National Library of Canada | Bibliothèque nationale du Canada

Canadian Theses Service | Services des thèses canadiennes

Ottawa, Canada
K1A 0N4

CANADIAN THESES

THÈSES CANADIENNES

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30.

THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED

LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS REÇUE

NL-339 (r.86/06)

Canada

STUDIES ON THE ENZYMATIC CONVERSION OF CELLULOSE TO FRUCTOSE

by

DEBORAH G. VANDENHOFF

A thesis
presented to the University of Ottawa
in fulfillment of the
thesis requirement for the degree of
MASTER OF APPLIED SCIENCE
in
THE DEPARTMENT OF CHEMICAL ENGINEERING

Ottawa, Ontario, 1986

© Deborah G. Vandenhoff, Ottawa, Canada, 1986.

Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

L'autorisation a été accordée à la Bibliothèque nationale du Canada de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur (titulaire du droit d'auteur) se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation écrite.

ISBN 0-315-36554-4

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

Concern about diminishing petroleum resources initiated studies into the use of renewable resources for the generation of fermentable sugars. Cellulose, the most abundant natural organic chemical in the world, can be hydrolyzed to glucose, a feedstock for liquid fuels and chemicals currently derived from petroleum.

The enzymatic hydrolysis of cellulose is impeded because of inhibition of the cellulase complex by glucose and the resulting accumulation of cellobiose. Fructose, however, has been found to have little or no inhibitory effect on β-glucosidase, the terminal enzyme in the cellulase complex responsible for the conversion of cellobiose to glucose. The conversion of glucose to fructose using glucose isomerase during cellulose hydrolysis could therefore reduce inhibition by glucose and, in turn, the inhibitory effect of cellobiose resulting in increased cellulolysis and a fermentable product.

The optimum temperature, and pH, for cellulose hydrolysis are 50°C, and pH 4.8, respectively. For simultaneous cellulose hydrolysis and glucose isomerization, it is therefore desirable to have a glucose isomerase active for the same conditions. A review of different possible sources of glucose isomerase resulted in the choice of Lactobacillus brevis NCDO 474 for the production of glucose isomerase for this work. There is a limited amount of literature available concerning the production of glucose isomerase ex Lactobacillus brevis. Because the sources

consulted provide no clear indication with regards to the temperature and composition of growth media for fermentation of Lactobacillus brevis, it was necessary to determine these as part of this work.

Glucose isomerase ex Lactobacillus brevis was found to have good enzyme activity in the pH range of 5.5 to 7.0 with the optimal pH being 6.5. The value of the Michaelis constant was found to be 0.87 M, indicating a high concentration of substrate required for isomerization.

Purification of glucose isomerase to increase the enzyme potency (activity/volume) was beyond the scope of this work. Therefore, only preliminary experiments to determine the effect of the addition of glucose isomerase during cellulose hydrolysis could be carried out. Production of reducing sugars during hydrolysis at pH 6.0 was shown to increase by 31% with the addition of glucose isomerase after a prehydrolysis step of 24 hours.

ACKNOWLEDGEMENTS

For all his help and valuable suggestions throughout this work, I wish to thank my research supervisor Dr. Gerald André.

I would like to acknowledge the help and suggestions of Roberto Leyva at the outset of this work and the kindness of Dr. Hayduk for reading the final draft copy.

I would also like to express my thanks to Messrs. G. Gasparetti, D. Lefebvre, and A. Bonaldo for their help whenever asked for assistance.

TABLE OF CONTENTS

Abstract ii

Acknowledgements iv

Chapter I: Introduction 1

Chapter II: Literature Survey 6

- Cellulose Hydrolysis 6
  - Endo-glucanase 8
  - Exo-glucanase 8
  - β-Glucosidase 9
  - Mode of action 9
  - Inhibition 13
  - Reduction of inhibition 15
    - Glucose to ethanol 15
    - Glucose to fructose 17
- Glucose isomerase 18
  - Microorganisms 21
  - Enzyme specificity 23
  - Xylose isomerization 24
- Immobilization 25
  - Techniques 25
  - Stabilization 30
  - Glucose isomerase 32

Chapter III: Methodology 33

- Glucose isomerase 33
  - Chemicals 33
  - Microorganism 33
  - Inoculum 33
  - Fermentation 34
  - Enzyme extraction 36
  - Properties of glucose isomerase 38
  - Analytical procedures 38
    - Cell growth 39
    - Protein content 39
    - Activity 39
    - HPLC analysis 42
- Cellulose hydrolysis 43
  - Microorganism 43

Inoculum . . . 43
Fermentation . . . 43
Hydrolysis . . . 45

Chapter IV: Results and Discussion . . . 47

Glucose isomerase . . . 47
Fermentation . . . 47
Cell growth . . . 47
Enzyme production . . . 52
Enzyme extraction . . . 57
Properties of glucose isomerase . . . 62
Effect of pH on glucose isomerase activity . . . 62
Effect of substrate concentration on glucose isomerase activity . . . 64
Enzyme stability . . . 66
Equilibrium isomerization . . . 68
Cellulose hydrolysis . . . 69
Fermentation . . . 69
Hydrolysis . . . 71
Effect of pH . . . 71
Effect of addition of glucose isomerase . . . 73

Chapter V: Conclusions and Recommendations . . . 75

Nomenclature . . . 79

Bibliography . . . 81

Appendix A: Taxonomic Properties of <u>Lactobacillus</u> <u>brevis</u> . . . 88

Appendix B: Purification of Glucose Isomerase . . . 91

Appendix C: Analytical Methods . . . 93

Protein content of fermentation broth by Biuret method . . . 93
Filter paper activity . . . 94
Determination of reducing sugars by DNS method . . . 95

Appendix D: Sample Outputs from HPLC Analysis . . . 98

Appendix E: Composition of Proteose Peptone and Yeast Extract . . 99

Appendix F: Experimental Data 102

LIST OF FIGURES

1. The overall process for the conversion of plant biomass to ethanol 4

2. The structure of cellulose 7

3. Schematic representation of synergistic action of enzymes in cellulolysis 12

4. Glucose isomerization reaction 20

5. A summary of techniques of enzyme immobilization 28

6. Schematic representation of the thermal unfolding of an enzyme 31

7. Effect of medium composition and incubation temperature on the total dry weight of cells 49

8. Effect of medium composition and incubation temperature on the optical density of the medium 50

9. The change in pH during the course of fermentation with the modified Yamanaka's medium 51

10. Profile of protein production during fermentation and the effect of medium composition and incubation temperature 53

11. The dependence of glucose isomerase activity on medium composition and incubation temperature 54

12. Comparison of the specific enzyme activity data for different operating conditions 56

13. Effect of the lyzing agent on the production of fructose during the extended isomerization 61

14. Effect of pH on glucose isomerase activity for extracted and cellular enzymes 63

15. Lineweaver-Burk plot for extracted and cellular enzymes . . . 65

16. Equilibrium isomerization of glucose isomerase at 50 °C 68

17. Cellulase production during fermentation 70

18. The effect of pH on the production of reducing sugars by cellulase 72

19. The final overall procedure established as a result of these investigations 77

20. The Hexose Monophosphate Shunt Pathway. 90

LIST OF TABLES

1. Properties of glucose isomerase produced from different microorganisms 22

2. Expected properties of immobilized glucose isomerase prepared by various procedures 29

3. Comparison of the two growth media for Lactobacillus brevis 35

4. Enzyme activity assay 41

5. Composition of the growth medium for Trichoderma reesei 44

6. Comparison of the effect of lyzing agent on extracted protein and enzyme activity 59

7. Effect of addition of glucose isomerase to hydrolysis vessel 74

8. Composition of proteose peptone and yeast extract 100

9. Data for fermentation at 30 °C with modified Yamanaka's medium 103

10. Data for fermentation at 37 °C with modified Yamanaka's medium 104

11. Data for fermentation at 30 °C with Yamanaka's medium . . . 105

12. Data for fermentation at 37 °C with Yamanaka's medium . . . 106

13. Effect of lyzing agent used in enzyme extraction 107

14. Effect of pH on glucose isomerase activity 108

15. Effect of substrate concentration on glucose isomerase . . . 109

16. Enzyme Stability 110

17. Equilibrium conversion data 111

18. Cellulase production and activity during fermentation 112

19. Effect of pH on the production of reducing sugars by cellulase 113

Chapter I

INTRODUCTION

Concern about diminishing petroleum resources initiated studies into the use of renewable resources for the generation of fermentable sugars. Cellulosic materials are available in large quantities which through hydrolysis and fermentation can yield fuels and chemicals currently derived from petroleum.

Acid hydrolysis of cellulose involves the use of concentrated sulphuric acid at low temperatures or diluted acid at high temperatures with the latter being the preferred method. While acid hydrolysis is fast and simple, and insensitive to the substrate, there are major drawbacks to this method of cellulose degradation. These include: corrosion of reactors, the high cost of acid recovery, the deactivation of lignin, and more importantly , the degradation of sugars to furfural, hydroxymethyl furfural, and humic substances.

Cellulose is found in plant biomass in a matrix of hemicellulose and lignin and, as such, is highly resistant to enzymatic degradation. Pretreatment of the substrate is therefore required to disrupt this matrix. Steam explosion, as a pretreatment, separates, without degrading, these three components and therefore allows for complete utilization of the lignocellulosic substrate (Saddler and Brownell, 1982b). The enzymatic hydrolysis of cellulose has the advantage that it occurs under mild operating conditions with no degradation of sugars.

The major setback to the commercial use of cellulase enzymes for cellulose hydrolysis is that of poor efficiency due to the increased resistance of the cellulosic residue, enzyme inactivation, and end-product inhibition. Much research has been done in the area of substrate pretreatment, optimization of the hydrolysis process, and isolation of microorganisms capable of producing higher levels of the enzymes (Bisaria and Ghose, 1981; Ladisch et al, 1983). Recently focus has turned towards the reduction of end-product inhibition by the removal or conversion of glucose. In the conversion of glucose, research has been directed towards the concurrent production of ethanol in the hydrolysis vessel. This requires that the operating temperature of the simultaneous saccharification and fermentation be reduced from 50 °C to 35 - 40 °C thereby reducing the rates of saccharification. This, coupled with inhibition by ethanol of the cellulase complex, does not appear to be the most beneficial solution towards the economic viability of the enzymatic hydrolysis of cellulose.

Woodward and Arnold (1981) found that β-glucosidase, the terminal enzyme in the cellulase complex responsible for the conversion of cellobiose to glucose, has a high specificity for the intact D-glucose with little or no inhibitory effect demonstrated by D-fructose. This work led to the suggestion by these researchers and by Leyva (1984) that glucose isomerase be used to convert glucose to fructose during the enzymatic hydrolysis of cellulose.

The overall process for the conversion of plant biomass to ethanol envisioned by the author is presented in Figure 1. This process includes pretreatment of the substrate, enzymatic hydrolysis of cellulose

with simultaneous conversion of glucose to fructose, and fermentation of these sugars to ethanol. The work of Leyva (1984) concentrated on the production of cellulase from Trichoderma reesei QM9414 and on cellulose hydrolysis while this work focusses on the production of glucose isomerase from Lactobacillus brevis and the combined cellulose hydrolysis and glucose isomerization.

Glucose isomerase (EC 5.3.1.5) is produced by a large number of bacteria as an intracellular enzyme with temperature and pH optima generally greater than 65 °C and pH 7.0 (Antrim et al, 1979). The higher temperature optimum of these enzymes is useful for the production of high fructose corn syrup; however, for simultaneous cellulose hydrolysis and glucose isomerization, it is desirable to operate close to the cellulolysis optima of 50 °C and pH 4.8. A review of reported data for the properties of glucose isomerase from different sources resulted in the choice of Lactobacillus brevis NCDO 474 for the production of glucose isomerase for this work.

There is a limited amount of literature available concerning the production of glucose isomerase ex Lactobacillus brevis. The sources consulted, however, provided no clear indication as to a suitable temperature and composition of growth media for fermentation of Lactobacillus brevis. Investigations were therefore considered necessary to determine these conditions.

Glucose isomerase is produced as an intracellular enzyme. Although it was considered beyond the scope of this work to purify the glucose isomerase, the enzyme extraction step of reported purification procedures (Kent and Emery, 1973; Yamanaka, 1963b) was investigated in

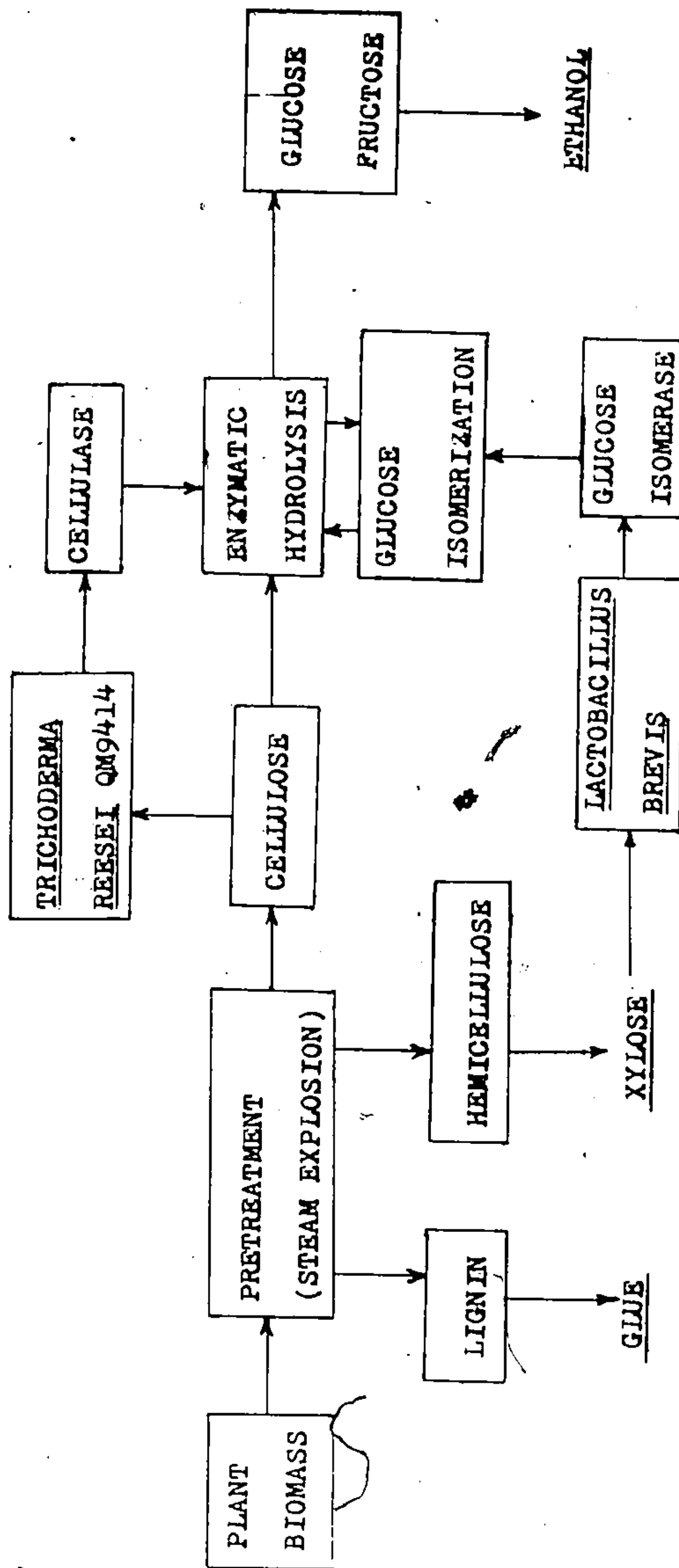


Figure 1: The overall process for the conversion of plant biomass to ethanol

this work. The suggestion of inhibition of glucose and xylose isomerases by Tris buffer (Antrim et al, 1979; Chen, 1980b; Slein, 1955), the lyzing agent used in these purification procedures, prompted studies on the effect of different lyzing agents on the extraction and activity of glucose isomerase *ex Lactobacillus brevis*.

The optimum pH for activity of glucose isomerase *ex Lactobacillus brevis* has been reported to be in the range of pH 6.0 to pH 7.0 (Chen, 1980a), while that of cellulose hydrolysis was found to be pH 4.8 for cellulase from *Trichoderma reesei* (Mandels, 1982). In this work, the effect of pH on enzyme activity was investigated. Other properties of glucose isomerase were also studied to determine the compatability of the two enzyme systems.

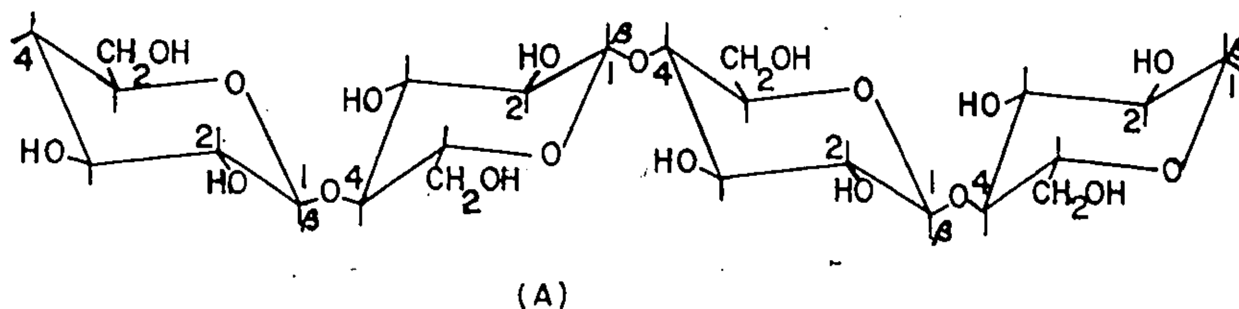
Chapter II

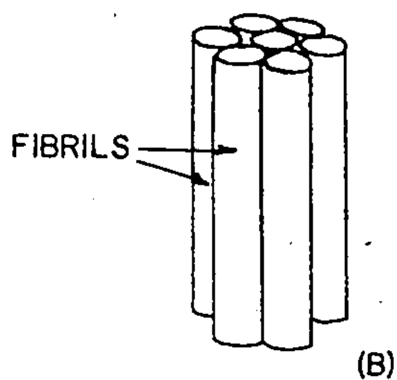
LITERATURE SURVEY

2.1 Cellulose Hydrolysis

The most abundant natural organic chemical in the world is cellulose, a linear polymer of glucose units bonded by β-1,4-glucosidic linkages with a degree of polymerization of 1,000 to 10,000. Strong intermolecular bonding is a characteristic of cellulose which results in the formation of fibrils of parallel polymer chains held firmly together by hydrogen bonding. Within these fibrils are areas of complete order, or crystalline regions, and amorphous regions where there is less order. Groups of these fibrils form fibres and a structure not readily accessible to breakdown. The structure of cellulose is illustrated in Figure 2.

The enzymatic hydrolysis of cellulose is catalyzed by an enzyme "complex" called cellulase. There are three major components of this complex: endo-glucanase, exo-glucanase, and β-glucosidase.





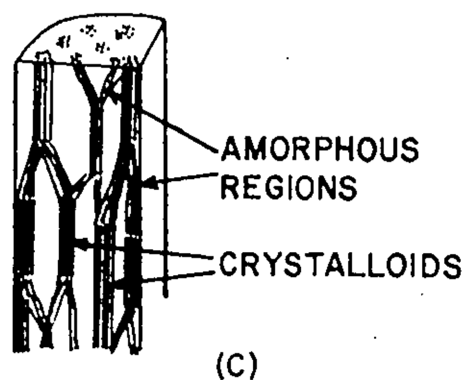


Figure 2: The structure of cellulose. Cellulose is a linear polymer of glucose units (A). Bundles of parallel fibrils are held together by hydrogen bonding (B). Each fibril is a group of parallel polymer chains with amorphous and crystalline regions (C).

2.1.1 Endo-glucanase

It is thought that, under the action of endo-glucanase, cellulose is subjected to a "random" hydrolysis of the internal β-1,4-glucosidic bonds to produce low molecular weight oligosaccharides, cellobiose, and some glucose.

Selby and Maitland (1967) found two enzymes in the endo-glucanase component in Trichoderma viride - a high molecular weight (HMW) and a low molecular weight (LMW) β-1,4-glucan glucanohydrolase. Mandels (1982) suggests that there are three to five of these enzymes which differ in physical properties and may also differ slightly in the mode of action. The molecular weights for these components have been reported by a number of researchers: Gong et al (1979) found that for Trichoderma reesei the LMW component had a molecular weight of 18,000 while that of the HMW component was 52,000; Mandels (1982) reported a range of 11,000 to 65,000 for the molecular weights of the components while Gritzali and Brown (1979) suggest that the range is 37,000 to 52,000. It has been suggested that the LMW endo-glucanase is more active towards crystalline cellulose because it is a smaller molecule (Gong and Tsao, 1979).

2.1.2 Exo-glucanase

The mode of action of the exo-glucanase or β-1,4-glucan cellobiohydrolase is an end-wise cleavage, by hydrolysis of the β-1,4-glucosidic bond, of cellobiose from the non-reducing end of a cellulose polymer. Another product of this cleavage is glucose, in the case of odd-numbered oligosaccharides.

Exo-glucanase is the major cellulase component produced by Trichoderma reesei with reported molecular weights varying to some degree: 72,000 (Gong et al, 1979); 42,000 (Berghem et al, 1975); 50,000 - 60,000 (Mandels, 1982); and 53,000 (Gritzali and Brown, 1979).

2.1.3 β-Glucosidase

The final step in the enzymatic hydrolysis of cellulose is the hydrolysis of the β-glucosidic linkage of a cellobiose molecule by β-glucosidase to give two molecules of β-D-glucose. Ninety percent of the glucose produced in cellulose degradation is the result of action by β-glucosidase.

