Enzyme Process Design for Water Treatment

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of
requirements for the degree
Master of Science

in

The Department of Chemical Engineering

by

Steven Wayne Johnston
U.S., Louisiana State University, 1973
December, 1976
ACKNOWLEDGEMENT

The author wishes to thank Dr. Frank Groves, Jr. under whose direction this research was conducted. Comments and suggestions from Drs. Philip A. Bryant, Clayton D. Callihan, and Bert Wilkins, Jr., my committee, have been helpful. I would also like to extend thanks for the assistance of various other personnel in the L.S.U. Department of Chemical Engineering.

I am grateful for the financial assistance provided by the U.S. Department of Interior, under P.L. 88-379, as part of project A-036-LA which was administered by the Louisiana Water Resources Research Institute.

Finally, I would like to thank Dr. James D. Johnston, Dr. Shaw S. Wang, and Diamond Shamrock Corporation for providing research materials that would have been otherwise very difficult to obtain.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>viii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I. STUDY OF THE FREE ENZYME</td>
<td>6</td>
</tr>
<tr>
<td>A. Theoretical Background</td>
<td>6</td>
</tr>
<tr>
<td>B. Experimental Procedures</td>
<td>9</td>
</tr>
<tr>
<td>C. Discussion of Results</td>
<td>22</td>
</tr>
<tr>
<td>II. STUDY OF THE ADSORPTION PROCESS</td>
<td>24</td>
</tr>
<tr>
<td>A. Theoretical Background</td>
<td>24</td>
</tr>
<tr>
<td>B. Experimental Procedures</td>
<td>27</td>
</tr>
<tr>
<td>C. Discussion of Results</td>
<td>36</td>
</tr>
<tr>
<td>III. STUDY OF THE COVALENTLY BOUND ENZYME</td>
<td>37</td>
</tr>
<tr>
<td>A. Theoretical Background</td>
<td>37</td>
</tr>
<tr>
<td>B. Experimental Procedures</td>
<td>43</td>
</tr>
<tr>
<td>C. Discussion of Results</td>
<td>82</td>
</tr>
<tr>
<td>SUMMARY OF RESEARCH AND RECOMMENDATIONS</td>
<td>84</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>88</td>
</tr>
<tr>
<td>APPENDIX I</td>
<td>92</td>
</tr>
<tr>
<td>II</td>
<td>93</td>
</tr>
<tr>
<td>Table of Contents---(Continued)</td>
<td>Page</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>III</td>
<td>94</td>
</tr>
<tr>
<td>IV</td>
<td>95</td>
</tr>
<tr>
<td>VITA</td>
<td>96</td>
</tr>
<tr>
<td>APPROVAL SHEETS</td>
<td>97</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Showing the Hydrolysis of the Free Enzyme</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Showing the pH Curve of the Free Enzyme</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>Showing the Loss of Oxygen from Waste Water</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>Showing the Loss of Oxygen from Waste Water</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>Equipment for the Adsorbed Invertase Tests</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>Activated Charcoal Adsorption Data</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>Activated Alumina Adsorption Data</td>
<td>31</td>
</tr>
<tr>
<td>8</td>
<td>Amberlite IRC-50 Adsorption Data</td>
<td>32</td>
</tr>
<tr>
<td>9</td>
<td>Amberlite IR-120 Adsorption Data</td>
<td>33</td>
</tr>
<tr>
<td>10</td>
<td>Resorcinol Resin Batch Data</td>
<td>53</td>
</tr>
<tr>
<td>11</td>
<td>Glutaraldehyde-treated Resorcinol Resin Data</td>
<td>54</td>
</tr>
<tr>
<td>12</td>
<td>Amberlite IRC-50 Batch Data</td>
<td>55</td>
</tr>
<tr>
<td>13</td>
<td>Tannic Acid Resin Batch Data</td>
<td>56</td>
</tr>
<tr>
<td>14</td>
<td>Glutaraldehyde-treated Tannic Acid Resin Data</td>
<td>57</td>
</tr>
<tr>
<td>15</td>
<td>Duolite ES-762 Resin Batch Data</td>
<td>58</td>
</tr>
<tr>
<td>16</td>
<td>Glutaraldehyde-treated Duolite Resin Data</td>
<td>59</td>
</tr>
<tr>
<td>17</td>
<td>Cross-linked Polystyrene Batch Data</td>
<td>60</td>
</tr>
<tr>
<td>18</td>
<td>Influence of Flow Rate on Degree of Conversion</td>
<td>62</td>
</tr>
<tr>
<td>19</td>
<td>Influence of the Reactor Volume on Degree of Conversion</td>
<td>63</td>
</tr>
<tr>
<td>20</td>
<td>Pooled Flow and Volume Reactor Data</td>
<td>64</td>
</tr>
<tr>
<td>21</td>
<td>Equipment for the Determination of Temperature Dependence of Conversion</td>
<td>65</td>
</tr>
<tr>
<td>FIGURE</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>22</td>
<td>Showing the Activity Dependence on Temperature for Duolite in Continuous Flow</td>
<td>67</td>
</tr>
<tr>
<td>23</td>
<td>Showing the Activity Dependence on pH for Duolite in Continuous Flow</td>
<td>68</td>
</tr>
<tr>
<td>24</td>
<td>Duolite Continuous Flow Data</td>
<td>69</td>
</tr>
<tr>
<td>25</td>
<td>Duolite Continuous Flow Data</td>
<td>70</td>
</tr>
<tr>
<td>26</td>
<td>Duolite Continuous Flow Data</td>
<td>71</td>
</tr>
<tr>
<td>27</td>
<td>Iodine Titration Data</td>
<td>74</td>
</tr>
<tr>
<td>28</td>
<td>Iodine-treated Duolite Continuous Flow Data</td>
<td>77</td>
</tr>
<tr>
<td>29</td>
<td>Equipment for the Continuous Flow Experiments</td>
<td>78</td>
</tr>
<tr>
<td>30</td>
<td>Iodine-treated Duolite Continuous Flow Data</td>
<td>79</td>
</tr>
<tr>
<td>31</td>
<td>Duolite pH Dependence Data</td>
<td>80</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Showing the pH of Glucose-Boric Acid Solutions at Various Temperatures</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>Comparison of Reaction Rates for the Free Enzyme</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Free Enzyme and Resin Experimental Data</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>List of Materials to Which Invertase has been Covalently Bound in Previous Research</td>
<td>38</td>
</tr>
</tbody>
</table>
ABSTRACT

Sucrose is often found in low concentrations in sugar mill waste water. When these wastes enter natural waterways, they cause a degradation of water quality; bacterial growth prompted by the sugar removes dissolved oxygen leading to stagnant water conditions. In this work we will examine one phase of a proposed process to remove the sucrose from the waste water.

Boric acid has long been known to react with certain compounds containing adjacent hydroxyl groups to form acidic complexes. This suggests the possibility of reacting sucrose with boric acid and then removing the complex by ion-exchange; unfortunately sucrose itself will not react with boric acid. For such a process to work, it is necessary that sucrose be converted to glucose and fructose by enzymatic hydrolysis with invertase. It is the purpose of this work to try to find an economical method for the hydrolysis of sucrose in very dilute solutions.

First the free enzymatic hydrolysis was examined, then adsorption tests were run on the following materials:

1. Activated Charcoal
2. Amberlite Ion-exchange Resin (IR-120)
3. Amberlite Ion-exchange Resin (IRC-50)
4. Activated Alumina

Then the properties of the covalently bound enzyme were examined on the following supports:

1. Resorcinol Resin
2. Glutaraldehyde-treated Resorcinol Resin
3. Tannic Acid Resin
4. Glutaraldehyde-treated Tannic Acid Resin
5. Duolite ES-762 Resin
6. Glutaraldehyde-treated Duolite ES-762
7. Amberlite IRC-50 Ion-exchange Resin
8. Polystyrene Beads
9. Poly(styrene-divinylbenzene) Copolymer Beads
10. Yeast-collagen Material

Duolite was examined further under simulated industrial conditions, then iodine-treated invertase was immobilized on Duolite ES-762 and also tested. The iodine treatment was shown to greatly improve enzyme activity and performance.

The prospects of using any of these enzyme systems were found to be rather poor for the proposed industrial process due to economic and/or environmental constraints.
INTRODUCTION

Sugar cane processing constitutes a major industry in Louisiana. Unfortunately, operation of the raw cane factories may result in degradation of water quality in adjacent areas due to discharges of sucrose contaminated water. This dissolved sucrose supports bacterial growth which results in the depletion of dissolved oxygen in the bodies of water receiving the discharge. Indeed in some areas of the state, stagnant water conditions have been observed to coincide with the start of the cane grinding season while high levels of dissolved oxygen were observed during the rest of the year. This pollution has had the effect of lowering dissolved oxygen levels to such an extent as to kill fish and preclude other aquatic life (1). As the body of water receiving the effluent becomes stagnant, anaerobic bacteria begin to produce hydrogen sulfide, giving the area an unpleasant odor. It also may be expected that sport and commercial fisheries would be affected adversely. All in all, the effects of the discharge are quite undesirable.

In order to design a chemical process for sucrose removal, it would be convenient to be able to ionize the sucrose molecule in some way. Boric acid has long been observed to form acidic complexes with organic compounds with hydroxyl groups on adjacent carbons. Sucrose itself, unfortunately, will not react; however, the hydrolysis products of sucrose, glucose and fructose, will quite readily bind with boric acid. This suggests a two stage process in which sucrose is first hydrolyzed by invertase and mixed with boric acid, and then
absorbed on an ion-exchange resin.

The traditional methods used by the sugar industry to deal with the waste water are to use spray pond aeration systems, large stagnant holding ponds, or simply discharges to natural bodies of water. From the large number of pollution instances in the past, it is clear that the treatment aspect of operation of the industry needs to be improved.

The waste water's sucrose content in a typical factory discharge will vary with the water's use. Condenser cooling water may pick up sucrose through faulty operation by plant personnel or leaking tubes; B.O.D. levels in this waste water would be 50-350 ppm. Water used to wash sugar cane would be expected to have levels of about 200-1000 ppm (2). Noting the oxygen required to complete the removal of the sucrose, we see that the waste waters contain about 45-890 ppm sucrose as from the following equation:

\[ 12 \text{O}_2 + C_{12}H_{22}O_{11} \rightarrow 12 \text{CO}_2 + 11 \text{H}_2\text{O} \]

Most of the enzyme testing was done with 250 or 200 ppm sucrose solutions in the following experimental work.

Sucrose hydrolysis by invertase is often spoken of as "inversion" because the sugar solution's optical rotation reverses during the reaction. The reaction itself may be thought of as a catalyzed hydrolysis:

\[ C_{12}H_{22}O_{11} + H_2O \rightarrow C_{6}H_{12}O_{6} + C_{6}H_{12}O_{6} \]

\( \text{(sucrose)} \) \( \text{(glucose)} \) \( \text{(fructose)} \)

A great deal of research has been done with the enzyme invertase; however, the problems encountered in designing a hydrolysis process
called for original research. Not only must the sucrose be hydrolyzed in rather dilute solutions, but this must be accomplished at a pH close to 7.0 - the pH activity maximum occurs at about 4.5 for invertase, and most invertase research has taken place with acidic, high-sucrose solutions. Most enzyme research also takes place with buffer solutions whose ionic strengths would be much greater than that of the sugar mill waste water. Ionic strength of the reactant solution has been shown with many enzyme systems to have a profound effect on the pH-activity curves over a wide range; the pH-activity peaks may shift or change shape, particularly for immobilized enzyme systems. In addition there may be problems with catalyst poisoning, and bacterial growth and interference. The literature very seldom gives enough information to design a process and certainly not enough to look at prospective systems with invertase under the conditions of interest. Since the hydrolysis products are known to compete for the enzyme's active site, we are dealing with a system of reactions. All the complications certainly justify detailed research with this specific enzyme process.

