Enhanced Ethanol Production from D-Xylose Using Co-encapsulated Xylose Isomerase and *Saccharomyces cerevisiae*

A Thesis
by
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Submitted in partial fulfillment of the requirements for the degree of

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Chemistry

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The undersigned have examined the thesis entitled ENHANCED ETHANOL PRODUCTION FROM D-XYLOSE USING CO-ENCAPSULATED XYLOSE ISOMERASE AND SACCHAROMYCES CEREVISIAE presented by Brian J. Frederick, a candidate for the degree of Chemistry, and hereby certify that it is worthy of acceptance:

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Craig Woodworth
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>5</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>7</td>
</tr>
<tr>
<td>TABLE OF ABBREVIATIONS</td>
<td>8</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>9</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>10</td>
</tr>
<tr>
<td>OBJECTIVES</td>
<td>11</td>
</tr>
<tr>
<td><strong>CHAPTER 1: INTRODUCTION</strong></td>
<td>12</td>
</tr>
<tr>
<td>1.1: ETHANOL AS AN ALTERNATIVE FUEL</td>
<td>12</td>
</tr>
<tr>
<td>1.2: APPROACHES TO D-XYLOSE FERMENTATION</td>
<td>16</td>
</tr>
<tr>
<td>1.3: APPLICATIONS OF ENCAPSULATION</td>
<td>20</td>
</tr>
<tr>
<td>1.4: ALGINATE AS A SHELL MATERIAL</td>
<td>23</td>
</tr>
<tr>
<td>1.5: HYPOTHESIS</td>
<td>24</td>
</tr>
<tr>
<td>1.6: OUTLINE</td>
<td>25</td>
</tr>
<tr>
<td><strong>CHAPTER 2: PREVIOUS RESULTS</strong></td>
<td>27</td>
</tr>
<tr>
<td>2.1: GLUCOSE FERMENTATION</td>
<td>27</td>
</tr>
<tr>
<td>2.2: XYLOSE FERMENTATION</td>
<td>29</td>
</tr>
<tr>
<td>2.3: IDENTIFICATION OF PROBLEMS</td>
<td>31</td>
</tr>
<tr>
<td><strong>CHAPTER 3: PROCEDURES</strong></td>
<td>33</td>
</tr>
<tr>
<td>3.1: CAPSULE FORMATION AND FERMENTATION</td>
<td>33</td>
</tr>
<tr>
<td>3.2: FERMENTATION ANALYSIS</td>
<td>35</td>
</tr>
<tr>
<td>3.2.1: pH</td>
<td>35</td>
</tr>
<tr>
<td>3.2.2: Reducing Sugar Assay</td>
<td>36</td>
</tr>
<tr>
<td>3.2.3: Ethanol Assay</td>
<td>37</td>
</tr>
<tr>
<td>3.2.4: Leaching Analysis</td>
<td>39</td>
</tr>
<tr>
<td>3.3: ENZYME ANALYSIS</td>
<td>40</td>
</tr>
<tr>
<td>3.4: ENZYME IMMOBILIZATION</td>
<td>43</td>
</tr>
<tr>
<td>3.4.1: Chitosan Immobilization</td>
<td>43</td>
</tr>
<tr>
<td>3.4.2: Microcrystalline Cellulose Immobilization</td>
<td>44</td>
</tr>
<tr>
<td><strong>CHAPTER 4: ENCAPSULATION AND FERMENTATION OPTIMIZATION</strong></td>
<td>45</td>
</tr>
<tr>
<td>4.1: SCALE-UP</td>
<td>45</td>
</tr>
<tr>
<td>4.2: BASELINE FERMENTATION AND EFFECTS OF NUTRIENTS</td>
<td>47</td>
</tr>
<tr>
<td>4.3: IMPROVED DIFFUSION</td>
<td>52</td>
</tr>
<tr>
<td><strong>CHAPTER 5: ENZYME IMMOBILIZATION AND EFFECTS ON ACTIVITY</strong></td>
<td>54</td>
</tr>
<tr>
<td>5.1: POLYMER IMMOBILIZED XYLOSE ISOMERASE</td>
<td>54</td>
</tr>
<tr>
<td>5.1.1: Effects of pH</td>
<td>56</td>
</tr>
<tr>
<td>5.1.4: Effects of Ethanol Concentration</td>
<td>56</td>
</tr>
<tr>
<td>5.1.3: Effects of Divalent Cations</td>
<td>57</td>
</tr>
<tr>
<td>5.1.2: Effects of Temperature</td>
<td>59</td>
</tr>
<tr>
<td>5.2: FREE XYLOSE ISOMERASE</td>
<td>60</td>
</tr>
<tr>
<td>5.2.1: Effects of pH</td>
<td>61</td>
</tr>
<tr>
<td>5.2.2: Effects of Temperature</td>
<td>61</td>
</tr>
<tr>
<td>5.3: XYLOSE ISOMERASE IMMOBILIZATION ON CHITOSAN</td>
<td>62</td>
</tr>
</tbody>
</table>
5.3.1: Effects of pH ...................................................................................................................... 64
5.3.2: Effects of Temperature ...................................................................................................... 64
5.4: Xylose Isomerase Immobilization on Cellulose .................................................................. 66
  5.4.1: Effects of pH ..................................................................................................................... 66
  5.4.2: Effects of Temperature ..................................................................................................... 67

CHAPTER 6: XYLOSE FERMENTATION ......................................................................................... 69
  6.1: Xylose Fermentation ........................................................................................................... 69
  6.2: With Polymer Immobilized Xylose Isomerase ...................................................................... 72
    6.2.1: Concentration and Optimized Conditions ........................................................................ 73
    6.2.2: Multiple Fermentation Cycles ....................................................................................... 75
  6.3: With Free Enzyme Xylose Isomerase .................................................................................. 77
  6.4: With Xylose Isomerase Immobilized on Cellulose ................................................................. 79

CHAPTER 7: SUMMARY .................................................................................................................. 81
  7.1: Conclusions .......................................................................................................................... 81
  7.2: Future Work .......................................................................................................................... 86

ACKNOWLEDGEMENTS .................................................................................................................. 87

REFERENCES ..................................................................................................................................... 88
List of Figures

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 1: Production profile of oil and gas liquids, showing a projected...</td>
<td>12</td>
</tr>
<tr>
<td>Fig. 2: Total consumption of oil by sector</td>
<td>12</td>
</tr>
<tr>
<td>Fig. 3: Energy price trends, as reported by BP</td>
<td>13</td>
</tr>
<tr>
<td>Fig. 4: Chemical synthesis of butanol</td>
<td>14</td>
</tr>
<tr>
<td>Fig. 5: Xylose to xylulose pathway found in <em>Candida</em></td>
<td>16</td>
</tr>
<tr>
<td>Fig. 6: Xylose fermentation via <em>Candida</em> pathway and xylitol...</td>
<td>17</td>
</tr>
<tr>
<td>Fig. 7: Xylose fermentation via XI or closed loop <em>Candida</em> pathway...</td>
<td>17</td>
</tr>
<tr>
<td>Fig. 8: Xylose fermentation results using GM <em>S. cerevisiae</em>...</td>
<td>18</td>
</tr>
<tr>
<td>Fig. 9: Xylose fermentation via xylose isomerase and the pentose...</td>
<td>19</td>
</tr>
<tr>
<td>Fig. 10: Encapsulation conceptualization for xylose and glucose...</td>
<td>22</td>
</tr>
<tr>
<td>Fig. 11: Steps of fermentation in a combined biomass plant...</td>
<td>22</td>
</tr>
<tr>
<td>Fig. 12: Composition of alginate</td>
<td>23</td>
</tr>
<tr>
<td>Fig. 13: 400× magnification of broth after anaerobic incubation...</td>
<td>28</td>
</tr>
<tr>
<td>Fig. 14: UV-Vis spectra of the ethanol assay of gravity filtered...</td>
<td>29</td>
</tr>
<tr>
<td>Fig. 15: Formation of alginate capsules</td>
<td>33</td>
</tr>
<tr>
<td>Fig. 16: Dinitrosalicylic acid reaction with reducing sugar...</td>
<td>36</td>
</tr>
<tr>
<td>Fig. 17: Alcohol dehydrogenase (AD) ethanol analysis (ADEA)...</td>
<td>37</td>
</tr>
<tr>
<td>Fig. 18: Coomassie Brilliant Blue dye...</td>
<td>39</td>
</tr>
<tr>
<td>Fig. 19: Autokit Glucose method...</td>
<td>41</td>
</tr>
<tr>
<td>Fig. 20: Interference of various solutions on glucose determination...</td>
<td>42</td>
</tr>
<tr>
<td>Fig. 21: Procedure for the immobilization of enzyme on chitosan...</td>
<td>43</td>
</tr>
<tr>
<td>Fig. 22: Procedure for the immobilization of enzyme on cellulose...</td>
<td>44</td>
</tr>
<tr>
<td>Fig. 23: Pump apparatus for forming alginate capsules...</td>
<td>45</td>
</tr>
<tr>
<td>Fig. 24: pH vs. time for the baseline glucose fermentation trial...</td>
<td>47</td>
</tr>
<tr>
<td>Fig. 25: Ethanol and sugar concentrations over time for the baseline...</td>
<td>48</td>
</tr>
<tr>
<td>Fig. 26: pH vs. time for the increased glucose fermentation trial...</td>
<td>49</td>
</tr>
<tr>
<td>Fig. 27: Ethanol and sugar concentrations over time for the increased...</td>
<td>49</td>
</tr>
<tr>
<td>Fig. 28: pH vs. time for the increased nutrient fermentation trial...</td>
<td>50</td>
</tr>
<tr>
<td>Fig. 29: Ethanol and sugar concentrations over time for the increased...</td>
<td>50</td>
</tr>
<tr>
<td>Fig. 30: pH vs. time for the improved diffusion fermentation trial...</td>
<td>52</td>
</tr>
<tr>
<td>Fig. 31: Ethanol and sugar concentrations over time for the increased...</td>
<td>53</td>
</tr>
<tr>
<td>Fig. 32: Preliminary polymeric XI activity results. UV-Vis spectra...</td>
<td>55</td>
</tr>
<tr>
<td>Fig. 33: Effect of pH on polymeric XI activity</td>
<td>56</td>
</tr>
</tbody>
</table>
Fig. 34: Effect of ethanol concentration on polymeric XI activity .............................................57
Fig. 35: Effect of Mg ions on polymeric XI activity .................................................................58
Fig. 36: Effect of Co ions on polymeric XI activity .................................................................58
Fig. 37: Effect of temperature on polymeric XI activity .........................................................59
Fig. 38: Effect of pH on free XI activity ....................................................................................61
Fig. 39: Effect of temperature on free XI activity .................................................................61
Fig. 40: Preliminary XI-Chitosan results, as activity compared to...........................................63
Fig. 41: Effect of pH on XI-chitosan vs. free XI activity .........................................................64
Fig. 42: Effect of temperature on XI-chitosan vs. free XI activity .........................................65
Fig. 43: Effect of pH on XI-cellulose vs. XI-chitosan and free XI...........................................67
Fig. 44: Effect of temperature on XI-cellulose vs. XI-chitosan and ........................................68
Fig. 45: pH vs. time for a xylulose fermentation trial ..............................................................70
Fig. 46: Ethanol and sugar concentrations over time for the xylulose.................................70
Fig. 47: pH vs. time for initial xylose fermentation trial.........................................................72
Fig. 48: Ethanol and sugar concentrations over time for the initial........................................73
Fig. 49: pH vs. time for the increased polymeric XI concentration ........................................74
Fig. 50: Sugar concentrations, as a percent of initial 15% xylose.........................................74
Fig. 51: pH vs. time for multiple fermentation cycles ..............................................................75
Fig. 52: Sugar concentrations, as a percent of initial 15% xylose........................................76
Fig. 53: pH vs. time of the free XI xylose fermentation trial .................................................77
Fig. 54: Sugar concentrations, as a percent of initial 15% xylose.......................................78
Fig. 55: pH vs. time of the microcrystalline cellulose-immobilized.......................................79
Fig. 56: Ethanol and sugar concentrations over time for the.................................................79
**List of Tables**

<table>
<thead>
<tr>
<th>Section:</th>
<th>Page:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1: Comparison of combustion energy, octane rating, and…</td>
<td>13</td>
</tr>
<tr>
<td>Table 2: Sugar composition of various plant biomass sources</td>
<td>15</td>
</tr>
<tr>
<td>Table 3: Ethanol yield from D-glucose fermentation based on…</td>
<td>28</td>
</tr>
<tr>
<td>Table 4: Ethanol yield from D-xylose fermentation based on…</td>
<td>30</td>
</tr>
<tr>
<td>Table 5: Preliminary XI activity results</td>
<td>55</td>
</tr>
</tbody>
</table>
Table of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADEA</td>
<td>Alcohol Dehydrogenase Ethanol Analysis</td>
</tr>
<tr>
<td>CMC</td>
<td>carboxymethyl cellulose</td>
</tr>
<tr>
<td>DNS</td>
<td>dinitrosalicylic acid</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>SAS I/II</td>
<td>Sugar Analysis Solution I/II</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-Visual</td>
</tr>
<tr>
<td>XI</td>
<td>xylose isomerase</td>
</tr>
<tr>
<td>XI-Cel</td>
<td>cellulose-immobilized xylose isomerase</td>
</tr>
<tr>
<td>XI-Chi</td>
<td>chitosan-immobilized xylose isomerase</td>
</tr>
</tbody>
</table>
Dedication

This thesis is dedicated to my girlfriend, Leticia Daconti. Without her insistence, this work would never have progressed beyond my senior thesis. I would like to additionally dedicate this thesis to the tree/trees that sacrificed their life/lives to print the multiple drafts, copies for my thesis committee, and final version of this paper. You will be missed.
Abstract

Currently, transportation is dependant on liquid fuels. With the era of petroleum coming to an end, one potential alternative is ethanol. However, to be economically feasible for producing enough fuel to sustain current demands requires greater production efficiency. To increase efficiency and produce more ethanol from the same amount of initial biomass, significant research has been devoted to fermenting pentose sugars, such as xylose, which are not effectively metabolized to ethanol via traditionally used fermenter organisms. The most successful approaches have employed genetic modification, despite the numerous difficulties and costs inherent to this technology. An alternative approach is the introduction of various enzymes to the ferment that are used for xylose fermentation, such as aldose reductase, xylitol dehydrogenase, and xylose isomerase. To lower the cost of ex vitro enzymes, encapsulation in biopolymers can be used to protect enzymatic activity and make them reusable. Previous research into this topic indicated that the co-encapsulation of xylose isomerase with yeast resulted in minor amounts of xylose fermentation. This thesis outlines advances in xylose isomerase and Saccharomyces cerevisiae co-encapsulation. These advances include scale-up of capsule production, optimization using glucose fermentation, analysis of the effects of various fermentation conditions on xylose isomerase activity with determination of activity enhancers, and improved fermentation of xylose.
Objectives

Work has previously been conducted into the co-encapsulation of *Saccharomyces cerevisiae*, brewer’s yeast, with D-xylose isomerase in artificial membranes\(^1\). This was done to improve the efficiency and lower the cost of five-carbon monosaccharide fermentation to ethanol, focusing exclusively on D-xylose as the monosaccharide source. However, this previous work had limited results, did not address key issues such as enzyme leaching, and did not sufficiently demonstrate ethanol production from D-xylose. This current study attempts to address these inadequacies. Specifically, this work endeavors to improve upon these past efforts with the following goals:

1. Develop a capsule shell composition with adequate diffusion rates to support ethanol production from glucose by encapsulated *S. cerevisiae* at rates identical or superior to free *S. cerevisiae*.
2. Scale-up capsule production and determine a set of analytical tools to more accurately determine the progression and efficiency of fermentation trials.
3. Characterize the activity of the xylose isomerase enzyme under a variety of fermentation related conditions so as to determine the optimal conditions for the conduction of xylose fermentation.
4. Prevent enzyme leaching from capsules, so as to render the enzyme reusable and protect activity.
5. Successfully co-encapsulate *S. cerevisiae* with xylose isomerase and improve ethanol production rates and yields from xylose fermentation over those previously reported\(^1\).
Chapter 1: Introduction

1.1: Ethanol as an Alternative Fuel

The era of petroleum, as we know it, is drawing to an end as less oil is discovered every year and the oil reserves we have developed dwindle with rising demand. It is projected that we will soon reach the peak of world oil production (Fig. 1). Gasoline, the blood of our society’s transportation infrastructure, is of particular importance to both industry and individuals. In fact, the vast majority of oil is consumed for transportation (Fig. 2). Drastic changes, chaotic markets, and potential societal instability will result from decreasing availability of this fuel, which is a highly inelastic commodity. This inelasticity means that if supply goes down, demand will remain the same, or decrease with extreme reluctance, leading to dramatic price increases. Even small supply issues, such as a storm delaying a tanker ship or the closure of a plant, can affect the price by tens of cents. After hurricane Katrina struck the Louisiana coast in 2005, damaging several offshore oil rigs and coastal refineries, the cost of gasoline rose to record highs. Eventually a point will be reached when the supply is decreasing so quickly that costs will be too great for normal transportation needs. It can be seen that in recent years, costs...
have already begun to drastically increase (Fig. 3). This trend would not only change our daily habits, but could completely collapse our current food and commodities distribution system. To maintain society’s standard of living, or even to simply sustain the growing global population, alternative sources of energy must be implemented.

