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Study of the production of fructose and ethanol from sucrose and molasses media using *Saccharomyces cerevisiae* ATCC 36858

by

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A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial
fulfillment of the requirement for the degree of Doctor of Philosophy in the
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Abstract

Fructose, the sweetest natural sugar, is of high commercial importance and is widely used in beverages, baking, canning, dairy and other food products. Due to its sweetness, smaller quantities of fructose are required to produce the same sweetness as sucrose, resulting in lower calorie intake. Fructose is available as a component of invert sugar and of high fructose corn syrups (HFCS) in which fructose and glucose are present in nearly equimolar concentrations. Since fructose is sweeter than glucose, the sweetness of these syrups can be enhanced by increasing their fructose content. The production of pure, solid fructose is carried out by crystallization of syrups containing 90-95% fructose. Existing industrial methods employ difficult and costly chromatographic techniques to separate glucose from fructose, which are isomers, to produce enriched fructose syrups from those with lower fructose concentrations.

An alternative to the costly chromatographic separation method is a bioseparation technique. This method involves a microbial, selective conversion of glucose to a product that is easier to separate from fructose than glucose. This study reports on a new mutant, *Saccharomyces cerevisiae* ATCC 36858, which is able to produce enriched and pure fructose syrups from synthetic glucose/fructose mixtures, sucrose and raffinose, and from molasses media. In general, most *S. cerevisiae* strains can hydrolyze sucrose by invertase and completely utilize the glucose and fructose parts of this disaccharide. They can also consume the fructose component of raffinose after hydrolyzing it with invertase, however, they do not degrade the remaining melibiose part due to the lack of melibiase. In this study, the results showed that *S. cerevisiae* ATCC 36858 can hydrolyze sucrose, raffinose and melibiose then selectively ferment glucose and galactose to ethanol.

In batch tests, the fructose yield was above 93% of the theoretical value in synthetic glucose/fructose media with a total sugar concentration of 300 g/L. The ethanol yield was about 76% of the theoretical value. The fructose yield was above 90% of the theoretical value in synthetic media with a sucrose concentration up to 726 g/L and in molasses media with a sugar concentration up to 323 g/L. The ethanol yield was between 72 and 85% in synthetic media with a sucrose concentration up to 451 g/L. A lower ethanol yield was obtained in molasses media. The strain of yeast used can be considered osmophilic because it can grow in synthetic media even with a sucrose concentration above 530 g/L. In synthetic media with initial raffinose concentrations between 30 and 242 g/L, fructose and ethanol yields were between 82 and 96%,

and 67 and 76%, respectively.

Syrups with more than 97% fructose based on the final total sugar concentrations can be produced from synthetic media with initial sucrose concentrations below 360 g/L. Even at a sucrose concentration of 574 g/L, the produced syrup contained 59% fructose and thus, it was richer in this carbohydrate than the ordinary 55% HFCS. The fructose fractions in the carbohydrate content of the produced syrups was more than 95% when the total initial sugar concentrations in sugar cane and beet molasses media were below 274 and 242 g/L, respectively. Syrups with more than 96% fructose based on the final total sugar concentrations can be produced in media with initial raffinose concentrations below 189 g/L.

The effects of initial biomass, yeast extract and ethanol concentrations, pH, and media sterilization on the production of fructose and ethanol in synthetic sucrose media were also examined. An increase in the biomass concentration in the fermentation media increased the bioreactor productivity. However, ethanol and fructose yields slightly changed. Increasing the initial yeast extract concentration resulted in higher growth rates of the mutant, biomass yields and ethanol productivities with insignificant changes in either ethanol or fructose yields. The mutant growth and fermentation capability considerably decreased as a result of increasing the initial ethanol concentration. When the effect of pH between 5.1 and 6.6 on the production of fructose and ethanol in sucrose media was considered, no significant changes in the mutant growth and fermentation capability were noticed. The results also showed that the use of either sterile or non-sterile media had an insignificant effect on the kinetics of the fermentation process or product yields.

The results from this research showed that *S. cerevisiae* ATCC 36858 has many advantages over other microbes such as *Zymomonas mobilis* and *S. cerevisiae* ATCC 36859 used by other researchers in the production of fructose and ethanol from media with high substrate concentration. These advantages include high sucrose hydrolysis efficiency, the ability to ferment in media with high substrate concentrations and high fructose and ethanol yields. *S. cerevisiae* ATCC 36859 has also demonstrated high fructose and ethanol yields, however it cannot utilize sucrose which limits its use to media with glucose/fructose mixtures.