As will be explained in the text to follow, though β-glucosidase does not degrade cellulose, it is important to take this enzyme into consideration when discussing cellulases because it removes the inhibitory cellobiose. Unfortunately, this enzyme is produced only in small amounts in Trichoderma sp. Gong and Tsao (1979) report that only 0.2% of the total extracellular protein of T. reesei, one of the best sources for extracellular cellulase, is β-glucosidase.

Mandels (1982) reports a molecular weight of 34,000 for β-glucosidase of T. reesei while Gong et al (1979) report 76,000 for the same enzyme.

2.1.4 Mode of action

Selby and Maitland (1967) isolated the three components of cellulase from a culture of Trichoderma reesei and found that on its own exo-

glucanase did not degrade cellulose but hydrolysis did occur in the presence of endo-glucanase.

Research conducted by Wood (1972, Wood and McCrae, 1979) supported Selby and Maitland. While exo-glucanase did not act on highly-ordered cellulose or carboxymethyl cellulose (the substrate used to determine endo-glucanase activity), it was able to degrade cellulose that had been swollen by phosphoric acid. Comparison of the effect of glucanohydrolases on the degree of polymerization of the same substrate indicated that the mode of action of these two enzyme components was very different. Over a period of time the degree of polymerization of the cellulosic substrate decreased only slightly when exo-glucanase acted alone while the degree of polymerization dropped drastically under the action of endo-glucanase alone. The findings of Pettersson et al (1972) also supported this research.

One of the main reasons that the mode of action of cellulase is so difficult to determine is the fact that there is a high degree of interaction between the components of the enzyme complex. The fact that each component working individually or in pairs is not as effective as the recombination of all three enzymes suggests a synergistic mechanism. The most important of these interactions is that between the endo- and exo-glucanases.

Wood (1972) found that when the endo- and exo-glucanases were recombined, 83% of the activity towards cellulose was restored. The addition of β-glucosidase however was necessary to fully recover the original activity.

Other researchers (Berghem et al, 1975; Gong and Tsao, 1979; Nisizawa et al, 1972) have also found that the addition of endo-glucanase enhanced the activity of exo-glucanase. A realistic explanation, and one that enjoys considerable support, for this is that by the hydrolyzing effect of endo-glucanase on internal bonds of the polymer, new chain ends are created which are susceptible to attack by exo-glucanases. This theory was first proposed by Eriksson (1969) and summarized by Bisaria and Ghose (1981):

1. Regions of low crystallinity in the cellulose fibre are attacked by endo-glucanases and free chain ends are created.
2. Exo-glucanases start the degradation from the chain ends by hydrolytically removing cellobiose.
3. Cellobiose is hydrolyzed to glucose through the action of β-glucosidase.

Cleavage of cellobiose units from the new chain ends then allows for enhanced access to internal β-1,4-linkages for the endo-glucanases. This synergism is best described by Figure 3.

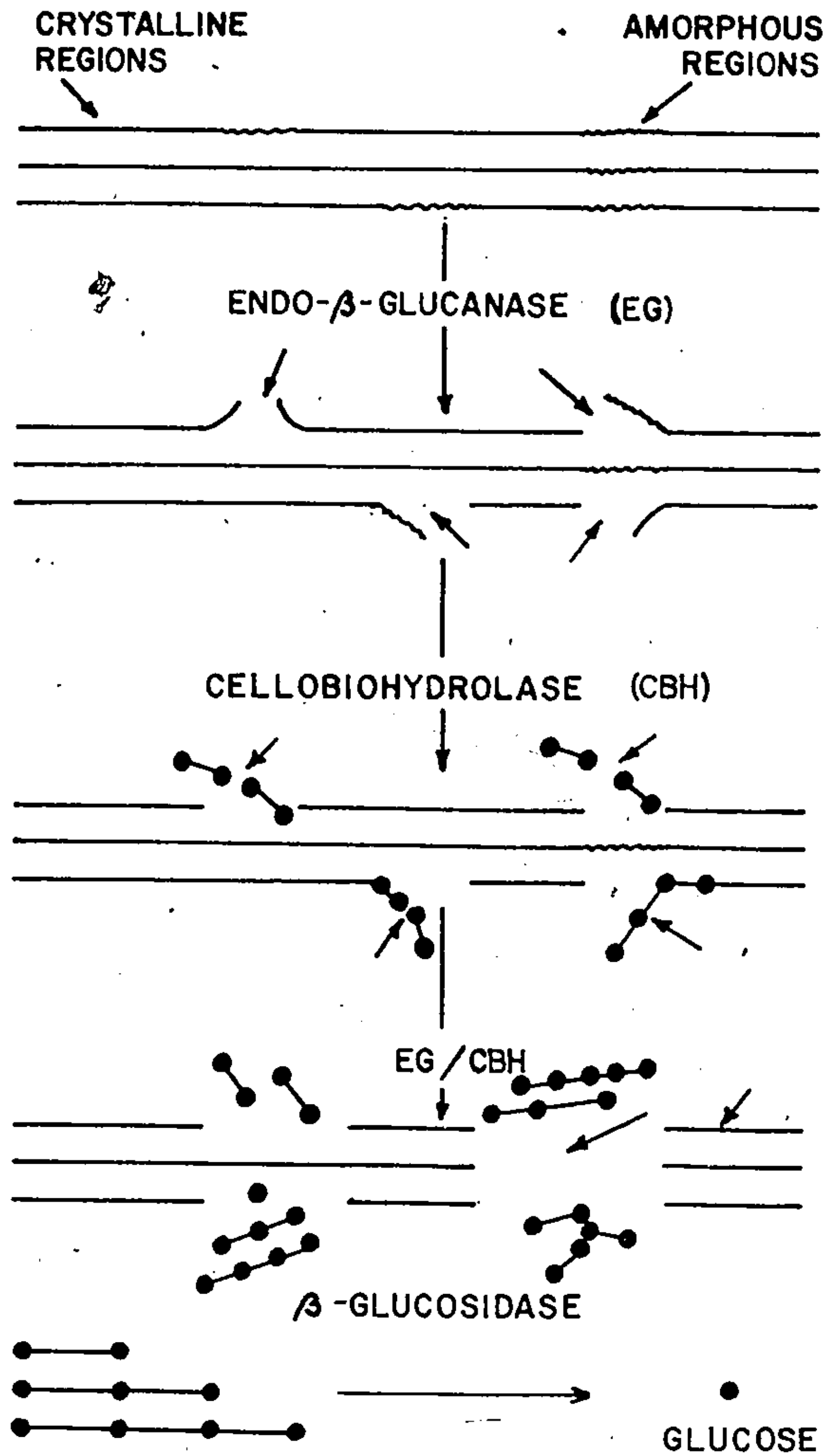


Figure 3: Schematic representation of synergistic action of enzymes in cellulolysis. (Bisaria and Ghose, 1981).

2.1.5 Inhibition

The reduction in reaction rates noticed during the enzymatic hydrolysis of cellulose can be attributed to a number of factors including enzyme age, changes in the structural properties of cellulose, changes in temperature and pH, the denaturing effect of shear, and inhibition by the intermediates and products of hydrolysis. Of particular interest in this research is the effect of end-product inhibition by the products cellobiose and glucose. The capability of these molecules to stop action of the enzymes is part of the feedback control mechanism found in normal cells to ensure efficient use of material and energy resources. In order to make the enzymatic hydrolysis of cellulose more economically viable, the degree of cellulose degradation reached before the enzyme is "turned off" or slowed down must be increased. This may be done in a variety of ways including genetic manipulation (implying modification of the genes producing cellulase) and removal of the inhibitory products.

The inhibition of the cellulase complex by cellobiose and glucose is complicated and magnified by the multiplicity of enzymes and the synergistic interactions between enzymes.

The importance of β-glucosidase in the cellulase complex becomes evident in the consideration of inhibition. β-Glucosidase acts by hydrolyzing cellobiose into glucose. Cellobiose has been found to be a strong competitive (Berghem et al, 1975; Halliwell and Griffin, 1973; Hsu et al, 1980) inhibitor of cellobiohydrolase (Berghem et al, 1975; Gong et al, 1979; Gong and Tsao, 1979; Halliwell and Griffin, 1973; Mandels, 1982; Sternberg, 1976). Endo-glucanase also suffers from inhibition by cellob-

iose (Mandels, 1982; Sternberg, 1976). Thus, by conversion of cellobiose to glucose, inhibition by cellobiose is reduced. However, glucose is an inhibitor of β-glucosidase (Fan and Lee, 1983; Gong et al, 1979; Gritzali and Brown, 1979; Mandels, 1982; Woodward and Arnold, 1981). The mode of inhibition is not agreed upon with some researchers reporting competitive inhibition (Fan and Lee, 1983; Woodward and Arnold, 1981) and others proposing a noncompetitive mode (Gong et al, 1979; Ladisch et al, 1980). This inhibition results in an accumulation of cellobiose and hence greater inhibition of the endo- and exo-glucanases.

Glucose also competitively inhibits the endo- and exo-glucanases (Gong and Tsao, 1979; Hsu et al, 1980; Ladisch et al, 1980) but to a lesser extent than cellobiose (Gong and Tsao, 1979; Hsu et al, 1980). Selby and Maitland (1967) found that the addition of glucose before incubation inhibited degradation of cellulose. Other polyhydrides including sorbitol, glycerol, xylose, fructose, galactose, and mannose were also tested for inhibitory effects but none was as great as that displayed by glucose. Due to the presence of β-glucosidase, the inhibitory effect of cellobiose as compared to glucose was not studied.

Woodward and Arnold (1981) also studied the extent of inhibition by isomers and derivatives of glucose towards β-glucosidase. They found that D-glucose produced greater inhibition than did L-glucose, glucose-6-phosphate, glucose-1-phosphate, and fructose indicating the high specificity of β-glucosidase for the intact D-glucose molecule.

From the findings discussed above it is therefore logical to conclude that the concurrent removal of glucose either directly or by conversion

would greatly decrease inhibition as even the effect of cellobiose would be lessened because of the increased activity of β-glucosidase.

2.1.6 Reduction of inhibition

Mandels (1982) suggests that the effect of product inhibition can be reduced by the removal of glucose from the hydrolysate using membrane reactors or by converting the glucose to ethanol during the hydrolysis. To the same end, Woodward and Arnold (1981) suggest the conversion of glucose to fructose during cellulose hydrolysis.

2.1.6.1 Glucose to ethanol

The reason that so much attention has been focussed on the degradation of cellulose is its potential as a raw material for the production of chemicals and liquid fuels. For most of these products the glucose produced therefore is ultimately to be fermented to ethanol which in turn is a feedstock for liquid fuels and chemicals currently derived from petroleum (Ladisch et al, 1983). Thus a logical solution to product inhibition is the direct conversion of glucose to ethanol during hydrolysis.

Simultaneous saccharification and fermentation (SSF) takes place in one vessel with the yeast or bacteria being added either at the same time as the cellulase or after a prehydrolysis for 20 to 24 hours. The advantage of SSF is that there is a reduced reactor volume and reportedly higher saccharification rates (Ghosh et al, 1982).

One of the most important considerations that must be made is the optimum temperature for SSF. Most yeasts have a temperature optimum of 30 - 35 °C for ethanol production (Emert and Katzen, 1981) at which the rate of hydrolysis would be greatly reduced. Zymomonas mobilis has maximum ethanol production at 30 °C. At 37 °C ethanol production is not significantly reduced while production is reduced by 30% at 40 °C and 60% at 45 °C (Viikari et al, 1981). All growth of this bacterium stopped above 37 °C. These fermentative yeast and bacteria utilize xylose and cellobiose aerobically but could not produce ethanol with either of the sugars (Meyers, 1978).

Ghosh et al (1982) combined cellulase from Trichoderma reesei QM9414 with Saccharomyces cerevisiae and found an increase in the effective reducing sugars concentration of 13 - 30%, depending on the enzyme concentration. This particular yeast however, has an optimal temperature for ethanol production of 35 °C and the increase in the reducing sugars concentration with SSF was compared only to hydrolysis, the temperature optimum of which is 50 °C, at 35 °C.

Emert and Katzen (1981) report a 25 - 40% increase in yield when comparing SSF at 40 °C to saccharification alone followed by subsequent conversion to ethanol.

The rate-determining step in a simultaneous saccharification and fermentation is the hydrolysis of cellulose. Saddler et al (1982a) allowed for a prehydrolysis which was carried out at its optimum temperature of 50 °C for 24 hours before reducing the temperature to 37 °C and adding the fermenter. They found that the higher glucose concentration obtained with a prehydrolysis enhanced the fermentation rate.

Meyers (1978) allowed the hydrolysis to proceed for 20 hours at 45°C and then added Candida utilis. Simultaneous saccharification and fermentation then occurred at 30 °C and pH 4.0, the optimum conditions for fermentation by this yeast. He found that, in terms of glucose equivalents, there was 22% more accumulation of product in SSF than in saccharification alone.

Blotkamp et al (1978) found that due to the initial cellulose concentration, the concentration of ethanol never attains an inhibitory level for both the yeast and cellulase. In fact, they report increases in cellulase activity with increasing ethanol concentrations up to 1%(w/v) and no further changes thereafter. They also claim a large increase in β-glucosidase activity for ethanol concentrations up to 10%(w/v).

These findings however are disputed by many including members of that research group (Ghosh et al, 1982; Meyers, 1978; Pemberton et al, 1980). Ghosh et al report significant inhibition of cellulase by ethanol at concentrations as low as 0.75%(w/v) with the saccharification rate halved at a concentration of 7.5%. Ethanol manifests itself in a general inhibition of cellulase, specifically towards β-glucosidase (Meyers, 1978; Pemberton et al, 1980). Meyers also found that the inhibitory effect of ethanol towards cellulase activity increased with the degree of crystallinity of cellulose.

2.1.6.2 Glucose to fructose

Woodward and Arnold (1981) reported that β-glucosidase has high specificity for the intact D-glucose molecule and that fructose has little or no inhibitory effect on the enzyme. It is hypothesized that conver-

sion of glucose to fructose during cellulose hydrolysis would result in the enhanced activity of β-glucosidase and therefore, by the subsequent reduction in accumulation of cellobiose, also of the endo- and exo-glucanases. The resulting sugars, glucose and fructose, are both fermentable sugars available for further processing to ethanol or other bio-products.

Except for the suggestions by Woodward and Arnold (1981) and Leyva (1984) to look into the use of glucose isomerase to convert glucose to fructose during cellulose hydrolysis, no other work was found towards this goal.

2.2 Glucose isomerase

Investigations into the development of an isomerization process which converts glucose to fructose to produce an alternative sweetener was inspired by the rising cost of sucrose. Glucose is only 70% as sweet as sucrose (Antrim et al, 1979) while fructose is 1.7 times as sweet (Chen, 1980a).

Several alkaline isomerization processes were developed but never commercialized, due to the formation of degradation products in syrups of greater than 40% fructose. These products reduced sweetness and developed colours and off-flavours that could not be easily removed.

Glucose isomerase, on the other hand, acts as a highly specific catalyst for glucose isomerization without producing degradation products. Glucose isomerase (EC 5.3.1.5) is now one of the most widely-used enzymes in industry. It is used after starch hydrolysis for the

conversion of glucose to fructose to produce high fructose corn syrup (HFCS). The isomerization reaction is depicted in Figure 4.

This enzyme is also capable of catalyzing the isomerization of xylose to xylulose. In fact, because the value of the Michaelis constant for the glucose substrate, ranging from 0.086 to 0.920 M, is much higher than for xylose, ranging from 0.005 to 0.093 M (Chen, 1980b), the ability of this enzyme to isomerize glucose was shadowed by its capability to catalyze the xylose isomerization.

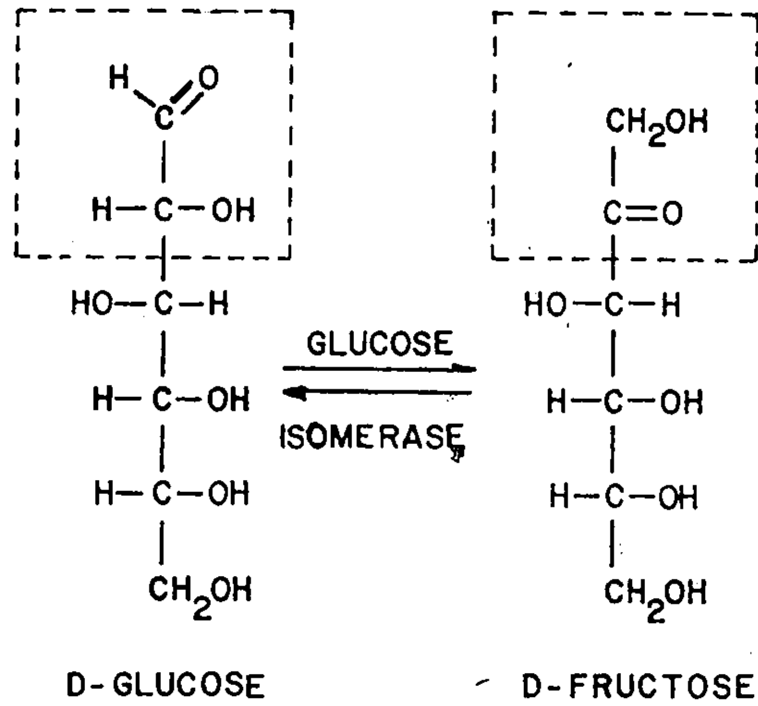


Figure 4: Glucose isomerization reaction

2.2.1 Microorganisms

Glucose isomerase is produced by a large number of bacteria as an intracellular enzyme. Table 1 lists some glucose isomerase- producing microorganisms. Most isomerases have optimal operating conditions of greater than 65 °C and greater than 7.0 for temperature and pH, respectively (Antrim et al ,1979).The optimal operating conditions of the isomerases of these microorganisms are also given in Table 1.

In the production of high fructose corn syrup, the glucose isomerase must be able to withstand high temperatures so as to reduce the cost of cooling the sugar solution before isomerization. The most commonly used microorganisms are Streptomyces sp., Actinoplanes missouriensis, and Bacillus coagulans which produce enzymes with both high optimal temperature and pH values. However, the combination of operation at high temperatures and higher values of pH results in the production of D-psicose, a non-metabolizable sugar, making a low pH optimum more desirable.

Lactobacillus brevis has the highest yield of glucose isomerase of the heterolactic bacteria (Yamanaka, 1963a). The low pH optimum of glucose isomerase from this microorganism makes it commercially interesting; however, because of its lower temperature optimum, it has not been used in the production of HFCS (Chen, 1980a).

In choosing a microorganism for the production of glucose isomerase, to be used in conjunction with cellulase it was important to consider the optimum operating conditions for hydrolysis. The optimal temperature and pH for cellulose degradation with Trichoderma reesei cellulase are

Table 1: Properties of glucose isomerase produced from different microorganisms

(Chen, 1980a)

MICROORGANISM	TEMPERATURE OPTIMUM (°C)	pH OPTIMUM
Actinoplanes missouriensis NRRL B-3342	90	7.0
Aerobacter cloacae	50	7.6
Bacillus coagulans HN-68	75	7.0
B. stearothermophilus	80	7.5-8.0
Escherichia intermedia	50	7.0 pH stability: 7-9
Lactobacillus brevis	50	6.0-7.0
Streptomyces sp. S41-10	75	8.5
Streptomyces sp.	80	7.0-8.0
S. albus YT-5	80	8.0-8.5
S. albus NRRL B-5778	70-80	-
S. bikiniensis	80	8.0-9.0
S. flavogriseus	70	7.5
S. flavovirens	85	8.5
S. olivochromogenes ATCC-21114	80	8.0-9.0
S. phaeochromogenes SK	90	9.3-9.5
S. phaeochromogenes NRRL B-3559	80	8.0

50 °C and 4.8 respectively (Mandels, 1982). A survey of the properties of glucose isomerase from different microorganisms listed by Chen (1980a) led to the choice of Lactobacillus brevis NCDO 474 for the production of glucose isomerase. Taxonomic properties of Lactobacillus brevis are listed in Appendix A.

2.2.2 Enzyme specificity

D-Glucose exists in two anomeric forms, α-D-glucopyranose and β-D-glucopyranose, and there are four anomers of D-fructose: α-D-fructopyranose, β-D-fructopyranose, α-D-fructofuranose, and β-D-fructofuranose. Glucose isomerase is specific for the α-pyranose forms of these sugars while cellulose hydrolysis yields β-D-glucose. McKay and Tavlarides (1979) suggest that at best the β-anomer is a weak substrate and/or inhibitor.

α-Glucose has a specific rotation of +110° while that of the β-anomer is +19°. In solution, both sugars will mutarotate to an equilibrium rotation of +52°. Hudson and Dale (1917) report that the temperature-dependent rate of mutarotation is the same for the α- and β-anomers. At 40 °C, the rate becomes difficult to measure on account of the rapidity. This suggests that at 50 °C, glucose isomerase should be able to convert the glucose produced during cellulose hydrolysis.

In general, glucose isomerases require a divalent cation such as Co^{2+}, Mn^{2+}, Mg^{2+}, or Cr^{2+} for catalytic activity. Some isomerases such as those from Aerobacter aerogenes, A. cloacae, Escherichia intermedia, and Pseudomonas hydrophilia require As^{3+}. Although Co^{2+} substitutes partially, glucose isomerase ex Lactobacillus brevis requires Mn^{2+} for activity (Chen, 1980b).

The fact that the metal is required for enzyme activation suggests the formation of an enzyme-metal-substrate bridge complex. Schray and Mildvan (1972) found that the sugar substrates and inhibitors of D-xylose isomerase form weak complexes with manganese(II). It is therefore logical to assume that the cation acts by forming a complex with the enzyme.

Young et al (1975) used proton resonance to investigate the effect of the enzyme-metal bridge complex. While metal-free D-xylose isomerase had no effect on the α- or β-anomers, the Mn^{2+}-xylose isomerase complex broadened the α-C1 resonance and had virtually no effect on the β-C1 proton resonance, confirming the anomeric specificity of the enzyme.

