Chemo-Enzymatic Route to Synthesis of Biodegradable Polymers and Glycolipid Analogs

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
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<td>$j$</td>
<td>Coupling constant</td>
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<td>MS</td>
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<td>PCL</td>
<td>Poly caprolactone</td>
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<td>PPL</td>
<td>Porcine pancreatic lipase</td>
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<td>Ring opening polymerization</td>
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<td>$R_f$</td>
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<td>Sophorolipid</td>
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New catalytic synthetic methods in organic chemistry that satisfy increasingly stringent environmental constraints are in great demand by the pharmaceutical and chemical industries. Studies over last 15 years have revealed that activity of enzymes can be increased in organic solvents rather than their natural aqueous environment. Because of their ease of use, high selectivity and environment friendliness, enzymes are enjoying increasing popularity in today’s synthesis world.

Chapter 1 describes chemo-enzymatic synthesis of various glycolipid analogs. A highly regioselective macrolactonization was achieved using lipase from *Candida antarctica* as a catalyst. It also describes evaluation of lipases from different source and their efficiency in catalyzing the macrolactonization reaction. These analogs were synthesized using commercially available agriculture based disaccharides (maltose, lactose, cellobiose, melibiose). These glycolipid analogues have potential applications in the cosmetic industry, formulation, food production, and pharmaceutical industry.

In Chapter 2, ring opening polymerization of ε-caprolactone in ionic liquid, [bmim][PF₆] was investigated. A comparative study of ROP in different solvents (toluene, Ionic liquid, and bulk condition) was conducted. Effect of time and enzyme concentration on molecular weight and % yield was investigated. It was concluded that enzymatic ring opening polymerization of ε-caprolactone in ionic liquid, [bmim][PF₆] is a very
competitive and environmental friendly way of synthesizing high molecular weight polyesters.
CHAPTER 1

Lipase-Catalyzed Synthesis of Glycolipid analogs

1.1 Introduction

Glycolipids are a particularly important class of cell membrane components. Principally they are only found in the exterior of the cell wall. Various bioactive structures possessing macrolicidic structures or an aliphatic chain attached to a sugar moiety are abundant in nature and are interesting synthetic targets due to challenges associated with the synthesis of highly functionalized glycolipid analogs from readily available synthons. Glycolipids are interesting group of natural products that have complex structures and interesting biological activities (Figure 1.1). Sophorolipids (SLs) belong to such a class of glycolipids which contain a ω-hydroxy acid as an agylcon attached to a saccharide backbone (Figure 1.1).
Figure 1.1 Various natural Glycolipids
Sophorolipids produced by the yeast *Candida bombicola* are amphiphilic molecules of growing commercial interest as biodegradable emulsifiers. Such molecules find applications in the petroleum, pharmaceuticals, and food processing industries where they can be used to reduce surface tension, stabilize emulsion, and promote foaming.\(^1\) Compared to their synthetic counterparts biosurfactants offer some distinct advantages: they are produced from renewable resources, they are nontoxic and biodegradable, they are effective under extreme conditions in small quantities. Sophorolipids consist of a sophorose molecule linked to a hydroxyl group at the penultimate position of, most often, a C-18 fatty acid.\(^2\) Native SL is rather complex mixture of up to 14 different compounds, both lactone and open chain acid form.\(^3\)

### 1.2 Bioactivity of glycolipids

Although the biological properties of glycolipids have not been fully assessed, a closer look at this class of natural products seems highly promising. They are typically involved in intercellular recognition processes like cell aggregation and dissociation and initiation of cell division. Globoside-3 (Figure 1.1) is a minor component of tissues, deficiency of which leads to Fabry’s disease.\(^4\) Yet another glycolipid, galactose cerebroside which has structure similar to globoside with galactose as head group is an important component of membranes of nerve tissue and is associated with disease such as multiple sclerosis.\(^4\) Tricolorin A (Figure 1.1) is isolated from a plant called *Ipomoea tricolor* which acts as a natural herbicide. It has been reported to have significant cytotoxicity against cultured P-388 and human breast cancer cell lines.\(^5-7\) Woodrosin I,
resin glycoside isolated from the stems of *Ipomoea toberosa* L, seems highly promising in view of the existing data on the use of glycolipids in general for the treatment of severe immune disorders.\(^8\)

The existing data indicates the potential of sophorolipids as immunomodulators for Parkinson's disease, Alzheimer's disease, psoriasis, AIDS treatment, as well as for antiviral immunostimulation.\(^9,10\) Also there have been reports of SLs causing differentiation and protein kinase C inhibition in the HL60 leukemia cell line.\(^11\) Glucolipsin, a macrolidic glycolipid produced by *Streptomyces purpurogenisclerotics*, is known to act as a glukinase inhibitor.\(^12\) Simonin 1 has been isolated from plant *Ipomoea batatas* is used in Brazilian folk medicine.\(^13\)

### 1.3 Traditional Syntheses of Glycolipids

Whole cell biocatalytic approaches have been investigated for synthesis of microbial sophorolipids. For instance, Gross et al. have used fermentation for the synthesis of Sophorolipids.\(^14\) In this strategy industrial waste containing fatty combined with glucose were evaluated both in batch and fed-batch processes for the production of sophorolipids using *Candida bombicola* ATCC 22214. Maximum sophorolipid yields of 120 g/L and productivity of 12.0 g/L per day was obtained by fed batch fermentation using tallow fatty acid residue. To improve the yields Feed of coconut fatty acid residues was used which resulted in increase of the cell production \(^14\) (Chart 1.1).
Daniel et al. have developed an improved two batch cultivation process (Chart 1.2) using deproteinized whey as feed. They used the yeast *C. curvatus* in the first and the yeast *C. bombicola* in second step. The two step strategy was important because *C. bombicola* was not able to consume lactose directly from the substrate deproteinized whey. This made it essential to use *C. curvatus* in the first cultivation step.
Chart 1.2. Flow chart of the coupled process including filtration.
It is reported that the composition of natural sophorolipid mixture can be altered by the selective-feeding of lipophilic substrates.\textsuperscript{16, 17} For example, changing sunflower to canola oil resulted in a large increase (50 to 73\%) of the lactonic portion of SLs.\textsuperscript{18} Unsaturated C-18 fatty acids such as oleic acid may be incorporated unchanged into SLs and result in dramatic change in their compositions.\textsuperscript{19,20} However, it is clear that this approach is largely limited to composition change or incorporation of select agylcons. Clearly, a different synthetic approach is needed but the intricate structure of these glycolipids poses many synthetic difficulties and consequently very little effort has been devoted to this area.

