National Library of Canada

Acquisitions and Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada

Direction des acquisitions et des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform 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.

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

AVIS

La qualité de cette microforme 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.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

Wood Blocks as a Carrier for *Saccharomyces cerevisiae* Cells Used in the Production of Fructose and Ethanol

by

Maryse Guénette

A thesis submitted to the School of Graduate Studies

in partial fulfilment of the requirements for the

degree of

MASTER OF APPLIED SCIENCE

in the Department of Chemical Engineering

University of Ottawa

October, 1993

ISBN 0-315-89623-X

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

Abstract

Saccharomyces cerevisiae ATCC 39859 was immobilized onto small cubes of wood in order to produce very enriched fructose syrup from synthetic glucose/fructose mixtures, through the selective fermentation of glucose. The kinetics of growth and ethanol production rates were measured. Several tests to assess the influence of substrate and product concentration on the production rates were carried out and appropriate rate equations were proposed as a design basis for continuous immobilized reactors. The growth and ethanol production rates were found to be inhibited linearly by both substrate and product concentrations.

A maximum ethanol productivity of 21.9 g/Lh was attained from a feed containing 10% (w/v) glucose and 10% (w/v) fructose. The ethanol concentration was 29.6 g/L, the glucose conversion was 78% and a fructose yield of 99% was obtained. This resulted in a fructose/glucose ratio of 2.7. At lower ethanol productivity levels the fructose/glucose ratio increases, as does the ethanol concentration in the effluent. The ethanol productivities obtained in this study were 70% higher than those obtained in a previous study using the same system, under the similar conditions, with the cell immobilized in algenate beads.

The addition of oleic acid, which is known as an anaerobic growth factor, increased the productivity by 13%.

The effect of reactor temperature on production rate was studied. Ethanol productivity peaked at 32.6°C and approached zero near 44°C.

Batch fermentations were carried out using the reactor effluent in an attempt to enhance the ethanol concentration in the effluent from the reactor. The productivity of these processes

was not very high due to the low biomass concentration leaving the reactor. The addition of yeast extract or active biomass increased the productivity substantially.

The immobilized cell bioreactor was also used to produce sorbitol continuously from fructose. Sorbitol is a polyol which is used as a sweetening agent for diabetics. It is also used in pharmaceutical, foods and chemical industry. A maximum productivity of 3.25 g/Lh was attained from a 133.8 g/L fructose feed, with a fructose conversion of 44%.

Acknowledgements

The author would like to thank Dr. Z. Duvnjak for his continuous guidance and financial support, Louis Tremblay for his countless technical services and Karol Lacki for his assistance and companionship in the laboratory.

Nomenclature

K_s	saturation constant (g/L)
K_1, K_2, K_i, B	empirical constants
$K_{\mu p}$	ethanol inhibition constant to growth
K_{vp}	ethanol inhibition constant to ethanol production
P	ethanol concentration (g/L)
P_{max}	ethanol concentration above which cells do not grow (g/L)
P'_{max}	ethanol concentration above which cells do not produce ethanol (g/L)
S	substrate concentration (g/L)
So	initial concentration of substrate (g/L)
S_{tc}	total carbohydrate concentration (g/L)
S_{tmax}	total carbohydrate concentration above which cells will not grow (g/L)
S'_{tmax}	total substrate concentration above which ethno will not be produced (g/L)
x	biomass concentration (g/L)
μ	specific growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})
ν	specific ethanol productivity (h^{-1})
ν_{max}	maximum specific ethanol productivity (h^{-1})

Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Fructose

Sweetness is the most important characteristic of high-fructose corn syrup (HFCS). Three HFCS products are commercially available; they contain 42, 55, and 90% fructose. In sweetness, the 42 HFCS is about equal to sucrose, while the 55 HFCS is about equal to medium sucrose invert and the 80-95% HFCS is 20-80% sweeter than sucrose (Long, 1991). The commercial success of HFCS has been remarkable due to the drastic increase in sugar prices in the early 1970s which opened the market for this industrial sweetener substitute.

The HFCS market has continued to grow and is replacing sucrose as well as dextrose in the industrial market. 42 HFCS is used in most food products that make use of a liquid sweetener. The development of second-generation syrups with higher fructose levels greatly extended the use of this sweetener. 55 HFCS is used as a replacement for sucrose in soft drinks. 90 HFCS has found limited applications in many new and specialty food products, such as calorie reduced jams and jellies (Coker and Venkatasubramanian, 1987). The penetration of these syrups into the market has almost reached a maximum. Therefore, further expansion of the industry is only possible if new methods are developed for the production of fructose in a form suitable for a wider range of applications. The production of a solid form of fructose would allow for the exportation of corn sweeteners in large quantities. This has not yet been achieved by HFCS producers (Hodgkin, 1987).

Solid fructose is produced by the crystallization of syrups containing 90-95% fructose

(Bateman et al., 1984). The glucose concentration in 42 and 55 HFCS must be substantially reduced in order to make use of these syrups for the production of a solid form of fructose. The separation of glucose and fructose is very difficult since they are isomers of the same molecular size. The current method available in industry uses chromatographic techniques (Coker and Venkatasabramanian, 1987). Since this process is complex and costly, the price of solid fructose is not competitive with other sweeteners.

1.2 Selective Conversion

The selective conversion of glucose to a product more easily separated from fructose would reduce the separation problem. In addition to cost reduction for fructose purification, the production of a valuable by-product would make the process more profitable. The selective conversion of glucose has been achieved by a number of microorganisms which often produce ethanol as a by-product. The following microorganisms have performed this selective fermentation: *Mucor sp. m105* and *Fusarium sp. F5* (Ueng et al., 1982) and *Zymomonas mobilis* (Bringer-Meyer et al., 1985; Doelle, 1986; Suntinanalert et al., 1986). Certain difficulties, such as substantial fructose consumption or the production of unwanted by-products, were encountered with the use of these microorganisms. Lamarche (1988) and Koren (1990) performed extensive studies on the use of a mutant strain of *Sacharomyces cerevisiae* for the selective fermentation of glucose. In batch fermentations, no fructose was consumed and no unwanted by-products were produced by the yeast. An ethanol yield of 91% was obtained. In continuous immobilized cell systems, the ethanol yield was 99% and fructose consumption began at glucose concentrations below 20 g/L.

1.3 Objectives

The results obtained by Koren (1990) show that *Saccharomyces cerevisiae* ATCC 36859 can be used for the selective fermentation of glucose from glucose/fructose mixtures. In free cell batch fermentations, the mutant had a lower ethanol productivity than the wild strain. Immobilization of the mutant yeast in Ca-alginate beads in a vertical packed bed reactor increased the ethanol productivity substantially. The advantages of immobilized cell systems depend on the method of immobilization. Systems employing cells entrapped in Ca-alginate possess excellent properties with respect to long term operational stability and high biomass loadings. However, these systems are subjected to mass transfer limitations imposed by the additional diffusion barrier created by the support matrix. The use of adsorbed yeast cells for continuous production employs a cheap and simple method of retaining high cell densities. This immobilization procedure maintains the cells in a viable state and does not create any barrier between the cells and the solution in the system. Wood blocks have been employed successfully as a support matrix for yeast cells in ethanol fermentations (Gencer and Mutharasan, 1981). The objective of this work was to explore the possibility of using wood blocks for immobilization of the *S. cerevisiae* cells in a vertical packed bed reactor and determine its benefits in the production of enriched or pure fructose syrup from 42% HFCS. A comparison with the results obtained by Koren (1990) by immobilizing the cells in alginate beads will be made. The system will be characterised by studying the effects of different carbohydrate feed concentrations, added oleic acid in the medium and reactor temperature. Models will be formulated for specific growth rate and ethanol productivity in order to describe the behaviour of the mutant under immobilized conditions. The use of batch fermentations on the reactor effluent will be studied

in order to enhance the product ethanol concentration, making ethanol recovery more profitable.

In previous batch fermentations with this mutant yeast, the production of sorbitol was observed in media containing only fructose as a carbohydrate source (Duvnjak et al., 1991a). A preliminary test of the continuous production of sorbitol from fructose will be performed with the mutant yeast immobilized on wood chips.

Chapter 2

Literature Review

2.1 High Fructose Corn Syrup

High-fructose corn syrup (HFCS) is a sweetener which has a higher sweetening power than sucrose; hence, less sweetener is needed to attain a desired sweetness level. This can benefit products containing sugar by making them less caloric for a similar degree of sweetness. The corn sweetener industry has been developing at a remarkable rate during the past few decades, capturing the majority of the U.S. industrial sugar and sweetener market by producing and offering a variety of HFCS products such as 42, 55 and 90% sweetener.

Glucose and fructose share the same chemical formula ($C_6H_{12}O_6$). However, they differ in their molecular structure (Figure 2.1).

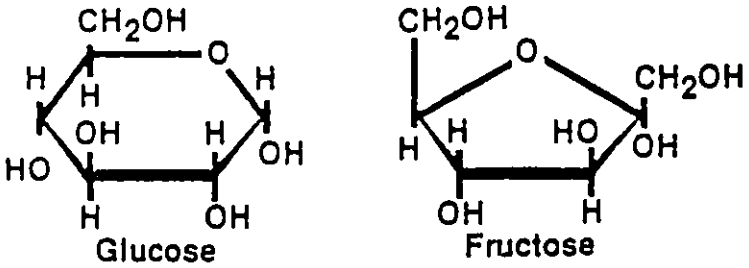

Figure 2.1: Glucose and fructose structure.

This difference in structure alters the sweetening qualities of the sugars, making fructose sweeter than glucose. The relative sweetness of fructose to other sweeteners is shown in Table 2.1 (Long 1991).

Table 2.1: Relative Sweetness of Nutritive Sweeteners (Long 1991)

Sweetener	Sweetness Relative to Sucrose
Sucrose	1.0
Crystalline fructose	1.2-1.8
90% HFCS	1.2-1.6
42% HFCS	1.0
55% HFCS	1.0+
Sucrose medium invert	1.0+
Glucose	0.7

High-fructose syrup can be produced from other sources of starch such as wheat, potatoes, rice and cassava, however, in North America it is produced mainly from corn. The main advantage of corn is that it is the most efficient converter of sunlight into food energy, thereby making it one of the lowest-cost and most abundant sources of usable carbohydrates.

HFCS is a product of the corn wet milling and corn refining industry. Takasaki and Tamade (1971) describe the basic process for the production of HFCS. The products of the corn wet milling process are corn oil, corn germ meal, animal corn feed and common corn starch. Corn refining, using starch as the substrate, yields corn syrups, HFCS, dextrose and maltodextrins as products of the process.

2.1.1 Corn Wet Milling Process

The corn wet milling process (Figure 2.2) consists of seven operations: cleaning, steeping, degerming, separation, milling, washing and centrifugal separation (Joglekar et al., 1983). Corn is cleaned, shelled and transferred to large, steep tanks where steeping is carried out for about 40 hours. The steep water, as a process by-product, can be sold with potential application as fermentation medium supplement. The steeped corn kernels are conveyed to degerminating mills, where their components are separated. Subsequently, the corn slurry with broken corn enters a hydroclone, which separates the germ. The recovered germ is washed and dried in preparation for oil recovery. Starch and hull are further ground in a mill and are screened. The resulting mill starch is passed to a continuous centrifuge for starch and gluten separation, while the hull (fibre) is processed as animal feed. The separated starch fraction is further purified by passing it through hydroclones to reduce the protein content to a minimum level of approximately 0.3%. The starch stream is rediluted with freshwater to the appropriate concentration for conversion to glucose and fructose through the refining process.

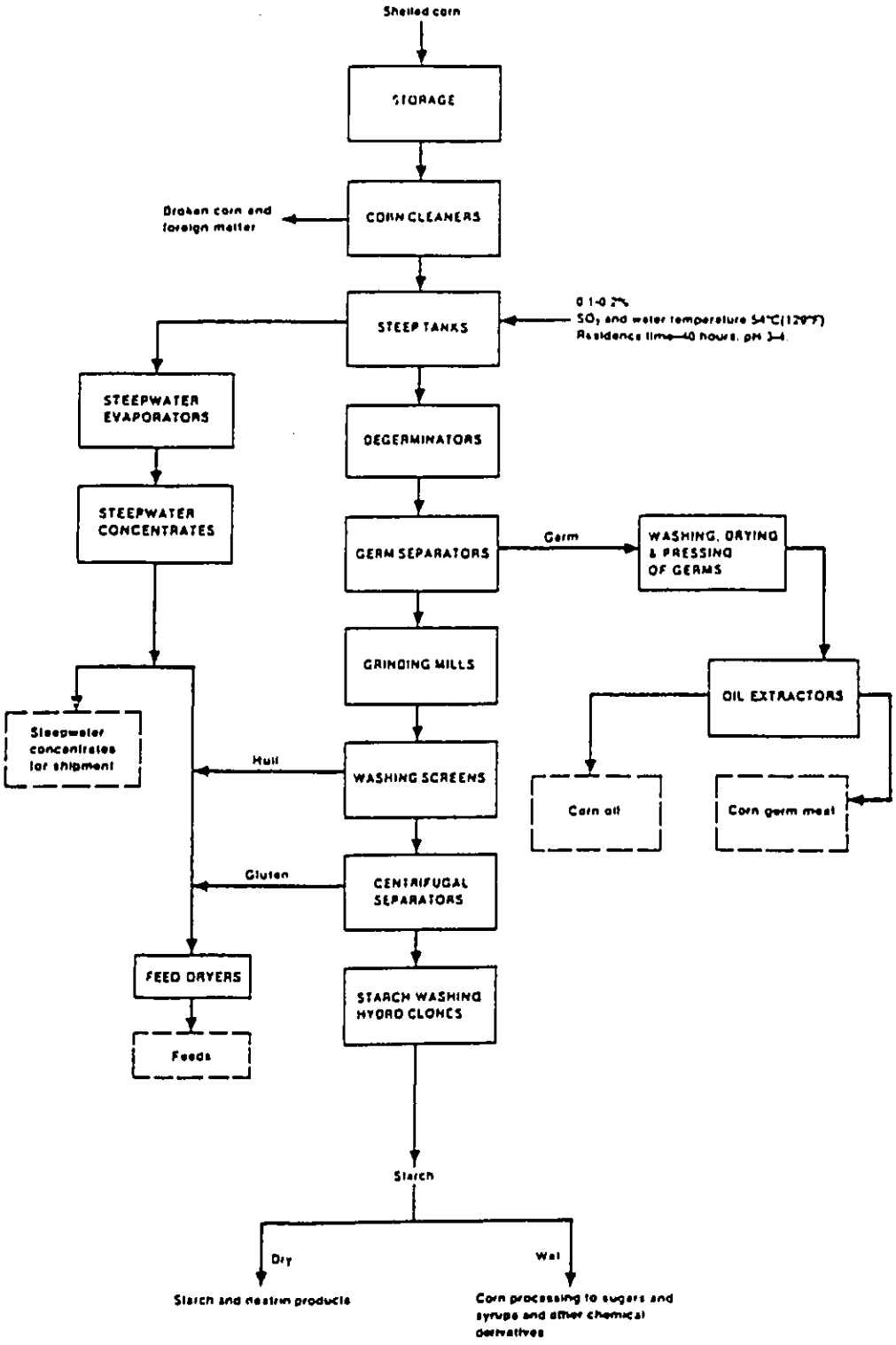

Figure 2.2: Corn wet milling process (Joglekar et al., 1983)

2.1.2 Refining Process

In the refining process (Figure 2.3), the starch slurry from the holding tank flows to one of the batch rectors, where continuous liquefaction is accomplished. The product from the liquefaction step is composed of soluble, higher saccharids, which are passed to the saccharification tank. Saccharification takes place in a batch reactor, where starch hydrolysis is completed to produce glucose. The saccharified liquor, or glucose syrup, is filtered to remove impurities. The resulting clarified liquor is passed through granular activated carbon and diatomaceous earth to remove remaining impurities and unacceptable colour. It is then cooled and passed through an ion-exchanger to remove unwanted salt. The liquor then enters the evaporator, where it is concentrated to 60% solids. Glucose-to-fructose conversion is achieved via isomerization through the action of the glucose isomerase enzyme. This is followed by carbon and ion-exchange refining and evaporation. The final product is known as 42% HFCS.

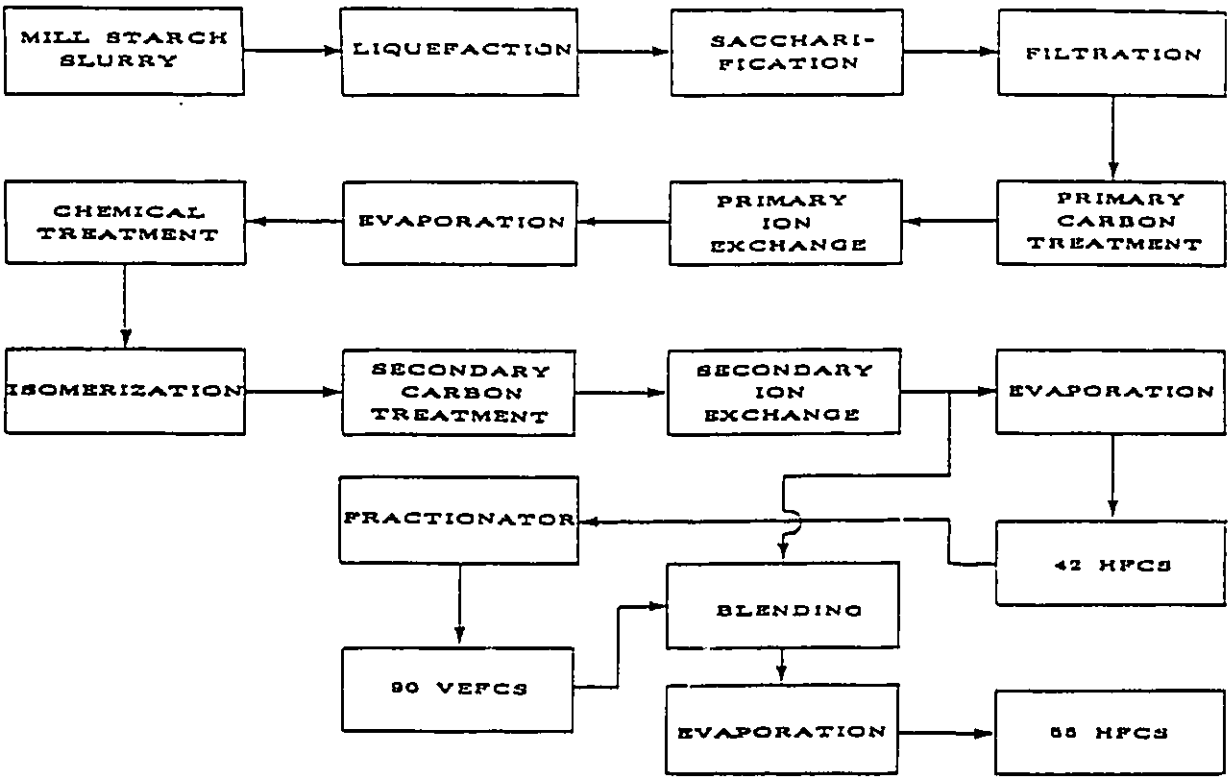

Figure 2.3: Refining process

Two other types of HFCS are also produced in North America: 55 and 90% HFCS. These are termed second-generation HFCS products. The 90% HFCS is obtained by ion-fractionation of 42% HFCS before evaporation. The 90% HFCS fructose is blended with 42% HFCS to obtain 55% HFCS.

HFCS has two major limitations: first, it is available only in liquid form, which limits its application to industrial use and increases the cost of transporting the product; secondly, if

HFCS is not properly stored it can partially crystallize.

HFCS has been used extensively in the beverage industry in products such as carbonated drinks, fountain syrups and flavour concentrates. The second largest use of HFCS is in cereal and bakery products, followed by the food processing industry and finally, dairy and miscellaneous food uses (Clerman, 1983).

Second-generation HFCS, containing 90% fructose, is much sweeter than the original 42 HFCS. This product is therefore potentially useful in low-calorie and dietetic products. However, the methods currently used in industry for the separation of glucose and fructose are expensive. Solid fructose and 90% HFCS would be more competitive with other sweeteners on the market if the cost of separation were reduced.

2.2 Separation of Glucose and Fructose

The isomerization of glucose to fructose is a reversible reaction with an equilibrium constant of about 1.0 at 60°C. Thus one would expect to obtain a fructose level of about 47-48% at equilibrium, starting from a feedstock containing 94-96% glucose. However, the reaction rate near the equilibrium value is so slow that it is prudent to terminate the reaction at a conversion level of 42% fructose to achieve practical reactor residence times (Coker and Venkatasubramanian, 1987). In order to obtain products with higher levels of fructose, it is necessary to selectively concentrate the fructose. A number of methods of chemical separation of glucose and fructose have been developed based on crystallization, precipitation, complex formation and on chromatographic procedures.

The separation of glucose and fructose can be achieved readily by the precipitation of

the sodium chloride-glucose double-salt from solutions containing fructose, glucose, and a stoichiometric amount of sodium chloride (Tatuki, 1972). This process requires careful monitoring of the pH and concentration of the components of the solution. The glucose can be recovered by dissolving the double-salt in water and deionizing. However, the pH required during the concentration procedure is 9.0, and it is possible that some alkaline isomerization may occur, as well as alkaline degradation, which contaminates the fructose solution.

Fructose can be precipitated as a calcium-sugar complex and then liberated with phosphoric acid (Bichsel et al., 1981). A similar type of separation has been carried out by using calcium chloride to complex with fructose and then removing the complex using electrodialysis. This process requires the use of ethanol to effect the separation of the fructose from the calcium chloride (Barker and Petch, 1985).

For the large-scale purification of fructose, there exits basically two different commercial processes. In both instances resins in the preferred cationic form are used in packed bed systems. One process employs an inorganic resin leading to a selective molecular absorption of fructose whereas the second uses chromatographic fractionation with organic resins (Coker and Venkatasubramanian, 1987).

The available methods for glucose and fructose separation are both complex and costly. These costs could be considerably reduced if the glucose were converted to a non-sugar compound which was more readily separated from fructose. This could be achieved by the use of microorganisms which selectively remove glucose only.

2.3 Selective Conversion of Glucose

The production of enriched fructose syrups from glucose-fructose mixtures can be achieved by the selective conversion of glucose. This has been achieved by a number of microorganisms often producing a valuable by-product during the process. In addition to a cost reduction for fructose purification, the production of a valuable by-product renders these processes more profitable.

One of the first microorganisms used for the selective conversion of glucose was *Tricholoma nudum*, which hydrolyses sucrose to glucose and fructose and then preferentially consumes glucose (Reusser et al., 1960). The by-product from this process is high protein cells which can by separated from solution by filtration. The microorganisms did consume a certain amount of fructose however, and this consumption increased when glucose was no longer present in the medium. Fructose consumption also increased with an increase in sucrose concentration.

Ueng et al. (1982) investigated the use of *Mucor sp. m105* and *Fusarium sp. F5* in the production of fructose from sucrose and glucose-fructose mixtures. *Mucor sp.* could not utilize sucrose for cell growth and fermentation. *Fusarium sp.*, on the other hand, could hydrolyse sucrose and produce ethanol. In mixtures containing equal concentrations of glucose and fructose, both microorganisms consumed glucose at a higher rate. However, 50% of the initial fructose was consumed by *Mucor sp.* in the time required for total consumption of the glucose.

Doelle (1982a) identified two separate enzymes responsible for glucose and fructose uptake in *Zymomonas mobilis*. These enzymes were identified as glucokinase and fructokinase respectively. Doelle (1982b) also found that fructokinase was inhibited in the presence of

glucose. *Zymomonas* grown in a glucose solution will produce a high concentration of ethanol with negligible amounts of by-products. However, in the presence of sucrose or glucose-fructose mixtures, the formation of sorbitol from fructose is observed, resulting in reduced ethanol yields. It was suggested that the formation of sorbitol was a consequence of the inhibition of fructokinase by glucose (Viikari, 1984a; 1984b). In the simultaneous production of fructose and ethanol from sucrose, *Z. mobilis* hydrolyses sucrose faster than either glucose or fructose are consumed. This results in an accumulation of glucose and fructose in the medium which subsequently enables the production of sorbitol. Mutants of *Z. mobilis* with higher glucose consumption rates have been developed by Doelle and Greenfield (1985a, 1985b) and Suntinanaler et al. (1986). These mutants consumed fructose at a much lower rate due to the reduced glucose accumulation in the medium. Bringer-Meyer et al. (1985) and Edye et al. (1989) studied mutants which preferentially consumed glucose over fructose. Bringer-Meyer et al. (1985) found that when the mutant was grown in media containing sucrose or a mixture of glucose and fructose, glucose was consumed at a much higher rate than fructose. However, a considerable amount of fructose was converted to sorbitol. The use of fed-batch cultures of the mutant enabled Edye et al. (1989) to obtain high concentrations of ethanol and fructose from sucrose with minimal production of sorbitol.

Another microorganism which selectively ferments glucose is a mutant of *Saccharomyces cerevisiae*. There are three enzymes present in the wild strain of *S. cerevisiae* which catalyze hexose phosphorylation: hexokinase A, hexokinase B, and glucokinase. Hexose phosphorylation is the first step in the metabolism of sugars in the cell. Hexokinase A and B have a phosphorylation ratio of fructose to glucose of 2.5 (Gancedo et al., 1977) and 1.3 (Colowick,

1973) respectively. Glucokinase exhibits no activity on fructose (Maitra, 1975). Mutants lacking hexokinase A and B were obtained by treating the wild strain with N-methyl-N'-nitro-N-nitroguanadine (Maitra, 1970). The mutant grew on glucose at a similar rate as the wild strain, however it was unable to grow on fructose. Preliminary tests with this mutant were carried out by Lamarche (1988). Ethanol and fructose were produced from media containing glucose and fructose concentration of up to 200 g/L each. The cells were also immobilized in a vertical packed bed reactor using Ca-alginate beads for the continuous production of ethanol and fructose. Fructose was not consumed by the mutants, however its presence inhibited the fermentation rate.

A more detailed study of the *S. cerevisiae* mutant was carried out by Koren (1990). Batch fermentations showed that the growth, glucose consumption and ethanol production rate for the mutant strain were lower than those of the wild strain. Fructose consumption was much lower than glucose consumption by the mutant strain, however both carbohydrates inhibited the fermentation rate equally. The production of fructose and ethanol was also carried out using hydrolysed Jerusalem artichoke juice supplemented with glucose or High Fructose Corn Syrup. Models formulated for these batch fermentations indicated a linear inhibition of the growth, glucose consumption and ethanol production rates by carbohydrate and ethanol. The substrate and product inhibition had a stronger effect on the growth than on the glucose consumption and ethanol production rates.

The mutant yeasts were also immobilised in Ca-alginate beads and placed in a vertical packed bed reactor. The reactor was fed with a glucose-fructose medium containing 100 g/L of each (Koren and Duvnjak, 1989) and with a solution of hydrolysed Jerusalem artichoke juice

supplemented with glucose (Koren and Duvnjak, 1990). Immobilization of the cells allowed for continuous production of very enriched fructose syrup for a long period of time with no loss in activity. Fructose consumption was not observed in the batch fermentations, however some fructose was consumed by the immobilized cells. The fructose/glucose consumption rate ratio increased with decreasing outlet glucose concentration. The formation of a product containing both a high ethanol and fructose concentration was not possible in the immobilized cell reactor due to substrate inhibition. These inhibitory effects were reduced by the use of a fed batch process. The process was fed with 42 HFCS and was found to be the best alternative for the formation of a product containing high fructose and ethanol concentrations.

A comparison of the microorganisms used for selective fermentation of glucose is summarised in Table 2.2. The most important factor to be considered is the fructose yield since it is the major product of this process. The highest fructose yields were obtained by *Fusarium sp F5*, *Z. mobilis* ATCC 53431, and *S.cerevisiae* ATCC 36859. The *Z. mobilis* ATCC 53431 was able to use a high sucrose concentration (390 g/L) and create a product with a high fructose concentration (172 g/L) with only 5% of the initial fructose consumed. The ethanol productivity was 2.2g/Lh , however, the yield was only 62% due to the formation of sorbitol. *F.sp F5* had a higher ethanol yield (70%) however the productivity was only 0.35 g/Lh and the fructose concentration in the product was much lower (50 g/L). *S. cerevisiae* ATCC 36859 had the highest fructose concentration in the product with an ethanol yield of 99% and an ethanol productivity of 12.6 g/Lh. This microorganism seems to be the best suited for the selective fermentation of glucose from a glucose-fructose mixture. As well, ethanol is produced at theoretical yields and in sufficient amounts to be recovered as a valuable by-product.