2.2.3 Xylose isomerization

Due to the presence of hemicellulose in cellulosic substrates, to the extent of 25 - 35% (Wang et al, 1980), hydrolysates contain xylose, the major five-carbon sugar produced during the hydrolysis of this fraction. Even in Solka Floc, a source of purified cellulose frequently used as a reference substrate, there is some hemicellulose present indicated by the significant xylose content of the hydrolysate. Leyva (1984), using Solka Floc SW-40, found 20 - 25% of the total sugars in the hydrolysate (xylose, glucose, and cellobiose) to be xylose.

The desire to maximize the use of the cellulosic substrate therefore demands that xylose be converted to ethanol. This aldopentose however, is not fermentable by most yeast and bacteria (Gong et al, 1981). *Shizosaccharomyces pombe* cannot ferment xylose but is one of the better

fermenters of its isomer xylulose (Wang et al, 1980). Wang et al suggest that the inclusion of glucose isomerase to convert D-xylose to D-xylulose will increase the production of ethanol to more efficiently make use of the total cellulosic substrate. Among the glucose isomerases tested by Wang is that of Lactobacillus brevis grown on a xylose medium, the microorganism used in this research.

2.3 Immobilization

Industrial use of enzymes has not been fully exploited due to the fact that enzymes do not have great stability at operational conditions and that they are difficult to separate from the product because of their solubility in water. Immobilization of cells or enzymes facilitates product recovery by making them water-insoluble. Recovery of the biocatalysts also means that they can be reused and that continuous production is possible. The effect of immobilization on enzyme stability will be discussed.

2.3.1 Techniques

There are a number of immobilization techniques in use today. One of the simplest of these is heat fixation which denatures the lytic enzymes to stop cell autolysis; thus, the enzymes of interest become cell-bound.

Chemical methods include adsorption of enzymes on solid supports, covalent bonding of the enzyme to a solid support, and cross-linking by multifunctional reagents.

Adsorption occurs due to ionic forces between the enzyme and a support such as activated carbon, alumina, silica gel, and substituted cellulose. This method is simple and requires no chemical modification but small changes in temperature, pH, and ionic strength can cause desorption of the enzyme.

Covalent bonding occurs between the amino or carboxyl groups of the enzyme and a support (such as porous glass and ceramics, stainless steel, sand, charcoal, cellulose, synthetic polymers, and metallic oxides) which has been activated. A multifunctional reagent such as glutaraldehdye, dimethyl suberimidate, and aliphatic diamines can bind enzymes and react with themselves to form polymers. It may be desirable to have the enzyme on the outer surface of an insoluble support by first adsorbing the enzymes and then cross-linking the enzymes. Enzymes are strongly bound by these methods and are less likely to be washed out or desorbed. Inactivation may occur by chemical modification of the enzyme's active site.

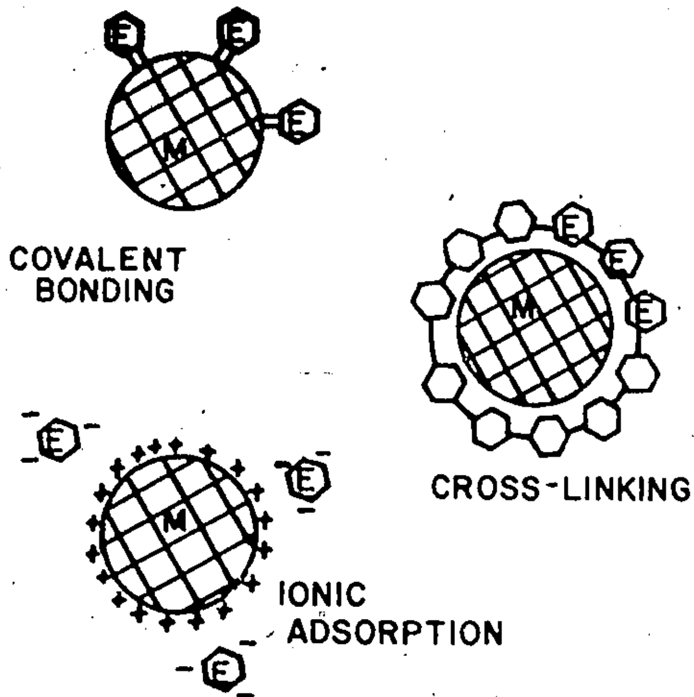
When molecules are linked to a support, some conformational changes which affect enzyme stability will occur. In fact, a preparation of immobilized enzymes can be quite heterogeneous in conformation thereby affecting experimental results. This effect, however, may be resolved by immobilizing an enzyme in such a way so that it will not interact with the support either by adsorption or chemically - i.e. by physical methods (Martinek and Mozhaev, 1985).

Physical methods include entrapment of enzymes in hollow or spun fibres and within an insoluble gel matrix or a microcapsule. An emulsion of an aqueous enzyme solution and a fibre-forming polymer is spun into

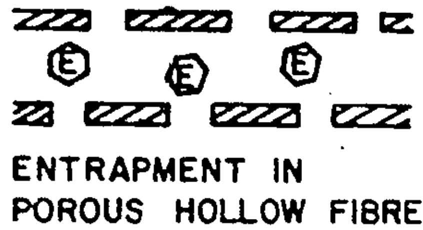
a hardening or coagulation bath forming fibre-entrapped enzymes. The polymerization of a solution of enzymes and monomers yields an immobilized enzyme within a gel matrix while interfacial polymerization in an organic phase containing enzymes produces microencapsulated enzymes. No chemical modification is involved and enzymes are better protected from microbial attack and denaturation. However, diffusion of substrate and products becomes important and some steps during polymerization could result in enzyme deactivation.

Schematic diagrams of these immobilization techniques are presented in Figure 5. These methods are also compared for cost and activity in Table 2.

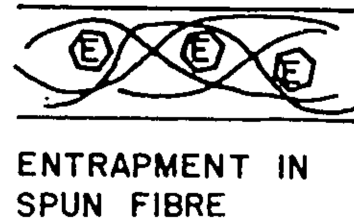
These immobilization techniques can be applied to either free enzymes or whole cells containing enzymes. While cost is reduced in the immobilization of whole cells because it does not require isolation or purification of the enzymes, the immobilized cell is not as stable as immobilized enzymes due to cell lysis. The best method for whole cell immobilization is gel entrapment because the lyzed enzyme remains within the matrix.



a) CHEMICAL METHODS

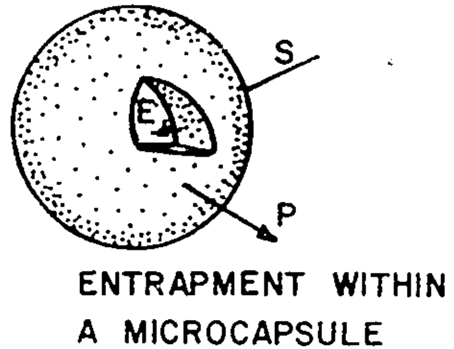


ENTRAPMENT IN POROUS HOLLOW FIBRE



ENTRAPMENT IN SPUN FIBRE

ENTRAPMENT WITHIN INSOLUBLE GEL MATRIX



ENTRAPMENT WITHIN A MICROCAPSULE

b) PHYSICAL METHODS

Figure 5: A summary of techniques of enzyme immobilization by a) chemical methods and b) physical methods. (Bailey and Ollis, 1977)

Table 2: Expected properties of immobilized glucose isomerase prepared by various procedures .

(Antrim et al, 1979)

IMMOBILIZATION PROCEDURE	EXPRESSED ACTIVITY*	POTENCY**	CARRIER COST	REUSABILITY OF CARRIER
Immobilized in bacterial cells	high	average	low	no
Adsorption on insoluble carrier	high to low	high	average to high	yes
Entrapped in insoluble matrix	low	average to low	high	no
Covalently bound to insoluble carrier	low	low	high	no

* $\frac{\text{Activity observed}}{\text{Activity bound}} \times 100$

** $\frac{\text{Activity}}{\text{weight}}$ or $\frac{\text{Activity}}{\text{volume}}$

2.3.2 Stabilization

Increased stability of the biocatalyst due to immobilization may occur depending on the technique used and the success of that technique. For example, entrapment of enzymes provides mechanical resistance.

Inactivation of enzymes occurs when enzymes are heated or exposed to denaturing agents. The mechanism of this inactivation is still not fully understood though the general consensus is that there are two steps involved. The first step is a reversible thermal denaturation (partial unfolding), followed by an irreversible (conformational or chemical) reaction (Zale and Klibanov, 1983).

Zale and Klibanov (1983) found that more stability against reversible denaturation will give an enzyme greater stability against irreversible denaturation so long as it is not exposed to very high temperatures. Bonding of the enzyme offers a constraint against partial unfolding in the molecule (Klibanov, 1983). This is illustrated in Figure 6.

One of the most interesting properties of immobilized enzymes is that of the formation of a micro-environment. Immobilization of enzymes with charged carriers can result in a shift in pH at the carrier surface. For example, the use of a polycationic carrier causes a local repulsion of hydrogen ions at the support surface resulting in a higher pH at the surface than in the bulk solution. Thus there is a shift in the optimum operational pH allowing the immobilized enzyme to function in more acidic solutions. The opposite occurs when an enzyme is immobilized in a negatively charged support. Shifts in pH-activity optima can be of 1 - 2.5 pH units. This electrostatic effect can also affect the value of the

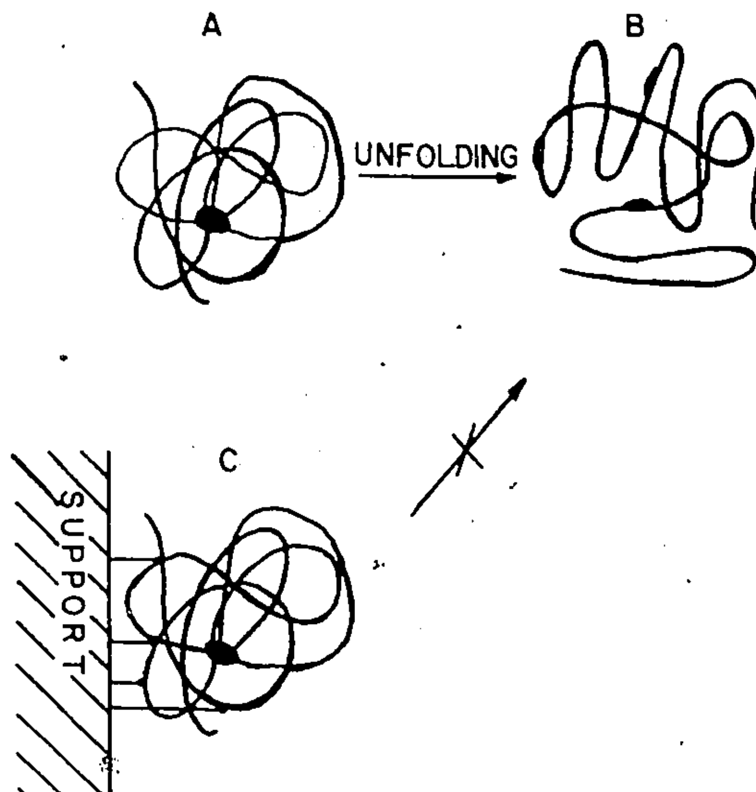


Figure 6: Schematic representation of the thermal unfolding of an enzyme. (A→B). For the enzyme attached by many links to a solid support (C), the unfolding becomes greatly hindered. The filled region depicts the enzyme's active centre. (Klibanov, 1983)

Michaelis constant with changes of more than an order of magnitude being reported (Bernath et al, 1977; Martinek and Mozhaev, 1985).

2.3.3 Glucose isomerase

The use of immobilized glucose isomerase for the production of HFCS is the most important commercial application of immobilized enzyme technology.

Glucose isomerase is an ideal enzyme for immobilization for a variety of reasons including:

1. Glucose isomerase is an intracellular enzyme.
2. On a weight basis, the amount of enzyme produced is considerably smaller than the amount of bacterial cells. Recovery of enzyme for reuse would be of value economically.
3. The enzyme is stable at relatively high temperatures.
4. The substrate and product are small molecules i.e. no diffusional problems.

Thus this enzyme has frequently been used in general immobilization studies.

The simultaneous use of glucose isomerase during cellulose hydrolysis would be enhanced by the formation of a micro-environment at the surface of a polycationic carrier. A possible shift in the pH optimum of the glucose isomerase towards the optimal operating pH of cellulose hydrolysis and a decrease in the value of the Michaelis constant for the conversion of glucose to fructose would result in a more efficient cellulose hydrolysis.

Chapter III

METHODOLOGY

3.1 Glucose isomerase

3.1.1 Chemicals

Lactobacilli MRS broth, Bacto-agar, proteose peptone, bacto yeast extract, lactalbumin hydrolysate, and dehydrated tomato juice broth were Difco Laboratories products.

3.1.2 Microorganism

The microorganism used in this study for the production of glucose isomerase was Lactobacillus brevis NCDO 474 obtained from the American Type Culture Collection (ATCC 8287). The bacterial cultures were maintained on agar slants made with Lactobacilli MRS broth (ATCC Medium 416). These slants were kept at room temperature and renewed biweekly.

3.1.3 Inoculum

The medium for the preparation of the inoculum was prepared, based on that of Kent and Emery (1973), with 1.0% proteose peptone, 1.0% lactalbumin hydrolysate, and 1.6% dehydrated tomato juice broth.

The pH was adjusted to 5.5 with 4N HCl and sterilized for 20 minutes at 121 °C and 15 psi. A spore suspension was made by suspending the spores from one of the slants in 3 ml of the same medium. After adding the spore suspension to the inoculum, it was inoculated at 30 °C or 37 °C for 24 hours.

3.1.4 Fermentation

Lactobacillus brevis has not been exploited commercially for the production of glucose isomerase due to the enzyme's relatively low optimum temperature. Consequently, there is a shortage of research papers on this particular microorganism. The most prominent of the few researchers is Yamanaka (1961, 1963a, 1963b, 1968) who found maximum glucose isomerase activity after 16 hours of incubation statically at 37°C. Kent and Emery (1973), who based their work on Yamanaka, incubated the bacteria at 30 °C with mild agitation to obtain maximum glucose isomerase activity at 21 hours.

Yamanaka (1963a) reported a much more gradual increase and then decrease in activity around 16 hours of incubation while Kent and Emery reported a very distinct maximum in activity at 21 hours. Also, Yamanaka's activity maximum corresponded to an optical density maximum while that of Kent and Emery was found to occur early in the retardation phase of cell growth.

These two research groups also differed with the fermentation media used. The same components were used but in different concentrations. The composition of Yamanaka's medium (Yamanaka, 1963a) and the modified Yamanaka's medium (Kent and Emery, 1973) is detailed in Table 3.

This disparity in the available literature therefore necessitated

Table 3: Comparison of the two growth media for *Lactobacillus brevis*

YAMANAKA'S MEDIUM (Yamanaka, 1963a) (%w/v)		MODIFIED YAMANAKA'S MEDIUM (Kent and Emery, 1973) (%w/v)
	BASAL SOLUTION	
1·0	Proteose peptone	5·0
1·0	Yeast extract	3·0
1·0	Sodium acetate crystal	1·0
0·03	$MnSO_4 \cdot H_2O$	0·03
0·01	$MgSO_4 \cdot 7H_2O$	0·01
0·012	$CoSO_4 \cdot 7H_2O$	0·012
	SUGAR SOLUTION	
1·0	Xylose	2·0
0·1	Glucose	0·5

investigations into the composition of the growth medium and the incubation temperature for fermentation of Lactobacillus brevis to produce glucose isomerase.

The basal and sugar solutions were prepared on the basis of total volume of the media. The solutions were autoclaved separately and mixed before inoculation with 5% inoculum. Incubation of the medium took place in a 37 °C water bath or in shake-flasks at 30 °C. When the cells were harvested they were first cooled in an ice-water bath to stop growth rapidly and then centrifuged at 4000 rpm for 10 minutes.

The effects of incubation temperature, the duration of incubation, and the composition of the growth medium on the growth of Lactobacillus brevis and on the production and activity of glucose isomerase were investigated.

3.1.5 Enzyme extraction

Glucose isomerase is produced as an intracellular enzyme in Lactobacillus brevis.

Yamanaka (1961) and Kent and Emery (1973) prepared cell-free enzyme extracts using tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.0). Yamanaka used 0.05M Tris buffer at 6°C while Kent and Emery used heat autolysis for enzyme extraction with 0.02M Tris buffer at 40 °C. However, Tris buffer has been documented as an inhibitor of glucose isomerase from Streptomyces flavovirens and S. phaeochromogenes (Chen, 1980b) and of xylose isomerase from Bacillus coagulans (Antrim et al, 1979).

Slein (1955) was investigating the effect of pH on xylose isomerase from Pasteurella pestis when he discovered that Tris buffer strongly inhibited the enzyme. Slein proposes a non-competitive mode of inhibition. Antrim et al (1979) suggest avoiding the use of this buffer for glucose isomerase.

Chen (1980a) suggested that glucose isomerase can be easily extracted by cell autolysis with cationic detergents yielding the same amount of glucose isomerase as mechanical disruption but with a higher specific activity.

The method of heat autolysis used by Kent and Emery (1973) was used for enzyme extraction. Centrifuged and washed cells from 100 ml of medium were resuspended in 2.4 ml of 0.05M Tris buffer, 0.05M phosphate buffer, or 0.1% (w/v) cetylpyridinium chloride in 0.05M phosphate buffer. Cell lysis occurred with incubation at 40 °C, 100 rpm for 16 hours, followed by centrifugation at 4000 rpm. Aside from that of centrifugation, all speeds refer to the rotational speed of the temperature-controlled shaker used in this research. The cell debris was discarded and the supernatant was the enzyme solution used in the enzyme activity assays and in investigations into the properties of glucose isomerase.

The different lyzing agents used in the enzyme extraction step were compared for their effects on protein release and enzyme activity.

Yamanaka (1963b) and Kent and Emery (1973) purified the extracted enzyme using very involved techniques. The purification technique used by Yamanaka is outlined in Appendix B. Purification, however, was beyond the scope of this work and all experiments were carried out with the crude extracts.

3.1.6 Properties of glucose isomerase

The dependence of glucose isomerase activity on pH and substrate was determined using the same composition as indicated in the enzyme activity assay of Table 4. Values of pH between 5.0 and 7.0 were obtained using mixtures of phosphate and citrate buffers. Solutions of 0.5, 1.0, and 2.0M glucose were used in the studies of the effect of substrate concentration on enzyme activity. To determine the necessity for enzyme extraction, the effects of pH and substrate concentration were also studied for cellular enzymes resuspended in the appropriate buffer volumes. In the Literature Survey, cell-bound enzymes are described as one of the simplest immobilization techniques. The "cellular enzymes" used in this research were simply the harvested bacterial cells. They were used to illustrate the effect of enzyme immobilization.

The stability of the enzyme was measured by incubating the enzyme solution with the metallic ion solution and buffer at 50 °C, 150 rpm for 0, 1, and 24 hours. After incubation, glucose was added and the isomerization reaction was allowed to proceed for 1 hour.

The concentration of fructose at equilibrium isomerization was also determined. Samples were taken at various times in a 48 hour period to determine the time required to reach equilibrium isomerization.

3.1.7 Analytical procedures

3.1.7.1 Cell growth

The extent of cell growth was monitored by measuring the optical density and the total dry weight. Optical densities of culture samples, diluted tenfold, were measured by the absorbance at 530 nm on a Turner Spectrophotometer, Model 380. The total dry weight of the bacteria was determined from a 30 ml sample which was centrifuged at 4,000 rpm for 15 minutes. The cells were resuspended in distilled water and recentrifuged. The cells were then dried at 80 °C to constant weight (4 hours).

3.1.7.2 Protein content

The protein content of the crude extracts was determined using the Biuret method (see Appendix C). It should be noted that this method measures the concentration of all proteins in solution not just the concentration of glucose isomerase.

3.1.7.3 Activity

The activity of glucose isomerase was determined using the enzyme activity assay described by Kent and Emery (1973) and Yamanaka (1963a). This method is outlined in Table 4. Fructose was analyzed by the cysteine-carbazole method by Yamanaka (1963, 1968) and Kent and Emery (1973) and also by the resorcinol method (Kent and Emery, 1973). These colorimetric analyses report that the effect of glucose, when in concentrations about equal to the concentration of fructose

present, is negligible. However, with the high concentration of glucose used in the enzyme activity assay (2M), this effect becomes quite substantial and therefore is no longer an accurate measure of the fructose present (Yaphe and Arsenault, 1965). Therefore, the concentration of fructose produced in the enzyme activity assay was determined by means of High Performance Liquid Chromatography. One unit of activity was defined as that which produces one micromole of fructose from glucose in 30 minutes at 50°C and pH 7.0.

Table 4: Enzyme activity assay

(Kent and Emery, 1973; Yamanaka, 1963a)

A solution consisting of

2.4 ml Enzyme solution

0.6 ml 10^{-2}M $MnSO_4$, 10^{-3}M $CoSO_4$ solution

and 1.0 ml buffer (pH 7.0) (0.05M phosphate or 0.05M Tris buffer) was incubated at 50°C, 150 rpm for 10 minutes,

after which

2.0 ml 2M glucose

was added and incubated at 50°C, 150 rpm for 60 minutes.

A 1.0 ml sample of the reaction mixture was pipetted into

0.067 ml 10% (w/v) trichloroacetic acid

to coagulate the proteins. The protein was allowed to settle overnight in the refrigerator. The supernatant was analyzed for fructose.

