HUMICOLA INSOLENS CUTINASE; A NOVEL CATALYST FOR POLYMER SYNTHESIS REACTIONS

DISSERTATION

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POLYTECHNIC INSTITUTE OF NEW YORK UNIVERSITY

By

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May 2013

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Vita

David Feder was born in New York and moved to Israel after completing high school. After 2 years in the Israel Defense Forces he began his studies in Control Engineering at Tel Aviv University where he remained for two years before returning to the United States to finish his Bachelors degree at Yeshiva University in New York. David earned his M. Sc. from Polytechnic University in Spring 2001 and joined Professor Richard Gross' research group in 2005 as a research assistant where he began to research the cutinase enzyme. He began his PhD studies in 2006 focusing his research on Humincola insolens cutinase and its potential as a biocatalyst for polycondensation reactions.
Dedication

I am dedicating this dissertation to the memory of my father, Rabbi Louis Feder (o.b.m).

My father worked hard all his life to provide for me and to educate me. He taught me the path in which to walk and he inculcated in me a love for learning Torah and pursuing higher education. My father was a role model not only for me, but for our entire community. People young and old would come to ask my father for advice in so many different areas and he would greet and relate to each individual with the utmost sensitivity and respect, giving them his undivided attention - as if at that moment, they were the most important person alive. Members of the community still come over to me and tell me how they attended my father’s Talmud class, and want me to know that he was such a good teacher – explaining each passage and every idea so clearly. It makes me so proud to hear that and I would like to think that my father (o.b.m) would be also be so proud of me with this accomplishment.
Acknowledgment

The years that I’ve spent at the Polytechnic Institute of NYU, studying, researching and working towards my PhD. degree have been wrought with extreme challenges; those that are conventional for any doctoral student of Material Science or Biomedical Engineering - Materials, and those that were quite unexpected and certainly unconventional. Through them all three people stood beside me every step of the way, and I know very well that without any one of them, I would not have been able to get to this point of submitting a dissertation and finishing my degree.

My mom, in a way is quite an ordinary Jewish mother, who managed her household with an iron hand, who took care of her two children as if they were more precious to her than anything else in her world, who together with my father (o.b.m) guided us and instilled in us the traditional value system of honesty, integrity and respect that has been in our family for generations and that please Gd I will transmit to my future children as well. My mom however is much more than that. She is a professional psychotherapist who has taught at a graduate level and who has a private practice to this day. She is there for me always, not only as I was growing up, but also in my adult life. She has been the crutch that has allowed me to get past every hard time and to get up on my feet every time that life has knocked me down. Throughout my PhD study, and throughout every chapter of my life, she has provided both financial support when needed and invaluable emotional support and advice. Mom, I know that I could never have accomplished this milestone (or any other) without your being there every step of the way.
When I came to see Professor Rich Gross for the first time, to enquire about the Biomedical Engineering program, I knew that I had met a very unique individual. Warm, soft spoken and modest despite immense scientific accomplishments, Professor Gross gained an admirer on that day, and I’ve been looking up to him as my mentor and adviser ever since. He is truly a mentor in every sense of the word, as he guided me with so much caring in a way that always made me feel important. In our weekly meetings when we analyzed my new data I always left with important and helpful advice as how to make my research proceed in a better and more efficient way. With most any problem that I would encounter, Professor Gross would guide me methodically in a way that I would end up solving the problem myself. During my illness, Professor Gross never gave up on me, but rather gently motivated me to continue, always assuring me that he was there to support me every step of the way. This dissertation, and all the research presented here would never have been possible without his professional mentorship and warm guidance.

I met YuFei during the course of my study for my PhD degree. It didn’t take long until we both realized that we were team – that we were meant to be together for life. During the course of our difficult long distance relationship our love only grew stronger each day. After she immigrated to the United States, like the biblical Ruth, she gave up her culture, language, religion, country and all she had and embraced a new lifestyle and a new religion in a country so foreign to her. Just as Ruth said to Naomi “Where you go I will go, where you live I will live, your people are my people, your god is my god, where you die I will die and there I will be buried…” so too my sweet YuFei said to me. During my illness YuFei was by my side every day, often sleeping in the hospital so that she could
spend more time with me. We are now both starting out, trying to find our way in this big and often scary world. I know that together we will succeed. I know that together we will make our dreams come true. YuFei, all that seemed impossible to me just few years ago is suddenly possible. You have given me strength and hope beyond my imagination. I hope with all my heart that I can be the best husband possible and give to all the love, support and happiness that you deserve.

I would also like to acknowledge all the members of our research group – my lab family – who provided much support during the years of my work in Professor Gross’ group. I would like to single out two people who were especially supportive to me:

Dr. Harold Mang - my first “supervisor” when I started working in the group. Harold took me under his wing and taught me so much with so much patience and caring. I couldn’t imagine a better teacher. He made the lab a second home for me; a place where I longed to be when I was elsewhere. The love that I have for my profession is largely due to the excellent training and the warm friendship that I received from Dr. Mang. Harold, although we have been out of touch for quite a few years, I think about you often and wish you only the best in life. You have given me so much and I will never forget it.

Dr. Kodandaraman Viswanathan, my colleague, my friend. Raman, you were my rock. You were always there for me - whenever I needed to talk, whenever I was upset, whenever I needed a friend it was you that I went to. If I had problems with a reaction at 10pm (or even later), it was you that I ran to for advice. How can I ever forget the hours that we spend talking on the roof during the cold winter months? For all your professional guidance and for your close friendship I am forever grateful.
ABSTRACT

**HUMICOLA INSOLENS CUTINASE; A NOVEL CATALYST FOR POLYMER SYNTHESIS REACTIONS**

by

David Feder

Adviser: Prof. Richard Gross

Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (Biomedical engineering)

May 2013

The cutinase class of enzymes, specifically *Humicola insolens* cutinase (HiC), was methodically studied for its use as a potential biocatalyst for various polymer synthesis reactions; as a substitute or possibly a better alternative to other commercially available biocatalysts.

Biocatalysis is an emerging field of research that has gained prominence in the past decade with greater emphasis on development of environmentally friendly processes and/or resources for development of materials with unique physical and chemical properties. This work focused on the development and optimization of immobilized HiC as it would allow for the optimal use of this enzyme in organic media, at substantially higher reaction temperatures and to facilitate removal of enzyme from the product at the termination of the reaction so that it may be re-used over multiple reaction cycles. This involved understanding how enzyme activity is affected by different methods of enzyme immobilization such as chemical verse physical immobilization; effects of the immobilization support such as porosity, surface chemistry and bead size; and the efficiency of enzyme loading onto immobilization supports. Furthermore, work was
carried out to determine effects of various organic solvents on immobilized HiC enzyme activity, optimal reaction temperature and catalyst concentration. Working at the highest possible reaction temperature where the enzyme remains active is crucial in order to limit diffusion constraints in reactions of polymer synthesis. In the first phase of this work, an HiC immobilized on Amberzyme oxirane resin (HiC-AO) system is optimized for use in polyester condensation reactions. In the second phase, HiC-AO was used to catalyze polycondensation reactions. The emphasis of this work was to gain an understanding of enzyme selectivity for different chain length diacid, diol and ω-hydroxyacid substrates. These reactions were also performed in parallel with CALB (N435) catalysis, and the results of the HiC-AO and CALB catalyzed reactions are compared. Moreover, a similar series of small molecule esterification experiments were carried out with both acids and alcohols of various chain lengths using both N435 (CALB) and HiC-AO. These products were analyzed and conclusions were drawn as to the chain length selectivity of the two enzymes in small molecule esterification reactions. Furthermore, comparisons were drawn between similar and dissimilar trends found in the two series of experiment.

In the third phase of this work, the HiC-AO was used to catalyze the synthesis of various polyol polyesters. Reactions were performed by changing the molar ratio of the alditol added to each reaction. Many of these reactions were repeated with N435 catalysis and enzyme activities as well as in-depth structural analysis of the products are compared. This work also includes an extensive thermostability study of 4 different wild type cutinase enzymes and preliminary data comparing the chain length selectivity of these enzymes in small molecule esterification reactions between octanol and acids of varying chain lengths.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AbC</td>
<td><em>Alternaria brassicicola</em> cutinase</td>
</tr>
<tr>
<td>AfC</td>
<td><em>Aspergillus fumigatus</em> cutinase</td>
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<tr>
<td>AoC</td>
<td><em>Aspergillus oryzae</em> cutinase</td>
</tr>
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<td>CALB</td>
<td><em>Candida Antarctica</em> lipase B</td>
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<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CL</td>
<td>caprolactone</td>
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<td>CLE</td>
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<td>crosslinked enzyme aggregates</td>
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<td>crosslinked enzyme crystals</td>
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<td><em>Candida rugosa</em> lipase</td>
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<td>DO</td>
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<td>DSC</td>
<td>differential scanning calorimetry</td>
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<td>GC-MAD</td>
<td>Gas chromatography–mass spectrometry</td>
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<tr>
<td>GPC</td>
<td>gel permeation chromatography</td>
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<tr>
<td>HiC</td>
<td><em>Humicola insolens</em> cutinase</td>
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<td>HiC-AO</td>
<td><em>Humicola Insolens</em> cutinase immobilized on Amberzyme Oxyrane resin</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<td>Polyvinyl acetate</td>
</tr>
<tr>
<td>ROP</td>
<td>ring opening polymerization</td>
</tr>
<tr>
<td>S-DVB</td>
<td>styrene di-vinyl benzyne</td>
</tr>
<tr>
<td>SEC-MALLS</td>
<td>size exclusion chromatography - multiangle laser light scattering</td>
</tr>
<tr>
<td>VOH</td>
<td>vinyl alcohol</td>
</tr>
<tr>
<td>ω-HA</td>
<td>omega hydroxy acid</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction

The text in this chapter has been written collaboratively by myself and Professor R. Gross.
1.1 Biocatalysis

As biocatalysis continues to mature and evolve, researchers seek to gain a better understanding of the potential range of reactions for which enzymes can be useful. Some examples of the known benefits of biocatalysis are the following: The enzyme catalysts, which are derived from renewable resources, are much more environmentally friendly than the harsh metal catalysts used. Reactions such as polycondensation reactions that are catalyzed with biological catalysts are performed at much lower temperatures than with their chemical counterparts and these reactions can be performed either in bulk, or in organic media. In addition, full advantage could be taken of the chemo, regio and stereo selectivity of different enzyme catalysts. Immobilized enzymes, unlike their chemical counterpart offer catalyst recyclability.\(^1\)\(^2\) In chemical catalysis of polyesters, organo-metallic catalysts are used, however, because of their toxicity these catalysts and their residues must be completely removed from the final product for use in any biomedical application which is practically impossible.\(^2\) Another drawback to the use of chemical catalysis in polycondensation reactions is concerning the high temperatures and acidic conditions that are often required to catalyze these reactions and the reduced pressure required to remove water which is often the byproduct of the reaction. At the high temperatures used in these polymerizations, side reactions such as dehydration of diols may take place thereby causing an unbalance in the stoichiometry of the reaction. Also, the strong acidic catalysts may cause discoloration of the polymer.\(^2\)

There are however drawbacks to the use of biocatalysts that don’t exist with conventional catalysis. As is mentioned by Bommarius et al,\(^3\) “…conformational changes
of less than an Angstrom can cause a precipitous decline in activity…” Hence, enzymes tend to be highly sensitive to variations in reaction conditions that can render them inactive. Examples of this could be 1) substrate – each biocatalyst is active with only a specific set of substrates. 2) solvent – an organic solvent which is not compatible with a specific biocatalyst/substrate set can substantially lower the catalytic activity or even cause the enzyme to be inactive. 3) Temperature – reactions incorporating substrates of high viscosity or solids of high melting points often require high reaction temperatures which will cause the denaturation of the enzyme catalyst and again render it inactive. An additional disadvantage in biocatalysis at the present time is the actual cost of the biocatalyst as opposed to a conventional chemical catalyst. This can make the implementation of a biocatalyst in an industrial process financially prohibitive.

1.2 Condensation Polymerization

Most polymerizations are carried out by one of two mechanisms, condensation polymerization also called step-polymerization or addition polymerization also called radical polymerization. The polymerization reactions carried out in this work have all been polycondensation reactions. Condensation polymerizations were first discussed by Wallace Carothers in his monumental work published in JACS in 1929. In this paper, Carothers postulates there exists two types or classes of polymers that are polymerized. The first is that of addition polymers; where the molecular formula of the monomer is identical with that of the structural unit and the polymer can be synthesized from the
monomer by self addition. The second class of polymers is that of condensation polymers; where the molecular formula of the monomer differs from that of the structural unit. The polymers of this class can be synthesized from the monomers by intermolecular condensation. Carothers defines condensation polymerization as the chemical union of many similar molecules with the elimination of simpler molecules (often H₂O). Carothers states that before his postulation about condensation polymerization, scientists believed that only small molecules could be formed by condensation reactions but when larger molecules are formed from these small molecules it could only be the result of addition polymerization which as Carothers mentions was the only form of polymerization which appears to have been generally recognized.

In condensation polymerizations, products that may be prepared are polyesters, polyamides, polyethers, etc. depending on the type of reaction involved (for example, esterification, amidation, etc.). In all condensation experiments presented here, the reaction is always esterification, as the COOH and OH react to form an ester bond causing a H₂O molecule to be released as the byproduct. There are two classes of condensation polymers that are presented in this work, linear polymers, which are the product of bifunctional reactants (such as ω-hydroxyacids [ω-HA’s] and diol/diacid) and branched polymers which are the product of reactants of a greater number of functional groups – such as the polyols incorporating glycerol and sorbitol. An important distinction between the mechanisms of addition and condensation polymerization as discussed by Paul Flory in his work on condensation polymerizations is that, addition polymerization is in effect a chain reaction.⁶
One molecule of monomer attaches to another, which attaches to the next molecule and so on. That is, addition polymerization is the addition of an individual molecule of monomer to an active polymer chain. At any given point in time, the reaction mixture will be comprised of higher polymer - which is found even at the beginning of the reaction - and unreacted monomer. There is almost no middle ground. However, condensation polymerization will begin with an intermolecular reaction between two molecules to form a dimer. That dimer might in turn react with either another molecule of monomer to form a trimer, or possibly it might react with another dimer to form a tetramer. These will in turn condense and can react with other species (i.e. monomer, dimer, trimer, etc.) causing the molecular weight to increase. At any given point in time there is mixture of monomer and polymer of differing chain lengths. As such, a condensation polymerization is accomplished by a series of independent condensation reactions which occur throughout the polymerization reaction. As the polymerization proceeds with time, the average chain size will increase exponentially and the reaction mixture will therefore become more viscous. Studies in polyester condensation reactions have shown that these reactions are catalyzed by hydrogen ions and would proceed uncatalyzed in the absence of an added catalyst. In that case, the second molecule of the carboxylic acid undergoing esterification, functions as the catalyst. However, uncatalyzed polyester condensation reactions are exceedingly slow at reaching high degrees of esterification. The use of catalysts, therefore, is extremely important as it accelerates the rate of polyesterification. Actually, the use of a viable catalyst is the only way to increase reaction rate. Lowering the pressure in the reaction to increase removal of the byproduct
of condensation polymerizations (e.g. water, methanol, etc.) is an important parameter to adjust to further accelerate the reaction rate.

1.3 CALB as a biocatalyst

In the field of polymer synthesis, Lipases, specifically, Lipase B from *Candida Antarctica* (known as CALB) has been the enzyme of choice.² This enzyme, a lipase isolated from the basidiomycetous yeast, has a vast number of desirable properties. It has very good thermostability in organic media when immobilized and is characterized by its broad substrate specificity.⁷ Also, CALB does not display any interfacial activations or conformational changes where the lid covering its active site must open for catalysis to take place - as with most other lipase enzymes which posses such a lid or flap that cover the catalytic triad.⁷ CALB has been genetically engineered to render it even more thermostable and to further improve its biological activity.⁸ A genetically engineered CALB immobilized on Lewatit VP OC 1600 PMMA beads is commercially available from Novozymes under the trade name Novozym435 or N435. There is a vast amount of work already reported with N435 and many patents already filed using N435 to catalyze polymer synthesis reactions of polyesters, polyethers, polycarbonates, polyamides and others.
1.4 Examples of N435 catalyzed polymer synthesis reactions

One example of an N435 catalyzed polymer synthesis reactions is the copolymerization of \( \omega \)-pentadecalactone (PDL) with \( p \)-dioxanone (DO); a novel and highly functional material with great potential importance in many biomedical applications.\(^9\) Other poly(\( DO \)), PDO, co-polymers have been previously synthesized in order to control and regulate the rate of biodegradation and functional properties of DO-based materials to be used for the preparation of surgical staples and sutures as well as other biomedical applications. Examples of DO containing copolymers are poly(\( DO-co\)-caprolactone), poly(\( DO-co\)-glycolide) and poly(\( DO-co\)-lactide); however, although these small ring size lactone monomers are somewhat successful in regulating the rate of biodegradation of PDO, in order to more dramatically enhance the lifetime of DO-containing polymers an attractive option is copolymerization with larger size lactones such as PDL.\(^{10}\) Using N435 catalysis, P(\( DO-co\)-PDL) has been successfully synthesized which was not possible by traditional chemical catalysis. In addition, upon hydrolysis and
degradation, PDL units would be converted to $\omega$-hydroxypentadecanoic acid which is a typical hydroxy-substituted fatty acid that is expected to be highly biocompatible and bioresorbable. \footnote{N435 was used to catalyze the synthesis of aliphatic polyesteramides (PEAs) containing siloxane units.}

Polyamides (ex. nylons) have superior mechanical properties to polyesters and they can also be easily modified. Furthermore, since the amide groups along polyamide chains are highly polar and can easily form hydrogen bonds, these polyamide segments will allow for the formation of a copolymer which has good thermal and mechanical properties even at low molecular weight. \footnote{Preparing PEAs with both ester and amide units on the polymer backbone has the potential to provide unique biocompatible and biodegradable polymers with superior mechanical properties which can be used for many biomedical applications. Preparing PEAs with silicone based repeat units can further enhance polymer properties. Silicones are extremely thermostable, posses a very low glass transition temperature (less then -120°C) and are flame retardant. One polysiloxane, polydimethylsiloxane (PDMS) has been extensively studied and is known to be biocompatible and to have good physical properties including low-surface tension/energy, extreme hydrophobicity, high-chain flexibility, good oxidative, thermal and UV stability. It also exhibits low chemical reactivity and high gas permeability. However, due to poor mechanical properties and cold flow even at very high-molecular weights, pure PDMS cannot be used directly to make medical devices. By blending PDMS block copolymers with other polymeric materials, the air-polymer surfaces of resulting systems are dominated by the low surface energy PDMS. Hence, even at low levels of PDMS, the resultant blends display}
completely silicone-like surface properties. However, by preparing PEAs with silicone repeat units, similar affects can be achieved without the problem of having pure PDMS remain on surfaces. Using N435 catalysis, our lab successfully copolymerized α,ω-(Diaminopropyl)polydimethylsiloxane, Diethyl Adipate, and 1,8-Octanediol under mild conditions with high enantio- and regioselectivity as opposed to the harsh conditions of chemical catalysis which can result in uncontrolled redistribution and unwanted side reactions. Also, high polymerization temperatures and acid/base catalysts cause decomposition of useful functional groups in siloxane monomers.

