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PRODUCTION OF FRUCTOSE AND ETHANOL BY SELECTIVE FERMENTATION OF GLUCOSE-FRUCTOSE MIXTURES

by

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Abstract

Fructose is a monosaccharide found along with other sugars in nearly all fruits, some vegetables and in honey. Certain properties that distinguish fructose from other natural sugars make it exceptionally well suited as a food additive. Chief among these is its sweetness; fructose is the sweetest naturally occurring sugar. Due to this, smaller quantities of fructose are required to produce the same sweetness resulting in fewer calories. In a calorie conscious world this is a very attractive characteristic for food and beverage producers.

Fructose is currently available as invert sugar or corn syrup; in these products fructose is present along with glucose in nearly equimolar concentrations. Since fructose is sweeter than glucose the sweetness of these syrups can be enhanced by increasing the fructose fraction in them. Manufacturers currently use chromatographic techniques to separate glucose and fructose. Due to the high costs associated with this operation very enriched syrups (i.e. syrups with greater than 90% fructose) are not available in large quantities.

To eliminate this costly separation step it has been proposed to convert glucose to another substance which is more easily separated from fructose. In this project the selective conversion of glucose to ethanol from glucose/fructose mixtures was investigated. The process was carried out using a mutant of *Saccharomyces cerevisiae*, batchwise, continuously with immobilized cells and semicontinuously.

The kinetic behaviour of *S. cerevisiae* ATCC 36859 was studied using batch fermentation data. The growth of the yeast is inhibited equally by glucose and fructose, even though fructose is not consumed by the yeast. Fermentation models were formulated in this work. These models include terms which account for the inhibition of growth, glucose consumption and ethanol production by the carbohydrate and ethanol. The models

predict that the growth rate of the yeast will be zero if the medium contains either 488 g/L total carbohydrates or 62 g/L ethanol. Glucose consumption and ethanol production are predicted to continue up to concentrations of carbohydrate and ethanol of 789 and 152 g/L respectively. It was found that the mutant strain had lower specific growth rates and ethanol productivities than the wild strain of *S. cerevisiae*.

Batch tests were carried out with hydrolyzed Jerusalem artichoke juice and High Fructose Corn Syrup; these are relatively inexpensive food grade materials that contain glucose and fructose. An ethanol productivity of 21 g/Lh was attained in a batch process using an initial biomass concentration of 94 g/L. It was found though that this process suffered from the inhibitory effects of high total carbohydrate concentrations, therefore products containing high fructose concentrations were produced with lower ethanol productivities. In addition, reuse of the biomass resulted in a reduction of 40% in its activity. In order to use the yeast cells for long periods of time, immobilization was carried out.

The cells were immobilized in calcium alginate beads and placed in a tubular reactor. In this form they were used for more than 1000 hours without a loss of activity. A syrup containing fructose as 99% of the reducing sugars was produced from synthetic as well as from food grade glucose/fructose mixtures. A maximum ethanol productivity of 13 g/Lh was attained. A product containing 76 g/L ethanol was also produced in this process. The productivity of the reactor was reduced as the total carbohydrate concentration was increased, therefore products with a high fructose concentration (>150 g/L) could not be formed without a significant drop in the productivity using the immobilized cells.

The inhibitory effects of high total carbohydrate concentrations were reduced by using a fed batch process. In this scheme, 42 High Fructose Corn Syrup was used with and without added nutrients as the feed solution; sterilization of the syrup was not necessary due to its high solids concentration. The syrup was continuously fed to a bioreactor, the glucose was converted to ethanol while the fructose accumulated. A syrup containing 257 g/L fructose and 68 g/L ethanol was produced in this process. In addition, a 347 g/L ethanol solution was collected outside of the reactor. For the formation of high fructose concentration products (>150 g/L). the fed batch process is the best scheme. The harsh

conditions (i.e. high total carbohydrate and ethanol concentrations) in the medium towards the end of the process results in a substantial loss in biomass activity therefore reuse of the biomass is not recommended.

The product formed in the process was purified with activated carbon and ion-exchange resin. A clear and colourless fructose syrup that visibly resembled HFCS was produced. The ethanol in the product allowed for storage of the product for long periods of time before its further treatment.

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Nomenclature

HFCS, HFS	High Fructose Corn Syrup, High Fructose Syrup
HJAJ	Hydrolyzed Jerusalem artichoke juice
HPLC	High Pressure Liquid Chromatograph
K_s	saturation constant (g/L)
K_1, K_2, K_p	inhibition constants
p	product concentration (g/L)
p_{max}	maximum ethanol concentration above which growth ceases (g/ L)
p'_{max}	maximum ethanol concentration above which glucose consumption and ethanol production cease (g/L)
P1	sugar consumption rate calculated at 0-80% sugar conversion (g/Lh)
P2	ethanol production rate calculated at 0-80% sugar conversion (g/Lh)
P3	maximum fructose consumption rate (g/Lh)
s	substrate concentration (g/L)
s_{max}	maximum carbohydrate concentration above which growth ceases (g/L)
s'_{max}	maximum carbohydrate concentration above which glucose consumption and ethanol production cease (g/L)
t	time (h)
μ	specific growth rate $=(\ln x_2/x_1)/(t_2 - t_1)$ (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})
ν	product formation rate (g/Lh)
ω	degree of substrate inhibition of growth rate (Eq. 3.25)

ω'	degree of substrate inhibition of product formation rate (Eq. 3.27)
x	biomass concentration (g/L)
$Y_{x/s}$	cell yield coefficient (g biomass produced/g carbohydrate consumed)
$Y_{p/s}$	product yield coefficient (g product produced/g carbohydrate consumed)
$Y_{p/x}$	product yield coefficient (g product produced/g biomass produced)

Subscripts

f	fructose
g	glucose
min	minimum concentration at which inhibition becomes measureable
o	initial condition
tc	total carbohydrate (glucose+fructose)
ye	yeast extract

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Chapter 1

Introduction

1.1 Fructose Syrup

The production of fructose based syrups have undergone substantial growth in recent years. The commercialization of High Fructose Corn Syrup (HFCS) in the early 1970's and its adoption by soft drink manufacturers in the 1980's led to a reduction in domination of the nutritive sweetener market by sucrose. Price and quality were the main reasons why companies made the switch from sucrose to HFCS (Luenser, 1987; Blanchard and Geiger, 1984). The scale of operations and the degree of mechanization are such that corn syrup can be offered at a significantly lower price and in a more consistent quality than sucrose (Blanchard and Geiger, 1984). Another reason for fructose's popularity is due to its better suitability as a food additive compared to other natural sweeteners (Bujake, 1986; Dziezak, 1987). Fructose's main advantage is its sweetness; it is 60% sweeter than sucrose and 150% sweeter than glucose (Blanchard and Geiger, 1984). Smaller quantities are thus required to produce the same sweetness, resulting in a reduction in caloric intake.

Fructose is currently available in large quantities only as a syrup. Two types are produced; a 42% fructose/52% glucose syrup (42 HFCS) and a 55% fructose/42% glucose syrup (55 HFCS). The development of second generation syrups, those with higher fructose contents (i.e. 55 HFCS) and their resultant higher sweetness levels has extended the use of HFCS's to many applications (Coker and Venkatasubramanian, 1987). The use of these

syrups has become so widespread that they have reached the maximum level of penetration in virtually every sector where a liquid sweetener is suited (Hodgkin, 1987). Further expansion of the industry is only possible if fructose can be made available in a form suitable for a wider range of applications, in particular in the form of a solid. This would allow the exportation of substantial tonnages of corn sweeteners, something that HFCS producers have never achieved (Hodgkin, 1987).

Solid fructose is produced by crystallization, this process requires the syrup to contain 90-95% fructose (10-5% glucose) (Forsberg et al., 1975; Bateman et al., 1984; Ito et al., 1987). If currently available syrups (i.e. 42 HFCS and 55 HFCS) are to be used, a substantial reduction in the glucose content is necessary. Since glucose and fructose are two isomers of the same molecular size, their separation is very difficult. On an industrial scale the purification is performed using chromatographic techniques (Coker and Venkatasubramanian, 1987). This is a very expensive enrichment process, therfore the price of solid fructose is not competive with those of other sweeteners.

1.2 Selective Conversion

The separation problem can be reduced if the glucose were converted to a substance more easily separated from fructose. Conversion to a substance which has economic value would also be beneficial to the overall viability of such a process. These criteria are met by the microbial conversion of glucose to biomass and/or ethanol. Various microorganisms can perform this selective fermentation; *Tricholoma nudum* (Reusser et al., 1960), a mycelial fungal system (Ueng et al., 1982), *Zymomonas mobilis* (Anonymous, 1984; Bringer-Meyer et al., 1985; Doelle, 1986; Suntinanalert et al., 1986) and *Pullularia pullulans* (Fan, 1988). In these processes however, problems such as substantial fructose consumption and/or production of unwanted by-products have been reported. Preliminary results were also obtained using a mutant strain of *Saccharomyces cerevisiae* (Lamarche, 1988). In these tests no fructose was consumed and no unwanted products were produced by the yeast.

1.3 Strategy (Objectives)

Although the results reported by Lamarche (1988) show that *Saccharomyces cerevisiae* ATCC 36859 can be used for the selective fermentation of glucose from glucose/fructose mixtures, certain limitations have yet to be overcome. One of these is the long time required to complete batch fermentations; this is partly due to the slow growth rate of the mutant strain. In addition, it is necessary to form products with higher fructose and ethanol concentrations in order to make their subsequent recovery more economical.

In order to address these difficulties the work was carried out in two stages. The first involved an investigation of the limitations of growth and ethanol production by the mutant strain. Although the wild strain of *S. cerevisiae* has been thoroughly investigated, little or no work concerning the mutant strain, which may be quite different, has been performed. This was necessary in order to fully exploit the selective fermentation capabilities of the mutant strain. Using these results, fermentation models will be formulated which will enable a comparison to the wild strain to be made. The models will also help to determine the capabilities of the yeast. In the second stage the performance of batch, continuous and semicontinuous processes will be examined in order to maximize the production of both fructose and ethanol. The feed materials to be used include hydrolyzed Jerusalem artichoke juice (HJAJ) and High Fructose Corn Syrup (HFCS). Both of these are food grade materials, contain fructose and glucose and are relatively inexpensive.

The goal of this work was to propose one or more operating schemes by which both fructose and ethanol could be produced from food grade glucose-fructose mixtures using the selective fermentation capabilities of *Saccharomyces cerevisiae* ATCC 36859.

Chapter 2

Literature Review

Before describing the research carried out here, the relevant literature will be reviewed. Four main subjects will be covered:

- fructose syrup production;
- glucose-fructose separation;
- selective conversion of glucose;
- ethanol production.

2.1 Fructose Syrups

2.1.1 Corn starch

The development of corn sweeteners can be traced back to the early 1880's and the discovery that starch yielded a sweet substance when heated in acid (Coker and Venkatasubramanian, 1987). It was later found that the hydrolytic end-product was glucose. Later, commercial production of syrups and crude sugars from starch became firmly established. As the demand for these types of syrups increased, corn emerged as the preferred source of starch because it was inexpensive and could easily be stored (Coker and Venkatasubramanian, 1987). The corn syrups available at that time contained only glucose; although this product

was useful in many applications, it lacked the sweetness of sucrose and thus commanded only a small share of the sweetener market.

In the 1950's Marshall and Kooi (1957) described an enzyme, glucose isomerase, that could catalyze the conversion of glucose to fructose. Application of this enzyme to the corn starch wet milling industry led to the production of High Fructose Corn Syrup (HFCS). In this process, corn starch is first hydrolyzed to glucose which is then converted with glucose isomerase to a syrup containing glucose and fructose. Since fructose is sweeter than glucose, the sweetness of this syrup was comparable to liquid sucrose and thus substitution was possible. The high cost associated with the production of the enzyme though slowed acceptance of this process. It was not until the advent of immobilization techniques which permitted reuse of the enzyme or whole immobilized cells in a continuous process that high fructose corn syrup was produced in large quantities (Joglekar et al., 1983).

The High Fructose Corn Syrup (HFCS) industry experienced remarkable growth from its inception. This was due in part to the rise in the price of sucrose produced from sugar cane. In 1971, the price of sucrose in the U.S. was 0.12 $/lb; by 1976, it rose to 0.19 $/lb and in 1981, a record price of 0.41 $/lb was reached (Coker and Venkatasubramanian, 1987). During this period of time the cost of syrups derived from corn starch remained relatively stable and their share of the sweetener market rose to 20% (Blanchard and Geiger, 1984). During times of reasonably balanced supply and demand, the price of HFCS is about 15-30% below the price of sucrose (Bujake, 1986).

The production of HFCS can be divided into two major stages; corn wet milling and corn refining. The milling process uses corn kernels as the raw material and through various cleaning and separation processes produces corn oil, corn germ meal, animal corn feed and corn starch. The corn starch is treated further in the refining stage which is described below.

In the first step of the refining process, thermostable enzymes of α-amylase are added to the starch slurry (40% solids) which is maintained at 95-107^{0}C for 5 to 10 minutes. A subsequent 1-2 hour hold at 95^{0}C stabilizes the starch; the enzymatic activity continues until the solution contains 10-15% glucose (Luenser, 1987). Once the corn starch is liquified,

glucoamylase is added to break the remaining starch bonds, the temperature is maintained at 60^{0}C until the maximum glucose concentration is attained. At this point 95% of the carbohydrate is glucose. The liquid is filtered, treated with ion exchange resin and concentrated to 40-50% solids. It is then preheated and magnesium which acts as a co-factor is added to the liquid before it passes to a bed of immobilized glucose isomerase which is kept at 60-65^{0}C (Luenser, 1987). Normally the isomerization equilibrium

$$glucose \Longleftrightarrow fructose \tag{2.1}$$

is established at a concentration of 55% fructose and 45% glucose (dry basis); to attain maximum productivity, most manufacturers produce a mixture containing 42% fructose (Bucke, 1979). The final solution after purification and evaporation contains 42% fructose, 55% glucose and 6% higher saccharides (42 HFCS) and is concentrated to 71% solids.

Commercial chromatographic separation can produce an enriched syrup containing 90% fructose (90 HFCS or very enriched fructose syrup). Manufacturers normally blend the 90 HFCS with the 42 HFCS to produce a syrup containing 55% fructose (55 HFCS). The composition and properties of the various high fructose corn syrups produced are given in Table 2.1 (Bujake, 1986; Osberger, 1986).

Table 2.1: Composition and properties of High Fructose Corn Syrups.

	42 HFCS	55 HFCS	90 HFCS
fructose (%)	42	55	90
glucose (%)	52	41	9
other saccharides (%)	6	4	1
solids (%)	71	77	80
sweetness (sucrose=100)	90-95	95-100	100-130

Table 2.1 shows that as the fructose content of the corn syrup increases, its sweetness relative to sucrose also increases. Since 55 HFCS is similiar in sweetness to sucrose it is used as a substitute in many foods and beverages. Manufacturers are interested in higher fructose

content syrups (e.g. 90 HFCS) because of their increased sweetness and the possibility of using them to produce crystalline fructose.

Since chromatographic separation is expensive, it would be beneficial if syrups containing higher fructose concentrations could be produced without it (Visuri and Klibanov, 1987). This would be possible if the isomerization reaction would provide higher yields of fructose. One method involves the displacement of the isomerization equilibrium in favour of fructose by adding ions that complex with fructose. Studies concerning this complexation with borate (Takasaki and Tanabe, 1971) and germanate oxyanions (Barker et al., 1978) demonstrated an increase in the fructose content to 87 and 93% respectively. The toxicity of these anions though precludes their use in the production of food grade syrups. The yield of the isomerization reaction can also be increased by operating at higher temperatures. Currently, isomerization is carried out 65^{0}C, if the temperature is increased, the equilibrium is shifted in favour of fructose. By carrying the reaction out at 100^{0}C, Tewari and Goldberg (1985) produced 55 HFCS. Unfortunately the half life of the enzyme at this temperature is only several hours. Another study found that the fructose yield could be increased if the isomerization were carried out in media containing ethanol (Visuri and Klibanov, 1987). It was reported that 55 HFCS could be produced at ambient temperatures in 85-90% ethanol media. This high ethanol concentration though reduces the enzymatic activity by up to 75%. Also due to the low solubility of the sugars in this media only dilute solutions could be treated.

Production of fructose syrups from corn starch involves hydrolysis of the starch to a glucose syrup, this is followed by isomerization which produces a glucose-fructose syrup. Fructose can also be produced from plants which store carbohydrates not as starch but as fructans (inulin). These are polymers of fructose which contain some glucose. Hydrolysis of this polymer yields a fructose rich syrup directly. The production of fructose syrups from inulin is discussed in the next section.

2.1.2 Inulin

Early attempts to produce fructose on a commercial scale centered on the use of plants in the *Compositae* family. These plants produce and then store fructose in the form of inulin and inulidins which are composed of multiple fructose units linked in a chain and terminated at one end by a glucose unit. Inulin is found in chicory, dahlia and Jerusalem artichoke tubers; the length of the inulin chain (i.e. degree of polymerization) varies in each. The Jerusalem artichoke has most often been studied as a raw material for fructose production because it has been shown to be among the highest producers of carbohydrates (Fleming and GrootWassink, 1979). The inulin in artichoke tubers contains an homologous series of polyfructans of degree of polymerization 2 to 35 (Edelman and Jefford, 1968). Hydrolysis of the inulin from Jerusalem artichoke tubers yields a syrup containing 75% fructose (dry basis). This is higher than the fructose content of 42 HFCS and 55 HFCS produced from corn starch. The hydrolysis of inulin can be performed chemically or enzymatically.

Chemical hydrolysis has been carried out with hydrochloric, sulphuric (McGlumphy et al., 1931; Conti, 1953), oxalic (Sandkuhl and Halbach, 1957) and phosphoric (Dykins et al., 1933) acids. The pH used for hydrolysis has varied from 1 to as high as 5. To increase the rate of hydrolysis, temperatures of up to 100^{0}C have been used, lower temperatures (e.g. 70-80^{0}C) are preferred because colour formation and fructose decomposition are reduced. Fleming and GrootWassink (1979) carried out hydrolysis with a variety of acids, no differences were noted in their abilities to hydrolyze the polyfructans. The most important consideration was the pH. At a pH of 1.5 and temperature of 80^{0}C total hydrolysis was achieved in 0.5 to 1.0 hours, whereas a pH of 3.5 resulted in hydrolysis of only 40% of the fructans in 3 hours (Fleming and GrootWassink, 1979).

Enzymatic hydrolysis is carried out with inulinase which is produced by yeast (e.g. *Kluyveromyces* and *Candida* species), fungi (e.g. *Aspergillus*, *Penicillium* and *Fusarium* species) and bacteria (Vandamme and Derycke, 1983). Hydrolysis takes place by the sequential cleaving of the fructose-fructose bonds, the fructose-glucose bond is usually severed last. Microbial enzymes are reported to exhibit optimum activity at pH values between 3.5 and 5.5 and in the temperature range between 45 and 55^{0}C (Vandamme and Derycke, 1983).

In order to carry out the process continuously immobilized techniques have been studied. Inulinase has been immobilized in agar (Bajpai and Margaritis, 1985a), calcium alginate (Bajpai and Margaritis, 1985b) and on tygon tubes and aminoethyl cellulose (Kim et al., 1979; Kim et al., 1982).

Following hydrolysis it is necessary to purify the extract, this involve the removal of particulate matter, soluble salts, nitrogen compounds and acids. Adjustments in pH and/or temperature have been used to coagulate some impurities which are then removed by filtration (Vergnaud and Pigeot, 1951). Decolourization is usually carried out using activated charcoal treatment (Dykins et al., 1933). Strong cation resins in the H^+ form are used to remove metallic constituents of salts, proteinaceous components (e.g. amino acids) and some colouring compounds. Anion resins in the OH^- form are used to remove odiferous and flavouring compounds and to improve colour stability (Nelson and Hinman, 1974).

In the past, many schemes have been proposed by which fructose syrups could be produced from Jerusalem artichoke tubers. The proposals may be classified into two types. In the first type, extraction of inulin from the tubers is carried out first, this is followed by precipitation of all other non-inulin material at a pH of 11.0 (Kierstan, 1980). Any free fructose will also be precipitated and lost, this usually amounts to about 2% of the total carbohydrate. This precipitation is followed by hydrolysis of the inulin. The drawback of this process is that two purification steps are necessary, one before hydrolysis and one after. Another scheme was proposed by Fleming and GrootWassink (1979) in which inulin is first hydrolyzed either chemically or enzymatically. The resulting solution is decolourized with activated carbon and deionized with ion exchange resin. The process yielded a clear and colourless syrup which was stable for several months at room temperature. Only 75% of the fructose was recovered in this process; losses occur during processing, primarily during ion exchange treatment (Fleming and GrootWassink, 1979).

Syrups produced from Jerusalem artichoke tubers have a higher fructose content than those produced by isomerization of glucose syrups derived from corn starch. In some cases syrups containing up to 90% fructose (dry basis) can be produced directly by the hydrolysis of Jerusalem artichoke juice. Due to the seasonal variability of the fructose-glucose ratios in

Jerusalem artichokes, standard production techniques yield, on average, syrups containing 75% fructose (Kierstan, 1978). Since crystallization techniques require a syrup with more than 90% fructose, separation of the glucose and fructose is still necessary. The techniques available to accomplish this are discussed in the next section.

2.2 Glucose-Fructose Separation

The classical method of glucose-fructose separation is precipitation. One such technique involves the precipitation of fructose as calcium fructosate by adding excess calcium hydroxide (Kieboom and vanBekkum, 1985). The precipitate is then treated with carbon dioxide to yield calcium carbonate and aqueous fructose. Another method involves the precipitation of the sodium chloride-glucose double salt from a solution of fructose, glucose and sodium chloride (Tatuki, 1972). Glucose is recovered by dissolution of the precipitate in water. Since the solution must be concentrated at a pH of 9 for salt precipitation, some fructose is lost due to alkaline degradation (Barker and Petch, 1985). A similiar type of precipitation has been carried out in which calcium chloride complexes with fructose, the complex is removed by electrodialysis (Ozaki and Hagiuara, 1973).

The most commonly used method for separation of glucose and fructose is chromatography. Much of the work in this area originates from Jones and Wall (1960) who discovered that simple sugars were attracted differently to ion exchange resin. It was learned that glucose is less strongly attached to the resin while fructose is more strongly attached. Therefore, when a mixture of both sugars is passed through a column containing the resin, glucose appears in the effluent first followed by fructose. Many types of resin have been tested but calcium based resins are predominantly used. Batch systems were initially used for this separation and are still suitable for the production of small quantities of fructose. Large High Fructose Corn Syrup producers utilize continuous processes (Teague and Arnold, 1983). In this scheme the adsorbent (i.e. resin) is placed into a number of compartments, the feed sugar mixture enters the process through a rotary valve and is directed to one of the compartments (Blanchard and Geiger, 1984). As the feed flows over the material, fructose

is retained on the resin while glucose passes through to the next compartment. After the first compartment is saturated, the rotary valve is altered so that the operation is moved one compartment downstream. Elution of the fructose from the first compartment then takes place. In this continuous chromatographic process a syrup containing 90% fructose (90 HFCS) is produced.

Since this process is expensive, manufacturers try to reduce the amount of material that is processed by it. This is done by diverting some of the material that is being fed to the process and blending it with 90 HFCS, the result is a syrup containing 55% fructose. Due to this, only small quantities of 90 HFCS are produced, making crystallization on a large scale nearly impossible. An alternative that has been suggested, eliminates this difficult and expensive glucose-fructose separation step. This process involves the selective conversion of glucose to another substance. This technique is described in the next section.

2.3 Selective Conversion of Glucose

The objective of this process is to convert glucose into another substance more easily separated from fructose. Conversion to a substance which itself has economic value would be beneficial in terms of the overall economics of the process. Separation would then yield pure fructose as well as a valuable by-product. The selective conversion has been carried out by a number of researchers.

Reusser et al. (1960) were among the first researchers to propose selective conversion for the production of very enriched fructose syrups (i.e. syrup with more than 90% fructose). In this proposal *Tricholoma nudum* was used, it hydrolyzes sucrose to glucose and fructose and then preferentially consumes the glucose. The glucose is used to produce cells which have a very high protein content and can be separated from the broth by filtration. Some fructose was consumed by the microbe, the rate of consumption increased when glucose was no longer present in the medium. In some cases as much as 25% of the fructose was consumed by the microbe.

Ueng et al. (1982) reported that two mycelial fungal systems, *Fusarium sp. F5* and

Mucor sp. M105, preferentially utilize glucose to produce ethanol. *Fusarium sp.* was able to utilize sucrose directly to produce fructose and ethanol. Results show that fructose consumption was nearly insignificant in the presence of glucose. In glucose-fructose mixtures *Mucor sp.* consumed glucose faster than fructose. When an equal concentration of both sugars is initially present only 50% of the original fructose is left unconverted after all the glucose was fermented to ethanol by *Mucor sp.*

The bacterium *Zymomonas mobilis* was used for the simultaneous production of fructose and ethanol from sucrose (Anonymous, 1984). This bacterium possesses two distinct and specific enzymes for carbohydrate uptake (Doelle, 1982a), one for glucose (glucokinase) and the other for fructose (fructokinase). It was found that glucose severely represses the activity of fructokinase (Doelle, 1982b). Since *Z. mobilis* hydrolyzes sucrose faster than either glucose or fructose are taken up, glucose and fructose accumulate in the medium. At higher sucrose concentrations substantial amounts of glucose and fructose accumulate in the medium; when the concentration of these two is sufficient *Z. mobilis* starts consuming fructose to produce sorbitol (Viikari, 1984; Leigh et al., 1984). In order to reduce fructose consumption Doelle and Greenfield (1985a; 1985b) and Suntinanalert et al. (1986) have developed mutants of *Z. mobilis* which have higher glucose uptake rates. Use of these mutants resulted in reduced glucose accumulation which was accompanied by a lower consumption of fructose. The consumption of fructose could be reduced to zero if the pH is controlled at 6.0, but this reduced the ethanol yield from 90 to 50% of the theoretical value. Bringer-Meyer et al. (1985) also produced a mutant of *Z. mobilis*. This mutant lacked fructokinase and thus could not utilize fructose for ethanol production. When both glucose and fructose are present in the medium, the mutant consumes glucose at a much faster rate than fructose. The fructose that is consumed is converted to sorbitol, the amount increases as the initial carbohydrate concentration is increased. Due to this Bringer and Sahm (1988) recommended concentrations of 75 to 100 g/L each of glucose and fructose for the efficient production of ethanol and fructose.

Another microorganism that selectively ferments glucose is *Pullularia pullulans* (Fan, 1988). When placed in a glucose-fructose mixture the microbe selectively converts glucose

to a biopolymer which may be separated from the broth by filtration. In this process up to 50% of the fructose was consumed.

A mutant of *Saccharomyces cerevisiae* has also been shown to possess selective fermentation capabilities (Lobo and Maitra, 1977). The wild yeast, *S. cerevisiae* has at least three enzymes for catalyzing hexose phosphorylation which is the first chemical transformation that sugars undergo in cells. These enzymes are: hexokinase A which has a phosphorylation ratio of fructose to glucose of 2.5 (Gancedo et al., 1977) hexokinase B whose ratio is 1.3 (Colowick, 1973) and glucokinase which exhibits no activity on fructose (Maitra, 1975). Isolation of mutants lacking hexokinase A and B and thus lacking fructose phosphorylating activity have been acquired by treating the wild strain with N-methyl-N'-nitro-N-nitroguanadine (Maitra, 1970). The mutant cells were then grown in media containing 2-deoxyglucose and those colonies which were characterized by a rapid growth rate were isolated. The fructokinase-negative mutant was unable to grow on fructose but grew on glucose nearly as well as the wild type. Preliminary work with this mutant was carried out by Lamarche (1988). Tests showed that media containing glucose and fructose concentrations of up to 200 g/L each could be used for the production of ethanol and fructose. In addition, the cells were immobilized in Ca-alginate beads and used in a vertical packed bed reactor to produce fructose and ethanol continuously. It was observed that although fructose was not consumed by the yeast, its presence resulted in a reduction in the fermentation rate.

The selective fermentation capabilities of the microorganisms discussed above are compared in Table 2.2. Since fructose is the major product of this process the most important factor to be considered when comparing the microbes is the fructose yield. The yield should be as high as possible, preferably over 95% if a process is to be developed incorporating the microbe. Using these criteria *Tricholoma nudum*, *Mucor sp. M105*, *Zymomonas mobilis* (ATCC 39676), *Zymomonas mobilis* (DSM 3126) and *Pullaria pullulans* are excluded. *Fusarium sp. F5* selectively converts glucose to ethanol and does not consume fructose, the ethanol yield reported though was only 70% of the theoretical value and the ethanol productivity was 0.35 g/Lh. *Z. mobilis* ATCC 53431 can utilize high substrate concentrations;

in the case shown in Table 2.2 a fructose concentration of 172 g/L can be produced with only 5% of the fructose consumed. The ethanol productivity was 2.2 g/Lh. The drawbacks of a process using this microbe are the low ethanol yield (62% of the theoretical value) and the production of sorbitol (35 g/L). Results obtained using *S. cerevisiae* ATCC 36859 provide the best results of those reported. The yeast has the capability of utilizing high substrate concentrations with no fructose consumption (Table 2.2). In addition, ethanol is produced with a theoretical yield and in large enough quantities to be recovered, the ethanol productivity is also relatively high (2.2 g/Lh). No sorbitol was produced by the yeast. The present study is thus concentrated on the use of *Saccharomyces cerevisiae* ATCC 36859 for the selective conversion of glucose.

Ethanol is also a valuable by-product in the process, therefore its production should be optimized. This can be done more easily once current production techniques are reviewed. The next section provides such a review.

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

Microbe	Feed[a] (g/L)	Product[a] (g/L)	Fructose yield (%)	Ethanol[b] yield (%)	P2[c] (g/Lh)	Reference
T. nudum	S-80	F-30	75	–	–	Reusser et al.(1960)
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 39676)	S-372	S-49 F-141 G-84 E-44 So-11	87	89	0.62	Suntinanalert et al.(1986)
Z. mobilis (ATCC 53431)	S-390	F-172 E-69 So-35	95	62	2.2	Suntinanalert et al.(1986)
Z. mobilis (DSM 3126)	F-99 G-100	F-73 E-43 So-25	74	66	1.4	Bringer and Sahm (1988)
P. pullulans	F-25 G-25	F-12.5	50	–	–	Fan(1988)
S. cerevisiae (ATCC 36859)	F-199 G-183	F-199 G-12 E-90	100	100	2.2	Lamarche(1988)

[a] S=sucrose; F=fructose; G=glucose; E=ethanol; So=sorbitol

[b] % of the theoretical value

[c] ethanol production rate

2.4 Ethanol Fermentation

2.4.1 Metabolic pathways

Currently yeasts are the only microorganisms used for large scale ethanol production. Some of the reasons for this are: they produce ethanol with a high selectivity (i.e. only traces of by-products are produced); they are very hardy; they can consume a variety of carbohydrates and they are relatively large which simplifies their handling (Maiorella et al., 1984). The microbes of primary interest are: *Saccharomyces cerevisiae*, *Saccharomyces uvarium* (*carlbergensis*), *Schizosaccharomyces pombe* and *Kluyveromyces* species (Kosaric et al., 1983).

Fermentation is a general term denoting the anaerobic degradation of glucose into various products for the purpose of obtaining energy in the form of ATP. The pathways for the fermentation of glucose by *Saccharomyces cerevisiae* are shown in Figure 2.1 (Lievense and Lim, 1982). The central pathway for the recovery of chemical energy is glycolysis (Embden-Meyerhof pathway). In this pathway glucose is converted to pyruvate in a sequence of 10 enzyme catalyzed reactions (Figure 2.1). After glycolysis, the pyruvate formed can take two routes. Aerobically the pyruvate can be oxidized, this results in the loss of its carbonyl group as CO_2 to form the acetyl group of acetyl-coenzyme A (Acetyl-CoA). The acetyl group is then oxidized completely to CO_2 and H_2O by the citric acid cycle (TCA pathway). The other major pathway leads to the production of ethanol. The pyruvate loses its carboxyl group by the action of pyruvate decarboxylase to form acetaldehyde. In the final step, acetaldehyde is reduced to ethanol through the action of alcohol dehydrogenase.

The fermentation of glucose to ethanol is induced while the oxidative metabolism is repressed (Crabtree effect) in the presence of high glucose concentrations (Lehninger, 1982). In this case, the reductive pathway from pyruvate to ethanol is important as a generator of NAD^+, a necessary cofactor in the Embden-Meyerhof pathway. The activity of the TCA cycle is greatly reduced, although it still serves as a source of intermediates for biosynthesis.

The overall stoichiometry of the ethanol production reaction is shown below

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + energy \qquad (2.2)$$

The energy produced is stored as adenosine triphosphate (ATP) and used for biosynthesis and maintenance of the cell. According to this equation 1.00 g of glucose will theoretically yield 0.51 g of ethanol. Reactions involving cell growth and maintenance also consume some glucose therefore ethanol yields usually range from 85-95% of this theoretical value. Depending on the substrate and the conditions of fermentation, various by-products are also produced. Some by-products that may be present in the medium after fermentation are lactic acid, acetic acid, acetaldehyde and fusel oils.

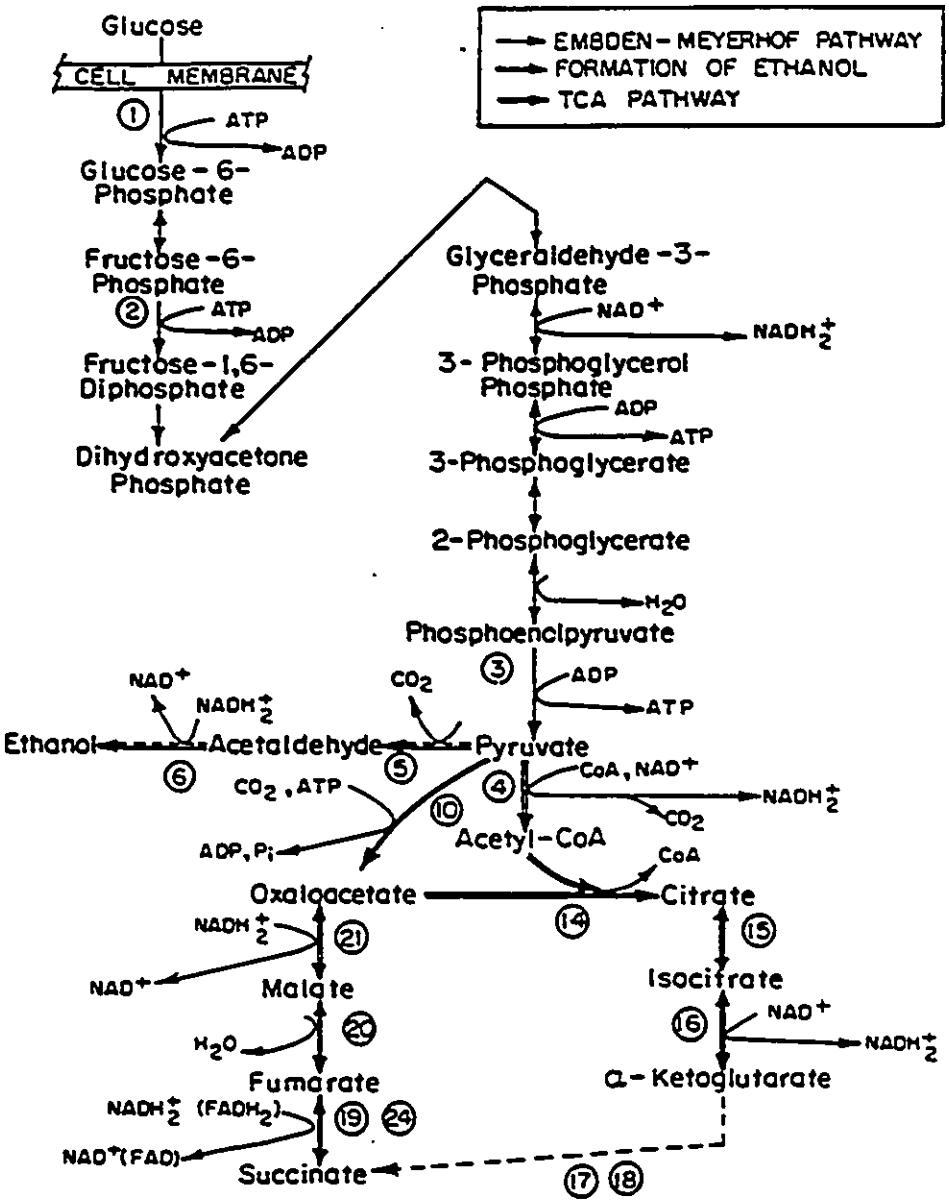

Figure 2.1: Metabolic pathways for the fermentation of glucose by *Saccharomyces cerevisiae*.

2.4.2 Ethanol Production

Industrial ethanol use may be divided into three categories: solvent, chemical intermediate and fuel. In these applications fermentation ethanol must compete directly with petroleum derived ethanol (Maiorella et al., 1984), thus low petroleum prices discourage production of fermentation ethanol while high prices encourage it. Continued growth of fermentation alcohol production also depends on the use of inexpensive raw materials as well as process improvements.

Three types of raw materials can be used for ethanol production, these are: saccharine materials, starch materials and cellulosics. Saccharine materials (e.g. sugar cane, sugar beet and molasses) contain fermentable sugars and thus require little or no preparation. Starch bearing materials such as corn, wheat and rice are often cheaper but they require initial processing to convert them into fermentable sugars. Materials containing inulin (fructose polymer) derived from Jerusalem artichoke also require conversion. Cellulosics are available as waste materials but require the most extensive pretreatment. Since 55-75% of the final ethanol price is due to raw material cost, selection of a suitable and cheap raw material is critical.

Other major economic factors that must be considered in the ethanol production industry are the cost of steam required for distillation and the size of the reactor (Ghose and Tyagi, 1979). Savings can be realized if highly concentrated sugar solutions could be fermented to high ethanol concentrations in fast rates. Different fermentation schemes have thus been developed in order to reach these objectives.

2.4.3 Free cell processes

Currently, 75% of the fermentation ethanol is produced by traditional batch processes (Margaritis and Merchant, 1984). In these processes a medium containing 100-150 g/L of sugar supplemented with nutrients is fed to a fermenter and inoculated to contain 10 %v/v yeast. The yeast is allowed to grow and produce ethanol until the maximum ethanol concentration is attained. This process takes anywhere from 24-36 hours and produces 50-75 g/L ethanol. Ethanol productivities for batch processes are usually 1.8-2.5 g ethanol/litre of reactor/hour

(Rose, 1976). By increasing the initial biomass concentration, higher ethanol productivities are possible. Ghose and Tyagi (1979a) attained an ethanol productivity of 21 g/Lh using an intial biomass concentration of 26 g/L. Even though productivities of batch processes are usually low, batch technology is preferred due to its ease of operation and the low financial risk involved (Kosaric et al., 1983).

The continuously stirred tank fermenter (CSTF) is most often used for the continuous production of ethanol. It resembles a batch fermenter except that feed is added and product withdrawn continuously. This process is useful because it enables microbial populations to be maintained at a particular growth rate for long periods of time. Since the microbe can be kept in the exponential phase of growth, unproductive lag and growth phases of the batch process can be avoided. This allows for higher output per unit volume of equipment, resulting in a cost saving since a smaller reactor can be used for the same amount of product formed. Ghose and Tyagi (1979b) and Cysewski and Wilke (1976; 1977; 1978) reported ethanol productivies of 4 and 6 g/Lh respectively using such a continuous system. Dilution rates though are limited by the growth rate of the cells, operation at dilution rates higher than the maximum specific growth rate of the microbe results in the washout of cells from the reactor. To overcome this limitation, CSTF's have been used in combination with a cell recycle system. In this scheme, cells in the product stream are separated by centrifugation or filtration and returned to the reactor. This increases the cell concentration in the reactor and allows operation at higher dilution rates. Ghose and Tyagi (1979a) maintained a cell concentration of 48 g/L in a CSTR using a cell recycle system. They attained a maximum ethanol productivity of 32 g/Lh at a steady state ethanol concentration of 60 g/L.

One of the major problems encountered when operating at high cell concentrations is the maintenance of cell viability. The replacement of dead cells through a low level of growth must also take place. These two objectives are difficult to attain because the ethanol produced by the yeast strongly inhibits cell viability and growth. This problem can be overcome by continuously removing ethanol from the reactor by operating it under vacuum conditions. Cysewski and Wilke (1977) achieved an ethanol productivity of 82 g/Lh using a feed containing 334 g/L glucose. The accumulation of toxic and non-volatile components

in the fermenter is a major drawback of vacuum fermentation systems.

The fermentation of highly concentrated substrate solutions although desirable imposes extreme osmotic constraints on the yeast cells (Panchal and Stewart, 1981). The high osmotic pressures result in the build-up of intracellular ethanol which is toxic to the yeast. In order to maintain the viability of the cells it is necessary to reduce the osmotic pressure of the medium. This can be accomplished while still treating concentrated substrate solutions by adding the substrate either in batches or continuously to the fermenter, this is known as fed batch processing. Constant fed batch processes are presently used in almost all Brazilian ethanol plants (Koshimizu et al., 1984). Panchal and Stewart (1981) showed that when the substrate was introduced in four batches, one at the beginning and three later during the fermentation, the ethanol concentration was higher than that attained in a single batch operation. Using a substrate concentration of 386 g/L a product containing 110 g/L ethanol was produced. The ethanol productivity was only 5 g/Lh due to the low inoculum concentration that was used. Koshimizu et al. (1984) was able to attain an ethanol productivity of 10 g/Lh with an initial biomass concentration of 75 g/L and a feed containing 230 g/L total reducing sugars.

Although high ethanol productivities have been achieved using novel free cell systems, the complexity and cost of these systems in most cases makes them unsuitable for ethanol production. An alternative process that is now being studied closely involves immobilized cells.

2.4.4 Immobilized cells

According to Abbott (1977) an immobilized cell system is one where microbial cells are confined within the bioreactor, thereby permitting their economical reuse. Immobilized cell technology offers advantages over conventional free cell systems. Cell immobilization increases fermenter productivity by increasing the quantity of cells in the reactor and by allowing the use of high flow rates in continuous operation without the risk of cell washout (Godia et al., 1987). In addition, many investigators have found that the useful lifetime of cells can be considerably enhanced by immobilization since they are less susceptible to the

effects of inhibitory compounds and nutrient depletion (Margaritis and Merchant, 1984).

The methods of immobilization are divided into three categories

- adsorption
- covalent coupling
- entrapment

Any surface in contact with a nutrient medium which contains suspended microorganisms will in time become biologically active due to cell adhesion (Margaritis and Merchant, 1984). This property has been exploited by a number of researchers to immobilize cells for ethanol production. Yeast cells have been adsorbed onto wood chips, sawdust, ion exchange resins, porous brick as well as other supports (Margaritis and Merchant, 1984). Moo-Young et al. (1980) adsorbed *S. cerevisiae* cells onto treated beech wood chips in a vertical packed column. Using a 130 g/L glucose medium, an ethanol productivity of 22 g/Lh was attained. Ryu et al. (1982) performed continuous ethanol fermentation with *S. cerevisiae* adsorbed on wood chips using a multistage system of six one litre mixed bioreactors. A final ethanol concentration of 130 g/L was achieved using a 300 g/L glucose feed. The maximum ethanol productivity was 5 g/Lh. The adsorption of *S. cerevisiae* on sawdust was evaluated by Michaux et al. (1982). Using a vertical packed bed, an ethanol productivity of 60 g/Lh was reached using a 100 g/L glucose feed. Anion exchange resins were also used to adsorb negatively charged cells of *S. cerevisiae* (Daugulis et al., 1981). When a packed column containing the resin and the adsorbed cells was operated with a 120 g/L glucose feed, a maximum ethanol productivity of 62 g/Lh was attained. The major disadvantage of adsorbed systems is the leakage of cells that occurs. Covalent coupling may help reduce cell leakage but this is scarcely applied to ethanol fermentations because bonding compounds are often toxic to living cells.

Immobilization methods based on the entrapment of cells in polymer matrices are the most widely applied methods used for ethanol production. Most of the work is directed towards the use of natural polymers such as alginate and carrageenan. The methods involved

are very simple and are carried out under very mild conditions. In addition, particles of different sizes and shapes with good porosity can be made.

Alginate is a natural polymer that has been used extensively to entrap cells. It is extracted from several different species of marine algae and made available, after processing, as water soluble sodium salts. In the presence of multivalent cations, the water soluble salt forms a gel. The immobilization procedure usually involves the addition of a mixture of sodium alginate and cell suspension dropwise into a $CaCl_2$ sclution. Upon contact with calcium ions, gelation occurs trapping the cells within it. The fermentation of glucose using alginate entrapped cells was first reported by Kierstan and Bucke (1977). In that work *S. cerevisiae* cells were encapsulated in fibres of Ca-alginate which were then packed into a vertical column. When a solution containing 100 g/L glucose was fed to the reactor a maximum ethanol productivity of 4.3 g/Lh was attained. Linko and Linko (1981) entrapped cells of *S. cerevisiae* in Ca-alginate beads, using cane molasses as feed, a 70 g/L ethanol effluent was maintained with a maximum ethanol productivity of 17.5 g/Lh. The inclusion of nutrients in the feed medium was essential in order to maintain ethanol productivities over long periods of time. Ca-alginate entrapped cells were also tested by Lee et al. (1983). The packed bed reactor produced an effluent ethanol concentration of 70 g/L (productivity of 38 g/Lh) using a feed containing 150 g/L glucose. At higher glucose concentrations (up to 250 g/L) lower conversions were obtained due to the inhibitory effects of substrate and product. In order to reduce carbon-dioxide hold-up and medium channelling which occur in vertical packed bed reactors, Shiotani and Yamane (1981) used a horizontal packed bed reactor. With *S. cerevisiae* cells entrapped in Ca-alginate beads, a feed containing 197 g/L glucose was fermented to ethanol with a maximum productivity of 46 g/Lh (Shiotani and Yamane, 1981). Cho et al. (1981) performed the fermentation in a fluidized bed using Ca-alginate entrapped yeast cells. With a 100 g/L glucose feed, the ethanol productivity of the fluidized bed was 21 g/Lh, more than 2 times higher than that produced in a fixed bed. The superior performance of the fluidized system was attributed to the absence of carbon-dioxide hold-up.

Another natural polymeric material frequently used for immobilization is carrageenan.

Wada et al. (1981) immobilized *S. cerevisiae* cells in carrageenan beads. When a feed containing 250 g/L glucose was fed to the reactor ethanol production was severely inhibited compared to its performance at lower glucose concentrations. However, with a stepwise increase in the glucose concentration, the inhibitory effects were reduced. An ethanol productivity of 44 g/L of gel beads/h and an effluent ethanol concentration of 110 g/L was attained.

The main drawbacks of these entrapment methods is the instability of the polymers in phosphate solutions and the disruption of the gel particles due to the formation of carbondioxide (Godia et al., 1987). The first problem can be avoided by minimizing phosphate concentration and by adding calcium ions to the feed solution. The second problem can be solved by the use of high porosity particles or by the addition of a protective coating around the beads (Margaritis and Merchant, 1984).

2.4.5 Ethanol productivities

When comparing the ethanol productivities of fermentation schemes special care must be taken because different methods of calculation are used. Productivity may be calculated on the basis of liquid phase volume, total reactor volume or solid bed volume. In some cases the void volume may be about 80% of the total reactor volume. Part of this volume may be occupied by CO_2 produced, therefore the liquid volume may be less than 40% of the total reactor volume. As a consequence, the productivities calculated using the liquid volume may be 2 to 3 times higher than the values based on the total reactor volume. In order to make comparisons between the immobilized and free cell systems easier, the total reactor volume will be used as the basis for productivity measurements. A comparison of productivities of some fermentation systems using this basis is provided in Table 2.3.

Batch processes although having low productivities are relatively simple to operate and are thus preferred by most manufacturers. Higher productivities can be obtained with higher initial biomass concentrations. The continuous free cell systems offer higher ethanol productivities but are more complicated since cell recycle may be necessary. Fed batch processes are advantageous for systems that suffer from substrate and product inhibition,

ethanol productivities though are less than those of other continuous processes. Immobilized systems can attain high ethanol productivities but experience inhibitory effects when processing high substrate or high product concentrations. The best process will therefore depend on the objectives of the individual ethanol fermentation and microbe used.

When a microorganism is to be used to produce ethanol its capabilities must be fully examined. In addition, the objectives (e.g. high ethanol concentration, high ethanol productivity, etc) of the process should be established. Once these steps are taken then recommendations may be made concerning the best process or processes which would meet these objectives given the capabilities of the microbe. In order to aid in this development, modelling is an invaluable tool. Once a fermentation model is obtained it can be applied in a generalized way, after appropriate modifications, to these different processes and thus help to suggest the type of processes best suited to a particular system. The use of models as they apply to biological systems is discussed in the next section.

Table 2.3: Comparison of ethanol productivities of different ethanol production schemes.

System	Feed sugars (g/L)	Conversion (%)	Dilution[a] rate (h^{-1})	Ethanol[a] productivity (g/Lh)	Reference
batch	100	100	-	1.8-2.5	Rose(1976)
batch (high inoculum)	220	87	-	21.0	Ghose and Tyagi(1979a)
continuous	100	100	0.17	7.0	Cysewski and Wilke(1976)
continuous + cell recycle	100	98	0.68	29.0	Cysewski and Wilke(1976)
continuous + cell recycle + vacuum	334	99	0.23	82.0	Cysewski and Wilke(1976)
fed batch	230	100	-	10.0	Koshimizu et al.(1984)
Ca-alginate vertical bed	175	78	0.22	17.5	Linko and Linko(1981)
Ca-alginate fluidized bed	150	98	0.29	20.0	Nagashima et al.(1984)

[a] calculated on the basis of total reactor volume

Chapter 3

Theoretical Aspects

3.1 Introduction

A fermentation model provides a generalized description of relevant aspects of the fermentation process. The factors that are termed relevant will depend on the model's intended use. Sikyta et al. (1977) summarized the main uses of models as:

- for elucidating the nature of the process, its biological, chemical or physical basis;
- in experimental planning, the determination of limiting experimental conditions;
- in interpolation and extrapolation of experimental results.

For fermentation systems it would be possible, in principle, to set out every possible reaction that cells catalyze and then write a set of equations to describe the rate of each. The resultant model might be of interest to biochemists and provide a theoretical basis but would be far too complex for practical purposes. Models that are less rigorous and only take into account components that are easy to measure, although revealing little about the biological mechanisms involved may be sufficient for technological purposes. These simplified models also known as unstructured models allow for the interpolation between experimental data. Since it is the purpose of the present work to determine its performance within the experimental range studied, unstructured models are used.

In an unstructured mechanism, the microorganism is regarded as a single reacting species which consumes the substrate to produce more cells and metabolic products. The central feature in this mechanism is the biomass concentration which affects the rates of substrate consumption and product formation. Models for cell growth are now discussed.

3.2 Cell Growth Rate

3.2.1 Substrate limiting models

Many kinetic models for growth and inhibition are based on those formulated for enzyme kinetics. This is not surprising since the cell is a chemical reactor in which a complex network of enzyme reaction pathways are occurring. The relationship between the specific growth rate and the concentration of a limiting medium constituent was thus fashioned after the standard rate equation for enzyme catalyzed reactions with a limiting substrate (Bailey and Ollis, 1986). Using this analogy Monod (1949) proposed the following expression for the specific growth rate as a function of the limiting substrate concentration

$$\mu = \mu_{max}\left(\frac{s}{K_s + s}\right) \tag{3.1}$$

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.

After a brief lag phase, in which the cells become adjusted to their environment, the number of cells and their mass doubles regularly with time. Under these circumstances the microbe is undergoing exponential growth which is described by the following expression

$$\frac{dx}{dt} = \mu x \tag{3.2}$$

where x is the biomass concentration and t is the time. Substitution of Eq. 3.1 into Eq. 3.2 yields an expression for the growth rate

$$\frac{dx}{dt} = \mu_{max}\left(\frac{s}{K_s + s}\right)x \tag{3.3}$$

Even though this model is a great oversimplification of the growth process, it expresses interelationships adequately in most instances. In some special cases physical significance may be attached to the parameters μ_{max} and K_s. The growth of some microorganisms is limited by the rate of active transport of substrate (Bailey and Ollis, 1986). Since active transport is an enzyme reaction, its rate varies with the external concentration of substrate in the same way as (dx/dt) varies with s according to Eq. 3.3. In this instance the values of K_s and μ_{max} can be considered to be parameters describing the active transport rate.

When growth is rapid, the exponential phase may not be present and thus the Monod equation may not be adequate to describe the growth rate. Under this circumstance other forms of the model have been proposed (Bailey and Ollis, 1986)

$$\mu = \mu_{max}\left(\frac{s}{K_s s_o + s}\right) \tag{3.4}$$

and

$$\mu = \mu_{max}\left(\frac{s}{K_1 + K_2 s_o + s}\right) \tag{3.5}$$

where s_o is the initial concentration of the substrate and K_1 and K_2 are empirical constants. These models indicate a deviation from the exponential phase as the initial substrate concentration is increased.

Other models for the specific growth rate have been proposed by Tessier (1936)

$$\mu = \mu_{max}(1 - e^{-s/K_s}) \tag{3.6}$$

and Contois (1959)

$$\mu = \mu_{max}\left(\frac{s}{Kx + s}\right) \tag{3.7}$$

where K is an empirical constant. The values of μ from Monod kinetics approaches its assymptote too slowly in some cases, the model proposed by Tessier (Eq. 3.6) is thought to fit experimental data more closely (Moser, 1985). At high biomass concentrations the relationship proposed by Contois (Eq. 3.7) predicts that μ becomes proportional to x^{-1}.

Although many models have been proposed, microbial kinetics is still dominated by Monod's expression since it fits most closely to the majority of observations and is intelligible in terms of physical and biological thinking (Moser, 1985).

3.2.2 Substrate inhibition

The expressions provided in the previous section predict that the growth rate will approach an assymptotic value (μ_{max}) at high substrate concentrations. In practice the growth rate declines at high substrate concentrations. Some of the causes of substrate inhibition and their respective kinetic equations have been reviewed by Edwards (1970). In most cases, equations for inhibition kinetics are derived from theories of the inhibition of a single enzyme.

One possible mechanism responsible for substrate inhibition is the reduction in the activity of an enzyme due to the complexing of it with excess substrate (Edwards, 1970). Yano et al. (1966) have generalized the case of the formation of an inactive enzyme-substrate complex by assuming the formation of multiple complexes

$$E + S \underset{k_{-1}}{\overset{k_1}{=}} ES \xrightarrow{k_2} E + P$$

$$ES + S \underset{k_{-2}}{\overset{k_2}{=}} ES_2(inactive)$$

$$ES_2 + S \underset{k_{-3}}{\overset{k_3}{=}} ES_3(inactive)$$

$$\vdots \qquad \vdots \qquad \vdots$$

$$ES_{n-1} + S \underset{k_{-n}}{\overset{k_n}{=}} ES_n(inactive)$$

By analogy to this mechanism an expression for the specific growth rate which accounts for substrate inhibition can be formulated

$$\mu = \mu_{max} \frac{s}{s + K_s + \sum_{j=1}^{n} \left(\frac{s}{K_j}\right)^j} \tag{3.8}$$

where K_j is an empirical constant.

An enzyme may also have more than one catalytically active center and the presence of a substrate molecule at one site may influence those at others (Edwards, 1970). The case

of two identical active sites was treated by Webb (1963). The scheme proposed was

$$E + S \underset{k_{-1}}{\overset{k_1}{=}} ES \xrightarrow{k_2} E + P \tag{3.9}$$

$$ES + S \underset{k_{-3}}{\overset{k_3}{=}} SES \xrightarrow{\beta k_2} ES + P \tag{3.10}$$

A steady state analysis yields the following

$$v = \frac{v_{max} s(1 + \beta s/K'_m)}{s + K_m + s^2/K'_m} \tag{3.11}$$

where $v = dp/dt$

$$K_m = (k_{-1} + k_2)/k_1$$

$$K'_m = (k_{-3} + \beta k_2)/k_3$$

When $\beta > 1$ the product formation rate is greater when both sites are occupied and when $0 < \beta < 1$, SES forms a product but at a slower rate than the ES complex. When the SES complex is inactive $\beta = 0$. By analogy to this case the specific growth rate would be

$$\mu = \mu_{max} \left(\frac{s}{K_s + s + s^2/K'_s} \right) \tag{3.12}$$

Other empirical models incorporating the effect of substrate inhibition have been proposed by Aiba et al. (1968)

$$\mu = \mu_{max} \left(\frac{s}{K_s + s} \right) e^{-s/K_i} \tag{3.13}$$

Moser (1985)

$$\mu = \mu_{max} (e^{-s/K_i} - e^{-s/K_s}) \tag{3.14}$$

Tseng and Wayman (1975) and Wayman and Tseng (1976)

$$\mu = \mu_{max} \left(\frac{s}{K_s + s} \right) - K_i (s - s_{min}) \tag{3.15}$$

where K_i is an empirical constant and s_{min} is the minimum substrate concentration at which inhibition becomes measurable.

Edwards (1970) compared a number of expressions used for substrate inhibition and found that no single expression is better than the rest. The choice between these expressions is partly a matter of convenience and requires reference to experimental results (Sinclair and Kristiansen, 1987).

3.2.3 Product inhibition

In many cases metabolic products act as inhibitors to the growth of microorganisms. Studies of product inhibition in ethanol fermentations often reveal a linear relationship between the specific growth rate and the ethanol concentration. Some of these expresssions were reported by Hinshelwood (1946)

$$\mu = \mu_{max} - K_p \tag{3.16}$$

and Holzberg et al. (1967)

$$\mu = \mu_{max} - a(p - p_{min}) \tag{3.17}$$

where p is the ethanol concentration, p_{min} is the ethanol concentration above which inhibition becomes measurable and K_p and a are empirical constants.

Aiba et al. (1968) have empirically correlated data from ethanol fermentations using an exponential relation

$$\mu = \mu_{max} \left(\frac{s}{K_s + s} \right) e^{-p/K_i} \tag{3.18}$$

Jerusalimsky and Neronova (1965) established a hyperbolic relationship which is analogous to that found for non-competitive inhibition of enzymes

$$\mu = \mu_{max} \left(\frac{s}{K_s + s} \right) \left(\frac{K_p}{K_p + p} \right) \tag{3.19}$$

Ghose and Tyagi (1979b) found non-competitive inhibition of growth by ethanol, the relationship between the specific growth rate and the ethanol concentration was modelled linearly

$$\mu = \mu_{max}\left(1 - \frac{p}{p_{max}}\right) \tag{3.20}$$

where p_{max} is the maximum ethanol concentration above which growth was no longer measurable.

It was reported (vanUden, 1985) that of the expressions found by researchers to model ethanol inhibition, those proposed by Ghose and Tyagi (Eq. 3.20) and Jerusalimsky and Neronova (Eq. 3.19) were used most often.

If common mechanisms are assumed, all equations proposed to take into account inhibition by ethanol could be used to describe inhibition by substrates and vice versa (Edwards, 1970).

3.3 Substrate Utilization and Product Formation

Substrate is used for the production of cell material and metabolic products, the rate of utilization is thus related stoichiometrically to the rates of formation of these materials. In some cases it is possible to write a chemical equation to represent this conversion, from this equation yield coefficients expressing the mass of cells or product formed per unit mass of nutrient consumed ($Y_{x/s}$ and $Y_{p/s}$) can be obtained. A general equation representing growth and product formation is

$$A(C_aH_bO_c) + B(O_2) + D(NH_3) \longrightarrow$$
$$M(C_\alpha H_\beta O_\gamma N_\delta) + N(C_{\alpha'}H_{\beta'}O_{\gamma'}N_{\delta'}) + P(CO_2) + Q(H_2O) \tag{3.21}$$

The carbohydrate is represented as $C_aH_bO_c$, the cells as $C_\alpha H_\beta O_\gamma N_\delta$ and the product as $C_{\alpha'}H_{\beta'}O_{\gamma'}N_{\delta'}$.

The cell yield coefficient on the carbon energy source is

$$Y_{x/s} = \frac{M(12\alpha + \beta + 16\gamma + 14\delta)/(1-r)}{A(12a + b + 16c)} \tag{3.22}$$

where r is the fraction of cell mass that is ash (r ≈ 0.07-0.10). Assuming a conversion of carbohydrate CH_2O to cellular material of composition $CH_{1.8}O_{0.5}N_{0.2}$, the approximate yield ($Y_{x/s}$) is 0.82 (Sinclair and Kristiansen, 1987).

Similarly for the product

$$Y_{p/s} = \frac{N(12\alpha' + \beta' + 16\gamma' + 14\delta')}{A(12a + b + 16c)} \tag{3.23}$$

In the case of glucose fermentation to ethanol a theoretical yield ($Y_{p/s}$) can be calculated. Since 1 mole of glucose produces 2 moles of ethanol, $Y_{p/s}$ is 0.51.

These yield coefficients are constants relating the formation of products from reactants in a chemical reaction. Experimental yields on the other hand are the ratios of the amount of product formed from one reactant. In fermentation processes the carbohydrate is used to produce cellular material as well as metabolic products. The calculated yields thus tend to be lower than the theoretical yields.

The concepts discussed concerning the growth, substrate consumption and product formation rates will now be combined in order to formulate a fermentation model.

3.4 Ethanol Fermentation Models

The first step in the formulation of a fermentation model is the determination of an expression for the growth rate. In most cases this involves the use of the Monod model with additional terms to take into account substrate and product inhibition. Kinetic models for the substrate consumption and product formation rates are then formulated using the yield coefficients just discussed. In ethanol fermentation the products result directly from the energy metabolism (Bailey and Ollis, 1986), therefore the model forms used are

$$\frac{dp}{dt} = -Y_{p/s}\left(\frac{ds}{dt}\right) = \frac{Y_{p/s}}{Y_{x/s}}\left(\frac{dx}{dt}\right) \tag{3.24}$$

Ghose and Tyagi (1979b) studied ethanol fermentation using *Saccharomyces cerevisiae* in a chemostat culture with glucose as the limiting substrate. The inhibition due to glucose and ethanol was investigated and separate kinetic expressions were obtained for each. These

relations were then combined with the Monod equation in order to formulate a model for the fermentation. The following expressions were obtained

$$\frac{dx}{dt} = \mu_{max} x \left(\frac{s}{s + K_s + s^2/K_s\omega} \right) \left(1 - \frac{p}{p_{max}} \right) \tag{3.25}$$

$$\frac{ds}{dt} = -\frac{1}{Y_{p/s}} \left(\frac{dp}{dt} \right) = -\frac{1}{Y_{x/s}} \left(\frac{dx}{dt} \right) \tag{3.26}$$

$$\frac{dp}{dt} = \nu_{max} x \left(\frac{s}{s + K'_s + s^2/K'_s\omega'} \right) \left(1 - \frac{p}{p'_{max}} \right) \tag{3.27}$$

where ω is the degree of substrate inhibition of growth and ω' is the degree of substrate inhibition of product formation. Estimates of the parameters were obtained from an integral analysis of data collected from batch and continuous experiments. The values obtained for ω and ω' were 427.5 and 455.0 respectively, since these values are nearly equivalent it indicates a similiar inhibitory effect of glucose on the growth and ethanol production rates. The parameter p_{max} represents the ethanol concentration above which cells no longer grow (87 g/L) while p'_{max} is the ethanol concentration above which the cells no longer produce ethanol (114 g/L). The values obtained for the parameters implies that fermentation is more resistant to ethanol than growth, a finding that has been confirmed by others (Brown et al., 1981; Lee et al., 1983). It was also found that the substrate concentration had little or no effect on the value of the yields, $Y_{p/s}$ and $Y_{x/s}$ which were 0.47 and 0.09.

Other fermentation models were proposed by Aiba et al. (1968) and Bazua and Wilke (1977). The analysis in these latter cases was carried out in a similiar fashion, the expressions obtained for ethanol inhibition though were different.

Since a mutant strain of yeast is used in the present work its fermentation may be quite different from that of the wild strains already reported. For this reason it is necessary to carry out studies on the growth of the mutant strain and the possible inhibitory effects of substrate and product in order to formulate a fermentation model. Currently, there is no such information concerning the particular yeast strain used in this work.

In the course of this investigation many models will be tested, the computational methods and the software packages that are to be used are described in the next section.

Chapter 4

Model Building Methodology

In order to understand the behaviour and to eventually propose a fermentation model for the mutant strain, the data obtained from batch experiments were analyzed using two different methods.

First, an integral analysis of the specific growth rate (μ) was performed. The rate equation defining μ (i.e. $\mu = (1/x)(dx/dt)$) was integrated, the integrated expression

$$\mu = \frac{ln(x_2) - ln(x_1)}{t_2 - t_1} \tag{4.1}$$

was then used to calculate μ in the exponential region of growth. From this analysis, mathematical relationships could be formulated relating the specific growth rates and the initial yeast extract, glucose, fructose and ethanol concentrations. The modelling of these results provide important information concerning the growth of the yeast in media containing different concentrations of these components.

Some of the batch data were also subjected to a differential analysis which involves an investigation into the dynamic behaviour of the culture. Equations relating the growth, glucose and fructose consumption and ethanol formation rates to the biomass, glucose, fructose and ethanol concentrations were formulated. The models include the Monod equation and terms to take into account inhibition by the substrate and product. The forms of the inhibitory functions were suggested from the results of the integral analysis.

4.1 Integral Analysis

The specific growth rates obtained from an integral analysis of the data were analyzed using SAS (1985) software. Depending on the form of the models tested, linear or non-linear regression analysis was carried out.

For models which were linear in the parameters, PROC REG was used. This procedure requires the data and a MODEL statement defining the dependent variable and the independent variables. The procedure then estimates an intercept (optional) and a coefficient for each independent variable. Output from this routine consists of an overall analysis of variance table, miscellaneous statistics and a report on the parameter estimates.

The NLIN procedure produces least squares or weighted least squares estimates of the parameters of a non-linear model (SAS, 1985). One must input the data, the regression expression, declare the parameters, provide a range of possible starting values of these parameters and specify derivatives of the model with respect to each of the parameters. The procedure first evaluates the residual sum of squares at each combination of starting values specified. The starting values with the lowest sum of squares then serves as the initial estimates of the parameters and as such a starting point for the iterative procedure. 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 (SAS, 1985). Output from NLIN consists of: analysis of variance table, parameter estimates along with upper and lower confidence intervals and the correlation matrix of the parameters. The model forms and the parameter estimates obtained are then used as a basis for the differential analysis.

4.2 Differential Analysis

Rate equations for the biomass, glucose, fructose and ethanol concentrations were formulated according to the methods described previously (Theoretical Aspects). These equations were then used in the analysis of a series of batch experiments. Since some of the parameters were common to two or more of the responses, more precise parameter estimates could be

obtained by combining information from all of the responses. An estimation criterion was developed by Box and Draper (1965) for this situation since the least squares criterion cannot be used. This is due to the fact that the observed values of the various responses at any point in time are not independent (Lozej and McLean, 1990). Software for Multiresponse Parameter Estimation was developed at the University of Ottawa's Chemical Engineering Department (Lozej and McLean, 1990).