In this study, the product formed in the process from molasses media was purified. Ethanol was removed from the broth by vacuum evaporation whereas colour was removed using ultrafiltration membranes and activated carbon. Two ultrafiltration membranes were used: polyethersulfone (PES) that had a molecular weight cut off (MWCO) of 10,000 Daltons and a

thin film composite (TFC) with a MWCO of 1,000 Daltons. The thin layer membrane was made of brominated sulfonated poly (2,6-dimethyl-1,4-phenylene oxide) and the supportive layer was made of polyethersulfone. The colour removal efficiencies were 94.2 and 98.7% with the PES and TFC membranes, respectively. Further treatments of the permeate of the PES membrane with 7.5% activated carbon in the slurry resulted in an increase in colour removal efficiency up to 99.7%. However, when the permeate of the TFC membrane was treated with 3% activated carbon in the slurry, the colour removal efficiency was up to 99.9%. When activated carbon alone was used to remove colourants from the broth containing fructose, a concentration of 33% activated carbon in the broth was needed to obtain a colour removal efficiency of 99.4%. These results demonstrated that combining the membrane separation technique and the activated carbon treatment for syrup purification considerably decreased the amount of activated carbon needed to obtain decolourization efficiencies above 99%. After the decolourization process, the demineralization of the final product was carried out using ion exchange resins. Clear and colourless fructose syrup that visibly looked like industrial HFCS was produced.

Kinetic models were proposed to describe the production of fructose and ethanol in sucrose and raffinose media. The results showed that the model predictions for biomass, sucrose, glucose, fructose and ethanol concentrations were in quite good agreement with the experimentally observed values in sucrose media below 257 g/L when individual parameter estimates were used. The model failed to adequately predict the exponential growth phase in media with sucrose concentration of 257 g/L or higher. However, it predicted very well the other variables and the stationary growth phase. In raffinose media, the results showed that the model predictions for biomass, raffinose, fructose and ethanol concentrations were quite good, especially in media with initial raffinose concentrations below 242 g/L and using individual parameter estimates. However, the proposed model for raffinose fermentation was unable to predict the experimentally observed trends for the production and consumption of melibiose.

Résumé

Le fructose, le sucre naturel le plus sucré, est d'une grande importance commerciale et est largement utilisé dans les boissons, la cuisson, la mise en boîte, les produits laitiers et d'autres produits alimentaires. Dû à son intensité, de plus petites quantités de fructose sont requises pour produire la même saveur que le sucrose, résultant en une réduction de calories consommées. Le fructose peut être séparé du sucre inverti et du sirop de maïs à haute concentration de fructose ("High Fructose Corn Syrup", HFCS) dans lesquels le fructose et le glucose sont présents en quantités presque équimolaires. Puisque le fructose est plus sucré que le glucose, ces sirops peuvent être plus sucrés en augmentant la concentration de fructose. La production du fructose pure et solide est réalisée par la cristallisation des sirops contenant 90-95% de fructose. Les méthodes industrielles courantes emploient des techniques chromatographiques difficiles et coûteuses pour séparer le glucose du fructose, qui sont des isomères, afin de produire des sirops riches en fructose à partir de sirops à basse concentration de fructose.

Une alternative à la séparation chromatographique est la bioséparation. Cette méthode emploie une conversion microbienne sélective du glucose en un produit qui est plus facile à séparer du fructose que du glucose. Cette étude rapporte qu'un nouveau mutant, *Saccharomyces cerevisiae* ATCC 36858, est capable de produire des sirops purs et riches en fructose à partir de mélanges synthétiques de glucose/sucrose et de raffinose, et à partir de mélasse. En général, la majorité des souches de *S. cerevisiae* peuvent hydrolyser le sucrose par invertase et utilisent complètement les composantes glucose et fructose de ce disaccharide. Elles peuvent aussi consommer la composante fructose du raffinose après son hydrolyse par invertase. Par contre, elles ne décomposent pas la composante mélibiose restante à cause d'une pénurie de mélibiase. Les résultats de cette étude démontrent que *S. cerevisiae* ATCC 36858 peut hydrolyser le sucrose, le raffinose et le mélibiose et, par la suite, fermenter de façon sélective le glucose et le galactose en éthanol.