Although invertase is being used in industry at the moment in free solution, it is not being used in the covalently bound immobilized form to the best of this author's knowledge. To use invertase in an economical way with dilute solutions containing unknown enzymatic inhibitors does, indeed, present a number of problems. The food processing industries, using less obnoxious solutions of higher sugar content and much greater economic value than industrial waste water, appear to be only using the free enzyme and a limited amount of the
adsorbed enzyme. It became apparent in the course of this research that something more sophisticated was called for.

This research work is divided into three sections. The first part deals with the properties of the free enzyme. As previously stated, much work has already been done on the free enzyme system, but various techniques for increasing the enzyme activity remained to be investigated as well as the enzyme behavior in the industrial environment. The second research phase involved the possibility of using the enzyme on fixed supports using adsorption techniques. Again research has already been done on similar systems, but not under reaction conditions relevant to this project. The third phase of the project involved the various fixed enzyme systems. A great many chemically-bound fixed enzyme systems have been investigated in recent years and a tremendous amount of research is going on at the moment. The problem in using and investigating this approach was largely involved with attempting to sort out all the possibilities. This part of the research involved some difficult decisions due to limitations of time and equipment. It is this writer's opinion that better enzyme support materials will quickly become commercially available making at least some of this work obsolete; the field is changing very quickly. It is hoped that this phase of the research has at least been able to outline the difficulties in applying fixed enzyme technology to the problem at hand and presenting some of the possible solutions.

Some unique enzyme supports were made and tested in this project, and iodine-treaded invertase was immobilized for the first time. The
iodine-treated enzyme was shown to have distinct advantages over the
normal immobilized invertase. Advantages of using a crosslinking
reagent were demonstrated for several resin supports. Enzyme inhibition
was found to be a major problem in the use of the immobilized
invertase and iodine treatment was shown to help. It is the author's
belief that the utility of iodine treatment for the enzyme's activity
retention is the most important finding in this work, if not for
this specific application, then perhaps elsewhere in the food processing
industries.
CHAPTER I
STUDY OF THE FREE ENZYME

Theoretical Background

The simplest way to accomplish the hydrolysis would be by adding the enzyme to form a solution with the waste water. Apart from any economic considerations, there are quite a number of possible problems with this method. First, the waste water is far from the optimum pH value for the enzyme activity (1). Second, we can expect the waste water would contain enzyme inhibitors (3). Also enzyme preparations are impure; the invertase used in this laboratory work was found to contain about 50% lactose. The final objective of an acceptable enzymatic hydrolysis process would be the removal of sucrose to reduce B.O.D., adding carbohydrate from the enzyme preparation is at odds with the ultimate process goal. If enough enzyme is added to the water, we may destroy water quality quite effectively even while removing sucrose completely.

The enzyme invertase (also called invertin, fructofuranosidase, saccharase, or sucrase) is, by definition, simply an enzyme which will hydrolyze sucrose and related glycosides. Although enzymes have been isolated from a large variety of organisms which satisfy this requirement, commercial and most laboratory preparations use invertase extracted from yeast. Even relatively pure invertase products may exhibit more than one active fraction or a rather broad band upon electrophoresis (4). Until rather recently, relatively pure and homogeneous laboratory preparations did not exist. It has been found
that invertase synthesis involves six different genes in yeast, for this reason we can expect that in any commercial material we would be dealing with a spectrum of enzymes capable of sucrose hydrolysis, but each having slightly different properties (5). The commercial materials are prepared from heteroploid baker's and brewer's yeasts; hence, even though in this research work we will speak of an enzyme preparation, we are in reality dealing with a mixture of invertase compounds and impurities. The invertase used in this work was Sigma grade V, prepared from baker's yeast (Saccharomyces carlsbergensis).

Invertase was one of the first enzymes to be identified and studied, having been extracted from yeast in 1860 (6). It was one of the enzymes studied by Sørensen during his work on hydrogen ion concentration and the pH-activity dependence of enzymes. Invertase kinetics were first examined by Michaelis and Menton while developing their well-known rate expression (7).

In order that we may evaluate the free enzyme's behavior, a rate expression must be chosen. It has been found that product competition (inhibition) and the formation of transfer products is insignificant as long as the substrate concentration is low (less than 0.3 M) (8). We can expect the treated wastes to be in the 45-900 ppm range which means solutions of 0.0001-0.0026 M. Assuming the typical enzyme reaction sequence we have:

\[ S + E \rightarrow ES \rightarrow GF + E \]

In the expression, \( S = \) sucrose, \( E = \) the enzyme, \( ES = \) the sucrose-enzyme complex, and \( GF = \) glucose and fructose. From this reaction sequence, we can get the familiar enzymatic rate equation:
\frac{d(s)}{dt} = -k(E)(s)/(K_m + s)

Here, k is a rate constant, and \(K_m\) is the Michaelis constant. When we examine actual experimental data; however, we see that this constant is of the order of 0.16 M (8). Consequently if we were to use a simple first-order rate expression instead, our error would be only 0.1-1.6%. Most of the experimental work done in this project was about 200 ppm which would result in a maximum error of 0.4% for a first-order rate expression; this is probably well within the experimental error for all the determinations that follow. In any event, it will be assumed that the rate of hydrolysis is essentially first-order with respect to both the invertase and sucrose concentrations with the concentration ranges we will deal with. The first-order rate may be converted to the appropriate Michaelis-Menten rate constants by assuming \(K_m\) values given in the literature. From now on, any rates derived in this paper for the free enzyme will be in reference to:

\frac{d(s)}{dt} = -k(E)(s)

Special forms of the rate constant will be needed for looking at the fixed enzyme's properties.
Experimental Procedures

The enzyme material used in these experiments was invertase (Sigma, grade V) from baker's yeast, rated at 53 units/mg activity; 1 unit hydrolyzed 1.0 micromole sucrose per minute at a pH of 4.5 at 55° C. Due to high substrate concentrations and product inhibition expected under the assay conditions, it is difficult to compare this rate to what was measured in this research work at much lower concentrations. The invertase was stored in a desiccator at 4° C; measurements over 2 year's time showed about 80% loss of activity.

In order to check that the reaction rate was indeed first-order toward substrate and give an accurate value for the enzyme activity at the substrate concentration range of interest, glucose-fructose concentration of a 1 liter batch of buffered 250 ppm sucrose solution was measured as a function of time after the introduction of enzyme with stirring to give 10 ppm invertase at 22° C. The buffer solution consisted of 10.2 ml of 1.142 M NaOH, 18.03 gm potassium hydrogen phthalate, and 0.250 gm sucrose made up to 1000 ml, pH = 4.5. Immediately after the addition of enzyme, the product concentration was sampled and continued to be sampled at 5 minute intervals. Results were graphed as follows:
ppm Invert Sugar

Invert Sugar Production by the Free Enzyme

Figure 1.

This gives a rate constant of $0.00972 \text{ minutes}^{-1} \text{ppm}^{-1}$. The activity was found from a linear least-square's fit of $\log_e(1 - \text{degree of conversion})$, with the constraint that the equation give zero conversion at time = 0.

$$\frac{dS}{dt} = -k(E)(S)$$

$$-\log_e(1 - \frac{CF}{CF_\infty}) = k(E)(t)$$

Determination of the pH optimum was accomplished with a KOH-acetate buffer made by diluting 0.01 M KOH with 1 M acetic acid until the desired pH was achieved. Assays were done at 24°C and invert
sugar was determined by the Folin-Wu method after a 40 minute incubation period. These results indicated a pH optimum at about 4.4. Glucose-fructose concentrations required correction for lactose present in the reagent invertase, the correction was easily found by doing Folin-Wu assays on invertase samples. The reducing ability of the invertase preparation was found to be equivalent to about a 50% lactose content. This correction was applied to all free enzyme data. Results of the pH-activity assays are graphed below:

![Graph showing variation of conversion with pH determined after 40 minutes]

**Figure 2.**

These results show that we will be using the enzyme at less than the optimum pH unless some means is devised to lower the pH of the sugar mill discharge. From the literature, we can see that for invertase there is a 40% loss of activity relative to the pH optimum when operating.
at a pH of 7.0 (9).

In order to lower the pH of the waste water, we may try several approaches. One possibility would be that boric acid could be mixed with the enzyme and waste water; boric acid would lower the pH somewhat and the boric acid–glucose–fructose complex would readily form for the second stage of the treatment process. Boric acid itself would be expected to change the acidity very little, the dissociation constant being $7.3 \times 10^{-10}$ for the first proton; this would give a pH of about 6.2 for a boric acid solution made up to be equimolar with 200 ppm sucrose. On the other hand, the ionization constant of the complex may be higher than that of boric acid. For this reason, it was decided to take pH measurements of an equimolar glucose–boric acid solution (1000 ppm glucose); if there is any significant change in pH with the addition of boric acid, it should be noticeable at these relatively high concentrations. In addition, these measurements were made over a range of temperatures to see if temperature would be an important consideration:

<table>
<thead>
<tr>
<th>Temperature ($^\circ$C)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.</td>
<td>6.34</td>
</tr>
<tr>
<td>30.</td>
<td>6.15</td>
</tr>
<tr>
<td>40.</td>
<td>6.12</td>
</tr>
<tr>
<td>50.</td>
<td>6.08</td>
</tr>
<tr>
<td>60.</td>
<td>6.02</td>
</tr>
</tbody>
</table>

Table 1. pH of an Equimolar Glucose–Boric Acid Solution

Measurements were taken with a Corning Model 12 Research pH meter. These results show that we may expect a change in pH of only
about 1 unit at the most by adding boric acid to the reaction mixture
during the hydrolysis, if we consider the glucose data representative.

Another possible method of altering pH would be to simply add
a mineral acid to the reactant mixture, an ordinary acid (such as
HCl) would be quite detrimental in that it would compete with the
acidic complex for positions on the ion-exchange medium in the second
stage of the waste water purification process. This suggests our
using some form of removeable acid such as carbonic acid. The acid
would be added by simply bubbling CO₂ through the waste water and
removed by raising the temperature, lowering the pressure, or running
the solution through an ion-exchange bed. Indeed, a saturated carbonic
acid solution at 25°C has a pH of about 3.8. To see if this was
a practical acidification method, four 400 ppm sucrose solutions were
made up; the pH being adjusted by equimolar boric acid, HCl and KOH, and
bubbling CO₂ respectively. Results at 25°C after 3.0 hours of
reaction with invertase are given below:

<table>
<thead>
<tr>
<th>Medium</th>
<th>pH</th>
<th>ppm GF</th>
<th>Rate Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H₂O</td>
<td>6.9</td>
<td>328.8</td>
<td>1.00</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>7.2</td>
<td>334.2</td>
<td>1.04</td>
</tr>
<tr>
<td>HCl and KOH</td>
<td>4.4</td>
<td>357.7</td>
<td>1.32</td>
</tr>
<tr>
<td>CO₂</td>
<td>4.1</td>
<td>364.1</td>
<td>1.39</td>
</tr>
</tbody>
</table>

Table 2. Comparison of pH Adjustment Methods

The "Rate Ratio" is defined to be the ratio of reaction rate
constants with the distilled water's in the denominator. From this
we see that a 39% increase in activity is possible with the use of
CO₂.
Another possible way to increase invertase activity while avoiding the introduction of undesirable cations from a mineral acid to the reaction mixture, would be to use a "fixed" acid, a cation-exchange resin. The resin beads would hopefully provide an acidic environment, adding to the enzyme's apparent activity despite a neutral bulk solution pH. It would seem relatively simple to test for this effect; hydrolysis of a sucrose solution could be examined with and without a cation-exchange resin present. It was decided, however, that the effective acidity within the resin beads might be strong enough to cause hydrolysis of the enzyme and a loss of activity. For this reason, it was decided to test a range of resin samples, titrating each with a different amount of KOH. The base would neutralize some of the acid sites in the ion-exchange resin matrix, providing a lower range of apparent acidity within the resin bead micro-environment of the samples and assure that enzyme destruction would be observable during the test if it occurs. In practice, it was found much simpler to first convert the resin to the K⁺ form and then add HCl.