There are numerous potential fuel sources that could be used to replace gasoline and other petroleum based products. Nuclear, hydroelectric, and wind power are all currently used and effective, but unless electric cars are implemented these alternatives are of no more use to a vehicle fleet than coal currently is. Unfortunately, at present, batteries appropriate for use in a vehicle do not have enough energy storage capacity for long distance travel and are too expensive for large-scale implementation. Photovoltaic cells are being used to power cars, but there are a plethora of problems associated with them, including a large input of petroleum based products for construction, poor efficiency, and the inconsistency derived from its dependence on the availability of sunlight. Hydrogen fuel cells have received a good amount of attention, but this is still a futuristic technology years or decades from implementation on a large scale.

Among the potential liquid alternative fuels are methanol, ethanol, and butanol. Some of the properties of these fuels are compared to gasoline in Table 1. As seen, methanol has significantly less BTU (British Thermal Units), or combustion energy, compared to gasoline and is therefore not being extensively explored as an alternative

<table>
<thead>
<tr>
<th>Table 1: Comparison of combustion energy (BTU), octane rating, and vapor pressure (VP) of several liquid fuels.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>BTU (K)</td>
</tr>
<tr>
<td>Motor Octane</td>
</tr>
<tr>
<td>VP (psi)</td>
</tr>
</tbody>
</table>

Fig. 3: Energy price trends, as reported by BP.
fuel. Ethanol has about 73% the BTU as gasoline and butanol combustion is the most comparable to that of gasoline at approximately 95%. This implies that butanol is the best gasoline alternative from this selection.

A further advantage of butanol is that it has a very low vapor pressure, making it easier to transport and store. However, butanol production is dependent on one of two industrial reactions that uses petroleum products as reactants (Fig. 4), or the use of *Clostridium acetobutylicium*. In *C. acetobutylicium*, butanol production serves as an emergency pathway to remove butyrate, a product of its metabolism, from the extracellular environment to protect its proton motive force (necessary for generating energy for metabolism)\(^4\). However, butanol is itself toxic to the cell at higher concentrations by fluidizing and disrupting the cell membrane, despite the best attempts of *C. acetobutylicium* to adapt through homeoviscousity adaptations\(^5\). This abolishes the cells’ proton gradient\(^6\), inhibits nutrient transport and halts ATP synthesis\(^7\), and stops cell growth\(^8\). The maximum butanol concentration that *C. acetobutylicium* can produce before death occurs is 1%\(^9\). Genetic modification has increased this concentration to 1.3%\(^10\) via hyperexpression of butanol production genes\(^11\), competitive pathway knockout\(^12\), and increased butanol resistance via membrane lipid composition alterations\(^13\). Combined, this makes butanol an energetically desirable yet economically and industrially infeasible alternative fuel.

Though ethanol has less combustion energy than butanol, an advantage is that the technology and production capabilities for the use of ethanol as an alternative fuel exist now. Since humans have been improving its production for thousands of years, strains of yeast used for ethanol fermentation, such as *Saccharomyces cerevisiae*, are capable of generating up to 23% (v/v) ethanol. This concentration is far greater than that which can be achieved in butanol production, and is economically feasible. Additionally, not only is ethanol compatible with gasoline and current engine designs, it is in fact a common additive. It is a solution right now and, as such, places it in a prime position to be
executed and used as either a final solution or a stepping stone to the ultimate solution to our transportation energy needs.

One of the few limitations to the implementation of ethanol as a fuel is its production at sufficient levels to meet future demands. Currently, plant matter is hydrolyzed by dilute acids or enzymatically digested into monosaccharide sugars that can then be used by yeast to produce ethanol. One drawback is that these yeast strains are limited to six-carbon monosaccharide (hexose sugar) fermentation. Though this is typically the most abundant type of monosaccharides found in plant matter, five-carbon monosaccharides (pentose sugars) including the second most abundant sugar in nature, xylose, comprise a significant proportion. While hexose sugars constitute from 29.6 to 40.6% of the carbohydrates in most plant matter, pentose sugars account for 11.7 to 40.4%, a lower yet very significant amount. Table 2 below gives the sugar composition of several different plant sources. To increase efficiency and yield of ethanol production to meet future demands, manners in which to ferment pentose sugars are actively being sought.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Corn Stover</th>
<th>Switch Grass</th>
<th>Poplar</th>
<th>Sugar Beets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>34.6</td>
<td>31.0</td>
<td>40.0</td>
<td>24.1</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.4</td>
<td>0.2</td>
<td>8.0</td>
<td>4.6</td>
</tr>
<tr>
<td>Galactose</td>
<td>1.0</td>
<td>0.9</td>
<td>0.0</td>
<td>0.9</td>
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<tr>
<td>Xylose</td>
<td>19.3</td>
<td>0.4</td>
<td>13.0</td>
<td>18.2</td>
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<tr>
<td>Arabinose</td>
<td>2.5</td>
<td>2.8</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Uronic Acid</td>
<td>3.2</td>
<td>1.2</td>
<td>NA</td>
<td>20.7</td>
</tr>
<tr>
<td>Lignin</td>
<td>17.7</td>
<td>17.6</td>
<td>20.0</td>
<td>1.5</td>
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<tr>
<td>Extractives</td>
<td>7.7</td>
<td>17.0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Ash</td>
<td>10.4</td>
<td>5.8</td>
<td>1.0</td>
<td>8.2</td>
</tr>
</tbody>
</table>
1.2: Approaches to D-Xylose Fermentation

Since D-xylose is the most abundant pentose sugar found in nature, it has garnered a lot of attention. Research into the development of xylose fermenters has been extensive and a multitude of different approaches to this issue have been explored. Several strains of yeast, including *Pichia stipitis* and *Candida shehatae* have been found that can ferment pentose sugars to ethanol and have served as the starting point for the majority of research efforts devoted to this topic. Part of this metabolic pathway in *Candida* is shown in Fig. 5, which uses the energy carriers ATP, NAD (nicotinamide adenine dinucleotide), and NADPH (NAD phosphate) to convert xylose to xylulose. Xylulose then enters the pentose phosphate pathway, the products of which are shuttled into the glycolysis pathway and are eventually converted to ethanol\(^{17}\), as shown in Fig. 9. Without renewal of the vital electron carriers NADPH and NAD through various additional metabolic pathways the organism will die, leading to poor efficiency of this pathway. Furthermore, these species have low ethanol resistance and grow poorly under fermentation conditions, rendering them unusable for industrial ethanol production\(^{18}\).

**Fig. 5: Xylose to xylulose pathway found in Candida.** 1) Aldose Reductase; requires NADPH. 2) Xylitol Dehydrogenase; requires NAD. 3) Xylulokinase; requires ATP.

An interesting solution to these poor fermentation abilities has been the forced adaptation of *Candida* and other species. By selecting for growth at low pH or elevated ethanol concentrations, strains within a species can be chosen, re-grown, and re-selected resulting in greater tolerance after many selection cycles. However, this process has limited results because when the genetic variety of a species for a particular trait is exhausted, any further improvements require the development of advantageous mutations, which complicates and prolongs the selection process\(^{19}\). Alternatively,
researchers have fused yeast cells from efficient fermenters, such as \textit{Saccharomyces cerevisiae}, with pentose fermenters, like \textit{Candida}. The hopes are that after the genetic reshuffling that occurs subsequent to fusion, the resultant cell will retain both five- and six-carbon fermentation abilities at high efficiencies. These hybrids are possible but have proven to be unstable over multiple cell cycles\(^{20}\).

The majority of research into the development of pentose fermenters has been devoted to genetic modification. Most of these attempts have revolved around expressing the \textit{Candida}-like xylose-to-xylulose conversion pathway, presented above, in \textit{S. cerevisiae}. A major obstacle to this pathway is that NAD and NADPH must be regenerated, or else the cell will not functionally express it. Though these cofactors can be regenerated through xylitol production\(^{21}\) (Fig. 6\(^{22}\)) or minor levels of mitochondrial respiration\(^{23}\), these lead to low ethanol conversion yields. Alternatively, a transhydrogenase can be used to convert NADH back to NAD, while converting NADP to NADPH. However, when heterologous genes for this enzyme are expressed in yeast, since homologous genes have yet to be found\(^{24}\), the opposite reaction is favored \textit{in vivo}, leading to an even greater cofactor imbalance\(^{25}\). A better solution is the use of site-directed mutagenesis to create enzymes in the \textit{Candida} pathway that rely on the same cofactor, thereby creating a closed loop (Fig. 7\(^{22}\)). This has been accomplished by creating an NADH-dependent aldose reductase, with NADH regenerated in the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6.png}
\caption{Xylose fermentation via \textit{Candida} pathway and xylitol production\(^{22}\).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{Xylose fermentation via XI or closed loop \textit{Candida} pathway\(^{22}\).}
\end{figure}
next step of the pathway by xylitol dehydrogenase\textsuperscript{26}. However, the efficiency of this alternative pathway for ethanol production has never been explored.

Fortunately, another pathway exists for converting xylose to xylulose that does not use any cofactors (Fig. 7\textsuperscript{22}). A single enzyme, called xylose isomerase (XI), converts xylose directly to xylulose. This enzyme has not been found in any yeast species but has been found in \textit{Piromyces}, a neocallimastigomycota (anaerobic fungi primarily found in the stomachs of ruminants) and various bacteria. In most cases, genes encoding XI have been successfully added into \textit{S. cerevisiae}, but have not been functionally expressed. Since these genes are from bacterial or distantly related fungal sources, the environment in which the proteins are synthesized and operate in can differ dramatically. This can lead to protein misfolding, altered posttranslational modification, and temperature and pH dependencies that render it inactive. However, there have been several successful attempts using XI from \textit{Piromyces} in \textit{S. cerevisiae}. These modified yeast strains produce ethanol from xylose at an 80% yield, but is too slow to be economically feasible (Fig. 8\textsuperscript{22}).

Though genetic modification has potential, it also has many complications. Successful modification has been impeded by the difficulty of finding appropriate donor DNA and inducing sufficient enough expression of the transplanted genes. Further, whenever genetically modified organisms are generated, they must have a built-in artificial nutrient requirement so that they will not spread in the environment. This dependency makes growth of these organisms slow and difficult, limiting the scale on which they can be implemented and increasing the costs of their use. Generally, genetic modification can be a lengthy and costly endeavor with limited results.

A simplistic alternative to genetic modification is the use of the XI enzyme in free solution with yeast during a fermentation batch, as diagrammed in Fig. 9. It has been
shown that when *S. cerevisiae* is placed under anaerobic conditions with D-xylose and a high concentration of XI, ethanol is produced\textsuperscript{27}. It is known that under such conditions, no imbalance in NAD or NADPH develops as a result of the xylulose-to-ethanol pathway. This has been demonstrated by inhibiting the primary route of cofactor re-synthesis, mitochondrial respiration, using substances such as azide, and showing that these cells remain viable. These cells produce good yields of ethanol, implying this pathway is in fact favorable *in vivo* by *S. cerevisiae*\textsuperscript{28}. Unfortunately, the high concentration of enzyme required to ensure efficient and timely conversion makes this procedure impractical. A potential solution to this dilemma is to trap the enzyme in close proximity to the yeast. This can be accomplished using microencapsulation technologies.

![Fig. 9: Xylose fermentation via xylose isomerase and the pentose phosphate, glycolysis, and fermentation pathways.](image-url)
1.3: Applications of Encapsulation

In the materials sciences, encapsulation is the enclosure of one phase or material within a second material, creating a closed environment. For instance, air is encapsulated inside a tennis ball. This technique is commonly utilized in the food and cosmetic industries and has potential applications such as drug delivery. One of the most common forms of encapsulation is the use of polymers as coating material. These polymers can be temporarily held together and adhered to a substrate via a variety of different interactions including hydrogen bonding, Van der Waals, and electrostatic attraction, dependent on the nature of the materials. Alternatively, chemical bonds can be formed between the polymer strands and with the substrate to create permanent, covalent crosslinking. An example of this is the use of glutaraldehyde to form links between polymers containing amino functional groups. Some common materials for encapsulation are alginate, chitosan, poly(styrene sulfonate), poly(allylamine hydrochloride), 4-(4-methoxycinnamonyl)phenyl methacrylate, 1,2-dihydroxypropyl methacrylate, and diazo resin. Despite the simplicity of these procedures, there are a plethora of applications for encapsulation.

A layer-by-layer polyelectrolyte deposition technique is used for the formation of films on top of various substrates, such as metal nanoparticles and colloids, serving to stabilize these dispersions. Depending on the type of polymers used, capsules or beads can be created that have specific responses to variations in light, pH, temperature, and other conditions that make them well suited for biosensors and biocomputing applications. A more common application is the creation of microreactors. Examples of this include capsules with intrinsic photocatalytic properties for pollutant destruction, photosynthetic abilities, or dialysis-like function for bioartificial liver assist devices. Alternatively, capsules can serve a more passive role as a carrier or protector. For instance, multilayered beads and microcapsules are used to contain, transport, and later release a variety of drugs. Even more common is the encapsulation of enzymes. This can protect and prolong activity of the enzyme by providing a surrounding support that can help maintain the functional conformation of the protein. Furthermore, the ability to regulate the permeability of these capsules is well documented. This
selectivity can be used to protect the encapsulated substance from harmful compounds, such as toxic substances produced from the hydrolysis of plant matter.