In the synthesis of macrolidic glycolipids, one of the major challenge resides in the regioselective formation of the macrolactone ring to synthesize lactonic analogs. The formation of the macrolactone thus far has been accomplished by Yamaguchi\textsuperscript{21}, Corey\textsuperscript{22}, or Mitsunobu\textsuperscript{23} macrolactonization conditions. More recently use of ring closing metathesis reaction has been explored for formation of the macrolides woodrosin I, sophorolipid lactone and Tricolorin A (Scheme 1.1).\textsuperscript{24, 25}
Heathcock et. al. have reported synthesis of tricolorin A by selective macrolactonization using Yonemitsu protocol\textsuperscript{26} (Scheme 1.2). Although very useful, these synthetic approaches to the formation of the macrolides require strategically placed reactive groups necessitating a number of protection/deprotection steps resulting in a long synthetic sequence.

Scheme 1.1 Retro-synthesis of a Tricolorin A intermediate by Furstner et al.\textsuperscript{24}
Our goal is to develop a chemoenzymatic strategy well suited to the synthesis of well defined macrolidic glycolipid analogs, which would otherwise be unavailable or difficult to synthesize, for subsequent evaluation of their properties and bioactivities. The main advantages of using this strategy would be

a) low cost starting materials.

b) changing number and variety of carbohydrate residue in the head group and using different aliphatic hydrophobic chain will provide a handle to different analogs having different physical properties.

**Scheme 1.2** Retrosynthesis of Tricolorin-A by Hitchcock et. al. 26
c) non-toxic reaction conditions and biodegradable products.

d) strategies applied are in accordance with the principles of green chemistry.

1.4 Experimental Section

1.4.1 Chemicals and enzymes.

All reagents were purchased from commercial sources and used as received. All solvents were purified and dried prior to use by known literature procedure. Porcine pancreatic lipase (PPL) Type II crude (activity = 61 units/mg protein) and Candida rugosa lipase (AYS) Type VII (activity = 4570 units/gm protein) were purchased from Sigma Chemical Co. The lipase PS-30 from Pseudomonas cepacia (20,000 units/g) was obtained from Amano Enzymes Co. Ltd. The carrier fixed lipase Novozyme 435 (10,000 units/g from Candida anatarctica, fraction B) was a gift from Novo Nordisk Inc.

1.4.2 Column chromatography

Column chromatographic separations were performed over silica gel 60 (Silicycle Inc.). In a typical separation, silica gel was used to pack a glass column (5cm X 50cm) in the eluent (ethyl acetate/hexane mixture). The compounds were dissolved in a minimal volume of eluent and loaded onto the top of the silica bed in the column. Different fractions were subsequently eluted and monitored by thin-layer chromatography (TLC). Fractions containing the purified compounds were pooled together, and the solvent was evaporated to give the pure compound.
1.4.3 Nuclear magnetic resonance

$^1$H-NMR and $^{13}$C-NMR spectra were recorded using Bruker ARX-250, INOVA 400, and INOVA 500 spectrometer. Chemical shifts in parts per million are reported downfield from 0.0 ppm using deuterated chloroform or deuterated DMSO with trimethylsilane (TMS) as the internal reference. Unambiguous assignments were derived from COSY and HMBC spectra.

1.4.4 General synthesis of octaacetate of lactose, maltose, melibiose and cellobiose

In a 250 ml round bottom flask equipped with reflux condenser 10 g (0.0277 mol) of disaccharide (maltose, cellobiose, melibiose, or lactose), 8 g (0.0975 mol) of sodium acetate and 50 ml (0.489 mol) of acetic anhydride were added. The reaction assembly was protected from atmospheric moisture by CaCl$_2$ guard tube. The reaction mixture was refluxed for 4 h, cooled to room temperature and worked up by precipitating out by adding it dropwise over stirring ice, the product precipitated out immediately which was then filtered, washed with ice-water, and dried overnight under pressure in a vacuum oven to yield octaacetate of sugar as a white solid (yield 96 %).

1.4.5 Synthesis of methyl 15-hydroxypentadecanoate

A 250 ml round bottom flask was charged with 10 g of $\omega$-pentadecalactone, 150 ml dry methanol and 2 ml 0.022 N freshly prepared sodium methoxide. Reaction mixture
was refluxed for 4 h and then cooled down to room temperature and then neutralized using glacial acetic acid. The reaction mixture was concentrated by rotoevaporation and poured over stirring crushed ice dropwise to yield the product. The white product was filtered, washed with ice-water and dried over night under pressure in a vacuum oven. (95% recovered Yield).