Dans des procédés en charge, le rendement du fructose était au-delà de 93% de la valeur théorique dans des milieux synthétiques de glucose/fructose ayant une concentration totale de sucre de 300 g/L. Le rendement de l'éthanol était environ 76% de la valeur théorique. Le rendement du fructose était au-delà de 90% de la valeur théorique en milieux synthétiques ayant une concentration de sucrose allant jusqu'à 726 g/L et en milieu de mélasse ayant une concentration allant jusqu'à 323 g/L. Le rendement de l'éthanol était entre 72 et 85% en milieux synthétiques ayant une concentration de sucrose allant jusqu'à 451 g/L. Un rendement inférieur a été obtenu en milieux de mélasse. La souche peut être considérée osmophilique puisqu'elle peut

croître en milieux synthétiques même avec une concentration de sucrose supérieure à 530 g/L. En milieux synthétiques ayant des concentrations initiales de raffinose entre 30 et 242 g/L, les rendements du fructose et de l'éthanol étaient entre 82 et 96% et entre 67 et 76%, respectivement.

Les sirops contenant plus de 97% de fructose peuvent être produits à partir de milieux synthétiques ayant une concentration initiale de sucrose inférieure à 360 g/L. Même à une concentration de sucrose de 574 g/L, le sirop produit contenait 59% de fructose et était donc plus riche en ce glucide que le HFCS ordinaire à 55%. Les fractions de fructose dans le contenu de glucide des sirops produits étaient supérieures à 95% lorsque les concentrations totales initiales de sucre dans les milieux de mélasses de canne à sucre et de betterave sucrière étaient inférieures à 274 et 242 g/L, respectivement. Les sirops contenant plus de 96% de fructose peuvent être produits à partir de milieux ayant une concentration initiale de raffinose inférieure à 189 g/L.

Les effets de biomasse initiale, de concentrations d'extrait de levure et d'éthanol, de pH, et de stérilisation du milieu sur la production de fructose et d'éthanol en milieux synthétiques de sucrose ont aussi été étudiés. L'augmentation de la concentration de biomasse dans le milieu de fermentation augmentait la productivité du bioréacteur. Par contre les rendements de l'éthanol et du fructose ne variaient que légèrement. L'augmentation de la concentration initiale d'extrait de levure résultait en des taux de croissance du mutant, des rendements de biomasse et des productivités d'éthanol plus élevés avec des changements négligeables dans les rendements d'éthanol et de fructose. La croissance du mutant et la capacité de fermentation diminuaient considérablement suite à une augmentation de la concentration initiale d'éthanol. Lorsque les effets du pH entre 5.1 et 6.6 sur la production de fructose et d'éthanol en milieu de sucrose étaient considerés, aucun changement significatif n'a été observé en ce qui concerne la croissance du mutant et la capacité de fermentation. Les résultats démontraient aussi que l'utilisation de milieux stériles ou non-stériles avait un impact négligeable sur la cinétique du procédé de fermentation ou des rendements de produits.

Les résultats de cette recherche ont démontré que *S. cerevisiae* ATCC 36858 possède plusieurs avantages par rapport à d'autres microbes tels que *Zymomonas mobilis* et *S. cerevisiae* ATCC 36859, utilisés par d'autres chercheurs dans la production du fructose et de l'éthanol à partir de milieux à concentration élevée de substrat. Parmi ces avantages figurent l'hydrolyse très efficace du sucrose, la capacité de fermenter en milieu à concentrations élevées de substrat, et des rendements élevés de fructose et d'éthanol. Le *S. cerevisiae* ATCC 36859 étudié a aussi démontré des rendements élevés de fructose et d'éthanol, mais il ne peut pas utiliser le sucrose, ce qui limite son utilisation avec les mélanges de glucose et de fructose.