Samples of Amberlite IRC-50 were first converted to the K⁺ form:

\[(\text{RCO}_2\text{H})_n + n\text{KOH} \rightarrow (\text{HCO}_2^- \text{K}^+)_n + n\text{H}_2\text{O}\]

This reaction was run with an excess of KOH. After the reaction, the bead samples were treated with various amounts of dilute HCl solution. Amberlite IRC-50 is specified to contain at least 10.0 millimoles per gram resin of exchange sites. Attempts to titrate the resin with KOH and determine the moles of active sites exactly were inconclusive; near the equivalence point in the titration the resin
acts like a weak organic acid - the system is highly buffered. Long after a portion of base solution was added to the resin, the pH would continue to drift. To carry out the fixed acid experiment, five 10.0 gm samples of IRC-50 were converted to the potassium form and washed with distilled water to remove excess base. Then each sample was titrated with HCl. 4 ml of 1000 ppm invertase in H₂O were added to each sample along with 25 ml of 300 ppm sucrose in distilled H₂O; enough water was then added to give a total of 40 ml liquid in each sample. After 5 minutes, 25 ml samples were drawn off of each respective resin-solution sample and kept separately for later analysis. Then after 3.0 hours of incubation time, Folin-Wu samples were taken and invert sugar analyzed. The results are listed as follows:

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>pH</th>
<th>Moles HCl</th>
<th>Resin</th>
<th>% Transmittance</th>
<th>ppm GF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.8</td>
<td>0.0</td>
<td>yes</td>
<td>42.2</td>
<td>234.2</td>
</tr>
<tr>
<td>2</td>
<td>6.2</td>
<td>0.01</td>
<td>yes</td>
<td>46.7</td>
<td>206.7</td>
</tr>
<tr>
<td>3</td>
<td>9.4</td>
<td>0.0004</td>
<td>yes</td>
<td>86.7</td>
<td>38.7</td>
</tr>
<tr>
<td>4</td>
<td>--</td>
<td>0.000016</td>
<td>yes</td>
<td>90.2</td>
<td>28.0</td>
</tr>
<tr>
<td>5</td>
<td>--</td>
<td>0.0000032</td>
<td>yes</td>
<td>83.3</td>
<td>49.6</td>
</tr>
<tr>
<td>6</td>
<td>--</td>
<td>0.0001</td>
<td>yes</td>
<td>90.2</td>
<td>28.0</td>
</tr>
<tr>
<td>7</td>
<td>4.8</td>
<td>0.0</td>
<td>no</td>
<td>37.6</td>
<td>265.5</td>
</tr>
<tr>
<td>8</td>
<td>6.2</td>
<td>0.01</td>
<td>no</td>
<td>43.2</td>
<td>227.8</td>
</tr>
<tr>
<td>9</td>
<td>9.4</td>
<td>0.0004</td>
<td>no</td>
<td>73.0</td>
<td>85.4</td>
</tr>
<tr>
<td>10</td>
<td>--</td>
<td>0.000016</td>
<td>no</td>
<td>81.0</td>
<td>57.2</td>
</tr>
<tr>
<td>11</td>
<td>--</td>
<td>0.0000032</td>
<td>no</td>
<td>78.6</td>
<td>65.4</td>
</tr>
<tr>
<td>12</td>
<td>--</td>
<td>0.0001</td>
<td>no</td>
<td>79.6</td>
<td>61.9</td>
</tr>
</tbody>
</table>

Table 3. Effect of a Resin upon Activity
Sample no. 1 contained an untreated portion of IRC-50 resin; no KOH or HCl had been added to it at any time. Sample no. 7 was the bulk solution sample drawn off of no. 1, sample no. 8 was the bulk solution sample drawn off of no. 2, etc. In this way, the 7-12 samples acted as controls for samples 1-6, allowing us to judge the increase in hydrolysis due only to the presence of the ion-exchange resin. In the table, moles HCl refer to the amount of acid used to create acid sites on the resin. Percent transmittance was determined from colorimetric analysis of the respective Folin-Wu samples and does provide a measure of invert sugar concentration. Glucose-fructose concentration was found from a Beer's law expression and the transmittance data.

Looking at these figures, we see that the ion-exchange resin seems to decrease the apparent enzyme activity, the opposite of the expected result. Also the untreated IRC-50 sample's (no.1 and no.7) were shown to have the highest activities. Since the control hydrolysis rates were consistently higher than those of the resin-containing samples, we may conclude that the resin appears to inhibit the free enzymatic hydrolysis. These results make the prospects of use of an ion-exchange resin as a "fixed" enzyme activity enhancing acid seem rather poor.

In order to make any economic evaluation of the free enzyme process, we need to determine the activity of a commercial preparation. Literature obtained from Universal Foods Corporation indicated that their invertase rich extract (Sacchara) had an activity of 1400-1800 inverton units per gram enzyme preparation. This
determination was done at a pH of 4.6 in a buffered 5.0% sucrose solution, conditions quite different from the higher pH, low ionic strength, and low sucrose concentrations of the waste water to be treated. So the invertase used in this project was assayed under the standard conditions used to determine inverton units (10). 25 ml of pH 4.6 Walpole acetate buffer with 25.0 gm of sucrose was made up to 500 ml with distilled water. At 25°C with stirring, 5.0 ml of a 100 ppm invertase solution was added. After Folin-Wu determination of invert sugar and correcting for traces of glucose-fructose in the reagent sucrose, it was found that reducing sugar was being formed at a rate of 8.10 ppm per minute. This means sucrose was consumed at a rate of 7.69 ppm per minute or 3.85 mg per minute. Since an inverton is the amount of activity which produces a 5.0 mg per minute hydrolysis rate, the 0.50 mg of enzyme in the reaction mixture contained 0.77 invertons. Therefore the Sigma V preparation had an activity of 1540 invertons per gram. Further tests showed that this preparation had a relatively low activity in simulated industrial conditions. Waste water was simulated by obtaining water samples from Bayou Teche at Breaux Bridge, Louisiana and adding sucrose. This water may be regarded as a good simulation of the industrial waste water since the bayou is a source of water for sugar mills in the area. Samples were collected along the bank at the boat landing between the Breaux Bridge factory and the La. 31 bridge; 5 gallon polyethylene bottles were used. The particular water sample used for this determination was collected on June 8th, 1976. With 200 ppm sucrose, the Sigma
enzyme was assayed to have an activity of \( 0.000163 \text{ ppm}^{-1}\text{ minute}^{-1} \) at an invertase concentration of 10. ppm at 25° C. From this we may deduce that the commercial product will have an activity of about \( 0.000148-0.000191 \text{ ppm}^{-1}\text{ minute}^{-1} \) in the sugar mill waste water at 25° C and a pH of 7.

The exact amount of enzyme required to give a certain degree of hydrolysis is, of course, dependent on the time allowed for the reaction. Experience with the simulated waste water preparations indicated that bacterial growth would become visible in a day or so at 25° C. This growth appeared as white filaments suspended in the solution. If the time required for hydrolysis with an economical amount of enzyme is so long that significant bacterial growth could occur, then several problems would arise. The bacteria would remove dissolved oxygen and necessitate aeration of the waste water before discharge. Also bacterial growth would be expected to cause problems by clogging the ion-exchange resin used in the second stage of the sucrose removal process. Attempts to use a bacteriaccide such as chlorine would be difficult since oxidizing agents destroy the enzyme. It was, therefore, desirable to get a quantitative estimate of the time allowable for hydrolysis before bacterial growth would start in the reaction mixture.

A sample of bayou water was filtered through a sand filter and then vigorously aerated by pouring between two beakers to insure saturated dissolved oxygen conditions. Sucrose was added to give a 200 ppm solution at 24° C. Samples of this solution were separately stored in 125 ml flasks which were immediately stoppered so
as to exclude air and air bubbles. These flasks were incubated at 24° C in the dark. At intervals, one flask would be removed for D.O. analysis, leaving the remaining flasks for later D.O. determinations. D.O. was measured by the unmodified Winkler method (11). Thiosulfate solution used in the titrations was 0.0104 M, standardized against potassium chromate. Results are given below:

![Graph showing the loss of oxygen from a 200 ppm sucrose waste solution over time.](image)

**Figure 3**
From this plot of D.O., it is apparent that some oxidation begins in the first 3 hours. Larger decreases begin after 7 hours. From this data with 200 ppm sucrose at 25° C, it is apparent that reaction time of the hydrolysis should be of the order of 7 or 8 hours in order to limit bacterial destruction of dissolved oxygen and prevent any further complications. The same tests were run with 210 ppm invert sugar.

![Dissolved Oxygen ppm](image)

Loss of Oxygen from a 210 ppm Glucose-fructose Solution

Figure 4.
200 ppm sucrose hydrolyzes to give 210 ppm glucose-fructose. The invert sugar D.O. test was run in the same way as the sucrose test except that the initial and incubation temperatures were slightly colder, 21°C for invert sugar. This was reflected in slightly higher D.O. levels for the invert sugar samples, 9.33 ppm, compared to 8.60 ppm for the sucrose samples.

Both sets of data show the same qualitative behavior. First there is an initial slow decrease in D.O. for the first 4 hours, then a sudden rapid decrease comes at 4-7 hours. D.O. levels remain the same for a period of 15 hours or slowly decrease. Then after about 24 hours, the rate of D.O. loss suddenly increases. This is about the time that bacterial filaments first become visible in the solutions. Perhaps what we are seeing is oxygen consumption from successive generations or populations of microorganisms.
Discussion of Results

From the bacterial consumption of dissolved oxygen, we can judge about 7 hours as an upper limit of hydrolysis time before significant bacterial growth begins in the reaction mixture. Using the commercial enzyme preparation (Sacarase), we know we will have an activity of about 0.000148-0.000191 ppm⁻¹ minute⁻¹, as shown by earlier experiments (p.17). In order to treat the waste water we will want T.O.D. (total oxygen demand) down below 8 ppm, at the least. This would mean that for the more concentrated wastes we would require about 99% removal of the sucrose. If we specify that the hydrolysis is to go to 99% of completion, then it may easily be shown from the equation describing the free enzyme hydrolysis (p.10) that an invertase concentration of 74.1-77.4 ppm would be required to do the reaction in the time allowed. The cost of the enzyme was 94¢ per pound delivered (1975). This would bring the enzyme cost to be 58-45¢ per 1000 gallons of waste water and represent 0.62-0.48 pounds preparation added per 1000 gallons. Both the enzyme cost and the amount added could be reduced by the use of carbonic acid, but this would require additional equipment be installed to remove CO₂. Calculations are given in appendix I.