The model in the multiresponse case can be written as

$$y_{nm} = f_m(\underline{\xi}_n, \underline{\theta}) + \epsilon_{nm} \tag{4.2}$$

where y_{nm} =observed value of the m^{th} response variable for the n^{th} run

$f_m(\underline{\xi}_n, \underline{\theta})$ =response function defining the expected value of the m^{th} response for the n^{th} run

$\underline{\xi}_n$ =vector of values of the operating (independent) variables for the n^{th} run

$\underline{\theta}$ =P×1 vector of values of the parameters

ϵ_{nm} =value of the random error associated with the measurement of the m^{th} response variable for the n^{th} run.

The residual matrix is:

$$\underline{Z} = \underline{Y} - \underline{H} \tag{4.3}$$

where $\underline{Y}$ =the N×M matrix of the measured responses for n runs

$\underline{H}$ =the N×M matrix of m predicted responses for n runs

If Z is normally distributed and independent with the same variance σ^2, then least squares is used and one finds the parameter values which minimize the sum of squared residuals of all NM responses (Bates and Watts, 1988). The estimation criterion would then be to minimize

$$d(\underline{\theta}) = det\underline{Z}^T\underline{Z} \tag{4.4}$$

One of the assumptions that is made about the error term is that the observed values of the responses from one observation to the next are independent (Lozej and McLean, 1990). In batch kinetics because observations are the results of taking samples from the same experimental run this assumption is not valid. Work by Podruzny and McLean (1987) indicate that point estimates of the parameters are not greatly affected but estimates of precision can be strongly influenced in this situation.

Convergence of the estimation algorithm is determined using one of two criteria. The first is based upon the value of the determinant, $d(\underline{\theta})$. Iterations are terminated when

$$\frac{d(\underline{\theta}_i) - d(\underline{\theta}_{i+1})}{d(\underline{\theta}_i)} < \tau_d \tag{4.5}$$

where τ_d is a user specified relative tolerance. The second criterion is one proposed by Bates and Watts (1984; 1985) in which the size of the proposed increment in the parameter vector is compared to the estimated size of the confidence region of the parameters. If the size of the increment is small relative to the precision of the estimates then convergence is declared.

The program requires as input the experimental data, the rate equations and the derivatives of the rate equations with respect to each of the responses and with respect to each of the parameters. The rate equations and their derivatives are written into Fortran subprograms. In this work two sets of equations are used, the programs are given in Appendix B. Output includes

- parameter estimates, determinants and Bates/Watts convergence criteria;
- predicted responses, observed responses and residuals at convergence;
- rough plots of residuals;
- correlation matrix for the parameters;
- converged parameter estimates and approximate standard errors.

The materials and methods used to carry out these batch experiments, as well as the other tests performed in this research are now discussed.

Chapter 5

Experimental Aspects

In this chapter various aspects of the experimental part of this research are discussed. The areas to be covered are:

- inoculum preparation;
- growth and ethanol production media;
- analytical procedures;
- cell immobilization and continuous fermentation;
- semicontinuous fermentation;
- purification.

5.1 Inoculum Preparation

Saccharomyces cerevisiae ATCC 36859 (mutant strain) and *Saccharomyces cerevisiae* (wild strain) were kept on agar slants (medium I). The yeasts were transferred to fresh slants every two months.

Tests were initiated by asceptically transferring a loopful of cells from test tube slants into liquid medium (medium II). Growth was carried out in 500 mL Erlenmeyer flasks containing 100 mL of medium. These were placed in a rotary shaker (200 rpm) at 33^{0}C

for 24-30 hours. This time was sufficient to ensure the cells were in the exponential growth phase.

5.2 Experimental Media

5.2.1 Synthetic

Several media were used during this research, the composition of these are given below

- Solid media for agar slants (medium I)
  - 10.0 g glucose
  - 3.5 g peptone
  - 4.5 g malt agar
  - 18.5 g agar
  - up to 1 litre distilled water
- Medium used for inoculum preparation (medium II)
  - 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 used for ethanol production (medium III)
  - variable glucose
  - variable fructose
  - variable 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 for ethanol production using the immobilized cell reactor (medium IV)
  - variable glucose
  - variable fructose
  - 3.0 g yeast extract
  - 1.0 g $MgSO_4.7H_2O$
  - 1.0 g $(NH_4)_2SO_4$
  - 1.5 g $CaCl_2.2H_2O$
  - up to 1 litre distilled water

Initially, tests were carried out with media containing glucose, fructose, or glucose and fructose and various concentrations of yeast extract (medium III). These were carried out in 500 mL Erlenmeyer flasks containing 100 mL of medium. The flasks were placed in a shaker (200 rpm) which was maintained at 33°C. Samples were taken asceptically at various times to determine the biomass, ethanol and sugar concentrations.

Medium IV was fed to the packed bed reactor. This medium is similiar to that used for inoculum growth and ethanol production (media II and III). The yeast extract concentration though was lower and free phosphates were avoided in this formulation since they are known to deteriorate alginate beads (Margaritis and Merchant, 1984). The $CaCl_2$ was added in order to maintain the structure of the beads.

Besides these glucose-fructose mixtures, food grade mixtures such as hydrolyzed Jerusalem artichoke juice and High Fructose Corn Syrup were used.

5.2.2 Hydrolyzed Jerusalem artichoke juice

Hydrolyzed Jerusalem artichoke juice was used because it is a food grade material and it contains glucose and fructose and nutrients that support growth and ethanol production by yeast cells. The juice was extracted from Jerusalem artichoke tubers which were obtained from Agriculture Canada. A known weight of tubers was thoroughly washed, sliced and macerated in a blender. The pulp was then squeezed through a triple layer of cheese cloth in a screw press. Approximately 500 mL of juice could be obtained from 1 kg of washed tubers.

The juice contained only a small amount of carbohydrates in the form of monosaccharides (i.e. glucose and fructose), the majority of the carbohydrates was in the form of inulin. Since the yeast cells used in this work could not utilize inulin, hydrolysis was necessary. Hydrolysis was carried out at a pH of 2.5 (adjusted with H_2SO_4) and a temperature of 80^0C for 135 minutes. This was followed by removal of suspended solids by centrifugation at 4000 g's for 20 minutes. The pH of the juice was then adjusted to 5.5 with 4N KOH. The final product contained 30-50 g/L glucose and 120-160 g/L fructose.

5.2.3 High Fructose Corn Syrup

Another food grade glucose-fructose mixture used was 42 High Fructose Corn Syrup (42 HFCS). This syrup was obtained from the Canada Starch Company (CASCO) in Cardinal, Ontario. It contained 410-460 g/L glucose, 330-350 g/L fructose and the rest water.

All media except 42 HFCS were sterilized at 115^0C for 15 minutes. The corn syrup was not sterilized because its high solids content prevented contamination.

5.3 Analysis

5.3.1 Ethanol

Ethanol concentrations were determined enzymatically using alcohol dehydrogenase (Bernt and Gutmann, 1974). The method involves oxidation of ethanol by Nicotinamide adenine dinucleotide (NAD) and Alcohol dehydrogenase (ADH) to form NADH and acetaldehyde

according to the following equation:

$$ethanol + NAD^+ \overset{ADH}{\Longleftrightarrow} acetaldehyde + NADH + H^+ \tag{5.1}$$

The concentration of NADH, which is proportional to the amount of ethanol present, is measured spectrophotometrically at 340 nm. The equilibrium is pushed completely to the right at alkaline pH's.

The reagents required 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 in 3 mL distilled water
- Alcohol dehydrogenase (ADH)
  - 30 mg in 1 mL distilled water

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

Ethanol yields are calculated on the basis of the total carbohydrates that were used at the maximum ethanol concentration.

5.3.2 Sugars

Fructose, glucose and sorbitol concentrations were determined with a Water's high performance liquid chromatograph (HPLC) using a refractometer as the detector. The columns used and their respective mobile phases were: Carbohydrate Analysis column (Waters) at room temperature with a mobile phase of acetonitrile/water (80/20) and Sugar Pak (Waters) and Polypore Calcium (Brownlee) columns at 80°C with deionized water as the mobile phase. The areas of the peaks obtained from the refractometer were measured with a Hewlett Packard 3380S integrator. Solutions containing between 1.0 and 4.0 g/L of the sugars or polyol were used to prepare a standard curve.

When only glucose or fructose was present in the medium, analysis was carried out using the DNS method (Nason, 1960). Dinitrosalicylic acid (5.0 g) is dissolved in 300 mL of distilled water. Another solution containing phenol (1.0 g), sodium bisulphite (0.25 g), sodium hydroxide (5.0 g) and sodium potassium tartarate (100 g) in 200 mL distilled water is also made. These two solutions are mixed and stored in a container that is protected from exposure to light. Samples are diluted to contain 1.0 to 2.5 g/L reducing sugars and are analyzed as follows. The diluted samples (1.0 mL) and 3.0 mL of the DNS reagent are placed in 30 mL test tubes. The tubes are mixed and placed in a boiling water bath (95°C) for 5 minutes. They are removed and quickly cooled to room temperature and then 20 mL of distilled water is added to each. The absorbance of the solution is measured against a reference at 600 nm. The reference is prepared by substituting 1.0 mL of distilled water for the 1.0 mL sample.

5.3.3 Biomass

Biomass concentrations were determined by the dry weight method. In this method a known volume of sample is centrifuged; the supernatant is collected and analyzed for sugar and ethanol content while the biomass is resuspended in distilled water and recentrifuged. This washed and centrifuged biomass is transferred to a preweighed aluminum dish and dried at 105°C for 24 hours. It is then weighed and the biomass weight is expressed on the basis of 1 L. This method provides the total biomass concentration.

Biomass yields are calculated on the basis of the total carbohydrates.

5.4 Continuous Fermentation

5.4.1 Cell immobilization

In this work cell immobilization was performed by entrapping the yeast cells in Ca-alginate beads. The inoculum was grown by the method described previously, the cells were then transferred and grown in a 15 L fermentor. When the cells were in the exponential growth phase, they were recovered from the reactor medium by centrifugation, resuspended in water and then added to an equal volume of a 40 g/L Na-alginate solution. The mixture was then extruded through a 3 mm glass tube and added dropwise to 0.2 M $CaCl_2$. Upon contact with the calcium chloride, spherical beads (3 mm diameter) of Ca-alginate were formed. They were left to harden in the $CaCl_2$ for at least 2 hours.

5.4.2 Immobilized reactor system

A schematic diagram of the immobilized reactor system is given in Figure 5.1. The reactor is a borosilicate glass tube (3.1 cm I.D.) surrounded by a plexiglass jacket. A temperature controller circulated water through the jacket in order to maintain a constant temperature (33^0C). The beads are confined to a 11.2 cm long section (84 mL) in the reactor by metal screens. Feed is introduced at the bottom of the reactor by means of a peristaltic pump and the product exits through the top of the reactor.

To initiate the experiment, the reactor is filled with beads so that the total volume occupied by the beads and the voids between them is 63 mL or 75% of the reactor volume. The extra space was left in order to accomodate later expansion of the beads. Approximately 1500 beads are added to the reactor under these conditions. The reactor is flushed with fresh feed and left to stand for 1 hour before the pump is turned on and the experiment begun. Since changes in the bed volume are observed during the experiment, the total reactor volume (i.e. 84 mL) is used as the basis for dilution rate and productivity calculations.

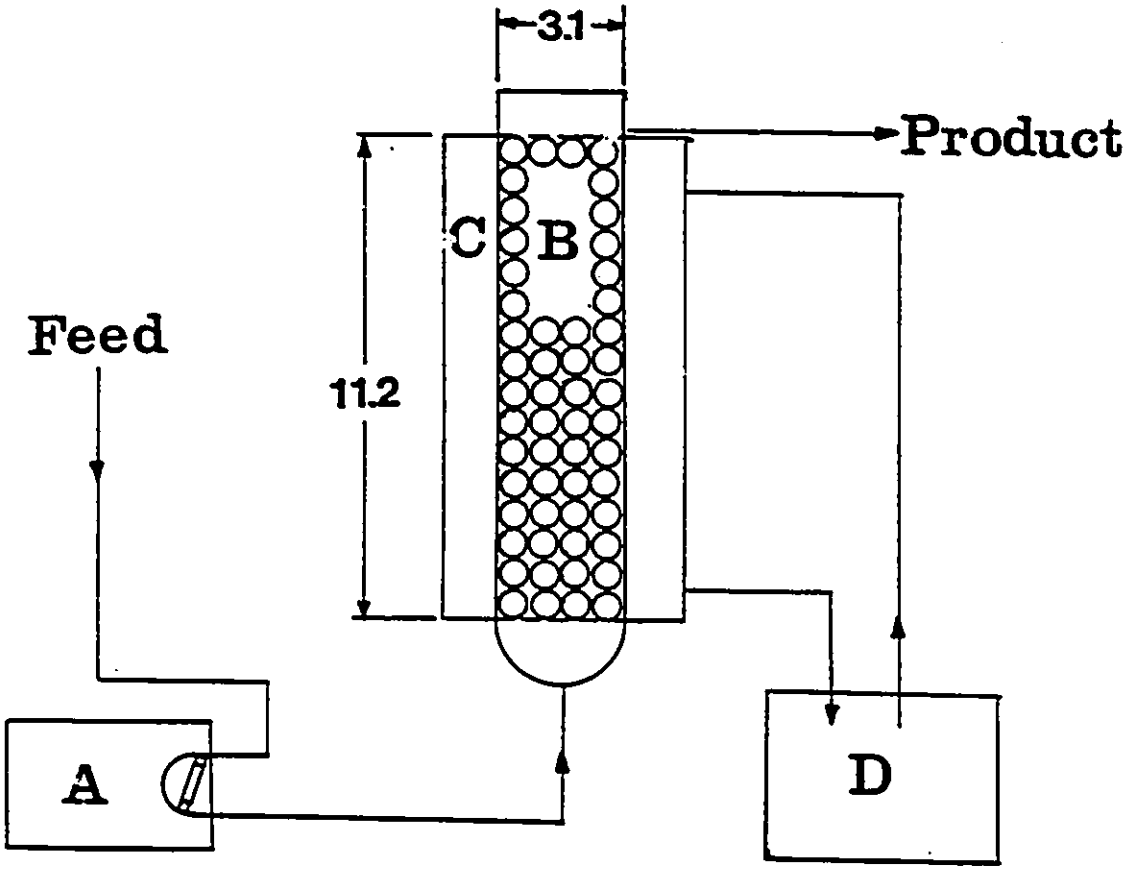

Figure 5.1: Schematic diagram of the immobilized yeast reactor system. A. perstaltic pump; B. immobilized cell reactor; C, water jacket; D, temperature controller (all dimensions in cm).

5.5 Fed Batch (Semicontinuous) Fermentation

The semicontinuous system used in this research is shown in Figure 5.2.

The bioreactor is a 1 litre glass vessel in which agitation is provided by a 6 bladed turbine. Air is introduced with a sparger located below the stirrer. The outlet from the reactor flows through a condenser, from which the condensate is collected in a 30 mL test tube (collector C) which is submerged in a 6^{0}C water bath. The noncondensed gases are directed to a liquid reservoir (collector A) also kept at 6^{0}C. After bubbling through the liquid, the gases then pass through a reservoir containing 8 litres of water at room temperature (collector B). The various reservoirs were used to collect ethanol that was evaporated from the reactor so that a material balance could be performed. The 42 HFCS was added to the reactor with a peristaltic pump. Its flow rate could be adjusted in order to maintain a low glucose concentration in the reactor.

The reactor initially contains 450 mL of medium II or hydrolyzed Jerusalem artichoke juice. An experiment is initiated by the addition of 45 mL of inoculum to the reactor. The air flow rate, agitation speed and 42 HFCS flow rate are then adjusted to their experimental values. Samples are withdrawn from the reactor in order to measure the biomass, glucose, fructose and ethanol concentrations. The ethanol concentrations of the reservoirs are also monitored by periodic sampling.

5.6 Purification

In order to produce a syrup with properties similiar to those of commercial syrups, decolourization and deionization were performed.

The product from the selective conversion process was first decolourized with activated carbon (Darco G-60). It was contacted with various concentrations of carbon at 33^{0}C in 500 mL Erlenmeyer flasks. Colour removal was monitored by the absorbance of the solution at various wavelengths. The carbon was removed from the solution by centrifugation at 4000 g's for 20 minutes. Deionization was then carried out with a mixture of cation and anion exchange resin (Rexyn I-300) which was packed into a 20 mL glass column. The

solution was passed through the column at a rate of 9 mL/min. At this rate the resin in the column was saturated after one pass of the liquid through it. Ion removal was measured by conductance using a YSI Model 31 Conductivity Bridge.

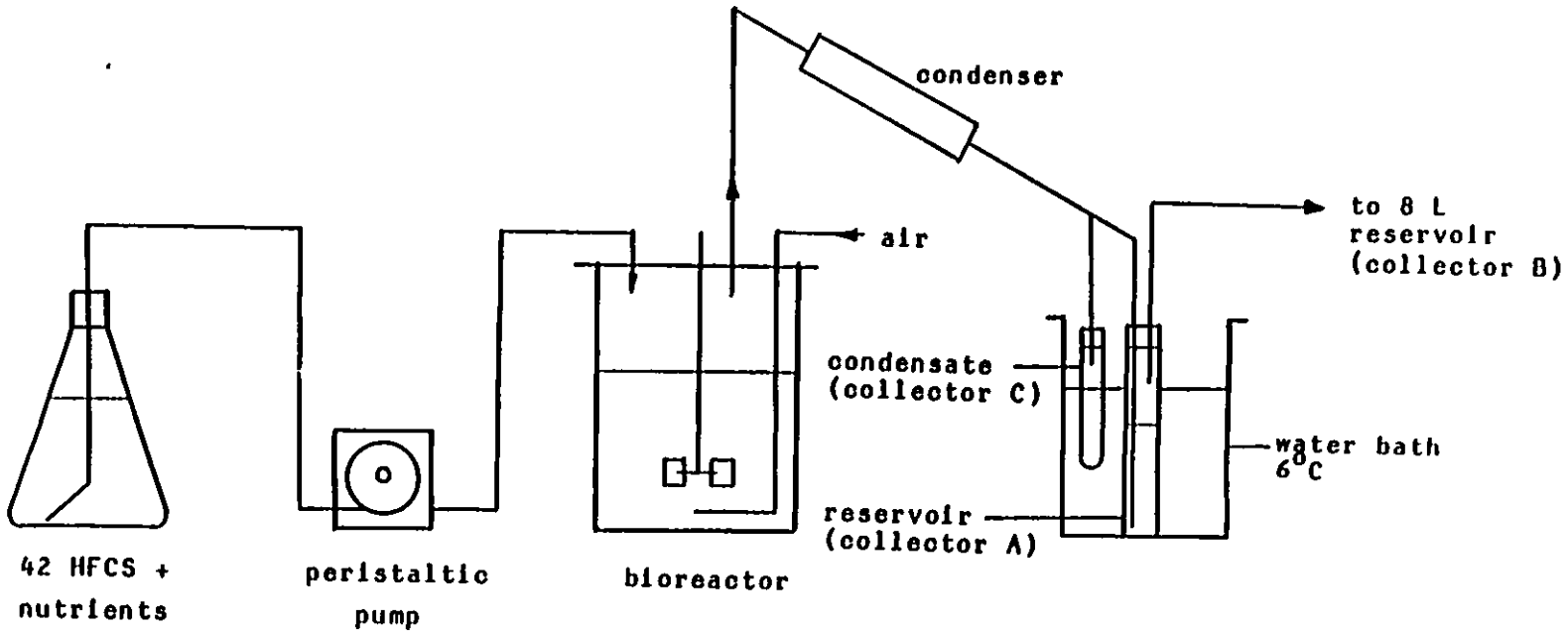

Figure 5.2: Schematic diagram of the fed batch (semicontinuous) system.

Chapter 6

Results and Discussion

6.1 Wild and Mutant Strains of *Saccharomyces cerevisiae*

Since the yeast used in this research is a mutant strain, its behaviour may be quite different from the wild strain. Preliminary studies by Lamarche (1988) have demonstrated the selectivity of the mutant strain toward glucose but other effects of the mutation have not been investigated. It is of particular interest to study the effects on the ethanol producing capabilities and the growth of the yeast. Therefore, in this section experiments with the wild and mutant strains were carried out in identical media in order to compare their performance. The effect of yeast extract on the behaviour of the two was also studied. Yeast extract is a source of vitamins and growth factors and as such is an important part of the medium (Cejka, 1985).

6.1.1 Wild strain

The growth, substrate consumption and ethanol production by the wild strain for two tests, one in a glucose medium and one in a fructose medium with 3.0 g/L yeast extract are compared in Figure 6.1. The figure shows that 103 g/L glucose is consumed by the wild strain in 9 hours; during this time the biomass increased from 2.8 to 8.4 g/L and 47.6 g/L ethanol (89% of the theoretical yield) is produced. Superimposed on these results are those obtained when the wild strain was inoculated into a medium containing 105 g/L fructose.

From the figure it is seen that the wild strain consumes fructose for biomass and ethanol production at the same rate that it consumes glucose. The only difference between the two is a 2 hour lag in ethanol production which occurs when the wild strain is in the fructose medium. A similar lag period was observed when other yeast extract concentrations were used. This occurs because the inoculum was grown in glucose medium, this period of time is necessary for the yeast to adjust to the new environment in which fructose is the only carbohydrate present.

The effects of the yeast extract on the fermentation by the wild strain in glucose media are shown in Figures 6.2–6.4. The figures compare the biomass growth, substrate consumption and ethanol production in media containing 3.0, 15.0 and 30.0 g/L yeast extract. An increase in the yeast extract concentration from 3.0 to 15.0 g/L results in a higher final biomass concentration (Figure 6.2). A further increase in the yeast extract concentration to 30.0 g/L has little or no effect on the fermentation or production of biomass. From Figure 6.3 it is seen that an increase in the yeast extract concentration from 3.0 to 15.0 g/L results in a reduction in the fermentation time from 9 to 4 hours. Whereas, there is very little difference between a fermentation carried out in 15.0 g/L and 30.0 g/L yeast extract. The final ethanol concentration is unaffected by the yeast extract concentration (Figure 6.4), although the times required to reach it were reduced as the yeast extract concentration was increased. Similiar effects were observed for the experiments carried out in media containing fructose.

The biomass and ethanol yields and the specific growth, sugar consumption and ethanol production rates for the tests carried out at different yeast extract concentrations are provided in Table 6.1. A comparison of the experiments carried out in glucose media with those carried out in fructose media reveal differences in these parameters of less than $\approx$10%, which is within the expected experimental error. The specific growth rates of the yeast in fructose are consistently higher (7%) than those in glucose media. The other parameters shown, reveal no clear trends.

In media containing glucose an increase in the yeast extract concentration from 3.0 to 30.0 g/L results in a 15% increase in the ethanol yield. Both the biomass yield and specific

growth rates increase when the yeast extract concentration is increased from 3.0 to 30.0 g/L, the bulk of the increase (70-80%) occurs when the concentration is increased from 3.0 to 15.0 g/L. The glucose consumption and ethanol production rates increased when the yeast extract concentration was augmented to 15.0 g/L, when it is increased to 30.0 g/L the rates decline.

Table 6.1: Ethanol and biomass yields and specific growth, sugar consumption and ethanol production rates for the wild strain of *S.cerevisiae* in media containing 3.0, 15.0 and 30.0 g/L yeast extract and either glucose or fructose.

Initial glu-fru (g/L)	Yeast extract (g/L)	Specific growth rate (h^{-1})	Biomass yield[a] (g/g)	Ethanol yield[a] (g/g)	P1[b] (g/Lh)	P2[c] (g/Lh)
103.2-0	3.0	0.344	0.055	0.453	16.01	7.08
100.3-0	15.0	0.400	0.101	0.472	23.31	10.72
91.4-0	30.0	0.429	0.115	0.519	21.01	10.61
0-105.0	3.0	0.362	0.060	0.418	15.73	6.54
0-100.8	15.0	0.435	0.101	0.475	22.83	10.74
0-97.6	30.0	0.465	0.107	0.483	21.75	10.20

[a] yields calculated at maximum ethanol and biomass concentrations

[b] glucose or fructose consumption rate at 0-80% carbohydrate conversion

[c] ethanol production rate at 0-80% carbohydrate conversion

The results indicate that there is very little difference between fermentations carried out in media containing glucose and those carried out in fructose. This is not surprising since both glucose and fructose are known to be taken up by the same membrane transport system (Cirillo, 1962; Cirillo, 1968) and metabolised by the yeast in a similiar manner.

In similar experiments carried out by Cason et al. (1987), it was found that glucose was consumed faster than fructose by a brewing strain of *S. cerevisiae*. The difference in utilization rates became smaller as the substrate concentration was increased above 20.0 g/L. An increase in the yeast extract concentration beyond 15.0 g/L had only minimal effects on the fermentation, indicating that sufficient vitamins and growth factors are provided by this concentration.

In order to compare the performance of the two strains, similar experiments were performed with the mutant strain of *S. cerevisiae*, these will now be described.

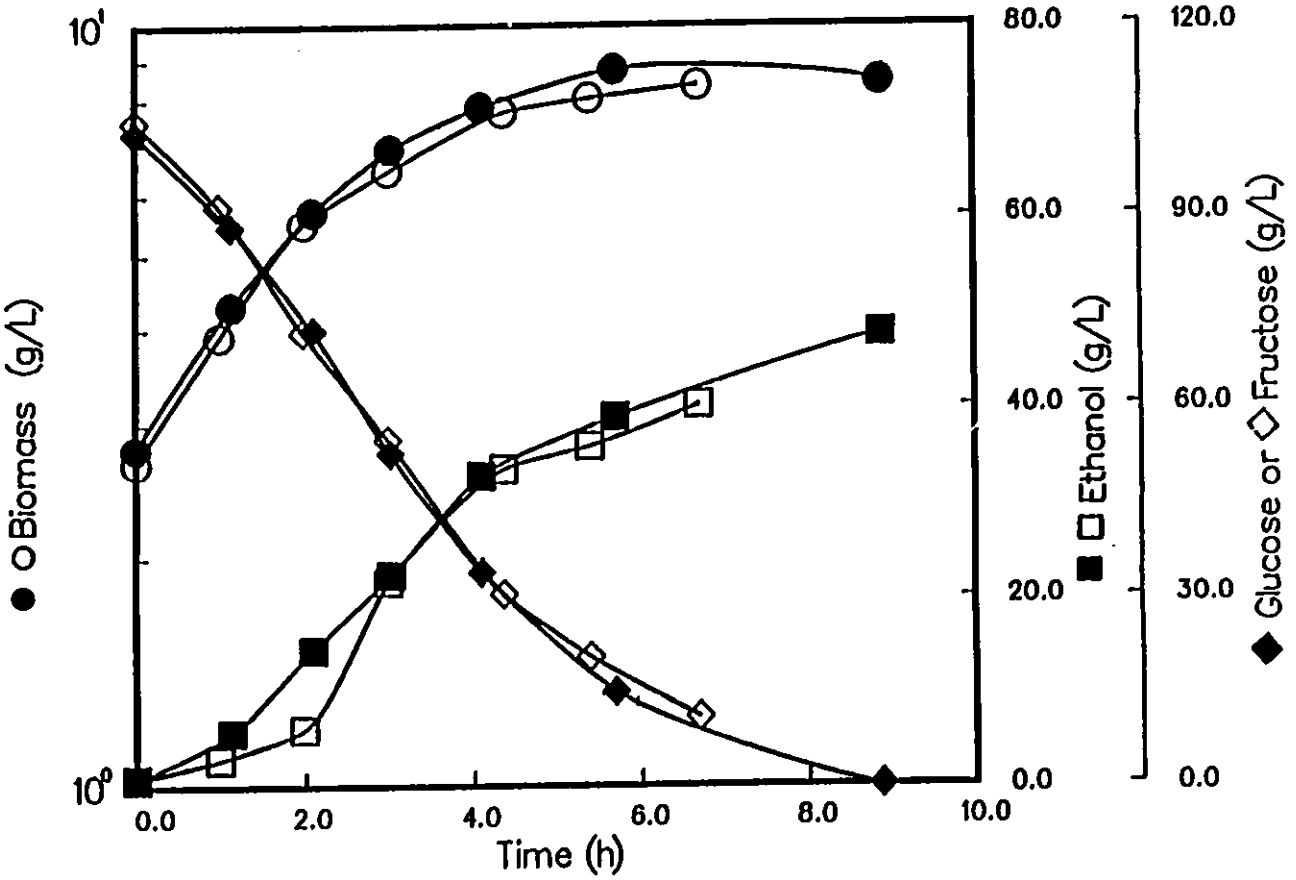

Figure 6.1: A comparison of the ethanol fermentation by the wild strain in a medium containing 3.0 g/L yeast extract and 103 g/L glucose with one containing 3.0 g/L yeast extract and 105 g/L fructose. Solid symbols represent data for glucose medium and open symbols for fructose.

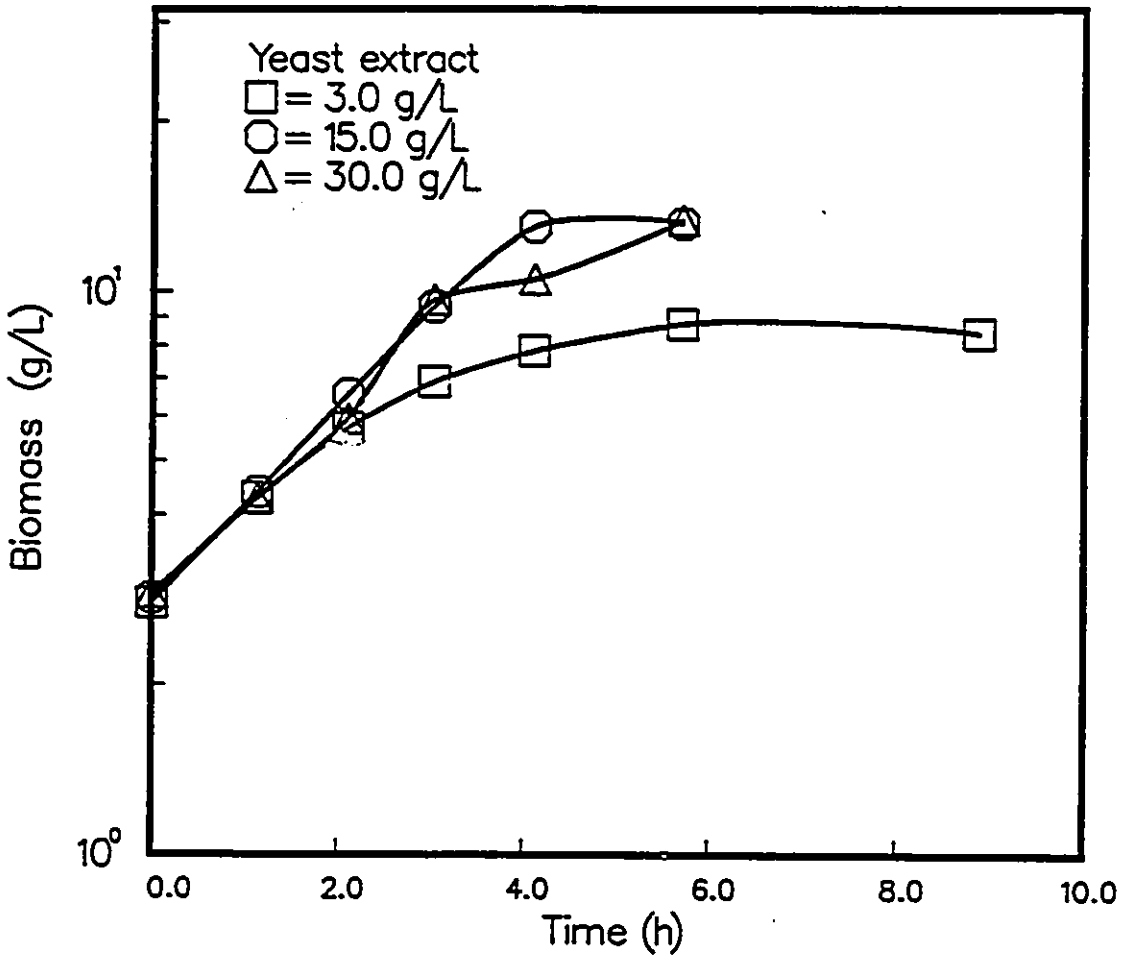

Figure 6.2: A comparison of the biomass production by the wild strain of *S. cerevisiae* at various yeast extract concentrations in media containing ≈ 95 g/L glucose.

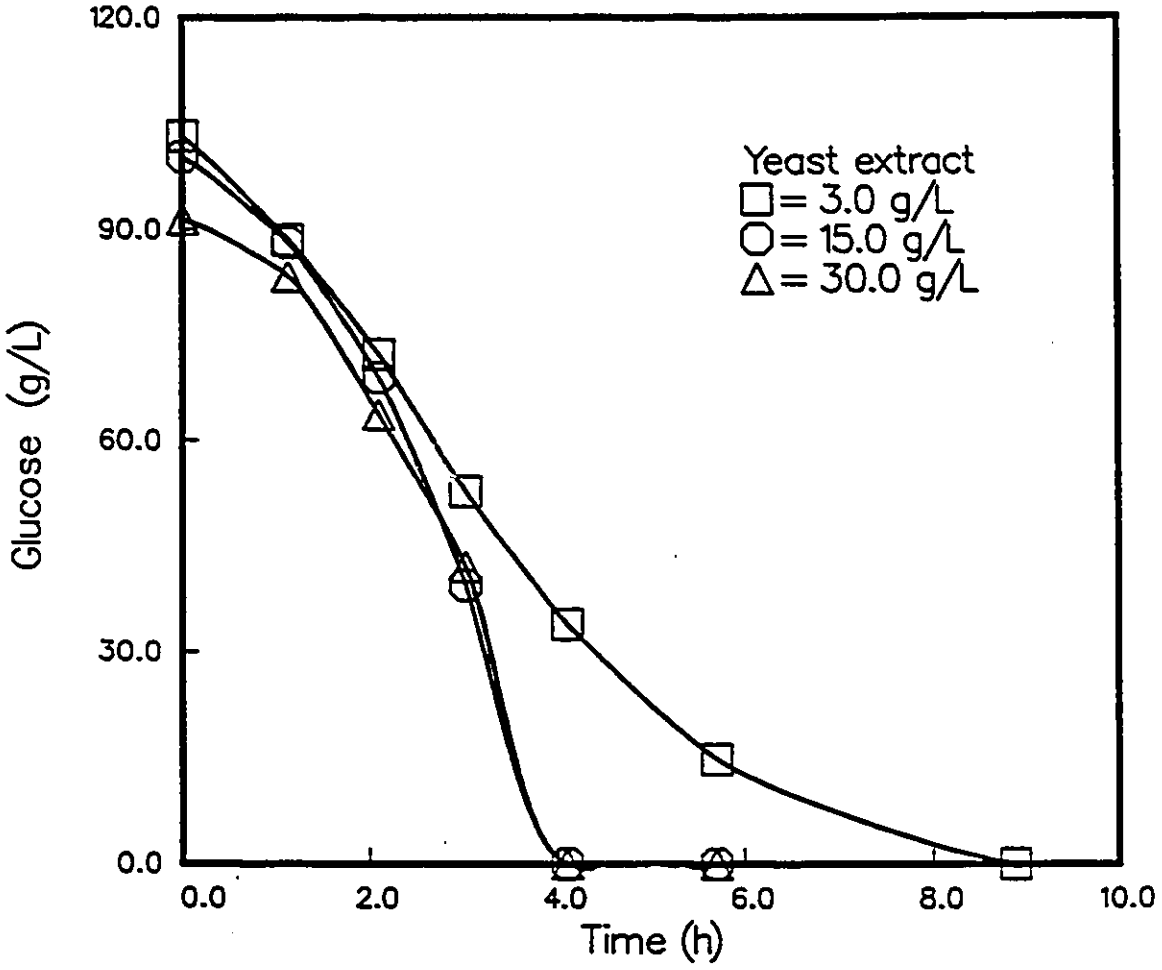

Figure 6.3: A comparison of the glucose consumption by the wild strain of *S. cerevisiae* at various yeast extract concentrations in media containing ≈ 95 g/L glucose.

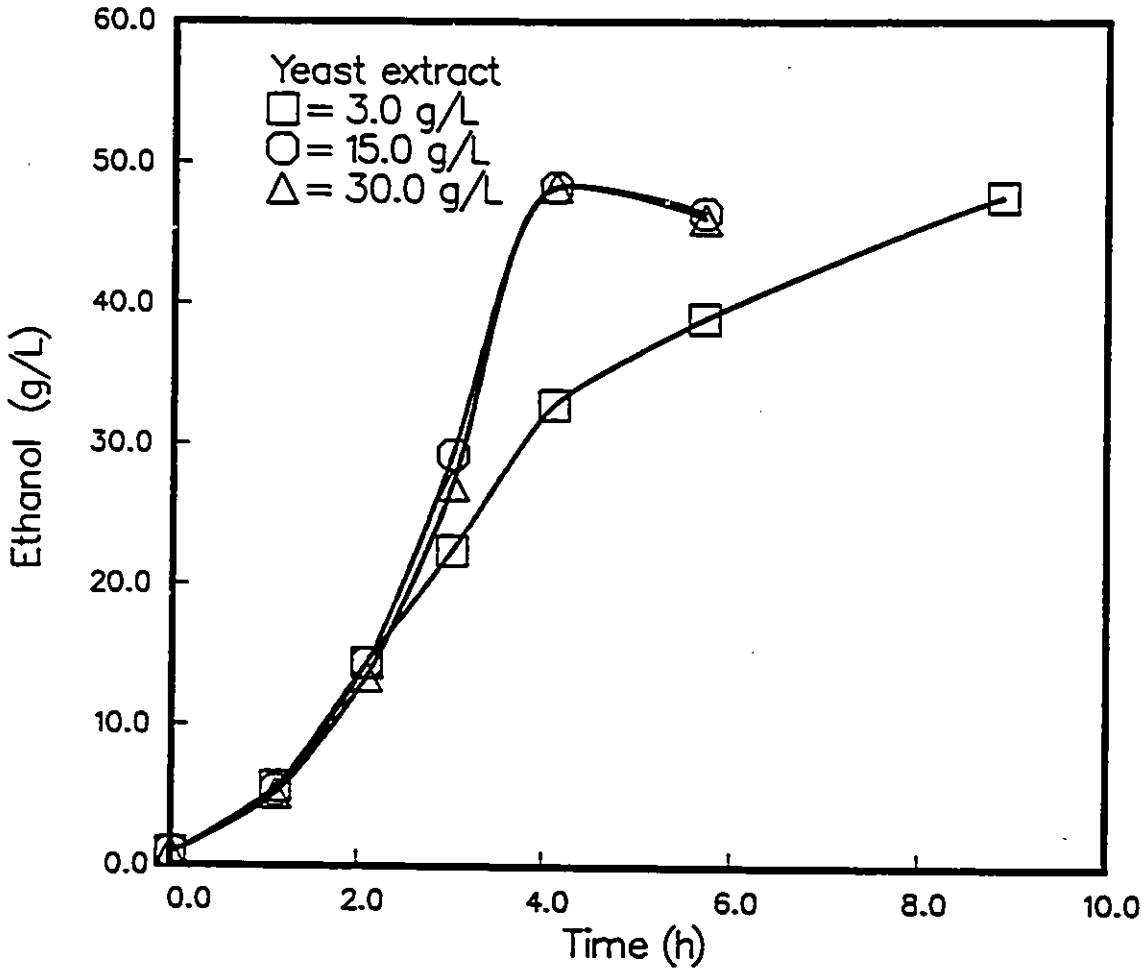

Figure 6.4: A comparison of the ethanol production by the wild strain of *S. cerevisiae* at various yeast extract concentrations in media containing ≈ 95 g/L glucose.

6.1.2 Mutant strain

Growth and ethanol production by the mutant strain of *S. cerevisiae* in media containing 3.0 g/L yeast extract and either glucose or fructose are compared in Figure 6.5. The mutant strain consumed 91 g/L glucose in 24 hours, increased the biomass from 2.2 to 5.8 g/L and produced 40.9 g/L ethanol. When it is inoculated into a medium containing fructose, only 33% of the fructose is consumed after 66 hours. During this time the biomass concentration increased to 6.5 g/L and 6.3 g/L of ethanol was produced. From the figure it is evident that the mutant strain consumes glucose to produce ethanol and biomass at a faster rate than fructose. Even though a smaller amount of fructose was consumed, the biomass produced was similar to that produced in the glucose medium, indicating a higher biomass yield.

Glucose media

A series of tests were carried out in media containing between 3.0 and 30.0 g/L yeast extract and $\approx$ 93 g/L glucose. The biomass, glucose and ethanol concentrations measured during these tests are compared in Figures 6.6–6.8. The specific growth rates, and the biomass and ethanol yields are provided in Table 6.2.

From Figure 6.6 it is seen that an increase in the yeast extract concentration results in an increased growth rate as well as higher final biomass concentrations. The specific growth rate increased from 0.105 to 0.192 h^{-1} when the yeast extract was increased from 3.0 to 30.0 g/L (Table 6.2). At the same time, the final biomass concentration increased from 5.8 to 13.1 g/L which represent biomass yields of 0.040 and 0.120 g/g respectively. This is expected since yeast extract may contain a substance that may be the limiting substrate for the microbe; as the substrate concentration is increased the amount of biomass produced will also be augmented.

The glucose consumption rates also increased as the yeast extract concentration was varied from 3.0 to 30.0 g/L. The fermentation time was reduced from 25 to less than 15 hours (Figure 6.7). The glucose consumption rates (P1) provided in Table 6.2 indicate increases of 29 and 17% when the yeast extract is increased from 3.0 to 7.5 and 7.5 to

Table 6.2: Biomass and ethanol yields and specific growth, glucose consumption and ethanol production rates for tests carried out in media with various yeast extract concentrations and glucose using the mutant strain.

Yeast extract (g/L)	Glucose (g/L)	Specific growth rate (h^{-1})	Biomass yield[a] (g/g)	Ethanol yield[a] (g/g)	P1[b] (g/Lh)	P2[c] (g/Lh)
3.0	92.8	0.105	0.040	0.444	3.83	1.83
7.5	93.6	0.118	0.064	0.446	4.95	2.29
15.0	93.6	0.145	0.090	0.464	5.81	2.50
20.0	93.2	0.163	0.102	0.459	6.17	2.77
30.0	90.9	0.192	0.120	0.455	6.39	2.80

[a] yields calculated at maximum ethanol and biomass concentrations

[b] glucose consumption rate at 0-80% carbohydrate conversion

[c] ethanol production rate at 0-80% carbohydrate conversion

15.0 g/L respectively. Further increases result in smaller changes (4-6%) in the glucose consumption rates.

Figure 6.8 compares ethanol production by the yeast in media containing various yeast extract concentrations. The rates of ethanol production increase as the yeast extract concentration is increased from 3.0 to 20.0 g/L, a further increase to 30.0 has only a minimal effect. The results provided in Table 6.2 for the ethanol formation rates substantiate this claim. When the yeast extract concentration is increased from 3.0 to 7.5 g/L, 7.5 to 15.0 and 15.0 to 20.0 g/L, the production rates increase by 25, 9 and 11% respectively. A further increase to 30.0 g/L results in an increase of only 1% in the ethanol production rate. The concentration of ethanol produced in each of these media is about 43 g/L (Figure 6.8), this represents a yield which is $\approx$ 90% of the theoretical value. In these tests the yield varies by

5%.

The results show that the glucose consumption and ethanol production rates are not significantly affected by increases in the yeast extract concentration above 20.0 g/L. The specific growth rate though continues to increase above this yeast extract concentration. Fermentations carried out in media containing fructose and various concentrations of yeast extract are shown in the next section.

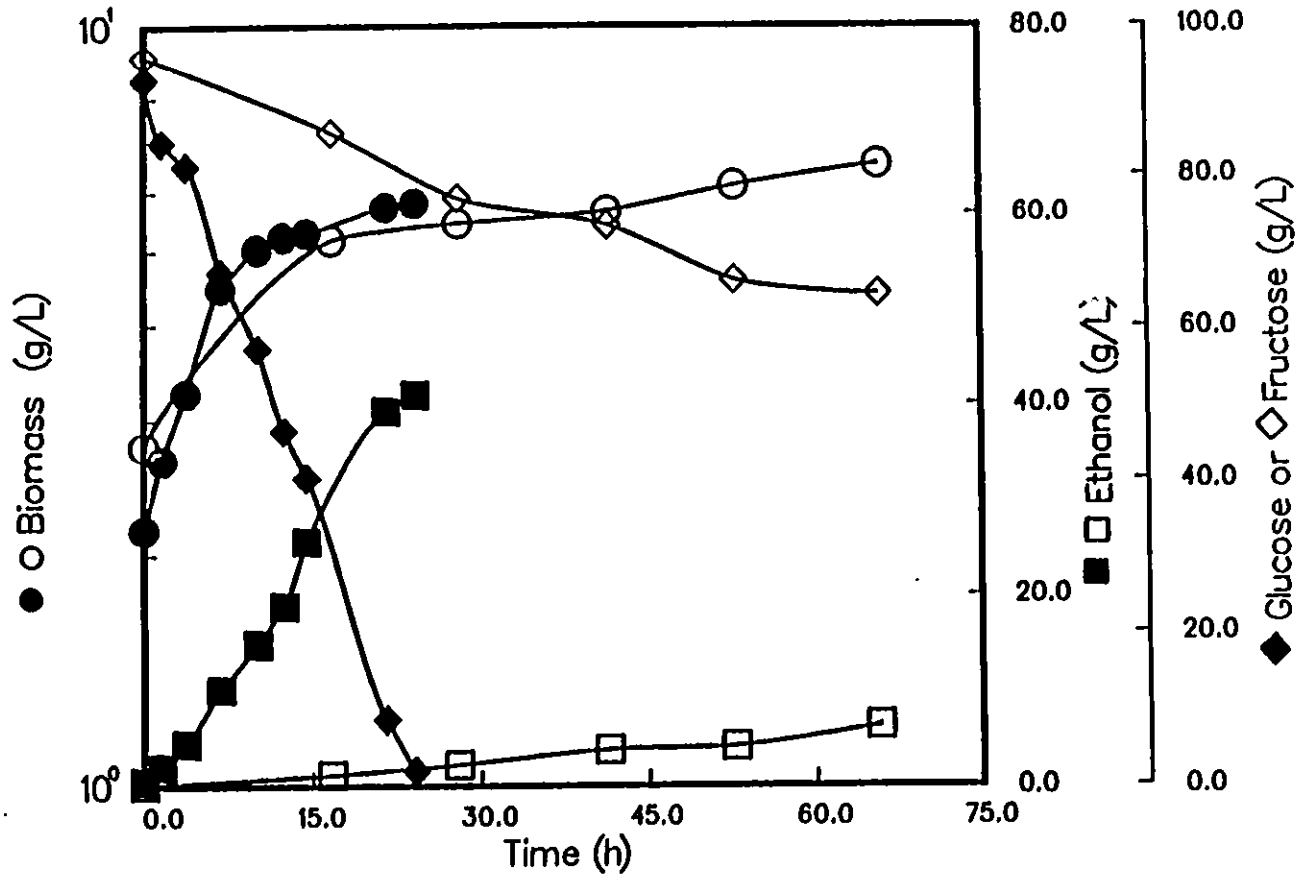

Figure 6.5: A comparison of the ethanol fermentation by the mutant strain in a medium containing 3.0 g/L yeast extract and 93 g/L glucose with one containing 3.0 g/L yeast extract and 96 g/L fructose. Solid symbols represent data for glucose medium and open symbols for fructose.

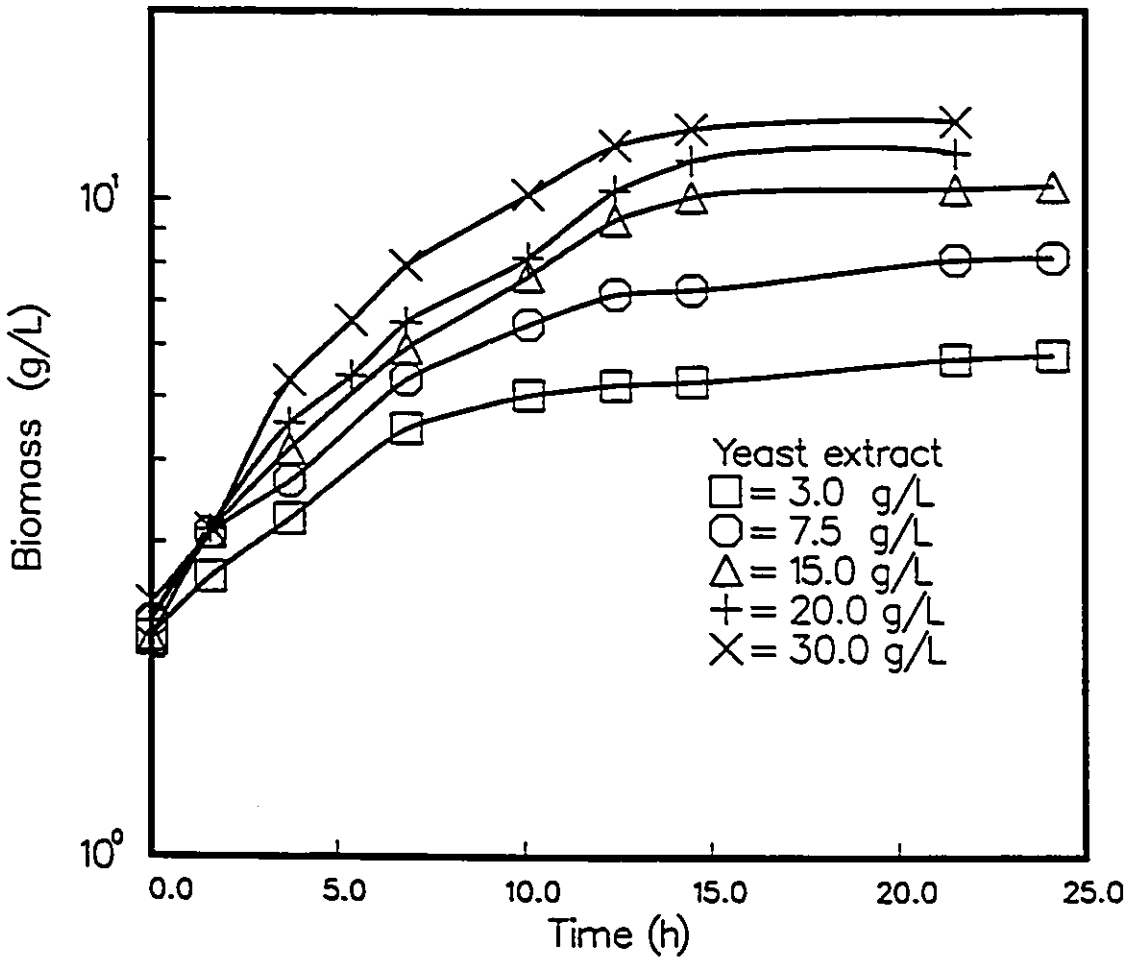

Figure 6.6: A comparison of the biomass production by the mutant strain of *S. cerevisiae* in media containing various yeast extract concentrations and ≈93 g/L glucose.

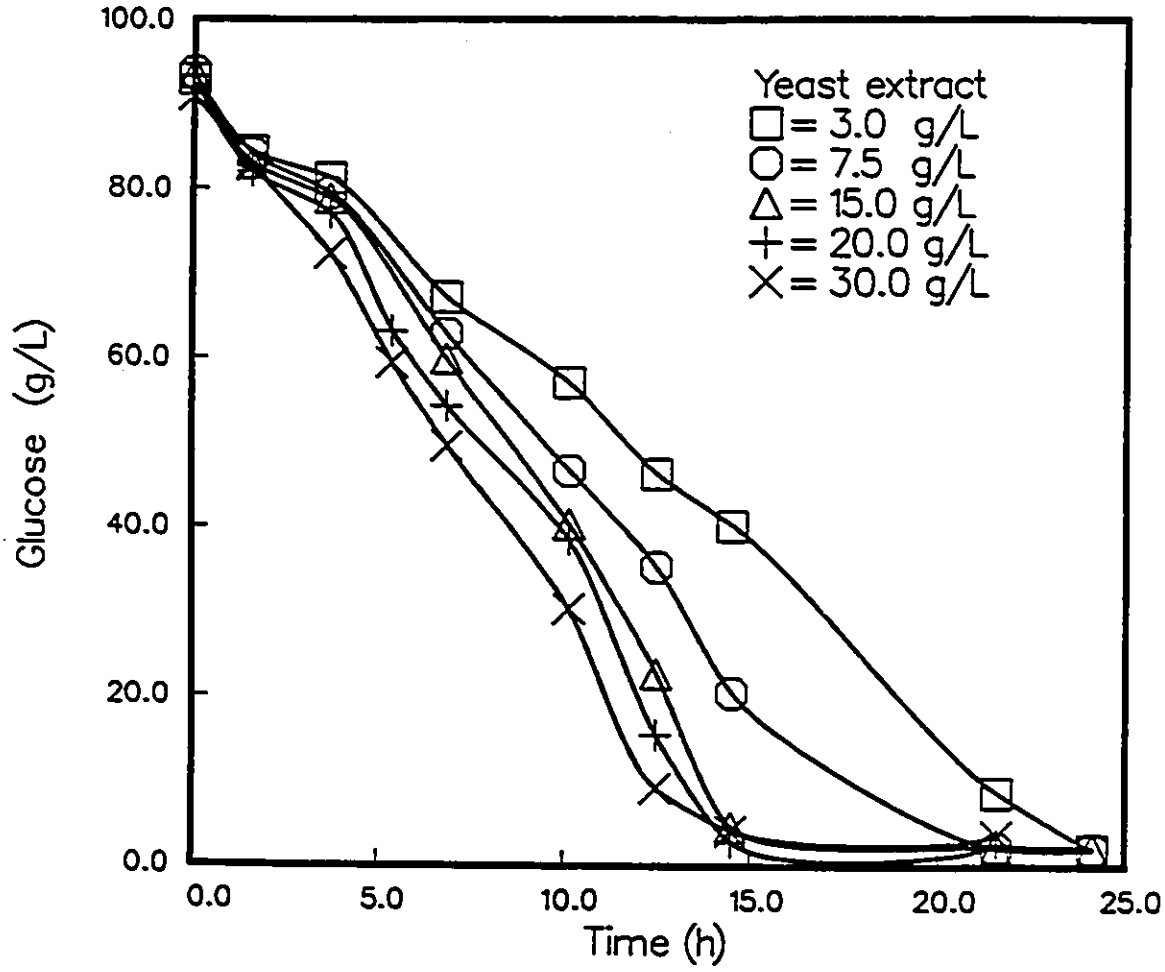

Figure 6.7: A comparison of the glucose consumption by the mutant strain of *S. cerevisiae* in media containing various yeast extract concentrations and ≈93 g/L glucose.

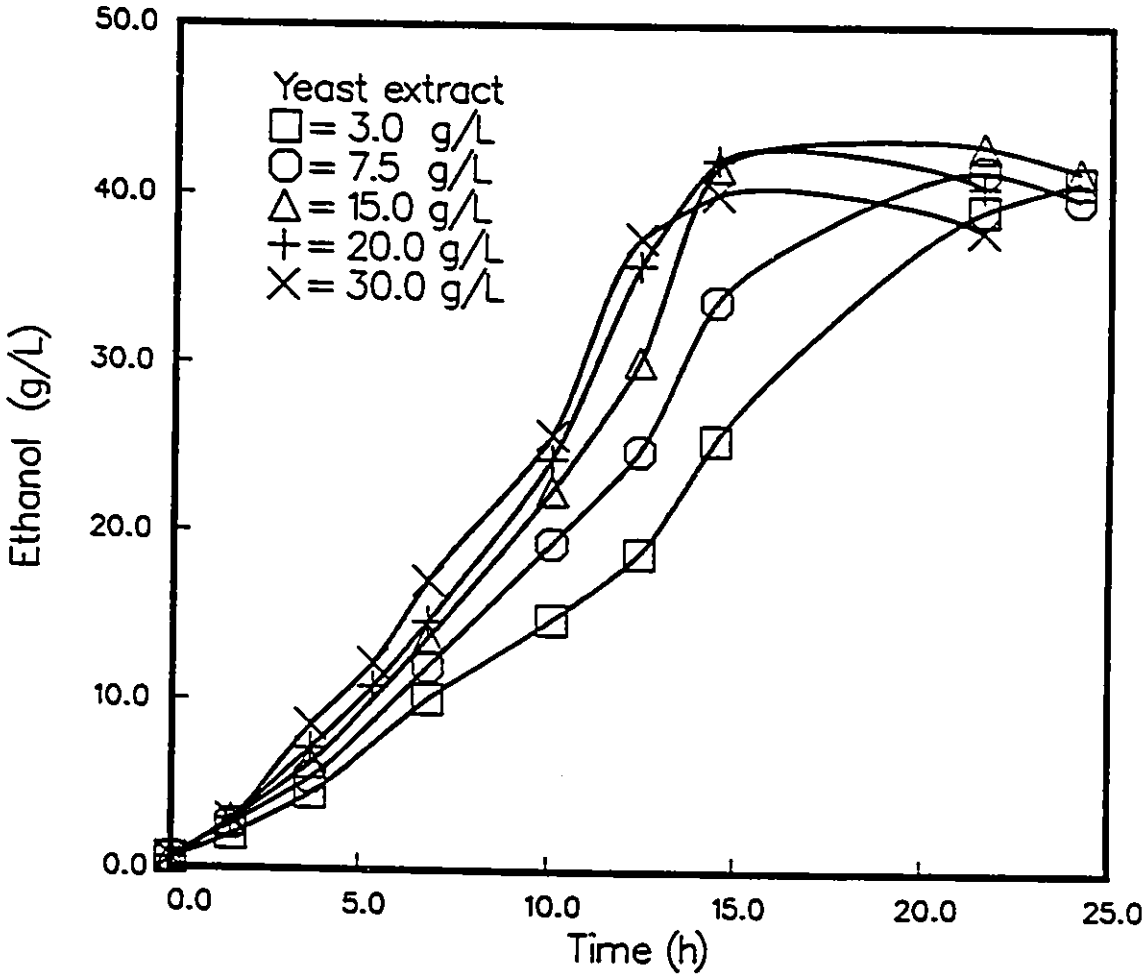

Figure 6.8: A comparison of the ethanol production by the mutant strain of *S. cerevisiae* in media containing various yeast extract concentrations and ≈93 g/L glucose.

Fructose media

Tests carried out at various yeast extract concentrations in media containing ≈ 92 g/L fructose are presented in Figures 6.9–6.12. Specific growth rates and biomass, ethanol and sorbitol yields are presented in Table 6.3.

Table 6.3: Specific growth rates and biomass, ethanol and sorbitol yields for fermentations carried out in media containing various yeast extract concentrations and ≈92 g/L fructose.

Yeast extract (g/L)	Fructose (g/L)	Specific growth rate (h^{-1})	Biomass yield[a] (g/g)	Ethanol yield[a] (g/g)	Sorbitol yield[a] (g/g)	P3[b] (g/Lh)
3.0	95.8	0.037	0.119	0.196	–	0.56
7.5	92.5	0.046	0.136	0.101	0.200	0.99
15.0	93.3	0.044	0.166	0.033	0.300	1.20
20.0	94.1	0.043	0.151	0.106	0.286	1.66
25.0	91.5	0.045	0.170	0.230	0.247	2.12
30.0	89.8	0.047	0.164	0.309	0.265	2.31

[a] yields calculated at maximum biomass, ethanol and sorbitol concentrations

[b] maximum fructose consumption rate

From Figure 6.9 it is seen that the initial growth rates are similar but the final biomass concentrations attained are higher as the yeast extract concentration is increased. The specific growth rate (Table 6.3) increased 24% when the yeast extract concentration is increased from 3.0 to 7.5 g/L. Higher yeast extract concentrations result in a change in the specific growth rate of less than 5%. The biomass yield is increased by 14 and 22% when the yeast extract concentration is increased from 3.0 to 7.5 and 7.5 to 15.0 g/L respectively. Further augmentation in the concentration of the yeast extract has only minimal effects (≈

10%) on the biomass yield.

The results from Figure 6.10 show an increase in the amount of fructose consumed as the yeast extract concentration is increased. Significant consumption of fructose occurs when the yeast is in the medium for more than 15 hours. The rate of consumption (Table 6.3) increased by 77% when the yeast extract increased from 3.0 to 7.5 g/L. Further increases in the concentration of yeast extract results in increases in the consumption rate ranging from 21 to 38%. An increase from 25.0 to 30.0 g/L changes the rate by only 9%. In the latter case the fructose consumption rate at 0-80% carbohydrate conversion was 1.53 g/Lh.

When fructose is the substrate, the yeast produces less than 10.0 g/L ethanol in media containing 3.0 to 20.0 g/L yeast extract (Figure 6.11). When the yeast extract is increased to 25.0 g/L the ethanol concentration is increased to 22 g/L. A product containing 28 g/L is formed when the medium contained 30.0 g/L yeast extract. The ethanol production rate (P2) was 0.25 g/Lh in this case. The ethanol yield first decreased to 0.033 when the yeast extract concentration is increased from 3.0 to 15.0 g/L (Table 6.3). Further increases in the yeast extract concentration to 30.0 g/L results in an increase in the ethanol yield to 0.309. This indicates that the yeast's metabolism is shifting from the production of ethanol to the production of another substance at lower yeast extract concentrations and then shifting back to ethanol production at higher yeast extract concentrations. Part of the shift is accounted for by the higher biomass yields in media containing low yeast extract concentrations. The production of sorbitol which was detected in some of these tests may also account for this phenomenon.

The sorbitol concentrations measured in the experiments are compared in Figure 6.12. In the medium containing 3.0 g/L yeast extract, sorbitol was not produced. When the yeast extract concentration was increased to 7.5 g/L, a maximum concentration of 9.0 g/L was attained after 35 hours. In a medium containing 20.0 g/L yeast extract a sorbitol concentration of 25.0 g/L was attained (0.286 g sorbitol produced per g of fructose consumed). When the yeast extract concentration was further increased to 25 g/L, the sorbitol concentration reached 21.4 g/L after 58 hours, after this point the sorbitol concentration decreased. This reduction in the sorbitol concentration may indicate consumption by the yeast, the amount

of consumption increased as the yeast extract concentration was increased to 30.0 g/L. At the same time the maximum sorbitol yield remained relatively constant (± 14%) at these higher yeast extract concentrations. Replicates that were carried out verified these trends.

The yeast (ATCC 36859) consumes fructose when it is the only carbohydrate present in the medium, at a rate that increases as the yeast extract concentration is increased. Ethanol and sorbitol are produced by the yeast, the yields of which increase as the yeast extract concentration is increased. At concentrations higher that 20.0 g/L, sorbitol is consumed by the yeast. The sorbitol may be converted to ethanol which would explain the higher ethanol yields measured at larger yeast extract concentrations.

Concerning sorbitol production, the yeast's behaviour is different from that of *Z. mobilis* which was also shown to produce sorbitol (Bringer-Meyer et al., 1985; Doelle and Greenfield, 1985b). In the case of the bacterium, sorbitol is produced when both glucose and fructose are present in the medium. Use of *Z. mobilis* in glucose/fructose media have produced sorbitol concentrations of up to 60 g/L (Bringer-Meyer et al., 1985). The yeast used in the present study produces sorbitol when fructose is the only carbohydrate present in the medium. Therefore the yeast is better suited for the production of a product containing only fructose and ethanol from glucose/fructose mixtures.

Since fructose is one of the main products of this process its use as a substrate must be minimized. In addition, the formation of sorbitol must be avoided since it is an unwanted by-product. From the results presented here, these objectives can be accomplished by using a low yeast extract concentration and/or by ensuring the yeast is not in contact with a medium containing fructose as the only carbohydrate source for long periods of time.

Unlike the wild strain of *S. cerevisiae* the mutant strain consumes glucose and fructose at significantly different rates and in some cases produce different products. These differences are summarized in the next section.

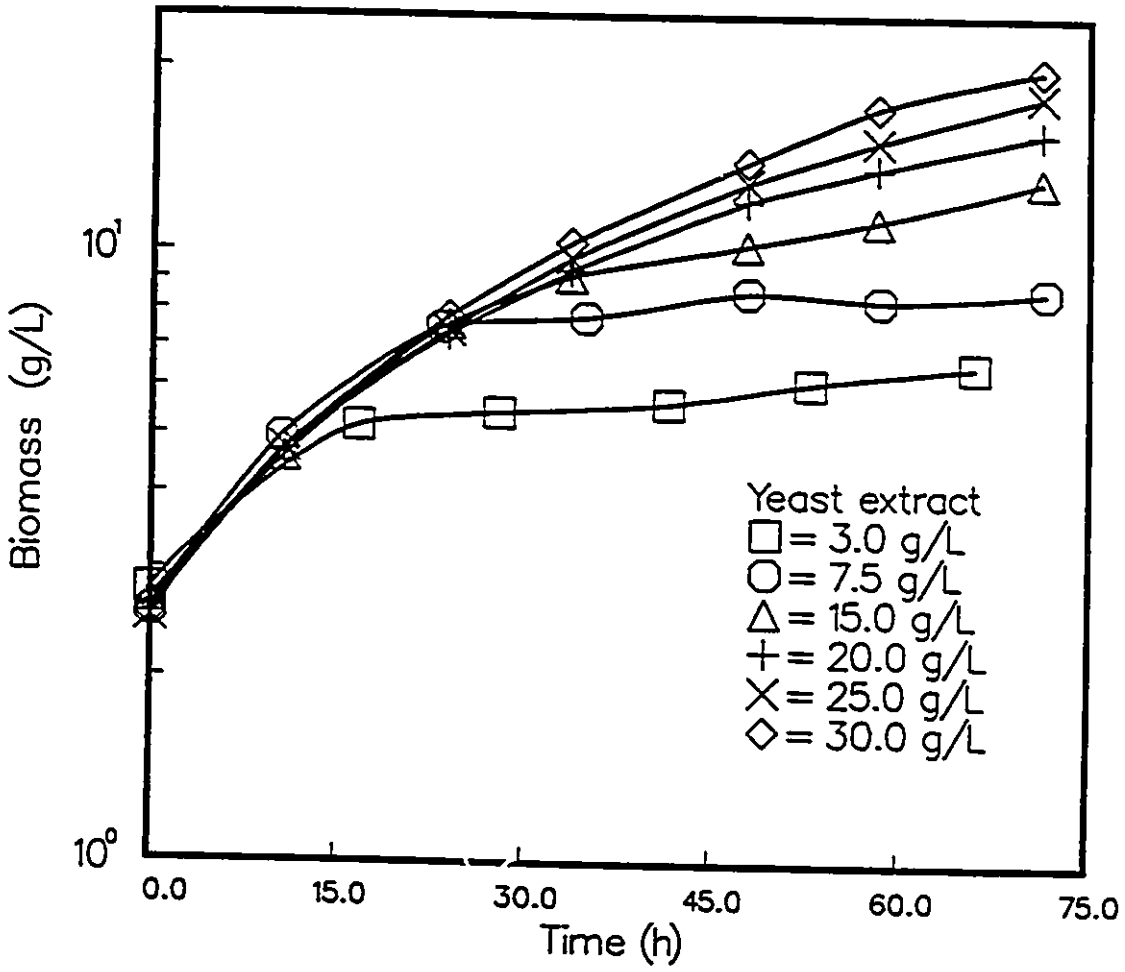

Figure 6.9: A comparison of the biomass production by the mutant strain of *S. cerevisiae* in media containing various yeast extract concentrations and ≈92 g/L fructose.

