composed of a CO_2 cylinder, a cooler for cooling of CO_2, a pump for delivery of pre-cooled carbon dioxide, several pressure gauges, a high pressure pump, and a stirred tank with a thermal controller, an indicator and a heating jacket. The reaction was started when all reactants, solvents and lipases were introduced into the reactor at the desired temperature and pressure with pre-cooled carbon dioxide.

Although supercritical carbon dioxide is a useful and promising substitute for synthesis of saccharide fatty acid, two main unavoidable disadvantages of must be overcome: the low solubility of non-polar compounds and the high capital and operating costs [96].

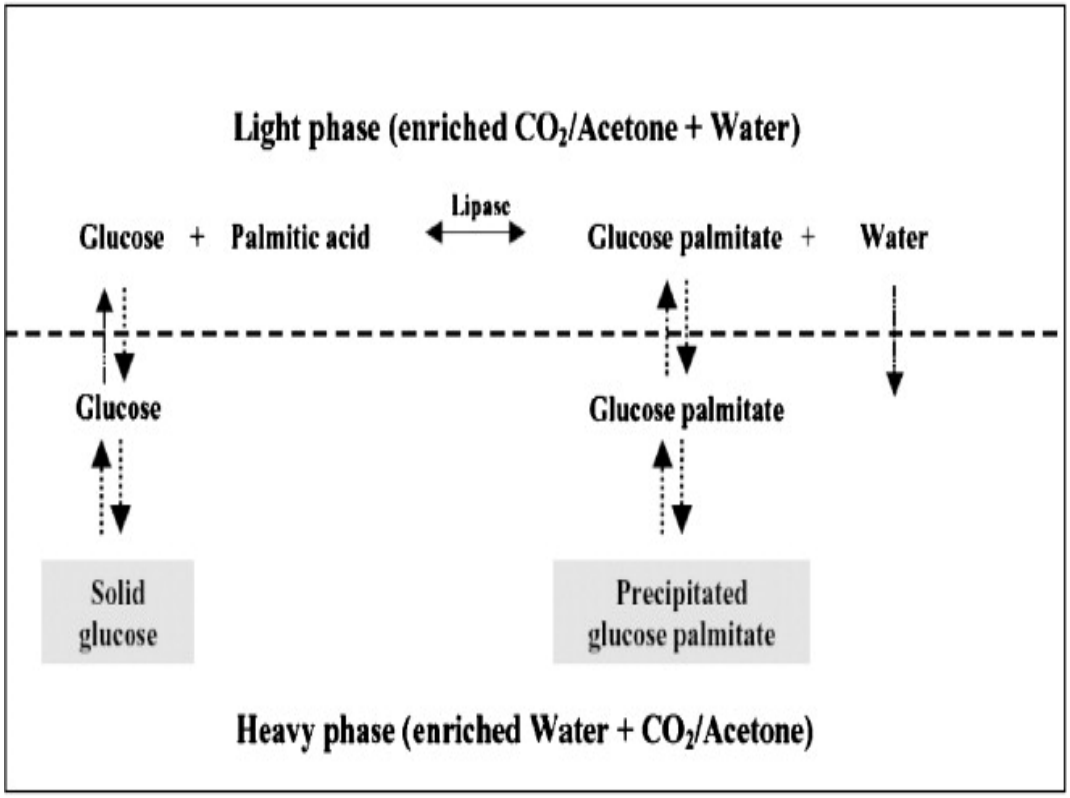


Fig.2.4. Reaction mechanism for the esterification of palmitic acid and glucose in a high-pressure acetone–CO2 system [92]

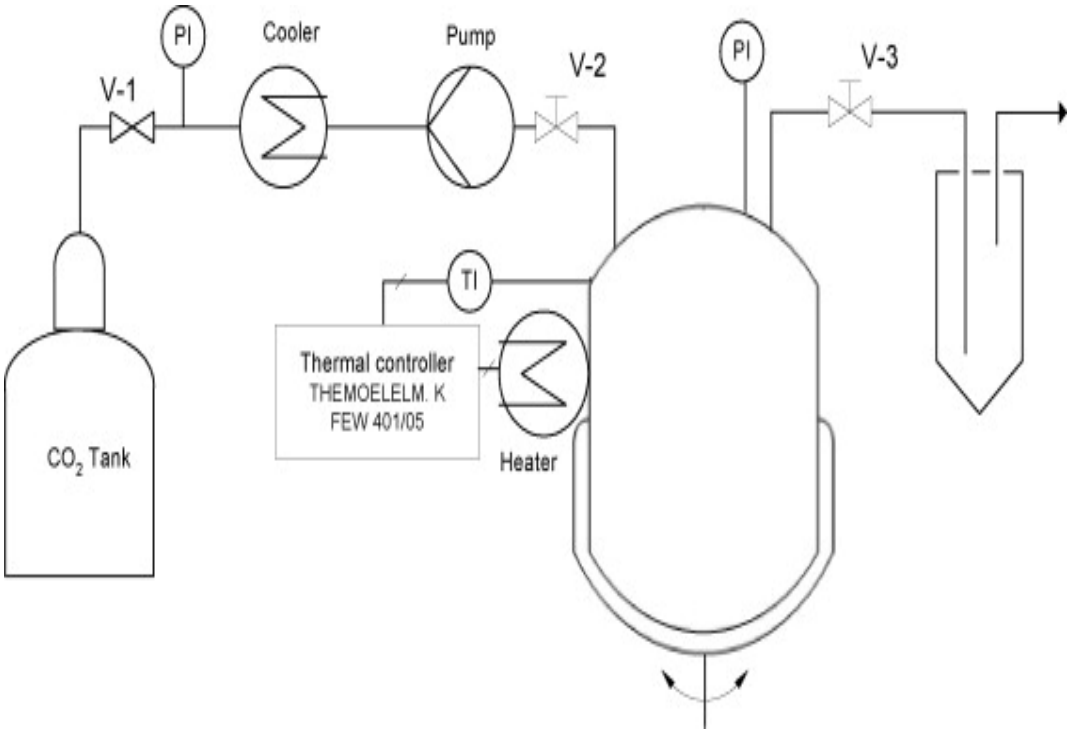


Fig. 2.5. Schematic diagram of the reactor unit for the esterification of palmitic acid and glucose in a high-pressure acetone–CO2 system. V-1: pressure valve; V-2: pressure valve; V-3: pressure valve; PI: pressure unit meter; T1: thermal controller [94]

2.7 Synthesis of Saccharide-Fatty Acid Esters in Ionic Liquids

Ionic liquids (ILs), consisting of organic salts that are liquid-phase at temperatures near ambient, represent a unique class of non-aqueous and polar solvents media receiving increasing attention for organic synthesis and biotransformations due to their absence of vapor pressure and excellent chemical and thermal stability [96-98]. Because of low melting points and non-volatile nature, ionic liquids, called "room-temperature environmental" or "green" solvents are attractive alternative for volatile organic solvents [48, 99]. More importantly, their widely tunable properties with regard to polarity, hydrophobicity and solvent miscibility behavior can be modified by selecting the chemical structure of their cation and anion moieties [100].

For most nonaqueous enzymology studies, relatively nonpolar ILs such as 1-butyl-3-methyl imidazolium hexafluorophosphate or 1-butyl-3-methyl imidazolium tetrafluoroborate (Bmim][PF_6], [Bmim][BF_4] respectively) can be used as the replacement of organic solvents. For an investigation of thermolysine and CALB, are no difference for the stability and selectivity of the enzymes in ILs compared to hexane, THF and acetonitrile, for biocatalytic reactions [98,177]. Many studies have been done on stability of enzymes in ILs, especially for lipase. For synthesis of saccharide-fatty acid esters, lipase activity and operational stability in [Bmim] [PF_6] was comparable to hexane and superior to other ILs, toluene, and THF [101-102]. The balance between kosmotropic and chaotropic ions of ionic liquids, natural properties of the enzyme, and water content in the system will be significant factors to influence the performance of enzyme activity in ILs [99]. In addition, ILs that consists of a "free" hydrophilic anion or cation and a hydrophobic alkyl chain are

structurally similar to surfactants and hence potentially valuable for enhancing solubility of acyl acceptor substrates. Plus, ILs can improve the enantioselectivity of lipase-catalyzed transesterifications [102-105].

Bornscheuer‘s group discovered that CALB modified by covalent attachment of poly (ethylene glycol) catalyzed the esterification of glucose and fatty acid in pure [Bmim] [BF_4] and [Bmim] [PF_6] with 30% and 35% conversion achieved, respectively [104]. Although 30% ~ 35% conversions are low, the addition of 40% *tert*-butanol to the ILs, producing a two-phase system, increased the conversion to 89% [104]. Another system consisting of an ionic liquid ([Bmim] [BF_4] or [BMIM] [PF_6]) and *tert*-butanol was prepared for lipase-catalyzed synthesis of glucose-fatty acid esters. The final conversion achieved was 60% in [Bmim][PF_6] and *tert*-BuOH when employing fatty acid vinyl ester as acyl donors in the presence of molecular sieves (10% w/v) and CALB at 60 °C [60]. This result presumably indicates *tert*-BuOH could improve the miscibility of substrates.

Zhao and co-works examined the solubility of D-glucose and sucrose in different ILs (Table 2.3). An IL (1.0g) in a glass was placed in an oil bath at 60^{o}C. D-glucose or sucrose (5 mg) was slowly introduced into IL. Then, the glass tube containing IL and sugar was clear after a vigorous agitation by hands. This procedure was repeated until the IL did not turn clear. It means the saturation limit was reached. [$Me(OEt)_3$-Et-Im][OAc] exhibits the good capability for the solubility of D-Glucose 80 wt% of ionic liquid shown in **Table. 2.3** [107]. Furthermore, Lee and coworkers created a novel procedure for the formation of supersaturated sugar/IL solution stirring a water-soluble saccharide solution and IL. Subsequently, the water was removed from the mixture solution by vacuum evaporation for 12 h at 60°C. Through this method, the glucose concentrations achieved in [Emim][TfO] and [Bmim][TfO] were 19 and 10-fold higher, respectively, compared to the solubility of glucose in

the ILs at room temperature. Furthermore, the stability of supersaturated glucose solutions in ILs was stable for 1 day and the glucose concentration was decreased by 87% compared to the initial concentration after 3 day [108]. The supersaturated solution when employed for lipase-catalyzed esterification produced a 96% yield of only monoester (6-*O*-lauroyl-D-glucose) in 1 day. Specifically, a high initial rate (15.0 μmol/ (min g)) in [Bmim] [TfO] was achieved when supersaturated glucose/IL solution was employed. These results indicated the utility of supersaturated solutions for lipase-catalyzed saccharide-fatty acid esterification [108].

Table 2.3. The solubility of D-glucose and sucrose in different Ionic liquids at 60^0C [107]

IL	Sugar	Solubility (wt%)
[BMIM][Tf_2N]	D-Glucose	<0.5
[EMIM][OAc]	D-Glucose	60
[Amm110][dca]	D-Glucose	4.5
	Sucrose	3.5
[Amm110][OAc]	D-Glucose	30
[$Me(OEt)_3$-Et-Im][OAc]	D-Glucose	80
[$Me(OEt)_7$-Et-Im][OAc]	D-Glucose	26
[$Me(OPr)_3$-Et-Im][OAc]	D-Glucose	45
[$Me(OEt)_3$-Et_3N][OAc]	D-Glucose	16
	Sucrose	16

2.8 Synthesis of Saccharide-Fatty Acid Esters in Solvent-Free Media

Solvent-free media is desirable for the lipase-catalyzed synthesis of sugar-fatty acid esters due to low cost, improved operational safety, and improved biocompatibility for food, cosmetics, and pharmaceutical applications [109]. Previously, sugar-fatty acid esters were synthesized at 1:1 and 2:1 molar ratios of fatty acid acyl group to sugar acyl acceptor at 75°C in open vials or under vacuum to remove byproducts (water or methanol produced when employing free fatty acid or fatty acid methyl ester as acyl donor, respectively) [110]. Solvent-free enzymatic synthesis of isopropylidene-derivatized xylofuranose fatty acid esters produced yields of 83–85% under optimal conditions using RML at 60°C. The enzyme was easily recovered after the reaction to allow for its reuse [72]. Evaporation of organic solvents from reaction mixtures during the initial phase of the time course of reaction is a related approach. This method was used for an initial reaction mixture containing 100 mM palmitic acid and 100 mM acyl acceptor (D-glucose, D-fructose, D-galactose, D-sorbitol, L-ascorbic acid or methyl-D-glucose) in 5.0 ml organic solvent (acetone, 2-methyl-2-butanol, *tert*-butanol, MTBE, or n-hexane) in the presence of molecular sieves (1.0g). The highest yield (76.0%) was obtained from palmitic acid and glucose using CALB at 45°C [112]. To achieve solvent-free conditions during the time course of reaction, solvents with low boiling points can be employed since they can be easily recovered and possibly reused [63, 113]. For this purpose, *tert*-butanol, a very effective solvent to co-solubilize fructose and oleic acid, was utilized by Hayes and co-workers to produce fructose-oleic acid ester at the initial stage of the reaction [83]. The reaction rate was enhanced over 10-fold higher at a *tert*-butanol concentration of 0.35–0.55 w/w and *tert*-butanol was removal during the middle-to-latter

stage of the reaction since the presence of *tert*-butanol impeded the reaction rate at latter stage [114]. For synthesis of saccharide-fatty acid ester, the poor miscibility of saccharide in the reaction media is the significant obstacle in solvent-free condition. The saccharide-fatty acid ester products greatly enhance the miscibility of the acyl donor and acceptor substrates. The apparent solubility of saccharide was significantly improved linearly from 0.002 to 0.13 g/g in the presence of mixtures of oleic acid substrate and fructose mono-and di-esters products at 60 °C [84]. A ternary phase diagram for the ternary system saccharide (fructose)/ oleic acid/fructose–oleic acid monoester (ME) at 60°C is illustrated in **Fig. 2.6** [115].

To further apply the enhanced miscibility imparted by saccharide-fatty acid ester products, Hayes and co-workers designed bioreactor systems for lipase-catalyzed esterification of saccharide fatty acid esters in solvent-free media at 65^{o}C [116-117]. Different bioreactor systems and modes of operation (e.g., fed-batch mode and employment of packed bed or continuously stirred tank bioreactors) in combination with a packed saccharide column for delivery of the acyl acceptor were developed, as depicted in **Fig. 2.7.** The fructose / silica gel packed column was believed to deliver saccharide at saturation concentration. The desorption of saccharide from the column increased as the concentration of the fructose-oleaic acid ester, FOE, in the liquid phase increased. The equilibrium partitioning of saccharide between the liquid and stationary phases was described by the Freundlich isotherm [116]. The highest conversion of 85% was obtained by utilization of a packed-bed bioreactor and operation through continuous recirculation. Control of water content was achieved by free evaporation during the entire time course of reaction plus the addition of 10 wt % molecular sieves during the latter period of the time course. However, the reaction rate was several-fold lower than the batch mode reactions due to the lower fructose concentrations [117]. The main goal of the

preliminary research is to understand why the reaction rate was too slow and make corrections in the bioreactor design accordingly.

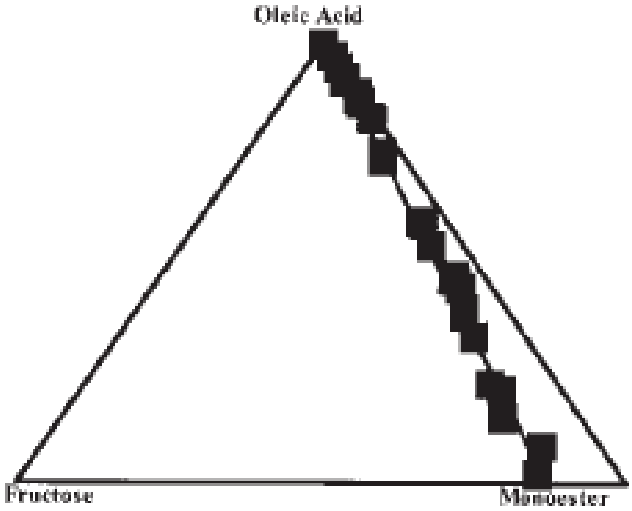


Fig.2.6. Ternary phase diagram for fructose/oleic acid/technical-grade monoester (5% diester and 95% monoester) at 60°C. A one-phase liquid mixture (saccharide suspended in the liquid phase) exists to the right of the phase boundary, two-phase media to the left of the boundary [84]

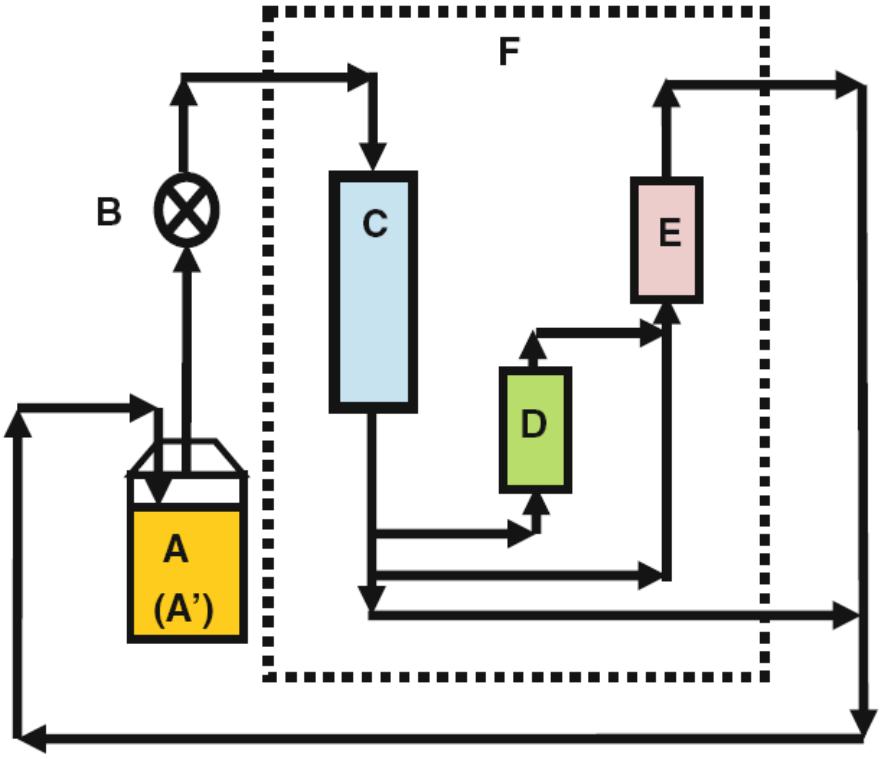


Fig. 2.7. Bioreactor systems for solventless synthesis of fructose–oleic acid ester, FOE, that undergo continuous recirculation and utilized a packed bed column of silica gel and saccharide A. Reservoir tank (no lipase), A'. Stirred tank bioreactor (STBR; with lipase) heated by hot plate to 65°C, B. Peristaltic pump (0.1 ml/min), C. Fructose desorption column (DC; 100 9 10 mm ID), D. Molecular sieves column (MSC; 1 g, 50 9 10 mm ID), E. Packed-Bed Bioreactor (PBBR; 50 9 10 mm ID) packed with RML, F; 65°C oven [117]

2.9 Kinetic Models of Lipase-Catalyzed Esterification of Saccharide-Fatty Acid Esters

Several different reactions are catalyzed by lipases: alcoholysis, transesterification, esterification, acidolysis, and hydrolysis [118]. In this section, kinetic models regarding esterification are described.

The Michaelis–Menten kinetic mechanism which is mathematically similar to Langmuir-Hinshelwood kinetics has been applied as a kinetic model for lipase-catalyzed reactions. The whole process is defined by two steps. Firstly, lipase opens the lid which covers its active site once it is activated at an interface. Secondly, product is generated by the hydrolysis of the enzyme-substrates intermediate eventually and the regenerated enzyme will be repeating the same action on the next recycle [118].

However, a primary underlying assumption is that the enzymatic reaction must take place in isotropic medium, i.e., substrates and enzymes are in the same phase. The Michaelis–Menten kinetic mechanism applies to irreversible reactions. Therefore, the Michaelis–Menten kinetic mechanism is obviously inadequate for the model to describe the action of lipolytic enzymes at the interface between the lipid (rich nonpolar) phase and the water phase [119]. The most common and accurate description of the catalytic process of lipases is a Ping-Pong Bi Bi model [119]. Two major steps constitute the entire mechanism. In the first step, nucleophilic attack occurs on the acyl bond through the lone-pair electrons of the oxygen atom contained on the hydroxyl group of serine at the active site after opening of the lid [119]. Then, the process causes the formation of an acyl-enzyme intermediate [119]. Subsequently, hydrolysis of the acyl-enzyme complexation occurs leading to production of the desired product and employment of recycled enzyme [119].

Due to many different species for lipases, different substrates, and non-enzymatic side reactions (e.g., deactivation of the enzyme or intramolecular migration of an acyl group) existing in lipase-catalyzed reactions reaction, it is challenging to develop a kinetic model [120]. As a consequence, most investigations of lipase kinetics have concentrated on model systems based on several assumptions which cover only a few specific reactions [120]. A simple kinetic model derived from a Ping-Pong Bi Bi mechanism to characterize the rate of acylation of glucose with lauric, palmitic, and stearic acids in the presence of CALB in acetone was successfully developed by Arcos *et al*., utilizing a few assumptions. First, reverse-direction enzymatic reactions were ignored. Second, the dissolved glucose concentration was assumed to be constant [120]. The mathematical model from this work has been verified by several sets of experimental data under different reaction conditions. The results indicated that the model fits the experimental data very well for temperatures from 30 to 60 degrees C, enzyme loadings from 90 to 180 mg, and fatty acid concentrations from 0.33M to 1M. Another research group proposed a full reversible kinetic model based on a Ping- Pong Bi Bi mechanism which represents the acylation of glucose by lauric acid in 2-methyl 2-butanol mediated by CALB at 60°C. The developed model displays a good fit of experimental results [121]. Dang and his coworkers developed a good linear kinetic Ping-Pong Bi Bi model regarding feed batch addition of saccharide during synthesis of fructose-oleic acid esters in agreement with measured data for the time course of the reaction [115].

2.10 Bioreactor Types for Lipase-Catalyzed Synthesis of Saccharide-Fatty Acid Esters

The production of saccharide-fatty acid esters needs to be economically feasible for practical industrial application. Bioreactors are vital components of scaling up of bioprocess. Several types of bioreactors have been employed: batch stirred-tank reactors (BSTRs), packed-bed bioreactors (PBBRs), fluidized-bed reactors (FBRs), and enzymatic membrane reactors (EMR) [122,137,138,139,140]. Three major processes associated with physicochemical processes have to be considered for bioreactors employing immobilized enzymes: 1) transport of the substrate (product) molecules of reactants from (to) the bulk phase to (from) the boundary layer surrounding the immobilized lipase, and transport across a boundary layer by diffusion 2) transformation of the substrate molecules by chemical reaction catalyzed by the immobilized lipase, and 3) deactivation of the immobilized lipase [122].

Generally, BSTRs are useful for preliminary screening of enzymatic reactions [123]. This configuration is the most commonly employed for of saccharide-fatty acid ester synthesis in literature reviews. BSTRs [124] consist of a vessel containing the reactant fluid mixture which is stirred by mechanical and physical methods (e.g., magnetic bars, reciprocal oscillators, submerged impellers, and end-over-end rotators) to avoid the formation of temperature and concentration of enzyme gradients. Immobilized enzyme is separated from the reaction medium at the end of the reaction by filtration or centrifugation. These reactors are easy to operate (e.g., to heat, cool, clean, and maintain) normally without the assistance of additional equipment. For example, monolauroyl maltose was selectively synthesized by an immobilized lipase in acetone in a BSTR or a continuous stirred tank reactor [125]. In addition, STBRs were employed for the synthesis of 6-O-stearoyl-D-glucose monoester in a mainly

solid phase system in the presence of a small amount of solvent, MEK which was regenerated by with a membrane-pervaporation separation unit [126]. Recently, scale-up has been investigated for lipase-catalyzed alcoholysis of palm oil with oleyl alcohol in *n*-hexane using a batch stirred-tank reactors with a single impeller mounted on the centrally located shaft. Compared to aluminum-hydrofoil and 2-bladed elephant ear impellers (2L, BiostatMD, B.Braun, Germany), rushton turbine impellers in batch mode was the most effective providing the highest reaction yield (95.8%) and homogenous enzyme particles suspension at 250 rpm. High stability and usability of RML was observed, with high yield (79%) occurring after 15 cycles of repeated batch reactions. Subsequently, the process was successfully scaled up to a 75 L stirred-tank reactor at constant impeller speed and a yield of 97.2% was obtained after 5 h reaction time [127]. Compared to only 70.1% for the 2L STBR in 2h, the product yield in 75 L stirred-tank reactor reached 88.8% in 2h, probably since the multiple impellers in large scale reactor improve effects of mass and heat transfers than single impeller in small scale reactor [127-128].

However, some undesirable properties of BSTRs limit their large-scale use in industry based on economic considerations. Firstly, the required operations of emptying, cleaning, and filling between batch runs will lead to dead times during the industrial process, which will decrease the productivity [122]. Secondly, additional steps would be required for separation of catalysis particles from final products. Thirdly, immobilized enzyme granules can be broken apart by impellers operating at high stir rates [122].

For packed bed bioreactors (PBBRs), the reaction mixture can be transported upward to avoid downward taking advantage of gravitational flow [122]. For two-phase media, the streams can be transported counter- or co-currently [122]. PBBRs have extensively investigated for industrial scale applications of enzyme-catalyzed reactions according to the literature. There are several

benefits for employing PBBRs. First, they assist downstream separation of product and biocatalyst. Second, the exit stream carries away possible enzyme inhibitors. Third, they facilitate reuse of the enzyme and improved enzyme stability. Fourth, they effectively catalyze streams possessing low substrate solubility. Fifth, their operation is simplified through automation [122]. Sixth, they are more appropriate for long-term and industrial-scale production than a stirred-tank reactor since mechanical shear stress of an impeller results in breakage of immobilized enzyme granules. Seventh, it is more cost effective than the batch operation [123]. The ratio between enzyme and substrates in a PBBR is low compared to other type of reactors resulting in the low cost. Therefore, a PBBR is suitable for most large-scale biocatalytic reactions [123,134,135].

Several research groups reported employment of PBBRs for the successful lipase-catalyzed synthesis of polyol-fatty acid esters. Mono- and dilauroyl arabitols, ribitols, xylitols and sorbitols were continuously synthesized at 50°C or 60°C using a system consisting of a packed column of sugar alcohol and a packed-bed reactor containing CALB in the presence of solvents (**Fig. 2.8**) [129]. Esters of L-ascorbic acid and several acyl donors were continuously produced at 50°C using a column packed with ascorbic acid powder and a PBBR containing CALB with the productivity of 1.6–1.9 kg/L achieved during an 11 day period [130]. Erythritol and medium-chain fatty acids in acetonitrile with 1% v/v water were esterified by CALB at 50°C using a PBBR at 70% conversion in a 10 day period [131]. In addition, PBBRs containing CALB were operated at 60 °C for the continuous production of 6-O-linoleoyl mannose, glucose and galactose in acetone. The high solubility of mannose in acetone among the hexoses yielded the highest production of ester [132]. Additionally, PBBRs have been investigated by the lipase-catalyzed synthesis of sugar-fatty acid esters in solvent-free media. Hayes and co-workers for the synthesis of fructose- and sucrose-oleic acid esters employed a bioreactor system

containing a packed "desorption" column which contained fructose crystals and silica gel for delivery of saccharide, and a PBBR, operated under continuous recirculation. The highest conversion (84.4%) was achieved in the presence of 10 wt % molecular sieves, added during the latter stage of reaction. However, the reaction rate was several-fold lower than achieved during batch mode reactions since concentrations of fructose provided by the desorption column were quite low [133].

EMRs were employed for the lipase-mediated esterification of sorbitol and fatty acid, in a two-phase media containing lipase immobilized at the inner fiber side of the membrane. Fatty acid was circulated in the internal circuit initially and a solution of sorbitol, 2-pyrrolidone, and buffer were circulated in the external circuit. Trans-membrane diffusion of 2-pyrrolidone occurred until equilibrium was reached. 80% yield was obtained after 74 h [136].

2.11 Water removal strategies during lipase-catalyzed synthesis of saccharide fatty acid esters

Water “content” refer to the quantity of water in a system or material [141]. Water “activity” (a_w), a thermodynamic property applying to single- or multi-phase systems at equilibrium, refers to the ratio of the partial pressure of water in the air headspace and vapor pressure of (pure) water at the reaction system’s temperature [142]. For enzyme-catalyzed reactions, water content and activity are importance parameters. A small amount of water is essential for the structural integrity, the active site polarity, and conformational stability of enzymes [143-145]. With an increase of water, flexibility and the expressed activity of the enzyme are enhanced [144]. Water facilitates bond formation

with the polar amino acids on its surface, leading to retention of its three-dimensional structural conformation. Otherwise, the absence of a proper amount of water may change bond formation, which could lead to an inactive conformation, intermolecular aggregation, or refolding [144-148]. However, an excess amount of water promotes the shift of the thermodynamic equilibrium toward the hydrolysis of esters, leading to low final conversions. Ideally, the reaction system will maintain an optimal water amount in the reaction mixtures. Therefore, many strategies have been applied for a continuous removal of the water generated during esterification [109,149-151].

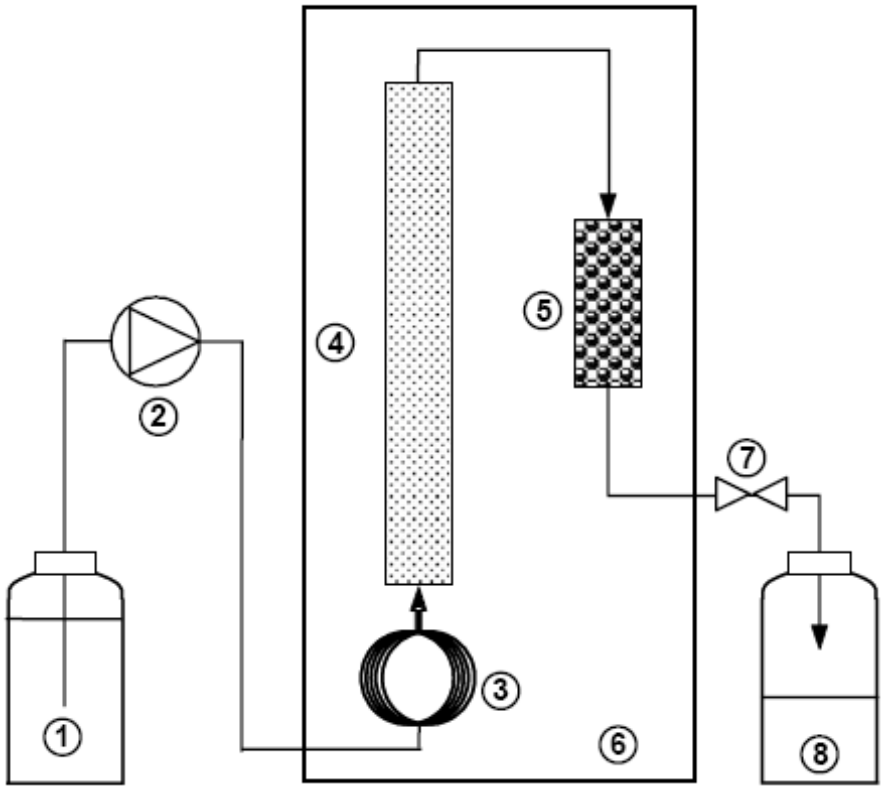


Fig. 2.8. Packed bed bioreactor system used for the continuous synthesis of biobased surfactant. 1 feed reservoir of fatty acid and acetone; 2 pump; 3 preheating coil; 4 a column packed with sugar alcohol powders; 5 a reactor packed with CALB; 6 temperature-controlled oven; 7 back-pressure regulator; 8 effluent reservoir [129]

Molecular sieves have been used frequently to removal excess water according to the literature. Sabeder *et al.* investigated the influence of molecular sieve concentration on fructose palmitate synthesis after 72 h of reaction (**Fig. 2.9**) [59]. The equimolar mixture (0.5 mmol) of fructose and palmitic acid was dissolved in 2-methyl 2-butanol at 60 °C and mixed with lipase at 600 rpm. The optimal concentration of molecular sieves was 12.1% (w/w of reaction mixture). With the increased amount of molecular sieves (**Fig. 2.9.**), the final yield of products decreased due to the excessive extraction of water from enzyme, resulting in the loss of enzyme activity [59, 93]. In another investigation, for the synthesis of fructose palmitate in 2-methyl 2-butanol, the steady- state concentration of the final product was improved by 100 % (from 16.5 g L^{-1} to > 32 g L^{-1}) and the initial rate of reaction is enhanced from 4.9 to 10.1 g L^{-1} h^{-1} through the addition of 4A molecular sieves to the reactor [67]. For the lipase-catalyzed synthesis of fructose oleate under solvent-free or nearly solvent-free conditions, Hayes *et al.* found the addition of molecular increased the conversion of final yield from 78.7% to 83.2% [117].

The type of molecular sieve employed has a strong effect on the removal of water. In 2-methyl-2-propanol and 2-methyl-2-butanol, 4A molecular sieve adsorbed slightly more water than 3A molecular sieves. Conversely, the performance of 3A molecular sieves was better than 4A molecular sieves in acetonitrile [153-154]. There are some disadvantages for utilization of molecular sieves. First, visual observations from experiments indicated that inhomogeneous stirring of a reaction mixture with molecular sieves led to limited mass transfer between liquid and solid phases [59, 93]. Consequently, molecular sieves are not suitable for the larger-scale production [155]. Secondly, the presence of molecular sieves can shift enzyme selectivity in favor of di- and poly-ester production due to the removal of water from the enzymes' active sites, leading to an increased hydrophobicity of the enzymes' microenvironment [67, 153].