The main objections to the use of the free enzyme process seem to be cost and environmental degradation. The levels of invertase preparation added to the waste water are too high to be environmentally acceptable. Invertase preparations contain large amounts of carbohydrate impurities which would be expected to result in the same problems that result from trace sucrose in the discharge. Even
though we may be able to remove the sucrose in the discharge water, our final objective, an environmentally acceptable waste water, would not be met. Also the cost of water treatment incurred by the use of the enzyme brings the minimum cost of the processed water above that of commercial process water (19). If the alternative to lack of water treatment is the shutdown of a sugar mill, this may be a minor consideration. These results do indicate, however, that free enzyme treatment of the waste water is relatively expensive.

All in all, the simple free enzyme hydrolysis process appears untenable. Therefore it would be desirable to minimize the amount of enzyme preparation required. This suggests that some form of fixed enzyme process would be more practical.
CHAPTER II
STUDY OF THE ADSORPTION PROCESS

Theoretical Background

The simplest method to prepare immobilized enzymes is by physical adsorption onto a solid carrier. This method has several great advantages. It requires no reagents other than the enzyme preparation and the support material. The adsorption step is relatively simple; the support is immersed in a concentrated enzyme solution and then used. An absorption process is relatively easily accomplished if a suitable adsorbent can be found. Within the carrier matrix, the enzyme is held by hydrogen bonding, salt linkages, and Van der Waal's forces. As a result, we can expect the enzyme to be left relatively intact by the immobilization process, in contrast to the various chemical immobilization methods which may involve harsh reaction conditions.

Quite a large number of substances have been found suitable to some degree for enzymatic adsorption, including: charcoal, alumina (12), cellulose (13), kaolin (14), silica (15), and collagen (16). The utilization of phenolic resins mentioned later in this research paper could be said to involve absorption immobilization to some degree, but since a cross-linking reagent (glutaraldehyde) was usually used and chemical reactions between the phenolic matrix and the enzyme are possible (17), the immobilization process was classified as basically chemical covalent bonding.
A few substances were studied for possible use in an enzyme adsorption process:

1. Darco activated charcoal, 12-20 mesh
2. Amberlite Ion-exchange Resin (IR-120), 20-50 mesh
3. Activated Alumina, 8-14 mesh
4. Activated charcoal, 100+ mesh
5. Amberlite Ion-exchange Resin (IRC-50), 20-50 mesh

Charcoal has been used to a very limited extent for invertase fixation in the food industry; it should be noted that since invertase constitutes only a minor cost and a certain amount of free invertase is desirable in the product syrup (in chocolate candy centers, for example) that leaching of enzyme from the support isn't a very important factor for this application. In short, although charcoal has been used industrially, it doesn't necessarily follow that it is suitable within the constraints of a water treatment process: high enzyme retention, high enzyme activity, and low enzyme concentration in the treated waters after the process.

The Amberlite resins were chosen for adsorption testing on the basis of experimental as well as theoretical grounds. Amberlite XE-97 (a carboxylic ion-exchange resin) has been shown suitable for catalase and lipase absorption. Indeed for these support-enzyme systems, the adsorbed enzyme appeared as strongly bound as the covalently linked enzyme in the same support material (17). In addition, it was hoped that the cation functional groups in the resin matrix would alter the bead microenvironment so as to shift the pH-activity curve, enhancing enzymatic activity for invertase at bulk
pHs near 7.0. The cations (in IR-120, sulfonic groups; in IRC-50, carboxylic groups) would be expected to attract protons, or at least cause an unequal distribution of hydrogen and hydroxyl ions between the bulk solution and the enzyme-polyelectrolyte phase. Experimental pH curves of chymotrypsin on progressively more cationic supports show shifts of several pH units to higher values (18). Similar results with invertase would result in a sharper activity peak at a higher pH and hopefully result in enhancement of enzymatic activity in near-neutral, low ionic strength solutions.

Alumina was chosen for study since its ability to absorb invertase is well known, the absorption being one step in the commercial purification of the enzyme. The activated alumina used was 8-14 mesh, obtained from Matheson Coleman and Bell Incorporated.
Experimental Procedures

The apparatus used in these experiments consisted of $\frac{1}{4}$ inch glass and plastic tubing, a Cole-Parmer "Masterflex" tubing pump with a 440 ml per minute head, and a chromatography tube with a porous glass bottom (40cm long, 20mm inside diameter). The system is illustrated below:

 Adsorbed Invertase Test Equipment

Column
Bubble Trap
Sample Collection
Feed Flask
Pump

Figure 5.
A flow rate of 10 cc per minute was maintained by adjusting a variable tubing clamp; this allowed satisfactory control despite the high flow rate of the pump. It was found necessary to provide an air trap to prevent bubbles from entering the system; the bubble trap also was found to be a convenient place in the apparatus to inject the enzyme. Column feed entered from below the column to provide a convenient means of washing out trapped air in the column material. When purging the column was needed, the bed in the column was fluidized at a flow rate of about 50 ml per minute. The powdered activated charcoal was found to require the feed entry at the top of the column to prevent fluidization at 10 ml per minute. This material was quickly discarded as the pressure required to maintain any flow was prohibitive.

The bottom feed mode was used in testing the 12-20 mesh charcoal, the Amberlite resins, and the alumina. The 10 ml per minute flow rate was too low to cause any fluidization in any of these materials. Stock feed solution consisted of 200 ppm sucrose in distilled water adjusted to a pH of 7.0 with dilute KOH and HCl. During the absorption experiments, a measured amount of support was first added to the column, the column and system purged of air and filled with feed solution, then enzyme was introduced by filling the air trap with 50 ml of 0.100% enzyme in distilled water. After the system flow was started 100 ml samples were taken of effluent at 10 minute intervals. Each sample was assayed for enzyme content by incubation of 5 ml in 50 ml of 1.0% sucrose, pH = 4.4, buffer (10.0 gm sucrose, 18.04 gm potassium hydrogen phthalate, and 9.3 ml of 1.142 M NaOH
made up to 1000 ml with distilled water) for 1.5 to 2.5 hours at 25° C.

After incubation, invert sugar was assayed by the Folin-Wu method. It was decided that it would be much too difficult to make quantitative enzyme assays of all the samples containing enzyme from the column, instead the light absorption at 450 millimicrons from the Folin-Wu assays was measured. From Beer's law this is proportional to the invert sugar content. Data giving relative absorptions and total effluent flow is graphed for the support materials below; the absorptions have been expressed in percents of maximum values.
Darco Activated Charcoal

38.1 gram column

Test Results
Amberlite IRC-50

30.3 gram column

Test Results

Figure 8
Amberlite IR-120
33.1 gram column
Test Results
All the efluent content plots were observed to peak very quickly at 50-150 ml. The Amberlite resins and the activated alumina show peaks in the 100-150 ml range. Since the 50 ml enzyme sample took at least 5 minutes to enter the system at the 10 ml per minute flow rate and the bubble trap was determined to be 100 ml in volume, column enzyme retention was obviously quite poor for all the materials tested. As a result of these test runs, it was decided that a process involving invertase immobilization by adsorption was probably impractical. Still, a semi-batch process hadn't been tested; this would involve the resin or support material being immersed in a concentrated enzyme solution and then being used in a continuous flow environment.

It should be noted that since activity tests with the covalently bonded enzyme-support samples do not distinguish between covalently bound and adsorbed enzyme, that these tests may also be regarded as setting upper limits for absorbed enzyme activity in the respective materials.

Careful examination of these test results show that IR-120 had the best results. The peak rises more slowly than the others in the 50-100 ml area. Also the IR-120 plot tapers off more quickly, perhaps indicating enzyme retention. This material was tested further with a continuous flow experiment. First, 1.00 gm of IR-120 was immersed in 25 ml of a 4% invertase solution with stirring for one hour. Then the resin was washed with 100 ml distilled water in a buret and 200 ppm sucrose bayou water was run through the column. Folin-Wu analysis showed essentially no invert sugar was
being produced. Since the flow was regulated through the column at 1.0 ml per minute and the glucose-fructose produced was less than 5 ppm, we may calculate the maximum activity compatible with the experimental results (see p. 61) as less than 0.008 minutes$^{-1}$. This may be compared to the Duolite assays to show that the IR-120's activity in the simulated waste water was less than 1.5% of that of the chemically bonded enzyme support's.
Discussion of Results

From these experiments, IRC-50 resin appeared to exhibit the most invertase retention in a packed column when compared to other support materials. Further tests showed that the activity itself of the invertase-resin system was negligible.

These tests results were so poor that considerations of a possible adsorption process were abandoned.
CHAPTER III

STUDY OF THE COVALENTLY BOUND ENZYME

Theoretical Background

Since an adsorption process appeared to be impractical, research was then done with invertase bound chemically to various support materials.

A wide range of materials have been investigated for the production of bound enzyme derivatives since the initial work of Grubhofer and Schleith (20). This early research dealt with diazotized polyaminostyrene and thionyl chloride treated polymers; it was the first research with synthetic covalently bound enzyme systems. There have evolved four basic methods for the synthesis of covalently bound derivatives since that time:

1) Reactive polymers, such as maleic anhydride copolymer, may be used:

\[
\left[ \begin{array}{c}
\text{CO}_2 \\
\text{NH-E}
\end{array} \right]_n + \text{NH}_2^\text{E} + \text{NaOH} \rightarrow \left[ \begin{array}{c}
\text{CO}_2 \\
\text{NH-E}
\end{array} \right]_n + \text{H}_2\text{O} + \text{Na}^+
\]

2) Polymer activation, such as the diazotization of polyamines, has been used:

\[
\text{R-NH}_2 + \text{NaNO}_2 \rightarrow \text{R-N}_2^+ \text{Cl}^- \rightarrow \text{R-N=Cl} \rightarrow \text{R-N=Cl}
\]

3) Polymer activation in the presence of the enzyme, such as carbodiimide coupling:

\[
\text{R-OH} + \text{BrCN} \rightarrow \text{R-O-C=NH} + \text{H}_2\text{N-E} \rightarrow \text{R-O-C=N-E}
\]
4) Polymer activation with a multifunctional reagent, such as the treatment of hydroxyl containing polymers with cyanuric acid derivatives, may be used:

\[
(R-\text{OH})_n + \overset{\text{Cl}}{\underset{\text{N}}{\text{N}}} \overset{\text{N}}{\underset{\text{N}}{\text{N}}} \overset{\text{Cl}}{\underset{\text{N}}{\text{N}}} \rightarrow (\overset{\text{R-O}}{\underset{\text{N}}{\text{N}}} \overset{\text{N}}{\underset{\text{N}}{\text{N}}})_n \rightarrow (\overset{\text{R-O}}{\underset{\text{N}}{\text{N}}} \overset{\text{HN-E}}{\underset{\text{N}}{\text{N}}})_n
\]

There has been a certain amount of interest in invertase bound by these different methods. The following is a list of support materials that have been used for covalent binding, the number of the methods used, and references giving details:

<table>
<thead>
<tr>
<th>Support</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyaminostyrene</td>
<td>2.</td>
<td>(9)</td>
</tr>
<tr>
<td>Bentonite</td>
<td>4. and 2.</td>
<td>(21)</td>
</tr>
<tr>
<td>Brick</td>
<td>4. and 2.</td>
<td>(21)</td>
</tr>
<tr>
<td>Cellulose</td>
<td>2.</td>
<td>(22)</td>
</tr>
<tr>
<td>Glass</td>
<td>4. and 2.</td>
<td>(21)</td>
</tr>
<tr>
<td>Phenol-Formaldehyde</td>
<td>4.</td>
<td>(23)</td>
</tr>
<tr>
<td>Resin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>4.</td>
<td>(24)</td>
</tr>
</tbody>
</table>

Table 4. Invertase Supports in the Literature

Of these materials, several are commercially available in forms that would be easily adapted to fixed enzyme use. Polyp-aminostyrene resins are commercially available and were used in the initial fixed enzyme research. CM-cellulose hydrazide may be obtained industrially and is readily used in enzyme fixation. Phenol-formaldehyde resins have long been used for ion-exchange
as well as other applications. Collagen is used in the food industry, and a technology exists for fabricating it in several forms. In addition to the materials previously listed, there have been a wide range of support materials used for the immobilization of other enzymes. To chose from the vast number of possibilities those which deserve to be investigated, it was necessary to pick some criteria. The prospective support material should be commercially available or easily synthesized, relatively inexpensive, and relatively successful in research already in the literature or at least not to have been shown to be impractical.