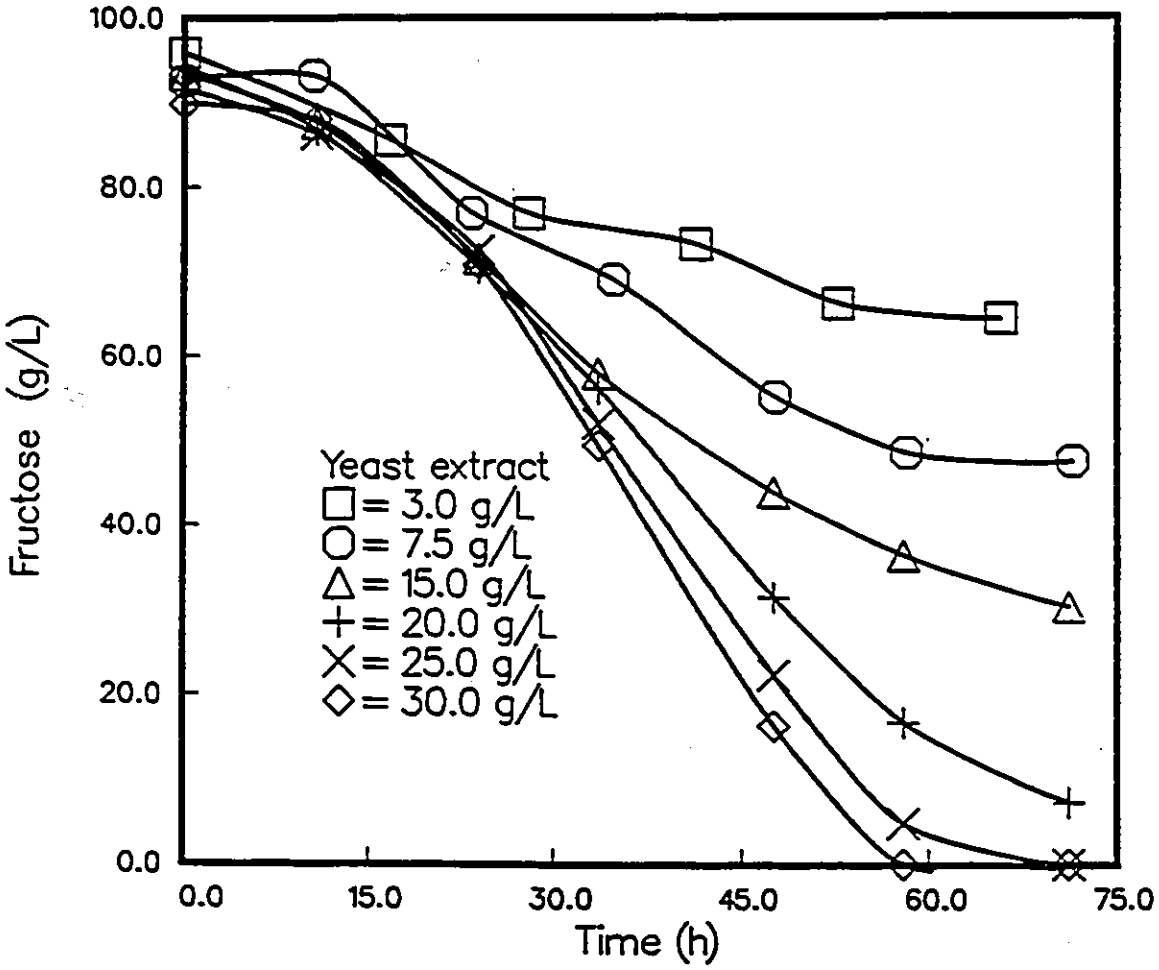

Figure 6.10: A comparison of the fructose consumption by the mutant strain of *S. cerevisiae* in media containing various yeast extract concentrations and ≈92 g/L fructose.

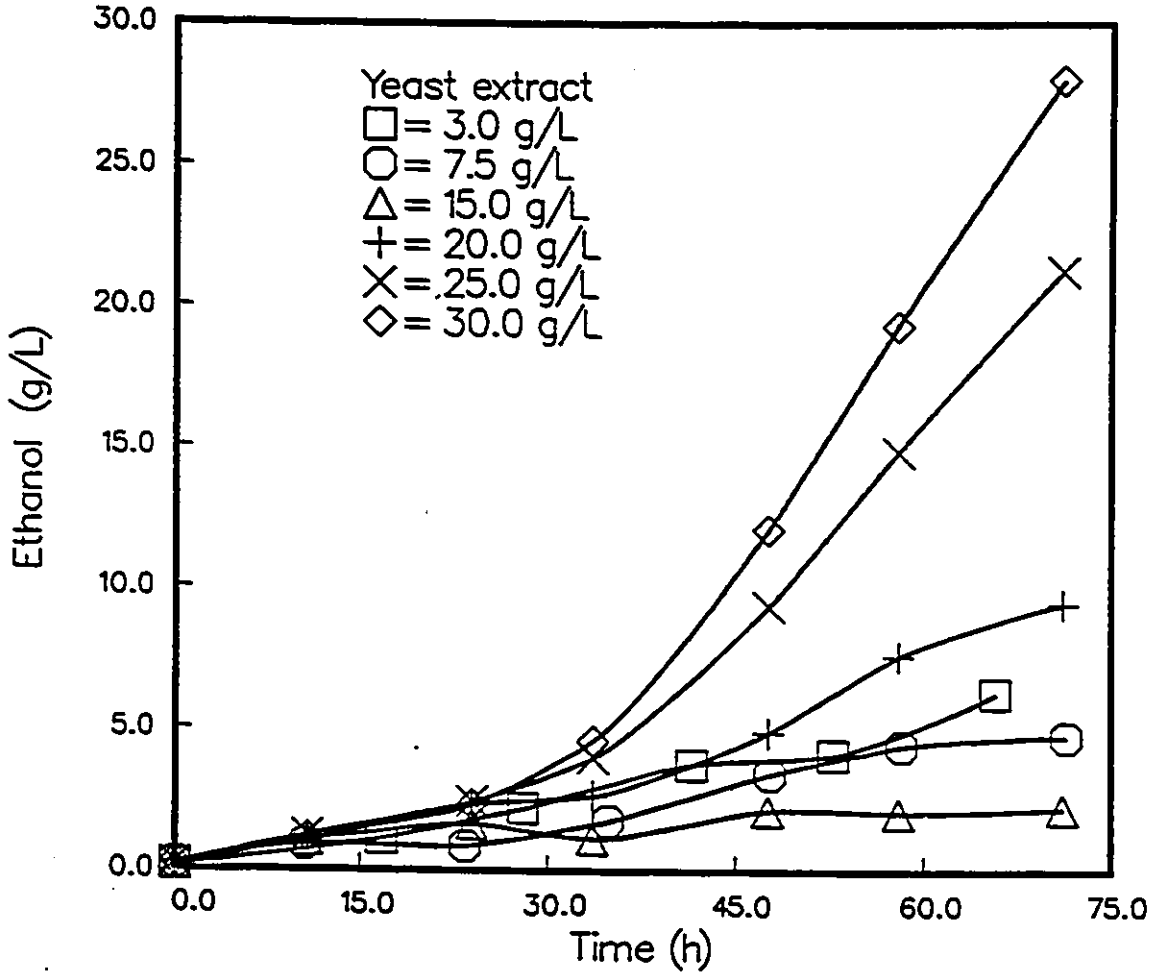

Figure 6.11: A comparison of the ethanol production by the mutant strain of *S. cerevisiae* in media containing various yeast extract concentrations and ≈92 g/L fructose.

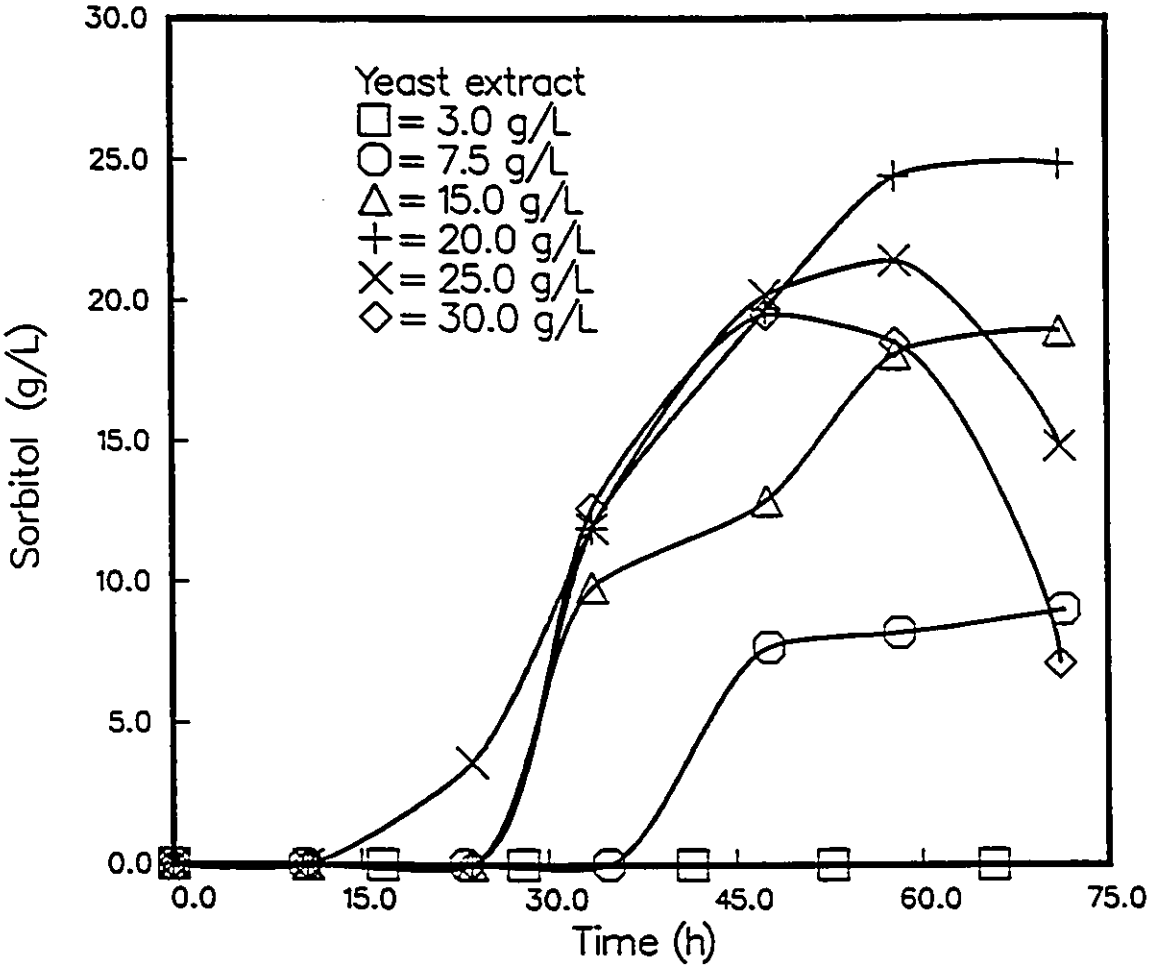

Figure 6.12: A comparison of the sorbitol production by the mutant strain of *S. cerevisiae* in media containing various yeast extract concentrations and ≈92 g/L fructose.

6.1.3 A comparison of the two strains

A comparison of the fermentation of glucose in media containing 30.0 g/L yeast extract using the wild and mutant strains is given in Figure 6.13. The wild strain is able to consume 91.4 g/L glucose in 4 hours, during this time the biomass increased from 2.9 to 10.5 g/L and 48.3 g/L ethanol was produced. Fermentation of a similiar concentration of glucose by the mutant strain was completed in 14.5 hours, the biomass that was produced was similiar in both cases but only 40.1 g/L ethanol was produced by the mutant strain. The differences in fermentation of glucose by the two strains can also be observed by reference to Tables 6.1 and 6.2. The ethanol yields for the experiments carried out in 3.0 and 15.0 g/L yeast extract were nearly equivalent, with 30.0 g/L yeast extract the yield for the wild strain was 14% higher. The biomass yields increased as the yeast extract concentration was increased for both strains but the increase for the mutant strain was larger. At 3.0 g/L yeast extract the biomass yield for the mutant strain was 38% lower, but in 30.0 g/L yeast extract the biomass yield for the mutant strain was 4% higher. The mutant strain had a specific growth rate which varied from 3.3 to 2.2 times lower than that of the wild strain. Glucose consumption and ethanol production were 3.3 to 4.3 times lower for the mutant strain. The difference between the two were reduced at higher yeast extract concentrations. This is expected since the rates for the mutant increased for concentrations up to 20.0 g/L yeast extract while those for the wild strain levelled off at 15.0 g/L. This would seem to indicate that the mutant strain requires a higher level of vitamins and growth factors which is present in yeast extract.

Fermentation with fructose using the wild and mutant strains in media containing 30.0 g/L yeast extract are compared in Figure 6.14. The wild strain consumes 98 g/L fructose in 4 hours to produce 48 g/L ethanol. The biomass increased from 2.5 to 13.0 g/L. On the other hand, the mutant strain consumes 90 g/L fructose in 58 hours. During this time 19.0 g/L ethanol was produced and the biomass increased from 2.5 to 17.0 g/L. In this instance, the fructose consumption rate at 0-80% carbohydrate conversion for the wild strain is 14 times larger than that of the mutant strain. The biomass produced by the mutant strain is higher though than that of the wild strain (Figure 6.14). The differences are also evident

by comparing the results reported in Tables 6.1 and 6.3. The growth rates of the mutant strain are about 10 times lower than the wild strain. The mutant strain produces more biomass as evidenced by the biomass yields which range from 53 to 100% higher than those of the wild strain. A comparison of the results also indicates lower ethanol yields by the mutant strain. This, as was shown previously is due to the production of sorbitol by the mutant strain. The wild strain did not produce sorbitol under the conditions tested.

In the paper concerning the mutation of *S. cerevisiae*, Lobo and Maitra (1977) reported that ATCC 36859 grew on glucose nearly as well as the wild strain. The results in this section show that the mutation has resulted in some significant differences to the yeast. The mutation has resulted in lower growth and ethanol fermentation rates in glucose media. In addition, the mutant strain consumes fructose at a much lower rate than glucose. Ethanol production in fructose media is substantially reduced, this is due to the production of sorbitol by the mutant strain in some of the cases. Although the longer fermentation times do not make the yeast suitable for the production of ethanol from glucose compared to the wild strain, its selectivity make it suitable for the production of fructose and ethanol from glucose/fructose mixtures. This selectivity is investigated further in the next section.

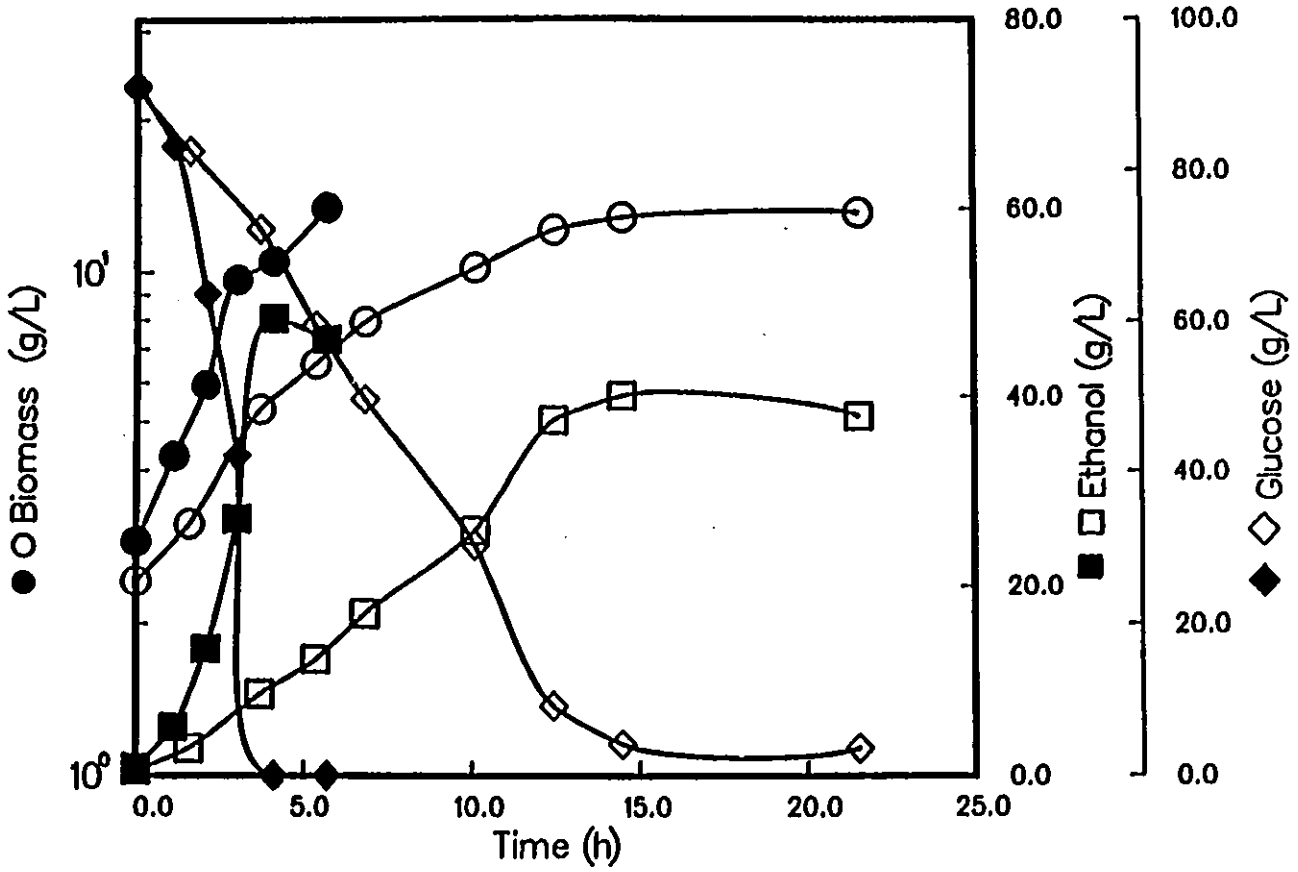

Figure 6.13: Growth and ethanol production using the wild and mutant strains of *S. cerevisiae* in glucose media. Solid symbols represent data for the wild strain and open symbols represent data for the mutant strain.

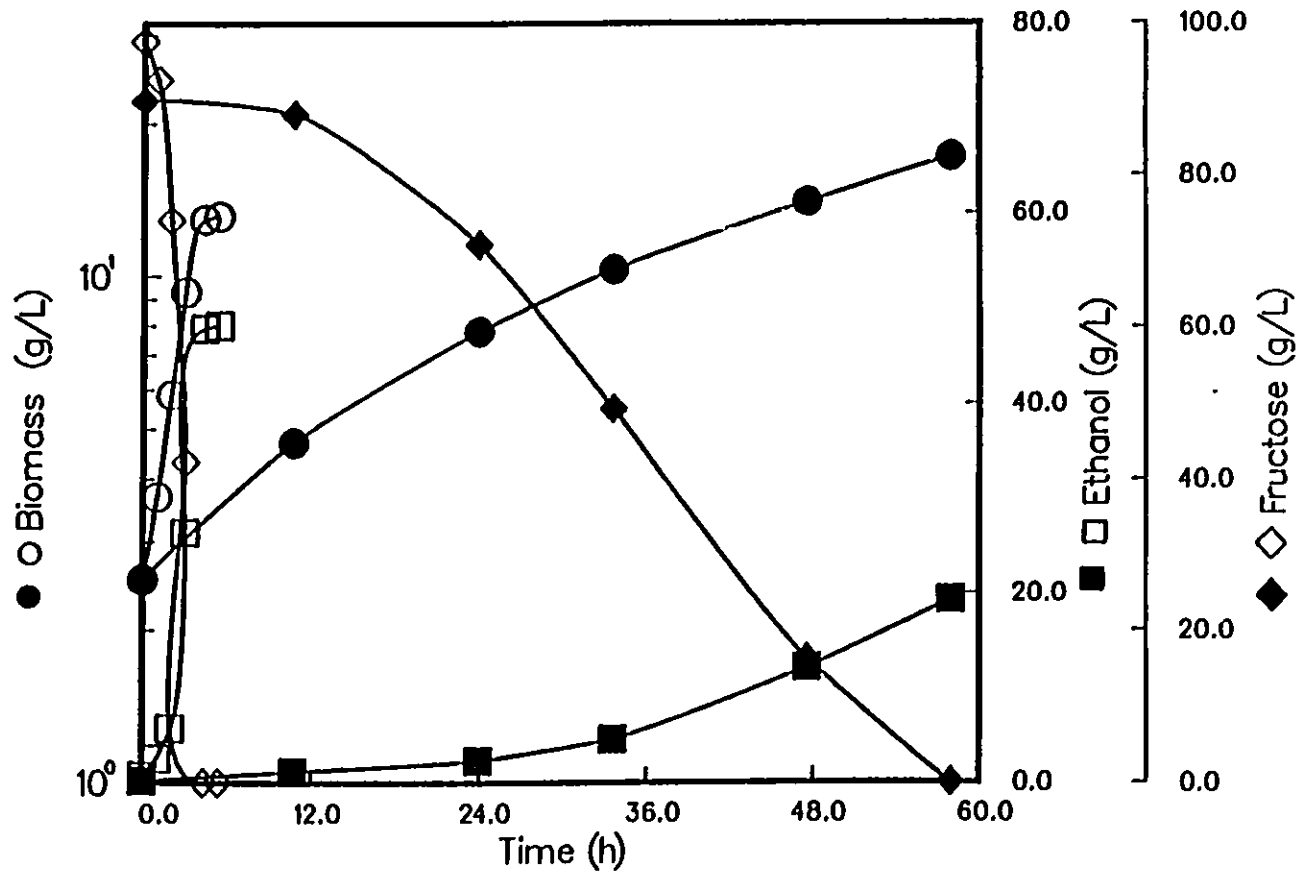

Figure 6.14: Growth and ethanol production using the wild and mutant strains of *S. cerevisiae* in fructose media. Solid symbols represent data for the wild strain and open symbols represent data for the mutant strain.

6.2 Batch Production of Ethanol and Fructose from Synthetic Glucose/Fructose Mixtures using *S. cerevisiae* ATCC 36859

In this section ethanol fermentations with *S. cerevisiae* ATCC 36859 are studied more closely. The effects of glucose concentration will be studied. In addition, inhibitory effects of fructose and ethanol, which are the two products of the process are also examined.

6.2.1 Effect of glucose concentration

The advantages of a high substrate concentration in the feed include reduced dilution water requirements, the reduction of osmosensitive contaminants and the potential of reduced distillation costs if ethanol inhibition can be limited (Jones et al., 1981). It has been shown that high substrate concentrations inhibit growth and may adversely affect product formation. It is thought that the effect is related to osmotic effects in which plasmolysis of the cells begin to occur at high substrate concentrations, the exact concentration is strain dependent. The effect of this phenomenon is studied more closely here.

Glucose serves as the primary carbon source for the yeast, its concentration influences both the growth and ethanol production process. A series of tests were carried out in medium III, which contained 30 g/L yeast extract and various concentrations of glucose. The biomass, glucose and ethanol concentrations were measured as a function of time in media containing 70.1, 96.4, 125.4 and 187.9 g/L glucose, the results are shown in Figures 6.15–6.18.

Figure 6.15 shows that 70.1 g/L glucose is consumed in 11 hours to produce 33.5 g/L ethanol (92% of the theoretical yield). The biomass increased from 1.4 to 11.6 g/L, which represents a biomass yield of 0.147 g/g. When placed in a medium containing 96.4 g/L glucose, the yeast consumes the glucose in 16 hours and produces 46.4 g/L ethanol (Figure 6.16). During this time the biomass concentration increased to 13.4 g/L from 1.3 g/L. Figure 6.17 provides the results obtained when the yeast is inoculated into a medium containing 125.4 g/L glucose. After 16 hours the biomass increased from 1.6 to 15.3 g/L (0.109 g/g

biomass yield). At the same time 60.2 g/L ethanol is produced. Finally in a medium containing 187.9 g/L glucose, fermentation is carried out in 24 hours (Figure 6.18). During this time 89.9 g/L ethanol is produced which is 93% of the theoretical value and the biomass is increased to 15.2 g/L from 1.5 g/L. Specific growth rates and biomass and ethanol yields for these tests are given in Table 6.4.

Table 6.4: Specific growth rates and biomass and ethanol yields for tests carried out in various glucose concentrations.

Glucose (g/L)	Specific growth rate (h^{-1})	Biomass yield[a] (g/g)	Ethanol yield[a] (g/g)	P1[b] (g/Lh)	P2[c] (g/Lh)
70.1	0.255	0.147	0.469	6.46	3.05
96.4	0.241	0.126	0.475	6.11	3.09
125.4	0.214	0.109	0.476	7.23	3.50
187.9	0.198	0.073	0.475	7.94	3.86

[a] yields calculated at maximum biomass and ethanol concentrations

[b] glucose or fructose consumption rate at 0-80% carbohydrate conversion

[c] ethanol production rate at 0-80% carbohydrate conversion

From the table it is seen that the specific growth rate is reduced by 22% as the intial glucose concentration is increased from 70.1 to 187.9 g/L. Monod's equation (Eq. 3.1) predicts that the specific growth rate will initially increase and then level off as the substrate concentration is increased. In the concentration range tested, glucose appears to have an inhibitory effect on the growth of the yeast. A 50% reduction in the biomass yield is also noticed as the glucose concentration is increased from 70.1 to 187.9 g/L. The higher glucose concentrations therefore discourages production of biomass. The repression of the energy producing metabolism (growth) is known as the Crabtree effect and is common for yeasts

(Lehninger, 1982). Use of the substrate for maintenance energy becomes more important as the fermentation times are increased. Margaritis and Bajpai (1983) also reported a sharp decline in the cell growth yield as the substrate concentration was increased using *K. marxianus*. The ethanol yields remain relatively constant at 92-93% of the theoretical yield for the glucose concentrations tested here. The ethanol yield was also found to be constant up to a substrate concentration of 250 g/L using *K. marxianus* (Margaritis and Bajpai, 1983).

It is also interesting to note that although the growth rate decreased, the glucose consumption and ethanol production rates increased as the glucose concentration was increased. The fact that these latter rates were not inhibited while the growth rate was, was also found by Ghose and Tyagi (1979b) using a wild strain of *S. cerevisiae*.

These tests show that the yeast was capable of consuming up to 188 g/L glucose to produce ethanol. The biomass yield decreased as the glucose concentration was increased to 188 g/L while the ethanol yield was unaffected by the glucose concentration in this range. As the glucose concentration was increased from 96 to 188 g/L the glucose consumption and ethanol production rates increased by 30 and 25% respectively. Unlike the growth rate the glucose consumption and ethanol production rates were not inhibited by an increase in the glucose concentration in the range tested.

The effect of fructose and ethanol which are the products of the process are studied in the next section.

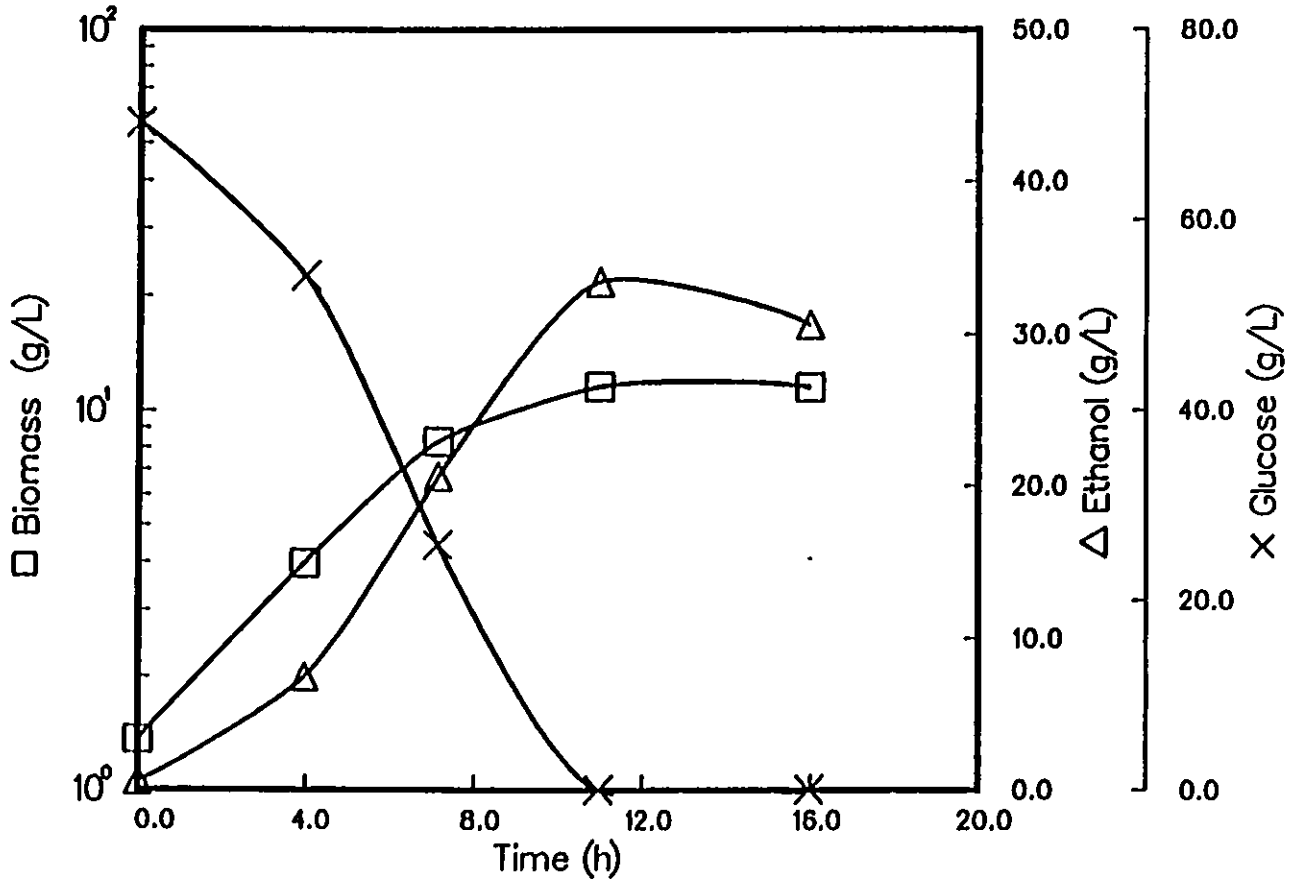

Figure 6.15: Growth and ethanol production by the mutant strain in a medium containing 30.0 g/L yeast extract and 70.1 g/L glucose.

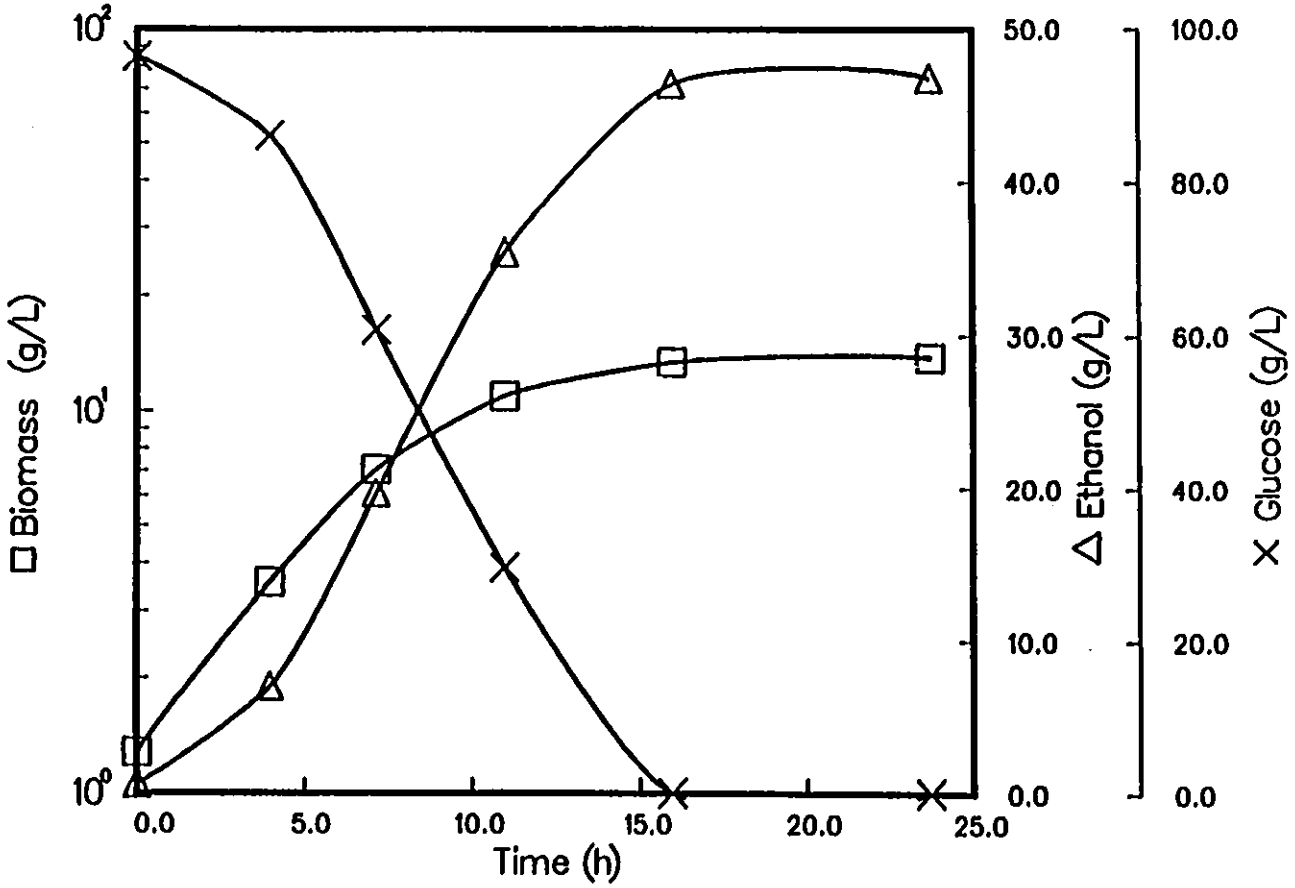

Figure 6.16: Growth and ethanol production by the mutant strain in a medium containing 30.0 g/L yeast extract and 96.4 g/L glucose.

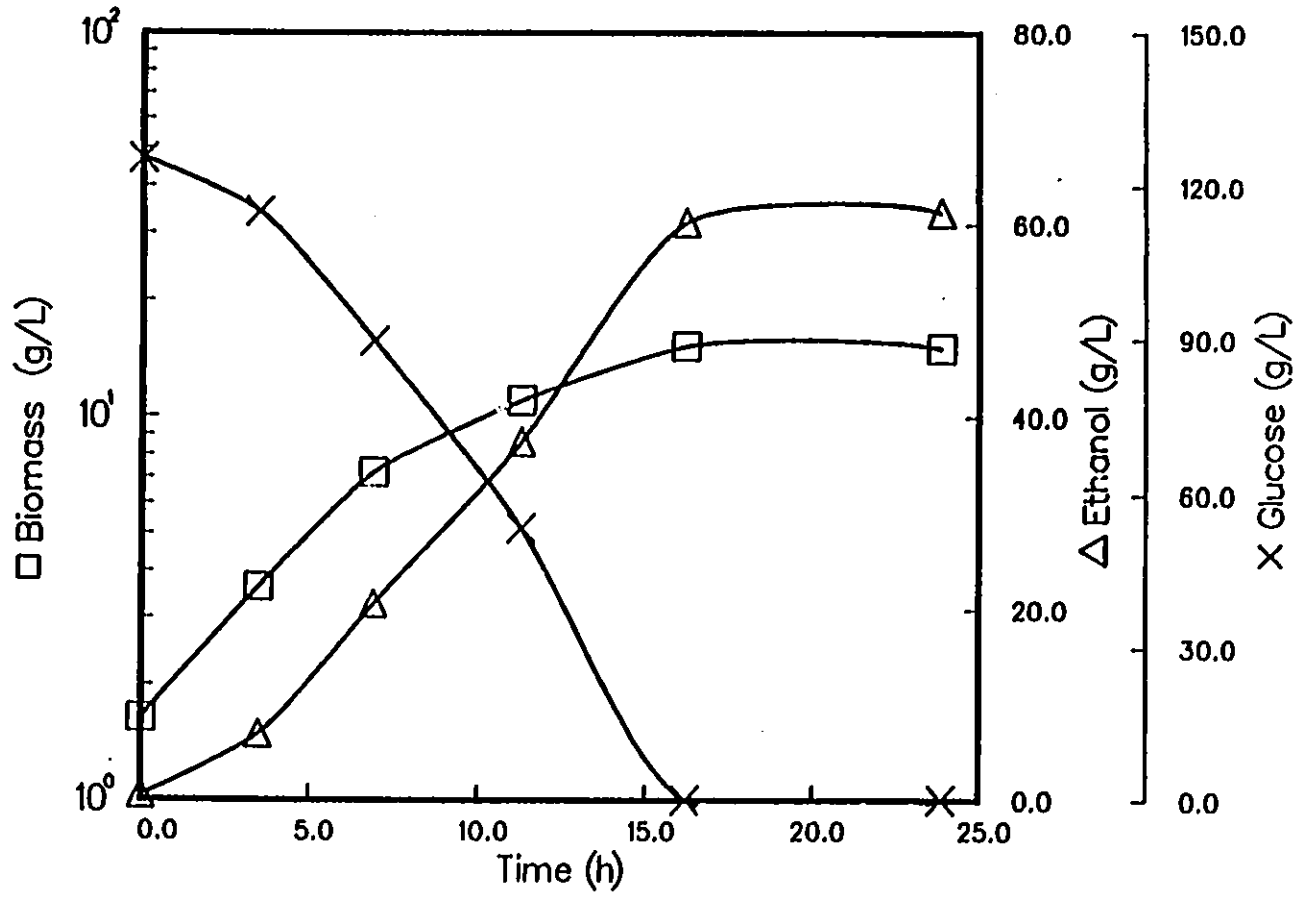

Figure 6.17: Ethanol fermentation by *S. cerevisiae* in a medium containing 30.0 g/L yeast extract and 125.4 g/L glucose.

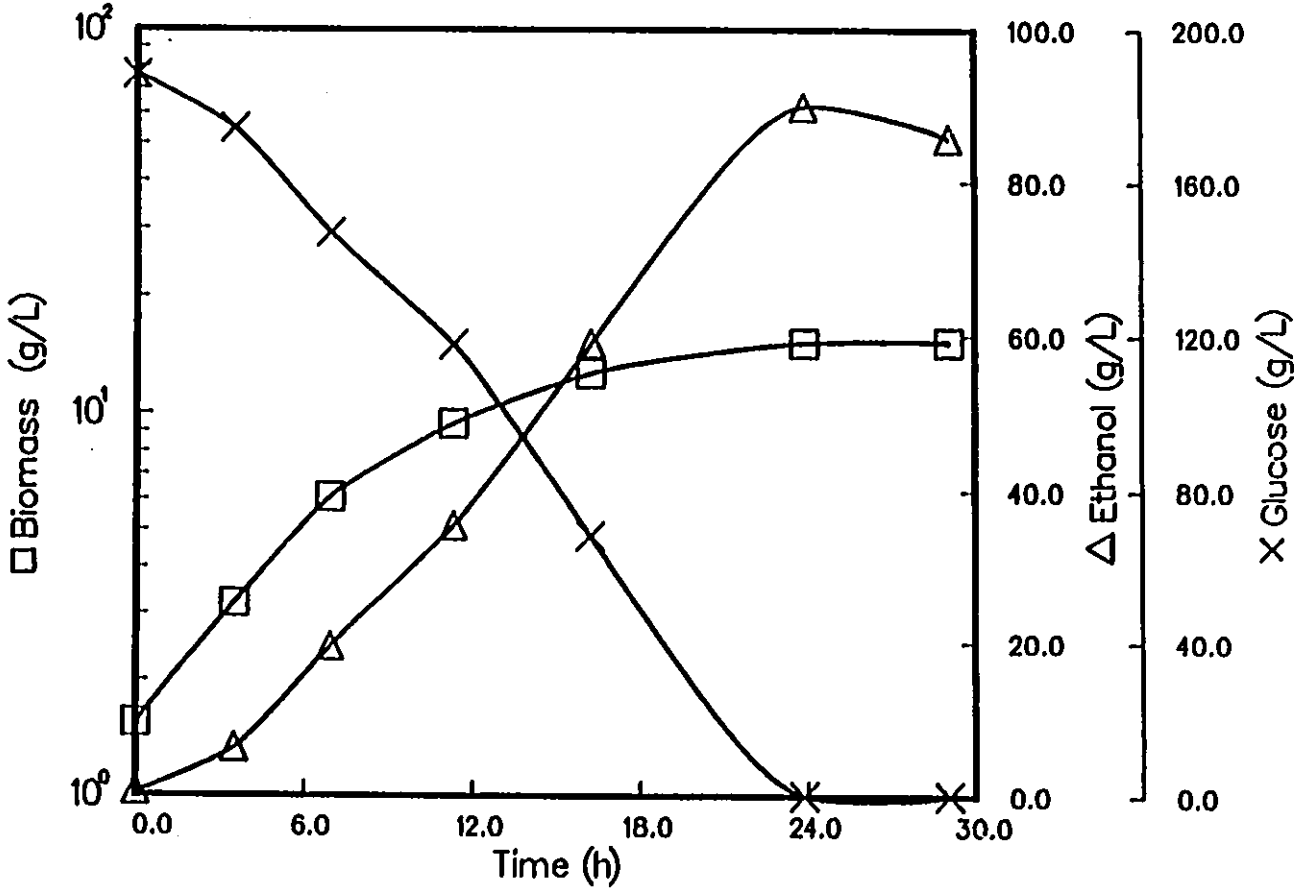

Figure 6.18: Ethanol fermentation by *S. cerevisiae* in a medium containing 30.0 g/L yeast extract and 187.9 g/L glucose.

6.2.2 Effect of product concentration

To reduce subsequent purification costs (i.e. distillation, evaporation), the concentrations of products formed in this process should be as high as possible. High concentrations though result in high osmotic pressures which leads to a reduction in the rate of growth and fermentation (Jones et al., 1981). The effect of increased fructose and ethanol concentrations on the particular yeast used in this study is now studied.

Fructose

Fermentation in media containing $\approx$ 65 g/L glucose, 30.0 g/L yeast extract and various concentrations of fructose from 0 to 129.3 g/L are shown in Figures 6.19–6.22. Results of tests carried out with a higher biomass concentration and fructose concentrations from 149.0 to 343.6 g/L are compared in Figures 6.23–6.26. Biomass and ethanol yields and specific growth, glucose consumption and ethanol production rates are shown in Table 6.5.

In the first set of experiments (i.e. fructose 0-129.3 g/L), increases in the fructose concentration to 129.3 g/L results in a reduction in the growth rate and a lower biomass concentration (Figure 6.19). When the fructose concentration is increased further (Figure 6.23), the growth of the yeast experiences an intial lag phase. In the medium containing 343.6 g/L fructose, the biomass concentration is reduced in the first 6 hours due to cell lysis, later the concentration increases as the yeast adapts to the high solids content environment. The specific growth rate was reduced by 62% in a medium containing 33.6 g/L fructose compared to a medium containing no fructose (Table 6.5). It is reduced a further 10% for each increase in the fructose concentration that was tested. The biomass yield was reduced by 68% in a medium containing 343.6 g/L fructose compared to that measured in a medium containing no fructose.

Glucose consumption is affected only minimally by increases in the fructose concentration to 129.3 g/L (Figure 6.20), the fermentation time remains about 12 hours. Further increases (Figure 6.24) result in increased conversion times, 27 hours was required to consume 69.5 g/L glucose from a medium which also contained 343.6 g/L fructose.

Ethanol production is also unaffected by an increase in the fructose concentration up to

Table 6.5: Biomass and ethanol yields and specific growth, glucose consumption and ethanol production rates for the fermentation of glucose/fructose mixtures with 30.0 g/L yeast extract.

Glu-Fru	Biomass	Biomass yield[a]	Ethanol yield[a]	Specific growth rate	P1[b]	P2[c]
(g/L)	(g/L)	(g/g)	(g/g)	(h^{-1})	(g/Lh)	(g/Lh)
70.1-0	1.27	0.147	0.469	0.255	6.46	3.05
65.8-33.9	1.40	0.143	0.494	0.231	5.80	2.91
67.3-66.5	1.60	0.124	0.446	0.216	6.31	2.84
62.6-129.3	1.60	0.101	0.467	0.190	5.69	2.76
66.4-149.0	2.16	0.096	0.437	0.147	4.18	2.17
67.8-222.2	2.25	0.071	0.456	0.150	3.96	1.92
66.5-290.1	2.05	0.050	0.443	0.108	2.74	1.37
69.5-343.6	2.24	0.047	0.428	0.098	2.34	1.14

[a] yields calculated at maximum biomass and ethanol concentrations

[b] glucose consumption rate at 0-80% carbohydrate conversion

[c] ethanol production rate at 0-80% carbohydrate conversion

129.3 g/L (Figure 6.21). The ethanol yields vary by only 10%. Results from Figure 6.25 and Table 6.5 show that beyond 222 g/L fructose the ethanol production rate is reduced significantly, 29% when the fructose concentration was increased to 290.1 g/L and 41% when it was increased to 343.6 g/L. The ethanol yield was affected only minimally (10%) for these tests.

Figures 6.22 and 6.26 show that fructose remains unconverted by the yeast during the course of these tests. Only small variations (± 7%) are observed.

The results indicate an inhibition of the growth rate and a reduction in the biomass yield

even when small concentrations of fructose are present in the medium. Even though fructose is not utilized by the yeast its presence results in the inhibition of growth. According to Stewart et al. (1983) the presence of a nonmetabolized sugar increases the osmotic pressure in the medium which results in an increase in the intracellular ethanol concentration. Since intracellular ethanol has a more damaging effect on the cell than extracellular ethanol, the growth rate is adversely affected. The rate of ethanol production is reduced by less than 10% up to fructose concentrations of 222.2 g/L. Higher fructose concentrations resulted in a reduction in the ethanol production rate by 30%. Similiar reductions were noted for the glucose consumption rates. From these results it is seen that the growth rate is more strongly inhibited by increased fructose concentrations than are the glucose consumption and ethanol production rates. This difference is expected since the growth rate is generally more sensitive to inhibitory compounds than the fermentation rate (vanUden, 1985). Ethanol yields vary by less than 10% in the fructose concentration range tested.

The effect on the fermentation by ethanol is studied further in the next section.

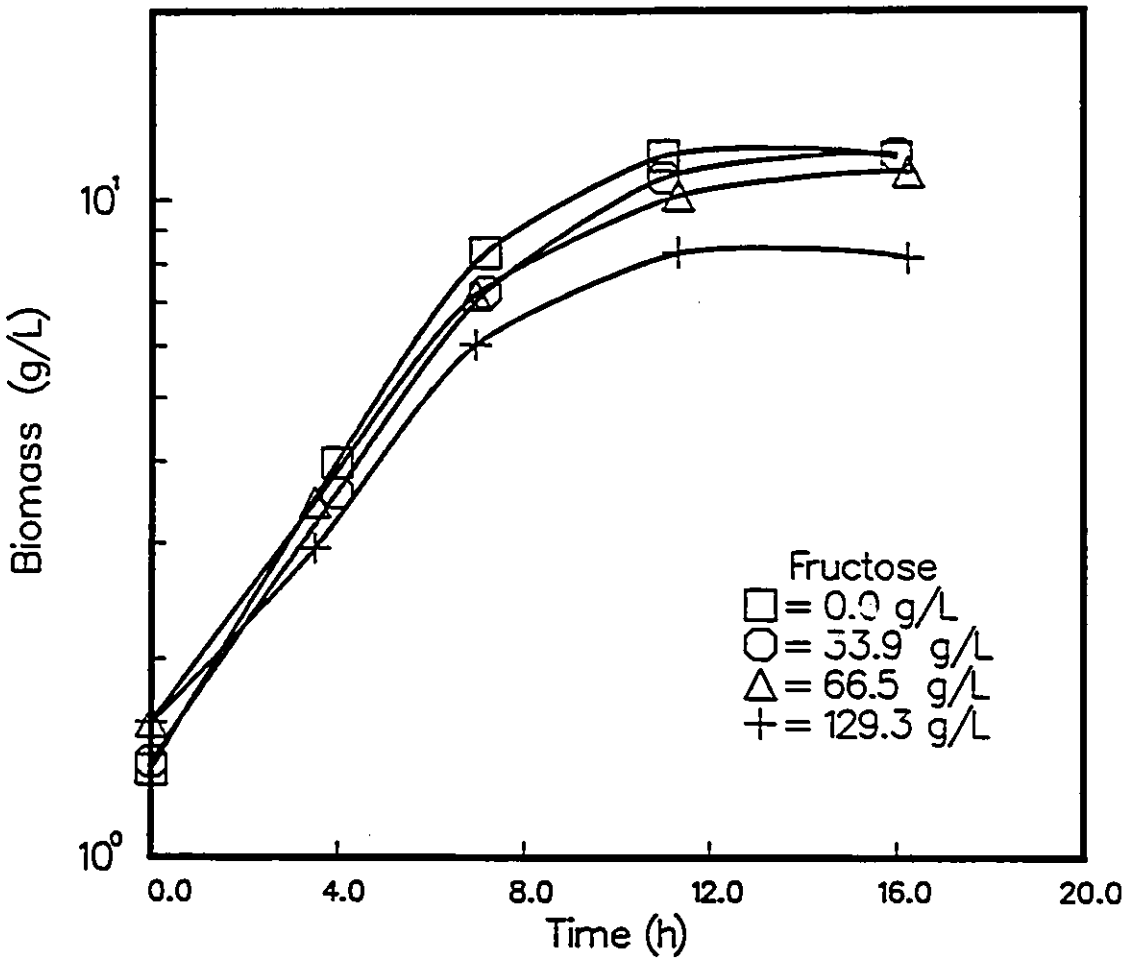

Figure 6.19: A comparison of the biomass production by the mutant strain of *S. cerevisiae* in media containing 1.5 g/L biomass, ≈65 g/L glucose and various concentrations of fructose.

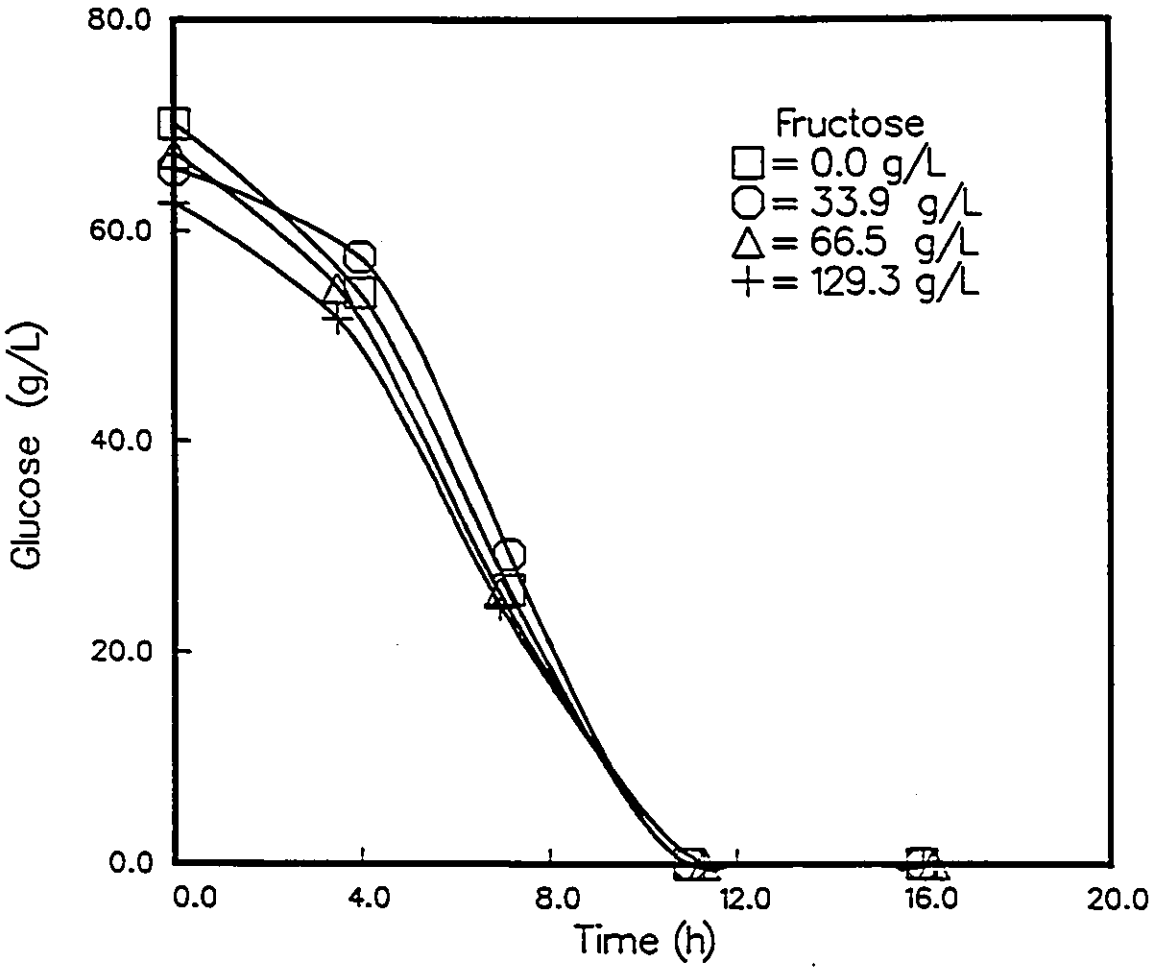

Figure 6.20: A comparison of the glucose consumption by the mutant strain of *S. cerevisiae* in media containing 1.5 g/L biomass, ≈65 g/L glucose and various concentrations of fructose.

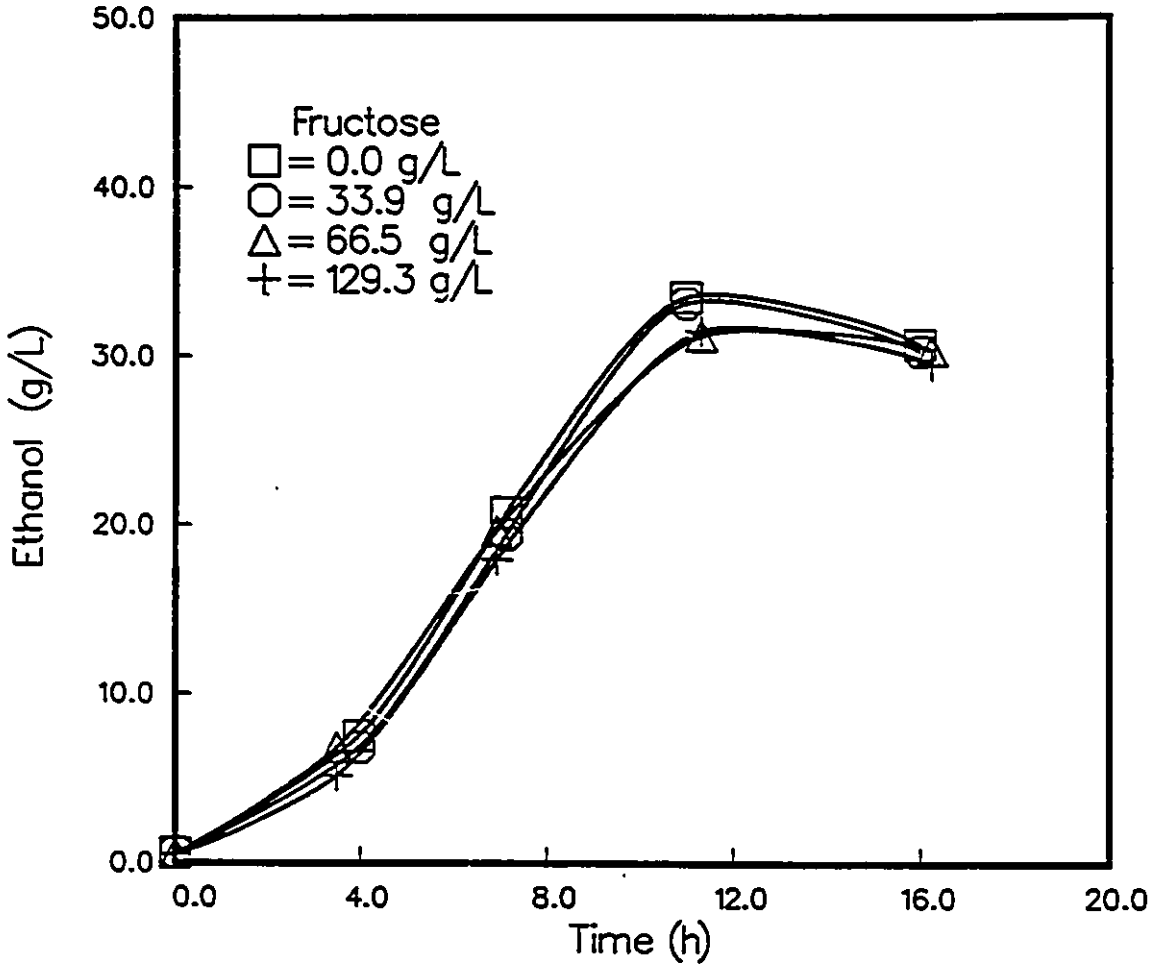

Figure 6.21: A comparison of the ethanol production by the mutant strain of *S. cerevisiae* in media containing 1.5 g/L biomass, ≈65 g/L glucose and various concentrations of fructose.

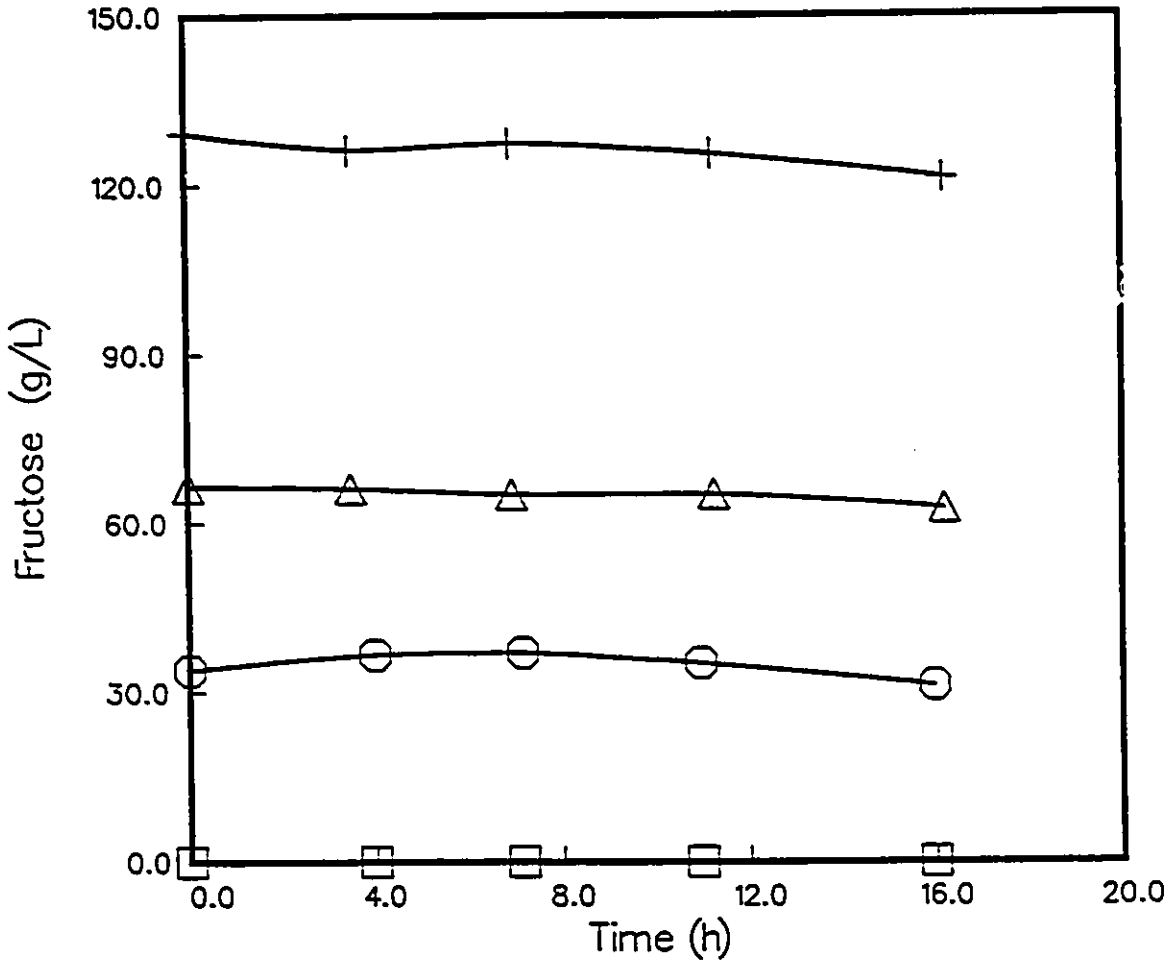

Figure 6.22: A comparison of the fructose consumption by the mutant strain of *S. cerevisiae* in media containing 1.5 g/L biomass, ≈65 g/L glucose and various concentrations of fructose.

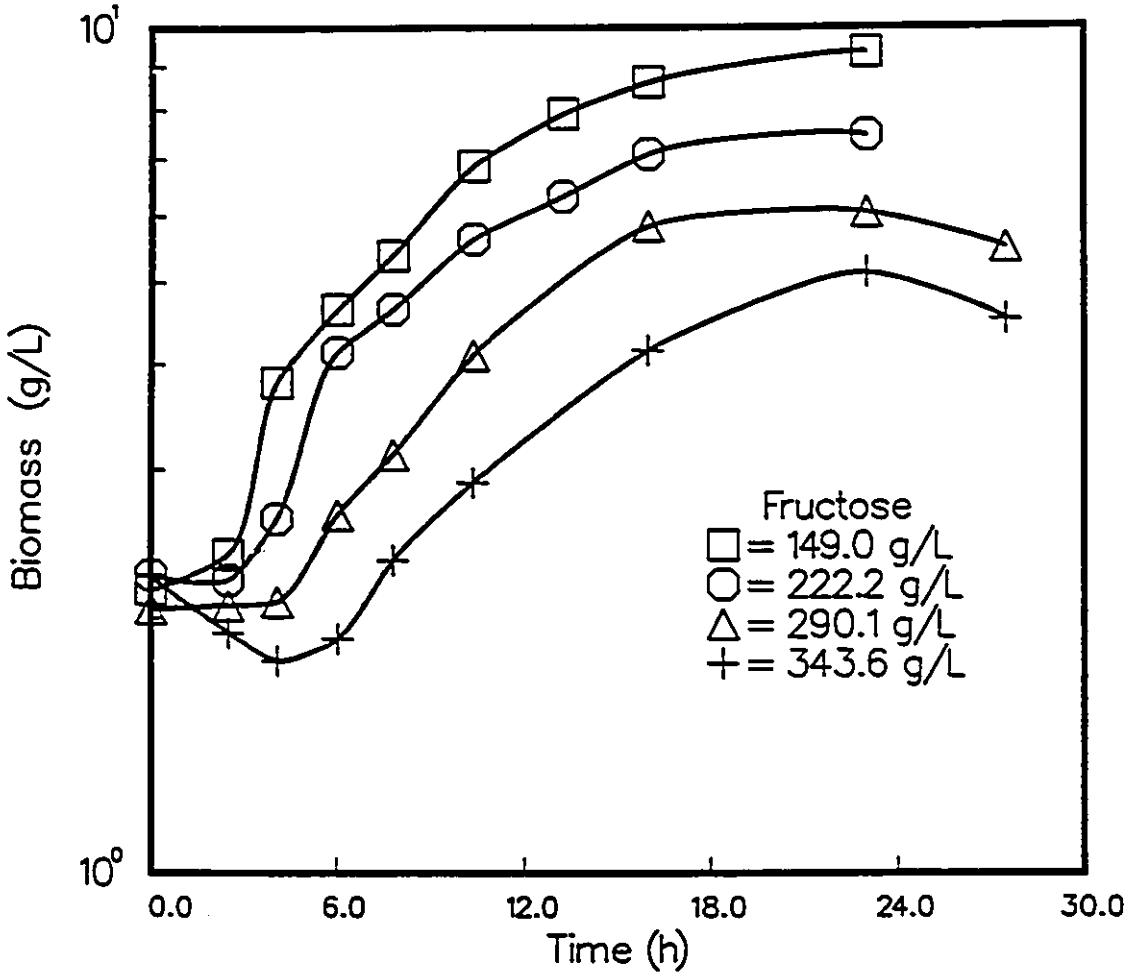

Figure 6.23: A comparison of the biomass production by the mutant strain of *S. cerevisiae* in media containing 2.1 g/L biomass. ≈65 g/L glucose and various concentrations of fructose.