1.4.6 Synthesis of compounds 3a-d

In 100 ml round bottom flask 2 g (0.0028 mole, 1 equiv.) of acetylated sugar and 1.168g (0.0043 mol, 1.5 equiv.) was taken. To this mixture 40 ml of freshly distilled dichloromethane was added to the flask using syringe. The reaction mixture was then cooled down to 0 °C by placing it in a ice-bath. Then to this cold stirring mixture, 2.03 ml (0.01 mol, 5 equiv.) of boron trifluoride etherate was added drop wise. After the addition was complete the reaction mixture was removed from ice bath and brought back to room temperature. It was stirred for 7 h at room temperature. The reaction mixture was protected from moisture using rubber septum and nitrogen was flushed through the round bottom flask during transferring solvent and catalyst. After 7 h, the reaction mixture was diluted by adding 20 mL of dichloromethane and then neutralized using saturated solution of sodium bicarbonate. The organic layer was washed 3 times with 20 mL de-ionized water, dried over sodium sulfate, and concentrated by rotoevaporation. The viscous liquid was then purified using column chromatography using hexane : ethyl acetate (60:40) eluent system. Same fractions were collected and dried under pressure in vacuum oven to yield pure coupled product 3a-d, 50% yield.
1.4.7 Synthesis of Compounds 4a-d

Herein we give general procedure for synthesis of 4a-d. In 100 ml round bottom flask 1 g of Compound 3 and 2 mL 0.022 N freshly prepared sodium methoxide in methanol were added. The reaction mixture was protected from moisture using rubber septum and nitrogen was flushed through the round bottom flask during transferring solvent and reagent. The reaction was stirred overnight at room temperature and then neutralized using glacial acetic acid. The reaction mixture was concentrated by rotoevaporation and poured over stirring crushed ice dropwise to yield the product. The white product was filtered, washed with ice-water and dried over night under pressure in a vacuum oven (92% recovered yield).

1.4.8 Screening of lipase and general procedure of lipase catalyzed mecro lactonization and lipase catalyzed ester hydrolysis

In 50 ml round bottom flask appropriate substrate (4a-d) was dried overnight in vacuum oven. Enzymes were dried over P₂O₅ overnight and enzymes were transferred to reaction flask in nitrogen bag to maintain strictly dry conditions. To the reaction flask then freshly distilled THF was added using syringe. Reaction was stirred for 96 h at 30 °C. Novozyme – 435 was found to be the only enzyme that catalyzed the lactonization reaction. The reaction mixture was then filtered through a bed of celite and concentrated by rotoevaporation. The resulting crude product was purified by wet column chromatography using methanol and dichloromethane as eluent.
1.5 Results and Discussion

Lipase catalyzed acylations and transesterification reactions have been evaluated for formation of lactones. The hydrolytic enzymes lipases are known to catalyze macrolactone formation by intra-esterification in absence of water or other nucleophiles such as alcohols. Stemming from our interest in biocatalysis, we developed efficient short synthetic route to compounds 5a-d (Figure 1.2), exploiting the regioselectivity of the lipases (Scheme 1.3).

Figure 1.2 Different glycolipid analogs
In a previous study by Bisht et. al. it was demonstrated that the sophorose, the glycon portion of sophorolipids, is able to fit in the active site of the lipase CA (from *Candida antarctica*) and that the lipase CA catalyzed macrolactone formation between 17-hydroxyoctadec-9-enoic acid subunit and the sophorose backbone.\(^{28}\) Utilization of readily available disaccharides maltose, lactose, cellobiose and mellibiose (Figure 1.3) is highly desired because of their origin in agriculture based feedstock and we have utilized them in synthesis of macrolidic glycolipids analogs.

![Disaccharide structures](image)

**Lactose**  
**Cellobiose**

**Maltose**  
**Melibiose**

*Figure 1.3* Different disaccharide used as starting material

The aglycon, 15-hydroxypentadecanoic acid was obtained from the \(\omega\)-pentadecalactone, which is a well known substrate for the *Candida antarctica* lipase.\(^{29}\)
Scheme 1.3. Synthetic approach to different glycolipid analogs.

Ring opening reaction of pentadecalactone using sodium methoxide in methanol provided methyl 15-hydroxy pentadecanoate in 93% yield (Scheme 1.4).

Scheme 1.4 Synthesis of 15-hydroxy pentadecanoate

Scheme 1.5 Acetylation of disaccharide
All the disaccharides (maltose, lactose, melibiose and cellobiose) were peracetylated using sodium acetate and acetic anhydride to form respective octaacetates 2a-d in nearly quantitative yield (Scheme 1.5). Taking advantage of the increased anomeric reactivity, 2a-d were directly glycosylated with methyl 15- hydroxypentadecanoate in the presence of boron trifluoride etherate in freshly distilled anhydrous dichloromethane to afford 3a-d in around 50% yields, respectively (Scheme 1.6). The stereochemical assignments were confirmed by $^1$H NMR, where the $J_{12}$ value for H-1 (8 Hz) indicated the β-D-configuration for the side chain. Global deprotection of the peracetates 3a-d was achieved in nearly quantitative yield upon stirring with sodium methoxide in anhydrous methanol for five hours at room temperature.

Structure assignments of 4a-d were confirmed using extensive spectrometric analysis. The absence of the acetoxy methyl resonances in the proton spectra at ~ 1.96-2.19 ppm and in the carbon-13 NMR spectra at ~75 ppm confirmed the global deacetylation. Also, absent were acetoxy ester carbonyl (C=O) resonances at ~170 ppm in the carbon-13 spectra. The resonance signal of the methyl ester was observed at ~3.5 ppm and ~50 in the proton and carbon-13 NMR spectra, respectively.
Scheme 1.6 Synthesis of 4(a-d)
1.5.1 Screening of Lipase

The screening for lipases were carried out in 50 mL round bottom flasks using 1:1 substrate to lipase ratio (w/w).