The silica-containing materials were rejected on the basis of the exotic reagents needed for enzyme binding and severe reaction conditions. Commercial scale use of these materials for other enzymes processes, however, might make these supports worth using for invertase in the future. Cellulose was rejected due to its low stability as an enzyme support; research had indicated a half-life of only 3.7 days in a continuous flow environment (25). Glass derivatives have a stability lower than would be desirable, researchers indicated a half-life of 42.5 days under continuous flow (25). These determinations were done with recirculating laboratory buffer solutions and probably represent upper limits not approached with the sucrose discharges to be treated in this project.

Since polyaminostyrene had already been shown to be a possible support material, it was the first support material to be considered for further testing in this research project. A search of the literature revealed that work with polystyrene was limited by the degree
of nitration possible. Excessively strong nitration conditions dissolved the support material (9). In order to get around this problem, the attachment was accomplished with both polystyrene and poly(styrene-divinylbenzene) copolymer with the hope that cross-linking would minimize the destruction of the support. In addition, a carboxylate-type cation exchange resin was tried in the hope that it would have increased activity due to the shift of the pH-activity optimum for the enzyme. Carboxylate anions would hopefully create an acidic micro-environment in the beads and enhance enzyme activity at bulk solution pHs close to neutral. In addition, the cation-exchange resin material (Amberlite IRC-50) was cross-linked and therefore expected to be more resistant to being dissolved by the nitration reagents; it is a polystyrene-type resin.

Simplicity of use suggested the investigation of various phenol-formaldehyde-type resins. These materials require immersion in an enzyme solution, perhaps followed by treatment with a cross-linking agent such as glutaraldehyde. This process works well with crude enzyme preparations and has been shown to result in a rather stable enzyme-phenol-formaldehyde complex (23). Research has been done on improving the resin material in several ways, a wide range of this type of resin has been examined for lactose immobilization (26). The commercially available resin (Duolite ES-762) is reported to be specifically designed for enzyme fixation (27). All in all, Duolite appeared to be a good material to test with invertase.

A similar resin material that had been shown suitable for lactose immobilization, resorcinol-formaldehyde resin, was also tested. The
material proved quite satisfactory for enzyme binding according to the literature and was quite easily synthesized (26).

A third phenol-type resin material, tannic acid-phenol-formaldehyde resin, was also chosen for testing. Tannins have been shown to form complexes with proteins and enzymes (28). In addition, the presence of the acid groups in the resin would be expected to lower the resin micro-environment and enhance activity at high bulk pH. Invertase itself has been shown to form a precipitate with tannic acid whose pH optimum is shifted to the higher pH range (29). Also the resin proved to be easily synthesized.

Glutaraldehyde treatment was used with the Duolite, resorcinol-formaldehyde, and the tannic acid-phenol-formaldehyde resins. By causing the enzyme strands to cross-link and by binding the enzyme to the support, invertase retention should be improved. While the exact mechanism involved in these reactions is not understood, the reaction has been shown to be relatively rapid (less than an hour to completion), dependent on temperature, dependent on pH and the reaction mixture ionic strength (30). All in all, we would expect glutaraldehyde treatment to increase retention of the enzyme in the resin supports.

The following support materials were finally chose for testing:

1) Resorcinol Resin
2) Glutaraldehyde-treated Resorcinol Resin
3) Tannic Acid Resin
4) Glutaraldehyde-treated Tannic Acid Resin
5) Duolite ES-762 Resin
6) Glutaraldehyde-treated Duolite ES-762
7) Amberlite IRC-50 Cation-exchange Resin
8) Polystyrene Beads
9) Poly(styrene-divinylbenzene) Copolymer
10) Yeast-collagen Material

A description of these support materials is given in appendix II.
Experimental Procedures

The following lists the procedures used to synthesize support materials and to fix the enzyme to them.

1, 2) **Resorcinol Resins** - 11 gm resorcinol and 100 ml of water were placed in a beaker with stirring. After the resorcinol dissolved to give a light-brown transparent solution, 15 ml of 37-38% formaldehyde was added, the mixture pH adjusted to 10.2 with 5 M potassium hydroxide, and then heated with stirring to 80°C. This solution quickly (in about ½ hour) became a dark-brown gel; the dark brown color is presumably due to oxidation of the resorcinol. The reaction mixture was cooled to 25°C and the gel broken up with a spatula. The gel slurry was made slightly acidic by the addition of 1 M HCl. The pieces of resin were then washed in a Büchner funnel with 1 liter of distilled water and then vacuum dried at low pressure at 40°C. Total yield after 1 day drying in the oven: 4.0 gm. This method is somewhat similar to that reported in the literature (26).

The dried resin was ground with mortar and pestle, then passed through a #14 mesh screen. Two 1.00 gm samples of the resin particles were placed in separate beakers. 5.00 gm invertase (Sigma, grade V) and 50 ml water were added with stirring to each sample. The enzyme was allowed to be adsorbed for another hour, then 5.0 ml of 25% glutaraldehyde was added to one beaker. After samples were treated for 2 hours, both were stored at 4°C. 15 hours later, the samples were each washed separately with 1 liter of distilled water in a Büchner funnel. The samples were labeled and stored separately at 4°C in 1 M NaCl.
3.4) **Tannic Acid Resins**— The synthesis of the tannic acid resin involved several false starts. First, 25 gm tannic acid (from M.C.B.) in 250 ml of 100% ethanol was placed in a flat-bottomed flask equipped with a condenser. 50 ml of 37-38% formaldehyde and 15 ml of concentrated HCl were mixed in a separate beaker, then added to the tannic acid solution. The mixture was refluxed for an hour and cooled to room temperature. Since the reaction mixture didn't become a gel and remained quite fluid, this method was abandoned. Perhaps the wrong form of tannic acid was used in this attempt.

Next, a synthesis paralleling the resorcinol resin's was tried. 25 gm of tannic acid in 200 ml of distilled water was adjusted to a pH of 10.5 with 5 M KOH, then 50 ml 37-38% formaldehyde was added with stirring. The mixture was heated to 70°C, but after several hours, there was still no sign of the formation of a gel. Upon cooling the reaction mixture was a dark brown fluid.

A successful synthesis was achieved upon trying to make a tannic acid-phenol-formaldehyde resin (31). 25 gm of NaOH were dissolved in 100 ml of distilled water. After cooling to room temperature, 50 gm of tannic acid was added and then 40 gm of sodium metabisulfite (anhydrous) with vigorous stirring and heat. To the reaction mixture was added 60 gm of phenol and 145 ml of 37-38% formaldehyde solution with stirring. The reaction mixture was boiled with magnetic stirring for 45 minutes until the surface began to form a gel. After being cooled to room temperature, this gel mixture was cut into $\frac{1}{2}$ cm thick slices and then dried in a drying oven at 60°C for 2 days. The dried resin was ground with mortar and pestle, then passed through a #14 mesh screen.
Two 1.00 gm tannic-phenol-formaldehyde resin samples were weighed out. The enzyme was fixed to both samples as with the resorcinol-formaldehyde resin, one sample having glutaraldehyde treatment as before. The two samples were stored at 4°C in 1 M NaCl after the 1 liter distilled water washes in a Büchner funnel.

4,6) **Duolite ES-762**– A sample of commercial phenol-formaldehyde resin prepared for enzyme attachment, Duolite, was obtained from Diamond Shamrock Chemical Company. The sample was dried in a vacuum at room temperature and then two 1.00 gm samples were weighed out. Invertase was attached to the samples as with the resorcinol and the tannic acid resins. Again one portion was treated with glutaraldehyde. Both Duolite samples were then stored at 4°C in 1 M NaCl.

7,8) **Amberlite IRC-50 Cation-exchange Resin**– To make a fixed enzyme material with this support involved an initial unsuccessful attempt.

The resin is a polystyrene carboxylate-type material which shall be represented as follows:

\[
\left[ \text{R} \quad \text{O} \quad \text{C-OH} \right]_n
\]

First, 10.0 gm Amberlite IRC-50 (wet weight) in 200 ml of anhydrous methanol with 5 ml of concentrated HCl were refluxed for 1 hour with vigorous magnetic stirring. This was to form the methyl ester of the polymer. The product was filtered with a Büchner funnel and washed with anhydrous methanol; the resin beads were dried at room temperature in a vacuum. Next, in a flask at 0°C, 200 ml of absolute methanol and the resin beads were stirred magnetically
while 20 ml of hydrazine monhydrate was slowly added. The reaction mixture was allowed to slowly warm to room temperature by removal from the ice bath used to regulate the reaction temperature. After 24 hours of stirring at 25°C, the resin was observed to have turned white like the Na⁺ or K⁺ forms of the resin. The beads were washed with anhydrous MeOH, then 100 ml of a 5% NaNO₂ solution was added at 0°C slowly on an ice bath. Vigorous bubbling was observed as an odorless gas was released. The mixture was allowed to react with stirring for 15 minutes on the ice bath, immediately followed by the beads being washed with 500 ml of ice water on a Büchner funnel. The washed beads were slowly added to a solution containing 20 gm of invertase in 100 ml of distilled water at 0°C with stirring; pH was adjusted to 8-9 with 5 M KOH. After reacting for 3 hours on the ice bath, the mixture was filtered and the beads washed with water. The beads were stored at 4°C in 250 ml of 1 M NaCl. The intended reaction sequence was as follows:

\[
\begin{align*}
\text{nMeOH} & + \left[ \begin{array}{c}
R \quad \text{O} \\
\text{COH}
\end{array} \right]_n \xrightarrow{H^+ \text{ MeOH}} \left[ \begin{array}{c}
R \quad \text{O} \\
\text{COMe}
\end{array} \right]_n + \text{nH}_2\text{O} \\
\text{nH}_2\text{NNH}_2 & + \left[ \begin{array}{c}
R \quad \text{O} \\
\text{COMe}
\end{array} \right]_n \xrightarrow{\text{MeOH}} \left[ \begin{array}{c}
R \quad \text{O} \\
\text{CNHNNH}_2
\end{array} \right]_n + \text{nMeOH} \\
\left[ \begin{array}{c}
R \quad \text{O} \\
\text{CNHNNH}_2
\end{array} \right]_n & \xrightarrow{\text{NaNO}_2 \text{ HCl}} \left[ \begin{array}{c}
R \quad \text{O} \\
\text{CN}_2^+ \text{Cl}^-
\end{array} \right]_n \\
\text{ENH}_2 & + \left[ \begin{array}{c}
R \quad \text{O} \\
\text{CN}_2^+ \text{Cl}^-
\end{array} \right]_n \xrightarrow{-\text{HCl}} \left[ \begin{array}{c}
R \quad \text{O} \\
\text{CNH-E}
\end{array} \right]_n + \text{N}_2
\end{align*}
\]
Where \( \text{ENH}_2 \) represents the enzyme with an amine group available. Subsequent testing of the beads revealed very little or no enzyme activity. Perhaps the evolution of gas when the sodium nitrite was added resulted from:

\[
\text{nH}_2\text{O} + \left[ \text{R} - \text{CN}^+ \text{Cl} \right]_n \rightarrow \left[ \text{R} - \text{COH} \right]_n + \text{nN}_2 \uparrow + \text{nHCl}
\]