Table 2.2: Microorganisms used for the production of fructose by the selective fermentation of glucose.

Microbe	Feed (g/L)	Product (g/L)	F yield (%)	E yield (%)	P2 (g/Lh)	Reference
T. nudum	S-80	F-30	75	-	-	Reusser et al. (1962)
F. sp F5	S-100	F-50 E-18	100	70	0.35	Ueng et al. (1982)
M. sp M105	F-70 G-70	F-38 E-42	54	81	0.88	Ueng et al. (1982)
Z. mobilis ATCC 53431	F-50 G-50	F-35 E-22 So-10	70	86	2.0	Bringer-Meyer et al. (1985)
Z. mobilis ATCC 39676	S-372	S-49 F-141 G-84 E-44 So-11	87	89	0.62	Suntinanalert et al. (1986)
Z. mobilis	S-158	F-70 E-49 S-6.5	78	108	4.1	Edye et al. (1989)
S. cerevisiae ATCC 36589 (batch)	F-129 G-63	F-129 E-24 G-12	100	91	2.8	Koren (1990)
S. cerevisiae ATCC 36859 (continuous)	F-98 G-101	F-97 E-35 G-33	99	99	12.6	Koren and Duvnjak (1989)

S=Sucrose
F=Fructose
G=Glucose
E=Ethanol
So=Sorbitol
P2=Ethanol productivity

2.4 Immobilized Cells

The application of immobilized living microbial cells as biocatalysts represents a rapidly growing trend in microbial technology. Whole-cell immobilization may be defined as the physical confinement or localization of intact cells to a certain defined region of space with the preservation of some desired catalytic activity. Immobilized cells exhibit many advantages over free cell systems. However, successful microbial cell immobilization and long term stability of biocatalyst during continuous operation greatly depends on proper support as well as on proper method chosen for cell immobilization.

2.4.1 Classification of Immobilized Cell Systems

Whole cell immobilization methods can be divided into four major categories based on the physical mechanisms involved in the immobilization: attachment to a surface; entrapment within a porous matrix; containment behind a barrier and self aggregation.

Any immobilization method in which cells are bound to a surface, regardless of the type of binding, can be classified as a surface-attached process. The thickness of the cell layer may range from a monolayer of cells to a film one millimetre or more in depth. Systems in which cells are immobilized by adsorption are popular due to the ease of this type of immobilization. The strength with which the cells are bonded to the support varies with cell type and support type (Fletcher et al., 1980), thus the method is more useful for some systems than for others. With the adsorption method, there is no barrier between the cells and the solution in the system, therefore the effluent may not be cell-free. The depth of the biofilm often varies, especially with feed flow rate and is not readily determined. Thus, it is often difficult to accurately control

biofilm processes, such as those used in sewage treatment. Even with these drawbacks this type of immobilization has been widely employed in industry. The process can by enhanced by chemically bonding the cells to a surface by a variety of methods which include cross-linking by glutaraldehyde, silanization to a silica support and chelation to metal oxides (Navarro and Durand, 1977). Many types of porous materials have been used to adsorb cells. These include cordierite, bricks, kieselguhr, volcanic rock and various types of ceramics (Gohmmidh et al., 1982; Parascandola et al., 1982).

The second major category of cell immobilization is entrapment within a porous matrix which is synthesized in situ around the cells to be immobilized. A variety of compounds can be gelled into hydrophillic porous matrices under conditions mild enough to allow cell entrapment with a minimal loss of viability. A variety of polymer matrices have been used to immobilize whole cells, including k-carrageenan, agar and alginate gels (Birnbaum et al., 1983). Gel entrapment is a relatively straightforward immobilization technique and can be used with a variety of systems.

The third major category of cell immobilization is containment behind a barrier. Again, this barrier can be preformed, or formed around the cells to be immobilized. The barrier which immobilizes the cells can be as simple as the liquid/liquid phase interface between two immiscible fluids. Mohan and Li (1975) immobilized the denitrifying bacteria, *Micrococcus denitrificans*, by emulsifying the cell suspension with a surfactant into a hydrocarbon solvent. Microencapsulation has been applied as a method for whole cell immobilization. This technique was first used in biotechnology for the immobilization of enzymes. The growth of cells behind a preformed semi-permeable membrane is another method of whole cell immobilization behind

a barrier which was pioneered with the immobilization of enzymes (Rony, 1971). Since the cells are retained behind a semi-permeable wall or membrane, this type of system is very similar to microencapsulation in an engineering sense.

Self-aggregating cells fall into the fourth major category of cell immobilization. Cells that naturally aggregate or flocculate can also be considered immobilized with the scope of the definition of immobilization presented earlier. The large size of the aggregates makes their use possible in reactors designed for immobilized cells, such as fluidized beds and continuous stir tank reactors. Cell types that belong in this category include yeasts and molds which naturally form pellets in culture. Artificial flocculating agents or cross-linkers may be added to enhance the process of aggregation for cells that do not naturally flocculate. It has been shown that similar mechanisms govern both artificially and naturally induced flocculation (Tenney and Verhoff, 1973).

2.4.2 Biological Properties of Immobilized Cells

There are many examples in the literature showing that cellular physiology and morphology sometimes change upon immobilization. There are a number of possible reasons for this. The immobilization procedure can alter the metabolic activity or viability of the cells since the microenvironment is often different than that of cells in a suspension culture. In addition, the physical stresses that closely packed growing cells exert on one another and on the support are not present in traditional fermentations.

A number of reports have appeared suggesting that certain unicellular microorganisms may exhibit an acceleration of their metabolic activities as a result of surface attachment.

(Helmstetter and Cooper, 1968; Bandyopdhyay and Ghose, 1982; Fletcher, 1979; Navarro and Durand, 1977). However, Fetcher and Marshall (1982) found that both increases and decreases in activity can be observed in undefined mixed cultures of surface attached cells. In a wide variety of cases the reaction behaviour of immobilized cells is essentially identical to the activity of free cells (Rittmann, 1982; Wang and Wang 1983; Scherer et al., 1981; Younes et al., 1984). Given the variability of the results reported, the only conclusion to draw is that under certain conditions the metabolic activity of cells at surfaces may be different from that of free cells.

The method of immobilization can reduce the culture viability. Some studies show that entrapment in a polyacrylamide gel destroys viability in 59-90% of cells (Wada et al., 1980; Koshcheyenko et al., 1983). Another investigation, however, indicates the respiration rate of a variety of cells was unchanged after entrapment in a variety of polymers (Nilsson et al., 1983). The retention of viability is dependent both on the microorganism and the gelation conditions (Koshcheyenko et al., 1983). Data on the retention of viability after entrapment behind a semipermeable membrane or adsorption onto a porous matrix show that the percent viability retained is very high. This is expected because the conditions of immobilization for both of these methods are very mild.

The permeability of the cell membrane can be changed upon immobilization, either as a side effect of the immobilization method or by design. The subject of cell permeabilization has been reviewed by Felix (1982). Intentional permeabilization of the cell membrane to increase production can succeed, but may also result in lower stability of the enzymes in the cell, perhaps due to the loss of enzyme from the cells through the membrane (Bang et al., 1983).

Immobilization affords whole cells some protection from physical and chemical

challenges. Takata et al. (1983a, 1983b) showed that the enzymatic activity of whole cells immobilized in k-carrageenan gel beads is more stable to environmental challenges than either free whole cells or native enzymes. These challenges include heat, pH changes, organic solvents of protein denaturing agents. It was suggested that the reason for the increased stability to the protein denaturing agents is the physical structure of the matrix which may hinder the action of the denaturing agents.

It is clear that immobilization affects cellular metabolism in at least some instances. The special stresses and environment to which an immobilized cell is subject need to be better understood so that the variables which affect the cell can be manipulated advantageously.

2.4.3 Advantages of Immobilized Cell Systems

Immobilized cell systems confer desirable properties to a biological process which are not readily achieved in conventional batch and continuous systems employing free cells. Although the advantages depend specifically on the method of immobilization, some generalizations can be made to illustrate potential benefits that may be achieved.

Immobilized cells offer the possibility of much greater cell concentrations within the bioreactor and therefore higher reaction rates may be obtained. Higher dilution rates can be achieved without fear of losing the microbial population by culture-washout, as in the case in a simple continuous stir tank reactor operating at similar dilution rates. Continuous processes employing immobilized cells are less susceptible to the effects of inhibitory compounds and nutrient depletion. The combined effects of high cell density and operation at high dilution rates reduces the risk of reactor shut-down due to microbial contamination. Depending on the method

of immobilization, the solution leaving the bioreactor may be relatively free of cells. This, therefore, eliminates the added energy and capital costs for the centrifuge unit, normally required in conventional free cell systems, for cell removal prior to distillation. In most immobilized cell systems, the presence of solid in dilute media are known to concentrate nutrients at the solid-liquid interface (Hattori, 1972). Thus the immobilized cells are exposed to much higher nutrient concentrations resulting in substantially higher reaction rates. Mass transfer may also be improved through an increase in the apparent density of microorganisms following immobilization. This results in greater differential velocities between the cells and the medium with attendant improvements in substrate diffusion rates and consequently higher reaction rates ensue (Engelbart and Engelbart, 1975).

It must be noted that not all of the above-mentioned merits are encountered in every immobilized cell bioreactor. In most cases, the advantages and limitations of a given bioreactor system depend on the method of immobilization and the reactor configuration used. The use of adsorbed yeast and bacterial cells for continuous production employs a cheap and simple method of retaining high cell densities without the use of expensive separation and recycle systems employed conventionally. Furthermore, the immobilization procedures maintain the cells in a viable state and do not require the use of toxic chemicals to induce adsorption. This process is, however, limited by two major constraints. Firstly, the amount of biomass that can be adsorbed by a unit of the carrier is limited by the surface area of the support particle. Secondly, the operational stability of the bioreactor system is restricted by the rate of desorption of cells from the support. The use of cross-linking agents has been used to minimise the desorption problem however, the toxic effects of some cross-linking agents may adversely affect cell activity or cell

viability.

Systems employing entrapped cells possess excellent properties particularly with respect to the overall performance of the bioreactor and its long term operational stability. Since high biomass loadings can be attained by cell entrapment, high ethanol productivities result. By the constant regeneration of cells, with nutrient addition and oxygen supply, high levels of ethanol productivities have been maintained for long periods of time (Linko and Linko, 1981). Alginate is also resistant to acidic and basic environments and is stable even at thermophilic fermentation temperatures (Windholz, 1976). The biomass loading that can be used in an entrapped cell system is however, limited by the fact that at high cell loadings there is a significant decease in gel strength (Cheetham et al., 1979; Krouwel et al., 1982). Entrapped cell systems are also subjected to mass transfer limitations imposed by the additional diffusion barrier created by the support matrix. In ethanol fermentation systems, CO_2 gas is also a major product. This gaseous product is characterized by its relatively low solubility in aqueous media and therefore the diffusion of the gaseous product out of the matrix can be rate-limiting (Krouwel and Kossen, 1980). If the diffusion of CO_2 out of the matrix is slower compared to its production, CO_2 will accumulate within the entrapment matrix and eventually result in its disruption. A major disadvantage of the use of calcium alginate as an immobilization support is that moderate concentrations of calcium chelating agents and certain cations such as phosphates, EDTA, Mg^{2+} and K^{+} disrupt the gel by solubilizing the bound Ca^{2+} (Cheetham, 1979). This results in a loss of the mechanical stability of the gel and finally to its complete disintegration.

Adsorption of yeast cells onto wood blocks provides an inexpensive and simple method of immobilization. The porosity and low lignin content of oak wood makes it a good candidate

for this purpose.

2.5 Ethanol Fermentation

Yeasts are the most commonly used microorganisms for ethanol production. Selected high-productivity strains come from the species *Saccharomyces cerevisiae*, *Sacch. uvarum*, *Sacch. anamensis*, *Schizosaccharomyces pombe* and *Candida utilis*. Although other microorganisms produce ethanol, they also yield significant quantities of undesirable byproducts. Yeasts generally produce ethanol with high selectivity with only traces of byproducts (Atkinson and Mavituna, 1983).

Raw material costs make up 70% of the final ethanol sale price, but substantial savings are still possible when improved methods are used superseding traditional batch fermentation.

2.5.1 Metabolic Pathway

The fermentation mechanism was first quantified by Gray-Lussac, based on the stoichiometric conversion of a hexose sugar into ethanol and carbon dioxide:

$$\underset{\substack{\text{hexosesugar}\\ 180}}{C_6H_{12}O_6} \rightarrow \underset{\substack{\text{ethanol}\\ 92}}{2C_2H_5OH} + \underset{\substack{\text{carbondioxide}\\ 88}}{2CO_2}$$

Therefore: 1.0g hexose sugar = .51g ethanol + .49g carbon dioxide

This theoretical ethanol yield at 51% by weight is termed the Gray-Lussac coefficient, and represents the basic datum in conversion efficiency (Jackman, 1987).

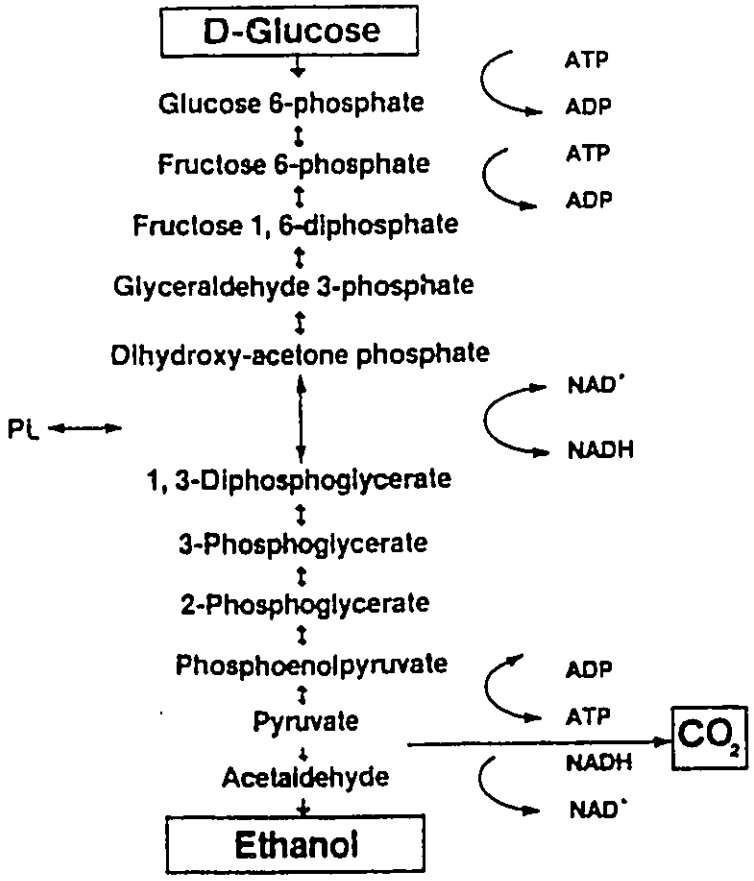

Figure 2.4: Biochemical pathway for the breakdown of glucose (Stewart et al., 1983).

The biochemical pathway for the breakdown of glucose can be divided into three major parts (Figure 2.4). The first part is a series of preparatory rearrangement reactions that do not

involve oxidation-reduction, and lead to the production of the key intermediate, glyceraldehyde-3-phosphate. In the second part, an oxidation-reduction reaction occurs, high-energy phosphate bond is produced in the form of ATP, and pyruvate is formed. In the third part, a second oxidation-reduction reaction occurs and the fermentation products ethanol and CO_2 are released. The biochemical pathway to pyruvate is called glycolysis, and it is also sometimes called the Embden-Meyerhof pathway, after two of its discoverers (Brock et al., 1984).

2.5.2 Free Cell Processes for Ethanol Production

Both batch and continuous processes are employed for ethanol production, and many of these incorporate some form of cell recycle. As well as reducing the fermentation time, cell recycle reduces the amount of substrate converted to cell matter. Conventional fermentation is by batch methods (Maiorella, 1984), which provide the best benchmark for comparison of newer process alternatives. Table 2.3 is a comparison based on a typical 10^9 L/h ethanol plant capacity and using molasses feed. A simple continuous stir tank reactor (CSTR) has only a slightly higher productivity than the batch fermentation. Recycle of cells to increase the CSTR cell density increases productivity and reduces costs (Cysewski and Wilke, 1978). A portion of the cells is retained, and the cell density in the fermenter is increased by a factor of 2.54. The use of CSTR fermenters arranged in series has been suggested to overcome end-product inhibition effects (Ghose and Tyagi, 1979; Rosen, 1978). For two reactors, the first is operated at an intermediate ethanol product concentration and high corresponding specific productivity. The second reactor, producing the final high concentration ethanol product, is left with less sugar to convert and the overall productivity may be increased. The perforated plate tower fermenter has

been suggested as a simple means to provide several fermenters in series (Falch and Gaden, 1969). A distillation column is divided into a series of fermentation zones separated by perforated plates and fitted with a single agitator shaft for all. Reaction takes place progressively as sugar ferments at the top level and sequentially overflows down to lower levels. In the plug flow fermenter (Moreno and Goma, 1979) cell mass and ethanol concentration both increase continuously along the reactor length. Cells must be recycled to the reactor inlet to initiate the reaction.

Table 2.3: Comparison of free cell ethanol fermentation processes

Process	Feed sugar concentration (g/L)	Volumetric productivity (g/Lh)	Reference
Batch	16.7	11.8	Maiorella (1984)
CSTR	15.6	14.1	Cysewski and Wilke (1978)
Recycle CSTR	16.7	42.5	Cysewski and Wilke (1978)
Partial recycle	15.6	35.9	Hough et al. (1962)
2 series CSTR	16.7	17.9	Ghose and Tyagi (1979)
Perforated plate column	16.9	9.0	Falch and Gaden (1969)
Plug Flow	16.7	52.7	Moreno and Goma (1979)

The advantages of continuous fermentation are: reduced fermenter size; greater ease in operation; utility requirements are constant, giving a greater economy in usage; high

productivity; and reduced manpower. Batch fermentation has the following benefits: higher final product concentration; less reliance upon automatic controls; and overall cheapness and simplicity in plant construction and maintenance (Jackman, 1987).

2.5.3 Immobilized Cell Systems for Ethanol Production

The various methods for immobilization of whole cells are widely utilised for the production of ethanol.

Berdelle-Hilge was issued a U.S. patent in 1973 for the continuous production of beer from wort using a bioreactor which consisted of a bed of yeast cells held between two porous support plates through which a fermentable substrate was pumped under pressure. The exit from the reactor was a yeast-free, fermented product. Grinbergs et al. (1977) employed a continuous bio-reactor, with a plug formed from yeast cell mass and diatomaceous earth, to produce ethanol from glucose. This plug fermenter could operate at fermentation rates several times higher than the simple batch systems. However, they do suffer from plugging problems and need constant regeneration of the yeast cells under aerobic conditions. High cell densities may also be maintained within dialysis bioreactors. A simple continuous dialysis bioreactor was developed by Gerhardt and Gallup (1963) which used a dialysis membrane to separate the fermentation zone from the nutrient reservoir. Another approach that has been employed to achieve high rate dialysis involves the use of hollow fibre bioreactors. The hollow fibre bioreactor consists essentially of several hundred fine hollow membrane fibres packed into a shell. The shell side is inoculated with growing cells. Workers with this system believe that cell concentration of up to 155 g/L and productivities as high as 70 g/L may be achieved when fermenting glucose to

ethanol (Wilke and Maiorella, 1981). Hollow fibre bioreactors are however complex and costly and carbon dioxide venting and membrane plugging may be encountered. Due to the inherent limitations of mechanical cell containment systems, other methods of cell immobilization are being investigated for the production of ethanol.

A variety of supports have been utilized for the adsorption of yeast for the continuous production of ethanol. Thus yeast cells have been adsorbed on to wood chips, sawdust, ion exchange resins, porous brick, silica gel, polystyrene beads and cotton fibres. Moo-Young et al. (1980) evaluated the suitability of beech wood chips for the adsorption of yeast cells and subsequently used it for the production of ethanol. *S. cerevisiae* cells were adsorbed on to treated beech wood chips by circulating a concentrated cell suspension through a vertical packed bed column. The bioreactor was operated continuously for at least 30 days and during this period cell retention was found to be virtually 100%. The continuous production of ethanol using yeast cells adsorbed on to wood chips was also studied by Gencer and Mutharasan (1981) and Ryu et al. (1982). Adsorption of *S. cerevisiae* cells on sawdust particles has been evaluated by Michaux et al. (1982) and anion exchange resins have also been employed to adsorb the negatively charged yeast cells (Daugulis et al., 1981). Minier and Goma (1982) performed ethanol fermentation with high sugar concentration (260 to 409 g/L) feeds by extractive fermentation employing yeast cells adsorbed onto porous brick particles in a vertical packed column.

In order to circumvent the problems associated in adsorbed cell systems, namely, cell desorption due to weak cell-solid interactions, the use of cross-linking agents and covalent bonding of cells to inert supports has been investigated. Glutaraldehyde, a common cross-

linking agent, has been known to react mainly with glycine residues within the protein structure and therefore may react readily with the protein present in the lipid-bilayer of cell membranes. By adsorbing gelatin to an inert support, a reactive base for glutaraldehyde is provided. This allows a covalent link to be formed between the microbial cells and the gelatin support, by way of glutaraldehyde. This method has been used successfully to immobilized yeast cells for the production of ethanol (Griffith and Compere, 1976; Sitton et al., 1981). However, it has been observed that live cells of *S. carlsbergensis* attached to glutaraldehyde treated silica appear to have lower cellular activity when compared to cells attached to untreated silica (Navarro and Durand, 1977). Although a number of other techniques of the covalent attachment of cells are known, most of these appear to cause cell death.

Many studies have been performed on the production of ethanol using entrapped cells. Ethanol production using alginate entrapped cells was reported by Kierstan and Bucke (1977). Cells of *S. cerevisiae* were entrapped in fibres of calcium alginate packed in a vertical column. The bioreactor was operated for a total of 24 days and the half life of the system was calculated to be approximately 10 days. Linko and Linko (1981) entrapped cells of *S.cerevisiae* in calcium alginate gel and employed a packed bed bioreactor for the continuous production of ethanol from sugar cane molasses and a 4.5% glucose medium. Williams and Munnecke (1981) also studied the continuous production of ethanol employing a packed bed bioreactor under optimized parameters derived from batch immobilized yeast cell experiments. Nutrient addition was found to be necessary after 18 days of operation in order to maintain high cell activity for a total period of 44 days. The incorporation of oxygen in high cell density fermentations is essential for maintenance of cell viability (Nagodawithana et al., 1974). However, under these

conditions, the rate of ethanol production is retarded presumably due to a shift towards aerobic metabolism. Using alginate entrapped yeast cells, Hahn-Hagerdahl and Mattiason (1982) have shown that by introducing a respiratory inhibitor (e.g., sodium azide) in the fermentation medium, the shift towards aerobic metabolism in the presence of oxygen is prevented.

Wada et al. (1980) studied the continuous production of ethanol employing carageenan entrapped yeast cells. A constant supply of nutrients was essential in maintaining a steady state ethanol productivity for a period of 3 months.

The continuous production of ethanol using entrapped cell systems has been largely restricted to the use of calcium alginate and carrageenan as the immobilization matrices. Other matrices that have been used for the entrapment of live yeast cells performing ethanol fermentation include, epoxide (Klein and Kressdorf, 1982) and gelatin (Siva Raman et al., 1982).

The ethanol productivities obtained using the different methods of immobilization are summarised in Table 2.4.

The adsorption of yeast cells onto wood chips provides an inexpensive and simple method of immobilization which has been used successfully for ethanol fermentation. The porosity and low lignin content of oak wood make it a good candidate for this purpose. Problems encountered in entrapped cell systems, due to the barrier created by the support matrix, are not present in adsorbed cell systems. The immobilization of yeast cells onto wood blocks should therefore created a good environment for high ethanol productivity.

Table 2.4: Ethanol productivities attained by the different methods of cell immobilization.

Process	Carbohydrate conc. in feed (g/L)	Productivity (g/Lh)	Reference
Hollow Fibres	-	70	Wilke and Maiorella (1981)
Wood chips in vertical packed column	130	25.2^{+}	Gencer and Mutharasan (1981)
Sawdust in vertical packed column	100	60$^{\bullet}$	Michaux et al. (1982)
Anion exchange resin	120	53.1$^{\bullet}$	Dauglis et al. (1981)
Gelatin coated Raschig rings + glutaraldehyde in vertical packed column	30	15.9^{+}	Sitton et al. (1981)
Ca-alginate beads in vertical packed column	127	17^{+}	Williams and Munneke (1981)
Ca-alginate beads in horizontal packed bed	197	46$^{\bullet}$	Shiotani and Yamane (1981)
Carrageenan beads in vertical packed column	100	43$^{\bullet}$	Wada et al. (1979)

$^{\bullet}$ based on packing volume
$^{+}$ based on total reactor volume

2.5.3 Growth Factors

Changes in ethanol concentration induce observable alterations in yeast membrane physiochemical properties. Central to understanding the biological effects of ethanol on a microbial system is knowledge of the cellular lipids. In yeast, it is suspected that free and

esterified fatty acids and sterols are principal components required for functional membranes and are largely responsible for the membrane physiochemical properties (Jones, 1989a). It has been shown that sterols and unsaturated fatty acids lead to improved ethanol tolerance of yeast by their incorporation in the cell membrane (Thomas et al., 1978), and that these growth factors were also necessary for fermentation to continue after yeast growth has ceased (Larue et al., 1980). These compounds allowed reacting cells to maintain their viability and hence their fermentative ability. In industry, aerobic "starters" are used in batch alcohol fermentation (Jones et al., 1981) to increase viability and overall fermentation rates. It is believed that these aerobic cells have built up reserves of unsaturated fatty acids and sterols. It may be deduced that the incorporation of unsaturated fatty acids and sterols into the cell membrane of the yeast, increased its permeability to ethanol. Where unsaturated fatty acids and sterols are not provided in the medium, it has been suggested that these could be synthesized by the yeast when oxygen was available but not in the total absence of oxygen (Cysewski, 1976). Hoppe et al. (1984) showed that micro-aerobic conditions enhanced the utilisation of substrate by increasing the ethanol tolerance of the yeast without any significant decrease in the ethanol yield per unit substrate consumed. Contrary to many earlier reports, Watson (1982) demonstrated that high ethanol production in yeasts was dependent on the presence of unsaturated fatty acid residues and not on the presence of ergosterol.

The maintenance of specific yeast lipid constituents is therefore important for continuous high alcohol productivity. The addition of oleic acid to an anaerobic system has been shown to increase the ethanol tolerance and productivity of yeast cells (Watson, 1982). This unsaturated fatty acid may therefore increase the glucose consumption in the production of fructose and

ethanol from glucose\fructose mixtures.

2.6 Sorbitol Production

Sorbitol, which was first isolated in 1872 by Joseph Boussingault, a French chemist, from the berries of the mountain ash tree, is found in a variety of fruit and marine vegetation. It has been used as a sweetening agent for diabetics since it was reported in the 1920s that moderate amounts of sorbitol taken by normal diabetic subjects caused only a small and insignificant rise in the blood sugar concentration. In addition, sorbitol is used in the production of polyethers for the manufacture of urethane foams and as a raw material for the synthesis of resins which are utilized as surface coating and printing-ink vehicles (Dwivedi, 1991).

Although sorbitol occurs in significant quantities in a number of fruit and berries, it is not commercially economical to extract them from these sources. Conventionally, polyalcohols are produced primarily by hydrogenation of corresponding reducing sugars. Glucose and starch are preferred raw materials for the production of sorbitol. If a material other than glucose is used, it is first hydrolysed to glucose. Glucose is then hydrogenated at 120-160°C at hydrogen pressure of 70-140 atm in the presence of a supported nickel catalyst (Dwivedi, 1991).