N435 catalysis, while having extraordinarily broad substrate specificity, is by no means universal. Examples of poor substrates for N435 catalysis of esterification or transesterification reactions include aromatic acids, maleic/fumaric acids, C2/C3 building blocks, secondary alcohols of [S]-stereochemical configuration and small lactones.

1.5 Cutinase in Nature

Most epidermal cells, representing the outermost layer of all higher plant surfaces, (e.g. leaves, petals, fruits and herbaceous stems) are covered by a continuous extra-cellular membrane of soluble and polymerized lipids called the cuticle. The cuticle is comprised of cutin – an insoluble lipid-polyester composed of hydroxy and epoxy fatty acids such as 16-hydroxyhexadecanoic acid, 10,16-dihydroxyhexadecanoic acid, and 9,10,18-trihydroxyoctadecanoic acid. In addition, the cuticle is comprised of integrated and superimposed waxes. The most important roles of the cuticle in plants are its function as a transpiration barrier and to regulate the plant’s water loss. The cuticle is
also crucial in protecting against mechanical, UV irradiation damage, and herbivore and pathogen attack. It serves as a barrier against the entry to the leaf of those pathogens which feed on plant leaves.\textsuperscript{18,19,24} Although both parts of the cuticle are instrumental in achieving the above functions, recent studies have shown that the wax portion of the cuticle is in fact, the prime transport limiting barrier, not cutin.\textsuperscript{19} Certain pathogens (fungi and bacteria) are able to secrete an enzyme - cutinase - which degrades cutin by hydrolyzing ester bonds which predominate in cutin, resulting in the release of cutin monomers. Scientists now know that the secretion of the cutinase is not the sole way by which phytopathogens penetrate the plant leaf. Fungi generally secrete a cocktail of hydrolytic enzymes, including cutinases, cellulases, pectinases, and proteases. Although the lack of cutinase can possibly be compensated by other enzymes in this cocktail along with other mechanisms for penetration into the leaf, cutinase is certainly a key player in the phytopathogen’s attack on the plant leaf.\textsuperscript{25} In one example, pea stem segments with an intact cuticle, when treated with cutinase enzyme inhibitors, were not penetrated by a Fusarium \textit{solani} pisi pathogen that was introduced to the sample. However, when a break was manually made in the cuticle the fungal pathogen was able to infect the plant segment.\textsuperscript{25}
Figure 2. A diagram of the cuticle of a mature plant showing the different components of the cuticle.

1.6 Cutinase Structure

Cutinase is a 214 residue protein in a compact one domain molecule that is 45x30x30 Å³ in size. Cutinases have molecular weights around 20KDa with highly conserved stretches, which include four cysteines, forming two disulfide bridges. X-ray crystallography studies of *F. solani pisi* cutinase (FsC) revealed that it belongs to the α/β hydrolase fold superfamily. FsC is one of the smallest members of the serine hydrolase family and contains a Ser-His-Asp catalytic triad. Interestingly, cutinases hydrolyze not only cutin, but also insoluble triglycerides and soluble esters such as *p*-nitrophenyl butyrate (*p*NPB). Thus, cutinases are considered as being intermediate between lipases and esterases. Only five beta strands (numbered 3 through 7) and four helices (labeled A, B, C and F) constitute the folded protein containing the active site triad Ser120, His188 and Asp175. Furthermore, the protein backbone at the active site Ser120
is highly rigid, while the other two active site residues, Asp175 and His188, are located in more flexible parts of the enzyme.\textsuperscript{28}

In all serine hydrolases, the active site serine is invariably located at the C-terminal end of one of the beta strands (number 5 in Figure 1) in an extremely sharp turn towards the next helix (in cutinase helix C), the so-called nucleophile elbow.\textsuperscript{23,28}

\textbf{Figure 3.} A diagram of the canonical/hydrolase fold of cutinase. The active site triad (Ser120, Asp175 and His188) is indicated together with five beta strands (black arrows labeled 3 to 7) and four helices (labeled A, B, C and F). Numbers at the right side of secondary structure elements point to start and finish of these elements.\textsuperscript{28}
The activity of most lipase enzymes is greatly increased at the lipid-water interface due to the presence of a lid that usually covers the catalytic triad. At the lipid-water interface, a conformational change occurs which causes the lid to open and expose the active site. Cutinases are different than lipases in that they don’t exhibit interfacial activation as there is no lid that blocks the active site.\textsuperscript{30} For cutinases, the active site is opened and exposed to the substrate and solvent as is illustrated in the ribbon diagram of \textit{Fusarium solani} cutinase (FsC) in Figure 2. Only a small helical flap comprising residues 81–85 and a binding loop involving residues 178–186 partially block the entrance of substrate molecules to the active site. As a consequence, the binding of cutinase at the interface doesn’t require the conformational change that lipases undergo, but, instead, a minor reorientation of a few lipophilic side chains.\textsuperscript{27,28}
In an interesting observation by Melo et al. is there are certain cutinases whose activation site opens and closes and possesses what Melo calls a “mini-lid”, indicating that cutinases might be more lipases than an esterases. Furthermore, this movement of the active site covering may explain the selectivity of the enzyme for different substrates. Another difference between cutinases and other lipases is that cutinases are active on both soluble and emulsified triglycerides while lipases are active only on emulsified triglycerides where there is a lipid-water interface.

The surface hydrophobicity characteristics and lipolytic activity of cutinases along with its stability in certain organic solvents have been exploited in industrial
applications of cutinases such as the food industry, cosmetics, laundry detergents and polymer chemistry.

**Figure 5.** 3D backbone fold of *Fusarium solanii* cutinase (FsC) showing accessibility of the active site region. The dynamics of the backbone is also indicated. Red, mobile; blue, rigid.²⁸
1.7 Synthetic Mechanism of Serine Hydrolase enzymes

The mechanism of cutinase catalyzed diol/diacid polyesterification (or homopolymerization of a bi-functional reagent) is quite similar to that of cutinase catalyzed hydrolysis. The arrangement of the residues in the catalytic triad, Asp, His, and Ser, causes a decrease in the pKa value of the serine hydroxy group. This enables it to perform a nucleophilic attack on the carbonyl group of the substrate which covalently bonds the acyl moiety of the substrate to the enzyme, forming an acyl enzyme intermediate with release of H₂O or another leaving group such as an alcohol (e.g. methanol or ethanol). In the second step, the alcohol, acting as the nucleophile, attacks the ‘acyl enzyme intermediate’ regenerating the enzyme and, in turn, causes the formation on an ester bond.

Figure 6. The Serine Hydrolase Mechanism³⁴
1.8 Previously reported work on cutinase enzymes

The emphasis of previous work by our laboratory with regards to cutinase-catalysis has primarily been on its potential for polyester degradation which will be discussed below.\textsuperscript{35,36} The work described in this dissertation was aimed at expanding the potential use of immobilized HiC for carrying out varying polyester synthetic reactions. The hope was that HiC could provide catalytic activities that would be unique and in some cases better than that observed using N435. After chemical immobilization of \textit{Humicola insolens} (HiC) on Amberzyme oxirane (AO) beads, this immobilized biocatalyst was used to catalyze various polymer synthetic transformations and small molecule model reactions. Earlier work that built the current foundation on the activity of cutinases for small molecule and polymer biotransformations are described below.

1.8.1 Triglyceride synthesis

In 1994, Melo and Ivanova\textsuperscript{37} reported the use of FsC to catalyze the reaction of glycerol and oleic acid to synthesize oleoyl glycerides of all three substitutions (monoolein, diolein, and triolein). The monolayer technique was used where the water subphase was replaced by glycerol, and a film of oleic acid was initially spread on the glycerol surface. A conversion of 50% acid to ester was obtained at pressure of 15.4 mN m\textsuperscript{-1} and low water content, while at higher surface pressures, lower degrees of conversion were observed. A preference of substitution was controlled by the surface pressure applied to the monolayer film with a higher amount at monoolein synthesized at higher surface pressure (24.5 mN m\textsuperscript{-1}), while at a lower surface pressure of 15.4 mN m\textsuperscript{-1} only triolein was detected.
1.8.2 Esterification and Transesterification

An early study of the synthetic activity of FsC was performed by Sebastiao, Cabral and Aires-Barros\textsuperscript{38} in 1992. They used a system by which a recombinant FsC was solubilized in reverse micelles where the enzyme is located inside water droplets. Reverse micelles containing cutinases are soluble in organic media and can be explored for reverse equilibrium chemistry. A study of chain length selectivity of their FsC system was conducted using small molecule esterification reactions between oleic acid and alkanols of varying chain lengths. There was an absence of enzyme activity in their study for butanol, while highest enzyme activity was seen for hexanol. When the alcohol chain length was increased from 6 to 8 and then to 10 carbons, there was a sharp decrease in activity and only a slight increase in activity as the alcohol chain length was then increased to 12 carbons. At this point the activity of the enzyme stabilized and was unchanged as the alkanol chain length was increased to 18 carbons. Furthermore, using esterification reactions between oleic acid and hexanol they found that for their system of reverse micelles containing FsC, the optimal temperature and pH is 30$^\circ$C and 10 respectively. Finally, using the parameters that they had optimized, they explored the effect of carbon chain length of the fatty acid in the esterification reaction, and found that the highest activity of FsC in reverse micelles was seen with butyric acid (C4) and a sharp decline in activity was seen as the acid chain length was increased to C8. The activity was fairly stable as the carbon chain length was increased from C8 to C18 with only a slight decrease in activity noted as the chain length was changed from C12 to C14.
Sereti et al.\textsuperscript{39} studied that activity of FsC for esterification reactions such as hexanol and hexanoic acid in supercritical CO\textsubscript{2}. They used a system of FsC adsorbed on Accurel EP100 macroporous polypropylene beads (enzyme loading 0.5\% w/w). In a reaction carried out at 45\textdegree C (0.25\% protein w/w) the hexanol hexanoate concentration increased linearly with time, reaching an almost constant value after 72 h of incubation time. No ester was formed in the absence of enzyme.

The effect of enzyme concentration was also studied and the rate of conversion to hexanol hexanoate was shown to increase linearly by increasing the amount of enzyme in the reaction system. The recyclability of immobilized FsC was also examined and even after three cycles the enzyme could catalyze the esterification reaction with the same efficiency.

Sarazin et al.\textsuperscript{40} studied FsC catalyzed esterification of octanoic acid and 1-butanol to give butyl octanoate. Reactions were performed directly in an NMR tube to allow on-line monitoring of the product formation without the need to withdraw samples. \textsuperscript{1}H and \textsuperscript{13}C spectra are seen throughout the course of the reaction, and the process of esterification is followed as resonances due to H\textsubscript{2}O and ester peaks increases in intensity and, likewise, resonances associated with the free alcohol decrease in intensity. It was shown by means of \textsuperscript{1}H NMR that the water produced by the esterification reaction is retained by the enzyme in the early phases of the reaction, however a distinct water phase is formed as the reaction proceeds. Furthermore, analysis by \textsuperscript{13}C NMR has shown the presence of two ester forms; an acid-ester complex and an ester proportion which is not associated with the acid and not accessible to the substrates, confirming that the ester produced in the reaction is not fully recovered in the organic phase.
The FsC-catalyzed transesterification (alcoholysis) reaction between hexanol and butyl acetate in isooctane was studied by Serralha and Lopez. The reaction was carried out at 30°C, with a 5:4 molar ratio of butyl acetate to hexanol. As opposed to the preceding studies where the enzyme was in its free form or solubilized in reverse micelles, here FsC was immobilized on zeolites; an inorganic solid support “with a very precise micro-porous structure”. The average loading of the enzyme on the zeolite supports was 1.5% w/w. Many different zeolites were explored and comparisons were made based on the cationic properties, the ability of the support to supply water to the enzyme, along with the amount of water needed for optimal catalysis and water activity. Supports with cations having a higher charge density can bind water more tightly and therefore will produce smaller $a_w$ for the same water content. It was not surprising that the highest enzymatic activity was found in reactions with the optimal water content which is a compromise between the necessary hydration of the enzyme while attempting to prevent hydrolytic activity.

Experiments have determined that aside from the cationic properties of the support, the silica:alumina (Si:Al) ratio has a clear influence on the catalytic activity of the enzyme with the sodium form of Y zeolite, NaY, showing the optimum Si:Al ratio of their structures. In a comparison of several different zeolites, NaY was shown to have the highest specific activity as a function of water content and to be the most promising zeolite support.

De Barros and co-workers explored the stability of the FsC enzyme using a model esterification reaction of hexanoic acid and ethanol. In their study they also
explored the effect on the stability of the enzyme with increased concentration of both acid and alcohol.

To test the stability of FsC in iso-octane, the enzyme was incubated in iso-octane for different periods of time prior to the start of the reaction. The reaction was started by the addition of a 2:1 ratio of hexanoic acid to ethanol. The reactions were repeated for two different enzyme concentrations (1.4 mg/mL and 2.3 mg/mL) and the conversion to ester was monitored for all reactions. Results of this study showed that the higher enzyme concentration did not provide a significant increase in conversion to ester. There was slightly higher yield in ester with the higher enzyme concentration for short incubation periods, but after 6h incubation the difference in yield disappears.

The effect of ethanol on FsC stability was assessed by a series of 24h esterification reactions after incubations of different time periods of FsC with various concentrations of ethanol. Here the molar concentration of hexanoic acid is held constant at 0.1mol/L. The results of this experiment showed that although the incubation time is of little consequence in relation to FsC specific activity, a change in the molar concentration of ethanol from 0.2mol/L to 0.4mol/L causes a substantial decrease in the yield of ethyl hexanoate from approximately 80% to below 40%. This decrease in enzyme activity has been explained to be due to the ethanol stripping off tightly bound water from the cutinase molecule which causes a structural change to the enzyme. A similar study was performed to assess the effect of hexanoic acid on FsC stability. FsC was incubated for various time periods together with different concentrations of hexanoic acid ranging from 0.1mol/L to 0.8mol/L. The 24h reactions are started with the introduction of 0.1 mol/L ethanol. The result of this experiment show that aside from a slight temporary decrease at
an incubation time of 1h the yield of ethyl caproate remains constant for all incubations 
times. In this experiment a molar concentration of 0.1 mol/L caproic acid (i.e. a 1:1 ratio 
of acid: alcohol) was optimal. This is possibly due to the affinity of FsC for C4 to C6 
chain length.

1.8.3 Hydrolysis

Considering the fact that the role of the cutinase enzymes in nature is hydrolysis 
of the ester bonds of cutin within the plant cuticle, therefore a logical use of the enzyme 
in-vitro is likewise to use this enzyme for polymer degradation. Murphy et al.\textsuperscript{22} 
demonstrated that poly(\(\varepsilon\)-caprolactone), PCL, a synthetic polyester formed by the ring-
opening polymerization (ROP) of \(\varepsilon\)-caprolactone (CL), was hydrolyzed to water-soluble 
products by FsC. In this work they found that PCL dimers and trimers, which are the 
byproducts of enzymatic degradation, are structurally similar to cutin degradation 
byproducts. Examples of cutin degradation by-product include C16 and C18 \(\omega\)-
hydroxyacids, which are also inducers of cutinase enzyme production by the 
corresponding microbial plant pathogenic species.\textsuperscript{22} This hydrolysis activity was found 
for FsC wild type enzyme and not for other cutinase negative mutant strains of \textit{Fusarium 
solani} that were studied in parallel.\textsuperscript{22} Research in this area is extremely important due to 
the pressing need for increased research in the area of biodegradable plastics.

Ronkvist and co-workers\textsuperscript{36} conducted a study in which they compared the 
hydrolytic activity of three different cutinase strains, HiC, FsC and Pseudomonas 
mendocina cutinase (PmC), using PVAc as the model substrate. The PVAc was coated 
within a macroporous S-DVB bead and the access of cutinase to the PVAc was assessed
by the distribution of OH groups on the PVAc covered surfaces both before and after incubation with cutinases as shown by IR microspectroscopy. The result of this study shows that both HiC and PmC exposed samples have a higher content of OH groups than the control sample, while the FsC exposed sample shows similar OH content to the control sample which might be due to FsC deactivation during the long incubation period. The deacetylation of PVAc was assayed using a pH-stat to measure NaOH consumption versus time. HiC was seen to be the most thermostable, with a peak activity at 70°C, followed by PmC and FsC (peak activity at 50°C and 40°C respectively). The same trend was found to be true for pH stability and for extents of PVAc deacetylation with HiC showing highest activity and FsC showing lowest hydrolytic activity. As HiC deacetylates the PVAc on S-DVB beads, it forms solubilized P(VAc-co-VOH) as well as continues in the hydrolysis of the VAc units of water-soluble P(VAc-co-VOH) which is already in the media. This research is significant in surface modifications as enzymatic deacetylation can be used to increase the hydrophilicity of polymeric surfaces that contain vinyl acetate units while leaving the bulk properties of the polymer unchanged.

Moreover, in a further study by Ronkvist et. al. the cutinase catalyzed hydrolysis of low crystalinity Poly(ethylene terephthalate) (PET) thin films was explored and once again a comparison was made between the hydrolytic activity of three cutinase strains, HiC, FsC and PmC. Cutinase activity was assessed using a pH-stat to measure NaOH consumption versus time. In this study as well, HiC was seen to have to highest hydrolytic activity of the three cutinases studied and resulted in a 97% weight loss in 96 hours at 70°C as opposed to a 5% weight loss with PmC and FsC at 50°C and 40°C respectively. An explanation of the significantly higher activity of HiC as compared with
the PmC and FsC is the ability of HiC to retain activity at 70°C giving HiC increased accessibility to PET ester groups.

Baker et al\textsuperscript{43} compared the residual activity and pH stability of 5 different cutinase enzymes. FsC, HiC, Alternaria brassicicola cutinase (AbC), Aspergillus fumigates cutinase (AfC), Aspergillus oryzae cutinase (AoC)

Residual activity was compared using pNPB hydrolysis as the model reaction. The enzymes were heated to various temperatures from 25 °C to 90 °C and then allowed to cool before the reactions were performed in parallel. It is clear from this experiment that the cutinase enzymes have wide range of residual activity, as AbC lost 90% of its activity when heated to 55 °C, however HiC retained 50% of its activity even when heated to 90 °C. Both AoC and AfC retained \textgreater 70% of their activity at 55 °C, however there was a significant drop in the residual activities of both enzymes at 65 °C.

All five cutinase enzymes were used to catalyze the degradation of polycaprolactone in various pH environments ranging from pH 8 down to pH 3. 1cm X 1cm PCL films (approx. thickness 250µm) were incubated in vials containing 2.5ml of 20mM buffer with a concentration of 8.8µM cutinase enzyme. HiC catalyzed degradation as weight percent was as follows: 100% at pH 8 (3h), 100% in pH 5 (6h) and 60% in pH 3 (6h). Both AoC and AfC catalyzed 100% weight loss after 6 hours in pH 8 and pH 5, while AoC showed a higher activity in pH 3 after 6 hours with a weight loss of 27% as opposed to no weight loss in the AfC sample. FsC and AbC showed much lower hydrolytic activity for PCL even at the more stable pH 8, as weight loss for PCL films after 6 hours was 50% and 43% respectively. At pH 5 there was 18% weight loss after 6
hours in the FsC catalyzed sample while there was no weight loss in the AbC catalyzed sample. At pH 3 there was no weight loss in either the FsC or the AbC catalyzed samples.