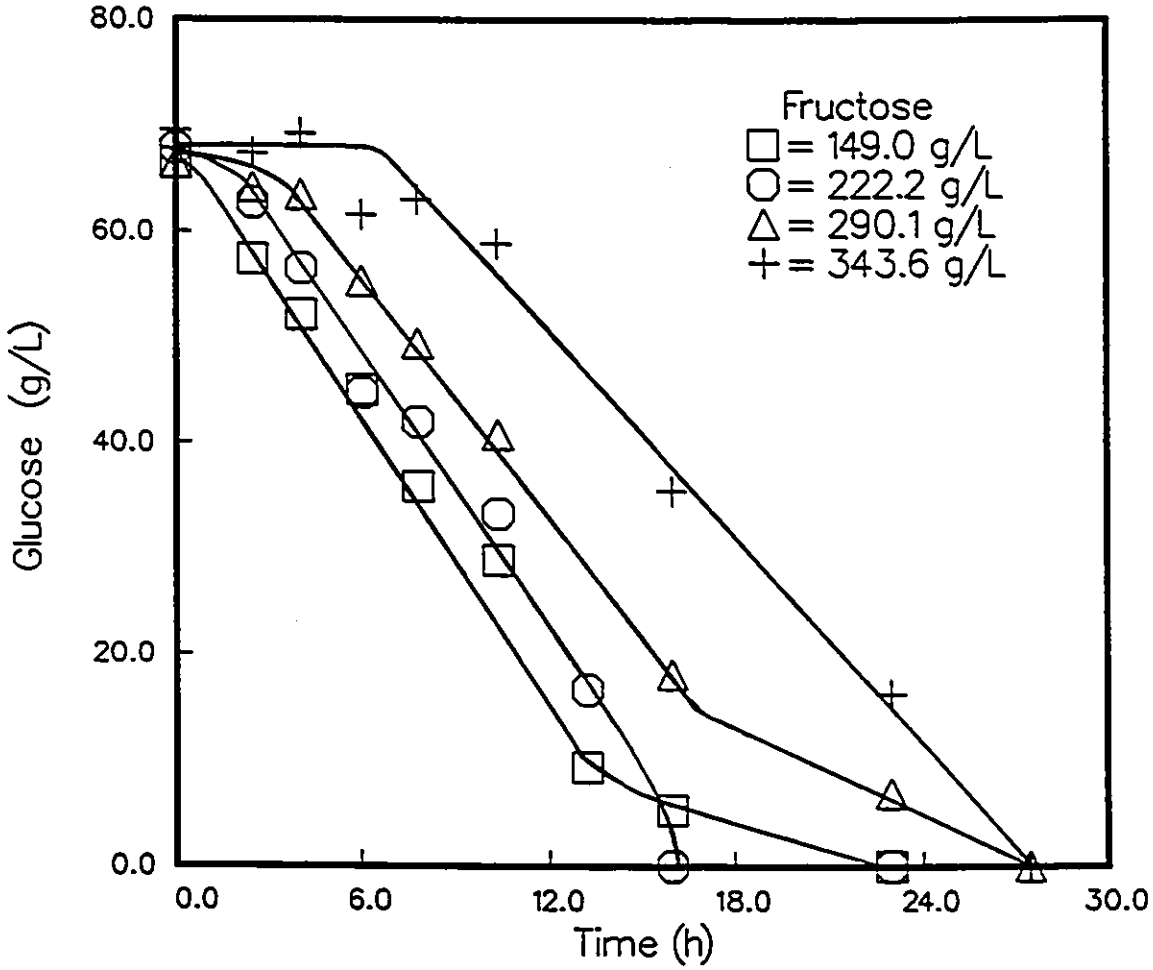

Figure 6.24: A comparison of the glucose consumption by the mutant strain of *S. cerevisiae* in media containing 2.1 g/L biomass, ≈65 g/L glucose and various concentrations of fructose.

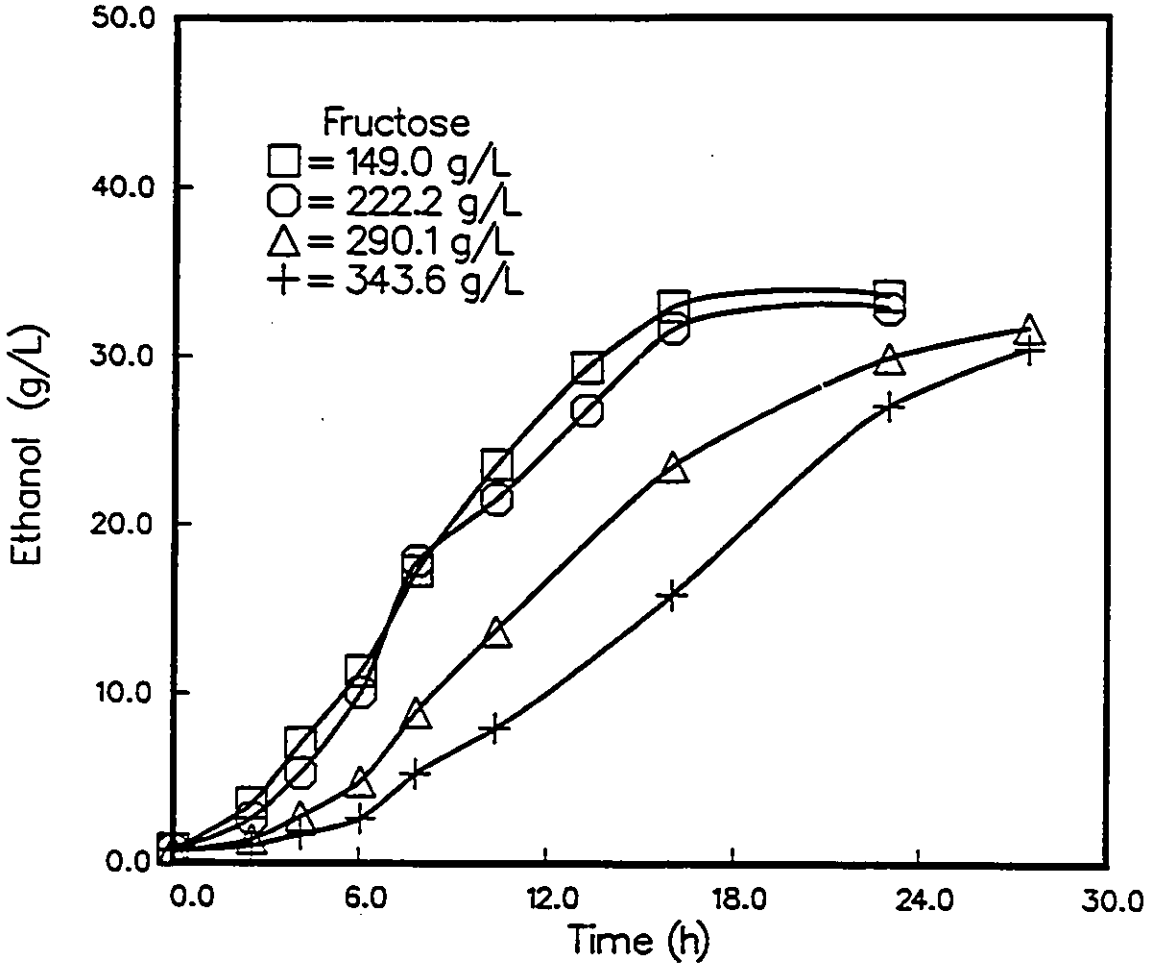

Figure 6.25: A comparison of the ethanol production by the mutant strain of *S. cerevisiae* in media containing 2.1 g/L biomass, ≈65 g/L glucose and various concentrations of fructose.

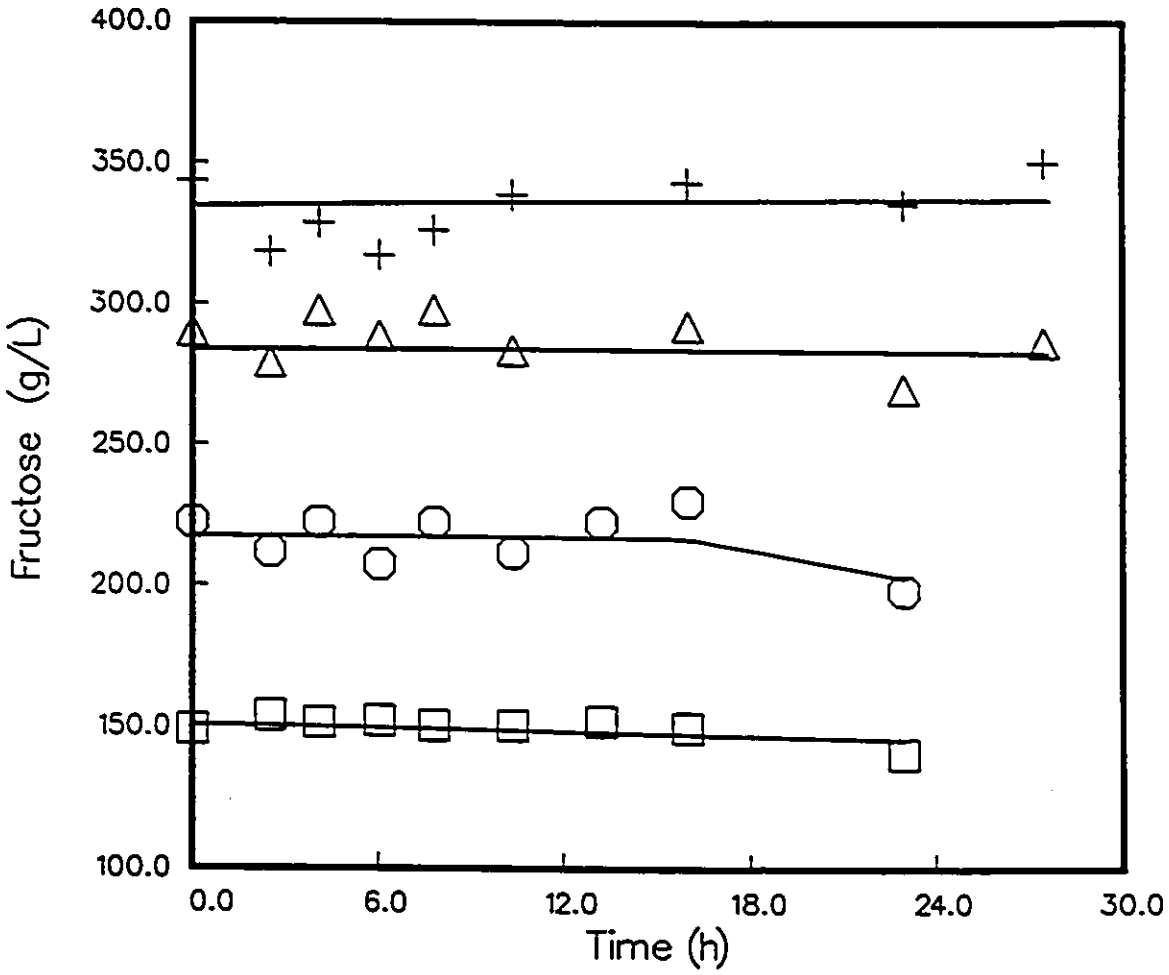

Figure 6.26: A comparison of the fructose consumption by the mutant strain of *S. cerevisiae* in media containing 2.1 g/L biomass, ≈65 g/L glucose and various concentrations of fructose.

Effect of ethanol

One of the most important limiting factors in ethanol fermentation is the effect of ethanol on the growth and fermentation by yeasts (Jones et al, 1981). The initial effects are usually most evident on the growth rate, as the ethanol concentration is increased the growth rate decreases. Since ethanol is one of the products of this process it would be advantageous to produce as high a concentration as possible. Therefore, the maximum concentration above which growth ceases is an important parameter.

The effect of ethanol on the fermentation was studied by carrying out tests with different initial ethanol concentrations. Results using media containing ≈ 90 g/L glucose, 30.0 g/L yeast extract and ethanol concentrations from 0.7 to 79.2 g/L are compared in Figures 6.27–6.29 and in Table 6.6.

Table 6.6: Biomass and ethanol yields and specific growth rates for the fermentation of glucose (≈90 g/L) in media containing various initial concentrations of ethanol and 30.0 g/L yeast extract.

Ethanol (g/L)	Glucose (g/L)	Biomass yield[a] (g/g)	Ethanol yield[a] (g/g)	Specific growth rate (h^{-1})
0.7	88.5	0.111	0.451	0.186
31.9	85.9	0.078	0.370	0.107
47.9	90.6	0.040	0.350	0.060
64.1	86.3	0.031	0.132	0.060
79.2	87.3	–	–	0.025

[a] yields calculated at maximum biomass and ethanol concentrations

The presence of ethanol in the medium reduces both the growth rate and the final biomass concentration of the yeast (Figure 6.27). When the medium contains an ethanol

concentration of 64.1 g/L or higher, little or no measurable growth takes place. The specific growth rate is reduced 68% and the biomass yield is reduced 72% in media containing 64.1 g/L ethanol compared to one containing 0.7 g/L ethanol (Table 6.6). Glucose consumption is also adversely affected as the ethanol concentration in the medium is increased (Figure 6.28). Only 17% and 8.4% of the glucose is consumed after 30 hours in media containing 64.1 g/L and 79.2 g/L ethanol respectively. The glucose that is consumed is used for cell maintenance since little or no growth is measured and no ethanol is produced in these tests. (Figure 6.29). The ethanol yield is reduced from 88 to 26% of the theoretical yield as the initial ethanol concentration is increased from 0.7 to 64.1 g/L.

The above results were obtained by adding ethanol to the medium. It has been found though that intracellular ethanol concentrations have a more damaging effect on the cell than that which exists in the bulk solution (Nagodawithana, 1976). Therefore, it would be more informative to study the inhibitory effects of ethanol that is produced by the yeast itself. It has been reported (Stewart et al., 1983) that the addition of extracellular ethanol up to 138 g/L was less damaging than 94 g/L ethanol produced by the cell during fermentation. The effect of ethanol produced by the cell is accounted for in a later section by formulating rate expressions for growth and ethanol formation which take into account inhibition due to the ethanol produced by the yeast. Before this can be done though, the relationships between the growth rate and the yeast extract, glucose, fructose and ethanol concentrations must be quantitated in the form of mathematical expresssions.

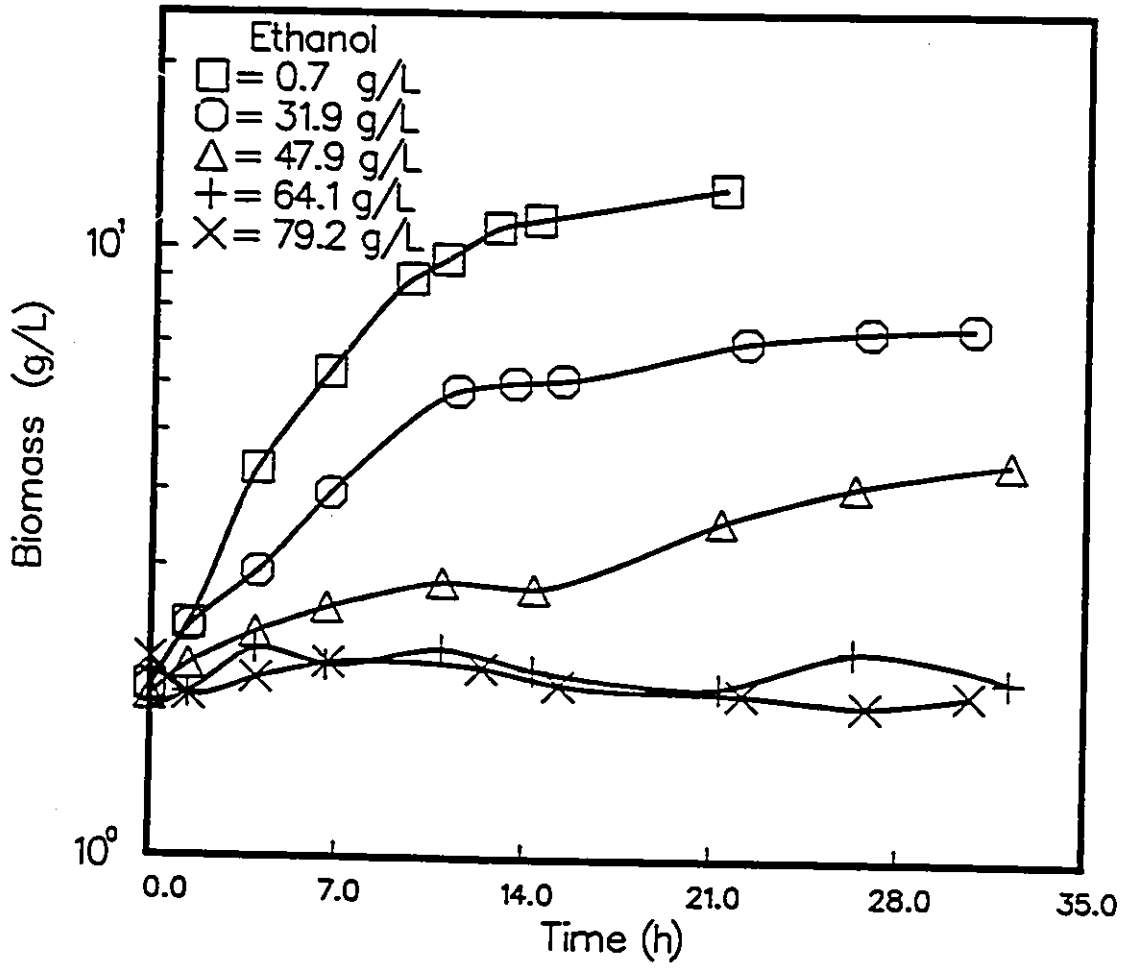

Figure 6.27: A comparison of the biomass production by the mutant strain of *S. cerevisiae* in media containing various initial ethanol concentrations and ≈87 g/L glucose.

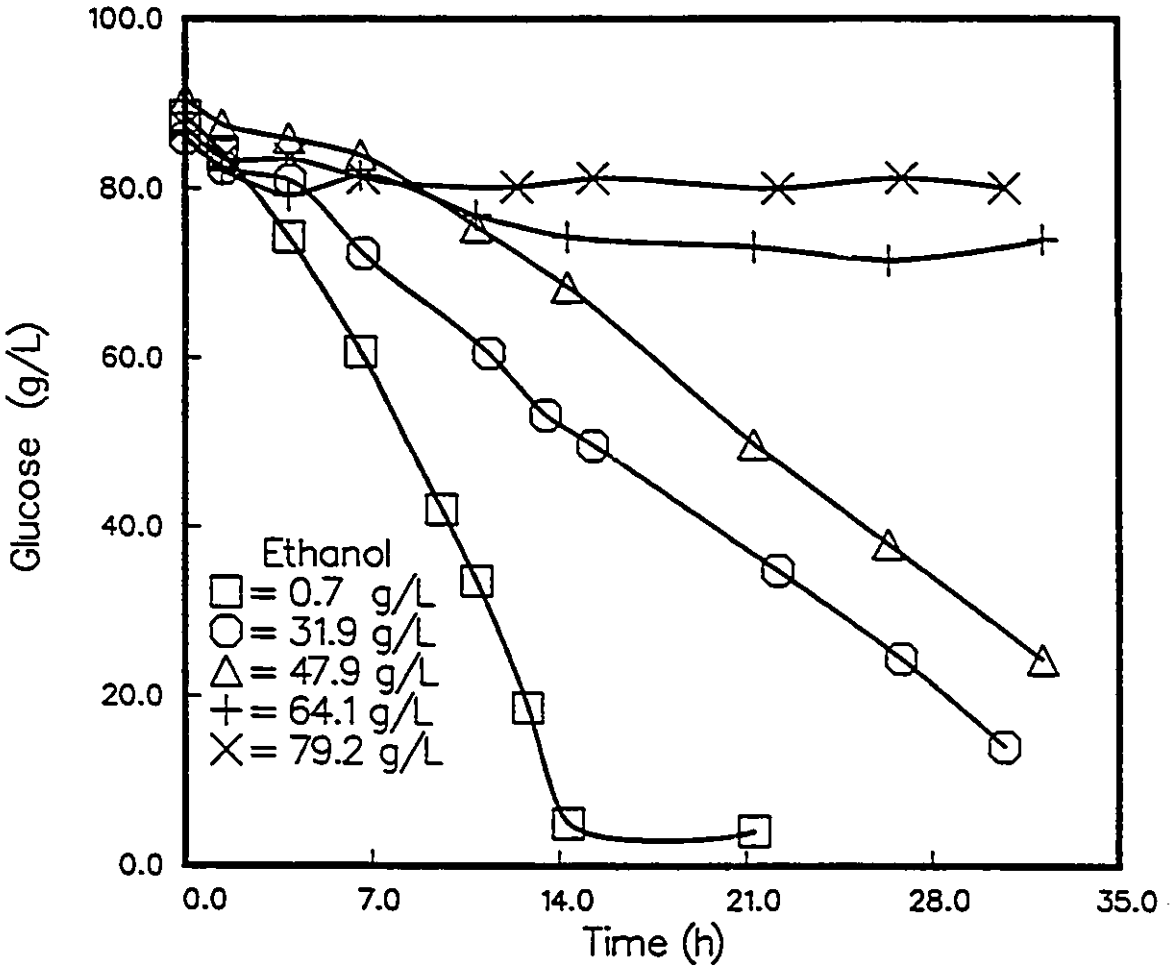

Figure 6.28: A comparison of the glucose consumption by the mutant strain of *S. cerevisiae* in media containing various initial ethanol concentrations and ≈87 g/L glucose.

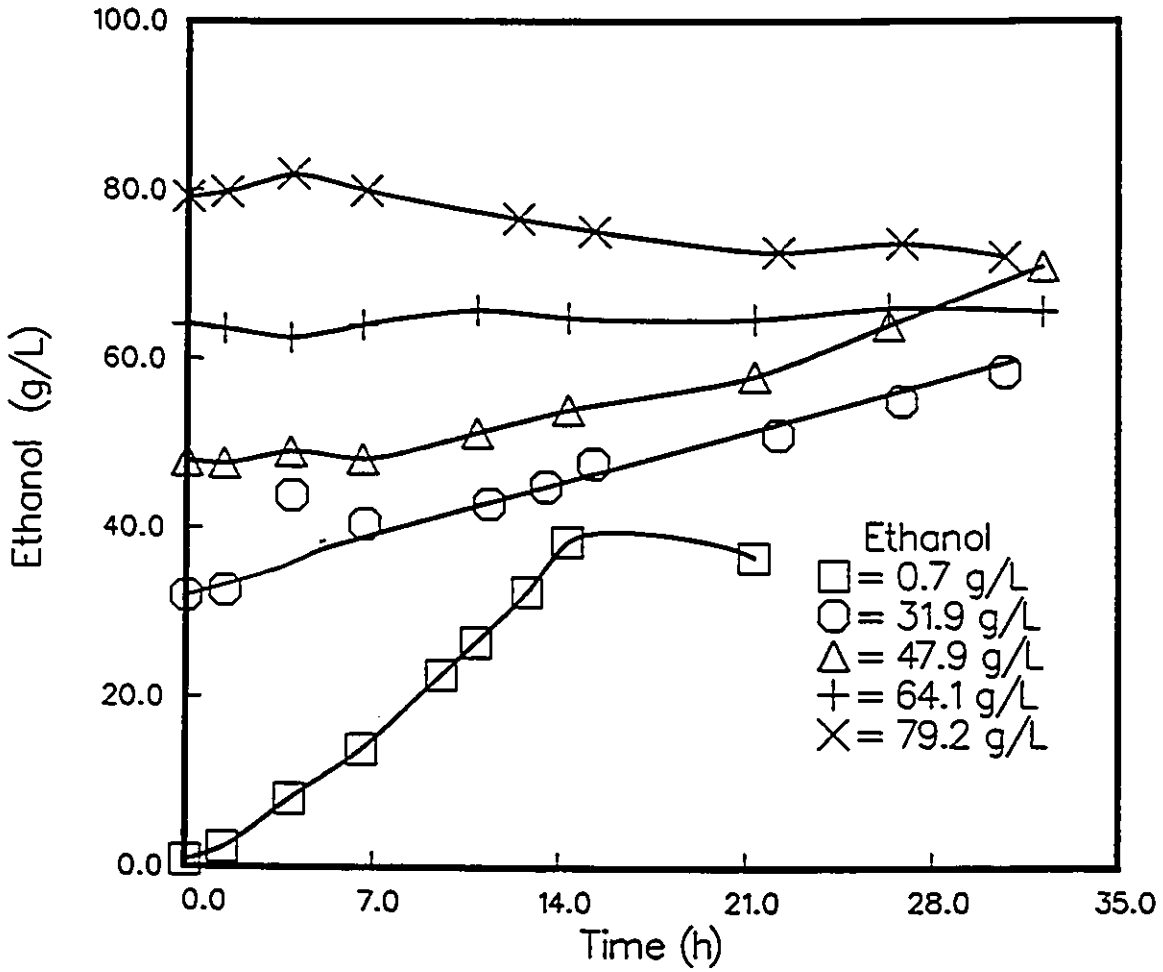

Figure 6.29: A comparison of the ethanol production by the mutant strain of *S. cerevisiae* in media containing various initial ethanol concentrations and ≈87 g/L glucose.

6.3 Analysis and Modelling of Batch Fermentations

To describe the relationship between the parameters just described, more tests were carried out and models were formulated. First, models for the growth rate were formulated. Once the growth rate was described as a function of the important variables (using an integral analysis of the data) the expression could then be combined with biomass and ethanol yields to predict the dynamic behaviour of the fermentation.

6.3.1 Integral analysis

Specific growth rate

Effect of carbohydrate concentration The specific growth rates of experiments carried out in either glucose or glucose/fructose media containing 3.0 or 30.0 g/L yeast extract are shown in Figure 6.30. Some data reported by Lamarche (1988) in media containing 3.0 g/L yeast extract are also shown. It is evident from the figure that an increase in the total carbohydrate concentration (glucose+fructose) results in a reduction in the specific growth rate, irrespective of the yeast extract concentration. Even though fructose is consumed only minimally by the yeast when glucose is present, the increase in the total carbohydrate concentration due to its presence results in a reduction in the specific growth rate.

As expected a higher yeast extract concentration (30.0 g/L) results in higher specific growth rates (Figure 6.30). An increase in the total carbohydrate concentration (glucose or glucose+fructose) resulted in a reduction in the specific growth rate for both yeast extract concentrations. The relationship between the specific growth rate and the total carbohydrate concentration appears to be linear, which is the same type of relationship that was found by Jones and Greenfield (1986; 1987). They proposed that inhibition of the growth is due to the reduction in the water activity caused by higher total carbohydate concentrations. To model these results the Monod model (Eq. 3.1) was used with an additional term to account for the inhibitory effects of the total carbohydrate concentration. The model proposed here was

$$\mu = \mu_{max}\left(\frac{s_g}{K_s + s_g}\right)(1 - B(s_g) - C(s_f)) \tag{6.1}$$

where s_g is the glucose concentration, s_f is the fructose concentration and B and C are empirical constants. This model tested whether it was necessary to treat the inhibition due to increased glucose and fructose concentrations separately. When the data were fitted to this equation the 95% confidence interval for K_s included zero. Since K_s should be positive and non-zero it will be assumed that it is too small to be accurately determined in this analysis. Therefore with the assumption that $K_s \ll s_g$, the model becomes

$$\mu = \mu_{max}(1 - B(s_g) - C(s_f)) \tag{6.2}$$

The values of the parameters μ_{max}, B and C and their respective 95% confidence levels when the data are fitted to Eq. 6.2. are indicated in Table 6.7.

Table 6.7: Converged parameter estimates for Eq. 6.2

	3.0 g/L yeast extract	30.0 g/L yeast extract
μ_{max} (±) h^{-1}	0.135 (0.016)	0.249 (0.020)
B (±) L/g	$1.86\times10^{-3}(6.74\times10^{-4})$	$1.57\times10^{-3}(6.47\times10^{-4})$
C (±) L/g	$1.79\times10^{-3}(2.76\times10^{-4})$	$1.68\times10^{-3}(2.31\times10^{-4})$

The parameter estimates (Table 6.7) suggest that the inhibition due to glucose (B) and that due to fructose (C) could be equal under the conditions tested. A simplified model could then be formulated between the specific growth rate (μ) and the total carbohydrate concentration (s_{tc}). It would also be useful to transform the linear model into the form suggested by Ghose and Tyagi (1979b) for ethanol inhibition (Eq. 3.20). The transformed model proposed here is

$$\mu = \mu_{max}\left(1 - \frac{s_{tc}}{s_{max}}\right) \tag{6.3}$$

where s_{max} is the maximum total carbohydrate concentration above which growth no longer takes place. The parameters μ_{max} and s_{max} obtained by fitting the above equation are given in Table 6.8. The fitted equation is shown in Figure 6.30 as the solid lines. The model was tested for its adequacy by comparing a pooled estimate of the pure error variance (Appendix A) with the residual sum of squares (Draper and Smith, 1966). The test indicates that any inadequacy in the fitted line is not significantly larger than the experimental error.

Table 6.8: Converged parameter estimates for Eq. 6.3

	3.0 g/L yeast extract	30.0 g/L yeast extract
μ_{max} (±) h^{-1}	0.134 (0.012)	0.251 (0.013)
s_{max} (±) g/L	554.1 (65.4)	602.8 (66.0)

The parameter estimates indicate that the specific growth rate will be equal to zero when the carbohydrate concentration is 554 and 603 g/L in media containing 3.0 and 30.0 g/L yeast extract respectively. When the confidence intervals are taken into consideration, the values obtained for s_{max} in media containing 3.0 and 30.0 g/L yeast extract could be equal. Thus the total carbohydrate concentration above which growth ceases is not dependent on the yeast extract concentration. On the other hand, the yeast extract concentration does affect the value of μ_{max} (Table 6.8).

Other models referred to in Section 3.2.3 were also fitted to the data. Of those tested only the model proposed by Aiba et al. (1968) fit the data adequately. It is shown on Figure 6.30 as the dashed line. Both models predict the data equally well at low and intermediate carbohydrate concentrations. At high concentrations the linear model proposed here (Eq. 6.3) predicts that the growth will stop at ≈560 g/L, which is more realistic than the assymptotic approach to zero predicted by Aiba's model.

The models relating the specific growth rate and the concentration of yeast extract are derived in the next section.

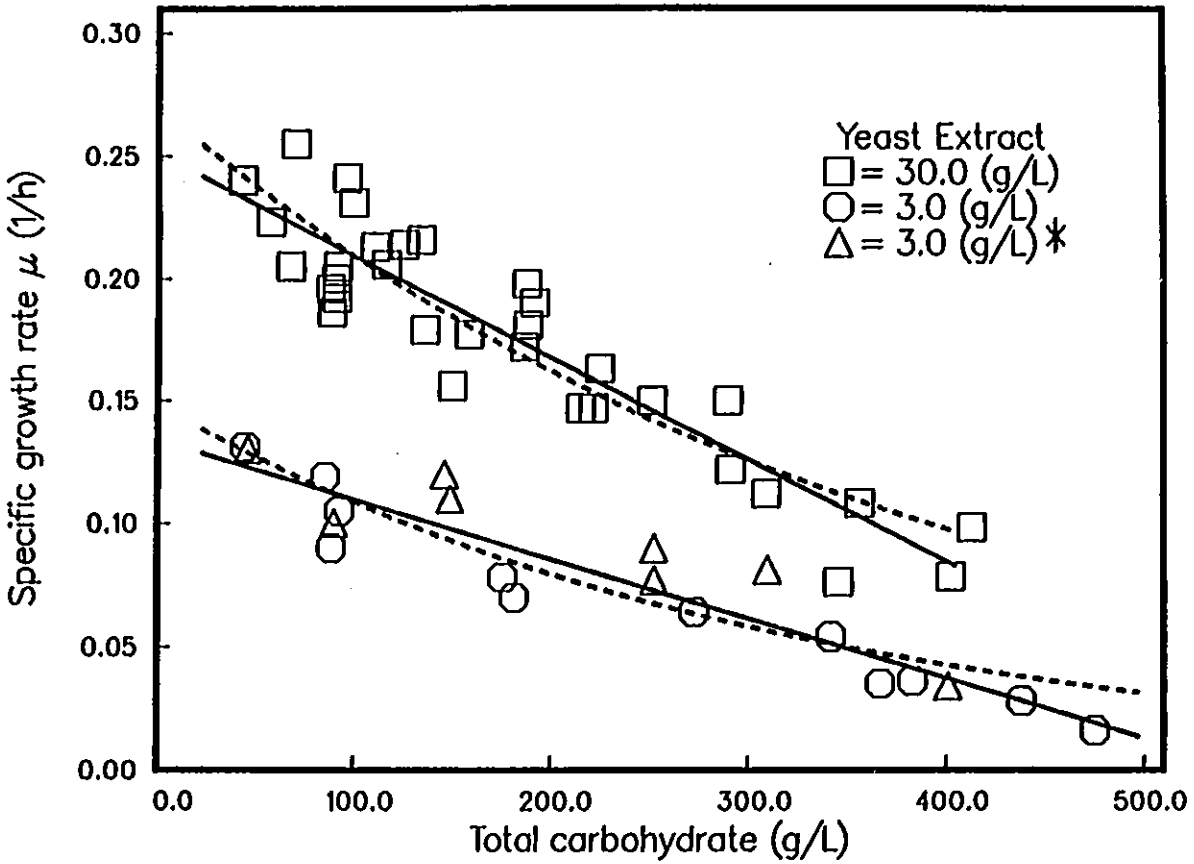

Figure 6.30: Specific growth rate as a function of total carbohydrate concentration. (—) Proposed model (Eq. 6.3). (- - -) Aiba model (Eq. 3.18). * Results obtained from Lamarche (1988).

Effect of yeast extract Yeast extract primarily serves as a source of nitrogen for the yeast. It is also rich in proteins and important vitamins (Cejka, 1985). Due to the importance of these compounds for the growth of the yeast, the effect of yeast extract on the specific growth rate is studied here.

Experiments were carried out at yeast extract concentrations between 3.0 and 30.0 g/L in media containing $\approx$ 85 g/L glucose. The specific growth rates are plotted as a function of the yeast extract concentration in Figure 6.31. The data show an increase in μ as the yeast extract concentration is increased. The relationship between these two variables is modelled using Monod's expression (Eq. 3.1) with the concentration of yeast extract as the limiting substrate, it is shown as the dashed line in Figure 6.31. It is seen that this model overpredicts the growth rate at intermediate concentrations of yeast extract. The solid line is a simple linear model which is seen to fit the data over the whole yeast extract concentration range tested. The model that is proposed here is

$$\mu = D + E(s_{ye}) \tag{6.4}$$

where s_{ye} is the yeast extract concentration and D and E are empirical constants. When the data are fitted to Eq. 6.4 the converged parameter estimates obtained for D and E are 9.47 $\times 10^{-2}$ ($\pm$ 1.35 $\times 10^{-2}$) h^{-1} and 3.56 $\times 10^{-3}$ ($\pm$ 6.34 $\times 10^{-4}$) L/gh respectively. In order to test the adequacy of the model the pure error variance calculated by pooling individual variance estimates (Appendix A) was compared to the sum of squares of residuals from the model. It was found that any inadequacy in the model is not significantly larger than the experimental error.

The linear relationship between the specific growth rate and the yeast extract concentration (Eq. 6.4) can be combined with the previous relationship obtained for the total carbohydrate concentration (Eq. 6.3). The combined expression

$$\mu = (D + E(s_{ye}))\left(1 - \frac{s_{tc}}{s_{max}}\right) \tag{6.5}$$

would be capable of modelling the effect of the yeast extract and the total carbohydrate concentration on the specific growth rate. When this model is fitted to the data, the

parameter estimates obtained are D = 0.115 (± 0.011) h^{-1}, E = 4.57 × 10^{-3} (± 4.75 × 10^{-4}) g/L and s_{max} = 585.5 (± 42.8) g/L.

The experimental versus predicted values of the specific growth rates are shown in Figure 6.32. For this analysis 66 experimental data points were used, these were tests carried out in media containing yeast extract (3.0 to 30.0 g/L) and glucose (43 to 253 g/L) or glucose and fructose (0 to 432 g/L). The inadequacy of the model was not significantly larger than the experimental error.

The model can predict the specific growth rate of the yeast at different carbohydrate and yeast extract concentrations. The relationship between the specific growth rate and the initial ethanol concentration is formulated in the next section.

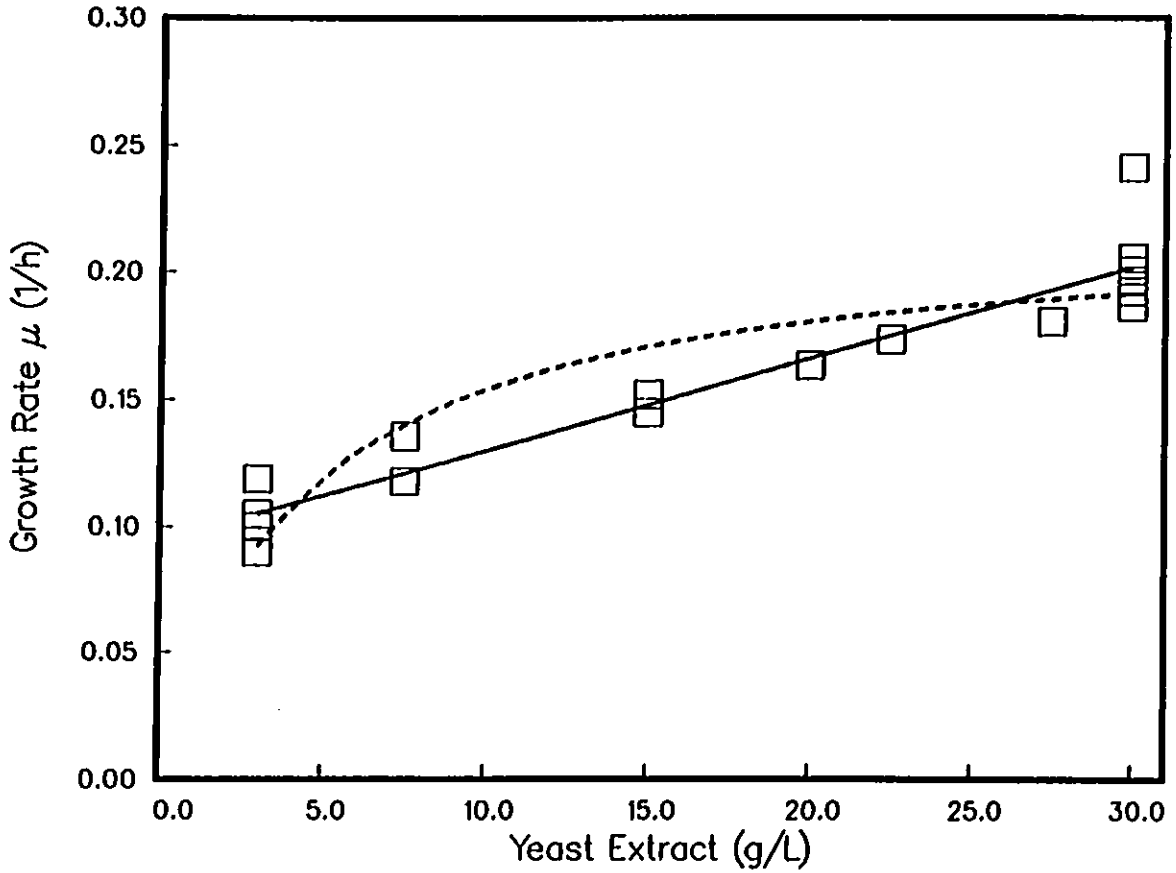

Figure 6.31: Specific growth rate as a function of yeast extract concentration in media containing ≈90 g/L glucose. (—) Eq. 6.4. (- - -) Monod model (Eq. 3.1).

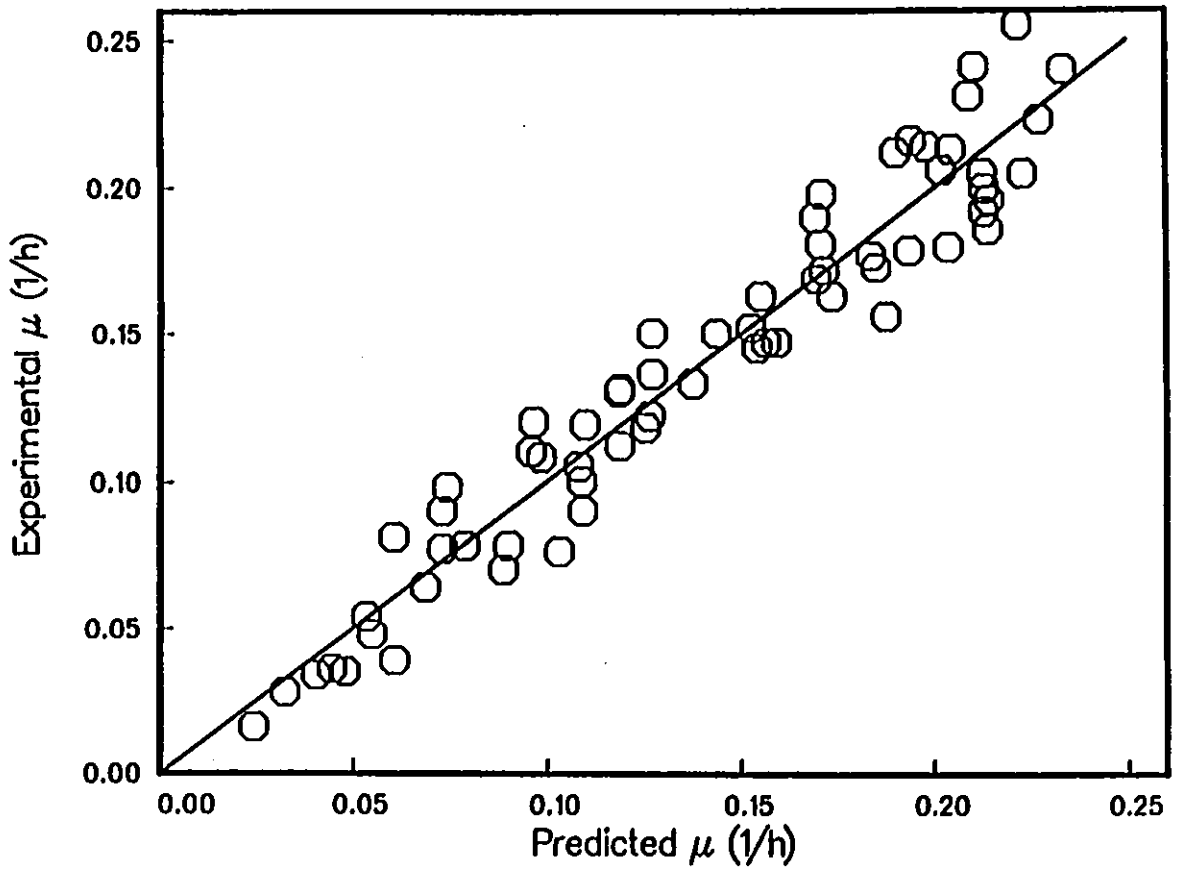

Figure 6.32: Experimental versus predicted specific growth rates from Eq. 6.5. The media contained 3-30 g/L yeast extract, 40-250 g/L glucose or glucose and 0-430 g/L fructose.

Effect of ethanol In this section the relationship between the specific growth rate and the ethanol concentration will be described quantitatively. Many researchers have studied the problem of ethanol inhibition using the wild strain of *S. cerevisiae*. These studies have yielded a large number of models which serve as a starting point for the work carried out here.

The specific growth rates were measured for a series of batch experiments with media containing 90 g/L glucose, 30 g/L yeast extract and various initial ethanol concentrations. The experimentally determined specific growth rates are plotted in Figure 6.33 as a function of the initial ethanol concentration. The growth rates were calculated when the yeast were in the initial exponential growth phase where additional ethanol produced by the yeast was minimal (less than 10 g/L).

Equations proposed by Ghose and Tyagi (Eq. 3.20), Aiba et al. (Eq. 3.18) and Jerusalimsky and Neronova (Eq. 3.19) were fitted to the data assuming that $K_s \ll s_g$. The only equations which adequately described the data were those formulated by Ghose and Tyagi (Eq. 3.20) and Aiba et al. (Eq. 3.18), these are shown in Figure 6.33 as the solid and dashed lines respectively. Both models predict the experimental data equally well at low and intermediate ethanol concentrations. The solid line implies that little or no growth will occur above 80 g/L ethanol while the dashed line predicts growth even above 90 g/L. Since the growth rate of the yeast reduces to zero at ≈90 g/L (Lamarche, 1988), the linear model is more realistic. The equation of this line is

$$\mu = \mu_{max}\left(1 - \frac{p}{p_{max}}\right) \tag{6.6}$$

where p is the ethanol concentration and p_{max} is the ethanol concentration at which the growth rate is zero. The parameter values obtained here are $\mu_{max} = 0.188 \pm 0.019$ (h^{-1}) and $p_{max} = 87.5 \pm 11.8$ (g/L). This model predicts little or no growth above 87.5 g/L ethanol which agrees with the data (Figure 6.33).

As was mentioned previously, these results were obtained by adding ethanol to the medium. The maximum concentration at which growth stops due to ethanol that is produced by the yeast is expected to be lower, as was found by Stewart et al. (1983).

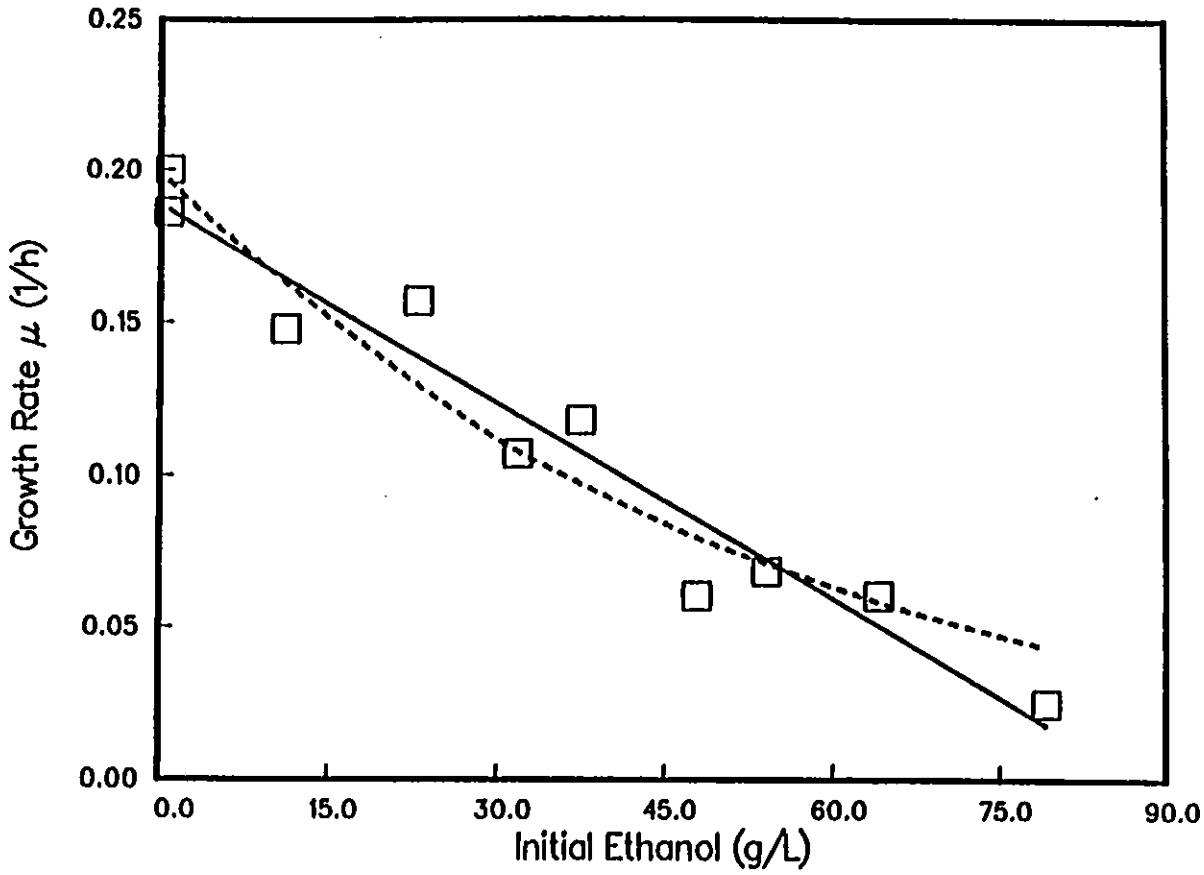

Figure 6.33: Inhibition of growth by ethanol. (—) Ghose and Tyagi (Eq. 3.20). (- - -) Aiba model (Eq. 3.18).

Biomass and ethanol yields

The biomass yields from experiments carried out in media initially containing 3 and 30 g/L yeast extract and various concentrations of glucose (43 to 253 g/L) or glucose (41 to 193 g/L) and fructose (43 to 432 g/L) are shown in Figure 6.34 as a function of initial total carbohydrate concentration. The results show that the biomass yield decreases as the initial total carbohydrate concentration is increased in media containing 30 g/L yeast extract. The trend is not so evident for the experiments carried out in media containing 3 g/L yeast extract. The linear dependency suggests that equations of the form of Eq. 6.2 could be fitted to the data. The equation derived for this relationship is

$$Y_{x/s} = Y'_{x/s}(1 - F(s_g) - G(s_f)) \tag{6.7}$$

where $Y'_{x/s}$ is the biomass yield as the carbohydrate concentration approaches zero and F and G are empirical constants. The values of the parameters and their respective 95% confidence levels are given in Table 6.9.

Table 6.9: Converged parameter estimates for Eq. 6.7.

	3.0 g/L yeast extract	30.0 g/L yeast extract
$Y'_{x/s}(\pm)$g/g	0.053 (0.010)	0.187 (0.015)
$F(\pm)$L/g	$3.87 \times 10^{-3}(7.02 \times 10^{-4})$	$3.53 \times 10^{-3}(5.09 \times 10^{-4})$
$G(\pm)$L/g	$7.22 \times 10^{-5}(6.48 \times 10^{-4})$	$1.60 \times 10^{-3}(2.21 \times 10^{-4})$

The results from this analysis indicate that an increase in the glucose concentration results in a reduction in the biomass yield; this reduction, F, is similar for both yeast extract concentrations. This behaviour is expected due to the repression of the oxidative metabolism that occurs at high substrate concentrations (Lehninger, 1982). In media containing 3.0 g/L yeast extract, the fructose concentration does not significantly affect the biomass yield (the value of the parameter G could be zero). When the media contains 30.0 g/L yeast extract, increases in the fructose concentration do not affect the biomass yield as strongly as do

increases in the glucose concentration (i.e. F> G). Therefore, unlike the growth rate which is affected equally by increases in the glucose and fructose concentrations, the biomass yield is more strongly affected by the glucose concentration than the fructose concentration. The values of experimental versus predicted biomass yields are shown in Figure 6.35. The predicted biomass yields are in good agreement with the experimentally measured ones.

The experimentally measured ethanol yields are plotted as a function of the initial total carbohydrate concentration in Figure 6.36. From the results in the figure it appears that the ethanol yield ($Y_{p/s}$) is independent of the total carbohydrate concentration. An equation similar to that used for the biomass yield was used to model the ethanol yields. The equation proposed was

$$Y_{p/s} = Y'_{p/s}(1 - H(s_g) - J(s_f)) \tag{6.8}$$

where $Y'_{p/s}$ is the ethanol yield as the total carbohydrate concentration approaches zero and H and J are empirical constants. The fitted parameter estimates (Table 6.10) indicate that a value of zero for H is possible, this implies that the glucose concentration does not significantly affect the ethanol yield in the concentration range tested (40 to 250 g/L). This was also found by Margaritis and Bajpai (1983) and Rogers et al. (1979).

Table 6.10: Converged parameter estimates for Eq. 6.8

	3.0 g/L yeast extract	30.0 g/L yeast extract
$Y'_{p/s}(\pm)$ g/g	0.467 (0.068)	0.426 (0.048)
$H(\pm)$ L/g	$1.49 \times 10^{-4}(9.94 \times 10^{-4})$	$8.56 \times 10^{-4}(1.14 \times 10^{-3})$
$J(\pm)$L/g	$-4.88 \times 10^{-4}(3.65 \times 10^{-4})$	$-3.60 \times 10^{-4}(3.38 \times 10^{-4})$

The parameter estimates obtained for J suggest that the fructose concentration affects the ethanol yield only minimally. In fact, when the residual sums of squares with and without the fructose term are compared, the difference is not significant. It was also found that

there was no significant difference between the ethanol yields measured from experiments carried out with 3.0 and 30.0 g/L yeast extract. An ethanol yield of 0.450 ($\pm$ 0.013) was found to fit all of the experimental data adequately. The solid line in Figure 6.36 represents this value.

From the results presented it is evident that increases in the glucose, fructose and ethanol concentrations reduce the growth rate of the yeast. This is due in part to the increased osmotic pressure resulting from an increase in the concentrations of these components. The inhibitory effect of glucose and fructose are identical, a finding that was also reported by Jones and Greenfield (1986; 1987) using a wild strain of *S. cerevisiae*. The inhibitory effects modelled in this section will be used along with the Monod model and the biomass and ethanol yields to formulate rate equations for the biomass, glucose, fructose and ethanol concentrations. These models will then be fitted to some of the experimental data in order to determine their applicability as well as estimates of the parameters. In this analysis, only experiments carried out with media containing 30 g/L yeast extract will be analyzed. Data collected with 3 g/L yeast extract are not analyzed since the growth rates in these media were too low for consideration in any future process.

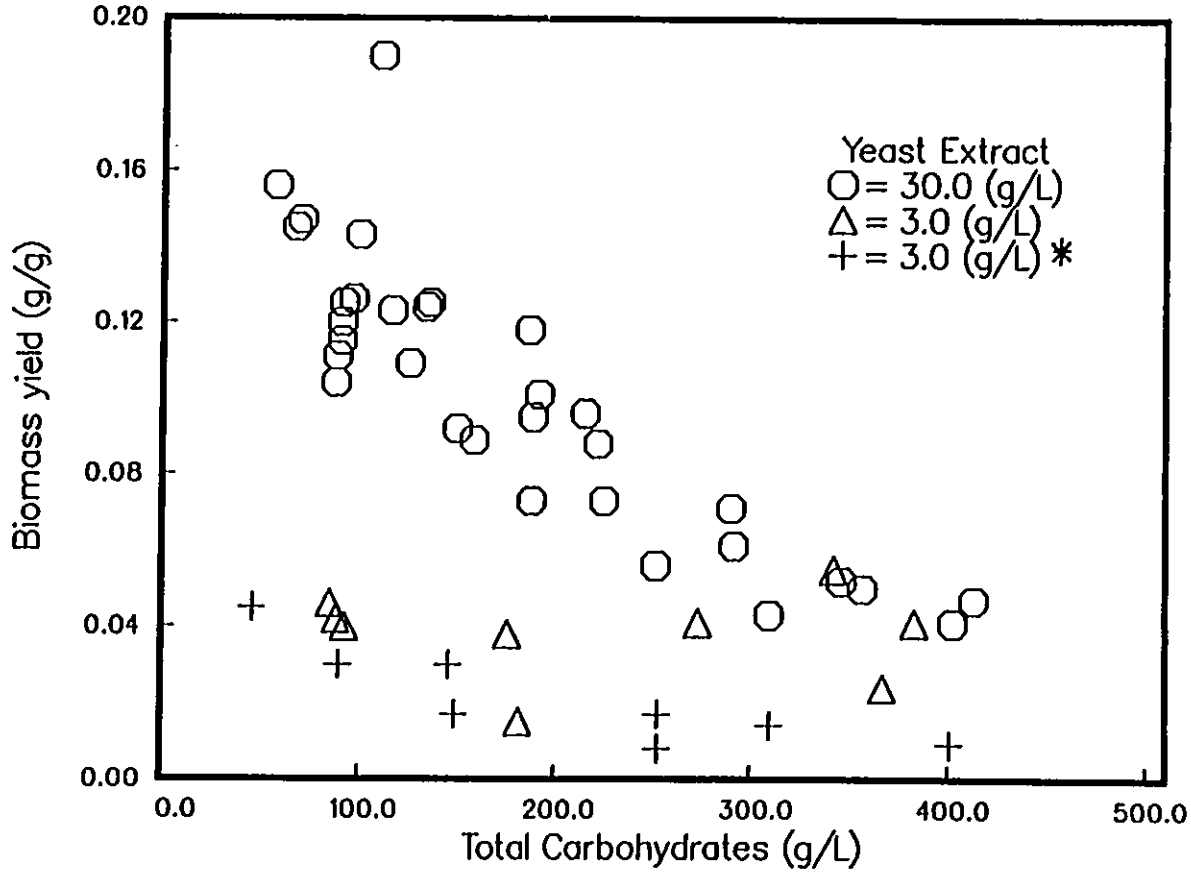

Figure 6.34: Biomass yield as a function of total carbohydrate concentration in various glucose and glucose/fructose mixtures. *Results obtained from Lamarche (1988).

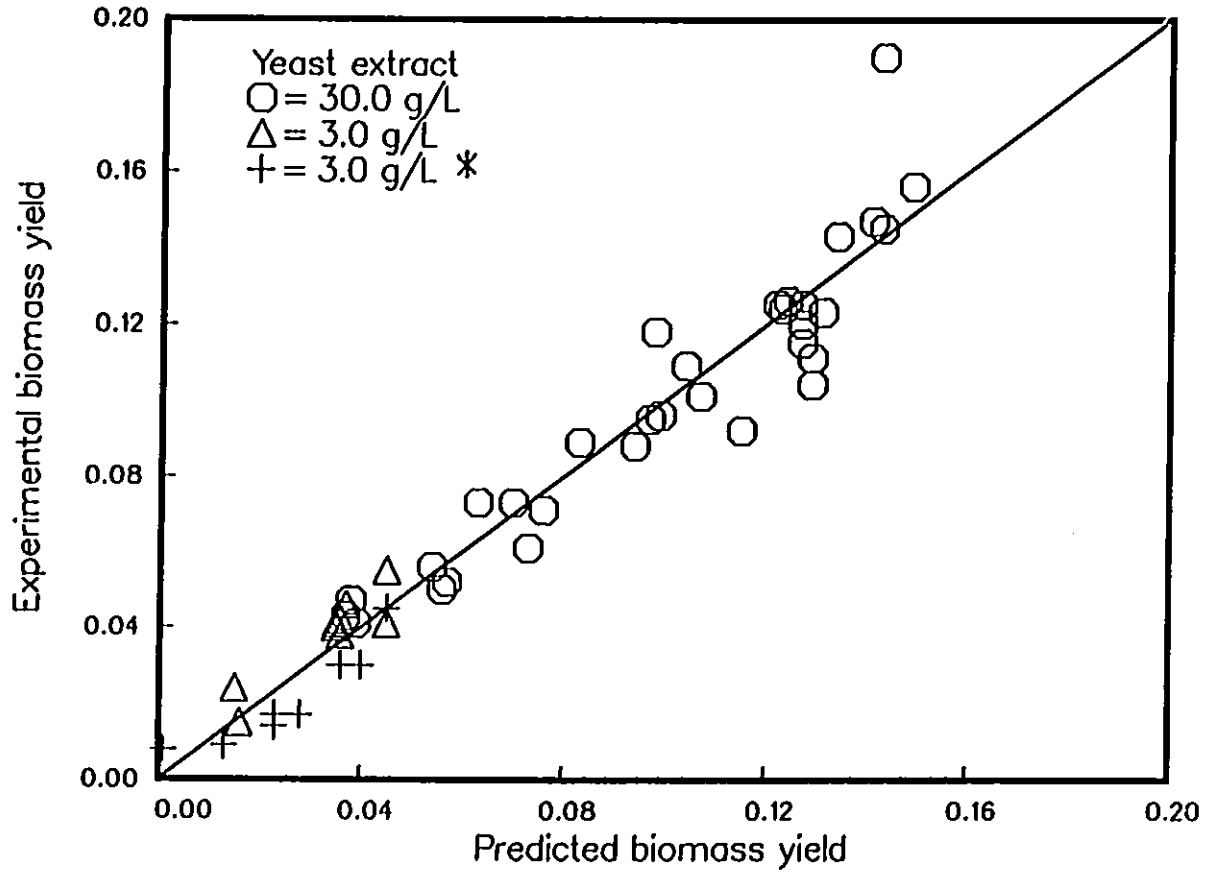

Figure 6.35: Experimental versus predicted biomass yields. *Results obtained from Lamarche (1988).

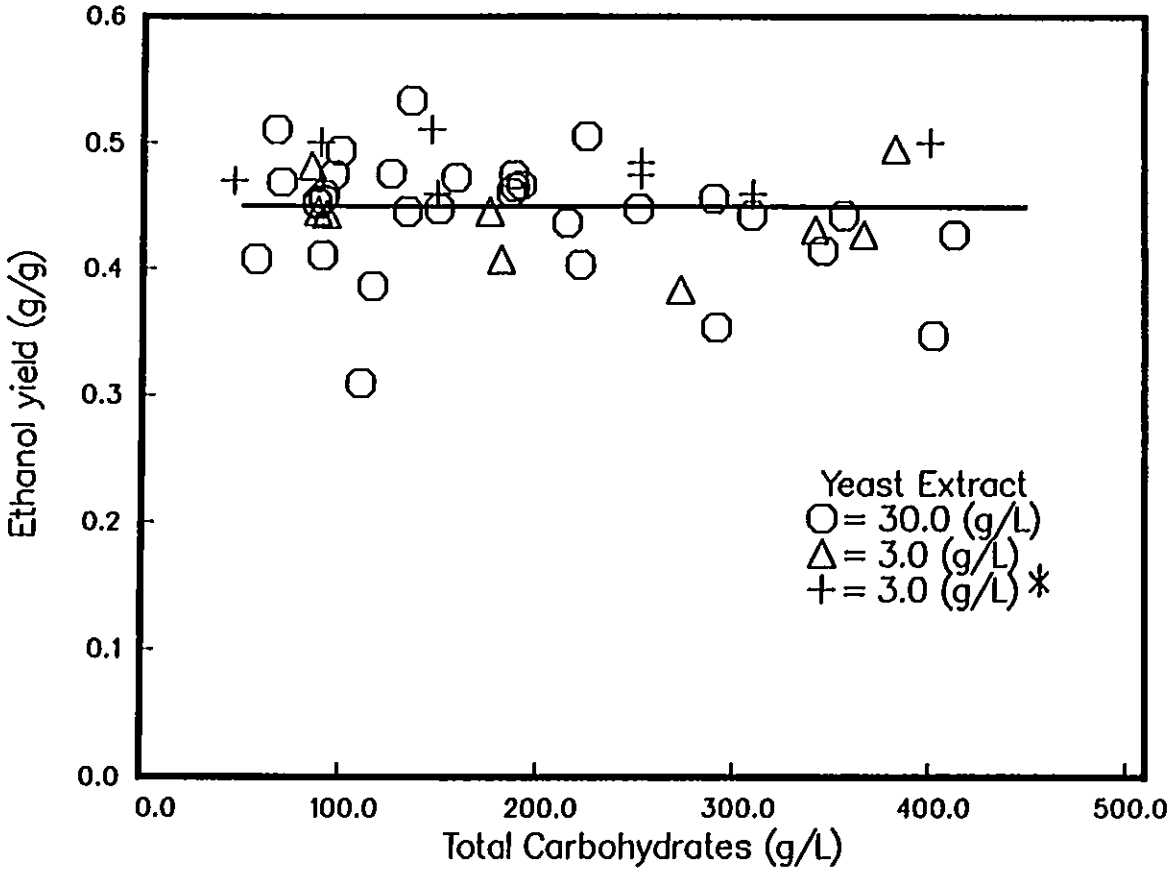

Figure 6.36: Ethanol yield as a function of total carbohydrate concentration in various glucose and glucose/fructose mixtures. *Results obtained from Lamarche (1988).

6.3.2 Differential analysis

Differential analysis requires differentiation of the data using graphical, numerical or analytical methods. The advantage of applying differential methods is that more information can be gathered concerning the relationships between the rates of reaction and the operating variables from fewer experimental runs.

For this analysis, rate equations for the biomass, glucose, ethanol and fructose concentrations are required. The Monod equation,

$$\frac{dx}{dt} = \mu_{max} x \left(\frac{s_g}{K_s + s_g} \right) \tag{6.9}$$

is the most commonly used expression for the growth rate From the previous integral analysis, the ethanol concentration was found to inhibit the growth rate in a linear fashion (Eq. 6.6). Taking this into consideration the Monod equation becomes

$$\frac{dx}{dt} = \mu_{max} x \left(\frac{s_g}{K_s + s_g} \right) \left(1 - \frac{p}{p_{max}} \right) \tag{6.10}$$

The rate expressions for the glucose and ethanol concentrations are directly related to the growth rate through yield coefficients (Eq. 3.24). The rate equations for these become

$$\frac{ds_g}{dt} = -\mu_{max} x \left(\frac{s_g}{K_s + s_g} \right) \left(1 - \frac{p}{p'_{max}} \right) \frac{1}{Y_{x/s}} \tag{6.11}$$

$$\frac{dp}{dt} = \mu_{max} x \left(\frac{s_g}{K_s + s_g} \right) \left(1 - \frac{p}{p'_{max}} \right) \frac{Y_{p/s}}{Y_{x/s}} \tag{6.12}$$

It should be noted that the ethanol concentration above which growth no longer occurs (i.e. p_{max}) is assumed to be different from the ethanol concentration above which glucose is no longer consumed and ethanol no longer produced (i.e. p'_{max}) as suggested by Ghose and Tyagi (1979b). The results already shown, indicate that the glucose consumption and ethanol production rates are not as strongly inhibited by the ethanol.

When glucose and fructose are present in the medium, an additional term to take into account the inhibition by the total carbohydrate concentration (Eq. 6.3) must be added to

the previous expressions. The rate equations for this case are

$$\frac{dx}{dt} = \mu_{max} x \left(\frac{s_g}{K_s + s_g}\right)\left(1 - \frac{p}{p_{max}}\right)\left(1 - \frac{s_{tc}}{s_{max}}\right) \tag{6.13}$$

$$\frac{ds_g}{dt} = -\mu_{max} x \left(\frac{s_g}{K_s + s_g}\right)\left(1 - \frac{p}{p'_{max}}\right)\left(1 - \frac{s_{tc}}{s'_{max}}\right)\frac{1}{Y_{x/s}} \tag{6.14}$$

$$\frac{dp}{dt} = \mu_{max} x \left(\frac{s_g}{K_s + s_g}\right)\left(1 - \frac{p}{p'_{max}}\right)\left(1 - \frac{s_{tc}}{s'_{max}}\right)\frac{Y_{p/s}}{Y_{x/s}} \tag{6.15}$$

The inhibitory effects of the total carbohydrate concentration on (ds_g/dt) and on (dp/dt) are assumed to be different than the effects on (dx/dt), therefore s'_{max} is introduced. The batch results already presented confirm this assumption.

Experimental continuous process results (Koren and Duvnjak, 1989) show that the ratio of glucose to fructose consumption rates is relatively constant, therefore a proportionality constant, R is introduced to relate the fructose rate to the glucose rate.

$$\frac{ds_f}{dt} = -\mu_{max} x \left(\frac{s_g}{K_s + s_g}\right)\left(1 - \frac{p}{p'_{max}}\right)\left(1 - \frac{s_{tc}}{s'_{max}}\right)\frac{1}{Y_{x/s}R} \tag{6.16}$$

The method that is usually used to solve such a system involves estimating the values of the parameters by minimizing the sum of deviations between the observed and predicted values of one response at a time. Using this type of analysis many of the individual parameters cannot be estimated, therefore lumped parameters are often reported. For example Ghose and Tyagi (1979b) lumped μ_{max}, $Y_{x/s}$ and $Y_{p/s}$ together into ν. Since some of the parameters are common to two or more of the responses (e.g. $\mu_{max}, K_s, s'_{max}, p'_{max}$ and $Y_{x/s}$) better estimates of the parameters can be obtained if all the responses are fit simultaneously. In this way all the parameters can be estimated. When several responses are used to estimate the parameters the procedure is known as "Multi response parameter estimation". Software has been developed at the University of Ottawa's Chemical Engineering Department (Lozej and McLean, 1990) to perform this analysis.

Media containing glucose

The kinetic data from a series of batch fermentations which contained media (medium III) with 30 g/L yeast extract and various concentrations of glucose were used for this analysis.

The initial conditions of the tests are given in Table 6.11

Table 6.11: Initial conditions for glucose tests used for the differential analysis.