The production of sorbitol by cells of the ethanol-producing bacterium *Z. mobilis* has been reported when *Z. mobilis* was grown in batch culture on sucrose or a mixture of glucose and fructose (Viikari, 1984; Barrow et al., 1984). Growth of *Z. mobilis* on sucrose in batch and continuous culture has shown that hydrolysis of sucrose is faster than utilization of glucose and fructose (Lyness and Doelle, 1981). Doelle (1982a) reported that inhibition of fructokinase occurs with glucose. This led Barrow et al. (1984) to suggest that due to the inhibition of

fructokinase, free intracellular fructose may be reduced to sorbitol via a dehydrogenase type enzyme. A possible metabolic pathway for the production of sorbitol was suggested by Leigh et al. (1984). An enzyme system described as a glucose/fructose transhydrogenase was identified which was capable of oxidizing glucose to gluconic acid concomitant with the reduction of fructose to sorbitol. This enzyme system was later identified as glucose-fructose oxidoreductase tightly coupled with the co-factor NADP (Zachariou and Scopes, 1986; Chun and Rogers, 1988).

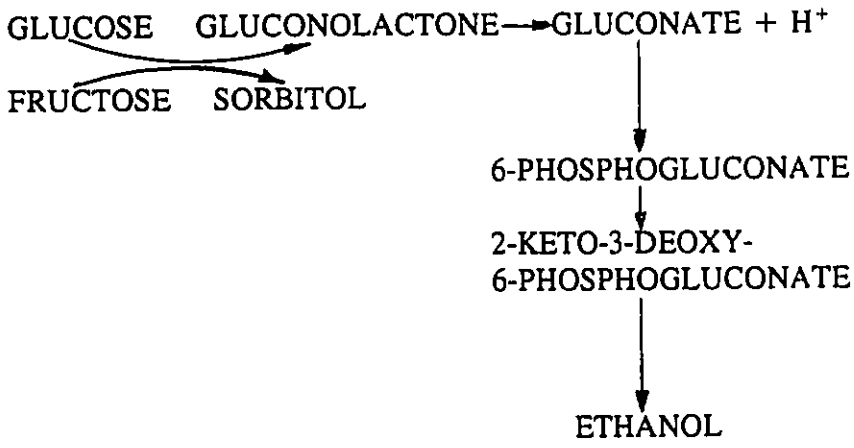

Figure 2.5: Mechanism of sorbitol/gluconic acid production by *Z. mobilis* (Chum and Rogers, 1988).

Chun and Rogers (1988) studied the production of sorbitol and gluconic acid by toluene-treated, permeabilized cells of *Z. mobilis*. The pathway from gluconate to ethanol is not functional if cells are fully permeabilized. High conversion efficiencies of glucose oxidation to gluconic acid and fructose reduction to sorbitol were obtained in both a free cell batch process and a continuous process with immobilized cells.

Duvnjak et al. (1991a) studied the production and consumption of sorbitol and fructose by *S. cerevisiae* ATCC 36859. The results showed that this mutant produced ethanol and sorbitol in a fructose medium but produced only ethanol in a glucose medium. When grown in a glucose-fructose medium, sorbitol was produced only after complete consumption of the glucose. Ethanol and fructose were produced from sorbitol by this strain when it was transferred from a fructose medium however, there was no sorbitol consumption when it was pregrown on glucose. Sorbitol was not produced by the wild strain of *S. cerevisiae* in either glucose or fructose medium. However, sorbitol was consumed and no fructose was produced by the wild strain regardless of whether it was pregrown on glucose or fructose. The use of Jerusalem artichokes for the production of sorbitol and ethanol by *S. cerevisiae* was also studied (Duvnjak et al., 1991b). Jerusalem artichokes contain a large amount of fructose and some glucose in the form of inulin. When the Jerusalem artichoke juice was supplemented with yeast extract, a considerable amount of sorbitol was produced from fructose. This production began only once the glucose was totally consumed.

2.7 Mathematical Modelling

Kinetic modelling is an essential tool for fermentation research and development. The behaviour of any fermentation is impossible to test under every possible set of conditions without the use of reaction kinetics and mathematical techniques.

As ethanol production is growth associated, most researchers have found that models for growth rate closely follow ethanol productivity, although it is often found that cell growth is more strongly inhibited than ethanol productivity by substrate and product concentrations.

2.7.1 Growth Kinetics

A functional relationship between the specific growth rate and an essential compound's concentration was proposed by Monod in 1942.

$$\mu = \frac{\mu_{max} S}{K_s + S} \qquad (2.1)$$

where μ is the specific growth rate, μ_{max} is the maximum specific growth rate, S is the substrate concentration and K_s is the saturation constant.

Other forms of the model have been proposed for cases when growth is rapid and the exponential phase may not be present (Bailey and Ollis, 1986).

$$\mu = \mu_{max}\left(\frac{S}{K_s s_o + S}\right) \qquad (2.2)$$

and

$$\mu = \mu_{max}\left(\frac{S}{K_1 + K_2 s_o + S}\right) \qquad (2.3)$$

where s_o is the initial concentration of the substrate and K_1 and K_2 are empirical constants.

The following is another related form of specific growth rate dependence which has been proposed by Contois (1959):

$$\mu = \mu_{max} \frac{S}{Bx + S} \qquad (2.4)$$

where B is an empirical constant and x is the biomass concentration.

The Monod equation is probably a great over simplification, however in many cases it reasonably expresses the growth kinetics.

The specific growth rate is often affected by medium constituents such as substrate and product. The following is a summary of models which have been proposed for these inhibitions.

2.7.2 Substrate Inhibition

The importance of inhibition by carbon sources on cellular activity has been stressed by many researchers in the past. Some actions that can cause a reduction in the metabolic activities of a cell are: modification of chemical potential of substrates, intermediates, or products, alterations in cell's permeability, change in activity of one or more enzymes, and dissociation of one or more enzyme or metabolic aggregates (Edwards, 1970). A fundamental work on substrate inhibition on whole cell activity of yeasts and bacteria was performed by Aiba and coworkers (1968;1969). The following models for substrate inhibition have been used by various authors attempting to construct a best fit for their experimental data:

Edwards (1970)

$$\mu=\mu_{max}\frac{S}{(K_s+S)\,(1+S/K_i)}; \qquad \nu=\nu_{max}\frac{S}{(K_s+S)\,(1+S/K_i)} \tag{2.5}$$

$$\mu=\mu_{max}\frac{S(1+S/K)}{K_s+S+S^2/K_i}; \qquad \nu=\nu_{max}\frac{S(1+S/K)}{K_s+S+S^2/K_i} \tag{2.6}$$

Moser (1985)

$$\mu = \mu_{max} [\exp(-S/K_i) - \exp(-S/K_s)];$$
$$\nu = \nu_{max} [\exp(-S/K_i) - \exp(-S/K_s)] \qquad (2.7)$$

and Ghose and Tyagi (1979b)

$$\mu = \mu_{max} \left(1 - \frac{S_{tc}}{S_{max}}\right); \qquad \nu = \nu_{max} \left(1 - \frac{S_{tc}}{S_{max}}\right) \qquad (2.8)$$

where ν is specific ethanol productivity, ν_{max} is maximum specific ethanol productivity, K_i is an empirical constant and S_{tc} is total carbohydrate concentration.

The first two functions were formulated using various assumptions about enzyme-substrate complexing, whereas the last two were selected to determine whether an exponential or linear dependence on substrate concentration would provide a better fit for the experimental data.

2.7.3 Alcohol Inhibition

Ethanol inhibition of growth and productivity has been extensively studied and a number of models describing this inhibition have been proposed. The relationships suggested by various authors to quantify the growth and productivity as a function of ethanol concentration are summarized below.

Holzberg et al. (1967)

$$\mu = \mu_{max}(1 - P/P_{m\mu}); \qquad \nu = \nu_{max}(1 - P/P_{m\nu}) \qquad (2.9)$$

Aiba et al. (1968)

$$\mu=\mu_{max}exp(-K_{\mu p})\ ; \qquad \nu=\nu_{max}exp(-K_{\nu p}) \tag{2.10}$$

Novak et al. (1981)

$$\mu=\ \mu_{max}\left(\frac{K_{\mu p}}{P+K_{\mu p}}\right); \qquad \nu=\ \nu_{max}\left(\frac{K_{\nu p}}{P+K_{\nu p}}\right) \tag{2.11}$$

Levenspiel (1980)

$$\mu=\ \mu_{max}\left(1-\frac{P}{P_{m\mu}}\right)^{\eta}; \qquad \nu=\nu_{max}\left(1-\frac{P}{P_{m\nu}}\right)^{\eta} \tag{2.12}$$

where P is the ethanol concentration, $P_{m\mu}$ is the ethanol concentration above which cells do not grow, $P_{m\nu}$ is the ethanol concentration above which no ethanol is produced, $K_{\mu p}$ is an ethanol inhibition constant to growth, $K_{\nu p}$ is an ethanol inhibition constant to ethanol productivity and η is an experimental constant.

2.7.4 Combined Substrate and Product Inhibition

In order to determine a kinetic relationship, an analytical equation must be formulated for the specific growth and ethanol production rate, taking into account both substrate and product inhibition.

$$\mu=f(S)g(P); \quad \nu=f'(S)g'(P)$$

The combined effects of inhibition exerted by ethanol and high sugar concentrations during ethanol fermentation was described with the following equation by Ghose and Tyagi (1979a).

$$\mu = \mu_{max}\left(1-\frac{P}{P_{m\mu}}\right)\frac{S}{S+K_s+S^2/K_s\omega}$$

$$\nu = \nu_{max}\left(1-\frac{P}{P_{m\nu}}\right)\frac{S}{S+K_s+S^2/K_s\omega} \qquad (2.13)$$

where ω is the magnitude of interference of one substrate molecule on the binding of the second. Ruggeri et al. (1988) used the following relationship to describe ethanol production from lactose by *Kluyveromyces Fragilis* in an immobilised yeast reactor:

$$\nu = \frac{\nu_{max}S}{K_s+S}\ \exp\left(-\frac{S}{K_i}\right)\left(1-\frac{P}{P_{m\nu}}\right)^{\eta} \qquad (2.14)$$

Koren (1990) found that a linear relationship for substrate and ethanol inhibition best described the behaviour of the mutant of *S. cerevisiae* in batch fermentations as shown in equation (2.15).

$$\mu = \mu_{max}\left(1-\frac{S_{tc}}{S_{max}}\right)\left(1-\frac{P}{P_{max}}\right) \qquad (2.15)$$

These models will be tested for their efficiency in describing the behaviour of the mutant of *S. cerevisiae* immobilized onto wood blocks at different carbohydrate and ethanol concentrations.

Chapter 3

Material and Methods

Experiments were carried out on the yeast *Sacharomyces cerevisiae* ATCC 36859 (mutant strain) immobilized onto small oak wood blocks in a vertical packed bed reactor. This system was used for the selective fermentation of glucose in the production of very enriched fructose syrup from glucose-fructose mixtures. Experiments were preformed to determine the effects of total carbohydrate concentrations, added oleic acid and temperature on the fermentation system.

3.1 Experimental Media

The composition of the media used during this research are listed below.

Medium I: Solid medium for the maintenance of the yeast

- 10.0 g glucose
- 3.5 g peptone
- 4.5 g malt agar
- 18.5 g agar
- up to 1 litre distilled water

Medium II: Medium for inoculum preparation

- 10.0 g glucose
- 30.0 g yeast extract
- 3.5 g peptone
- 2.0 g KH_2PO_4
- 1.0 g $MgSO_4.7H_2O$
- 1.0 g $(NH_4)_2SO_4$
- up to 1 litre distilled water

Medium III: Medium for high-fructose syrup production in reactor

- variable glucose
- variable fructose
- 3.5 g peptone
- 2.0 g KH_2PO_4
- 1.0 g $MgSO_4.7H_2O$
- 1.0 g $(NH_4)_2SO_4$
- up to 1 litre distilled water

The media were sterilized in an autoclave at 115°C for 15 minutes. The initial pH of the media was 5.5.

Saccharomyces cerevisiae ATCC 36859 (mutant strain) was kept on agar slants (medium I) and transferred to fresh slants every two months.

Inoculum for the reactor was prepared by aseptically transferring a loopfull of cells from the agar slant into 500 mL Erlenmyer flasks containing 100 mL of medium II. These were placed into a rotary shaker at 33°C for 24-30 hours, at which point the cells were in the exponential growth phase.

3.2 Immobilized Cell Reactor

3.2.1 Immobilized Reactor System

Figure 3.1 is a schematic diagram of the immobilized reactor system used in this research. The reactor is a borosilicate glass tube (3.1 cm I.D.) surrounded by a plexiglass jacket. The temperature was kept constant at 33°C (except for temperature variation experiment) by circulating water through the jacket using a temperature controller. A probe was placed in the placed in the center of the reactor to monitor temperature. Since the feed was not preheated, there was a slight temperature variation from the bottom to the top of the reactor. However, the temperature difference was assumed to be insignificant due to the low dilution rate in the reactor. Metal screens confined wood blocks (0.5cm X 0.5cm X 0.5cm) to a 11.2 cm long section (84 mL) in the reactor. The feed was introduced through the bottom of the reactor using a peristaltic pump and the effluent exited from the top of the reactor. Due to the increase in biomass concentration in the reactor throughout the experiments, the dilution rates were calculated based on the total reactor volume (84 mL). The total reactor volume was also used in the study conducted by Koren (1990) with the mutant yeast immobilized in Ca-alginate beads.

3.2.2 Inoculation of Immobilized Cell Reactor

The reactor filled with wood blocks was sterilized at 120°C for 20 minutes. 400 mL of concentrated yeast suspension (50 g/L), prepared as described in section 3.1 for inoculum preparation, was recirculated through the reactor at a dilution rate of 0.1 h^{-1} for 24 hours in order to adsorb the cells onto the wood blocks. A feed containing 50 g/L glucose was then passed through the reactor in order to build up biomass. When a high biomass concentration was reached in the reactor and the glucose consumption and ethanol productivity reached steady state, the experiments were started by passing medium III through the reactor for the production of high fructose syrup and ethanol.

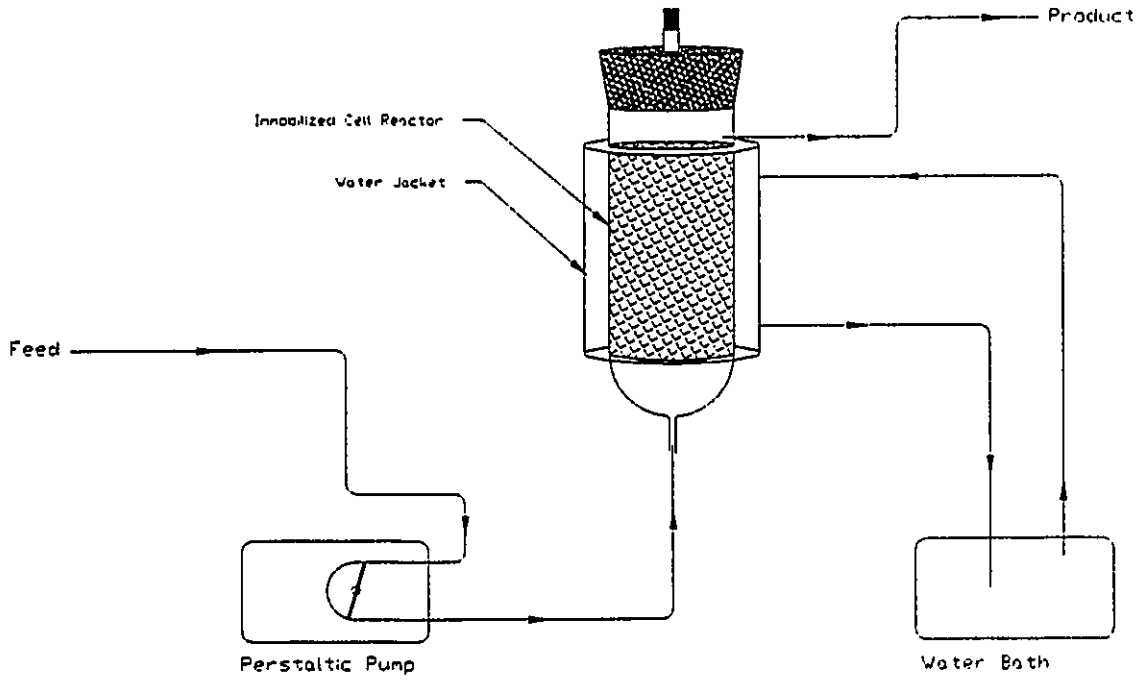

Figure 3.1: Schematic diagram of the immobilized cell reactor.

3.3 Analysis

3.3.1 Ethanol

Ethanol concentrations were determined enzymatically using alcohol dehydrogenase (Bernt and Gutmann, 1974). Nicotinamide adenine dinucleotide (NAD) and Alcohol dehydrogenase (ADH) oxidize ethanol to form acetaldehyde and NADH according to the following equation:

$$ethanol + NAD^{+} \overset{ADH}{\rightleftharpoons} acetaldehyde + NADH + H^{+} \qquad (3.1)$$

The reaction is pushed completely to the right at alkaline pH.

The amount of NADH in solution, which is proportional to the concentration of ethanol, was measured spectrophotometrically.

The required solutions for this analysis are:

Buffer

- 10 g $Na_4P_2O_7.10\ H_2O$
- 2.5 g semicarbazide hydrochloride
- 0.5 g glycine
- up to 250 mL distilled water
- pH adjusted to 8.7 with 4N KOH
- Diluted to 300 mL

Nicotinamide adenine dinucleotide (NAD)

- 50 mg of NAD in 3 mL distilled water

Alcohol dehydrogenase (ADH)

- 30 mg of ADH in 1 mL distilled water

Samples for analysis were diluted to contain between 0.047 and 1.15 g/L ethanol. Test tubes were then filled with the following: 3 mL buffer, 0.10 mL NAD, 0.20 mL sample and 0.02 mL ADH. The tubes were well mixed and incubated in a water bath at 37°C for 25 minutes. The absorbance of the solution was measured against a reference at 340 nm. The reference was prepared by replacing the sample with 0.20 mL distilled water.

Ethanol yields from the reactor were calculated on the basis of the total initial carbohydrates concentration in the solution. The standard curve for ethanol determination is found in the appendix (Figure A1).

3.3.2 Sugars

Glucose, fructose and sorbitol concentrations were determined using a Water's 600E high performance liquid chromatograph (HPLC) with a refractometer as the detector. The samples were diluted to contain sugars concentrations below 4.0 g/L. A Brownlee polypore CA column was used at 80°C with deionized water as the mobile phase. At a flow rate of 0.2 mL/min the retention times for glucose and fructose were 5.45 min and 6.41 min respectively. The standard curve for sugar analysis is found in the appendix (Figure A2). The peak areas obtained from

the refractometer were measured with a Hewlett Packard 3380S integrator.

3.3.3 Biomass

The total biomass concentration leaving the reactor was measured by the dry weight method. A known volume of sample was centrifuged in order to separate the biomass from the supernatant. The biomass was then resuspended in distilled water and centrifuged once again. The washed biomass was then transferred to a pre-weighed aluminum dish and dried at 105°C for 24 hours. The dish was then weighed and the total dry biomass weight was determined.

The total biomass in the reactor was determined at the end of each set of experiments by emptying the total contents of the reactor. The yeast cells were separated from the support by washing the wood blocks with distilled water and centrifuging the collected liquid. The total biomass removed from the wood blocks was then dried at 105°C for 24 hours and weighed to determine the total biomass present in the reactor. This method assumes that the biomass concentration remains constant with time. However, the total biomass in the reactor actually increased slightly throughout the experiments. It would be very difficult to determine the actual biomass concentration in the reactor at all times. Removing blocks from the reactor during an experiment to measure biomass would not be indicative of the total biomass. Each block had a different biomass concentration adsorbed onto the surface, depending on its position in the reactor. Biomass concentration was higher near the bottom of the reactor. Therefore, measuring the final biomass in the reactor was determined to be the most accurate method available for biomass determination.

3.3.4 Viability

The viability of the yeast cell in the effluent from the reactor was determined using the plate count method. The samples were diluted several times in a saline solution (0.08% NaCl), and 0.1 mL from each dilution was spread on agar plates containing medium I. After 48 h incubation at 33°C, the colonies were counted and counts of the replicates were averaged.

Chapter 4

Results and Discussion

Saccharomyces cerevisiae ATCC 36859 was immobilized onto wood chips in a vertical packed bed reactor. The application of this system for the continuous production of high fructose syrup and ethanol was studied. The effects of initial total carbohydrate, reactor temperature and oleic acid were observed. The reactor was also used in the continuous production of sorbitol from fructose. Sample calculations are found in the appendix.

4.1 Reactor Start-up

The initial feed for the reactor described in chapter 3 was the medium used for the production of high fructose syrup (medium III) with a 5.2% w/v glucose concentration. This low carbohydrate feed was used immediately after inoculation in order to accumulate biomass in the reactor. The dilution rate was increased after 200 hours because the glucose concentration in the reactor was low, and continuing the decrease. The higher dilution rate allowed for steady state to be attained. The reactor reached steady state approximately 400 hours after inoculation (Figure 4.1). Once steady state was attained, the biomass concentration leaving the reactor remained constant at a given dilution rate. The biomass in the reactor increased slightly throughout the experiments. However, the total biomass in the reactor was assumed to be constant for the purposes of growth rate calculations, which were based on the effluent biomass concentration. The final biomass concentrations varied from one start-up to the other and are listed in the title of each table of results in this chapter.

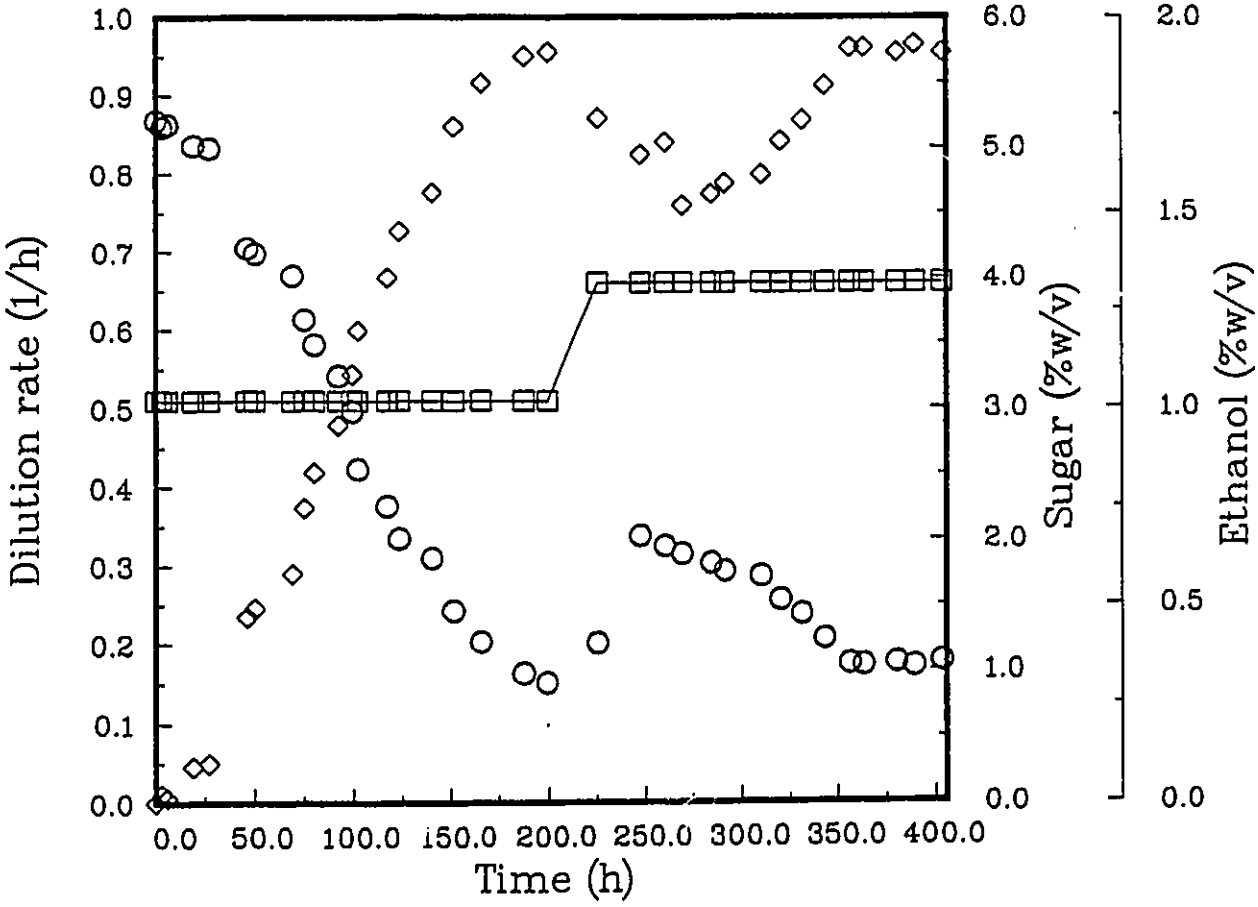

Figure 4.1: Conditions in an immobilized cell reactor during biomass accumulation using a feed containing 52.1 g/L glucose: □, dilution rate; ○ glucose; ◇, ethanol.

4.2 Effect of Feed Carbohydrate Concentration on Fermentation Rate

Once the reactor attained steady state the feeding began with media containing various initial glucose and fructose concentrations to determine the effect of sugar concentration on the growth rate and ethanol productivity. The media were fed at different dilution rates in order to determine the optimum conditions for the production of high fructose syrup with a high ethanol productivity, a reasonable concentration of ethanol and the lowest possible concentration of glucose in the effluent.

A medium containing 97.8 g/L glucose was fed to the packed bed reactor at four different dilution rates to determine the ethanol productivity and glucose conversion in the reactor (Figure 4.2). As the dilution rate was increased from 0.34 to 0.74 h^{-1} the glucose concentration in the effluent increased from 2.9 to 28.0 g/L, while the ethanol concentration decreased from 46.5 to 34.0 g/L (Table 4.1). The maximum ethanol productivity, 25.16 g/Lh, was obtained at a dilution rate of 0.74 h^{-1} (Figure 4.3). The glucose conversion decreased with increasing dilution rate.

Table 4.1: Product concentrations from the immobilized cell reactor with a biomass of 69.0 g/L and using a feed containing 97.8 g/L glucose.