This would mean that the invertase was never bound to the resin. It was decided to attempt a second approach:

First, 36 ml of concentrated sulfuric acid was cooled on an ice bath, 28 ml of concentrated \( \text{HNO}_3 \) was added. After evolution of heat, the acid solution was further cooled on an ice bath and 50 gm (wet weight) \( \text{IRC}-50 \) was added with stirring. A great deal of heat was evolved during the nitration, but the temperature was kept to 40-45°C. Then a 10% \( \text{NaOH} \) solution was added carefully until the reaction mixture was neutral. The resin was washed with distilled water in a Büchner funnel. These dried beads were a bright yellow-orange color. The reaction is as follows:

\[
\text{nHNO}_3 + \left[ \text{R} - \text{COH} \right]_n \xrightarrow{\text{H}_2\text{SO}_4} \left[ \text{R} - \text{NO}_2 \text{COH} \right]_n + \text{nH}_2\text{O}
\]

The ferrous hydroxide test revealed that the compound was indeed nitrated:

\[
\left[ \text{R} - \text{COH} \right]_n \xrightarrow{(\text{blue})} 6\text{nFe(OH)}_2 \rightarrow \left[ \text{R} - \text{NH}_2 \text{COH} \right]_n \xrightarrow{(\text{brown})} 6\text{nFe(OH)}_3
\]

Next, the beads were divided into 2 samples of equal weight. To one sample was added 400 ml of 6% \( \text{Na}_2\text{S}_2\text{O}_4 \) in distilled water and 45 gm
of KOH. After 3 hours of stirring at 70° C, the reaction was stopped by the addition of HCl to give a neutral solution. The beads were washed on a Büchner funnel with 500 ml of 0.1 M HCl. The product resin beads were pure white and smelled of H₂S. The resin was stored in 0.6 M HCl at 7° C overnight. The reaction is given below:

\[
\begin{align*}
\text{[R-} & \text{O-COCH}_n + 3n\text{KOH} \rightarrow [R-} \text{O-COK}_n + n\text{Na}_2\text{SO}_4 \\
\text{NO}_2 & + n\text{H}_2\text{O} + n\text{K}_2\text{SO}_4
\end{align*}
\]

A solution of 20 gm sodium nitrite in 100 ml of distilled water was cooled to 0° C, and then was added to the resin beads with stirring on an ice bath. Only a slight evolution of gas was observed at this stage. The mixture was filtered in a Büchner funnel and washed with 0.1 M HCl. The presumed reaction was as follows:

\[
\begin{align*}
\text{[R-} & \text{O-COK}_n \xrightarrow{\text{NaNO}_2/\text{HCl}} [R-} \text{O-COH}_n \\
\text{NH}_2 & \text{N}_2\text{Cl}^-
\end{align*}
\]

For the attachment of the enzyme, 20 gm of enzyme preparation was dissolved in 100 ml of distilled water and cooled to 0° C. Then the resin beads were added and the reaction mixture was allowed to warm up to room temperature by removal from the ice bath. The mixture was kept at 25° C for 14 hours with stirring and then filtered and washed with distilled water. The product was stored in 1 M NaCl at 7° C. The attachment reaction is listed below:

\[
\begin{align*}
\text{[R-} & \text{O-COH}_n + n\text{ENH}_2 \rightarrow [R-} \text{O-COH}_n + n\text{N}_2 + n\text{HCl} \\
\text{N}_2\text{Cl}^- & \text{NHE}
\end{align*}
\]
8) **Polystyrene**— Polystyrene pellets (about 10 mesh) were obtained from Aldrich Chemical Company. 10.0 gm of pellets were mixed with 36 ml of concentrated sulfuric acid and 28 ml concentrated nitric acid on an ice bath. The reaction mixture was allowed to warm to 25°C and stirring continued for 15 hours. The reaction was stopped by dilution with water and washing of the reaction mixture on a Büchner funnel. The polystyrene nitration product was stored in 0.1 M HCl at 7°C. This reaction was somewhat more severe than that given in the literature (9); the beads appeared slightly etched after the reaction and had aquired a light-orange color.

Next, the polynitrostyrene was mixed with 400 ml water, 24 gm Na₂S₂O₄, and 45 gm KOH. The reaction mixture was heated to 70°C for 3 hours. Then the polyaminostyrene was washed with 0.1 M HCl on a Büchner funnel. The beads were stored at 7°C in 0.1 M HCl.

Finally, the beads in 300 ml of 0.6 M HCl were cooled on an ice bath, then 60 ml of 0°C 20% aqueous sodium nitrite solution was added slowly. The reaction mixture was stirred vigorously for 30 minutes, then the polystyrenediazide chloride beads were filtered and washed on a Büchner funnel with 0.1 M HCl. 20 gm of enzyme material and 22 gm of Na₄P₂O₇ were dissolved in 100 ml of water, the solution was cooled to 0°C. The enzyme mixture and the resin beads were combined and left to react for 4 hours with the pH adjusted to 8.0 with KOH and HCl. The reaction was stopped with the filtering of the reaction mixture on a Büchner funnel and washes with distilled water. The product was a light, translucent orange color and was stored in 1 M NaCl at 7°C.
9) **Cross-linked Polystyrene**- Polystyrene-2% divinylbenzene copolymer beads (200-400 mesh) were obtained from Eastman Kodak Company. The reaction steps were essentially the same as in the previous synthesis except that the initial nitration step was carried out under more severe conditions. Cross-linking should be expected to inhibit the solution of the support in the acid mixture.

10.0 gms of poly(styrene+2%divinylbenzene) copolymer beads were placed in a solution containing 36 ml concentrated sulfuric acid and 28 ml concentrated nitric acid at 25°C. After 22 hours at room temperature with stirring, the beads had turned orange-yellow. The nitrated copolymer was separated from the reaction mixture by washing on a Büchner funnel; this was followed by further washes with distilled water.

The remaining reaction steps were the same as for the polystyrene resin procedure. The final product was a deep orange-brown color and was stored in 1 M NaCl at 7°C.

10) **Collagen-Yeast Material**- Collagen was obtained from the Engineering and Development Laboratory of the U. S. Department of Agriculture. 66 gms of collagen dispersion (15.2% solids) were combined with 20 gms of brewer’s yeast obtained from Fisher Scientific Company. A pH = 6.5 buffer was made by diluting 8.0 gms potassium hydrogen phosphate and 1.1 ml of 1.14 M NaOH to 500 ml with distilled water. 60 ml of the buffer and the yeast and collagen were mixed thoroughly in a Waring blender. A portion of this material was dried in a vacuum at 40°C on a polyethylene film. The net result of the vacuum treatment was a 8 by 8 cm piece of leathery, brown material about
1 mm thick. This material was cut into 0.5 cm squares. These pieces of collagen film were added to 125 ml of a 10% glutaraldehyde solution. The membrane material was tanned in the glutaraldehyde solution for 3 minutes and immediately washed with a great deal of water. The chips were dried at 25°C in a vacuum.

In order to compare these various enzyme-support materials, it was necessary to devise a procedure to subject the materials to severe operation conditions and then detect any losses of activity. The following procedure was used to achieve a comparison:

1) 1.00 gm samples of each of the support-enzyme materials were obtained.

2) These materials were assayed by stirring at a controlled rate in 50 ml of 1000 ppm sucrose buffer. The buffer was made by diluting 18.03 gm of potassium hydrogen phthalate, 10.2 ml of 1.142 M NaOH solution, and 1.000 gm of sucrose to 1000 ml with distilled water, giving a pH of 4.5. Activity was measured by an assay of invert sugar at intervals after the introduction of the resins to the sucrose buffer.

3) After removal from the assay solutions by filtration in a Büchner funnel, the support samples were each placed in 1 liter volumes of 1 M NaCl with vigorous magnetic stirring. The samples were stirred in their respective NaCl solutions for a 24 hour period.

4) Samples were recovered from the NaCl solutions by filtration. These were then assayed, as previously, and NaCl solution treatment was repeated. Assays and NaCl treatment were alternated as long as it was deemed useful to do so.
Graphs of enzyme activity as a function of NaCl immersion time are presented as figures on the following pages. Collagen-yeast material was not plotted since the initial assay showed negligible enzyme activity. The polystyrene bead sample showed such a low initial activity that no subsequent testing was done with that material either. The assays were performed by the graphing of invert sugar concentration against time of hydrolysis and visually determining the slope of a line passing through the origin which best described the reaction data points. The slope of the line was simply the activity of the preparation and was expressed in ppm invert sugar per minute.
Figure 11.
Amberlite IRC-50 Resin

Test Results

Figure 12.
Cross-linked Polystyrene

Test Results
The NaCl solution would be expected to greatly increase leaching of the enzyme from the support. The tannic acid resins were observed to rapidly lose activity despite high initial values. IRC-50 beads slowly lost their already low activity, and the resorcinol resins showed very low activities. By far the best retention of activity was displayed by the glutaraldehyde-treated Duolite sample. Indeed after about 1.5 days of NaCl solution treatment, this Duolite sample had the highest activity of any of those tested. Upon 2 days of treatment, activity loss was negligible for glutaraldehyde-treated Duolite, in contrast to all the other samples measured. Therefore it seemed reasonable to select this support-enzyme system as the most suitable for further testing.

To make any consistent activity measurements, it was necessary to obtain a rate expression for the Duolite-enzyme beads. It was decided that all activity determinations should be done under conditions likely to occur in an industrial process. During the previous testing, all the support materials were observed to physically degrade somewhat by breaking down into fine particles during stirring. It seemed reasonable that an industrial reactor would use a packed bed to minimize physical support attrition. Therefore all subsequent support assays were done with packed-bed columns. The behavior of the packed-bed columns were examined with a plug-flow model with a first-order reaction rate (with respect to enzyme and substrate). These assumptions may be easily shown to result in an expression in the following form:

$$-\ln\left(1 - \frac{GF}{G_\infty}\right) = \beta V/\omega$$
where

\[ GF_\infty = \text{invert sugar concentration at complete conversion in ppm} \]

\[ V = \text{the column volume in ml} \]

\[ \omega = \text{flow rate in ml per minute} \]

\[ \beta = \text{rate constant in minutes}^{-1} \]

To find if the above expression indeed applied to the Duolite support system, the flow rate of a 10.6 ml column was varied and the invert sugar concentration produced assayed. A 10 mm diameter titration column with gravity feed was found to be sufficient. Flow rate was varied with the buret's valve and measured by weighing the product stream flow weight over a given time period. The buffer feed used was made by diluting 0.200 gm sucrose, 11.75 gm NaH₂PO₄·H₂O, and 43.7 ml of 1.233 M NaOH to 1000 ml with distilled water, giving a 200 ppm sucrose pH = 7.0. If the experimental results agree with the previously listed model, then a plot of \(-\ln(1 - GF/\text{GF}_\infty)\) against inverse flow rate should be linear. The data in this form is graphed below:

![Graph showing the dependence of conversion on flow rate](image-url)
These results appear reasonably linear over the flow range tested. Further testing of the rate expression was done by varying the column volume while using a fixed flow rate of 10.0 ml per minute through the column. This was done by varying the amount of Duolite resin beads in the column while carefully controlling flow with a Sigmanotor finger pump with a variable pump rate adjustment. Flow rate was simply measured by simultaneously observing the time indicated on a stopwatch and the height of liquid in a graduated cylinder receiving the product stream; flow during the sample time was easily controlled to within 1%. A graph of \( \ln(1 - \frac{GF}{GF_\infty}) \) is given below as a function of \( V \), column volume.

\[
\begin{align*}
2.0 & \quad -\ln(1 - \frac{GF}{GF_\infty}) \\
1.0 & \\
0.0 & \\
0.0 & \quad 10.0 \quad 20.0 \quad \text{ml volume}
\end{align*}
\]

Figure 19.