![Scheme 1.7](image)

**Scheme 1.7** Screening different lipases for the regioselective macrolactonization of substrate 4a.

These reactions were carried out in dry THF using different lipases for 96 h. Strictly anhydrous conditions were maintained. Since the size of scissile fatty acid binding pocket in lipase is known to vary considerably\(^{30}\), five different enzymes were used namely, PS-30 (from *Pseudomonas cepacia*), PPL (porcine pancreatic lipase), AK, AYS (from *Candida rugosa*) and Novozyme-435 (immobilized preparation of *Candida antartica*).
While no activity was seen for PPL, PS-30, AK and AYS in the tested reaction media, formation of a prominent product (different Rf values compared to the substrate) was observed within 96 h upon incubation of 15-(4-O-α-D-glucopyranosyl-D-glucose)-pentadecanoate 4a with lipase from Candida antarctica (Novozyme–435). With these observations further studies were done using Novozyme-435.

1.5.2 Lipase-catalyzed macrolactonization

The final step of our synthetic scheme was the regioselective formation of macrolactone resulting in compound 5a and 5b. In a previous study Bisht et. al. have reported enzyme catalyzed formation of macrolactone ring at C-6” position when sophorolipid methyl ester was taken as a substrate.\textsuperscript{28} With 4a and 4b in hand, the lipase catalyzed macrolactonization was attempted. The reactions were performed in anhydrous THF at 30\textdegree C for 96 hours. Control reactions were setup similarly but without added lipase. Of all the lipases tested only the lipase CAL led to lactonic analogs 5a and 5b (Scheme 1.8) which had a Rf higher than the starting compound when compared on a thin layer chromatography in methanol: CH\textsubscript{2}Cl\textsubscript{2} (1:3). Detail spectral analysis, described later in this chapter, was undertaken to establish the structure as the compounds as 5a and 5b. Control reactions set similarly but without added lipase did not catalyze formation of the compounds 5a and 5b. This observation clearly establishes the process being lipase catalyzed macrolactonization.
Scheme 1.8 Lipase catalyzed macrolactonization.

1.5.31 Lipase catalyzed hydrolysis of the methyl ester

Unlike the reaction of the lipase with compounds 4a and 4b, in reaction set up with compounds 4c and 4d, the lipase catalyzed reaction with Novozym 435 (Scheme 1.9) lead to a product with lower Rf in a thin layer chromatography experiment in methanol: CH$_2$Cl$_2$ (1:3).
Scheme 1.9 Lipase catalyzed hydrolysis of methyl ester

The $^1$H NMR spectrum of the products $5c$ and $5d$ did not have the resonance for methyl ester group which shows up at ~3.5 ppm in the precursors but a new resonance at ~12 ppm was present, possibly for a free carboxylic acid group. The $^{13}$C-NMR experiment also did not contain a resonance for the ester methyl (~50 ppm) and the carbonyl resonance was present at ~168 ppm. The HMBC experiment, to probe long range $^{13}$C-$^1$H couplings revealed that the carbonyl carbon (C-1) of the aliphatic chain did not show coupling with any of the hydrogens of the disaccharide head group. In the DEPT -135 NMR spectrum of compound $5(c,d)$, resonance for methoxy methyl group was missing but there was no movement in C6” resonance or any other carbon when compared with the starting material(Figure 1.4). These facts suggested the hydrolysis of the methyl ester of the aliphatic chain yielding acidic analog $5(c,d)$. Which was confirmed by the mass spectral analysis of the compounds $5c$ (ESIMS m/z 582 (M+H)) and $5d$, (ESIMS m/z 582 (M+H); calculated M+ 582).
1.5.4 Structure elucidation of macrolactone lipids 5a and 5b

Extensive structural analysis of products isolated from the lipase catalyzed reactions was undertaken using $^1$H, $^{13}$C and 2D-NMR spectra. Detailed NMR analysis was carried out on similar glycolipid like structures by Bisht et al. $^{28}$ and noted limited utility of the $^1$H NMR spectra in establishing the structure and assignments of various signals. $^{13}$C NMR spectrum because of it’s wider range (0-200 ppm) was better suited for assignment of different carbons in these complex glycolipid analogs structures. COSY, $^{13}$C-NMR, DEPT and HMBC (carbon- proton long range correlation) were utilized in assignments of different resonances.
The spectra of the compounds 5a and 5b were compared to their corresponding starting materials, 4a and 4b. In the $^1$H – NMR spectra the resonance for methyl ester protons (~3.5 ppm) was not present in the products 5a and 5b, and also a resonance for the free carboxylic acid (~ 12 ppm) was not observed, suggesting the formation of macrolactone. Though the proton resonances of the sugar skeleton appeared perturbed it was not possible to decipher any useful observation due to overlapping of the various resonances. The carbon 13 NMR has been previously used by Bisht et al$^{28}$ and we decided to utilize it in our case. The carbon spectra was edited using a DEPT pulse sequence and the DEPT-135 of products were compared with starting materials. The wide distribution of the resonance frequencies in the DEPT spectra allowed for assignment of each carbon resonance using COSY, HETCOR and HMBC correlations. Comparison of the DEPT spectra of the product and the respective starting compound allowed for interesting observation to be made (Figure 1.5). For example, in DEPT for compound 5a signal for methyl ester group was missing and downfield shift of 4 ppm was observed for C-6” carbon. The HMBC spectra also showed three bond coupling between the carbonyl carbon (C1, in the side chain) and C-6” hydrogens, confirming the formation of macrolactone between the carbonyl carbon of the side chain and C-6” of the maltose head group (Figure 1.6). These observations unequivocally established the formation of lactone ring at C-6” position and the structure of the compounds were established as 5a and 5b.
Figure 1.5 DEPT-135 showing lipase catalyzed macrolactonization at C6” position.
1.6 Conclusion