D (h^{-1})	Final Glucose (g/L)	Final Ethanol (g/L)	Glucose conversion (g/g)	Ethanol Yield (g/g)	Ethanol Productivity (g/Lh)
0.34	2.9	46.5	0.970	0.490	15.8
0.42	6.5	44.6	0.934	0.488	18.7
0.48	12.1	41.9	0.876	0.489	20.1
0.74	28.0	34.0	0.714	0.487	25.1

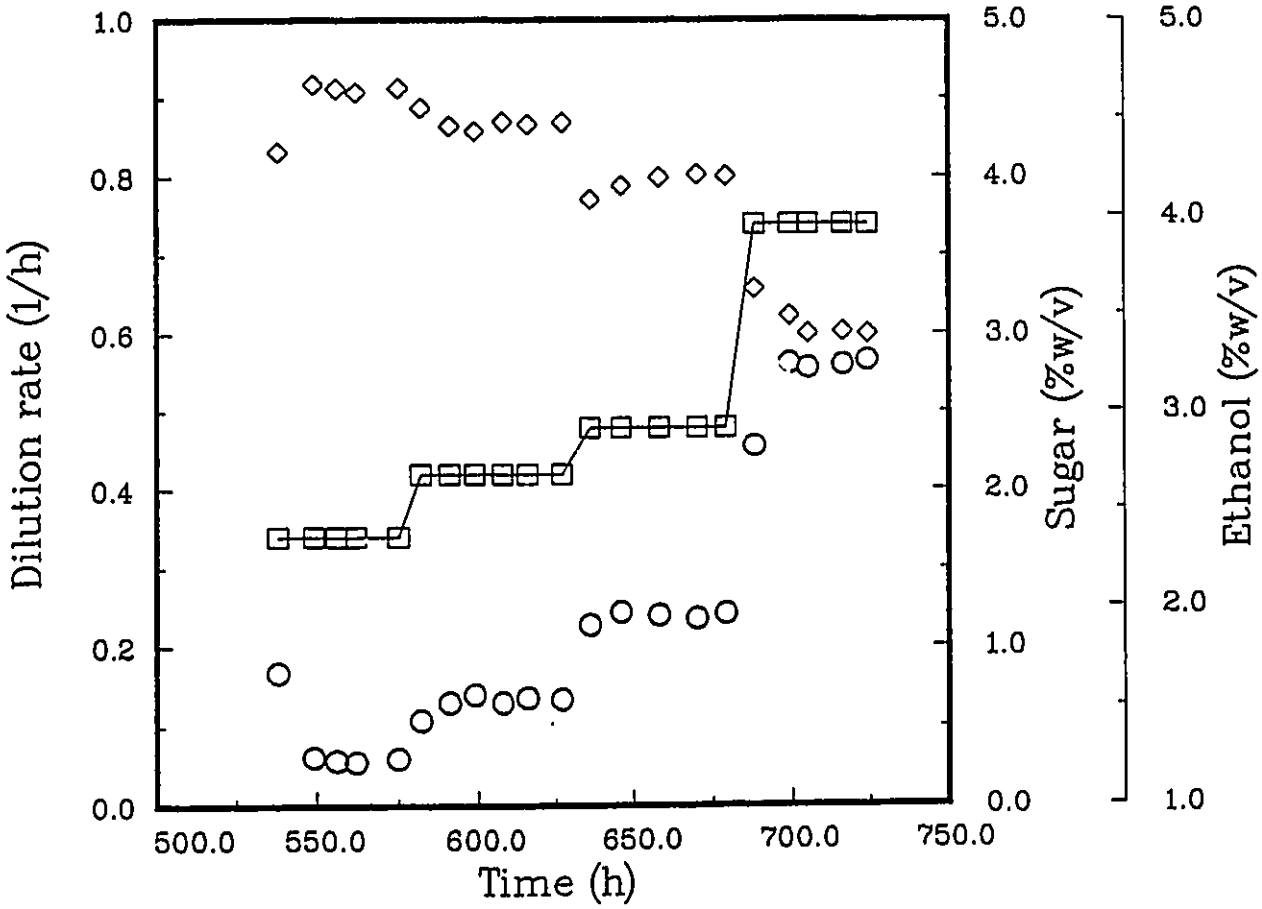

Figure 4.2: Ethanol production using a feed containing 97.8 g/L glucose: □, dilution rate; ○, glucose; ◇, ethanol.

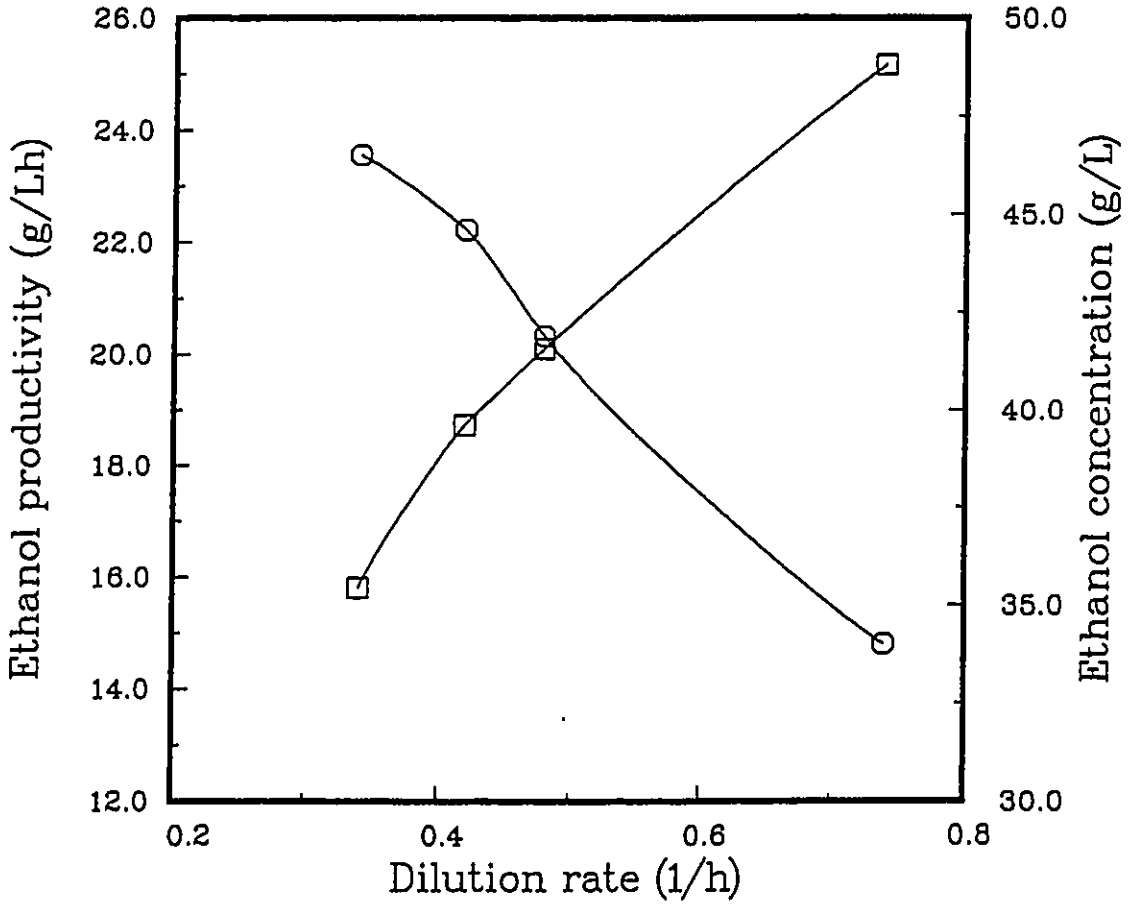

Figure 4.3: Ethanol productivity using a feed containing 97.8 g/L glucose: □, ethanol productivity; ○, ethanol concentration.

The feed was changed to a medium containing 97.2 g/L glucose and 98.8 g/L fructose for the production of high fructose syrup and to determine the effect the added fructose had on the system (Figure 4.4). The glucose concentration in the outlet increased from 12.5 to 36.5 g/L with a change in dilution rate from 0.34 to 0.74 h^{-1} while the ethanol concentration decreased from 41.9 to 29.6 g/L (Table 4.2). Fructose consumption was very low with only 1% being consumed at the lowest dilution rate. The maximum ethanol productivity, 21.9 g/Lh, was obtained at a dilution rate of 0.74 (Figure 4.5). However, at this high dilution rate the ethanol concentration is only 29.6 g/L and only 62.4% of the glucose is consumed, resulting in a fructose/glucose ratio of 2.7. At the lowest dilution rate (0.34 h^{-1}) the ethanol productivity is only 14.25 g/Lh, but the ethanol concentration is 41.9 g/L and the fructose/glucose ratio increases to 7.8.

The maximum ethanol productivities obtained with this reactor were 25.2 g/Lh using a feed containing only glucose and 21.9 g/Lh when fructose was added to the feed at a 1:1 ratio with glucose. The ethanol productivity and concentration decreased by 13% with the added fructose due to a higher substrate inhibition.

In previous studies, this mutant was immobilised in Ca-alginate beads in the same vertical reactor and fed with similar media (Koren and Duvnjak, 1989). With a feed containing 100 g/L glucose, the maximum productivity obtained by Koren and Duvnjak was 15.1 g/Lh at a dilution rate of 0.366 h^{-1} and an ethanol concentration of 41.3 g/L. When the feed was changed to one containing 101 g/L glucose and 98 g/L fructose, the maximum productivity fell to 12.7 g/Lh at the same dilution rate and an ethanol concentration of 34.5 g/L.

The ethanol productivities for both methods of immobilization were compared for similar

carbohydrate feed concentrations and effluent ethanol concentrations. The ethanol productivity in the wood chip reactor at an ethanol concentration of 41.0 g/L and a feed of 97.8 g/L glucose was 33% higher than in the Ca-alginate system. With a feed containing glucose and fructose and an effluent ethanol concentration of 35 g/L the ethanol productivity in the wood chip reactor was 42% higher than in Ca-alginate immobilized cell reactor (Table 4.6).

Table 4.2: Product concentrations from the immobilized cell reactor with a biomass of 69.0 g/L and using a feed containing 97.2 g/L glucose and 98.8 g/L fructose.

D (h^{-1})	Final Gluc. (g/L)	Final Fruc. (g/L)	Final EtOH (g/L)	Gluc. Conv. (g/g)	EtOH Yield .(g/g)	EtOH Prod. (g/Lh)	Growth rate (g/Lh)
0.34	12.5	97.8	41.9	0.871	0.489	14.2	0.070
0.42	16.6	97.9	39.8	0.829	0.488	16.7	0.081
0.48	21.0	98.1	37.5	0.784	0.488	18.0	0.092
0.74	36.5	98.5	29.6	0.624	0.485	21.9	0.117

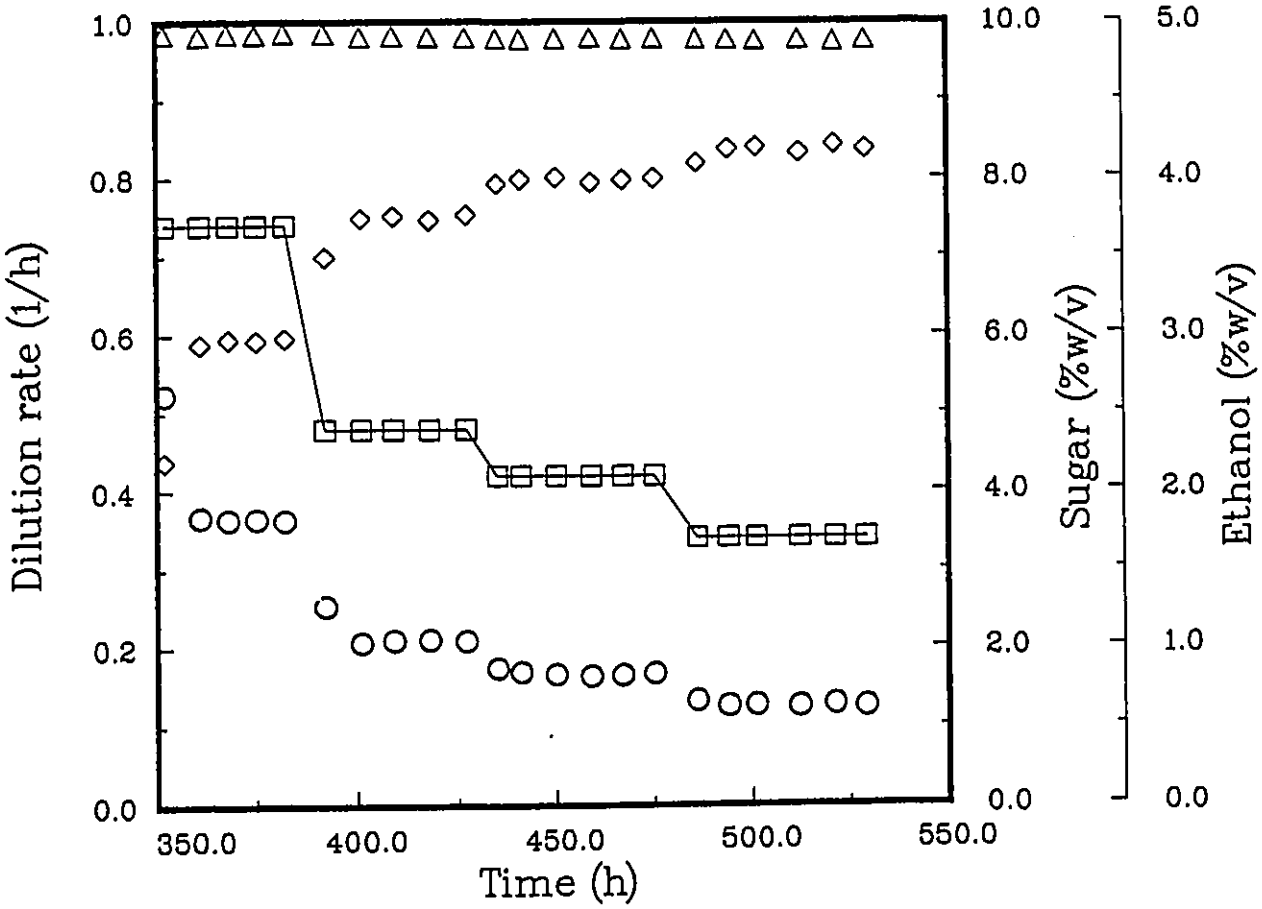

Figure 4.4: Ethanol production using a feed containing 97.2 g/L glucose and 98.8 g/L fructose: □, dilution rate; ○, glucose; △, fructose; ◇, ethanol.

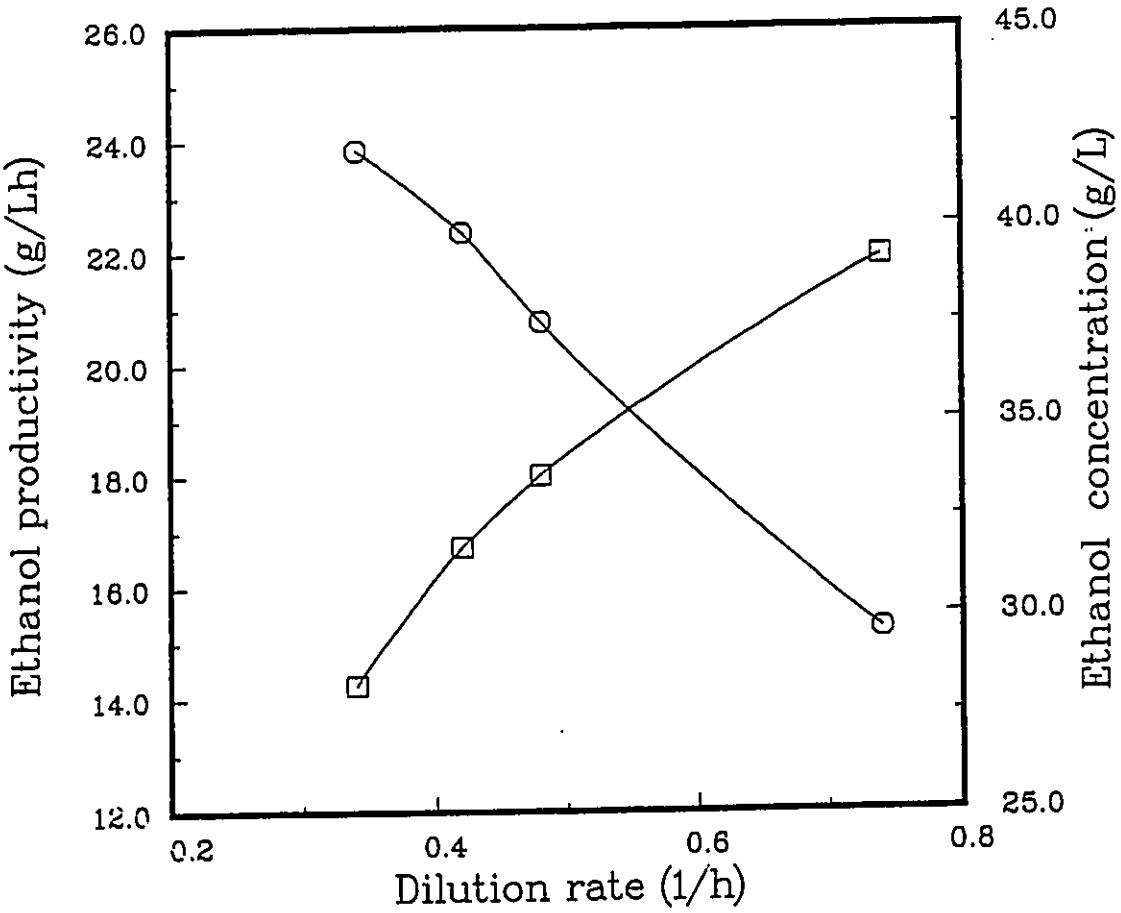

Figure 4.5: Ethanol productivity using a feed containing 97.2 g/L glucose and 98.8 g/L fructose: □, ethanol productivity; ○, ethanol concentration.

A medium containing 50.9 g/L glucose and 111.2 g/L fructose was fed to the reactor in order to simulate hydrolysed Jerusalem artichoke juice (HJAJ) (Figure 4.6). HJAJ has been used for the production of ethanol (Kosaric et al., 1983) and fructose (Fleming et al., 1979).

The simultaneous production of ethanol and fructose from hydrolysed Jerusalem artichoke juice by this yeast was studied by Koren and Duvnjak (1990) in free and immobilized cell systems. In the free cell batch fermentations conducted by Koren and Duvnjak, the maximum ethanol productivity obtained was 1.07 g/Lh with a final ethanol concentration of 17.8 g/L. No fructose was consumed by the mutant yeast. In the immobilized cell system, the maximum ethanol productivity reached 5.1 g/Lh at an ethanol concentration of 20.1 g/L. Glucose consumption was at 88% with a 3% consumption of fructose.

In the wood block immobilized cell reactor, an increase in dilution rate from 0.25 to 0.51 h^{-1} resulted in an increase in effluent glucose concentration from 0.9 to 8.0 g/L. Fructose consumption of 15% was observed at a dilution rate of 0.25 due to the low concentration of glucose in the effluent (Table 4.3). The maximum productivity obtained with this feed was 11.83 g/Lh at the highest dilution rate, at an ethanol concentration of 23.2 g/L (Figure 4.7). A 132% increase in ethanol productivity was observed by adsorption of the cells onto wood block, as opposed to entrapment in Ca-alginate beads (Table 4.6).

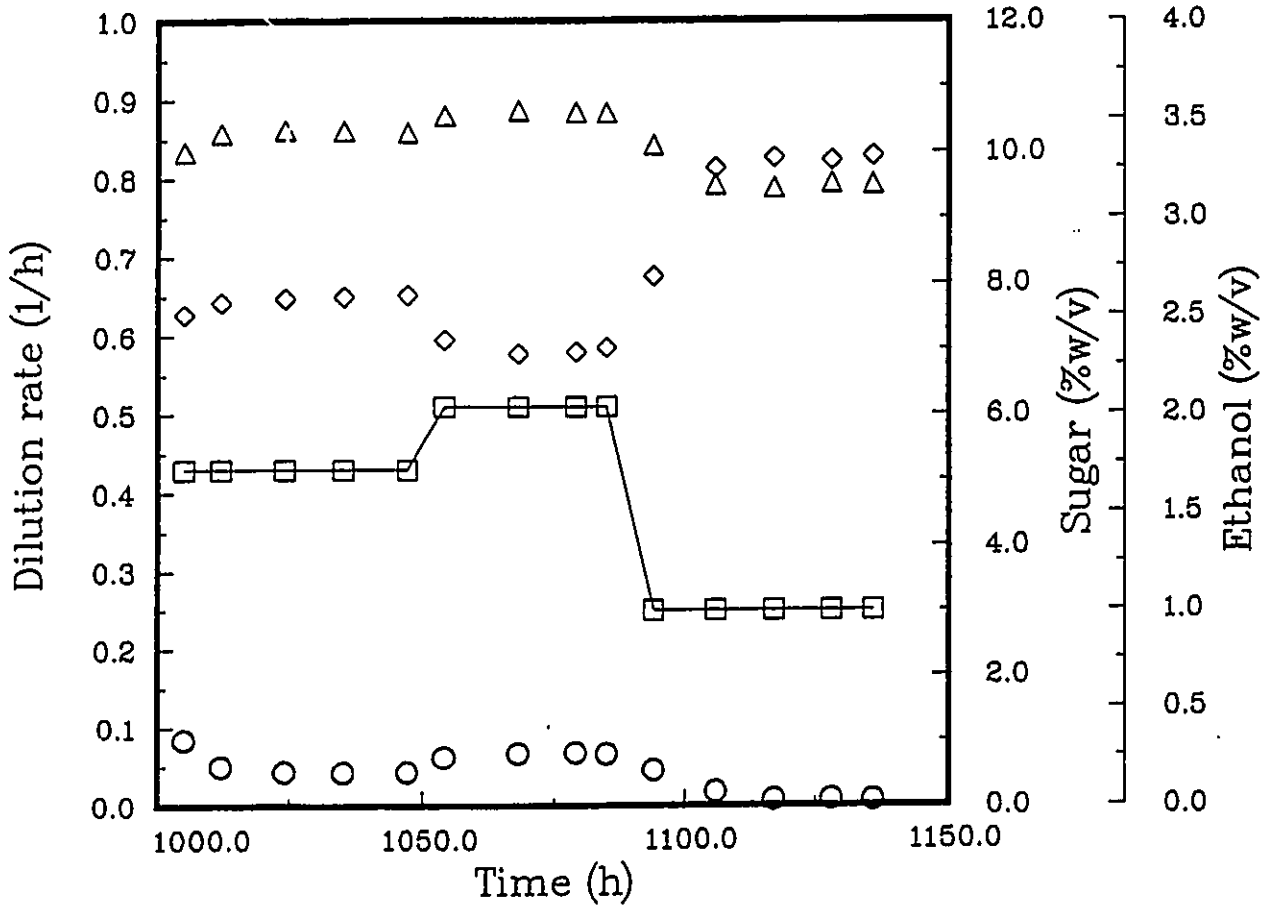

Figure 4.6: Ethanol production using a feed containing 50.9 g/L glucose and 111.2 g/L fructose: □, dilution rate; ○, glucose; △, fructose; ◇, ethanol.

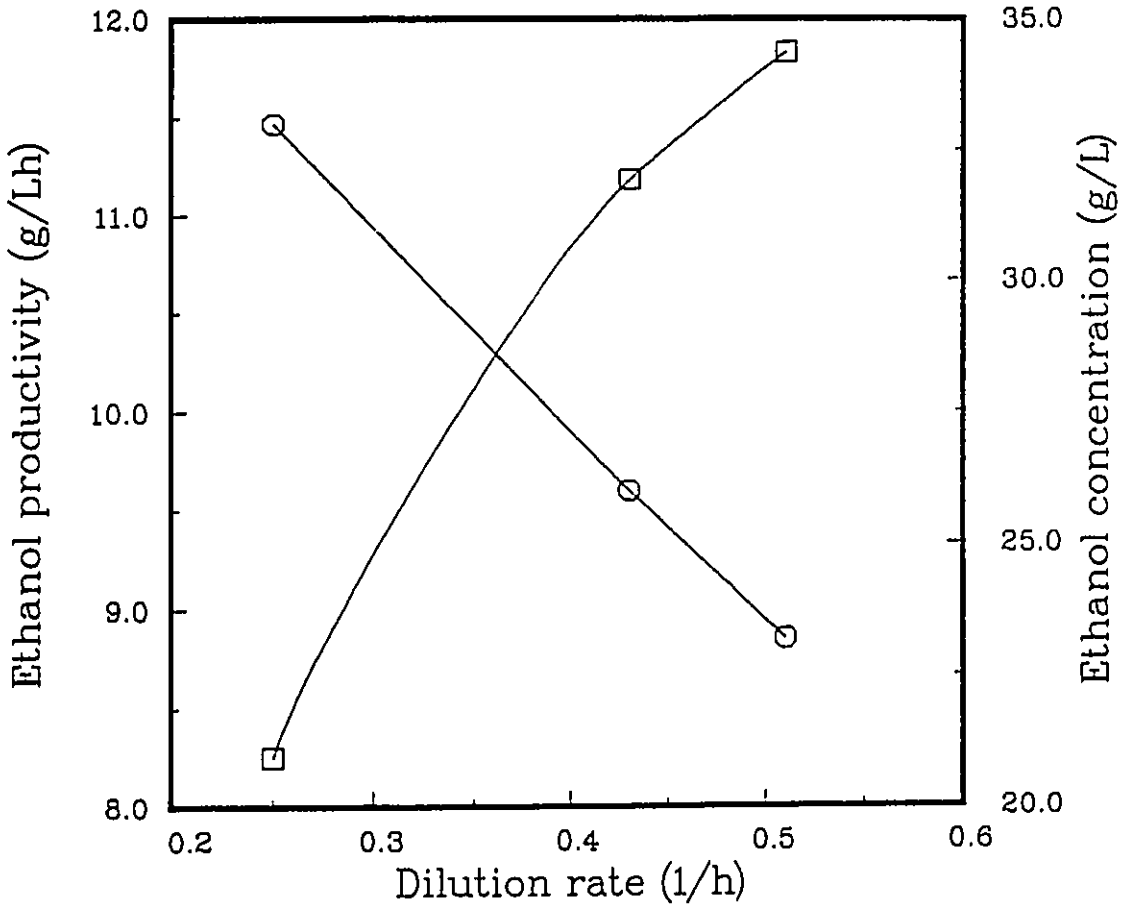

Figure 4.7: Ethanol productivity using a feed containing 50.9 g/L glucose and 111.2 g/L fructose: □, ethanol productivity; ○, ethanol concentration.

Table 4.3: Product concentrations from the immobilized cell reactor with a biomass of 80.0 g/L and using a feed containing 50.9 g/L glucose and 111.2 g/L fructose.

D (h^{-1})	Final Gluc. (g/L)	Final Fruc. (g/L)	Final EtOH (g/L)	Gluc. Conv. (g/g)	EtOH Yield (g/g)	EtOH Prod. (g/Lh)	Growth rate (g/Lh)
0.25	0.9	95.0	33.0	0.982	0.498	8.2	0.015
0.43	5.2	103.3	26.0	0.898	0.485	11.1	0.069
0.51	8.0	106.2	23.2	0.843	0.484	11.8	0.092

The following experiment was designed to simulate HJAJ supplemented with glucose (Figure 4.8). The reactor was fed with a medium containing 85.2 g/L glucose and 129.0 g/L fructose. As expected, an increase in dilution rate resulted in an increase in effluent glucose concentration and a decrease in ethanol concentration (Table 4.4). The maximum productivity of 14.33 g/Lh was obtained at a dilution rate of 0.51 h^{-1} and an ethanol concentration of 28.1 g/L (Figure 4.9). The fructose/glucose ratio was 4.4. This ratio increased to 7.2 at a dilution rate of 0.25 h^{-1} and the ethanol concentration in the product was 45.9 g/L. The ethanol productivity, however, dropped to 11.48 g/Lh.

Koren (1990) conducted a similar test using the mutant yeast immobilised in Ca-alginate beads and a feed of glucose supplemented HJAJ (84.9 g/L glucose and 140.6 g/L fructose). An ethanol productivity of 8.2 g/L was obtained at a dilution rate of 0.25 h^{-1} and an ethanol concentration of 32.2 g/Lh.

Ethanol productivity was 49% higher in this latest study at a similar outlet ethanol concentration (Table 4.6).