Less often employed yet intriguing is the encapsulation of living cells within polyelectrolyte shells. For this application, alginate and chitosan are most often used since the procedure for forming capsules using these polymers is generally milder, they are biocompatible, and they are biodegradable\textsuperscript{50}. In fact, there is a significant history of yeast immobilization within these capsules. For example, \textit{Saccharomyces cerevisiae} entrapment has been used to study the mechanical properties of such capsules\textsuperscript{51}, nutritional requirements for encapsulated yeast has been studied\textsuperscript{52}, and such systems have been used for fermentation. Studies using encapsulated yeast for ethanol production from glucose\textsuperscript{53} and dilute acid hydrolysis mixtures\textsuperscript{54} have been done, as well as for producing xylitol from xylose (employing \textit{Candida guilliermondii})\textsuperscript{55}. It has been shown that yeast encapsulation can protect the cells from some of the toxic substances found in hydrolysate solutions while still providing access to monosaccharides and nutrients\textsuperscript{53}. Overall, this increases the efficiency and life-span of the yeast. Moreover, studies into continuous flow fermentation with encapsulated yeast\textsuperscript{56} and for the protection and reusability of genetically modified yeast\textsuperscript{57} have been conducted with positive results. The continuous flow strategy is more economically and industrially desirable for ethanol production, as compared to traditional batch fermentation approaches. In many cases, these capsules have a liquid core, allowing for free movement of the encapsulated material and increased efficiency of nutrient/material uptake as compared to fully immobilized cells in polymeric beads. An additional advantage is that the capsules can be reused, reducing the amount of waste per fermentation batch.

Though not significantly explored, encapsulation of materials is not limited to single component systems such as those listed above. Multi-cultures, solutions of enzymes, and mixtures of the two can easily be performed. In this manner, the high enzyme concentration requirement for the efficient fermentation of xylose to ethanol via a free solution of \textit{S. cerevisiae} and xylose isomerase (XI), presented in section 1.2, can be overcome. This makes the fermentation of xylose more economically feasible. The premise of this study is that a very high localized concentration of XI can be created, at low cost, in close proximity to \textit{S. cerevisiae} cells, allowing for the efficient production
and cellular uptake of xylulose, converted from xylose (Fig. 9). *S. cerevisiae* will be used in this study due to its high fermentation efficiency and ability to ferment xylulose\textsuperscript{17}.

Encapsulation of these two components eliminates the need for genetic modification, is more stable than yeast hybrids, employs inexpensive materials, and should efficiently ferment up to 90% of the sugar found in most plant biomass. Since the capsules are reusable and potentially more efficient, costs will be further diminished, as will waste. Specifically, in the overall scheme of cellulosic ethanol production (Fig. 11\textsuperscript{58}), encapsulation has the prospect of reducing enzyme and fermentation process costs. Furthermore, since the hemicellulose used as a source of xylose in this study is co-processed with cellulose, and thermoprocessing of the ferment after ethanol production will be the same, there are no additional costs associated with this approach, other than the necessary costs of capsule materials, which are very cheap, and purchase/production of the XI enzyme. In the future, additional enzymes can be incorporated into the capsule interior or immobilized on the exterior to perform one or more steps in the biomass hydrolysis process, thereby combining stages of the overall production pathway, decreasing processing time and increasing efficiency. Lastly, in addition to lowered costs, decreased waste, increased ethanol production, increased fermentation efficiency, and reusability, capsules can be used for the more favorable continuous flow fermentation approach mentioned earlier.
1.4: Alginate as a Shell Material

As mentioned above, alginate is one of the polymers of choice for the encapsulation of living cells due to its biocompatibility, biodegradability, and mild synthesis procedure. Alginate is a polysaccharide isolated from seaweed that is composed of mannanuronate and guluronate monomers. These monomers contain carboxylic acid functional groups that can be electrostatically crosslinked with divalent cations such as calcium ions (Fig. 12). By dropping a solution with calcium ions and the right viscosity into a solution of alginate, the alginate molecules will form a meshwork around the droplet. The thickness of this meshwork depends on how long the newly formed capsules are left in the alginate solution and the concentration of calcium ions in the droplet solution. By bathing in a CaCl₂ solution after formation, additional crosslinking will occur, forming a durable, sturdy, spherical shell with a liquid-filled core. Porosity of this shell can be adjusted by using different amounts of surfactants in the alginate solution, while pore size can be altered via the use of different types of surfactant. These pores allow for diffusion of materials below a certain size, which can be adjusted to allow free diffusion of ferment nutrients while blocking the escape of yeast cells and enzymes. Furthermore, these pores can prohibit the entrance of toxic substances, above a certain size, that could cause cell death and/or enzyme inactivation. Toxins include phenolics, fufurals and vanillins, resulting from biomass hydrolysis.

![Fig. 12: Composition of alginate: A) β-D-mannuronate B) α-L-guluronate. C) Initially disordered strands become D) ordered layers crosslinked with calcium ions as sodium is replaced.](image_url)
1.5: Hypothesis

Previous efforts at the co-encapsulation of yeast with xylose isomerase for the efficient fermentation of xylose to ethanol encountered numerous shortcomings\(^1\). The premise of this work is that these shortcomings can be overcome through modification of the capsule shell porosity to allow more adequate nutrient diffusion, using immobilized enzyme to prevent leaching, and enhancing enzymatic activity by fixing key fermentation parameters such as pH and temperature.
1.6: Outline

The following chapters outline all previously reported work on this project as well as the progress made in this study to date. An overview of the procedures used to acquire the presented results and speculations as to what direction this project should take in the future are also provided. The following is a brief description of all subsequent chapters:

- Chapter 2: The final outcome of all previous work is presented in this chapter. This is broken down into the most successful results for glucose fermentation, and those for xylose fermentation. Lastly, the major problems left unsolved by this previous work are outlined to set the foundation for the work conducted in this study.
- Chapter 3: Various procedures employed in this endeavor are described in this chapter. This includes a general overview of the encapsulation process as well as how fermentation batches were conducted. Techniques employed for analysis of both fermentation batches and enzyme activity are also presented.
- Chapter 4: One of the problems identified in the previous work on this topic was sub-optimal glucose fermentation as compared to free yeast solutions. This section describes how the encapsulation procedure was optimized to support mass-production of capsules, as well as how the porosity of these capsules was altered to improve nutrient diffusion rates. The efficiency of these capsules was measured against their ability to perform glucose fermentations at par with free yeast solutions.
- Chapter 5: After the diffusion rates of capsules had been optimized, the enzymatic activity of the xylose isomerase was examined. Several different types of enzyme were used, including a polymeric immobilized form, free solution enzyme, and enzyme covalently bound to chitosan and microcrystalline cellulose. Activity of these types of enzyme under a variety of conditions, including varying pH values, temperatures, and concentrations of ethanol, was examined. The effect of divalent cations on activity was also determined.
• Chapter 6: With capsules optimized for diffusion, a variety of fermentations were conducted on xylose using the co-immobilized yeast and xylose isomerase. Each of the types of enzyme discussed in Chapter 5 were used in these trials, with the progression of experiments demonstrating the necessity for developing the chitosan- and cellulose-immobilized enzymes. Outlined in this section is the pinnacle of the research conducted in this study.

• Chapter 7: This chapter serves as a general summary of the work outlined in previous sections. It will draw conclusions from the results and indicate where further advancements must be made for the ultimate success of ethanol production from xylose using a co-immobilized yeast-xylose isomerase system.
Chapter 2: Previous Results

In all previously reported trials, capsules were created using a 0.2% alginate, 0.06% Tween 20 shell solution, and all fermentation media contained 4% sugar. Trials conducted with glucose as a carbon source contained 10 capsules, while xylose trials used 5 capsules, both in 30 mL of fermentation medium. During these trials, only ethanol concentration was analyzed, using the alcohol dehydrogenase ethanol analysis (ADEA) procedure described in Chapter 3.2.3.1, and this was only done at the end of a fermentation batch instead of periodically throughout. What follows are the best results for ethanol yield from trials conducted on glucose and on xylose. Lastly, the shortcomings of these results and what this current study has strived to amend are discussed.

2.1: Glucose Fermentation

Fermentation of glucose was initially carried out so as to optimize the capsule structure to prevent leaching of yeast and enzyme, while allowing for the greatest possible diffusion of nutrients and fermentation products. Visual examination of the fermentation medium from these trials showed that yeast cells were successfully inhibited from escaping the capsule interior (Fig. 13). The solution was examined for ethanol content via the ADEA procedure, in which ethanol is oxidized to acetaldehyde thereby generating the UV-Vis detectible compound NADH from NAD as discussed in the next chapter. It was found that the capsule batch produced a yield of 23.0%, whereas the free solution control produced 35.4% after 24 hrs (Table 3). This was calculated by taking the initial amount of glucose in solution (30 mL of 40 g/L broth plus 50 g/L added; 2.7 g total of glucose) and calculating the theoretical amount of ethanol that could be produced from that amount of glucose (2.7 g glucose × 1 mol/180 g × 2 EtOH/1 glucose = 30 mmol EtOH), then comparing it to the actual amount of ethanol in solution. This does not take into account the amount of sugar remaining in solution at the end of the 24 hrs of fermentation.
Stated differently, after 24 hrs of fermentation the free yeast solution control produced 154% the amount of ethanol that was produced in the encapsulated yeast batch. This is a clear indication that the diffusion rate of nutrients, primarily glucose, through the capsule shell is significantly reduced compared to a free solution. However, overall yields for both capsule and control batches were significantly low. It was initially speculated that this low yield was due to an initial aerobic growth period in which the unpurged reaction beakers contained significant amounts of oxygen, until microbial activity finally eliminated it. During this period of aerobic growth, glucose would have been converted to acetyl-CoA and enter the citric acid cycle followed by mitochondrial respiration, ultimately being converted to carbon dioxide and water instead of ethanol. This could greatly reduce the ethanol yield. Alternatively, if oxygen leaked into the system, this too would lead to a certain level of mitochondrial respiration and lowered yields. Another speculated cause for the low yield was that the fermentation had not gone to completion. Since at this time no analysis was conducted to determine the amount or presence of sugar in solution, this could not be verified.

<table>
<thead>
<tr>
<th>Source</th>
<th>Absorbance</th>
<th>EtOH (mmol)</th>
<th>EtOH Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>capsules</td>
<td>0.734</td>
<td>6.90</td>
<td>23.0%</td>
</tr>
<tr>
<td>control</td>
<td>0.566</td>
<td>10.6</td>
<td>35.4%</td>
</tr>
</tbody>
</table>

Fig. 13: 400× magnification of broth after anaerobic incubation for 24 hrs. A) capsule sample and B) control. Yeast cells are ~ 3.5 µm in diameter.

Table 3: Ethanol yield from D-glucose fermentation based on absorbance at 340 nm.
2.2: Xylose Fermentation

With some results for glucose fermentation using capsules, it was decided to attempt co-encapsulation of the xylose isomerase enzyme with *Saccharomyces cerevisiae*, followed by xylose fermentation trials. This was despite a significant issue with the co-encapsulation. Copious amounts of the enzyme leached from the alginate capsules, as determined by the Bradford method described in the next chapter. It was hoped that the leaching would occur slowly enough that some initial improvement in ethanol production could be seen from the co-encapsulation before the enzyme become too diluted.

In this instance, two trials of varying length were conducted, one for 24 and one for 72 hrs. Again, the ADEA procedure for ethanol determination was employed. Here, a significant improvement in ethanol yield was seen for the capsule batch after 24 hrs of fermentation, with nearly twice as much ethanol produced compared to the free solution control (Fig. 14). After 72 hrs of fermentation, the amount of ethanol in both batches was nearly identical. These results have several implications. First, ethanol was produced from xylose, even though a solution of free *S. cerevisiae* is incapable of such metabolism. Secondly, there appears to be a clear short-term advantage of using the developed encapsulation procedure.

![Fig. 14: UV-Vis spectra of the ethanol assay of gravity filtered fermentation mediums from two trials. A) After 24 hrs of anaerobic conditions, and B) after 72 hrs. Free solution refers to the unencapsulated yeast and enzyme control.](image)

Specifically, using the same type of calculations employed for determining the ethanol yields for glucose fermentation, after 24 hrs of fermentation, the capsule batch produced an ethanol yield of 10.5%, whereas the free solution yield was 5.6% (Table 4).
For these calculations, it was assumed that for every three xylose molecules consumed, five ethanol molecules will be produced, compared to the one to two ratio of glucose to ethanol molecules. These results show that over a short time span, co-encapsulated enzyme and yeast produced 187% the amount of ethanol that the free solution did. Though yield for both batches was the same after 72 hrs, it was speculated that this was due to the enzyme leaching from the capsules, resulting in decreased activity as a consequence of dilution and allowing the free solution to ‘catch-up’ as the fermentation came to an end at some point before 72 hrs. Overall low yields, ~26%, were speculated to be caused by the same factors effecting glucose fermentation yields; oxygen contamination and incomplete fermentation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Source</th>
<th>Absorbance</th>
<th>EtOH (mmol)</th>
<th>EtOH Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hrs</td>
<td>Free Yeast</td>
<td>0.110</td>
<td>0.75</td>
<td>5.6%</td>
</tr>
<tr>
<td>24 hrs</td>
<td>Capsules</td>
<td>0.212</td>
<td>1.44</td>
<td>10.5%</td>
</tr>
<tr>
<td>72 hrs</td>
<td>Free Yeast</td>
<td>0.513</td>
<td>3.51</td>
<td>26.3%</td>
</tr>
<tr>
<td>72 hrs</td>
<td>Capsules</td>
<td>0.533</td>
<td>3.58</td>
<td>26.9%</td>
</tr>
</tbody>
</table>
2.3: Identification of Problems

One of the most troubling results from this previous work was that the encapsulated yeast cells performed very poorly at glucose fermentation compared to free yeast. The only plausible explanations are that the encapsulation procedure results in decreased viability of the yeast cells or there are diffusion restrictions caused by the alginate shell of the capsules. During the encapsulation procedure, yeast cells are surrounded by carboxymethylcellulose (CMC), calcium ions, and chloride ions, which are then coated with alginate, as described in the following chapter. CMC and alginate are both biologically inert compounds and would have no effect on yeast cells. Ions may have some effect, but calcium can help stabilize cell membranes and both calcium and chlorine ions are common and necessary for cell survival. As such, it is unlikely that any significant loss in cell viability occurred due to encapsulation. It is far more likely that the alginate shell is simply not porous enough to allow free diffusion of nutrients.

These trials did demonstrate that ethanol could be produced from xylose via the co-encapsulation of xylose isomerase and *Saccharomyces cerevisiae*, and that this system outperforms a free solution of yeast and enzyme. However, these trials consisted of five capsules. To confirm the validity of these results, further trials must be conducted and at larger scales, with hundreds of capsules, to mitigate confounding fluctuations that commonly occur at small scales. This necessitates that capsule production be scaled-up, with several engineering obstacles inherent to such an endeavor that must be overcome. Furthermore, though it was shown that ethanol was produced using the co-encapsulation procedure, there was a significant amount of enzyme leaching. To retain the enhanced activity, faster xylose-to-ethanol conversion rates, and reusability that encapsulation confers to the system thereby decreasing costs, this enzyme leaching must be prevented.

Another serious flaw in this work was that the only factor examined was ethanol concentration, and only at the end of a particular fermentation trial. To better understand the progression and efficiency of the fermentation, a variety of factors must be tracked, and done so over the course of the entire fermentation trial. Two key sensors that should be monitored in addition to the ethanol concentration are pH values and sugar concentration. pH serves as a good indication of the progression of fermentation while
the amount of sugar in the fermentation medium will allow for more accurate calculation of ethanol yield.