The enzyme activity was calculated as follows:

$$U/ml = \frac{\text{Fructose concentration(mg/ml)} \times DF}{0.180 \text{ mg/umol} \times 2} \qquad DF = \frac{6 \text{ ml}}{2.4 \text{ ml}} = 2.5$$

where DF=Dilution Factor.

3.1.7.4 HPLC analysis

High Performance Liquid Chromatography was used to measure the concentrations of sugars in solution. This analysis was made with a Waters Associates ALC-200 system with a Model 600A Solvent Delivery System, a Model U6K Universal Liquid Chromatograph Injector, and a Model R401 Differential Refractometer. A Brownlee Labs H2-GU Amino guard column protected the Amino Spheri-10 H2-10A 4.6 mm x 25 cm column. Sugar peaks and retention times were recorded and peak areas calculated by a Hewlett-Packard 3380A Integrator. This recorder's capability of log attenuation was especially advantageous with the high glucose concentration (a 2M glucose solution was used in the enzyme activity assay) and the lower fructose concentration.

The carrier solvent was a mixture of acetonitrile and water (80:20) filtered through a 0.45 micron Millipore filter. After filtration, the solvent was put in a sonic bath for 1 hour and then allowed to equilibrate overnight before use.

Because of the similarity between the molecular structures of glucose and fructose, their retention times in the column were also similar. It was therefore necessary to operate the column at a flow rate of 1.0 ml/min to achieve satisfactory separation of the two sugars.

All samples were filtered prior to injection through Acro LC13 (Gelman Sciences) 0.45 micron filters. Sample volumes of 20 microlitres were injected in the HPLC using a high precision syringe (Hamilton 802). The HPLC was calibrated by injecting solutions of glucose and fructose of known concentrations. Included in the standards were mixtures of

fructose and glucose of approximately the same concentrations of the samples being analyzed. Samples of the output from the Hewlett-Packard Integrator for standard solutions of glucose and fructose are presented in Appendix D.

3.2 Cellulose hydrolysis

3.2.1 Microorganism

The microorganism used for cellulase production was Trichoderma reesei QM9414 (formerly Trichoderma viride), ATCC 26921, kept on potato dextrose agar slants.

3.2.2 Inoculum

A spore suspension was prepared using 1% (v/v) Tween 80 solution and was transferred, aseptically, to an inoculum containing modified Mandel's medium (Leyva, 1984), trace elements, and 1% lactose as the carbon source. The inoculum was incubated at 28 °C with shaking (200 rpm) for 5 days.

3.2.3 Fermentation

A 5 litre stirred-tank fermentor (New Brunswick Scientific Inc., Model MF-105) was charged with modified Mandel's medium, trace minerals, Tween 80, proteose peptone, and 2.0% Solka Floc SW-40 (Brown Co., Berlin, N.H.) and sterilized before being inoculated. The composition of the growth media is listed in Table 5.

Table 5: Composition of the growth medium for *Trichoderma reesei*

COMPONENT	CONCENTRATION
MINERALS	
$(NH_4)_2SO_4$	4·12 g/l
KH_2PO_4	4·0 g/l
$CaCl_2$	0·2 g/l
$MgSO_4$	0·2 g/l
TRACE ELEMENTS	
$FeSO_4 \cdot 7H_2O$	10·00 mg/l
$MnSO_4 \cdot H_2O$	3·12 mg/l
$ZnSO_4 \cdot H_2O$	2·80 mg/l
$CoSO_4 \cdot H_2O$	8·60 mg/l
ADDITIVES	
Proteose peptone	0·2 %
Tween 80	0·2 %
CARBON SOURCE	
Cellulose	20·0 g/l

The temperature was maintained at 28 °C while the pH was allowed to drop to 3.0 and then controlled at that point with a pH controller unit (NBS Model pH 22). The air flow rate was set at 3.0 litres/minute and mixing was provided by turbine impellers operating at 400 rpm. Dissolved oxygen was monitored using a Dissolved Oxygen electrode (NBS, 900 series) and maintained above 20% of the saturation concentration. Foam was controlled automatically with Antifoam B (Dow Chemicals).

The filter paper activity was determined for samples withdrawn throughout the fermentation and the protein content was measured using the Biuret method. These assay procedures are outlined in Appendix C.

3.2.4 Hydrolysis

Hydrolysis was carried out in shake-flasks with 7% Solka Floc SW-40 and equal parts of buffer and whole broth using a mixing speed of 200 rpm and a temperature of 50 °C. The effect of pH on hydrolysis was studied using citrate and phosphate buffers to obtain the desired pH values of 5.0, 5.5, and 6.0. Hydrolysis was monitored by measuring the concentration of reducing sugars using the DNS method (see Appendix C).

Glucose isomerase requires a relatively high concentration of glucose; therefore, all subsequent hydrolyses were carried out using commercially-prepared cellulase: Novo Enzymes Celluclast 1,5 L prepared from a strain of Trichoderma reesei and cellobiase, Novozym 188, produced by a strain of Aspergillus niger. The suggested enzyme concentration was increased three-fold to produce enough glucose for the glucose isomerization reaction.

Hydrolysis of cellulose was allowed to proceed for 24 hours before the addition of crude extracts of glucose isomerase and the appropriate amount of 10^{-2} M $MnSO_4$, 10^{-3} M $CoSO_4$ solution (according to the proportions in the enzyme activity assay). The hydrolysis was allowed to proceed for 3 hours.

All hydrolysis reactions were stopped by immersion of the shake-flasks in a boiling-water bath for 5 minutes. Remaining cellulose was filtered from the hydrolysate and samples were kept for reducing sugar analysis.

Chapter IV

RESULTS AND DISCUSSION

4.1 Glucose isomerase

There were a number of aspects concerning the production of an active enzyme solution which required attention. The final overall procedure that was established as a result of these investigations is presented in Chapter V of this report. Therefore, unless otherwise stated, the procedure followed was as according to Figure 19.

4.1.1 Fermentation

4.1.1.1 Cell growth

Growth of *Lactobacillus brevis* was found to be dependent on incubation temperature and medium composition. As suggested in the ATCC catalogue, cell growth was greater at 30°C than at 37 °C. However, the composition of the growth medium was found to have the greatest effect on cell growth with the modified Yamanaka's medium (see Table 3 for composition of growth media) enhancing growth at either temperature by approximately 75%.

The growth phases were also found to be dependent on the medium composition. The lag phases lasted for 4 hours and 6 hours for Yamanaka's and modified Yamanaka's media respectively. With Yamanaka's medi-

um the exponential cell growth phase lasted for 8 hours with a maximum cell concentration at 12 hours while with the modified Yamanaka's medium this phase was 10 hours long with a maximum at 16 hours of fermentation.

These phases and the effect of temperature and medium composition on the total dry weight of cells and the optical density are depicted in Figure 7 and Figure 8. Very good agreement exists between the total dry weight and optical density measurements (correlation coefficients were calculated by linear regression and are presented with the raw data in Tables 9-12 in Appendix F).

The pH of the modified Yamanaka's medium was found to decrease during the course of fermentation as illustrated in Figure 9. The greatest decrease in pH occurred after 6 hours of fermentation corresponding to a sharp increase in cell growth (Figure 7 and Figure 8). The pH starts to level off after 16 hours of fermentation, again corresponding to the levelling off of cell growth.

This decrease in pH can be attributed to the fact that Lactobacillus brevis is a heterofermentative bacteria which converts glucose to a mixture of lactic acid, ethanol, and carbon dioxide in the molar ratio of 1:1:1 and xylose to lactic acid and ethanol through the hexose monophosphate shunt pathway (Stanier et al, 1970). It is interesting to note that the change in pH of the fermentation medium during the course of the fermentation was very similar for growth at 30 °C and 37 °C even though cell growth was greater at 30°C.

In Appendix A, it is mentioned that growth at higher values of pH could result in an extension of the lag phase or a reduction in the total

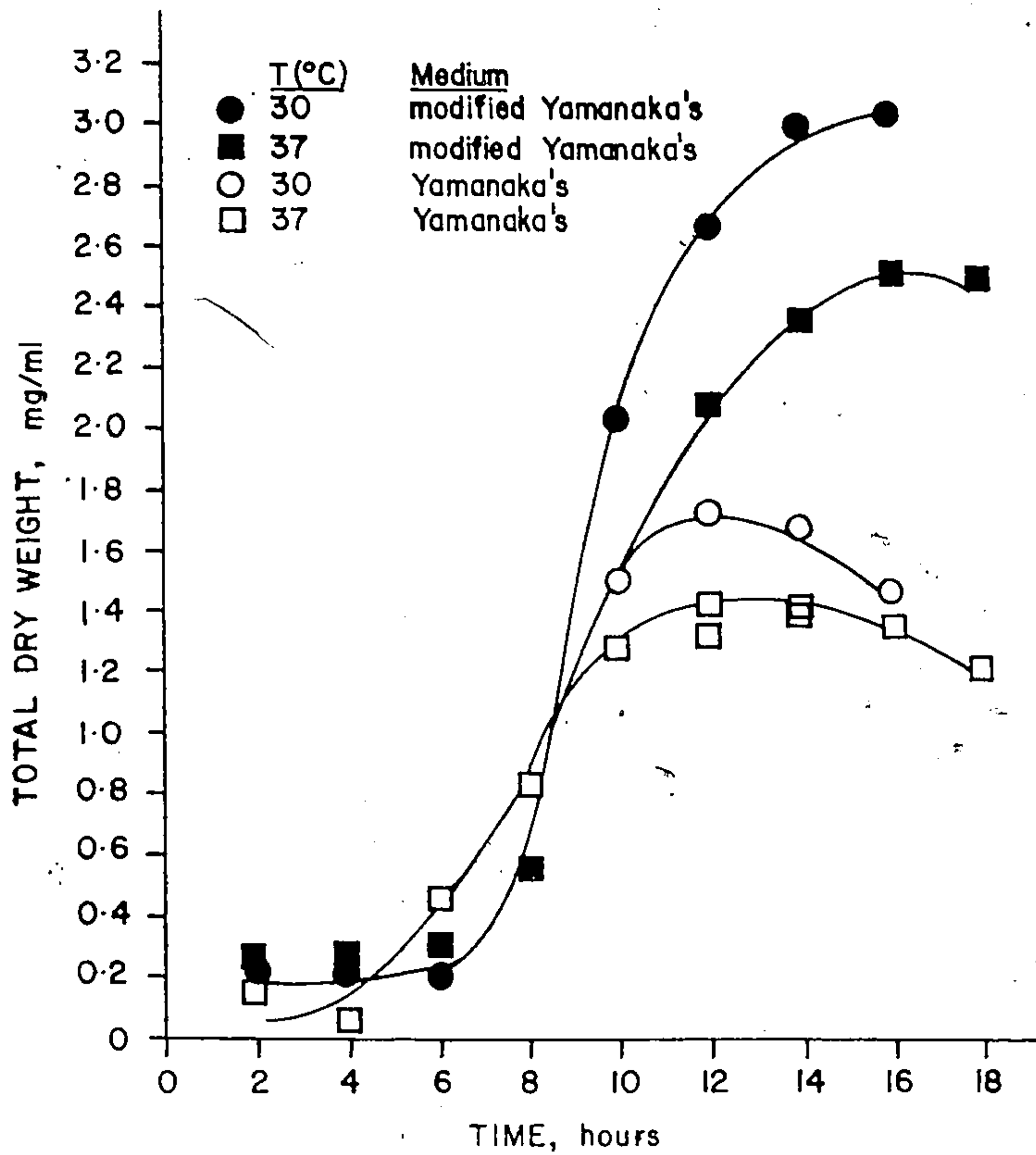


Figure 7: Effect of medium composition and incubation temperature on the total dry weight of cells

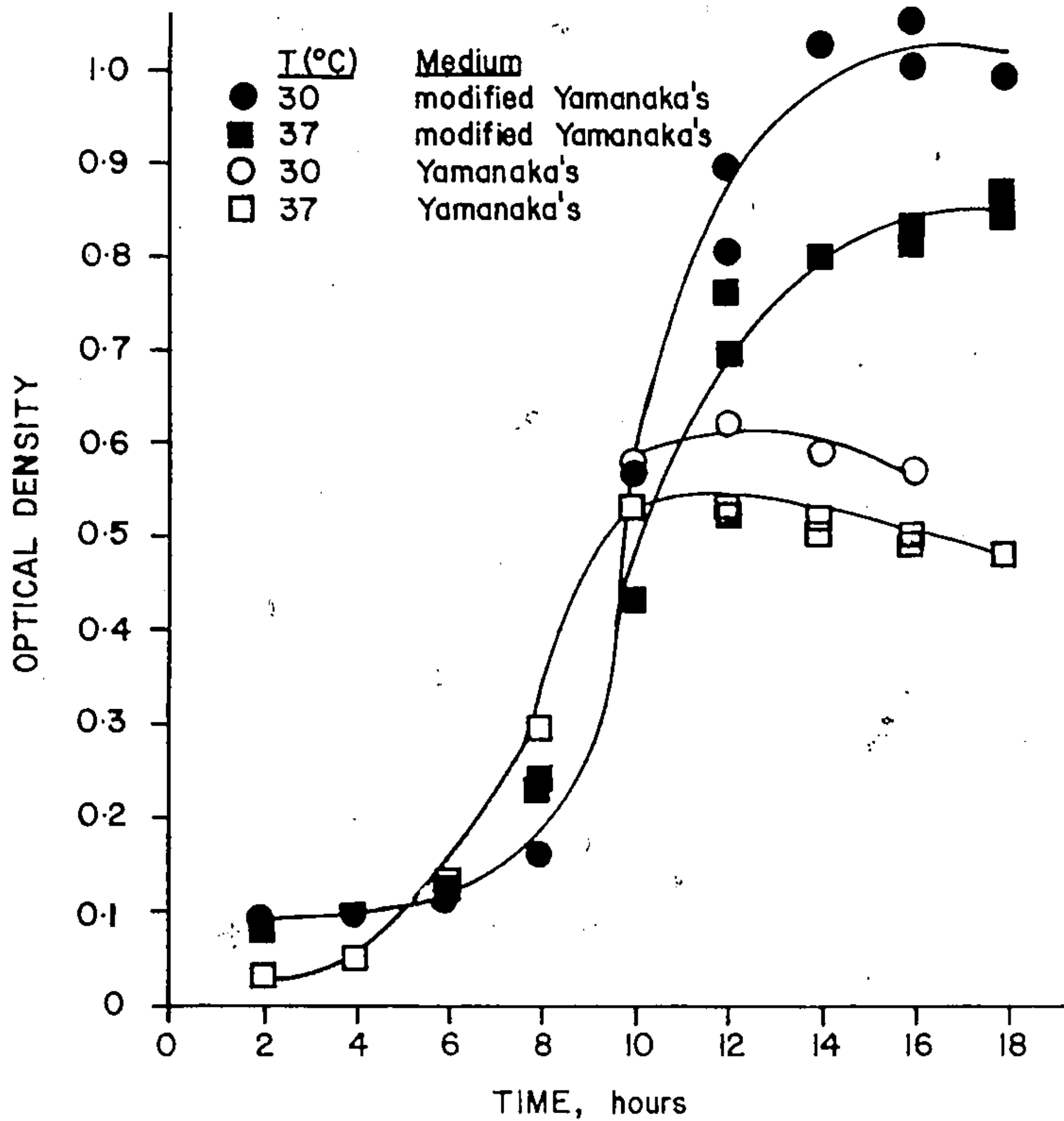


Figure 8: Effect of medium composition and incubation temperature on the optical density of the medium

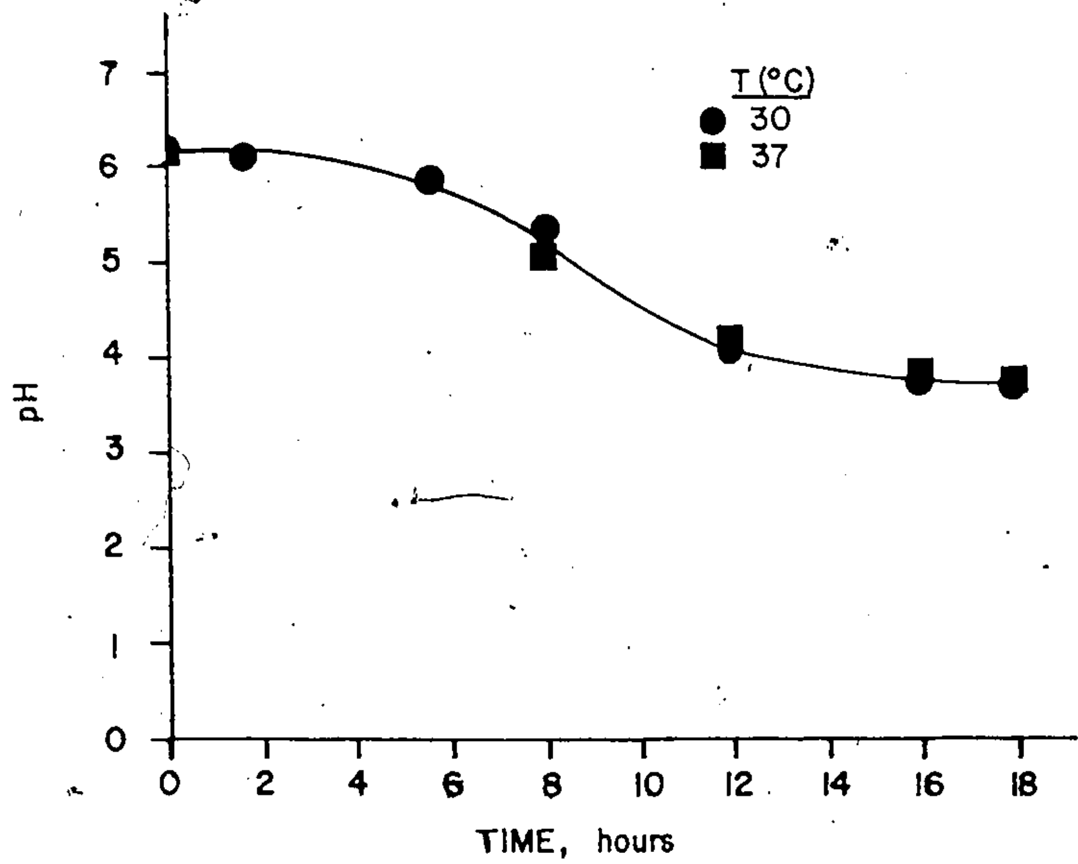


Figure 9: The change in pH during the course of fermentation with the modified Yamanaka's medium

growth yield. This, however, could not be the cause of either the longer lag phase with the modified Yamanaka's medium or the lower growth experienced with Yamanaka's medium because the initial pH values of the media were essentially equal: pH 6.21 for Yamanaka's medium and pH 6.19 for the modified medium.

4.1.1.2 Enzyme production

As with cell growth, the protein content of cell extracts was greatest for growth at 30 °C with the modified Yamanaka's medium (see Figure 10). The first maxima of protein content occurred approximately 4 hours before the cell concentration maxima. The protein content then increased with declining growth.

Though the exact concentration of glucose isomerase cannot be found with the protein content assay, the protein content of cell extracts is useful for comparing enzyme extraction methods and for comparing the specific activity of glucose isomerase.

Figure 11 illustrates the dependence of enzyme activity of glucose isomerase on the growth temperature and medium. Once again, the greatest activity was obtained by incubation at 30 °C with the modified Yamanaka's medium. However, the difference between the maximum enzyme activity was not as great as for other measurements made for the other three sets of operating conditions.

As previously mentioned, Yamanaka (1963a) achieved a maximum in enzyme activity at 16 hours of fermentation while Kent and Emery (1973) obtained it at 21 hours. The corresponding maxima for this work occurred at 12 and 16 hours respectively. Maximum enzyme activities

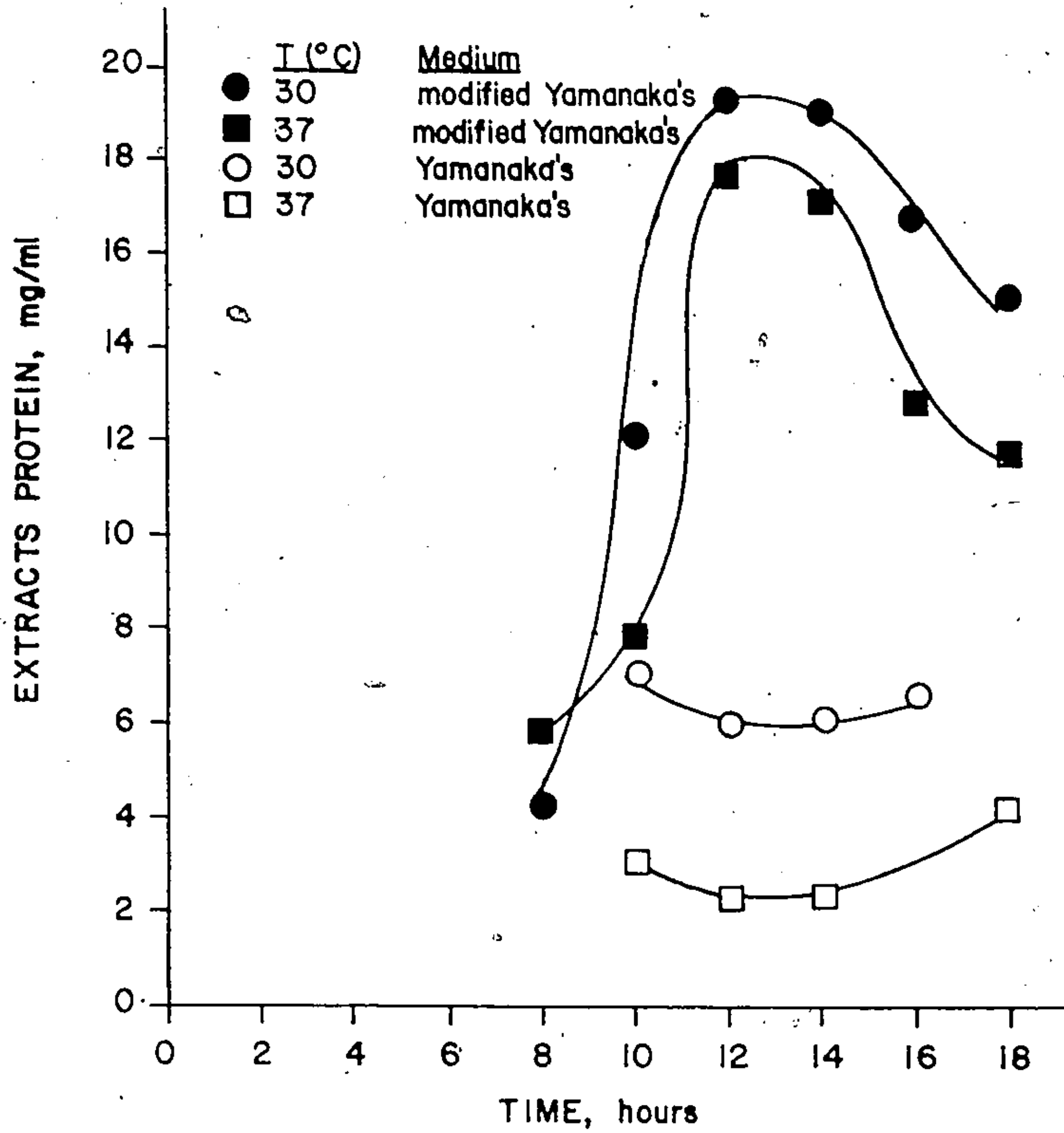


Figure 10: Profile of protein production during fermentation and the effect of medium composition and incubation temperature. Crude extracts were prepared with phosphate buffer.