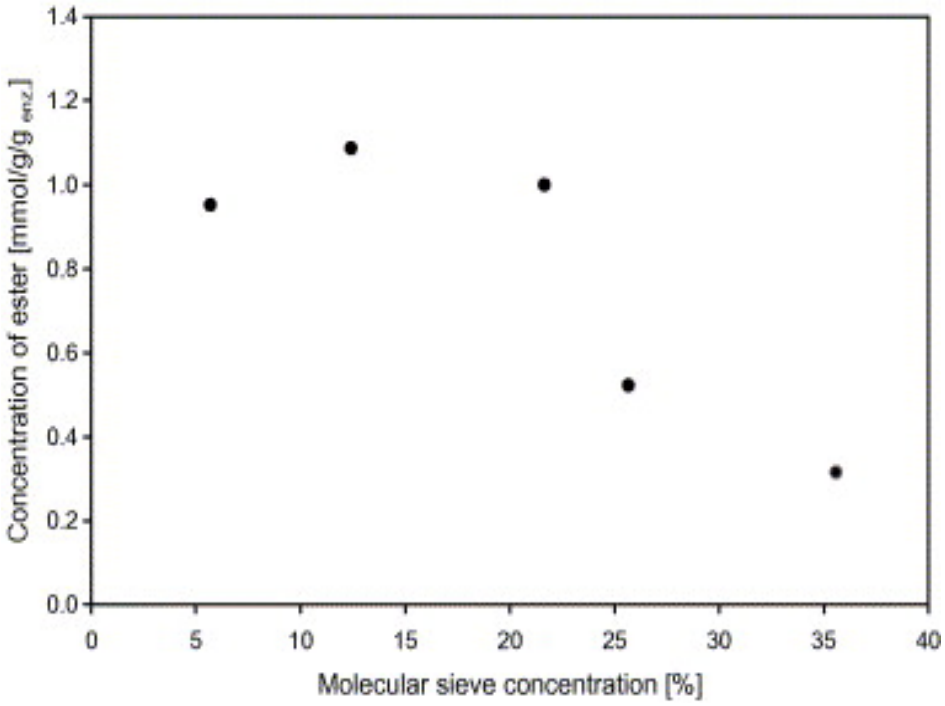


Fig. 2.9. Influence of molecular sieve concentration on ester concentration after 72 h of reaction performance. Reaction conditions: 0.5 mmol fructose, 0.5 mmol palmitic acid, 0.6 mL 2-methyl 2-butanol, 22.5 mg lipase SP 435, 60°C, 600 rpm [59].

Many research groups have employed silica gel to remove the excess water during the lipase-catalyzed reactions [156-158]. In the early literature, one group demonstrated the capability of silica gel to extract water continuously formed from esterification, leading to a shift in the equilibrium efficiently toward synthesis [158]. 60% palmitate conversion was achieved in the presence of silica gel. In contrast, only 20% conversion of palmitate was obtained in the absence of silica gel [158]. Later investigations tested different adsorbents for water removal. From the experimental screening, silica gel had the highest capacity to adsorb water on a weight basis [157-160].

Azeotropic distillation is another alternative approach to remove water from reaction mixtures. An azeotrope is defined as a mixture consisting of two or more liquids in a certain ratio. In general, azeotropic mixtures are introduced to the esterification reaction as a media to lower the boiling temperature compare to either of its constituents and remove the water by distillation [161]. Taking advantage of the lower boiling temperature, the synthesis of sorbitan esters catalyzed by CALB was carried out in azeotropic mixtures of binary solvent systems (n-Hexane / *tert*-Butanol 78: 22 v/v) at 64°C with 89% conversion achieved [161]. Yan *et al*. developed a practical process potentially useful on an industrial scale for removal of by-products (water and methanol) by azeotropic distillation during lipase-catalyzed sugar fatty acid ester synthesis. Acetone was introduced to remove methanol during transesterification since the boiling point of the azeotrope of acetone / methanol 86/14 v/v is 54.6°C. MEK was employed for the removal of water due to the azeotrope of 67% MEK and 33% water v/v at a boiling point of 73.5°C.The pretreatment of the reaction mixture was operated in a 50-ml two-necked round-bottom flask equipped with a Soxhlet extractor. A condenser with a vacuum controller was placed on the top of this apparatus. Molecular sieves were activated by heating overnight to 250°C under reduced pressure. With addition of activated

molecular sieve placed in the Soxhlet extractor for the removal of byproducts (3 A for water and 5 A for; methanol,), the condensed solvent was pre-dried by mixing with activated molecular sieves before pumping back to the reaction system. This method constantly removed water and methanol generated in the reaction and shifted the equilibrium position towards sugar ester synthesis. Under above conditions, the highest conversion of 90% was achieved by conducting the reactions under reduced pressure at 60°C [155]. By the same principle, the synthesis of sugar-fatty acid ester catalyzed by CALB was performed in a mixture of MEK and hexane (4:1, v/v) to remove water efficiently at 59°C. The process is shown in **Fig.2.10.** [162]. The highest yield of glucose stearate was 93% after 48h using an equimolar ratio of substrates via this approach [162].

However, the employment of organic solvents is unavoidable for use of azeotropic distillation. Therefore, some implications, for instance, process cost, toxicity and catalytic activity should be considered [161]. Another major drawback of this method is that the reaction temperature should be maintained above the boiling point of the azeotrope [163].

A convenient and rather simple method for maintaining a constant water activity during esterification is the introduction of a suitable salt hydrate pair to the reaction medium [164], which can maintain the water activity during the reaction at a certain value of water activity at a given temperature [164]. It acts as “a buffer” by adjusting (releasing or extracting) water molecules as required in the presence of some of both salt hydrate pairs in the reaction medium [164]. The yield of sucrose ester versus water activity by direct addition of salt hydrate pair in closed reaction system is given in Table 5. The 13.8% yield of final products was obtained in the presence of $Ba(OH)_2$ octahydrate-monohydrate [$Ba(OH)_2$,8/1 H_2O] pair (water activity; a_w=0.44) and 0.6 g of $Ba(OH)_2$ per 3.57 g of the reaction mixture was the most favorable content of

salt hydrate. In addition, the authors concluded that monoester was formed only with water activity in the range of 0.3–0.5. Although the conversion (13.8%) is low in the presence of salt hydrate pairs to control this water activity during the reaction, it is still a useful approach for maintaining the water content [165].

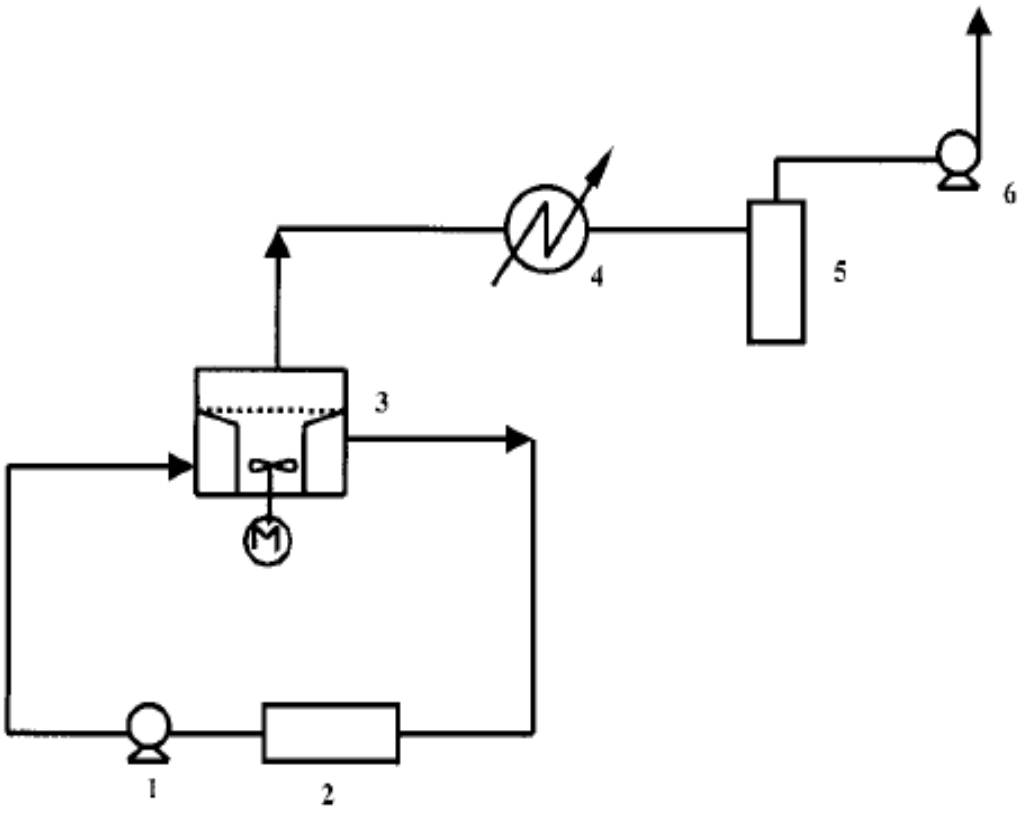


Fig.2.10. Schematic diagram of the process of production of CALB lipase-catalyzed synthesis of glucose stearate in the mixture of ethyl methylketone and hexane 4/1 v/v (1, pump; 2, water bath; 3, membrane reactor; 4, condenser, 5, permeate container; 6, vacuum pump) [162]

Another method for the adjustment of water activity in the reactor was used by circulating saturated salt solutions contained silicone tubing [178] or hollow fibers [179].Mattiasson with coworkers developed a thin silicone tubing filled in saturated salt solutions to control water activity in the reaction medium by transporting water through the wall of the tubing [178]. The highest conversion of lipase-catalyzed esterification of decanoic acid and dodecanol in diisopropyl ether was approximately 80% for 50h at 0.33 water activity [178]. Kim *et al.* investigated various salt saturated solutions for the optimal water activity control in a solvent-free system (Table 2.6) [165]. Desiccant ($CaSO_4$) was introduced to the pretreatment on reduce moistures of enzyme, fructose, and oleic acid. The results clearly indicated that the reaction rate decreased with dehydration of lipase in comparison to a control experiment. Water removal from fructose and oleic acid prior to the initiation of reaction was found that it is had little or no influence on the time course of esterification under solvent-free conditions [114]. Using saturated aqueous solutions to control water activity has one shortcoming. This method is not effective enough, particularly at a larger scale, due to the slow mass transfer of water between three phases: saturated salt solution, air, and organic phase. [151].

A solution for controlling water content in a system is the introduction of dry air or nitrogen into the reactor to remove water from the reaction mixture [151,166]. Air has a relatively high capacity for water removal and a limited direct effect on enzyme activity and stability [167]. An online system was developed to monitor water activity and utilize dry air to favor water removal and during the lipase-catalyzed esterification reaction of capric acid and *n*-decyl alcohol in solvent-free media (**Fig. 2.11.**). The final yield is up to nearly 100% at the optimal water activity (a_w=0.6) after 1600 min [167].

Table.2.4. Reaction yield at different initial water activities[1] [165]

Initial a_w (at 20°C)	Salt saturated solution	Reaction yield (%)
0.75	NaCl	0
0.67	$CuCl_2$	0
0.57	NaBr	5.1
0.43	KCO_3	6.4
0.33	$MgCl_2$	6.2
0.22	KAc	2.3
0.11	LiCl	0

[1]Enzyme and substrate were preequilibrated over saturated salt solution in closed vessel for 7 days at room temperature before the reaction. Reaction mixture consisted of 2.92 mmol of sucrose, 0.1 g of *M. miehei* lipase, and 14.6 mmol of capric acid in a stoppered vial. Reaction condition was 50°C, 350 rpm, and 48 hr.

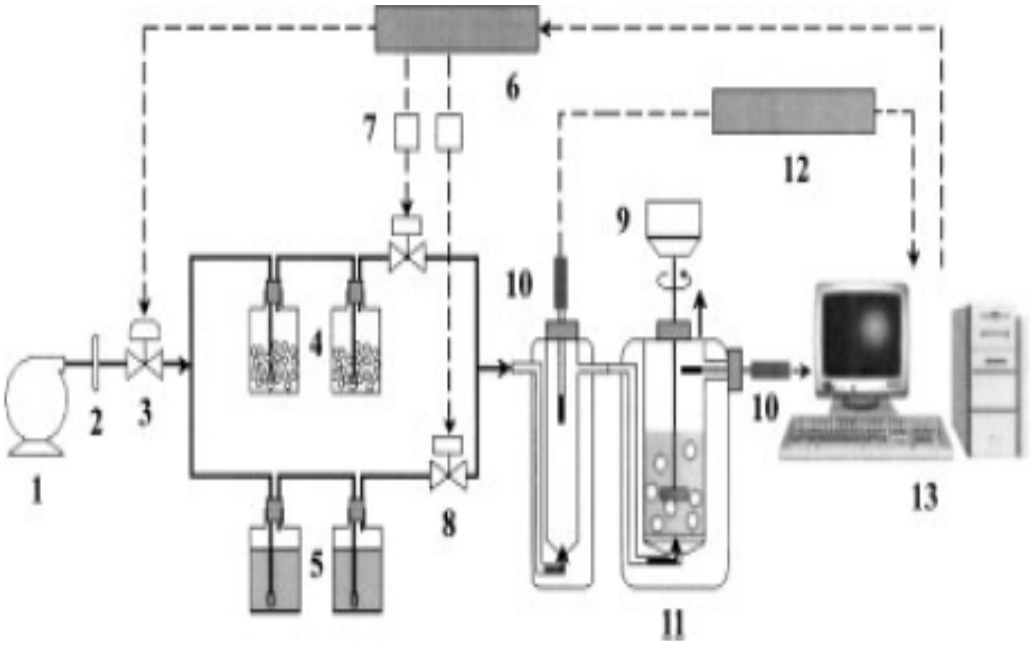


Fig. 2.11. Schematic diagram of the computer-based system for water activity control and on-line conversion estimation. (1) Air pump; (2) air filter; (3) mass flow controller; (4) silica gel; (5) distilled water; (6) digital to analog converter; (7) solid state relay; (8) solenoid valve; (9) stirrer; (10) relative humidity sensor; (11) enzyme reactor; (12) analog-to-digital converter; (13) computer [167].

Vacuum pressure has been employed to remove water from a system during reaction. For example, an air-bled evacuated-headspace reactor was designed and constructed under a vacuum of 0.7 bar [168]. It would appear that the quality of the vacuum pressure employed did not have any positive or negative influence on the initial rate of esterification [168]. However, combination of vacuum and air leakage was utilized to improve the performance of synthesis of organic-phase enzymatic esterification. A yield of 96% ester was achieved in a reactor operated under a vacuum of 0.7 bar and air leakage at 50 °C during the RML-catalyzed esterification of a solvent-free sorbitol-decanoic acid mixture when air at 20 °C with a relative humidity of 54% was leaked into the headspace [168]. Furthermore, another study illustrated that the application of even a moderate vacuum (20 mbar) led to an increase in the reaction yield 80% from 75% [110].

The water content of the reaction mixture can also be controlled by a pervaporation, defined as a chemical separation method in which one of the components in a solution selectively passes through a semi-permeable membrane. A pervaporation process was applied to the lipase-catalyzed synthesis of *n*-butyl oleate to selectively separate water from the reaction mixture using a nonporous polymeric membrane [169]. In addition, pervaporation enhanced the enantioselective esterification of (R, S)-2-chloropropanoic acid with *n*-butanol using *Candida rugosa* lipase in IL. The highest conversion 36.6% was obtained at 0.5 % w/w constant water content in [Bmim] [PF_6] [170]. The yield 36.6% under optimal water content (0.5% w/w) was increased by nearly 10% compared to 26.3% for the control [170]. Furthermore, it was demonstrated that water removal at the beginning phase of the reaction has a negative effect on optimization of the water content for the enzyme because too small amount of water content (e.g. 0.2% w/w) reduced the water content in the enzymes' microenvironment, leading to a reduction of the biocatalytic activity [170]. This conclusion was verified by the early

literature in the field [171]. Enzymatic triacylglycerol synthesis was conducted at 25 °C catalyzed by an immobilized lipase under pervaporation. 26% activity of enzyme remained after 600 hours with initial a_w equal to 0.1 and 71% when a_w was equal to 0.45 [171].

Free evaporation utilized for water removal during lipase-catalyzed synthesis of sugar fatty acid esters at super-ambient temperatures (50°C-65°C) is well documented in the literature [58,175,176]. The effect of water control on the solvent-free equilibrium conversion of fructose-oleic acid esterification was investigated by as part of this dissertation (described in Ch.3) [115].

In conclusion, water activity plays a significant role in lipase-catalyzed esterification reaction since it is formed as a byproduct. Insufficient amount water inactivates and destabilizes an enzyme since water has a significant influence on the enzyme structure by non-covalent bonding and disruption of hydrogen bonds. In contrast, excess water in reaction system lead to the lower yield due to thermodynamic equilibrium. Therefore, water at the proper amount in the reaction system is crucial for successful synthesis of sugar fatty acid esters. Although numerous methods have been generated, the efficient and economical approach for water removal has not been fully developed to meet the requirement of industrial scale-up.

CHAPTER 3

CHARACTERIZATION OF SUSPENSIONS AND BIOREACTOR SYSTEM DESIGN FOR SOLVENT-FREE LIPASE CATALYZED SYNTHESIS OF SACCHARIDE-FATTY ACID ESTERS

3.1 Abstract

Saccharide-fatty acid esters, important biobased and biodegradable biobased in foods, cosmetics, and pharmaceuticals, were synthesized by immobilized *Rhizomucor miehei* lipase-catalyzed esterification in solvent-free systems at 65^{o}C in a 2-step process: rapid mixing (e.g., at 800 rpm) of acyl donor, acceptor, and a mixture of mono- and di-ester at an elevated temperature (e.g., 85^{o}C) for several hours (≥ 6 h), followed by centrifugation at a low angular velocity (e.g., 800 rpm) for a short duration (e.g., 0.5-1.0 min, to remove large particles), a supersaturated solution of 1.5-2.0 wt % saccharide was obtained that remained stable for ≥10-12 h, either in the presence or absence of acetone. The solvent-free suspensions were used in a bioreactor system at 65^{o}C, consisting of a reservoir open to the atmosphere that contained molecular sieves, a peristaltic pump, and a packed bed bioreactor, operated under continuous recirculation. At 10 h intervals, suspensions were re-formed in the substrate/product mixture by adding additional acyl acceptor and applying strong agitation. Using this system and approach, a product mixture containing 88% fructose oleate was formed, of which 92% was monoester, within 6 days. This equates to a productivity of 0.2 mmol h^{-1} g^{-1}, which is similar to values reported for synthesis in the presence of solvent.

3.2 Introduction

In this chapter, the content was taken from a recently published paper [181]. Note that the contents of this chapter are a component of Objective 1 (given in Chapter 1). Saccharide fatty acid esters, biocompatible and biobased

surfactants, are valuable emulsifiers in a variety of food, cosmetic, and pharmaceutical commercial products such as chocolate, toothpaste, lotions, shampoo, and lipstick [41-43]. They possess excellent antimicrobial activity as well [50-52]. Although they are currently produced chemically (at high pressure and temperature [39]), their synthesis catalyzed by enzymes (lipases primarily) has received great interest due to the enhancement of sustainability: near-ambient pressure and temperature (leading to lower energy usage and hence lower CO_2 production), the absence of alkaline or acidic conditions (leading to lower amounts of waste products), and a more narrow product distribution, with mono- and di-esters formed selectively via the primary hydroxyl groups of the acyl acceptor. The major hurdle to overcome for lipase-catalyzed synthesis is the poor miscibility of the lipophilic acyl donor and hydrophilic acyl acceptor, leading to unacceptably slow reaction rates. Several different approaches have been employed to overcome this barrier. The most common approach to achieve the highest miscibility is to employ immobilized thermophilic lipases with polar organic solvent or solvent mixtures near their boiling points or under reflux (e.g., *tert*- butyl or *tert*-amyl alcohol, methyl ethyl ketone, or acetone; or their mixtures with very polar solvents [octanol-water partition coefficients, or log P values, of ≤1], such as dimethylsulfoxide, DMSO, at < 20 vol %) , with great care being used to minimize their inactivation of the biocatalyst (particularly by polar solvents) and their accumulation of water, which reduces yields [63-73]. Recently, the use of ionic liquids [99, 100] and pressurized solvent systems near or above their critical points (e.g., supercritical CO_2 / solvent mixtures) [92-95] have applied for the improvement of rates of reaction.

Our group is focusing upon the RML-catalyzed production of saccharide-fatty acid esters under solvent-free conditions, taking advantage of the presence of saccharide-fatty acid esters to enhance the miscibility of acyl donor and acceptor with a nearly stoichiometric substrate ratio (1:1) by fed-batch addition

of saccharide. In the early work, *tert*-Butanol, as a cosolvent for the increase of solubility of saccharide, was utilized in the initial phase at 65°C at stirred batch mode. *tert*-Butanol was totally evaporated away at around 25% conversion of the product. The final conversion reached 80-93% in 250h [115]. Subsequently, bioreactor systems were developed consisting of initial reaction medium (fructose-oleic acid ester/ oleic acid 25/75 w/w) and stirred-tank or packed bed reactors with a packed column of the mixture of saccharide crystals and silica gel at 65°C reaching upon 80-85% conversion without any employment of solvent [133].

However, the reaction rate for bioreactor systems utilizing saccharide packed columns for the delivering of saccharide was relatively slow due to the low concentration of saccharide yielded [115]. In contrast, the best reaction rate occurred when suspensions were employed with *tert*-butanol during batch mode reactions. Accordingly, we decided to revisit the use of solvent to assist in the formation of suspensions. Acetone was selected because it is a food-grade solvent in the EU (Chapter 2). Therefore, an investigation of using acetone as solvent between 30 and 65^{o}C the RML-catalyzed formation of saccharide in solvent-free media was undertaken. With the employment of acetone, results indicated that the reaction rate and yield were promoted when the reaction temperature was nearly or above the boiling point (56°C) temperature of acetone [181]. Under the latter conditions, acetone evaporated away from the reaction medium within a few hours, suggesting the enhancement was not due solely to the presence of the solvent. It was hypothesized that the underlying reason for the promotion of acetone-based reaction rate were suspensions of saccharide were formed in the reaction medium [181].

3.3 Materials and Methods

3.3.1 Materials

Technical grade oleic acid, 90 % pure, as determined by HPLC (14), and Lipozyme®IM, lipase (EC 3.1.1.3) from *Rhizomucor miehei* immobilized onto macroporous anionic beads, or "RML," the latter a product manufactured by Novozymes, Inc. (Franklinton, NC), were purchased from Sigma-Aldrich (St. Louis, MO). Fructose (>98% purity), sucrose (> 99%), acetone (HPLC-grade), acetonitrile (HPLC-grade), molecular sieves (Type 3A, 4-8 mesh, Grade 562) were obtained from Fisher Scientific (Pittsburgh, PA). All materials were used without further purification. The saccharide crystals were ground into a fine powder using a mortar and pestle. Technical grade fructose-oleic acid ester, FOE, the reaction product and a component of the initial charge to the bioreactor system, was synthesized by the RML-catalyzed fed batch reaction protocol given in our modified from previous report [18], as described elsewhere [21]. The purity of FOE was 89.6% (and the remaining fraction being oleic acid), with its composition being 83.6% monoester (ME) and 16.4% diester (DE). This product was mixed with oleic acid to obtain the desired proportions of oleic acid and ester, and fed to the bioreactor system.

3.3.2 Preparation of supersaturated solutions of fructose in oleic acid / fructose oleic acid ester mixtures

The preparation of a slurry of fructose crystals and a mixture of oleic acid and FOE was conducted in a 20 mL scintillation vial placed on a 4-position hot plate-magnetic stirrer (Super-Nuova from Barnstead, Dubuque, IA USA) open to the atmosphere, to allow for the free evaporation of water and acetone (when

used). Typical conditions consisted of adding 0.5 g of crystalline fructose to 2 g of oleic acid + FOE, and in some instances acetone, at a specified stir rate and temperature, for a specified time. The slurry was centrifuged at a specified angular velocity and for a specified time. The model Centrific™ table-top centrifuge and model AccuSpin™ microcentrifuge, both from Fisher, were used for angular velocities of ≤ 3000 rpm and > 3000 rpm, respectively. The supernatant was collected and employed in enzymatic reactions, and/or was analyzed for fructose content, particle size, water content and absorbance at 1000 nm. For experiments which analyzed the stability of the supersaturated solutions, the suspensions prepared as described above were allowed to settle without stirring applied at a controlled temperature.

3.3.3 Operation of a Packed-Bed Bioreactor System Undergoing Continuous Recirculation

The bioreactor system consisted of a 20 mL scintillation vial open to the atmosphere which served as reservoir, a peristaltic pump (BioLogic LP® from Bio-Rad, Hercules, CA USA), and a packed bed bioreactor (PBBR; 50mm L × 10mm ID Omnifit® chromatography column packed with 0.025-0.10 g of RML per gram of oleic acid + FOE) in **Fig 3.1**. The frit restrictors contained within the endcaps of the Omnifit® column were not used, but were replaced a small piece of a 100 denier,156 mesh polyester net manufactured by SiamDutch Mosquito Netting Co., Ltd., Bangkok, Thailand, determinedgravimetrically to possess an areal density of 29 g m^{-2}. C-FLEX® 1.6 mm ID tubing made of a styrene-ethylene-butylene modified block copolymer from Cole-Parmer (Vernon Hills, IL), was used to connect the reservoir to the pump, the pump to the PBBR, and the PBBR to the reservoir, to form a closed-loop system that underwent continuous recirculation. PharMed® BPT 1.6 mm ID tubing (Saint-Gobain Performance Plastics Corp., Akron, OH) was used within the peristaltic

pump apparatus. The PBBR and associated tubing were placed within a convection oven to yield a constant temperature of 65^{o}C for the recirculating liquid phase. The reservoir's contents were maintained at 65^{o}C and stirred gently at 200 rpm using the above-mentioned hot plate / stirrer. Molecular sieves (0.10 g per g of oleic acid + FOE) were added to the reservoir for some experiments after reaching 60~70% ester content to reduce the water content and hence the degree of conversion.

The reaction for carried out using supersaturated solution of fructose prepared by the method described above, by stirring a slurry or saccharide crystals (1.5 g) in an oleic acid/ saccharide-oleic acid mixture (10 g) at 80^{o}C and 800 rpm for 6 hr, followed by centrifugation at 800 rpm for 30 s, and then collecting the supernatant. The initial charge to the bioreactor systems typically consisted of 25% FOE and 75% oleic acid, equivalent to a reaction mixture that has achieved 25% conversion. At 10 hour intervals, the suspensions contained within the reservoir were re-treated as described above to incorporate additional saccharide. For the retreatment to occur, the bioreactor system was temporarily disabled by stopping the pump, the reservoir's contents removed to a separate container, 1.5 g of saccharide added, and the suspension reformed by the 2-step method given above and then returned to the reservoir.

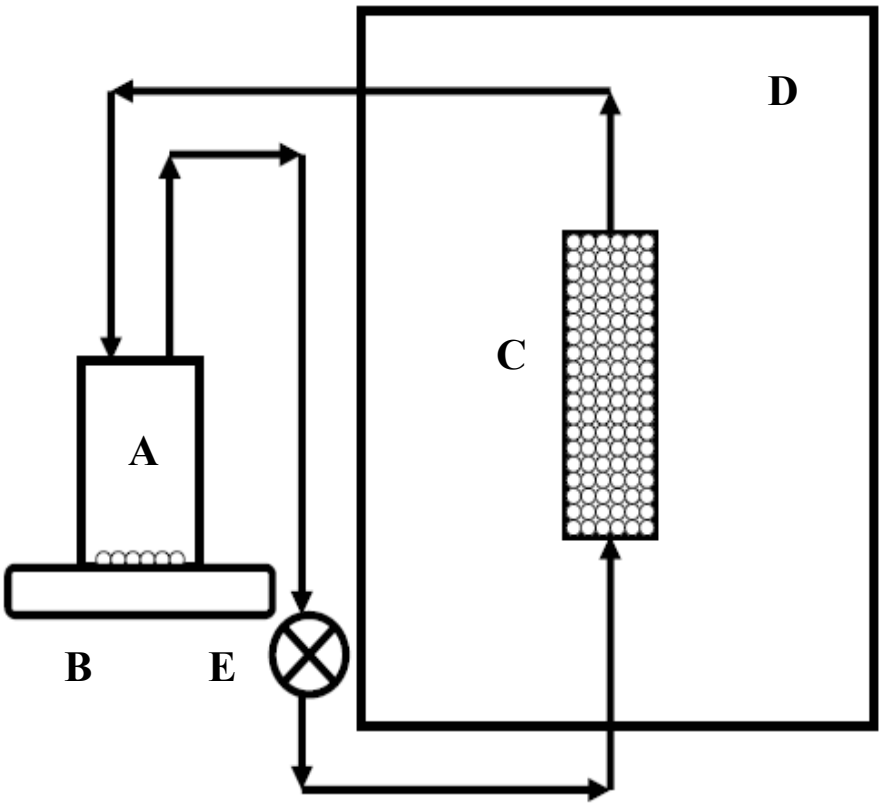


Fig. 3.1. The diagram of packed-bed based bioreactor system A. 20ml vessel opened to the atmosphere B. Hot plate maintained at 85^{o}C C. PBBRs D. Oven maintained at 65^{o}C E. Pump (0.5ml/min)

3.3.4 Monitoring of water

The water content for an aliquot of the reaction mixture, after being diluted with methanol, was analyzed by Karl-Fischer titration using a Coulometric KF Titrator (Denver Instrument Company, Aurora, CO). The working principle is following the formulae below:

$H_2O+I_2+SO_2+3C_5H_5N \rightarrow 2(C_5H_5NH)I^- + C_5H_5N+SO_3 \rightarrow C_5H_5NSO_3+$
$CH_3OH \rightarrow (C_5H_5NH)OSO_2OCH_3$

The working principle utilizes the reaction of water with iodine and sulfur dioxide in the presence of a lower alcohol such as methanol or other organic solvents. In this dissertation, methanol was used as a medium to dissolve the reaction medium due to its lower price compared with other organic solvents [219].

3.3.5 Monitoring of oleic acid, ester, and fructose concentration

Quantitative analysis of oleic acid and its mono- and di-esters on a fructose-free basis was performed using a dual-pump system from Varian (Walnut Grove, CA) and a model Mark III evaporative light scattering detector from Alltech Associates, a division of WR Grace (Deerfield, IL) . An analytical reversed phase (4.6 * 250 mm, pore diameter 5 μm) C_{18} column from Alltech was employed using separation conditions consisting of a column temperature of 25^{o}C and an isocratic solvent system, acetone / acetonitrile / acetic acid (45 / 45 / 10 v/v/v) at flow rate of 1.0 mL min^{-1}. Response factors were measured and employed to convert peak areas into concentrations.

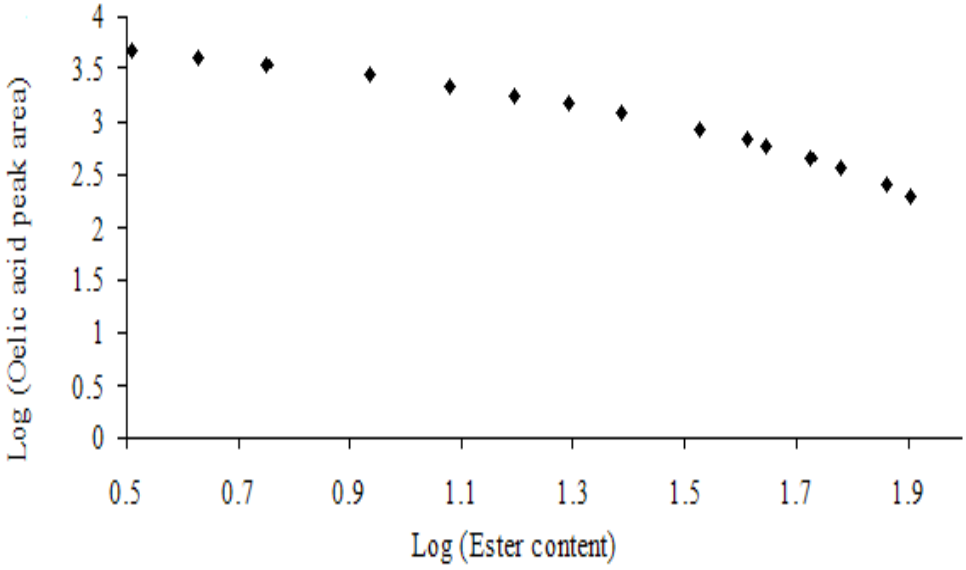


Fig. 3.2. Log of oleic acid peak area vs Log of ester content

To analyze the fructose content, 40 mg-sized aliquots of column effluent were subjected to liquid-liquid extraction by the system of *n*-hexane and water (500 μL of each). The extraction was carried out 3 X at 35^{o}C for 2 hr using a thermomixer (Eppendorf AG, Germany). The aliquots from the pooled aqueous extraction solutions were diluted with acetonitrile to match the composition of the HPLC mobile phase to prevent peak broadening in the HPLC analysis. An analytical Prevail Carbohydrate ES column (4.6 * 250 mm, pore diameter 5 μm) from Alltech was employed using a column temperature of 25^{o}C and an isocratic solvent system, acetonitrile / deionized water (80 / 20 v/v) at flow rate of 1 mL min^{-1}. Standard curves for fructose concentration in an oleic acid / fructose oleate liquid phase versus peak area were obtained and found to be independent of the reaction mixture's composition.

3.3.5 Measurements of absorbance and particle size for suspensions

The absorbance of solutions between 500 and 1000 nm was performed to provide a measure of turbidity using a model UV-1700 instrument from Shimadzu (Japan) and either a 1.0 or 0.2 cm pathlength quartz length cuvette by Hellma (Plainview, NY USA), with all reported values normalized to a 1.0 cm pathlength. Light scattering is a method to determine the distribution of small size particles in suspension. In this dissertation, photometric mode was used to measure absorbance (ABS) at an arbitrary fixed wavelength (1000nm). Absorbance values at 1000 nm are reported since at this wavelength the values were often < 1.0 units, meaning that the linear Beer-Lambert law is applicable. The Beer-Lambert law is a principle to describe the linear relationship between absorbance and concentration of an absorbing sample. The Beer-lambert law is usually written as:

$$A = a(\lambda) * b * c$$

where A is the measured absorbance, a(lambda) is a wavelength-dependent absorptivity coefficient, b is the path length, and c is the analyte concentration.

When working in concentration units of molarity, the Beer-Lambert law is written as:

$$A = epsilon * b * c$$

where *epsilon* is the wavelength-dependent molar absorptivity coefficient with units of M^{-1} cm^{-1}

Differences of absorbance values between samples at this wavelength are representative in trend of differences at the other wavelengths. The particle size distribution of the dispersions present in the above-mentioned solutions was analyzed by Zeta potential Analyzer, Zeta PALS (Brookhaven Instruments Corporation, Holtsville, NY USA).

3.4 Results and Discussion

3.4.1 Formation of Metastable Suspensions of Saccharide in Solvent-free Media

To justify the above-mentioned hypothesis, reaction medium was prepared which simulated the reaction conditions used for the RML-catalyzed reactions in the presence of acetone. The (apparent) concentration of fructose and particle size distribution in the liquid medium was analyzed.