Dans cette étude, le produit formé à partir de mélasse a été purifié. L'éthanol a été séparé du milieu par évaporation sous vide, et la couleur a été enlevée par membranes à ultrafiltration et par charbon activé. Deux membranes à ultrafiltration ont été utilisées: le polyéther sulfoné (PES) qui a un seuil de coupure moléculaire de 10 000 Daltons, et un composé à couche mince (CCM) qui a un seuil de coupure moléculaire de 1 000 Daltons. La membrane à couche mince était composée de poly (oxide de 2,6-diméthyl-1,4-phenylène) sulfoné brominé et la couche de support était composée de polyéther sulfoné. Les efficacités d'enlèvement de couleur étaient 94,2 et 98,7% avec les membranes de PES et de CCM, respectivement. D'autres traitements du perméat de la membrane de PES avec 7,5% de charbon activé dans la suspension a résulté en une augmentation dans l'efficacité d'enlèvement de couleur jusqu'à 99,7%. Par contre, quand le perméat de la membrane de CCM était traité avec 3% de charbon activé dans la suspension, l'efficacité d'enlèvement de couleur était aussi élevé que 99,9%. Quand le charbon activé seul était utilisé pour enlever la couleur du milieu de fructose, 33% de charbon activé dans le milieu était nécessaire pour obtenir une efficacité d'enlèvement de couleur de 94,4%. Ces résultats ont démontré que la combinaison de la technique de séparation par membrane avec le traitement au charbon activé pour la purification du sirop a diminué considérablement la quantité de charbon activé nécessaire pour obtenir une efficacité d'enlèvement de couleur supérieure à 99%. Après le procédé de décoloration, le produit final a été déminéralisé en utilisant des résines d'échange ionique. Du sirop de fructose sans couleur qui ressemblait visuellement au HFCS industriel a été produit.

Des modèles de cinétique ont été proposés afin de décrire la production du fructose et de l'éthanol dans des milieux de sucrose et de raffinose. Les résultats ont démontré que les prédictions de ces modèles pour les concentrations de biomasse, de sucrose, de glucose, de fructose et d'éthanol étaient en accord avec les valeurs obtenues expérimentalement dans des milieux de concentration de sucrose inférieure à 257 g/L lorsque les estimés de paramètres individuels étaient utilisés. Le modèle n'a pas bien prédit la phase de croissance exponentielle dans des milieux ayant une concentration égale ou supérieure à 257 g/L. Par contre il a très bien prédit les autres variables et la phase stationnaire. En milieux de raffinose, les résultats ont démontré que les prédictions de ces modèles pour les concentrations de biomasse, de raffinose, de fructose et d'éthanol étaient assez bonnes, surtout dans des milieux ayant une concentration initiale de raffinose inférieure à 242 g/L et en utilisant les estimés de paramètres individuels. Par contre le modèle proposé pour la fermentation du raffinose était incapable de prédire les tendances obtenues expérimentalement pour la production et la consommation du mélibiose.

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Nomenclature

Abbreviations

ADH	Alcohol Dehydrogenase
ATCC	American Type Culture Collection
Brix	A measurement of total solids in sugar liquor or syrup using a Brix hydrometer. Brix is equal to the percentage of sugar in solution containing only sugar and water.
DSM	Deutsche Sammlung von Mikroorganismen
EDTA	Ethylene Diamine Tetraacetic Acid
HFCS	High Fructose Corn Syrup
HFS	High Fructose Syrup
HPLC	High Performance Liquid Chromatography
ICUMSA	International Commission for Uniform Methods of Sugar Analysis
IMRI	Industrial Membrane Research Institute at the University of Ottawa
IU	ICUMSA colour units
MWCO	Molecular Weight Cut Off, molecular weight of particles rejected by the membrane with a 90% or higher efficiency.
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced form of Nicotinamide Adenine Dinucleotide
PES	Polyethersulfone
RBU	Reference Base Units, which are used by the soft drink industry and are approximately equivalent to ICUMSA colour units
SPPOBr	Poly (2,6-dimethyl-1,4-phenylene oxide) Brominated Sulfonated
TFC	Thin Film Composite
v/v	Volume per volume
w/v	Weight per volume

Symbols

A	Effective membrane area	m^2
A_{420}	Absorbance at 420 nm	
b	Path length of the light (1 cm)	cm