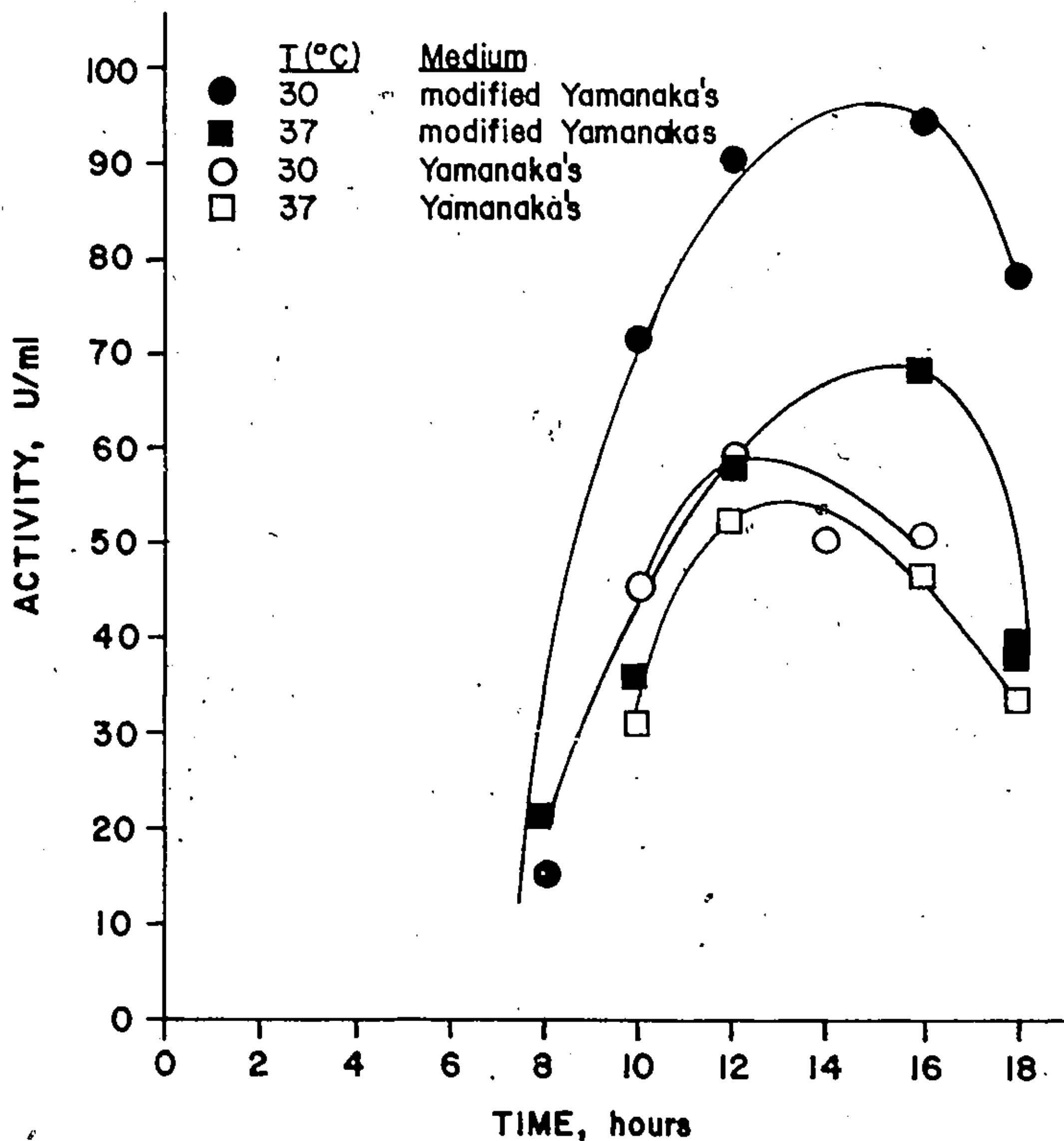


Figure 11: The dependence of glucose isomerase activity on medium composition and incubation temperature. Crude extracts were prepared with phosphate buffer.

corresponded to the maxima in cell growth regardless of the medium composition and incubation temperature. These results are in agreement with those of Yamanaka (1963a), who found maximum enzyme activity during the period of maximum cell growth, but not with those of Kent and Emery (1973), whose findings indicated a maximum early in the retardation phase of cell growth. Also, the more gradual increase and decrease in enzyme activity about the maximum is consistent with the results of Yamanaka but conflicts with those of Kent and Emery.

It is interesting to compare the specific enzyme activity data for different operating conditions (see Figure 12). By far, the greatest specific activity was for fermentation at 37 °C with Yamanaka's medium with a value of 22.7 U/mg protein at 12 hours, compared with 5.6 U/mg protein at 16 hours incubation at 30 °C with the modified medium. Thus, while more enzyme was produced by growth of *Lactobacillus brevis* at 30 °C with the modified Yamanaka's medium, the glucose isomerase produced by the growth of *Lactobacillus brevis* at 37 °C with Yamanaka's medium was probably of better quality.

Production of *Lactobacillus brevis* and glucose isomerase was dependent on the medium composition and incubation temperature. Cell growth, protein content, and enzyme activity were greatest for incubation at 30 °C with the modified Yamanaka's medium. Maximum enzyme activity occurred at 16 hours of fermentation in contrast with the 21 hours reported by Kent and Emery (1973). A comparison of the enzyme activity profile and the cell growth curves indicated that glucose isomerase production is growth-associated. This result confirmed that of Yamanaka (1963a). Specific activity was greatest for incubation at 37 °C

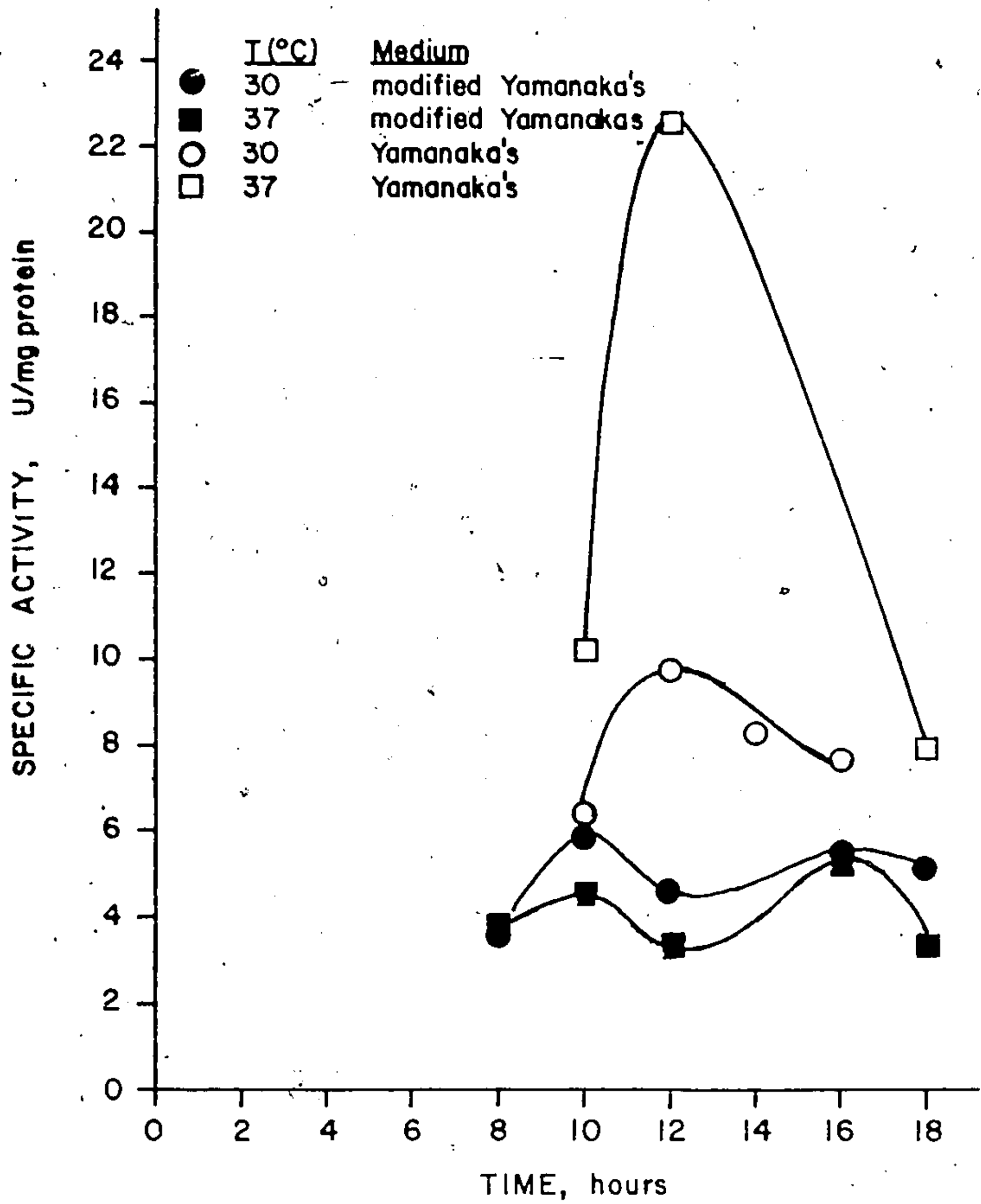


Figure 12: Comparison of the specific enzyme activity data for different operating conditions. Crude extracts were prepared with phosphate buffer.

with Yamanaka's medium; however, fermentation at 30 °C with the modified medium seems to be the most economically viable operation.

The composition of the growth medium was found to have the greatest effect on cell growth. The ingredients of each of the media were the same but the concentrations were different. Using the analysis of proteose peptone and yeast extract (presented in Appendix E), the carbon to nitrogen ratio for the modified Yamanaka's medium was calculated to be 1.1. The C:N ratio for Yamanaka's medium was 2.2; double that of the modified medium.

4.1.2 Enzyme extraction

Of the number of mechanical, physical, and chemical techniques developed for the extraction of intracellular enzymes, the most commonly used technique for large-scale disruption of microorganisms is mechanical. Mechanical disruption of cell walls, however, is energy-intensive and requires high capital investment. Also, to prevent protein denaturation or enzyme inactivation due to the high conversion of energy input to heat, adequate cooling is required. The cost of mechanical disruption techniques, therefore, account for a significant portion of the total production costs (Engler, 1985).

There are a number of non-mechanical methods for cell wall disruption that are, at present, suitable only for laboratory use. Chemical treatment and autolysis, however, are being used industrially to some degree. These methods are particularly promising as a low cost cell disruption technique.

The three lyzing agents used for heat autolysis - 0.02 M tris(hydroxymethyl)aminomethane, 0.05 M phosphate buffer, and 0.1% (w/v) cetylpyridinium chloride in 0.05 M phosphate buffer - varied to some degree with respect to the protein released during the extraction but showed greater variation in their effect on enzyme activity. Data for extracted protein, enzyme activity, and specific enzyme activity for each lyzing agent is presented in Table 6. The initial enzyme activity for the Tris buffer and cetylpyridinium chloride solution extracts was reduced to 24% and 12%, respectively, of that obtained with the phosphate buffer.

Figure 13 illustrates the effect of the lyzing agents on the amount of fructose produced when the isomerization reaction is allowed to continue. While the enzyme extracted with the Tris buffer appears to recover somewhat from the inhibition exhibited early in the isomerization, the cetylpyridinium chloride solution-extracted enzyme does not seem to overcome the inhibition. Thus the inhibition exhibited by Tris buffer is, to a certain degree, reversible while that shown by cetylpyridinium chloride is irreversible. Activity of the glucose isomerase extracted by Tris buffer was recovered to 77% of that of the phosphate-extracted enzyme.

Cellular enzyme was used to determine the effect of Tris buffer when used as a buffer in the enzyme activity assay so as not to confuse this result with the effect of the enzyme extraction buffer. When phosphate buffer was used in the enzyme activity assay, the cellular enzyme demonstrated an activity of 33.0 U/ml. A negligible amount of fructose was produced however, when the Tris buffer was used in the assay.

Table 6: Comparison of the effect of lyzing agent on extracted protein and enzyme activity

Conditions for growth of Lactobacillus brevis: 30°C, modified Yamanaka's medium.

LYZING AGENT	PROTEIN EXTRACTED (mg/ml)	ENZYME ACTIVITY	
		(U/ml)	(U/mg)
0·02 M Tris(hydroxymethyl) aminomethane buffer	12·8	27·2	2·1
0·05 M Phosphate buffer	14·7	112·6	7·7
0·1% (w/v) cetylpyridinium chloride	10·8	13·3	1·2

In further enzyme extractions and enzyme activity assays therefore, 0.05 M phosphate buffer was used instead of the Tris buffer suggested by Kent and Emery (1973) and Yamanaka (1961, 1963a).

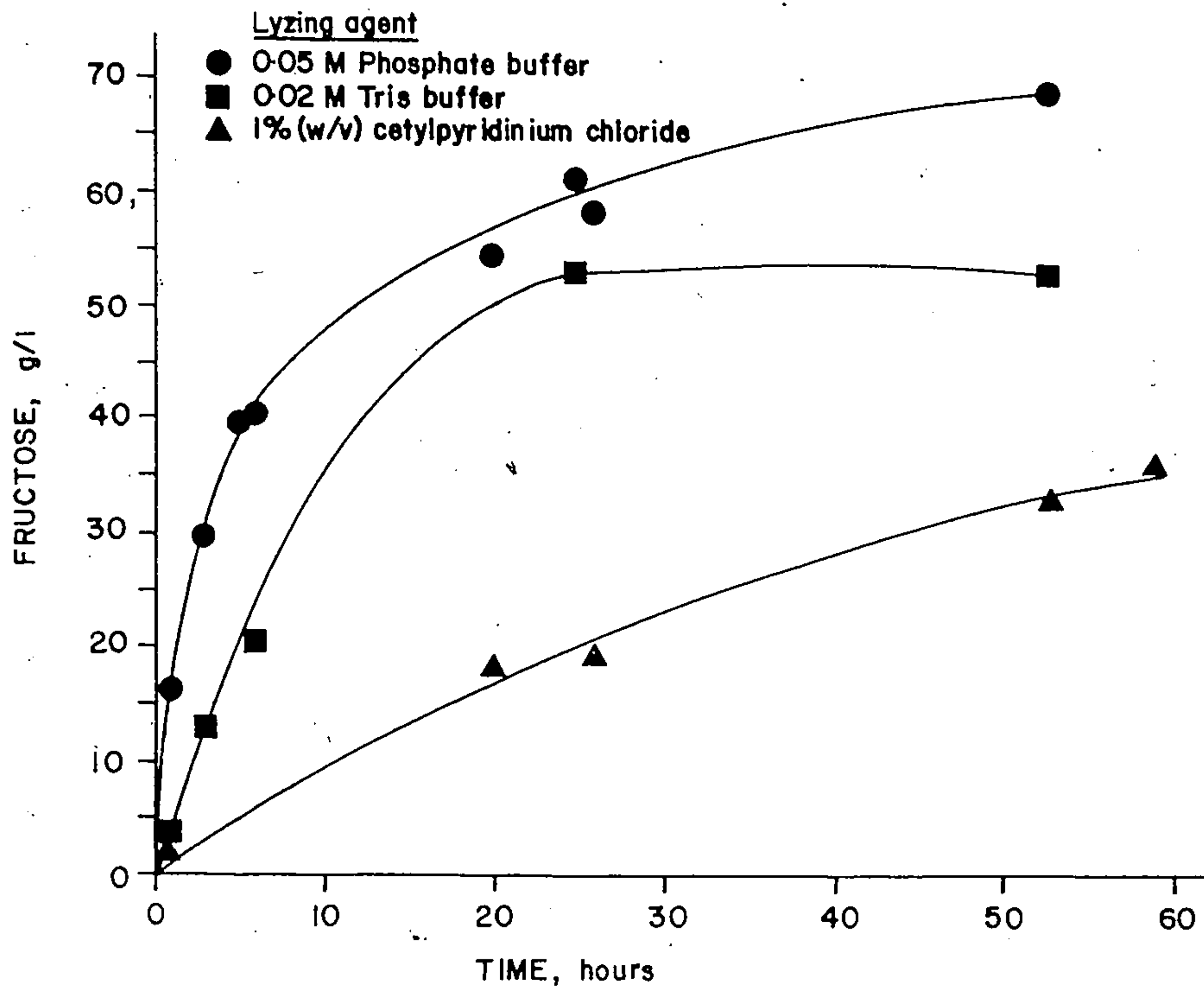


Figure 13: Effect of the lyzing agent on the production of fructose during the extended isomerization. Conditions for growth of *Lactobacillus brevis*: 30°C, modified Yamanaka's medium.

4.1.3 Properties of glucose isomerase

4.1.3.1 Effect of pH on glucose isomerase activity

Figure 14 illustrates the effect of pH on the glucose isomerizing activity of both extracted and cellular enzymes. The pH range for activity for the extracted enzyme was from 5.5 to 7.0 with maximum activity occurring at pH 6.5. The cellular enzyme however, had very little activity from pH 5.0 to 6.5 showing greatest activity at pH 7.0 for the range of pH examined. The pH optimum for the cellular enzyme may have actually been greater than 7.0 but was outside the range of interest in this work.

The broad range of enzyme stability with respect to pH and particularly the high activity found at pH 5.5 was encouraging for glucose isomerization during cellulose hydrolysis.

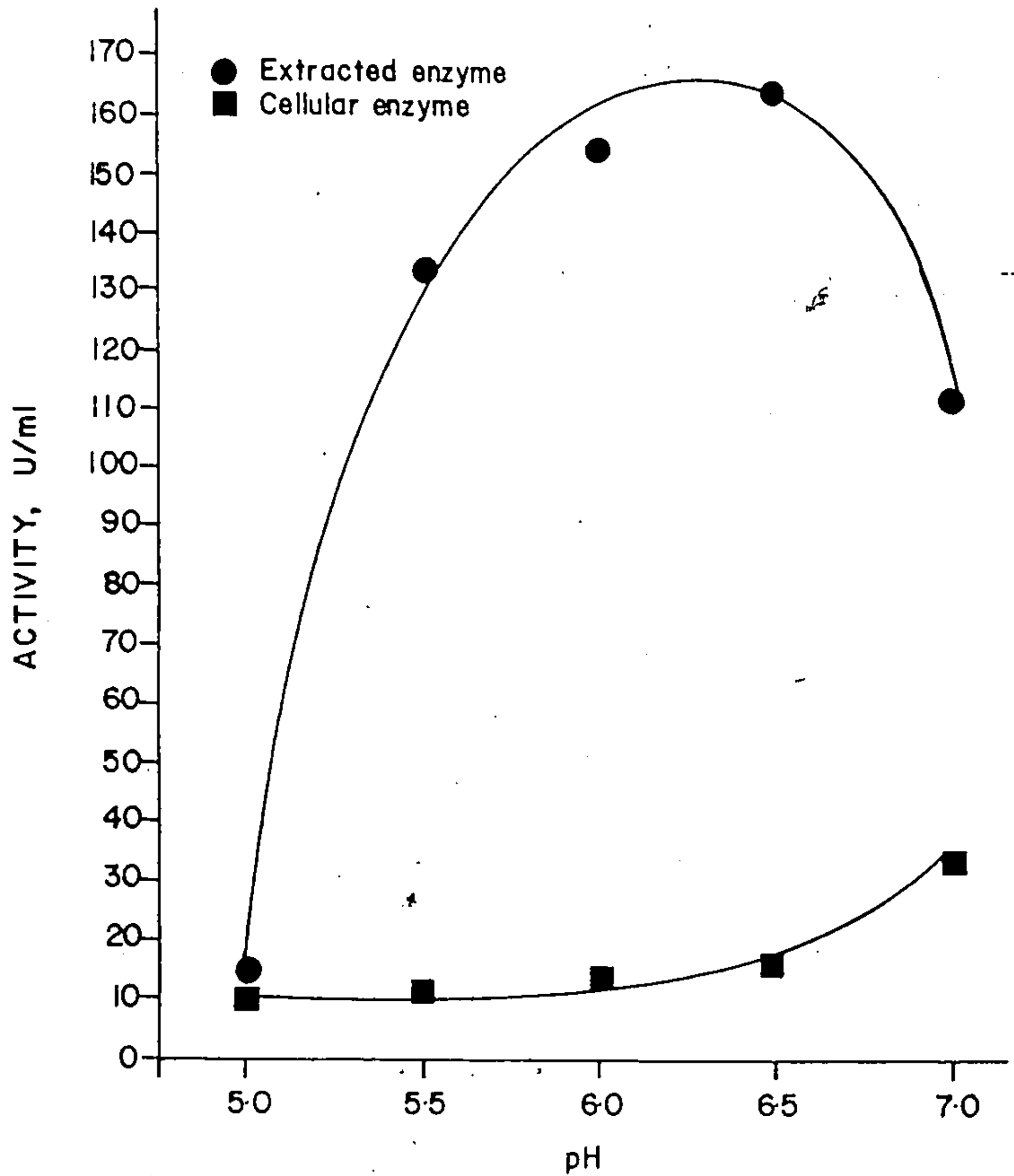


Figure 14: Effect of pH on glucose isomerase activity for extracted and cellular enzymes. Conditions for growth of *Lactobacillus brevis*: 30°C, modified Yamanaka's medium. Crude extracts were prepared with phosphate buffer.