6.0 g of FOE/oleic acid 25/75 w/w was mixed with 1.5 g fructose and either 0.0 or 6.0 g acetone for 20 h at 800 rpm in a 20 mL scintillation at a temperature near or above the boiling point of acetone, 56°C. After centrifugation for 2.0 min, the larger particles were removed and the supernatant was collected and analyzed. **Fig. 3.3** illustrates that the fructose concentration, absorbance at 1000 nm, and average particle size significantly increased with the increase of fructose oleic ester (FOE) concentration and the decrease of centrifugation angular velocity. Absorbance reflects the scattering

of light by the suspended particles, and is proportional to the size multiplied by the number density of the particles. The detection of particles of the size range 5-70 μm confirms that suspensions were formed, in agreement with the hypothesis put forth. Compared with controls, the employment of acetone significantly increased the saccharide concentration of all three measurements but only slightly increased the average size of particles.

Next, several parameters related to the formation of suspensions were investigated to optimize the concentration of suspended saccharide crystals in FOE / oleic acid (25 / 75, w/w). **Fig. 3.4** depicts the effect of the mass ratio of acetone to the oleic acid / FOE mixture and stirring time with the stirring rate (800 rpm), temperature (65°C), and centrifugation conditions (3,000 rpm, 2 min) held constant. The stirring time was set in accordance to the time required nearly 100% remove of acetone by free evaporation at 65°C. Results illustrate that absorbance for both cases (acetone and solvent-free) increased with the stirring time. There was no significant increase of absorbance when the acetone /FOE +oleic acid ratio was increased from 3:1 to 5:1. However, the presence of acetone did not increase suspension formation after 6h of stirring time; hence, acetone is not necessary for formation of the suspension of saccharide crystals, particularly if a longer stirring time is employed.

Fig. 3.5 describes the effect of stirring rate for a constant stirring time (3 hr), with the other conditions being identical to those described for **Fig. 3.4.** The results demonstrate that the concentration of suspended fructose increases linearly with stir rate from 200 rpm to 800 rpm. In addition, acetone (at a 1:1 mass ratio per oleic acid + FOE) did not increase the concentration of fructose distinctly. **Fig. 3.6** illustrates that an increase of temperature from 65°C to 85°C slightly increased the liquid-phase fructose concentration.

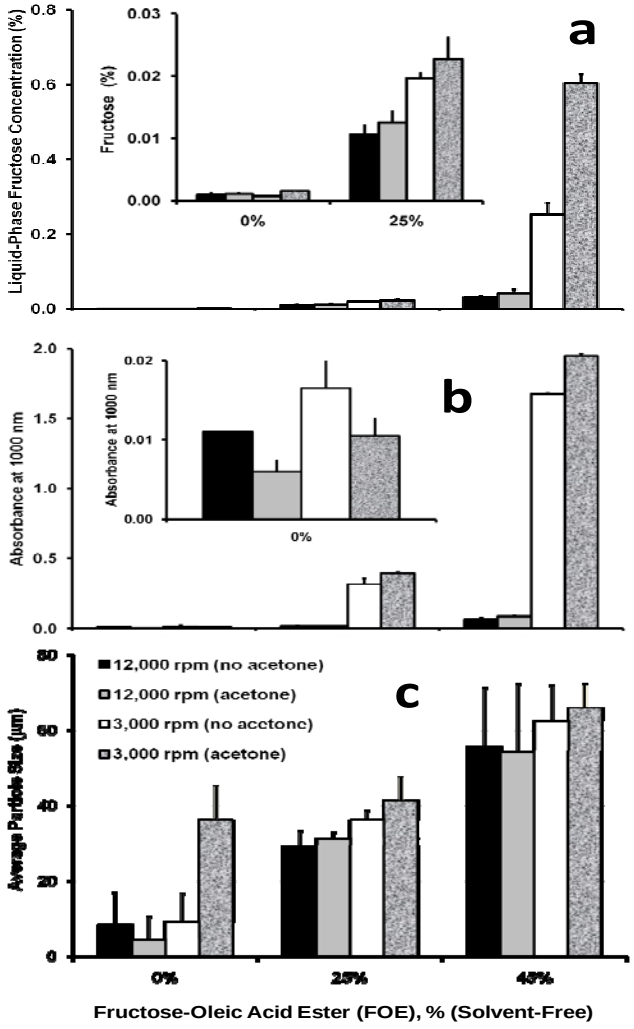


Fig.3.3 Effect of acetone, wt % fructose-oleic acid ester, and sedimentation angular velocity on (a) the apparent solubility, (b) the absorbance at 1000 nm, and (c) the average particle size of the suspensions of fructose in oleic acid/FOE mixtures at 65°C.

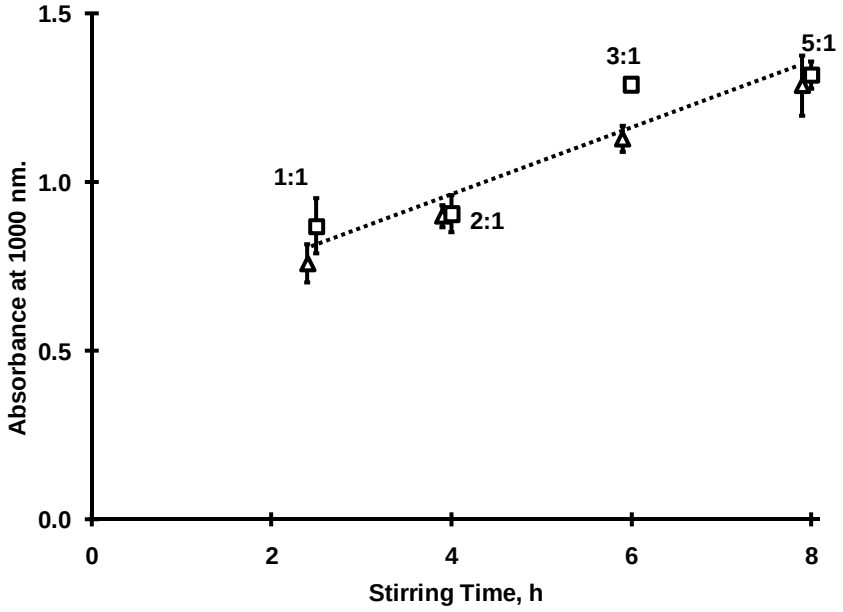


Fig.3.4 Effect of stirring time on the absorbance at 1000 nm for a suspension of fructose in (Δ) solvent-free media and (□) in the presence of acetone. [181]

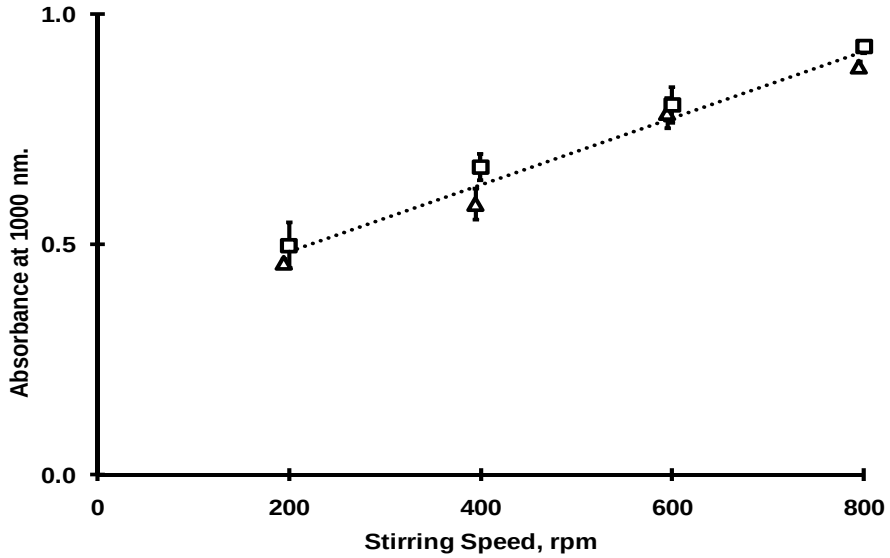


Fig.3.5 Effect of stirring rate on the absorbance at 1000 nm for a suspension of fructose in (Δ) solvent-free media and (□) in the presence of acetone. [181].

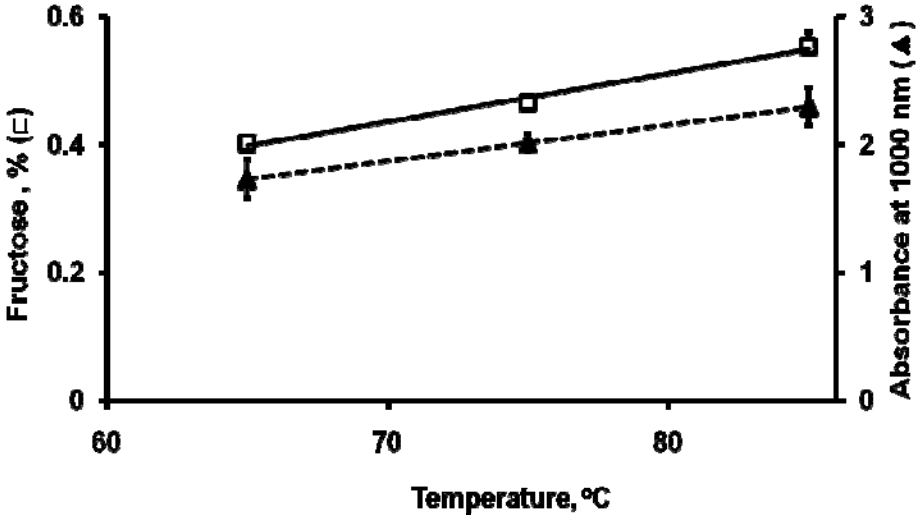


Fig. 3.6 Effect of temperature on the apparent fructose concentration and absorbance at 1000 nm for a suspension of fructose in a solvent-free medium. [181].

Centrifugation is an essential component of the procedure for forming suspensions since it removes excessively large size fructose particles that can potentially create blockage within the bioreactor system or can adsorb to the immobilized lipase particles, imparting mass transfer limitations. **Fig. 3.7** indicates that the concentration, absorbance, and average particle size increased linearly with the decrease of angular velocity and centrifugation time. The highest concentration was obtained at minimal angular velocity value for the apparatus (800 rpm) and 1.0 min for centrifugation time, leading to the formation of 100-200 μm sized particles. **Fig. 3.8** illustrates that the suspensions formed are reasonably stable over 12 h, especially under solvent-free conditions. Therefore, a suspension solution containing ~10-100 μm – sized fructose crystals can be created using mixtures of FOE and oleic acid under solvent-free condition.

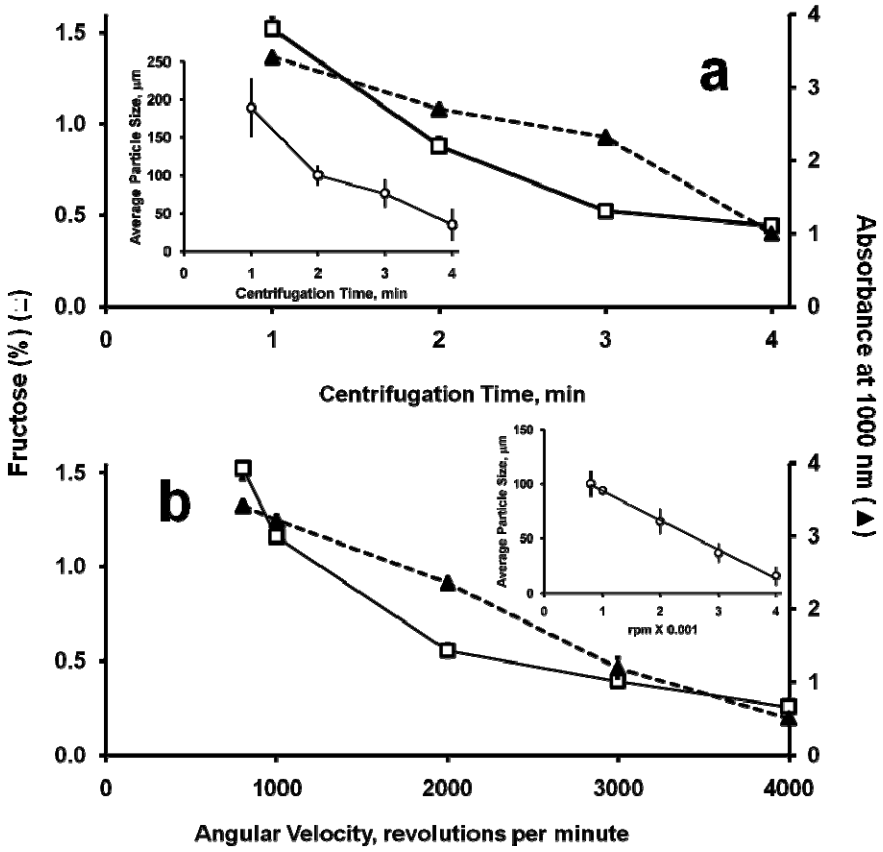


Fig.3.7 Effect of centrifugation parameters on the apparent fructose concentration, absorbance at 1000 nm, and particle size (insets) for a suspension of fructose in a solvent less medium. [181]

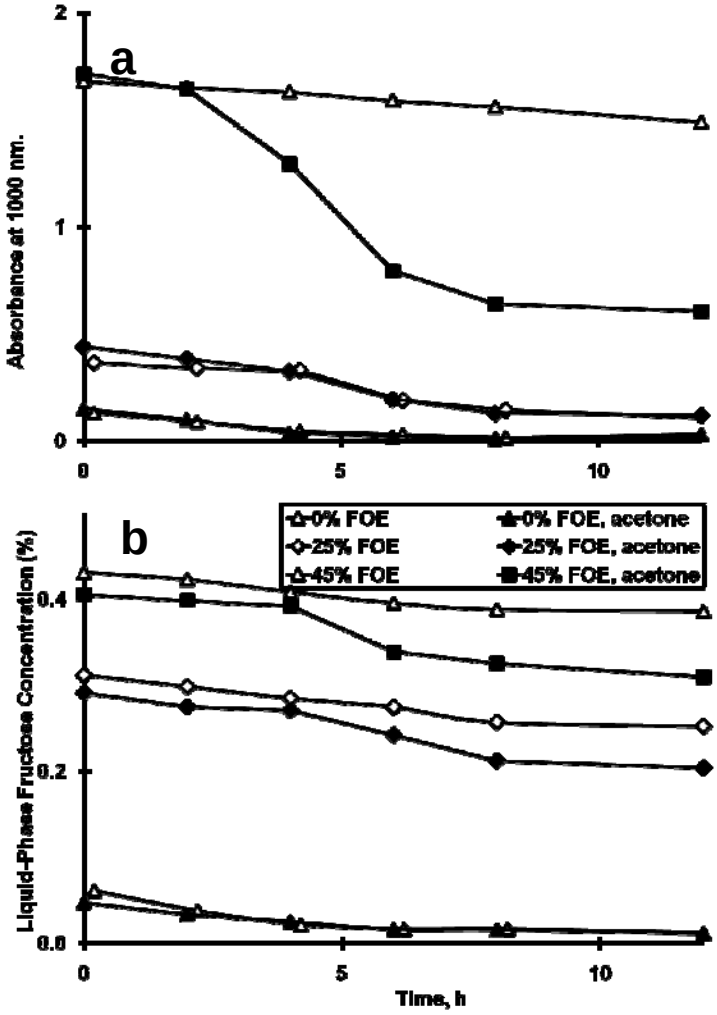


Fig. 3.8 Change of (a) absorbance at 1000 nm and (b) apparent fructose concentration of liquid phase for a suspension of fructose crystals and fructose-oleic acid esters (FOE)/ oleic acid prepared in the presence or absence of acetone at 65^{o}C. [181].

3.4.2 Assessment of the Performance of a Bioreactor System with Saccharide Suspension Crystals

A bioreactor system composed of a packed bed bioreactor (PBBR) containing RML particles, an open reservoir and a peristaltic pump was created. The liquid phase consisting of the mixture of oleic acid / FOE 75/25 w/w with suspended fructose crystals underwent continuous recirculation under a fixed flow rate at 65°C. The system was stopped at 10 h intervals, at which point the reservoir's liquid phase was isolated by centrifugation and additional fructose was introduced to form a new suspension. Finally, the new suspension medium was returned to the reservoir and the pump re-initiated.

From **Fig. 3.9a**, the reaction rate increased with the mass of RML in the PBBR, or equivalently, the residence time of the liquid phase in the PBBR. High repeatability of the experimental results occurred. One of the experiments used molecular sieves at 70 h to remove water and therefore increase the final conversion. Moreover, the high water content (0.7%) in the absence of molecular sieves led to a slow reaction rate after the product yield reached ~60%, after 2 days of reaction time. The employment of molecular sieves decreased the water content from ~0.7% to ~0.5 % (Fig 3.8b), resulting in a slight enhancement in the conversion of FOE, **Fig. 3.9.a**. It indicates that further research is required to optimize the water activity in the bioreactor system to increase the rate and conversion of reaction. This work is a component of this proposal's research plan.

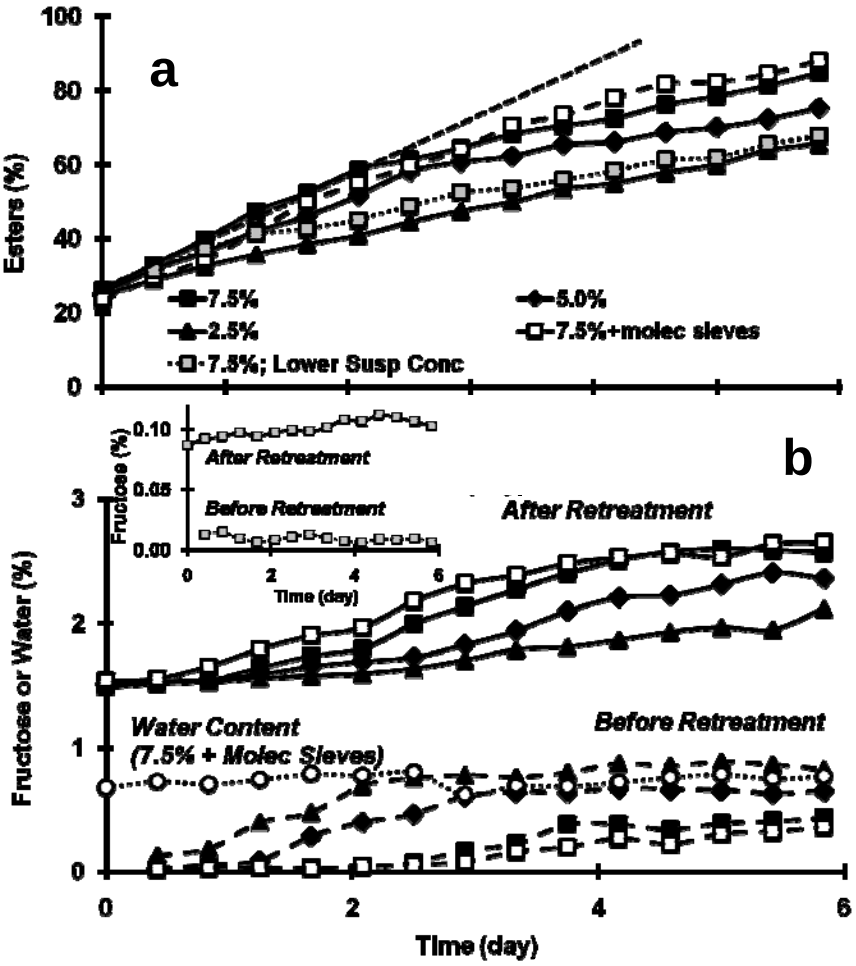


Fig.3.9. Effect of bioreactor volume on the change of (a) fructose-oleic acid esters and (b) fructose concentration before (solid lines) and after (dashed lines) retreatment for solvent-free RML-catalyzed fructose-oleic acid ester [181].

Fig. 3.9b illustrates the changes of fructose concentrations of the reservoir in the bioreactor system between the beginning and ending period for each 10 h interval cycle. For the reactions performed with 7.5% (w/w) RML, fructose was almost completely consumed at the end of each cycle during the first 60 h of reaction. The ~0.3% interval concentration of the residual fructose between "before retreatment" and "after retreatment" demonstrated low concentration the reaction rate. In addition, the distance between the "before retreatment" and "after retreatment" in the time course is consistent with the conversion of ester synthesis, **Fig 3.9a**. The increase of fructose concentration "after retreatment" as a function of time is consistent with the increase of saccharide concentration with increased FOE proportion.

The initial rate reaction data between 0 and 40 h are described as a function of residence time per pass through the bioreactor system, evaluated by dividing the volume of the PBBR by the volumetric flow rate, Q (Fig. 3.10). Further experiments were conducted using a constant PBBR volume (3.46 mL; 0.075 g_{RML} per g of oleic acid + fructose) with Q varied between 0.3 and 1.2 mL min^{-1} for determination of an optimal average residence time. The maximal initial rate was 15.5-16.0 % conversion of oleyl acyl groups per day using a residence time of 10 min or $Q \leq 0.3$ mL min^{-1}. The initial rate increased linearly with residence time for residence times below 6 min, following classical Michaelis-Menten kinetics model [116].

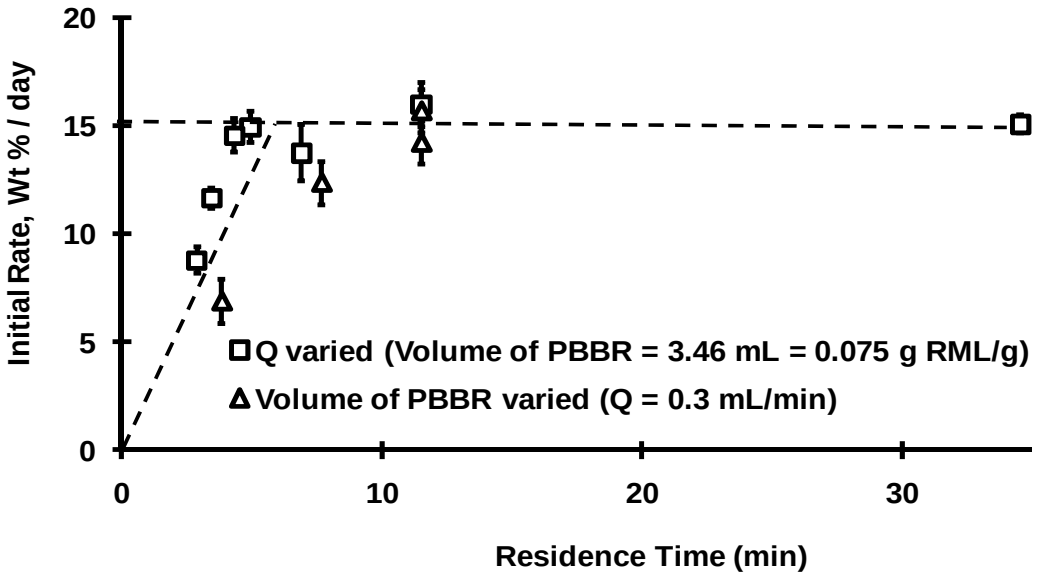


Fig. 3.10 Effect of residence time in packed bed bioreactor (PBBR) per pass through the bioreactor system on the initial rate of esterification (wt % FOE /day) for a series of experiments [181].

3.5 Conclusion

In conclusion, in this chapter, it was found that 22.6g/L supersaturated solution of suspensions of saccharide in the mixture of monoester and acyl donor (25/75 w/w) was created under solvent-free condition under the following conditions: stirring at 800rpm for 6h at 85^{o}C and then, centrifugation at 800 rpm for 0.5-1.0 min. Subsequently, solvent-free suspensions solution was introduced to a bioreactor system undergoing continuous recirculation at 65 ^{o}C (**Fig. 3.1**). At 10h interval time, suspensions were reformed after addition of additional saccharides. The reaction rate was slowed at approximately 60% conversion due to the relatively high water content (0.7%). Molecular sieves were employed into the bioreactor system during time course as water removal method. 88% yield of saccharide-fatty acid esters was achieved in 6 days.

As noted, when water content in the bioreactor system was decreased from ~0.85% to ~0.5% through addition of molecular sieves, a slight increase in the level of esters occurred. This suggests optimal control of the bioreactor system's water content will increase reaction rate and yield, performed in Chapter 4.

CHAPTER 4

OPTIMIZATION OF SOLVENT-FREE LIPASE-CATALYZED SYNTHESIS OF SACCHARIDE-FATTY ACID ESTER USING SUSPENSIONS THROUGH WATER REMOVAL AND OPTIMIZED INTERVAL TIME

4.1 Abstract

The synthesis of saccharide-fatty acid esters, environmentally-friendly biobased surfactants, via immobilized *Rhizomucor miehei* lipase (Lipozyme®IM, Novozymes, Franklinton, NC USA) at 53 ^{o}C using a packed bed bioreactor (PBBR)-based bioreactor system utilizing suspensions of saccharide in a mixture of acyl donor and monoester-rich product (solvent-free conditions), was optimized to enhance reaction rate and conversion by programming the control of water activity and the interval time for re-forming suspensions. When the bioreactor system reached 57.75 % w/w conversion, water was removed by the combination of nitrogen gas bubbling and vacuum pressure at 2.16 mg_{H2O} h^{-1} to maintain at 0.4% w/w plus/minus 0.05 % w/w level in the liquid phase. Also, the optimization of the interval time for re-treatment of suspensions decreased the reaction time by 23 h compared to the arbitrary use of a 10 h interval time. Additionally, the initial ester concentration in the solvent-free suspensions media was reduced from 25% w/w to 5% w/w without any loss of reaction rate. In conclusion, the optimized PBBR-based bioreactor system operated at optimal conditions employing an initial charge of fructose suspensions in oleic acid / fructose oleate 95/5 w/w yielded 92.6% w/w conversion within 132 h and a productivity (0.297 $mmol_{FOE}$ h^{-1} g_{lipase}^{-1}) 2-fold higher than the control. RML displayed excellent stability during four successive reactions (528 h) in the PBBR based bioreactor system. No loss of Initial rates was observed.

4.2 Introduction

In this chapter, the content was taken from a recently accepted paper [220]. Note that the contents of this chapter are a component of Objective 2 (given in Chapter 1).

Saccharide-fatty acid esters, biodegradable, environmental friendly nonionic biobased surfactants prepared from inexpensive renewable agricultural feedstocks, are employed as emulsifiers in foods, pharmaceutical and cosmetics [41-46]. In addition, they possess antimicrobial activity, leading to their potential employment in food preservation [42, 43] and insecticides [50-51]. Traditionally, saccharide-fatty acid esters are produced by chemical methods under harsh conditions, for instance, high pressure and temperature, yielding undesirable byproducts and unsafe operation condition. In contrast, biocatalytic synthesis of saccharide-fatty acid esters employs near-ambient operation conditions and leads to a narrow product distribution. However, the presence of significant hurdles hinders the application of biocatalysts for industrial utilization is the poor miscibility of polar and non-polar substrates, resulting in slow reaction rates. Although several methods have been successfully utilized to enhance the miscibility, particularly the employment of co-solvents: polar organic solvents [63-73] supercritical CO_2 [92, 95] and ionic liquids [99, 100], their use involves several disadvantages, including the high cost of solvent and/or their recovery or disposal, reduction of process safety, and the environmental impact of solvent utilization.

The aim of our group is to develop “green” approaches for the lipase-catalyzed synthesis of saccharide-fatty acid esters, utilizing the ester product to improve the dissolution of saccharide into the acyl donor (oleic acid), the latter acting as substrate and solvent [114-117]. Recently, we have employed suspensions of

saccharide crystals 10-200 μm in length dispersed in solvent-free media, formed by stirring the solid-phase saccharide and the liquid-phase media (acyl donor plus fatty acid ester) for several minutes, followed by sedimentation to remove larger particles [181]. In conjunction with a closed-loop bioreactor system, consisting of a reservoir open to the atmosphere (for free evaporation of the reaction product, water), a peristaltic pump, and packed bed bioreactor (PBBR), operated under continuous recirculation, this medium has led to an increased saccharide concentration, therefore, to a high conversion and an enhanced reaction rate: 88% w/w conversion into fructose oleate (92% w/w monoester and 8% w/w diester) within 6 days, starting with a reaction medium containing 75% w/w oleic acid and 25% w/w fructose oleate [181]. The suspension-based medium required retreatment with additional fructose at 10 h intervals, requiring a temporary stoppage of the bioreactor system. Molecular sieves were added to the reservoir upon achieving 60% w/w conversion of oleic acid to remove water, to enable further conversion [181]. The primary objective of this study is to improve the performance of the bioreactor system by controlling and optimizing the water concentration in reaction medium and the interval time for re-treatment and reformation of suspensions. Different approaches for water removal were compared. Also, the water removal strategies were employed for stirred tank bioreactor systems to test their universality.

4.3 Methods and Materials

4.3.1 Materials

Technical grade oleic acid, 90% pure, and Lipozyme®IM, lipase (EC 3.1.1.3) from *Rhizomucor miehei* immobilized onto macroporous anionic beads, or

"RML," the latter a product manufactured by Novozymes, Inc. (Franklinton, NC USA), were purchased from Sigma-Aldrich (St. Louis, MO USA). Fructose (>98% purity), acetone (HPLC-grade), acetonitrile (HPLC-grade), and molecular sieves (Type 3A, 4-8 mesh, Grade 562) were obtained from Fisher Scientific (Pittsburgh, PA USA). All materials were used without further purification. The saccharide crystals were ground into a fine powder using a mortar and pestle. Technical grade fructose-oleic acid ester, FOE, the reaction product and a component of the initial charge to the bioreactor system, was synthesized by the RML-catalyzed fed batch reaction protocol given in our modified from previous report [8], as described elsewhere [19]. The purity of FOE was 89.6% (and the remaining fraction being oleic acid), with its composition being 83.6% monoester (ME) and 16.4% diester (DE). This product was mixed with oleic acid to obtain the desired proportions of oleic acid and ester, and fed to the bioreactor system.

4.3.2 Methods

4.3.2.1 Formation of supersaturated solutions of fructose in oleic acid / fructose-oleic acid ester mixtures

Typically, the initial reaction medium, a suspension of saccharide crystals in solvent-free media, was formed by mixing 1.5 g fructose crystals and 10 g oleic acid / fructose-oleic acid ester (FOE), 75/25 w/w, equivalent to a reaction mixture that has achieved 25% conversion, in a 20 mL scintillation vial open to the atmosphere on a magnetic stirrer plate (Super-Nuova from Barnstead, Dubuque, IA USA) at 80^{o}C and 800 rpm (radius of 1.5 cm) for 6 h. The slurry was centrifuged at 800 rpm for 0.5-1.0 min, with the supernatant collected.

4.3.2.2 Operation of Bioreactor Systems Undergoing Continuous Recirculation

The bioreactor system included three major components: a 20 mL scintillation vial open to the atmosphere serving as a reservoir, a peristaltic pump (BioLogic LP® from Bio-Rad, Hercules, CA USA), and a packed bed bioreactor (PBBR; 50mm L × 10mm ID Omnifit® chromatography column packed with 0.75 g of RML per g of oleic acid + FOE). The three major components were connected in series, forming a closed-loop system that underwent continuous recirculation The PBBR and associated tubing were placed in an oven at constant temperature, 53^{o}C. The reservoir was kept at 65^{o}C and stirred at 200 rpm. Other details of the bioreactor system are described in our previous paper [19]. The stirred-tank bioreactor (STBR) system utilized the same apparatus as described above except for the replacement of the PBBR with a STBR, a 20 mL scintillation vial equipped with magnetic stirring (300 rpm). To enable the re-treatment of suspensions for the latter, the reaction medium was treated by microfiltration to remove the immobilized lipase as an initial step.

At specified intervals (typically 10 h), the suspensions contained within the reservoir were re-treated as described above through introduction of additional saccharide (1.5 g). During the re-formation of suspensions, the bioreactor system was temporarily paused by stopping the pump; the mixtures in the reservoir were transferred to another 20 mL vial and the pumps re-initiated. Molecular sieves, MS were added to the reservoir or incorporated into a packed column (MSC) inserted between the reservoir and pump for several experiments.

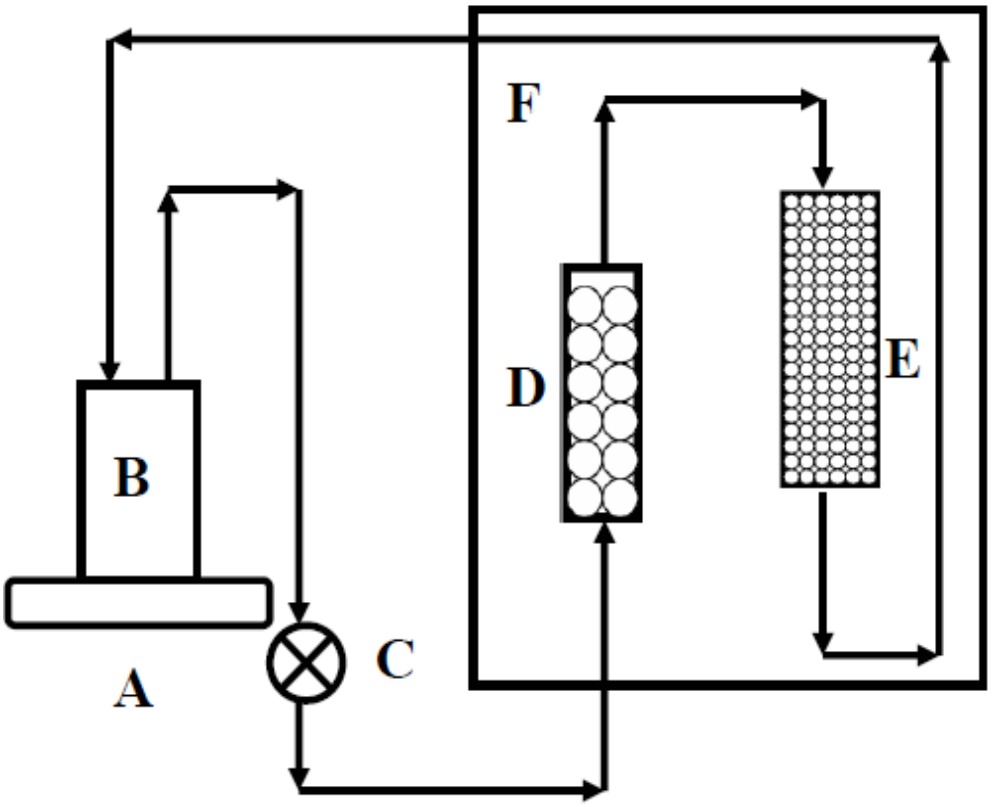


Fig.4.1. The diagram of bioreactor system with molecular sieve column A. Hot plate maintained at 85^{o}C **B.** 20ml vessel opened to the atmosphere **C.** Pump (0.5ml/min) **D.** Molecular sieves column (MSC) **E.** PBBRs **F**. Oven maintained at 53^{o}C

4.3.2.3 Operation of Bioreactor Systems Undergoing Continuous Recirculation with In Situ Water Control

A Wheaton Celstir spinner double-side arm flask was used to replace the 20 mL scintillation vial acting as a reservoir and *in situ* water control place. The formation of supersaturated solutions of fructose in oleic acid / fructose-oleic acid ester mixtures was identical with section **4.3.2.1.** Operation of bioreactor system was same as section **4.3.2.2** except that water control was conducted *in situ* in a Wheaton Celstir spinner double-side arm flask using the combination of nitrogen gas bubbling and vacuum pressure. After one cycle of fructose oleate synthesis, the immobilized lipase was isolated from the PBBR, washed with a small amount of acetone quickly, and then reused for the next reaction. Replicated experiments were operated at four times.