Exp. no.	Biomass (g/L)	Glucose (g/L)	Ethanol (g/L)
F33	1.36	70.1	0.57
F35	1.27	96.4	0.58
F36	1.64	125.4	0.54
F39	1.55	187.9	0.49
F73	2.67	91.6	0.73
F 75	2.82	67.4	0.58
F134	1.90	90.8	0.84
F137	1.90	88.5	0.73
F159	2.43	90.9	0.61

The rate equations (Eq. 6.10, 6.11 and 6.12) were then fitted to the data; the parameter estimates and their confidence intervals are given in Table 6.12.

Table 6.12: Converged parameter estimates and confidence intervals using data collected from glucose experiments.

Parameter estimates		Confidence interval	
		upper	lower
μ_{max}	0.235 h^{-1}	0.259	0.210
K_s	10.2 g/L	17.8	2.60
p_{max}	72.6 g/L	76.0	69.1
$Y_{x/s}$	0.155 g/g	0.164	0.147
$Y_{p/s}$	0.462 g/g	0.471	0.454
p'_{max}	161.9 g/L	232.9	90.9

The results from the differential analysis were first examined on the basis of the reason-

ableness of the parameter estimates. This was done by comparing the results obtained here with those obtained using the integral analysis carried out previously. In addition, they were compared to values reported by other researchers. Since no work has been performed with the yeast used in this work, results reported for the wild strain of *S. cerevisiae* by Ghose and Tyagi (1979b) and Bazua and Wilke (1977) were compared.

The value obtained for μ_{max} in this analysis is 0.235 ($\pm$0.025) h^{-1}, this range for the maximum specific growth rate falls within that found previously for the integral analysis shown in Table 6.8 (i.e. 0.251 $\pm$ 0.013 h^{-1}). These values though are lower than the values of 0.4 and 0.448 h^{-1} reported by Ghose and Tyagi (1979b) and Bazua and Wilke (1977) respectively. This was expected since the mutant yeast used here was already shown to have a lower growth rate than the wild strain of the yeast.

The value of the saturation constant K_s (10.2 $\pm$7.6 g/L) is considerably higher than those predicted by Ghose and Tyagi (1979b) (0.48 g/L) and Bazua and Wilke (1977) (0.24 g/L). This may also be due to the lower growth rate of the yeast, the large uncertainty in the converged value of the parameter makes it difficult to form any firm conclusions.

Concerning ethanol inhibition, it was previously shown that the growth rate would be reduced to zero when 87.5 g/L of ethanol was added to the medium. In this analysis the ethanol concentration above which growth is predicted to be zero is somewhat lower than this value (i.e. 72.6 $\pm$3.4 g/L). This may be due to the difference in the measurement technique that was used. Previously, the inhibition due to externally added ethanol was measured; the models presented here measure the inhibition due to the ethanol that is produced by the yeast. Therefore, externally added ethanol does not inhibit growth as strongly as does ethanol produced by the yeast. This was also reported by Hoppe and Hansford (1982) using the wild strain of the yeast. Ghose and Tyagi (1979b) and Bazua and Wilke (1977) reported values of 87 and 93.6 g/L respectively for the value of p_{max}. These values were obtained by testing the inhibitory effects of externally added ethanol.

The fact that $p'_{max} > p_{max}$ indicates that the growth rate is more strongly inhibited by ethanol than are the glucose consumption and ethanol production rates, which was found to occur for most microbes (van Uden, 1985). Ghose and Tyagi (1979b) reported values of

87 and 114 g/L for p_{max} and p'_{max} respectively.

The biomass yield is expected to be relatively constant as was reported by Ghose and Tyagi (1979b) and Bazua and Wilke (1977). Using this assumption a value of 0.155 (±0.008) g/g was estimated here. This is higher than the values of 0.09 and 0.10 reported by Ghose and Tyagi (1979) and Bazua and Wilke (1977) respectively. The higher value is a result of the higher yeast extract concentration used. In this work a concentration of 30.0 g/L was used while the other researchers used 10.0 g/L. As was shown previously the yeast extract concentration affects the biomass yield significantly.

The parameter estimate for the ethanol yield ($Y'_{p/s}$) obtained in this analysis is 0.462 (±0.008) g/g this is comparable to the 0.450(±0.013) g/g estimated previously from the integral analysis. This is also similar to the ethanol yield of 0.47 reported by Ghose and Tyagi (1979b).

The next step was to analyze the predicted values of the responses. Plots comparing the experimental and predicted results are provided in Figures 6.37 to 6.45. The experiments are presented in the order in which they were performed, the first 6 were carried out initially, the other 3 were carried out about 12 months later.

Figure 6.37 compares the experimental and predicted values of a fermentation carried out in media containing 70.1 g/L glucose. From the figure it is seen that the model initially underpredicts the ethanol production curve. The maximum biomass and ethanol concentrations are also reached at a later time than was observed experimentally. In a medium containing 96.4 g/L glucose (Figure 6.38) the model predicts final biomass and ethanol concentrations that are within 6 and 4% respectively of the observed values. In the exponential phase of growth (i.e. 5-10 hours) the model underpredicts the experimental behaviour by ≈ 25%. The results in Figure 6.39 show that there is a good agreement between experimental and predicted fermentation curves in a medium containing 125.4 g/L glucose. Figure 6.40 shows a fermentation carried out in a medium with 187.9 g/L glucose. The model predicts that growth stops after 16 hours, the growth actually continues though up to 24 hours. The final biomass and ethanol concentrations are predicted within 2% of the observed values. Fermentation in a medium containing 91.6 g/L glucose and a higher biomass concentration

than was used previously is shown in Figure 6.41. Although the final biomass and ethanol concentrations predicted by the model are within 2% of the observed values, agreement in the exponential phase is not as good. The predicted curves seem to lag the experimental values by $\approx$ 4 hours. These same trends are noted in Figure 6.42 for a fermentation in a medium containing 67.4 g/L glucose. In this case the final biomass and ethanol concentrations are predicted to within 5 and 8% respectively. The next two figures (i.e. Figure 6.43 and 6.44) are repeated experiments carried out with $\approx$ 90 g/L glucose. In these cases the experimental results are overestimated by the models that were formulated. The final biomass and ethanol concentrations predicted are within 3% of the experimental values. The last test was carried out in a medium containing a similiar concentration of glucose but with a higher biomass concentration (Figure 6.45). As was the case for the previous two tests, the model overestimated the biomass and ethanol concentrations. The final biomass and ethanol concentrations are overpredicted by 4.5 and 12.4% resepectively.

In the first set of data the models tend to underpredict the biomass formation, glucose consumption and ethanol production rates. The final concentrations of biomass and ethanol are predicted within $\approx$8%. For the tests carried out more recently (i.e. Figures 6.43, 6.44 and 6.45), the biomass, glucose and ethanol concentrations are overpredicted. In addition, the final ethanol concentration predicted by the model varies from 12 to 21% higher than the experimental values. Since these two sets of data behave differently it is suspected that the yeast's activity may have changed between the time that the two sets of data were collected.

When the analysis was performed with the data from the most recent tests only, convergence was not reached. These three sets of data did not contain enough information for all the parameters to be estimated.

The analysis performed here provided reasonable estimates of the parameters in the models tested. Final biomass and ethanol concentrations predicted are within 8% of the experimental values. The fitted equations though did not adequately represent the data. This may be due to the changing activity of the yeast.

The next set of data to be analyzed are from experiments which were conducted in media

containing glucose and fructose. The effect of the presence of fructose on the fermentation can be analyzed by comparing the parameter estimates obtained with those obtained in glucose media. The tests were also be carried out over a shorter period of time and a greater number of samples were taken in order to overcome the difficulties encountered in the first analysis.

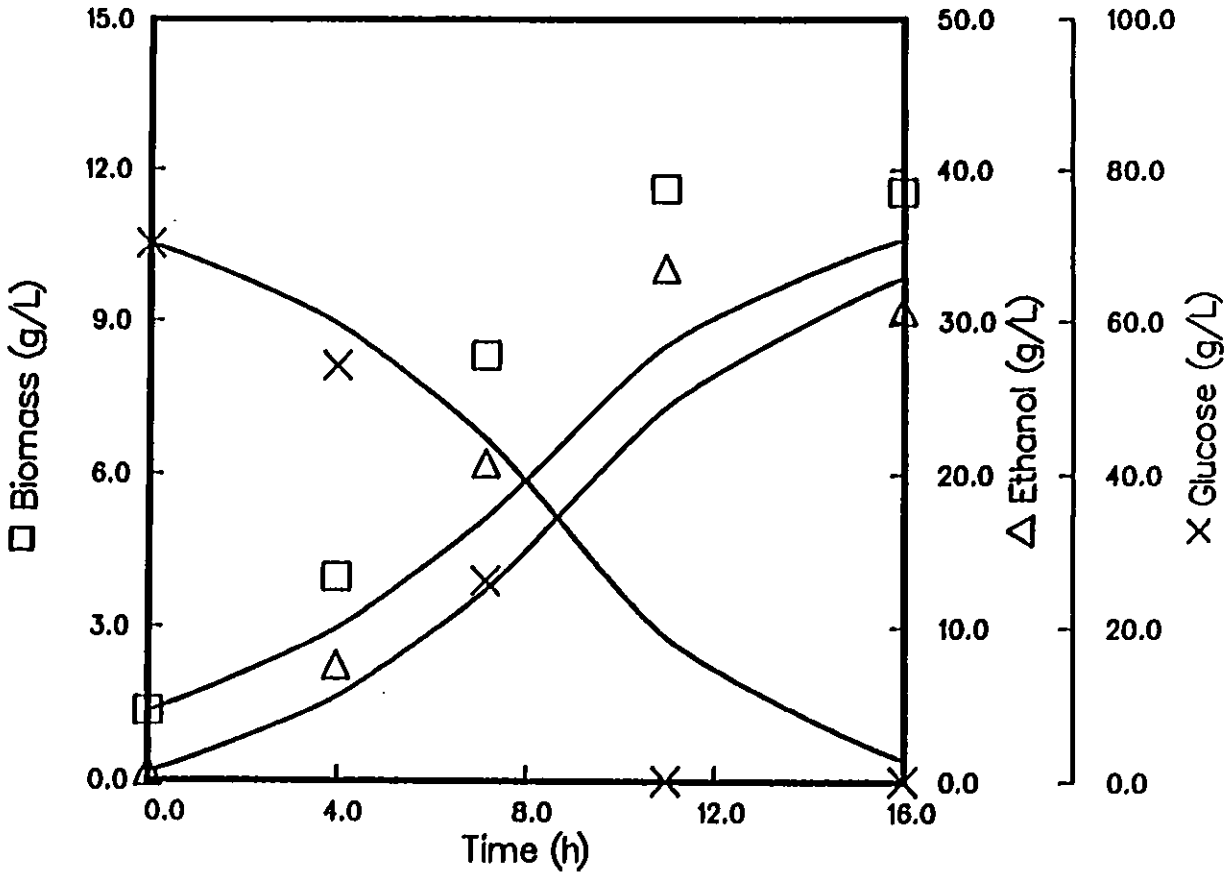

Figure 6.37: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 70 g/L glucose.

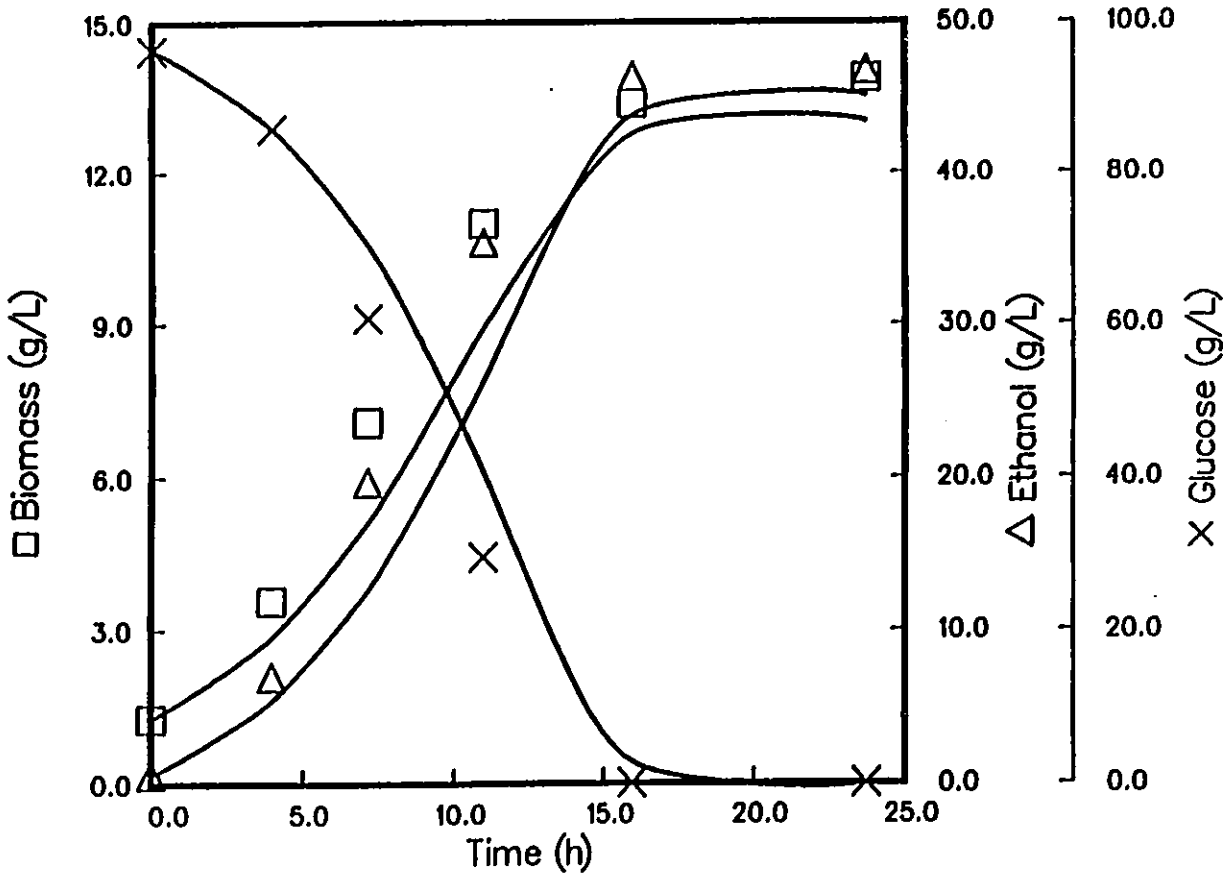

Figure 6.38: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 96 g/L glucose.

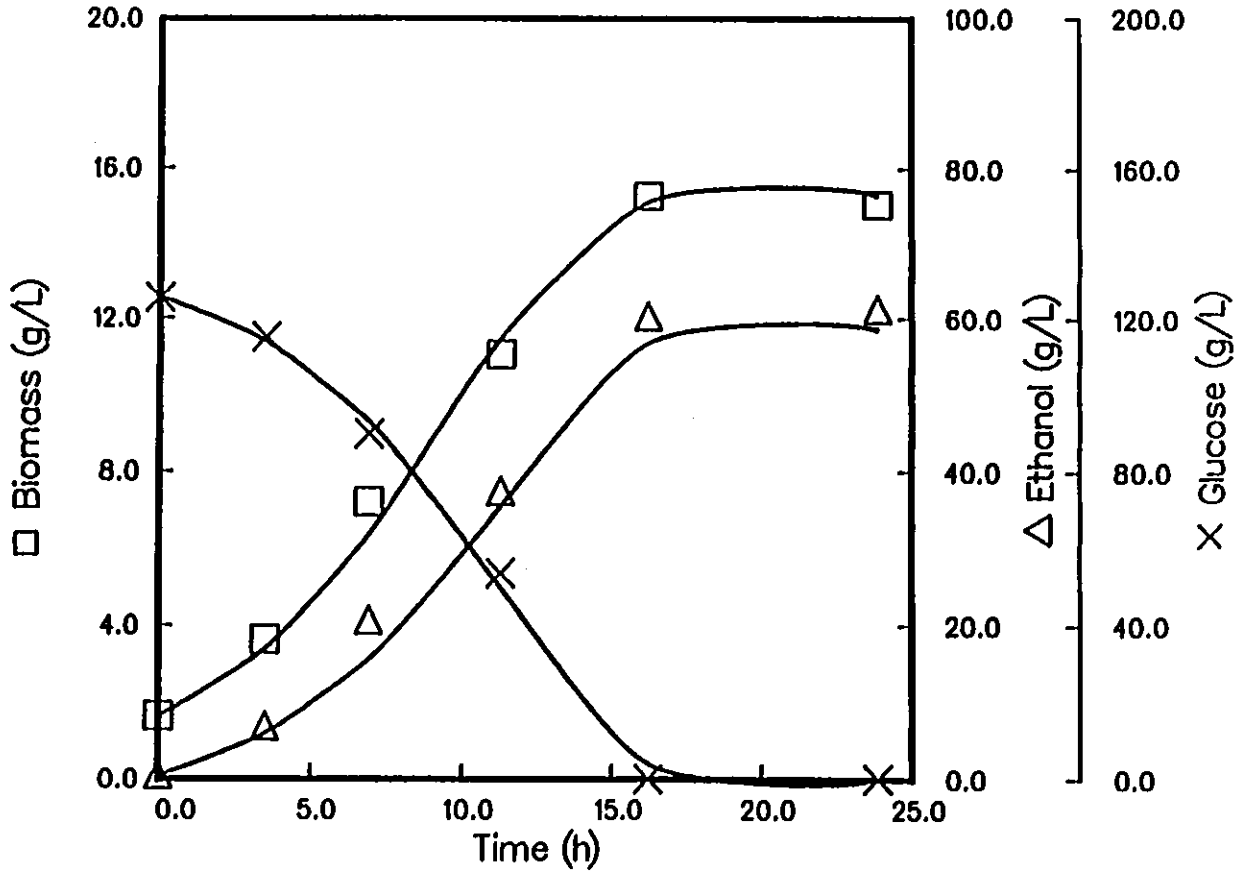

Figure 6.39: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 125 g/L glucose.

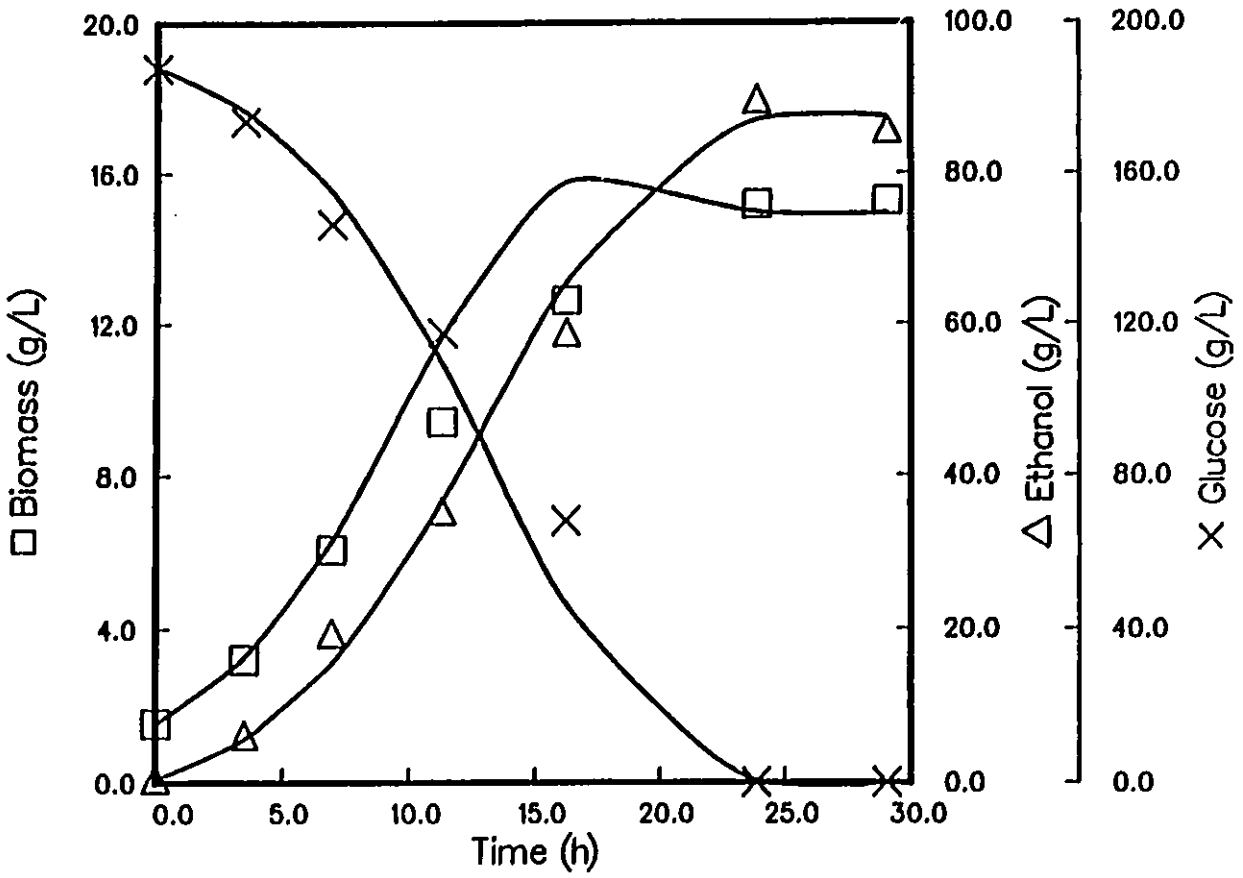

Figure 6.40: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 188 g/L glucose.

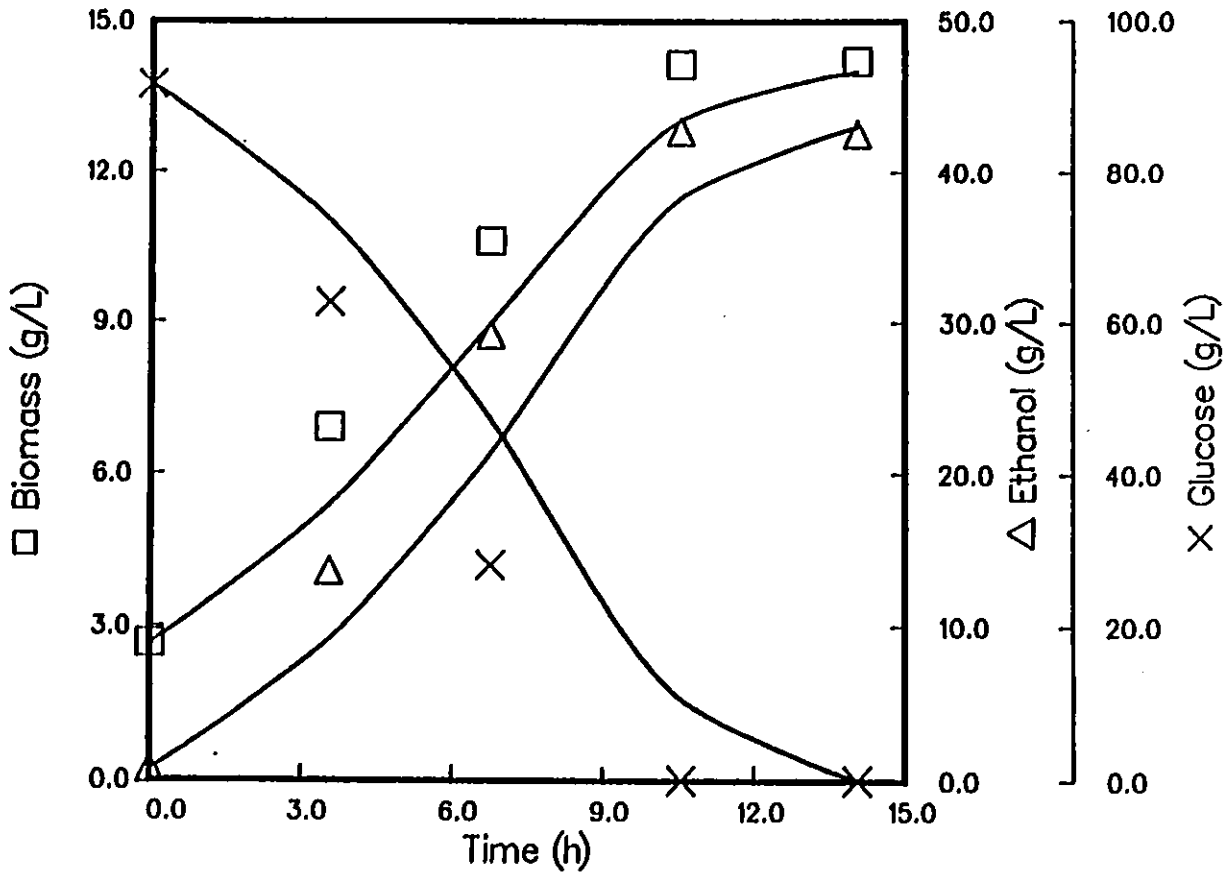

Figure 6.11: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 92 g/L glucose.

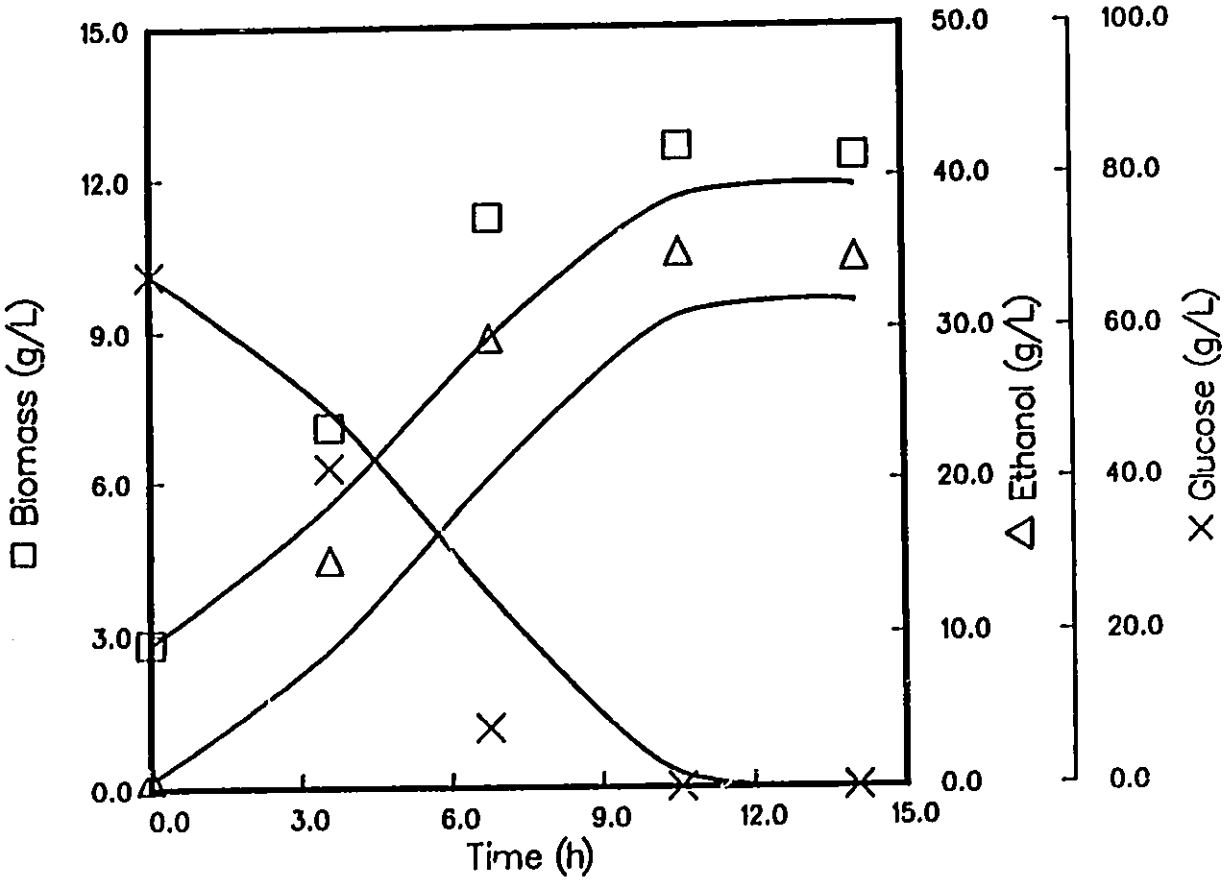

Figure 6.12: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 67 g/L glucose.

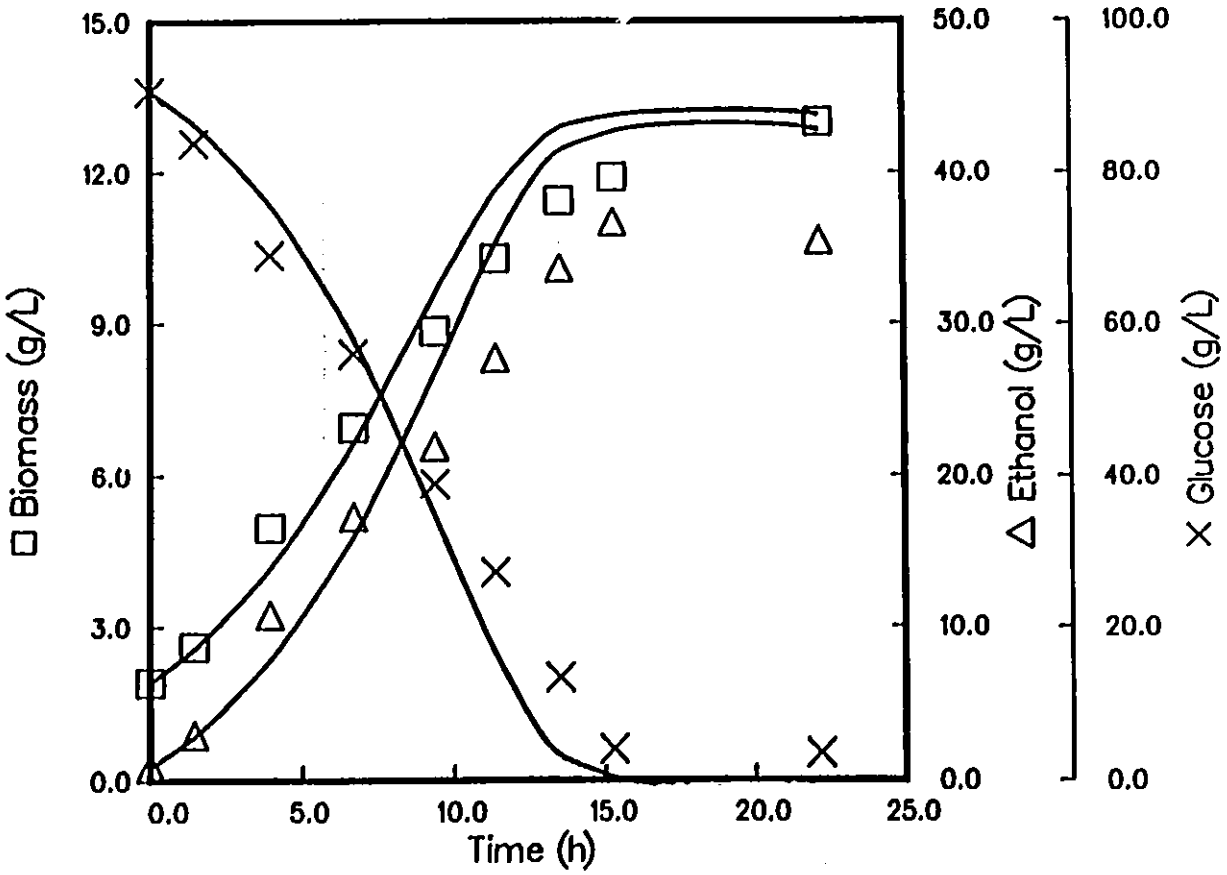

Figure 6.13: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 91 g/L glucose.

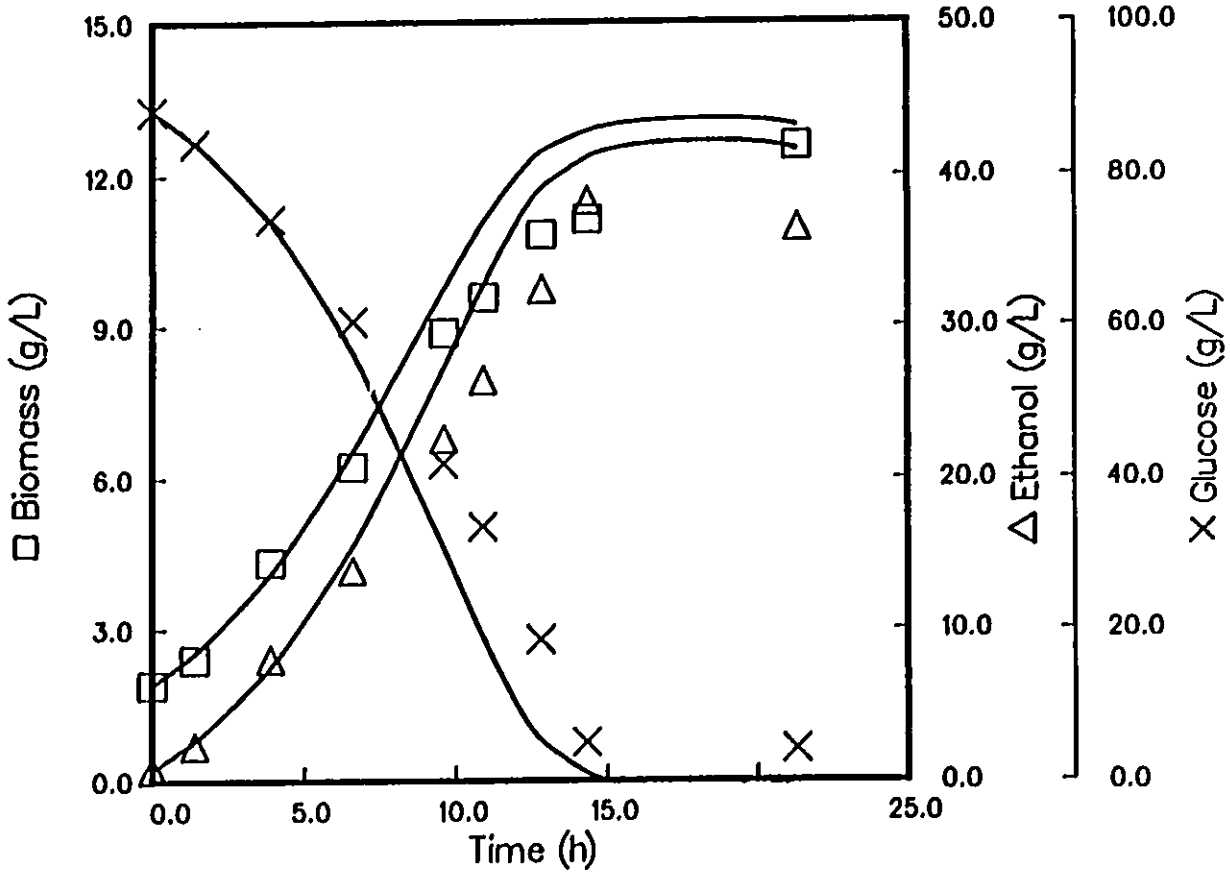

Figure 6.14: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 89 g/L glucose.

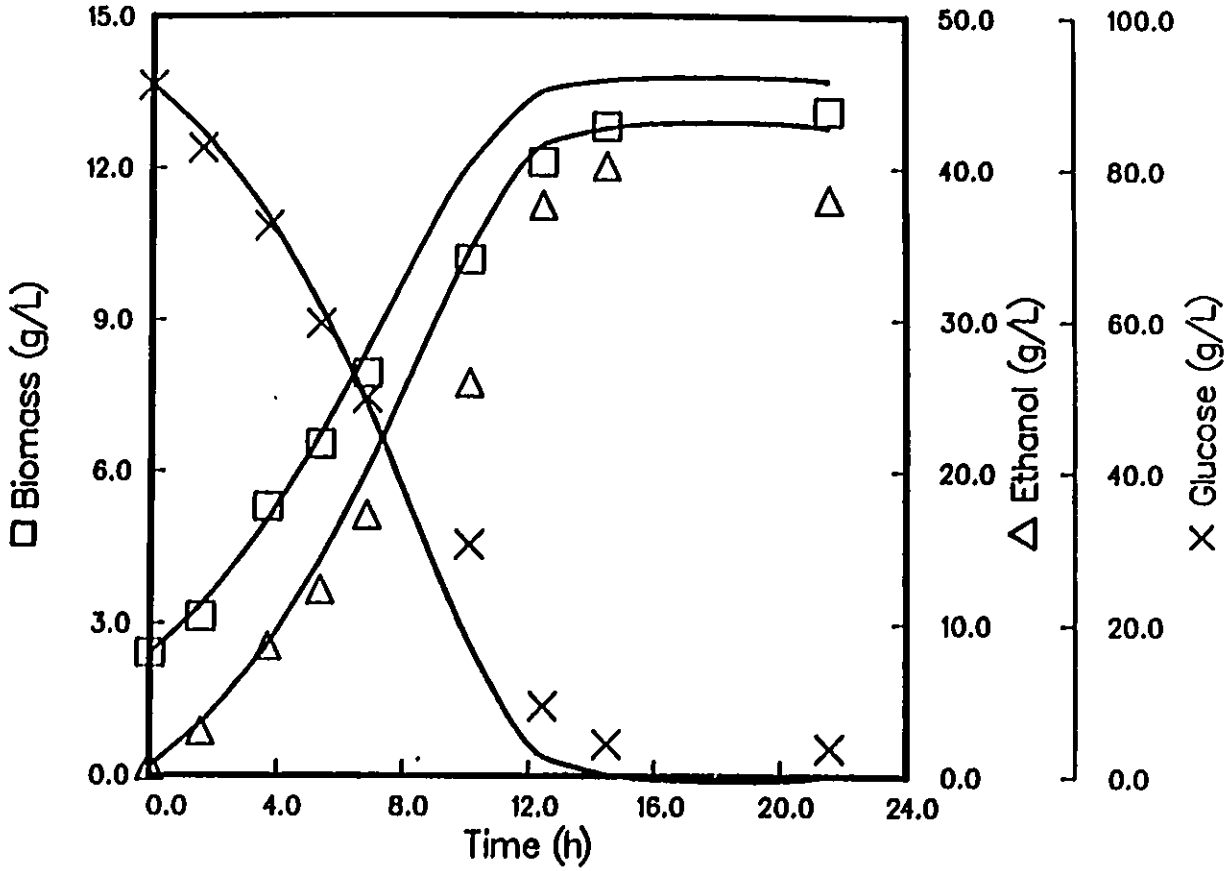

Figure 6.45: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 91 g/L glucose.

Media containing glucose and fructose

In this section data collected from experiments with glucose and fructose are fitted using the equations described previously (Eqs. 6.13-6.16). The initial conditions of the experiments used in this analysis are provided in Table 6.13

Table 6.13: Initial conditions for glucose-fructose tests used for the differential analysis.

Exp. no.	Biomass (g/L)	Glucose (g/L)	Fructose (g/L)	Ethanol (g/L)
F149	1.96	74.2	147.8	0.91
F162	2.61	137.0	88.1	0.47
F160	2.90	160.7	91.0	0.42
F161	2.72	157.8	151.7	0.43
F153	2.16	66.4	149.0	0.80
F150	2.25	67.8	222.2	0.72
F151	2.05	66.5	290.1	0.71
F148	2.04	75.9	326.6	0.75
F152	2.24	69.5	343.6	0.72

The parameter estimates and the 95% confidence intervals obtained when Eqs. 6.13-6.16 are fitted to the data are provided in Table 6.14.

Comparing the parameter estimates in this analysis with those obtained previously (Table 6.12) it is seen that the values of μ_{max}, $Y_{x/s}$, $Y_{p/s}$ and p'_{max} differ by less than 11%, this is within the expected error of batch tests (15-20%). The values of K_s obtained using the two sets of tests are significantly different but their large confidence interval suggests that they may be equal. Taking into account the confidence intervals obtained for p_{max} a difference is indicated. The lower value obtained when fructose is present in the medium indicates that the inhibitory effect is higher in this case. The presence of fructose results in a higher osmotic pressure due to the higher solids concentration in the medium. As expected by Jones and Greenfield (1986; 1987) this increase makes it more difficult for the

Table 6.14: Converged parameter estimates and confidence intervals using data collected from glucose/fructose experiments.

	Parameter estimates	Confidence intervals	
		upper	lower
μ_{max}	0.241 h^{-1}	0.260	0.222
K_s	4.56 g/L	7.43	1.69
p_{max}	62.0 g/L	65.8	58.2
s_{max}	488.4 g/L	510.6	466.3
$Y_{x/s}$	0.140 g/g	0.157	0.124
$Y_{p/s}$	0.466 g/g	0.475	0.457
p'_{max}	152.0 g/L	191.8	112.2
s'_{max}	788.9 g/L	899.9	677.9
R	22.7	41.1	4.29

ethanol produced in the cell to be transported out thereby increasing its inhibitory effect.

The value of s_{max} here is 19% lower than the value obtained from the integral analysis (Table 6.8). The value obtained from the present analysis is thought to be more precise because of its smaller confidence interval. The total carbohydrate concentration above which glucose consumption and ethanol production cease is 62% higher than the value for total cessation of growth. This implies that growth is more strongly inhibited by the total carbohydrate concentration than is the glucose consumption and ethanol production rates. This was also observed from the batch results already presented.

The model predicts that the yeast consumes glucose 23 times faster than it consumes fructose when both are present in the medium. At the end of the fermentation when glucose is no longer present in the medium the consumption of fructose is expected to increase, as was the case for tests carried out in media containing only fructose (Table 6.5). The model therefore indicates that fructose is being consumed by the yeast even when glucose is present in the medium. The selectivity of the yeast though is such that fructose consumption is insignificant compared to glucose consumption.

Plots comparing the experimental and predicted results of the fermentation used in this analysis are shown in Figures 6.46-6.54. Figure 6.46 shows the results of an experiment carried out in a medium containing 74 g/L glucose and 148 g/L fructose. The final biomass concentration is underpredicted by 11% since the model predicts no further growth after 16 hours whereas experimentally, growth continued. Final ethanol and fructose concentrations predicted by the models are within 5 and 9% respectively of the experimentally observed values. The next figure shows the results of a test carried out in a medium containing 137 g/L glucose and 88 g/L fructose (Figure 6.47). From the figure it is seen that there is good agreement between the predicted and experimental values. The final biomass, ethanol and fructose concentrations predicted are within 6% of the observed values. An increase in the glucose concentration to 161 g/L with a fructose concentration of 91 g/L results in the appearance of a lag phase (Figure 6.48). Since the model assumes an exponential growth, this lag phase is not followed. Due to this, the biomass concentration is overpredicted for most of the test, the final biomass concentration predicted is within 2% of the observed value. The ethanol and fructose concentrations are predicted within 4% of the observed values. Experimental and predicted results for a fermentation carried out in a medium containing 158 g/L glucose and 152 g/L fructose is shown in Figure 6.49. As was the case in the previous test a short lag phase occurs, resulting in an overprediction by the model (12%). The final ethanol and biomass concentrations predicted by the model are within 3% of the experimental values. Figure 6.50 compares predicted results for a test carried out in a medium containing 66 g/L glucose and 149 g/L fructose. The model predicts the experimentally determined biomass and ethanol concentrations to within 5%. Using a similar glucose concentration but increasing the fructose concentration to 222 g/L the results in Figure 6.51 are obtained. A short lag phase was observed for the experimental results, this was not followed by the model. After this lag period the biomass concentration is followed closely, the final predicted concentration is 3% higher than the experimental value. The final ethanol and fructose concentrations predicted are within 2 and 11% of the experimental values. The results of a test carried out in a medium containing 67 g/L glucose and 290 g/L fructose are shown in Figure 6.52. At the end of the test the biomass concentration

decreased due to cell lysis, the predicted results did not follow this trend therefore the model overpredicted the biomass concentration by 16%. The ethanol and fructose concentrations predicted are in good agreement with the experimental values (within 0.2%). In a medium containing 76 g/L glucose and 327 g/L fructose, the lag and exponential regions of the growth curve are not followed by the models (Figure 6.53). The final biomass concentration predicted is only 1% higher than the experimental value. The final experiment contains 70 g/L glucose and 344 g/L fructose (Figure 6.54). It is evident from the figure that a lag phase occurs in both the growth and ethanol production curves which are not followed by the models. The differences between the predicted and experimental final biomass and ethanol concentrations are 21 and 5% respectively. The final fructose concentration is only 8% lower than the experimental value.

An examination of the plots comparing the experimental and predicted values shows that the model predicts the biomass, glucose, fructose and ethanol concentrations quite well (Figure 6.46–6.54). The predicted values diverge from the experimental values when a lag phase is present, this is especially evident when the initial total carbohydrate concentration is above 310 g/L (i.e. Figures 6.53 and 6.54). This is expected since the model used here assumes exponential growth. The final biomass concentrations predicted by the model are within 12% of the experimental values when the total carbohydrate concentration is less than 310 g/L, above this concentration an error of up to 21% is measured. The final ethanol and fructose concentrations predicted are within 12% of the experimental values for all the tests examined.

The analysis shows that the batch fermentation of glucose by *S. cerevisiae* ATCC 36859 can be modelled using Monod kinetics. The use of Multiresponse Parameter Estimation techniques allows for the determination of all the parameters in the models without the need for experiments to be carried out in a continuous stirred tank reactor.

The presence of fructose in the medium has an inhibitory effect on the glucose fermentation. Both the total carbohydrate and ethanol concentrations inhibit the growth, substrate consumption and ethanol production rates in a linear manner. As suggested by Jones and Greenfield (1986; 1987) the inhibition is due to the increased osmotic pressure caused by the

increased concentration of these constituents. The fact that the inhibition due to glucose and that due to fructose are similar adds further evidence for the application of this theory in this case.

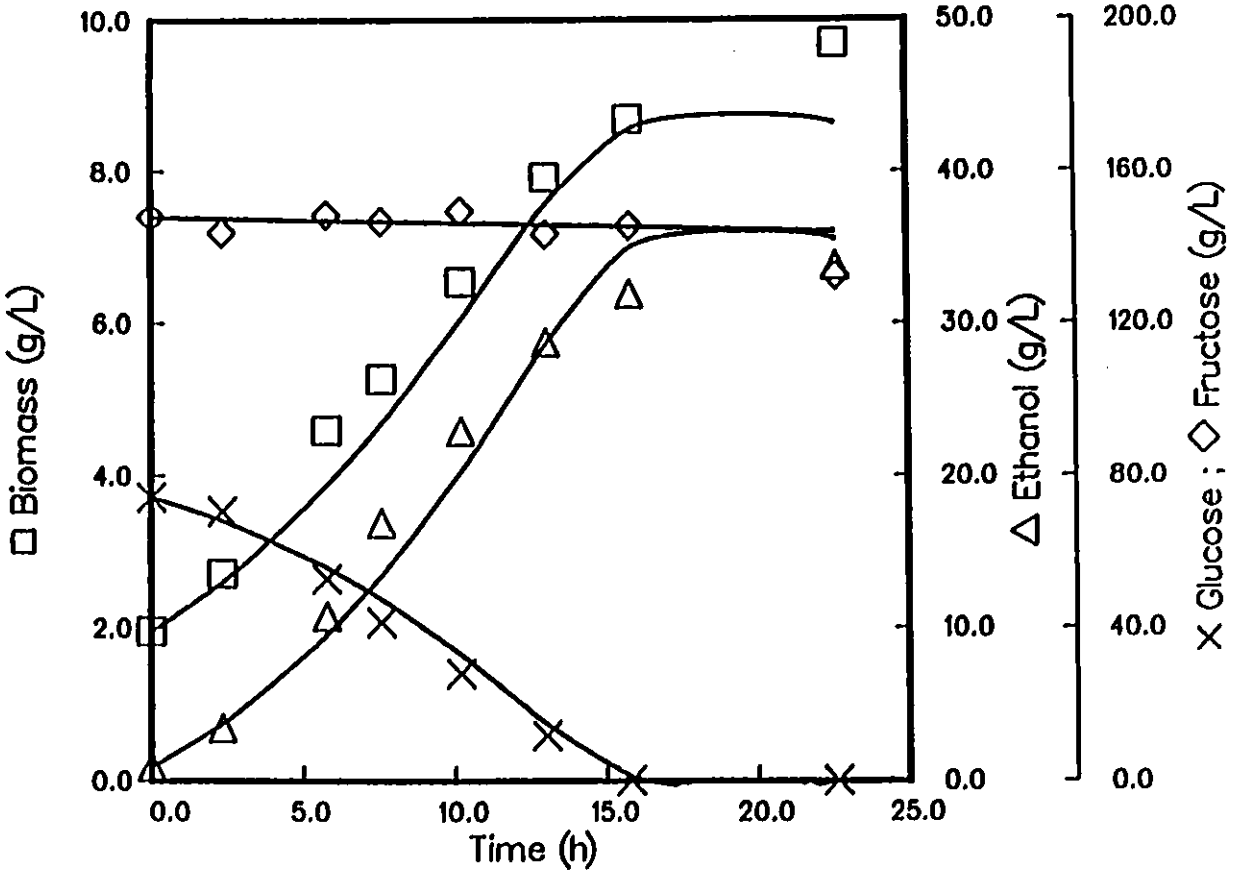

Figure 6.16: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 7.1 g/L glucose and 14.8 g/L fructose.

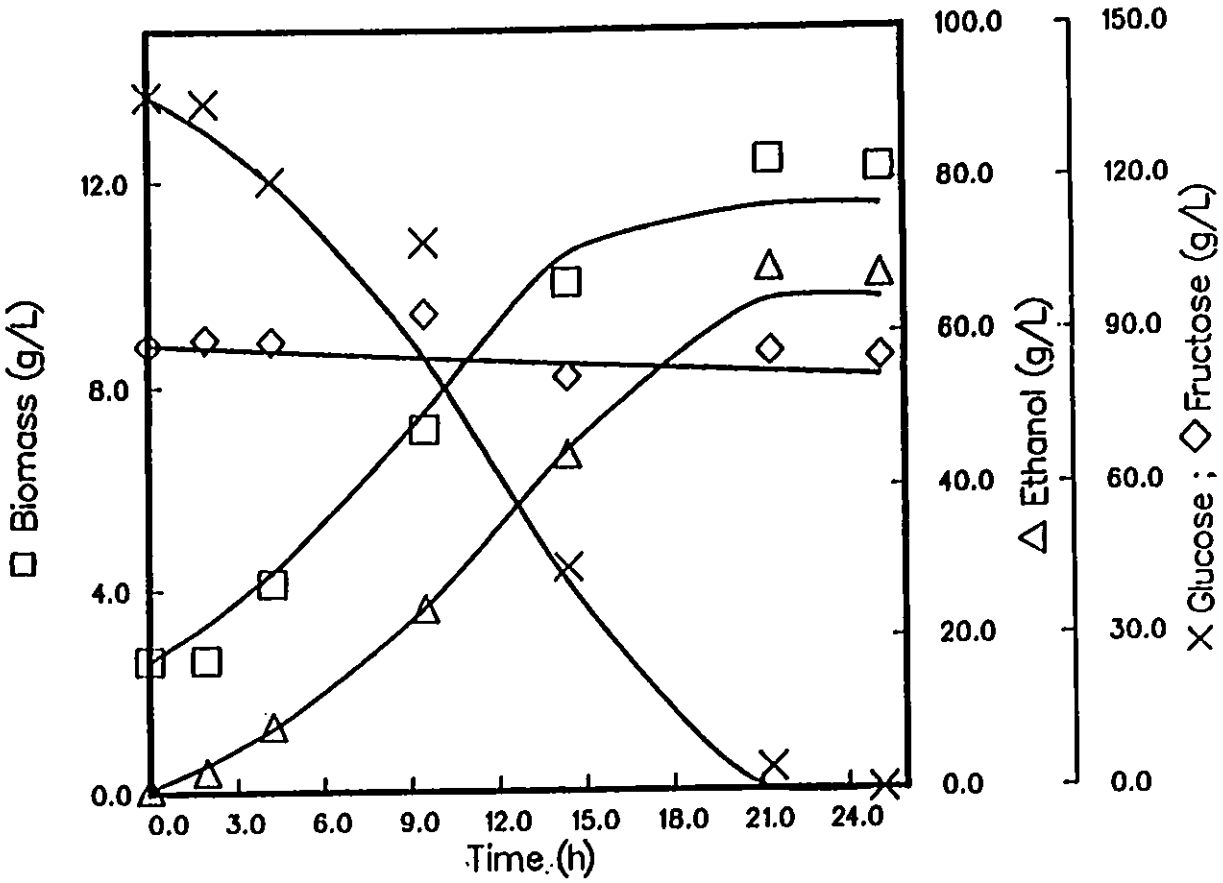

Figure 6.47: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 137 g/L glucose and 88 g/L fructose.

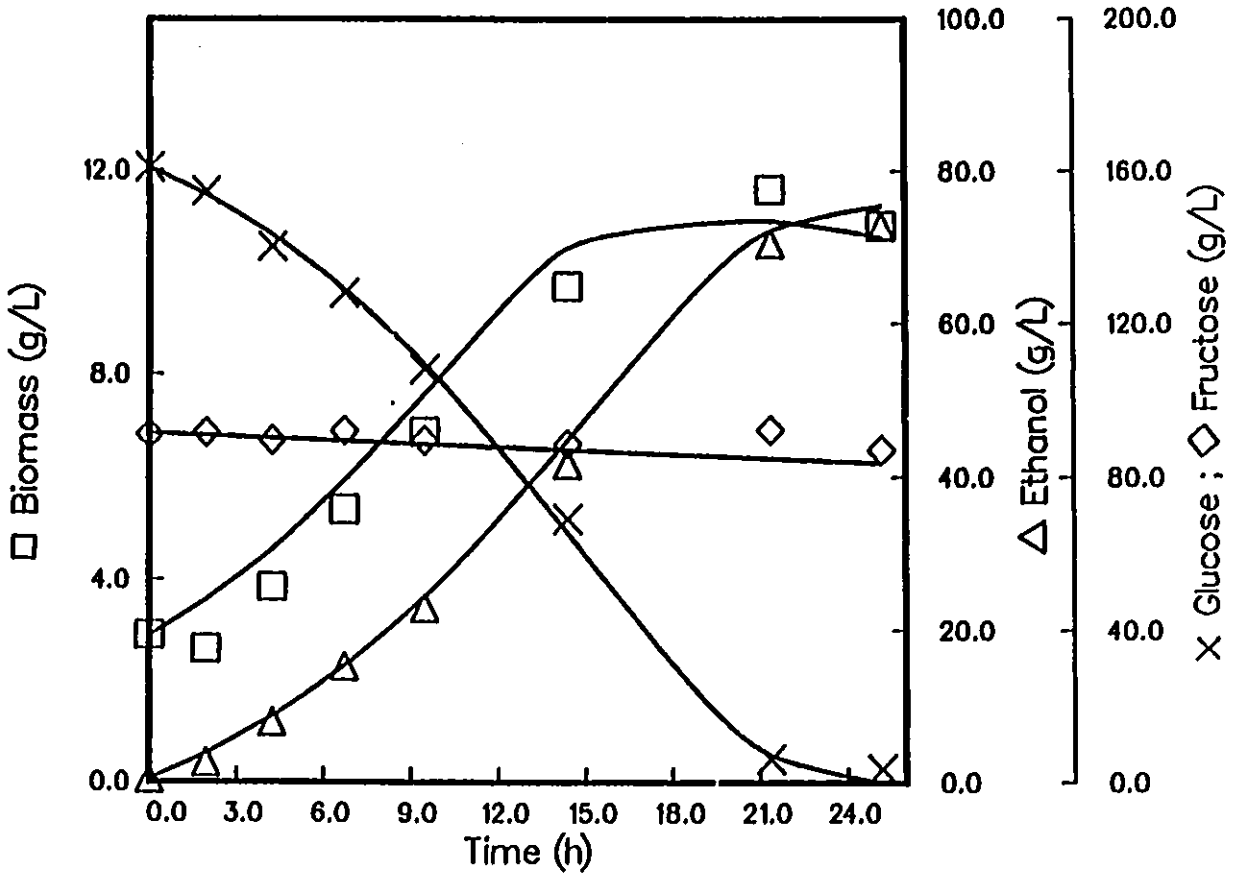

Figure 6.48: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 161 g/L glucose and 91 g/L fructose.

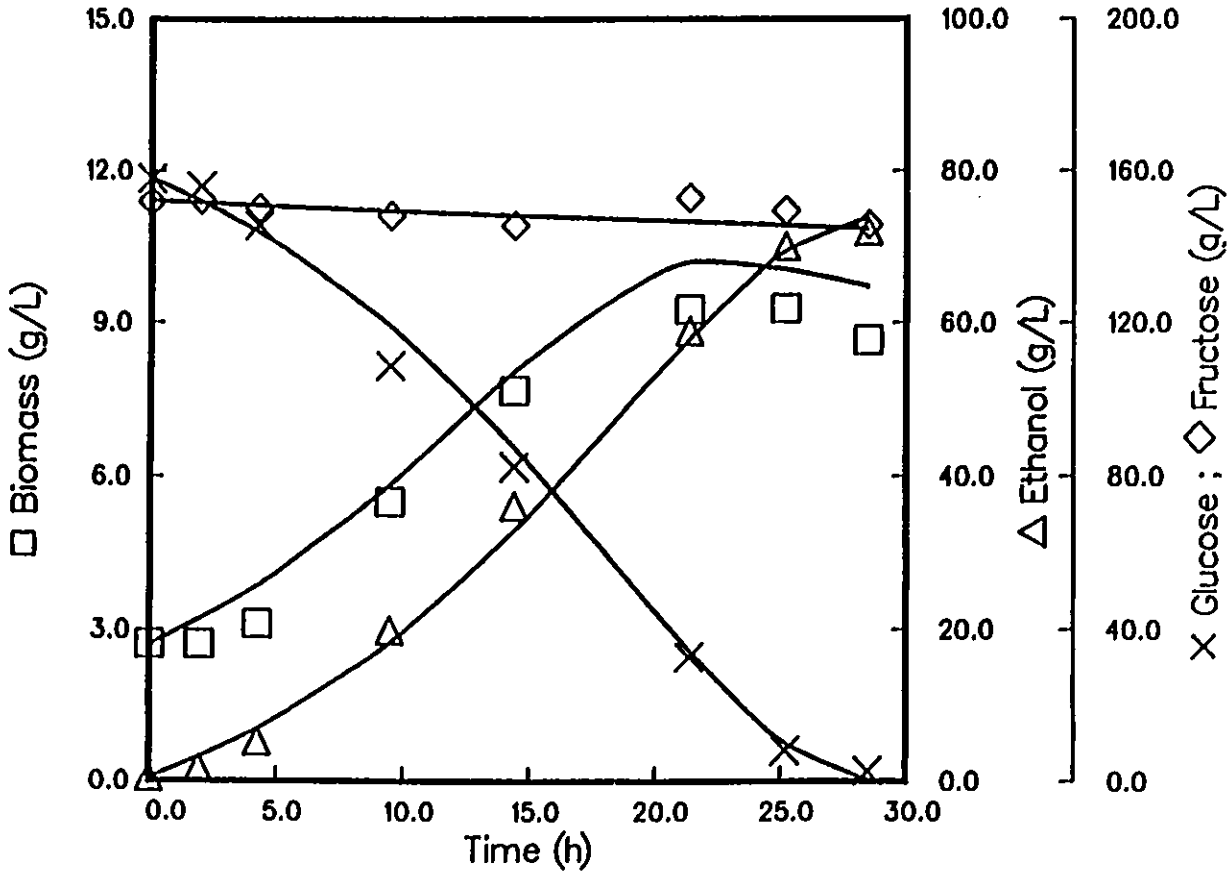

Figure 6.49: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 158 g/L glucose and 152 g/L fructose.

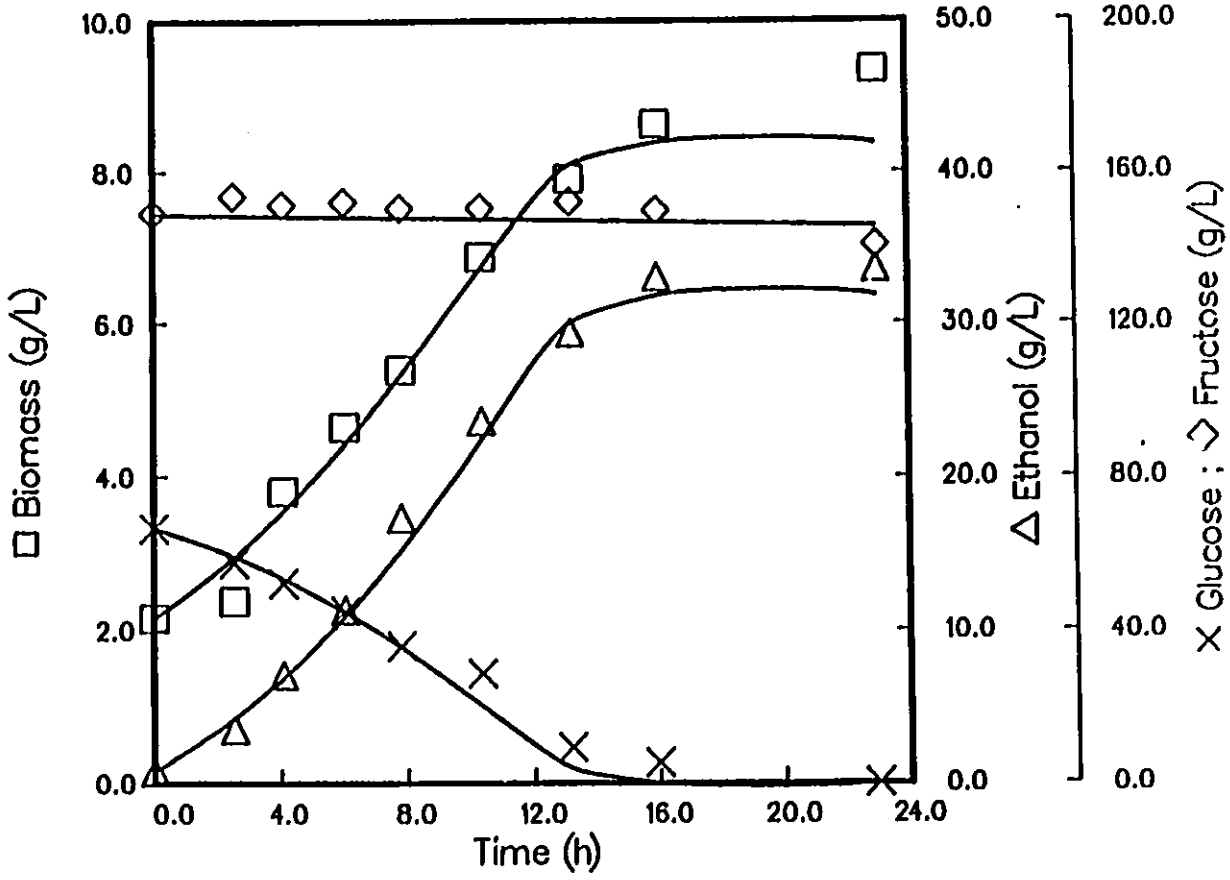

Figure 6.50: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 66 g/L glucose and 149 g/L fructose.

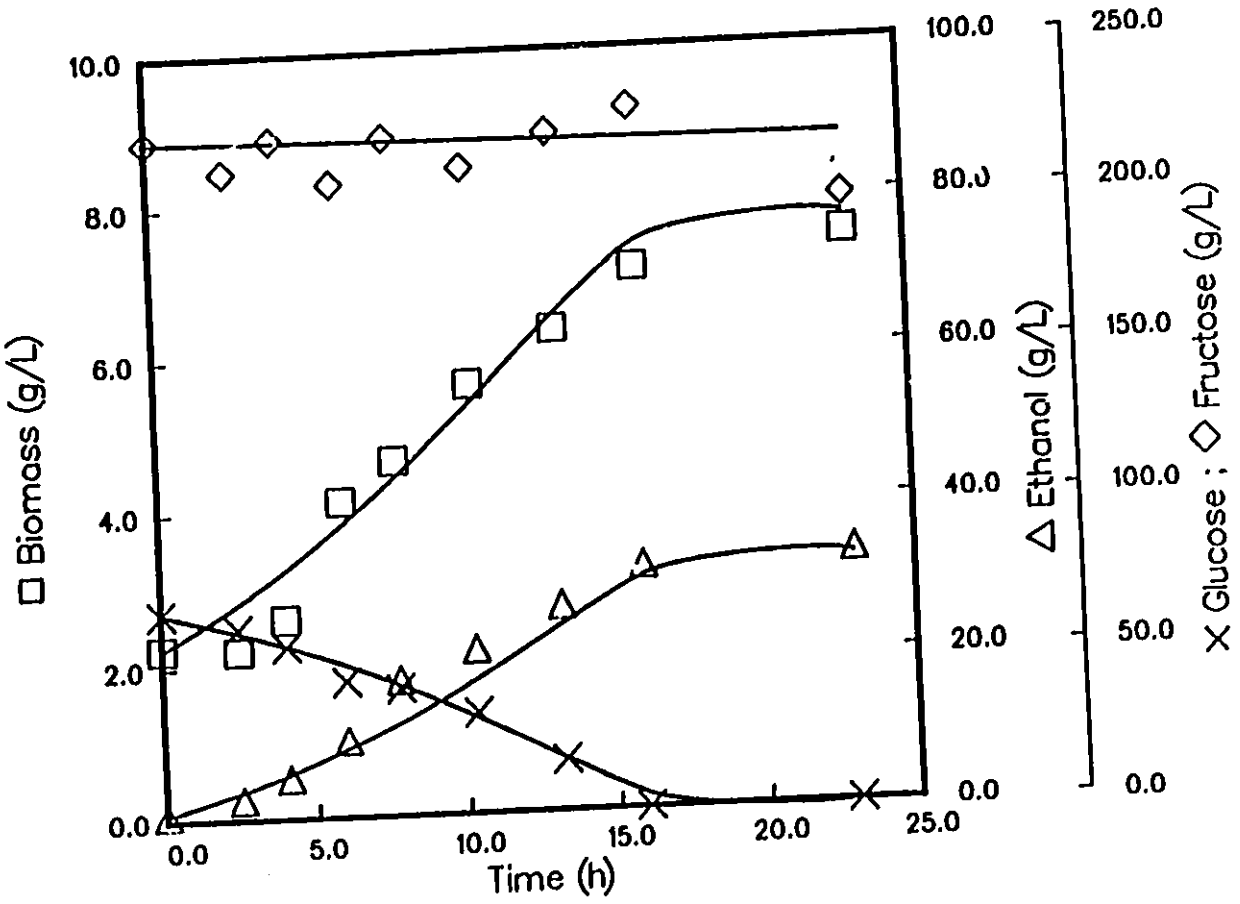

Figure 6.51: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 68 g/L glucose and 222 g/L fructose.