4.1.3.2 Effect of substrate concentration on glucose isomerase activity

Michaelis-Menten kinetics are used in the treatment of enzyme kinetics in this report. The intention is not to make a rigorous treatment of the data but rather to obtain an indication of the enzyme properties.

The effect of the glucose concentration on enzyme activity for extracted and cellular enzymes was used for the determination of the Michaelis constant for each enzyme. The value of this constant was 1.29, and 0.87 M, for the cellular, and extracted enzyme, respectively. The values of the Michaelis constants were determined graphically using the Lineweaver-Burk plot shown in Figure 15. The relatively high values of these constants indicate the high concentration of substrate required for the isomerization reaction.

This requirement is a drawback in the isomerization of glucose during cellulose hydrolysis because a high concentration of glucose is very inhibitory towards the cellulase complex. As discussed in the Literature Survey, however, immobilization and the subsequent formation of a micro-environment could shift the pH optimum closer to pH 5.0 and more importantly cause a change in the Michaelis constant of more than an order of magnitude. The effect of the formation of a micro-environment is illustrated in the cellular enzyme activity assays. Although the shift in the pH optimum and the higher value of the Michaelis constant with the cellular enzyme were not favourable changes for the conversion of glucose to fructose during cellulose hydrolysis, the results do show that the formation of a micro-environment can cause changes and that experimentation is required to find the appropriate carrier for immobilization.

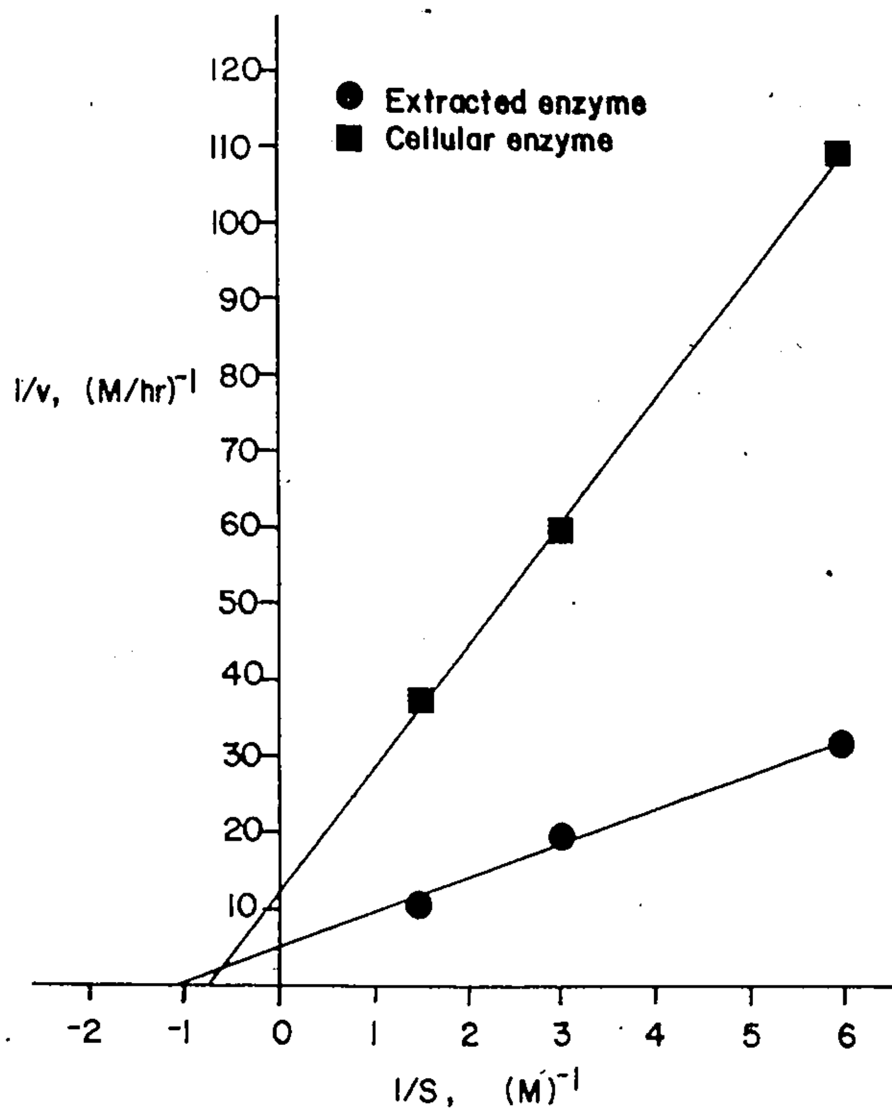


Figure 15: Lineweaver-Burk plot for extracted and cellular enzymes. Conditions for growth of *Lactobacillus brevis*: 30°C, modified Yamanaka's medium. Crude extracts were prepared with phosphate buffer.

4.1.3.3 Enzyme stability

After incubation of glucose isomerase at 50 °C, and with a rotational speed of 150 rpm for 24 hours, the enzyme activity was found to be reduced to 55% of the original activity.

Irreversible thermal denaturation is considered to follow a first-order decay law:

$$de/dt = -k_d e \qquad (1)$$

In the above equation, "e" is the amount of enzyme and "k_d" is the decay constant. For large concentrations of substrate the rate of reaction is given by:

$$v = k_2 e = k_2 e(0)\exp(-k_d t). \qquad (2)$$

By definition, v=dp/dt and therefore by integration:

$$p = k_2 e(0)(1 - \exp(-k_d t))/k_d \qquad (3)$$

The above expression applies for constant temperature, where "k_2" is the rate constant for dissociation of the enzyme-substrate complex into product "p" and free enzyme. Using this equation the decay constant was estimated to be 1.8hr.$^{-1}$ at 50 °C.

4.1.3.4 Equilibrium isomerization

In the isomerization of glucose to fructose using glucose isomerase, the equilibrium reaction mixture normally contains 55% fructose. For example, for glucose isomerase ex *Actinoplanes missouriensis* the con-

version ratio ranges from 51.0% to 53.6% while for glucose isomerase from Bacillus coagulans the ratio is 50%. For Streptomyces albus, and S. cinnamonensis, the conversion ratios of glucose to fructose are 45%, and 59%, respectively (Chen, 1980b). Commercial isomerization reaction mixtures contain 42% fructose for economic reasons (Bucke, 1983).

The conversion to fructose, as a percentage of the initial concentration of glucose present, is presented as a function of time in Figure 16, for glucose isomerase ex Lactobacillus brevis. An equilibrium isomerization concentration of 57% fructose was achieved after approximately 45 hours of incubation at 50 °C. This result is comparable to the equilibrium concentration found by Kent and Emery (1973).

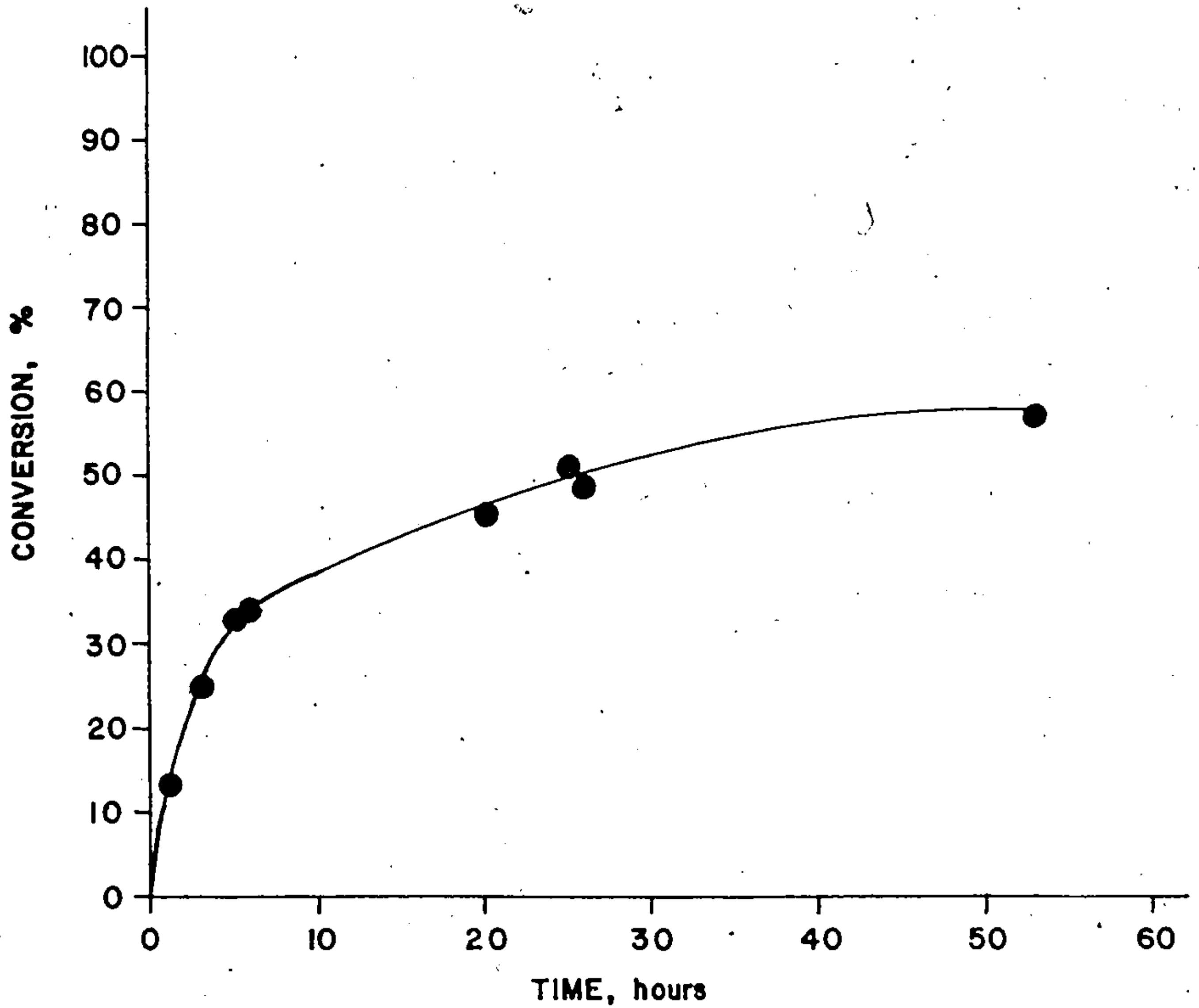


Figure 16: Equilibrium isomerization of glucose isomerase at 50 °C. Conditions for growth of Lactobacillus brevis: 30°C, modified Yamanaka's medium. Crude extracts were prepared with phosphate buffer.

4.2 Cellulose hydrolysis

4.2.1 Fermentation

Enzyme production started relatively early during the fermentation with a maximum intracellular protein content at approximately 72 hours of fermentation. Filter paper activity however, started to increase rapidly only after the maximum intracellular protein was achieved. The maximum filter paper activity was attained at 145 hours of fermentation with a value of 2.5 IU/ml. Figure 17 shows the changes in intracellular protein content and enzyme activity with time.

The reason for the increase in cellulase activity after the maximum intracellular protein content occurred may be attributed to the fact that as the fermentation proceeds cell lysis occurs, releasing the enzymes into the fermentation broth. This is consistent with the drop in intracellular protein content illustrated in Figure 17.

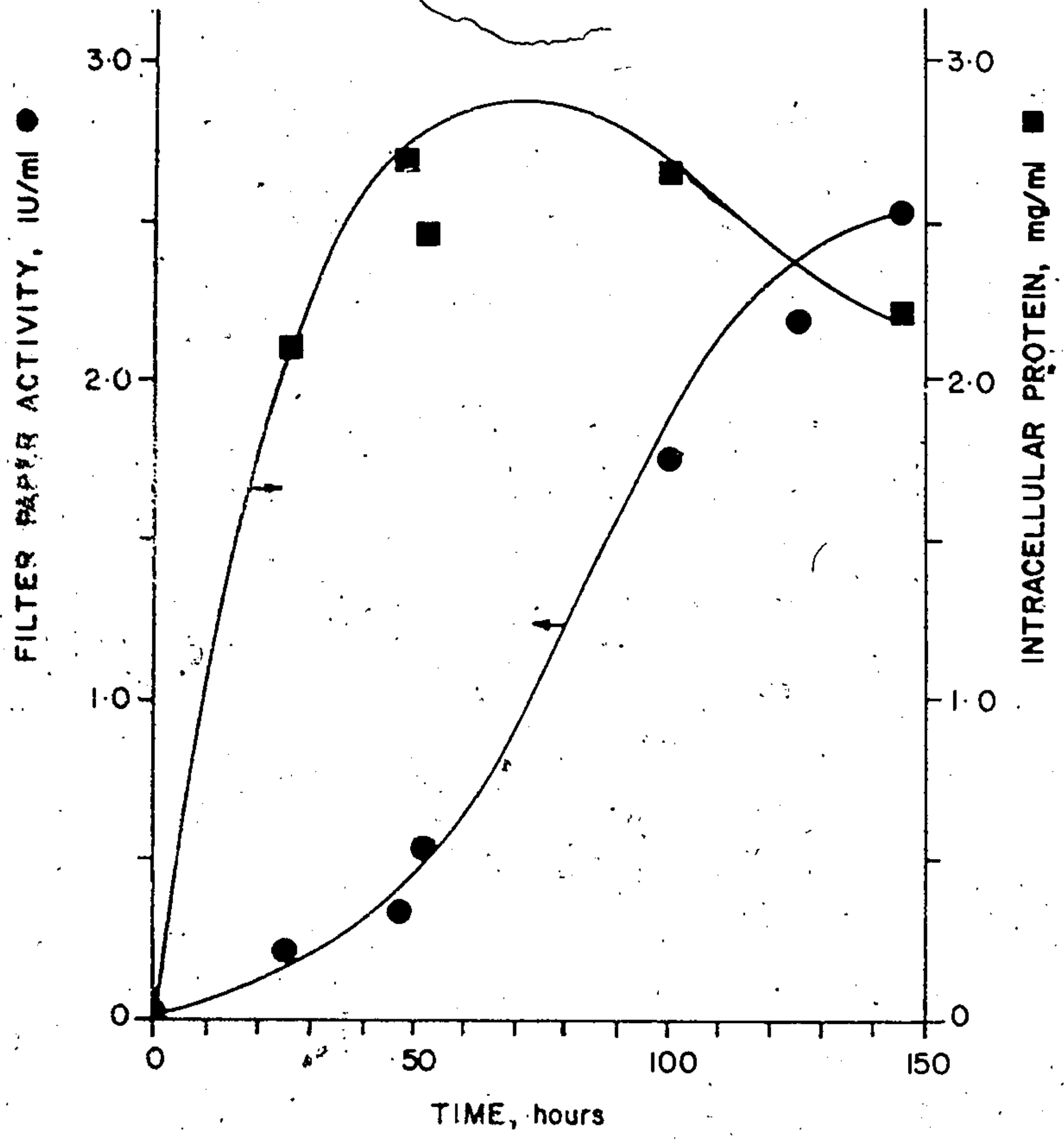


Figure 17: Cellulase production during fermentation. Conditions for growth of Trichoderma reesei: 28°C, pH 3.0, 400 rpm.

4.2.2 Hydrolysis

4.2.2.1 Effect of pH

The production of reducing sugars levelled off at 18 to 24 hours of hydrolysis by cellulase. The maximum cellulose degradation to reducing sugars occurred with hydrolysis at pH 5.0. Hydrolysis at pH 5.5 and pH 6.0 resulted in decreases in the production of reducing sugars. The effect of pH on cellulose hydrolysis is depicted in Figure 18.

The inhibitory effect of pH for the production of reducing sugars increased as the hydrolysis proceeded. It is considered that the effects of end-product inhibition and of increased crystallinity of the cellulose residue are greatest at the higher values of pH. It is of interest that hydrolysis at pH 6.5 and 7.0 resulted in a very low concentration of reducing sugars after 2 hours of reaction, and that negligible cellulose degradation occurred after that.

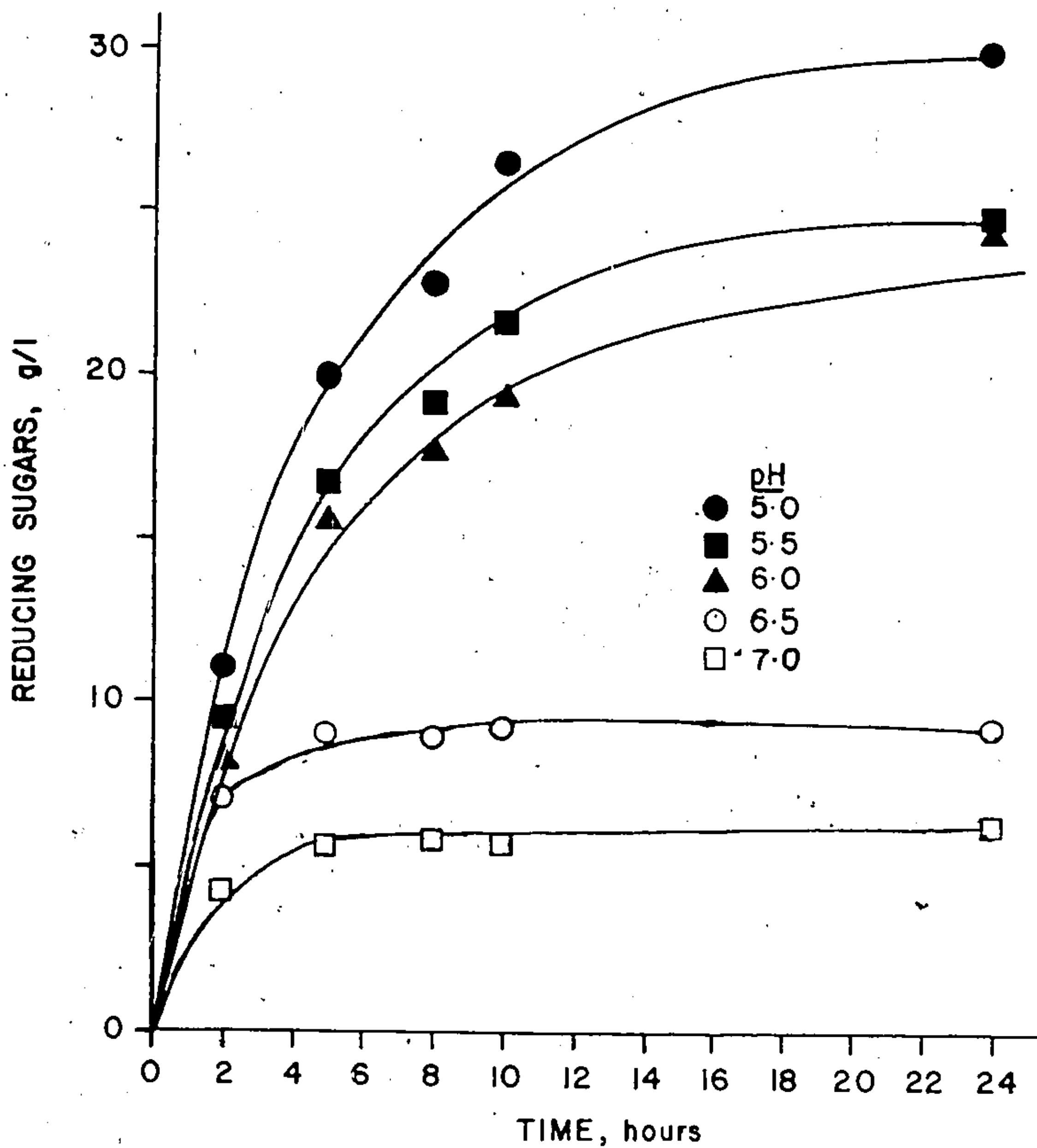


Figure 18: The effect of pH on the production of reducing sugars by cellulase. Hydrolysis: 7% Solka Floc, 200 rpm, 50°C.

4.2.2.2 Effect of addition of glucose isomerase

As indicated in Table 7, the addition of glucose isomerase after 24 hours of hydrolysis increased the production of reducing sugars at pH 6.0 by 31%.

However, the reducing sugars concentration did not reach that of hydrolysis at pH 5.0. Addition of more glucose isomerase solution to attain greater isomerizing activity with the hydrolysis was not feasible because of the high increase in volume and therefore a decrease in the glucose concentration. It is expected that purification of the glucose isomerase solution would result in a greater enzyme potency (activity/ volume).

Table 7: Effect of addition of glucose isomerase to hydrolysis vessel

Hydrolysis: 7% Solka Floc, 200 rpm, 50°C. Conditions for growth of Lactobacillus brevis: 30°C, modified Yamanaka's medium. Crude extracts were prepared with phosphate buffer.