1.9 HiC as Catalyst for Polymer Synthesis

Hunsen et al. in 2007\textsuperscript{44} first reported a successful cutinase-catalyzed polyester synthesis reaction. An engineered HiC from Novozymes was first immobilized on Lewatit – a poly methyl methacrylate resin. Sufficient data was presented that demonstrated that HiC immobilized on Lewatit was highly active for both condensation and ROP reactions to form polyesters. Substrates with various carbon chain lengths or ring sizes were used to show the potential broader utility of this newly discovered polyester synthesis catalyst. HiC catalyzed polycondensation reactions of 1,4 cyclohexanediol and diacids with a chain lengths ranging from 4 to 10 carbons were successfully carried out (bulk, 70°C, HiC 1% w/w, 48h) yielding polymers with molecular weights ($M_n$) increasing from 0.9 to 19 Kg/mol.

HiC immobilized on Lewatit was also successfully used to carry out ring opening polymerization (ROP) reaction in bulk and in toluene (70°C, 0.1% enzyme w/w, 24h). ROP reactions of ε-caprolactone in bulk formed poly(ε-caprolactone) with $M_n$ 16 Kg/mol and a conversion >99%. The same reactions in toluene formed a polymer with $M_n$ 25 Kg/mol and conversion >99%. HiC catalyzed ROP reactions of ω-pentadecalactone in toluene gave poly(ω-pentadecalactone) with $M_n$ 47 Kg/mol and <99% conversion.

As anticipated for a selective enzyme catalyst, the molecular weights of polyesters formed varied as a function of substrate chain length or lactone ring size.\textsuperscript{44}
Building on the previous communication, in 2008, Hunsen et al.\textsuperscript{16} conducted a more detailed study of HiC immobilized on Lewatit to catalyze ROPs. While HiC was highly active for CL and PDL ROPs giving high molecular weight polymers, HiC was inactive for catalysis of L-lactide (L-LA) and (R,S)-β-butyrolactone ROP’s. Interestingly, HiC showed poor activity on lactones with 6-membered rings – polymerization of δ-valerolactone (VL) gave a $M_n$ of 2700 – while HiC was highly active on 7-membered ring lactone CL to give PCL of $M_n$ 24,900. In contrast, N435 catalyzed ROPs of VL and CL gave PVL and PCL with $M_n$ values of 10,000 and 19,000, respectively. Thermostability of HiC-Lewatit was determined at temperatures from 50 to 90°C in bulk. The results of this work showed that HiC-Lewatit had optimal activity at 70°C. Raising the reaction temperature just a few degrees higher resulted in large loses in activity. A study of molecular weight as a function of enzyme water content was also performed for HiC-catalyzed reactions. By carefully controlling the water content products with predictable molecular weight can be prepared.\textsuperscript{16} This ability to use water content in reactions to control product molecular weight was similarly observed using N435.\textsuperscript{45}

![Diagram](image-url)

\begin{align*}
m = 1 & \quad \delta\text{-valerolactone (VL)} \quad m = 1 & \quad P(VL) \\
m = 2 & \quad \varepsilon\text{-caprolactone (CL)} \quad m = 2 & \quad P(CL) \\
m = 11 & \quad \omega\text{-pentadecalactone (PDL)} \quad m = 11 & \quad P(PDL)
\end{align*}
Motivated by the above results, this thesis focused on gaining additional knowledge on the potential use of HiC to catalyze polyester synthesis. The catalytic activity of immobilized HiC was explored for polycondensation reactions using various substrates. Furthermore, reaction conditions promoting improved activity of immobilized HiC were studied. There are three distinct chapters, each leading into the next. The first, deals with the optimization of the HiC catalysis system for polycondensation reactions; more specifically, the section describes how the immobilization support and parameters of HiC immobilization affect the result in biocatalysts activity for polymer synthesis. In the second section, the chain length specificity of immobilized HiC is explored by performing a series of step-condensation polymerization reactions. Results obtained with
immobilized HiC were compared to those obtained with N435. Finally, in the third section, immobilized HiC was used to catalyze the synthesis of polyol polyesters using glycerol and sorbitol as multifunctional monomers. The resulting polyester polyols were analyzed to determine molecular weights, polydispersity and degree of branching as compared with their N435 catalyzed counterparts.

1.10 Enzyme immobilization

By immobilizing an enzyme on a macroporous support, the enzyme is deposited as thin layers (possibly monolayers) on a large surface area that is exposed to reactants and products. Furthermore, as long as the reaction medium can diffuse into macroporous supports that present enzymes at surfaces, reactions can be conducted in a range of organic media.\textsuperscript{46} Furthermore, immobilization potentially enhances the thermostability, catalytic activity and selectivity of the enzyme catalyst.\textsuperscript{47,48} It also facilitates easy separation of the enzyme from the product which can minimize or eliminate protein contamination of the product.\textsuperscript{46,48-50} By permitting catalyst re-use over multiple reaction cycles the cost of catalyst can become sufficiently low so that it may be used to produce commodity chemicals.\textsuperscript{47,49,51}

1.10.1 Immobilization by cross-linking

This includes either crosslinked enzyme crystals (CLECs) or cross linked enzyme aggregates (CLEAs). Crosslinking of enzymes was originally developed in the 1960s however, this method of producing cross-linked enzymes (CLEs) had several drawbacks, such as low activity retention, poor reproducibility, low mechanical stability, and
difficulties in handling the cross linked enzymes.\textsuperscript{52,53} Crosslinking is an advantageous method of immobilization because the final product is a relatively pure protein with high concentration of enzyme per unit volume, unlike other methods of immobilization where there is dilution of the catalytic activity resulting from the introduction of a large proportion of non-catalytic mass.\textsuperscript{53,54} CLECs have been shown to be stable and highly active biocatalysts. Furthermore, they are uniform and microporous in contrast to soluble enzymes, and remain monodisperse upon reconstitution even in organic solvents.\textsuperscript{54} Moreover, CLECs can easily be separated from the product by simple filtration and can be recycled and reused many times without significant decrease in enzyme activity. They are also said to be robust in the sense that they will not break under that stress of industrial high speed mixing.\textsuperscript{54} However, it is unlikely that a protein CLEC can be developed that would have the durability of a properly designed synthetic macroporous support.

A disadvantage to this method is the high cost and difficulty of crystallization and, therefore, CLEA was developed as a more accessible option.\textsuperscript{55} This simpler method which consists of covalently crosslinking a precipitated enzyme that is not crystallized, is based on the understanding that proteins in general can be precipitated by inorganic salts (such as (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}) or certain types of organic media (acetone) without undergoing denaturation. Enzyme purification via precipitation in ammonium sulfate can easily be integrated into the preparation of CLEA.\textsuperscript{53} The group of Cao \textit{et al.}, who developed the method of CLEA, was successful in immobilizing lipases, precipitating the enzyme with (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} or acetone and crosslinking with glutaraldehyde.\textsuperscript{55}
Figure 8. CALB CLEA particle can contain up to 8 million enzyme molecules (magnification 3500X).\(^53\)

1.10.2 Entrapment or Microencapsulation in an Insoluble Polymer

This is a type of physical enzyme immobilization where chemical bonds are not formed between the enzyme and the matrix. This is advantageous for certain uses such as biomedical applications as well as for bioanalytical assays.\(^56\) Entrapment, which can be defined as the physical restriction of an enzyme within a confined space or network, occurs as the enzyme is brought into the gel matrix as a monophasic solution and is then entrapped in the matrix as the enzyme is polymerized.\(^57\) The most commonly used gels for this kind of immobilization are polyacrylamide, alginate based gels and urethane prepolymer. Each gel matrix has its unique advantage. Polyacrylamide, for example, has the advantage of having non-ionic properties so that enzymes are only minimally
modified in the presence of the gel matrix. Furthermore, diffusion of charged substrates and products are not affected. Alginate is the major structural polysaccharide of marine brown algae. This polysaccharide forms viscous solutions in the presence of monovalent cations, whereas, in the presence of divalent cations, especially Ca\[^{2+}\], gelation occurs. Furthermore, increasing the concentration of the alginate solution will form a tighter and more cross linked matrix. This method of entrapment can easily be used for enzymes since it can be carried out under mild conditions.\(^{58}\)

A less popular method of enzyme entrapment inside a matrix of urethane prepolymer was developed by Fukushima et al in the late 1970s as a safer and more conventional alternative to the widely used polyacrylamide method which will be applicable to entrapment of not only enzymes but also microbial cells and organelles.\(^{59}\)

In all the above methods, immobilization of enzyme by entrapment can be performed by simple procedures. Moreover, the cost of immobilization is low as compared to other methods. There are however three major drawbacks to this method. First; continuous leaching of the enzyme due to the wide pore size distribution of the gel matrix. Second; there is significantly lower substrate accessibility associated with poor diffusion to the entrapped enzyme, and third; in some instances there is loss of enzyme activity due to free radicals generated in the polymerization of the gel.\(^{56,60}\)

Encapsulation is another method of enzyme immobilization very similar to entrapment. In this method, the enzyme is enclosed within a semi-permeable polymer membrane capsule. Originally, the membrane was formed by either interfacial polymerization leading to nylon microcapsules or by interfacial precipitation leading to cellulose nitrate microcapsules.\(^{61}\) Since nylon was shown to cause denaturation of the
enzyme, cellulose nitrate is therefore preferred.\textsuperscript{61} Recently, much work has been done to optimize the method of enzyme encapsulation, and silica based matrices (usually alkyl-alkoxysilanes) have been demonstrated to be very promising.\textsuperscript{62} These highly porous silica materials made by low-temperature sol-gel process have many benefits for enzyme immobilization. The aqueous character of sol-gel processing and the fact that the synthesis can be carried out in mild conditions are important for biomolecules to retain their native structure, dynamics and function. The rigid framework of sol-gel polymers, while preventing leaching of the entrapped enzyme, will furthermore stabilize the enzyme’s structure and protect the enzyme from aggregation and unfolding.\textsuperscript{62,63} These silica matrixes are chemically inert, hydrophilic, and inexpensive to synthesize. Moreover, the mesoporous structure, having pore widths between 2 and 50 nm\textsuperscript{64} allow free and easy access of low to medium molecular weight substrates to enzyme.

Reetz et al.\textsuperscript{65} developed a unique method of immobilization by changing the matrix in which the enzyme (lipase) is encapsulated from SiO\textsubscript{2}, which is highly polar, to a non-polar mixture (approx 5:1) of RSi(OCH\textsubscript{3})\textsubscript{3} and Si(OCH\textsubscript{3})\textsubscript{4}, where R is a non-hydrolyzable alkyl group. Furthermore, an additional porous solid support is used during the sol-gel process on which the lipase containing gels are bound in the pours of this solid support during gelation. By using this method in model esterification reactions, the group was able to substantially improve relative enzyme activity by a factor ranging from 2 to 8 with respect to the traditional use of the corresponding lyophilized lipase powder. Moreover, the enzyme thermostability was significantly improved as well.\textsuperscript{65} Even better results were obtained when the gel was tailored with surfactants and/or crown ethers.
Sun et al.\textsuperscript{66} reviewed several approaches to prepare biomimetic silica. He explains that, in nature, unicellular organisms such as diatoms use structuring and templating biomolecules to produce silica shells with highly structured pores with dimensions ranges from nanometer to micrometer.\textsuperscript{66,67} Moreover, this material has remarkable mechanical and structural properties. The natural process of silica formation (i.e. silica biomineralization) is receiving increasing attention, since it holds the key to the formation of silica morphologies with a dedicated organization of hierarchically structured elements and the ability to synthesize such silicas under ambient conditions.\textsuperscript{66} This research into silica biomineralization is very pertinent to enzyme encapsulation as the novel silica-based material (e.g. silica gels) can further improve enzyme activity, thermostability and recyclability by offering the enzyme an optimum catalytic environment with favorable enzyme/matrix interactions and an optimal balance between molecular restraint and conformational mobility. In recent work using biomimetic silica Chen et al.\textsuperscript{67} encapsulated \textit{Pseudomonas Cepacia} lipase together with magnetic nanoparticles to facilitate enzyme recovery. Their novel encapsulation process can be completed within minutes, much faster than the conventional sol-gel approach which requires hours. Furthermore, by adding silanes and changing the precursor, enzyme activity can be increased by as much as 450\% relative to free enzyme. In addition, the thermostability and pH stability can also be drastically improved.

Vidinha et al.\textsuperscript{62} studied sol-gel encapsulation to immobilize FsC (\textit{Fusarium solani} cutinase) and found that cutinase activity increases with increasing alkyl chain length of the precursor up to the point where the matrix formed lacks the strength to withstand the drying process. That is, alkylated precursors with a higher alkyl chain length are
expected to yield more highly ductile matrices, with lower mechanical strength. Moreover, they also found that by incorporating select additives into the alkyl-alkoxysilane matrix there was a marked increase in enzyme activity. This suggests that the structural characteristics of the matrix are not the only reason for the observed differences in cutinase activity. By reacting with silanol groups, the additives decrease the number of those available for binding to residues on the enzyme, thus reducing constraints on enzyme conformational mobility.⁶²

**Figure 9.** SEM micrographs of various sol–gel matrices with encapsulated cutinase. The bar represents 50 µm.⁶²

### 2.10.3 Immobilization by Physical Adsorption.
In a recent work on the adsorption of the hyperthermophilic enzyme, LamA, on both hydrophobic and hydrophilic supports, Koutsopoulos et al.\textsuperscript{68} studied adsorption of the hyperthermophilic enzyme LamA on both hydrophobic and hydrophilic supports. The authors point out that physical adsorption is nature’s method of enzyme immobilization as LamA remains active and retains its conformation in the extreme temperatures of its natural environment by adsorption on the surface of rocks and other materials.

Exposure of an aqueous protein solution to a solid surface typically results in the adsorption of the protein on the interface.\textsuperscript{68,69} Much research has been done to find the optimum solid support for any given enzyme, as different proteins have different surface chemistry requirements to achieve optimum performance. Furthermore, although adsorption is a mild method to immobilize proteins on a carrier as compared with chemical (covalent) immobilization, enzymes that are active in solution may deactivate in the adsorbed state. For enzyme adsorption to be a beneficial method of immobilization for biocatalysis the enzyme must have a strong affinity for the particular solid support yielding a high loading of the enzyme on the support, it must remain folded with enhanced catalytic activity on that support. Other desired outcomes would be to increase the enzyme thermostability upon adsorption. In addition the solid support must be of a compatible material (organic or inorganic), with an optimal particle and pore size for porous resins. In their study of the effect of pore size and surface characteristics of the solid support, Takahashi et al.\textsuperscript{70} experimented with horseradish peroxide (HRP) adsorbed on mesoporous silica with varying pore size. They concluded that when the average pore size just matches the molecular diameters of the enzyme, the immobilized HRP exhibits peak activity and best stability in that organic media.
**Figure 10.** a) Structural model of an HRP molecule. b-d) Image models of immobilized HRP in mesoporous silica (FSM-16) with various sizes using a computer schematic model. The FSM-16 model was constructed in accordance with the folded sheet formation mechanism. The pore diameters of the FSM-16 model selected were 30, 50, and 90 Å for models b-d, respectively.70

Thermodynamically, the extent of protein adsorption is determined by the Gibbs free energy of adsorption, which depends both on the enthalpy and the entropy of adsorption. The forces of enthalpy include either positive (repulsing) or negative (attractive) electrostatic forces, and attractive van der Waals interactions between protein molecules and the interface. The entropy forces are from any changes in the conformation of the protein and from hydrophobic dehydration which is due to the removal of water molecules surrounding hydrophobic surfaces of the solid support and hydrophobic areas on the protein. Since these water molecules are more ordered as compared to those in the
bulk phase, displacement of water from hydrophobic surfaces leads to increased entropy of water and of the entire system. Furthermore, entropy forces also come from any conformational change where hydrogen bonds are broken in the protein core, resulting in a greater structural freedom.49,68,71

Sugiura and Isobe72 in 1975 asserted that lipases have exceptionally high affinity for hydrophobic supports and are readily adsorbed onto the surfaces of hydrophobically activated solids.