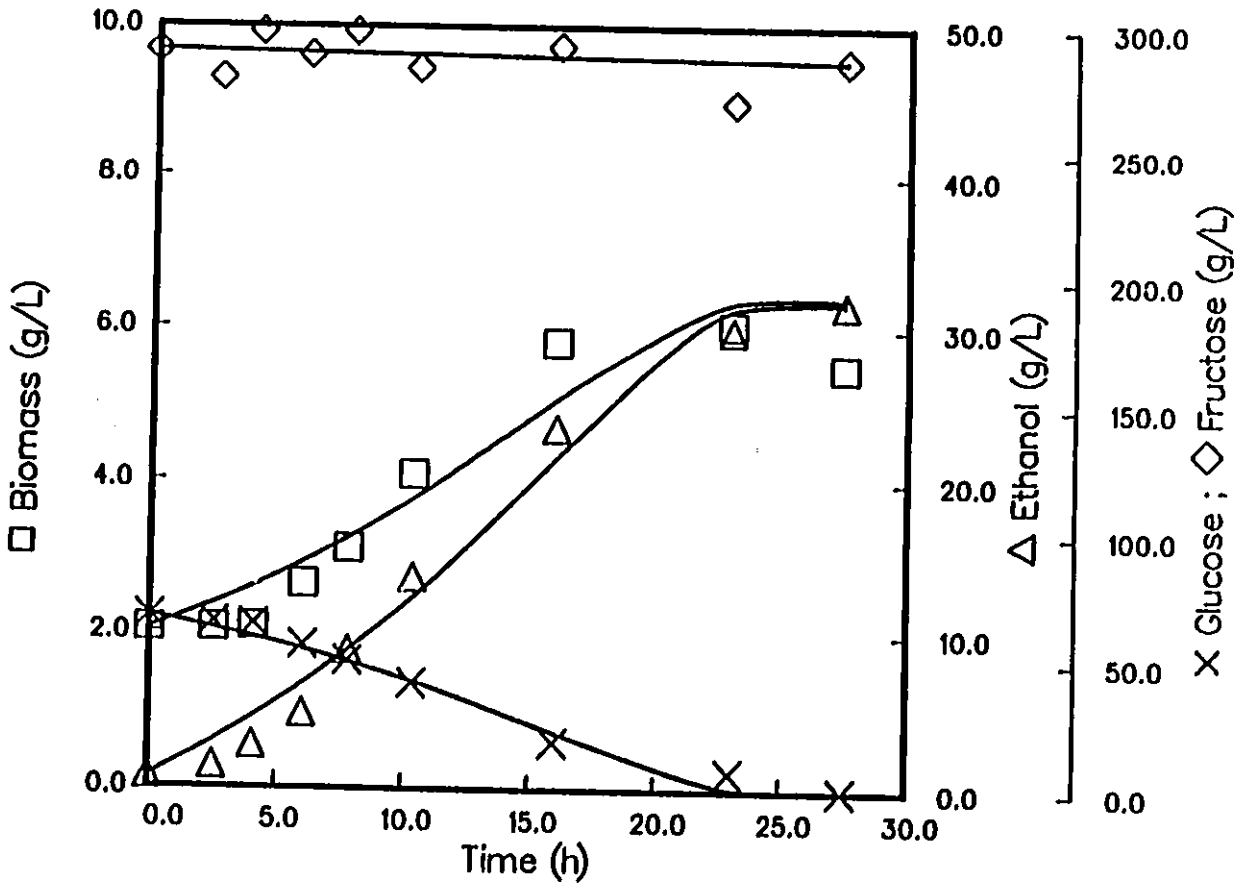

Figure 6.52: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 67 g/L glucose and 290 g/L fructose.

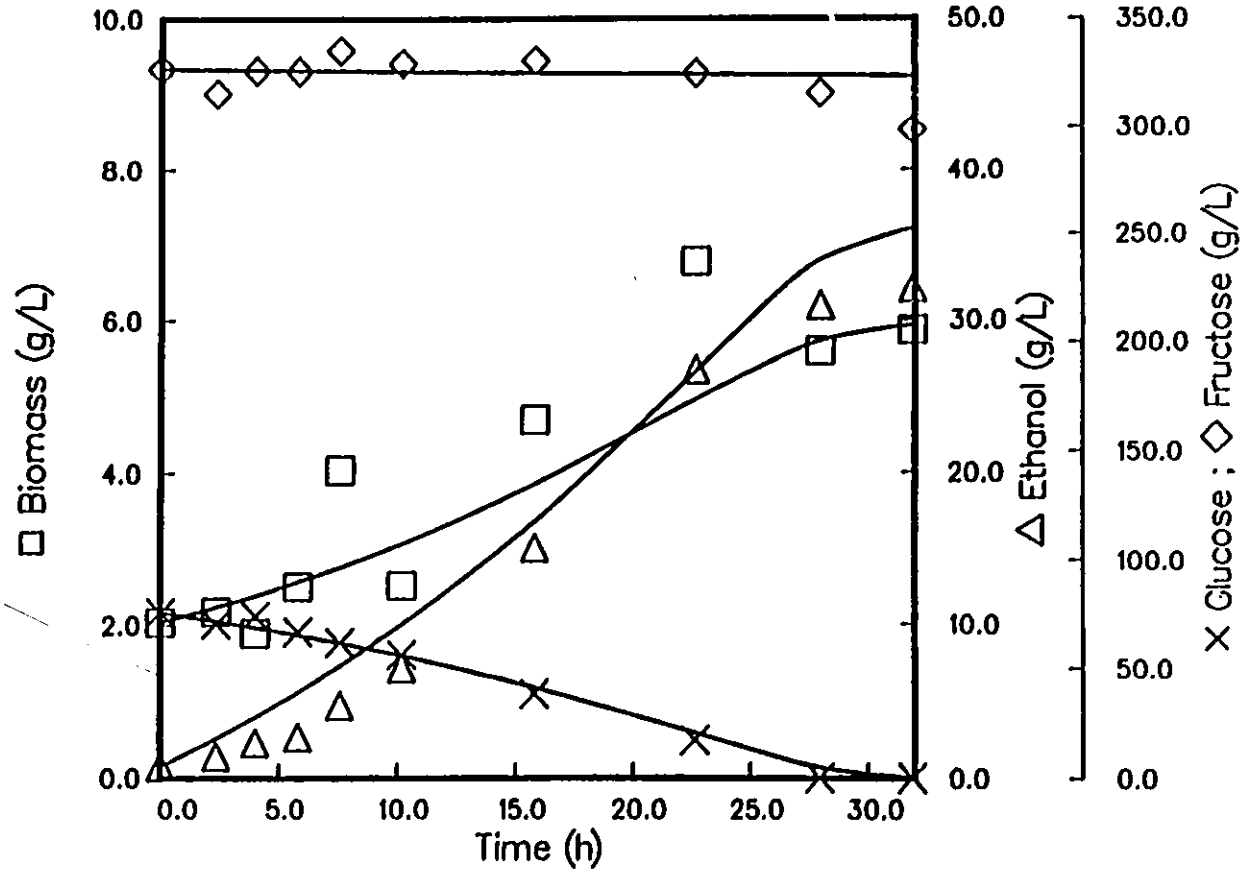

Figure 6.53: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 76 g/L glucose and 327 g/L fructose.

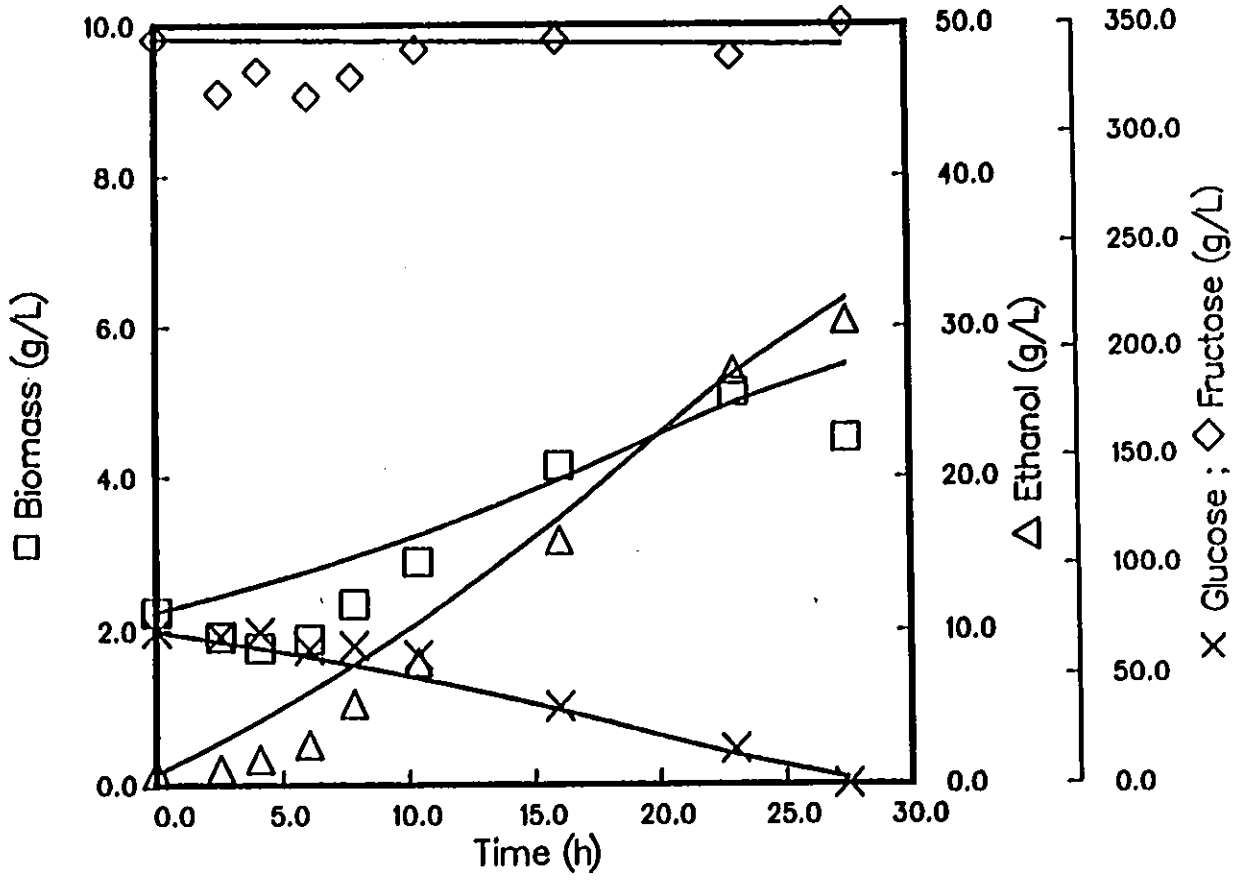

Figure 6.54: Experimental (symbols) and predicted (curves) results from a test carried out in a medium containing 70 g/L glucose and 344 g/L fructose.

Predicted growth and ethanol production rates

Since the fermentation models derived from tests carried out in media containing glucose and fructose mixtures provide realistic estimates of the parameters and provide a reasonable fit to the experimental data (±10%), they can be considered to be valid for the experimental range studied here.

The specific growth and ethanol production rates predicted by the models are shown in Figures 6.55 and 6.56 as a function of the glucose concentration at different fructose concentrations. From Figure 6.55 it can be seen that increases in the glucose concentration initially result in an increase in the specific growth rate, irrespective of the fructose concentration as expected from the Monod expression. The rate eventually levels off and decreases as the glucose concentration is increased further due to substrate inhibition effects. The figure also shows the effect of increasing fructose concentration. If an initial medium contains 150 g/L glucose and either 100, 200 or 300 g/L fructose, the specific growth rate will be reduced by 30, 59 and 89% respectively compared to a medium that does not contain fructose. Under the same conditions the specific ethanol production rate (ν) is reduced only 16, 31 and 47% respectively (Figure 6.56). Therefore high fructose concentrations significantly inhibits growth while ethanol production is affected to a smaller degree. The same observations can be made for the inhibition by ethanol.

In order to produce a syrup with a high concentration of fructose and ethanol the inhibitory effects on the growth rate must be taken into consideration. Several schemes can be envisaged in order to reduce these inhibitory effects. The first involves the growth of a large amount of biomass in a medium containing a low substrate concentration. The biomass is then transferred to a medium containing high glucose and fructose concentrations for rapid ethanol production. Another scheme that takes advantage of a high biomass concentration for ethanol production involves the use of immobilized cells. In this process the biomass can be retained in a reactor so that it can be used on a continuous basis. This process enables the retention a high concentration of biomass which can be used for the continuous production of ethanol. Lastly, the inhibitory effects can be reduced by using a fed-batch process. In this process high concentrations of products can be produced by adding the

substrate in batches or continuously (Koshimizu et al., 1984).

Based upon these recommendations further tests are carried out batchwise, continuously using immobilized cells and semicontinuously (fed-batch). Since the final product is to be used for human consumption these tests will also evaluate different food-grade glucose/fructose mixtures for the production of fructose and ethanol.

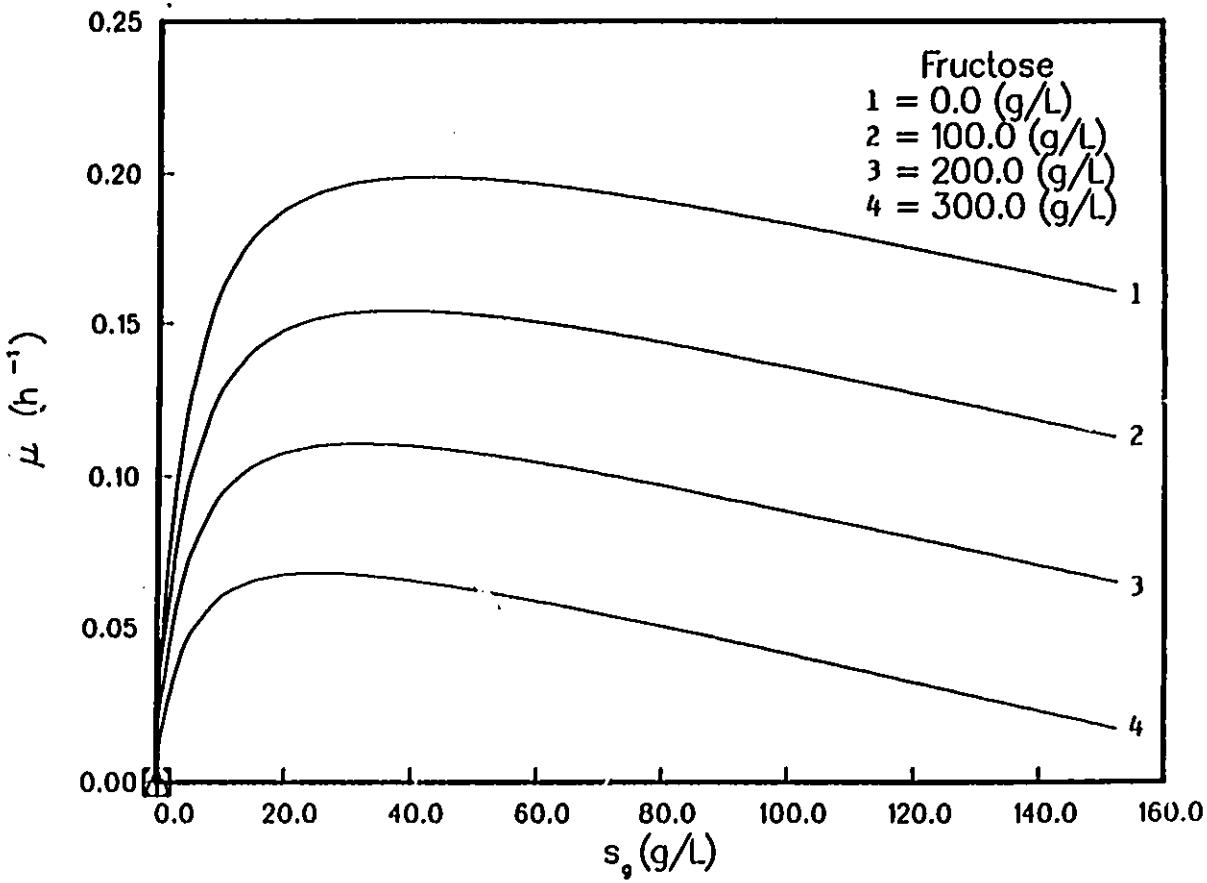

Figure 6.55: Predicted specific growth rate for the mutant strain of *S. cerevisiae* as a function of glucose concentration at various fructose concentrations

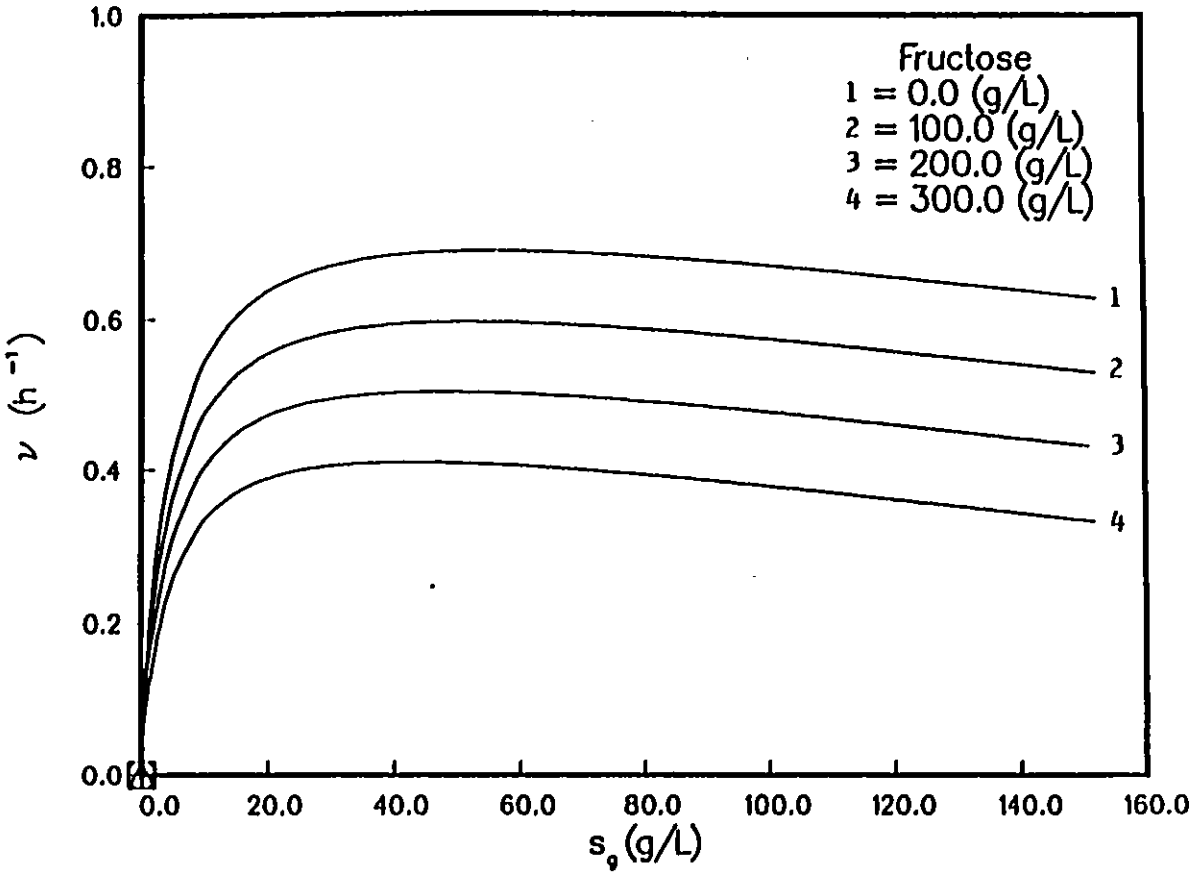

Figure 6.56: Predicted specific ethanol production rate for the mutant strain of *S. cerevisiae* as a function of glucose concentration at various fructose concentrations

6.4 Batch Production of Ethanol and Fructose from HJAJ and/or HFCS

The objective of this work is to use *S. cerevisiae* ATCC 36859 to produce pure fructose syrup and ethanol from food grade glucose/fructose mixtures. These mixtures are to be relatively inexpensive and readily available. Two materials which meet these criteria are hydrolyzed Jerusalem artichoke juice (HJAJ) and High Fructose Corn Syrup (HFCS). The juice contains nutrients other than carbohydrates that support growth and ethanol fermentation by the yeast while HFCS contains only carbohydrates (glucose and fructose).

6.4.1 Hydrolyzed Jerusalem artichoke juice

Hydrolyzed Jerusalem artichoke juice has been used for the production of ethanol (Kosaric et al., 1985) or fructose (Fleming and GrootWassink, 1979). In this study it is used for the simultaneous production of ethanol and fructose. Since the glucose concentration in hydrolyzed juice is low ($\approx$ 40 g/L) the ethanol concentration produced by its fermentation will also be low, too low for economic recovery. To increase the final ethanol concentration, additional glucose is added to the juice in some cases.

Results of tests carried out with Jerusalem artichoke juice are shown in Figures 6.57, 6.58 and 6.59 and in Table 6.15 (Duvnjak and Koren, 1987). With an initial biomass concentration of 0.27 g/L the glucose in hydrolyzed Jerusalem artichoke juice (39.2 g/L) is fermented to 17.6 g/L ethanol (71% of the theoretical yield) in 27 hours (Figure 6.57). The specific growth rate is 0.101 h^{-1} and the biomass yield is 0.073 g/g. The fructose was left unconverted by the yeast. When the initial biomass was increased from 0.27 to 0.47 g/L (Figure 6.58) the glucose was eliminated from the broth in 22 hours. During this time the biomass increased to 3.4 g/L (biomass yield 0.077 g/g) and 17.8 g/L ethanol was produced. The fructose concentration stayed relatively constant, the final broth contained 122 g/L fructose. A further increase in the initial biomass concentration to 1.4 g/L resulted in the production of a broth containing 17.8 g/L ethanol (84% of the theoretical yield) in 14.5 hours (Figure 6.59). The biomass concentration increased to 4.7 g/L which represents a

biomass yield of 0.087 g/g.

By increasing the initial biomass concentration from 0.27 to 1.4 g/L, the ethanol productivity increased from 0.54 to 1.07 g/Lh and the glucose consumption increased from 1.29 to 2.50 g/Lh (Table 6.15). The reduced fermentation time resulted in a smaller fraction of the carbohydrate being used for maintenance energy which in turn results in higher fractions being used for ethanol and biomass production. Due to this the ethanol and biomass yields increased 35 and 19% respectively. The specific growth rate is virtually unchanged by increases in the biomass concentration.

Table 6.15: Biomass and ethanol yields and specific growth and ethanol production rates for fermentations in HJAJ.

Initial glu-fru (g/L)	Initial biomass (g/L)	Biomass yield[a] (g/g)	Ethanol yield[a] (g/g)	Specific growth rate (h^{-1})	P1[b] (g/Lh)	P2[c] (g/Lh)
39.2-135.9	0.27	0.073	0.361	0.101	1.29	0.54
37.8-124.5	0.47	0.077	0.424	0.090	1.62	0.69
37.8-141.9	1.40	0.087	0.428	0.103	2.50	1.07

[a] Yields were calculated at maximum biomass and ethanol concentrations

[b] glucose consumption rate at 0-80% carbohydrate conversion

[c] ethanol production rate at 0-80% carbohyrate conversion

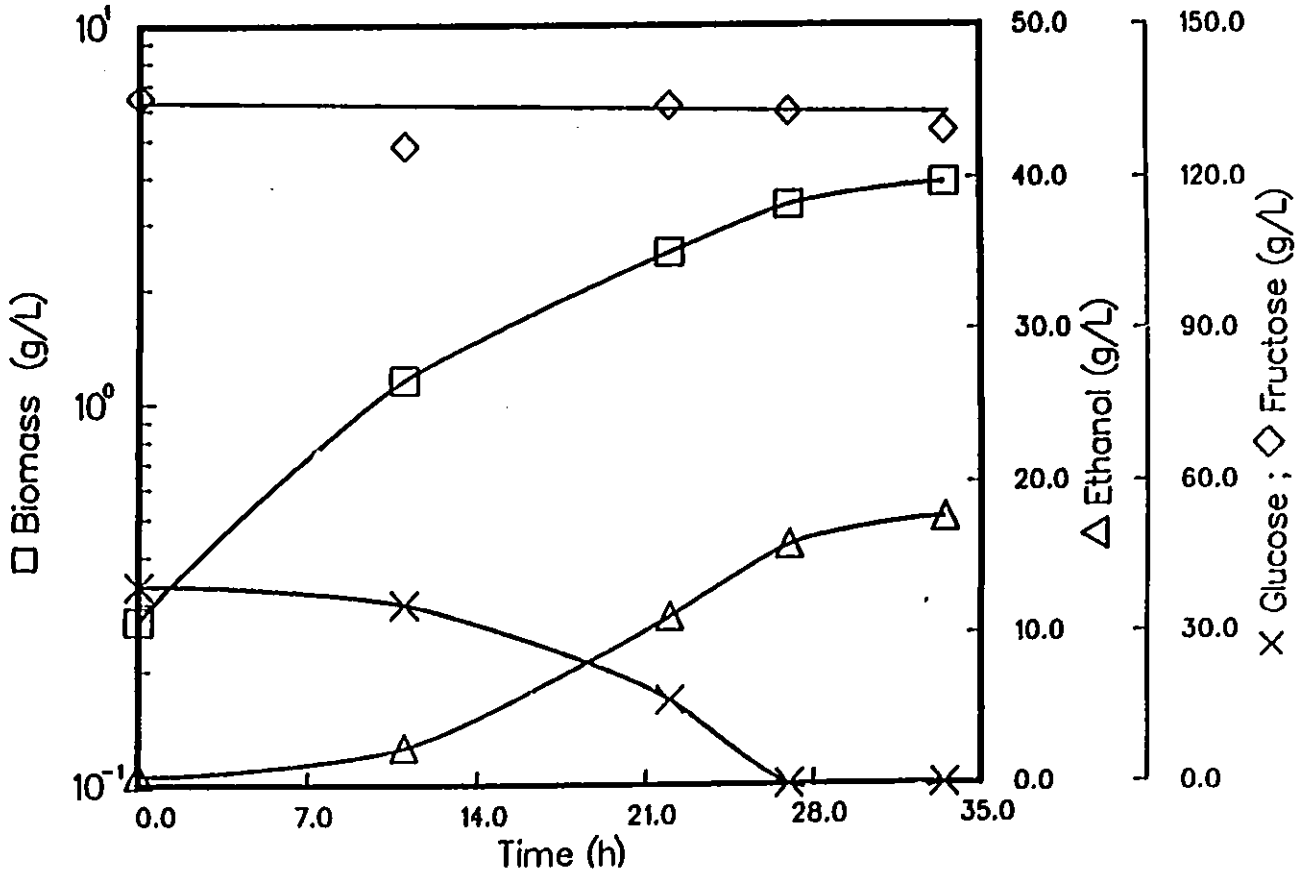

Figure 6.57: Batch fermentation of hydrolyzed Jerusalem artichoke juice using an initial biomass concentration of 0.27 g/L.

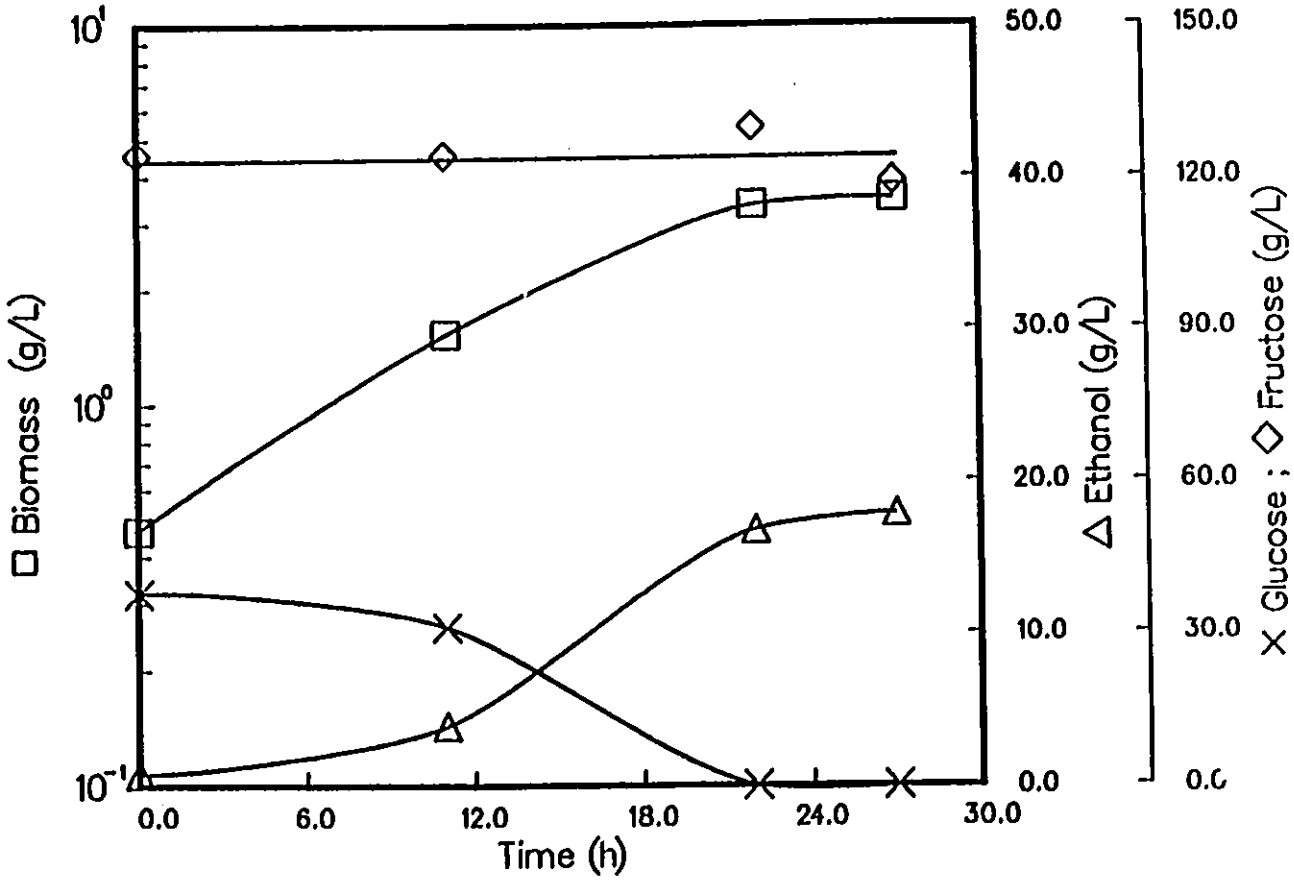

Figure 6.58: Batch fermentation of hydrolyzed Jerusalem artichoke juice using an initial biomass concentration of 0.47 g/L.

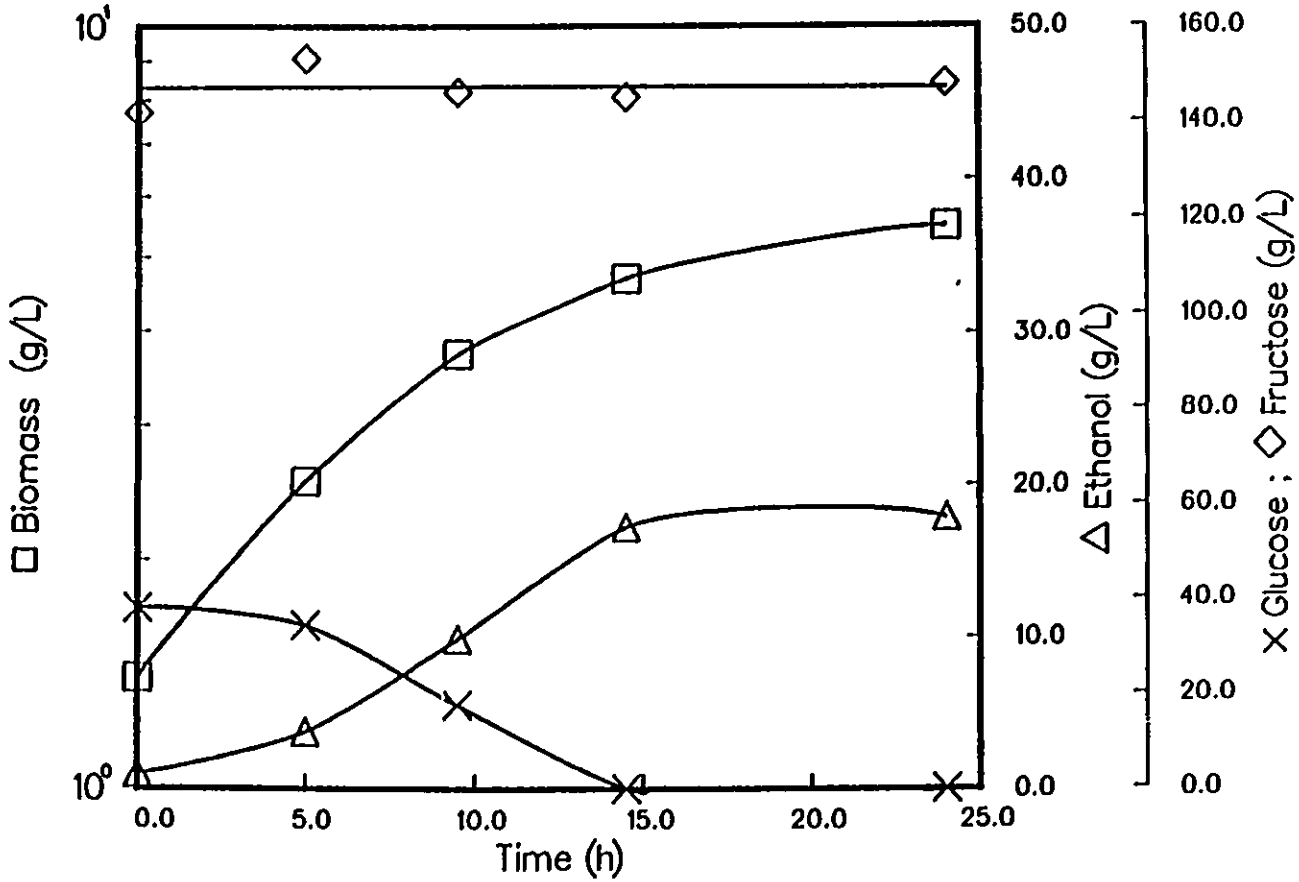

Figure 6.59: Batch fermentation of hydrolyzed Jerusalem artichoke juice using an initial biomass concentration of 1.40 g/L.

Although glucose was entirely consumed, the ethanol concentration was too low in these cases for it to be recovered economically. In order to increase the final concentration of ethanol in the broth, glucose was added to the hydrolyzed juice. Tests were carried out with batches of juice in which the final glucose concentrations were increased to 73.2, 126.7 and 157.9 g/L. The results are shown in Figures 6.60 and 6.61 and in Table 6.16. In Figure 6.60, 73.2 g/L glucose is consumed by the yeast in 24 hours. During this time the biomass increased from 1.4 to 5.8 g/L and 32.0 g/L ethanol was produced (83% of the theoretical yield). The specific growth rate was reduced to 0.089 h^{-1} compared to 0.103 h^{-1} which was measured in pure juice (Table 6.16). When the initial glucose concentration was increased to 126.7 g/L, the fermentation time was increased to 57.5 hours (Figure 6.61). The specific growth rate was 0.059 h^{-1}, while the biomass concentration increased to 5.1 g/L which is a biomass yield of 0.030 g/g. The final ethanol concentration was 49.0 g/L. A further increase in the glucose concentration to 157.9 g/L resulted in a final ethanol concentration of 56.4 g/L. In this case the specific growth rate was reduced to 0.069 h^{-1} while the biomass yield decreased to 0.028 g/g.

As expected, an increase in the glucose concentration from 38 to 158 g/L results in higher final ethanol concentrations. The higher total carbohydrate concentrations though result in lower specific growth rates and biomass yields (Table 6.16). These trends have also been reported by Margaritis and Bajpai (1983) using *Kluyveromyces* species. They reported that the specific growth rate fell from 0.44 to 0.13 as the total carbohydarate concentration was increased from 50 to 300 g/L. The biomass yields in that case fell from 0.07 to 0.01 for the same increase. The glucose consumption and ethanol production rates increased by 18 and 23% respectively when the glucose concentration was increased from 37.8 to 73.2 g/L (Table 6.16). A further increase in the glucose concentration to 126.7 g/L results in a 21 and 29% reduction in the rates. When the medium contains 157.9 g/L glucose the ethanol productivity is reduced by an additional 15%. The specific growth rates and the inhibitory effects of the total carbohydrate concentration are similiar to those in synthetic media which contain 3.0 g/L yeast extract (Figure 6.30).

Instead of adding glucose to the juice to increase the ethanol concentration in the final

juice. HFCS which is a food grade material can be added. in this manner both the ethanol and fructose concentration in the final product can be increased. The results of these tests are discussed in the next section.

Table 6.16: Biomass and ethanol yields and specific growth and ethanol production rates for fermentations in media containing HJAJ supplemented with glucose.

Initial glu-fru (g/L)	Initial biomass (g/L)	Biomass yield[a] (g/g)	Ethanol yield[a] (g/g)	Specific growth rate (h^{-1})	P1[b] (g/Lh)	P2[c] (g/Lh)
73.2-144.3	1.37	0.061	0.425	0.089	2.96	1.32
126.7-139.5	1.22	0.030	0.377	0.059	2.34	0.94
157.9-140.1	0.84	0.028	0.413	0.069	1.93	0.80

[a] Yields were calculated at maximum biomass and ethanol concentrations

[b] glucose consumption rate at 0-80% carbohydrate conversion

[c] ethanol production rate at 0-80% carbohydrate conversion

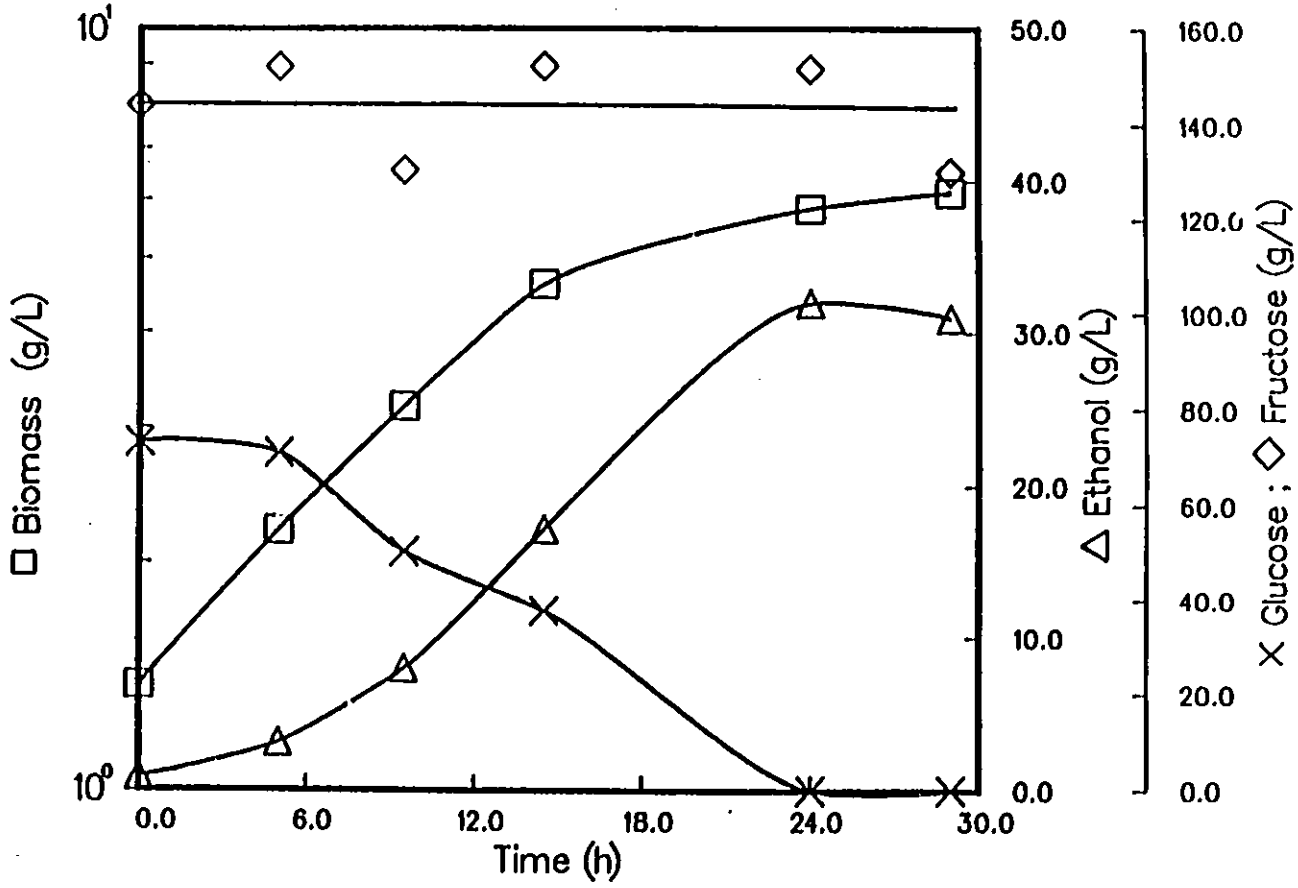

Figure 6.60: Batch fermentation of hydrolyzed Jerusalem artichoke juice and added glucose to 73.2 g/L.

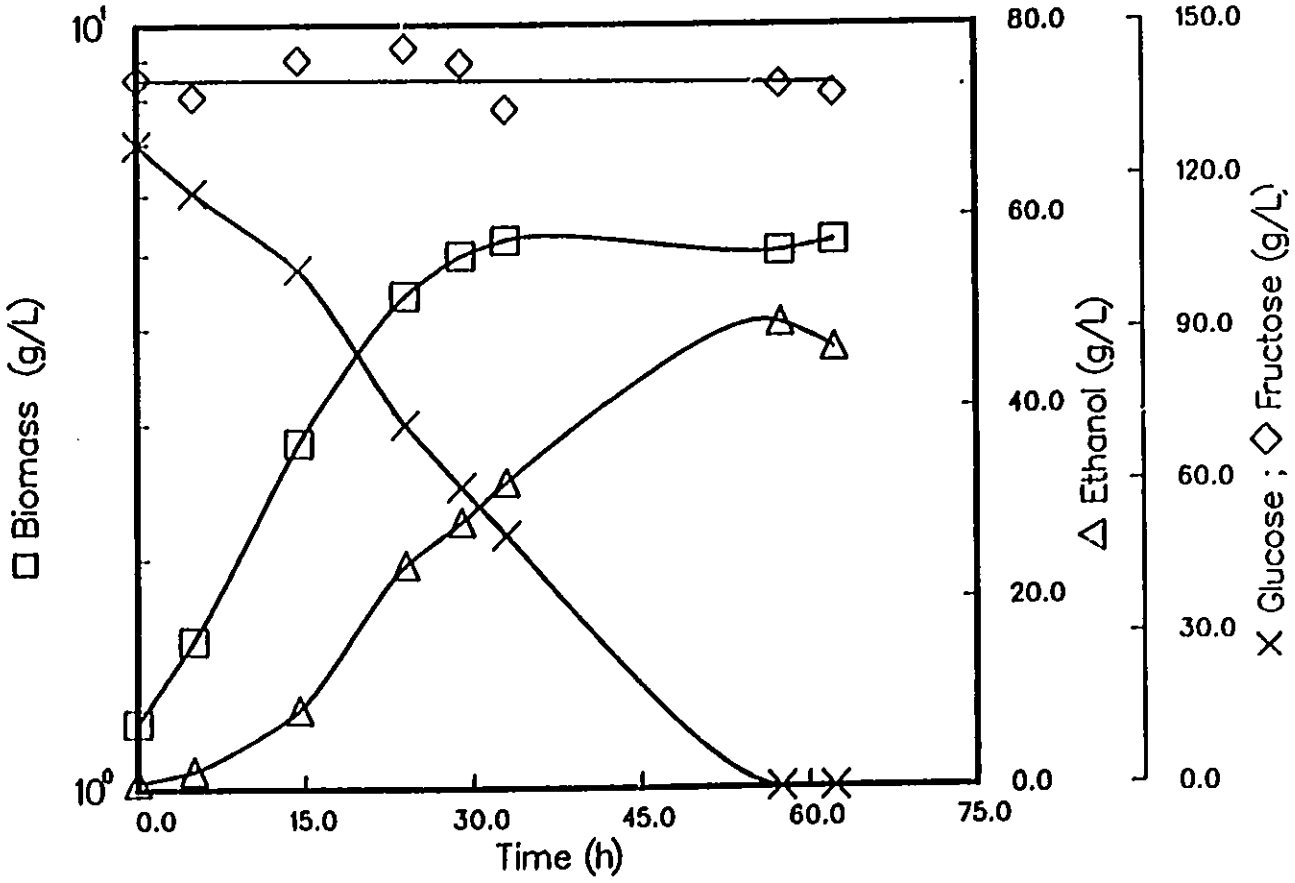

Figure 6.61: Batch fermentation of hydrolyzed Jerusalem artichoke juice and added glucose to 126.7 g/L.

6.4.2 Hydrolyzed Jerusalem artichoke juice and High Fructose Corn Syrup

The most widely available glucose/fructose mixtures are High Fructose Corn Syrup (HFCS) and invert sugar. In this study 42 HFCS is used as a carbon source for the yeast. It is diluted so that the glucose concentration was approximately 100 g/L. To support growth of the yeast in the diluted syrup, hydrolyzed Jerusalem artichoke juice (HJAJ) is used because it has been shown to contain nutrients for growth and ethanol production.

Three concentrations of juice were tested, 5, 15 and 25 %v/v. The biomass and ethanol yields and the specific growth and ethanol production rates that were measured are provided in Table 6.17 (Koren and Duvnjak, 1990). The results of a fermentation carried out in medium containing 5 %v/v HJAJ and 22 %v/v HFCS are shown in Figure 6.62. The medium contained 107.8 g/L glucose and 81.5 g/L fructose. The glucose was consumed in 68 hours, during this time the biomass concentration was increased to 4.3 g/L and 34.4 g/L of ethanol (62% of the theoretical yield) was produced. When the hydrolyzed juice concentration was increased to 15 %v/v the initial medium contained 111.4 g/L glucose and 101.8 g/L fructose, with 25 %v/v juice the medium contained 115.7 g/L glucose and 118.6 g/L fructose (Table 6.17). Bearing in mind that the initial total carbohydrate concentrations differ by about 20%, the time taken to convert the first 80 g/L glucose was used as the basis for a comparison of the results (Table 6.17). This time was 46 hours when the medium contained 5 %v/v juice, it was reduced to 31 hours when the medium contained 25 %v/v juice. A reduction in the process time with an increase in the juice concentration is obvious. An increase in the juice concentration is also beneficial from the point of view of the ethanol yield; the ethanol yield in the medium with 15 %v/v juice was 12% higher than that measured in a medium containing 5 %v/v juice. A further increase in the juice concentration increased the ethanol yield by only 2%. The specific growth rate was reduced by 21% when the juice concentration was increased to 15 %v/v. This was caused by the increased total carbohydrate concentration present in the latter instance. As the juice content is increased, the fermentation time is reduced and the ethanol and biomass yields are increased as would be expected by an increase in the nutrient concentration.

Another factor that should be taken into consideration is the appearance of the product. Since fructose syrups must be colourless, subsequent purification steps are necessary. Jerusalem artichoke juice contains a number of colour producing compounds (Fleming and GrootWassink; 1979) that are difficult to remove, thus lower concentrations of juice would reduce purification costs. Taking this into account as well as the fact that 5 %v/v juice is sufficient to support total glucose conversion, this concentration would be recommended for the simultaneous production of fructose syrup and ethanol. To increase the ethanol productivity using this concentration of juice, the initial biomass concentration was increased tenfold. The results are shown in Figure 6.63 and in Table 6.17. The increase in inoculum resulted in a decrease in the process time from 60 hours (Figure 6.62) with the previous lower inoculum concentration to 11 hours in this test (Figure 6.63). The increase in inoculum also resulted in a higher ethanol yield. In the present test 46.6 g/L of ethanol was produced which is 90% of the theoretical yield while previously only 34.1 g/L of ethanol was produced (62% of the theoretical yield). The biomass yield was also increased in the present instance. Due to the shorter fermentation time, the amount of carbohydrate used as a source of maintenance energy was greatly reduced, resulting in an increase in the ethanol yield. The ethanol productivity was increased from 0.58 to 4.22 g/Lh.

Table 6.17: Biomass and ethanol yields and specific growth and ethanol production rates for fermentations carried out in media containing HJAJ and HFCS

Substrate (%v/v)	Initial glu-fru (g/L)	Biomass yield[a] (g/g)	Ethanol yield[a] (g/g)	Specific growth rate (h^{-1})	Fermentation time[b] (h)	P2[c] (g/Lh)
HJAJ(5) + HFCS(22)	107.8-85.1	0.028	0.316	0.094	46	0.58
HJAJ(15) + HFCS(22)	111.4-101.8	0.034	0.353	0.073	36	0.78
HJAJ(25) + HFCS(22)	115.7-118.6	0.034	0.364	0.072	31	0.97
*HJAJ(5) + HFCS(22)	101.6-84.7	0.037	0.458	0.027	9	4.22

[a] Yields were calculated at maximum biomass and ethanol concentrations

[b] time required to reduce glucose concentration by 80 g/L

[c] ethanol production rate at 0-80% carbohydrate conversion

* high initial inoculum concentration

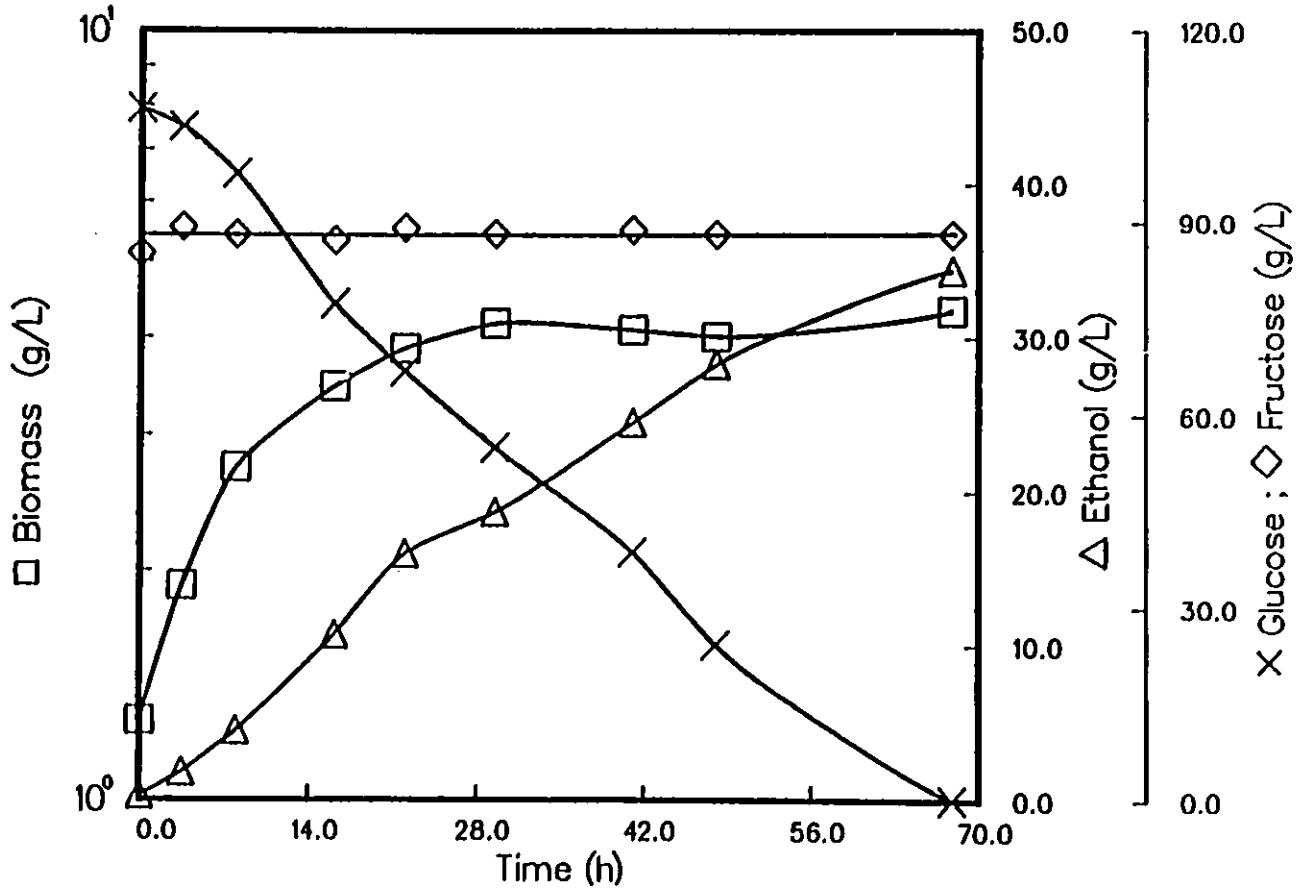

Figure 6.62: Batch fermentation in a medium containing 5%v/v hydrolyzed Jerusalem artichoke juice, 22%v/v High Fructose Corn Syrup and 1.3 g/L biomass.

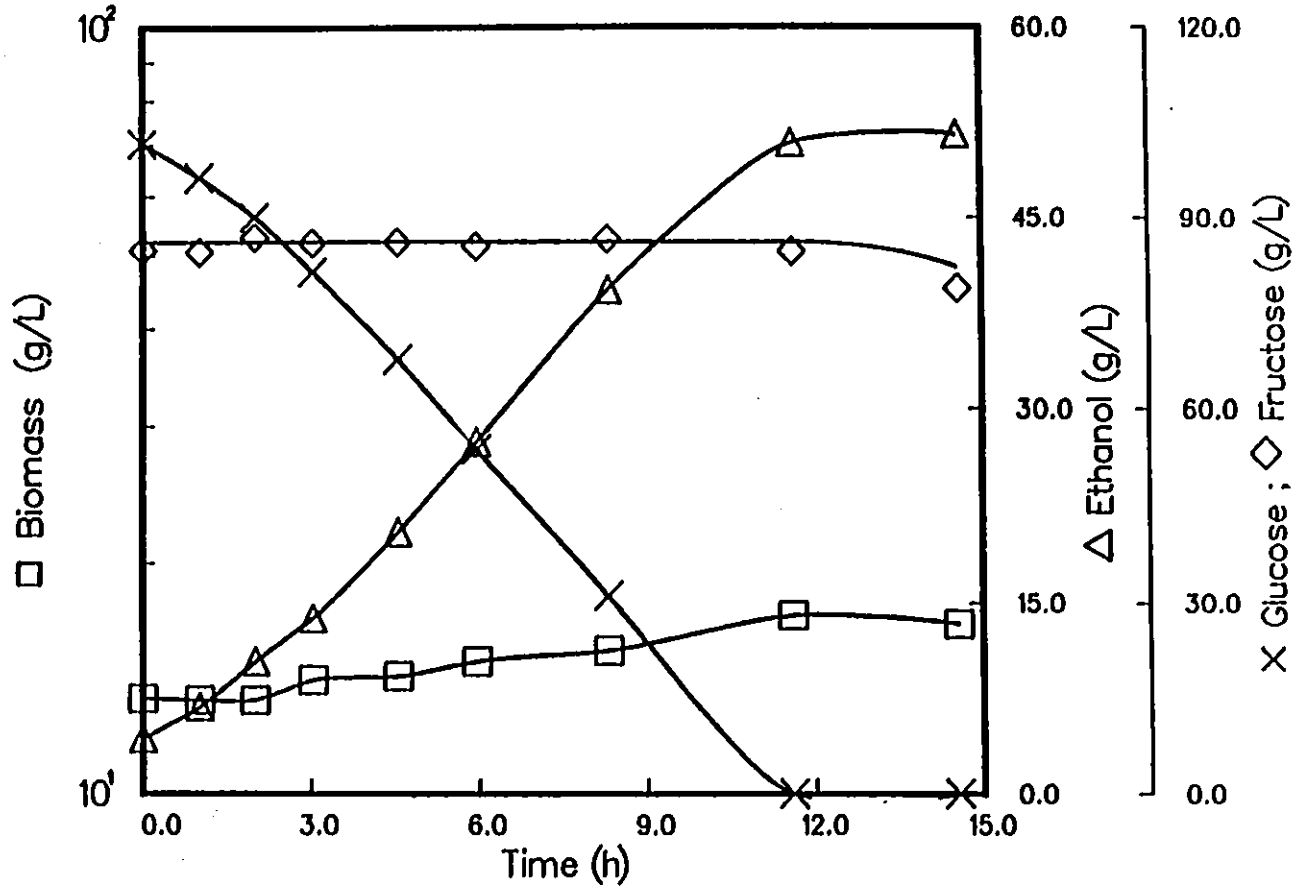

Figure 6.63: Batch fermentation in a medium containing 5%v/v hydrolyzed Jerusalem artichoke juice, 22%v/v High Fructose Corn Syrup and 13 g/L biomass.

To increase the ethanol concentration in the final product the HFCS content of the medium was increased to 60 %v/v. A larger amount of nutrients are required for this conversion therefore the HJAJ content was doubled to 10 %v/v. In order to increase the rate of fermentation, the initial biomass concentration was increased to 94 g/L. This large inoculum was grown in a medium containing a low glucose concentration in order to reduce substrate inhibitory effects. The results are shown in Figure 6.64 and in Table 6.18. From an initial medium of 183.3 g/L glucose and 165.2 g/L fructose a product containing 81.4 g/L ethanol was formed in 4.3 hours. Taking into account the fact that 8.0 g/L ethanol was transferred with the inoculum, the ethanol yield was 78% of the theoretical yield. The ethanol productivity was 21.1 g/Lh which is comparable to the productivity reported by Ghose and Tyagi (1979a) using the wild strain of *S. cerevisiae* (Table 2.3).

When the fermentation was completed, the biomass was separated from the spent medium by centrifugation and used again in fresh medium. Although all of the biomass was transferred to the new medium the biomass concentration was lower. This was due to loss of biomass as a result of samples that were taken during the first fermentation. The initial medium contained 77.4 g/L biomass, 185.4 g/L glucose, 165.2 g/L fructose and 15.3 g/L ethanol. After 9.4 hours the ethanol concentration was increased to 98.2 g/L (Figure 6.65) which represents a yield of 82% of the theoretical yield. The biomass concentration remained constant througout the experiment. When the lower biomass concentration is taken into consideration the ethanol productivity is still 40% lower when it was reused.

Table 6.18: Batch ethanol and fructose production from 60 %v/v HFCS and 10 %v/v HJAJ.

Initial glu-fru (g/L)	Initial biomass (g/L)	Ethanol yield[a] (g/g)	P1[b] (g/Lh)	P2[c] (g/Lh)	Max. ethanol (g/L)
183.3-165.2	93.7	0.400	52.2	21.1	81.4
185.4-165.2	77.4	0.420	27.9	10.1	98.2

[a] Yield was calculated at maximum ethanol concentration

[b] glucose consumption rate at 0-80% carbohydrate conversion

[c] ethanol production rate at 0-80% carbohydrate conversion

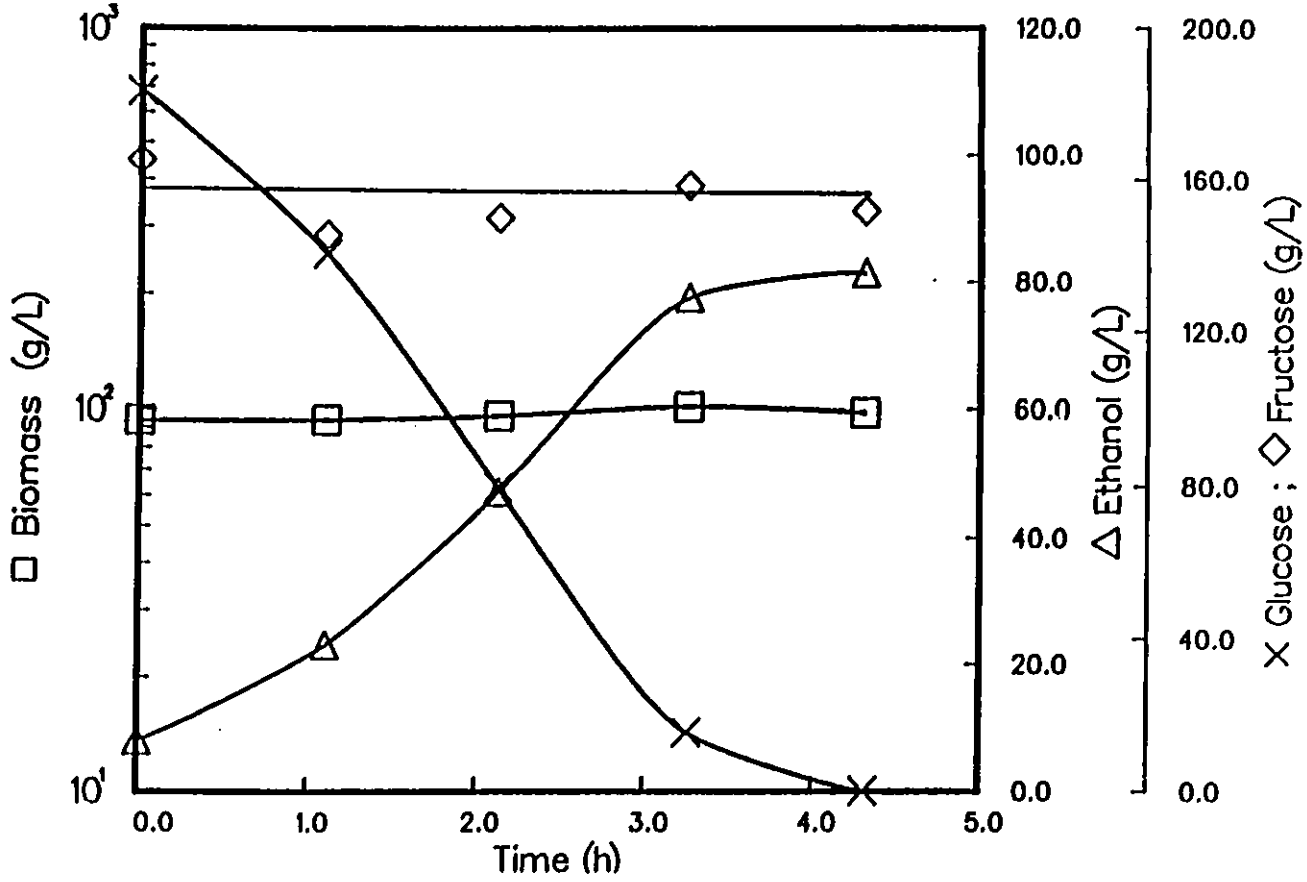

Figure 6.61: Batch fermentation of hydrolyzed Jerusalem artichoke juice and High Fructose Corn Syrup which contains 183 g/L glucose and 165 g/L fructose.

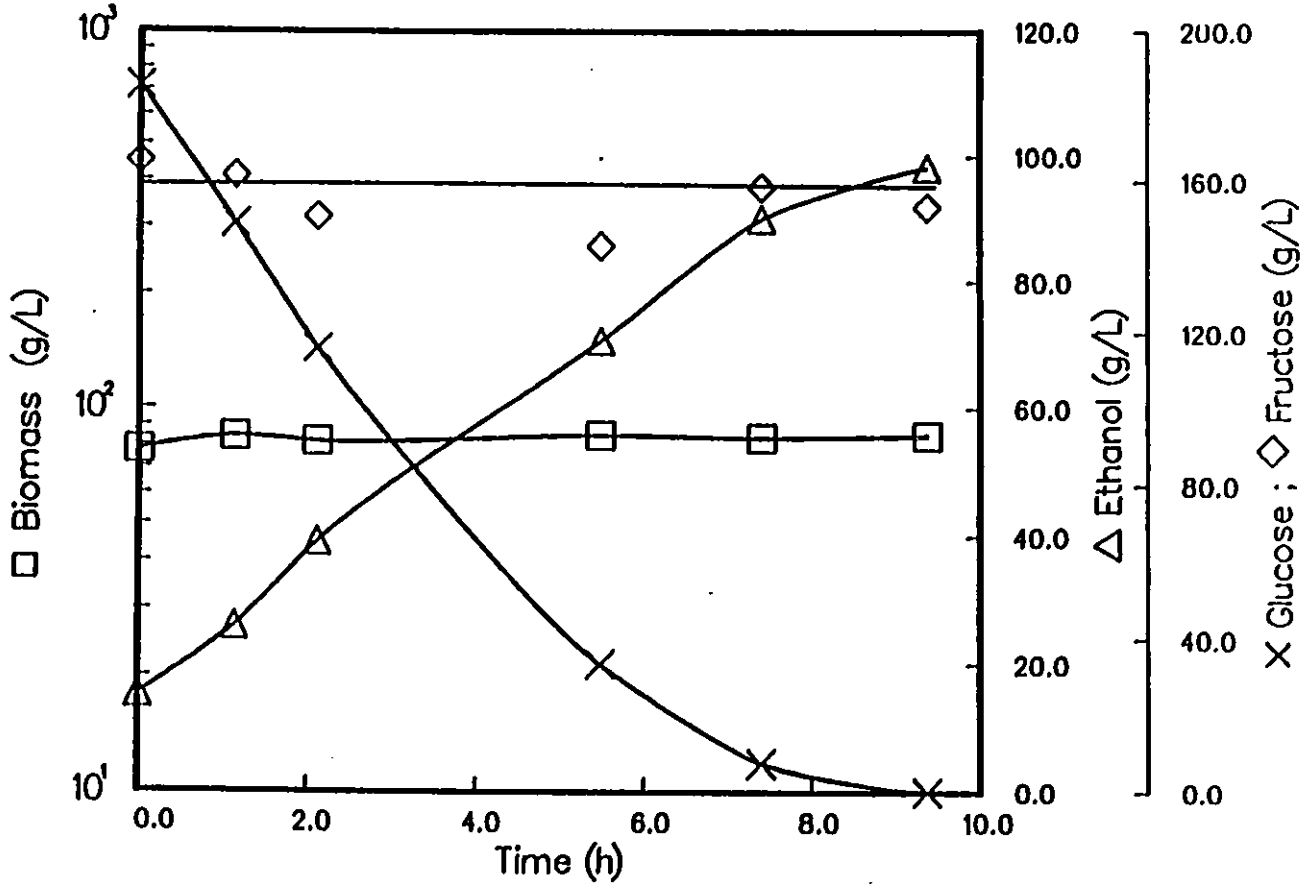

Figure 6.65: Batch fermentation of hydrolyzed Jerusalem artichoke juice and High Fructose Corn Syrup which contains 185 g/L glucose and 165 g/L fructose (reused biomass).

The results shown indicate that food grade glucose/fructose mixtures can be used for the production of pure fructose and ethanol by selective fermentation of glucose. An ethanol productivity of ≈ 1.0 g/Lh can be expected when the initial biomass concentration is ≈ 1.7 g/L. Higher ethanol productivities can be attained when the biomass concentration is increased. An increase in the initial glucose concentration in the juice results in higher final ethanol concentrations but the rate of fermentation is reduced due to inhibitory effects. An ethanol productivity of 21 g/Lh and a final ethanol concentration of 81.4 g/L was reached using an initial biomass concentration of 93.6 g/L. The reuse of this biomass results in a 40% reduction in the productivity. This loss in activity was due to the high ethanol concentration that were reached in the first fermentation.