In summary we have achieved highly regioselective formation of macrolactone catalyzed by lipase. The selectivity demonstrated by the lipase is unparalleled in organic synthesis and without involving extensive protection/deprotection chemistries. These sophorolipid-type glycolipid analogues have potential applications in the cosmetic industry, formulation, food production, and even utility in technical purpose such as oil pollution abatement of sea water can be envisaged as these biosurfactants can enhance the emulsification of hydrocarbons increasing their availability for microbial degradation. These analogs will be evaluated for their biological and surface properties.
1.7 References


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CHAPTER 2

Ring opening polymerization of ε-Caprolactone in ionic liquid

2.1 Introduction

Today, polymers find applications not only in commodities or structural items but also in biomaterials, i.e. implants, artificial limbs, drug delivery, etc. Polymers have nearly replaced traditional materials such as wood, steel, aluminum, and glass in many applications because of rather simpler process of polymerization and the vast variety of polymers that can be synthesized with entirely different properties. Degradable polyesters, for their biocompatibility, have wide range of uses in medical applications and other environmental applications. The ester linkage is susceptible to hydrolysis and has been shown to degrade both hydrolytically and enzymatically. The degradation products of the biodegradable polyesters are typically hydroxy acids, such as glycolic acid and lactic acid that are generally recognized as being non-toxic. For these reasons there has been a large volume of research into the biodegradable polyester family in the past few decades. One of the most widely used monomer in synthesis of polyesters is ε-caprolactone. Poly (ε-caprolactone), for it’s biodegradability and permeability into various molecules\(^1\), can be used in drug delivery systems. PCL can also be potentially used as herbicide encapsulator for slow delivery of herbicide over a period of time.\(^2\) Biocompatibility, slow degradation and higher strength makes polycaprolactone fibers especially suitable for tissue engineering.\(^3\)
Although polymers are virtue of the modern world, the environmental pollution caused by the methodologies used has raised increasing concerns. The increasing need for sustainable growth and development makes it imperative to find ways to limit production of unwanted side-products, to lower energy requirements and costs, and to design and introduce biodegradable polymers where environmental disposal is required. There has been an exponential increase in interest in area of in vitro enzyme catalyzed organic reactions, since many families of enzymes can be utilized for transformation of not only their natural substrates but a wide range of unnatural compounds, yielding a variety of useful materials. Isolated enzymes are being used as ‘off-the-shelf’ catalysts because of the ease of handling and product isolation. Enzymes offer promising substrate conversion, high selectivity (enantio- and regioselectivity), recyclability, and biocompatibility (non-toxic catalysts). In addition to the aforementioned remarkable features, enzymes are part of a sustainable environment. They come from natural systems and when they are degraded, their constituent amino acids are recycled back into natural substances, which makes them particularly attractive to replace potentially toxic heavy metal catalysts for polymer synthesis.

All naturally occurring polymers are produced in vivo by enzymatic catalysis. In last few years, in vitro synthesis of polymers through enzymatic catalysis (“enzymatic polymerization”) has been extensively developed. Besides being very selective, enzymes are dynamic and sometimes very generous in recognizing variety of substrates in vitro. This property of enzymes allows them to catalyze the synthesis of not only some natural polymers but variety of unnatural polymers too. Thus, the target macromolecules for
Enzymatic polymerization have been polysaccharides\textsuperscript{7}, polyesters, polycarbonates, poly(amide acids)\textsuperscript{8}, vinyl polymers, etc.

Enzymatic polymerization has been receiving increasing attention as a new environmentally friendly method of polymer synthesis, in contrast to the chemical methods, which generally need harsh conditions and metallic catalysts that must be completely removed especially for medical applications. Furthermore, enzymatic polymerization can offer a novel method to produce polymers that are difficult to be synthesized by conventional polymerization.\textsuperscript{9} Among the various polymerization methods, ring-opening polymerization is an important alternative route because leaving groups that can limit monomer conversion or degree of polymerization are not generated during polymerization.\textsuperscript{10} Enzymatic ring-opening polymerization of lactones has been investigated, for lactone of small-size (4-membered)\textsuperscript{11}, medium-size (6- and 7-membered) lactones\textsuperscript{12-14}, and large size (12-, 13-, 15-, 16-membered).\textsuperscript{15-18} It has been observed that enzymatic ring opening of the macrolactones yield much higher rate of polymerization and molecular weight of the polyester formed compared to the polymerization catalyzed by traditional chemical catalysts.\textsuperscript{19}

In spite of all the benefits of enzymatic catalysis their utility in polymerization reaction is greatly limited due insolubility of the monomer and the growing polymer chain in the non polar solvents, such as toluene and hexanes, which are typically employed in such reactions. Although, enzymatic catalysis has been reported in polar nonprotic solvents, e.g., DMF, DMSO, pyridine, etc. but with significant loss of enzyme activity.\textsuperscript{20, 21} To avoid inactivation of the enzyme in polar solvents, polymerizations reaction especially of liquid or low melting monomers have been carried out without
added solvent, i.e., in bulk. However, ever increasing viscosity of the reaction mixture, as a result of the growing polymer chain, has been held responsible for low molecular weight of the resulting polymer.\(^{22}\) There clearly exists a need to evaluate other solvent systems for enzymatic polymerization reaction.