Again we see a linear relationship, as would be expected from the model. To determine the activity of glutaraldehyde-treated Duolite under these reactor conditions, the data points from the
flow rate and the reactor volume experiments were pooled together and a least-squares fit done on \( \ln(1 - GF/GF_\infty) \) as a function of \( V/\omega \) with the constraint that the line pass through the origin. The slope of this line, \( \beta \), was found to be 0.881 minutes\(^{-1}\) at 25\(^\circ\) C. It was assumed in all future experiments that the plug-flow model was accurate, and the resin support reaction rate constant in each of the determinations that followed was found using the expression on p. 61. The pooled data for the activity determination is given below in graphical form:

![Graph](image)

**Figure 20**
Next, the behavior of glutaraldehyde-treated Duolite resin was determined as a function of temperature; this would be quite important in designing a process to treat condenser cooling water discharge as well as giving a basis for comparing behavior of the enzyme-support at temperatures other than 25° C where most of these experiments were run.

The apparatus used consisted of a 1 liter flask used as a feed solution reservoir, a thermometer required for measuring feed temperature, various lengths of glass and plastic ¼ inch tubing for air intake and solution flow, a 9 mm inside diameter resin-packed column for the actual hydrolysis, and a tubing pump to regulate flow. Immersion of the feed reservoir, tubing, and resin column in a jar bath assured control of temperature. The apparatus is illustrated below:

Temperature Dependence Apparatus

Figure 21
(a) Temperature Control
(b) Air Inlet
(c) Bath Thermometer
(d) Feed Thermometer
(e) Feed Flask
(f) Enzyme Resin Column
(g) Sample Collection
(h) Pump

The column resin volume was determined to be 4.6 ml. The feed buffer contained 200 ppm sucrose and had a pH of 7.0 (see p. 62). Flow rate was simply measured by simultaneously observing the time indicated on a stopwatch and the level of liquid in the sample collection graduated cylinder. Throughout the experiment, the flow was kept at 10.0 ml per minute. The jar bath was equipped with a magnetic stirring plate and a thermostatically controlled heater element, all made by Blue M Electric Company. Ice was added to the bath to obtain the lower temperatures.

The temperature-activity determinations were run as follows: first, ice was added to the bath and the bath and feed temperatures allowed to equilibrate. Then the column was flushed with feed for 10 minutes - this was to assure that the reactor had achieved steady state, and then a sample for invert sugar assay was taken. The column and bath were warmed by the bath's heater system until both the bath and feed stock had reached the next desired temperature. Another sample was then taken and the process repeated. Altogether, only about 15 minutes time were required at each temperature to make an
activity determination. When the highest temperature run was completed (56°C), an assay was run back at 25°C. This assay showed that about 50% of the enzyme activity had been lost. Since the enzyme-support system appears to be unstable at the higher temperatures, the upper (35°C + ) portion of the data should be regarded with caution. Activity in that part of the data has probably been underestimated due to the thermal deactivation of the enzyme. The temperature data was graphed below:

![Graph showing the dependence of activity on temperature](Figure 22)
In this graph, the activities have been normalized to that at 25°C, the temperature at which most fixed-enzyme experiments took place.

Next, the pH-activity curve of the support-invertase material was determined. A buffer was chosen to allow comparison to other pH-activity curves for invertase systems in the literature (9). A stock equimolar citric acid, NaH₂PO₄, boric acid solution was adjusted to the approximate pH required with 1 M HCl and 1 M NaOH. This solution was added to a volumetric flask with sufficient sucrose to give a 200 ppm solution, and the flask was made up to volume with distilled water; all buffer ions had a resultant 0.020 M concentration.

A column was filled with 14.5 ml of enzyme-support, and the column feed filled with the appropriate buffer. After suitable rinsing of the column, determinations of invert sugar were done with a column flow of 10.0 ml per minute. A graph of the pH-activity results is given below:

![Graph showing the dependence of activity on pH](Figure 23)
These results have been normalized to the activity at pH = 7.0. These results indicate a pH optimum near 4.5-5.0, about the same as the free enzyme.

All the previous work at this point for the fixed-enzyme determinations was done with buffer solutions made up with distilled water. To obtain more realistic and useful information, waste water was simulated with Bayou Teche water (p. 17) with 200 ppm sucrose. A 1 gm (3.0 ml) sample of glutaraldehyde-treated Duolite was placed in a 10 mm inside diameter column with gravity feed. Flow rate was very difficult to control due to blockage of the column by microscopic growth and sediment; activity as a function of total flow is given below:

![Diagram showing loss of activity with continuous flow](image)

Figure 24
The activity of the support-enzyme system appears to have a half-life of about 1000 reactor volumes; this is obviously inadequate for a treatment process using this material. An attempt was made at understanding the cause of the rapid loss of activity by the use of various models. First, if the activity loss is due to simple hydrolysis of the enzyme or temperature deactivation, we should expect to see an exponential decay of the rate constant. Plotting the natural logarithm of the rate constant against the total volume treated should give a linear relationship if this was the case. The data in this form are given below:

![Graph showing Loss of Activity with Continuous Flow](loss_activity_continuous_flow.png)

Figure 25.
This relationship is clearly not linear. It is interesting to note that the decay of activity may be approximated by a parabolic expression:

\[
\frac{d(\mathcal{A})}{d\tau} = -a \mathcal{A} \quad \mathcal{A}_0 \\
- \ln(\mathcal{A}/\mathcal{A}_0) = \frac{a}{2} \tau^2
\]

Loss of Activity with Continuous Flow

(Liters Treated)²

Figure 26.
There are several possible models that would predict this behavior. During the experiments, a couple of models were developed involving various reactions between the enzyme, the substrate, and enzymatic inhibitors in the waste water. Whatever the exact mechanism of deactivation, the rapid loss of activity of the enzyme and the way in which the loss took place suggest that inhibition of the enzyme is a major problem for this particular enzyme system.

A search of the literature revealed a possible solution; early in this century there was a great deal of research with iodine-treated invertase. It was shown that iodine treatment left the enzyme relatively unreactive with various inhibitors while still allowing some activity toward sucrose hydrolysis to remain (32). Perhaps if the enzyme could be reacted with iodine and then fixed to the resin, we would see a greatly improved performance.

The exact mechanism involved in the reaction of iodine with invertase is not known. Early researchers assumed that an iodine-invertase complex was formed which would not react with inhibitors, more recent work suggests invertase undergoes a reduction reaction (33). Initial attempts in this research to produce a fixed iodine-treated invertase proved unsuccessful. The initial trials involved first fixing invertase to Duolite, treating the material with glutaraldehyde, and then treating the resultant material with iodine. All the resultant materials proved to be inert toward sucrose hydrolysis; probably because the exact amount of iodine needed could not be accurately gauged.
These problems led to another attempt at synthesis of the fixed iodine-treated compound by a pathway in which the amount of iodine necessary could be easily found. In order to do this, it was first necessary that the iodine-treated invertase be synthesized; this compound would then be fixed to Duolite just as its untreated counterpart was.

A buffer solution was made by making 18.0 gm potassium hydrogen phthalate, 0.250 gm sucrose, and 4.7 ml of 2.476 M NaOH up to 1000 ml in a volumetric flask with distilled water. The resultant solution had a pH of 4.5 and 250 ppm sucrose. An iodine solution was made by diluting 1.00 gm iodine to 100 ml volume with absolute methanol. Various amounts of the iodine solution were then placed in beakers, sufficient water added to bring the total liquid volume to 20 ml in each. 10 ml of invertase solution (0.1% in distilled water) was added with stirring to each beaker and allowed to react for 1 hour. Then enzymatic activity was assayed by adding 100 ml of the sucrose buffer solution to each beaker with stirring and allowing another hour hydrolysis time. At the end of this final incubation period at 25° C, invert sugar was assayed by the Folin-Wu method. When the activity of the enzyme samples were plotted against the ratio of iodine to invertase in the initial preparations, an interesting relationship appeared. Such a graph is given on the following page:
Titration of Invertase with Iodine

Figure 27.
If we assume the iodine and enzyme reacted to completion, it appears that we are seeing two kinds of reactions between iodine and the enzyme. First, there is a linear relationship between the amount of iodine added and the decrease in enzyme activity observed; when the ratio of iodine to enzyme increases over 0.05, another kind of reaction resulting in a slower decrease in activity results. It was assumed that the first linear decrease is due to the formation of the iodine-treated invertase compound and that the further decrease was simply due to inhibition of the enzyme by iodine. From the data after making these assumptions, it is apparent that a ratio of 20 parts invertase to 1 part iodine is sufficient to produce the iodine derivative; also the iodine-treated enzyme appears to have about one third the activity of the untreated enzyme. This is more or less consistent with the literature data which claim the iodine-treated enzyme has 50% the activity of the untreated enzyme (33).

5.0 ml of absolute methanol containing 50 mg of iodine was added to 20 ml of distilled water in a beaker at 25°C. Then 1.00 gm of invertase was added with stirring and allowed to react for 60 minutes. Then 1.00 gm of Duolite resin was added (dry weight, 3.0 ml wet volume) with stirring and allowed to react further for 60 minutes. At the end of this time period, 1.0 ml of 25% glutaraldehyde was added. After 22 hours in the glutaraldehyde reaction mixture, the product was obtained by filtration on a Buchner funnel, followed by washes with 500 ml of distilled water. The final product beads were a dark brown-orange color.
Continuous flow apparatus was used as in previous experimental work (p. 69) to determine the behavior of the iodine-glutaraldehyde-treated Duolite system. The apparatus was the same as before except that for the first 20.5 liters of simulated waste water run through the column, a sand filter was installed between the feed flask and the column to remove fine sediment particles. It became apparent during the experiment that this filter was causing some very strange results to be observed. The activity data is graphed on the next page; at the point (a), the apparatus was taken apart and the components sterilized by heating at 120° C overnight and the resin transferred to a new clean glass column. All pieces of plastic tubing were replaced. This obviously had some influence the invert sugar and activity assays that resulted. At the point (b), the filter was removed altogether and the system (except for the resin beads) was sterilized over 5 liters or so. It appears that bacterial growth in the system and on the sand filter resulted in activity readings both above and below that of the actual enzyme-support. With the filter removed, it was found necessary to backwash the resin every 5 liters or so and a pump was used to maintain relatively uniform flow. The experimental apparatus that was used in the latter part of the experiment is shown on page 78.
Activity Loss with Continuous Flow

Equation 1

Equation 2

Figure 28.
(a) Feed Flask
(b) Enzyme Column
(c) Pump
(d) Sample Collection

Continuous Flow Apparatus

Figure 29.
Equation 1 in figure 28 was derived earlier (p. 71), and was given here for comparison. Equation 2 was found by making a linear least-squares fit of the log$_e$ of the enzymatic activity against the volume of waste water treated, using those data points after 22.1 liters. The fitted relationship was the following:

$$-\ln(\beta) = 0.0367v_t - 0.0679$$

The last data points have been graphed below as the log$_e$ of the activity against volume treated, the relationship appears somewhat linear over this range with wide scatter:

![Graph showing loss of activity with continuous flow](image.png)

**Figure 30.**
From the empirical fits to the continuous flow experiments, it is easy to show the glutaraldehyde-treated Duolite has a half-life of about 2.3 liters. The iodine-glutaraldehyde-treated Duolite has, assuming the fit is appropriate, an activity half-life of 18.9 liters—an 8.2-fold increase in activity retention. In addition the iodine treatment results in a higher initial activity, perhaps because the iodine treated compound is better incorporated in the support, or excess iodine alters the resin matrix micro-environment favorably.