It has been reported that the effects of inhibitory compounds can be reduced by immobilization (Margaritis and Merchant, 1984). This technique also permits the maintenance of high cell concentrations in a reactor which can operate on a continuous basis, thereby increasing its productivity. For these reasons the production of pure fructose and ethanol using immobilized cells will be studied.

6.5 Continuous Production of Ethanol and Fructose using Immobilized Cells

Yeast cells were grown in a fermenter and immobilized in Ca-alginate beads. The beads were placed in the reactor which was then fed with synthetic media (medium IV) or HJAJ and HFCS on a continuous basis.

6.5.1 Synthetic feed

In this set of tests the beads contained 55 g biomass/L of bead. To determine the yeast's capability to convert glucose to ethanol in a continuous process the reactor was fed with a glucose medium at various dilution rates (Figure 6.66 and Table 6.19) (Koren and Duvnjak, 1989). At a dilution rate of 0.106 h^{-1} the glucose concentration was reduced from 103.3 g/L in the feed to 1.1 g/L in the product. The ethanol concentration reached a value of 50.5 g/L. Increasing the dilution rate from 0.106 to 0.254 h^{-1} had little or no effect on the amount of glucose or ethanol in the product. When the dilution rate was increased to 0.366 h^{-1} the glucose concentration in the product increased substantially to 18.4 g/L while the ethanol was reduced to 41.3 g/L (Table 6.19). The ethanol yield varied from 97 to 95% of the theoretical value from the lowest to the highest dilution rates.

Table 6.19: Product concentrations from the immobilized cell reactor using a feed containing 103 g/L glucose.

Dilution rate (h^{-1})	Final glucose[a] (g/L)	Final ethanol[a] (g/L)	Ethanol yield (g/g)
0.106	1.1	50.5	[illegible]
0.180	1.3	50.2	0.493
0.254	1.8	50.1	0.494
0.366	18.4	41.3	0.487

[a] Steady state values are average of two or three samples

Table 6.20: Product concentrations from an immobilized cell reactor using a feed containing 101 g/L glucose and 98 g/L fructose

Dilution rate (h^{-1})	Final glucose[a] (g/L)	Final fructose[a] (g/L)	Final ethanol[a] (g/L)	Fructose yield (%)
0.106	0.7	83.3	50.4	85
0.180	4.0	95.2	49.9	97
0.254	13.9	97.2	43.4	100
0.366	32.9	98.0	34.5	100

[a] Steady state values are average of two or three samples

After 148 hours of the reactor's operation, the feed solution was changed to one containing 100.7 g/L glucose and 97.7 g/L fructose (Figure 6.67 and Table 6.20) (Koren and Duvnjak, 1989). The results show that at a dilution rate of 0.106 h^{-1}, a product containing fructose (83.3 g/L) and ethanol (50.4 g/L), with only a small amount of glucose (0.7 g/L) was produced. As expected, the glucose concentration increased and the ethanol concentration decreased with increases in the dilution rate. At the same time the fructose concentration in the product increased to its inlet value; a fructose yield of 100% was measured at dilution rates of 0.254 and 0.366 h^{-1}.

The effect of feed composition and dilution rate on the ethanol productivities and glucose conversions are shown in Figure 6.68. With only glucose in the feed, the conversion remained above 98% for dilution rates up to and including 0.254 h^{-1}. The glucose conversion decreased to 82% with an increase to 0.366 h^{-1}. Ethanol productivity increased as the dilution rate increased; the maximum productivity attained was 15.1 g/Lh at a dilution rate of 0.366 h^{-1}. Using *S. cerevisiae* NRRL Y135 immobilized on alginate beads, Cho et al. (1981) reported a productivity of 12.2 g/Lh and a conversion of 70% at a dilution rate of 0.35 h^{-1} with a 100 g/L glucose feed solution. The results reported by Godia et al. (1987) using carrageenan beads and a glucose medium, are simliar to those obtained in this work. The performance of the immoblized cells in this work with a 100 g/L glucose feed are thus

comparable to those reported using *S. cerevisiae* strains which ferment both glucose and fructose equally.

When the glucose feed was replaced by a glucose-fructose one, the productivity and glucose conversion was not affected at dilution rates of 0.106 and 0.180 h^{-1}. From the results it can be speculated that at these dilution rates the process was likely to be diffusion controlled (i.e. transport of reactants or products between the bulk solution and the yeast was the slowest step). Since the glucose concentration was not changed, the driving force for the diffusion was not changed therefore the rate was unaffected. With an increase in the dilution rate the presence of fructose reduced productivity and conversion (Figure 6.68). The increased turbulence in this latter case would reduce the importance of diffusion and increase control by the fermentation reaction. As was shown previously rates of glucose consumption and ethanol production decrease as the total carbohydrate concentration is increased. This is caused by the higher osmotic pressure present in the medium in the latter case (Stewart et al., 1983).

In the continuous system some fructose was consumed by the mutant yeast cells (Table 6.20) in contrast to the previous batch results where no fructose was consumed from glucose/fructose mixtures. The consumption appears to be dependent on the dilution rate. The rate of fructose consumption is the highest at the smallest dilution rate while it is reduced to near zero at the highest dilution rates. This phenomenon may be related to the concentration of glucose in the outlet stream. As was shown previously, significant fructose consumption took place when it was the only carbohydrate available to the yeast (Table 6.3). It is clear therefore that the reactor must be run at less than 100% glucose conversion in order to avoid fructose consumption. In the batch system, there was no fructose consumption because the glucose concentration was low only for a short time at the end of the experiment.

A continuous process for ethanol production which employs the mutant strain of *S. cerevisiae* is comparable to processes which use the wild strain of *S. cerevisiae*. A syrup containing fructose as 99% of the reducing sugars was produced from a 101 g/L glucose and 98 g/L fructose feed at a dilution rate of 0.106 h^{-1}. When the dilution rate is increased to

0.366 h^{-1} the ethanol productivity was 12.7 g/Lh which is 16% less than the value attained in a similar medium containing only glucose.

The feasibilty of this process was then tested on a medium containing food grade glucose/fructose mixtures.

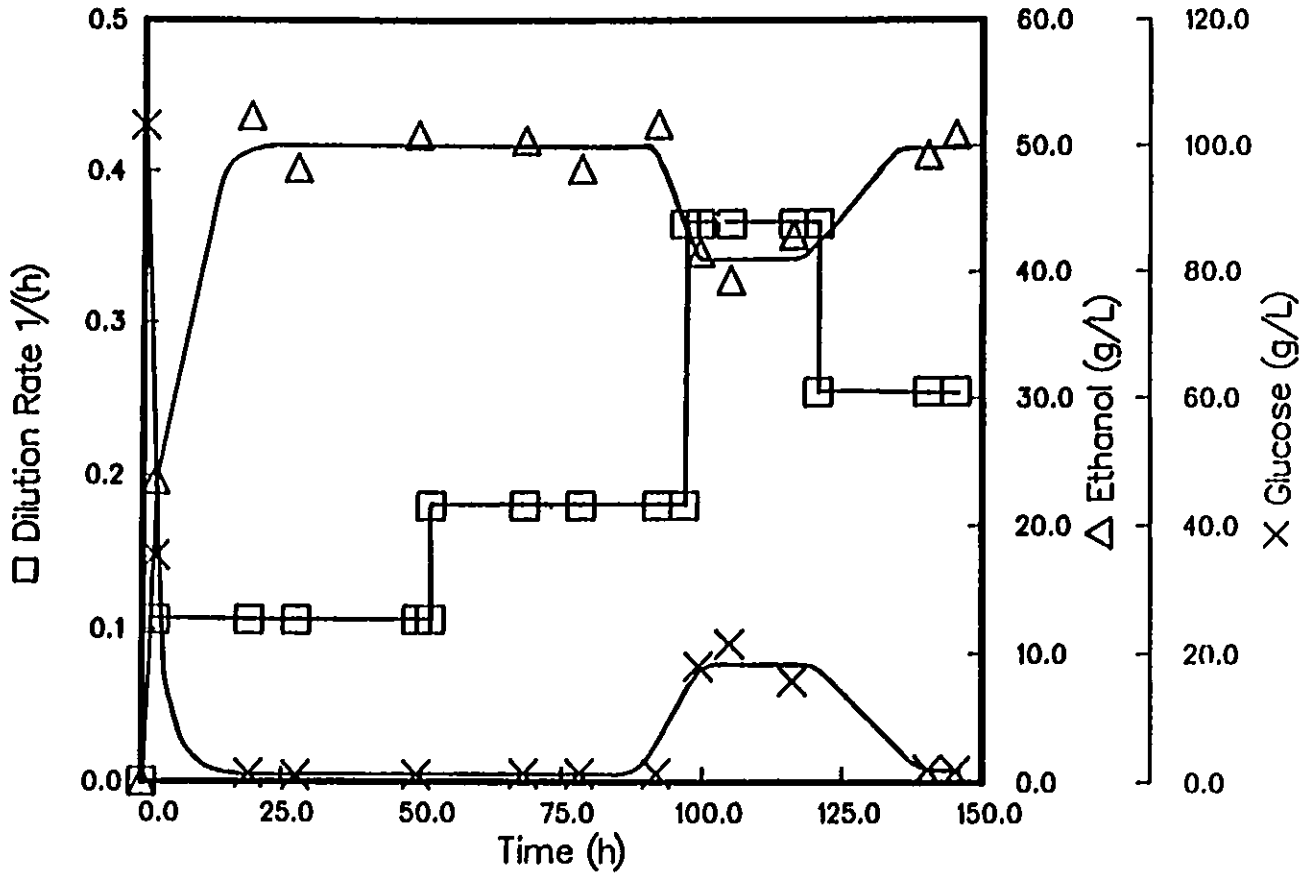

Figure 6.66: Ethanol production using an immobilized cell reactor with feed containing 103 g/L glucose

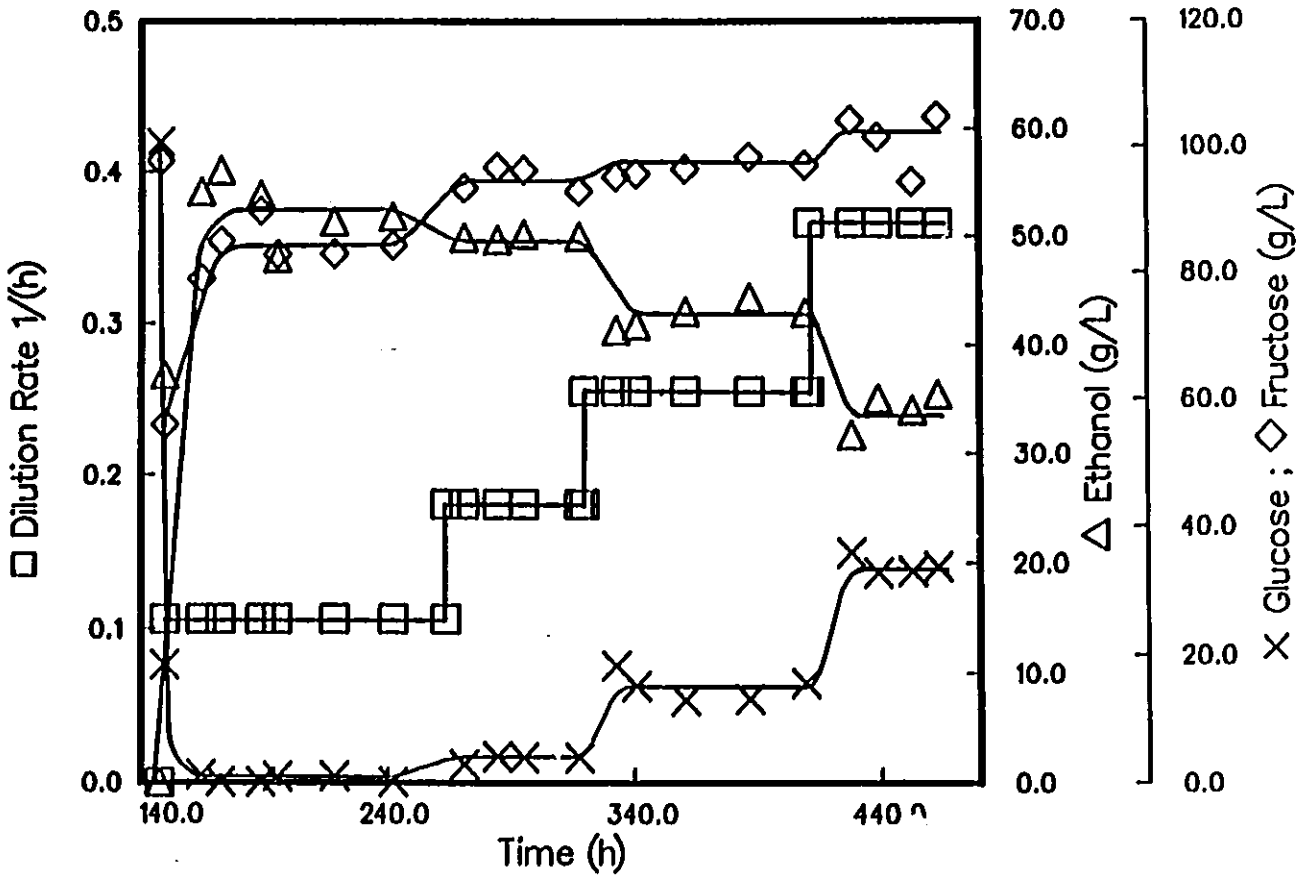

Figure 6.67: Ethanol production using an immobilized cell reactor with feed containing 101 g/L glucose and 98 g/L fructose

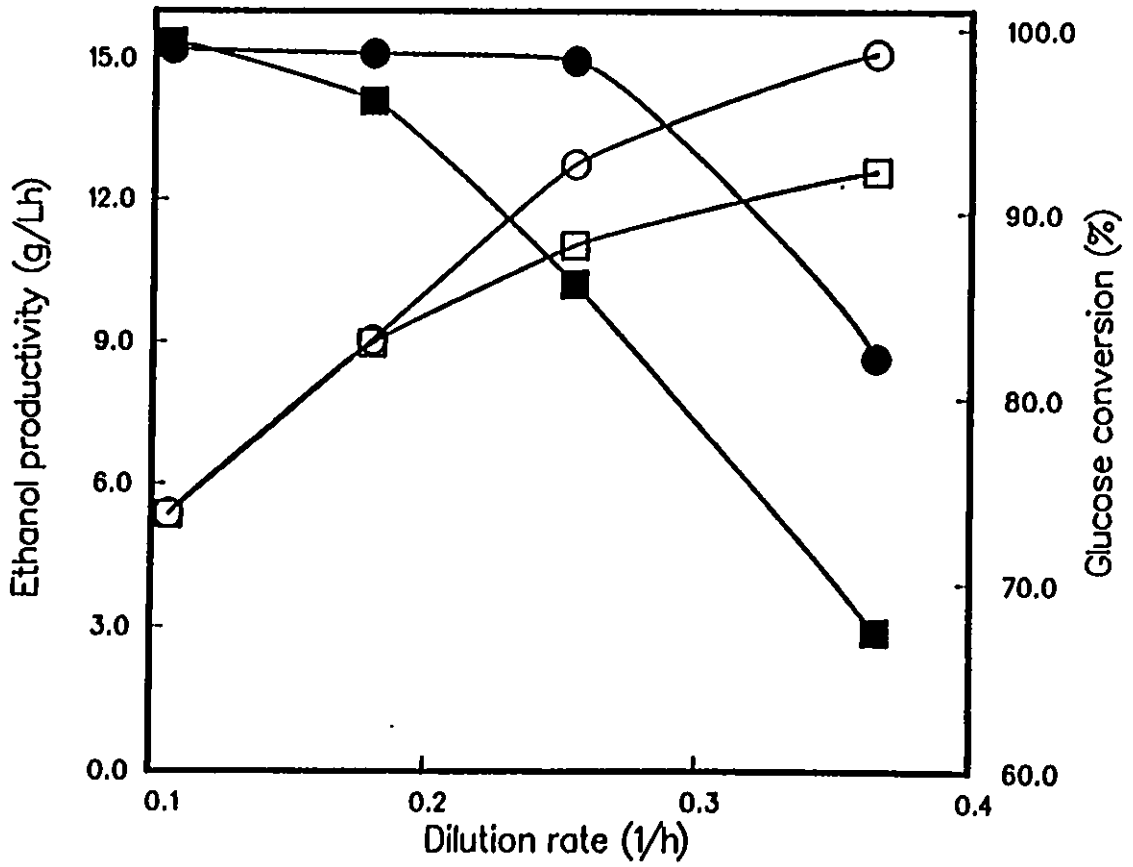

Figure 6.68: Ethanol productivity and glucose conversion as a function of dilution rate. Ethanol productivity (○) and glucose conversion (●) for a glucose feed and ethanol productivity (□) and glucose conversion (■) for a glucose-fructose feed.

6.5.2 Food grade glucose/fructose mixtures

In order to test the feasability of this continuous process on readily available glucose-fructose mixtures, media containing HFCS and/or HJAJ were used. The beads contained 47 g/L biomass which is similar to the concentration used in the previous test.

Hydrolyzed Jerusalem artichoke juice

The performance of the immobilized cell reactor at different flow rates using a feed of pure hydrolyzed Jerusalem artichoke juice (HJAJ) is shown in Figure 6.69 and Table 6.21. At a dilution rate of 0.106 h^{-1} the glucose concentration was reduced to 1.0 g/L from 45.5 g/L, producing 25.8 g/L ethanol. The ethanol concentration was higher than it was theoretically possible to produce from the glucose used because some fructose was consumed, reducing its concentration to 132.7 g/L from 144.0 g/L. The ethanol yield when the total amount of carbohydrate consumed is taken into consideration is 89% of the theoretical value. At this dilution rate the carbohydrate content of the product was 99.3% fructose and 0.7% glucose. When the dilution rate was increased to 0.254 h^{-1} the fructose concentration approached its inlet value. The product contained 96% of the total sugars as fructose and 4% as glucose.

Tests were also carried out at other dilution rates; the whole process lasted for 420 hours, the steady state product concentrations are provided in Table 6.21. The results show that even at the highest dilution rate (0.625 h^{-1}) the product contained 93% of the reducing sugars as fructose (7% as glucose). Comparing this carbohydrate composition with that in the feed (77% fructose and 23% glucose) it can be concluded that a substantial increase in the fructose/glucose ratio can be obtained from Jerusalem artichoke juice applying this technology. Therefore, the sugar mixtures produced from these treated juices would contain much less glucose than those which could be obtained from untreated ones.

In the following set of tests, a study of the performance of the reactor at higher glucose concentrations was carried out. Attention was paid to the productivity of the reactor and to the effect of the outlet glucose concentration on the ratio of the fructose to glucose consumption rates.

Tests of the performance of the reactor at different dilution rates were carried out with

Table 6.21: Product concentrations from the immobilized cell reactor using a feed containing hydrolyzed Jerusalem artichoke juice

Feed glu-fru (g/L)	Dilution rate (h^{-1})	Final glucose[a] (g/L)	Final fructose[a] (g/L)	Final ethanol[a] (g/L)
45.5-143.3	0.106	1.0	132.7	25.7
	0.143	2.5	131.8	22.3
	0.180	3.6	133.2	21.7
	0.254	5.3	138.4	20.1
41.1-139.0	0.329	5.6	135.5	18.3
	0.477	7.9	135.9	16.2
	0.625	10.4	138.2	14.6

[a] Steady state values are average of two or three samples

Jerusalem artichoke juice supplemented with glucose to concentrations of 85 and 129 g/L. The results from these tests concerning the relationship between the dilution rates and the ethanol, glucose and fructose concentrations in the outlet stream are shown in Table 6.22. With a glucose concentration of 85 g/L in the feed, a product containing 5.3 g/L glucose, 38.0 g/L ethanol and 121.6 g/L fructose was produced at a dilution rate of 0.106 h^{-1} (Table 6.22). When the glucose concentration was increased to 128.7 g/L, the outlet glucose concentration increased to 48.6 g/L at the same dilution rate. In the latter case the outlet stream contained 28.4 g/L glucose and 126.8 g/L fructose. It is evident that higher ethanol concentrations can be attained when Jerusalem artichoke juice is supplemented with glucose. With increases in the glucose concentrations and dilution rates though increasing amounts of glucose were passing unconverted through the reactor.

The ethanol productivities of the immobilized cell reactor versus the total carbohydrate concentration at different dilution rates are shown in Figure 6.70. At the lowest dilution rate (0.106 h^{-1}), increases in the total carbohydrate concentration caused by increases in the glucose concentration result in a linear increase in the ethanol productivity. This would

Table 6.22: Product concentrations from the immobilized cell reactor using a feed containing Jerusalem artichoke juice supplemented with glucose.

Feed glu-fru (g/L)	Dilution rate (h^{-1})	Final glucose[a] (g/L)	Final fructose[a] (g/L)	Final ethanol[a] (g/L)
84.9-140.6	0.106	5.3	121.6	38.0
	0.143	10.6	129.1	36.8
	0.180	14.4	130.8	35.9
	0.254	22.4	136.1	32.2
128.7-133.5	0.106	28.4	126.8	48.6
	0.143	47.5	133.0	39.3
	0.180	56.3	129.7	35.7
	0.254	73.9	131.9	26.5

[a] Steady state values are average of two or three samples

be expected if the reaction rate were diffusion controlled. At higher dilution rates, increases in the total carbohydrate concentration result in an initial increase in the productivity, this is followed by a levelling off and eventual reduction in the productivity at higher carbohydrate concentrations. This type of behaviour would occur if the controlling reaction rate were initially diffusion controlled which is linearly dependent on the carbohydrate concentration. As the carbohydrate concentration is increased the fermentation rate becomes the controlling rate, this rate is reduced when the carbohydrate concentration is increased due to inhibition as was discussed previously. Therefore the effect of substrate inhibition is more significant at higher dilution rates. This type of behaviour was also observed by Lee et al. (1983) using immobilized cells of the wild strain of *S. cerevisiae*.

The immobilized system was operated for more than 1100 hours without aeration using hydrolyzed Jerusalem artichoke juice with and without added glucose (Figure 6.71). During this time the activity of the cells remained relatively constant. Some loss in activity was observed after 1150 hours of operation; 145 hours after this period the ethanol productivity

dropped to 4.6 from 5.5 g/Lh. The decline in activity may be due to the fact that the yeast cells were in contact with a solution containing little or no oxygen and high ethanol and carbohydrate concentrations for long periods of time. For the most part, the beads remained spherical throughout the experiment, only a small number were broken.

To increase the economic viability of the process it is necessary to increase the conversion rate and the outlet concentration of ethanol. These objectives were attained by increasing the yeast concentration by ≈2 times in the alginate beads and by using a feed containing a high glucose concentration. The beads contained 102 g/L biomass. Jerusalem artichoke juice supplemented with glucose was used as the feed, it contained 156.8 g/L glucose and 118.6 g/L fructose. The results show (Table 6.23) that pure fructose syrup was produced at a dilution rate of 0.086 h^{-1}. About 17% of the fructose was consumed in the process, this high amount of fructose consumed was due to the fact that the glucose concentration in the outlet was zero. When the dilution rate was increased to 0.113 h^{-1}, the amount of fructose consumed was reduced to 6% but the ethanol concentration in the product stream was reduced from 76 to 67 g/L. When the dilution rate was increased to 0.113 h^{-1} fructose consumption was reduced further. Under these conditions only 77% of the glucose was converted resulting in a drop in the ethanol concentration to 56.5 g/L. At a dilution rate of 0.086 h^{-1} the ethanol productivity was 6.5 g/Lh, when the dilution rate was increased to 0.113 h^{-1} the productivity rose to 7.6 g/Lh. A further increase in the dilution rate to 0.130 h^{-1} resulted in a slight drop in the productivity to 7.4 g/Lh. This may be due to the effects of the total carbohydrate concentration which are more evident at higher dilution rates.

The increased biomass concentration in the beads enabled the production of higher ethanol concentrations than were produced previously (Table 6.22). The ethanol productivities though were low due to the high total carbohydrate concentration in the feed medium. From these tests it was seen that fructose consumption is also a disadvantage of this continuous process. This is discussed in more detail in the next section.

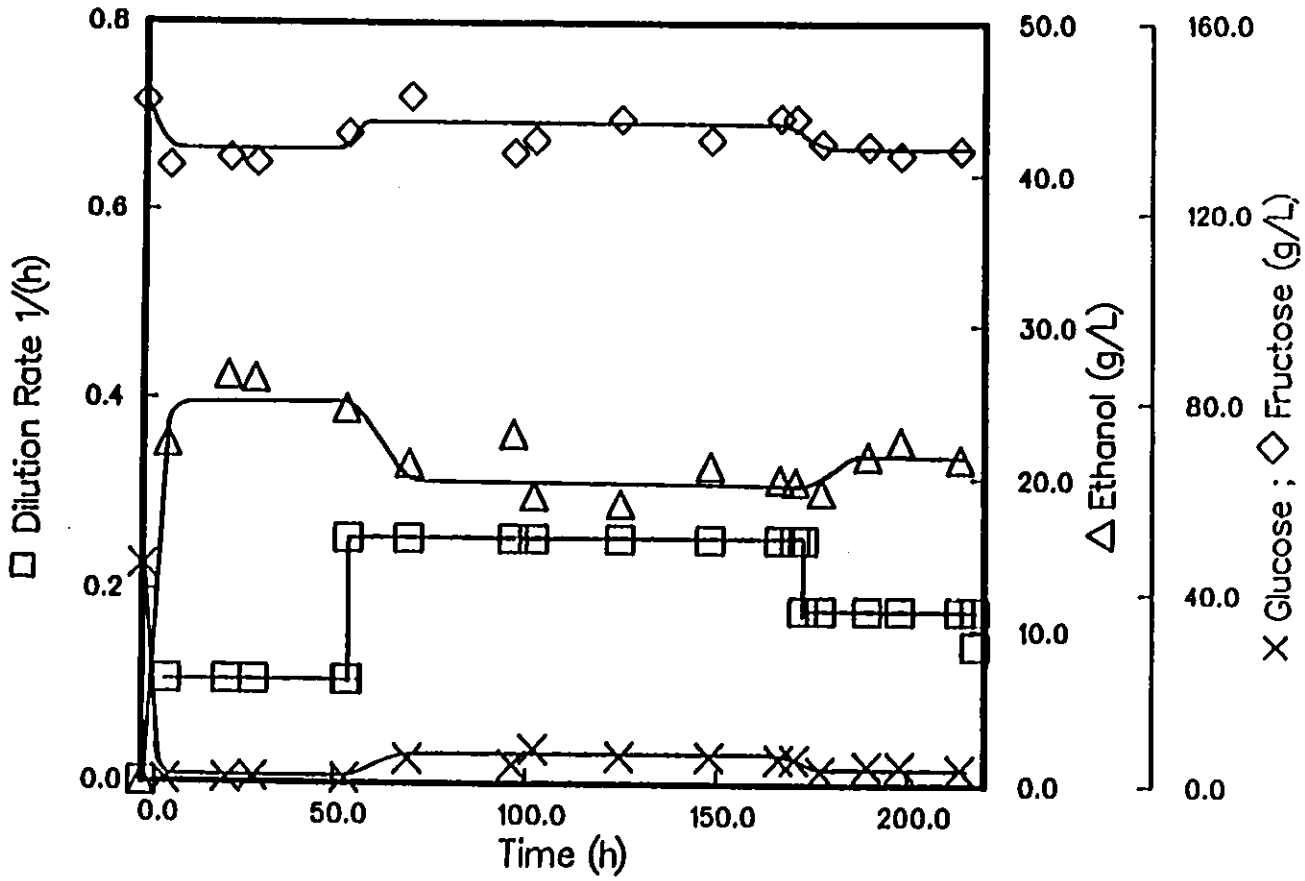

Figure 6.69: Ethanol production by the immobilized cell reactor using a feed containing hydrolyzed Jerusalem artichoke juice

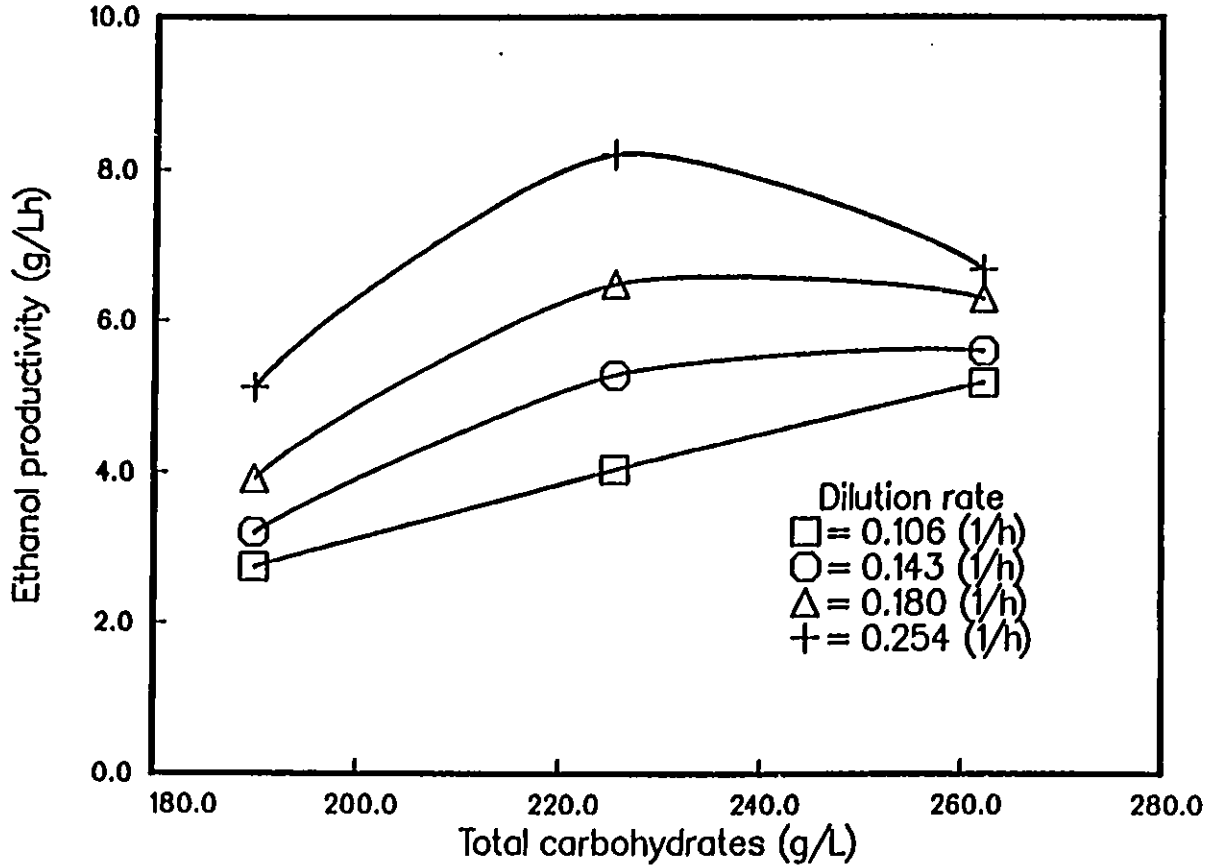

Figure 6.70: Ethanol productivity as a function of total carbohydrate concentration at different dilution rates using a feed containing hydrolyzed Jerusalem artichoke juice supplemented with glucose

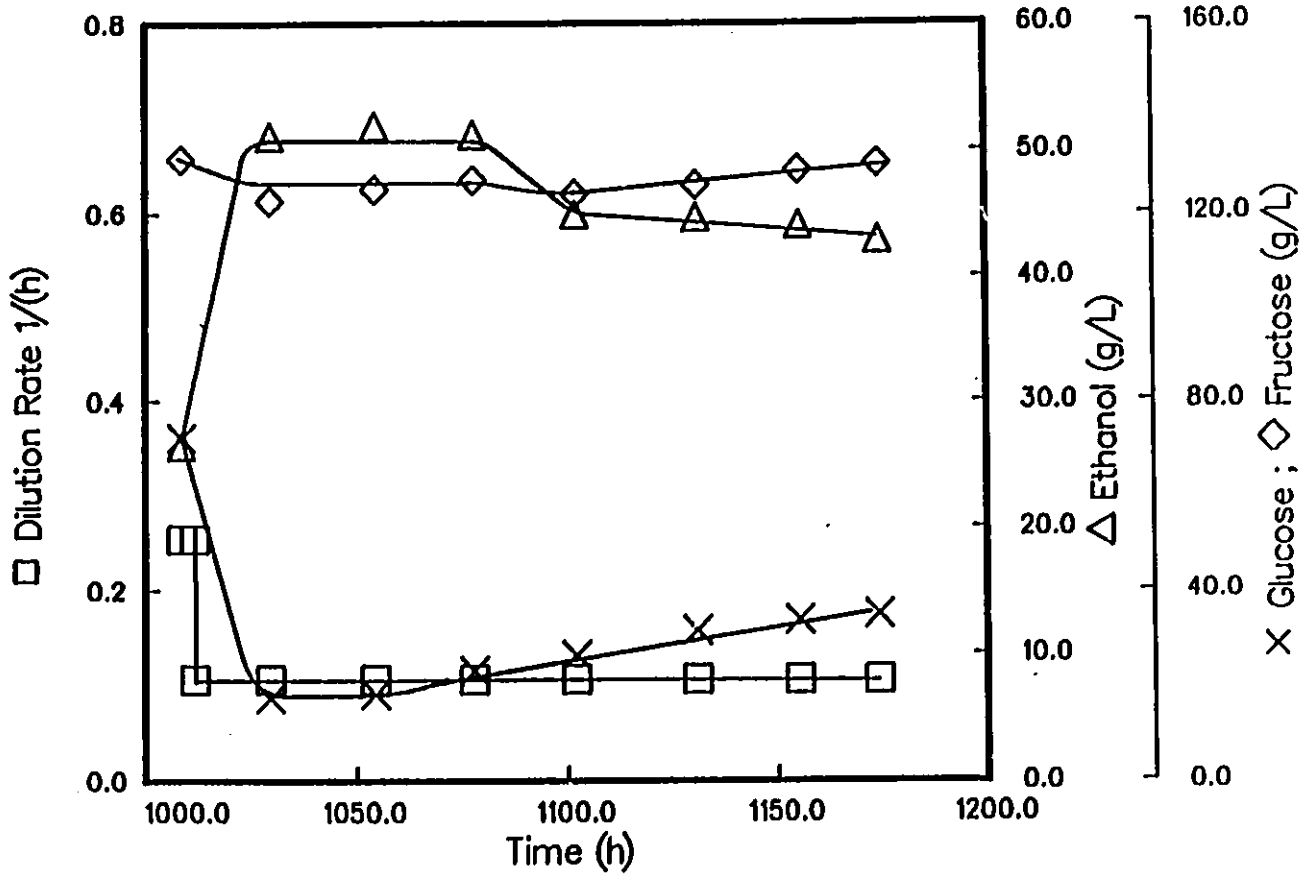

Figure 6.71: Ethanol production by the immobilized cell reactor using a feed containing hydrolyzed Jerusalem artichoke juice supplemented with glucose.

Table 6.23: Product concentrations from the immobilized cell reactor using a feed containing hydrolyzed Jerusalem artichoke juice and High Fructose Corn Syrup

Dilution rate (h^{-1})	Final glucose[a] (g/L)	Final fructose[a] (g/L)	Final ethanol[a] (g/L)
0.086	0.0	98.6	75.6
0.113	0.0	111.6	67.0
0.130	36.1	112.6	56.5

[a] Steady state values are average of two or three samples

Fructose Consumption

The data obtained from the experiments using the immobilized cell reactor indicate that some fructose was consumed by the yeast. An analysis of the data obtained using HJAJ and/or HFCS shows that there is a relationship between the ratio of the fructose to glucose consumption rates and the outlet glucose concentration (Figure 6.72). At glucose concentrations below 20 g/L, the ratio is reduced in a linear fashion as the glucose concentration is increased. For this region the equation proposed is

$$\frac{(ds_f/dt)}{(ds_g/dt)} = A + B\, s_g \tag{6.17}$$

where (ds_f/dt) is the fructose consumption rate, (ds_g/dt) is the glucose consumption rate and A and B are empirical constants. By fitting the equation to the data, the parameters and their confidence intervals are $A = 0.263$ (± 0.091) and $B = -0.014(\pm 0.012)$ L/g. This equation is shown in Figure 6.72 as the solid line.

When the outlet glucose concentration is greater than 20 g/L the ratio of fructose to glucose consumption rates appear to be constant. When an equation similar to that used in the previous analysis is fitted to this data, the value obtained for B was -6.23×10^{-4} ($\pm 1.36 \times 10^{-3}$) L/g. Due to the high uncertainty in the value of the parameter, the effect of glucose is insignificant in the range tested. A constant value of 0.060 for the ratio of fructose to glucose consumption rates adequately fits the data in this region (Figure 6.72). The differential analysis indicated this value to be 0.043. These are similiar to the value of 0.05 found for the ratio of fructose to glucose phosphorylation measured for glucokinase (Maitra, 1970). This would lead one to suggest that the limiting reaction for consumption of fructose by the yeast in glucose/fructose mixtures may be its phosphorylation by glucokinase.

When the outlet glucose concentration is below 20 g/L the ratio of fructose to glucose consumption decreases as the glucose concentration increases. In this range glucose is acting as an inhibitor to the fructose consumption. When the outlet glucose concentration is larger than 20 g/L, the ratio is unaffected by the outlet glucose concentration. Therefore to reduce fructose consumption the reactor should be run so that it is converting less than 100% of

the glucose, in the case examined here the outlet glucose concentration should be at least 20 g/L to minimize fructose consumption by the immobilized yeast cells.

The hydrolysis and fermentation processes introduced impurities into the glucose/fructose mixtures. Before the syrup can be used as a food grade material some of these impurities must be removed in order to improve the product's appearance and taste. The purification of the fructose/ethanol product formed in this process is discussed in the next section.

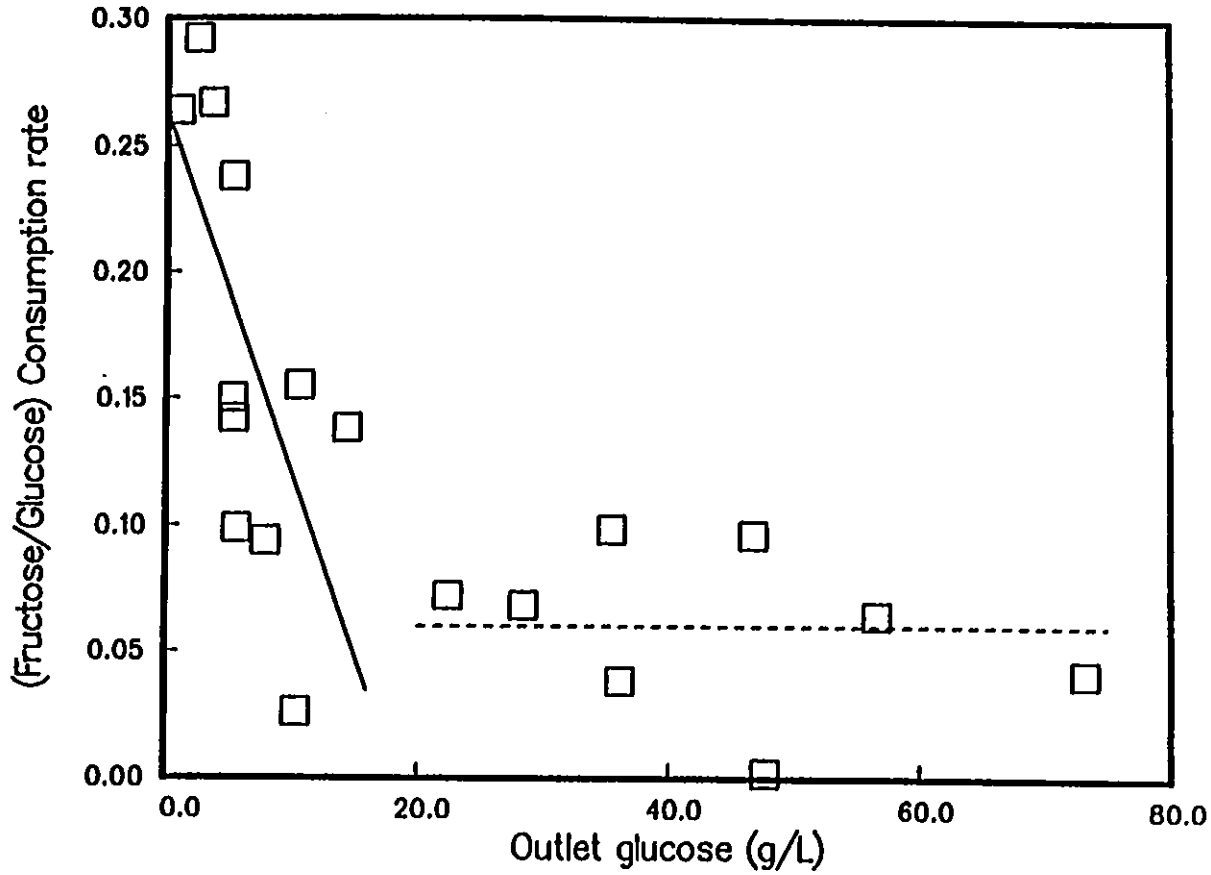

Figure 6.72: Ratio of fructose to glucose consumption rate as a function of outlet glucose concentration using immobilized cells.

Purification

A high fructose syrup should be colourless, have no flavour other than sweetness, no objectionable odour and should be clear in appearance (Fleming and GrootWassink, 1979). If the product formed in this process is to have these qualities some purification is necessary. Purification in this case will consist of decolourization and deionization as is normal industrial practice (Coker and Venkatasubramanian, 1987).

The main function of carbon treatment is the removal of colour but it also helps to eliminate colour precursors and sugar degradation products (Blanchard and Geiger, 1984). Decolourization of a food grade product from the continuous reactor was performed with activated carbon. The feed to the reactor consisted of 19 %v/v HFCS and 15 %v/v HJAJ, it contained 99.7 g/L glucose and 94.1 g/L fructose. The product was light brown in colour and contained fructose, ethanol and glucose. Some of the product (100 mL) was placed in an Erlenmeyer flask and various amounts of activated carbon was added, the flask was then placed in a shaker kept at 33°C. Samples were taken at various times and absorbances were measured. The absorbance at 460 nm which best shows the effects of browning is shown in Figure 6.73. The results show that decolourization is virtually complete (90%) after only 5 minutes of contact with the activated carbon. The minimum absorbance is measured after 45 minutes of contact. The results also show a reduction in the absorbance as the activated carbon concentration is increased to 2 g per 100 mL of solution. Similiar results were measured at 500, 550 and 600 nm. Further experimentation revealed that 30 g of activated carbon per L of solution was required to reduce the absorbance of the solution to near zero in the visible region (Table 6.24). The process produced a stable and colourless solution with no loss of fructose or ethanol.

In addition to the removal of colour compounds, the production of a fructose syrup requires the elimination of ionic matter. Some colour compounds which are not adsorbed by the carbon, are ionized and therefore retained on the ion exchange resin. It has been reported that up to 70% of the nitrogenous materials such as amino acids are removed by cation exchange resin (Blanchard and Geiger, 1984). The anion resin then absorbs the H^+ ions, leaving the syrup free of ions. In this case deionization was carried out using a mixture

Table 6.24: Decolourization of reactor product with activated carbon.

	Wavelength (nm)			
	460	500	550	600
Reactor product	0.577	0.343	0.200	0.128
20 g/L activated C	0.033	0.023	0.016	0.012
30 g/L activated C	0.004	0.002	0.000	0.000

of anion and cation exchange resin. Since a mixture of resins was used, any alkalinity formed by the anion resin is quickly removed by the cation resin and vice versa. Therefore adverse conditions which may cause degradation of fructose are eliminated. Some of the decolourized solution (200 mL) treated with 30 g/L activated carbon was passed through a 20 mL bed of resin. At a flow rate of 9 mL/min the resin in the column was saturated after one passage of the 200 mL sample. The product was then passed through three other resin containing columns. The conductivity, pH and the ethanol, glucose and fructose concentrations of the products from each bed are shown in Figure 6.74. From the figure it is seen that the conductivity is reduced from 0.9×10^5 to 19 μmhos. The pH was initially reduced from 4.48 to 2.67 after passage through 2 beds of resin, passage through the next bed had no effect on the pH. The reduction is due to the exchange of H^+ ions from the resin with cations in the solution. Upon passage through another bed the pH rose sharply to 5.60. At this stage the OH^- ions from the resin are exchanged with anions in the solution resulting in an increase in the pH. Unlike the decolourization step, some glucose, fructose and ethanol was lost in the process. After deionization 13.6 and 12.5% of the fructose and glucose respectively were lost, similiar losses were reported by other researchers (Fleming and GrootWassink, 1979).

These losses can be reduced by washing the resins and recovering the glucose and fructose that was absorbed. The final product contained 25.5% less ethanol than was present in the initial feed. This large loss can be avoided if the ethanol were recovered before the deionization process.

The purification processes produced a fructose syrup that is clear and colourless and visibly resembled HFCS. It contained fructose as 94% of the reducing sugars and ethanol. The final pH of the solution would allow for storage of the syrup without the risk of fructose degradation. In addition, the ethanol that is present prevents contamination of the syrup and thus allows for longer storage times before its further treatment.

The results show that an immobilized reactor can be used for the production of fructose and ethanol by selective fermentation of glucose/fructose mixtures. The immobilized cells could be used on a continuous basis for more than 1000 hours without a loss in activity. Using Jerusalem artichoke juice supplemented with glucose, a product containing 75 g/L ethanol and 99 g/L fructose was produced. Purification of the product with activated carbon and ion exchange resin resulted in the formation of a clear, colourless and stable fructose/ethanol solution. When the initial total carbohydrate concentration is increased beyond 220 g/L, the ethanol productivity is reduced, the effect is more pronounced as the dilution rate is increased.

To produce a highly concentrated fructose product using *S. cerevisiae* ATCC 36859 the inhibitory effects of the carbohydrates must be reduced. This can be done by avoiding high initial carbohydrate concentrations and allowing fructose to slowly accumulate in the reactor while glucose is converted to ethanol. This type of process is known as fed batch or semicontinuous fermentation. Experimental results obtained using this process are discussed in the next section.

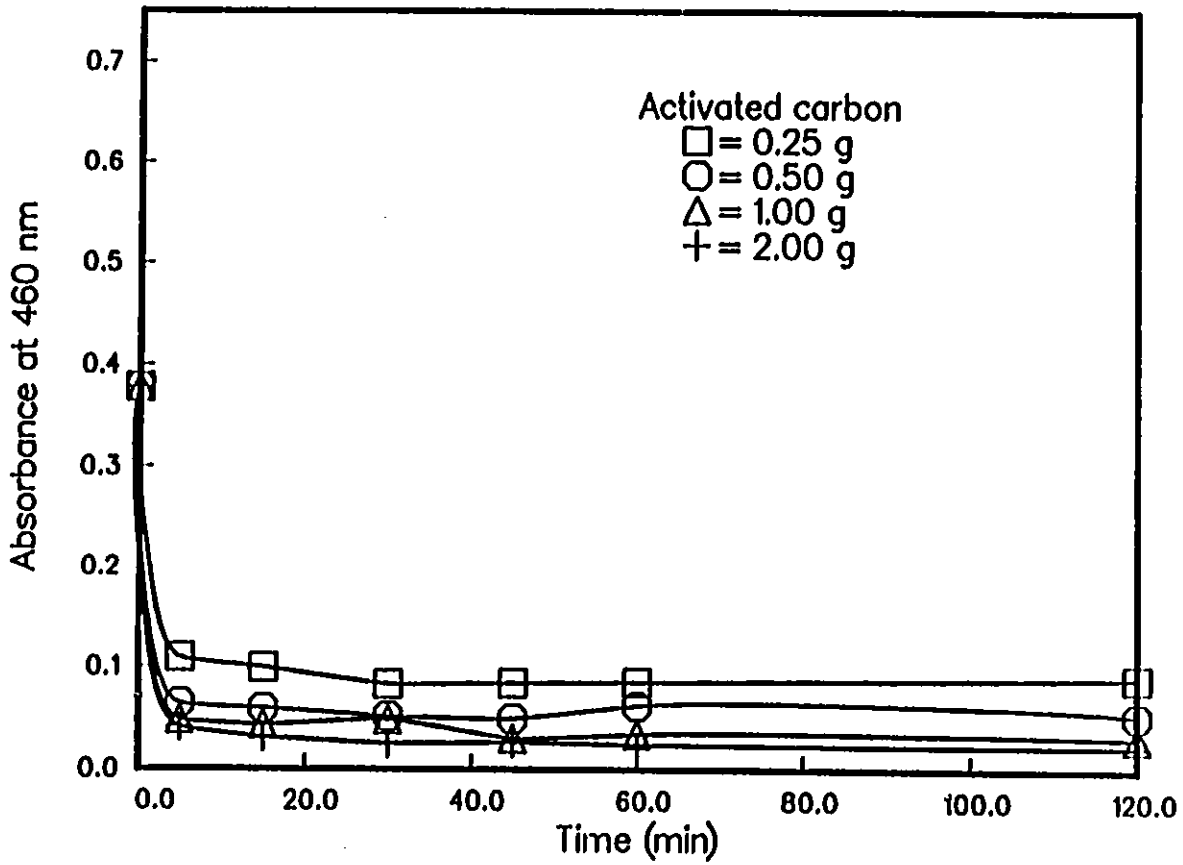

Figure 6.73: Decolourization of a fructose/ethanol product with various concentrations of activated carbon.

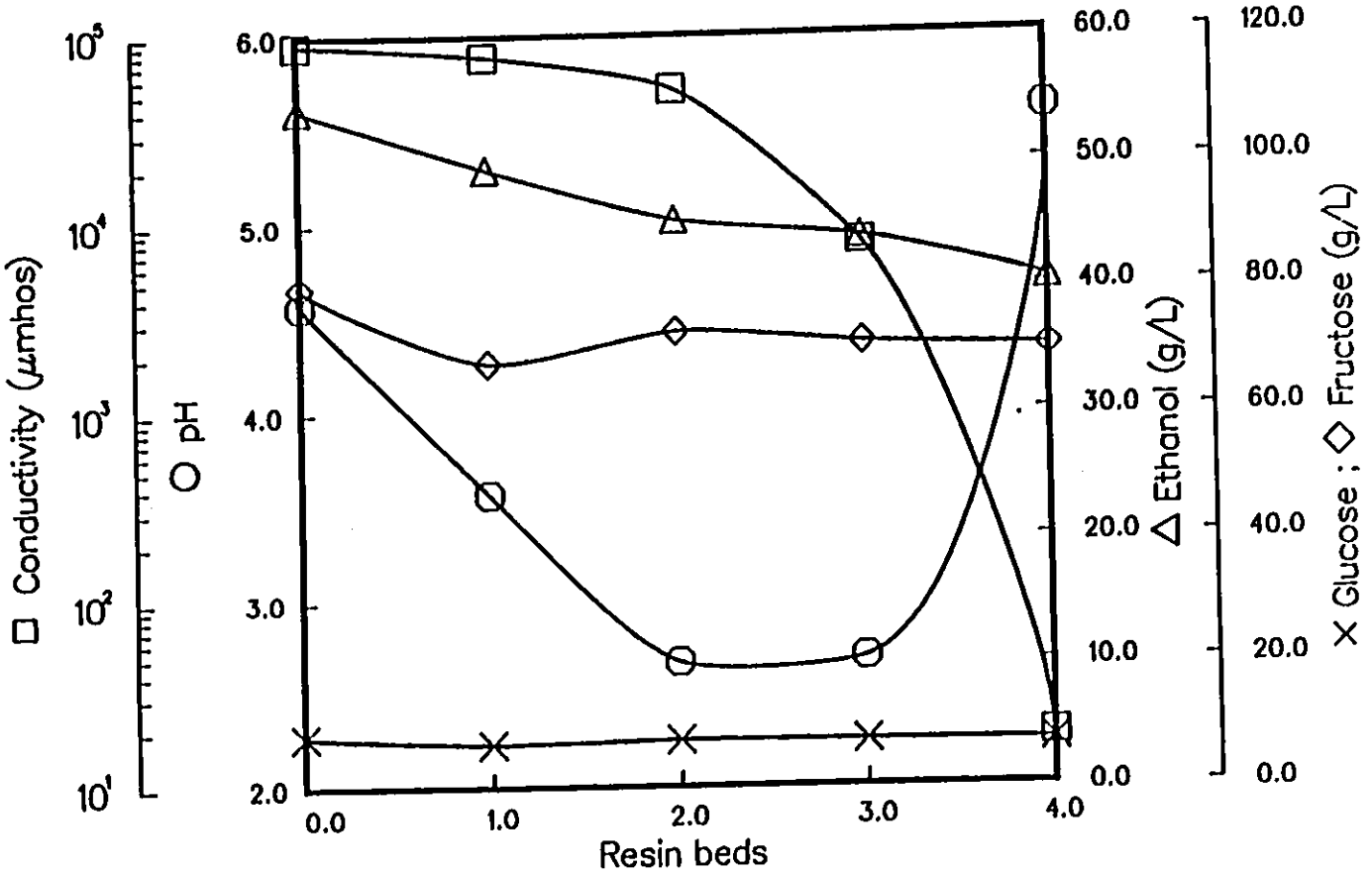

Figure 6.7-4: Deionization of a fructose/ethanol product with a mixture of anion and cation exchange resin.

6.6 Fed Batch Production of Fructose and Ethanol

The fermentation of highly concentrated substrate solutions although desirable imposes extreme osmotic conditions on the yeast and results in a reduction in the growth and fermentation rates (Jones et al., 1981). This effect was evident when solutions containing high total carbohydrate concentrations were used as the initial medium in batch and continuous fermentations already carried out. In order to reduce the problem of substrate inhibition but at the same time employing highly concentrated substrate solutions, fed batch processing will be carried out.

The scheme proposed here involves the feeding of a glucose/fructose syrup (i.e. 42 HFCS) to a fermenter which contains the mutant strain of *S. cerevisiae*. The glucose that is added is fermented to ethanol while the fructose remains unconverted and accumulates in the reactor. In this way the osmotic effects of the highly concentrated syrup are not imposed on the yeast at all times, instead the build-up in concentration is gradual. Another advantage of the process is that it can process a high solids concentration feed which does not require prior sterilization.

The objectives of the tests carried out here are to produce a syrup containing a high concentration of fructose (>200 g/L) and ethanol (>70 g/L) using a fed batch process with *S. cerevisiae* ATCC 36859. In the first set of tests the initial solution in the reactor was made of a synthetic medium. Later hydrolyzed Jerusalem artichoke juice, which is relatively inexpensive and a food grade glucose/fructose mixture is used.

6.6.1 Synthetic medium

In the first test approximately 150 mL of medium III, containing 8.3 g/L glucose and 15 g/L yeast extract was placed in a stirred tank reactor. The medium was then inoculated with the yeast; the initial biomass concentration was 6.9 g/L (Figure 6.75). Undiluted and unsterilized HFCS containing 463 g/L glucose and 353 g/L fructose was then added to the reactor. The rate of addition was varied in order to balance the consumption rate of glucose by the yeast, in this way the glucose concentration in the reactor was kept low

thus reducing the inhibitory effects of high total carbohydrate concentrations. To encourage growth of the yeast a high aeration rate was used (771 mL/min). This high aeration rate increases ethanol evaporation therefore the ethanol concentration in the reactor may not reflect the total ethanol produced by the yeast. The glucose consumption rate is thus used as a measure of the fermentation rate. Later when the ethanol was collected, the ethanol productivities were also calculated. In 11.8 hours, 34.4 mL of 42 HFCS was added to the reactor. The glucose was totally consumed by the yeast while 32.4 g/L of ethanol and 65.9 g/L of fructose had accumulated in the reactor (Figure 6.75). The fructose and ethanol concentrations reached 155.9 and 48.4 g/L respectively after 23 hours. Some of the glucose that was added was not consumed, resulting in an increase in its concentration to 29.8 g/L. To prevent the build-up of glucose, the addition of HFCS was stopped at this point. Five hours later the glucose was reduced to 12.3 g/L so the addition of HFCS was begun. The ethanol concentration reached a maximum of 53.5 g/L after 27.4 hours but later was reduced since the evaporation rate was higher than its formation rate. At a point 33.9 hours after the beginning of the test, the fructose concentration reached a maximum of 201.2 g/L, the ethanol concentration was 39.3 g/L. The productivity of the reactor was 7.2 g of glucose consumed per L of reactor volume per hour. It was not beneficial to continue the experiment because the productivity was reduced considerably after this point.

If a batch test was carried out using 42 HFCS which was diluted so that a final product containing 201 g/L fructose were to be produced. the initial medium would contain 264 g/L glucose and 201 g/L fructose. At this total carbohydrate concentration (465 g/L) the growth rate of the yeast would be reduced to nearly zero (Figure 6.30). The glucose consumption and ethanol production rates would also be reduced substantially (Table 6.14). The advantages of this process for the production of a high fructose syrup are therefore clear.

To prolong the life of the cells so that higher fructose concentrations could be attained, nutrients were added to the 42 HFCS. In addition, the final ethanol concentration in the reactor will be increased so that it may be recovered economically.

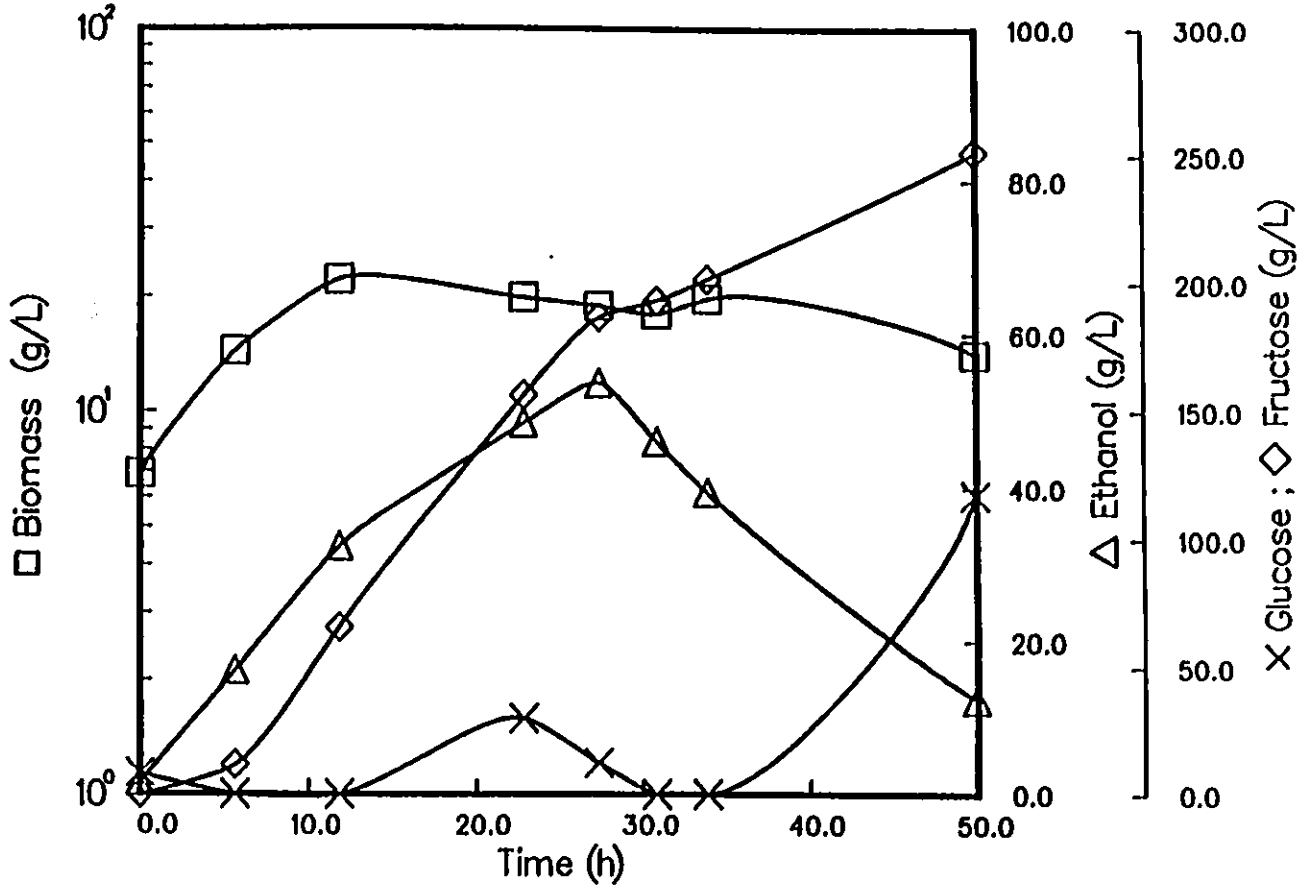

Figure 6.75: Fed batch production of fructose and ethanol by adding HFCS to a bioreactor which initially contains 6.9 g/L biomass and 8.3 g/L glucose.

Nutrients were added to the HFCS in the following concentrations: yeast extract (15 g/L), peptone (3.5 g/L), KH_2PO_4 (2.0 g/L), $MgSO_4.7H_2O$ (1.0 g/L) and $(NH_4)_2SO_4$ (1.0 g/L). The ethanol concentration in the reactor can be increased by stopping the air flow to the reactor, this would reduce the loss of ethanol from the reactor due to evaporation. The results of this test are shown in Figure 6.76. In the first 23.2 hours, 100.4 mL of 42 HFCS was added to the reactor, during this time the fructose concentration increased to 215.8 g/L. Some of the added glucose was not consumed, therefore its concentration increased to 41.6 g/L, the glucose consumption rate up to this point was 7.1 g/Lh. To reduce the glucose concentration, the addition of HFCS was stopped; after 5.3 hours the glucose concentration was reduced to 13.9 g/L while the ethanol concentration increased to 48 g/L. At this point the product contained fructose as 94% of the reducing sugars (223 g/L) and glucose as 6% (13.9 g/L). The glucose consumption rate was 7.1 g/Lh. In order to increase the ethanol concentration in the reactor, aeration was stopped at a point 35.4 hours after the fermenatation was begun. The ethanol concentration was increased to 91.4 g/L after 144 hours. During this time the glucose concentration reached a maximum value of 69.4 g/L, it was reduced to 39.3 g/L, 144 hours after the test was begun. The overall glucose consumption rate was 2.0 g/Lh. The low glucose consumption rate made it necessary to reduce the addition rate of 42 HFCS in order to avoid the build-up of glucose in the reactor. Due to this the fructose concentration only increased from 248 to 264 g/L between the times 35 and 144 hours. The high ethanol, fructose and glucose concentrations were likely the cause of the low fermentation rate in the present instance.

To reduce ethanol inhibition, its concentration in the reactor is to be maintained at a low level by employing high aeration rates. The ethanol lost due to evaporation could be collected in another vessel. Subsequent experiments were performed in a large fermenter which offered better air dispersion, this is essential if high ethanol evaporation rates are to be maintained. In order to collect the ethanol, the outlet gases from the reactor are passed through a condenser the condensate of which is collected in a separate vessel outside of the reactor.

When 450 mL of medium (medium III + 6.5 g/L glucose + 30.0 g/L yeast extract) was

inoculated with the same amount of yeast as was used previously the initial concentration was 2.9 g/L (Figure 6.77). The better aeration provided by the present reactor favours growth therefore a lower initial biomass concentration was used. During the first 34.6 hours, 340.1 mL of 42 HFCS was added to the reactor, the fructose concentration increased to 155.7 g/L while the glucose concentration was 13.2 g/L and the ethanol concentration was 57.6 g/L. The biomass concentration increased to 14.4 g/L and remained constant for the rest of the experiment. A product containing 261.2 g/L fructose, 16.1 g/L glucose and 30.8 g/L ethanol was produced after 82.8 hours. During the experiment 52.5 mL of condensate was collected, it contained 159 g/L ethanol. Using this process two separate products were formed, a syrup containing fructose as 94% of the reducing sugars and a liquid containing 159 g/L ethanol. The ethanol yield calculated using the ethanol that was collected was only 32% of the theoretical value. This low value is due to the inefficient collection system that was used. In order to increase the collection efficiency in subsequent experiments, the collection vessel was placed in a water bath that was kept at 6°C. The overall glucose consumption rate was 3.1 g/Lh.

To increase the rate of fermentation the initial biomass concentration was increased to 12.1 g/L (4.3 times greater than the previous test). The initial medium contained 86.7 g/L glucose and 92.3 g/L fructose (+ medium III + 30.0 g/L yeast extract), no HFCS was added until the initial glucose was consumed (Figure 6.78). These concentrations were chosen in order to simulate a medium that might be made from diluted 42 HFCS. Since the medium already contained some fructose the time required to reach 250 g/L is also expected to be shorter. The initial glucose was consumed in 7.7 hours (rate 15.6 g/Lh), at this point the addition of HFCS was started. After 20.1 hours of fermentation the fructose concentration increased to 257.2 g/L and the ethanol concentration was 52.8 g/L. During this time the glucose concentration increased to 74.0 g/L. To reduce the glucose concentration in the reactor, the HFCS addition rate was reduced. A further 25 hours (45.4 hours in total) was required to reduce the glucose to zero. The overall glucose consumption rate was 6.2 g/Lh, this is double the rate in the previous test. The final product contained 238 g/L fructose and 50.9 g/L ethanol. In the collection vessel 27.5 mL of condensate containing 367 g/L ethanol

accumulated. If this liquid were added to the reactor, the final product would contain 64.5 g/L ethanol and 227 g/L fructose. Higher concentrations of fructose could not be reached because the glucose consumption rates were very low when the fermentation was continued.

In order to reach a higher fructose concentration new biomass was added to the medium after a fructose concentration of 200 g/L was reached. The results of this test are shown in Figure 6.79. Starting with a medium containing 10.6 g/L biomass, 84.4 g/L glucose and 85.9 g/L fructose, a product containing 190.8 g/L fructose and 63 g/L ethanol was obtained after 26.5 hours. Up to this point the glucose consumption rate was 7.3 g/Lh. The biomass was then separated from the medium and fresh biomass was added. When a further 26.5 hours passed, the fructose concentration was increased to 287.3 g/L. Fructose made up 92.4% of the reducing sugars in the solution, this is higher than the 47% present in HFCS. The overall glucose consumption rate was 5.6 g/Lh. while the ethanol productivity was 2.3 g/Lh. To recover more of the ethanol that was evaporated from the fermenter, collector A contained ≈50 mL of water which was kept at 6°C (Figure 5.2). The outlet from this container was bubbled through a large reservoir which contained 8 L of water at room temperature (collector B). After 53 hours collector A had 55 mL of liquid containing 289 g/L ethanol. The product in the reactor contained 287 g/L fructose, 24 g/L glucose and 42 g/L ethanol. If the liquid in collector A were added to the reactor, the final product would contain 259.8 g/L fructose, 65.6 g/L ethanol and 21.3 g/L glucose. The fructose concentration was higher than that obtained in the previous test. In this test 36% of the ethanol that was collected was absorbed into collector B, the final ethanol concentration was 4.7 g/L. The low concentration would make it uneconomic to recover the ethanol from this reservoir. The ethanol yield is 80.7% of the theoretical value.