In recent years, room temperature ionic liquids have received increasing attention as green solvents for wide range of reactions.\(^{23}\) Room temperature ionic liquids are organic salts whose ions do not pack well and remain liquid at room temperature.\(^{24}\) Ionic liquids provide advantages with respect to catalyst recovery, product separation, low toxicity, non-flammability, and re-usability. Ionic liquids are able to dissolve a wide variety of relatively insoluble organic and inorganic compounds to very high concentrations. Because of their above stated properties ionic liquids (ILs) have been evaluated as alternative solvents for lipase catalyzed reactions.\(^{25-27}\) They have also been widely employed for synthetic organic reactions.\(^{28-30}\) It has been acknowledged that ionic liquids can be engineered to make them process-compatible by selecting appropriate cation and anion.\(^{31, 32}\) Although a variety of ionic liquids have been synthesized and studied; ionic liquid field continues to be extensively dominated by imidazolium salts with fluorine containing anions (Scheme 2.1).
Scheme 2.1 Synthesis of ionic liquid [bmim][PF₆]

An important research focus of our laboratories continues to be the syntheses of biodegradable polymers through environment friendly methodologies. The enzymatic ring opening polymerization of ε-caprolactone (Scheme 2.2) in ionic liquid [bmim][PF₆] 1 (Scheme 2.1) is an approach to efficiently synthesize high molecular weight biodegradable polymers using bio-friendly procedures, which is yet another step towards ‘green chemistry’. Ionic liquids have polarities similar to polar organic solvents yet they do not inactivate lipases. In fact the polarity of the ILs is expected to enhance the solubility of the monomers and the oligomers, and thus facilitate the enzymatic reaction and lead to possibilities of higher molecular weights. Several lipases are known to have enhanced catalytic activity in 1-butyl-3-methylimidazolium hexafluorophosphate ionic liquid.33 This prompted us to attempt enzyme-catalyzed polyester synthesis in [bmim][PF₆].
2.2 Experimental Section

Lipase Novozyme-435 was kindly provided by Novo Nordisk Bioindustrial, Inc. \( \varepsilon \)-caprolactone monomer was bought from Sigma Aldrich and was distilled before use.

2.2.1 Procedure for polymerization.

The monomer and lipase were dried (in drying pistol over \( \text{P}_2\text{O}_5 \), at 50 °C/0.1 mm of Hg; 15 h) separately in 6 mL reaction vials. In a glove bag maintained under nitrogen atmosphere, enzymes were transferred to the vials containing monomers and vials were capped using rubber septa. Then ionic liquid was added using 500 µL syringe and vials were placed in constant temperature oil bath maintained at 60 °C for predetermined time. Reactions were terminated by adding cold chloroform to the reaction vial and removal of enzyme by filtration (glass fritted filter, medium pore porosity). The filtered enzyme was washed with chloroform and the filtrates were combined. Polymers were precipitated by adding chloroform solution drop wise to stirring methanol placed in ice bath. Polymers were separated by vacuum filtration using Hirch funnel and Millipore filter paper (0.45 µm). The polymers were dried under vacuum to constant weight and dissolved in chloroform (HPLC grade) and chloroform-d for GPC analyses and NMR spectral data, respectively.

2.2.2 Preparation of 1-butyl-3-methyl imidazolium hexafluorophosphate:

A) 1-Butyl-3-methylimidazolium bromide. A 250 ml, three-necked, round-bottom flask was equipped with a heating oil bath, a nitrogen inlet adapter, an internal thermometer adapter, and a reflux condenser. The flask was flushed with nitrogen and charged with 50 ml (0.06 mol) of freshly distilled N-methylimidazole, 50 mL of
acetonitrile and 67 mL (0.06 mol) of 1-bromobutane, and brought to gentle reflux (75-80°C internal temperature). The solution was heated under reflux for 48 h and then cooled to room temperature. The volatile material was removed from resulting yellow solution under reduced pressure. The remaining light yellow oil was re-dissolved in dry acetonitrile (50 mL) and added dropwise to well stirred 200 mL dry ethyl acetate. The imidazolium salt began to crystallize almost immediately. After the addition was complete the flask was put in an ice bath and cooled down to 0°C for 2 h. The supernated solution was removed via filtration and the resulting white solid was dried under reduced pressure for 6 h to yield butyl-3-methylimidazolium bromide in 85% yield.

B) 1-Butyl-3-methylimidazolium hexafluorophosphate. A 200 mL, one-necked, round bottom flask was charged with 10 g (0.046 mol, 1 equiv) of 1-butyl-3-methylimidazolium bromide and 8.9 g (0.0506 mol, 1.1 equiv) of sodium hexafluorophosphate in 16 mL distilled water. The reaction mixture was stirred at room temperature for 2 hr affording a two-phase system. The organic phase was washed with 3 X 10 mL of water and dried under reduced pressure. Then 20 mL of dichloromethane and anhydrous sodium sulfate were added. After 1 hr the suspension was filtered and volatile material was removed under reduced pressure. The resulting ionic liquid a light yellow viscous liquid (80% yield) was the dried under vacuum for 6 hr.

2.2.3 Molecular weight Measurements.

Molecular weights were measured by gel permeation chromatography (GPC) using a Shimadzu HPLC system equipped with a model LC-10ADvp pump, model SIL 10A auto injector, model RID 10A refractive index detector (RI), model SPD-10AV UV-vis detector, and waters HR 4E styragel column. Chloroform (HPLC grade) was used as
eluent at a flow rate of 1.0 ml/min. The sample concentration and injection volumes were 0.5% (w/v) and 50 µL, respectively. EzChrome Elite, Scientific Software Inc., was used to calculate molecular weights based on a calibration curve generated by narrow molecular weight distribution polystyrene standards.

2.3 Results and Discussion

![Scheme 2.2 Synthesis of polycaprolactone in ionic liquid [bmim][PF₆]](image)

Poly ε- caprolactone (PCL) (Scheme 2.2) is one of the most investigated environmentally biodegradable synthetic polymers due to facile accessibility, variable biodegradability and good mechanical properties.³⁴ It has been shown in our lab previously that Candida antarctica lipase (Lipase CA; Novozyme-435®) exhibits promising result with respect to conversion, molecular weight and substrate selectivity in ring opening polymerization.³⁵ Lipase CA is known to have high catalytic activity for transesterification in [bmim][PF₆].³⁶, ³⁷ We investigated lipase catalyzed ring opening polymerization of ε-caprolactone in ionic liquid, [bmim][PF₆].