4.3.2.4 Water removal via vacuum pressure and N_2 (g) bubbling

A Wheaton celstir spinner double-side arm flask (50 mL) with a tight screw cap was placed on a hot plate/stirrer at 80 ^{o}C and stirred gently at 200 rpm (radius = 2.5 cm). The right side of the flask was connected to N_2 (g) and a flowrate meter when needed; and, the left side was interfaced with a vacuum pump and a vacuum meter when needed. Subsequently, the fructose suspensions were placed into the flask. After water control treatment, the reaction media containing fructose suspensions was transferred to the bioreactor system.

4.3.2.5 Operation of Bioreactor Systems Undergoing Continuous Recirculation with In Situ Water Control

A Wheaton Celstir spinner double-side arm flask was used to replace the 20 mL scintillation vial acting as a reservoir and in situ water control. Other components and operation conditions remain the same as described above.

4.3.2.6 Monitoring of Water, Oleic Acid, Ester, and Fructose Concentration

The water content for the reaction mixture was analyzed using a Coulometric Karl-Fischer Titrator (Denver Instrument Company, Aurora, CO) after its dilution with methanol. The relative amounts of oleic acid and its mono- and di-esters formed with fructose was determined using a dual-pump system from Varian (Walnut Grove, CA) and a model Mark III evaporative light scattering detector from Alltech Associates, a division of WR Grace (Deerfield, IL) [18] . An analytical reversed phase (4.6 x 250 mm, pore diameter 5 μm) C_{18} column from Alltech was employed using separation conditions consisting of a column temperature of 25^{o}C and an isocratic solvent system, acetone / acetonitrile / acetic acid (45 / 45 / 10 v/v/v) at flow rate of 1.0 mL min^{-1}. Response factors were measured and employed to convert peak areas into concentrations [8].

To analyze the fructose content, 40 mg-sized aliquots of column effluent were subjected to liquid-liquid extraction by the system of *n*-hexane and water (500 μL of each) [18]. The extraction was carried out 3 X at 35^{o}C for 2 hr using a thermomixer (Eppendorf AG, Germany). The aliquots from the pooled aqueous extraction solutions were diluted with acetonitrile to match the composition of the HPLC mobile phase to prevent peak broadening in the HPLC analysis. An analytical Prevail Carbohydrate ES column (4.6 x 250 mm, pore diameter 5 μm) from Alltech was employed using a column temperature of 25^{o}C and an

isocratic solvent system, acetonitrile / deionized water (80 / 20 v/v) at flow rate of 1 mL min^{-1}. Standard curves for fructose concentration in an oleic acid / fructose oleate liquid phase versus peak area were obtained and found to be independent of the reaction mixture's composition [8].

4.4 Results and Discussion

4.4.1 Effect of Molecular Sieve Concentration on Production of Fructose-Oleic Acid Esters Using a Packed-Bed Bioreactor System

For maintaining water concentration in the liquid phase in the reaction medium at the optimal level, molecular sieve column (MSC) at different concentrations were introduced into the bioreactor system developed by Obejctive1, consisting of a reservoir, peristaltic pump, and a PBBR, undergoing continuous recirculation at 0.50 mL/min and at 70 h during the time course of fructose oleate, using suspensions of fructose crystals in solvent-free media 53^{o}C as described in Fig. 4.1. MSC were utilized other than scattered molecular sieve preventing mass transfer limitations for transport of water between the liquid phase and solid phases [59] and decreased the possibility of molecular sieve absorbing water away from enzyme directly [116]. At the initial phase, the conversion of fructose oleate increased with the amounts of molecular sieve. The maximum conversion (89.9 wt %) was obtained at the employment of 12 wt % MS (i.e., 0.12 g molecular sieves per g of reaction mixture) in a MSC, equal to a residence time of 6.21 min per pass (**Fig. 4.2A**). Higher or lower amounts of molecular sieves have a negative effect on the final yield (**Fig. 4.2**). The amount of removed water was directly proportional to the concentration of MSC, as shown when the water concentration decrease at 70 h in **Fig. 4.2B** is plotted versus the molecular sieve concentration (**Fig. 4.3**). Hence, it appears that the optimal water concentration is approximately at 0.4wt % with 12 wt %

molecular sieve (**Fig. 4.2B**). Lower amounts of molecular sieve (i.e., 7wt %) removed insufficiently water from reaction medium (from 0.775 wt % to 0.611 wt %) resulting in the hydrolysis of the desire products. In contrast, higher amount of molecular sieve (i.e., 16 and 20 wt %) reduced excessive water (0.236 wt % to 0.092 wt %) leading to the loss of enzyme activity [182, 183].

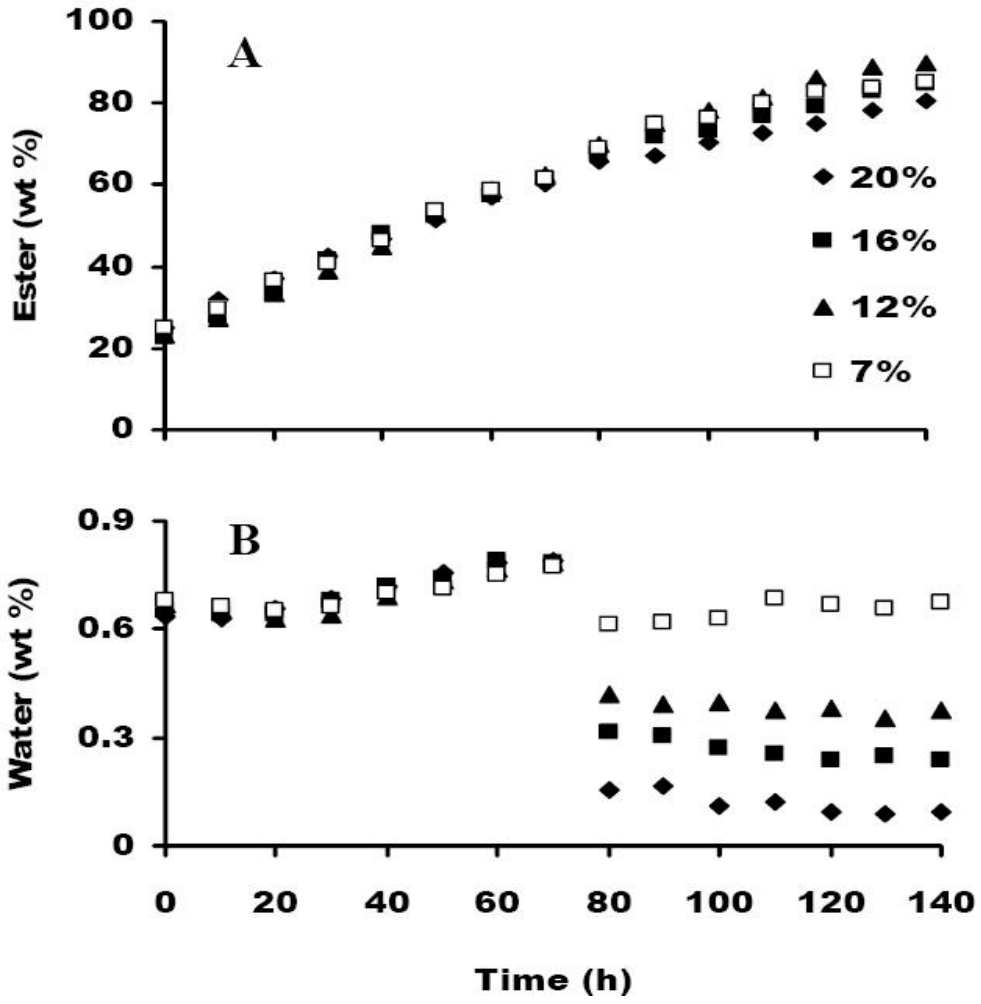


Fig.4.2. Effect of molecular sieve concentration (g per g oleic acid+FOE, given in legend) on a. immobilized Rhizomucor miehei lipase- (RML-) catalyzed synthesis of fructose -oleic acid esters and b. liquid phase water concentration for a bioreactor system

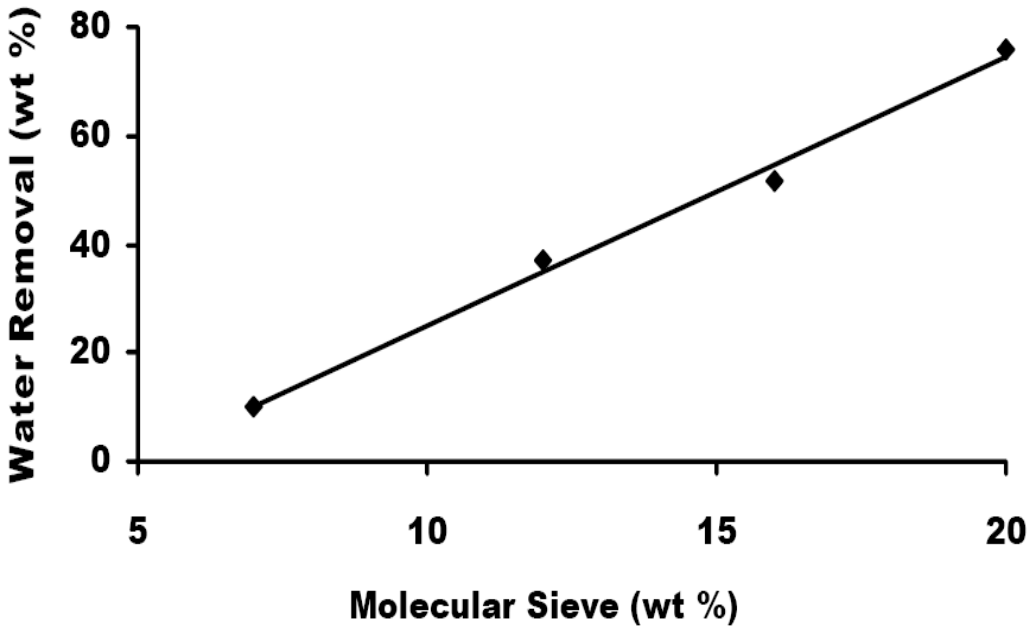


Fig.4.3. Effect of molecular sieve concentration (g per g of reaction medium*100 %) on water removal (g per g of reaction medium*100 %) in the liquid phase of reaction medium.

4.4.2 Effect of Timing for Incorporating Molecular Sieve Column into the Packed-Bed Bioreactor System

As mentioned in previous reports of our group [114, 115, and 160], inappropriate introduction time of water removal method results in a decrease reaction rate. Up to this point in the dissertation, timing for the incorporating MSC into the bioreactor system is still unclear and need to be investigated for optimization of bioreactor system. Fig. 4.4 compare the different introduction times for incorporating the MSC (12 wt % sieves) into the PBBR-based bioreactor system utilizing suspensions. The maximum rate and conversion (89.1 wt %) was achieved when incorporating the MSC at 70 h, equivalent to approximately 60 wt % conversion into esters in the liquid phase (**Fig. 4.4A**). The 70 h incorporation time created the optimal water concentration 0.35-0.40 wt % in the liquid phase (**Fig.4.4B**). Furthermore, **Fig. 4** demonstrates although the early introduction of molecular sieves into the reaction (i.e., at 30 or 50 h, equivalent to 39.5 wt % and 52.9 wt % conversion, respectively) also obtained the optimal water concentration (0.4 wt %), the rate of reaction was decreased, presumably due to extraction of water from the lipase, and hence reduced rates and yields(Fig 3). However, with employment of molecular sieves into the reaction during the latter stage of reaction (90 and 110 h, or equivalently 68 and 77% conversion, respectively), they lead to the lower conversions since the high water contents (~0.6 wt %) cause the hydrolysis of esters (**Fig. 4A & B**). The time course of reaction for the experiments in **Figs 4.4** are in strong consistent with each other and with **Fig. 4.3** experiments, indicating the high repeatability of the bioreactor performance.

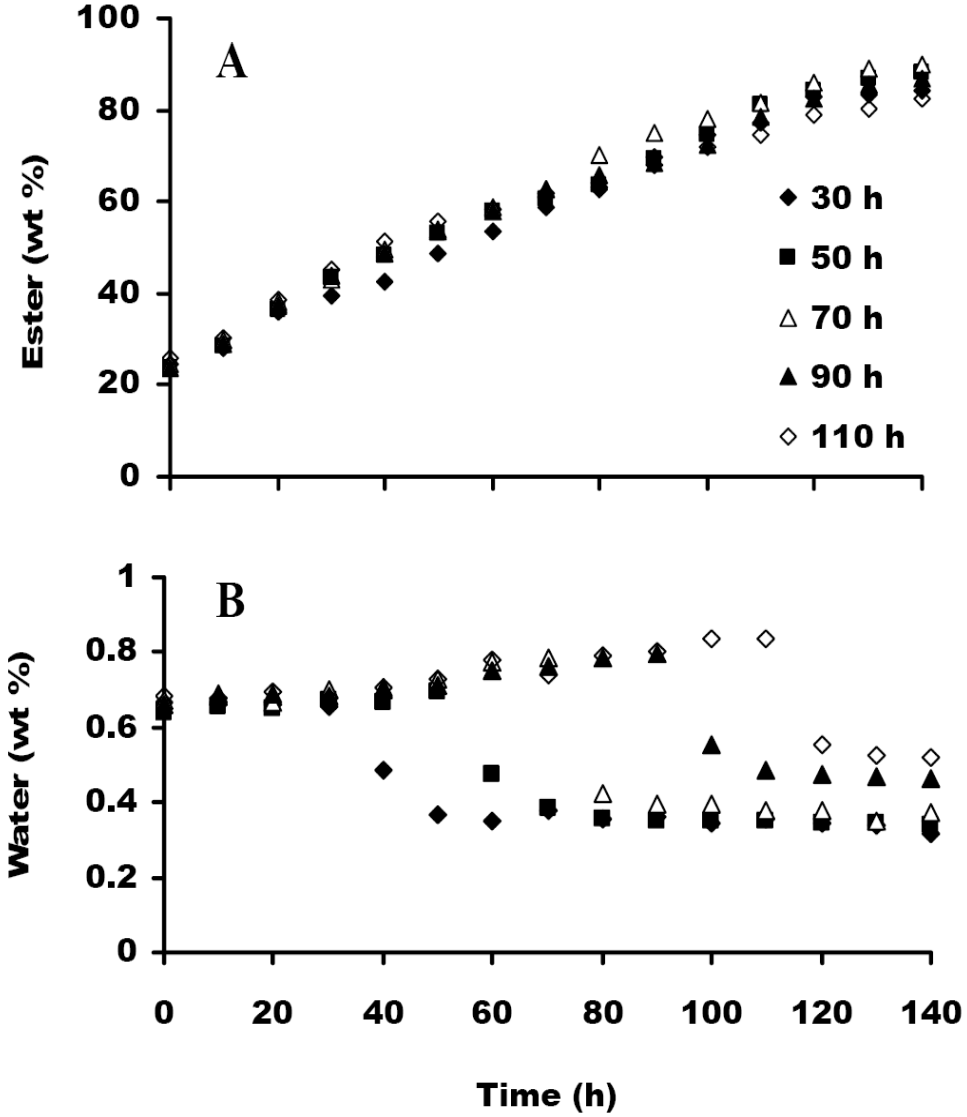


Fig.4.4. Effect of introduction time for the molecular sieve column (given in legend) on the RML-catalyzed esterification of fructose and oleic acid employing a suspension of fructose crystals in solvent-free media and a bioreactor system containing a packed-bed bioreactor undergoing continuous recirculation.

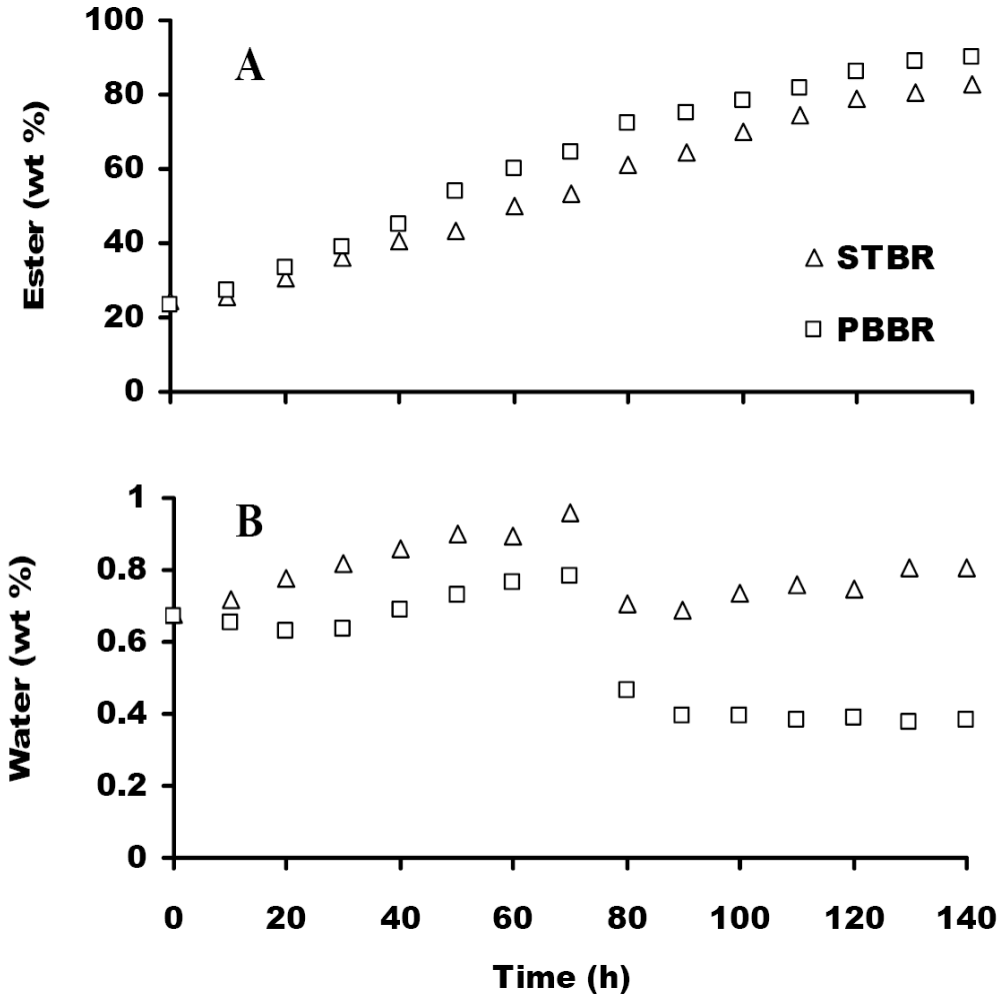


Fig. 4.5 Effect of different bioreactor types on the RML-catalyzed fructose-oleic acid ester synthesis utilizing a suspension of fructose crystals undergoing continuous recirculation at 65^{o}C using biosystem.

4.4.3 Effect of Bioreactor Type on Production of Fructose Oleate

In the previous publication [116], the different bioreactor types using saturation saccharide concentrations were investigated and demonstrated that the rate and extent of reaction was higher for PBBRs compared to stirred-tank bioreactors (STBRs). However, the comparison of PBBRs and STBRs using suspensions has not been studied yet. **Fig. 4.5** compared the two typical bioreactor types with the employment of suspensions, with identical molecular sieve concentrations and programming of the introduction time of the MS for both bioreactor systems. The results indicated that the highest productivity (0.193 $mmol_{FOE}\ h^{-1}\ g_{lipase}^{-1}$), and 90.2 wt % FOE, were obtained in PBBR system. Only 0.171 $mmol_{FOE}\ h^{-1}\ g_{lipase}^{-1}$ with 82.5 wt % ester was achieved In STBRs. This result is strongly in agreement with the previous paper in our group [116]. The difference of water contents (**Fig.4.5B**, 0.42 wt % in PBBRs and 0.817 wt % in STBRs, respectively) in the liquid phase of two bioreactor types resulted in the difference of ester productivity and conversions. Although the MS particles did not undergo breakage as expected, an adsorbed saccharide layer was formed on the MS particles during the later phase of reaction, hindering the ability of MS particles to remove water from reaction medium, further leading to a higher water concentration in STBR liquid phase.

4.4.4 Comparison of Water Removal Methods

In the Chapter 2, numerous water removal methods were reviewed for the lipase catalyzed synthesis of saccharide fatty acid esters, commonly including molecular sieves [59,67, 93, 117], silica gel [156-158], azeotropic distillation [161,162], vacuum pressure [110,168], pervaporation [169-171], free evaporation [58,115,175,176], dry air or N_2 [184,185], salt hydrate pairs

[186,187]. However, few publications have been reported and compared various water removal methods for lipase-catalyzed reactions in nonaqueous media. **Fig. 4.6** indicates the effect of four different water removal approaches: vacuum pressure (20 in Hg), N_2 bubbling (2.5 L min^{-1} at standard pressure and temperature, 101.3 kPa and 273.15 K, respectively), MS (20 wt% overall), and a combination of vacuum pressure and N_2 bubbling, applied to a suspension of fructose crystals in oleic acid/FOE 75/25 w/w at 80 °C during the time course. The water removal methods are listed in their order of effectiveness to remove water as followings: vacuum+ N_2 bubbling (2.16 mg_{H2O} h^{-1} > molecular sieves (1.42 mg_{H2O} h^{-1}) > N_2 bubbling (0.68 mg_{H2O} h^{-1}) > vacuum (0.23 mg_{H2O} h^{-1}). Consequently, the combination of vacuum and nitrogen was chosen as an effective water removal method to strip water from the reaction medium for optimizing the performance of bioreactor in subsequent experiments. Additionally, the water convective mass transfer rates of air or/and N_2 bubbling is relatively high with minimal influence on enzyme activity and stability [184]. On the other hand, with employment of the combination of vacuum and N_2, lowered the water contents from 0.67 wt % to 0.24 wt % in 20 h, and to 0.16 wt % in 140 h (**Fig. 4.6**) were obtained, which is far below the optimal level, 0.4 wt % plus/minus 0.05 wt % in **Fig. 4.1**. Hence, when using this approach, excessive removal of water should be avoided.

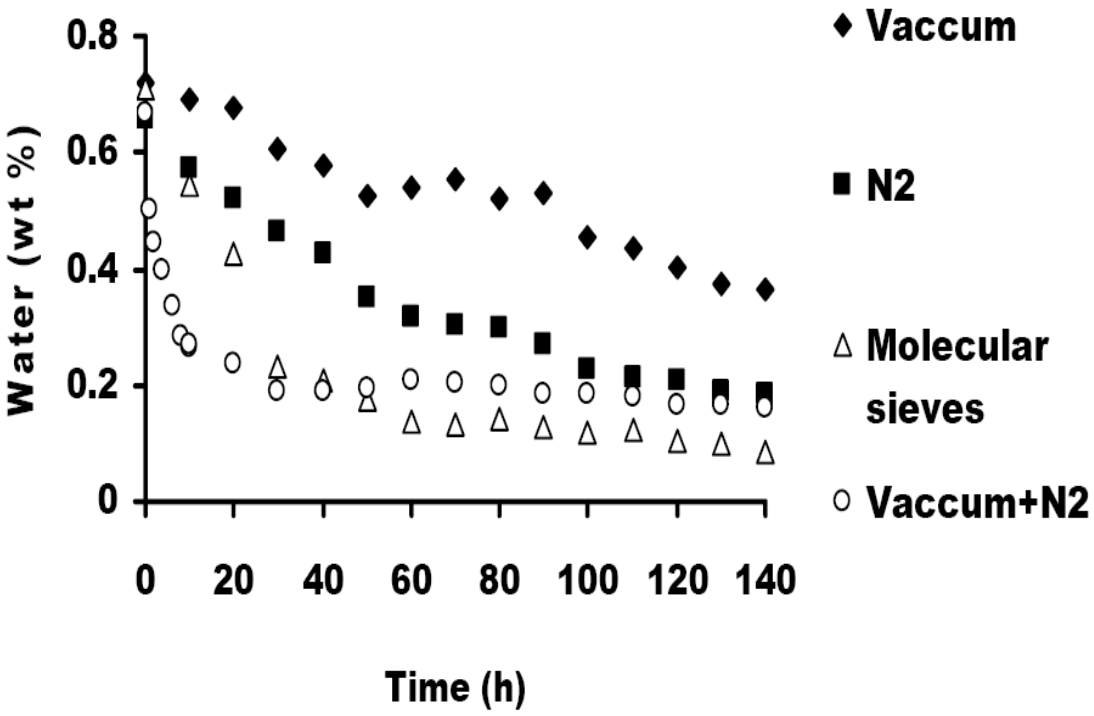


Fig.4.6. Effect of water removal methods on the water concentration for a suspension of fructose crystals in FOE/oleic acid 25/75 w/w at 80 ^{o}C.

4.4.5 Determination of Required Interval Time for the Reformation of Suspensions of Fructose Crystals in Solvent-Free Media

For the PBBR-based bioreactor system employed in our previous work [17] and for **Figs 4.2**, **4.3**, and **4.4** of this dissertation, 10 h intervals were employed for the removal of the liquid phase and re-formation of suspensions through the addition of solid-phase fructose under stirring, followed by sedimentation to remove the larger particles. However, we observed that saccharide was frequently 100% consumed before the end of the 10 h cycle was reached, particularly during the initial period of the reaction where the rate is highest [17].Therefore, the interval times during lipase-catalyzed synthesis of saccharide-fatty acid esters reaction can be further optimized. **Fig. 4.6** explores the time course of reaction for each interval using the PBBR-based bioreactor system. For the first cycle, it is clear that the formation of ester is nearly complete since the conversion slows at the approach of 30% esters (dashed arrows). Therefore, utilization of 4.0 h is sufficient for operation of the first cycle (solid downward-pointing arrows of **Fig. 4.6**) rather than the 10 h interval employed previously. Likewise, the second and third cycles can be shortened to 4.0 h, the fourth cycle to 5.0 h, and the fifth cycle to 8.0 h (**Fig. 4.6**). Additionally, the total reaction time is decreased from 140 h to 117 h when the interval time is optimized. Of note, the final conversion achieve at the end of each interval of **Fig. 4.6** strongly agrees with the conversions achieved at the end of each 10 h interval in **Fig. 4.2**, further demonstrating the repeatability of the PBBR-based bioreactor's performance.

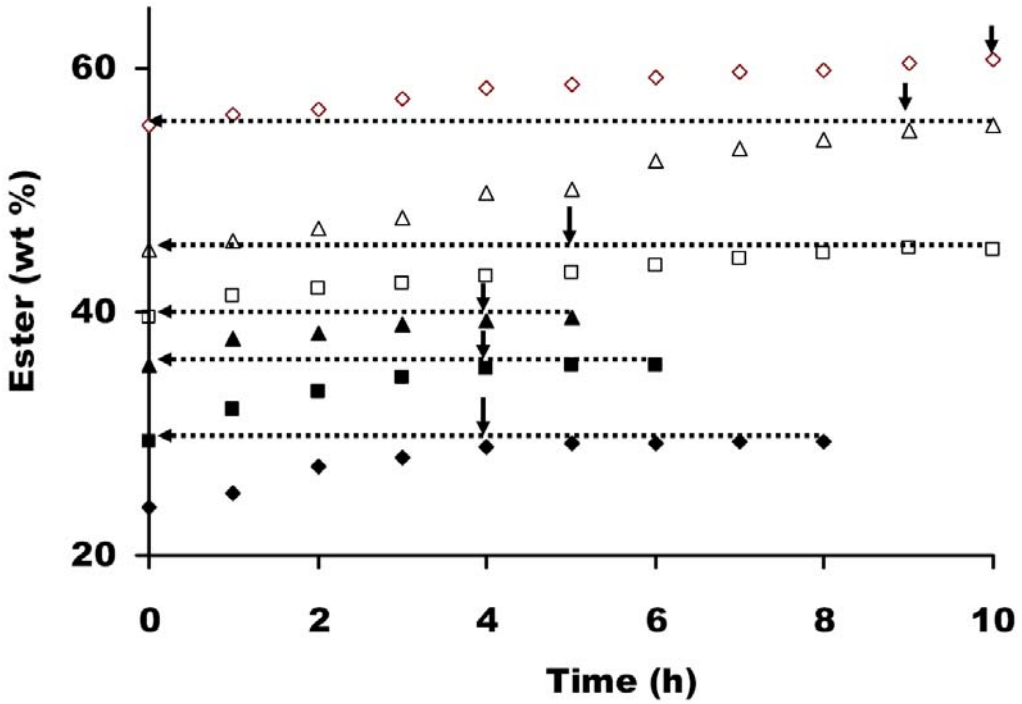


Fig.4.7. Determination of required interval time for the reformation of suspensions of fructose crystals in solvent-free media for the RML-catalyzed esterification.

4.4.6 Employment of Lower Initial Ester Concentrations

Up to this point in my dissertation, the mixture of 25% w/w ester/75 % w/w oleic acid was used as the starting materials for lipase catalyzed synthesis of saccharide fatty acid esters. In this section, we explored the possibility of minimizing the ester content in initial phase to form the stable, metastable suspensions. **Fig. 4.7** indicated that the initial reaction rate did not vary with ester concentration between 5 and 25 wt %, illustrating oleic acid/FOE 95/5 w/w could act as the initial reaction medium, requiring 40 h (i.e., 4 cycles) of additional reaction time. Furthermore, the fructose concentrations were reasonably high: 0.69 wt% initially, increasing to 0.79 wt %, 1.18 wt %, and 1.49 wt % after the second, third, and fourth 10 h cycles, respectively. The latter value is strongly consistent with the initial fructose concentration for 25% FOE / 75% oleic acid as initial medium, 1.45% (e.g., **Fig. 4.2**). However, FOE concentrations < 5 wt %, for instance, 3 wt % and 0 wt %, resulted in the poor initial reaction rates since the low concentration of saccharide suspensions were obtained (unpublished data).

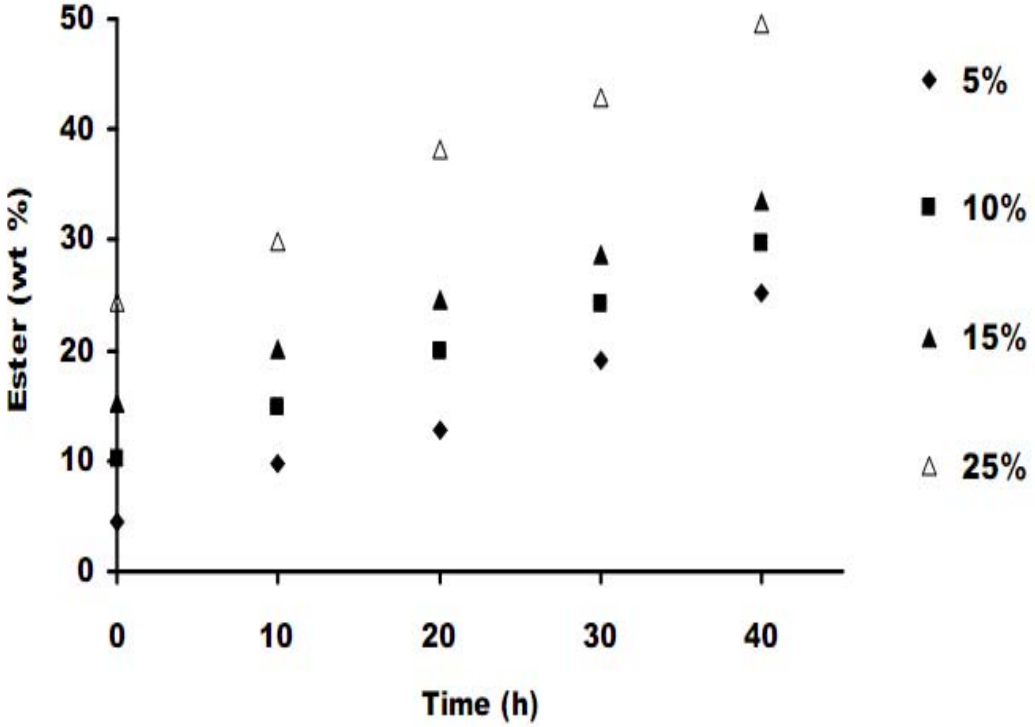


Fig.4.8. Effect of the initial ester content of the solvent-free reaction medium on the initial rate of RML-catalyzed esterification.

4.4.7 Operation of Solvent-Free Packed-Bed Bioreactor System Using Suspensions under Optimized Interval Time and Water Removal Programming

With the goal being to optimize the performance of the PBBR-based bioreactor system for synthesis of the solvent-free lipase-catalyzed saccharide-fatty acid esters, combining the knowledge learned on controlling the methodology, the appropriate amount and timing of water removal (vacuum pressure, 20 in Hg + N_2 bubbling, 2.6 L min^{-1} selected, introduced upon reaching 60% conversion) bioreactor type (with PBBR selected, due to the lower water content), and the interval time (programmed as described in **Fig. 4.7**). Initial medium contained oleic acid/FOE 95/5 w/w containing suspensions of solid-phase fructose. In **Fig. 4.9A**, 92.6 wt % conversion was yielded in 5.5 days. The reaction product consisted of 93 wt % monoester and 7 wt % diester in **Fig. 4.10**, consistent with our previous results [181]. The control experiment obtained < 60 wt % conversion over the same time period (employing 10 h intervals for re-formation of suspensions and only free evaporation for water removal). For the average productivity rate, 9.13% conversion per day conversion for the control is much lower than optimal conditions, 15.9% conversion per day. **Fig. 4.9B** indicates that the water concentration of the optimized process was maintained at the optimal water content, approximately 0.4 % w/w, which is much lower than control's water concentration ~0.9 % w/w, leading to the hydrolysis of desired products. **Fig. 4.11** showed that the saccharide concentration was consistently increased with the increase of ester content and finally reached upon ~2.5 wt

4.4.8 Operation of Solvent-Free Packed-Bed Bioreactor System Using Suspensions under Optimized Interval Time and Water Removal Programming

Furthermore, the reusability and operational stability of RML was investigated using the PBBR based bioreactor system developed in Chapter 3 with a Wheaton Celstir spinner double-side arm flask serving as a reservoir and allowing for *in situ* water control place by the combination of nitrogen gas bubbling and vacuum pressure at 2.16 mg_{H2O} h^{-1} to maintain water concentration at nearly 0.4% w/w. In sum, four successive reactions were operated without essentially any loss of activity, demonstrated by minimal change in the time course of reaction, including the initial rate (**Fig. 4.12** and **Table 4.1**). The final ester content was consistently 84.8-90.2 % w/w for the four successive reactions in **Fig. 4.12a** and **Table 4.1**. The water concentration was maintained at ~0.4 w/w in **Fig. 4.12b**, consistent with the previous results in this Chapter. The excellent stability of RML may benefit from the solvent-free reaction medium and water control method. The lack of organic solvent in the reaction medium during the time course reduces deactivation of lipase. Air or/and N_2 have a minimal influence on enzyme activity and stability [184]. In conclusion, RML maintained a high activity for four successive reactions, equal to 528h (22 day) of operation.