	REDUCING SUGARS (g/l)
pH 5·0	56·5
pH 6·0	34·8
pH 6·0 + Glucose isomerase	45·7

Chapter V

CONCLUSIONS AND RECOMMENDATIONS

The limited amount of literature available concerning the production of glucose isomerase ex Lactobacillus brevis and the conflicting information from the available sources necessitated the investigations into conditions for growth of Lactobacillus brevis and extraction of the intracellular glucose isomerase.

Growth of Lactobacillus brevis was found to be dependent on incubation temperature and medium composition with the greatest effect being the composition of the growth medium. Regardless of the temperature and medium used for fermentation, the enzyme activity was found to be growth-associated. The best cell growth and enzyme activity was attained with fermentation at 30 °C with the modified Yamanaka's medium.

The tris(hydroxymethyl)aminomethane buffer and the cetylpyridinium chloride solution, as lyzing agents, were found to have inhibitory effects towards enzyme activity. The cetylpyridinium chloride caused irreversible inhibition while the inhibition exhibited by the Tris buffer was found to be reversible with 77% recovery of activity as compared to the phosphate buffer-extracted glucose isomerase. Even though the protein content of the enzyme extract solution was virtually the same for all lyzing agents, the activity of the extracted enzyme was greatest for the phosphate buffer. The initial enzyme activity for the Tris buff-

er and cetylpyridinium chloride solution extracts was reduced to 24% and 12%, respectively, of that obtained with the phosphate buffer. Because of the inhibition by Tris buffer, a change was made to phosphate buffer for the enzyme activity assay.

The final overall procedure that was established as a result of this investigation is presented in Figure 19.

Glucose isomerase ex Lactobacillus brevis was found to have good enzyme activity in the pH range of 5.5 to 7.0 with the optimal pH being pH 6.5. The cellular enzyme, however, appeared to have a pH optimum of greater than 7.0.

The values of the Michaelis constant for extracted and cellular enzymes were found to be 0.87 M and 1.29 M, respectively, indicating the high concentration of substrate required for isomerization.

After 24 hours of incubation at 50 °C, the glucose isomerase was found to lose 45% of its original activity. The decay constant was estimated to be 1.8 hr^{-1} at 50 °C for glucose isomerase ex Lactobacillus brevis. The equilibrium isomerization concentration was found to be 57% fructose at 50 °C. This equilibrium was achieved after approximately 45 hours of incubation.

The maximum filter paper activity was attained at 144 hours of fermentation while the maximum intracellular protein of Trichoderma reesei occurred at 72 hours of fermentation.

While a very low concentration of reducing sugars was produced by hydrolysis at pH values of 6.5 and 7.0, reasonable production occurred with hydrolysis at pH 5.5 and 6.0 with the maximum concentration achieved with hydrolysis at pH 5.0 after 24 hours of reaction.

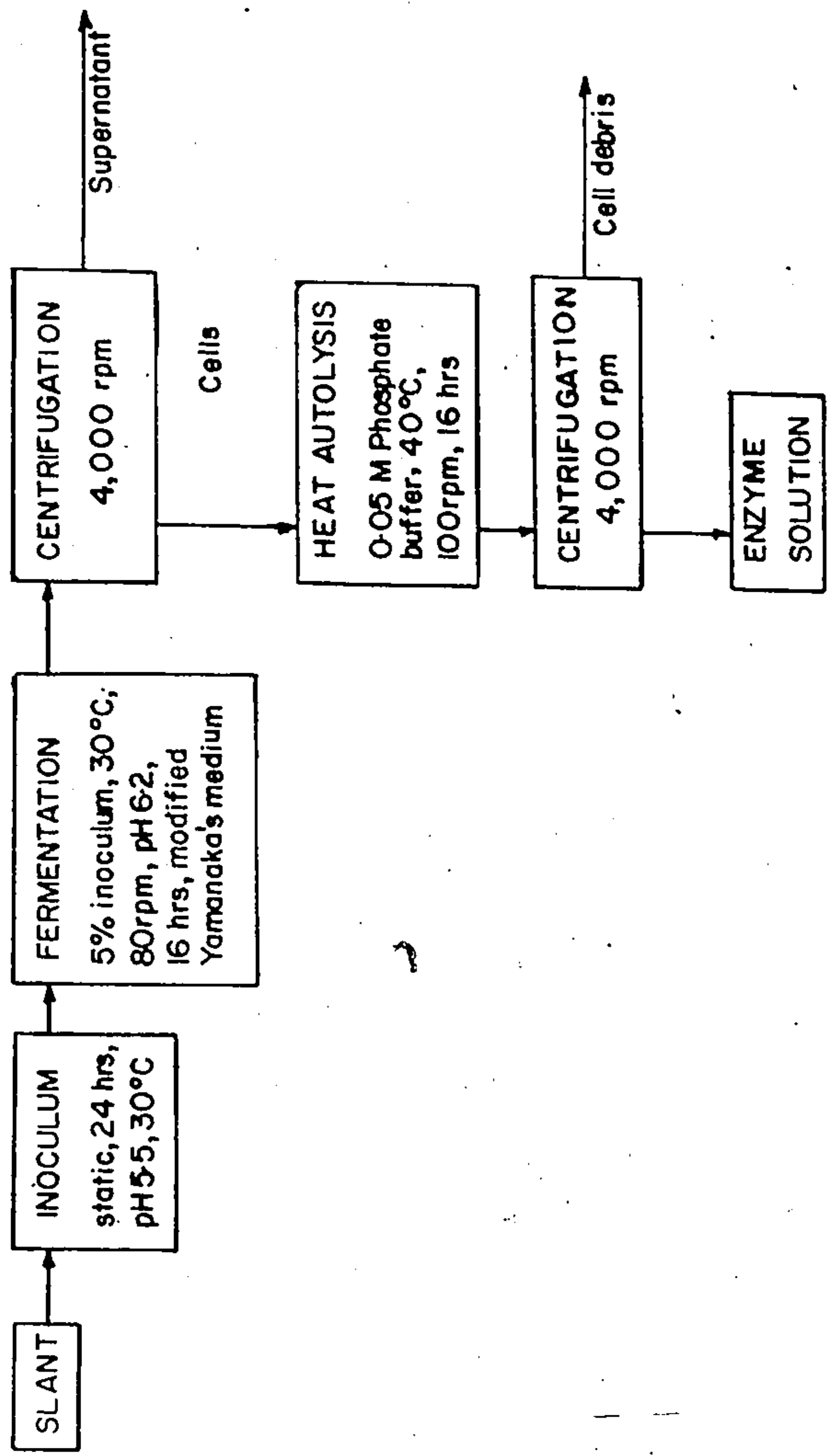


Figure 19: The final overall procedure established as a result of these investigations

The addition of glucose isomerase after 24 hours of hydrolysis with a commercial enzyme preparation at pH 6.0 resulted in an increase in the concentration of reducing sugars by 31% after a subsequent 3 hours of hydrolysis. It is considered that the rate of cellulose degradation was constrained by the low potency of the enzyme solution.

The differences in enzyme activity as a result of changes in pH and in the values of the Michaelis constant for cellular and extracted glucose isomerase, demonstrate the effect of the formation of a micro-environment that can be achieved with immobilization. Experimentation with different immobilization techniques and carriers could result in the discovery of an immobilized glucose isomerase with a pH optimum closer to that of cellulase. Perhaps a lower value of the Michaelis constant could also be obtained, enabling conversion of glucose before a very high concentration occurred with hydrolysis.

The cellulase enzyme and glucose isomerase systems were found to be very compatible with respect to the conditions of the optimum temperature and pH required for enzyme activity.

NOMENCLATURE

DF	Dilution factor
e	Amount of enzyme
FPA	Filter paper activity. A measure of cellulase activity. Units: IU/ml
HFCS	High fructose corn syrup
k_2	Rate constant for dissociation of the enzyme-substrate complex to product and free enzyme.
k'_d	Decay constant
K_M	Michaelis constant
OD	Optical density
p	Product of enzyme-catalyzed reaction
SSF	Simultaneous saccharification and fermentation
Solka Floc	A source of purified cellulose frequently used as a reference substrate.
TDW	Total dry weight

U/ml Units of activity for glucose isomerase. One unit of activity is defined as that which produces one micromole fructose from glucose in 30 minutes.

v Rate of reaction

BIBLIOGRAPHY

Antrim, R. L.; Collilla, W.; and Schnyder, B.J. "Glucose isomerase production of high-fructose syrups" Appl Biochem Bioeng Vol. 2; L. M. Wingard, E. Katchalski-Katzir, and L. Goldstein, eds.; Academic Press, N.Y.; 97-155; 1979

Bailey, J.E. and Ollis, D.F. Biochemical Engineering Fundamentals; McGraw-Hill Book Co.; N.Y.; 1977

Berghem, L.E.R.; Pettersson, L.G.; and Axio-Fredriksson, U.B. "The mechanism of enzymatic cellulose degradation: Characterization and enzymatic properties of a β-1,4-glucan cellobiohydrolase from Trichoderma viride" Eur J Biochem 53: 55-62; 1975

Bernath, F.R.; Venkatasubramanian, K. and Vieth, W.R. "Immobilized enzymes" in Annual Reports on Fermentation Processes vol. 1, D. Perlman, ed., Academic Press, N.Y., 235-266; 1977

Bisaria, V.S. and Ghose, T.K. "Biodegradation of cellulosic materials: substrates, microorganisms, enzymes, and products" Enzyme Microb Technol 3: 2: 90-104; 1981

Blotkamp, P.J.; Takagi, M.; Pemberton, M.S. and Emert, G.H. "Enzymatic hydrolysis of cellulose and simultaneous fermentation to alcohol" AIChE Symposium Series 74: 181: 85- 90; 1978

Buchanan, R.E. and Gibbons, N.E. Bergy's Manual of Determinative Bacteriology 8th ed., Williams and Wilkins Co., Baltimore, 1974

Bucke, C. "Glucose-transforming enzymes" in Microbial Enzymes and Biotechnology W.M. Fogarty, ed., Applied Science Publishers, London; 93- 129; 1983

Chen, W.P. "Glucose isomerase (A review)" Process Biochem 15: 5: 30-35; 1980a

Chen, W.P. "Glucose isomerase (A review)" Process Biochem 15: 6: 36-41; 1980b

Enari, T.M. "Microbial cellulases" in Microbial Enzymes and Biotechnology W.M. Fogarty, ed., Applied Science Publishers, London; 183-223; 1983

Engler, C.R. "Disruption of microbial cells" in Comprehensive Biotechnology vol. 2, M. Moo-Young, ed., Permagon Press, Toronto; 305-324; 1985

Emert, G.H. and Katzen, R. "Chemicals from biomass by improved enzyme technology" in Biomass as a Nonfossil Fuel Source D.L. Klass, ed., American Chemical Society Symposium Series 144: 213-225; 1981

Eriksson, K.E. "New methods for the investigation of cellulases" Advances in Chemistry Series 95: 83- 104; 1969

Fan, L.T. and Lee, Y.H. "Kinetic studies of enzymatic hydrolysis of insoluble cellulose: Derivation of a mechanistic mechanical model" Biotechnol Bioeng 25: 2707- 2783; 1983

Ghosh, P.; Pamment, N.B. and Martin, W.R.B. "Simultaneous saccharification and fermentation of cellulose: effect of β-D-glucosidase activity and ethanol inhibition of cellulases" Enzyme Microb Technol 4: 425- 430; 1982

Gong, C.S.; Chen, L.F.; Flickinger, M.C.; Chiang, L.C. and Tsao, G.T. "Production of ethanol from D-xylose by using D-xylose isomerase and yeasts" Appl Environ Microb 41: 2: 430- 436; 1981

Gong, C.S.; Ladisch, M.R. and Tsao, G.T. "Biosynthesis, purification, and mode of action of cellulases of Trichoderma reesei" Advances in Chemistry Series 181: 261- 287; 1979

Gong, C.S. and Tsao, G.T. "Cellulase and biosynthesis regulation" in Annual Reports on Fermentation Processes vol. 3, D.Perlman, ed., Academic Press, 111- 139; 1979

Gritzali, M. and Brown, R.D. "The cellulase system of Trichoderma. Relationships between purified extracellular enzymes from induced or cellulose grown cells" Advances in Chemistry Series 181: 237- 260; 1979

Halliwell, G. and Griffin, M. "The nature and mode of the cellulolytic component C_1 on Trichoderma koningii on native cellulose" Biochem J 135: 587- 594; 1973

Herbert, D.; Phipps, P.J. and Strange, R.E. Methods in Microbiology vol. 5B, J.R. Norris and D.W. Ribbons, eds., Academic Press, London, 244-249; 1971

Hsu, T.A.; Gong, C.S. and Tsao, G.T. "Kinetic studies of cellodextrins hydrolyses by exoglucanase from Trichoderma reesei" Biotechnol Bioeng 22: 2305- 2320; 1980

Hudson, C.S. and Dale, J.K. "Studies on the forms of D-glucose and their mutarotation" J A C S 39: 2: 320- 328; 1917

Kent, C.A. and Emery, A.N. "The preparation of an immobilized glucose isomerase I. The production and selected properties of the partially-purified enzyme from Lactobacillus brevis" J Appl Chem Biotechnol 23: 689- 703; 1973

Klibanov, A.M. "Immobilized enzymes and cells as practical catalysts" Science 219: 722- 727; 1983

Ladisch, M.R.; Gong, C.S. and Tsao, G.T. "Cellobiose hydrolysis by endo-glucanase (glucan glucanhydrolase) from Trichoderma reesei Kinetics and mechanisms" Biotechnol Bioeng 22: 1107- 1126; 1980

Ladisch, M.R.; Lin, K.W.; Voloch, M. and Tsao, G.T. "Process considerations in the enzymatic hydrolysis of biomass" Enzyme Microb Technol 5: 3: 82- 102; 1983

Leyva, R. "Study of the production of cellulases with Trichoderma reesei and investigation of the enzymatic hydrolysis of cellulosic substrates" M.A.Sc. Thesis, University of Ottawa; 1984

Mandels, M. "Cellulases" in Annual Reports on Fermentation Processes vol. 5, G.T. Tsao, ed., Academic Press, N.Y., 35- 78; 1982

Mandels, M.; Andreotti, R. and Roche, C. "Measurement of saccharifying cellulase" Biotechnol Bioeng Symp no. 6: 21- 33; 1976

Martinek, K. and Mozhaev, V.V. "Immobilization of enzymes: An approach to fundamental studies in biochemistry" in Advances in Enzymology A. Meister, ed., John Wiley and Sons, N.Y., 57: 179-249; 1985

McKay, G.A. and Tavlarides, L.L. "Enzymatic isomerization kinetics of D-glucose to D-fructose" J Molecular Catalysis 6: 57- 69; 1979

Meyers, S.G. "Ethanolic fermentation during enzymatic hydrolysis of cellulose" AIChE Symposium Series 74: 181: 79- 84; 1978

Miller, G.L. "Use of dinitrosalicylic acid reagent for determination of reducing sugar" Analyt Chem 31: 3: 426- 428; 1959

Nisizawa, K.; Tomita, Y.; Kanda, T.; Suzuki, H.; and Wakabayashi, K. "Substrate specificity of C_1 and C_x cellulase components from fungi" in Proc IVth Int Fermentation Symp G. Terui, ed., March 19- 25, 1972; Kyoto, Japan; 719- 725; 1972

Pemberton, M.S.; Brown, R.D. and Emert, G.H. "The role of β-glucosidase in the bioconversion of cellulose to ethanol" Can J Chem Eng 58: 723; 1980

Pettersson, L.G.; Axio-Fredriksson, U.B. and Berghem, L.E.R. "The mechanism of enzymatic cellulose degradation" in Proc IVth Int Fermentation Symp G. Terui, ed., March 19- 25, 1972; Kyoto, Japan; 727- 729; 1972

Saddler, J.N.; Hogan, C.; Chan, M.K.H. and Louis-Seize, G. "Ethanol fermentation of enzymatically hydrolyzed pretreated wood fractions using Trichoderma cellulases, Zymomonas mobilis, and Saccharomyces cerevisiae" Can J Microb 28: 12: 1311- 1319; 1982

Saddler, J.N. and Brownell, H.H. "Pretreatment of wood cellulosics to enhance enzymatic hydrolysis to glucose" presented at the International Symposium on Ethanol from Biomass sponsored by the Royal Society of Canada, Winnipeg, Canada; October 13-15, 1982b

Schray, K.J. and Mildvan, A.S. "Kinetic and magnetic resonance studies of the mechanism of D-xylose isomerase I. Binary and ternary complexes with manganese (II), substrates, and inhibitors" J Biol Chem 247: 7: 2034- 2037; 1972

Selby, K. and Maitland, C.C. "The cellulase of *Trichoderma viride*. Separation of the components involved in the solubilization of cotton" *Biochem J* 104: 716- 724; 1967

Slein, M.W. "Xylose isomerase from *Pastuerella pestis*, strain A-1122" *J A C S* 77: 1663- 1667; 1955

Stanier, R.Y.; Doudoroff, M. and Adelberg, E.A. *The Microbial World* 3rd ed.; Prentice-Hall Inc., New Jersey; 1970

Sternberg, D. "β-Glucosidase of *Trichoderma*: Its biosynthesis and role in saccharification of cellulose" *Appl Environ Microb* 31: 5: 648- 654; 1976

Viikari, L.; Nybergh, P. and Linko, M. "Hydrolysis of cellulose by *Trichoderma reesei* enzymes and simultaneous production of ethanol by *Zymomonas* sp." in *Advances in Biotechnology* vol. 2, M. Moo-Young ed., Permagon Press, Toronto, 137- 142; 1981

Wang, P.Y.; Johnson, B.F. and Schneider, H. "Fermentation of D-xylose by yeasts using glucose isomerase in the medium to convert D-xylose to D-xylulose" *Biotechnol Bioeng* 2: 6: 273- 278; 1980

Wood, T.M. "The C_1 component of the cellulase complex" in *Proc IVth Int Fermentation Symp* G. Terui, ed.; March 19- 25, 1972; Kyoto, Japan; 711- 718; 1972

Wood, T.M. and McCrae, S.I. "Synergism between enzymes involved in the solubilization of native cellulose" *Advances in Chemistry Series* 181: 181- 209; 1979

Woodward, J. and Arnold, S.L. "The inhibition of β-glucosidase activity in *T. reesei* C30 cellulase by derivatives and isomers of glucose" *Biotechnol Bioeng* 23: 1553- 1562; 1981

Yamanaka, K. Agr Biol Chem 25:272;1961

Yamanaka, K. "Sugar isomerases Part I. Production of D-glucose isomerase from heterolactic acid bacteria" Agr Biol Chem 27: 4: 265-270; 1963a

Yamanaka, K. "Sugar isomerases Part II. Purification and properties of D-glucose isomerase from Lactobacillus brevis" Agr Biol Chem 27: 4: 271- 278; 1963b

Yamanaka, K. "Purification, crystallization and properties of the D-xylose isomerase from Lactobacillus brevis" Biochim Biophys Acta 151: 670- 680; 1968

Yaphe, W. and Arsenault, G.P. "Improved resorcinol reagent for the determination of fructose and of 3,6-anhydrogalactose in polysaccharides" Analyt Biochem 13: 143- 148; 1965

Young, J.M.; Schray, K.J. and Mildvan, A.S. "Proton magnetic relaxation studies of the interaction of D-xylose and xylitol with D-xylose isomerase" J Biol Chem 13: 143- 148; 1975

Zale, S.E. and Klibanov, A.M. "On the role of reversible denaturation (unfolding) in the irreversible thermal inactivation of enzymes" Biotechnol Bioeng 25: 2221- 2230; 1983

Appendix A

TAXONOMIC PROPERTIES OF LACTOBACILLUS BREVIS

References: Buchanan and Gibbons, 1974; Stanier *et al.* 1970

- Isolated from milk, kefir, cheese, sauerkraut, spoiled tomato products, sourdough, certain soils, cow manure, feces, and the mouth and intestinal tract of humans and rats.
- Gram-positive; non-sporing.
- Rods, generally short and straight, 0.7 - 1.0 x 2.0- 4.0 microns occurring singly or in short chains.
- Absence of cytochromes - growth has a chalky white appearance.
- Growth at 15 °C; no growth at 45 °C, optimum about 30°C.
- Aciduric; optimum pH usually 5.5 - 5.8 or less and generally growing at 5.0 or less; at neutral or initial alkaline conditions, lag phase may be lengthened or total growth yield reduced.
- Complex nutritional requirements; usually cultivated on media containing peptone, yeast extract, or other digests of plant or animal material.
- Facultative anaerobe; grows readily on the surface of solid media exposed to air, even though they cannot derive energy from respiration.

- Growth yields are the same in air and in anaerobiosis, the fermentation of sugars being the source of energy under both conditions.
- Heterofermentative lactic acid bacteria; glucose metabolized through the hexose monophosphate shunt pathway (Figure 20).