Cutinases, like lipases, have a high affinity for hydrophobic supports. Previous work on HiC-catalyzed ROPs as well as early studies on HiC-catalyzed step-condensation polymerizations utilized the enzyme physically immobilized on Lewatit (hydrophobic) PMMA beads. The rationale for experimenting with physical adsorption to immobilize cutinase enzymes is based on the assumption that the large hydrophobic area that surrounds the active sites of cutinase (as well as that of lipase), is the region of the enzyme which is mainly involved in their adsorption on strongly hydrophobic solid surfaces. Thus, the enzymes recognize these surfaces similarly to those of their natural substrates which are similarly strongly hydrophobic.73 Physical adsorption has indeed proven to promote enhanced enzyme activity and thermostability in cutinases.49,74 Some simple modifications to this straightforward process have been suggested to further improve this immobilization technique such as modification of the protein by a soluble, hydrophobic reagent (e.g. PEG activated with p-nitrophenyl chloroformate) prior to adsorption which considerably enhances the esterification activity of the immobilized enzyme,75 or modification of the soluble protein by reacting with an excess of a hydrophobic imidoester.76
2.10.4 Immobilization by Covalent Attachment

Covalent immobilization of enzymes dates back to the 1960s. However, these early studies generally were plagued by problems such as the use of toxic reagents, complicated activation of the solid support and low stability of the reactive groups. The more recent covalent immobilization techniques, however, have rectified the aforementioned flaws and provide for a stable and reusable enzyme, prepared using favorable non contaminant solvents. Protocols for covalent enzyme immobilization are quite simple and in fact similar to that of physical adsorption, however, there is substantially less enzyme leaching or desorption when the protein is covalently bound to the solid support. Covalent immobilization utilizes a solid support with an oxirane (epoxy) or other group which can form a covalent bond with an accessible functional group of exposed amino acids in the protein. Epoxy supports are indeed an ideal matrix to use for protein immobilization, as they are readily available in an activated form and the immobilization reaction can take place under mild conditions which is crucial for proteins which can easily denature and thus reduce their activity. Epoxy based resins are commonly used for enzyme immobilization due to their compatibility with a wide range of enzymes, and can react with the amino group in lys and arg, the carboxyl group in asp and glu, the hydroxyl group in ser, and thr, the mercapto (or thiol) group in cys, the phenol group in tyr, -the imidizol group in his and the indole group in trp, dependent on the specific protein to be immobilized (See fig 11). However, since lys, which is very often the reactive residue, is basic with a side chain pKa of approx 10.3, the reactivity during the immobilization of many soluble proteins performed at a neutral pH is rather
low. Moreover, it is most probable that the reactive groups of the enzyme and of the solid support are not correctly aligned. In fact they both have a rigid and dissimilar structure which further compromises the efficiency of the reaction and the ability to form multipoint covalent bonds needed for an optimal biocatalyst. Because of this, a two-step mechanism is adopted. First, there is rapid physical adsorption of the enzyme onto the solid support followed by covalent attachment that occurs between the epoxy group of the solid support and the amino group of an available lys residue. For this reason, most commercially available solid supports for the purpose of enzyme immobilization are hydrophobic in order to adsorb the protein during the first phase of the immobilization process. Moreover, to compensate for the low lysine reactivity, a longer reaction time is also necessary to form multipoint attachments. Slightly elevated incubation temperatures (~25 °C) can increase the flexibility of the enzyme during immobilization and thereby further improve its reactivity towards epoxy groups on the support. Only a multipoint immobilization will stabilize or rigidify the enzyme sufficiently in order to for it to be used as an optimal biocatalyst in various reactions. The immobilization is called a multipoint covalent immobilization when multiple lysine residues on the surface of the enzyme are all covalently bound to the solid support thereby greatly reducing the protein mobility (See fig. 12). This is accomplished by using a porous solid support with a high density of reactive epoxy groups. For these reasons we evaluated a commercially available epoxy based solid support manufactured by Rohm & Haas known as Amberzyme Oxirane (235 μm particle size, 220 Å pore size) which would potentially be a good choice as a solid support for enzyme immobilizations for use as a biocatalyst as it is hydrophobic to allow for adsorption, macroporous to allow for the formation of
multipoint attachments and has been shown to be reusable making it economically viable.\textsuperscript{71}

\textbf{Figure 11.} Residues on the exterior of the protein chain that can react with epoxy groups on solid supports to form a covalently immobilized enzyme.\textsuperscript{81}
Figure 12. Mechanism of two step multipoint covalent attachment of a protein (such as CALB or HiC) to an epoxy activated solid support.\textsuperscript{77,85}

1.11 Chain length selectivity

Selectivity of hydrolase enzymes such as lipases and esterases is normally strongly dependent on the chain length of simple small molecule substrates such as n-alkyl carboxylic acid and n-alkanols.\textsuperscript{86-88} The modification of chain length selectivity through genetic engineering has been a topic widely discussed in the literature.\textsuperscript{89-92} Genetic engineering may be undertaken in order to decrease the chain length specificity of the enzyme, thereby increasing the range of acyl chain lengths that a biocatalyst is active on. In the case of Candida rugosa lipase, the enzyme is engineered to increase the specificity of the acyl chain length so that the enzyme might hydrolyze only the short chain length fatty acids and, thereby, lead to enrichment of long polyunsaturated fatty acids in certain foods which is highly beneficial.\textsuperscript{93}
Unlike the literature on small molecules, other than a 1995 paper by Linko et al.\textsuperscript{94} which briefly mentions chain length selectivity of a free lipase for a polytransesterification reaction, there is scarcely any literature on chain length selectivity of an immobilized lipase or cutinase enzyme for a polymer synthesis reaction. This thesis describes for the first time a systematic study to define HiC specificity during step condensation polymerization reactions. For this work, three series of monomers were studied: \textit{i}) \textit{ω-hydroxyalkanoic acids (HAs)} with 6, 10, 12 and 16-carbons, \textit{ii}) copolymerizations of sebacic acid (C10-diacid, C\textsubscript{10}H\textsubscript{18}O\textsubscript{4}) with \textit{α,ω-n-alkane diols} with 3, 4, 5, 6 and 8-carbon chain lengths, and \textit{iii}) copolymerizations of 1,8-octanediol with \textit{α,ω-n-alkane diacids} with 6, 8, 9, 10 and 13-carbon chain lengths. HiC selectivity was evaluated by monitoring increases in polymer chain length vs. reaction time as a function of monomer structure. Similarities and differences in activity and selectivity between CALB and HiC were determined by performing all reactions above using immobilized CALB catalysis (N435) under identical conditions (in bulk, in vacuo, 70 °C).

\textbf{1.12 HiC catalyzed polyester polyols}

One of the subjects in this thesis is the preparation of hyperbranched polyester polyols (HBPs) using immobilized HiC as catalyst. HBPs are extensively used in a wide range of applications. In the field of biomedical materials, HBPs are being explored for use as carriers for MRI contrast agents and synthetic vectors for gene transfection.\textsuperscript{95} The relative ease of synthesis of HBPs relative to dendrimers, the overall charge on HBPs and the large number of functional groups on the periphery make HBPs very interesting for use in drug delivery.\textsuperscript{95,96} Furthermore, glycerol polyesters in hydrogel sealants have been
reported to have been used in vivo for optical tissue repair and orthopedic applications. Because of their high degree of solubility and low viscosity, HBPs are being used in the manufacture of polyurethane foams and coatings.

One way to achieve a polyurethane coating with the desired properties is to change the macromolecular architecture of the polymer. One such class of polymer architecture that is widely used is HBPs which have various functional groups at the periphery which are used to obtain the desired material characteristics. Changes in the melt viscosity of HBPs is easily regulated by modifying the degree of branching along chains. An excellent example of how nature uses branching is the production in plants of linear amylose and highly branched amylopectin that regulate starch physical, chemical and biological properties.

HBPs are actually a subclass of the dendritic architecture class. As opposed to dendrimers and dendronized polymers - also subclasses of the same dendritic architecture class but exhibit perfect branching with a degree of branching (DB) of 1.0 (or 100% branching) - HBP exhibit a randomly branched structure. The common methods for synthesis of HBPs employ chemical catalysis – often acid catalysis. In this work we have used immobilized HiC to catalyze the synthesis of both glycerol and sorbitol based polyesters. Experiments were conducted with varying polyol contents up to 60% glycerol and 40% sorbitol. Terpolymerization condensation reactions were carried out in bulk between sebacic acid, 1,8-octanediol and polyol. Samples were taken at different time intervals to study the evolution of the polyol structure and progression of molecular weight as a function of time. The reactions reported here have been repeated with N435 using the same reaction conditions and the results of both sets of reactions are compared.
and contrasted. HiC is shown to be a beneficial alternative to CALB catalysis for the synthesis of polyol polyesters.
CHAPTER 2

Materials and Methods

The analytics and characterization team of Dr. Minmin Cai, Dr. Wenchun, Xie and Dr. Chunxiao Han assisted in the characterization of polymers and esters. Dr. John Decatur assisted with the NMR studies of polyols which was performed at Columbia University.
2.1 Materials

Novozym 435 (specific activity 10500 PLU/g), abbreviated as N435, was a gift from Novozymes (Bagsvaerd, Denmark) and consists of Candida antarctica Lipase B (CALB) physically adsorbed within the macroporous resin Lewatit VPOC 1600 (poly[methyl methacrylate-co-butyl methacrylate], supplied by Bayer). N435 contains 10 wt % CALB that is located on the outer 100 μm of 600 μm average diameter Lewatit beads.\textsuperscript{16,79} HiC was a kind gift from Novozymes (Bagsvaerd, Denmark) received in liquid media.

Rohm and Haas’ Amberlite® XAD 1180 (poly[styrene-divinyl benzene]) was purchased from Acros Organics. Resindion EC-EP and EC-EP 403/S were kind gifts of Resindion (Binasco, Italy). Lewatit VPOC 1600 (poly[methyl methacrylate-co-butyl methacrylate]), Amberzyme oxirane (AO) resin as well as the experimental resins WJZ-5346 and WJZ-5349 was a kind gifts of Rohm & Haas/Ion Exchange Resins (Philadelphia, PA).

10-Hydroxydecanoic acid (C10-HA), 12-hydroxydodecanoic acid (C12-HA), adipic acid (C6), brassylic acid (C13), propanediol (C3), and butanediol (C4) as well as C3, C5, C6, C8, C10 Mono-alcohols and hexanoic acid (C6), octanoic acid (C8), decanoic acid(C10), myristic acid (C14) and palmitic acid (C16) were purchased from Sigma-Aldrich, while 16-hydroxyhexadecanoic acid (C16-HA), suberic acid (C8), azelaic acid (C9), 1,5-pentanediol (C5), and 1,6-hexanediol (C6) 1-butanol (C4) and lauric acid (C12) were purchased from Acros. 1,8-Octanediol (C8-diol) and sebatic acid (C10-
diacid) were purchased from TCI and 6-hydroxyhexanoic acid was synthesized in our lab from ε-caprolactone following a literature procedure. Sorbitol and glycerol were both purchased from Sigma-Aldrich. All other solvents and reagents were obtained commercially at the highest purity available and used as received without further purification.

2.2 Methods

Synthetic Methods:

Covalent immobilization of HiC on Amberzyme oxirane (AO) beads

HiC was first purified by several cycles of ultrafiltration [Millipore model 2000 high-performance Ultrafiltration Cell] through a 10 KDa membrane and lyophilized. Enzyme purity, determined by SDS-PAGE, showed it was >95% pure. Lyophilized HiC (50 mg) was dissolved in 20 mL potassium phosphate buffer (0.1M, PH 7.8). Various epoxy based resins (500 mg) were first activated with EtOH and then added to the 20 ml of enzyme solution 2.5 mg/mL HiC. Immobilization was performed in 50 mL polypropylene tubes at room temperature, with shaking (150 RPM), for 48 h. Subsequently, the supernatant was removed, beads were washed 3 times with 10 mL of potassium phosphate buffer (0.1M, PH 7.8) to remove loosely bound protein, and the resin was dried by lyophilization for 24 h. From aliquots taken both prior to and after HiC immobilization, HiC concentration was measured by the BCA method to determine loading on the beads. Below, HiC immobilized on AO resin will be abbreviated as HiC-AO.
Physical immobilization of HiC by adsorption on various polymer resins

HiC was first purified by several cycles of ultrafiltration [Millipore model 2000 high-performance Ultrafiltration Cell] through a 10 KDa membrane and lyophilized. Enzyme purity, determined by SDS-PAGE, showed it was >95% pure. Lyophilized HiC (100 mg) was dissolved in 25 mL ammonium bicarbonate buffer (0.1M, PH 7.8). Various polymer resins (500 mg) were first activated with EtOH and then added to the 25 ml of enzyme solution 4 mg/mL HiC. Immobilization was performed in 50 mL polypropylene tubes at room temperature, with shaking (150 RPM), for 72 h. Subsequently, the supernatant was removed, beads were washed 3 times with 10 mL of ammonium bicarbonate buffer (0.1M, PH 7.8) to remove loosely bound protein, and the resin was dried by lyophilization for 24 h. From aliquots taken both prior to and after HiC immobilization, HiC concentration was measured by the BCA method to determine loading on the beads.

Homopolymerization of ω-hydroxyalkanoic acids (Ha) - Parallel reactor:

HA (300 mg) and diphenylether (1.2 mL) were transferred to reaction tubes [100 mm x 15 mm] that were placed in a Radley Greenhouse Plus Parallel Synthesizer (Brinkmann). Contents of reaction tubes were maintained at 100 °C for 30 min with magnetic stirring to obtain a monophasic solution. Subsequently, the temperature was reduced to 70 °C and immobilized catalyst (HIC-AO or N435, 1% w/w protein relative to monomer) was added. After 2 h, vacuum (10 mg/Hg) was applied. Aliquots were removed after 15 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h and prepared for GPC analysis. Reaction
products were isolated by addition of chloroform, removing immobilized enzyme by filtration and roto-evaporation of solvent.

*Homopolymerization of ω-hydroxyalkanoic acids (Ha) – toluene-water azeotrope:*

HA (300 mg) and toluene (5 mL) were transferred to a 250 ml rb flask that was placed in an oil bath. The flask was fitted with a dean-stark trap and a coiled reflux condenser. Contents of the flask were maintained at 100 °C for 30 min with magnetic stirring to obtain a monophasic solution. Subsequently, the temperature was reduced to 70 °C and immobilized catalyst (HIC-AO or N435, 1% w/w protein relative to monomer) was added. After 2 h, an additional 15 ml of toluene was added and vacuum (360 mg/Hg) was applied. The temperature was adjusted to 100 °C and the contents of the flask were observed to be boiling and condensing properly. Aliquots were removed after 15 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h and prepared for GPC analysis. (Care was taken to remove flask from oil bath before vacuum is stopped when removing aliquots, in order to maintain enzyme conformation.) Reaction products were isolated by addition of chloroform, removing immobilized enzyme by filtration and roto-evaporation of solvent.

*General method for diol/diacid condensation copolymerizations:*

Diol (2 mM) and diacid (2 mM) were transferred to reaction tubes (140 mm x 25 mm) that were placed in an Eyela Process Station PPS-5510 (Tokyo Rikakikai Co., LTD.). Contents of reaction tubes were maintained at 115 °C for 30 minutes with magnetic stirring to obtain a monophasic solution. Subsequently, the temperature was reduced to 70 °C and immobilized catalyst (HIC-AO or N435, 1% w/w protein relative to monomer) was
added. After 2 h, vacuum (20 mg/Hg) was applied. Aliquot removal, preparation of samples for GPC, and isolation of products follows exactly as described above for poly(HA) synthesis.

General method for diol/polyol/diacid condensation terpolymerizations:

Diol + polyol (2 mM) and diacid (2 mM) were transferred to reaction tubes (140 mm x 25 mm) that were placed in an Eyela Process Station PPS-5510 (Tokyo Rikakikai Co., LTD.). Contents of reaction tubes were maintained at 120 °C for 45 minutes with magnetic stirring to obtain a monophasic solution. Subsequently, the temperature was reduced to 70 °C and immobilized catalyst (HiC-AO or N435, 1% w/w protein relative to monomer) was added. After 2 h, vacuum (100 mm/Hg) was applied and the vacuum pressure was reduced 20 mm/Hg after each subsequent 2 hrs. until a vacuum pressure of 20 mm/Hg is attained (i.e. 100mm/hg at 2 hrs, 80mm/Hg at 4 h, 60mm/Hg at 6 h, 40mm/Hg at 8 h and 20mm/Hg at 10 h). Aliquot removal, preparation of samples for GPC, and isolation of products follows exactly as described above for poly(HA) synthesis.

General method for small molecule esterification reactions:

Alcohol (2 mM), acid (2 mM) and diphenylether (1:1 ratio to monomer weight) were transferred to reaction tubes [100 mm x 15 mm] that were placed in a Radley Greenhouse Plus Parallel Synthesizer (Brinkmann). Contents of reaction tubes were heated to 70 °C with magnetic stirring and under N₂. (Reactions with wild type strains of cutinase were performed at 50°C.) Subsequently, immobilized catalyst (HIC-AO or N435,
1% w/w protein relative to monomer) was added. Reaction was terminated after 30 min. (Reactions with WT strains of cutinase were terminated after 25 minutes.) Aliquot was taken and prepared for Gas chromatography (GC)-mass spectrometry (MS) analysis. Immobilized enzyme was removed from reaction products by filtration.

Statistical significance:
All experiments were run in either duplicate or triplicate and the data presented is the average of those trials. If a run was clearly out of range due to human error, it was disregarded and the experiment was repeated. For those plots where error bars are present, the average value is plotted and the ± error is calculated using standard deviation.

Instrumental methods

Gel Permeation Chromatography (GPC):
The number- and weight-average molecular weights ($M_n$ and $M_w$, respectively) were determined by size exclusion chromatography using a Waters HPLC system equipped with a model 510 pump, model 717 autosampler, and model 410 refractive index detector with 500, $10^3$, $10^4$, and $10^5$ Å Ultrastyragel columns in series. Waters Empower GPC software (Version 3, Viscotek Corp.) was used for data analysis. Chloroform was used as eluent with a flow rate of 1.0 mL min$^{-1}$ at room temperature. Sample concentrations of 5 mg/mL and injection volumes of 35 µL were used. Molecular weights were determined on the basis of a conventional calibration curve generated by narrow molecular weight polystyrene standards obtained from Aldrich Chemical Co.
SEC-MALLS:

Molecular weight and degree of branching of certain polyol prepolymer samples was determined by light scattering (SEC-MALLS) using the identical Shimadzu/Waters/Wyatt system and experimental method as was described elsewhere.\textsuperscript{104} The identical Wyatt ASTRA 5.3.4.14 software was used as well.

GC-MS analyses:

Conversions of small molecule esterification reactions were determined by GC-MS. Analyses were performed at 70ev using a ThermoFinnigan TraceGC Ultra gas chromatograph coupled with a Trace DSQ mass spectrometer. GC-MS analyses were performed with injector, ion source and interface temperatures of 200, 250 and 280 °C respectively. Samples in hexane (1 µL) were injected in PTV split mode and run on a capillary column (Varian CP8944 VF-5MS, 0.25 mm x 0.25 µm x 30 m). The oven temperature was programmed at 120 °C for 1 min increasing to 300 °C at 20 °C/min, and then maintained at 300 °C for 4 mins. Standard esters were used to construct standard curves that allowed quantification of percent conversion values. These compounds were synthesized in an Eyela Process Station PPS-5510 (Tokyo Rikakikai Co., LTD.) at 90°C with N435 catalysis, in toluene, following the general method for small molecule esterification reactions given above. Ester standards were purified by silica column chromatography using a 10:1 mixture of n-hexane and ethyl ether as eluent.
The glycerol polyesters formed were characterized using inverse gated (quantitative) carbon (\(^{13}\)C) NMR spectroscopy recorded on a Bruker NMR spectrometer (model DPX300) at 300 MHz, in deuterated chloroform (CDCl\(_3\)) as solvent. \(^{13}\)C NMR chemical shifts were referenced relative to chloroform-d at 77.0 ppm as the internal reference. The concentration of the polymer was 40% w/v in chloroform-d. The instrument parameters were as follows: acquisition time 1.89 s, temperature 300 K, spectral width 18 000 Hz, 65000 data points, relaxation delay 10 s, 15000-20000 transients.
CHAPTER 3

Optimization of the HiC Catalyst System

The text in this chapter was written by myself. Dr. Wenhua Lv and Dr. Peter J. Baker assisted with fermentation and purification of FsC, AoC, AbC and wildtype HiC. Dr. Asa Ronkvist provided assistance with the purification of PmC.
3.1 Model Reaction for Optimization Study

In order to efficiently develop an HiC catalytic system for polyester synthesis it is necessary to study and to optimize the various parameters of this system. To that end, a homopolymerization of C16 ω-hydroxy fatty acid was used as a model reaction for the majority of the optimization study.

\[
\text{Scheme 1: Homopolymerization reactions of C16 ω-hydroxyalkanoic acid.}
\]

3.2 Protein to monomer ratio and Protein loading

Two parameters that must be explored when optimizing an immobilization system are enzyme activity as a function of protein loading on the solid support, and the effect on the polymerization when varying the amount of protein used to catalyze a polycondensation reaction. To better understand these parameters, the model reaction described above was carried out in parallel first altering the amount of protein used and then using immobilized enzyme with different protein loadings. In the first part of this study, the amount of protein used in relation to the monomer weight was varied in each reaction (0.1, 0.25, 0.5, 0.75 and 1% protein w/w respectively) while holding the protein loading constant at 10% w/w. Molecular weight as a function of time was carefully monitored. Based on the results obtained, we can see that a substantial increase in the molecular weight (100%) is observed as the protein content is increased from 0.75% to
1%. Therefore, 1% HiC relative to total monomer is optimal for most efficient synthesis of high molecular weight polyester.