4.5 Conclusion

In this Chapter, the performance of the lipase-catalyzed synthesis of saccharide-fatty acid esters employing solvent-free suspensions of solid-phase saccharide in a packed bed bioreactor system was optimized in this study. The results illustrated that water removal method should be introduced to the bioreactor system upon reaching approximately 60% w/w conversion, at an optimal liquid-phase water concentration of ~0.4 % w/w. In addition, the combination of nitrogen gas bubbling + vacuum pressure was tested to be the most efficient water removal method. Plus, the performances of the stirred tank bioreactor and packed bed bioreactor under the uniform experimental condition were examined. Results indicated that the rate and yield of ester were significantly higher using a PBBR compared to a STBR, due to lower water content in the former. Furthermore, the interval time for re-treatment of suspensions was optimized leading to a decrease of the reaction time by 23 h. Additionally, the initial ester concentration for formation of the solvent-free suspensions could be decreased from 25% w/w to 5% w/w without any loss of reaction rate. By incorporating the optimal condition mentioned above, a yield of 92.6% w/w fructose oleate was achieved in the PBBR-based bioreactor system within 132h, increasing the productivity (0.297 $mmol_{FOE}$ h^{-1} g_{lipase}^{-1}), more than a 2-fold increase compared to the control (0.129 mmol h^{-1} g^{-1}).

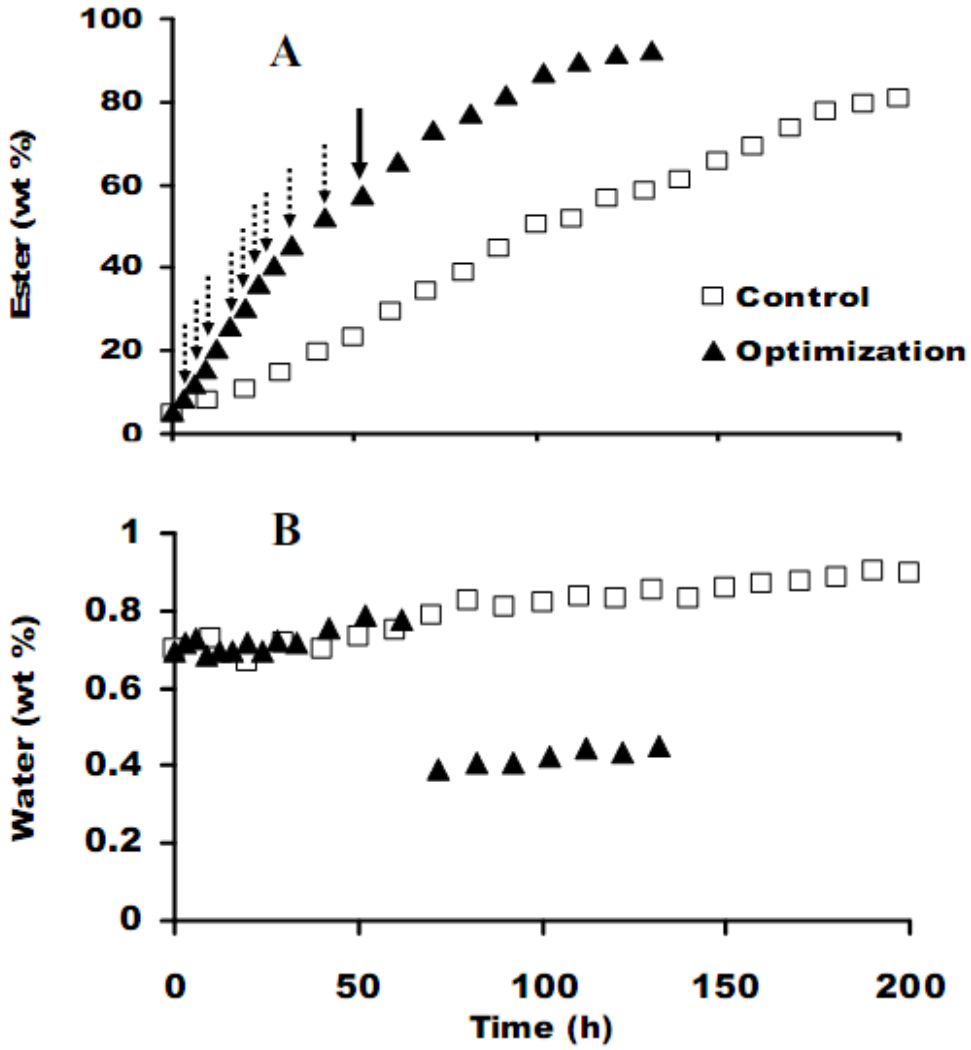


Fig.4.9. The effect of optimization of interval time for retreating of suspensions and removal of water for the solvent-free RML-catalyzed synthesis of fructose oleate using bioreactor system.

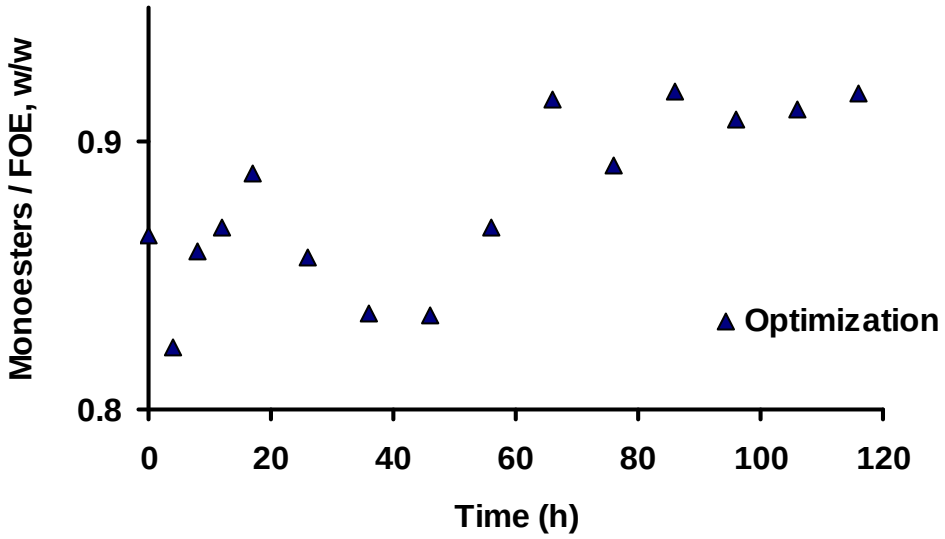


Fig. 4.10. Mass fraction of monoesters among fructose-oleic acid esters (FOE) for the reaction depicted in Fig. 4.9 that was operated under optimal conditions.

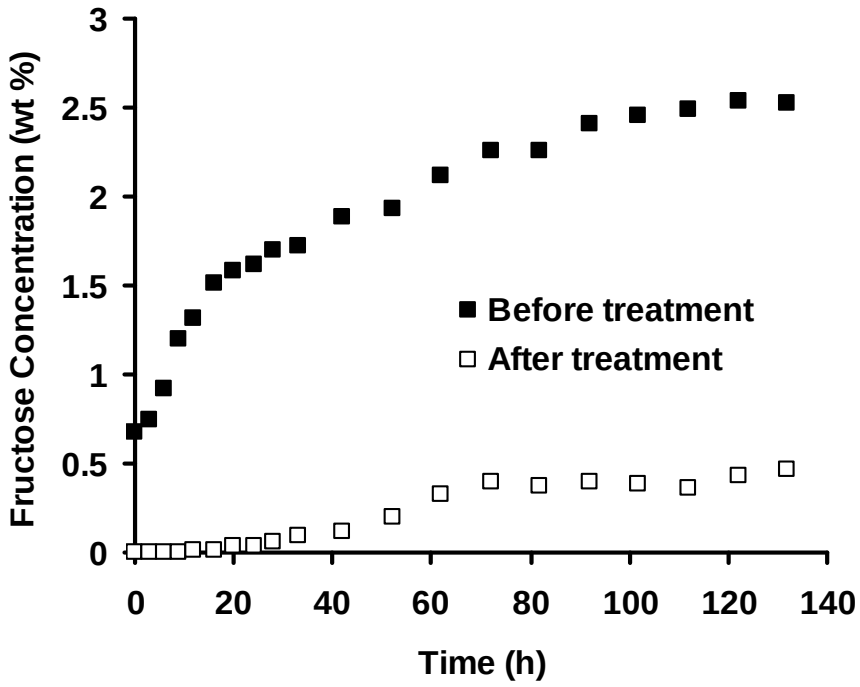


Fig.4.11. Concentration of fructose in the liquid phase before and after re-treatment of the liquid-phase medium through additional fructose, to produce metastable fructose suspensions, for the reaction depicted in Fig. 4.9.

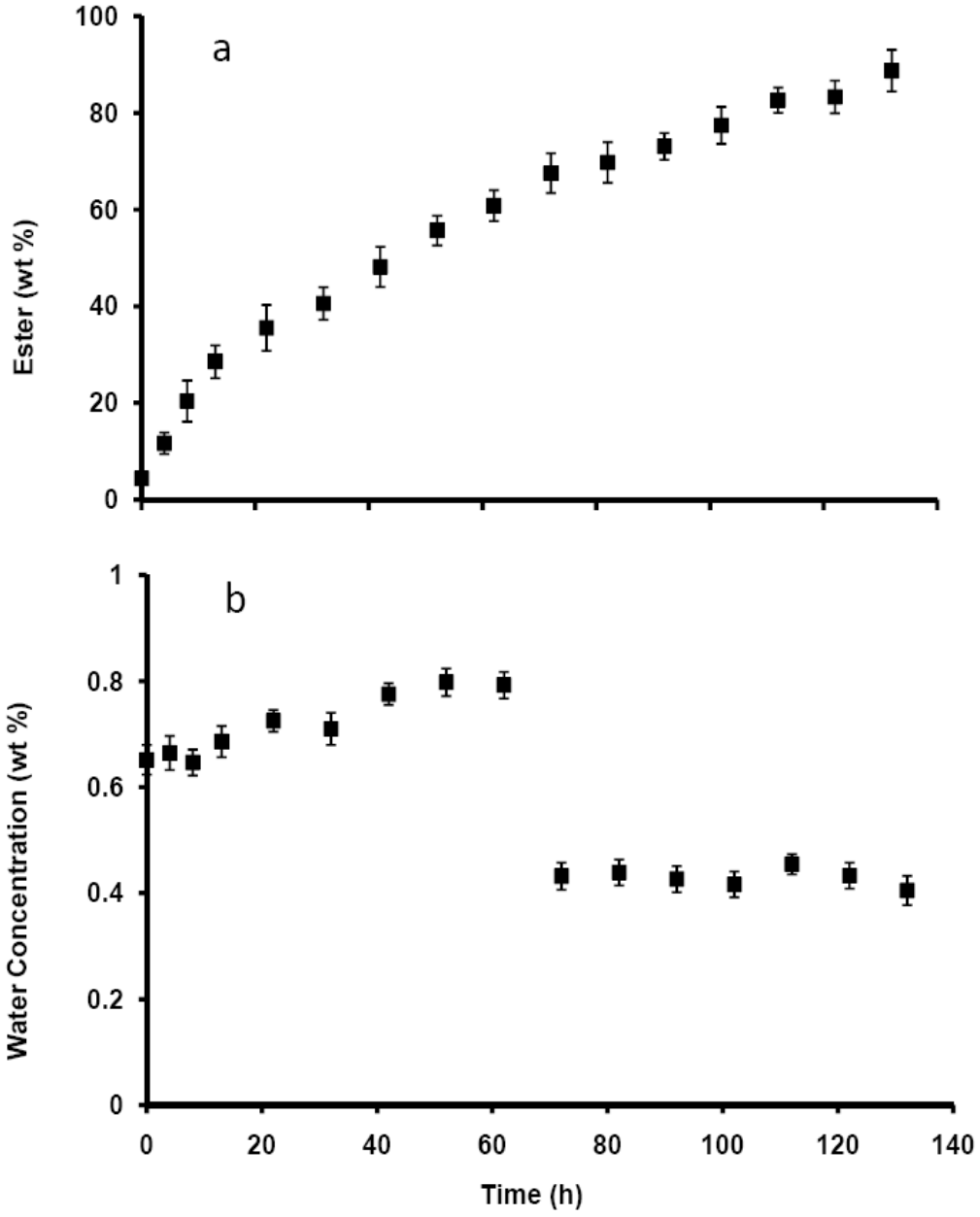


Fig.4.12. The examination of enzyme activity retention for RML for the solvent-free synthesis of fructose oleate at 65 ^{o}C in bioreactor system.

Table 4.1 The Change of Initial Reaction Rate & Yield of Fructose Oleate for Four Successive Runs Employing the Same Enzyme [a]

Run number	**Initial rate (% Ester h^{-1})**	**% Ester @ 132h**
1	1.27	87.5
2	1.32	91.2
3	1.25	86.1
4	1.29	89.6

[a] Reaction conditions: The reaction was operated under optimized condition described in Chapter 4 in a packed bed based bioreactor system with *in situ* wheaton celstir spinner double-side arm flask using the combination of nitrogen gas bubbling and vacuum pressure at 2.16 mg_{H2O} h^{-1} to maintain water concentration at nearly 0.4% w/w .

CHAPTER 5

EFFECT OF ACYL DONORS AND ACCEPTORS ON LIPASE-CATALYZED SYNTHESIS OF SACCHARIDE-FATTY ACID ESTERS UTILIZING SOLVENT-FREE SUSPENSIONS

5.1 Abstract

The effect of acyl donor (oleic, caprylic, lauric and myristic acids) and acceptor (fructose, sucrose, glucose and xylose) were investigated for the solvent-free lipase-catalyzed synthesis of saccharide-fatty acid esters, biodegradable and biocompatible biobased surfactants. The reactions were conducted using a packed bed (PBBR)-based bioreactor system at 53 ^{o}C or a stirred tank bioreactor (STBR) at 65^{o}C using immobilized *Rhizomucor miehei* lipase (or "RML," Lipozyme®IM, Novozymes, Franklinton, NC USA) and solvent-free (50-200 μm-sized) suspensions of saccharide crystals in a mixture of fatty acid / fructose oleate (90 wt % monoester and 10 wt % diester) at a ratio of 75/25 w/w initially. The highest conversion and initial rate occurred when the saccharide concentration was highest. Suspensions containing the highest saccharide concentration coincided with saccharide crystals of the smallest average size, since large-sized crystals sedimented out during the workup for formation of the suspensions. The best performance was achieved using fructose and oleic acid as substrates (92.3 wt % ester, 92 wt % of which was monoester) with a PBBR; and, all other acyl acceptors and donors achieving at least 47.6 wt % conversion of acyl donor, or equivalently, a solvent-free product mixture containing at least 69 wt % ester.

5.2 Introduction

In this chapter, the content was taken from a recently accepted paper [201]. Note that the contents of this chapter are a part component of Objective 2 (given in Chapter 1).

As described in Chapter 3 and 4, I successfully developed a two-step bioreactor system undergoing continuous recirculation at 0.5 mL min^{-1} using the fructose or sucrose suspensions crystals was. However, it is unclear for me to use other common acyl donors and acceptors for this bioreactor system. Hence, in this Chapter, the effect of acyl donor (oleic, caprylic, lauric and myristic acids) and acceptor (fructose, sucrose, glucose and xylose) were investigated to examine the universality of the bioreactor system. Furthermore, another aim of this section is to better understand the difference of the performances of acyl acceptors, dependent on inherent selectivity of acyl acceptor substrate or/and attributable to differences in saccharide concentration. To complete this aim, determining the underlying reason for difference in sacchariade concentration between suspensions formed by different acyl donors and acceptors was studied in this section.

5.3 Methods and Materials

5.3.1 Materials

Technical grade oleic (*cis*-9-octadecenoic) acid, 90% pure (melting point temperature, or T_{MP}, of 16.3^{o}C, D-(+)-glucose (>99.5% pure), lauric (dodecanoic) acid (>99% pure, T_{MP} = 43.2^{o}C) and myristic (tetradecanoic) acid

(>99% pure, T_{MP}, = 54.4°C) and Lipozyme®IM were purchased from Sigma-Aldrich (St. Louis, MO USA). The latter, referred to as "RML" in this Chapter, is lipase (EC 3.1.1.3) from *Rhizomucor miehei* immobilized onto macroporous anionic resin beads (140 U g^{-1}, where 1 U refers to the amount of enzyme which releases 1 μmol stearic acid per minute from tristearin at pH 8.0 and 70 °C), manufactured by Novozymes, Inc. (Franklinton, NC USA). D-Fructose (>98% pure), D-xylose (>98% pure), sucrose (>98% pure), acetone (HPLC-grade), acetonitrile (HPLC-grade), and molecular sieves (Type 3A, 4-8 mesh, Grade 562) were obtained from Fisher Scientific (Pittsburgh, PA USA). Caprylic (octanoic) acid (>99.5% pure) was purchased from MP Biomedicals, Inc. (Solon, OH USA). All materials were used without further purification. The saccharide crystals were ground into a fine powder using a mortar and pestle. Technical grade fructose-oleic acid ester, the reaction product and a component of the initial charge to the bioreactor system, was synthesized enzymatically. The reaction product consisted of 89.6 wt % ester, or FOE (of which 83.6 wt % is monoester and 16.4 wt % diester), and 10.4 wt% oleic acid. This product was mixed with fatty acid to obtain the desired proportions of acyl donors and ester, and fed to the bioreactor system.

5.3.2 Methods

5.3.2.1 Formation of Supersaturated Solutions of Saccharides in Fatty Acid / Fructose-Oleic Acid Ester Mixtures (Initial Reaction Medium)

Generally, the initial reaction medium introduced into the bioreactor system, a suspension of saccharide crystals in solvent-free media, was formed by mixing 1.5 g saccharide crystals and 10 g of a fatty acid / FOE 75/25 w/w mixture in a 20 mL scintillation vial open to the atmosphere on a magnetic stirrer plate (Super-Nuova from Barnstead, Dubuque, IA USA) at 800 rpm (or equivalently,

at a radial acceleration of 11 g) and 80^{o}C for 6 h. The slurry was centrifuged at 800 rpm (or equivalently, 71 g) for 0.5-1.0 min, with the supernatant collected. The temperature of the resultant suspension after removal from the centrifuge was 25^{o}C. For suspension-based media formed using lauric or myristic acid as acyl donor, centrifugation was not employed because the centrifugation instrumentation possessed by us could not retain the media above the melting point temperature, leading to solidification. Therefore, for these acyl donors, larger particles were removed by allowing the medium to remain stagnant on a hot plate for 30 min at 65^{o}C; then, the supernatant was isolated by careful use of a transfer pipette.

5.3.2.2 Operation of Packed-Bed Bioreactor System Undergoing Continuous Recirculation

The closed-loop bioreactor system, employed for reactions using oleic and caprylic acid as acyl donor, consisted of four main components connected in series: a 20 mL scintillation vial open to the atmosphere serving as a reservoir, a peristaltic pump (BioLogic LP® from Bio-Rad, Hercules, CA USA), a packed column of molecular sieves (1.36 g of MS packed into a 50 mm × 10 mm ID Omnifit® chromatography column) a packed bed bioreactor (PBBR; 50mm L × 10mm ID Omnifit® chromatography column packed with 0.76 g of RML per g of reaction medium). The outlet stream from the PBBR was returned to the reservoir to create a loop, which underwent continuous recirculation at 0.5 mL min^{-1}; moreover, the average residence time for the MSC and PBBR were 6.2 min per pass and 7.6 min per pass, respectively. The MSC and PBBR were maintained at 53^{o}C via a convection oven. The enzyme concentration and recirculation rate employed herein were determined previously to be optimal [181]. The initial charge to the reactor consisted of 10 g of acyl donor / fructose oleate 75/25 w/w. Other details of the bioreactor system have been

mentioned in my previous paper [181]. All reactions were performed in duplicate, with data reported in the figures and table representing average values.

At specified intervals determined previously to be optimal, the suspension-based solution contained within the reservoir was re-treated as described above through introduction of additional (1.5 g) saccharide, to replenish the consumed substrate: 3.0 h for intervals 1-3, 4.0 h for intervals 4-6, 6.0 h for interval 7, 9.0 h for interval 8, and 10 h for subsequent intervals (Ye and Hayes, submitted). During the re-formation of suspensions, the bioreactor system was temporarily paused by stopping the pump; the mixture in the reservoir was transferred to another 20 mL vial where additional saccharide was added and suspensions reformed, as described above. Then the retreated suspension-based medium was returned to the reservoir and the pump re-initiated. Initially, the MSC was not contained in the closed loop bioreactor system (since the free evaporation of water in the reservoir was found to be sufficient for removal of the water generated by the reaction), but was incorporated into the system when the ester content of the medium reached 60% to further reduce the liquid—phase water content, since this approach was found to be optimal in our previous work [201].

5.3.2.3 Operation of Stirred-Tank- Bioreactor

For reactions employing lauric and myristic acid as acyl donors, reactions were conducted in a stirred-tank bioreactor (STBR), a 20 mL scintillation vial containing 0.756 g of RML particles per g or reaction medium operated under magnetic stirring at 300 rpm (equivalently, 1.5 g) at 65^{o}C through use of a hot plate-stirrer with 13 wt% molecular sieves for water concentration control. The mass of the initial charge to the STBR is identical to that used for the PBBR bioreactor systems, with its composition consisting of 72.1% lauric or myristic acid, 2.9% oleic acid, and 25% FOE; i.e., the acyl donor composition

was 96.1% lauric or myristic acid and 3.9% oleic acid. The suspension-based medium was retreated with additional acyl acceptor periodically, as described above; however, RML particles were removed as a first step through microfiltration conducted in a 65 ^{o}C oven. All reactions were performed in duplicate, with data reported in the figures and table representing average values.

5.3.2.4 Monitoring of Water, Fatty Acid, Ester, Saccharide Concentration and Particle Size

The water content for the reaction mixture was analyzed using a coulometric Karl-Fischer Titrator (Denver Instrument Company, Aurora, CO, USA) after its dilution with methanol. The relative amounts of fatty acid and its mono- and di-esters formed with saccharides was determined using a dual-pump system from Varian (Walnut Grove, CA) and a model Mark III evaporative light scattering detector from Alltech Associates, a division of WR Grace (Deerfield, IL) [7] . An analytical reversed phase (4.6 x 250 mm, pore diameter 5 μm) C_{18} column from Alltech was employed using separation conditions consisting of a column temperature of 25^{o}C and an isocratic solvent system, acetone / acetonitrile / acetic acid (45 / 45 / 10 v/v/v) at flow rate of 1.0 mL min^{-1}. Response factors were measured and employed to convert peak areas into concentrations [7]. Chromatograms of mono- and diester species obtained by us agree with those reported in Ref [19]

To analyze the saccharide content, 40 mg-sized aliquots of column effluent were subjected to liquid-liquid extraction by the system of *n*-hexane and water (500 μL of each) [19]. The extraction was carried out 3 X at 35^{o}C for 2 hr using a thermomixer (Eppendorf AG, Germany). The aliquots from the pooled aqueous extraction solutions were diluted with acetonitrile to match the composition of the HPLC mobile phase to prevent peak broadening in the

HPLC analysis. An analytical Prevail Carbohydrate ES column (4.6 x 250 mm, pore diameter 5 μm) from Alltech was employed using a column temperature of 25^{o}C and an isocratic solvent system, acetonitrile / deionized water (80 / 20 v/v) at flow rate of 1 mL min^{-1}. Standard curves for saccharide concentration in a fatty acid / saccharide-fatty acid esters liquid phase versus peak area were obtained and found to be independent of the reaction mixture's composition [19].

The particle size distribution of the dispersions present in the above-mentioned suspension solution following the method in the section of formation of supersaturated solutions of saccharides in fatty acid was analyzed by a Zeta potential Analyzer, Zeta PALS (Brookhaven Instruments Corporation, Holtsville, NY, USA) [9]. All measurements were performed in triplicate, with data reported in the figures and table representing average values.

5.4 Results and Discussion

5.4.1 Effect of Acyl Acceptor Type on Lipase-Catalyzed Solvent-Free Production of Saccharide-Oleic Acid Esters in a Packed-Bed Bioreactor System

As mentioned in the prior publications [115, 133, 181, 182], fructose serving as the main acyl acceptor for was employed for the formation of suspensions, further to synthesize the saccharide fatty acid ester in the bioreactor system. Here, sucrose, glucose, and xylose were examined for their capabilities acting as acyl acceptors. Fructose oleic acid ester (FOE) was used as a cosolvent promoting the miscibility of acyl acceptors and donors. From the previous section, it is known that 5 wt% ester is adequate for fructose to form metastable suspensions in oleic-rich solvent free media at 53^{0}C. However, for glucose and

xylose, starting FOE (< 25 wt %) generated the poor concentration of glucose and xylose suspensions leading to slow initial reaction rates. Therefore, all the reactions in this section employed the mixture of oleic acid/FOE 75/25 w/w initially on a saccharide-free basis. These reactions were operated at the optimal conditions in the bioreactor system, as described in the Section 4**.7.** The performances of four different acyl acceptors were compared during the time course of lipase-catalyzed esterification reaction for synthesis of saccharide-oleic acid esters in PBBR-based bioreactor system using suspensions in **Fig. 5.1a**. The order of the final ester concentrations and initial reaction rates of four common acyl acceptors are as follows: fructose (92.3 wt % ester) > sucrose (85.5 wt %) > glucose (82.6 wt %) > xylose (74.9 wt %). The maximum fructose-oleic acid ester content is agreement with our prior publications [181]. Since water concentrations were maintained at the optimal level (~0.4 wt %) by the molecular sieve column in **Fig. 5.1a**, the saccharide concentrations were attributed to be the primary reason for the differences of performances of bioreactor system. The saccharide concentrations in the PBBR at the beginning and end of each interval were plotted in **Fig. 5.1b** (i.e., before and reformation of suspensions after adding additional saccharide crystals), demonstrating saccharide was completely consumed during the initial 4-hour stage for each acyl acceptor. During the first 40 h period of the reaction, the saccharide concentrations increased gradually with the improvement of ester concentration. The highest saccharide concentration was obtained using fructose as acyl acceptor, followed by sucrose, glucose and xylose (**Fig. 5.1b**), reflecting the rate and extent of esterification versus acyl acceptors relationship listed above. The identical trend of saccharide concentration with ester conversion indicated the relative concentration of acyl acceptor was more influential than the inherent acyl acceptor substrate selectivity of RML under these conditions. In general, the rate of lipase-catalyzed synthesis of saccharide-fatty acid esters increases with the increase of acyl acceptor (saccharide) concentration in publications for reactions conducted employing

polar solvents [188, 189] , ionic liquids [190], and supercritical carbon dioxide [191]. More importantly, for the reactions employing glucose, xylose, and sucrose as acyl donor, no free fructose was detected, suggesting the absence of lipase-catalyzed alcoholysis between FOE and acyl acceptor.

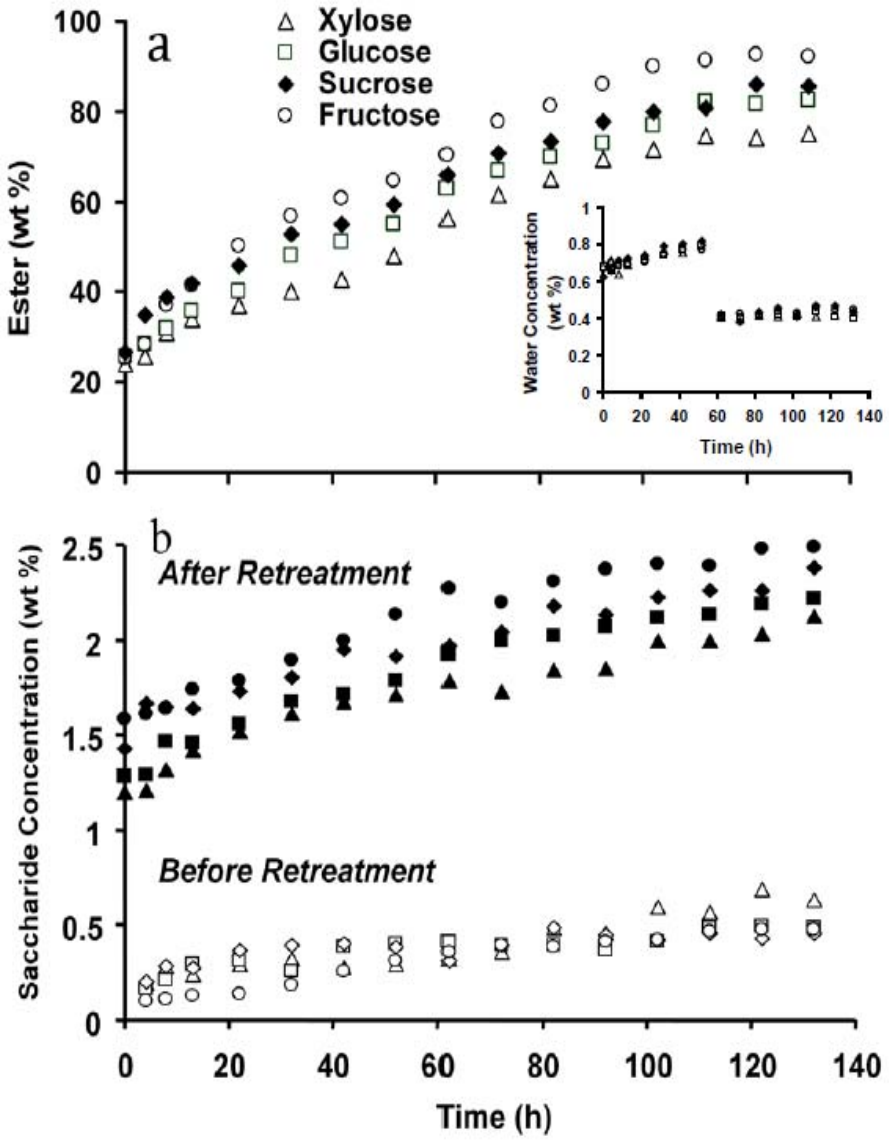


Fig. 5.1 Effect of different acyl acceptors (given in legend) on the time course of reaction for immobilized *Rhizomucor miehei* lipase- (RML-) catalyzed synthesis of saccharide -oleic acid esters in a packed-bed bioreactor (PBBR)-based bioreactor system at 53^{o}C.

5.4.2 Effect of Acyl Donor Type on Lipase-Catalyzed Solvent-Free Production of Saccharide-Oleic Acid Esters in a Packed-Bed Bioreactor System

In our previous work [115, 133, 181, 182], oleic acid (C_{18}) has been used as acyl donor. In this section, other acyl donors, for instance, caprylic, lauric, and myristic acids, have been employed for lipase- catalyzed synthesis of saccharide-fatty acid esters. Caprylic acid was utilized in the PBBR-based bioreactor system described above, employing the same operating conditions as described for **Fig. 5.1**, including the presence of 25 wt % FOE initially. However, lauric and myristic acids possessed too high of a melting point for the PBBR-based system, leading to frequent precipitation when employed. The precipitation caused the blockage of system, particularly in the tubing and entries and exits of the system components. Therefore, a stirred-tank bioreactor, or STBR, operated at 65^{o}C were employed for the latter two donors. **Fig. 5.2a** compares the time courses of lipase-catalyzed solvent-free synthesis of glucose and fructose-caprylic acid esters in the PBBR-based bioreactor system. The final reaction medium contained 79.3 wt % glucose-caprylic acid ester and 72.6 wt % fructose-caprylic acid ester, respectively. It appears the difference in ester yield and initial rate between glucose and fructose, and the lower ester yield and rate for caprylic acid employed as acyl donor compared to oleic acid is attributable to their respective saccharide concentration. **Fig.5.2b** indicates the same principle as **Fig.5.1b**: the saccharide concentrations increased gradually with the enhancement of ester concentration. The highest saccharide concentration was obtained using fructose as acyl acceptor. As shown in **Table 5.1**, the nearly linear relationship between the initial rate and yield of esters versus the initial saccharide concentration is consistent with the two other different acyl donors in the PBBR-based bioreactor system. The final product distribution for the caprylic acid esters is in the range from 85 to 91 wt % monoester among the esters. For both the oleic

and caprylic acid reactions, the fraction of monoesters is lowest for glucose and highest for fructose and sucrose, probably reflecting a difference in the inherent substrate selectivity of RML. Additionally, according to early investigations [192-194], it was indicated different acyl donors have an influence on the activity of lipase. More specifically, the activity of RML is promoted with the increase of chain length of fatty acids [192-194] . Hence, the activity of RML is supposed to be higher than caprylic acid (C_8) during the period of using the chain length of oleic acid (C_{18}) as an acyl donor, partly leading to the difference on the time course of ester yields.