c	Solid content	g/mL
F	Total flux	L/ m^2 h
h_R	Specific raffinose uptake rate	g raffinose/g biomass h
h_S	Specific sucrose uptake rate	g sucrose/g biomass h
IU	ICUMSA colour units	IU
K	Empirical constant	
K_1, K_2	Empirical constants (Eqs. 3.11, 3.14 and 3.16)	L/g
K_i	Inhibition constant	
K_R	Saturation constant for raffinose uptake	g/L
K_S	Saturation constant for growth	g/L
K'_S	Saturation constant for product formation	g/L
K_{SS}	Saturation constant for sucrose uptake	g/L
P	Product (ethanol) concentration	g/L
P_{min}	Ethanol concentration above which inhibition becomes measurable	g/L
P_{max}	Lowest ethanol concentration above which growth is not measurable	g/L
P'_{max}	Lowest ethanol concentration above which cells no longer produce ethanol	g/L
R	Ratio of glucose to fructose consumption rate	
S	Substrate concentration	g/L
S_{max}	Lowest total carbohydrate concentration above which growth is not measurable .	g/L
S'_{max}	Lowest total carbohydrate concentration above which cells no longer produce ethanol	g/L
t	Time	h
t_P	Permeation time	h
T_s	Transmittance	
V_P	Permeation volume	L
X	Biomass concentration	g/L
$Y_{F/R}$	Fructose yield coefficient	g fructose/g raffinose
$Y_{G/SUC}$	Glucose/fructose yield coefficient	g glucose or fructose/g sucrose
$Y_{M/R}$	Melibiose yield coefficient	g melibiose/g raffinose
$Y_{P/M}$	Ethanol yield coefficient	g ethanol/g melibiose

$Y_{P/S}$	Ethanol yield coefficient	g ethanol/g carbohydrate
$Y_{X/M}$	Biomass yield coefficient	g biomass/g melibiose
$Y_{X/S}$	Biomass yield coefficient	g biomass/g carbohydrate

Greek symbols

μ	Specific growth rate	h^{-1}
μ_{max}	Maximum specific growth rate	h^{-1}
ν_{max}	Maximum specific ethanol formation rate	h^{-1}
ω	Degree of substrate inhibition of growth rate	
$\omega^{'}$	Degree of substrate inhibition of ethanol formation	
λ	Empirical constant	

Subscript

F	Fructose
G	Glucose
M	Melibiose
R	Raffinose
SUC	Sucrose
TC	Total carbohydrates (glucose and fructose)

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Chapter 1

Introduction

1.1 Fructose syrup

Fructose is the sweetest natural sugar. It is a constituent of invert sugar and of high fructose corn syrup (HFCS), which are produced industrially in large quantities. In these products, fructose and glucose are present in nearly equimolar concentrations. The commercialization of high fructose corn syrup started in early 1970's. Three HFCS containing 42, 55 and 90% fructose are commercially available. In sweetness, the 55 HFCS is about equal to sucrose, while the 90 HFCS is 20-60% sweeter than sucrose (Long, 1991). The major use of HFCS is in beverages, baking, canning and dairy products.

Production of pure, solid fructose is carried out by the crystallization of syrups containing 90-95% fructose. The main advantage of using pure fructose over sucrose is that fructose is about 60% sweeter than sucrose and 150% more than glucose (Blanchard and Geiger, 1984). Therefore, a much smaller amount of fructose is needed to produce the same degree of sweetness, which leads to a reduction in calorie intake. The use of currently available syrups (i.e. 42 HFCS and 55 HFCS) for the production of pure fructose requires a substantial reduction in their glucose content. Separating glucose from fructose, which are isomers, using existing industrial methods requires difficult and costly chromatographic techniques in order to produce 90 HFCS from syrups with a lower fructose concentration (Coker and Venkatasubramanian, 1987).

1.2 Alternative methods for the production of fructose syrup

An alternative to the chromatographic separation process is microbial conversion of glucose from glucose/fructose mixtures or sucrose media to ethanol, a product that is easier to separate from fructose than glucose. Mutants of various microorganisms such as *Tricholoma nudum* (Reusser et al., 1960), *Fusarium sp.* F5 (Ueng et al., 1982), *Pullularia pullulans* (Fan, 1988) and *Zymomonas mobilis* (Suntinanalert et al., 1986; Edye et al., 1989; Doelle and Doelle, 1991; Kirk and Doelle, 1994) have been used for the production of fructose from sucrose media. In glucose/fructose mixtures, mutants of *Mucor sp.* M105 (Ueng et al., 1982), *Zymomonas mobilis* (Bringer and Sahm, 1988; Doelle and Doelle, 1991) and *Saccharomyces cerevisiae*

ATCC 36859 (Duvnjak and Koren, 1987; Koren and Duvnjak, 1989; Koren and Duvnjak, 1992; Remize et al., 1998) have been used to produce fructose and ethanol. However, a substantial fructose consumption and/or production of byproducts such as sorbitol were noticed in processes with a majority of the above microorganisms.