![Diagram showing Mn vs. time for HiC catalyzed ω-hydroxyhexadecanoic acid homopolymerization comparing various amounts of protein relative to total monomer weight used to catalyze the reaction. Data represents an average of 2 trials (n=2).](image)

**Figure 13.** Plot of $M_n$ vs. time for HiC catalyzed ω-hydroxyhexadecanoic acid homopolymerization comparing various amounts of protein relative to total monomer weight used to catalyze the reaction. Data represents an average of 2 trials (n=2).

To study the optimal protein loading, HiC was immobilized under different conditions to obtain four different protein loadings (10, 7, 4 and 1% w/w respectively). Model homopolymerization reactions were performed in parallel each catalyzed by immobilized HiC with a different protein loading. The results of this experiment show us that by decreasing the loading on the resin, the catalytic activity of the immobilized HiC will increase proportionately. This is due to enzyme aggregation on the resin particle. Furthermore, overloading of enzyme on a solid support will cause conformational changes to the enzyme. It has been clearly shown that changes in the natural enzyme
conformation will markedly alter the kinetic activity of the enzyme.\textsuperscript{3,105-108} Despite the observed results however, the relatively small increase in $M_n$ doesn’t warrant large quantities of resin to be used for condensation reactions. Moreover, excess resin will increase the diffusion constraints in the reaction which must be addressed in enzyme catalyzed polycondensation reactions. It was therefore concluded that the optimal protein loading for HiC immobilization systems is 10% w/w.

![Graph](image-url)

**Figure 14.** Plot of $M_n$ vs. time for HiC catalyzed $\omega$-hydroxyhexadecanoic acid homopolymerization comparing various amounts of protein loadings on the Amberzyme resin. Data represents an average of 2 trials (n=2).

### 3.3 Immobilization and resin studies

Following the successful results of the preliminary HiC-Lewatit catalyzed polymer synthesis reactions, the same method of immobilization was chosen as a starting point to study solid supports. To begin, two hydrophobic polymer resins have been selected for comparison of HiC synthetic activity: Lewatit VP OC 1600 (poly(methyl
methacrylate co-divinylbenzene)) and Amberlite XAD-1180 (styrene di-vinyl benzene). Bosley et al\textsuperscript{48} (1993) in their work on the lipase immobilization reported that the surface chemistry of the support and the pore size are of greatest significance and can greatly affect the synthetic activity of the enzyme. The optimal pore size is essential since if it is not large enough, the entry of the enzyme can be restricted and the efficiency of any immobilized enzyme would be low. Moreover, the surface area of the resin particle, although important is not as critical, as this merely controls the maximum amount of enzyme that can be deposited.\textsuperscript{48} Efficiency however, was found to increase in their studies with decreasing particle size. Therefore, the resins chosen for comparison are of both different physical and chemical characteristics.

\textbf{Figure 15} Chemical structure and pore distribution of Amberlite XAD1180 polymeric adsorbent. Image taken from Rohm & Haas Product Data Sheet – Amberlite XAD 1180

(As Lewatit VP 1600 is proprietary, the exact chemical structure is not available; however the physical characteristics are shown in table 1. )
<table>
<thead>
<tr>
<th></th>
<th>Surface Area (m²/g)</th>
<th>Bead size (mm)</th>
<th>Pore Diameter (Å)</th>
<th>Pore Volume (cc/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lewatit VPOC 1600</td>
<td>130</td>
<td>0.315 – 1.0</td>
<td>150</td>
<td>~0.5</td>
</tr>
<tr>
<td>Amberlite XAD-1180</td>
<td>&gt;450</td>
<td>0.350 - 0.600</td>
<td>400-450</td>
<td>~0.6</td>
</tr>
</tbody>
</table>

Table 1. Physical properties of hydrophobic resins for HiC adsorption (immobilization)

To test the catalytic activity of HiC physically adsorbed on the two solid supports, two model homopolymerization reactions (C16 ωHA) were carried out in parallel, each one catalyzed by HiC adsorbed on one of the supports. Both catalysts were successfully able to catalyze the reaction, and at the earlier time points the molecular weights of the sample were identical (see fig. 16). However, after 4h the sample catalyzed by Lewatit already started to show a significantly higher $M_n$. One explanation for the disparity in the enzyme activity between the two catalysts is that the loading on the Amberlite resin used in this experiment was considerably higher than that on the Lewatit (21% vs. 10%) and as previously discussed, overloading of enzyme on a solid support will reduce the efficacy of the catalyst. However, since Lewatit had been successfully used both previously in our lab and in this study as well, it will continue to be used as an adsorbent solid support for HiC immobilization.
Figure 16. Plot of $M_n$ vs. time for HiC catalyzed $\omega$-hydroxyhexadecanoic acid homopolymerization. Enzyme physically adsorbed on 2 different polymeric resins. Data represents an average of 2 trials (n=2).

While physical immobilization (adsorption) is a low cost, simple and effective method for enzyme immobilization, there are drawbacks that are commonly associated with physically immobilized enzymes such as the leaching of the enzyme into the reaction product due to the weak forces attaching the enzyme to the solid support, and also the adsorption of non specific proteins that might interfere with the reaction and can certainly lower the loading of our desired enzyme during the immobilization process. Although, the later is less problematic when using a purified enzyme, enzyme leaching is a serious problem, as enzyme leached into the product can hydrolyze the product in the presence of water, by degrading the ester bonds that were just formed – as is the
mechanism of HiC in nature. Furthermore, due to enzyme leaching, the reusability of the catalyst is greatly reduced. Moreover, the problem of enzyme leaching can most certainly preclude a product from being used for most any biomedical application, as certain enzyme catalysts (including cutinases) are not biocompatible. These problems are substantially reduced by using covalent immobilization whereby the enzyme is attached to the resin by strong covalent bonds which greatly reduces the amount of enzyme leaching. Several resins with epoxy functional groups have been chosen to test the synthetic efficiency of the covalent immobilization of HiC.

Parallel C16 ωHA homopolymerization reactions were carried out with five different epoxy based resins. The epoxy resins tested here are; Resindion EC-EP, Resindion EC-EP 403/S, Rohm & Haas WJZ 5349, Rohm & Haas WJZ 5346 and Rohm & Haas Amberzyme Oxirane. The epoxy resins differed in physical characteristics (i.e. bead size and pore size) and chemical composition (i.e. epoxy content) with the Rohm & Haas resins WJZ 5349 and WJZ 5346 containing a substantially higher epoxy content. The result obtained here showed that after 24h there was an increase of approximately 66% in molecular weight ($M_n$) in the HiC/Amberzyme catalyzed sample over the Resindion EC-EP 403/S catalyzed sample which had next highest $M_n$. Amberzyme Oxirane proved to be a better resin for HiC immobilization than Lewatit, both having a higher synthetic activity while reducing the possibility of enzyme leaching into the reaction. The polydispersities ($M_w/M_n$) of all the samples were similar.
Figure 17. Plot of Mn vs. time for HiC catalyzed ω-hydroxyhexadecanoic acid homopolymerization comparing various epoxy based resins using covalent and physical immobilization. Data represents an average of 3 trials (n=3). Error bars signify the standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Amberzyme</th>
<th>WJZ 5349</th>
<th>WJZ 5346</th>
<th>RESINDION EC-EP403/S</th>
<th>RESINDION EC-EP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn</td>
<td>1.37</td>
<td>1.67</td>
<td>1.44</td>
<td>1.70</td>
<td>1.25</td>
</tr>
</tbody>
</table>

Table 2. Polydispersity of C16 ω-HA polymer catalyzed by HiC immobilized on various epoxy based resins
### Table 3. Epoxy resins of different pore size and bead size.

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Resin</th>
<th>Pore size</th>
<th>Bead size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resindion</td>
<td>EC-EP</td>
<td>350 Å</td>
<td>150 -300μm</td>
</tr>
<tr>
<td>Resindion</td>
<td>EC-EP 403/S</td>
<td>1000 Å</td>
<td>200 -600μm</td>
</tr>
<tr>
<td>Rohm &amp; Haas</td>
<td>WJZ 5349</td>
<td>1000 Å</td>
<td>70μm</td>
</tr>
<tr>
<td>Rohm &amp; Haas</td>
<td>WJZ 5346</td>
<td>1000 Å</td>
<td>70μm</td>
</tr>
<tr>
<td>Rohm &amp; Haas</td>
<td>Amberzyme</td>
<td>220 Å</td>
<td>235μm</td>
</tr>
</tbody>
</table>

### 3.4 Solvent Systems for HiC Catalyzed Reactions

The choice of the organic solvent for biocatalytic conversions is critical, and conversion is affected by physical properties of the solvent such as; solvent polarity, water solubility and dielectric constant\(^{109,110}\). Kobayashi et al conducted a study comparing 11 different solvents for use in lipase catalyzed ring opening polymerization of \(\varepsilon\)-caprolactone, with results showing conversion from monomer to polymer ranging from 1% to 100\(^{111}\).

In their work on Lipase catalyzed polycondensations, it was determined by Mahapatro et al, that diphenyl ether is the optimal solvent for N435 catalyzed aliphatic polycondensation reaction\(^{112}\). Likewise, it is important to determine the optimal solvent for HiC catalyzed reactions. To this end, the small molecule butanol and lauric acid (C12
saturated fatty acid) esterification reaction was run in parallel with 12 different solvents, and the conversion from acid to ester was monitored by GC analysis. In addition, the esterification reactions in the same solvents were repeated using N435 catalysis and the results are compared. The results of this experiment showed a vast difference between the two enzyme catalysts. After the 2 hour reaction, the conversion from acid to ester among the 12 N435 catalyzed samples ranged from 36.4% to 77.3% however reactions in only 3 solvents had conversion less than 64%. In the N435 samples, the esterification reaction in diphenylether indeed had the highest conversion at 77%. Within the series of HiC reactions however, there is a vast disparity between the catalytic activities in different solvents. HiC was much more solvent selective as HiC was active in only six of the solvents used with a conversion ranging from 43% (diphenylether) to 73% (cyclohexane). The reactions performed in the other six solvents showed little or no activity (2% to 5%).

Although the reaction in diphenylether did not have the highest conversion, diphenylether does have the added important benefit of an extremely high boiling point (259 °C) which makes it a very desirable solvent for polycondensation reactions which are run at higher temperatures under reduced pressure. Despite this being a small molecule esterification reaction, and not a polyesterification reaction, the comparison of HiC catalyzed reactions and N435 catalyzed reactions is important, as we can start to see the specificity of HiC as compared with N435. We do not however expect to see the exact same behavior of HiC (or N435) in polycondensation reactions.
**Scheme 2.** Esterification of butanol and lauric acid to give butyl laurate

![Chemical structure](image)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>B. Pt.</th>
<th>Log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>80</td>
<td>2.1</td>
</tr>
<tr>
<td>cyclohexane</td>
<td>80</td>
<td>3.4</td>
</tr>
<tr>
<td>dibutylether</td>
<td>143</td>
<td>3.2</td>
</tr>
<tr>
<td>dioxane</td>
<td>101</td>
<td>-0.3</td>
</tr>
<tr>
<td>diphenylether</td>
<td>259</td>
<td>4.2</td>
</tr>
<tr>
<td>DMF</td>
<td>153</td>
<td>-0.8</td>
</tr>
<tr>
<td>DMSO</td>
<td>189</td>
<td>-1.4</td>
</tr>
<tr>
<td>isopropylether</td>
<td>69</td>
<td>1.5</td>
</tr>
<tr>
<td>nonane</td>
<td>151</td>
<td>5.3</td>
</tr>
<tr>
<td>THF</td>
<td>66</td>
<td>0.5</td>
</tr>
<tr>
<td>toluene</td>
<td>110</td>
<td>2.7</td>
</tr>
<tr>
<td>o-xylene</td>
<td>144</td>
<td>3.1</td>
</tr>
</tbody>
</table>

**Table 4.** Solvents used in esterification experiment their respective boiling points and Log P.
Figure 18. butyl laurate esterification. Plot of conversion as a function of solvent used in reaction. Reactions catalyzed by both HiC (blue) and N435 (purple). Data represents an average of 2 trials (n=2)

Although the high boiling point of diphenyl ether is a beneficial characteristic for HiC catalyzed polycondensation reactions, it also causes a significant problem. Because of the high boiling point, it is extremely difficult to remove the solvent from the finished product which combined with the extreme toxicity of diphenyl ether prevents its use for any industrial application. Even in the lab, the use of diphenyl ether as a solvent can pose a problem as it limits the methods of characterization available for certain samples. It is therefore important for us to explore other systems for our polycondensation reactions as possible alternatives to the solvent system.

An alternative system that was explored is the toluene:water azeotrope system. This system is useful since it will efficiently remove water (the byproduct of polyester
condensation reactions) without highly reduced pressure. Moreover, it limits diffusion constraints by providing a low viscosity environment. As opposed to diphenylether, toluene is very easy to remove in a rotary evaporator after the reaction is complete. It is important to note that in the azeotrope system the set temperature must be calculated based on the vacuum pressure in order to maintain a proper reaction temperature in the flask.

A model HiC catalyzed homopolymerization reaction (C16 ω-hydroxy acid) was performed both in a Greenhouse parallel reactor using diphenyl ether as the solvent, and also in a toluene:water azeotrope system. Although the reaction in diphenyl ether produced a higher molecular weight with 52.1 Kg/mol as opposed to 26.3 Kg/mol in the azeotrope system (see fig.19), because of the disadvantages of diphenyl ether as previously discussed, the success using the toluene azeotrope system is an important step in our research. Given the gap in the molecular weights, the polydispersity in both diphenyl ether and azeotrope systems were very similar with 1.67 and 1.42 respectively.
Figure 19. Plot of $M_n$ vs. time for HiC catalyzed $\omega$-hydroxyhexadecanoic acid homopolymerization comparing reactions in toluene azeotrope system to diphenyl ether. Data represents an average of 2 trials (n=2).

3.5 Thermostability of HiC

Enhancing enzyme thermal stability by immobilization has widely been reported in the literature. While experimental methods for determining the thermal stability (or $T_m$) of a free (soluble) enzyme include differential scanning calorimetry (DSC), fluorescence spectroscopy or circular dichroism (CD), determining the stability of an immobilized enzyme catalyst is often accomplished by performing parallel enzyme catalyzed reactions at various temperatures and noting the effect of the reaction temperatures on the polymerization rate. A previously reported study from our laboratory regarding the effect of temperature in HiC catalyzed ring opening polymerization reactions showed that even slight changes in temperature can greatly affect the activity of the HiC enzyme.
To determine the optimal reaction temperature for an HiC catalyzed polycondensation reaction, HiC catalyzed 1,8-octanediol/Sebacic acid polycondensation reactions were performed in parallel at various temperatures ranging from 60°C to 90°C, both in bulk (without solvent) and in diphenyl ether. This reaction was chosen as opposed to the model C16 ωHA homopolymerization, since unlike the homopolymerization reaction, this reaction can be performed in solventless conditions at lower temperatures.

![Scheme 3](image)

**Scheme 3.** Polycondensation reaction of Sebacic acid and 1, 8-octanediol

It can be seen from the results of the above reactions that there is a slight disparity in the optimal reaction temperature for HiC catalyzed reactions in solvent and in bulk. For the diol/diacid reaction that we performed here HiC is more thermostable in bulk reactions then in reactions carried out in diphenyl ether.

In bulk the enzyme activity is greatest at 75°C, but denaturation of the enzyme takes place between 75°C and 80°C. The reactions that were run at both 80°C and 90°C show little or no enzyme activity. In diphenyl ether, however, the enzyme is slightly less thermostable. The highest enzyme activity is seen between 60°C and 70°C, while denaturation of the enzyme occurs between 70°C and 75°C.
It is known that conformational changes take place when an enzyme is placed in organic solvent and the enzyme is more flexible in aqueous media than in organic solvent.\textsuperscript{3,117} Moreover, it can easily be found in the literature in various studies that reactions have been modified by performing them in an organic solvent. Pogorevc et al.\textsuperscript{118} discuss how the catalytic performance of an enzyme can be altered in organic solvents via modification of solvent properties, and region- and enantio- selectivities can be “tuned” by choice of an appropriate organic solvent. Furthermore, we can find reports of similar enzymes behaving radically different in the presence of the same organic solvent. Masomian et al.\textsuperscript{119} report that when comparing the change in activity of two similar lipases, \textit{Aneurinbacillus thermoaerophilus} lipase showed a 23\% increase in activity in DMF, while \textit{bacillus sphaericus} lipase showed a decrease of 80\% activity in DMF – both as compared to the activity in phosphate buffer pH 7.0. Thus, reaction parameters are certainly known to change in the presence of an organic solvent which would also be a cause of the lower thermostablity of HiC in organic solvent than in bulk.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Mn_vs_time.png}
\caption{Plot of Mn vs. time for HiC catalyzed polycondensation reaction of 1,8-}
\end{figure}
Octanediol and sebacic acid in bulk comparing enzyme activity at various temperatures in a bulk system. Data represents an average of 2 trials (n=2).

Figure 21. Plot of Mn vs. time for HiC catalyzed polycondensation reaction of 1,8-Octanediol and sebacic acid in diphenyl ether comparing enzyme activity at various temperatures in a diphenyl ether system. Data represents an average of 2 trials (n=2).

3.6 Comparison of Thermostability and Synthetic Activity of Various Cutinase Enzymes

Within the cutinase family of enzymes there are many enzymes which possess catalytic properties. Enzymes from the cutinase family have a similar backbone structure; however, various differences on the enzyme structure cause variations in the enzyme properties such as substrate specificity, hydrophobicity and optimal temperatures for catalytic activity. Here, the thermostability of four WT cutinases, Alternaria brassicicola cutinase (AbC), Aspergillus oryzae cutinase (AoC), FsC and HiC, all immobilized on Lewatit VOC 1600 is compared extensively in a small molecule octyl laurate esterification. The
reactions were carried out with all four WT strains in parallel at 8 different reactions with temperatures ranging from 30°C to 100°C in 10 degree increments. A shorter reaction time was used in order to terminate the reactions before equilibrium was reached, thus enabling us to note a greater disparity between the reactions carried out at different temperatures, as well as those at the same temperature with different enzymes. It is clear that the WT HiC enzyme showed both a higher catalytic activity than any of the other cutinase enzymes and a wider range of temperatures where the enzyme is substantially active, with a conversion of >50% in reactions from 30°C to 80°C.

There are several points that are interesting to note regarding the other cutinase enzymes used in this experiment. AbC, which has the lowest catalytic activity is seen on average to have a peak activity at 60°C, which is higher than the other enzymes where the peak activity is seen to be at 50°C (or 40°C for FsC). Notwithstanding the overall low catalytic activity, at 60°C to 70°C the rate of conversion is higher than that of FsC and similar to that of AoC. Moreover, at 70°C the conversion is not substantially less than that of HiC with a 38% and 47% conversion respectively.

It is also interesting to note that the catalytic activity of AoC remains relatively stable from 40°C to 80°C. In that wide range of 40 degrees the rate of conversion changes only ± 8% which could possibly be quite useful in a situation with large temperature fluctuations.
Figure 22. Temperature stability study of four wild-type cutinase enzymes (AbC, AoC, FsC and HiC). Octyl laurate esterification. Data represents an average of 3 trials. Error bars signify the standard deviation.