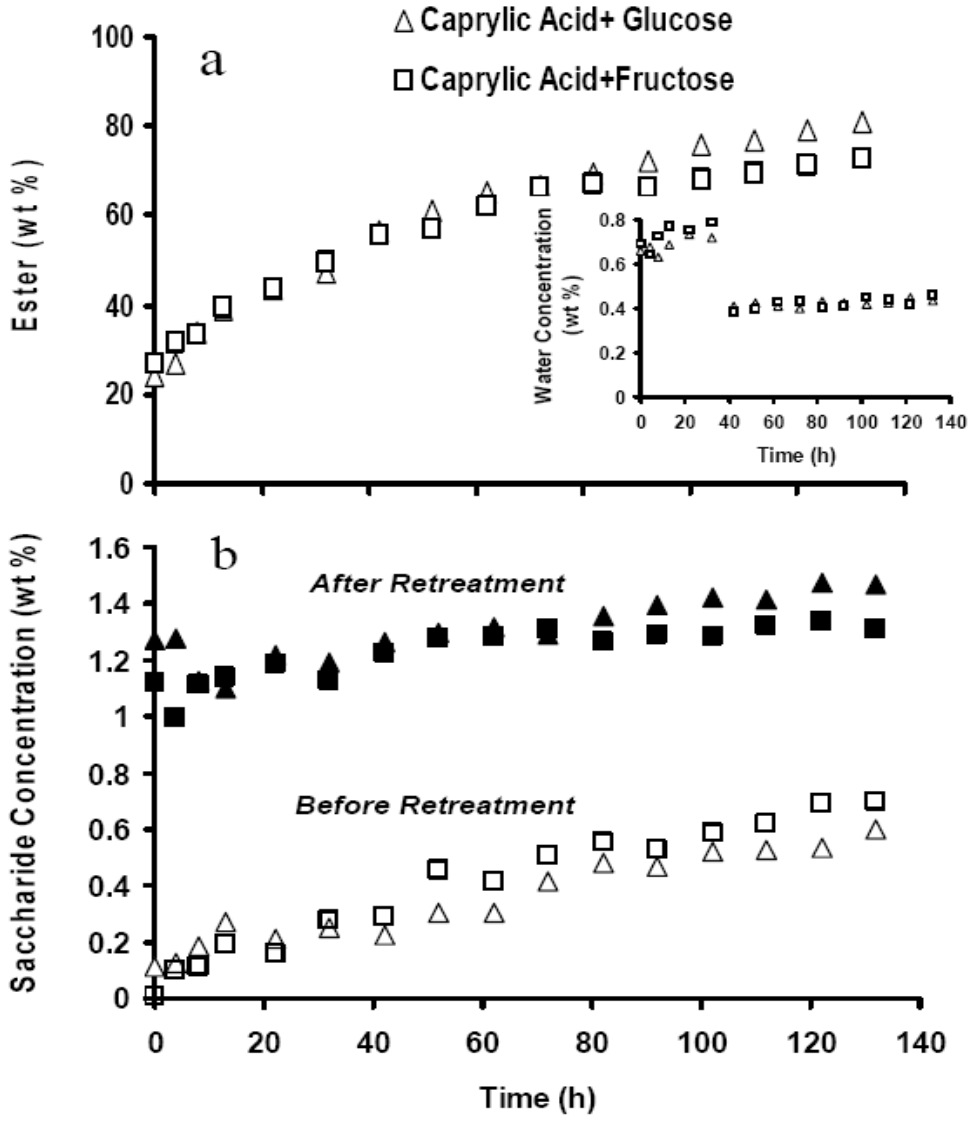


Fig. 5.2 Effect of acyl acceptor type on the time course of reaction for RML-catalyzed synthesis of saccharide –caprylic acid esters in a packed-bed bioreactor (PBBR)-based bioreactor system at 53^{o}C.

Fig. 5.3 demonstrates the time course of reaction for RML-catalyzed solvent-free esterification between lauric and myristic acids as acyl donor and fructose and sucrose as acyl acceptors using a STBR. The final ester concentration for all reactions was more than 75 wt % (in **Table 5.1**). The greater yields and rates of reaction occur when employing lauric acid as acyl donor and fructose as acceptor. The ester yield was listed following: lauric acid + fructose (86.7 wt %) > lauric acid + sucrose (85.1 wt %) > myristic acid + fructose (81.2 wt %) > myristic acid + sucrose ester (76.8 wt %). The relationship between initial rate and final ester concentrations for the STBR data plotted in **Fig. 5.4** is consistent in trend with the PBBR-derived data of **Figs 5.1** and **5.2**, with both quantities increasing with the saccharide concentration (**Table 5.1**). However, **Fig. 5.4** states clearly that the rate and ester production of reactions in the STBR are slightly lower than the PBBR bioreactor system, although the former bioreactor system was operated at a slightly higher temperature, 65^{o}C, versus 53^{o}C for the PBBR-based system. The difference water concentration between the two bioreactor systems is considered to be the primary reason. Molecular sieves (0.13 g per g of reaction medium) were introduced into both bioreactor systems, when the ester concentration reached upon 60 wt %, to augment the removal of produced water via free evaporation, using a MSC for the PBBR bioreactor system and adding MS directly to the STBR. For the PBBR bioreactor system, the liquid-phase water concentration increased from 0.65 to 0.8 wt% during the initial reaction period, then decreased to 0.4 wt % upon the incorporation of the MSC, with the latter water concentration deemed optimal. In contrast, the water concentration for all STBR reactions increased from 0.65 to 0.9 wt %, then decreased to 0.7 wt % upon the addition of MS. Therefore, the water concentration was significantly higher during operation of the STBR than PBBR. This agrees with previous investigations by us where the two bioreactors were compared for the solvent-free RML-catalyzed synthesis of FOE [133] and in Chapter 4. The difference is partly attributed to enhanced mass transport in PBBR. Furthermore, contact between MS and the

reaction medium is more efficient for the MSC than the stirring of the MS in the STBR. Moreover, the literature suggests that lipases are more efficient biocatalysts in a PBBR compared to a STBR [195, 196]. Importantly, no transesterification between FOE and the acyl donor and acceptor substrates occurred due to the absence of free fructose (for reactions using sucrose as acyl acceptor) or fructose laurate or myristate at detectable levels by HPLC. The monoester fraction among the esters ranged between 85 and 92 wt%, but was slightly lower than for fructose and sucrose oleate (**Table 5.1**).

Table 5.1. Summary of final yield and product distribution for saccharide-fatty acid esters in solvent-free bioreactor systems

Bioreactor system	Acyl donor	Acyl acceptor	Initial Saccharide (wt %)	Initial rate (% Ester h^{-1})	Final ester content (wt %)
PBBR[a]	Oleic acid [c]	Fructose	1.67 ± 0.06 [g]	1.31 ± 0.02 [g]	91.3 ± 1.01 [g]
		Sucrose	1.46 ± 0.03 [g]	1.16 ± 0.03 [g]	85.9 ± 0.41 [g]
		Glucose	1.33 ± 0.06 [g]	1.08 ± 0.02 [g]	81.4 ± 1.20 [g]
		Xylose	1.21 ± 0.02 [g]	0.93 ± 0.07 [g]	72.4 ± 2.53 [g]
	Caprylic acid [d]	Fructose	1.14 ± 0.02 [g]	0.86 ± 0.05 [g]	69.1 ± 3.56 [g]
		Glucose	1.31 ± 0.03 [g]	1.05 ± 0.01 [g]	77.8 ± 1.51 [g]
STBR [b]	Lauric acid [e]	Fructose	1.63 ± 0.08 [g]	1.23 ± 0.04 [g]	87.1 ± 0.31 [g]
		Sucrose	1.51 ± 0.02 [g]	1.19 ± 0.02 [g]	85.9 ± 0.83 [g]
	Myristic acid [e]	Fructose	1.43 ± 0.02 [g]	1.12 ± 0.03 [g]	81.7 ± 1.09 [g]
		Sucrose	1.19 ± 0.03 [g]	1.01 ± 0.05 [g]	76.4 ± 0.45 [g]

DE saccharide-fatty acid diester, *ME* saccharide-fatty acid monoester;

[a] Packed-bed bioreactor system undergoing continuous recirculation

[b] Stirred-tank bioreactor

[c] Reaction displayed in Fig.1

[d] Reaction displayed in Fig.3

[e] Reaction displayed in Fig.4

[f] Fraction of monoesters among the saccharide-fatty acid esters; beginning reaction mediums containing 25 wt % fructose oleate with 90 wt% monoester

[g] Standard deviation in each column equivalent to error bars

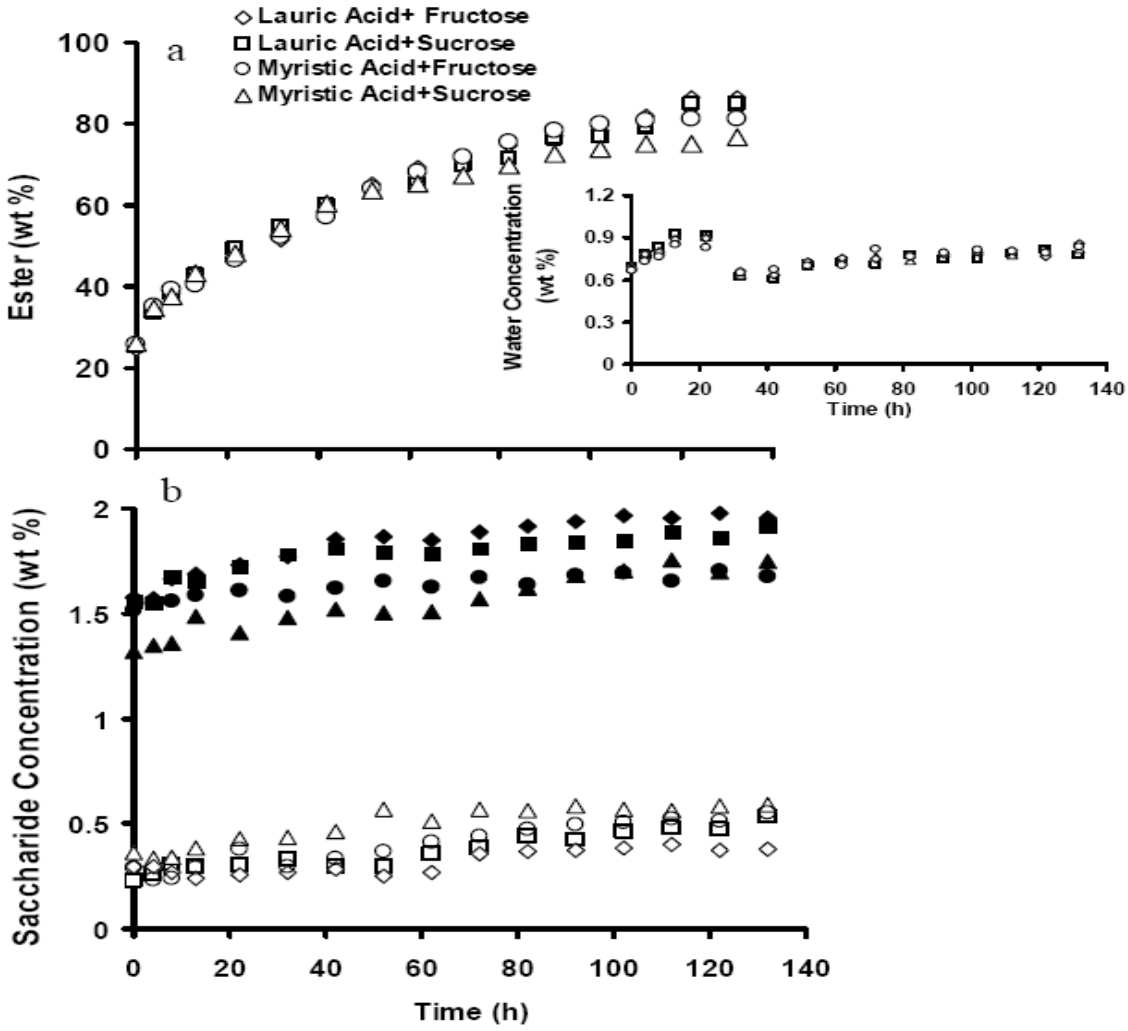


Fig.5.3. Effect of different acyl acceptors and donors (given in legend) on the time course of reaction for RML-catalyzed solvent-free synthesis of saccharide -oleic acid esters in a stirred bioreactor (STBR) at 65oC that utilizes solvent-free suspensions of saccharide crystals.

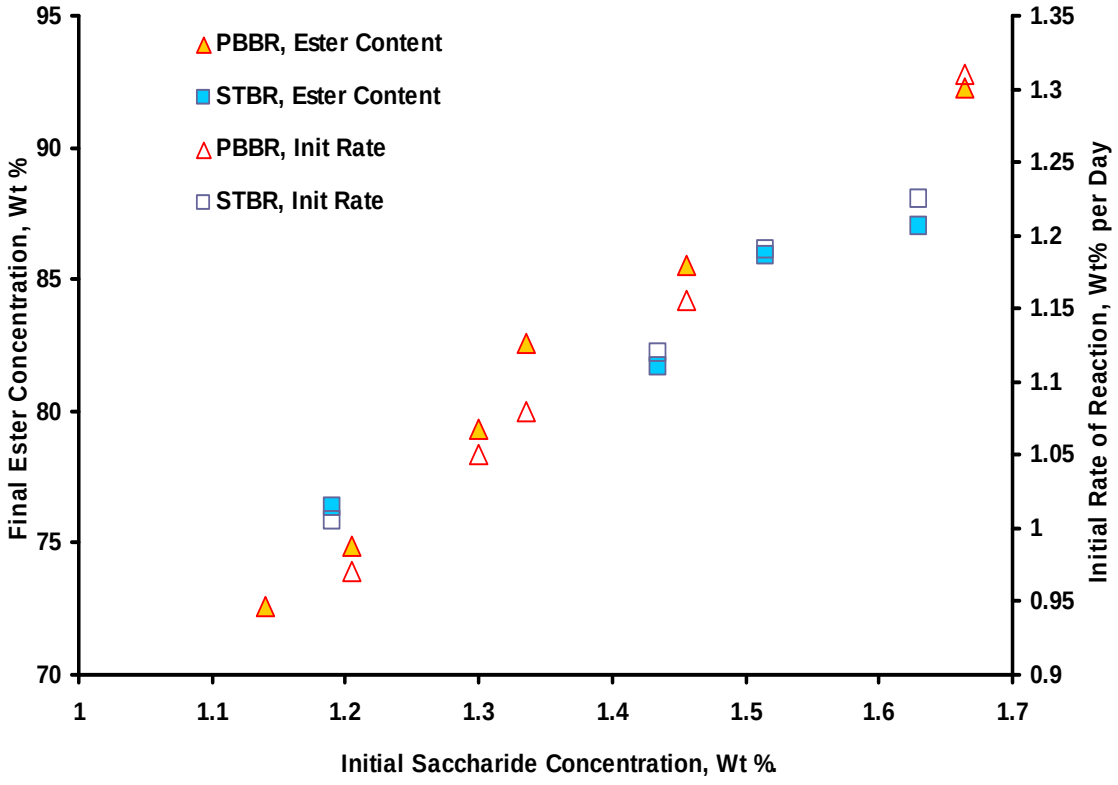


Fig.5.4. Relationship between initial saccharide concentration, final ester concentration, and initial rate of reaction for the solvent-free lipase-catalyzed synthesis of saccharide-fatty acid esters in either a packed-bed bioreactor (PBBR) system at 53°C or a stirred-tank bioreactor (STBR)at 65°C.

5.4.3 Characterization of the Solvent-Free Suspensions Formed by Different Acyl Acceptors and Donors and Fructose Oleate

As mentioned above in Section **5.1-5.3**, the most significant factor for operating lipase-catalyzed solvent-free saccharide-fatty acid ester synthesis is the maximization of the saccharide concentration in the suspension-based medium. Therefore, for better understanding the most significant factors for maximizing saccharide concentration, the physicochemical properties of the suspensions were characterized and studied. This investigation was limited to the starting media containing 75 wt % acyl donor (oleic or caprylic acid) and 25 wt % FOE at 25^{o}C, i.e., conditions resembling the initial conditions in the reactions of **Figs. 5.1** and **5.2.** The characterization of solvent-free medium containing lauric acid or myristic acid could not be conducted due to the absence of heating remaining capability in the characterization instrumentation; moreover, it would be quickly solidified without heating.

Fig. 5.5 illustrates the average particle size, achieved by light scattering, and saccharide concentration of suspensions formed as per the initial reaction media of **Figs 5.1** and **5.3**. The saccharide concentrations in **Fig. 5.5a** are strongly consistent with those of **Figs 5.1a** and **3a**, indicating the highest saccharide concentrations occurred for fructose and sucrose as acyl acceptors and oleic acid as acyl donor. Additionally, the rapid and simple method was performed previously by our group to assess relative differences in saccharide concentration at 1000 nm absorbance values [181], reflecting the trends between acyl donors and acceptors in **Fig. 5.6.** The light scattering results of **Fig. 5.6** indicate the average particle sizes of suspensions formed from oleic acid / FOE increased slightly, from 30-43 μm, in the order: xylose >glucose > sucrose > fructose, the opposite trend as compared to saccharide concentration in **Fig. 5.5**. The range of average particle size agrees with those reported previously by us for fructose/oleic acid/FOE-based suspensions [181]. In agreement, for suspensions formed from caprylic acid, glucose as acyl acceptor yielded the highest saccharide concentration and lowest average particle size in **Fig.**

5.5. Also, there is consistent with the relationship between increasing saccharide concentration and decreasing particle size when comparing acyl donors for a common acyl acceptor, glucose or fructose in **Fig. 5.5**. In conclusion, suspensions consisting of smaller average particle size result in higher saccharide concentration, reflecting the relative stability of the smaller particles to resist settling out of solution when subjected to centrifugation during the processing of the suspensions.

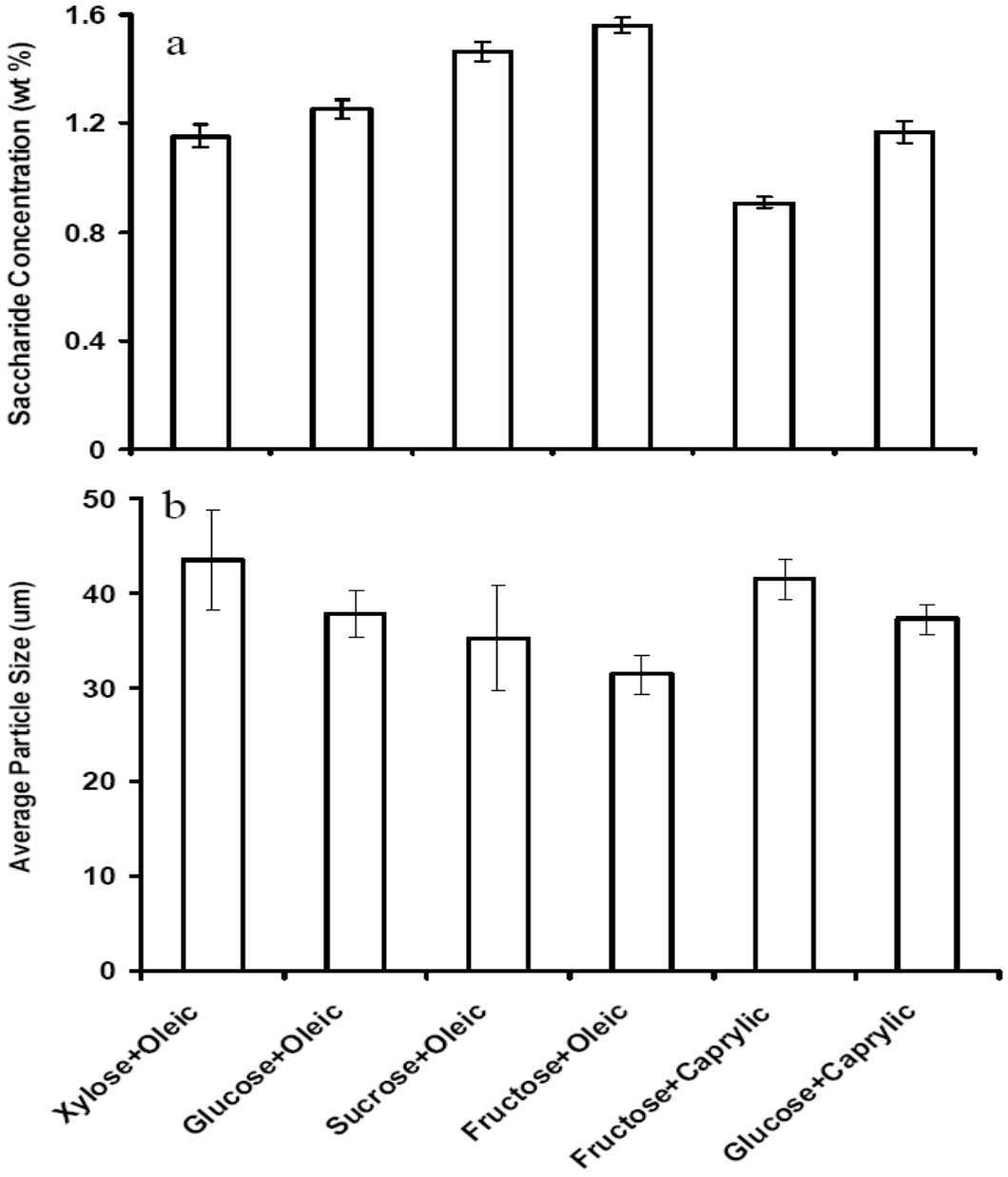


Fig.5.5. Effect of acyl donor types and acceptor types on a. liquid phase saccharide concentration, and b. the average particle sizes of suspensions at 25^{o}C formed by mixing saccharide and oleic acid / fructose oleate (FOE) under the conditions given in Fig. 5.1.

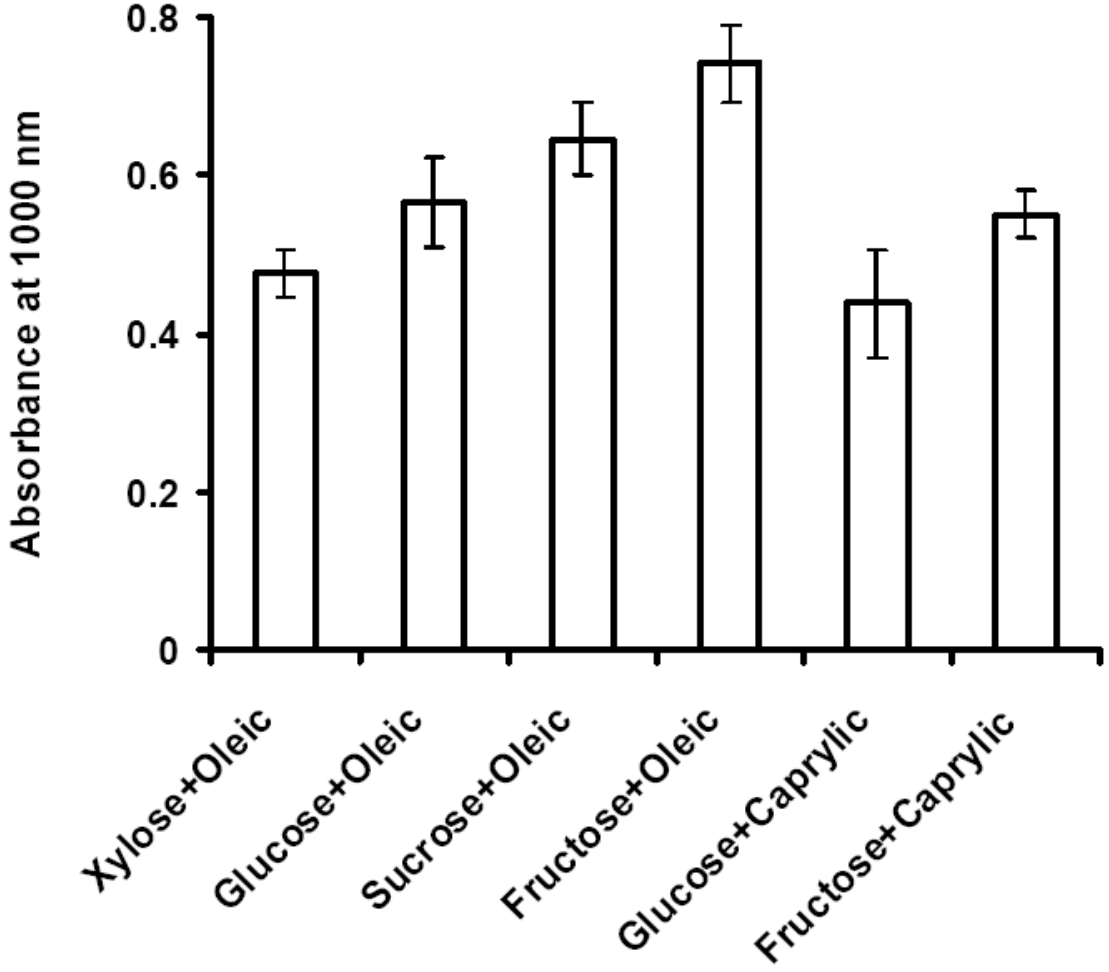


Fig.5.6. Comparison of suspensions of different acyl acceptors and donors in solvent-free media on the absorbance at 1,000 nm for the suspension samples listed in Fig. 5.2

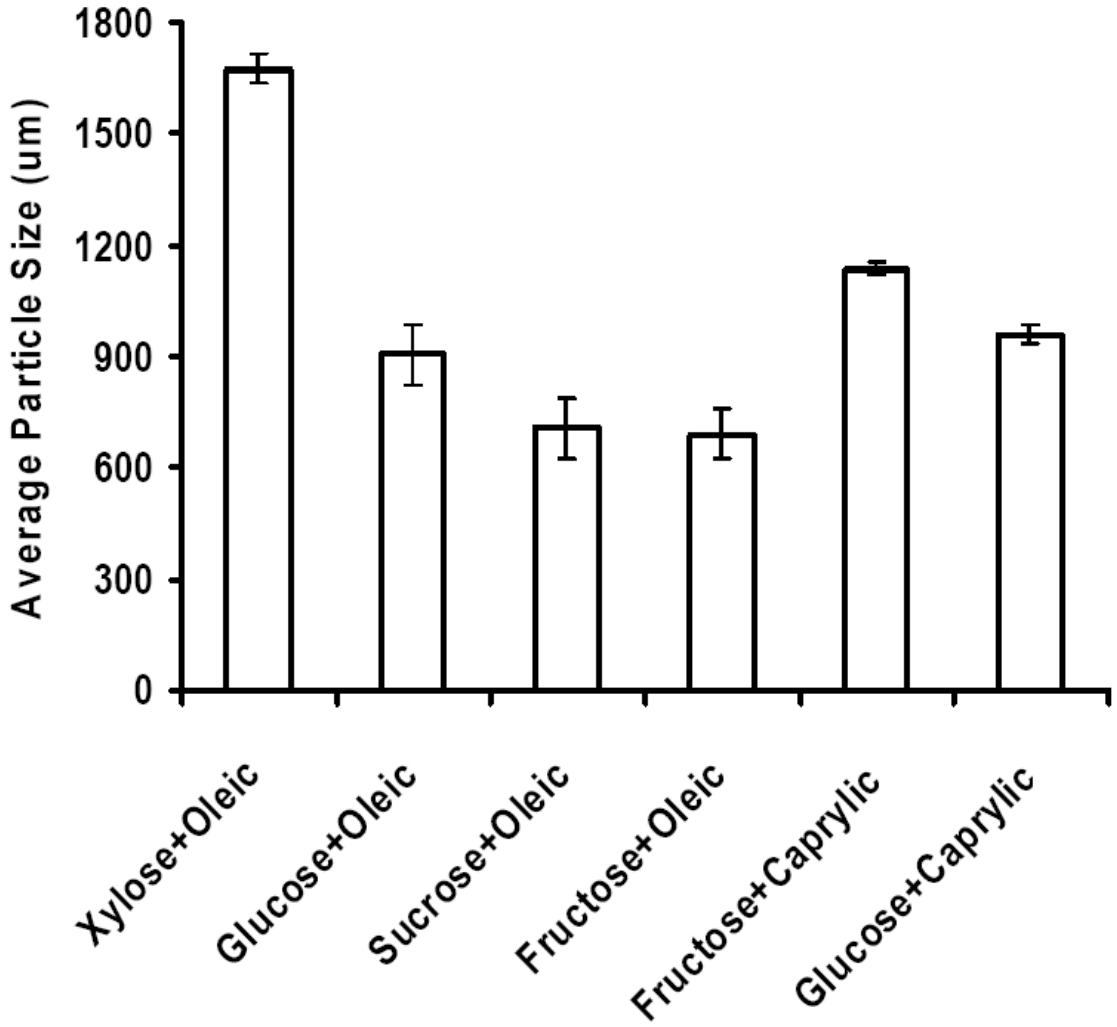


Fig.5.7. Effect of different acyl acceptors and donors on the average particle sizes of suspensions of saccharide crystals in solvent-free media at 80^{o}C after forming suspensions as mentioned in Figs 5.1 and 5.2, but before the centrifugation step is invoked.

As described above, the average size of suspended particles has a direct relationship with the size of the saccharide crystals in suspensions. Light scattering analysis for the oleic acid/FOE 75/25 w/w suspensions prior to centrifugation show particles for xylose are quite large, 1650 μm, followed by glucose, 900 μm, sucrose, 700 μm, and fructose, 650 μm in Fig. 5.7, reflecting the same trend obtained for the particle sizes of the suspensions after centrifugation in Fig. 5.5. For the better understanding visually, light microscopy was utilized for obtaining optical images, presenting the same trend with the light scattering results: suspensions of sucrose and fructose in oleic acid/FOE (prior to centrifugation) contain relatively small particles; glucose particles in the same medium are larger, and particles for xylose suspensions are even larger (Fig. 5.8); in contrast, after centrifugation, for all samples, very few particles were detected by light microscopy (Fig. 5.9). Additionally, optical microscopy images for saccharide-caprylic acid –based suspensions follow trends mentioned above for oleic acid-based suspensions (Fig. 5.10). Accordingly, it is concluded that the suspensions formed by larger sized crystals lose a larger fraction of their saccharide content through centrifugation due to their lower settling velocities. Of note, the formation of suspensions is anticipated to impact the size of the suspended crystal particles minimally since the forming environment is relatively benign toward influencing the formation of the suspended crystals. First, due to free- evaporation by the pretreatment in a vial opened to the atmosphere at 80^{o}C and low water contents of initial materials, this medium offers nearly-anhydrous environment (water concentration, 0.65 wt %±0.04 wt %), which is considered to be a distinct barrier for crystallization or dissolution of large size crystals [197]. Second, the high viscosity of the reaction medium inhibits crystallization since it offers the high energy hurdle for crystallization process [108].

5.5 Conclusion

In conclusion, the performances of common acyl donors (oleic acid, caprylic acid, lauric acid and myristic acid) and acceptors (fructose, sucrose, glucose and xylose) on solvent-free lipase (RML)-catalyzed synthesis of saccharide-fatty acid esters using acyl donor/ester-based suspensions in PBBR and STBR bioreactor systems were investigated and compared. The results presented that all of the donors and acceptors can act as substrates effectively, with conversion and initial rate being highest for fructose, followed by sucrose, glucose, and xylose among acyl acceptors, and oleic acid, followed by lauric, myristic, and caprylic acid among acyl acceptors. Significantly, an increase of acyl acceptor (saccharide) concentration was considered to be the primary factor for maximizing conversion and initial rate. It was demonstrated that the suspension formed from a saccharide starting material consisting of abundant smaller size saccharide particles yielded stable suspended saccharide particles of average size 30-45 mm, and therefore higher saccharide concentration, resulting in the higher conversion.

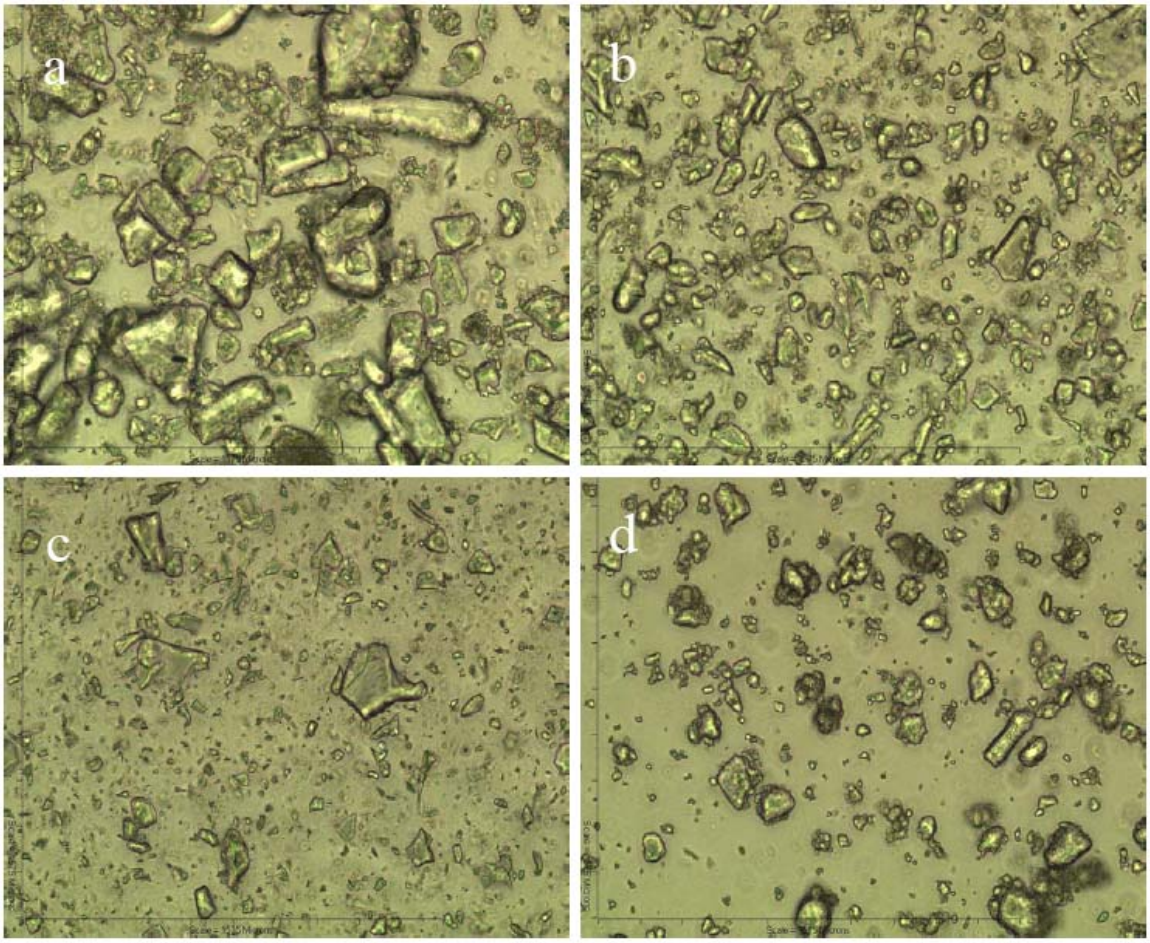


Fig.5.8. Images from light microscopy, 40X magnification, of suspensions for 4 different acyl acceptors (a. Xylose, b. Glucose, c. Sucrose, and d. Fructose) in solvent-free media (Fructose-oleic acid esters/ oleic acid, 25/75, w/w) at 25^{o}C after forming suspensions as mentioned in Figs 5.1 and 5.3.