Different reaction conditions were studied. For example to investigate the effect of the quantity of enzyme used, polymerization was done using different monomer to enzyme ratio. (Table 2.1 and Table 2.2). It was noticed that with increasing amount of
the enzyme the yield was slightly lower than compared to when less enzyme was used and reaction was run for 40h. Also, the molecular weight of the polymer was higher when larger amount of the lipase was used (Table 2.2). To evaluate the performance of enzymes in catalyzing ring opening polymerization in ionic liquid ([bmim][PF$_6$]), polymerization in toluene and in absence of any added solvent was also investigated. Result of the comparative study in toluene, bulk and ionic liquid ([bmim][PF$_6$]) are included in Tables 2.1 and 2.2. It was observed that % yield was slightly higher in ionic liquid than other reaction medium studied (i.e. bulk and toluene) (entries 1 and 2 in Table 2.1 and table 2.2). Polymerization in bulk resulted in lower conversion (entries 3 and 4 in Table 2.1; entry 3 Table 2.2) partially because of the increased viscosity of the reaction medium. Higher yield in ionic liquid in comparison to polymerization in toluene could be attributed to the polar nature of ionic liquid enhancing the solubility of growing polymer chains. Toluene being less polar solubilizes the polymer chains in moderation resulting in slightly lower conversions. The results of reactions carried out at 75 °C for 40 h are summarized in Table 2.1 and Table 2.2.
Table 2.1 Comparison of polymerization in different mediums with monomer:enzyme:solvent ratio being 2:1:2 by weight at 75 °C for 40 h..

<table>
<thead>
<tr>
<th>SN</th>
<th>Monomer</th>
<th>Vol.(Mon.)</th>
<th>Solvent</th>
<th>Volume</th>
<th>Novozyme(g)</th>
<th>Yield(%)</th>
<th>M_n</th>
<th>M_w/M_n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ε-CL</td>
<td>200 µL</td>
<td>[bmim][PF_6]</td>
<td>200 µL</td>
<td>0.104</td>
<td>79</td>
<td>11884</td>
<td>2.3</td>
</tr>
<tr>
<td>2</td>
<td>ε-CL</td>
<td>200 µL</td>
<td>[bmim][PF_6]</td>
<td>200 µL</td>
<td>0.104</td>
<td>81</td>
<td>11548</td>
<td>2.2</td>
</tr>
<tr>
<td>3</td>
<td>ε-CL</td>
<td>200 µL</td>
<td>Bulk</td>
<td>-------</td>
<td>0.104</td>
<td>48</td>
<td>14812</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>ε-CL</td>
<td>200 µL</td>
<td>Bulk</td>
<td>-------</td>
<td>0.104</td>
<td>64</td>
<td>13119</td>
<td>2.3</td>
</tr>
<tr>
<td>5</td>
<td>ε-CL</td>
<td>200 µL</td>
<td>Toluene</td>
<td>200 µL</td>
<td>0.104</td>
<td>75</td>
<td>12749</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>ε-CL</td>
<td>200 µL</td>
<td>Toluene</td>
<td>200 µL</td>
<td>0.104</td>
<td>70</td>
<td>13285</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Table 2.2 Comparison of polymerization in different mediums with monomer:enzyme:solvent ratio being 4:1:4 by weight at 75 °C.

<table>
<thead>
<tr>
<th>SN</th>
<th>Monomer</th>
<th>Vol.(Mon.)</th>
<th>Solvent</th>
<th>Volume</th>
<th>Novozyme(g)</th>
<th>Yield(%)</th>
<th>M_n</th>
<th>M_w/M_n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ε-CL</td>
<td>200 µL</td>
<td>[bmim][PF_6]</td>
<td>200 µL</td>
<td>0.051</td>
<td>60</td>
<td>13219</td>
<td>2.2</td>
</tr>
<tr>
<td>2</td>
<td>ε-CL</td>
<td>200 µL</td>
<td>[bmim][PF_6]</td>
<td>200 µL</td>
<td>0.051</td>
<td>45</td>
<td>12374</td>
<td>2.2</td>
</tr>
<tr>
<td>3</td>
<td>ε-CL</td>
<td>200 µL</td>
<td>Bulk</td>
<td>-------</td>
<td>0.051</td>
<td>40</td>
<td>16793</td>
<td>2.6</td>
</tr>
<tr>
<td>4</td>
<td>ε-CL</td>
<td>200 µL</td>
<td>Toluene</td>
<td>200 µL</td>
<td>0.051</td>
<td>45</td>
<td>14384</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>ε-CL</td>
<td>200 µL</td>
<td>Toluene</td>
<td>200 µL</td>
<td>0.051</td>
<td>50</td>
<td>14678</td>
<td>2.7</td>
</tr>
</tbody>
</table>

The lower yield in table 2.1 and table 2.2 polymerization reactions run at 75 °C were somewhat disappointing. The lower catalytic activity of the lipase, due to denaturation of enzymes at this high temperature could be the culprit and in fact survey of the literature revealed that CA lipase is known to show lower activity at higher temperature.38, 39 Hence these results of higher temperature on yields instigated us to run
the polymerization reactions at lower temperatures and subsequently studies with the lipase were conducted at 60 °C.

The reaction time was another important influencing factor and to study the progress of the polymerization reaction, the monomer conversion and the polymer molecular weight of the polymer were monitored with time (Table 2.3). A time related study on polymerization of ε-CL in ionic liquid was carried out at 60 °C.