To summarize, this previous work can be improved upon by: 1) increasing fermentation efficiency, 2) ensuring that there is free nutrient diffusion through capsule shells, 3) using additional analytical tools to more closely and accurately monitor a fermentation batch, and 4) preventing enzyme leaching. Specifically, encapsulated yeast should be able to produce ethanol from glucose at the same rate and overall yield as a free yeast solution. The xylose isomerase must be prevented from exiting the capsule by aggregation, immobilization, or some other manner to sterically block its escape, so as to fully reap many of the benefits of encapsulation. To increase the accuracy and usefulness of fermentation data, capsule production must be scaled-up and sugar concentration and pH of the fermentation medium must be tracked, in addition to ethanol concentration. Lastly, the ability and efficiency of co-encapsulated xylose isomerase and S. cerevisiae to produce ethanol from xylose must be verified by large-scale fermentations and optimized.
Chapter 3: Procedures

3.1: Capsule Formation and Fermentation

The process for capsule formation is schematically represented in Fig. 15. Freeze dried *Saccharomyces cerevisiae*, available from SuperYeast Liquor Quik, was reconstituted and grown using a sterile 1% BactoPeptone (BD), 15% glucose (Sigma) solution. After 24 hrs growth at room temperature with gentle agitation, a portion of this yeast slurry was gently centrifuged at 3,000 rpm for 1 min. The required amount of pelleted yeast was then recovered and mixed with a core mixture. This core mixture consisted of 1.3% carboxymethylcellulose (Sigma), 1.3% CaCl$_2$ desiccant (Sigma-Aldrich), and material to be encapsulated. Yeast cells were added to the mixture at a concentration of 50 mL per L of mixture. In some trials, xylose isomerase was also added to this mixture, varying both in concentration and composition (free, polymeric entrapped, or immobilized).

Core mixture was dropwise added to the shell solution, consisting of 0.2% alginate (Aldrich) with varying amounts of Tween 20 (Acros), ranging from 0.06 to 0.3% (w/v). After sitting in this solution for 10 min, newly formed capsules were washed with dH$_2$O and transferred to a 1.5% CaCl$_2$ solution to further crosslink the alginate shell,

![Diagram](image)

*Fig. 15:* Formation of alginate capsules. A) Mixture of CMC/CaCl$_2$/yeast/enzyme is dropped into alginate with Tween 20. B) After hardening in additional CaCl$_2$, capsules are introduced to fermentation medium, in which nutrients freely diffuse through shell.
assuring durability. Resultant capsules had a diameter or approximately 2.5 mm.

A certain amount of these capsules were then added to a sterile ferment broth, consisting of 1% BactoPeptone, 4 to 15% glucose or xylose (Sigma-Aldrich), and, dependent on the trial, 50 mM MgCl$_2$ (JT Baker). These batches were placed under anaerobic conditions using an ‘S’-shaped gas trap with a plugged hole through which samples were taken. Incubation was conducted at room temperature or 35 °C in a Lab-Line Incubator-Shaker set to 200 rpm. A control batch containing an equivalent amount of the core mixture with the same amount of fermentation medium was concurrently conducted. Samples from these ferments were periodically taken, gravity filtered, and analyzed in the manners described in section 3.2.
3.2: Fermentation Analysis

Periodically retrieved samples taken from batch fermentations were analyzed using several techniques to determine the progression and efficiency of the fermentation. Specifically, pH, sugar concentration, ethanol concentration, and the amount of leached yeast and enzyme were tracked for each fermentation trial, as presented below.

3.2.1: pH

The process of fermentation has several key stages. At the initiation of fermentation there is a lag period. This is due to the adaptation of the yeast cells to a novel environment. Specifically, nutrient and oxygen availability were altered by introduction of the cells to the anaerobic ferment batch from an aerobic, high nutrient growth medium. After sufficient time has passed, the yeast cells enter an exponential phase, in which they rapidly consume nutrients and produce ethanol at high rates. Lastly, as nutrients become depleted, yeast cells enter a stagnant stage, in which little metabolic activity takes place.

pH of the fermentation medium can be used to track these different changes. During the lag period, pH remains nearly constant. Exponential phase is accompanied by a sharp decrease in pH to a value of between 3 and 4. After this drop-off, pH remains fairly constant, therefore the final stationary stage cannot be detected by alterations in pH values. However, this remains a useful method for tracking fermentation progression. For this reason, the pH of every fermentation mixture sample was recorded. These measurements were taken using an Orion model 420A pH meter.
3.2.2: Reducing Sugar Assay

In order to determine the yield and efficiency of a given reaction, the amount of reactant and product must be known. For fermentation, this necessitates the analysis of the amount of sugar, reactant, in each sample. To accomplish this, a well established procedure using dinitrosalicylic acid (DNS) to detect reducing sugars was used\(^6\).

In DNS colorimetric analysis, DNS is reduced by the aldehyde group of a sugar molecule (Fig. 16). This elicits a color transition from orange to brown. By observing changes in absorbance at a wavelength of 575 nm and via comparison to a calibration curve, the concentration of sugar can be determined. A drawback of this procedure is that it cannot distinguish between reducing species, and is therefore nonspecific for both pentose and hexose sugars.

![Fig. 16: Dinitrosalicylic acid reaction with reducing sugar.](image)

For analysis, two stock solutions were made. Sugar Analysis Solution I (SASI) consisted of 40% sodium potassium tartrate (Sigma-Aldrich), while SASII was 1% DNS (Aldrich), 0.05% sodium sulfite (Sigma-Aldrich), and 1% NaOH. SASI and SASII were mixed in a 1:4 ratio just prior to analysis. A certain amount of sample, dependent on the original sugar concentration of the fermentation medium, was mixed with 3 mL of this combined solution, then placed in a 90 °C hot water bath for 15 min. Absorbance at 575 nm was recorded using a UV-2401 PC Shimadzu spectrophotometer, after samples were cooled to room temperature. This absorbance was correlated to sugar concentration via the Beer-Lambert Law and a related calibration curve.
3.2.3: Ethanol Assay

The procedure providing the most relevant and important information on the success of a particular fermentation trial will obviously be one that examines the amount of ethanol produced per batch over time. In this study, two different methods were used for this analysis. The first, alcohol dehydrogenase ethanol analysis (ADEA), used the enzyme alcohol dehydrogenase and a colorimetric reaction to determine the amount of ethanol in solution. A second method was later developed to improve accuracy and create a more simplified procedure, employing gas chromatography and analysis of the headspace of samples. Both of these approaches are outlined below.

3.2.3.1: ADEA

ADEA is, as with DNS analysis of sugar, a well-established procedure\textsuperscript{61,62}. In this method, a solution of alcohol dehydrogenase, nicotinamide adenine dinucleotide (NAD), and sample containing ethanol is made. In the subsequent reaction, ethanol is oxidized to acetaldehyde while the NAD is reduced to NADH, mediated by the enzyme. To prevent the reverse reaction, a high pH is used to drive the reaction towards NADH, NAD is used in excess, and semicarbazide is added to the solution to eliminate acetaldehyde by forming semicarbazone and water. Each step of this process is shown in Fig. 17. NADH is detectable using UV-Vis spectroscopy, with an absorbance peak at 340 nm, thereby

![Fig. 17: Alcohol dehydrogenase (AD) ethanol analysis (ADEA).](image)
allowing one to determine the amount of ethanol in the original sample by how much NADH was produced.

For analysis, 3.5 mL per sample of a buffer solution containing 75 mM sodium pyrophosphate (Fisher), 22 mM glycine (Fluka), and 75 mM semicarbazide (Eastman) adjusted to a pH value of 8.7 using NaOH was used. To this, 0.2 mL per sample of 16 mM NAD (Sigma) and 0.1 mL per sample of 1.2 kU/L alcohol dehydrogenase (Sigma) were added. Lastly, between 5 and 200 µL of sample was added, dependent on the approximate expected ethanol concentration. After 30 min of incubation at 30 °C, or until the UV-Vis spectrophotometer signal at 340 nm stabilized, the absorbance value at 340 nm was recorded. Via comparison to a calibration curve, ethanol concentration in the original sample was thereby determined.

3.2.3.2: GC Headspace Analysis

Since the ADEA procedure relies on an enzymatically driven reaction that can be problematic due to loss of enzyme activity, gas chromatography headspace analysis was developed as a simpler approach to determining the amount of ethanol present in solution. There are numerous references to use of this approach for ethanol analysis. It is considered a fast, reproducible, and reliable procedure for analyzing ethanol.

During fermentation trials, all samples were stored in GC vials with a septa and cap securely fastened. For prolonged trials, samples were frozen to prevent ethanol vapor escape. Standards using ethanol mixed with unused, sterile fermentation medium were concurrently made and stored along with all samples. The headspace of these vials was sampled using a 10 µL syringe, which was injected into the GC. Between each ferment sample injection run, an external standard injection run was conducted. Comparison of the peak area at retention time 1.3 min. between samples and the external standards was used to determine the ethanol concentration in each sample. A 5890 Series II Hewlett-Packard GC equipped with a flame ionization detector (FID) was used for analysis, running the method entitled BRIAN.M with the following parameters:
Manual injection
Injection Temperature: 200 °C
Detector A Temperature (FID): 280 °C
Oven Initial Temperature: 65 °C
Temperature Program: 65 °C for first 1.4 min., increase at 70 °C/min. to 110 °C, hold at 110 °C for 0.75 min, reset

3.2.4: Leaching Analysis

Since one of the greatest benefits of the encapsulation procedure being explored in this study is the ability to reuse the biocatalyst material, it was necessary to detect if the encapsulated material was escaping, or leaching, into the bulk solution. For the detection of yeast, the bulk solution was sampled and a Micromaster light microscope (Fisher) was used for visual detection of cells. If deemed necessary, sample was placed on microscope slides and heat-fixed, followed by ethidium bromide staining to enhance detection of cells. To test for enzyme leaching in trials using encapsulated xylose isomerase, bulk fermentation medium was analyzed using the Bradford Method\textsuperscript{64}. Under acidic conditions, the dye, Coomassie Brilliant Blue (Fig. 18), will form complexes with protein protonated amino groups, which then absorbs well at 555 nm. 3 mL of commercially available Bradford Reagent (Sigma) was mixed with 60µL of the sample. After a 5 min incubation at room temperature, absorbance at 595 nm was recorded using a UV-Vis spectrophotometer. If enzyme was present, indicating that leaching had occurred, there would be a significant absorbance at that wavelength.

Fig. 18: Coomassie Brilliant Blue dye.
3.3: Enzyme Analysis

The preceding procedures are all related to running and analyzing the efficiency of a fermentation trial. However, to ensure that xylose fermentation would proceed at optimal rates, the activity of the xylose isomerase (XI) enzyme, which is vital for this process, was directly analyzed under a variety of conditions. By tracking the enzyme activity over these sets of conditions, the effects of pH values, temperature, nutrients, ions, ethanol concentration, and various immobilization protocols on the enzyme performance were established.

Activity analysis was carried out by using the conversion of fructose to glucose, mediated by the XI enzyme, followed by glucose assay using a commercial kit (Autokit Glucose from Wako). This procedure used the enzyme to convert fructose to glucose, instead of xylose to xylulose, since there are no readily available analysis procedures for xylulose concentrations. Additionally, this approach has previously been used to determine XI activity towards xylose\textsuperscript{65}. Furthermore, it has been shown that XI activity towards xylose is greater than that towards fructose\textsuperscript{66,67}, meaning all results of this procedure are valid for the optimization of xylose conversion conditions while specific activity values err on the conservative side.

The glucose assay kit used in this procedure uses a series of reactions that result in the activation of a UV-Vis spectroscopy detectable pigment (Fig. 19). Mutarotase converts all α-D-glucose in solution to β-D-glucose. Glucose oxidase then converts the β-D-glucose to gluconic acid, generating hydrogen peroxide. Peroxidase facilitates the reaction of hydrogen peroxide with phenol and 4-aminoantipyrine in solution to synthesize a red pigment. This pigment absorbs maximally at 505 nm, with concentration calculable by comparing absorbance at this wavelength to a calibration curve. Ascorbate oxidase is also included in the kit buffer to eliminate any ascorbic acid interference that may occur, if ascorbic acid is present in the sample being analyzed (such as in a blood sample).

For determining XI activity, a certain amount of enzyme was added to an eppendorf tube with 15% fructose. Parameters of this fructose solution were varied, specifically pH, ethanol concentration, temperature, and the presence and amount of a
variety of divalent cations. After this enzyme and fructose solution was mixed using a vortex, it was left to incubate for a period of time at room temperature. Once this incubation period was over, the tubes were centrifuged and some amount of the supernatant, based on the expected glucose concentration, was added to 3 mL of the Autokit Glucose buffer. Full pigment development within this solution was complete after a 5 min incubation at 37 °C. A UV-Vis spectrophotometer was then used to determine the absorbance of the solution at 505 nm, allowing the amount of fructose that was converted to glucose by the enzyme to be resolved. This allows the activity of the XI towards fructose to be determined.

![Fig. 19: Autokit Glucose method. A) α-D-glucose conversion to β-D-glucose via mutarotase. B) Gluconic acid and hydrogen peroxide production from β-D-glucose via glucose oxidase. C) Synthesis of red pigment from hydrogen peroxide, phenol, and 4-aminoantipyrine via peroxidase.](image-url)

Since activity of XI was being tested under varying pH conditions, ethanol concentrations, and cation concentrations, it was necessary to determine if these conditions also affected the kit analysis since a variety of enzymes are vital to the function and accuracy of the kit. As such, all solutions being used for enzyme activity were analyzed with the kit after addition of a standard amount of glucose (without
addition of XI or an incubation period). These results are outlined in Fig. 20. It can be seen that there was no statistically significant variation for cation solutions. Solutions of varying pH values did show minor variations, most significantly at a pH of 3. Ethanol also showed some interference, with absorbance at 15% (v/v) ethanol approximately 2% lower than the absorbance at 0% (v/v) ethanol. For all solutions, the level of interference was miniscule enough to be deemed insignificant interferences for the kit analysis.

**Fig. 20:** Interference of various solutions on glucose determination using commercial Autokit. A) Effect of Mg$^{2+}$ and Co$^{2+}$ compared to standard. CoI was 0.5 mM, CoII 10 mM, MgI 5 mM, and MgII 50 mM. B) Effect of pH from 7 to 3. C) Effect of ethanol concentration, 15% (v/v) vs. 0% (v/v). All standards were 500 mg/L glucose.
3.4: Enzyme Immobilization

To prevent enzyme leaching from the capsule interior, two immobilization approaches were taken. The resultant enzyme-substrate molecule is too bulky to fit through the pores in the alginate shell, while the immobilization may serve to enhance enzyme activity by protecting the protein conformation. Below are the procedures for the two forms of immobilization explored, chitosan and microcrystalline cellulose immobilization. Characterization of and fermentation with these forms of immobilized enzyme are presented in Chapters 5 and 6, respectively.

3.4.1: Chitosan Immobilization

Immobilization on chitosan was done by using glutaraldehyde crosslinking, a well established procedure for covalently linking molecules via amino groups\(^\text{29,68}\). This reaction is schematically presented in Fig. 21. 5 g of chitosan (Aldrich) was mixed with 10 mL 12.5% glutaraldehyde (Acros) and left covered with magnetic stirring for a minimum of 12 hrs. The reaction mixture was then vacuum filtered, rinsed with 20 mL of dH\(_2\)O, rinsed with acetone, then air dried. From this, a portion was mixed in a 1:1 ratio with a suspension of glucose isomerase from Hampton Research. After an additional 6 hrs of

![Diagram of chitosan immobilization process]

Fig. 21: Procedure for the immobilization of enzyme on chitosan.
stirring at room temperature, 1 mL of 0.2 M NaBH₄, a reducing agent, per 5 mL of reaction mixture was added to reduce the chitosan-glutaraldehyde and enzyme-glutaraldehyde Schiff bases. This was stirred for 18 hrs, followed by the addition of 1 mL 1 M Tris buffer pH 8 per 2 mL of reaction mixture and a further 6 hrs of mixing. The Tris served to block any remaining active glutaraldehyde sites. Lastly, the reaction mixture was filtered and the filtrate was stored after air drying. No organic solvent was added, as in the activation step to expedite drying, so as to avoid enzyme denaturation.