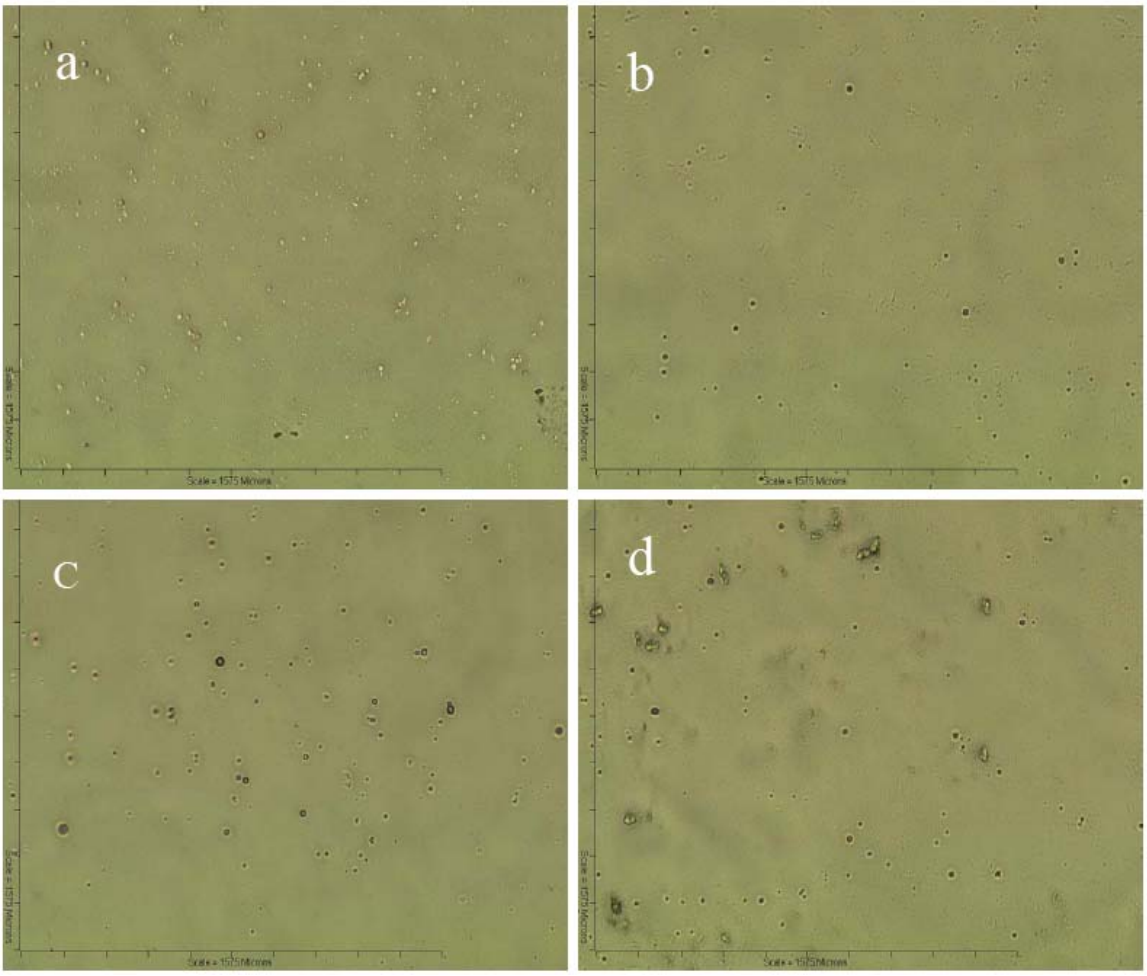


Fig.5.9. Images from light microscopy, 40X magnification, of suspensions for 4 different acyl acceptors (a. Xylose, b. Glucose, c. Sucrose, and d. Fructose) in solvent-free media (fructose-oleic acid esters/ oleic acid, 25/75, w/w) at 25^{o}C after completion of centrifugation.

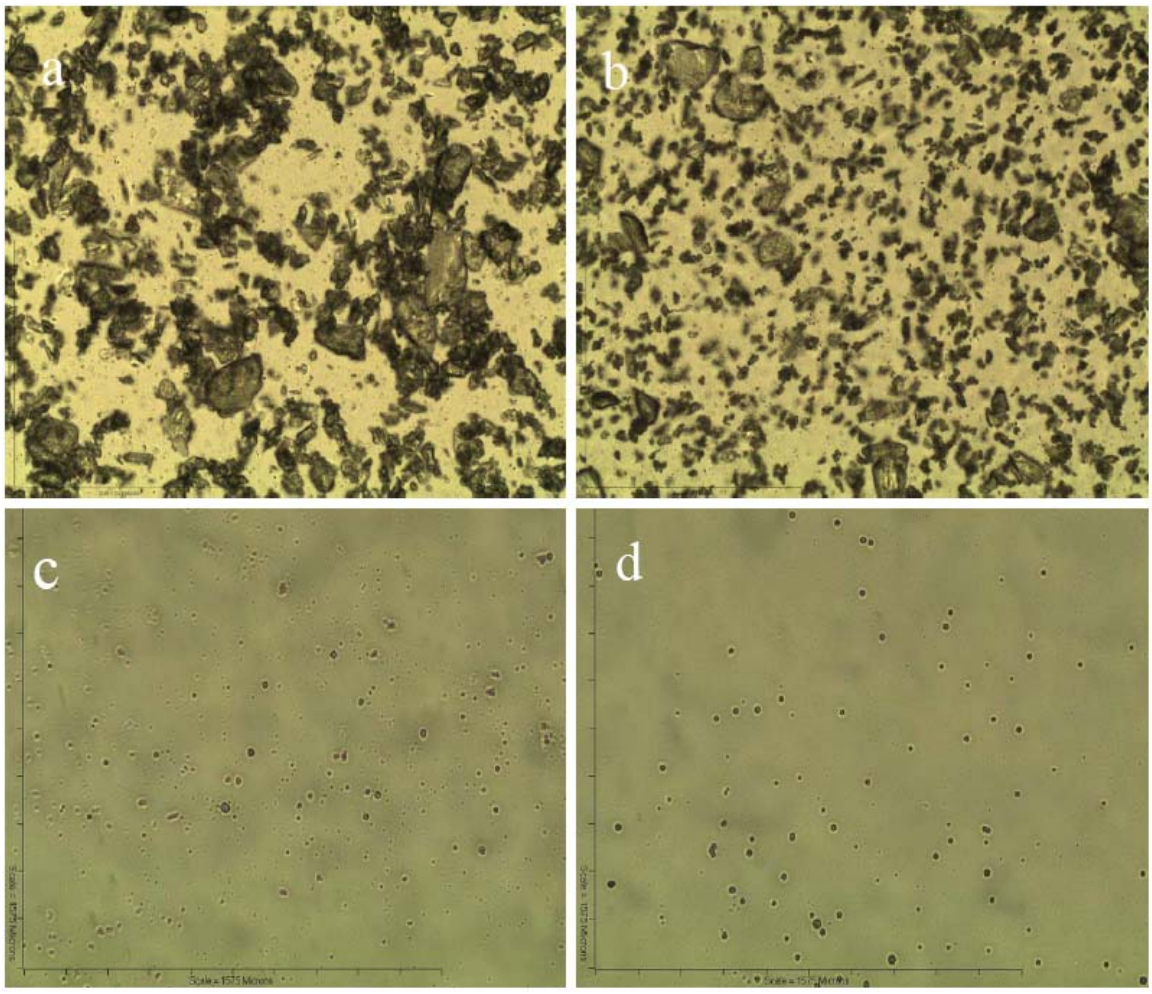


Fig. 5.10 Images from light microscopy, 40X magnification, of suspensions for two different acyl acceptors before centrifugation (80^{o}C): a. Fructose and b. Glucose; and after centrifugation 25^{o}C: c. Fructose and d. Glucose in solvent-free media (Fructose oleic acid esters/ caprylic acid, 25/75, w/w).

CHAPTER 6

MATHEMATICAL MODELING OF THE TIME COURSE OF SOLVENT-FREE FRUCTOSE OLEATE SYNTHESIS FOR AN IMPROVED BIOREACTOR SYSTEM

6.1 Abstract

Saccharide-fatty acid esters, biodegradable, biocompatible and nonionic biobased surfactants derived from inexpensive renewable agricultural sources, utilized in foods, cosmetics, and pharmaceuticals. An improved design of the bioreactor system was developed through the combination of Nitrogen + Vacuum applied to suspensions *in situ* with an in-line filter allowing for operation of forming the suspensions and production of ester synchronously, utilizing stable 10-200 μm suspensions of saccharide in solvent-free media in packed bed bioreactor (PBBR).

The reaction started with the initial reaction medium consisting of oleic acid/ fructose-oleic acid esters 95/5 w/w, using the addition of saccharide periodically to a reservoir where water is removed via vacuum pressure and nitrogen gas bubbling and suspensions is stirred at 80 ^{o}C and 800rpm continuously. The rate of water removal was programmed to retain an optimal liquid-phase water concentration of ~0.4 wt %. The bioreactor system yielded a final conversion of 84 wt % with ~90 wt % of the ester consisting of monoester with a productivity of 0.195 mmol h^{-1} g^{-1}. The resultant technical grade product can potentially be used directly, without further purification. RML exhibits the good stability for two times runs of the enzymatic reaction. A mathematical model was successfully developed to predict the concentration of substrates (oleic acid and saccharide concentration) during the time course of reaction, which utilizes mass balances and a Ping-Pong Bi Bi kinetic model.

6.2 Introduction

Saccharide-fatty acid esters are important biodegradable, environmental friendly nonionic biobased surfactants that are utilized in foods, pharmaceuticals and cosmetics. They can be synthesized from cheap agricultural renewable materials and feedstocks [41-46]. In addition to emulsification and stabilization, they can be employed as food preserves and insecticides since they possess antimicrobial activity. Conventionally, saccharide-fatty acid esters are produced by chemical methods under harsh conditions, for instance, high pressure and temperature, and undesirable byproducts. In contrast, biocatalytic synthesis of saccharide-fatty acid esters employs mild, near-ambient operation conditions and leads to a narrow product distribution. However, the presence of significant hurdles hinders the application of biocatalytic production of biobased surfactants for industrial utilization. The major problem is the poor miscibility of polar and non-polar substrates, resulting in slow reaction rates. To overcome this obstacle, a novel packed-bed based bioreactor system that utilizes solvent-free media was successfully developed in Chapter 3. Typically, the initial reaction medium, a suspension of saccharide crystals in solvent-free media, was formed by mixing 1.5 g fructose crystals and 10 g oleic acid / fructose-oleic acid ester (FOE), 3/1 w/w, in a 20 mL reservoir open to the atmosphere on a magnetic stirrer plate at 80^{o}C and 800 rpm (radius of 1.5 cm) for 6 h. The slurry was centrifuged at 800 rpm 71.57g's for 0.5-1 min, with the supernatant collected. Subsequently, the solvent-free suspensions, stable for over 12 h, were employed in a closed-loop bioreactor system, consisting of a reservoir open to the atmosphere (for free evaporation of the reaction byproduct, water), a peristaltic pump, and packed bed bioreactor (PBBR), operated under continuous recirculation at 0.5 L/min. The suspensions were retreated at 10 h intervals, through stopping the pump, removing the liquid phase, retreating the latter with additional saccharide to reform the suspensions using the methodology described above, and returning the liquid phase to the bioreactor system. In Chapter 4, this bioreactor system was optimized to enhance reaction rate and conversion by

programming the control of water activity and the interval time for re-forming suspensions.

However, this approach has suffered from the need to reform the suspension media at regular time intervals (to replenish saccharide consumed by the reaction), requiring the stoppage of the recirculation and removal of the reaction medium for several hours at a time. Therefore, this approach is not robust for scale-up. Therefore, the bioreactor system was redesigned for one-step process for the continuous production of saccharide fatty acid esters. One-step process refers to the continuous formation of suspensions (with the periodic addition of saccharide), water control and production of ester within the bioreactor system during its operation. This development was enabled by re-designing the system to contain a reservoir serving as a place for continuous formation of suspensions and water removal (via free evaporation), an in-line filter for preventing larger aggregates to be transported through the remainder of the bioreactor system. Specifically, the redesigned closed-loop bioreactor system consists of the reservoir, in-line filter, a peristaltic pump, and a packed bed bioreactor (PBBR), with the latter's effluent returned to the reservoir. This system was operated under continuous recirculation.

The most common and accurate description of the catalytic process of lipases is a Ping-Pong Bi Bi model [119]. Many reports have investigated and developed mathematic models for lipase-catalyzed reaction based on a Ping-Pong Bi Bi mechanism. A simple kinetic model derived from a Ping-Pong Bi Bi mechanism to characterize the rate of acylation of glucose with lauric, palmitic, and stearic acids in the presence of CALB in acetone was successfully developed by Arcos *et al*., utilizing a few assumptions. First, reverse-direction enzymatic reactions were ignored. Second, the dissolved glucose concentration was assumed to be constant [120]. The mathematical model from this work has been verified by several sets of experimental data under different reaction conditions. The results indicated that the model fits the experimental data very well for temperatures from 30 to 60 degrees C, enzyme

loadings from 90 to 180 mg, and fatty acid concentrations from 0.33M to 1M. Another research group proposed a full reversible kinetic model based on a Ping- Pong Bi Bi mechanism which represents the acylation of glucose by lauric acid in 2-methyl 2-butanol mediated by CALB at 60°C. The developed model displays a good fit of experimental results [121]. Dang and his coworkers developed a good linear kinetic Ping-Pong Bi Bi model regarding feed batch addition of saccharide during synthesis of fructose-oleic acid esters in agreement with measured data for the time course of the reaction [115]. In this chapter, a mathematical model was developed understand the relationship between the significant variables in the system and the observed time course of reaction. The model is based on mass balances and an enzymatic kinetic model (Ping-Pong bi bi mechanism), the latter developed previously by Hayes and co-workers.

6.3 Materials and Methods

6.3.1 Materials

Lipozyme RM IM®, lipase from *Rhizomucor miehei* immobilized onto macroporous anionic resin beads ("RML"), was purchased from Novozymes, Inc. (Franklinton, NC USA). D-fructose (> 98%) and solvents employed for HPLC analysis (HPLC-grade) were purchased from Fisher Scientific (Pittsburgh, PA). Technical grade oleic acid (90% pure) was purchased from Sigma-Aldrich (St Louis, MO). All materials were used without further purification. The saccharide crystals were ground into a fine powder using a mortar and pestle. Technical grade fructose-oleic acid ester (Ester/Oleic acid, 8.8/1.2 w/w), a component of the initial charge to the bioreactor system was synthesized by RML in batch mode.

6.3.2 Methods

6.3.2.1 Closed-Loop Bioreactor System (Fig. 6.1)

A Wheaton Celstir spinner double-side arm flask (25 mL) with a tight screw cap was placed on a hot plate (Super-Nuova from Barnstead, Dubuque, IA USA) at 80 ^{o}C and its contents stirred at 800 rpm. It served as a reservoir. The right arm of the flask was connected to purified nitrogen gas and a flowmeter; the left arm was interfaced with a vacuum pump and a vacuum gauge. Fluid was withdrawn from the reservoir through tubing connected to an in-line nylon filter of nominal size 180 microns (Millipore, Billerica, CA) and subsequently to the peristaltic pump, a BioLogic® LP model purchased from Bio-Rad (Hercules, CA). A packed bed bioreactor (PBBR; 50mm L × 10mm ID Omnifit® chromatography column packed with 7.5g of RML) was enclosed in a temperature–regulated oven (Isotemp® Economy Lab Incubator from Fisher Scientific) and maintained at 78^{o}C. The PBBRs exit stream was returned to the reservoir.

6.3.2.2 Operation of the Bioreactor System

The bioreactor system consisted of three major components connected in series, forming a closed-loop system that underwent continuous recirculation: a Wheaton Celstir flask serving as a reservoir, a peristaltic pump (BioLogic LP® from Bio-Rad, Hercules, CA USA), and a packed bed bioreactor (PBBR; 50mm L × 10mm ID Omnifit® chromatography column packed with 0.75 g of RML per g of oleic acid + FOE). The frit restrictors contained within the endcaps of the Omnifit® column were not used, but were replaced a small piece of a 100 denier,156 mesh polyester net manufactured by SiamDutch Mosquito Netting Co., Ltd., Bangkok, Thailand, determined gravimetrically to possess an areal density of 29 g m^{-2}. C-FLEX® 1.6 mm

ID tubing made of a styrene-ethylene-butylene modified block copolymer from Cole-Parmer (Vernon Hills, IL), was used to connect the in-line filter in the reservoir to the pump, the pump to the PBBR, and the PBBR to the reservoir, to form a closed-loop system that underwent continuous recirculation. PharMed® BPT 1.6 mm ID tubing (Saint-Gobain Performance Plastics Corp., Akron, OH) was used within the peristaltic pump apparatus. The PBBR and associated tubing were placed within a convection oven at 78 ^{o}C to yield a constant temperature of 65^{o}C for the recirculating liquid phase. The reservoir's contents were maintained at 80^{o}C and stirred at 800 rpm using the above-mentioned hot plate / stirrer. The reaction medium charged to the bioreactor system's reservoir consisted of the solvent-free media, formed by mixing 1.5 g fructose crystals and 10 g of a oleic acid / FOE 95/5 w/w mixture, equivalent to a reaction medium that has achieved 5% w/w conversion of oleic acid at 80^{o}C. 1.5g grounded saccharide crystals were periodically added into the reservoir (every 6h). The in-line filter was placed between the reservoir and the pump. Initially, free evaporation was the only means of removal for the co-product water to be removed. Vacuum and N_2 were introduced into the bioreactor system after 40h to augment the removal of water by free evaporation, a step deemed necessary to enhance the rate and extent of reaction (Chapter 4). After one run, RML was washed by a small amount of acetone for reuse. Two replicate experiments were performed.

6.3.2.3 Monitoring of Water

The water content for an aliquot of the reaction mixture, after being diluted with methanol, was analyzed by Karl-Fischer titration using a Coulometric KF Titrator (Denver Instrument Company, Aurora, CO). The working principle utilizes the reaction of water with iodine and sulfur dioxide in the presence of a lower alcohol such as methanol or other organic solvents. Please find more details regarding Karl-Fischer titration in Chapter 3. In this dissertation, methanol was used as a medium to dissolve the reaction medium due to its lower price compared with other organic solvents [219].

6.3.2.4 Monitoring of Oleic Acid, Ester, and Fructose Concentration

Quantitative analysis of oleic acid and its mono- and di-esters on a fructose-free basis was performed using a dual-pump system from Varian (Walnut Grove, CA) and a model Mark III evaporative light scattering detector from Alltech Associates, a division of WR Grace (Deerfield, IL). An analytical reversed phase (4.6 * 250 mm, pore diameter 5 μm) C_{18} column from Alltech was employed using separation conditions consisting of a column temperature of 25^{o}C and an isocratic solvent system, acetone / acetonitrile / acetic acid (45 / 45 / 10 v/v/v) at flow rate of 1.0 mL min^{-1}. Response factors were measured and employed to convert peak areas into concentrations.

To analyze the fructose content, 40 mg-sized aliquots of column effluent were subjected to liquid-liquid extraction by the system of *n*-hexane and water (500 μL of each). The extraction was carried out 3 X at 35^{o}C for 2 hr using a thermomixer (Eppendorf AG, Germany). The aliquots from the pooled aqueous extraction solutions were diluted with acetonitrile to match the composition of the HPLC mobile phase to prevent peak broadening in the HPLC analysis. An analytical Prevail Carbohydrate ES column (4.6 * 250 mm, pore diameter 5 μm) from Alltech was employed using a column temperature of 25^{o}C and an isocratic solvent system, acetonitrile / deionized water (80 / 20 v/v) at flow rate of 1 mL min^{-1}. Standard curves for fructose concentration in an oleic acid / fructose oleate liquid phase versus peak area were obtained and found to be independent of the reaction mixture's composition.

6.3.2.5 Measurements of Absorbance and Particle Size for Suspensions

The absorbance of solutions between 500 and 1000 nm was performed to provide a measure of turbidity using a model UV-1700 instrument from Shimadzu (Japan) and either a 1.0 or 0.2 cm pathlength quartz length cuvette by Hellma (Plainview, NY USA), with all reported values normalized to a 1.0 cm pathlength. Light scattering is a

method to determine the distribution of small size particles in suspension. Differences of absorbance values between samples at this wavelength are representative in trend of differences at the other wavelengths. The particle size distribution of the dispersions present in the above-mentioned solutions was analyzed by Zeta potential Analyzer, Zeta PALS (Brookhaven Instruments Corporation, Holtsville, NY USA).

6.3.2.6 Phase Diagram Determination

During the lipase catalyzed esterification reaction in an improved bioreactor system as described in Sect. **6.3.2.2**, the samples were very carefully collected every 10 h from the bioreactor system for determining the mass fraction of ester, oleic acid and fructose in the liquid phase by HPLC analysis (Sect.**6.3.2.4)**. The mass fractions of ester, oleic acid and fructose in the liquid phase were calculated and plotted a yield a triangular phase diagram of the saccharide fructose/oleic acid/fructose-oleic acid monoester ternary system at 65 ^{o}C. The boundary line between one-phase and two-phase was plotted according to the change of concentration of saccharide and ester over the reaction time. Phase boundary represents metastable suspensions; it does not represent a true phase boundary between two homogenous phases at thermodynamic equilibrium.

6.4 Results and Discussion

6.4.1 Improved Design of Bioreactor System

The primary objective in the Chapter 6 is to find the best approach to create and operate the reactor system to minimize the labor required by an operator during the interval time. Specifically, since the formation of suspensions was performed in a

reservoir, where 10-200 micron-sized saccharide aggregates were suspended in solvent-free media formed at high stir rate, separation steps have to be taken to prevent large size saccharide crystals blocking the system's tubing. Second, it is always a major challenge for researchers to find the superior way for controlling the water content efficiently in the esterification reaction. As mentioned in Chapter 4, the most efficient water removal method (the combination of nitrogen gas bubbling + vacuum pressure applied to suspensions off-line) was found to successfully maintain water content at the optimal level (~0.4 % w/w). However, this water control method is required to be *in situ*, allowing for the more efficient operation of the bioprocess suitable for scale up. Consequently, the bioreactor system of Chapter 4 requires improvement.

To accomplish these objectives, a bioreactor system was redesigned for one-step process for the continuous production of saccharide fatty acid esters under optimal operation condition in **Fig. 6.1**. The suspensions formed in the reservoir resided at the optimal water concentration. A Wheaton Celstir spinner double-side arm flask (25 mL) with a tight screw cap placed on a hot plate serves as a reservoir for formation of suspensions and a reactor for water removal. The suspension-based solvent-free medium, after passing through the in-line filter of nominal size 180 microns (to prevent passage of large saccharide crystals effectively to be transported through the bioreactor system, making the bioreactor system operated under recirculation) were transported to the PBBR by the peristaltic pump. Subsequently, the reaction medium leaving the PBBR with the enhanced monoester content was returned into the reservoir.

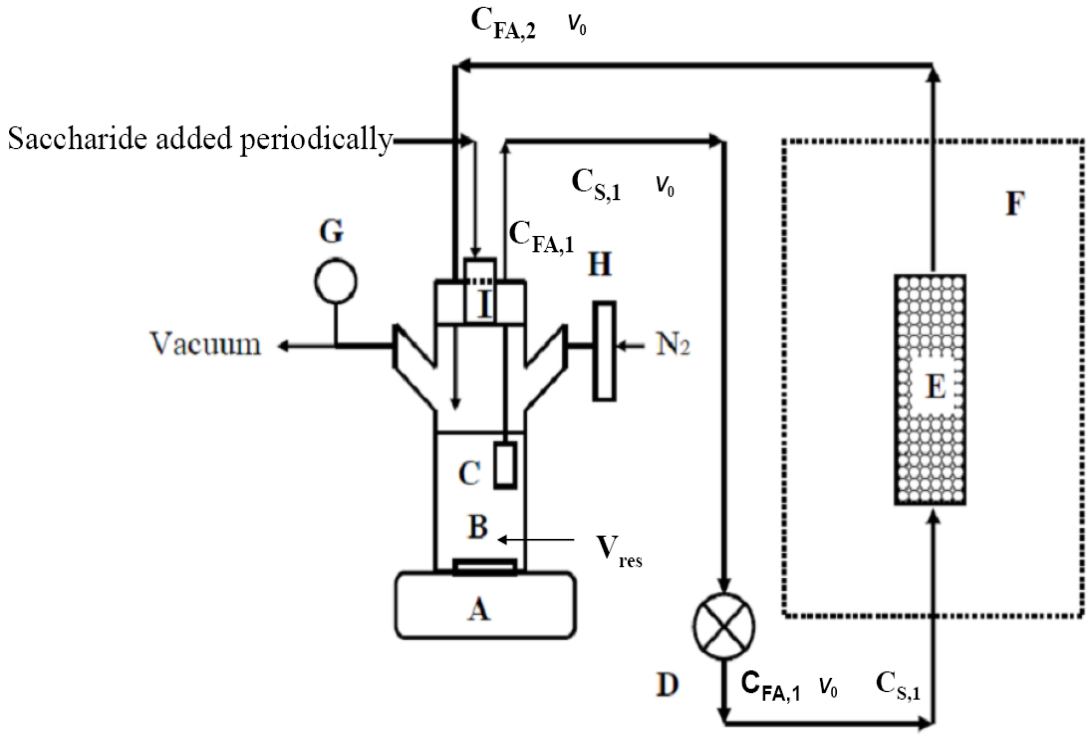


Fig.6.1. The diagram of improved bioreactor system and parameters for mathematic model included. (A**.** Hot plate maintained at 85^{o}C **B.** A 25 ml Wheaton Celstir spinner double-side arm flask with stirred bars **C**. An in-line filter **D.** Peristaltic pump (0.5mL/min) **E.** PBBRs **F**. Oven maintained at 78^{o}C **G**. A vacuum pressure gauge **H.** An air flow meter **I**. A vent for periodical addition of saccharide. Symbols represent parameters used in mathematical modeling).

6.4.2 Effect of the Improved Bioreactor System for Lipase-Catalyzed Synthesis of Saccharide-Fatty Acid Esters Utilizing Solvent-free Medium

From Chapter 5, it was illustrated that when the oleic acid and fructose were utilized as acyl donor and acceptor, respectively, the reaction obtained the highest conversion and initial rate compared with other common acceptors and donors (sucrose, glucose, and xylose among acyl acceptors, and oleic acid, followed by lauric, myristic, and caprylic acid among acyl acceptors) due to the higher saccharide concentration produced by this substrate system. Hence, fructose and oleic acid were selected for employment in this chapter. The reservoir initially contained the mixture of fructose oleate / oleic acid 5/95 w/w, and suspensions of 1.5 g fructose crystals dispersed by magnetic stirring (800 rpm) on the hot plate at 80^{o}C. Additional fructose crystals were added periodically: 1.5 g (0.5 mmol) added at 6 h intervals. The new bioreactor system was operated successfully. For the in-line filter, it effectively blocked larger aggregates into the bioreactor system as determined from visual observation. The time course of reaction for the improved bioreactor system is given in **Fig. 6.2**. A linear increase of ester content was obtained with respect to time up to about 84% w/w ester concentration within 8.4 days using completely solvent-free reaction media (i.e., a productivity of 0.195 mmol h^{-1} g^{-1}). The productivity is lower than the optimal productivity (0.297 mmol h^{-1} g^{-1}) obtained in Chapter 4. However, the pretreatment time for forming suspensions was not included when calculating for the productivity of optimal bioreactor system in Chapter 4. The "corrected" optimal Chapter 4 productivity is 0.226 mmol $h^{-1}g^{-1}$, slightly higher than the productivity obtained for the improved bioreactor system design of this chapter. Secondly, the initial saccharide concentrations for these two experiments are significantly different. **Fig. 6.3** represents the saccharide concentration for the new bioreactor system during the entire time course. Saccharide concentration during the time course increased with the increase of monoester concentration in **Fig. 6.3**. However, the final saccharide concentration of the optimal biosystem (~2.4 wt %) in Chapter 4 is much higher than for the improved

bioreactor system (~1.68 wt %) in **Fig. 6.3**. The pore size of the in-line filter (180 μm) is not sufficiently large to allow all appropriately sized suspensions to be pumped into the bioreactor system, preventing the higher saccharide concentration from occurring. Since the higher saccharide concentration leaded to higher initial reaction rate (shown in Table 5.1), the lower saccharide concentration limited by the pore size of filter is the reason for the lower productivity. In addition, there may not be sufficient time in the improved bioreactor system for formation of the suspensions. Moreover, using the approach of Chapters 3-5, a minimum of 6 hours stirring was required to maximize the concentration of saccharide in the suspension-based medium.

During the initial phase of the time course of reaction, water was removed through free evaporation. After 40h, the combination of vacuum+N_2 bubbling was introduced into the bioreactor system since the water concentration increased quickly due to its formation from the reaction and is accumulated up to ~0.8 wt% after 40h. Water content in the reaction medium was remained at the previously-determined optimal level ~ 0.4 % w/w during the entire time course (described in Chapter 4, displayed in **Fig. 6.4**). This result indicates that the *in situ* water control is equally as effective as the off-line water control method of Chapter 4.

For evaluating the effect of the water concentration in the improved bioreactor, the value of the concentration-based equilibrium constant, K_c, was calculated according to the following equation:

$$K_c = [ME]_{eq} [H_2O]_{eq} [S]_{eq}^{-1} [ME]_{eq}^{-1} \qquad [115]$$

Where $[ME]_{eq}$ is 1.74 $x10^{-2}$; $[H_2O]_{eq}$ is 0.25 $x10^{-2}$; $[S]_{eq}^{-1}$ is 7.05 x 10^{-4}; $[ME]_{eq}^{-1}$ is 9.2 $x10^{-2}$. From the previous report [115] in the literature, solvent (*tert*-butanol) was present only during the initial phase of the time course of the esterification reaction to enhance fructose solubility and was allowed to evaporate away completely on reaching 25 wt % conversion. Free evaporation was used into this reaction as a water

control method and K_c was equal to 6.4 [115]. In this chapter, the K_c value for the improved design bioreactor system was calculated to be 6.7, similar to the value in the previous report (6.4) [115], but much higher than other lipase (CAL) catalyzed synthesis of saccharide fatty acid esters in organic solvents (0.55 [199], 0.30 [200]). This suggests the conversion limitation that occurred for the reaction upon reaching 80-90% w/w yield in **Fig. 6.2** due to the thermodynamic equilibrium. It suggests excess water occurs in the bioreactor system. Hence, some works pertaining to water control are required to be performed in the future work to remove excess water from bioreactor system for the improvement of ester conversion.

Regarding the product distribution, the selectivity toward fructose–oleic acid monoester (ME) over fructose–oleic acid diester (DE) occurred at a ratio of approximately 9:1 throughout the majority of the time course of the reaction (in **Fig. 6.5)**, highly consistent with the results from Chapters 3-5.

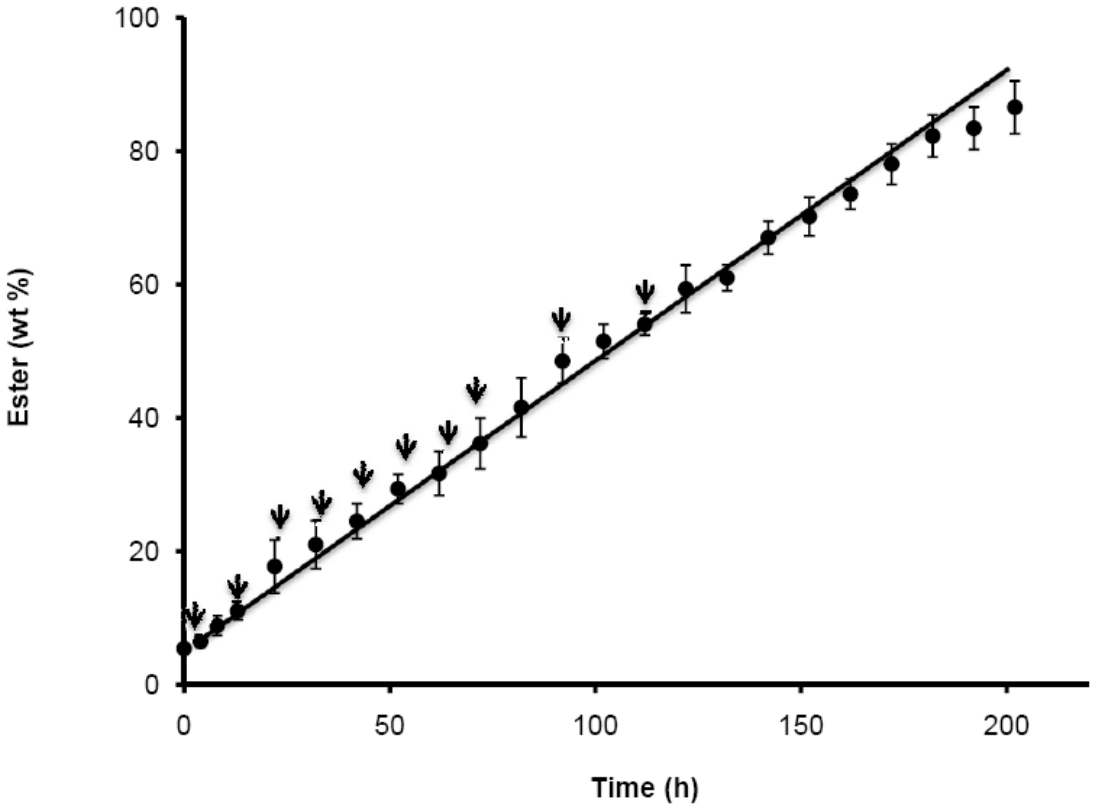


Fig. 6.2 The effect of improved design for the bioreactor system (Fig. 6.1) for the solvent-free RML-catalyzed synthesis of fructose oleate using bioreactor system containing a packed-bed bioreactor operated under continuous recirculation at 65^{o}C under optimization condition and reservoir was maintained at 78 ^{o}C.