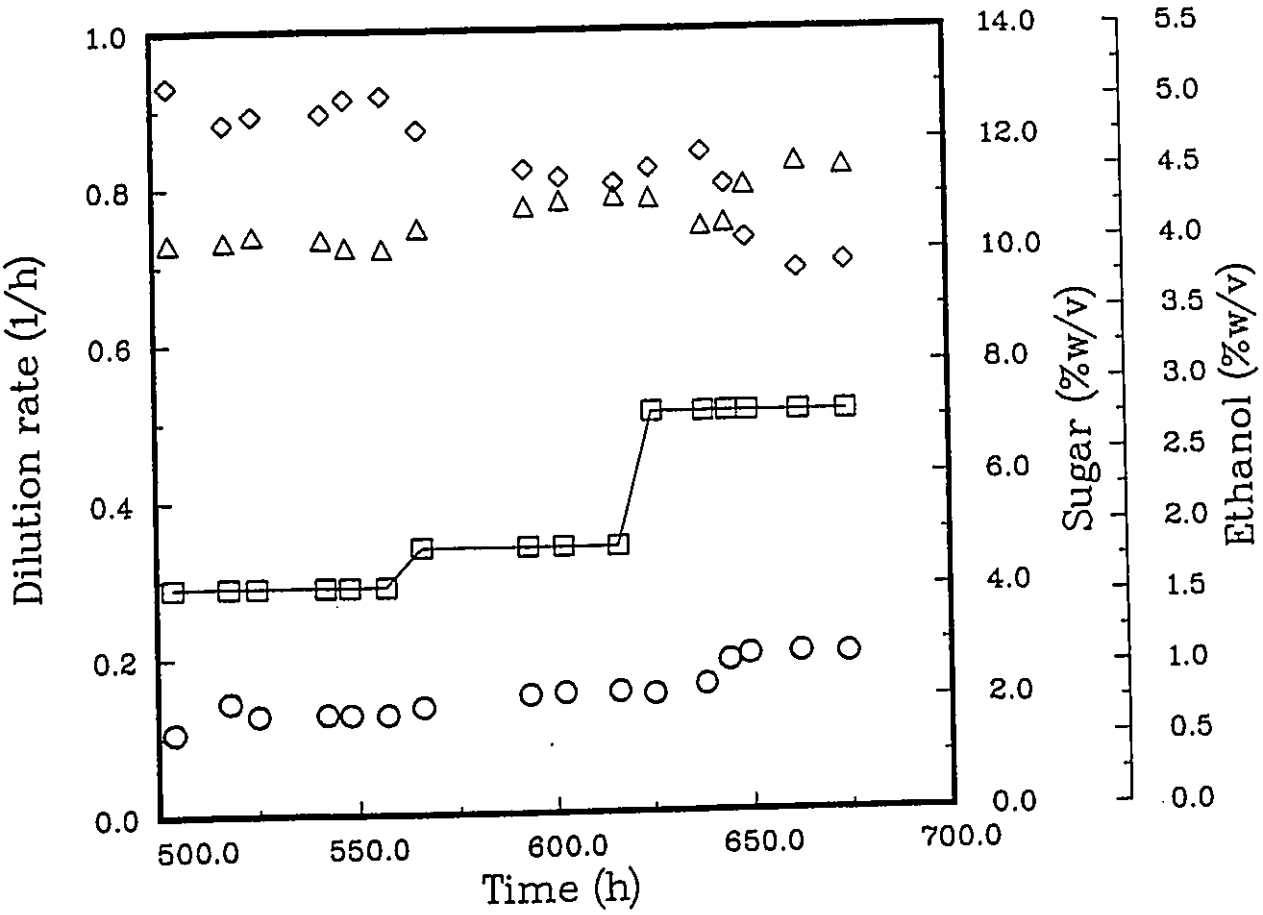

Figure 4.8: Ethanol production using a feed containing 85.2 g/L glucose and 129.0 g/L fructose: □, dilution rate; ○, glucose; △, fructose; ◇, ethanol.

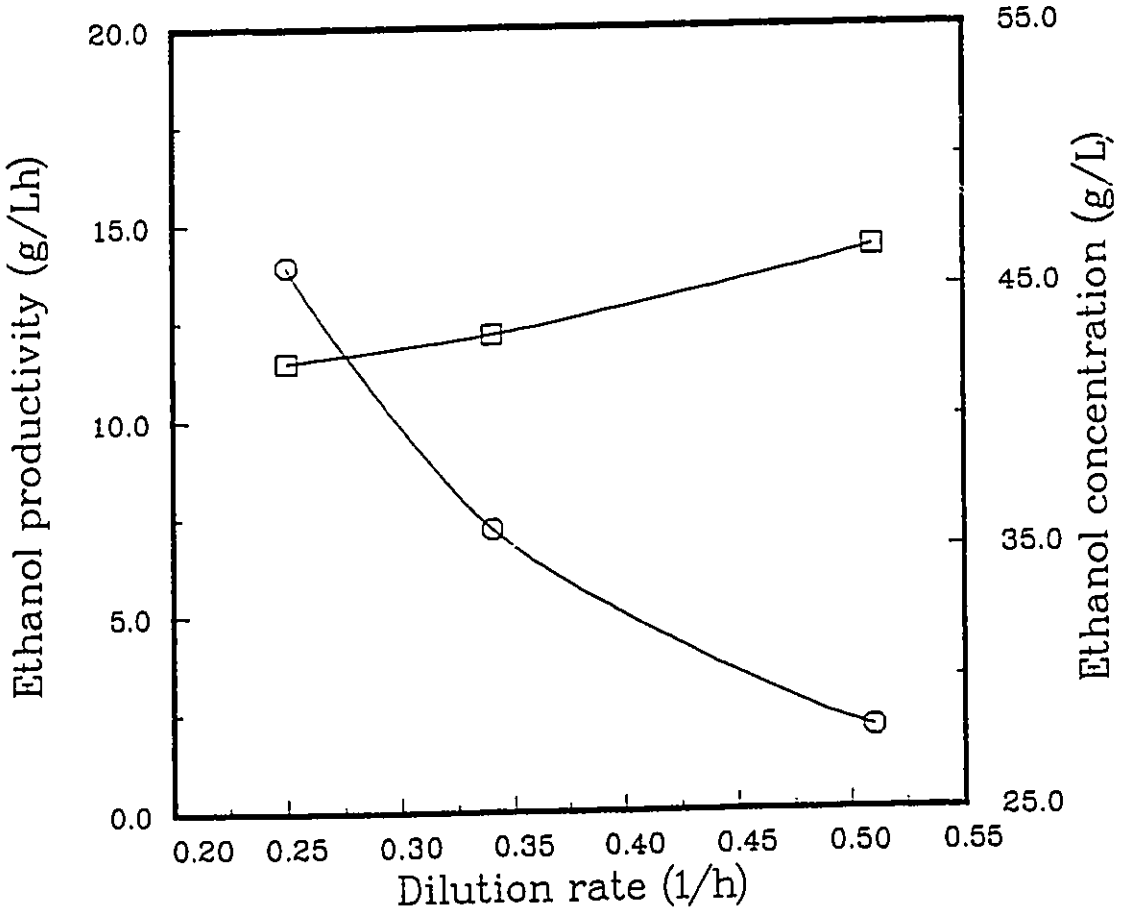

Figure 4.9: Ethanol productivity using a feed containing 85.2 g/L glucose and 129.0 g/L fructose: □, ethanol productivity; ○, ethanol concentration.

Table 4.4: Product concentrations from the immobilized cell reactor with a biomass of 45.0 g/L and using a feed containing 85.2 g/L glucose and 129.0 g/L fructose.

D (h^{-1})	Final Gluc. (g/L)	Final Fruc. (g/L)	Final EtOH (g/L)	Gluc. Conv. (g/g)	EtOH Yield (g/g)	EtOH Prod. (g/Lh)	Growth rate (g/Lh)
0.25	14.5	105.0	45.9	0.830	0.485	11.4	0.043
0.34	21.4	119.0	35.8	0.749	0.485	12.1	0.058
0.51	29.0	127.2	28.1	0.660	0.484	14.3	0.068

A medium containing 145.5 g/L glucose and 118.5 g/L fructose was also fed to the reactor in order to determine the effects of very high carbohydrate concentrations (Figure 4.10). With an increase in dilution rate from 0.1 to 1.56 h^{-1} the glucose concentration in the effluent increased from 35.2 to 133.6 g/L, while the ethanol concentration decreased from 61.9 to 6.0 g/L (Table 4.5). The productivity reached a maximum of 9.79 g/Lh at a dilution rate of 0.64 h^{-1}, and levelled off at higher dilution rates due to substrate inhibition (Figure 4.11). The fructose/glucose ratio was only 1.0 at these conditions and the ethanol concentration was 15.3 g/L. At a dilution rate of 0.1 h^{-1} the ethanol productivity decreases to 6.19 g/Lh, but the ethanol concentration reaches 61.9 g/L and the fructose/glucose ratio is 3.0. This ratio and the ethanol concentration could be increased further with batch fermentations carried out on the effluent. This possibility will be explored in section 4.6.

Table 4.5: Product concentrations from the immobilized cell reactor with a biomass of 32.0 g/L and using a feed containing 145.5 g/L glucose and 118.5 g/L fructose.

D (h^{-1})	Final Gluc. (g/L)	Final Fruc. (g/L)	Final EtOH (g/L)	Gluc. Conv. (g/g)	EtOH Yield (g/g)	EtOH Prod. (g/Lh)	Growth rate (g/Lh)
0.10	35.2	105.0	61.9	0.758	0.500	6.1	0.022
0.29	84.2	116.5	31.3	0.421	0.494	9.0	0.045
0.64	115.5	117.2	15.3	0.206	0.489	9.7	0.050
0.91	124.7	117.8	10.5	0.143	0.488	9.5	0.051
1.56	133.6	118.1	6.0	0.082	0.488	9.3	0.051

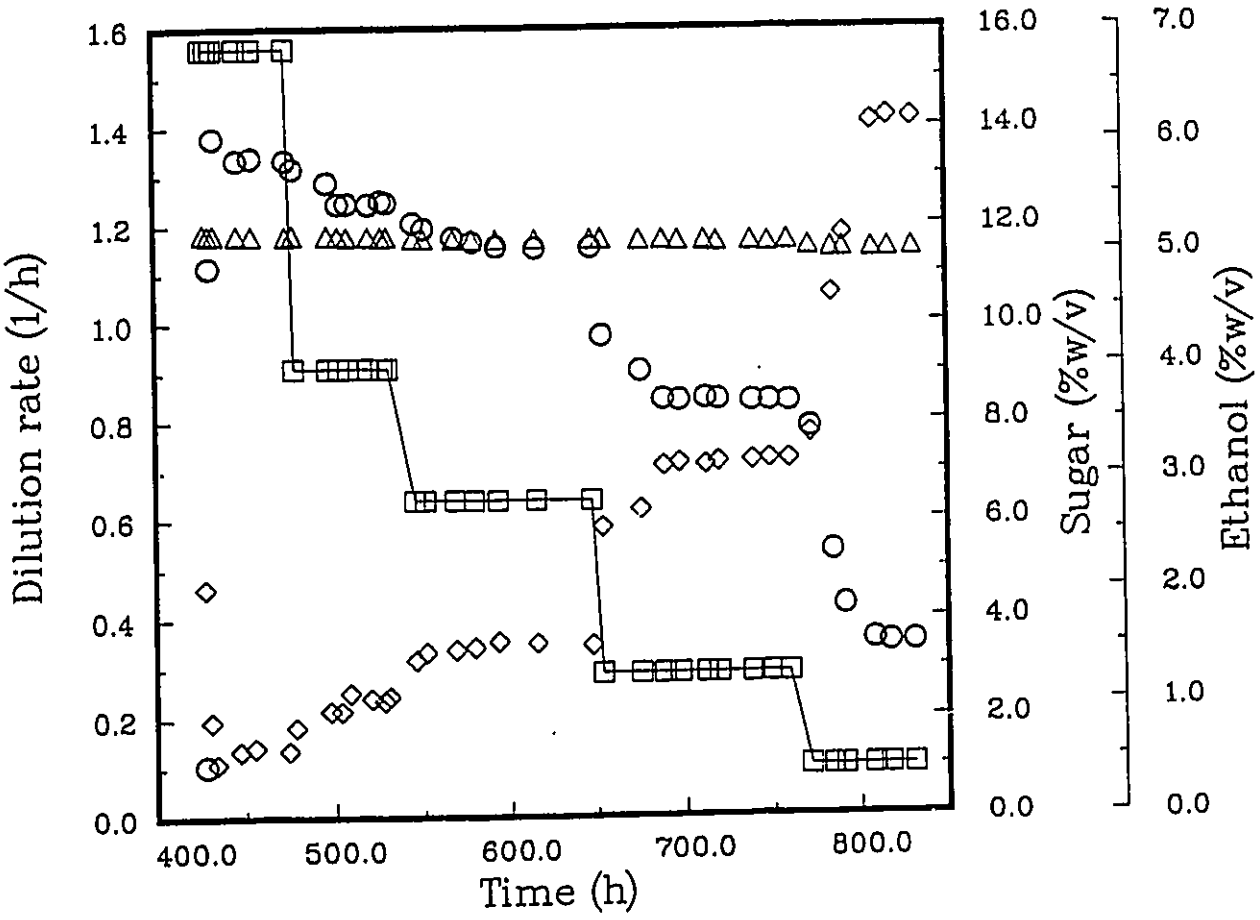

Figure 4.10: Ethanol production using a feed containing 145.5 g/L glucose and 118.5 g/L fructose: □, dilution rate; ○, glucose; △, fructose; ◇, ethanol.

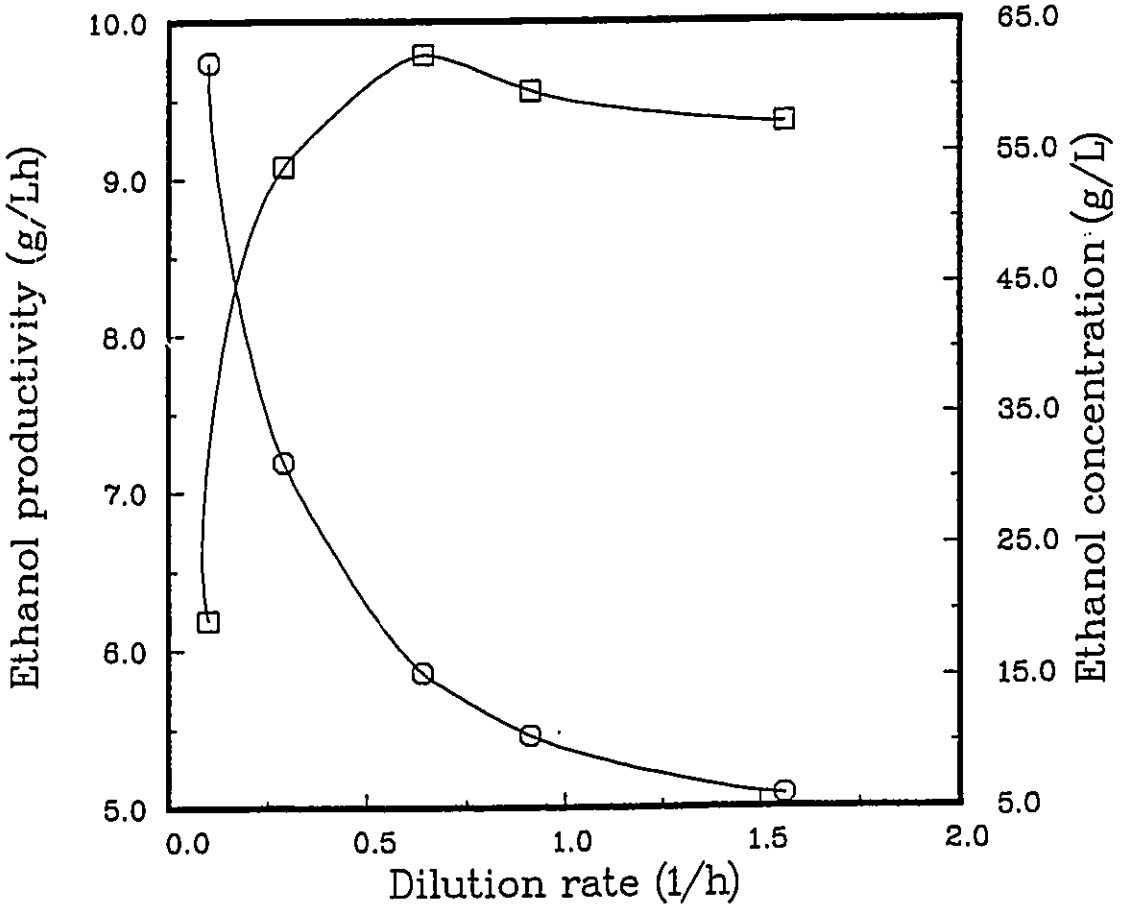

Figure 4.11: Ethanol productivity using a feed containing 145.5 g/L glucose and 118.5 g/L fructose: □, ethanol productivity; ○, ethanol concentration.

Immobilized cell systems confer desirable properties to a biological process which are not readily achieved in conventional free cell processes. however the advantages depend specifically on the method of immobilization. Entrapped cell systems are subjected to mass transfer limitations imposed by the additional diffusion barrier created by the support matrix. CO_2, one of the products of fermentation, is characterized by its relatively low solubility in aqueous media and therefore the diffusion of the gaseous product out of the matrix can be rate-limiting (Krouwel and Kossen, 1980). With the adsorption of yeasts onto wood blocks these additional mass transfer limitations are not encountered. Other problems may however be encountered with this immobilization process. The amount of biomass that can be adsorbed by unit gram of carrier is limited by the surface area of the wood. Also, the operational stability is restricted by the rate of desorption of the cells from the support. At high dilution rates excess desorption may occur. Some cells settled in the bottom of the reactor following desorption from the wood chips.

The productivities obtained by the mutant yeast were comparable to those observed in similar systems using the wild strain. Gencer and Mutharasan (1981) obtained an ethanol productivity of 25.2 g/Lh by immobilizing the wild strain of *S. cerevisiae* onto wood blocks, using a feed containing 130 g/L of glucose. An ethanol productivity of 17.0 g/Lh was obtained with a feed of 127 g/L glucose, by immobilizing *S. cerevisiae* in Ca-alginate beads in a vertical packed bed reactor (Williams and Munnede, 1981).

The mutant yeast used in this study did consume a certain amount of the fructose present in the feed. This was also observed by Koren and Duvnjak (1989) with the mutant immobilized in Ca-alginate beads. This yeast is known to consume fructose when no other source of

carbohydrate is present (Koren, 1990), therefore at low concentrations of glucose the mutant will begin to consume the fructose. To reduce fructose consumption, the reactor must therefore be run at less than 100% glucose conversion if no fructose consumption is desired.

The ethanol productivities obtained in the wood block reactor were compared with those obtained with the same mutant yeast immobilized in Ca-alginate beads. At similar ethanol concentrations in the effluent, the productivities were 33% to 132% higher in the wood block reactor. The productivities may be lower in the Ca-alginate system due to the additional mass transfer limitations imposed by the support matrix. With the adsorption method, the reactor solution is in direct contact with the yeast cells, resulting in a higher ethanol productivity.

Table 4.6: Comparison of ethanol productivities obtained in a vertical packed bed reactor with cells immobilized in Ca-alginate beads and on wood blocks.

Feed	Ca-alginate beads		Wood blocks		Increase in productivity with wood blocks (%)
	EtOH Conc. (g/L)	EtOH Prod. (g/Lh)	EtOH Conc. (g/L)	EtOH Prod. (g/L)	
100 g/L glucose	41.3	15.1	41.9	20.1	33
100 g/L glucose 100 g/L fructose	34.5	12.7	37.5	18.0	42
50 g/L glucose 111 g/L fructose	20.1	5.1	23.2	11.8	132
85 g/L glucose 120 g/L fructose	32.2	8.2	35.8	12.2	49

4.3 Modelling of Growth and Ethanol Productivity

The data, obtained from each of the experiments in the previous section, were assembled in order to create mathematical models for growth and ethanol production rates in the immobilized cell reactor. The growth rates and ethanol productivities from each run were converted to specific growth and ethanol production rates by dividing these values by the biomass concentration in the reactor. The data are summarised in Table 4.7.

A number of mathematical models representing growth and ethanol productivity were examined for their validity in describing the behaviour of the mutant yeast immobilised on wood blocks in this study.

Ethanol production is a growth-associated-type fermentation. If there is no net cell accumulation in the reactor, then the shed rate of cells from the reactor is equal to the growth rate. If we assume that all immobilized cells have the same random chance of being shed regardless of age, viability, or of position in the reactor, a steady-state analysis of the fraction of live cells in a reactor at any point of constant environmental conditions may be performed. Results obtained by Dale et al. (1990) support the assumption of random shedding of cells from an adsorbed cell, continuous immobilised cell reactor, as it was found that the shed cell viable fraction over time was linearly correlated to the reactor productivity rates. The random shedding of cells was assumed for growth rate modelling in this study.

Table 4.7: Specific ethanol productivity and cell growth rate in the immobilized cell reactor at different glucose concentrations.

Glucose concentration (g/L)	Specific ethanol productivity (ν) (h^{-1})	Specific growth rate (μ) (h^{-1})X10^{-3}
0.9	0.103	0.19
5.2	0.139	0.86
8.0	0.148	1.15
12.5	0.206	1.02
14.5	0.255	0.95
16.6	0.244	1.18
21.0	0.260	1.33
21.4	0.270	1.28
29.0	0.315	1.52
35.2	0.193	0.68
36.5	0.317	1.69
84.2	0.284	1.40
115.5	0.307	1.57
124.7	0.298	1.59
133.6	0.293	1.60

From the models presented earlier the equation which best described the yeast growth rate in the packed bed reactor was obtained by combining the Monod equation for growth (2.1) with equation 2.8 (Ghose and Tyagi, 1979b) for substrate inhibition and equation 2.9 (Holzberg et al.,1967) for product inhibition:

$$\mu = \mu_{max}\frac{S_g}{K_s+S_g}\left(1-\frac{P}{P_{max}}\right)\left(1-\frac{S_{tc}}{S_{tmax}}\right) \qquad (4.1)$$

where μ is the specific growth rate, K_s is the saturation constant, μ_{max} is the maximum specific growth rate, S_g is the glucose concentration, S_{tc} is the total carbohydrate concentration (glucose and fructose), P is the ethanol concentration, P_{max} is the ethanol concentration above which cells will not grow, and S_{tmax} is the total substrate concentration above which cells will not grow. The ethanol productivity follows the same model.

$$\nu = \nu_{max}\frac{S_g}{K'_s+S_g}\left(1-\frac{P}{P'_{max}}\right)\left(1-\frac{S_t}{S'_{tmax}}\right) \qquad (4.2)$$

where P'_{max} is the ethanol concentration above which ethanol will not be produced and S'_{tmax} is the total substrate concentration above which ethanol will not be produced.

The substrate associated with the growth and ethanol productivity is glucose whereas the fructose is considered only in the substrate inhibition portion of the equation. These equations indicate that the mutant yeast follows the Monod equation for growth and productivity with linear substrate and product inhibition. The kinetic parameters were determined by the NLIN procedure (SAS,1985) which produces least squares estimates of the parameters of a non-linear model. NLIN uses iterative methods (Marquardt) to regress the residuals onto the partial derivatives of the model with respect to the parameters until the iterations converge. The parameters determined by NLIN for equations 4.1 and 4.2 are listed in Table 4.8.

Table 4.8: Converged parameter estimates for ethanol production and cell growth rates.

Parameter	Estimate	95% Confidence Interval	
		Lower	Upper
μ_{max}	0.0051 h^{-1}	0.0046	0.0056
K_s	10.3 g/L	5.2	15.4
P_{max}	85.1 g/L	78.9	91.3
S_{tmax}	402.0 g/L	366.5	437.5
ν_{max}	0.699 h^{-1}	0.629	0.769
K_s'	14.1 g/L	8.8	19.4
P'_{max}	195.9 g/L	174.9	216.9
S'_{tmax}	496.0 g/L	460.8	531.2

According to these parameters, growth will continue up to a substrate concentration of 402.0 ± 35.5 g/L whereas the ethanol productivity will continue until a substrate concentration of 496.0 ± 35.2 g/L is reached. The maximum ethanol concentration at which growth will occur is 85.1 ± 6.2 g/L while ethanol will continue to be produced until an ethanol concentration of 195.9 ± 21.0 g/L is attained.

The data along with the mathematical models are shown in Figures 4.12 and 4.13. At low glucose concentrations an increase in the glucose concentration results in a linear increase in both the ethanol productivity and the growth rate. Further increases in the glucose concentrations result in a levelling off and eventual reduction in the ethanol production and cell growth rates. The controlling reaction rate appears to be initially the diffusion across the cell membrane, resulting in a linear dependence on the carbohydrate concentration. As the carbohydrate concentration increases, the fermentation rate becomes the controlling rate. A

reduction in the fermentation rate is observed with an increase in carbohydrate concentration due to substrate inhibition (Figure 4.11). This phenomenon is believed to be related to osmotic effects in which plasmolysis of the cells begins to occur at a high substrate concentration. The repression of the energy producing metabolism (growth) is known as the Crabtree effect and is common for yeasts (Lehninger, 1982). The increased osmotic pressure in the medium due to the presence of non-metabolized sugars, also results in an increase in the intracellular ethanol concentration (Stewart et al., 1983). Since intracellular ethanol has a more damaging effect on the cell than extracellular ethanol, the growth rate and productivity are adversely affected. The presence of ethanol in the medium also has an inhibitory effect on the growth and ethanol productivities.

The parameters indicate that growth is more strongly inhibited by product and substrate than the ethanol productivity. This phenomenon also occurred with the mutant yeast in free cell batch processes (Koren, 1990). The parameters determined for the free cell system using a similar model were: μ_{max}=0.241 h^{-1}, K_s=4.56 g/L, P_{max}=62.0g/L, S_{max}=488.4 g/L, P'_{max}=152.0 g/L, S'_{max}=788.9 g/L. It has been reported that the effect of inhibitory compounds can be reduced by certain methods of immobilisation (Margaritis and Merchant, 1984). A number of reports have also suggested that microorganisms may exhibit an acceleration of their metabolic activities as a result of surface attachment (Bandyopdhyay and Ghose, 1982; Navarro and Durand, 1977). The permeability of the cell membrane can be changed upon immobilisation (Felix, 1982). Permeabilization of the cell membrane may increase production but may also result in lower stability of the enzymes in the cell, perhaps due to the loss of enzyme from the cells through the membrane (Bang et al., 1983). The results obtained in this study indicate that

immobilization of the yeast onto wood blocks increases the tolerance for ethanol. The substrate inhibition remains unchanged for growth rate and is increased for ethanol productivity.

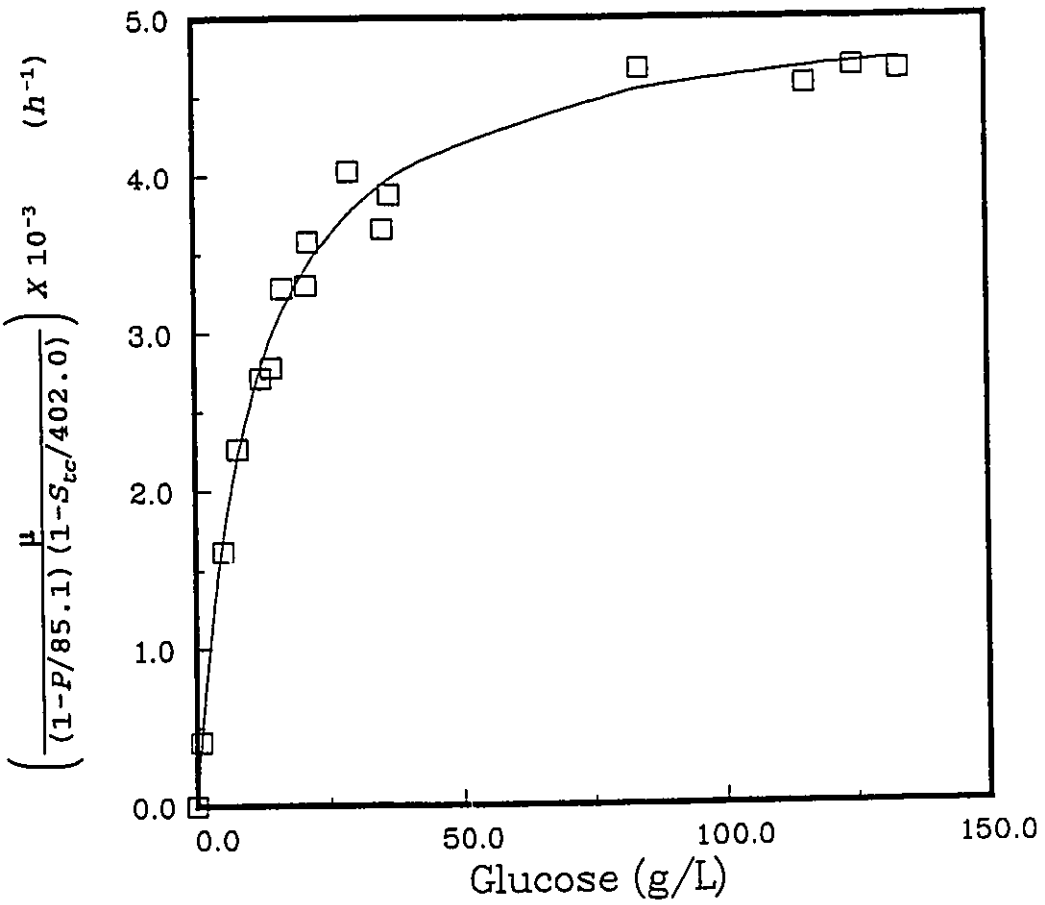

Figure 4.12: Model for growth rate in the immobilized cell reactor. The symbols (□) represent the experimental data and the line represents the model formulated with equation 4.1.