The ethanol content of the large vessel (collector B) increased after the ethanol concentration in collector A became greater than 265 g/L. Therefore losses of ethanol to the large reservoir can be reduced if a larger amount of ethanol were allowed to accumulate in the reactor instead of in collector A. This can be accomplished by stopping aeration when fermentation is near completion to allow the ethanol concentration in the reactor to increase. This strategy will be followed in a subsequent test which is carried out with an

initial medium consisting of hydrolyzed Jerusalem artichoke juice and 30.0 g/L yeast extract. Use of this medium will reduce the ethanol collection problems somewhat since the initial glucose concentration is lower than in the previous tests. In addition, hydrolyzed Jerusalem artichoke juice is a relatively inexpensive food grade glucose/fructose mixture.

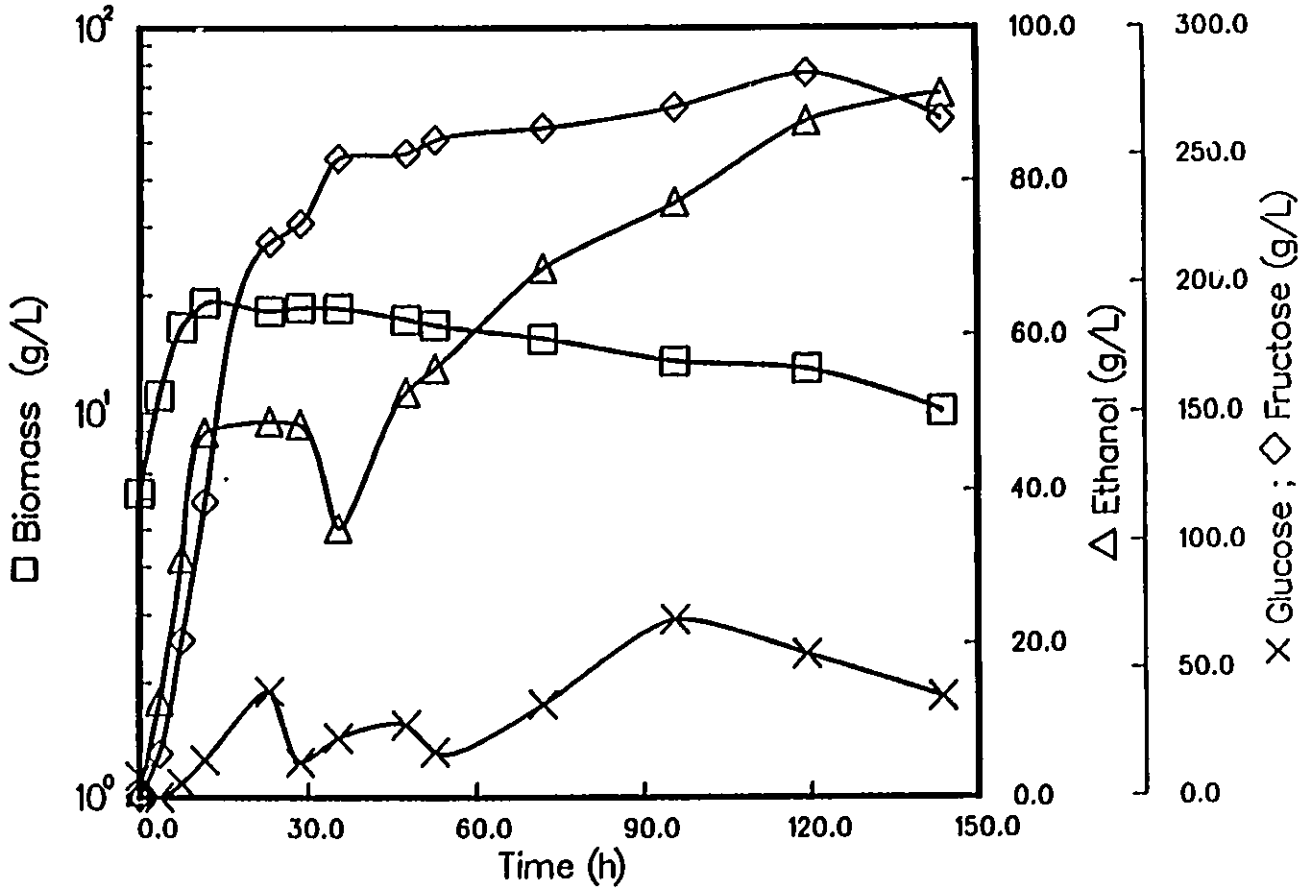

Figure 6.76: Fed batch production of fructose and ethanol by adding HFCS and nutrients to a bioreactor which initially contains 6.2 g/L biomass and 8.3 g/L glucose

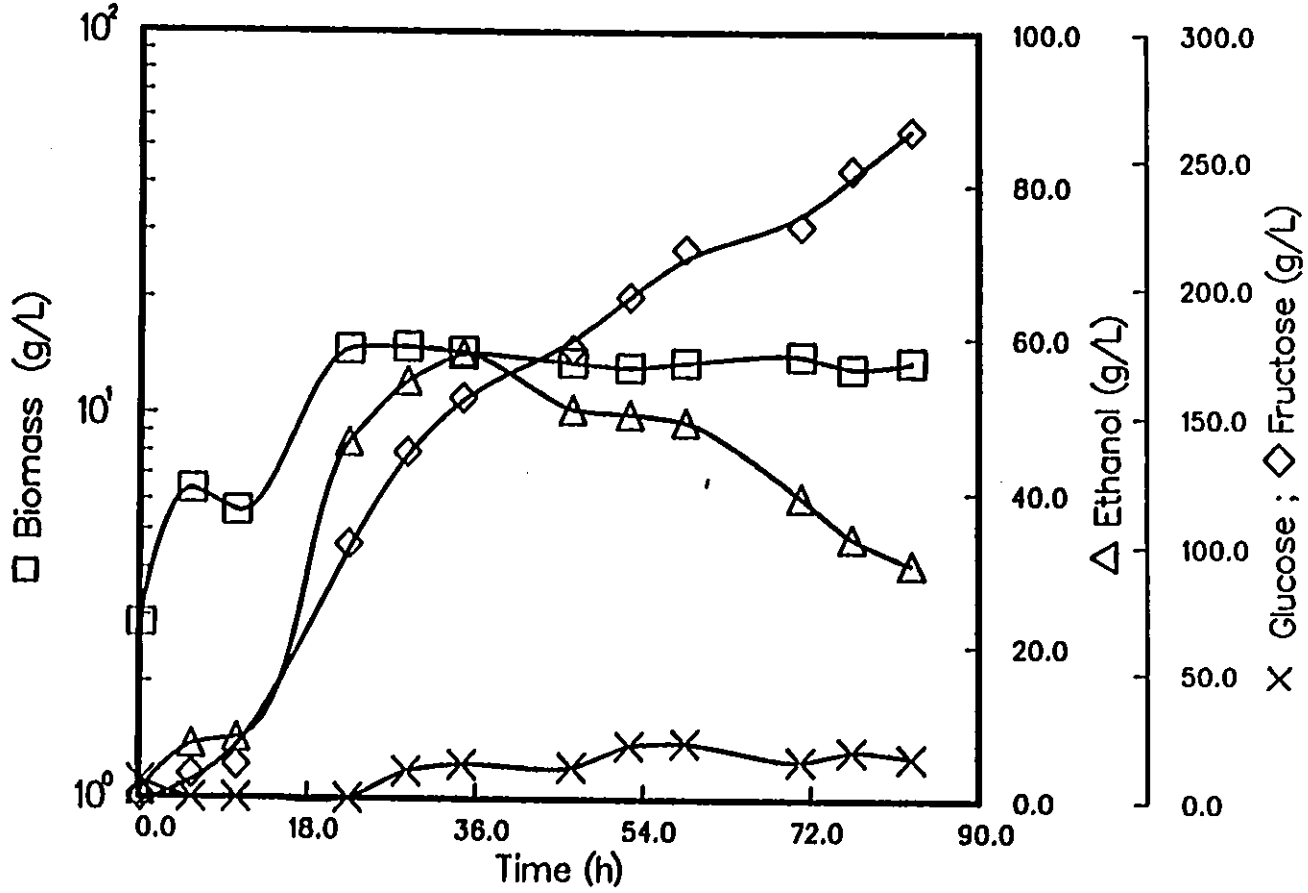

Figure 6.77: Fed batch production of fructose and ethanol by adding HFCS, and nutrients to a bioreactor which initially contains 2.9 g/L biomass and 6.5 g/L glucose.

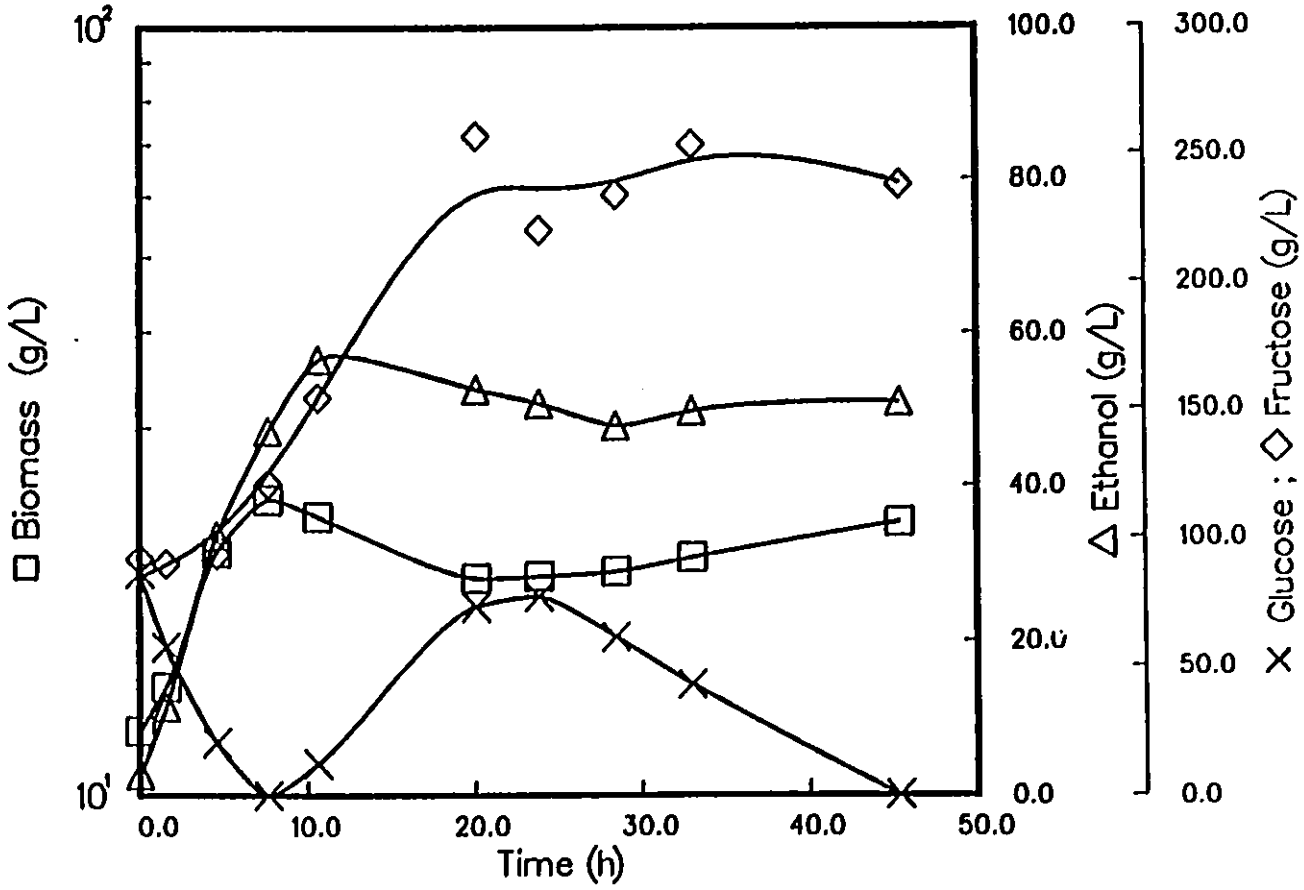

Figure 6.78: Fed batch production of fructose and ethanol by adding HFCS and nutrients to a bioreactor which initially contains 12 g/L biomass, 87 g/L glucose and 92 g/L fructose.

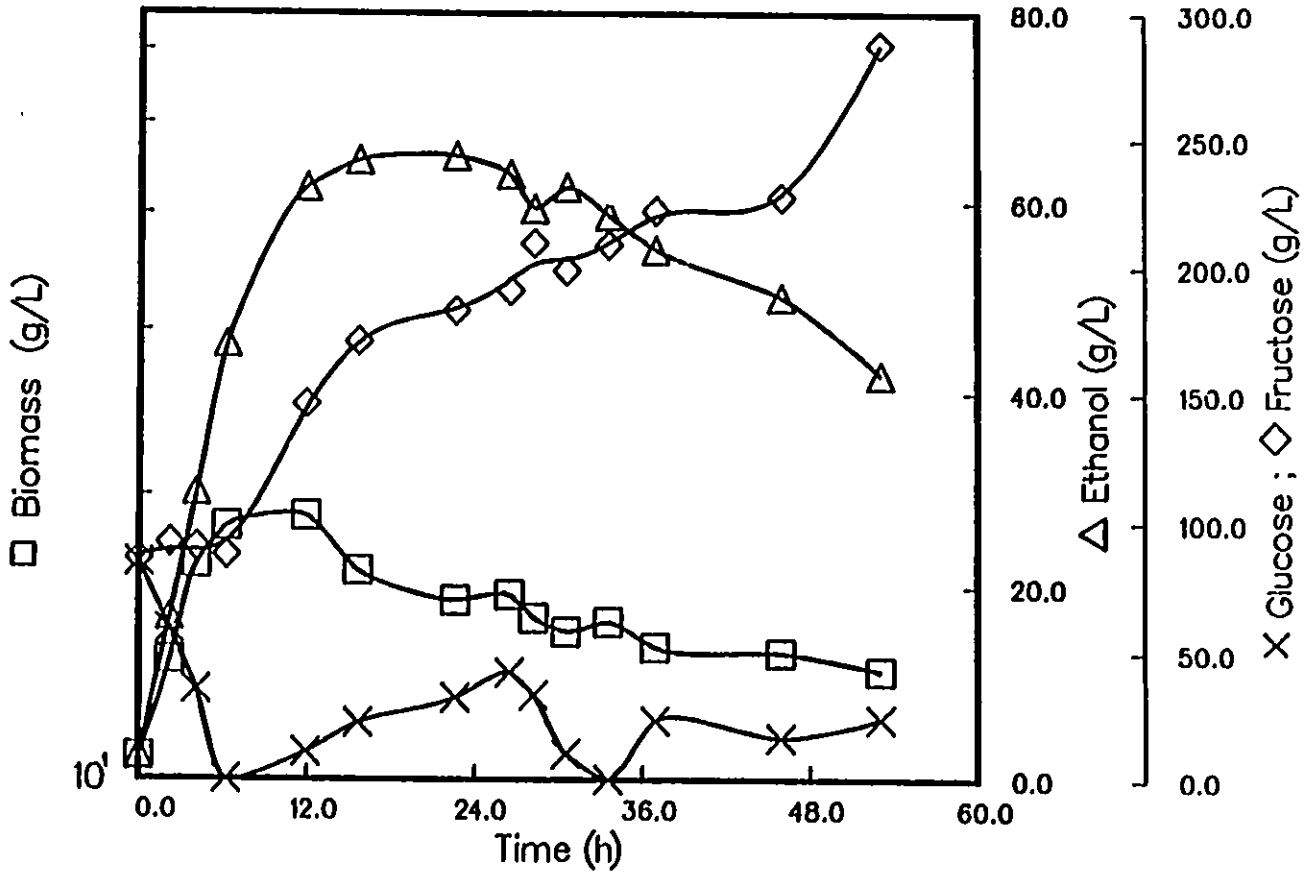

Figure 6.79: Fed batch production of fructose and ethanol by adding HFCS and nutrients to a bioreactor which initially contains 11 g/L biomass, 84 g/L glucose and 86 g/L fructose.

6.6.2 Hydrolyzed Jerusalem artichoke juice

In this test hydrolyzed Jerusalem artichoke juice supplemented with 30.0 g/L yeast extract was used as the initial medium in the reactor. After inoculation the biomass concentration was 27.6 g/L (Figure 6.80). Since the initial glucose concentration was low, the addition of 42 HFCS to the reactor was begun immediately after inoculation. A high aeration rate was maintained for the first 26.2 hours, up to this time the ethanol concentration was kept below 50 g/L. After this time aeration was stopped, this allowed the ethanol concentration in the reactor to increase to 67.5 g/L after 46.5 h. During this time the fructose concentration increased to 256.7 g/L, no glucose was present in the final product. The overall glucose consumption rate was 4.9 g/Lh and the ethanol productivity was 2.2 g/Lh. The rate is lower than those measured in the previous tests because HJAJ is not as rich in nutrients as was the synthetic medium but it is still about the same as classical batch fermentations (Rose, 1976). The ethanol that was collected was distributed as follows: 65.5% (67.5 g/L) in the reactor, 24.0% (346.5 g/L) in collector A and 10.5% (1.0 g/L) in collector B. The ethanol yield for this process was 88% of the theoretical value. If the liquid in collector A were combined with the reactor's contents the product would contain 240 g/L fructose and 86 g/L ethanol. This test is an improvement on the previous test because only 10.5% of the ethanol is lost to the large reservoir (collector B) this results in a higher ethanol concentration in the final product.

In the previous test a high biomass concentration was used, it would be beneficial to the process if it could be reused. In order to do this the biomass should be kept in a viable state. This can be accomplished by ensuring that the ethanol concentration in the reactor is kept low. The following scheme is proposed here. The fed batch process is carried out with a high initial biomass concentration and a high aeration rate. After reaching a fructose concentration of $\approx$ 250 g/L the medium is centrifuged and the biomass is transferred to a fresh medium and the process is repeated. The spent medium is then placed in collector A where it can absorb ethanol produced from the next fermentation. In this way the ethanol content of the spent medium can be increased. In this scheme the condensed part of the reactor outlet vapour is collected separately (collector C) before it passes to collector A (see

Figure 5.2).

In Figure 6.81 the results of a fed-batch process carried out with HJAJ (31.4 g/L glucose, 116.9 g/L fructose), 30.0 g/L yeast extract and 29.3 g/L biomass are shown. After 45.1 hours the medium contains 247 g/L fructose and 48.2 g/L ethanol. Due to the high aeration rate the ethanol concentration in the reactor is kept below 51 g/L. The ethanol that is collected is distributed as follows: 36% (48.2 g/L) in the reactor, 11% (271.6 g/L) in collector C, 49% (45.8 g/L) in collector A (827 mL) and 5.0% (0.5 g/L) in collector B. The ethanol yield was 96% of the theoretical value. The glucose consumption rate was 5.5 g/Lh and the ethanol productivity was 2.7 g/Lh.

The reactor contents were then centrifuged; the biomass was put into fresh medium in the reactor while the supernatant was placed in collector A. The reactor initially contained 37.1 g/L glucose, 147.0 g/L fructose and 27.2 g/L biomass. By the addition of HFCS to the reactor, the fructose concentration was increased to 250.1 g/L after 69.8 hours (Fig. 6.82). The glucose consumption rate was 3.3 g/Lh (ethanol productivity 1.55 g/Lh). The ethanol that was collected was distributed as follows: 20% (33.8 g/L) in the reactor, 19% (275.1 g/L) in collector C, 33% (102.3 g/L) in collector A and 28% (2.6 g/L) in collector B. The high fraction of ethanol lost to collector B indicates that the liquid in collector A became saturated during the test. Further use of this system, therefore would dictate the replacement of the liquid in collector A with some fresh solution. For this process the ethanol yield was 92% of the theoretical yield. The rate of glucose consumption was 39% lower than the previous test. Since the conditions of both tests were nearly identical it is suspected that the reduction in productivity was due to the lower biomass activity in the latter case. In addition, it was noticed that 12% of the fructose was consumed by the yeast during this second test. The final product of this process is the liquid in collector A, it contains 250 g/L fructose and 102 g/L ethanol.

The biomass in the reactor liquid was separated by centrifugation and placed in fresh medium. The supernatant was transferred to collector A so that its ethanol concentration could be increased. Reuse of the biomass a third time resulted in a further 30% drop in its activity, while 22% of the fructose was consumed by the yeast.

Although reuse of the biomass would be beneficial to the process, the reduction in its activity as well as its increased fructose consumption makes the reuse of the biomass uneconomic. It is recommended therefore that the biomass be used only once in the fed batch process. Ethanol would be evaporated from the reactor in order to keep the cells in a viable state and towards the end of the fermentation the aeration would be stopped in order to allow the ethanol concentration in the reactor to increase.

A fed batch process offers the best alternative for the formation of a product with high fructose and ethanol concentrations. The results indicate that a product containing 240 g/L fructose can be produced using a fed batch process. This is higher than the fructose concentration obtained using *Z. mobilis* (Suntinanalert et al., 1986) and *S. cerevisiae* ATCC 36859 (Lamarche, 1988). In addition, the final product contains no residual glucose and no sorbitol as was the case for the previous results. The ethanol concentration and productivity in these tests are the same or higher than other selective fermentations (Table 2.2).

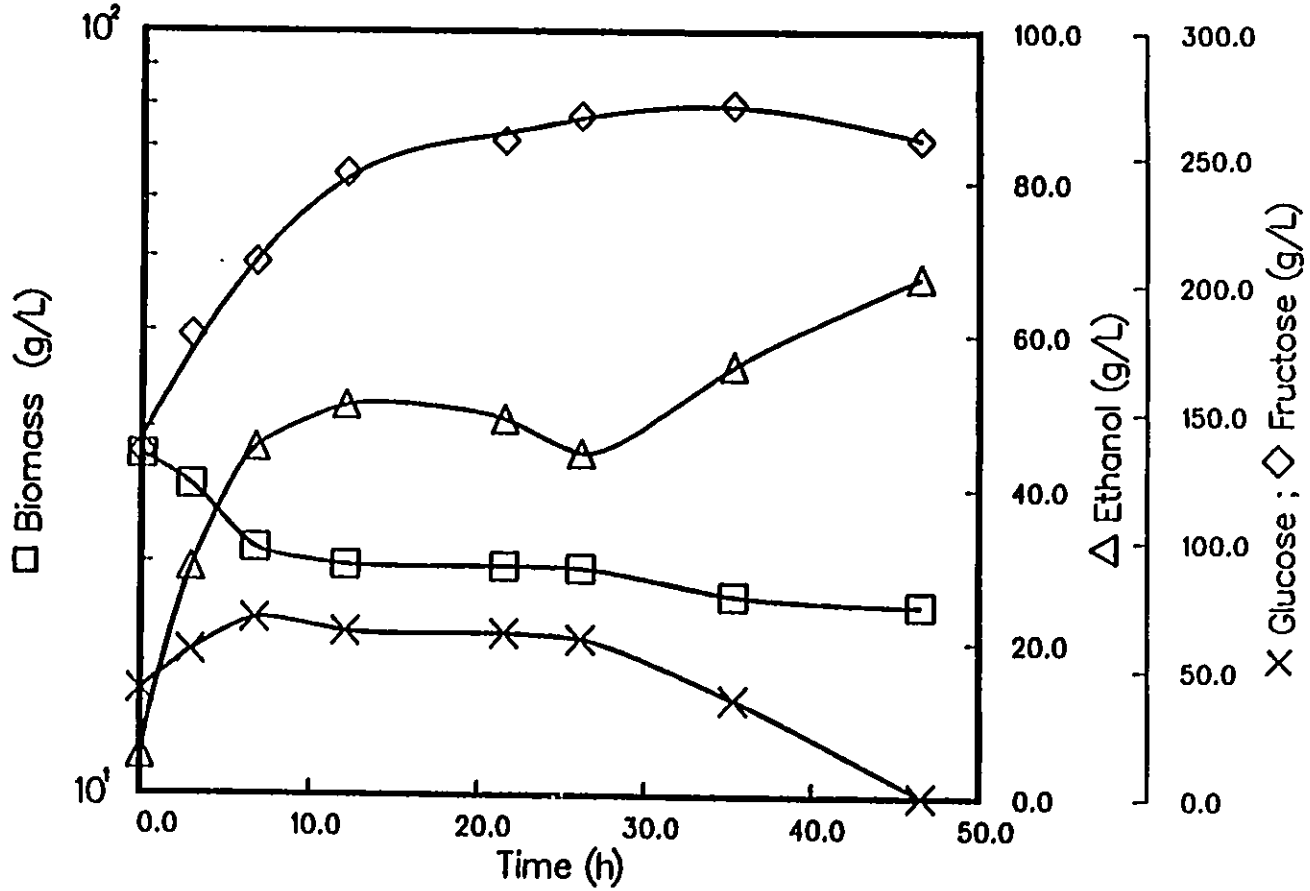

Figure 6.80: Fed batch production of fructose and ethanol by adding HFCS and nutrients to a bioreactor which initially contains 28 g/L biomass, 41 g/L glucose and 133 g/L fructose.

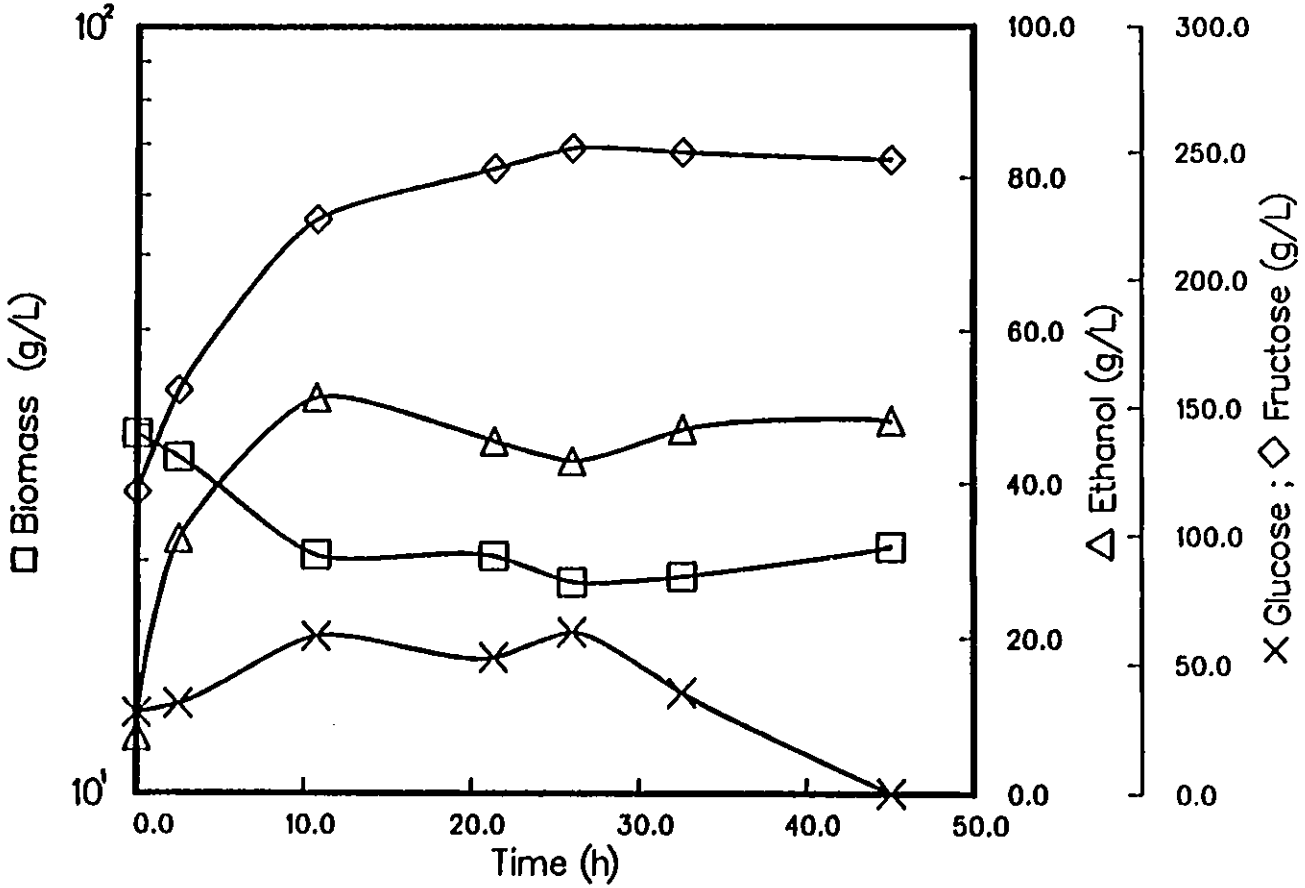

Figure 6.81: Fed batch production of fructose and ethanol by adding HFCS and nutrients to a bioreactor which initially contains 29 g/L biomass, 31 g/L glucose and 117 g/L fructose.

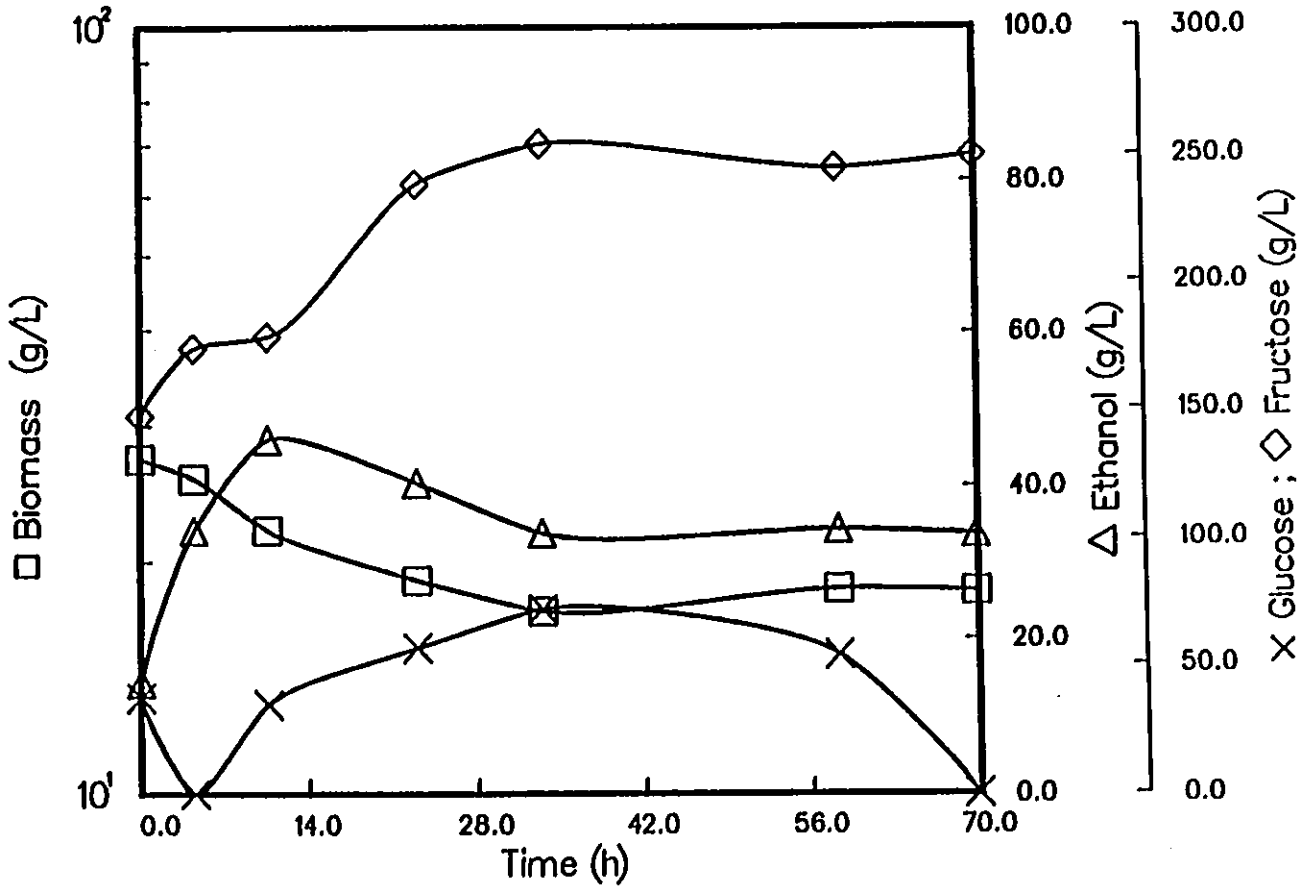

Figure 6.82: Fed batch production of fructose and ethanol by adding HFCS and nutrients to a bioreactor which initially contains 27 g/L biomass, 37 g/L glucose and 147 g/L fructose.

Chapter 7

Conclusions

The conclusions that were reached in this project concerning the production of fructose and ethanol are listed below.

- The growth, glucose consumption and ethanol production rates for the mutant strain are lower than those of the wild strain. Unlike the wild strain the mutant strain consumes fructose at a much lower rate than glucose and produces sorbtol in some cases. The selectivity of the mutant strain makes it suitable for the production of fructose and ethanol from glucose/fructose mixtures.

- The mutant strain consumes fructose at a much lower rate than glucose, but it was found that both carbohydrates inhibit the growth of the yeast equally. Fermentation up to a total carbohydrate concentration of 413 g/L was demonstrated but the fermentation rate was reduced due to substrate inhibition.

- The effects of initial total carbohydrate and yeast extract concentrations on the growth rate was modelled. The model indicated that the maximum carbohydrate concentration above which growth ceases is 586 g/L. This value is independent of the yeast extract concentration.

- Rate equations were formulated for the growth, glucose consumption, ethanol production and fructose consumption for the mutant yeast. Parameter estimates for these

equations were obtained from batch experiments using a Multiresponse Parameter Estimation technique.

- The models indicate that the inhibition by total carbohydrate and ethanol can be accounted for by linear terms. The glucose consumption and ethanol production rates are not as strongly inhibited by the total carbohydrate and ethanol concentrations as is the growth rate.

- Hydrolyzed Jerusalem artichoke juice was used for the simultaneous production of fructose and ethanol. To make ethanol recovery economically feasible the final ethanol concentration was increased by the addition of glucose or High Fructose Corn Syrup to the initial juice.

- Batch fermentation allows high ethanol concentrations (>75 g/L) to be produced at a fast rate when a large biomass concentration is used. A maximum ethanol productivity of 21 g/Lh was attained in this process. Due to substrate inhibition, products containing high ethanol and fructose concentrations cannot be produced. Reuse of the biomass is not recommended because its activity is substantially reduced due to its sensitivity to high ethanol and carbohydrate concentrations.

- Immobilization of the cells in calcium alginate beads enabled their continuous utilization for a long period of time. A product with fructose as 99% of the reducing sugars was produced using a feed which contained 100 g/L glucose and 100 g/L fructose. The maximum ethanol productivity was 13 g/Lh.

- Hydrolyzed Jerusalem artichoke juice supplemented with glucose was also used for the production of very enriched fructose syrup using the immobilized cells. With a feed containing 157 g/L glucose and 119 g/L fructose, a product containing 99 g/L fructose and 76 g/L ethanol was formed.

- Using the immobilized cells it was found that some fructose consumption was taking place. The fructose/glucose consumption rate ratio was reduced as the outlet glucose

concentration was increased. Fructose consumption can be practically avoided by operating the immobilized cell reactor at less than 100% glucose conversion.

- A clear and colourless very enriched syrup can be produced by treating the product of this process with activated carbon and ion exchange resin. The ethanol present in the solution prevents contamination of the juice and allows for long storage times before its further treatment.

- The immobilized cell systems enable the yeast to be used for long periods of time without a loss in activity. When a feed containing a high total carbohydrate concentration is used (>250 g/L), the ethanol productivity is reduced due to substrate inhibition. Therefore, the formation of a product containing both a high ethanol and fructose concentration is not possible using an immobilized cell reactor.

- The inhibitory effects of high carbohydrate concentration solutions were reduced by the use of a fed batch process. In this process unsterilized 42 HFCS with or without nutrients was continuously fed to a bioreactor. The glucose that was added was converted to ethanol by the yeast while the fructose remained unconverted and accumulated in the reactor.

- With the addition of 42 HFCS to an initial medium containing 40 g/L glucose and 133 g/L fructose (hydrolyzed Jerusalem artichoke juice), a syrup with 257 g/L fructose and 68 g/L ethanol was produced using a fed batch process. A separate product containing 347 g/L (34.7 %w/v) ethanol was also produced. If these two are combined the solution would contain 240 g/L fructose and 86 g/L ethanol (8.6 %w/v).

- The fed batch process offers the best alternative for the formation of a product containing both a high fructose (240 g/L) and a high ethanol (86 g/L) concentration. An ethanol productivity of 2.2 g/Lh was attained. Using this scheme 42 HFCS can be processed without the need for dilution or sterilization.

Chapter 8

Recommendations

In this work, fructose and ethanol were produced by selectively fermenting the glucose from a variety of glucose/fructose mixtures. Based on the results obtained here, the following recommendations are made:

- Examine the effect of temperature on the tolerance of the yeast cells to high substrate and product concentrations.
- Different reactor configurations such as a horizontal packed bed or fluidized bed should be investigated in order to reduce CO_2 hold-up in the reactor.
- In the fed batch processing scheme the productivity of the reactor should be increased by increasing the initial biomass concentration. The biomass could be grown in a separate vessel with HJAJ as the medium. The low glucose concentration in the juice would ensure a low ethanol concentration and thus a high biomass activity.
- Incorporation of viable and non-viable cell concentrations into the batch fermentation models. This would allow for their application to the semicontinuous and continuous processes.

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Appendix A

Repeated Results

The following are the pooled estimates of the pure error variance obtained from the results of the repeated experiments shown in Table A.1:

μ 8.458×10^{-5} (6 degrees of freedom)

$Y_{p/s}$ 2.72×10^{-4} (3 degrees of freedom)

$Y_{x/s}$ 9.52×10^{-5} (3 degrees of freedom)

Table A.1: Experiments used to estimate pure error variance.

Exp. no.	Yeast extract (g/L)	Biomass (g/L)	Glu-Fru (g/L)	$Y^a_{p/s}$ (g/g)	$Y^a_{x/s}$ (g/g)	μ (h^{-1})
F155	3.0	2.16	92.8-00.0	0.444	0.040	0.105
F179	3.0	2.08	85.4-00.0	-	-	0.119
F156	7.5	2.28	93.6-00.0	0.446	0.064	0.118
F178	7.5	2.04	86.7-00.0	-	-	0.136
F157	15.0	2.13	93.6-00.0	0.464	0.090	0.145
F177	15.0	2.04	98.7-00.0	-	-	0.152
F73	30.0	2.67	91.6-00.0	0.459	0.125	0.205
F159	30.0	2.43	90.9-00.0	0.455	0.120	0.192
F134	30.0	1.90	90.8-00.0	0.411	0.115	0.200
F137	30.0	1.90	88.5-00.0	0.451	0.111	0.186
F74	30.0	2.72	93.4-95.3	0.465	0.095	0.181
F171	30.0	2.30	92.3-94.4	0.461	0.118	0.172

[a] yields calculated at maximum ethanol and biomass concentration

Appendix B

Function Sub-Programs for Multiresponse Parameter Estimation Routine

B.1 Program used for Differential Analysis of the tests in Glucose Media

```
FILE: REDUK5E  FORTRAN  *   UNIV D'/OF OTTAWA CMS

      SUBROUTINE EQN(NEQNS,X,Y,YPRIME)                                  RED00010
C                                                                       RED00020
C                                                                       RED00030
C  THIS SUBROUTINE SUPPLIES THE RESPECTIVE FUNCTION VALUES AS NEEDED    RED00040
C  BY DVERK. THE PROGRAM "MULTI FORTRAN" REQUIRES THAT THE DERIVATIVES  RED00050
C  BE ENTERED IN THAT WAY IN ORDER TO CORRECTLY USE THE OUTPUT.         RED00060
C                                                                       RED00070
C                                                                       RED00080
      IMPLICIT REAL*8(A-H,O-Z)                                          RED00090
      PARAMETER(MAXOBS=62,MAXRES=09,MAXPAR=10,MAXRUN=10,MAXEQN=30,      RED00100
     &          IZ=61,ID=2,NW=30,IV=300,IX=9,IQ=360,IC=24,IL=3)         RED00110
      CHARACTER*80 TITLE,TITLE1,TITLE2,TITLE3,TITLE4,NAMRSP(MAXRES),    RED00120
     &             NAMPAR(MAXPAR),NAMES(MAXPAR),NAME(MAXPAR)            RED00130
      CHARACTER*80 YNAME                                                RED00140
      CHARACTER*9 XNAME                                                 RED00150
      CHARACTER*1 LINE(IZ),YAXIS(IZ)                                    RED00160
      COMMON/COM/HX(MAXOBS,MAXRES),H(MAXOBS,MAXRES),                    RED00170
     &           SP(MAXOBS,MAXEQN,MAXPAR),                              RED00180
     &           HP(MAXOBS,MAXRES,MAXPAR),T(MAXOBS),                    RED00190
     &           YOBS(MAXOBS,MAXRES),                                   RED00200
     &           CORR(MAXPAR),HS(MAXOBS,MAXRES,MAXEQN),                 RED00210
     &           YP(MAXOBS,MAXRES),                                     RED00220
     &           FP(MAXOBS,MAXRES,MAXPAR),                              RED00230
     &           SPIN(MAXEQN,MAXPAR,MAXRUN),                            RED00240
     &           COR(MAXPAR),CONDIN(MAXRUN,MAXRES)                      RED00250
      COMMON/NUM/NPARAM,N,M,MAXIT,IRUNS,NRUNS,NSTEQ,                    RED00260
     &           IRUN(MAXRUN),IRESP(MAXRES),NRESVA                      RED00270
      COMMON/PARAM/PA(MAXPAR),SIMPA(MAXPAR)                             RED00280
      COMMON/WRK/WORK2(MAXPAR)                                          RED00290
      COMMON/QRDEC/QRAUX(MAXRES)                                        RED00300
      COMMON/CONV/HESS(MAXPAR,MAXPAR),GRAD(MAXPAR),XGRAD(MAXPAR),       RED00310
     &            HES(MAXPAR,MAXPAR),STRHES(MAXPAR,MAXPAR)            RED00320
      COMMON/FCN/S(MAXOBS,MAXEQN)                                       RED00330
      COMMON/CRT/TEMP                                                   RED00340
      COMMON/INITIL/IUNKNO,NUNKNO,NRUN(MAXRUN),NRES(MAXRES)             RED00350
      COMMON/TRAN/THETA(MAXPAR)                                         RED00360
C                                                                       RED00370
C                                                                       RED00380
      DIMENSION Y(MAXEQN),YPRIME(MAXEQN)                                RED00390
C_______________________________________________________________________RED00400
      AP1 = THETA(2) + Y(2)                                             RED00410
      AP2 = 1-(Y(3)/THETA(3))                                           RED00420
      AP3 = 1-(Y(3)/THETA(6))                                           RED00430
C                                                                       RED00440
      US11 = (THETA(1)*Y(2)/(THETA(2)+Y(2)))*AP2                        RED00450
      US12 = (THETA(1)*THETA(2)*Y(1)/((THETA(2)+Y(2))**2))*AP2          RED00460
      US13 = -(THETA(1)*Y(1)*Y(2)/(THETA(2)+Y(2)))/THETA(3)             RED00470
      US21 = -(THETA(1)*Y(2)/(THETA(2)+Y(2)))*AP3/THETA(4)              RED00480
      US22 = -(THETA(1)*THETA(2)*Y(1)/((THETA(2)+Y(2))**2))*AP3         RED00490
     +       /THETA(4)                                                  RED00500
      US23 = (THETA(1)*Y(1)*Y(2)/(THETA(2)+Y(2)))/(THETA(6)*THETA(4))   RED00510
      US31 = (Y(2)*THETA(5)*THETA(1)/(THETA(2)+Y(2)))*(AP3/THETA(4))    RED00520
      US32 = (THETA(2)*Y(1)*THETA(5)*THETA(1)/((THETA(2)+Y(2))**2))*    RED00530
     +       (AP3/THETA(4))                                             RED00540
      US33 = -(Y(1)*Y(2)*THETA(5)*THETA(1)/(THETA(2)+Y(2)))/(THETA(6)   RED00550
```

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FILE: REDUKSE  FORTRAN  *   UNIV D'/OF OTTAWA CMS

     +       *THETA(4))                                                    RED00560
C                                                                          RED00570
C 1ST RESPONSE                                                             RED00580
C                                                                          RED00590
      YPRIME(1) = (THETA(1)*Y(1)*Y(2)/(THETA(2)+Y(2)))*                    RED00600
     +            (1-(Y(3)/THETA(3)))                                     RED00610
C                                                                          RED00620
C 2ND RESPONSE                                                             RED00630
C                                                                          RED00640
      YPRIME(2) = -(THETA(1)*Y(1)*Y(2)/(THETA(2)+Y(2)))*                   RED00650
     +            (1-(Y(3)/THETA(6)))/THETA(4)                            RED00660
C                                                                          RED00670
C 3RD RESPONSE                                                             RED00680
C                                                                          RED00690
      YPRIME(3) = (Y(1)*Y(2)*THETA(5)*THETA(1)/(THETA(2)+Y(2)))*           RED00700
     +            (1-(Y(3)/THETA(6)))/THETA(4)                            RED00710
C                                                                          RED00720
C 1ST DERIVATIVES                                                          RED00730
C                                                                          RED00740
      YPRIME(4) = US11*Y(4)+US12*Y(10)+US13*Y(16)+                         RED00750
     +            (Y(2)*Y(1)/(THETA(2)+Y(2)))*AP2                         RED00760
C                                                                          RED00770
      YPRIME(5) = US11*Y(5)+US12*Y(11)+US13*Y(17)-                         RED00780
     +            (THETA(1)*Y(1)*Y(2)/((THETA(2)+Y(2))**2))* AP2          RED00790
C                                                                          RED00800
      YPRIME(6) = US11*Y(6)+US12*Y(12)+US13*Y(18)+                         RED00810
     +            (Y(2)*Y(1)*THETA(1)*Y(3)/                               RED00820
     +            (AP1*(THETA(3)**2)))                                    RED00830
C                                                                          RED00840
      YPRIME(7) = US11*Y(7)+US12*Y(13)+US13*Y(19)                          RED00850
C                                                                          RED00860
      YPRIME(8) = US11*Y(8)+US12*Y(14)+US13*Y(20)                          RED00870
C                                                                          RED00880
      YPRIME(9) = US11*Y(9)+US12*Y(15)+US13*Y(21)                          RED00890
C                                                                          RED00900
      YPRIME(10)= US21*Y(4)+US22*Y(10)+US23*Y(16) -                        RED00910
     +            ((Y(2)*Y(1)/(THETA(2)+Y(2)))*AP3/THETA(4))              RED00920
C                                                                          RED00930
      YPRIME(11)= +US21*Y(5)+US22*Y(11)+US23*Y(17)+                        RED00940
     +            (THETA(1)*(Y(1)*Y(2)/((THETA(2)+Y(2))**2))*AP3/         RED00950
     +            THETA(4))                                               RED00960
C                                                                          RED00970
      YPRIME(12)= US21*Y(6)+US22*Y(12)+US23*Y(18)                          RED00980
C                                                                          RED00990
      YPRIME(13)= US21*Y(7)+US22*Y(13)+US23*Y(19)+                         RED01000
     +            ((Y(2)*Y(1)*THETA(1)/(THETA(2)+Y(2)))*AP3/              RED01010
     +            (THETA(4)**2))                                          RED01020
C                                                                          RED01030
C                                                                          RED01040
      YPRIME(14)= US21*Y(8)+US22*Y(14)+US23*Y(20)                          RED01050
C                                                                          RED01060
C                                                                          RED01070
      YPRIME(15)= US21*Y(9)+US22*Y(15)+US23*Y(21)-                         RED01080
     +            (THETA(1)*Y(2)*Y(1)*Y(3)/((THETA(2)+Y(2))*              RED01090
     +            (THETA(6)**2)*THETA(4)))                                RED01100
```

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FILE: REDUKSE  FORTRAN  *   UNIV D'/OF OTTAWA CMS
                                                                      RED01110
                                                                      RED01120
C                                                                     RED01130
      YPRIME(16)= US31*Y(4)+US32*Y(10)+US33*Y(16) +                   RED01140
     +            (Y(1)*Y(2)*THETA(5)/(THETA(2)+Y(2)))*AP3/THETA(4)   RED01150
C                                                                     RED01160
      YPRIME(17)= US31*Y(5)+US32*Y(11)+US33*Y(17)-                    RED01170
     +            (Y(1)*Y(2)*THETA(5)*THETA(1)/((THETA(2)+Y(2))**2))  RED01180
     +            *(AP3/THETA(4))                                     RED01190
C                                                                     RED01200
      YPRIME(18)= US31*Y(6)+US32*Y(12)+US33*Y(18)                     RED01210
C                                                                     RED01220
      YPRIME(19)= US31*Y(7)+US32*Y(13)+US33*Y(19)-                    RED01230
     +            (THETA(1)*Y(1)*Y(2)*THETA(5)*AP3/(AP1*(THETA(4)**2)))RED01240
                                                                      RED01250
C                                                                     RED01260
      YPRIME(20)= US31*Y(8)+US32*Y(14)+US33*Y(20)+                    RED01270
     +            (Y(2)*Y(1)*THETA(1)/(THETA(2)+Y(2)))*(AP3/THETA(4)) RED01280
C                                                                     RED01290
      YPRIME(21)= US31*Y(9)+US32*Y(15)+US33*Y(21)+                    RED01300
     +            (Y(2)*Y(1)*THETA(5)*Y(3)*THETA(1)/((THETA(2)+Y(2))* RED01310
     +            (THETA(6)**2)))/THETA(4)                            RED01320
C                                                                     RED01330
      RETURN
      END
```

B.2 Program used for Differential Analysis of the tests in Glucose/Fructose Media

```
FILE: REDUK5YC FORTRAN  *   UNIV D'/OF OTTAWA CMS

      SUBROUTINE EQN(NEQNS,X,Y,YPRIME)                                  RED00010
C                                                                       RED00020
C                                                                       RED00030
C  THIS SUBROUTINE SUPPLIES THE RESPECTIVE FUNCTION VALUES AS NEEDED    RED00040
C  BY DVERK. THE PROGRAM "MULTI FORTRAN" REQUIRES THAT THE DERIVATIVES  RED00050
C  BE ENTERED IN THAT WAY IN ORDER TO CORRECTLY USE THE OUTPUT.         RED00060
C                                                                       RED00070
C                                                                       RED00080
      IMPLICIT REAL*8(A-H,O-Z)                                          RED00090
      PARAMETER(MAXOBS=79,MAXRES=10,MAXPAR=20,MAXRUN=20,MAXEQN=75,      RED00100
     &          IZ=61,ID=2,NW=30,IV=300,IX=9,IQ=360,IC=50,IL=3)         RED00110
      CHARACTER*80 TITLE,TITLE1,TITLE2,TITLE3,TITLE4,NAMRSP(MAXRES),    RED00120
     &             NAMPAR(MAXPAR),NAMES(MAXPAR),NAME(MAXPAR)            RED00130
      CHARACTER*80 YNAME                                                RED00140
      CHARACTER*9 XNAME                                                 RED00150
      CHARACTER*1 LINE(IZ),YAXIS(IZ)                                    RED00160
      COMMON/COM/HX(MAXOBS,MAXRES),H(MAXOBS,MAXRES),                    RED00170
     &           SP(MAXOBS,MAXEQN,MAXPAR),                              RED00180
     &           HP(MAXOBS,MAXRES,MAXPAR),T(MAXOBS),                    RED00190
     &           YOBS(MAXOBS,MAXRES),                                   RED00200
     &           CORR(MAXPAR),HS(MAXOBS,MAXRES,MAXEQN),                 RED00210
     &           YP(MAXOBS,MAXRES),                                     RED00220
     &           FP(MAXOBS,MAXRES,MAXPAR),                              RED00230
     &           SPIN(MAXEQN,MAXPAR,MAXRUN),                            RED00240
     &           COR(MAXPAR),CONDIN(MAXRUN,MAXRES)                      RED00250
      COMMON/NUM/NPARAM,N,M,MAXIT,IRUNS,NRUNS,NSTEQ,                    RED00260
     &           IRUN(MAXRUN),IRESP(MAXRES),NRESVA                      RED00270
      COMMON/PARAM/PA(MAXPAR),SIMPA(MAXPAR)                             RED00280
      COMMON/WRK/WORK2(MAXPAR)                                          RED00290
      COMMON/QRDEC/QRAUX(MAXRES)                                        RED00300
      COMMON/CONV/HESS(MAXPAR,MAXPAR),GRAD(MAXPAR),XGRAD(MAXPAR),       RED00310
     &            HES(MAXPAR,MAXPAR),STRHES(MAXPAR,MAXPAR)            RED00320
      COMMON/FCN/S(MAXOBS,MAXEQN)                                       RED00330
      COMMON/CRT/TEMP                                                   RED00340
      COMMON/INITIL/IUNKNO,NUNKNO,NRUN(MAXRUN),NRES(MAXRES)             RED00350
      COMMON/TRAN/THETA(MAXPAR)                                         RED00360
C                                                                       RED00370
C                                                                       RED00380
      DIMENSION Y(MAXEQN),YPRIME(MAXEQN)                                RED00390
C_______________________________________________________________________RED00400
      AP1 = THETA(2) + Y(2)                                             RED00410
      AP2 = 1-(Y(3)/THETA(3))                                           RED00420
      AP3 = 1-(Y(3)/THETA(6))                                           RED00430
      AP4 = 1-((Y(4)+Y(2))/THETA(7))                                    RED00440
      AP5 = 1-((Y(4)+Y(2))/THETA(9))                                    RED00450
C                                                                       RED00460
      US11 = (THETA(1)*Y(2)/AP1)*AP2*AP4                                RED00470
      US12 = THETA(1)*Y(1)*AP2*AP4/AP1 - THETA(1)*Y(1)*Y(2)*AP2*AP4/    RED00480
     +       (AP1**2) - THETA(1)*Y(1)*Y(2)*AP2/(AP1*THETA(7))           RED00490
      US13 = -(THETA(1)*Y(1)*Y(2)/(AP1*THETA(3)))*AP4                   RED00500
      US14 = -(THETA(1)*Y(1)*Y(2)/(AP1*THETA(7)))*AP2                   RED00510
      US21 = -(THETA(1)*Y(2)/(AP1*THETA(4)))*AP3*AP5                    RED00520
      US22 = -(THETA(1)*Y(1)*AP3*AP5/(THETA(4)*AP1)) + THETA(1)*Y(1)*   RED00530
     +       Y(2)*AP3*AP5/(THETA(4)*(AP1**2)) + THETA(1)*Y(1)*Y(2)*AP3  RED00540
     +       /(THETA(4)*AP1*THETA(9))                                   RED00550
```

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FILE: REDUK5YC FORTRAN  *   UNIV D'/OF OTTAWA CMS

      US23 = (THETA(1)*Y(1)*Y(2)/(THETA(4)*THETA(6)*AP1))*AP5            RED00560
      US24 = (THETA(1)*Y(1)*Y(2)/(THETA(4)*THETA(9)*AP1))*AP3            RED00570
      US31 = (Y(2)*THETA(5)*THETA(1)/(AP1*THETA(4)))*AP3*AP5             RED00580
      US32 = THETA(1)*Y(1)*THETA(5)*AP3*AP5/(THETA(4)*AP1) -             RED00590
     +       THETA(1)*Y(1)*Y(2)*THETA(5)*AP3*AP5/(THETA(4)*(AP1**2)) -   RED00600
     +       THETA(1)*Y(1)*Y(2)*THETA(5)*AP3/(THETA(4)*AP1*THETA(9))     RED00610
      US33 = -(Y(1)*Y(2)*THETA(5)*THETA(1)/(AP1*THETA(4)*THETA(6)))*AP5  RED00620
      US34 = -(Y(1)*Y(2)*THETA(5)*THETA(1)/(AP1*THETA(4)*THETA(9)))*AP3  RED00630
      US41 = -(THETA(1)*Y(2)/(AP1*THETA(8)*THETA(4)))*AP3*AP5            RED00640
      US42 = -(THETA(1)*Y(1)*AP3*AP5/(THETA(8)*THETA(4)*AP1)) +          RED00650
     +        THETA(1)*Y(1)*                                            RED00660
     +       Y(2)*AP3*AP5/(THETA(8)*THETA(4)*(AP1**2)) +                RED00670
     +       THETA(1)*Y(1)*Y(2)*AP3                                     RED00680
     +       /(THETA(8)*THETA(4)*AP1*THETA(9))                          RED00690
      US43 = (THETA(1)*Y(1)*Y(2)/(THETA(8)*THETA(4)*THETA(6)*AP1))*AP5   RED00700
      US44 = (THETA(1)*Y(1)*Y(2)/(THETA(8)*THETA(4)*THETA(9)*AP1))*AP3   RED00710
C                                                                        RED00720
C 1ST RESPONSE                                                           RED00730
C                                                                        RED00740
      YPRIME(1) = (THETA(1)*Y(1)*Y(2)/AP1)*AP2*AP4                       RED00750
C                                                                        RED00760
C 2ND RESPONSE                                                           RED00770
C                                                                        RED00780
      YPRIME(2) = -(THETA(1)*Y(1)*Y(2)/(AP1*THETA(4)))*AP3*AP5           RED00790
C                                                                        RED00800
C 3RD RESPONSE                                                           RED00810
C                                                                        RED00820
      YPRIME(3) = (Y(1)*Y(2)*THETA(5)*THETA(1)/(AP1*THETA(4)))*AP3*AP5   RED00830
C                                                                        RED00840
C 4TH RESPONSE                                                           RED00850
C                                                                        RED00860
      YPRIME(4) = -(THETA(1)*Y(1)*Y(2)/(AP1*THETA(8)*THETA(4)))*AP3*AP5  RED00870
C                                                                        RED00880
C 1ST DERIVATIVES                                                        RED00890
C                                                                        RED00900
      YPRIME(5) = US11*Y(5)+US12*Y(14)+US13*Y(23)+US14*Y(32)+            RED00910
     +            (Y(1)*Y(2)/AP1)*AP2*AP4                                RED00920
C                                                                        RED00930
      YPRIME(6) = US11*Y(6)+US12*Y(15)+US13*Y(24)+US14*Y(33)-            RED00940
     +            (THETA(1)*Y(1)*Y(2)/(AP1**2))*AP2*AP4                  RED00950
C                                                                        RED00960
      YPRIME(7) = US11*Y(7)+US12*Y(16)+US13*Y(25)+US14*Y(34)+            RED00970
     +            (Y(2)*Y(1)*THETA(1)*Y(3)/(AP1*(THETA(3)**2)))*AP4      RED00980
C                                                                        RED00990
      YPRIME(8) = US11*Y(8)+US12*Y(17)+US13*Y(26)+US14*Y(35)             RED01000
C                                                                        RED01010
      YPRIME(9) = US11*Y(9)+US12*Y(18)+US13*Y(27)+US14*Y(36)             RED01020
C                                                                        RED01030
      YPRIME(10)= US11*Y(10)+US12*Y(19)+US13*Y(28)+US14*Y(37)            RED01040
C                                                                        RED01050
      YPRIME(11)= US11*Y(11)+US12*Y(20)+US13*Y(29)+US14*Y(38)+           RED01060
     +            (THETA(1)*Y(1)*Y(2)/((THETA(7)**2)*AP1))*AP2*          RED01070
     +            (Y(2)+Y(4))                                            RED01080
C                                                                        RED01090
      YPRIME(12)= US11*Y(12)+US12*Y(21)+US13*Y(30)+US14*Y(39)            RED01100
```

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FILE: REDUKSYC FORTRAN  *   UNIV D'/OF OTTAWA CMS

C                                                                       RED01110
      YPRIME(13)= US11*Y(13)+US12*Y(22)+US13*Y(31)+US14*Y(40)           RED01120
                                                                        RED01130
C                                                                       RED01140
C                                                                       RED01150
C 2ND DERIVATIVES                                                       RED01160
C                                                                       RED01170
      YPRIME(14)= US21*Y(5)+US22*Y(14)+US23*Y(23)+US24*Y(32)-           RED01180
     +            (Y(1)*Y(2)/(AP1*THETA(4)))*AP3*AP5                   RED01190
C                                                                       RED01200
      YPRIME(15)= US21*Y(6)+US22*Y(15)+US23*Y(24)+US24*Y(33)+           RED01210
     +            (THETA(1)*Y(1)*Y(2)/((AP1**2)*THETA(4)))*AP3*AP5      RED01220
C                                                                       RED01230
      YPRIME(16)= US21*Y(7)+US22*Y(16)+US23*Y(25)+US24*Y(34)            RED01240
C                                                                       RED01250
      YPRIME(17)= US21*Y(8)+US22*Y(17)+US23*Y(26)+US24*Y(35)+           RED01260
     +            (THETA(1)*Y(1)*Y(2)/((THETA(4)**2)*AP1))*AP3*AP5      RED01270
C                                                                       RED01280
      YPRIME(18)= US21*Y(9)+US22*Y(18)+US23*Y(27)+US24*Y(36)            RED01290
C                                                                       RED01300
      YPRIME(19)= US21*Y(10)+US22*Y(19)+US23*Y(28)+US24*Y(37)-          RED01310
     +            (THETA(1)*Y(3)*Y(1)*Y(2)/(THETA(4)*(THETA(6)**2)      RED01320
     +            *AP1))*AP5                                            RED01330
C                                                                       RED01340
      YPRIME(20)= US21*Y(11)+US22*Y(20)+US23*Y(29)+US24*Y(38)           RED01350
C                                                                       RED01360
      YPRIME(21)= US21*Y(12)+US22*Y(21)+US23*Y(30)+US24*Y(39)           RED01370
C                                                                       RED01380
      YPRIME(22)= US21*Y(13)+US22*Y(22)+US23*Y(31)+US24*Y(40)-          RED01390
     +            (THETA(1)*Y(1)*Y(2)/(THETA(4)*(THETA(9)**2)           RED01400
     +            *AP1))*AP3*(Y(2)+Y(4))                                RED01410
C                                                                       RED01420
C                                                                       RED01430
C 3RD DERIVATIVES                                                       RED01440
C                                                                       RED01450
      YPRIME(23)= US31*Y(5)+US32*Y(14)+US33*Y(23)+US34*Y(32)+           RED01460
     +            (Y(1)*Y(2)*THETA(5)/(AP1*THETA(4)))*AP3*AP5           RED01470
C                                                                       RED01480
      YPRIME(24)= US31*Y(6)+US32*Y(15)+US33*Y(24)+US34*Y(33)-           RED01490
     +            (THETA(1)*Y(1)*Y(2)*THETA(5)/((AP1**2)*THETA(4)))*    RED01500
     +            AP3*AP5                                               RED01510
C                                                                       RED01520
      YPRIME(25)= US31*Y(7)+US32*Y(16)+US33*Y(25)+US34*Y(34)            RED01530
C                                                                       RED01540
      YPRIME(26)= US31*Y(8)+US32*Y(17)+US33*Y(26)+US34*Y(35)-           RED01550
     +            (THETA(5)*THETA(1)*Y(1)*Y(2)/((THETA(4)**2)*AP1))*    RED01560
     +            AP3*AP5                                               RED01570
C                                                                       RED01580
      YPRIME(27)= US31*Y(9)+US32*Y(18)+US33*Y(27)+US34*Y(36)+           RED01590
     +            (THETA(1)*Y(1)*Y(2)/(THETA(4)*AP1))*AP3*AP5           RED01600
C                                                                       REDu1610
      YPRIME(28)= US31*Y(10)+US32*Y(19)+US33*Y(28)+US34*Y(37)+          RED01620
     +            (THETA(5)*THETA(1)*Y(3)*Y(1)*Y(2)/(THETA(4)*         RED01630
     +            (THETA(6)**2)*AP1))*AP5                               RED01640
C                                                                       RED01650
      YPRIME(29)= US31*Y(11)+US32*Y(20)+US33*Y(29)+US34*Y(38)
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C                                                                       RED01660
      YPRIME(30)= US31*Y(12)+US32*Y(21)+US33*Y(30)+US34*Y(39)           RED01670
C                                                                       RED01680
      YPRIME(31)= US31*Y(13)+US32*Y(22)+US33*Y(31)+US34*Y(40)+          RED01690
     +            (THETA(5)*THETA(1)*Y(1)*Y(2)/((THETA(9)**2)          RED01700
     +            *AP1*THETA(4)))*AP3*(Y(2)+Y(4))                      RED01710
C                                                                       RED01720
C                                                                       RED01730
C                                                                       RED01740
C 4TH DERIVATIVES                                                       RED01750
C                                                                       RED01760
      YPRIME(32)= US41*Y(5)+US42*Y(14)+US43*Y(23)+US44*Y(32)-           RED01770
     +            (Y(1)*Y(2)/(AP1*THETA(8)*THETA(4)))*AP3*AP5           RED01780
C                                                                       RED01790
      YPRIME(33)= US41*Y(6)+US42*Y(15)+US43*Y(24)+US44*Y(33)+           RED01800
     +            (THETA(1)*Y(1)*Y(2)/((AP1**2)*THETA(8)*THETA(4)))     RED01810
     +            *AP3*AP5                                              RED01820
C                                                                       RED01830
      YPRIME(34)= US41*Y(7)+US42*Y(16)+US43*Y(25)+US44*Y(34)            RED01840
C                                                                       RED01850
      YPRIME(35)= US41*Y(8)+US42*Y(17)+US43*Y(26)+US44*Y(35) +          RED01860
     +            (THETA(1)*Y(1)*Y(2)/((THETA(4)**2)*AP1*               RED01870
     +            THETA(8)))                                            RED01880
     +            *AP3*AP5                                              RED01890
C                                                                       RED01900
      YPRIME(36)= US41*Y(9)+US42*Y(18)+US43*Y(27)+US44*Y(36)            RED01910
C                                                                       RED01920
      YPRIME(37)= US41*Y(10)+US42*Y(19)+US43*Y(28)+US44*Y(37)-          RED01930
     +            (THETA(1)*Y(3)*Y(1)*Y(2)/(THETA(8)*THETA(4)*          RED01940
     +            (THETA(6)**2)                                         RED01950
     +            *AP1))*AP5                                            RED01960
C                                                                       RED01970
      YPRIME(38)= US41*Y(11)+US42*Y(20)+US43*Y(29)+US44*Y(38)           RED01980
C                                                                       RED01990
      YPRIME(39)= US41*Y(12)+US42*Y(21)+US43*Y(30)+US44*Y(39) +         RED02000
     +            (THETA(1)*Y(1)*Y(2)/((THETA(8)**2)*AP1*THETA(4)))     RED02010
     +            *AP3*AP5                                              RED02020
C                                                                       RED02030
      YPRIME(40)= US41*Y(13)+US42*Y(22)+US43*Y(31)+US44*Y(40)-          RED02040
     +            (THETA(1)*Y(1)*Y(2)/(THETA(8)*THETA(4)*(THETA(9)**2)  RED02050
     +            *AP1))*AP3*(Y(2)+Y(4))                                RED02060
      RETURN                                                            RED02070
      END                                                               RED02080
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