### 3.4.2: Microcrystalline Cellulose Immobilization

Enzyme immobilization on microcrystalline cellulose followed a similar chemistry. The cellulose was activated by using NaIO₄ to form ketones, performed by Dr. Maryna Ornatska using an established procedure⁶⁹ and shown in Fig. 22. After activation, the material was washed with acetone and air dried. This was mixed in a 1:1 ratio with a glucose isomerase suspension from Hampton Research and left stirring at room temperature for 6 hrs. 1 mL of 0.2 M NaBH₄ per 5 mL of reaction mixture was added to reduce the cellulose-enzyme Schiff bases that had formed and this mixture was stirred for an additional 12 hrs. Next, 1 mL of 1 M Tris buffer pH 8 per 2 mL of reaction mixture was added to block any remaining activated sites, followed by 6 hrs of stirring. The microcrystalline cellulose immobilized enzyme was then recovered via vacuum filtration, air dried, and stored for future use. As with the chitosan immobilization, acetone was not used to increase the drying rate since it would denature the enzyme.
Chapter 4: Encapsulation and Fermentation Optimization

4.1: Scale-up

One objective of this study was to scale-up capsule production using a pump system to replace hand micropipetting. This was to demonstrate that the capsules could be produced at the amounts necessary for industrial use and to allow larger fermentation batches to be conducted. A larger batch results in increased accuracy of results by decreasing the large effects minor fluctuations can have on a small system.

As such, the pump system shown in Fig. 23 was developed. In this system, the core solution, containing CMC, CaCl$_2$, and material to be encapsulated, is well-mixed and drawn through a tube by a peristaltic pump (Watson Marlow SciQ 400). The suspension is dropwise added from a height of approximately 2 cm into a gently stirring solution of 0.2% alginate with 0.06 to 0.3% (w/v) Tween 20. From here, the encapsulation procedure is identical to that presented in Chapter 3. After stirring in the alginate solution for 10 min, the newly formed capsules were washed with dH$_2$O and further hardened in 1.5% CaCl$_2$ for an additional 15 min. Resultant capsules had a diameter of approximately 2.5 mm.

Initially, this procedure resulted in elongated capsules because droplets would sit on the alginate surface and slowly drop into the shell solution. To overcome this,
magnetic particles were added to the core solution suspension. 0.125% magnetic Fe$_2$O$_3$ nanoparticles (prepared by Dr. Daniel Andreescu; Clarkson University) was added to the CMC/CaCl$_2$ solution and sonicated for 20 min to disperse the particles in solution. Since these particles slightly increased the mass of the droplets without effecting the size or volume, these droplets strike the alginate shell solution surface with greater momentum. Dropping the solution from a higher distance would also allow the droplets to gain greater momentum, but this typically results in malformation of the capsules whereas the magnetic particles confer further advantages to prevent this. Specifically, the magnet at the bottom of the alginate solution, used to stir the solution, applies some force on the magnetic particles to help draw them into the solution. This also pulls the rest of the core solution suspension droplet in, allowing capsule malformations to be avoided since smaller momentums can be used. The end result is the production of well formed, uniform, spherical capsules. In addition to simplifying mass production of the capsules, these magnetic particles allow for easy recovery of the capsules from large fermentation batches, thereby providing a convenient manner in which to transfer capsules to the next batch fermentation.

Another advantage of this pump system is that a variety of parameters can be altered to form capsules of different sizes and shapes. The speed at which the core solution is pumped, the angle and height of the pipette tip from which it is dropped, the size of the pipette tip, and the speed at which the alginate shell solution is being mixed can all easily be adjusted. This is useful because when the viscosity of either the core solution or the shell solution is altered, a new set of conditions must be found that will consistently yield well formed capsules.
4.2: Baseline Fermentation and Effects of Nutrients

A series of analytical procedures, presented in Chapter 3, were tested on initial fermentation trials to evaluate their ability to track progression and efficiency of fermentation batches. These trials were also done to set the baseline from which to make improvements to rates of ethanol production and nutrient diffusion. Glucose was used as the carbon source since it is well known that yeast will ferment this to ethanol. As such, any ethanol production differences between a free yeast solution control and encapsulated yeast will be solely due to restrictions in nutrient diffusion or cell viability brought about by the encapsulation procedure. Likewise, in fermentation trials that vary the amounts and types of nutrients within the fermentation medium, comparison of fermentation rates will not be confounded by other factors, such as the ability of an enzyme to produce a useable fermentation feedstock under the divergent conditions.

First, media nutrients and sugar concentrations were varied to determine if any improvement to the fermentation was seen. In one of the first trials, 500 capsules containing an internal concentration of 2.5% *Saccharomyces cerevisiae* were added to a 100 mL ferment broth containing 1% BactoPeptone and 4% glucose and left under anaerobic conditions at room temperature with agitation for 48 hrs. A control using the same amount of yeast cells was prepared and left under identical conditions. Periodically through the trial, sugar concentration, ethanol content, and pH were determined through DNS analysis, GC chromatography headspace analysis, and using a pH meter, respectively. It was found that pH did not drop below a value of 4, whereas typical fermentation conditions range from a value of 3 to 4 (Fig. 24). Under these conditions contaminant microbial growth could have occurred. Sugar analysis showed that

![Fig. 24: pH vs. time for the baseline glucose fermentation trial.](image-url)
sugar was metabolized at a fast, steady rate and disappeared in both fermentation batches by 12 hrs of fermentation (Fig. 25). There was no significant difference between the rates of sugar metabolism between the two fermentation batches. Ethanol analysis showed erratic ethanol concentrations over time, with values well below those used for development of the calibration curve (Fig. 25). These results demonstrated that the fermentation carried out on glucose was problematic, with likely microbial contamination and problematic GC ethanol analysis (drift in calibration).

After this trial, it was decided to increase the amount of initial sugar from 4% to 15%. With extra glucose in solution, the final amount of ethanol produced should be significantly higher. This would help to alleviate the variability seen in the GC ethanol analysis by increasing the signal to noise ratio. More stringent protocols, including autoclaving of all glassware and performing all fermentation medium samplings using flame sterilized glass pipettes, were also enforced so as to prevent microbial contamination. With these improvements, a second fermentation was carried out, identical in setup to the previous except 15% glucose with 1% BactoPeptone was used as the fermentation medium instead of 4% glucose with 1% BactoPeptone. This trial was again conducted for 48 hrs with periodically recovered samples analyzed for sugar concentration, ethanol content, and pH value. To further improve the accuracy of the GC analysis, a 5% (v/v) ethanol in fermentation medium was used as an external standard.

In this trial, the pH dropped to a value of ~3.5, typically seen for fermentation, with no significant lag period (Fig. 26). Sugar was metabolized at a fairly consistent rate, after a minor lag period, ending up around 30 and 35% of the initial sugar for the capsule

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**Fig. 25**: Ethanol and sugar concentrations over time for the baseline glucose fermentation trial. The primary Y-axis is GC peak ht. for ethanol and the secondary is sugar as a percent of initial 4% glucose.
and free yeast batches, respectively (Fig. 27). Though values are different, the rate and overall trend for both batches closely mirrored each other, indicating that there may have been interferences present in one solution during the DNS analysis. Ethanol production was much more significant than that seen in the previous fermentation trial, as expected given the much greater concentration of glucose in the fermentation medium (Fig. 27). To summarize this trial, ethanol was being produced at a yield of roughly 80%, after taking into account the amount of residual sugar in solution after 48 hrs of fermentation and estimating ethanol content from GC analysis via comparison to the external standard that was used. This is in accordance with published and accepted values for glucose fermentation, indicating that the system is working on glucose solutions. These results are also superior to those seen in the previously reported work presented in Chapter 2, indicating that the larger scale fermentation batches perform better. However, the free yeast cells were still outperforming the encapsulated yeast cells, and the overall yield and rate could be improved.

Fig. 26: pH vs. time for the increased glucose fermentation trial.

Fig. 27: Ethanol and sugar concentrations over time for the increased glucose fermentation trial. The primary Y-axis is GC peak ht. for ethanol and the secondary is sugar as a percent of initial 15% glucose.
To address this, another trial was conducted that adjusted the type and amount of nutrients present in the fermentation medium. It was proposed that the addition of certain key nutrients would improve fermentation rate and yield of ethanol from glucose. As such, a syringe-filtered aliquot of yeast energizer, containing diammonium phosphate, magnesium sulfate, and vitamin B complex, was injected into 100 mL of the 15% glucose with 1% BactoPeptone fermentation medium. This yeast energizer is made commercially available as a way of initiating fermentation or restarting a halted fermentation. 500 capsules containing an internal concentration of 2.5% yeast cells were added to the fermentation medium. A second control batch was made with an equivalent amount of yeast, and both batches were placed under anaerobic conditions at room temperature with gentle agitation for 48 hrs.

pH results for this trial were similar to trials without added nutrients, indicating that there was no significant decrease in the lag period of fermentation. Capsule and free yeast solutions were consistently similar and ending at the expected value of approximately 3.5 (Fig. 28). Sugar analysis results were similar to that seen for

![Fig. 28: pH vs. time for the increased nutrient fermentation trial.](image_url)

![Fig. 29: Ethanol and sugar concentrations over time for the increased nutrient fermentation trial.](image_url)

Enhanced Xylose Fermentation, Page 50
previous glucose fermentation trials as well. Free yeast and capsule batches had a nearly identical, steady decrease in sugar concentration ending at approximately 35% of the initial sugar concentration after 48 hrs of fermentation (Fig. 29). The interference seen in the sugar analysis of previous fermentation trials was overcome by extending incubation time of the DNS reaction, allowing all samples to fully react. Ethanol content was also similar to previous fermentation trials, but with a more pronounced initial lag period and a later plateau period (Fig. 29). Compared to the 5% (v/v) ethanol external standard, both batches approach approximately 4% ethanol, again giving a rough overall yield of 80% based on the amount of residual sugar.

Overall, this fermentation trial shows no major improvement due to the inclusion of the additional nutrients into the ferment broth. Markedly similar results to previous glucose fermentation trials were seen, as presented above, though results were improved by overcoming the sugar analysis interference. After this fermentation trial, it was apparent that minor modifications to the capsule shell composition were still necessary. This was in part due to minor differences still seen in the fermentation rate of encapsulated yeast cells compared to free yeast. However, a more prominent issue was that in all these trials a significant amount of capsules burst. The “capsule mortality rate” for these trials was as high as 25% after 48 hrs of incubation. It was assumed that this was due to the nucleation of carbon dioxide, a product of fermentation, within capsules. Though dissolved carbon dioxide and very small bubbles can escape from a capsule, nucleation can lead to the fast development of carbon dioxide gas bubbles that are too large to diffuse through the alginate shell. As these bubbles increase in size, they begin to exert internal pressure on the capsule shell, eventually leading to rupturing. If there is a high mortality rate seen in the capsules, the advantages they gain from being reusable quickly diminish. Therefore, the capsule shell composition needed to be further adjusted to prevent the formation of carbon dioxide bubbles as well as increase nutrient diffusion rates.
4.3: Improved Diffusion

As discussed in section 4.2, the composition of the capsule shell material needed to be further adjusted so as to increase nutrient diffusion and decrease “capsule mortality rate” via carbon dioxide rupturing. This was to ensure that the full benefits of capsule reusability could be realized. To determine the effectiveness of these improvements, fermentation trials were conducted on glucose. This was, as previously explained, to directly compare the fermentation of encapsulated to free yeast cells without any complicating factors such as interference of enzymatic activity.

To increase diffusion rates of gasses and nutrients, the capsule shell material was altered by increasing permeability via increasing the porosity. Specifically, porosity was increased by increasing the amount of Tween 20, a surfactant, in the shell alginate solution from 0.06 to 0.3% (w/v). Tween 20 serves a two-fold purpose. First, it increases the number of pores in the capsule shell, allowing more avenues of carbon dioxide escape and nutrient entry. Second, since Tween 20 is a surfactant, it will act to disperse carbon dioxide, thereby preventing its nucleation into non-diffusible gas bubbles. A batch of 500 of these modified shell capsules, with an internal yeast cell concentration of 2.5%, were placed in 100 mL of 15% glucose with 1% BactoPeptone fermentation medium and left under anaerobic conditions at room temperature for 48 hrs. Additionally, a control batch with the same amount of yeast and fermentation medium was concurrently run.

As was routine for all previous glucose fermentation trials, pH values dropped to between 3.5 and 4 during fermentation (Fig. 30). Sugar analysis results were also similar to previous trials, as well as similar for the capsule batch compared to

![Fig. 30: pH vs. time for the improved diffusion fermentation trial.](image)

![Fig. 31: Ethanol and sugar concentrations over time for the increased diffusion fermentation trial. The primary Y-axis is GC peak ht. for ethanol and the secondary is sugar as a percent of initial 15% glucose. The external standard used for GC was 5% (v/v) ethanol.](image)
the free solution batch. The capsule and free batches contained 35.3 and 29.4% of the initial sugar, respectively, after 48 hrs of fermentation (Fig. 31). This showed that the free solution yeast had slightly higher metabolic rates than the encapsulated yeast. Ethanol concentration reached 4.5% for capsules and 4.7% for free solution after 48 hrs (Fig. 31). Therefore, free solution yeast produced more ethanol than encapsulated yeast. However, when examining sugar and ethanol concentrations together, it was found that encapsulated yeast had a yield of 90.7% compared to 86.8% for free yeast. Therefore, though the rate of ethanol production was slightly lower for encapsulated yeast, fermentation was more efficient than in the free solution. Furthermore, the yield for 0.3% Tween capsules was 10% higher than that for previous 0.06% Tween trials.

Lastly, the “capsule mortality rate” for this fermentation trial was zero. The new shell material composition allows for sufficient diffusion of fermentation-generated gasses, thereby preventing the capsule bloating and rupturing that was seen in initial fermentation trials. Overall, this new shell composition of 0.2% alginate with 0.3% Tween 20 was successful at eliminating previously encountered problems while improving fermentation rates and yields to levels comparable to, or better than, those seen in industrial grade yeast strains. As a consequence, it was assumed that the system was fully optimized for fermentation on glucose. Consequently, the capsule composition was assumed to be optimized for fermentation conditions, regardless of whether glucose or xylose is used as the carbon source. This accomplished one of the main objectives of this study, by creating capsules that produce ethanol from glucose at the same or greater yields than free yeast and at industrially acceptable values.
Chapter 5: Enzyme Immobilization and Effects on Activity

With capsule structure optimized for nutrient diffusion, the next step in this work was to determine how the activity of the enzyme xylose isomerase (XI) would perform under a variety of conditions. The conditions tested were those most relevant to fermentation: pH, temperature, and ethanol concentration. Also tested was the effect of various divalent cations on activity, since literature suggested that several of these cations enhanced XI activity. These tests were conducted using the procedure outlined in section 3.3. Several different types of enzyme were used in this study, primarily a commercially available polymeric enzyme and a free enzyme used as an x-ray crystallography standard. This free enzyme was used as it arrived, and immobilized in the lab. The immobilization techniques used were covalent binding on chitosan and on microcrystalline cellulose. These procedures were discussed in section 3.4.