A study comparing the polysynthetic activity of the genetically modified HiC (which we are studying here) to various other cutinase enzymes; wt AbC, wt AoC, wt FsC and another genetically modified cutinase, *Pseudomonas mendocina* cutinase (PmC) - a bacterial cutinase modified by Genencor - was also conducted. It’s worthwhile to note here that both the HiC and the PmC have been modified to optimize hydrolysis reactions; not synthesis reactions.

A homopolymerization of 16 carbon ω-hydroxyalkanoic acid (ωHA) – was again performed in parallel using the various cutinase enzymes to catalyze the reactions. The molecular weight ($M_n$) buildup as a function of time was followed for 24h and compared to see the catalytic activity of each of the cutinase enzymes.
The Novozymes HiC can be seen to have far greater catalytic activity than any of the other cutinase enzymes tested. Although at 2h all the samples have more or less the same molecular weight, already at the 4 hour point the HiC catalyzed sample has double the $M_n$ of the AoC catalyzed sample and almost 4X the $M_n$ of the AbC catalyzed sample. At the end of the 24h reaction the catalytic activity of HiC is 2X and 5X greater than that of AoC and AbC respectively. The PmC and FsC catalyzed samples were of much lower molecular weight with 2817 Kg/mol and 3177 Kg/mol respectively. All of the cutinases produced samples with relatively low polydispersities ($M_w/M_n$), ranging from 1.13 to 1.52.

![Plot of Mn vs. time for ω-hydroxyhexadecanoic acid homopolymerizations catalyzed by several various cutinase enzymes comparing synthetic activity in the various enzymes. Data represents an average of 3 trials. Error bars signify the standard deviation.](image)
<table>
<thead>
<tr>
<th>HiC</th>
<th>AoC</th>
<th>AbC</th>
<th>PmC</th>
<th>FsC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2605</td>
<td>1.528245</td>
<td>1.134324</td>
<td>1.183589</td>
<td>1.164004</td>
</tr>
</tbody>
</table>

*Table 5.* Polydispersities (Mw/Mn) of 24h samples catalyzed by various cutinase enzymes.
CHAPTER 4

Exploring Chain Length Selectivity in HIC-Catalyzed Polycondensation Reactions

4.1 Homopolymerization of ω-Hydroxyalkanoic Acids (HAs).


In the first set of experiments, ω-HAs with 6, 10, 12, and 16 carbons were used as monomers for HiC-AO- and N435-catalyzed homopolymerization reactions (See Scheme 4). Figure 22a shows that HiC-AO is highly active for polymerizations of C16-ωHA. For polymerizations of C12-ωHA, a lag period for up to 2 h was observed. However, by 6 and 10 h reaction times, poly(C12-ωHA) and poly(C16-ωHA) reached similar Mn values (at 10 h, 32.1 and 40.3 kg/mol, respectively). Polydispersity (Mw/Mn) values were likewise similar at 1.7 and 1.4, respectively. By further decreasing the monomer chain length by only two carbons (C10-ωHA), polymer formation was not observed. Similarly, HiC-AO was not active for polymerizations of C6-ωHA. Figure 22b displays the results of N435-catalyzed polycondensation reactions with the same set of ωHA monomer substrates. N435-catalyzed polymerization of C16-ωHA and C12-ωHA occur at similar rates giving polyesters with nearly identical Mn values (25.5 kg/mol and 24.5 kg/mol, respectively, at 8 h). Again, polydispersity was likewise similar at 2.0 and 2.3, respectively. Furthermore, whereas HiC-AO was inactive for C10-ωHA polymerizations, N435 accepts C10-ωHA as a substrate, forming poly(C10-ωHA) with Mn values of 5.0 and 11.1 kg/mol at 1 and 8 h, respectively (PDI 1.4 for both time points). However, as was observed with HiC-AO,
N435 was inactive for polymerization of C6-ωHA. Thus, HiC-AO’s activity for ωHA substrates was as follows: C16 > C12, where C10-ωHA and C6-ωHA were not polymerized. In contrast, N435’s activity for ωHA substrates was as follows: C16 > C12 > C10, where C6-ωHA was not polymerized. In a set of control experiments where no catalyst was used, Mn values of under 1 kg/mol were attained for each of the substrates.
Figure 24. Enzyme-catalyzed polyesterification of linear aliphatic $\omega$-hydroxyacids (HA) (70 °C, in diphenylether, 1% wt/wt enzyme HiC-to-monomer, with application of vacuum after 2 h): (a) HiC-AO catalysis, extent of $M_n$ as a function of time; (b) N435 (immobilized CALB) catalysis, $M_n$ as a function of time. Data represents an average of 2 trials (n=2).
4.2 Copolymerization of Sebacic Acid with Diols of Varying Chain Lengths

Scheme 5. HiC-AO and N435-catalyzed copolymerizations of (a) sebacic acid and \(\alpha,\omega\)-n-alkane diols of varying chain lengths, (b) 1,8-octanediol with \(\alpha,\omega\)-n-alkane diacids of varying carbon chain lengths

For this second set of experiments, polymerizations were performed in bulk. Sebacic acid and diols with chain lengths of 3, 4, 5, 6, and 8 carbons were used as monomers for HiC-AO- and N435-catalyzed copolymerization reactions (see Scheme 5a). Figure 25a shows that HiC-AO is active for copolymerizations of both C6- and C8-diols, although activity is higher for the latter. Indeed, by 2 h, \(M_n\) values for poly(hexylsebacate) and poly(octylsebacate) are 1.7 and 6.6 kg/mol, respectively (PDI 1.5 and 1.7, respectively), and at 8 h, \(M_n\) values are 6.5 and 10.2 kg/mol, respectively (PDI 1.9 and 2.9, respectively). By decreasing the diol carbon chain length by only one methylene unit, from C6 to C5, HiC-AO no longer converted these substrates to polyesters. Similarly, C4 and C3 diols were not copolymerized with sebacic acid by HiC-AO. Figure 25b displays results of N435-catalyzed polycondensation reactions with the
same set of diol monomer substrates. In contrast to HiC-AO, N435 is active on diols of all chain lengths. Diol monomers with C5, C6, and C8 chain lengths are polymerized at similar rates, hence N435 does not differentiate between these substrates. Copolymerizations of C3 and C4 diols proceed relatively slower. By 1 h, Mn values for copolymerizations of sebacic acid with C3, C4, C5, C6, and C8 diols are 2.0, 3.0, 3.8, 4.0, and 4.2 kg/mol, respectively, with PDI values of 1.9, 1.7, 1.6, 1.7, and 1.8, respectively. Thus, for HiC-AO, its activity for diol substrates was as follows: C8>C6, where C3, C4, and C5 diols were not polymerized. In contrast, N435 showed greater substrate promiscuity such that all diol chain lengths studied were converted to polyesters. N435’s relative activity for diol substrates was as follows: C8=C6=C5>C4>C3. In a set of control experiments where no catalyst was used, Mn values of under 1 kg/mol were attained for each of the substrates.
**Figure 25.** Enzyme-catalyzed polyesterification of linear diols with sebacic acid (70 °C, in bulk, diol diacid 1:1 mol/mol, 1% w/w of enzyme-to monomer, application of vacuum after 2 h): (a) HiC-AO catalysis, $M_n$ as a function of time; (b) N435 catalysis (immobilized CALB), $M_n$ as a function of time. Data represents an average of 2 trials (n=2).
4.3 Copolymerization of 1,8-Octanediol with Diacids of Varying Chain Lengths

Reactions were performed without solvent, at 70°C, under vacuum as above. 1,8-Octanediol and diacids with chain lengths of 6, 8, 9, 10, and 13 carbons were used as monomers for HiC-AO- and N435-catalyzed copolymerization reactions (see Figure 24). Figure 26a shows HiC-AO has similar activity for C10 and C13 diacids. For 2 h, Mn values for poly(octylsebacate) and poly(octylbrassylate) are 6.6 and 5.1 kg/mol, respectively, while at 8 h, Mn values are 10.2 and 11.1 kg/mol, respectively (PDI 2.9 and 1.8, respectively). In agreement with the above results, HiC-AO activity for polyester synthesis is extraordinarily sensitive to diacid chain-length. HiC-AO showed little activity for conversion of C9-diacid to polyester, whereas, for C10-diacid, polyester synthesis was rapid. In accord with the low activity found for C9-diacid, a further decrease in diacid chain length to C8 and C6 resulted in similarly low activity for conversion to polyester.

Figure 4b displays the results of N435-catalyzed polycondensation reactions with the same set of diacid monomer substrates. In agreement with studies of diols with differing chain lengths, N435 shows very different selectivity then HiC-AO as it is active on diacids of all chain lengths studied herein. By 1 h, Mn values for N435-catalyzed copolymerizations of 1,8-octanediol with C6, C8, C9, C10, and C13 diacids are 4, 4, 5, 5, and 6 kg/mol, respectively. Therefore, N435 does not differentiate between these substrates even though they differ in chain length by up to seven carbons. Thus, for HiC-AO, its activity for diacid substrate copolymerization with 1,8-octanediol was as follows:

C13 = C10, where C6, C8, and C9 diacids showed little activity for copolymerization.

N435 showed greater substrate promiscuity such that all diacid chain lengths studied were converted to polyesters. Furthermore, within experimental error, N435 co-polymerizations
in Figure 26b proceeded with equivalent rates of Mn increase as a function of reaction time. In the set of control experiments where no catalyst was used, Mn values of under 1 kg/mol were once again attained for each of the substrates.

**Figure 26.** Enzyme-catalyzed polyesterification of 1,8-octanediol and linear diacids (70 °C, in bulk, diol diacid 1:1 mol/mol, 1% w/w of enzyme-to monomer, application of vacuum after 2 h): (a) HiC-AO catalysis, extent of $M_n$ as a function of time; (b) N435 catalysis (immobilized CALB), $M_n$ as a function of time. Data represents an average of 2 trials ($n=2$).
4.4 Small Molecule Model Systems

4.4.1 Esterification of Lauric Acid with \( n \)-Alkanols of Varying Chain Lengths

Scheme 6: HiC-AO and N435-catalyzed esterifications (70 °C, in diphenyl ether) between: i) lauric acid and \( n \)-alkanols with chain lengths of 3, 4, 5, 6, 8 and 12 carbons, and ii) hexanol was reacted with \( n \)-alkanoic acids having chain lengths of 6, 8, 10, 12, 14 and 16 carbons.

This work compares the chain length selectivity of HiC-AO and N435 for a series of small molecule esterification reactions and determines the extent that this selectivity correlates with that observed above for polycondensation polymerizations. Reactions were performed in diphenylether (1:1 ratio) in capped tubes at 70 °C. Lauric acid and \( n \)-alkanols with chain lengths of 3, 4, 5, 6, 8, and 12 carbons were used as substrates for HiC-AO- and N435-catalyzed esterification reactions.

Results of reactions conducted for 30 min are displayed in Figure 27a. HiC-AO shows a preference for the C6 alcohol relative to alcohols of longer and shorter chain length. Percent conversion for HiC-AO-catalyzed esterifications between lauric acid and 3-, 4-, 5-, 6-, 8-, and 12-carbon \( n \)-alkanols are 18, 37, 36, 72, 38, and 6, respectively. Comparison to N435-catalyzed esterifications between lauric acid and \( n \)-alkanols of
differing chain length shows similar trends. Hence, hexanol is the preferred substrate and alcohols of shorter and longer chain lengths reach lower conversion values. Percent conversion for N435-catalyzed esterifications between lauric acid and 3-, 4-, 5-, 6-, 8-, and 12-carbon \( n \)-alkanols are 41, 63, 58, 84, 73, and 46, respectively. For all \( n \)-alkanol chain lengths, N435 catalysis results in higher ester \%\)-conversion values. Thus, N435 has higher reactivity than HiC-AO for this series of substrates. Furthermore, consistent with results discussed above for polycondensation reactions, N435 shows a broader specificity as a function of \( n \)-alkanol chain length. That is, using N435, the maximum difference in \%\)-conversion between any two substrates is a factor of 2X whereas, for HiC-AO, a difference in \%\)-conversion of 12X is observed between \( n \)-hexanol and \( n \)-dodecanol. Comparison between HiC-AO catalysis as a function of alcohol chain length for the small molecule model system and polymerization results in Figure 27a shows interesting contrasts. Whereas \( n \)-propanol and \( n \)-butanol show significant extents of esterification over 30 min reactions with lauric acid, 1,3-propane diol and 1,4-butanediol show no significant chain growth over 8 h. Furthermore, whereas \( n \)-hexanol reacts more rapidly than \( n \)-octanol with lauric acid, chain growth occurs much more rapidly for copolymerizations between sebacic acid and 1,8-octanediol relative to 1,6-hexanediol. In contrast, there is good agreement found when comparing N435 catalysis as a function of alcohol chain length for the small molecule model system and polymerization results (Figure 27b). For polycondensations between sebacic acid and \( n \)-alkanediols, as the chain length increases from C3 to C4 and C6, there is an increase in the rate at which chain growth occurs. Furthermore, C6 and C8 \( n \)-alkanediols form polyester with sebacic acid at identical rates. Similarly as \( n \)-alkanol chain length increases from C3 to C4 and C6,
esterification with lauric acid occurs to higher extents. Moreover, C6 and C8 \( n \)-alkanols form ester with lauric acid at similar rates.

4.4.2 Small Molecule Model System: Esterification of Hexanol with \( n \)-Alkanoic Acids of Varying Chain Lengths

In addition to the above model, studies between \( n \)-alkanols and lauric acid, a series of esterification reactions catalyzed by HiC-AO and N435 were performed where hexanol was reacted with \( n \)-alkanoic acids having chain lengths of 6, 8, 10, 12, 14, and 16 carbons. Results of reactions conducted for 30 min are displayed in Figure 27b. HiC-AO shows similarly high reactivity with \( n \)-alkanoic acids having 8, 10, 12, and 14 carbons. However, a small decrease and increase in \( n \)-alkanoic acid chain length to C6 and C16, respectively, results in a large decrease in ester formation. Percent conversion for HiC-AO-catalyzed esterifications between \( n \)-hexanol and 6-, 8-, 10-, 12-, 14-, and 16- carbon \( n \)-alkanoic acids are 14, 70, 73, 70, 65, and 13%, respectively. Comparison to N435-catalyzed esterifications between \( n \)-hexanol and \( n \)-alkanoic acids of differing chain length shows that, unlike HiC-AO, N435 does not differentiate between any of the \( n \)-alkanoic acid chain lengths studied. Percent conversion for N435-catalyzed esterifications between hexanol and 6-, 8-, 10-, 12-, 14-, and 16-carbon \( n \)-alkanoic acids are 74, 70, 75, 76, 76, and 83%, respectively. Thus, consistent with results discussed above for polycondensation reactions, N435 shows a broader specificity as a function of \( n \)-alkanoic acid chain length. That is, using N435, there is little difference in \% -conversion between any two substrates, whereas, for HiC-AO, a difference in \% -conversion of 5X is observed between decanoic acid and both \( n \)-hexanoic and palmitic acids. Comparison between
HiC-AO catalysis as a function of \( n \)-alkanoic acid chain length for the small molecule model system and polymerization results in Figure 4a shows interesting contrasts. Whereas octanoic acid showed significant extent of esterification over 30 min reactions with \( n \)-hexanol, suberic acid (C8)/1,8- octanediol copolymerization showed no significant chain growth over 8 h. However, there is excellent agreement for HiC-AO catalyzed esterification of C6 and C10 \( n \)-alkanoic acids and polyester synthesis with corresponding C6 and C10 diacids. That is, the C6 \( n \)-alkanoic acid and diacid (adipic acid) were poor substrates, whereas the C10 \( n \)-alkanoic acid and diacid (sebacic acid) were very good substrates for ester and polyester synthesis, respectively. In contrast, when comparing N435 catalysis as a function of \( n \)-alkanoic acid and diacid chain lengths for the small molecule model system (Figure 27b) and polymerization reactions (Figure 26b), respectively, excellent agreement is found. That is, for polycondensations between 1,8-octanediol and diacids of chain lengths C6 (adipic) to C13 (brassylic acid), all diacids were good substrates with similar rates of molecular weight increase. Similarly, for \( n \)-alkanoic acids of chain lengths C6-C14, esterification reactions with \( n \)-hexanol occurred to similar extents (\( \sim 70\% \)). Again, these results for small molecule and polyester synthesis for HiC and N435 catalysis with \( n \)-alkanoic acid and diacids of different chain lengths supports the generally broader range of substrates accepted by N435.

It is not surprising to discover there are differences between chain length selectivity for small molecule model systems and polycondensation reactions. Such variations may originate due to inherent differences in model and polycondensation reactions. Small molecule condensation reactions have well-defined acyl donor and acceptor species. In contrast, after conversion of monomers to dimers and higher
molecular weight species, polycondensation reactions may have an array of acyl acceptor and donor species differing in oligomer and polymer chain lengths. Furthermore, small molecule substrates are monofunctional $n$-alkanol and $n$-alkanoic acids, whereas polyester synthesis is performed with difunctional acid and hydroxyl monomers. Nevertheless, determining the extent of agreement between small molecule esterification and polycondensation reactions as a function of precursor chain length is important since similarities in substrate specificities can greatly simplify investigations by modeling to explain enzyme selectivity. For this study, what is undoubtedly true is that comparisons of N435 and HiC-AO first for polycondensation reactions and then for small molecule substrates differing in chain length provides insights into similarities and deviations in substrate selectivity.
Fig. 27. HiC-AO and N435 catalyzed esterification reactions (30 min, 70 °C, in diphenylether, molar ratio of alcohol-to-acid is 1:1, 1 % by-wt enzyme to total monomer substrates): (a) extent conversion of reactions between lauric acid and n-alkanols of varying chain length. (b) extent conversion of reactions between hexanol and n-alkanoic acids of varying chain length. Data represents an average of 3 trials (N=3). Error bars signify the standard deviation.
4.5 Further Comments on Relative Activities of HiC and N435 for Polycondensation Reactions

Review of results above show that, for both N435 and HiC-AO, the preferred diacid, diol, and ω-hydroxyalkanoic acid substrates are brassylic acid (C13-diacid), 1,8-octanediol, and ω-hydroxyhexadecanoic acid (C16-ωHA), respectively. To facilitate careful comparison of N435 and HiC-AO activities using these preferred substrates, $M_n$ and $M_w/M_n$ (PDI) values are listed in Tables 6 and 7 for C16-ωHA homopolymerizations and copolymerizations of 1,8-octanediol with C13-diacid, respectively. For both polymerization reactions, N435 catalyzes more rapid chain growth from 0 to 1 h. Values of $M_n$ at 1 h for N435- and HiC-AO-catalyzed C16-HA homopolymerizations are 8.6 and 4.7 kg/mol, respectively. Similarly, using these catalysts, $M_n$ values at 1 h for 1,8-octanediol/C13-diacid copolymerizations are 5.7 and 3.0 kg/mol, respectively. For both the C16-HA and 1,8-octanediol/C13-diacid polymerizations, observation of Tables 6 and 7 shows that, from 2 to 8 h, chain growth occurs more rapidly using HiC-AO as catalyst. For example, for C16-HA polymerizations using HiC-AO and N435, the $M_n$ increase from 2 to 8 h is 9.7 to 40.4 kg/mol and 11.7 to 25.5 kg/mol, respectively. PDI values for 1,8-octanediol/C13-diacid copolymerizations catalyzed by HiC-AO and N435 are similar and generally have values of about 1.7. However, PDI values for C16-HA polymerizations catalyzed by HiC-AO remain at $\leq 1.5$, whereas, with N435 catalysis, PDI reaches 2.0 for products formed at 4 and 8 h, respectively. Narrow PDI values have previously been reported by our
laboratory for both N435 and HiC catalysis\textsuperscript{16,120} and this phenomenon was attributed to “chain selectivity” of these catalysts during propagation steps.