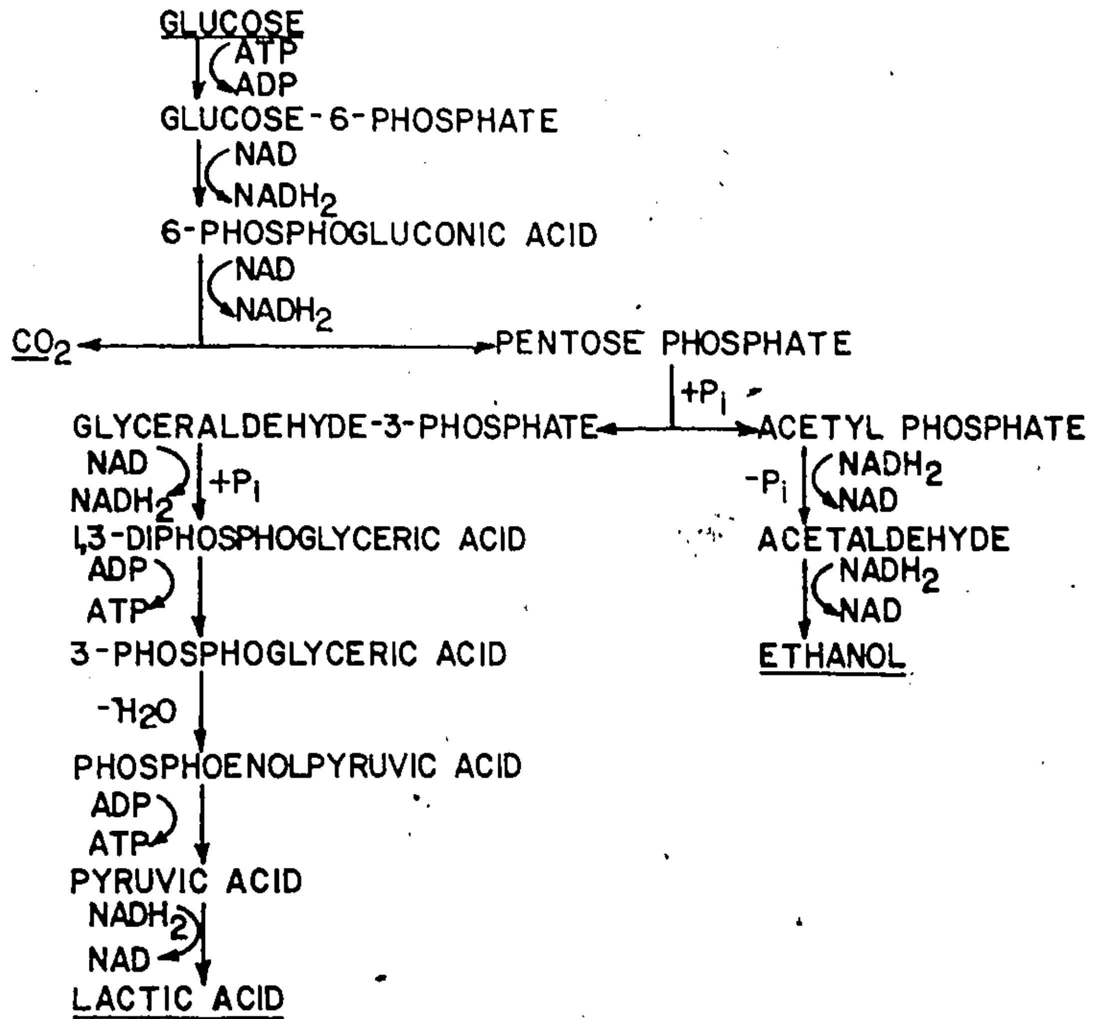


Figure 20: The Hexose Monophosphate Shunt Pathway.

Appendix B

PURIFICATION OF GLUCOSE ISOMERASE

Reference: Yamanaka, 1963b

- Cell-free extracts were prepared by grinding the cells with alumina and enzymes were extracted with Tris buffer. Precipitated alumina was removed by decantation, a $MnCl_2$ solution was added dropwise with stirring, pH 6.8 to 7.2. After having been allowed to stand overnight, precipitates were collected by a bath rotor of a Spinco centrifuge at 15,000 rpm and discarded.
- Solid ammonium sulfate was added with stirring to achieve 100% saturation, maintaining pH at 6.8 to 7.2 by ammonia solution. Solution was allowed to stand two hours, and precipitates were collected by filtration.
- Proteins were dissolved in Tris buffer; insoluble matters were removed by centrifugation. Solid ammonium sulfate was added slowly to bring saturation to 45%, and precipitated protein was discarded by centrifugation.
- Enough salt was added to supernatant fluid to bring the saturation to 95%. Precipitate was collected by centrifuge, then dissolved in a buffered solution and dialyzed for 44 hours.

- Solution of cobalt chloride was added to this fraction at pH 7.0 and the enzyme solution was immersed immediately in a bath at 80 °C. When the temperature of solution was raised to 53 °C, the flask was transferred to a bath at 55 °C and maintained for 10 minutes at 55 °C. The flask was cooled in an ice-bath and the coagulated proteins were removed by centrifugation.
- Protein was further fractionated by adding solid ammonium sulfate to bring the saturation to 50%, 50 to 85%, and 85 to 95%. Each fraction was dissolved and dialyzed.
- DEAE-Sephadex G-50 was suspended in a solution of Tris-acetate buffer, adjusting its pH to 7.0, and washed three times by the same buffered solution. Sephadex was poured into a column and then the enzyme (50- 85% fraction) was poured into this column.
- Chromatography was developed by stepwise elution of Tris buffer. Fractions were collected. Active fractions were obtained by elution using KCl solution. Appropriate fractions were subsequently combined.
- Enzyme was precipitated by adding suitable amounts of ammonium sulfate, and subsequently dialyzed.

Appendix C

ANALYTICAL METHODS

C.1 Protein content of fermentation broth by Biuret method

Reference: Herbert et al, 1971

Procedure:

- Pipet two 2 ml samples of fermentation broth into two centrifuge tubes.
- Add to each tube about 6 ml of distilled water and shake tube gently.
- Centrifuge for 5 minutes at 2000 rpm.
- Withdraw 5 to 6 ml of supernatant with aspirator. Shake tube gently and add equivalent volume of water. Recentrifuge.
- Repeat step d) at least once. Make up volume to 2 ml.
- Prepare standards (2 ml volumes) at different concentrations (e.g., 1.0, 2.0, and 4.0 g/l) with bovine albumin in centrifuge tubes.
- Pipet 2 ml of water in last centrifuge tube (to be used as blank).
- Add 1.0 ml of 3.0 N NaOH solution to each tube, cover and and place in a boiling water bath for 10 minutes. Cool in cold water.
- Add 1.0 ml aqueous $CuSO_4$-$5H_2O$ solution (2.5% (w/v)) , mix thoroughly with vortex mixer. Allow to stand 5 minutes and centrifuge 10 minutes at 2000 rpm.

- Read absorbance of supernatant at 555 nm against a blank.

Calculation: Determine the concentration of protein in the sample as g/l using linear regression of the standards. Report the average of the two readings. Comments:

- This procedure has been developed for measuring the protein content of fresh samples of broth.
- The absorbance should be read within one hour.
- To determine the protein content of the enzyme extract solution, follow the procedure from step f) using 2 ml samples of appropriately diluted enzyme extract solution.

C.2 Filter paper activity

Reference: Mandels _et al_ , 1976

Reagents:

- Citrate buffer 0.1 M (see DNS method)
- 3,5-Dinitrosalicylic acid reagent (see DNS method)
- Standard solution: glucose 1.0 g/l

Procedure:

- Place 1 ml of diluted sample in test tube (try several dilutions).
- Add 1 ml of 0.1 M citrate buffer.
- Coil a 1cm x 6cm strip of filter paper (Whatman No.1).
- Vortex, add coiled filter paper and vortex again.
- Cover tubes and incubate at 50 °C.
- After 60 minutes, add 3 ml of DNS reagent.
- Put tubes in 95 °C water bath for 10 minutes.
- Cool and centrifuge (if necessary).

- Read absorbance at 600 nm against a blank.

Notes

- A substrate blank is included for each sample to correct for the presence of reducing sugars. 1 ml sample + 1 ml buffer + 3 ml DNS reagent. Do not add filter paper.
- The blank is prepared with 1 ml distilled water + 1 ml buffer + 3 ml DNS reagent.
- A series of standards are included with each run (e.g. 0.2, 0.4, 0.6, 0.8, and 1.0 g/l).
- Substrate blanks, blanks, and standards are treated in the same way as the samples.

Calculation: From regression analysis, determine the concentrations for samples and substrate blanks; and determine activity by:

FPA (IU/ml)=(Csample - Csubs. blank) x DF/10.8

where C is the apparent glucose concentration.

C.3 Determination of reducing sugars by DNS method

Reference: Miller, 1959

Reagents:

- Citrate buffer (0.5 M): Dissolve 35 g citric acid monohydrate and 98 g sodium citrate dihydrate in distilled water. Complete volume up to 1 litre. Keep refrigerated.
- Citrate buffer (0.1 M): Dilute 200 ml of the citrate buffer stock solution (0.5 M) to 1 litre with distilled water. Keep refrigerated.

- 3,5-Dinitrosalicylic acid: Dissolve 64.0 g NaOH in 3 litres of distilled water. Add 40.0 g of 3,5-dinitrosalicylic acid and 1,200 g of NaK tartrate. Bring the volume to 4 litres with water. Warm while stirring. Keep refrigerated.
- Glucose standard solution: 1.0 g/l (keep refrigerated).

Procedure:

- Place 1 ml of appropriately diluted sample in test tube.
- Add 1 ml 0.1 M citrate buffer and mix with vortex mixer.
- Add 3 ml DNS reagent.
- Cover tubes and incubate at 95 °C for 10 minutes.
- Cool and centrifuge if necessary (if solution is cloudy).
- Read absorbance at 600 nm against a blank prepared with 1 ml of distilled water instead of sample.

Notes:

- For dark or colored samples include another series of "background" samples replacing DNS reagent with distilled water. The absorbance of these samples is read against a blank prepared with 4 ml of water and 1 ml of buffer.
- Include a series of standards with each run (e.g., 0.2, 0.4, 0.6, 0.8, 1.0 mg/ml).

Calculation: Determine the concentration of glucose as g/l by linear regression of the standards. For samples requiring background samples:

$$\text{Corrected OD} = \text{OD}_{sample} - \text{OD}_{background}$$

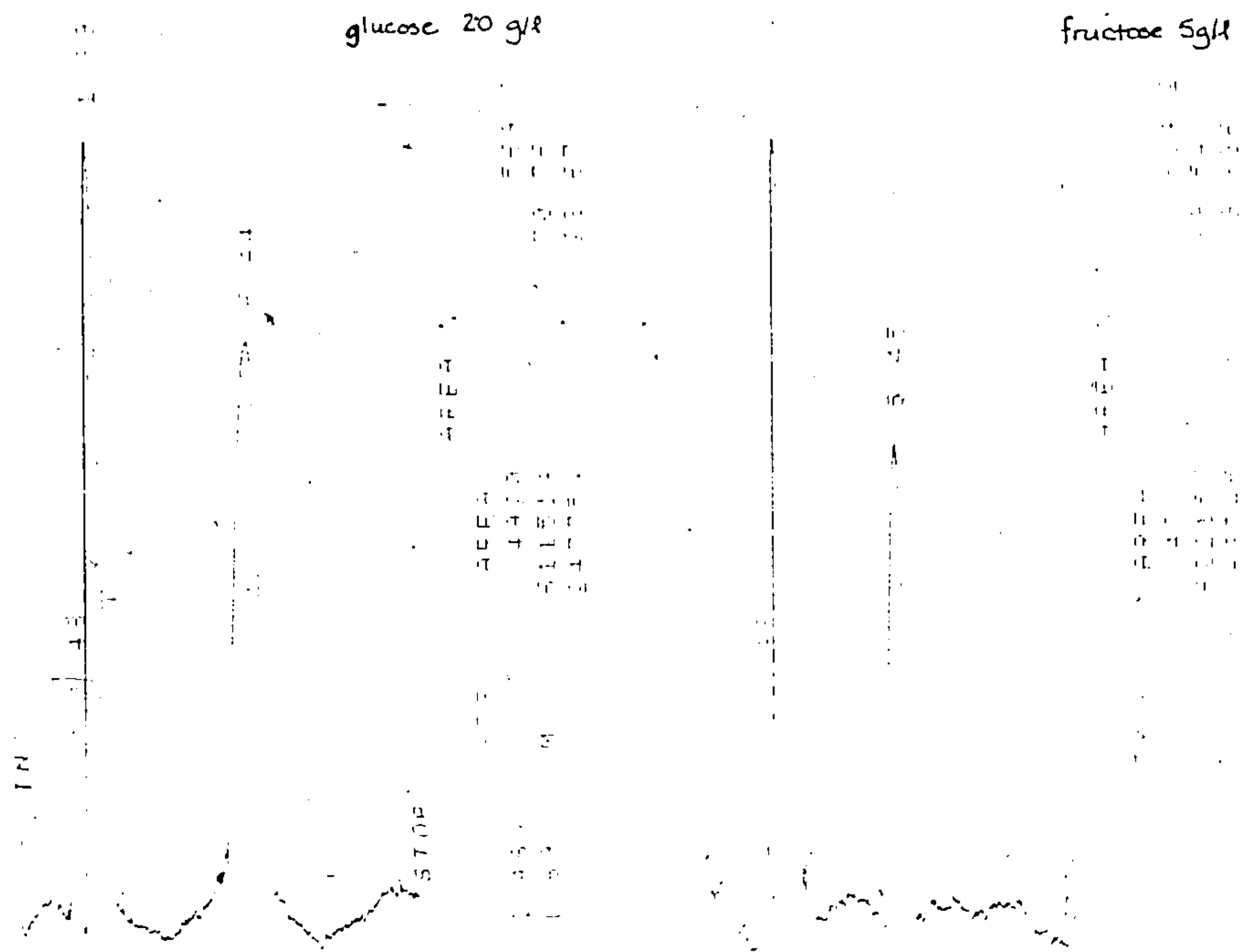
Standard solutions of glucose and fructose of the same concentration gave the same absorbance readings when analyzed by the DNS method for determination of reducing sugars.

Standard Concentration (g/l)	Absorbance (600 nm)	
	Glucose	Fructose
0.2	0.029	0.030
0.4	0.070	0.066
0.8	0.128	0.131
1.0	0.228	0.211

Appendix D

SAMPLE OUTPUTS FROM HPLC ANALYSIS

Samples of the output from the Hewlett-Packard integrator for standard solutions of fructose and glucose are presented below.



Appendix E

COMPOSITION OF PROTEOSE PEPTONE AND YEAST EXTRACT

Lactobacillus brevis has complex nutritional requirements and is grown on media containing peptone and yeast extract. An analysis of the proteose peptone and yeast extract (products of DIFCO Laboratories) used for the growth media in this investigation was provided by personal communication with a representative of DIFCO Laboratories and is presented in Table 8.

Table 8: Composition of proteose peptone and yeast extract

Percent	Proteose Peptone	Yeast Extract
Ash	9.61	10.1
Ether soluble extract	0.32	
Total N	14.37	9.81
Primary proteose N	0.60	
Secondary proteose N	4.03	
Peptone N	9.74	
Ammonia N	0.00	
Free amino N (Van Slyke)	2.66	
Amide N	0.94	
Mono-amino N	7.61	
Di-amino N	4.51	
Arginine	6.8	0.78
Aspartic acid	7.4	5.1
Cystine (Sullivan)	0.56	
Glutamic acid	12.0	6.5
Glycine	11.6	2.4
Histidine	1.7	0.94
Isoleucine	3.3	2.9
Leucine	6.4	3.6
Lysine	5.3	4.0
Methionine	2.0	0.79
Phenylalanine	3.3	2.2
Threonine	3.5	3.4
Tryptophane	0.72	0.88
Tyrosine	3.4	0.60
Valine	4.4	3.4
Organic sulfur	0.60	
Inorganic sulfur	0.04	
Phosphorus	0.24	0.29
Iron	0.0038	0.028
SiO_2	0.078	0.052
Potassium	0.70	0.042
Sodium	2.84	0.32
Magnesium	0.118	0.030
Calcium	0.137	0.040
Chlorine	3.95	
Chloride	3.95	0.190
PPM		
Manganese	5.3	7.8
Lead	5.00	16.00
Arsenic	0.25	0.11
Copper	31.00	19.00
Zinc	44.00	88.00

Micrograms per gram	Proteose Peptone	Yeast Extract
Pyridoxine	3.0	20.0
Biotin	0.43	1.4
Thiamine	3.0	3.2
Nicotinic acid	131.00	279.00
Riboflavin	11.00	19.00

Appendix F

EXPERIMENTAL DATA

Table 9: Data for fermentation at 30 °C with modified Yamanaka's medium

t (h)	TDW (mg/ml)	O.D. (530 nm)	pH	Protein (mg/ml)	Activity (U/ml)	Activity (U/mg protein)
0	-	-	6.19	-	-	-
2	0.23	0.095	6.10	-	-	-
4	0.22	0.094	-	-	-	-
6	0.21	0.116	5.87	-	-	-
8	-	0.163	5.33	4.29	15.3	3.57
10	2.03	0.565	-	12.19	71.5	5.87
12	2.66	0.809 0.897	4.05	19.44	90.3	4.65
14	3.00	1.028	-	19.21	-	-
16	3.03	1.000 1.051	3.77	16.91	94.4	5.58
18	-	0.991	3.72	15.17	78.5	5.17

The correlation coefficient between data for Total Dry Weight and Optical Density was calculated to be 0.994 using linear regression analysis.

Table 10: Data for fermentation at 37 °C with modified Yamanaka's medium

t (h)	TDW (mg/ml)	O.D. (530nm)	pH	Protein (mg/ml)	Activity	
					(U/ml)	(U/mg protein)
0	-	-	6.19	-	-	-
2	0.26	0.082	-	-	-	-
4	0.28	0.096	-	-	-	-
6	0.31	0.129	-	-	-	-
8	0.57	0.239 0.245	5.06	5.88	21.5	3.66
10	-	0.436	-	7.92	36.1	4.56
12	2.08	0.696 0.765	4.23	17.80	58.3	3.28
14	2.36	0.803	-	17.24	-	-
16	2.51	0.815 0.836	3.84	12.90 13.26	68.1	5.28
18	2.49	0.845 0.873	3.77	11.83	39.9 38.2	3.37

The correlation coefficient between data for Total Dry Weight and Optical Density was calculated to be 0.997 using linear regression analysis.

Table 11: Data for fermentation at 30°C with Yamanaka's medium

t (h)	TDW (mg/ml)	O.D. (530 nm)	Protein (mg/ml)	Activity	
				(U/ml)	(U/mg protein)
10	1.50	0.575	7.07	45.8	6.48
12	1.72	0.623	6.02	59.0	9.80
14	1.68	0.591	6.06	50.7	8.37
16	1.46	0.572	6.64	51.4	7.74

The correlation coefficient between data for Total Dry Weight and Optical Density was calculated to be 0.892 using linear regression analysis.

Table 12: Data for fermentation at 37°C with Yamanaka's medium

t (h)	TDW (mg/ml)	O.D. (530 nm)	Protein (mg/ml)	Activity	
				(U/ml)	(U/mg protein)
2	0.16	0.034	-	-	-
4	0.06	0.052	-	-	-
6	0.46	0.135	-	-	-
8	0.84	0.298	-	-	-
10	1.29	0.536	3.07	31.3	10.20
12	1.43 1.33	0.525 0.538	2.30	52.2	22.70
14	1.42 1.40	0.522 0.505	2.35	-	-
16	1.36	0.503	-	47.2	-
18	1.22	0.486	4.24	34.0	8.02

The correlation coefficient between data for Total Dry Weight and Optical Density was calculated to be 0.990 using linear regression analysis.

Table 13: Effect of lyzing agent used in enzyme extraction

	LYZING AGENT					
	Phosphate Buffer		Tris Buffer		Cetylpyridinium Chloride	
t (h)	Activity (U/ml)	Fructose (g/l)	Activity (U/ml)	Fructose (g/l)	Activity (U/ml)	Fructose (g/l)
1	112.6	16.2	27.2	3.91	13.3	1.91
3		29.9		13.0		
5		39.6				
6		40.5		20.6		
20		54.4		-		18.3
25		61.3		53.2		-
26		58.2		-		19.4
53		68.9		53.0		33.3
59		-		-		36.0

Table 14: Effect of pH on glucose isomerase activity

	Cellular Enzyme		Extracted Enzyme	
pH	Fructose (g/l)	Activity (U/ml)	Fructose (g/l)	Activity (U/ml)
5.0	2.15	14.9	1.35	9.4
5.5	19.22	133.5	1.59	11.0
6.0	22.3	154.9	1.93	13.4
6.5	23.75	164.9	2.35	16.3
7.0	16.22	112.6	4.75	33.0

Table 15: Effect of substrate concentration on glucose isomerase

		Cellular Enzyme		Extracted Enzyme	
Glucose Solution(M)	1/S $(M)^{-1}$	Fructose (g/l)	1/v $(M/hr)^{-1}$	Fructose (g/l)	1/v $(M/hr)^{-1}$
0.5	6.0	1.63	110.4	5.64	31.9
1.0	3.0	3.01	59.8	8.94	20.1
2.0	1.5	4.75	37.9	16.22	11.1
			K_M=1.29 M		K_M=0.87 M

Table 16: Enzyme Stability

Hours of Incubation before Addition of Glucose	Activity (U/ml)
0	112.6
1	103.4
24	61.4

Table 17: Equilibrium conversion data

Initial Glucose Concentration = 120 g/l

t (h)	Fructose produced (g/l)	%
1	16.2	13.5
3	29.9	24.9
5	39.6	33.0
6	40.5	33.8
20	54.4	45.3
25	61.3	51.1
26	58.2	48.5
53	68.9	57.4

Table 18: Cellulase production and activity during fermentation

t (h)	Filter Paper Activity (IU/ml)	Intracellular Protein (mg/ml)
0	0.022	-
27	0.225	2.1
47	0.335	2.68
52	0.541	2.44
100	1.756	-
125	2.196	2.62
144	2.530	2.21

Table 19: Effect of pH on the production of reducing sugars by cellulase

	REDUCING SUGARS (g/l)				
t (h)	pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0
2	11.08	9.59	8.3	7.13	4.39
5	19.90	16.76	15.51	8.99	5.65
8	22.81	19.22	17.7	8.99	5.80
10	26.51	21.59	19.27	9.29	5.76
24	29.90	24.98	24.54	9.21	6.32