5.1: Polymer Immobilized Xylose Isomerase

This commercial form of enzyme was purchased from Sigma as small grains and was originally isolated from Streptomyces murinus. This enzyme was entrapped or immobilized in an unknown polymeric substance. These grains could be ground to a fine powder using a pestle and mortar. A preliminary study of this polymeric XI tested the activity of powdered and un-powdered enzyme, then the activity of powdered enzyme in the presence of 5% (v/v) ethanol, 10 mM MgCl₂, 1 mM CoCl₂, 10 mM MgCl₂ + 1 mM CoCl₂, and 1.5% CaCl₂ (the highest possible Ca²⁺ concentration that could be seen in a normal capsule interior given that a solution of 1.5% CaCl₂ is used in the final step of capsule formation).

It was found that the presence of 5% (v/v) ethanol in the sample inhibited the activity, translating to a lowered production of glucose. As expected, the activity of the powdered enzyme was slightly higher that that of the un-powdered immobilized enzyme. The Ca²⁺ slightly enhanced activity, while the 1 mM Co²⁺ approximately doubled
activity, compared to the powdered solution. Most successful of the solutions was the 10 mM Mg$^{2+}$, which nearly tripled the activity, whereas a combination of 1 mM Co$^{2+}$ and 10 mM Mg$^{2+}$ resulted in an activity level between that of the cobalt and magnesium independently (Fig. 32). This implies that the increased activity is due to binding of the cations to an activation or enhancer site on the enzyme, and magnesium is more effective at this activation. When magnesium and cobalt are both present, cobalt competes for the binding site, resulting in an intermediate amount of activity. Conversion rate values for these trials are presented in Table 5. The success of these results led to a more in depth characterization of the XI activity under the conditions represented here, as well as at a variety of temperatures, which is presented below.

**Table 5**: Preliminary XI activity results. Rates for 50 µg per mL enzyme in various solutions in 15% fructose. Unless otherwise specified, the enzyme was powdered.

<table>
<thead>
<tr>
<th>Trial Solution</th>
<th>µg Fructose / min</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% (v/v) EtOH</td>
<td>3.7</td>
</tr>
<tr>
<td>Unpowdered</td>
<td>4.8</td>
</tr>
<tr>
<td>Powdered</td>
<td>6.4</td>
</tr>
<tr>
<td>10 mM Mg$^{2+}$</td>
<td>17.2</td>
</tr>
<tr>
<td>1 mM Co$^{2+}$</td>
<td>11.8</td>
</tr>
<tr>
<td>1.5% (w/v) Ca$^{2+}$</td>
<td>7.4</td>
</tr>
<tr>
<td>10 mM Mg$^{2+}$ + 1 mM Co$^{2+}$</td>
<td>14.0</td>
</tr>
</tbody>
</table>

**Fig. 32**: Preliminary polymeric XI activity results. UV-Vis spectra with the peak at 505 nm equating to glucose concentration in solution after incubation. All samples were with powdered enzyme except the one specified as unpowdered. When specified, ethanol was 5% (v/v), magnesium 10 mM, cobalt 1 mM, and calcium 15%.
5.1.1: Effects of pH

Within a fermentation medium, pH will range from values around 6 at the initiation of a batch to as low as 3 after the fermentation is well underway. As such, the activity of the polymeric XI must be known over this range of values to determine if the fermentation medium must be buffered to a particular value so as to prevent loss of activity. To accomplish this analysis, 0.1 M phosphate buffer saline solutions containing 15% fructose and pH values adjusted to 3, 3.5, 4, 5, 6, and 7 were mixed. Polymeric XI was incubated in these solutions and the procedure outlined in section 3.3 was performed to determine the enzyme activity. From pH 7, there was a steep decrease in activity to pH 6, then a nearly steady activity to pH 3 (Fig. 33). Overall, there was approximately a 35% decrease in conversion rate between pH 7 and 3. These results indicate that pH does have a significant effect on polymeric XI activity, but implies that, under fermentation conditions, a significant conversion rate should occur. Since buffering a fermentation medium can lower the rate and efficiency of ethanol production, a sacrificial 35% decrease in activity is deemed acceptable.

5.1.4: Effects of Ethanol Concentration

Since ethanol is produced during a fermentation trial, the effect of ethanol concentration on polymeric XI activity should be determined. If it is found that activity sharply drops off after a certain value, for instance after 10% ethanol, the fermentation trial can be setup so that it will not accumulate ethanol above that concentration. For
example, this can be done by starting with no more than 20% sugar in the fermentation medium so that the theoretical ethanol yield is below that of the activity limit. To accomplish this analysis, polymeric XI was incubated in 15% fructose solutions containing 1, 5, 10, and 15% (v/v) ethanol. Results showed a steady decline in activity by increasing ethanol from 1 to 10% (v/v), with a further but shallower decrease from 10 to 15% (Fig. 34). Specifically, from 1 to 15% ethanol there was a 21% drop in activity. The fermentation solutions to be used in this project are expected to be 15-20% sugar, with an expectation of yielding between 7 and 10% ethanol. As such, based on these results, it can be expected that the polymeric XI activity will drop by up to approximately 27% during future fermentations due to the presence of ethanol. However, there was still a significant conversion rate even at 15% (v/v) ethanol. Since little can be done to avoid this decrease in activity, it must simply be sacrificed.

5.1.3: Effects of Divalent Cations

Review of literature revealed that the XI enzyme functions in a homodimeric form and that it may operate via an enzyme-divalent cation-substrate binding mechanism. This demonstrates that divalent cations must be present in solution for the function of XI. Though the polymeric XI being used may originally have bound cations when introduced into the capsules, in previously conducted fermentation trials the only other source of cations was the calcium crosslinking the alginate shell. It is possible that if the fermentation medium contains divalent cations in addition to the calcium from the capsules, the polymeric XI activity may be significantly enhanced. This was supported
by the preliminary results presented above with Mg\textsuperscript{2+} and Co\textsuperscript{2+}. Therefore, it was decided that the optimal concentrations of these divalent cations should be determined for implementation in future fermentation trials.

For Mg\textsuperscript{2+}, concentrations of 5, 10, 15, 20, 25, 30, 40, and 50 mM were used, while 0.5, 1, 1.5, 2, 2.5, 3, 4, and 5 mM solutions were used for Co\textsuperscript{2+}. Activity was determined via 45 min. incubation of the solutions in 15% fructose, with subsequent UV-Vis determination of glucose concentration performed as previously discussed in section 3.3. For the magnesium, activity followed a logarithmic pattern (Fig. 35). Activity was not expected to improve significantly above the maximum tested magnesium concentration of 50 mM. Cobalt solution activity followed a linear relationship, but with a low slope indicating that high concentrations of cobalt would be necessary to significantly improve enzyme activity (Fig. 36).

Based on these results, it was decided to use magnesium in future fermentation trials to enhance the polymeric XI activity. This was largely since these and previous results demonstrated that magnesium enhances the enzyme activity to a much greater extent than cobalt. Furthermore, magnesium is an important micronutrient for yeast, and therefore not harmful at the concentrations being examined. It is not know whether these concentrations of cobalt would affect yeast cell viability. For maximum enzyme
enhancement, all future fermentation mediums contained 50 mM Mg\textsuperscript{2+}. This concentration was chosen since little improvement in activity was expected at higher Mg\textsuperscript{2+} concentrations and it was anticipated that the metabolism of some magnesium by the yeast would take place.

5.1.2: Effects of Temperature

All enzymes have a temperatures optimum. In order to fully optimize polymeric XI activity, this optimum was sought. To do so, enzyme activity tests were performed at 25, 30, 35, and 40 °C. These values were chosen since they are favorable for yeast survival and fermentation. A perfect linear relationship between activity and temperature was seen, with a 295% improvement in activity from 25 to 40 °C (Fig. 37). Previous fermentation trials were conducted at room temperature (near 25 °C), so these data suggested that by increasing the temperature to 35 °C, near the optimal temperature for fermentation, a 229% increase in polymeric XI activity over previous trials should be seen. Therefore, enzyme activity could be improved by increasing temperature while also increasing the rate and yield of fermentation. Consequently, it was decided that all future fermentations with the polymeric XI enzyme would be conducted at this optimal temperature.

Fig. 37: Effect of temperature on polymeric XI activity.
5.2: Free Xylose Isomerase

Due to limitations from the polymeric XI outlined in Chapter 6, a solution of free XI was also tested. This material was purchased from Hampton Research under the name of glucose isomerase as a crystal suspension. The XI crystals were mostly 50-150 microns in size and came in a solution at a concentration of 33 mg/mL with 6 mM Tris hydrochloride pH 7.0, 120 mg/mL ammonium sulfate, and 1 mM magnesium sulfate. These additional materials in solution serve to stabilize the XI crystals. Importantly, it should be noted that this XI was isolated and purified from Streptomyces rubiginosus, as opposed to the Streptomyces murinus used for the polymeric XI, and will therefore have slightly different activities and tolerances. Needless to say, gram per gram the free XI has a much higher concentration of enzyme than the polymeric XI and, consequentially, higher activity.

This free XI is small enough that it will leach from capsules, as shown in the previously presented work on this topic\(^1\). However, since it is not immobilized or trapped in a polymeric matrix, it can be incorporated into capsules at a much higher concentration. It must be remembered that, due to leaching, this free XI cannot be used in the end product of this study. However, working from this free XI, two approaches were taken to prevent enzyme leaching while using higher concentrations of XI than could be possible with the polymeric XI. These two approaches are outlined shortly. Consequently, for comparison to these methods, the key parameters of pH and temperature tolerances were tested for the free enzyme. It is likely, since magnesium ions are present at far greater concentrations in the fermentation medium than necessary for polymeric enzyme activity improvement, that the same will hold true for the free XI. Ethanol will have an effect on activity, but this is unavoidable. Both pH and temperature, however, are adjustable to some extent, and therefore are vital to characterize for free XI activity optimization. These results are outlined below.
5.2.1: Effects of pH

As with polymeric XI characterization, pH of a 15% fructose solution was adjusted to values between 3 and 7 with activity of a 10 µL aliquot of free XI determined (Section 3.3). Unlike the previous trial, a 0.2 mM acetate buffer was used to adjust the pH values, instead of phosphate, since it was determined that phosphate forms insoluble salts with the divalent cations vital to the enzymatic function of XI. Results are shown in Fig. 38, with a 32% decrease in activity seen when pH dropped from 7 to 6. Activity remained constant below this level, a similar trend as seen for the polymeric XI (Fig. 33). This is a difficult obstacle to overcome since significant improvements in XI activity will only be seen above pH 6, conditions not conducive to fermentation.

5.2.2: Effects of Temperature

Free XI activity was tested at temperatures ranging from 25 to 40 °C using 10 µL aliquots of enzyme. There was a steady increase in activity with increasing temperature over this range, with a 183% improvement in activity seen between the two tested extremes (Fig. 39). This is substantial but lower than the 295% improvement seen over the same range for polymeric XI activity. From 25 to 35 °C there was a 148% improvement, validating the conduction of xylose fermentations at 35 °C.
5.3: Xylose Isomerase Immobilization on Chitosan

To prevent enzyme leaching, free XI was covalently bound to chitosan. Chitosan is similar to alginate in that it is a natural polymer of sugar monomers. Unlike alginate, chitosan has amino functional groups, composed of D-glucosamine and N-acetyl-D-glucosamine, and is synthesized by deacetylating the chitin recovered from crustacean shells. It is attractive for use as an immobilization substrate because it has a high concentration of functional groups allowing a large number of enzymes to be immobilized per polymer molecule. Additionally, chitosan is a long linear polymer that cannot easily escape through capsule shell pores and has amino groups that would interact with the carboxyl groups of the capsule alginate shell if the polymer did try to escape, thereby trapping it. The immobilization of XI on chitosan was carried out using a well established glutaraldehyde crosslinking reaction\(^{29,68}\), as presented in section 3.4.1.

In order to covalently immobilize the enzyme on chitosan, the chitosan must first be activated so that it will react with and bind the enzyme. This is accomplished by mixing the chitosan with glutaraldehyde, which binds to the chitosan and exposes an aldehyde group at the end of a molecular arm. This aldehyde will form Schiff bases with the N-terminus of the enzyme. After reduction of this Schiff base with a reducing agent such as sodium borohydride, the enzyme is effectively covalently bound to the chitosan (Fig. 20). Under actual conditions, many activated chitosan functional groups may remain unbound to the enzyme. To prevent binding of these groups to other molecules and yeast, they are blocked by introducing Tris buffer to the reaction mixture. This serves the additional purpose of adjusting the pH of the mixture to 8, at which value the chitosan remains undissolved. The solid chitosan suspension, with bound XI, can then easily be recovered via filtration.

After biofunctionalization, activity of the recovered XI-Chit was tested using the procedure presented in section 3.3 (Fig. 40). The measured activity was lower than that seen for an equal amount of free XI however direct comparison between the immobilized and free enzyme is difficult. The reason for this difficulty is that chitosan may not have bound the entire amount of XI it was mixed with, or may have bound a certain amount in an orientation inhibiting the enzyme activity. More significantly, equal weights of
immobilized and free enzyme were used in this comparison, whereas the immobilized enzyme is actually a mixture of chitosan and enzyme. As such, half of the immobilized mass, at most, would be XI given the 1:1 ratio used in the immobilization procedure. This means that there was likely far less enzyme in the XI-Chit than in the free solution. Lastly, the free enzyme contained the activity enhancer Mg$^{2+}$ from its storage solution and was more evenly distributed in the solution used for determining activity since it was dissolved in the solution. The XI-Chit was not dissolved and settled out in solution over time, providing it with a lower local availability of substrate during the test. However, these results show that the XI was successfully immobilized on chitosan and expressed high levels of enzyme activity, making it a promising solution to the enzyme leaching problem.

One drawback to this approach is that commercially available chitosan comes as large grains. By performing the immobilization procedure on these grains, the material cannot be mixed with the CMC/CaCl$_2$ core solution for incorporation into capsules since the chitosan will not pass through the pipette tip used to drop the core suspension into the alginate shell solution. Grinding with a pestle and mortar was not effective at sufficiently reducing the chitosan grain size. A ball grinder could be employed but requires significant amounts of material and has yet to be attempted.

As an alternative solution, the chitosan can be dissolved at low pH values then precipitated using a base. If the solution is dilute, small particles of chitosan will be created. This was attempted using 0.25% chitosan in 0.1 M acetic acid. pH was adjusted to 12 using concentrated NaOH and the precipitate was filtered and washed with dH$_2$O. Glutaraldehyde activation and XI immobilization were carried out as described in section 3.4.1 and the material was recovered via filtration. Resultant material was small enough for incorporation into capsules and was used for a fermentation trial discussed in Chapter

\[
\begin{array}{c}
\text{Absorbance} \\
\end{array}
\]

\[
\begin{array}{c}
300 & 400 & 500 & 600 & 700 \\
0 & 0.05 & 0.1 & 0.15 \\
\end{array}
\]

Fig. 40: Preliminary XI-Chitosan results, as activity compared to XI-free.
This material was also tested for activity at various pH values and temperatures to
determine tolerances and for comparison to results for the free enzyme activity, as
presented below.

5.3.1: Effects of pH

Due to limited availability of the free XI enzyme, only a small amount of
chitosan-immobilized XI was used for characterization trials. For testing pH
tolerances, 10 mg of XI-chitosan was mixed with 15% fructose buffered to pH
values between 3 and 6 using an acetate buffer. Activity was determined as outlined in
section 3.3. pH 7 was not tested due to an inadequate amount of enzyme material. As
seen in Fig. 41, activity of the XI-chitosan was slightly greater than that of the free
enzyme at pH values 3 through 6. This is to be expected, since immobilization can help
stabilize enzymes and protect their activity. If these data are more closely examined, it
can be seen that the activity of the XI-chitosan did slightly change over this range, with a
conversion rate of 14.5 and 14.2 µg Fru/hr at pH 6 and 3, respectively, a decrease of
approximately 2.3%. This immobilization therefore improves the XI activity over the
free solution at pH 3-6.