<table>
<thead>
<tr>
<th>SN</th>
<th>Monomer</th>
<th>Amount</th>
<th>Solvent</th>
<th>Time</th>
<th>yield (%)</th>
<th>MWn</th>
<th>Mw/ Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>Caprolactone</td>
<td>200 µL</td>
<td>[bmim][PF₆]</td>
<td>40min</td>
<td>41.2%</td>
<td>9615</td>
<td>1.9</td>
</tr>
<tr>
<td>2)</td>
<td>Caprolactone</td>
<td>200 µL</td>
<td>[bmim][PF₆]</td>
<td>1.30h</td>
<td>62.6%</td>
<td>11225</td>
<td>2.2</td>
</tr>
<tr>
<td>3)</td>
<td>Caprolactone</td>
<td>200 µL</td>
<td>[bmim][PF₆]</td>
<td>1.30h</td>
<td>58.35%</td>
<td>12576</td>
<td>2.0</td>
</tr>
<tr>
<td>4)</td>
<td>Caprolactone</td>
<td>200 µL</td>
<td>[bmim][PF₆]</td>
<td>2.30h</td>
<td>61.95%</td>
<td>13308</td>
<td>2.0</td>
</tr>
<tr>
<td>5)</td>
<td>Caprolactone</td>
<td>200 µL</td>
<td>[bmim][PF₆]</td>
<td>2.30h</td>
<td>65.40%</td>
<td>13286</td>
<td>2.0</td>
</tr>
<tr>
<td>6)</td>
<td>Caprolactone</td>
<td>200 µL</td>
<td>[bmim][PF₆]</td>
<td>4.5h</td>
<td>77.15%</td>
<td>11018</td>
<td>2.0</td>
</tr>
<tr>
<td>7)</td>
<td>Caprolactone</td>
<td>200 µL</td>
<td>[bmim][PF₆]</td>
<td>4.5h</td>
<td>81.65%</td>
<td>11018</td>
<td>2.0</td>
</tr>
<tr>
<td>8)</td>
<td>Caprolactone</td>
<td>200 µL</td>
<td>[bmim][PF₆]</td>
<td>25h</td>
<td>78.9%</td>
<td>11300</td>
<td>2.0</td>
</tr>
<tr>
<td>9)</td>
<td>Caprolactone</td>
<td>200 µL</td>
<td>[bmim][PF₆]</td>
<td>25h</td>
<td>77.95%</td>
<td>11171</td>
<td>2.0</td>
</tr>
<tr>
<td>10)</td>
<td>Caprolactone</td>
<td>200 µL</td>
<td>[bmim][PF₆]</td>
<td>96h</td>
<td>91.50%</td>
<td>10614</td>
<td>1.9</td>
</tr>
<tr>
<td>11)</td>
<td>Caprolactone</td>
<td>200 µL</td>
<td>[bmim][PF₆]</td>
<td>96h</td>
<td>91.25%</td>
<td>11191</td>
<td>2.0</td>
</tr>
</tbody>
</table>

It was observed that the monomer conversion increased with increasing reaction time and a linear relationship between reaction time and conversion was established (Chart 2.2).
It is important to point out that in polymerization reactions without added solvent the monomer conversion increases with time reaching a certain maximum (~90%) and does not increase with further increase in the reaction time. It has been attributed to the fact that molecular weight of the polymer increases with increasing conversion leading to higher viscosity, which makes the monomer inaccessible to the active site of the enzyme. In the ionic liquid, however, no such viscosity limitation exists and the linear increase in conversion with time is observed (Chart 2.2). Molecular weight of the polycaprolactone formed increased with increasing conversion and reaction time and was 9615 g/mol within 40 minutes at 41.2 % conversion (entry 1 in Table 2.3). The molecular weight increased further to ~13300 g/mol after 2.3 h at 63 % conversion (Chart 2.1). However, the molecular weight did not show a continuous increase beyond 13300 g/mol even with increasing conversion to 90 %. In fact the molecular weight decreased somewhat to with progress of the polymer reaction leading to molecular weight of ~11000 g. mol at 91 % conversion (Chart 2.1). Decrease in the molecular weight with the progress of the reaction beyond 63 % conversion is somewhat intriguing but not unexpected. Work in our laboratories and others have made similar observation in reaction carried out in bulk and in solvents. It is believed at beyond a certain conversion the monomer availability to the enzyme decreases where as its accessibility to the polymer chain increases. With increasing accessibility to the growing polymer chain the enzyme catalyzed transesterification become more prevalent than polymer forming reaction leading to decrease in molecular weight.
Chart 2.1 Plot of molecular weight as function of time.
The ionic liquids clearly are suited for enzymatic polymerization providing good polymer yield and easy enzyme recovery. The solvent recovery was easy and quantitative. The reaction catalyzed in IL lead to higher polymer yield compared to the reaction performed in bulk conditions. The are similar to observation made in solvents such as toluene and are in agreement with previous reports.\textsuperscript{14,34} In essence it was observed that polymerization in ionic liquid [bmim][PF\textsubscript{6}] was competitive with the polymerization in toluene with better bicompatibility and easier solvent recovery.

\textbf{Chart 2.2} Plot of \% yield as a function of time.
2.4 Conclusion

Novozyme-435 catalyzed polymerization of $\varepsilon$-caprolactone in ionic liquid was found to be highly efficient for polyester production in an environment friendly strategy. By using this system a high % conversion and higher molecular weight polyesters were synthesized. It was concluded from above study that using lower equivalents (4:1, monomer : enzyme) of enzyme resulted in slightly higher molecular weight but with lower yields. Reaction time and higher temperature does not affect enzyme catalysis in ionic liquid as much as it does in organic solvent under same conditions.

With their vast array of applications, synthesis of high molecular weight, biodegradable polyesters in environment friendly medium is an absolute necessity and ionic liquids serve as one of the best reaction medium for effective enzyme catalysis and production of high molecular weight polymers.
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Appendix 1
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