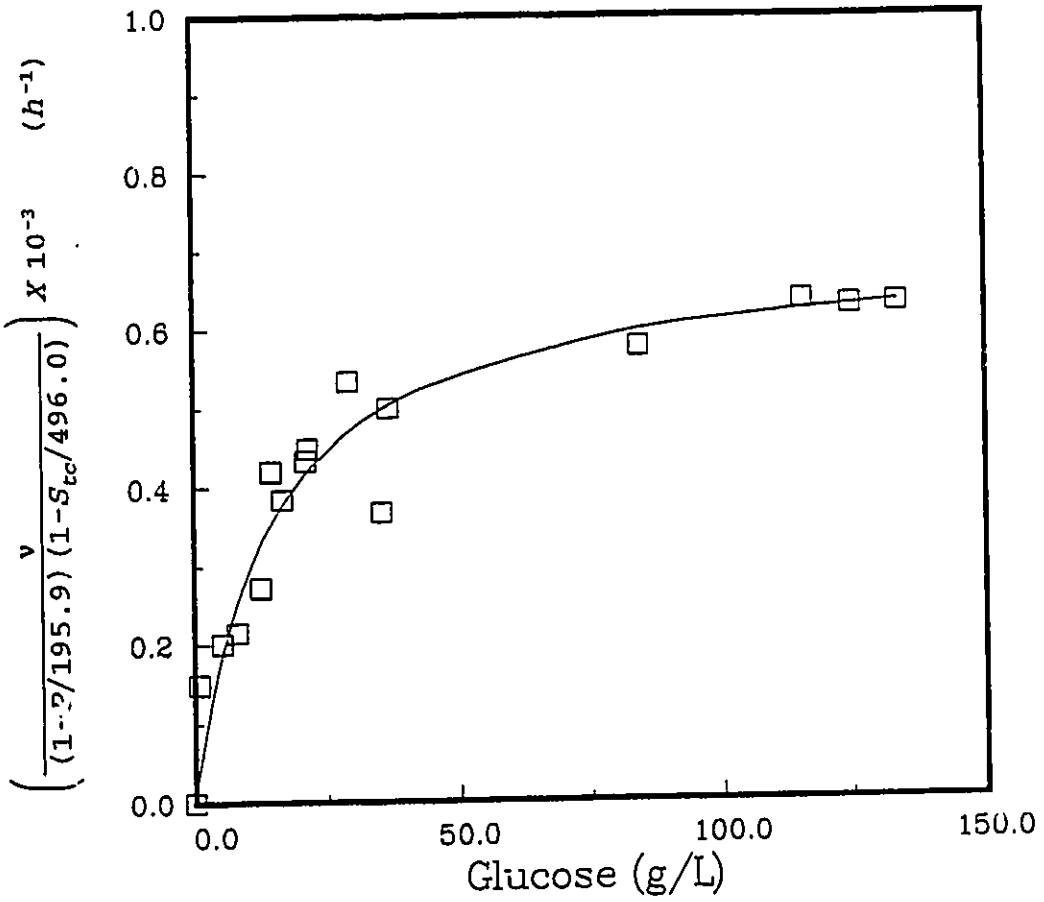

Figure 4.13: Model for ethanol productivity in the immobilised cell reactor. The symbols (□) represent the experimental data and the line represents the model formulated with equation 4.2.

4.4 Effect of Oleic Acid

Unsaturated fatty acids and sterols are suspected to be principal components required for functional membranes and are largely responsible for the membrane physiochemical properties in yeasts (Jones, 1989). It has been shown that the incorporation of such compounds in the cell membrane improved ethanol tolerance of yeast (Thomas et al., 1978). In aerobic conditions unsaturated fatty acids could be synthesised by the yeast; however, in the total absence of oxygen this is not possible (Cysewski, 1976). The feed in Table 4.2 (97.2 g/L glucose and 98.8 g/L fructose) was followed by a feed containing 97.5 g/L glucose, 97.8 g/L fructose and 30 mg/L oleic acid (Figure 4.14). As the dilution rate increased from 0.42 to 0.74 h^{-1} with the oleic acid enriched feed, the glucose concentration in the effluent increased from 9.0 to 29.1 g/L and the ethanol concentration decreased from 43.8 to 33.5 g/L (Table 4.9).

Table 4.9: Product concentrations from the immobilized cell reactor with a biomass of 69.0 g/L and using a feed containing 97.5 g/L glucose and 97.8 g/L fructose, and 30 mg/L oleic acid.

D (h^{-1})	Final Gluc. (g/L)	Final Fruc. (g/L)	Final EtOH (g/L)	Gluc. Conv. (g/g)	EtOH Yield (g/g)	EtOH Prod. (g/Lh)
0.42	9.0	97.2	43.8	0.908	0.492	18.4
0.48	12.4	97.4	41.8	0.873	0.489	20.0
0.74	29.1	97.5	33.5	0.702	0.488	24.8

Figure 4.15 is a comparison of the productivities obtained with and without oleic acid in the feed. The maximum productivity in the presence of oleic acid was 13% higher than without the unsaturated fatty acid and the ethanol concentration was increases by 10%. The glucose

conversion was increased by 9% in the presence of the unsaturated fatty acid. These results suggest that oleic acid does indeed act as a growth factor for the mutant yeast.

Results obtains by Watson (1982) showed that Saccharomyces cells enriched in oleic acid residues had a substrate conversion efficiency of between 86-95%, whereas cells with a relatively low content of oleic acid residues had a substrate conversion efficiency of less than 70%. The increase in efficiency in this study may have been lower due to the ethanol concentration in the effluent. The was significantly lower than the maximum ethanol concentration beyond which the cells will no longer produce ethanol. Since oleic acid increases ethanol tolerance, it should be more efficient at higher ethanol concentrations.

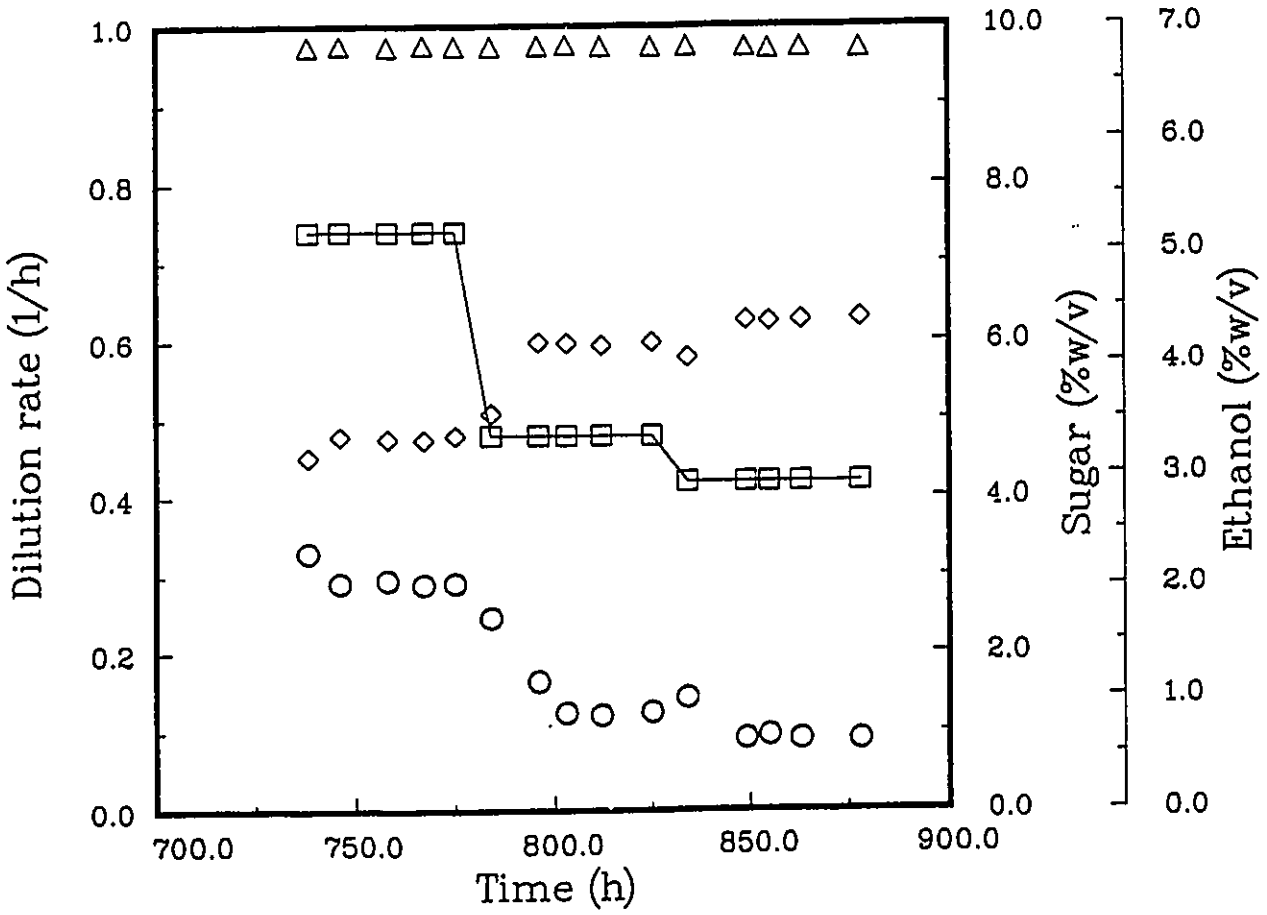

Figure 4.14: Ethanol production using a feed containing 97.5 g/L glucose, 97.8 g/L fructose and 30 mg/L oleic acid: □, dilution rate; ○, glucose; △, fructose; ◇, ethanol.

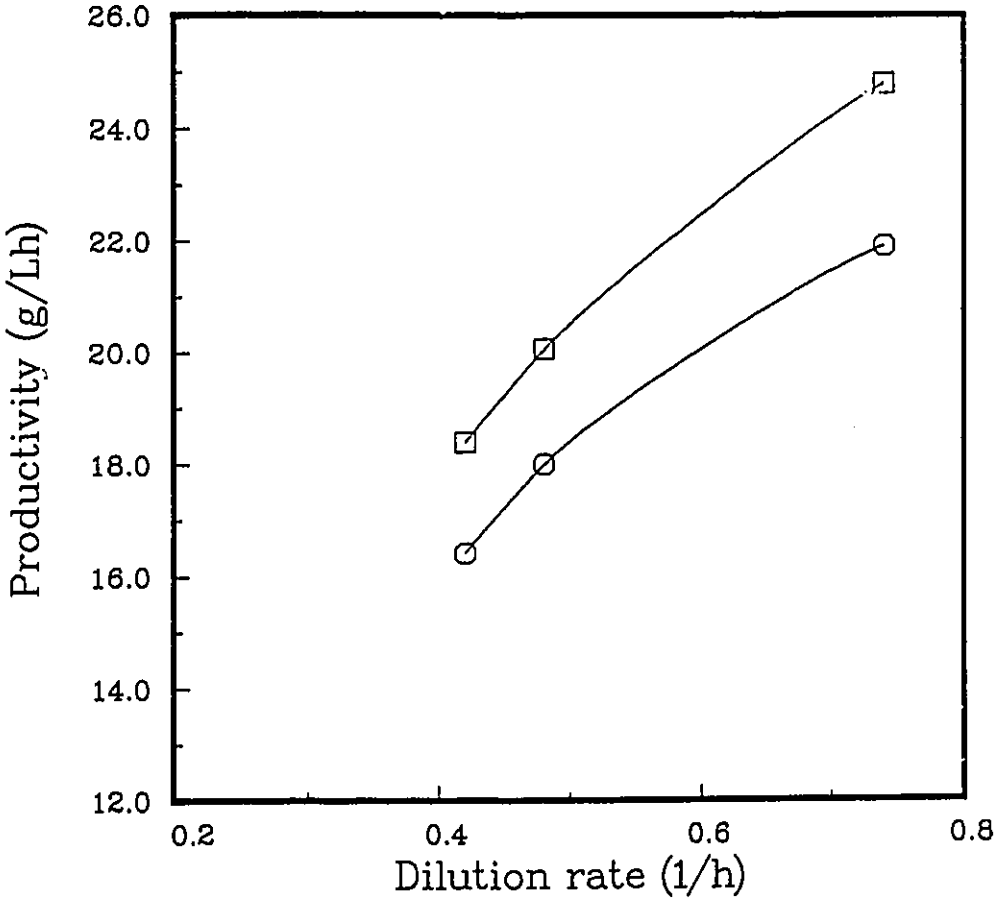

Figure 4.15: Comparison of ethanol productivities with and without oleic acid: □, 30 mg/L oleic acid; ○, no oleic acid.

4.5 Temperature Effect

The effect of temperature on the immobilized yeast was determined by changing the temperature of the reactor water jacket from 29.8 to 44.4 °C. A medium containing 101.4 g/L glucose and 97.3 g/L fructose was fed to the reactor (Figure 4.16). As the temperature increased from 29.8 to 32.6 °C the glucose concentration in the effluent decreased from 28.2 to 19.5 g/L, while the ethanol concentration increased from 3.78 to 4.25 g/L (Table 4.10).

Table 4.10: Product concentrations from the immobilized cell reactor at various temperatures with a dilution rate of 0.51 h^{-1} and a feed containing 101.4 g/L glucose and 97.3 g/L fructose.

Temp. (°C)	Final Gluc. (g/L)	Final Fruc. (g/L)	Final EtOH (g/L)	Gluc. Conv. (g/g)	EtOH Yield (g/g)	EtOH Prod. (g/Lh)
29.3	28.2	93.5	37.8	0.722	0.491	19.3
32.0	21.1	92.5	41.6	0.792	0.489	21.2
32.6	19.5	92.6	42.5	0.808	0.491	21.7
34.4	24.5	93.5	39.3	0.758	0.487	20.0
36.5	32.0	94.2	35.2	0.684	0.486	18.0
38.5	39.5	94.8	31.0	0.610	0.481	15.8
40.1	48.2	95.7	26.4	0.525	0.482	13.5
42.3	66.9	96.8	16.5	0.340	0.471	8.4
44.4	94.5	96.8	3.4	0.068	0.459	1.7

The ethanol productivity reached a maximum of 21.7 g/Lh at 32.6°C (Figure 4.17). As the temperature was further increased to 44.4°C the glucose concentration increased to 94.5 g/Lh and the ethanol concentration decreased to 3.4 g/L. The productivity at this temperature was

1.7 g/Lh. Figure 4.18 shows that the amount of biomass being released into the effluent began to increase when the temperature reached 40.1 °C, however the viability of these cells decreased with increasing temperature. The increase in biomass released from the reactor is due to the cell's loss of adhesive qualities upon its death. After leaving the reactor at 44.4 °C for several hours all of the cells had detached from the wood and had either settled to the bottom of the reactor or been carried out with the effluent.

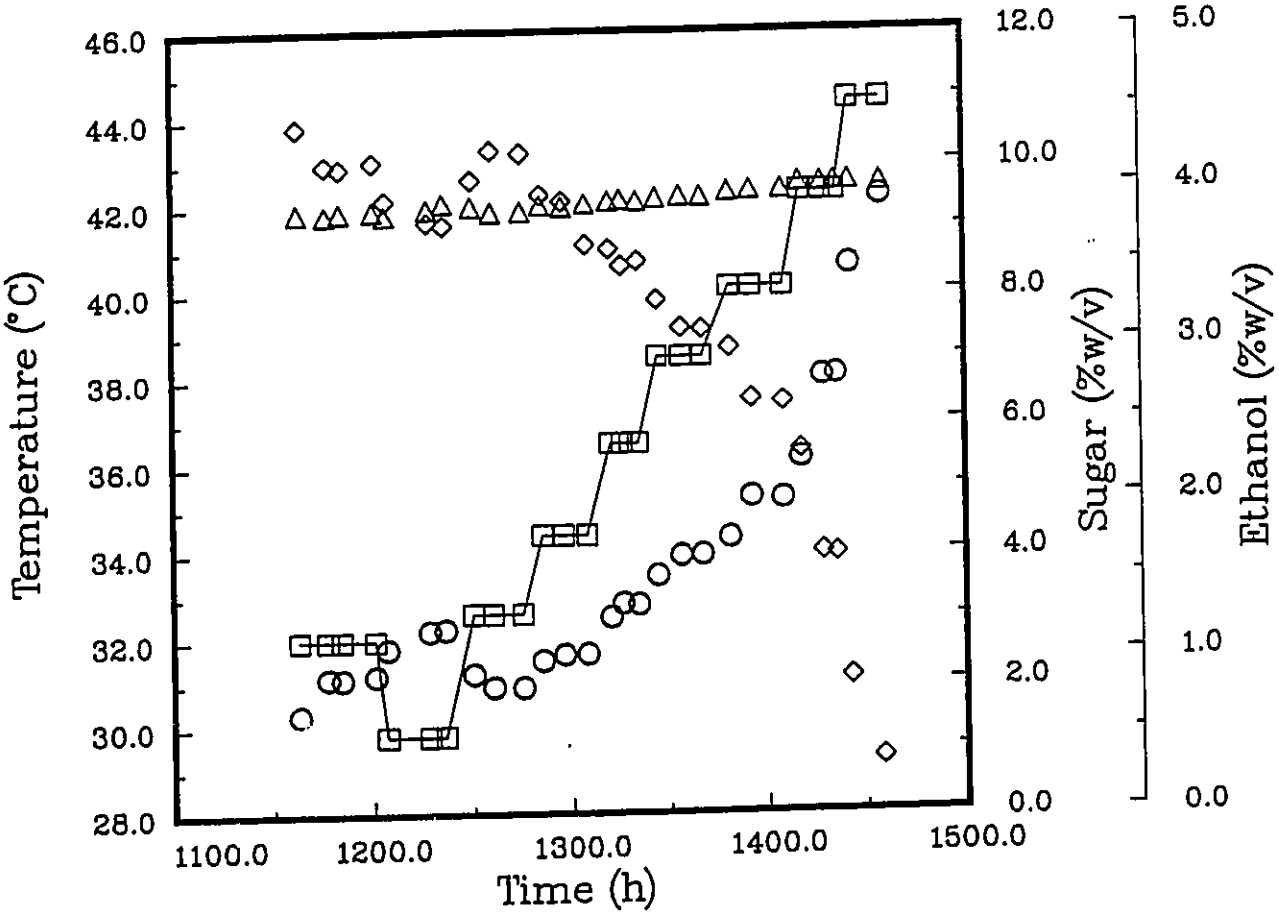

Figure 4.16: Ethanol production at varying temperatures using a feed containing 101.4 g/L glucose and 97.3 g/L fructose and a dilution rate of 0.51 h^{-1}: □, temperature; ○, glucose; △, fructose; ◇, ethanol.

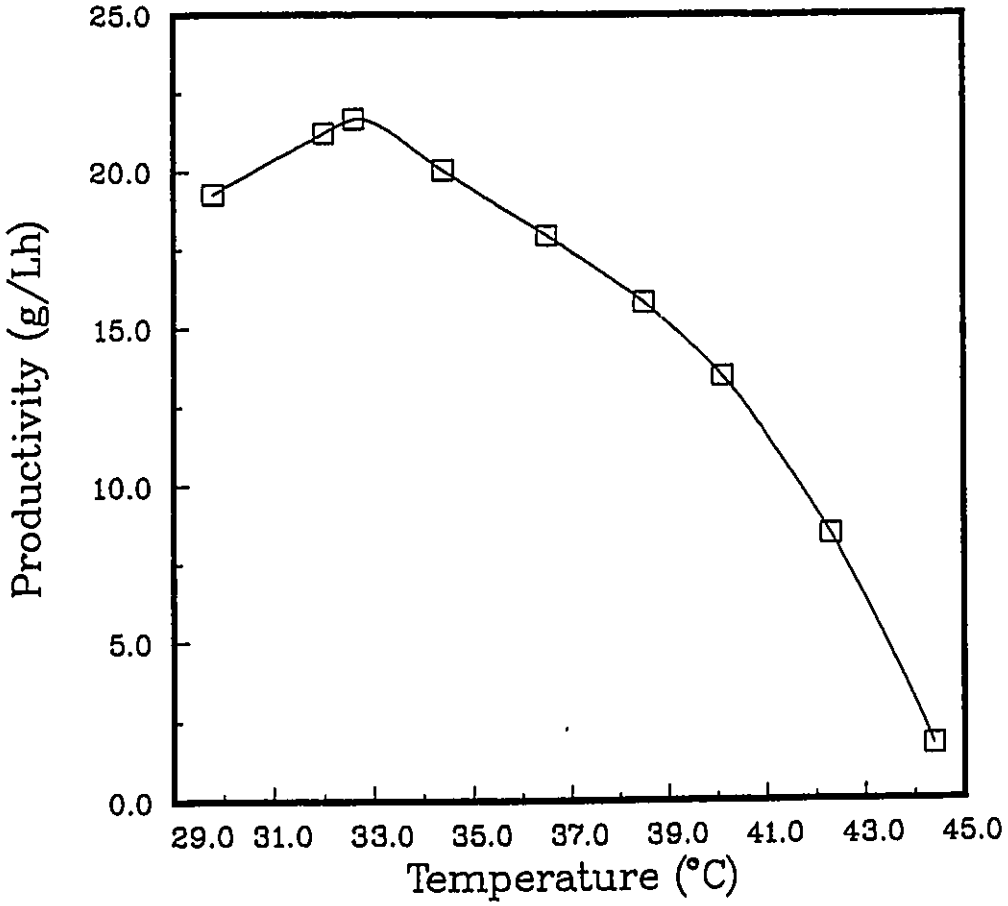

Figure 4.17: Ethanol productivity at various temperatures using a feed containing 101.4 g/L glucose and 97.3 g/l fructose and a dilution rate of 0.51 h^{-1}.

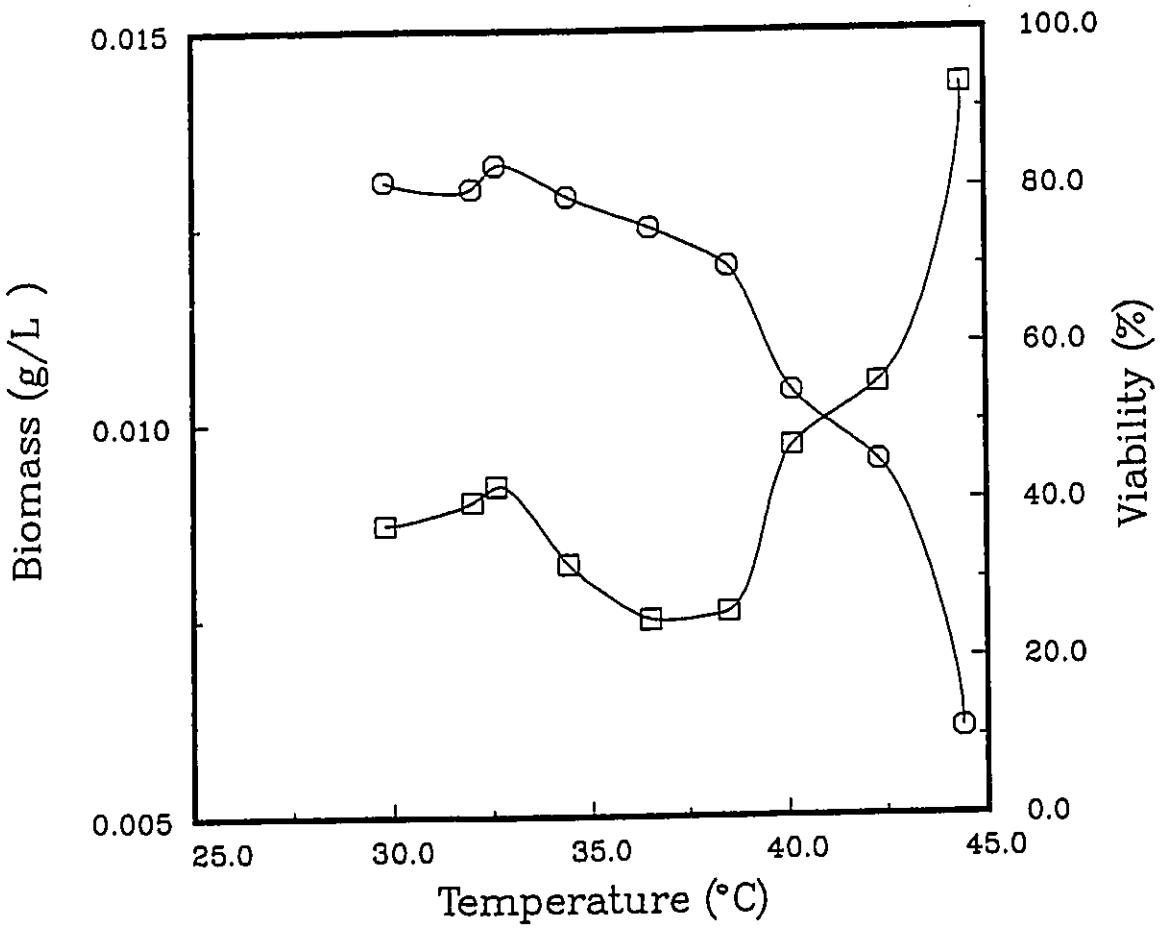

Figure 4.18: Production and viability of biomass in the effluent of the immobilized cell reactor with a change in temperature: □, biomass; ○, viability.

4.6 Effluent Fermentations

Previous experiments indicated that the reactor could not be run at 100% glucose consumption due to considerable fructose consumption at low glucose concentrations. To enhance the ethanol concentration, batch fermentations were carried out using the effluent from the reactor. The fermentations were carried out in 500 mL Erlenmyer flasks containing 100 mL of effluent.

In the first set of experiments (Table 4.11), the effects of added concentrated yeast suspension and glucose were studied. The addition of 5.0 mL of yeast suspension increased the ethanol productivity and glucose consumption by 30 and 50% respectively. Adding 50 g/L of glucose increased the ethanol productivity from 0.222 to 0.433 g/Lh and the glucose consumption from 0.463 to 0.886 g/Lh. A comparison of the media containing an additional 50 g/L of glucose showed an increase in ethanol productivity and glucose consumption of 33% and 12% respectively with the addition of 5.0 mL yeast suspension. The addition of 10 mL yeast suspension increased the productivity by 88% and the consumption by 90%. This final case increased the ethanol concentration of the original effluent by 80% however the ethanol productivity in this batch fermentation is only 8% of that in the continuous reactor.

The second set of experiments determined the effect of yeast extract on the batch fermentations (Table 4.12). The addition of 3.0 g/L of yeast extract to the effluent increased the ethanol productivity from 0.335 to 0.502 g/Lh. The glucose consumption was increased from 0.812 to 1.01 g/Lh. The addition of 15.0 g/L of yeast extract increased the ethanol production and glucose consumption by another 129 and 122% respectively. Adding 50 g/L of glucose increased the productivity by 101% and the consumption by 113%. The addition of

15.0 g/L of yeast extract to the medium containing an added 50 g/L of glucose gives an additional increase of 84 and 42 % for the production and consumption respectively. These last conditions gave the maximum ethanol productivity of 2.47 g/Lh. This in only 11% of the productivity obtained in the reactor, however the ethanol concentration was increased by 130%.

Table 4.11: Effluent batch fermentations from the immobilized cell reactor with an effluent containing 21.7 g/L glucose, 98.5 g/L fructose, 36.6 g/L ethanol and 0.446 g/L biomass. Productivity in the reactor was 21.9 g/Lh.

Flask content	F_G	F_F	F_E	P1	P2	Spec. growth rate (h^{-1})
Effluent treated batchwise without any additives	4.3	96.6	45.1	0.222	0.463	0.0253
Effluent + 5.0 mL yeast suspension	4.1	96.4	45.6	0.289	0.625	0.0295
Effluent + 50.0 g/L glucose	14.2	97.9	65.4	0.433	0.886	0.036
Effluent + 5.0 mL yeast suspension + 50.0 g/L glucose	14.4	98.0	65.8	0.578	0.988	0.064
Effluent + 10.0 mL yeast suspension + 50.0 g/L glucose	14.3	97.8	65.5	0.815	1.683	0.085

F_G= Final Glucose (g/L)
F_F=Final fructose (g/L)
F_E=Final ethanol (g/L)
P1=Ethanol productivity (g/Lh)
P2=Glucose consumption (g/Lh)

Table 4.12: Effluent batch fermentations from the immobilized cell reactor with an effluent containing 38.1 g/l glucose, 96.0 g/L fructose, 26.9 g/l ethanol and 0.546 g/L biomass.