5.3.2: Effects of Temperature

When the effect of temperature was examined for XI-chitosan, a range of 30 to 40
°C was used, excluding 25 °C due to lack of material. Again, for each value 10 mg of
XI-chitosan was incubated in 15% fructose and activity was determined via the procedure
in section 3.3. In this particular case, there was not a significant improvement seen in activity with increasing temperature (Fig. 42). An increase in temperature from 30 to 40 °C resulted in a 6% improvement in activity. The overall activity was also significantly lower than that of the free XI. This may have been due to by errors caused by the difficulty of comparing the activity of free to immobilized enzyme, as previously outlined, specifically the inability to insure that the same amount of enzyme was used for both trials during activity analysis. This originates from the difficulty in adequately quantifying the exact amount of immobilized and functional enzyme per unit of XI-chitosan material. Since there is no indication that a temperature of 35 °C inhibits XI activity, it will continue to be used in future fermentation trials.

![Graph showing effect of temperature on XI-chitosan vs. free XI activity.](image)

**Fig. 42:** Effect of temperature on XI-chitosan vs. free XI activity.
To avoid the difficulties in the incorporation of the enzyme material into the capsule interior encountered in the XI-chitosan procedure, cellulose was exploited as an alternative immobilization substrate. This cellulose was purchased in crystalline form and activated using an established procedure employing NaIO₄, followed by reductive alkylation similar to that used for XI-chitosan immobilization⁶⁹, as presented in section 3.4.2. Since the crystals are large enough to prevent leaching and allow for easy recovery via filtration, yet small enough to pass through the pipette tip used for capsule production (see section 3.1), it serves as a useful alternative to chitosan immobilization. Additionally, cellulose is inert and stable under a variety of conditions.

To perform immobilization on cellulose, NaIO₄ is used to open the rings of the glucose monomers, creating a polyaldehyde with two exposed aldehyde functional groups per altered monomer. At this point, the procedure is identical to that used for glutaraldehyde-activated chitosan immobilization. The enzyme is added, forming a Schiff base through its N-terminus. After reduction of this Schiff base with a reducing agent such as sodium borohydride, the enzyme is covalently bound to the cellulose (Fig. 21). Lastly, remaining activated groups are blocked via the introduction of Tris buffer to block open aldehyde sites. The modified cellulose crystals with immobilized XI are then recovered using filtration or centrifugation.

After this procedure was performed, the XI-cellulose was examined for enzyme activity under various pH and temperature conditions. These results were compared to those of the alternative immobilization method, XI-chitosan, and of the free enzyme. This material was also used to run a xylose fermentation trial, as will be discussed in Chapter 6.6

**5.4.1: Effects of pH**

pH values between 3 to 7 were tested using 15% fructose solutions buffered with acetate. The enzyme was used in 10 µg aliquots to maintain consistency with XI-chitosan
and free XI characterization trials and activity was determined using the procedure in section 3.3. It was found that at pH values from 4 to 6, the XI-cellulose behaved similarly to the free enzyme. At values below 4, the enzyme activity quickly dropped to levels below that of the other forms of the enzyme (Fig. 43). At pH 7, it was also found that activity only slightly increased over that of other pH values, in contradiction to results for the free XI. In all, there was a 11.5% decrease in enzyme activity from pH 7 to 3. This is a larger drop than that for the XI-chit. Though small amounts of enzyme were used in these trials and there were likely many complications, requiring that these experiments be repeated, these results indicate that the XI-chitosan outperforms both the XI-cellulose and free XI at pH values below 6.

5.4.2: Effects of Temperature

A temperature range of 25 to 40 °C was used for testing the temperature tolerance of XI-cellulose, with 10 mg aliquots of enzyme incubated in 15% fructose. The XI activity was then determined as previously described in section 3.3. Results of these trials are provided, with comparison to those for free XI and XI-chitosan in Fig. 44. Data for this trial are similar to those gathered for the XI-chitosan, demonstrating that temperature does not have as great of an effect on the immobilized enzyme as compared to a free solution (Fig. 44). Specifically, there was a 6% increase in XI-chitosan activity, and a 6.7% increase in XI-cellulose activity over this range. This is consistent with immobilization results. Since immobilization may be able to lock the conformation of an enzyme, temperature, as well as pH, should have less of an effect on altering that

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**Fig. 43:** Effect of pH on XI-cellulose vs. XI-chitosan and free XI activity.
conformation. Since these immobilizations were conducted at room temperature, the conformation of the enzyme at that temperature would be locked, and little improvement should be seen with increasing temperature. Activity of the immobilized enzyme should remain approximately at levels seen for the free enzyme at room temperature. In fact, if the free XI temperature data is extrapolated using a linear best-fit trendline \( y = 1.2391x - 7.8723; R^2 = 0.989 \), the average value of the XI-cellulose and XI-chitosan activity rates over all tested temperatures (14.56 µgFru/hr), corresponds to a temperature of 18.1 °C, slightly below room temperature. This provides a potentially novel approach to XI activity optimization.

If immobilization is carried out at temperatures well above those conducive for fermentation, but at which XI exhibits much greater activity, it may be possible to lock the conformation to provide much greater activity at temperatures optimal for fermentation.

![Fig. 44: Effect of temperature on XI-cellulose vs. XI-chitosan and free XI activity.](image)
Chapter 6: Xylose Fermentation

Periodically during the optimization of capsules and fermentation conditions, fermentation trials using the co-encapsulation of *Saccharomyces cerevisiae* and xylose isomerase (XI) were carried out. This was done to test the progress of the study. Also tested was the ability of *S. cerevisiae* to directly ferment xylulose to ethanol, as a proof that if the XI enzyme is functioning correctly, ethanol can be produced from xylose. In the following chapter, xylulose fermentation will be presented first, followed by several xylose fermentation attempts via co-encapsulation. Ultimately, over the course of this project, four different types of XI were used for xylose fermentation, as presented in the previous chapter. Initially, polymeric XI was used to avoid enzyme leaching. Fermentation results from these trials are presented second in this chapter. Due to limitations inherent to the polymeric XI, despite increases in concentration and the optimization of fermentation conditions, free-XI was used and the XI-cellulose approach was developed, which will be presented thirdly, and fourthly, respectively.

6.1: Xylulose Fermentation

The premise of this project is that xylose will be converted to xylulose via XI, and it is this xylulose that is taken up by *S. cerevisiae* and converted to ethanol via the pentose phosphate pathway and glycolysis. However, it had not been demonstrated in our lab that the yeast being used could ferment xylulose. If it could not, no amount of optimization of capsules and enzyme activity would produce positive results. Furthermore, knowing the rate and ability of this xylulose-to-ethanol metabolic process is vital for critical analysis of the efficiency of the XI-mediated xylose-to-ethanol conversion being explored. To verify and characterize this activity, a trial was conducted using free yeast and xylulose as the carbon source.

Since xylulose is not readily commercially available, it was synthesized in the lab from xylose. To that end, a solution of 50% xylose was mixed with polymeric XI and heated to 70 °C for 5 hrs. This mixture was vacuum filtered to remove the polymeric XI.
According to literature, the theoretical composition of this remaining solution should have been 35% xylose and 15% xylulose\(^7\). Since isolation of these sugars is highly difficult and xylose is not metabolized by yeast under fermentation conditions, it was decided that further purification of the reaction mixture was not necessary for testing xylulose fermentation. BactoPeptone was added to this solution to a concentration of 1% to provide the necessary nutrients for yeast growth and fermentation. Free yeast was added to this and the solution was left under anaerobic conditions at 35 °C in a shaker with periodic sampling conducted for 144 hrs.

Due to the high viscosity of the solution (50% sugar between the xylose and xylulose), pH measurements were highly variable. However, it was seen that there was not a dramatic drop in pH values (Fig. 45). Sugar analysis was also compounded by the significant amount of sugar in solution (Fig. 46). Since, of the 50% sugar solution, xylulose theoretically constituted only 15%, and the xylose was not utilizable by the yeast, any consumption of xylulose would easily be masked by the stronger xylose signal. Ethanol analysis was the least confounded of the measurements taken since it analyzes the volatiles above the solution. The only

![Fig. 45: pH vs. time for a xylulose fermentation trial.](image)

![Fig. 46: Ethanol and sugar concentrations over time for the xylulose fermentation trial. The primary Y-axis is GC peak ht. for ethanol and the secondary is sugar as a percent of initial 50% xylose/xylulose. The external standard used for GC was 5% (v/v) ethanol.](image)
potential error was the matrix effect, which was taken into consideration and eliminated by the standards that were injected along with the actual samples. This analysis showed the production of ethanol over time with a peak of 0.57% (v/v) ethanol. Although this value was small, it is likely that the high viscosity of the solution greatly affected the abilities of the yeast to ferment, likely leading to high levels of osmotic lysis. This trial demonstrates that the yeast being used in this project do ferment xylulose to ethanol, validating the final undemonstrated step in the overall process of ethanol production from xylose. Unfortunately, to gather adequate and accurate information on yields and rates, additional fermentation trials on xylulose need to be conducted using a more purified sample, so that the viscosity of the fermentation medium can be adjusted to more physiologically acceptable levels for the yeast while maintaining an adequately high xylulose concentration.
6.2: With Polymer Immobilized Xylose Isomerase

An initial xylose fermentation trial was run after the optimization of capsule diffusion on glucose fermentation, presented in section 4.3. Since preliminary polymeric enzyme activity results showed that divalent cations provide an increase in activity, shown in section 5.1, magnesium ions were added to the fermentation medium. In this trial, the 100 mL fermentation medium consisted of 15% xylose, 1% BactoPeptone, and 50 mM MgCl$_2$. A batch of 500 capsules was used with shells constructed from a 0.2% alginate, 0.3% Tween 20 solution for enhanced diffusion, and internal capsule concentrations of 2.5% yeast cells and 2.5% powdered polymeric XI. A second control batch contained the same amount of yeast and powdered polymeric XI, and both batches were kept under anaerobic conditions at room temperature with gentle agitation. After 48 hrs, a 20 mL injection of 15% glucose with 1% BactoPeptone was made to determine if the yeast were still viable, followed by an additional 24 hrs of fermentation.

pH results for this trial showed very little difference in pH until after the glucose injection. Before glucose injection, the pH values were around 5.5, above typical fermentation conditions. After glucose injection, pH characteristically dropped to around 4 (Fig. 47). This implies that fermentation may not have been taking place with xylose as the sole carbon source. Sugar analysis showed a greater initial xylose metabolism in the capsule batch compared to the free solution that quickly flat-lined before slowly decreasing. After the glucose injection, there was a fast decrease in sugar concentration, as expected (Fig. 48). Ethanol analysis fluctuated a little during the pure xylose period, peaking at just over 1% (v/v) for the free solution and just under 1% (v/v) for the capsule solution. After glucose injection, there was an expected spike in ethanol,
which was higher for the free solution than for the capsule solution (Fig. 48). At the end of the 72 hr fermentation period, it was discovered that the alginate solution used for forming the capsules had been contaminated with fungi. As such, the solutions were severally contaminated, resulting in the high fluctuation in ethanol concentrations that were seen. Though ethanol amounts were low and can be improved, and the contamination necessitated that this trial be viewed only qualitatively, it can be seen that the co-encapsulation produced some ethanol from xylose.

![Fig. 48: Ethanol and sugar concentrations over time for the initial xylose fermentation trial. The primary Y-axis is GC peak ht. for ethanol and the secondary is sugar as a percent of initial 15% xylose. The external standard used for GC was 5% (v/v) ethanol.](image)

### 6.2.1: Concentration and Optimized Conditions

The above fermentation trial showed that despite optimized capsule diffusion and some improvements to fermentation conditions, ethanol was not readily produced from xylose. A possible cause was that the polymeric XI being used had insufficient activity even though additional steps were taken to improve activity. It may be that the enzyme concentration was simply too low for timely xylose conversion. To address this possibility, a fermentation trial was run using a much higher concentration of polymeric enzyme within the capsules than was previously conducted. In order to further improve the fermentation, results from further polymeric XI characterization, found in section 5.2.4, were employed by increasing the fermentation temperature to 35 °C.
A batch of 250 enhanced capsules containing 200 mg/mL powdered polymeric XI, four times the concentration used in previous batches, and 50 µL/mL yeast was placed in 50 mL of a 15% xylose, 1% BactoPeptone, and 50 mM MgCl$_2$ solution. This polymeric enzyme concentration was the highest possible while still producing consistent, durable, spherical capsules (above this concentration and the capsules fall apart). For a control, an equivalent amount of yeast and enzyme was added to an additional 50 mL of the same fermentation medium. These were placed under anaerobic conditions at 35 °C and left with gentle agitation for 120 hrs with periodic sampling.

According to pH tracking (Fig. 49), there was a gradual decrease in pH, indicating a prolonged lag period. Final values of around 4 were seen, at the border of fermentation conditions. Sugar results indicated that there was very limited metabolism of xylose over time (Fig. 50). After an initial drop in concentration caused by the addition of capsules/free solution control to the fermentation medium, there was no significant additional decrease. Ethanol results were not attainable, though based on the information from the sugar analysis it is likely that little or no detectable ethanol was produced.

![Fig. 49: pH vs. time for the increased polymeric XI concentration xylose fermentation trial.](image)

![Fig. 50: Sugar concentrations, as a percent of initial 15% xylose, over time for the increased polymeric XI concentration xylose fermentation trial.](image)
According to this information, little or no fermentation took place on xylose. This could be due to insufficient XI activity, even though four times the concentration previously used was encapsulated. Another plausible explanation is that there was insufficient time. The yeast used in this trial were initially grown on glucose and therefore possessed a high level of enzyme and protein expression for glucose metabolism when first introduced to the xylose solution and fermentation conditions. Significant proteomic expression changes, mediated by various signaling pathways and requiring altered gene expression with new protein synthesis, are necessary before the yeast are capable of utilizing the xylulose produced by the XI. Furthermore, these changes are triggered by the presence of xylulose. If the enzyme activity is low, resulting in a slow build up in xylulose concentration, these changes will take much longer to occur. It was speculated that a prolonged fermentation period or a second cycle may yield better results. Additionally, if a second cycle shows improved efficiency, it demonstrates a further advantage of encapsulation.

6.2.2: Multiple Fermentation Cycles

Since the previously presented fermentation trial indicated that a prolonged fermentation period or multiple cycles of fermentation may be required to condition yeast for xylose fermentation, the capsules used for that fermentation were recovered, washed, placed in 50 mL of fresh 15% xylose, 1% BactoPeptone, and 50 mM MgCl$_2$ fermentation medium, and placed back under anaerobic conditions at 35 °C. After 170 hrs with periodic sampling, the fermentation was stopped. pH over this time period was initially lower than the first cycle, but had a similar final value around 4 (Fig. 51). This
indicated that the conditions were similar from one cycle to the next. Sugar analysis showed that much less sugar was metabolized over time (Fig. 52). Lastly, ethanol analysis was not available for this trial. Based on sugar results and the previous fermentation, however, it is unlikely detectable levels of ethanol were produced.