Next the pH-activity curve of the iodine-treated resin was determined. Assays were done under continuous flow as were done for the glutaraldehyde-treated invertase (p. 68) determinations. The resultant curve is given below:

![Figure 31](image_url)

Comparison of Activity Dependence on pH for $I_2$ and non-$I_2$ Treated Duolite Resin.
The glutaraldehyde-treated Duolite results have also been given here for comparison. Both sets of data have been normalized to the $I_2$-treated pH = 7.0 activity. Iodine treatment gives a product with a broader peak and higher activity values throughout the pH range tested, although the untreated product shows enhanced activity at its pH optimum and around 7.3 relative to its activity at 7.0.
Discussion of Results

From these experiments, glutaraldehyde-treated Duolite ES-762 appeared to exhibit the greatest activity retention of all the materials examined in the initial tests. Subsequent tests characterized this material's behavior with respect to temperature and pH. Continuous flow experiments with simulated waste water indicated that enzymatic inhibition was a major problem, the glutaraldehyde-treated Duolite losing half of its initial activity after 770 reactor volumes treated (from the graph on p. 71).

In order to improve the activity retention, iodine-treated invertase was synthesized and bound to Duolite, followed by glutaraldehyde treatment to prevent leaching. This material was found to have a greatly improved resistance to inhibition, having a half-life of 6300 reactor volumes (from the equation on p. 79).

From these figures, economic constraints may be calculated. The manufacturer of Duolite (1975) specifies that the resin costs $15 per moist pound (800 moist gm per liter); assuming that commercial use of the material may bring the price down to $3 per moist pound, it may be easily calculated that the minimum treatment cost would be about $3.20 per 1000 gallons waste water treated assuming that the support may only be used once. Details of this calculation may be found in appendix III. Although lack of available time precluded any experimental attempts to show that the resin wasn't reusable, it seems a reasonable assumption considering the nature of the enzyme binding. In any event, the cost of water treatment with iodine-treated invertase fixed to Duolite ES-762 seems much too high for waste water treatment.
Time limitations for this project prevented further attempts to improve fixed enzyme performance. Observations of iodine-glutaraldehyde-treated Duolite over 2 months at 25°C in a standing column (wetted with distilled water) showed that activity loss with time was negligible. Therefore the major problem with fixed enzymes in the process environment seems to be the presence of inhibitors.

A broad range of compounds have long been known to inhibit invertase activity. The identification of specific ones for each process environment would be expected to be necessary before means of protecting the enzyme could be developed. Some possible treatment processes might involve filtration and adsorption with activated charcoal before any enzyme treatment of the feed stream. Also heavy metal absorbents may prove to be useful. All in all, the unknown nature of the inhibitors and the likelihood that their concentrations and compositions may likely vary with time and plant water source make it very difficult to approach the problem. If an enzyme support is developed that is much cheaper than Duolite or one is found that is easily reusuable, then the fixed enzyme process might prove to be economically feasible for waste water treatment as long as the support showed properties at least as favorable as the material tested here. With the rapid changes in fixed enzyme technology in recent years, such materials might be soon developed, especially as the industrial use of fixed enzymes increases.
SUMMARY OF RESEARCH AND RECOMMENDATIONS

Of the enzyme processes examined, the free enzyme process appeared to be the closest to present-day economic feasibility. Problems with bacterial growth in the reaction mixture were shown to be quite major, also the impurity of the commercial preparation imposes environmental constraints. Probably the quickest way to develop a free-enzyme process would center around a purification step for the commercial enzyme before it is added to the hydrolysis mixture and a method for controlling bacterial growth without destroying the enzymatic activity. Also the development of cheaper invertase preparations would help.

The adsorption process in itself shows promise due to its simplicity and potential low cost. All of the materials examined in this project, however failed to live up to this potential, losing activity and/or enzyme rather quickly. The most promising materials for an absorption process were examined with the covalently-bound enzyme systems, Duolite and tannic acid-phenol-formaldehyde resin. Any further work with an absorption process should probably involve some form of phenolic-type resin.

Although tannic acid-phenol-formaldehyde resin showed the highest initial activity in the initial covalently-bound enzyme experiments, its activity retention was not very good. Glutaraldehyde-treated Duolite showed superior activity retention in the NaCl solution experiments, but was found to lose activity rather quickly in simulated waste water. In order to improve the performance of this support system, iodine-treated invertase was synthesized and attatched to Duolite followed by glutaraldehyde treatment to cause cross linking and prevent leaching.
This material was shown to be far superior to the Duolite-enzyme system without iodine treatment, when used with simulated waste water. The presence of inhibitors in the waste water proved to be a major problem with both support systems. Also bacterial growth was found to result in major fluctuations in the apparent enzymatic activity. To make the fixed-enzyme process practical would require development of a method to prevent or limit bacterial growth without destroying the support activity. Also a much cheaper support material must be used and/or the problem of enzymatic inhibition be solved. As enzymes are used to a greater extent in industrial processes and as applied enzymatic research progresses, we can expect the fixed-enzyme process to appear more favorable.

All in all, at the present moment the free-enzymatic process would be the easiest to develop into an environmentally sound, if not economical, hydrolysis process. A fixed-enzyme process, however, has the most promise in the long run. Fixed-enzyme research is progressing very quickly; a review of this research work in the light of the new technology will quite likely reveal better and more practical approaches in a couple of years.

A listing of further research recommendations is given below:

1.) Investigate the possibility of purifying the industrial preparation.
2.) Examine methods to reduce bacterial growth in the waste water without destroying enzymatic activity in the hydrolysis step.
3.) Look at future invertase prices, development of higher invertase yeasts may bring down prices.
4.) See if the most promising support materials in this report (Duolite and tannic acid-phenol-formaldehyde resin) may be reusable. One possibility would be treatment with a hot mineral acid to hydrolyze and break up the enzyme. Another possibility would involve the use of a broad specificity protease to remove the invertase.

5.) Also tannic acid-phenol-formaldehyde resin should be examined as a possible support in a packed column with waste water.

6.) Look at the fixed enzyme literature in the future for superior support-enzyme systems and test them in the process environment.

7.) Examine enzyme inhibition to see if the use of absorbants such as activated charcoal or heavy metal absorbants are effective in protecting the enzyme.

8.) Examine other support-invertase systems to see if they are less vulnerable to inhibition than Duolite-invertase; it is possible that functional groups in the support matrix (such as carboxalate groups in the tannic acid resin) may interact with inhibitors and protect the invertase.

9.) See if the enzyme inhibition may be made to be reversible, by treatment with thymol, for example.

10.) Examine the possibility of using the best material developed during this project, iodine-glutaraldehyde-treated invertase-Duolite ES-762, for use in the food processing industries. One possible use would be in the manufacture of molasses from cane syrup.

Recommendations 1-3 deal with the free enzymatic hydrolysis; research along these lines would probably be the quickest way to develop a working process with today's technology.
Recommendations 4-9 deal with making a fixed enzyme process workable. Of primary importance are 7-9 which deal with aspects of the problem of enzymatic inhibition. From the research done in this project enzymatic inhibition appears to be the major problem with any prospective fixed enzyme process; even if inhibition appears unavoidable, it must be quantified for process water in order to examine the economics of any fixed system.

Recommendation no. 10 is an outgrowth of this research more than a critical issue in the treatment of waste water. Iodine-glutaraldehyde-treated invertase-Duolite ES-762 has been shown to be a superior material for treating sugar mill waste water. Although economics of this process appear rather bad, the treatment of high sucrose solutions in the food processing industries might prove to be quite practical with this material.
BIBLIOGRAPHY


27. Personal correspondence with Diamond Shamrock Chemical Company.


APPENDIX I

CALCULATION OF ENZYME COST FOR THE FREE ENZYME PROCESS

The enzymatic activity constant was found to be \( 0.000148 \pm 0.000191 \) ppm\(^{-1}\) minutes\(^{-1}\) for the commercial preparation.

To get 99% conversion in 7.0 hours we have:

\[
\log_e(1 - 0.99) = (0.000148 \text{ or } 0.000191)(E)(7.0)(60.)\text{ppm}
\]

\[E = 74.1 \text{ or } 57.4 \text{ ppm}\]

At a cost of 94¢ per pound for the enzyme, the cost of treatment of 1000 gallons of water (8350 pounds) for the enzyme itself would be:

\[
(94\text{¢})(74.1 \text{ or } 57.4)(8350.)(10^{-6}) =
\]

58¢ or 45¢ per 1000 gallons

Reducing the degree of hydrolysis requirement to 90% gives a reduction of enzyme cost and concentration of:

\[
\frac{\log_e(0.1)}{\log_e(0.01)}(100\%) = 50\%
\]
APPENDIX II

LIST OF SUPPORT MATERIALS

<table>
<thead>
<tr>
<th>Support Material</th>
<th>Size</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resorcinol-formaldehyde</td>
<td>#14+ mesh</td>
<td>synthesized</td>
</tr>
<tr>
<td>Tannic Acid</td>
<td>#14+ mesh</td>
<td>synthesized</td>
</tr>
<tr>
<td>Duolite ES-762</td>
<td>#30-120 mesh</td>
<td>Diamond Shamrock Co.</td>
</tr>
<tr>
<td>Amberlite IRC-50</td>
<td>#20-50 mesh</td>
<td>Mallinckrodt Chem. Works</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>#10 mesh</td>
<td>Aldrich Chem. Co.</td>
</tr>
<tr>
<td>Cross-linked Polystyrene</td>
<td>#200-400 mesh</td>
<td>Eastman Kodak Chem. Co.</td>
</tr>
<tr>
<td>Collagen</td>
<td>1 x 5 x 5 mm</td>
<td>U. S. Dept. of Agri.</td>
</tr>
</tbody>
</table>
APPENDIX III
CALCULATION OF MINIMUM COST
OF FIXED ENZYMES TREATMENT

Assume that the enzyme reactor is designed to treat the waste water until the support has lost 50% of activity. This means 6300 reactor volumes may be treated or for 1000 gallons we would require:

\[
(1000 \text{ gal.})(1./6300.)(3.785 \text{ liters/gal.}) = 0.601 \text{ liters}
\]

At 800 gm per liter this means that 1.06 pounds is needed to treat 1000 gallons. Therefore the cost under these assumptions and at a resin price of $3.00 per pound would be $3.20 per 1000 gallons.
VITA

S. W. Johnston was born on May 7\textsuperscript{th}, 1952 in Bellefonte Pennsylvania. He got his high school education at Broadmoor Senior High School in Baton Rouge and entered Louisiana State University in May of 1970. In December 1973, he graduated from the College of Chemistry and Physics at Louisiana State University with a B. S. in Chemical Physics. As an undergraduate he was awarded two academic scholarships. In August of 1974, he joined the Graduate School and he is now a candidate for the degree of Master of Science in Chemical Engineering.
EXAMINATION AND THESIS REPORT

Candidate:  Steven Wayne Johnston

Major Field:  Chemical Engineering

Title of Thesis:  Enzyme Process Design for Water Treatment

Approved:

Frank R. Groves, Jr.
Major Professor and Chairman

James E. Truesdell
Dean of the Graduate School

EXAMINING COMMITTEE:

Clayton D. Fielding

Philip A. Bryant

Date of Examination:

November 16th, 1976