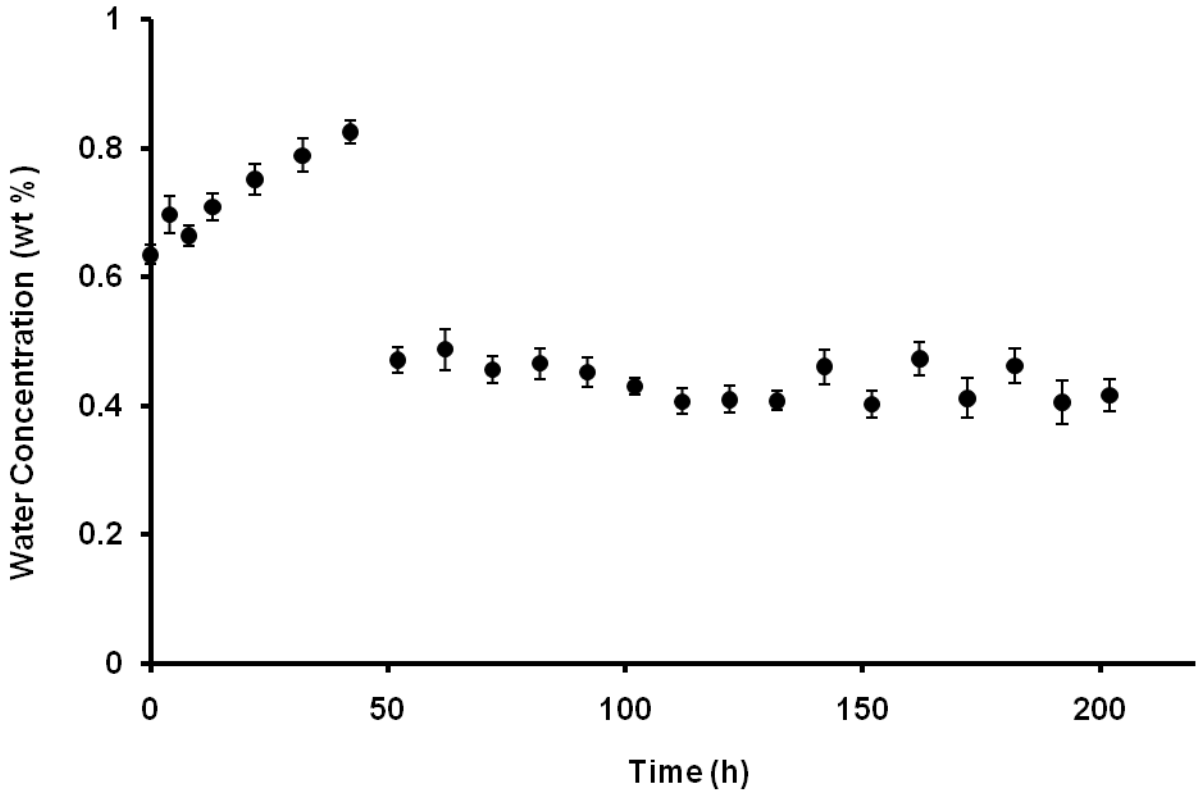


Fig. 6.4. The water concentration versus time for the experiment in Fig. 6.2. (Error bars represent the standard deviation from replicate runs of the enzymatic reaction).

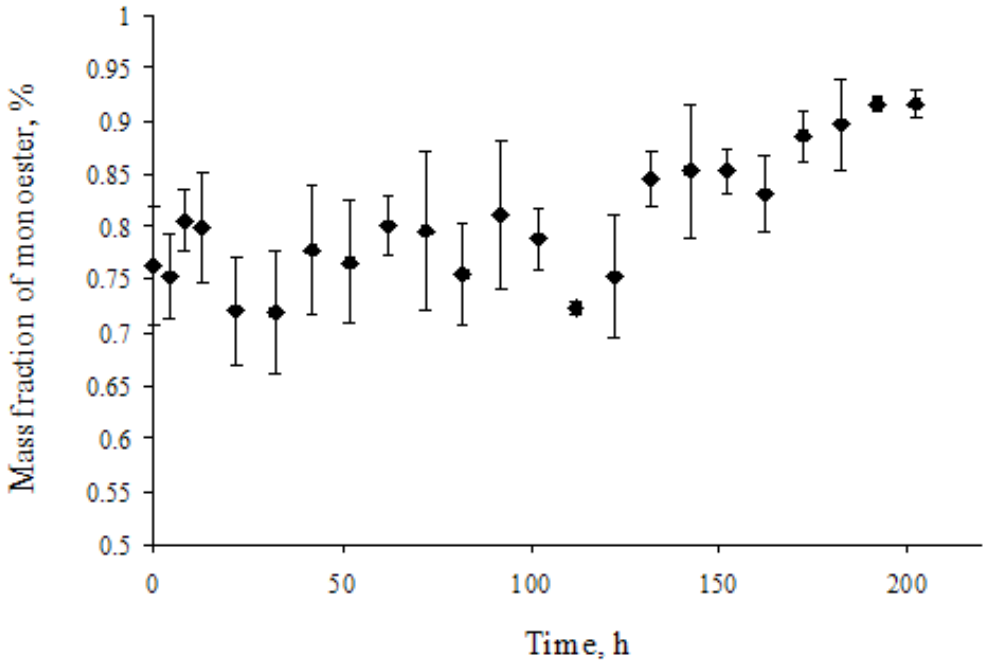


Fig.6.5. Mass fraction of monoester among the esters for the experiment in Fig. 6.2. (Error bars represent the standard deviation from replicate runs of the enzymatic reaction).

6.4.3 A Mathematical Model for the Improved Bioreactor System for Lipase-Catalyzed Synthesis of Saccharide-fatty Acid Esters Utilizing Solvent-free Medium

6.4.3.1 A Ternary Phase Diagram

A ternary phase diagram for the saccharide (fructose, S)/ (oleic acid, FA)/fructose-oleic acid monoester (ME) ternary system at 65 ^{o}C is described in **Fig. 6.6**. A one-phase mixture is located at the right of the phase boundary and two-phase media to the left of the boundary. "One phase" in this experiment does not mean true solubilization of saccharide. It means the formation of metastable suspensions mixtures of fructose particle, oleic acid and fructose oleate. The suspension media was formed via a specific protocol in Chapter 6 (**6.3.2.2).** Moreover, Fig. 6.6 replots the data of Figs 6.2 and 6.3 in the form of a three phase diagram. **Fig. 6.6** demonstrates that with the increase of ester content, the area of one phase region increases. Mass fraction of fatty (oleic) acid acyl donor, saccharide (fructose) acyl acceptor at the saturation, and fructose-oleic acid monoester product in the reservoir unit of the bioreactor system (Fig. 6.1), were defined as ω_{FA}, ω_s, and ω_{ME}, respectively. With the increase of ω_{ME} from 0.045 to 0.802, ω_s increased from 0.0029 to 0.016. The trend of the increase of ω_s with the increase of ω_{ME} is in agreement with a triangular phase diagram in previously published results for fructose [115]. However, ω_s of the new bioreactor system controlled by the in-line filter is apparently lower than Dang's work (ω_s =0.13) [115] since the in-line filter blocked many of suspensions particles. Compared with the previous work of Dang [115], the area of the "one phase" region is slightly smaller.

The phase boundary of **Fig. 6.6** can be described by the following linear equation:

$$\omega_s = A - B \cdot \omega_{FA} \qquad (6.1)$$

where *A* and *B* equal 0.638 and 0.163, respectively.

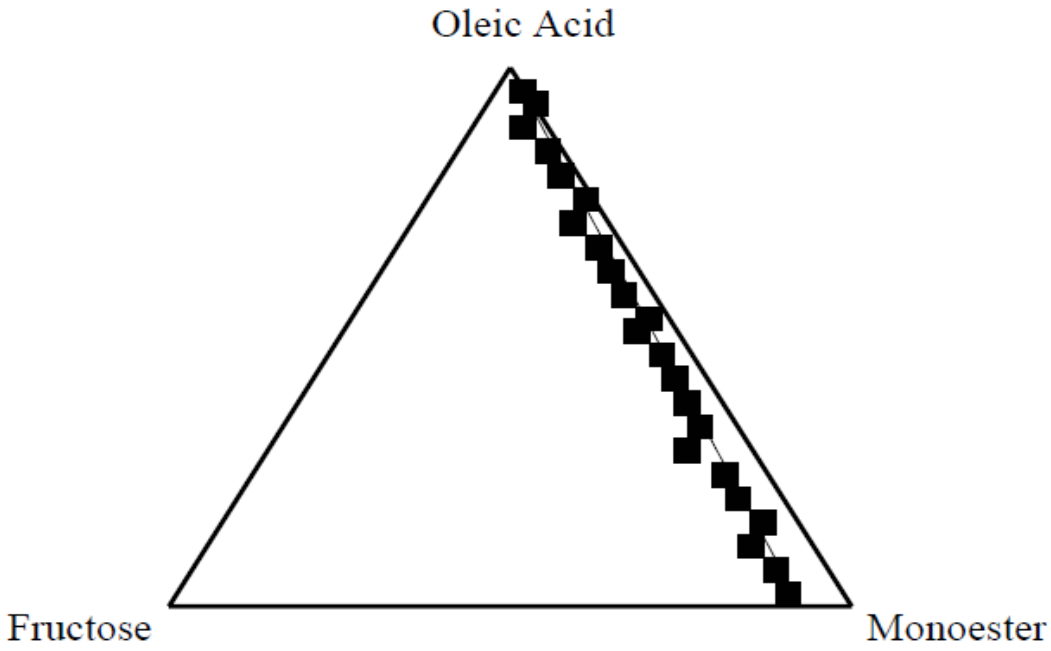


Fig.6.6. Ternary phase diagram for fructose/oleic acid/technical-grade monoester (8% diester and 92% monoester) in the stream of leaving in-line filter in improved bioreactor system at 65°C.

6.4.3.2 Mathematic Model for the Time Course of Reaction of Solvent-Free Fructose Oleate Synthesis in the Improved Bioreactor System

A mathematical model, based on a model developed previously by Hayes and coworkers [115], was derived to predict the concentration change of two substrates (oleic acid as acyl donor, and saccharide as acyl acceptor) over the time course of reaction. Some assumptions were incorporated: First, the effect of water and inhibition by substrates and products were neglected. Second, although diester was also formed, mono- and di-ester were combined together and referred to collectively as "monoester" (ME). These assumptions were also employed successfully for the batch-mode model [115].

A mole balance for fatty acid on the reservoir (see Fig. 6.1) yields the following equation:

$$C_{FA,2} \cdot \nu_0 - C_{FA,1} \cdot \nu_0 + 0 = \frac{dC_{FA,1}}{dt} \cdot V_{res} \tag{6.2}$$

This equation assumes the reservoir is well mixed, moreover, that the exit stream's composition equals the composition at all positions inside of the researvoir. Rearrangement of this equation yields the following:

$$\frac{\nu_0}{V_{res}} \cdot \left(C_{FA,2} - C_{FA,1} \right) = \frac{dC_{FA,1}}{dt} \tag{6.3}$$

where $C_{FA,1}$, and $C_{FA,2}$ refer to the concentration of fatty acid in the reservoir, the stream leaving the reservoir and the recycle stream returning to the reservoir from PBBR respectively; t refers to the reaction time; and v_0 refers to the volumetric flow rate of reaction medium circulating throughout the system (0.5 ml/min); V_{res} refers to the volume of reaction medium in the reservoir. V_{res}, is treated as a constant, which assumes the mass and density of the reservoir's contents remain constant. This assumption is valid as a first approximation since the change of density from beginning to end of the reaction was small, from 907 $g\ L^{-1}$ to 928 $g\ L^{-1}$, calculated using the density values for pure components published previously [115] and neglecting the volume change upon mixing, less than a 2.5% difference. The assumption also neglects the loss of mass due to water evaporation.

A mole balance for fatty acid in the packed-bed (bio-) reactor, PBBR, yields the following equation:

$$v_0 \cdot \frac{dC_{FA,1}}{dW} = -\left(-r_{FA}{}'\right) \tag{6.4}$$

where W refers to the cumulative weight of lipase in the PBBR encountered during travel in the axial direction and $-r_{FA}{}'$ to the rate of reaction for fatty acid in dimensions of moles per mass unit of biocatalyst per unit of time. The time course of reaction for lipase-catalyzed saccharide-fatty acid ester synthesis, and many other lipase-catalyzed reactions, has been effectively described by the Ping-Pong bi bi kinetic model [115, 201]. Previously, the following kinetic equation was derived by Hayes and coworkers for describing fructose-oleic acid esterification in batch mode [115]:

$$r_{FA}' = \frac{\frac{V_{\max}}{C_{ENZ}} \cdot C_{FA} \cdot C_S}{K_S \cdot C_{FA} + K_{FA} \cdot C_S + C_{FA} \cdot C_S} \tag{6.5}$$

where $\frac{V_{\max}}{C_{ENZ}}$ is the maximal velocity in dimensions of moles$_{FA}$ g_{lipase}^{-1} $time^{-1}$, and K_S and K_{FA} are Michaelis constants. For the batch-mode reaction under the conditions employed, is was determined that $r_{FA}' \simeq \frac{V_{\max}}{C_{ENZ}}$ [115]. C_{ENZ} in this PBBR based bioreactor system refers to the ration of mass of RML in the PBBR to the volume of reaction medium. However, motivated by the nearly-constant time course of reaction (**Fig. 6.2**), it was assumed that the rate of reaction was equal to an "apparent" value of the maximal velocity; moreover:

$$r_{FA}' \simeq \left(\frac{V_{\max}}{C_{ENZ}} \right)_{app} \tag{6.6}$$

Substitution of Eq. 6.6 into Eq. 6.4, followed by separation of variables followed and integration of both sides of the equation leads to:

$$\nu_0 \cdot \left(\frac{V_{\max}}{C_{ENZ}} \right) \cdot \int_{C_{FA,1}}^{C_{FA,2}} dC_{FA,1} = \int_0^W dW \tag{6.7}$$

Integration of Eq. 6.7 yields the following:

$$\nu_0 \left(\frac{C_{FA,2} - C_{FA,1}}{\left(\frac{V_{\max}}{C_{ENZ}} \right)} \right) = W_{tot} \tag{6.8}$$

Substitution of Eq. 6.8 into Eq. 6.3 leads to:

$$\frac{dC_{FA,1}}{dt} = -\frac{W_{tot}}{v_0}\cdot\left(\frac{V_{\max}}{C_{ENZ}}\right) \tag{6.9}$$

Integration of Eq. 6.9 leads to the following:

$$C_{FA,1} = C_{FA,res,0} - \alpha \cdot t \tag{6.10}$$

where:

$$\alpha \equiv \frac{W_{tot}}{v_0}\cdot\left(\frac{V_{\max}}{C_{ENZ}}\right)_{app} \tag{6.11}$$

and W_{tot} = total mass of immobilized lipase in the PBBR , and $C_{FA,res,0}$ is the initial concentration of fatty acid in the reservoir. The mathematical model derived above was successful in describing the time course of reaction, as demonstrated by the reasonably close fit to the experimental data in **Fig. 6.7**

An experimentally-determined triangular phase diagram for the fructose/oleic acid/fructose monooleate system (**Fig. 6.7)** yields the following relationship in, indicating the phase boundary. Eq. 6.12 is an alternate form of Eq. 6.1:

$$C_{s,1} = A_1 - B_1 \cdot C_{FA,1} \tag{6.12}$$

where $C_{s,1}$ refers to the concentration of saccharide in the stream of leaving the in-line filter. A_1 and B_1 represent the density of reaction medium (ρ_{mix}) multiplied by the constants A and B of Eq. 6.1, respectively. A_1 and B_1 are equal to 0.58 and 0.15, respectively. The density of the mixture is calculated assuming no change of volume due to mixing:

$$\rho_{mix} = \frac{\rho_{ME} + [FA] \cdot MW_{FA} \cdot \left(1 - B - \frac{\rho_{ME}}{\rho_{ME}}\right)}{1 - A} \qquad [115]$$

where ρ_{FA} and ρ_{ME} are the density of oleic acid (0.895 g/ml) and fructose monoleate (0.936g/ml), respectively [115].

Substitution of Eq. 6.10 into Eq. 6.12

$$C_{s,1} = A_1 - B_1 \cdot C_{FA,res,0} + B_1 \cdot \alpha \cdot t \qquad (6.13)$$

The mathematical model derived above was successful in describing the time course of reaction, as demonstrated by the reasonably close fit to the experimental data in **Fig. 6.8**. **Fig. 6.8** depicts the saccharide content in the reservoir. The equation predicting the fructose concentration (Eq. 6.3) fits to the experimental data very well during the time course of reaction (**Fig. 6.2**). Parameters employed in the mathematical model, obtained through measurement, consisted of V_{res} = 11 *mL*, W_{tot} = 0.75 *g*, $C_{FA,res,0}$ = 3.17 *mol* L^{-1}, and the constants *A* and *B* for the solubility of fructose as 0.638 *mol* L^{-1} and 0.163, respectively, obtained from **Fig. 6.6**. The only adjustable parameter in the model was $\left(\frac{V_{max}}{C_{ENZ}}\right)_{app}$, determined to be 0.21*mmol* g_{lipase}^{-1} hr^{-1} from Chapter 3. This value is significantly lower, by a factor of ~2, than corresponding value obtained for the same reaction operated in batch mode under similar operating conditions, 0.46 *mmol* g_{lipase}^{-1} hr^{-1} [115]. The inherent RML activity between Reference [115] and this work was assumed to be different. To confirm this hypothesis two experiments from the previous report [115] were repeated using the same RML preparation employed throughout this dissertation to prepare a Lineweaver–Burk plot, **Fig. 6.9**. This plot also contains a plot obtained from [115] obtained under comparable conditions. The slopes for two reactions are similar indicating michaelis constants are roughly identical. The

intercept of Lineweaver–Burk plot refers to $1/V_{max}$. In **Fig. 6.9**, the intercept ($1/V_{max}$) of this work in Chapter 6 is 2-fold higher than that of [115], demonstrating the inherent RML activity in the latter was ~2-fold higher than the RML preparation employed throughout this dissertation. Moreover, the RML preparation purchased from Novozymes through Sigma-Aldrich is less active than the RML preparation donated by Novozymes Inc for the work of [115].

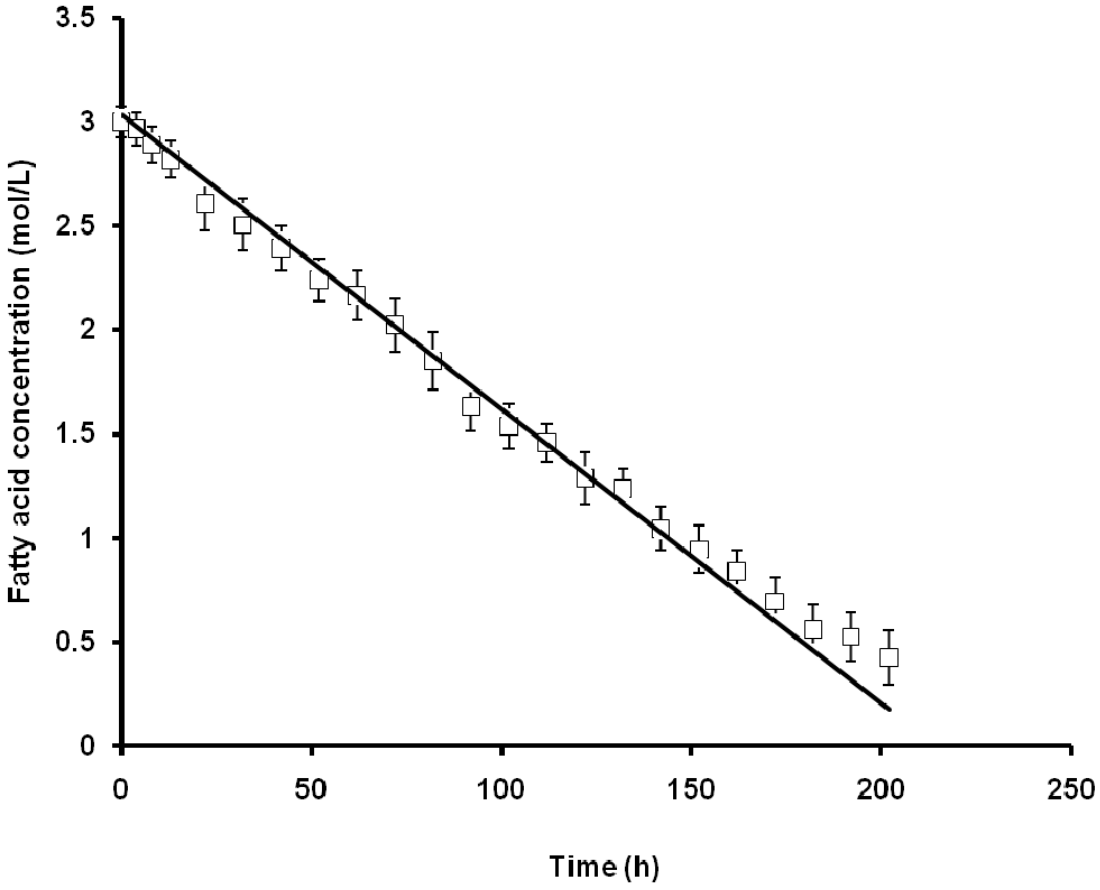


Fig.6.7. The change of the concentration of oleic acid in the mixture of ester and oleic acid over the time course for RML lipase-catalyzed synthesis of fructose oleate at 65 oC employing improved bioreactor system. (The straight line represents mathematical model fit assuming the concentration of oleic acid in the reservoir (Eq. 6.12). This is a re-plotting of data from Fig. 6.2. Error bars represent the standard deviations from replicate runs of the enzymatic reaction).

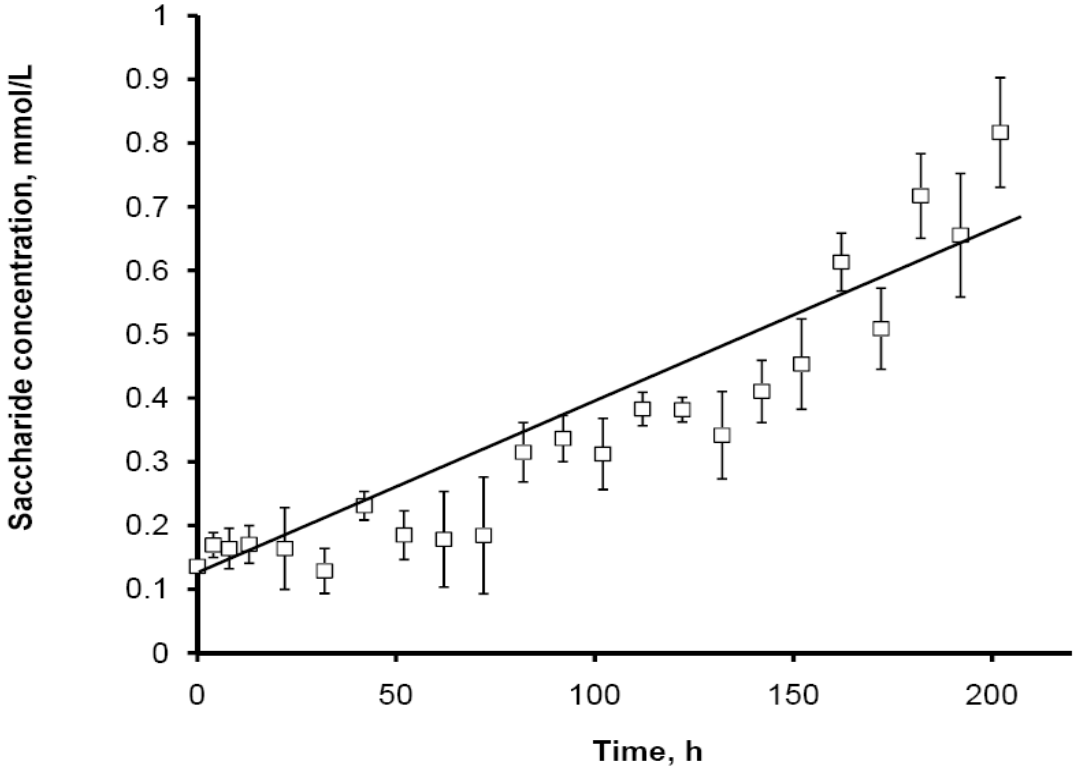


Fig.6.8. The change of the concentration of saccharide in the mixture of ester and oleic acid over the time course for RML lipase-catalyzed synthesis of fructose oleate at 60oC employing improved bioreactor system. (The straight line represents mathematical model fit (Eq, 6.13). This is a re-plotting of data from Fig. 6.3. Error bars represent the standard deviations from replicate runs of the enzymatic reaction).

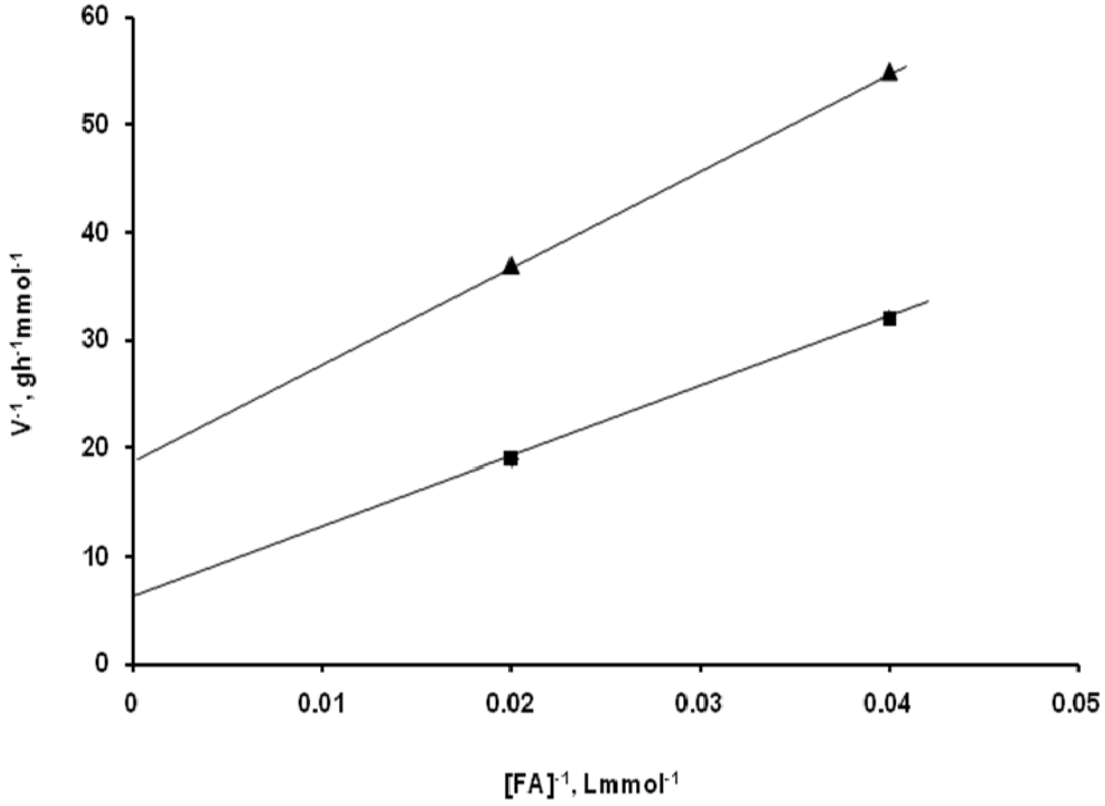


Fig.6.9. Lineweaver–Burk plots for comparison of RML inherent activity. Reaction conditions: 127.5 mM fructose (constant), 23.4 g (30 mL) *t*-BuOH, 0.3 g RML, 65°C, stirring rate of 350 rpm. *t*-BuOH and water were allowed to freely evaporate during the time course of esterification. (■) Data taken from [115]; (▲) RML Data obtained from experiments performed by the author of this dissertation.

6.5 Conclusion

In conclusion, an improved bioreactor system was contained through re-designing the system to contain a reservoir serving as the site for continuous formation of suspensions and water removal (via free evaporation during the initial phase, and the combination of N_2+vaccum during the later phase of the time course of reaction) and to include an in-line filter for preventing larger aggregates to be transported throughout the bioreactor system. Specifically, the redesigned closed-loop bioreactor system consists of the reservoir, in-line filter, a peristaltic pump, and a packed bed bioreactor (PBBR), with the latter's effluent returned to the reservoir. This system was successfully operated under continuous recirculation. A linear increase of maximum conversion was obtained with respect to time up to about 84 % w/w ester concentration within 8.4 days using completely solvent-free reaction media (i.e., a productivity of 0.195 mmol h^{-1} g^{-1}). RML exhibits the good stability for two times runs of the enzymatic reaction. In addition, a mathematical model was successfully developed to understand the relationship between the significant variables in the system and the observed time course of reaction. The model based on mass balances and an enzymatic kinetic model (Ping-pong bi bi mechanism) for predicting the substrate concentrations fits the experimental data very well.

CHAPTER 7

CONCLUSIONS AND FUTURE PERSPECTIVES

7.1 Conclusions for This Dissertation

In sum, several achievements for this dissertation have been achieved as listed below:

1. Designed and developed a simple, inexpensive and environmentally friendly packed-bed based bioreactor system successfully using metastable suspensions of small, 10~200μm-sized, saccharide crystals in a fatty acid ester/ saccharide ester mixture, to synthesize saccharide fatty acid esters at high reaction rate under solvent-free condition in Chapter 3[181]. By this approach, the initial reaction medium, a suspension of saccharide crystals in solvent-free media, was formed by mixing 1.5 g fructose crystals and 10 g oleic acid / fructose-oleic acid ester (FOE), 3/1 w/w, in a 20 mL reservoir open to the atmosphere on a magnetic stirrer plate at 80^{o}C and 800 rpm (radius of 1.5 cm) for 6 h. The slurry was centrifuged at 800 rpm for 0.5-1 min, with the supernatant collected. Subsequently, the solvent-free suspensions, stable for over 12 h, were employed in a closed-loop bioreactor system, consisting of a reservoir open to the atmosphere (for free evaporation of the reaction product, water), a peristaltic pump, and packed bed bioreactor (PBBR), operated under continuous recirculation at 0.5 L/min. The suspensions were retreated at 10 h intervals, through stopping the pump, removing the liquid phase, retreating the latter with additional saccharide to reform the suspensions using the methodology described above, and returning the liquid phase to the bioreactor system [181]. The technical-grade product was then collected from the reservoir by this sustainable and environmentally friendly method without the need for downstream purification. Due to the absence of organic solvents and ionic liquids, the process is also greatly simplified and less expensive. This accomplishment has been published by the Journal of the American Oil Chemists Society [181]

2. Optimized the overall performance of the developed bioreactor system for the enhancement of the reaction rate and conversion in Chapter 4. Water as byproduct of esterification is a significant factor for obtaining the high conversion of reaction.

However, excess water removal will lead to the loss of enzyme activity since water is essential for maintaining the three dimensional conformations of enzymes. An efficient water removal method was discovered using the combination of vacuum+N_2 bubbling in a Wheaton Celstir spinner double-side arm flask with a tight screw cap was placed on a hot plate at 2.16 mg_{H2O} h^{-1} to maintain at 0.4% w/w plus/minus 0.05 % w/w level in the liquid phase when the bioreactor system reached 57.75 % w/w conversion. With the employment of this approach, the excess water can be removed efficiently. This approach can be widely applied for the chemistry or chemical engineering industry when water control needed, for instance, the manufacture of biodiesel. In addition, the overall performance of the developed bioreactor system has been optimized for the promotion of the reaction rate and yield, including the optimal water concentration in the reaction medium and the initial ester concentration. In conclusion, the optimization of the interval time for re-treatment of suspensions decreased the reaction time by 23 h compared to the arbitrary use of a 10 h interval time. Additionally, the initial ester concentration in the solvent-free suspensions media was reduced from 25% w/w to 5% w/w without any loss of reaction rate. Conclusively, the optimized PBBR-based bioreactor system operated at optimal conditions employing an initial charge of fructose suspensions in oleic acid / fructose oleate 95/5 w/w yielded 92.6% w/w conversion within 132 h and a productivity (0.297 $mmol_{FOE}$ h^{-1} g_{lipase}^{-1}) 2-fold higher than the control. This accomplishment has produced a manuscript and been accepted by the Journal of the American Oil Chemists Society for publication [201]. In addition, enzyme (RML) activity retention was examined in the bioreactor system described above but with *in situ* Wheaton Celstir spinner double-side arm flask by the combination of vacuum+N_2 bubbling. In addition, the activity of RML using suspensions media exhibits excellent stability without any loss of activity in four successive runs in 22days.

3. Examined the universality of the developed bioreactor system using different acyl donors (oleic, caprylic, lauric and myristic acids) and acceptors (fructose, sucrose, glucose and xylose) and discovered the underlying reason for difference in saccharide

concentration in Chapter 5. The results revealed that the highest conversion and initial rate occurred when the saccharide concentration was highest. Suspensions containing the highest saccharide concentration coincided with saccharide crystals of the smallest average size, since large-sized crystals sedimented out during the workup for formation of the suspensions. In addition, the underlying mechanism for effect of formation of suspensions on size of the suspended crystal particles was investigated. The formation of suspensions is anticipated to affect the size of the suspended crystal particles minimally since the environment present is relatively benign toward influencing the formation or decomposition of the crystals. First, due to the pretreatment in a vial opened to the atmosphere at 80^{o}C and low water contents of starting materials, this medium offers nearly-anhydrous environment (water concentration, 0.65 wt %±0.04 wt %), which is considered to be a distinct barrier for crystallization or dissolution of large size crystals. Second, the high viscosity of the reaction medium inhibits crystallization since it offers the high energy hurdle for crystallization process. A manuscript is in preparation based on this work.

4. Improved the design of the bioreactor system and derived a mathematic model to describe the change of two substrates over the time course in the improved bioreactor system in Chapter 6. For the bioreactor developed in accomplishment 1, the need to reform the suspension media at regular time intervals (to replenish saccharide consumed by the reaction) requires the stoppage of the recirculation and removal of the reaction medium for several hours at a time, leading to potentially the increase cost of production, and it is unsuitable for scale-up. To improve the bioreactor system's performance, I re-designed the system to contain a reservoir serving as a place for continuous formation suspensions and water removal, an in-line filter for preventing larger aggregates to be transported through the bioreactor system, making the bioreactor system operated under recirculation. This system was successfully operated under continuous recirculation. A linear increase of maximum conversion was obtained with respect to time up to about 84 % w/w ester concentration within 8.4 days using completely solvent-free reaction media (i.e., a productivity of 0.195mmol

h^{-1} g^{-1}). RML exhibits the good stability for two times runs of the enzymatic reaction. In addition, a mathematical model was successfully developed to understand the relationship between the significant variables in the system and the observed time course of reaction. The model based on mass balances and an enzymatic kinetic model (Ping-pong bi bi mechanism) for predicting the substrate concentrations fits the experimental data very well.

7.2 Recommendations for Future Work

My recommendations for the next phase of this investigation are as follows:

1. In Chapter 6, an improved bioreactor system with an in-line filter was successfully developed. However, this system is suffering from the lower saccharide concentration than optimal system's in Chapter 4. Consequently, a suitable in-line filter with larger pore size is recommended to achieve the higher saccharide concentration.

2. For achieving more stable, specific and higher activity of lipase, genetic engineering e.g. mutagenesis strategies, could be applied for the enhancement of the enzyme stability and activity. More specifically for lipase, thermostability is a key factor in successful bioprocesses, because typically reaction rates and conversion increase exponentially with temperature, until the denaturation of enzyme. Therefore, the development and programming of thermostable lipases from thermophilic microorganisms is significant for use in scale-up industrial reaction.

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Vita

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