By more closely examining sugar consumption, it can be seen that 82% of the xylose remained at the end of the second cycle, compared to 75% after the first cycle. There were 4.4% fewer capsules at the beginning of the second cycle due to capsules rupturing in the first cycle, which accounts for a 1.1% decrease in consumption. The remaining disparity is likely caused by enzyme inactivation and/or yeast death due to nutrient starvation (without being able to use xylose, there is no carbon source in the fermentation solution). As such, the best approach towards future fermentations would be to increase the enzymatic activity. Since, at this point, the polymeric enzyme activity had already been examined and optimized, and the interior capsule concentration of polymeric XI was at the physical limit, it was decided that an alternative enzyme would be required.
6.3: With Free Enzyme Xylose Isomerase

Due to the shortcomings of the polymeric XI, in terms of concentration and activity limitations, free XI was examined since much higher concentrations of this enzyme could be easily incorporated into capsules. By using a higher enzyme concentration, xylulose will be produced from xylose at a faster rate, thereby providing yeast with a more abundant carbon source for fermentation. Previous trials with this free enzyme provided promising results, though a full analysis of the fermentation (sugar content, pH) was not conducted and the trials were not as tightly regulated\(^1\). The drawback to use of the free enzyme is that it is small enough to diffuse from the capsules, thereby eliminating its reusability potential. An initial trial was conducted to determine if initial free XI concentrations create a sufficient enough enzyme activity for efficient ethanol fermentation from xylose, while later trials using immobilized XI addressed the issue of enzyme leaching.

To that end, 50 µL/mL of a 33 mg/mL suspension of XI was mixed with 50 µL/mL of yeast and core solution, and encapsulated in alginate. 60 of these capsules were placed in a 30 mL solution of 15% xylose, 1% BactoPeptone, and 50 mM MgCl\(_2\), under anaerobic conditions for 144 hrs with periodic sampling. pH values for this fermentation showed a slow but steady decline in value until a pH of ~4 was achieved at the end of the trial, at the upper boundary of fermentation (Fig. 53). These results were slightly different from polymeric enzyme fermentation trial data because the value decline was gradual, compared to the less steady decrease seen in the later (compare to Fig. 51). Sugar analysis was improved for this trial to account for the initial dilution seen from capsule addition. These results also showed a steady decrease, ending up at ~80% of the initial xylose after 144 hrs of fermentation (Fig. 54). This appears to be higher than the amount
of xylose remaining for the polymeric enzyme fermentation trial presented above but in reality, since dilution was accounted for, the total amount of sugar consumed was higher for this trial. After ethanol analysis, it was found that only minimal amounts of ethanol were produced over the course of the fermentation (not shown).

This fermentation demonstrated a higher xylose metabolism rate by using the free XI as compared to the polymeric enzyme. These are promising results, but after 144 hrs of fermentation there was still 80% of the initial sugar remaining. Furthermore, given that sugar was consumed but only small amounts of ethanol were produced, it follows that xylose was made available to the yeast via xylulose production but may have been utilized in alternative pathways to fermentation, such as the formation of structural components or for mitochondrial respiration. Alternatively, the consumption may have been slow enough that any carbon dioxide produced by the fermentation adequately diffused out of the reaction chamber and was replaced by oxygen. Under these conditions, the sugar would have been consumed and the yeast would have undergone the more energy favorable process of respiration. An added problem was that the free enzyme leached from the capsules. This trial showed that the free XI will work and is superior to the polymeric XI, however leaching had to be prevented via immobilization of the XI

Fig. 54: Sugar concentrations, as a percent of initial 15% xylose, over time for the free XI xylose fermentation trial.
6.4: With Xylose Isomerase Immobilized on Cellulose

As an alternative to immobilization on chitosan, the free XI was also covalently linked to microcrystalline cellulose, as discussed in section 3.4.2. Following immobilization, 50 mL/mL XI-cellulose was mixed with 50 µL/mL of yeast and core solution, and encapsulated in alginate. 35 of these capsules were placed in a 30 mL solution of 15% xylose, 1% BactoPeptone, and 50 mM MgCl₂, under anaerobic conditions for 118 hrs with periodic sampling. For a control, an equivalent amount of free XI was added to a second, 30 mL ferment batch and treated identically to the XI-cellulose batch.

Both batches had an initial lag period, lasting 46 hrs in the XI-free batch and 33 hrs in the Xi-cellulose, followed by a gradual drop in pH to values at the upper limit of fermentation conditions (Fig. 55). These results indicate that immobilization can lower the lag period of time in a fermentation trial (section 6.4). This shortened lag period is an indication of the success and value of preventing XI leaching. With higher localized concentrations of XI, as compared to a free solution, xylulose will be in greater abundance around the yeast, triggering faster adaptation of the

![Fig. 55: pH vs. time of the microcrystalline cellulose-immobilized XI xylose fermentation trial.](image1)

![Fig. 56: Ethanol and sugar concentrations over time for the XI-cellulose xylose fermentation trial. The primary Y-axis is GC peak ht. for ethanol and the secondary is sugar as a percent of initial 15% xylose. The external standard used for GC was 5% (v/v) ethanol.](image2)
yeast to the xylose fermentation environment. Sugar analysis of the batch samples revealed a gradual metabolism of the xylose in the free enzyme batch with a shallower decrease in sugar concentration in the XI-cellulose batch (Fig. 56). Ethanol results revealed that the peak ethanol concentration in the XI-cellulose batch was ~0.8% (v/v), compared to a peak of slightly above 1% (v/v) in the XI-free control (Fig. 58). Higher levels of ethanol were maintained throughout the fermentation period in the XI-cellulose batch compared to the XI-free batch, but a decline was seen over time.

Contamination was the likely cause of decreasing ethanol concentration with time. Black spots were observed on the surface of the alginate shells at the end of the fermentation, indicating that something may not only have been contaminating the solution, but may have been consuming the ethanol as it was being produced by the capsules, providing an explanation for why it was localized to the surface of the capsules. The alginate shell material itself was not contaminated, because these black spots were not observed throughout the shell layer or on the capsule internal surface. Regardless, these are promising results, demonstrating that ethanol was produced from the XI-cellulose in conjunction with *S. cerevisiae*. This trial must be repeated, preferably with a higher concentration of XI per unit of XI-cellulose material, and these results should be compared to those from future XI-chitosan xylose fermentation trials, so as to determine which is the more advantageous system.
Chapter 7: Summary

7.1: Conclusions

This study was a continuation of a senior/honors thesis and had five main goals. The success of this current thesis is judged by how successful the work herein was at achieving these objectives. As such, each goal will be individually discussed below in the context of this work. In the next section, it is speculated as to how the shortcomings of this current study can be overcome in future works and where it is most likely that progress will and should be made.

1. Develop a capsule shell composition with adequate diffusion rates to support ethanol production from glucose by encapsulated *S. cerevisiae* at rates identical or superior to free *S. cerevisiae*.

Previous work had shown that nutrient diffusion through the alginate capsule shell was limited. Consequently, rates of ethanol production on a glucose substrate were not optimal compared to free yeast suspensions. To improve this diffusion, the porosity of the shell was increased in this study by increasing the amount of Tween 20, a surfactant, in the alginate shell solution from 0.06% to 0.3%. This greatly improved diffusion, with ethanol yields of greater than 90% achieved using these enhanced capsules. The rate of production was slightly slower than that of a free yeast suspension, however with a third of sugar consumed from a 15% glucose fermentation medium within 48 hrs, and greater ethanol yields, this slight decrease in time is not very significant. Furthermore, the addition of greater concentrations of Tween 20 greatly reduced the “capsule mortality rate” per fermentation batch. With heightened amounts of glucose in the batch, fermentation progressed readily, producing copious amounts of carbon dioxide as a side-product. This carbon dioxide proved to be produced at a greater rate than the rate of diffusion from the capsules, nucleated into small bubbles that could not escape, and ultimately ruptured the capsule shell. By addition of the Tween 20, the shell contained...
greater porosity, facilitating greater diffusion of the carbon dioxide from the capsule interior, and, as a surfactant, prevented nucleation. As such, this particular goal was achieved and in doing so greatly improved the durability and longevity of the alginate capsules being used.

2. Scale-up capsule production and determine a set of analytical tools to more accurately determine the progression and efficiency of fermentation trials.

In order to garner more accurate results, larger fermentation batches were necessary. Accomplishment of this necessitated the development of a method for the mass production of capsules, since hundreds to thousands of capsules would be required for these larger batches and the previously employed method of hand pipetting could not meet this demand. A pump system was developed that extrudes core solution through a pipette tip, allowing the solution to drop into a gently stirring alginate solution. Magnetic particles were incorporated into the core solution so that consistent, spherical capsules would more easily form. This also provides the potential for easier manipulation of the system in future scale-ups when the capsules are used for multiple fermentation batches, requiring an easy method for recovery and transfer. This system is flexible, with a variety of alterable parameters to allow one to form capsules of different sizes, shapes, and compositions.

More information was also required from each ferment batch to determine its progression, efficiency, and ultimate success over time. Previous work only analyzed ethanol concentration, using ADEA, and this only at the end of a fermentation trial. To truly understand how a particular fermentation behaves, it must be tracked over time. This was easily implemented by taking ferment samples periodically over the course of each trial. In order to gain more information from these samples, pH and sugar concentration were analyzed in addition to the ethanol concentration. pH can be used to track the progression of a fermentation batch, since pH drops as yeast initiate fermentation. Sugar concentration provides information about the metabolic activity of the yeast and allows one to more accurately determine the ethanol yield. For these parameters, a pH meter and simple dinitrosalicylic acid colorimetric analysis were
employed. Ethanol tracking is the ultimate measure of the success of a fermentation, and an analysis method using gas chromatography was used in addition to the ADEA method, since this GC method was to be more accurate, less costly, and simpler to run. Combined, the analytical tools brought to bear on each fermentation batch were successful and effective at meeting the second objective of this work.

3. Characterize the activity of the xylose isomerase enzyme under a variety of fermentation related conditions so as to determine the optimal conditions for the conduction of xylose fermentation.

Ultimately, four different types/configurations of XI were used in this study. At the initiation of this work, a polymeric XI was employed. This was to prevent leaching from the capsules, which would render the enzyme unusable for subsequent fermentation batches run using the same capsules. To optimize the efficiency of fermentation systems employing this enzyme, the effects of divalent cations, ethanol concentration, pH, and temperature on enzyme activity were analyzed. It was found that magnesium ions can triple the activity, where as an increase in temperature from 25 to 35 °C increases activity by over 200%. Based on these results, all subsequent fermentation trials using XI have been conducted using 50 mM MgCl in the fermentation medium with the temperature held at 35 °C. This increased temperature is also known to improve the fermentation since the yeast cells find it more favorable. It was found that a 27% decrease in XI activity can be expected, as a result of the production of ethanol up to a concentration of 10% (v/v). Lastly, a drop in pH from 7 to 3 caused a 35% decrease in activity, though buffering of the fermentation medium could limit this effect.

The remainder of the types of XI used in this work were based on a free XI from a crystallography standard. The free XI itself was used for several fermentation trials, though it was known that it would leach out of the capsules. This was for comparison and to demonstrate that the co-encapsulation system could work, so long as enzyme leaching was prevented. This free XI was also immobilized on both chitosan and microcrystalline cellulose, as will be discussed under the next objective. These three XI types were analyzed for activity at varying pH values and temperatures, then compared.
The free XI had a 32% decrease in activity between pH 7 and 3, but a 183% increase from 25 to 40 °C. XI-cellulose was more susceptible to pH changes than XI-chitosan, with a 11.5% decrease in activity compared to 2.3%. Neither of the immobilized XI demonstrated a large increase in activity due to temperature, with approximately a 6% increase from 25 (30 in the case of XI-chitosan) to 40 °C. These results showed the benefit of immobilization, by protection of the enzyme from pH changes, as well as demonstrating the detriments, such as by preventing the enhancement of activity from increased temperatures. It may be possible that immobilization at higher temperatures may render increased enzyme activity, though this has yet to be explored. The combination of these results gave greater insight into how these various types of XI behave under a variety of conditions, allowing conditions to be altered to improve XI activity during xylose fermentation trials.

4. Prevent enzyme leaching from capsules, so as to render the enzyme reusable and protect activity.

As briefly mentioned, two approaches were taken to prevent enzyme leaching. Both employed the immobilization of the enzyme on a support via covalent bonds. The first method used chitosan immobilization by way of glutaraldehyde crosslinking, and the second exploited microcrystalline cellulose, activated by creating aldehyde groups by ring cleavage of sugar monomers, that would react with and bind the enzyme. In both cases, the attempts were successful, showing significant enzyme activity and no leaching. Characterization was carried out as discussed above and they were employed for xylose fermentation trials as will be elaborated upon below. In addition to the work that has already been done on this immobilization, it should be determined what the saturation limits for these substrates are, so that the most amount of enzyme as possible can be immobilized and incorporated into the capsules.

5. Successfully co-encapsulate *S. cerevisiae* with xylose isomerase and improve ethanol production rates and yields from xylose fermentation over those previously reported\(^1\).
Though it is easy to co-encapsulate materials, such as yeast cells and enzymes, it is notably more difficult to develop a functional system. In trials with encapsulated *S. cerevisiae* and XI, fermentation on xylose was carried out and the ability of the system to metabolize xylose while producing ethanol was examined. Based on these trials, improvements to capsule structure, fermentation medium composition, and fermentation conditions, specifically temperature, were made. Minor improvements were seen in these trials, but a roadblock has been encountered. The XI initially used, as a polymeric entrapped enzyme, did not have high enough activity to be effective, and attempts at increasing rates of xylose conversion were limited by the amount of polymeric XI that could be incorporated into the capsule interior. For this reason, alternative forms of XI that could have higher concentrations within the capsules were sought. A free enzyme was examined to demonstrate that fermentation from xylose was possible and this endeavor was successful, despite XI being small enough to diffuse out of the capsules. When immobilized on microcrystalline cellulose, this free enzyme was prevented from leaching from the capsules and was shown to not only contain enzymatic activity but facilitated the production of ethanol when co-immobilized with yeast. These systems provide great opportunity for future fermentation trials since the material used was not fully saturated with enzyme. It may be possible to use this immobilization to not only increase XI activity by locking the enzyme in a more optimal conformation (immobilization at heightened temperatures), but to incorporate a far higher concentration of enzyme into the capsule interior than previously attainable. In all, these results have again demonstrated that the system studied in this thesis works, though further optimization is required.
7.2: Future work

As outlined above, this work has been fully or moderately successful in fulfilling all of the proposed objectives, though work remains to be done. Further optimization of the system is required, focused mainly on increasing XI activity. It may be possible that the immobilization techniques explored in this work can be implemented in a manner to overcome this obstacle by locking the conformation of enzymes in a manner expressing greater activity and by increasing the amount of enzyme that can be encapsulated. Alternatively, sources of XI from microorganisms that have functionally superior forms of the enzyme might be sought. Given the flexibility of the developed and scaled-up encapsulation procedure, a multi-culture of microorganisms could be explored that contains species such as *Saccharomyces cerevisiae* for hexose fermentation, *Pichia stipitis* for pentose fermentation, and *Streptomyces murinus* for production of xylose isomerase. After the vital “xylose conversion to a species readily fermentable by efficient fermenting microorganisms” step in the fermentation process has been optimized, the system is already optimized for other conditions such as nutrient diffusion and is fully capable of being scaled-up. A further advancement that might be explored is the incorporation of additional enzymes into the capsule interior or expressed on the exterior capsule shell surface to further expand the capabilities of these capsules. For instance, cellulase and hemicellulase enzymes might be included to combine the plant biomass feedstock hydrolysis step with fermentation, creating a more continuous and economically favorable feedstock-to-ethanol production process. So long as ethanol and other products from the metabolism of microorganisms are in demand, the encapsulation procedure enhanced in this current work can find an economically favorable application.
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