Flask content	F_G	F_F	F_E	P1	P2	Spec. growth rate (h^{-1})
Effluent treated batchwise without any additives	7.62	93.2	41.8	0.335	0.812	0.0125
Effluent + 3.0 g/L yeast extract	7.58	92.9	42.0	0.502	1.01	0.0155
Effluent + 50.0 g/L glucose	17.4	95.0	62.1	1.15	2.23	0.0495
Effluent + 15.0 g/L yeast extract	7.65	92.8	41.8	0.673	1.73	0.0142
Effluent + 15.0 g/L yeast extract + 50.0 g/L glucose	17.7	94.8	62.4	1.24	2.47	0.0444

F_G= Final Glucose (g/L)
F_F=Final fructose (g/L)
F_E=Final ethanol (g/L)
P1=Ethanol productivity (g/Lh)
P2=Glucose consumption (g/Lh)

The ethanol productivities and glucose consumption rates obtained with the batch fermentations were very low relative to those obtained in the immobilized cell reactor. This is mainly due to the low biomass concentration and the ethanol present in the effluent. The addition of biomass increased the rates considerably however the addition of yeast extract appeared to be more effective. The effluent is likely to contain very little yeast extract since most of it would have been consumed in the reactor. Koren (1990) demonstrated that the mutant

yeast needed high concentrations of yeast extract relative to the wild strain, in order to obtain similar ethanol production and glucose consumption rates.

No fructose consumption occurred until more than 80% of the glucose present in the medium was consumed.

Batch fermentations could be used to enhance the ethanol concentration of the product and make ethanol recovery more profitable. However, even with added yeast extract or yeast suspension, the ethanol productivity is quite low.

4.7 Sorbitol Production

S. cerevisiae ATCC 36859 produced sorbitol and ethanol from fructose when no glucose was present in the medium (Duvnjak et al., 1991a). In previous experiments, using free cell batch fermentations, a maximum sorbitol concentration of 35.0 g/L and a maximum sorbitol production rate of 0.49 g/Lh were obtained. In order to determine the effect of cell immobilisation on the production of sorbitol by this mutant, a feed containing 133.9 g/L fructose was fed to the vertical packed column (Figure 4.19). With an increase in dilution rate from 0.25 to 0.62 h^{-1} the fructose concentration in the effluent increased from 31.6 to 108.5 g/L and the ethanol concentration decreased from 45.6 to 10.5 g/L (Table 4.13).

The sorbitol concentration in the effluent reached a maximum of 8.51 g/L at a dilution rate of 0.34 h^{-1} and decreased to 4.5 g/L at a dilution of 0.62 h^{-1}. Ethanol productivity reached a maximum of 12.4 g/Lh at a dilution rate of 0.34 h^{-1} whereas the sorbitol productivity peaked at 3.24 g/Lh at a dilution of 0.43 h^{-1} (Figure 4.20). As the dilution rate increased, the amount of consumed fructose used for ethanol production decreased while the amount used for sorbitol

production increased (Table 4.14).

Table 4.13: Product concentrations from immobilized cell reactor with feed containing 133.8 g/L fructose.

D (h^{-1})	Final Fruc. (g/L)	Final Sorb. (g/L)	Final EtOH (g/L)	Fruc. Conv. (g/g)	EtOH Prod. (g/Lh)	Sorb. Prod. (g/Lh)
0.25	31.6	8.51	45.6	0.764	11.4	2.13
0.34	50.8	8.52	36.6	0.621	12.4	2.90
0.43	74.9	7.53	25.6	0.441	11.0	3.24
0.62	108.5	4.50	10.5	0.190	9.51	2.79

Table 4.14: Fructose used for ethanol and sorbitol production in immobilized cell reactor with a feed containing 133.9 g/L.

D (h^{-1})	Fructose consumed (g)	Fe/Fc (g/g)	Fs/Fc (g/g)	(Fe+Fs)/Fc (g/g)
0.25	10.23	0.873	0.083	0.956
0.34	8.32	0.864	0.101	0.965
0.43	5.90	0.848	0.125	0.973
0.62	2.56	0.805	0.178	0.983

Fc: fructose consumed
Fe: fructose used to produce ethanol
Fs: fructose used to produce sorbitol

Therefore, the higher the concentration of fructose in the medium, the higher the relative amount of fructose used for sorbitol production. This trend was also observed in the free cell batch

fermentations carried out with this mutant (Duvnjak et al., 1991a). Duvnjak and colleagues (1991a) determined that ethanol was responsible for the suppression of sorbitol production. When the ethanol concentration was reduced by sparging sterile air into the medium a higher sorbitol concentration was obtained. Sparging air into the packed bed reactor could therefore increase the production of sorbitol by decreasing the ethanol concentration.

This is only a preliminary test of sorbitol production in a continuous process and it is not surprising that a relatively small amount of sorbitol was produced. More tests should be carried out to optimize the process, however, this is not within the scope of this research.

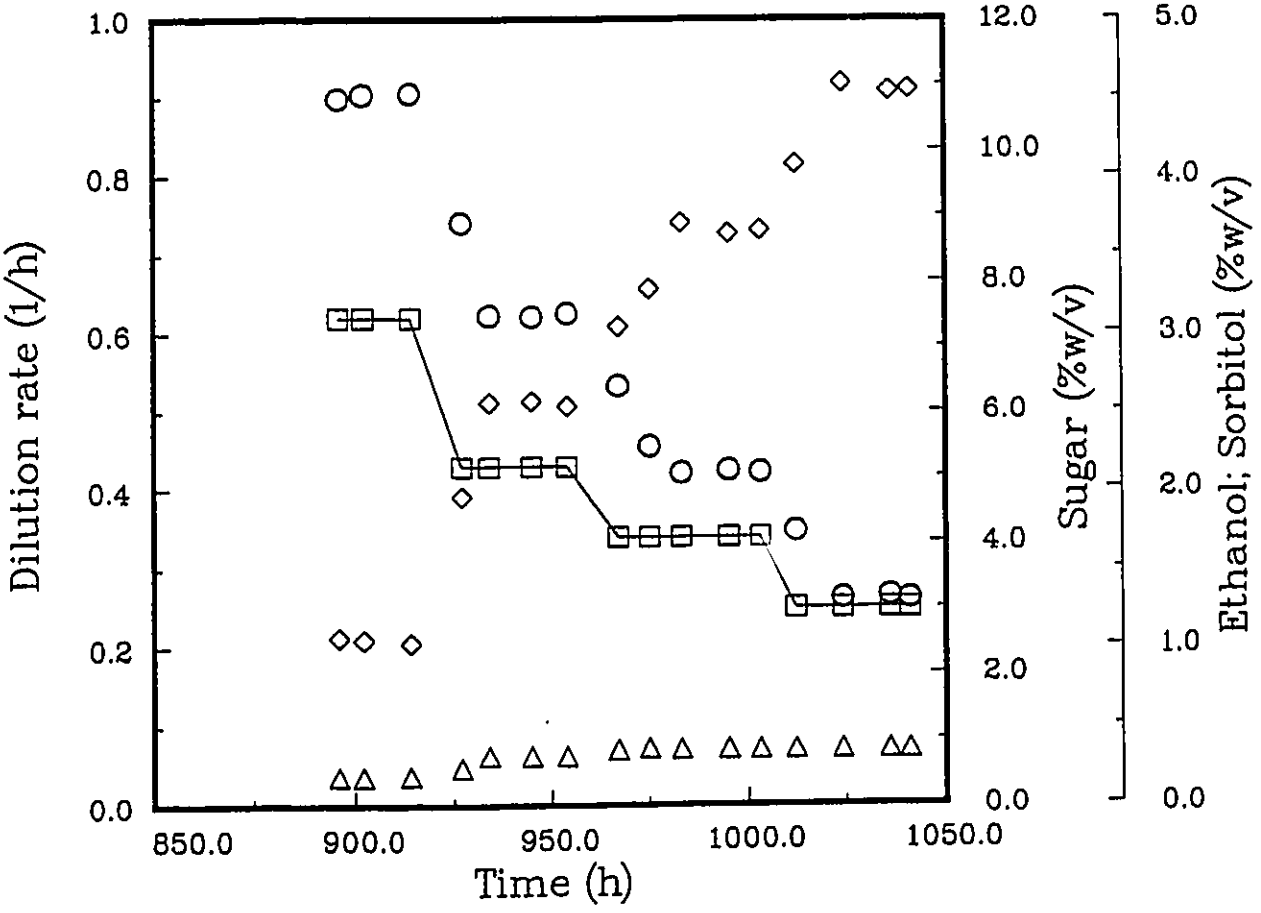

Figure 4.19: Ethanol and sorbitol production using a feed containing 133.9 g/L fructose: □, dilution rate; ○, fructose; △, sorbitol; ◇, ethanol.

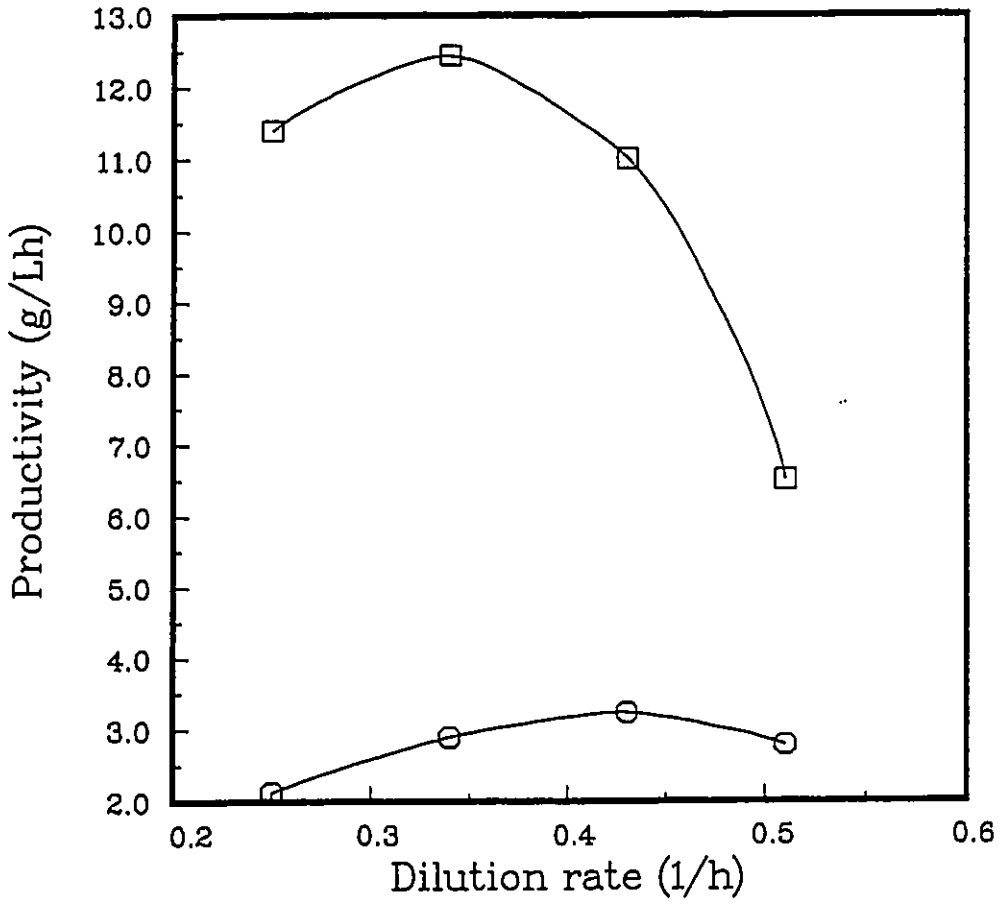

Figure 4.20: Ethanol and sorbitol productivities using a feed containing 133.9 g/L fructose: □, ethanol; ○, sorbitol.

Chapter 5

Conclusions

The following conclusions were drawn from the results obtained on the production of fructose, ethanol and sorbitol in the immobilized packed bed reactor.

- Immobilization of the yeast cells onto wood blocks allowed for a good quantity of cells to be adsorbed and enabled the continuous production of fructose and ethanol in a vertical packed bed reactor. A maximum ethanol productivity of 21.9 g/Lh was obtained with a feed containing 97.2 g/L glucose and 98.8 g/L fructose, at a dilution rate of 0.74 h^{-1}. The fructose consumption was less than 1% at glucose concentrations above 50 g/L. Fructose consumption reached 15% with a glucose concentration of 0.9 g/L in the effluent.

- The ethanol productivities in this wood block packed reactor were 33-130% higher than the ethanol productivities obtained in a similar reactor, under the same conditions, with the cells entrapped in Ca-alginate beads.

- The addition of 30 mg/L oleic acid to a medium containing 97.5 g/L glucose and 97.8 g/L fructose increased the ethanol productivity by 13%.

- The optimum reactor temperature for the conversion of glucose to ethanol was 32.6 °C. The ethanol productivity and cell viability approached zero at 44.4°C. The cells lost

their adhesive properties upon death at high temperatures.

- In batch fermentations to enhance the ethanol concentration in the effluent from the reactor, the maximum benefit was obtained when the medium was supplemented with glucose, yeast extract or a concentrated yeast suspension. The final ethanol concentration was 130% higher than that in the original effluent, however, the ethanol productivity in the batch fermentation was only 11% of the productivity in the reactor.

- Sorbitol and ethanol were produced in the reactor using a feed containing 133.8 g/L fructose. The maximum sorbitol productivity obtained was 3.25 g/Lh whereas the ethanol productivity reached 12.44 g/Lh.

- Models for the growth rate and ethanol productivity for the selective fermentation of glucose by the mutant yeast in the immobilized cell reactor were formulated. The models indicated that the growth rate and ethanol productivity had a Monod type dependence on glucose concentration and were linearly inhibited by total carbohydrate and ethanol concentrations. The cell growth rate was more strongly inhibited than the ethanol productivity by carbohydrate and ethanol concentration.

Chapter 6

Recommendations

The production of fructose and ethanol from synthetic glucose/fructose media was studied using an immobilized cell reactor. Tests were also carried out on the production of sorbitol and ethanol from fructose in the same reactor. Based on the results obtained in this work, the following recommendation are brought forward.

- Additional unsaturated fatty acids or sterols could be added to the medium in order to increase cell viability through higher ethanol tolerance.

- Other methods of immobilization could be explored in order to increase biomass concentration in the reactor and improve the cell characteristics, such as consumption and productivity rates and tolerance to various inhibitors.

- Ethanol inhibits the production of sorbitol by the mutant yeast. Ethanol concentrations could be reduced during sorbitol production by sparging air into the reactor.

References

Aiba, S., Shoda, M. and Nagatani, M., "Kinetics of product inhibition in alcohol fermentation", *Biotechnol. Bioeng.* **10**, 845-864 (1968).

Aiba, S. and Shoda, M., "Reassessment of the product inhibition in alcohol fermentation". *J. Ferment. Technol.* **47**, 790-794 (1969).

Atkinson B. and Mavituna F., "Biochemical Engineering and Biotechnology Handbook", Macmillan Publishers Ltd., 397, (1983).

Bailey, J.E. and Ollis, D.F., "Biochemical Engineering Fundamentals", McGraw-Hill Book Company, New York, (1986).

Bandyopadhyay K.K. and Ghose T.K., "Studies on immobilized *Saccharomyces cerevisiae* III. Physiology of growth and metabolism on various supports", *Biotechnol. Bioeng.* **24**, 805- 815 (1982).

Bang W.G., Behrendt U., Lang S. and Wagner F., "Continuous production of L-Tryptophan from indole and L-Serine by immobilisation of *Escherichia coli* cells", *Biotechnol. Bioeng.* **25**, 1013-1025 (1983).

Barker, S.A. and G.S. Petch, "Enzymatic processes for high fructose corn syrup", Enzymes and Immobilized Cells in Biotechnology; Ed. A.I. Laskin, Benjamin Cummings (1985).

Barrow, K.D., Collins, J.G., Leigh, D.A., Rogers, P.L., Warr, R.G., "Sorbitol production by *Zymomonas mobilis*", *Appl. Microbiol. Biotechnol.* **20**, 225-232 (1984).

Bateman, J.R., Coodacre, B.C., Smithson, A., European Patent Application 0,116,389 (1984).

Bernt, E. and Gutmann, I., "Ethanol determination with alcohol dehydrogenase and NAD", Methods of Enzymatic Analysis; Ed. H.U. Bergermeyer, Academic Press (1974).

Berdelle-Hilge, Ph., "Wort under hydraulic pressure passed through a layer of yeast", U.S. Patent, 3,737,323, (1973).

Bichsel, S.E., Y. Wang and A.M. Sandre, U.S. Patent 4,263,052 (1981).

Birnbaum S., Larsson P.O. and Mosbach K., in Solid phase Biochemistry: Analytical and Synthetic Aspects (Edited by Scouten W.H.), Wiley, New York 1983.

Brock, T.D., Smith D.W. and Madigan M.T., "Biology of Microorganisms", Prentice-Hall, (1984).

Bringer-Meyer, S., Scollar, M., and Sahm, H., "Zymomonas mobilis mutants blocked in fructose utilization", *Appl. Microbiol. Biotechnol.* **23**, 134-139 (1985).

Cheetham, P.S.J., "Physical studies on the mechanical stability of columns of calcium alginate gel pellets containing microbial cells", *Enzyme Microb. Technol.* **1**, 183-188 (1979).

Cheetham, P.S.J., Blunt, K.W., and Bucke, C., "Physical studies on cell immobilization using calcium alginate gels", *Biotechnol. Bioeng.* **21**, 2155-2168 (1979).

Chun, U.H., and Rogers P.L., "The simultaneous production of sorbitol from fructose and gluconic acid from glucose using an oxidoreductase of Zymomonas mobilis", *Appl. Microbiol. Biotechnol.* **29**, 19-24 (1988).

Coker, L.E. and K. Venkatasubramanian, "Corn Sweeteners", Food Biotechnology; Ed. D. Knorr, Marcel Dekker (1987).

Colowick, S.P., "The Enzymes"; Ed. P.D. Boyer, Academic Press (1973).

Contois, D.E., "Kinetics of bacterial growth: Relationship between population density and specific growth rate of continuous cultures", *J. Gen. Microbiol.* **21** 40-50 (1959).

Cysewski, G.R. and Wilke, C.R., "Utilizaiton of cellulosic materials throught enzymatic hydrolysis to ethanol and single-cell protein", *J. Gen. Microbiol.* **21**, 40-50 (1959).

Cysewski, G.R. and Wilke, C.R., "Process design and economic studies of alternative fermentation methods for the production of ethanol", *Biotechnol. Bioeng.* **20**, 1421- 1444 (1978).

Dale, M.C., Chen, C. and Okos, M.R., "Cell growth and death rates as factors in the long-term performance, modelling, and design of immobilized cell reactors", *Biotechnology and Bioengineering*, **36**, 983-992 (1990).

Daugulis, A.J., Brown, N.M., Cluett, W.R. and Dunlop, D.B., "Production of ethanol by adsorbed yeast cells", *Biotechnol. Lett.*, 3, 651-656 (1981).

Doelle, H.W., "Kinetic characteristics and regulatory mechanisms of glucokinase and fructokinase for *Zymomonas mobilis*", *Eur. J. Appl. Microbiol. Biotechnol.* **14**, 241-246 (1982a).

Doelle, H.W., "The existence of two separate constitutive enzymes for glucose and fructose in Zymomonas mobilis", *Eur. J. Appl. Microbiol. Biotechnol.* **15**, 20-24 (1982b).

Doelle, H.W., "Sucrotech: Alternative ethanol production", *Proc. Control Eng.* **8**, 54, 56 (1986).

Doelle, H.W. and P.F. Greenfield, "The production of ethanol form sucrose using Zymomonas mobilis", *Appl. Microbiol. Biotechnol.* **22**, 405-410 (1985a).

Doelle, H.W. and P.F. Greenfield, "Fermentation pattern of Zymomonas mobilis at high sucrose concentrations", *Appl. Microbiol. Biotechnol.* **22**, 411-415 (1985b).

Duvnjak, Z., Turcotte, G. and Duan, Z.D., "Production and Consumption of Sorbitol and Fructose by *Saccharomyces cerevisiae* ATCC 36859", *J. Chem. Tech. Biotechnol.*, **52**, 527-537 (1991a).

Duvnjak, Z., Turcotte, G. and Duan, Z.D., "Production of sorbitol and ethanol form Jerusalem artichokes by *Saccharomyces cerevisiae* ATCC 36859", *Appl Microbiol. Biotechnol.* **35**, 711-715 (1991b).

Dwivedi, B.K., "Sorbitol and Mannitol, Alternative Sweeteners"; Eds. L.O. Nabors and G.C. Gelardi, Marcel Dekker (1991).

Edwards, V.H., "Influence of high substrate concentrations on microbial kinetics", *Biotechnol. Bioeng.* **12**, 679-712 (1970).

Edye, L.A., M.R. Johns and D.N. Ewings, "Fructose production by *Zymomonas mobilis* in fed-batch culture with minimal sorbitol formation", *Appl. Microbiol. Biotechnol.* **31**, 129-133 (1989).

Engelbart, W. and Engelbart, F., "Apparatus and method for optimizing chemical reactions and biological fermentations", U.S. Patent, 3,880,716, (1975).

Falch, E. and Gaden, E., "A continuous, multistage tower fermentor. I Design and performance tests", *Biotechnol. Bioeng.* **11**, 927-943 (1969).

Felix H., "Review: Permeabilized cells", *Anal. Biochem.* **120**, 211-234 (1982).

Fleming, S.E. and GrootWassink, J.W.D, "Preparation of high-fructose syrup from the tubers of the Jerusalem artichoke *Helianthus tuberosus*" *CRC Critical Reviews in Food Science and Nutrition.* **12** 1-28 (1979).

Fletcher M., "A microantoradiographic study of the activity of attached and free-living bacteria", *Arch. Microbiol.* **122**, 271- 274 (1979).

Fletcher M., Latham M.J., Lynch J.M. and Rutter P.R., in "Microbial Adhesion to Surfaces" (Edited by Berkeley R.C.W., Lynch J.M., Melling J., Rutter P.R. and Vincent B.). Ellis Horwood, Chichester 1980.

Fletcher M. and Marshall K.C., "Are solid surfaces of ecological significance to aquatic bacteria?", *Adv. Microb. Ecol.* **6**, 199-236 (1982).

Gancedo, J.-M., D. Clifton and D.G. Fraenkel, "Yeast hexokinase mutants", *J. Biol. Chem.* **252**, 4443-4444 (1977).

Gencer, M.A. and Mutharasan, R., "Ethanol fermentation in a yeast immobilized column fermenter", *Advances in Biotechnology*, Vol. 1, Ed. M. Moo-Young, Pergamon Press, Toronto, 627-633 (1981).

Gerhardt, P. and Gallup, D.M., "Dialysis flask for concentrated culture of microorganisms", *J. Bacteriol.*, **86**, 919-924 (1963).

Ghommidh G., Navarro J.M. and Durand G., "A study of acetic acid production by immobilized *Acetobacter* cells: oxygen transfer", *Biotechnol. Bioeng.* **24**, 605-617 (1982).

Ghose,T.K. and Tyagi, R.D., "Rapid ethanol fermentation of cellulose hydrolysate II. Product and substrate inhibition and optimization of fermentor design", *Biotechnol. Bioeng.* **21**, 1401-1420 (1979a).

Ghose, T.K. and Tyagi, R.D., "Rapid ethanol fermentation of cellulose hydrolysate II. Product and substrate inhibition and optimization of design", *Biotechnol. Bioeng.* **21**, 1401-1420 (1979b).

Griffith, W.L. and Compere, A.L., "A new method for coating fermentation tower packing so as to facilitate microorganism attachment", *Dev. Ind. Microbiol.*, **17**, 241-249 (1976).

Grinbergs, M., Hildebrand, R.P. and Clarke, B.J., "Continuous fermentations of glucose solutions", *J. Inst. Brewing*, **83**, 25-31 (1977).

Hahn-Hagerdal, B. and Mattiason, B., "Shift in metabolism towards ethanol production in *Saccharomyces cerevisiae* by addition of metabolic inhibitors", *Biotechnol. Bioeng.*, Symp. No. 12, 193-197 (1982).

Hattori, R., "Growth of *Escherichia coli* on the surface of an anion-exchange resin in continuous flow system", *J. Gen. Appl. Microbiol.*, **18**, 319-325 (1972).

Helmstetter C.E. and Cooper S., "DNA synthesis during the division cycle of rapidly growing *Escherichia coli B/r*", *J. Molec. Biol.* **31**, 507-518 (1968).

Hodgkin, J.A., "High furctose: A growing world role?", *Sugar y Azucar* **82**, 15,18,19,22,23 (1987).

Holzberg, I., Finn, R.K., and Steinkraus, K.H., "A kinetic study of the alcoholic fermentation of grape juice", *Biotechnol. Bioeng.* **9**, 413-427 (1967).

Hoppe, G.K. and Hansford, G.S., "The effect of micro-aerobic conditions on continuous ethanol production by *Saccharomyces cerevisiae*", *Biotechno. Letts.*, **6**(10), 681-686 (1984).

Jackman E.A., "Industrial Alcohol", Basic Biotechnology; Ed. J. Bu'lock and B. Kristiansen, Academic Press (1987).

Joglekar,R. R.J. Clerman, R.P. Ouellete and P.N. Cheremisinoff, "High Fructose Corn Syrup", Biotechnology in Industry; Ann Arbor Science Publishers (1983).

Jones, R.P., Pamment N. and Greenfield, P.F., "Alcohol fermentation by yeasts - the effect of environmental and other variables" *Process Biochem.* **16**, 42-49 (1981).

Jones, R.P., "Biological principles for the effects of ethanol", *Enzyme Microb. Technol.* **11**, 130-158 (1989).

Kierstan, M. and Bucke, C., "The immobilization of microbial cells, subcellular organelles, and enzymes in calcium alginate gels", *Biotechnol. Bioeng.*, **19**, 387-397 (1977).

Klein, J. and Kressdorf, B., "Immobilization of living whole cells in an epoxy matrix", *Biotechnol. Lett.* **4**, 375-380 (1982).

Koren, D.W., "Production of fructose and ethanol by selective fermentation of glucose-fructose mixtures", PhD. Thesis, University of Ottawa (1990).

Koren, D.W. and Z. Duvnjak, "Continuous production of very enriched fructose syrup by the conversion of glucose to ethanol form glucose-fructose mixtures in an immobilized cell reactor", *Int. J. of Food Sci. and Technol.* **24**, 429-437 (1989).

Koren, D.W. and Z. Duvnjak, "Pure fructose syrup and ethanol production form High Fructose Syrup supplemented with Jerusalem artichoke juice", *J. Chem. Technol. Biotechnol.* **47**, 117-125 (1990).

Kosaric, N., Wieczorek, A., Cosentino, G.P., Magee, R.J., Prenosil, J.E., "Ethanol Fermentation", Biotechnology vol.: Eds. H.J. Rehm and G. Reed, Verlag-Chimie (1985).

Koshcheyenko K.A., Turkina M.V. and Skryabin G.K., "Immobilization of living microbial cells and their application for steroid transformations" *Enzyme Microb. Technol.* **5**, 14-21 (1983).

Krouwel, PG., Harder, A., and Kossen, N.W.F., "Tensile stress-strain measurements of materials used for immobilization", *Biotechnol. Lett.* **4**, 103-108 (1982).

Krouwel, P.G. and Kossen, N.W.F., "Gas production by immobilized micro-organisms: theoretical approach", *Biotechnol. Bioeng.* **22**, 681-687 (1980).

Lamarche, K., "Production of fructose and ethanol from sucrose", MASc. Thesis, University of Ottawa (1988).

Larue, F., Lafond-Lafourcade, S. and Ribereau-Gayon, P.,"Relationship between the sterol content of yeast cells and their fermentation activity in grape must", *Appl. Environ. Microbiol.* **39(4)**, 808-811 (1980).

Lee, T.H., Ahn, J.C. and Ryu, D.D.Y., "Performance of an immobilized yeast reactor system for ethanol production", *Enzyme Microb. Technol.* **5**, 41-45 (1983).