<table>
<thead>
<tr>
<th>rxn. time</th>
<th>HiC-AO catalysis</th>
<th>N435 catalysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$M_n$</td>
<td>PDI</td>
</tr>
<tr>
<td>15 min</td>
<td>1 800</td>
<td>1.3</td>
</tr>
<tr>
<td>30 min</td>
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<td>4 h</td>
<td>26 000</td>
<td>1.4</td>
</tr>
<tr>
<td>8 h</td>
<td>40 400</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Table 6.** $M_n$ and PDI ($M_w/M_n$) values for homopolymerizations of C16-ωHA catalyzed by HiC-AO and N435

<table>
<thead>
<tr>
<th>rxn. time</th>
<th>HiC-AO catalysis</th>
<th>N435 catalysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$M_n$</td>
<td>PDI</td>
</tr>
<tr>
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<td>1 000</td>
<td>1.6</td>
</tr>
<tr>
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</tr>
<tr>
<td>8 h</td>
<td>11 000</td>
<td>1.8</td>
</tr>
</tbody>
</table>

**Table 7.** $M_n$ and PDI ($M_w/M_n$) values for copolymerizations of 1,8-octanediol and brassyllic acid catalyzed by HiC-AO and N435

It is noteworthy that, during the first 2 h of reactions, vacuum is not applied. Hence, one explanation for more rapid chain growth at earlier and later reaction times with N435 and HiC-AO, respectively, may be differences in CALB and HiC activities as a function of
reaction water content. HiC may have optimal activity at lower reaction water contents, whereas CALB may exhibit the opposite behavior. Indeed, previous work performed by our laboratory showed that, for N435-catalyzed reactions where no vacuum is applied, polymer chain growth is quite rapid at early reaction stages.\textsuperscript{121} To the best of our knowledge, there are no literature reports that describe the relationship between water concentration in organic media and its effect on HiC activity for ester formation.
CHAPTER 5

HiC catalysis of hyperbranched polyester polyols

5.1 HiC Catalyzed Glycerol Polyester Reactions

In the first set of experiments sebacic acid (2mM) was reacted with a varying proportion of 1,8-octanediol and glycerol with a 1:1 ratio. The glycerol content was increased in 10% increments from 0 mol% to 60 mol%.

Scheme 7. HiC-AO and N435-catalyzed terpolymerization reaction of sebacic acid with 1,8-octanediol and glycerol

Reactions with glycerol content ranging from 0 to 20 mol% appear to be identical in the increase of their molecular weight ($M_n$) as a function of time for the first 8 hours of the reaction (see figure 28). At the 24h time point the 10 mol% and 20 mol% glycerol samples show only a slight increase in molecular weight from the 8h time point (3.2 Kg/mol and 4.2 Kg/mol increase respectively), while the reaction with no glycerol content continued to build molecular weight from 8h to 24 h (6.9 Kg/mol increase).
Figure 28. HiC-AO catalyzed terpolymerization reaction of sebacic acid with 1,8-octanediol and glycerol. Ratio of glycerol to 1,8-octanediol varying from 0:1 to 0.2:0.8 Data represents an average of 2 trials (n=2).

Polydispersity of all 3 reactions were also almost identical for the first 4 hours of the reaction (see figure 29). The PDi of the two reactions containing 10 and 20% glycerol content, continue to grow dramatically at the 8h time point and even more so at the 24h time point while the PDi of the sample with no glycerol content remains basically unchanged after 4 hours.
Figure 29. Polydispersity (Mw/Mn) of HiC-AO catalyzed terpolymerization reaction of sebacic acid with 1,8-octanediol and glycerol. Ratio of glycerol to 1,8-octanediol varying from 0:1 to 0.2:0.8

At 4h the polydispersities of the 3 reactions (0, 10 and 20 mol% glycerol) are 1.8, 1.8 and 2.0 respectively, while at 24h the polydispersities of the same three reactions are 1.8, 3.3 and 3.3 respectively. When comparing the HiC-AO catalyzed terpolymerization reactions with 30 mol% and 40 mol% glycerol, it is apparent that these two samples once again show the same trend in molecular weight (Mn) build up as a function of time during the first four hours of the reaction, after which the 30 mol% glycerol sample continues to build molecular weight while the 40 mol% sample plateaus (see figure 30).
Figure 30. HiC-AO catalyzed terpolymerization reaction of sebacic acid with 1,8-octanediol and glycerol. Ratio of glycerol to 1,8-octanediol varying from 0.3:0.7 to 0.4:0.6 (a) extent of Mn as a function of time, (b) Polydispersity (Mw/Mn) as a function of time. Data represents an average of 3 trials (n=3). Error bars signify the standard deviation.

The molecular weight ($M_n$) increase in the 30 mol% reactions from 4h to 24h was 5.3 Kg/mol while for the same points the increase in the 40 mol% reactions was only 3.3 Kg/mol. This same trend was seen previously when studying the reactions containing 0 mol% to 20 mol% glycerol. There is a reciprocal relationship between the increased glycerol content in the reaction and the final molecular weight of the product. As the
glycerol content in the reaction is increased, the molecular weight of the product is decreased. This can be explained by the fact that increases in glycerol shift the stoichiometry of the reaction. An equimolar reaction solely between sebacic acid and 1,8-octanediol has a 1:1 ratio between COOH groups and OH groups. With each addition of 10 mol% glycerol, this reaction stoichiometry will shift by 0.1, i.e., a reaction with 10 mol% glycerol with have a 2:2.1 ratio between COOH and OH groups, a reaction with 20 mol% glycerol the stoichiometry will shift again to 2:2.2 and in a reaction with 30 mol% glycerol the ratio will shift again to 2:2.3 and so on. Polydispersity, on the other hand will increase as the glycerol content in the reaction increases. When examining the HiC-AO catalyzed terpolymerization reaction with 50 and 60 mol% glycerol it is apparent that only low molecular weight polymer (2.8 kg/mol) can be achieved with 50 mol% and no polymerization occurred with 60 mol% glycerol (see figure 31).
Figure 31: HiC-AO catalyzed terpolymerization reaction of sebacic acid with 1,8-octanediol and glycerol. Ratio of glycerol to 1,8-octanediol varying from 0.5:0.5 to 0.6:0.4 (a) extent of Mn as a function of time, (b) Polydispersity (Mw/Mn) as a function of time. Data represents an average of 2 trials (n=2).

Likewise, little or no change in polydispersity is observed in these reactions. Looking at the Mn and PDi values as a function of increasing glycerol content (8h time point), the trends in Mn and PDi are apparent (see table 8).
<table>
<thead>
<tr>
<th>Mol% Glycerol</th>
<th>Mn</th>
<th>Mw</th>
<th>PDi</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>9900</td>
<td>27000</td>
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<td>1500</td>
<td>2900</td>
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</table>

Table 8. $M_n$, $M_w$, and PDI ($M_w/M_n$) values for HiC-AO catalyzed terpolymerizations of sebacic acid with 1,8-octanediol and glycerol. Reaction time is 8h for all samples.

5.1.1 Comparison with N435 Catalyzed Samples

Three of the proceeding reactions were repeated using N435 catalysis, while all the reaction conditions remained otherwise the same in order to create a fair comparison between the two enzyme catalysts (see figure 32). It is interesting to note that the HiC catalyzed reactions with lower glycerol content (i.e. 20 and 30 mol%) produced a polymer with a higher molecular weight and a more stable polydispersity than their N435 catalyzed counterparts. At 24h the $M_n$ of the HiC catalyzed product with 20 mol% and 30 mol% glycerol are 12.6 kg/mol (PDi 3.3) and 12.0 kg/mol (PDi 2.9) respectively. The same reactions with N435 catalysis at 24h yielded $M_n$ of 10.8 kg/mol (PDi 2.8) and 8.0 kg/mol (PDi 5.4). The reaction with higher glycerol content (i.e. 50 mol%) however, was more successful with N435 yielding a polymer at 24h with $M_n$ 5.6 kg/mol (PDi 4.3) as opposed to the HiC catalyzed reaction which at 24h had an $M_n$ of only 2.9 kg/mol.
Figure 32. HiC-AO and N435 catalyzed terpolymerization reactions of sebacic acid with 1,8-octanediol and 20, 30 and 50 mol% glycerol.

### 5.1.2 Glycerol Polyester Polymer Structure Analysis

A study was conducted to analyze and compare the structure of the HiC and N435 catalyzed polymer samples with varying glycerol content, specifically 20, 30 and 50 mol% glycerol. The reactions were performed in parallel and a control experiment without any enzyme catalyst was also performed yielding an $M_n < 1000$ and no ester peak was observed in the $^1H$ NMR spectra. Structural analysis was performed on these six samples after 4h reaction time by $^{13}C$ NMR in d-CHCl$_3$. From the NMR spectra it is possible to determine the observed molar ratio of octyl sebacate units to glycerol sebacate units (OS: GS) as described by Hu et al$^{122}$ and listed here in table 9. Comparing both the observed OS: GS ratio and the actual feed ratio of 1,8- octanediol to glycerol in a given reaction (i.e. 20, 30 and 50 mol% glycerol) using the different catalysts, we can see that the observed ratio after 4 hours is identical in both HiC and N435 catalyzed samples. Furthermore, this observed ratio is also almost identical to the actual feed ratio in all
samples. Therefore, it is apparent that already at 4h the majority of the glycerol
introduced in the feed, has been reacted. Kulshrestha et al\textsuperscript{123} have reported a similar
result with N435 catalyzed glycerol polyol reactions using adipic acid (C6) in place of the
sebacic acid (C10), while Hu et al\textsuperscript{122} show that for that same reaction using adipic acid,
less than half of the glycerol introduced in the reaction has been incorporated in the
polymer at the 2h time point.

The percent of branching is classically calculated using one of the two following
equations.

Frechet’s equation\textsuperscript{124}:

\[
(1) \quad \% \text{ branching} = \frac{\text{(no. of dendritic units)} + \text{(no. of terminal units)}}{\text{total no. of units}} \times 100
\]

Frechet explains that as terminal units as well as the dendritic units contribute to the
branched character of the polymer, both are considered in the calculation of percent
branching, as opposed to the linear units, which in fact decrease the degree of
branching.\textsuperscript{124}

Frey’s equation\textsuperscript{125}:

\[
(2) \quad \% \text{ branching} = \frac{2 \times \text{no. of dendritic units}}{(2 \times \text{no. of dendritic units}) + \text{(no. of linear units)}} \times 100
\]

Frey explains that “the degree of branching of a hyperbranched molecule may be
expressed in terms of the number of actual growth directions compared to the maximum
number of possible growth directions”. In the Frechet equation however, “already the
linear direction is counted as a branching direction, which leads to an overestimation for
small or little branched molecules (i.e. in low generations).\textsuperscript{125}” Furthermore, the Frey
equation does not require the determination of the terminal units, which specifically in
the case of the COOH terminal units is not trivial and the assignment of their
corresponding peaks on the $^{13}$C NMR spectra is not reliable.$^{125,126}$ Therefore, the Frey
equation was used here to calculate percent branching.

As was expected given the relatively short reaction time (4h), the percent
branching for all our samples was quite low ranging from 1.7% in both 20 mol% glycerol
samples to 4.7% in the N435 catalyzed 50 mol% glycerol sample. In the work by
Kulshrestha et al$^{123}$ the higher percent branching that was reported with N435 (19% and
36% branching for 20 mol% glycerol and 50 mol% glycerol respectively) was only after
a reaction time of 42h.

The regioselectivity of acylation at the primary OH sites of the glycerol units was
calculated based on the relative intensity of $^{13}$C NMR peaks. The peaks considered were
those corresponding to the methine carbon in glycerol in each of the various substitution
scenarios (L$_{1,2}$, Terminal, Den., and L$_{1,3}$). The percentage values of regioselectivity were
determined by the following equation$^{101}$:

$$
\text{Regioselectivity at primary hydroxyl sites (\%)} = \frac{[L_{1,2}] + [\text{Terminal}] + 2[\text{Den}] + 2[L_{1,3}]}{2[L_{1,2}] + [\text{Terminal}] + 3[\text{Den}] + 2[L_{1,3}]} \times 100
$$

(3)

All of our samples proved to have almost an identical regioselectivity ranging only from
84.2 to 87.8% (a difference of only 3.6%) regardless of the glycerol content or the
catalyst used. A high regioselectivity for primary hydroxyls has previously been show for
CALB and reported by Kumar et al and others in our group.$^{122,123,127}$ We can now show
the same high degree of regioselectivity for primary hydroxyls in our HiC catalyst.
The percent of glycerol hydroxyls that are substituted is also determined by the $^{13}$C NMR spectra using the following equation\textsuperscript{122}:

\begin{equation}
\text{Substitution at glycerol sites (\%)} = \frac{2[L_{1,2}] + [\text{Terminal}] + 3[\text{Den}] + 2[L_{1,3}]}{3([L_{1,2}] + [\text{Terminal}] + [\text{Den}] + [L_{1,3}])} \times 100
\end{equation}

It is likewise apparent that at 4h, despite changes in the glycerol content the percent of hydroxyls substituted in the glycerol unit remains rather constant for all samples except for the HiC catalyzed 50 mol\% glycerol samples where the substitution is slightly lower. This is understandable given the substantially lower activity of HiC in reactions with higher glycerol content as apparent from the low molecular weight of that sample (see figure 32 and table 9).

Since the dendritic repeat units play an essential role in the hyperbranched character of the polymer, it is important to compare the mol\% of dendritic substituted units in N435 and HiC samples with the same glycerol content. In both the 20 and 50 mol\% glycerol reactions the N435 catalyzed samples showed a higher percent of dendritic substituted units (23 and 31\% higher respectively). In the 30 mol\% glycerol reactions, however, HiC showed a substantially higher percentage of dendritic substituted units over the N435 catalyzed counterpart (51\% higher). This can also be explained by the enzyme activity in each reaction at the relatively early 4h time point. In the 20 mol\% glycerol reaction, HiC is more active than CALB in longer reaction durations (24H) (see figure 32) however, at the early stages of the reaction (4h) CALB has a higher level of
catalytic activity than does HiC. HiC has shown to have maximum activity in glycerol copolymerization reactions with 30 mol% glycerol – substantially higher than CALB - while still retaining high activity at 40 mol%. At 50 mol% glycerol, however, the catalytic activity of HiC drops significantly and does not increase with longer reaction times.

<table>
<thead>
<tr>
<th></th>
<th>GH20</th>
<th>GN20</th>
<th>GH30</th>
<th>GN30</th>
<th>GH50</th>
<th>GN50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed (OS:GS)</td>
<td>80:20</td>
<td>80:20</td>
<td>70:30</td>
<td>70:30</td>
<td>50:50</td>
<td>50:50</td>
</tr>
<tr>
<td>Mn (mol/g)</td>
<td>5850</td>
<td>7336</td>
<td>6565</td>
<td>4100</td>
<td>1645</td>
<td>2570</td>
</tr>
<tr>
<td>Mw (mol/g)</td>
<td>10330</td>
<td>15917</td>
<td>23134</td>
<td>11532</td>
<td>3560</td>
<td>6038</td>
</tr>
<tr>
<td>PDi</td>
<td>1.76</td>
<td>2.16</td>
<td>3.52</td>
<td>2.81</td>
<td>2.16</td>
<td>2.35</td>
</tr>
<tr>
<td>Branching (%)</td>
<td>1.71</td>
<td>1.72</td>
<td>3.22</td>
<td>2.85</td>
<td>3.55</td>
<td>4.69</td>
</tr>
</tbody>
</table>

**Table 9:** HiC-AO and N435 catalyzed glycerol polycondensations: Polyol Composition, Molecular Weight Averages, and Degree of Branching. Reaction time is 4h for all samples.
Table 10. HiC-AO and N435 catalyzed glycerol polycondensations: Relative percentages of linear, terminal and dendritic glycerol units and average values for percent substitution and regioselectivity. Reaction time is 4h for all samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>L1,2 (mol%)</th>
<th>Terminal (mol%)</th>
<th>Dendritic (mol%)</th>
<th>L1,3 (mol%)</th>
<th>Average substitution at glycerol site (%)</th>
<th>Regioselectivity for Primary hydroxyl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiC 20%</td>
<td>19.29%</td>
<td>25.21%</td>
<td>8.24%</td>
<td>47.25%</td>
<td>61.01%</td>
<td>84.95%</td>
</tr>
<tr>
<td>N435 20%</td>
<td>18.50%</td>
<td>29.02%</td>
<td>10.14%</td>
<td>42.34%</td>
<td>60.37%</td>
<td>84.19%</td>
</tr>
<tr>
<td>HiC 30%</td>
<td>12.78%</td>
<td>27.93%</td>
<td>11.99%</td>
<td>47.30%</td>
<td>61.35%</td>
<td>86.54%</td>
</tr>
<tr>
<td>N435 30%</td>
<td>21.18%</td>
<td>25.16%</td>
<td>7.95%</td>
<td>45.70%</td>
<td>60.93%</td>
<td>84.06%</td>
</tr>
<tr>
<td>HiC 50%</td>
<td>14.19%</td>
<td>37.89%</td>
<td>6.35%</td>
<td>41.57%</td>
<td>56.15%</td>
<td>87.81%</td>
</tr>
<tr>
<td>N435 50%</td>
<td>17.77%</td>
<td>24.83%</td>
<td>8.36%</td>
<td>49.04%</td>
<td>61.17%</td>
<td>85.77%</td>
</tr>
</tbody>
</table>

Additional analysis was performed on the 4h and 24h samples of both the HiC-AO and N435 catalyzed 20 mol% glycerol polyester. The samples were analyzed by static light scattering (SEC-MALLS) as well and viscometry and the relevant plots were generated to further help us compare the degree of branching of these samples. From the $^{13}$C NMR the mol% of dendritic units was calculated for the 4h samples discussed here and the results obtained for the HiC-AO and N435 catalyzed samples were 8.24% and 10.14% respectively (See Table 10). Although the numbers are very close, the N435 catalyzed sample on average – as NMR gives an average – is slightly more branched. From the static light scattering plot of molar mass vs. volume (or molecular weight vs. time) we can see that in the 4h sample, for the same molecular weight, the N435 sample has greater retention time. Since a polymer which has a higher degree of branching will have a smaller hydrodynamic size, the retention time will be greater. Therefore, the light scattering result of the 4h sample concurs with the result from the $^{13}$C NMR. After a 24h
reaction time, however, the HiC-AO sample is distinctly more branched than its N435 catalyzed counterpart, as the retention time of the HiC-AO sample is greater throughout the whole sample spectrum.