Lehninger, A.L., "Principles of Biochemistry", Worth Publishers (1982).

Leigh, D., Scopes R.K. and Rogers P.L., "A proposed pathway for sorbitol production by *Zymomonas mobilis*", *Appl. Microbiol. Biotechnol.*, **20**, 413-415 (1984).

Levenspiel, O., "The Monod equation: A revisit and a generalization to product inhibition situations", *Biotechnol. Bioeng.* **22**, 1671-1687 (1980).

Linko, Y.Y. and Linko, P., Continuous ethanol production by immobilized yeast reactor, *Biotechnol. Lett.* **3**, 21-26 (1981).

Long, John, E., "High Fructose Corn Syrup", Alternative Sweeteners; Eds. L.O. Nabors and G.C. Gelardi, Marcel Dekker (1991).

Lyness E., Doelle, H.W., "Fermentation pattern of sucrose to ethanol conversions by *Zymomonas mobilis*", *Biotechol. Bioeng.* **23**, 1449-1460 (1981).

Maitra, P.K., "Glucokinase from *Saccharomyces cerevisiae*" *J. Biol. Chem.* **245**, 2423-2431 (1970).

Maitra, P.K., "Glucokinase from yeast", *Methods in Enzymol.* **42**, 25-30 (1975).

Maiorella, B.L., "Ethanol", Comprehensive Biotechnology, Volume 2, Ed. M. Moo-Young, Pergamon, Oxford, (1984).

Margaritis, A. and Bajpai, P., "Effect of sugar concentration in Jerusalem artichoke extract on *Kluyeromyces marxiamus* growth and ethanol production", *Appl. Environ. Microbiol.* **45**, 723-725 (1983).

Margaritis, A., Merchant, F.J.A., "Advances in ethanol production using immobilized cell systems" *CRC Crit. Reviews in Biotechnol.*, **1**, 339-393 (1984).

Michaux, M., Paquot, M., Baijot, B. and Thonart, Ph., "Continuous fermentation: improvement of cell immobilization by Zeta potential measurement", *Biotechnol. Bioeng.*, Symp. No. 12, 475 (1982).

Minier, M. and Goma, G., "Ethanol production by extractive fermentation", *Biotechnol. Bioeng.*, **24**, 1565-1579 (1982).

Mohan R.R. and Li N.N., "Nitrate and Nitrite reduction by liquid membrane-encapsulated whole cells", *Biotechnol. Bioeng.* **17**, 1137- 1156 (1975).

Moo-Young, M., Lamptey, J. and Robinson, C.W., "Immobilization of yeast cells on various supports for ethanol production", *Biotechnol. Lett.*, **2**, 541-548 (1980).

Moreno, M., Goma, G., "Alcohol fermentation in strict anaerobiosis in a plug flow fermentor: effect of cell recycling:, *Biotechnol. Lett.* **1**, 483-488 (1979).

Moser, A., "Kinetics of Batch Fermentation", Biotechnology vol.2; Eds. H.-J. Rehm and G. Reed, VCH Publishers (1985).

Nagodwithana, T.W., Castellano, C. and Steinkraus, K.H., "Effect of dissolved oxygen, temperature, initial cell count, and sugar concentration on the viability of *Saccharomyces cerevisiae* in rapid fermentations", *Appl. Microbiol.* **28**, 383-391 (1974).

Navarro J.M. and Durand G., "Modification of yeast metabolism by immobilization onto porous glass", *Eur. J. Appl. Microbiol.* **4**, 243-254 (1977).

Nilsson K., Birnbaum S., Flygare S., Linse L., Mosback K. and Brodelius P., "A general method for the immobilization of cells with preserved viability" *Eur. J. Appl. Microbiol. Biotechnol.* **17**, 319-326 (1983).

Novak, M., Strehaiano, P., Moreno, M., and Goma, G., "Alcoholic fermentation: on the inhibitory effect of ethanol", *Biotech. Bioeng.*, **23**, 201-211 (1981).

Parascandola P., Salvadore S. and Scardi V., "Tuff as a convenient material for supporting immobilized invertase - active whole cells of *Saccharomyces cerevisiae*", *J. Ferment. Technol.* **60**, 477-480 (1982).

Reusser, F., P.A.J. Gorin and J.F.T. Spenser, "The production of fructose as a residue of sucrose fermentation by Tricholoma nudum", *Can. J. Microbiol.* **6**, 17-20 (1960).

Rittmann B.E., "The effect of shear stress on biofilm loss rate", *Biotechnol. Bioeng.* **24**, 501-506 (1982).

Rony P.R., "Multiphase catalysis II. Hollow fiver catalysts", *Biotechnol. Bioeng.* **13**, 431-447 (1971).

Rosen, K., "Continuous production of alcohol", *Process Biochem.* **13**, 25-27 (1978).

Ruggeri, B., Specchia, V., Gianetto, A., "Ethanol production from lactose by *Kluyveromyces fragilis*: Kinetic study on an immobilized yeast reactor", *Chem. Eng. J.*, **37**, B23-B30 (1988).

Ryu, V.W., Navarro, J.M. and Durand, G., "Comparative study of ethanol production by an immobilized yeast in a tubular reactor and in a multistage reactor", *Eur. J. Appl. Microbiol. Biotechnol.*, **15**, 1-8 (1982).

Scherer P., Kluge M., Klein J. and Sahm H., "Immobilization of the methanogenic bacterium *Methanosareina barkeri*", *Biotechnol. Bioeng.* **23**, 1057-1065 (1981).

Shiotani, T. and Yamane, T., "A horizontal packed bed bioreactor to reduce CO_2 holdup in the continuous production of ethanol by immobilized yeast cells", *Eur. J. Appl. Microbiol. Biotechnol.*, **13**, 96-101 (1981).

Sinclair, C.G. and Kristiansen, B., "Fermentation Kinetics and Modelling", Taylor & Francis, New York, (1987).

Sitton, O.C., Magruder, G.C. and Gaddy, J.L., "Production of ethanol with immobilized *Saccharomyces cerevisiae*", Advances in Biotechnology, Vol.2, Ed. Moo-Young, Pergamon Press, Toronto, 231-237 (1981).

Siva Raman, H., Seetarama Rao, B., Pundle, A.V. and Siva Raman, C., "Continuous ethanol production by yeast cells immobilized in open pore gelatin matrix", *Biotechnol. Lett.* **4**, 359-364 (1982).

Stewart, G.G., Panchal, C.J., Russell, I., Sills, A.M., "Biology of ethanol producing microorganisms", *CRC Crit. Reviews in Biotechnol.* **3**, 161-188 (1983).

Suntinanalert, P., J.P. Pemberton and H.W. Doelle, "The production of ethanol plus fructose sweetener using fructose utilization negative mutants of *Zymomonas mobilis*", *Biotechnol. Letts.* **8**, 351-356 (1986).

Takasaki, Y. and O. Tanabe, U.S. Patent 3,616,221 (1971).

Takata I., Tosa T. and Chibata I., "Stabilization of fumarase activity of *Brevibacterium flavum* cells by immobilization with k-carrageenan" *Appl. Biochem. Biotechnol.* **8**, 31-38 (1983a).

Takata I., Tosa T. and Chibata I., " Reasons for the high stability of fumarase activity of *Brevibactirium flavum* cells immobilized with k-carrageenan gel" *Appl. Biochem. Biotechnol.* **8**, 39-54 (1983b).

Tatuki, R. (1972) U.S. Patent 3,671,316. (EICB)

Tenney M.W. and Verhoff F.H., "Chemical and autoflocculation of microorganisms in biological wastewater treatment", *Biotechnol. Bioeng.* **15**, 1045-1073 (1973).

Thomas, D.S., Hossack, J.A. and Rose A.H.,"Plasma-Membrane lipid composition and ethanol tolerance in *Saccharomyces cerevisiae*", *Arch. Microbiol.*, **117**, 239-245 (1978).

Ueng, P.P., L.D. McCracken, C.S. Gong and G.T. Tsao, "Fructose production from sucrose and High Fructose Syrup: A mycelial fungal system", *Biotechnol. Letts.* **4**, 353-358 (1982).

Viikari, L., "Formation of levan and sorbitol from sucrose by *Zymomonas mobilis*", *Appl. Microbiol. Biotechnol.* **19**, 252-255 (1984a).

Viikari, L., "Formation of sorbitol by *Zymomonas mobilis*", *Appl. Microbiol. Biotechnol.* **20**, 118-123 (1984b).

Wada, M., Kato, J. and Chibata, I., "Continuous production of ethanol using immobilized growing yeast cells", *Eur. J. Appl. Microbiol. Biotechnol.* **10**, 275-287 (1980).

Wang G. and Wang D.I.C., "Production of acetic acid by immobilized whole cells of *Clostridium thermoaceticum*" *Appl. Biochem. Biotechnol.* **8**, 491-503 (1983).

Watson, K., "Unsaturated fatty acid but not ergosterol is essential for high ethanol production in *Saccharomyces*", *Biotechnol. Letts.*,**4(6)**, 397-402 (1982).

Wilke, C.R. and Maiorella, B., "High productivity anaerobic fermentation with dense cell culture", *Advances in Biotechnology*, Vol. 1, Ed. M. Moo-Young, Pergamon Press, Toronto, 539-545 (1981).

Williams, D. and Munnecke, D.M., "The production of ethanol by immobilized yeast cells", *Biotechno. Bioeng.*, **23**, 1813-1825 (1981).

Windholz, M., "Algin and alginic acid", The Merck Index, Merck and Co., New Jersey, 1976, 34.

Younes G., Nicaud J.M. and Guespin-Michel J., "Enhancement of extracellular enzymatic activities produced by immobilized growing cells of *Myxococcus xanthus*", *Appl. Microbiol. Biotechnol.* **19**, 67-69 (1984).

Zachariou M. and Scopes R.K., "Glucose-fructose oxidoreductase, a new enzyme isolated from *Zymomonas mobilis* that is responsible for sorbitol production.", *J. Bacteriol.* **167**, 863-869 (1986).

APPENDIX

Raw data from the immobilized cell reactor

Table A1: Feed - 5.21% Glucose

Time (h)	D (h^{-1})	Glucose (% w/v)	Ethanol (% w/v)
0	0.51	5.21	0.0
3	0.51	5.16	0.022
6	0.51	5.18	0.013
19	0.51	5.02	0.092
27	0.51	5.00	0.10
46	0.51	4.23	0.47
50	0.51	4.19	0.49
69	0.51	4.02	0.58
75	0.51	3.68	0.75
80	0.51	3.49	0.84
92	0.51	3.25	0.96
99	0.51	2.98	1.09
102	0.51	2.54	1.30
117	0.51	2.26	1.43
123	0.51	2.01	1.55
140	0.51	1.86	1.65
151	0.51	1.46	1.82
165	0.51	1.22	1.93
187	0.51	0.98	2.05
199	0.51	0.91	2.08
225	0.66	1.21	1.84
247	0.66	2.03	1.55
260	0.66	1.95	1.58
269	0.66	1.89	1.62
284	0.66	1.82	1.65
291	0.66	1.76	1.68
310	0.66	1.72	1.70
320	0.66	1.54	1.78
331	0.66	1.43	1.83
343	0.66	1.24	1.93
356	0.66	1.05	2.02
363	0.66	1.04	2.02
380	0.66	1.06	2.01
389	0.66	1.03	2.03
403	0.66	1.07	2.01

Table A2: Feed-9.78% glucose

Time (h)	D (h^{-1})	Glucose (% w/v)	Fructose (% w/v)	Ethanol (% w.v)
538	0.34	0.84	4.33	0.10
549	0.34	0.31	4.67	0.30
556	0.34	0.29	4.65	
562	0.34	0.28	4.63	0.48
575	0.34	0.30	4.65	0.59
582	0.42	0.54	4.55	0.62
591	0.42	0.65	4.46	0.60
599	0.42	0.70	4.43	0.80
608	0.42	0.65	4.48	0.95
616	0.42	0.68	4.50	0.94
627	0.42	0.67	4.48	1.10
636	0.48	1.14	4.09	1.05
646	0.48	1.22	4.16	1.03
658	0.48	1.20	4.20	1.07
670	0.48	1.18	4.21	1.39
679	0.48	1.21	4.20	1.45
688	0.74	2.28	3.63	1.48
699	0.74	2.81	3.49	1.50
705	0.74	2.78	3.40	1.55
716	0.74	2.80	3.41	1.53
724	0.74	2.83	3.40	1.52

Table A3: Feed- 9.72% glucose and 9.88% fructose

Time (h)	D (h^{-1})	Glucose (% w/v)	Fructose (% w/v)	Ethanol (%w/v)
352	0.74	5.24	9.85	2.19
361	0.74	3.67	9.83	2.94
368	0.74	3.64	9.85	2.97
375	0.74	3.65	9.84	2.98
382	0.74	3.63	9.86	2.98
392	0.48	2.54	9.85	3.50
401	0.48	2.07	9.81	3.74
409	0.48	2.10	9.82	3.76
418	0.48	2.11	9.81	3.73
427	0.48	2.09	9.80	3.76
435	0.42	1.74	9.79	3.96
441	0.42	1.69	9.78	3.99
450	0.42	1.66	9.79	4.00
459	0.42	1.64	9.80	3.97
467	0.42	1.65	9.78	3.99
475	0.42	1.67	9.79	4.00
486	0.34	1.32	9.78	4.09
494	0.34	1.25	9.77	4.18
501	0.34	1.26	9.76	4.19
512	0.34	1.24	9.77	4.16
521	0.34	1.27	9.75	4.21
529	0.34	1.24	9.77	4.18

Table A4: Feed - 9.75% glucose, 9.78% fructose, 30 mg/L oleic acid

Time (h)	D (h^{-1})	Glucose (% w/v)	Fructose (% w/v)	Ethanol (% w/v)
738	0.74	3.30	9.75	3.17
746	0.74	2.91	9.76	3.35
758	0.74	2.95	9.74	3.33
767	0.74	2.89	9.76	3.32
775	0.74	2.91	9.75	3.36
784	0.48	2.46	9.74	3.56
796	0.48	1.64	9.75	4.19
803	0.48	1.24	9.76	4.19
812	0.48	1.21	9.74	4.16
825	0.48	1.25	9.74	4.19
834	0.42	1.43	9.74	4.05
849	0.42	0.91	9.73	4.38
855	0.42	0.95	9.71	4.37
863	0.42	0.90	9.72	4.38
878	0.42	0.89	9.71	4.40

Table A5: Feed - 5.09% glucose and 11.12% fructose

Time (h)	D ($^{-1}$)	Glucose (% w/v)	Fructose (% w/v)	Ethanol (% w/v)
1005	0.43	1.01	10.02	2.51
1012	0.43	0.60	10.30	2.57
1024	0.43	0.52	10.35	2.59
1035	0.43	0.51	10.34	2.60
1047	0.43	0.52	10.31	2.61
1054	0.51	0.74	10.56	2.38
1068	0.51	0.80	10.64	2.31
1079	0.51	0.81	10.61	2.32
1085	0.51	0.79	10.60	2.34
1094	0.25	0.54	10.10	2.70
1106	0.25	0.21	9.50	3.25
1117	0.25	0.09	9.45	3.30
1128	0.25	0.10	9.53	3.29
1136	0.25	0.08	9.51	3.31

Table A6: Feed - 8.52% glucose and 12.90% fructose

Time (h)	D (h^{-1})	Glucose (% w/v)	Fructose (% w/v)	Ethanol (% w/v)
504	0.25	2.01	10.63	5.12
518	0.25	1.48	10.55	4.55
525	0.25	1.42	10.56	4.61
542	0.25	1.40	10.48	4.62
548	0.25	1.49	10.53	4.58
557	0.25	1.48	10.50	4.59
566	0.34	1.91	10.86	4.80
593	0.34	2.11	11.84	3.53
602	0.34	2.14	11.94	3.61
616	0.34	2.15	11.91	3.58
625	0.51	2.16	11.97	3.02
638	0.51	2.26	12.47	2.86
644	0.51	2.88	12.68	2.80
649	0.51	2.89	12.75	2.81
662	0.51	2.92	12.71	2.80
674	0.51	2.90	12.70	2.85

Table A7: Feed - 14.55% glucose and 11.85% fructose

Time (h)	D (h^{-1})	Glucose (% w/v)	Fructose (% w/v)	Ethanol (% w/v)
428	1.56	1.06	11.85	2.03
431	1.56	11.16	11.82	0.85
434	1.56	13.80	11.83	0.48
447	1.56	13.35	11.82	0.59
455	1.56	13.40	11.81	0.62
474	1.56	13.34	11.80	0.60
478	0.91	13.17	11.82	0.80
497	0.91	12.89	11.81	0.95
503	0.91	12.45	11.79	0.94
508	0.91	12.46	11.78	1.10
520	0.91	12.44	11.78	1.05
527	0.91	12.51	11.77	1.03
530	0.91	12.48	11.78	1.07
545	0.64	12.06	11.74	1.39
551	0.64	11.94	11.73	1.46
568	0.64	11.75	11.72	1.48
579	0.64	11.66	11.72	1.50
593	0.64	11.57	11.71	1.55
615	0.64	11.53	11.71	1.53
647	0.64	11.55	11.72	1.51
653	0.29	9.75	11.72	2.56
675	0.29	9.03	11.69	2.71
688	0.29	8.45	11.70	3.10
697	0.29	8.42	11.68	3.13
712	0.29	8.46	11.68	3.11
719	0.29	8.43	11.65	3.13
738	0.29	8.40	11.67	3.14
748	0.29	8.39	11.64	3.15
759	0.29	8.38	11.67	3.15
771	0.10	7.85	11.55	3.38
784	0.10	5.34	11.52	4.63
791	0.10	4.25	11.52	5.16
808	0.10	3.55	11.50	6.16
817	0.10	3.49	11.50	6.20
831	0.10	3.50	11.51	6.19

Table A7: Feed - 13.39% Fructose (for sorbitol production)

Time (h)	D (h^{-1})	Fructose (% w/v)	Sorbitol (% w/v)	Ethanol (% w/v)
896	0.62	10.79	0.447	1.06
902	0.62	10.85	0.440	1.05
914	0.62	10.87	0.453	1.03
927	0.43	8.90	0.581	1.96
934	0.43	7.48	0.756	2.56
945	0.43	7.46	0.749	2.57
954	0.43	7.51	0.751	2.54
967	0.34	6.40	0.847	3.04
975	0.34	5.47	0.873	3.28
983	0.34	5.06	0.850	3.70
995	0.34	5.10	0.854	3.63
1003	0.34	5.07	0.851	3.65
1012	0.25	4.17	0.849	4.07
1024	0.25	3.15	0.855	4.58
1036	0.25	3.18	0.852	4.54
1041	0.25	3.14	0.850	4.55

Taule A8: Temperature variation - Feed: 101.4 g/L glucose, 97.3 g/L fructose
D=0.51 h^{-1}.

Time (h)	Temperature (°C)	Glucose (% w/v)	Fructose (% w/v)	Ethanol (% w/v)
1163	32.0	1.53	9.25	4.40
1177	32.0	2.10	9.22	4.16
1184	32.0	2.08	9.26	4.14
1201	32.0	2.13	9.28	4.18
1207	29.8	2.54	9.21	3.93
1228	29.8	2.81	9.30	3.79
1236	29.8	2.83	9.39	3.77
1250	32.6	2.15	9.34	4.06
1260	32.6	1.95	9.25	4.25
1275	32.6	1.94	9.26	4.23
1285	34.4	2.35	9.35	3.96
1296	34.4	2.44	9.33	3.92
1308	34.4	2.45	9.37	3.64
1320	36.5	3.01	9.41	3.61
1326	36.5	3.21	9.43	3.50
1334	36.5	3.19	9.40	3.53
1344	38.5	3.64	9.45	3.28
1356	38.5	3.94	9.48	3.10
1367	38.5	3.95	9.47	3.09
1381	40.1	4.21	9.54	2.97
1392	40.1	4.83	9.56	2.64
1408	40.1	4.80	9.57	2.62
1417	42.3	5.42	9.68	2.31
1428	42.3	6.68	9.67	1.66
1435	42.3	6.70	9.69	1.65
1442	44.4	8.40	9.70	0.86
1458	44.4	9.45	9.67	0.34

Table A9: Effluent biomass with temperature change

Temperature (°C)	Biomass (% w/v)	Viability (%)
29.8	0.0087	81
32.0	0.0090	80
32.6	0.0092	83
34.4	0.0082	79
36.5	0.0075	75
38.5	0.0076	70
40.1	0.0097	54
42.3	0.0105	45
44.4	0.0143	11

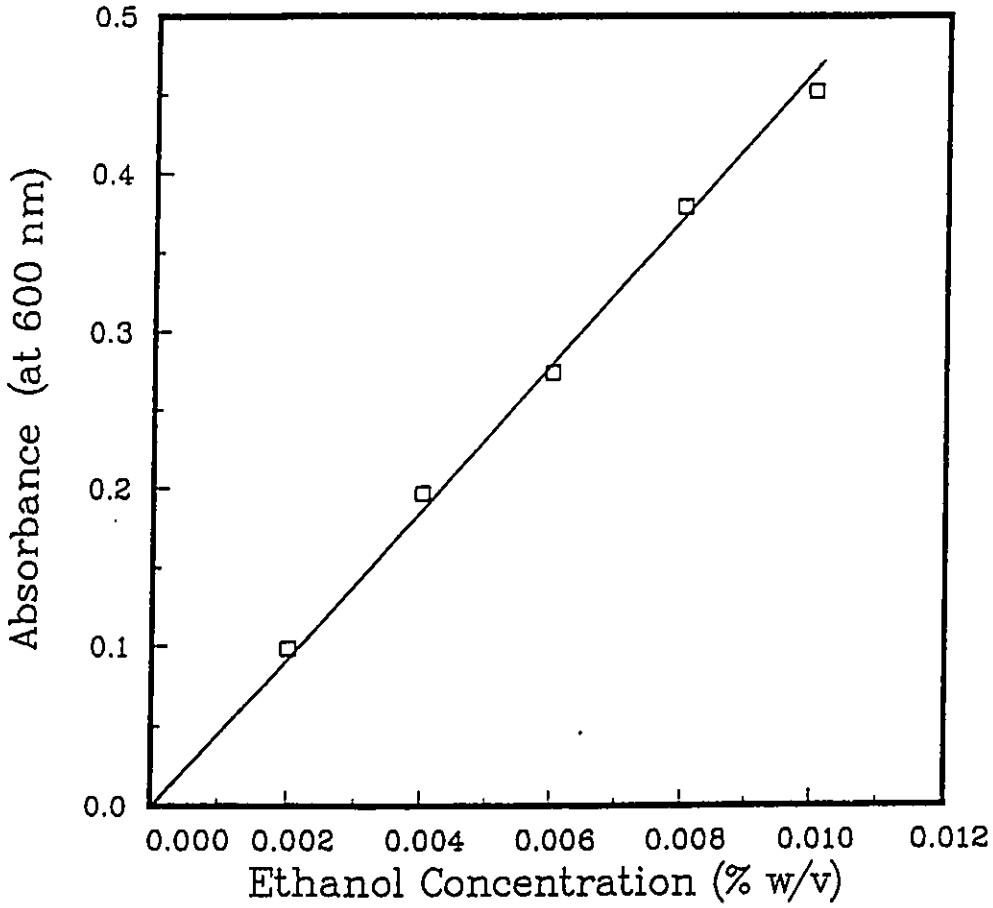

Figure A1: Standard curve for ethanol determination using alcohol dehydrogenase.

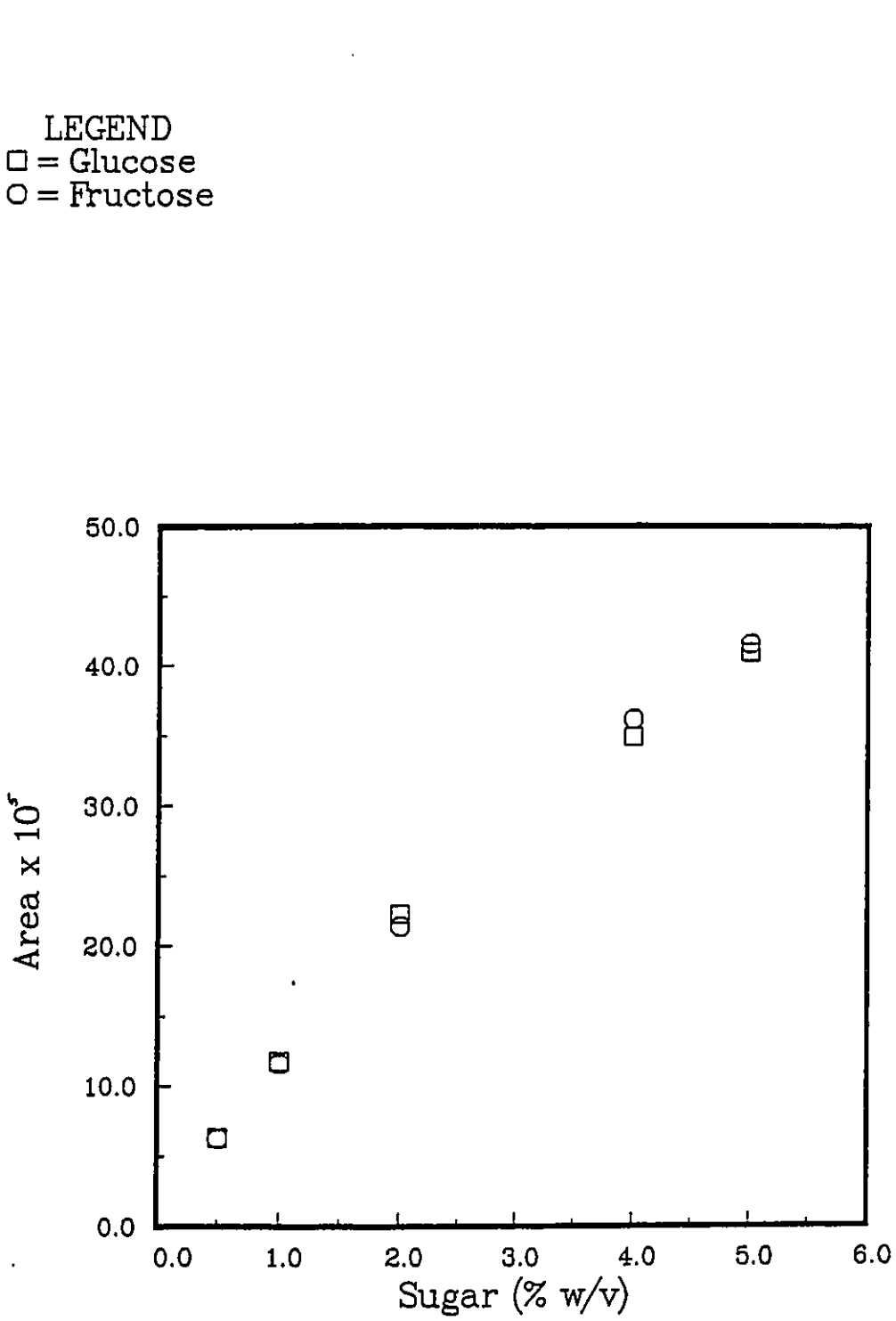

Figure A2: Standard curve for glucose and fructose determination with the Water's high performance liquid chromatograph.

Sample Calculations

1. Dilution rate:

   Effluent flow rate / reactor volume

   $D = 28.6 \text{ mL/h} / 84 \text{ mL} = 0.34 \text{ h}^{-1}$

2. Ethanol productivity:

   EtOH conc. x D

   Prod. $= 46.5 \text{ g/L} \times 0.34 \text{ h}^{-1} = 15.8 \text{ g/Lh}$

3. Specific ethanol productivity:

   EtOH prod. / total biomass in reactor

   $\nu = 15.8 \text{ g/Lh} / 69.0 \text{ g/L} = 0.229 \text{ h}^{-1}$

4. Growth rate:

   Biomass leaving reactor x D

   Growth rate $= 0.21 \text{ g/L} \times 0.34 \text{ h}^{-1} = 0.07 \text{ g/Lh}$

5. Specific growth rate:

   Growth rate / total biomass in reactor

   $\mu = 0.07 \text{ g/Lh} / 69.0 \text{ g/L} = 1.01 \times 10^{-3}$