![Graph](image.png)

**Figure 33.** Static light scattering (SEC-MALLS) spectra of 4h and 24h samples of HiC-AO and N435 catalyzed 20 mol% glycerol polyester.

The result of the viscometry analysis as shown by the Mark-Houwink plot, which plots intrinsic viscosity as a function of molar mass, further concurs with the above analysis of these samples. For the same molecular weight, the viscosity of a polymer with a higher degree of branching is less than that of a linear polymer or than the viscosity of a polymer with a lower degree of branching. This is so as a branched polymer has an uncoiled architecture which limits polymer chain interaction resulting in a lower viscosity. A linear polymer however, is more tightly coiled and therefore there is more
interaction between polymer chains causing a higher viscosity. As seen in the Mark-Houwink plots, after a 4h reaction time, the viscosity is smaller for the N435 catalyzed sample. In the 24h sample, however, the opposite is true and the HiC-AO sample is less viscous along the entire sample, proving that it is more highly branched than its N435 catalyzed counterpart.

Figure 34. Mark-Houwink plot of HiC-AO and N435 catalyzed 20 mol% glycerol polyester. 4h and 24h reaction times.
5.2 HiC-AO Catalyzed Sorbitol Polyester Reactions

In the second set of experiments sebacic acid (2mM) was reacted with a varying combination of 1,8-octanediol and sorbitol with a diacid to polyol ratio of 1:1. The sorbitol content was increased in 10% increments from 0 mol% to 40 mol%.

Scheme 8. HiC-AO and N435-catalyzed terpolymerization reaction of sebacic acid with 1,8-octanediol and sorbitol

Reactions with sorbitol content ranging from 0 to 20 mol% appear to be identical in their molecular weight buildup as a function of time only for the first hour of the reaction (see figure 35). After that time, a trend develops - similar to that seen in the glycerol reactions – where the addition of polyol (sorbitol) into the reaction will lessen the molecular weight buildup as a function of time. In the HiC catalyzed reactions (figure 35a) we can see a proportional gap in the molecular weight increase as a function of time. At 8h the $M_n$ values for 0, 10 and 20 mol% sorbitol are 9.4Kg/mol, 7.2 Kg/mol and 5.3 Kg/mol respectively, which is a difference of 2 Kg/mol between each sample. At 24h the $M_n$ values of these same samples are 16.3 Kg/mol, 12.1 Kg/mol, and 8.3 Kg/mol respectively – a difference of 4Kg/mol between each sample. In the N435 catalyzed
samples (figure 35b) we can see the 10 and 20 mol% sorbitol reactions to have the same molecular weight growth for the first two hours of the reaction. After that time - from 4h to 8h - the gap in \( M_n \) growth between these 2 samples increases rapidly with 4h \( M_n \) values of 5.1 Kg/mol and 3.5 Kg/mol respectively, and \( M_n \) values at 8h of 7.0 Kg/mol and 3.3 Kg/mol respectively.

Figure 35. Terpolymerization reaction of sebacic acid with 1,8-octanediol and sorbitol. Extent of \( M_n \) as a function of time. Ratio of sorbitol to 1,8-octanediol varying from 0:1 to 0.2:0.8 (a) HiC-AO catalyzed reaction  (b) N435 catalyzed reaction

Data represents an average of 3 trials (n=3). Error bars signify the standard deviation. The polydispersity \( (M_w/M_n) \) of all the N435 and HiC catalyzed reactions from 10 to 20 mol% sorbitol is almost identical for the first 8h, ranging only from a low of 2.0 to a high of 2.7 (see figure 36). At 24h, however, the polydispersity increases greatly for all samples and is 5.2 and 6.1 respectively for the HiC catalyzed reactions, and 4.1 and 3.3 respectively for the N435 catalyzed reactions. In contrast, the PDi of the sample with no sorbitol content remained unchanged throughout the course of the reaction. These results
are especially interesting for the HiC catalyzed reactions where the molecular weight build up with time was so consistently incremental. It is obviously not possible to correlate the changes in Mn and PDI as a function of time.

**Figure 36:** Terpolymerization reaction of sebacic acid with 1,8-octanediol and sorbitol. Polydispersity (Mw/Mn) as a function of time. Ratio of sorbitol to 1,8-octanediol varying from 0: 1 to 0.2:0.8 (a) HiC-AO catalyzed reaction (b) N435 catalyzed reaction
HiC catalyzed reactions with both 30 and 40 mol% sorbitol increased incrementally throughout the 24h course of the reaction with little difference between the $M_n$ of the two samples (see figure 37). In contrast to this result, the N435 catalyzed reactions with 30 mol% sorbitol increased rapidly for the first 2h of the reaction, and thereafter only a gradual increase in $M_n$ was noted for the remainder of the 24h reaction time, giving a product with an $M_n$ similar to that of the HiC catalyzed reaction (3.7 Kg/mol and 3.3 Kg/mol, respectively). The N435 catalyzed reaction with 40 mol% sorbitol however, increased only gradually throughout the reaction and substantially lower molecular weights were achieved, giving a product with an $M_n$ lower than that of the HiC catalyzed reaction (2.3 Kg/mol and 3.7 Kg/mol, respectively). This is the same trend that was observed in the first series of sorbitol reactions with 10 mol% and 20 mol% sorbitol content. There too, both HiC catalyzed reactions showed similar molecular weights, while a large disparity was present in the molecular weights of the two N435 catalyzed reactions.
**Figure 37.** Terpolymerization reaction of sebacic acid with 1,8-octanediol and sorbitol. Extent of $M_n$ as a function of time. Ratio of sorbitol to 1,8-octanediol varying from 0.3:0.7 to 0.4:0.6 (a) HiC-AO catalyzed reaction (b) N435 catalyzed reaction

Data represents an average of 3 trials ($n=3$). Error bars signify the standard deviation.

Comparing the polydispersity in the 30 and 40 mol% sorbitol reactions, we can see a gradual but steady increase in the PDi as a function of time in the HiC catalyzed samples. The increase is more rapid in the 30 mol% sorbitol reaction, however after a
jump in the PDI of the 40 mol% sorbitol sample at 8h both 30 and 40 mol% samples have the same polydispersity at that time point (3.3), and a similar polydispersity after 24h (4.4 and 3.5 respectively). Likewise, both N435 catalyzed samples have a similar trend in polydispersity increase; however the polydispersities are slightly smaller than those of the same HiC catalyzed reactions.

**Figure 38.** Terpolymerization reaction of sebacic acid with 1,8-octanediol and sorbitol. Polydispersity (Mw/Mn) as a function of time. Ratio of sorbitol to 1,8-octanediol varying from 0.3: 0.7 to 0.4:0.6 (a) HiC-AO catalyzed reaction (b) N435 catalyzed reaction
5.2.1 Further analysis of sorbitol polyesters

The comparative study of the HiC-AO and N435 catalyzed polyol reactions with various sorbitol contents was plotted on the same graph to show the markedly higher activity of HiC throughout the time course study in both the 20 and 40 mol% sorbitol samples, while the 30 mol% samples of both catalysts were almost identical.

Additional analysis was performed on the 4h samples using static light scattering (SEC-MALLS) instead of refractive index GPC to determine the molecular weight of the samples. The result of this analysis shows almost identical molecular weight for the HiC-AO catalyzed 20 and 30 mol% samples and a slight decrease in the molecular weight for the 40 mol% sample. This same trend repeats exactly for the N435 catalyzed samples. Once again we can see from this analysis the higher activity of HiC-AO as compared to N435 in sorbitol polyol reactions.

Figure 39. Reaction of sebacic acid with 1,8-octanediol and sorbitol. Extent of $M_n$ as a function of time. 20, 30 and 40 mol% HiC-AO and N435 catalyzed reactions.
Figure 40. Molecular weight ($M_n$) as analyzed by SEC-MALS of 20, 30 and 40 mol% sorbitol 4h sample. HiC-AO vs. N435.

As with the glycerol based polyols, a comparative study of polymer branching was conducted by SEC-MALLS and by Viscometry. Once again the HiC-AO and N435 catalyzed 20 mol% sorbitol samples with both 4h and 24h reaction times were analyzed. In the static light scattering plot of molar mass vs. volume, the results of both the 4h reaction time and the 24h reaction time were very close. In the 4h reaction time plot, the HiC-AO sample has a slightly greater retention time than does the N435 sample which is caused by its smaller hydrodynamic size, meaning that the HiC-AO sample has a slightly higher branched architecture. The exact same is true for the 24h samples. The HiC-AO once again is seen to be slightly more branched.
**Figure 41.** Static light scattering (SEC-MALLS) spectra (molar mass vs. time) of 4h and 24h samples of HiC-AO and N435 catalyzed 20 mol% sorbitol polyester.

The results of the Viscometry analysis as shown by the Mark-Houwink plots for these samples reinforce and support the results from the light scattering analysis. In both the 4hr and 24h reaction times, the HiC-AO catalyzed samples have just slightly lower viscosity than those catalyzed by N435 and therefore are of a slightly more highly branched architecture.
Figure 42. Mark-Houwink plot (intrinsic viscosity vs. molar mass) of HiC-AO and N435 catalyzed 20 mol% sorbitol polyester. 4h and 24h reaction times.

It is interesting to note the difference in the behavior specifically in the HiC-AO catalyzed glycerol and sorbitol polyols. The HiC-AO catalyzed glycerol based polyol becomes substantially more branched as the reaction progresses, while the N435 catalyzed polyol remains roughly unchanged. In the case of the sorbitol based polyol, the degree of branching of both the HiC-AO and N435 catalyzed polyols basically does not change with time.
CHAPTER 6

Conclusion
This work explores the use of enzymes from the cutinase family - specifically HiC – as a biocatalyst for polyester condensation reactions. Here we are experimenting with a genetically modified HiC from Novozymes. Although the enzyme was modified for hydrolysis, we show that it is an excellent catalyst for polysynthesis reactions as well. Enzymes from the cutinase family had been previously used for hydrolysis and small molecule reactions but not for polymer synthesis reactions. A viable system of immobilized HiC has been developed here and extensively optimized for use in polycondensation reactions. During the optimization stage of this work, the resin and the immobilization technique has been carefully selected. The thermal stability of HiC-AO has also been studied to determine the range of optimal activity for HiC catalysis in a polycondensation reaction with various solvent systems. Results of experiments showed that HiC was optimally covalently immobilized on Amberzyme oxirane beads with an optimal protein loading of approximately 10% while 1% w/w protein is used for polycondensation catalysis. HiC has been shown to be most active at 70°C in most reactions. HiC-AO has been shown to be active in solventless reactions, in diphénylether as well as in a toluene azeotrope system.

In a parallel homopolymerization reaction of ω-hydroxyhexadecanoic acid, HiC-AO was shown to be considerably more synthetically active than other cutinase enzymes, yielding a polymer with >2X the molecular weight of AoC and > 5X the molecular weight of AbC.

The thermal stability of WT HiC was tested along with 3 other wt cutinase enzymes using a small molecule esterification reaction. It was found that wt-HiC and AoC are similarly thermostable at 70°C and even 80°C with a conversion of
approximately 50%, while AbC and FsC had a very low rate of conversion (~10%) at 80°C.

In an in-depth study the chain length selectivity of the HiC-AO catalyst system was assessed in polycondensation reactions between various substrates. The chain length of these substrate types was varied to assess chain length selectivity of HiC-AO and to compare this with that of N435 (CALB immobilized on Lewatit beads). Results of experiments proved that HiC-AO has higher chain length selectivity than N435. For the series of ωHA substrates studied, N435 catalyzed the homopolymerization of 3 out of the 4 substrate chain lengths – all except the shortest, while HiC-AO was active on only the longer ωHAs. HiC-AO was likewise highly selective in catalyzing polycondensation reactions with diols of varying chain lengths. In contrast, N435 was active in polymerizing all diol chain lengths with only small differences in activity between the different chain length substrates. The same experiment, but with varying diacid chain lengths, showed unequivocally the high selectivity of HIC for longer chain length substrates, as HIC was highly active with C13 and C10 substrates, but was not active with substrates with chain length below C10. For this substrate series, N435 was not selective, showing almost the same activity for all diacids used. The list of substrates for which HiC-AO showed little or no activity included: i) C6 and C10 ωHAs, ii) C3, C4 and C5 diols for copolymerizations with C10-diacid, and iii) C6, C8 and C9 diacids for copolymerizations with 1,8-octanediol. However, N435 was active on all these substrates except C6 ωHA.

Activities of HIC-AO and N435 for step-condensation reactions using longer chain length substrates C16-HA and 1,8-octanediol/C13-diacid were compared. During
the first 2 h of reactions, N435 more rapidly builds chains giving higher $M_n$s. However, after 2 h, the relative activities of these two catalysts reverse and, ultimately, higher molecular weight polyesters were obtained by HiC-AO catalysis. It is noteworthy that, during the first 2 h of polymerizations, reactions must contain relatively high water contents since vacuum was not applied during this period. Hence, differences in CALB and HiC activities at earlier and later stages of polymerizations may be due to reaction water content. HiC might require lower reaction water contents to have optimal activity whereas CALB may exhibit the opposite behavior.

To further study affects of substrate chain length on HiC-AO and N435 activity, two series of model small molecule esterification reactions was studied where either the $n$-alkanol or $n$-alkanoic acid chain length was varied. Despite inherent differences between model esterification reactions and polycondensation reactions, N435 showed greater substrate promiscuity for a majority of reactions performed, and conversion results from substrate to ester were similar for most substrate chain lengths. This is in contrast to HiC-AO which showed large differences in percent conversion of preferred and non-preferred substrates.

In an additional in-depth study, HiC-AO was used to catalyze a series of glycerol and sorbitol polyol condensation reactions with sebacic acid, 1,8-octanediol and varying amounts of alditol. Both the molecular weight and the polydispersities of the samples were studied and a comparison was made to identical reactions catalyzed by N435. A study of branching was also conducted and a comparison was again drawn between the HiC-AO and N435 catalyzed samples.
Results of the glycerol experiments proved that HiC-AO successfully catalyzed reactions containing \( \leq 40\% \) glycerol and the polydispersity of these samples increased incrementally with the addition of glycerol. Samples with higher glycerol content however, remained at a lower molecular weight.

A study of 3 samples (20, 30 and 50 mol\% glycerol) was conducted in parallel with HiC-AO and N435 catalysis. In comparison with N435, in longer reactions (24h) the HiC-AO proved to be more active in samples with lower glycerol content producing higher molecular weight as well as a more stable polydispersity then the N435 catalyzed samples. In reactions of shorter lengths N435 catalyzed samples had slightly higher molecular weights. \(^{13}\)C NMR was performed on these six samples after 4h reaction time. From the fact that the observed ratio of OS:OG is identical in all samples and almost identical to the feed ratio we can see that the majority of glycerol has indeed been incorporated into the polymer after 4h. Calculating the branching using Frey’s equation we can see that the branching on all samples is quite low which is understandable given the short duration of the reaction time. The higher branching that had been reported previously with N435 was after a 42h reaction. The regioselectivity of acylation at the primary hydroxyl sites was calculated and show a high degree of regioselectivity regardless of catalyst. This was previously seen in N435 samples and we have now shown the same is true for HiC-AO catalyzed samples. The percent of hydroxyls substituted in the glycerol unit is likewise determined by the \(^{13}\)C NMR and is seen to remain constant for all samples except for the HiC-AO catalyzed 50 mol\% glycerol sample where the substitution is somewhat lower. This of course is understandable given the lower degree of synthetic activity of HiC-AO in reactions with higher glycerol
content. As calculated from the $^{13}$C NMR study we see that the mol% of dendritic substituted units which is also indicative of the hyperbranched character is higher for the N435 catalyzed 20 and 50 mol% glycerol samples, while substantially lower for the 30 mol% sample.

Both the HiC-AO and N435 catalyzed 20 mol% glycerol samples (4h and 24h) were additionally analyzed by SEC-MALLS and Viscometry. The SEC-MALLS plots for the two 4h samples show the N435 catalyzed sample with slightly higher molar mass and therefore slightly more branched, while the 24h samples clearly show higher branching in the HiC-AO catalyzed samples. Intrinsic viscosity measured by the Mark-Houwink plot clearly shows lower viscosity (higher branching) for the N435 catalyzed sample after 4h and for the HiC-AO sample after 24h.

All of the sorbitol experiments from 10 to 40 mol% sorbitol were catalyzed by HiC-AO and in parallel by N435. HiC-AO proved to be more active then N435 in the 10 and 20 mol% sorbitol samples with a slightly higher polydispersity but showed similar activity to N435 in the 30 and 40 mol% samples and likewise the samples had similar polydispersity.

As with the glycerol samples both the HiC-AO and N435 catalyzed 4h and 24h samples were analyzed by SEC-MALLS and Viscometry to study and compare the hyperbranching of the polymers. Both the SEC-MALLS plot and the Mark-Houwink intrinsic viscosity plot concur that both HiC-AO catalyzed samples are very slightly more branched then their N435 catalyzed counterpart.

This knowledge that we have presented here with the research in this work is just a beginning in the potential contribution of HiC catalysis of synthetic polymer reactions.
Indeed the whole field of biocatalysis has taken on a new importance in times of global warming and vast environmental damage caused by industry today. The success of the HiC-AO catalyst in polycondensation reactions warrants further experimentation towards the goal of developing this system for possible industrial use. Given the immensely lucrative polyurethane industry today, the success in using HiC-AO for alditol prepolymers is undeniably an important discovery.

In future work it would be important to determine the extent of protein leaching and catalyst reusability of the HiC-AO system. Furthermore, it would interesting to assess the activity of cutinases for reactions where CALB has thus far generally shown poor reactivity, such as reactions with aromatic acids/esters or secondary alcohols of [S]-stereochemical configuration. Aromatic Polyesters are especially of great importance in the polymer industry (ex. PET, aromatic polyol polyesters) and it would be significant to explore HiC catalysis if these polymers.

One key limitation of HiC-AO catalysis is the thermostability of the enzyme. As discussed previously, both in this work and by Hunsen et al\textsuperscript{16,44} the HiC enzyme is most active for condensation polymerizations as well as ROP at a temperature of 70\textdegree C (as opposed to N435 which can catalyze polymerization reactions at temperatures above 90\textdegree C\textsuperscript{112}). Increasing the temperature in an HiC catalyzed reaction, even slightly, will cause denaturation of the enzyme. This poses a serious problem for many polymerization reactions which require a higher temperature. Many high melting monomers and polymers that are of great value to the polymer industry can only be catalyzed at this point by chemical catalysis. Moreover, the success of reactions that are currently possible with HiC, specifically in bulk conditions, is limited due to diffusion constraints during
chain growth at lower temperatures. With more advanced knowledge that is currently available in the field of protein engineering, the modification of the HiC thermal stability would greatly increase the versatility and functionality of HiC as a biocatalyst in polymer synthesis reactions.
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APPENDIX
Fig. A-1 $^{13}$C spectra of 20% glycerol reaction – 4h sample a) HiC-AO, b) N435
Fig. A-2. $^{13}$C spectra of 30% glycerol reaction – 4h sample a) HiC-AO  b) N435
Fig. A-3. $^{13}$C spectra of 50% glycerol reaction – 4h sample a) HiC-AO b) N435