1.3 Objectives and strategy of the research

The strain *Saccharomyces cerevisiae* ATCC 36859 showed high fructose and ethanol yields in batch fermentations with glucose/fructose mixtures (Koren and Duvnjak, 1993). However, that strain could not utilize sucrose, which limited its use to media with glucose/fructose mixtures. The present work reports on a new mutant, *Saccharomyces cerevisiae* ATCC 36858, which is able to produce fructose and ethanol from media that contain sucrose. A literature review did not show that such a strain of *S. cerevisiae* was previously reported.

The objective of this research was to study the ability of *S. cerevisiae* ATCC 36858 to produce fructose and ethanol from sucrose and molasses media. The work was carried out in several stages. The first stage involved an investigation of biomass growth and ethanol production in synthetic media with glucose, fructose or galactose as the only carbon source, to examine the ability of the new mutant to ferment various sugars. In the second stage, the characteristics of the new mutant to produce ethanol and fructose in synthetic media with glucose/fructose mixtures were studied to compare this mutant with other microorganisms, in particular with *S. cerevisiae* ATCC 36859. The third stage examined the fermentation of *S. cerevisiae* ATCC 36858 in synthetic sucrose media. In this stage, the effects of initial sucrose, yeast extract, ethanol, and biomass concentrations, pH and media sterilization were examined to see the limitations of yeast growth and fructose and ethanol production of this strain. The fourth stage was to investigate the ability of the new mutant to produce fructose and ethanol in synthetic media with raffinose and in complex media such as sugar cane and sugar beet molasses. These media were chosen since molasses contains sucrose, fructose, glucose and raffinose at a total carbohydrate concentration of 50-60% (w/v), of which more than 60% is sucrose. It also contains minerals and other nutrients that support the growth of yeast cells. The fifth stage explored the purification of fructose syrups produced from molasses media using activated carbon, ultrafiltration membranes and ion-exchange resins. The sixth stage was to develop mathematical models to describe the production of ethanol and fructose from sucrose and raffinose media.

Chapter 2

Literature Review

Relevant literature to the production of fructose and ethanol will be reviewed in the following sections. This will include fructose syrup production, glucose-fructose separation, selective conversion of glucose and ethanol production.

2.1 Fructose syrups production

High fructose syrups (HFS) are produced around the world from a variety of raw materials. Sucrose from sugar cane and sugar beet as well as starch raw materials including rice, tapioca, wheat, cassava, potato and corn can be used for the production of HFS (Hanover and White, 1993; Vuilleumier, 1993). Inulin, a polymer in which many plants such as Jerusalem artichokes store fructose, is considered another source for producing high fructose syrup (Fleming and GrootWassink, 1979; Kosaric et al., 1985; Duvnjak and Koren, 1987; Koren and Duvnjak, 1990; Remize et al., 1998). However, the most widely used source for HFS production is corn starch because of its abundance and low cost (Vuilleumier, 1993). When corn starch is used for HFS production, the product is called high fructose corn syrup (HFCS).

The development of corn sweeteners can be traced back to the early nineteenth century with the discovery that starch yielded a sweet substance when heated with acid. It was later found that the sweet substance was glucose. This established the basis for the commercial production of syrups and crude sugars from starch. As the demand for starch-derived syrups increased, corn emerged as the preferred source of starch because it was inexpensive, plentiful and easily stored (Coker and Venkatasubramanian, 1987). Previously, the corn syrups contained only 42% glucose. Although, this product was useful in many applications, it lacked the sweetness of sucrose and could not compete effectively with it.

The discovery, isolation and application of various carbohydrase enzymes resulted in the development of many new corn syrups with different properties. Marshall and Kooi (1957) used glucose isomerase to catalyze the conversion of glucose to fructose. The enzymatic transformation of glucose to fructose was first introduced to corn sweetener production on an industrial level in 1967 (Bhosale et al., 1996). Application of this enzyme to corn starch industry led to the production of HFCS.

A typical process of producing HFCS is shown in Figure 2.1. In this process, corn starch is first hydrolyzed to glucose by α-amylase and glucoamylase, followed by converting glucose to fructose using the enzyme glucose isomerase. A thermostable α-amylase is added to the starch slurry (35% solids) which is maintained at 104 to 107°C for 5 to 8 minutes. A subsequent 1 to 2 hour hold at 95°C stabilizes the starch and enables enzymatic activity to continue until a solution containing 10-15% glucose is attained (Coker and Venkatasubramanian, 1987; Luenser, 1987).

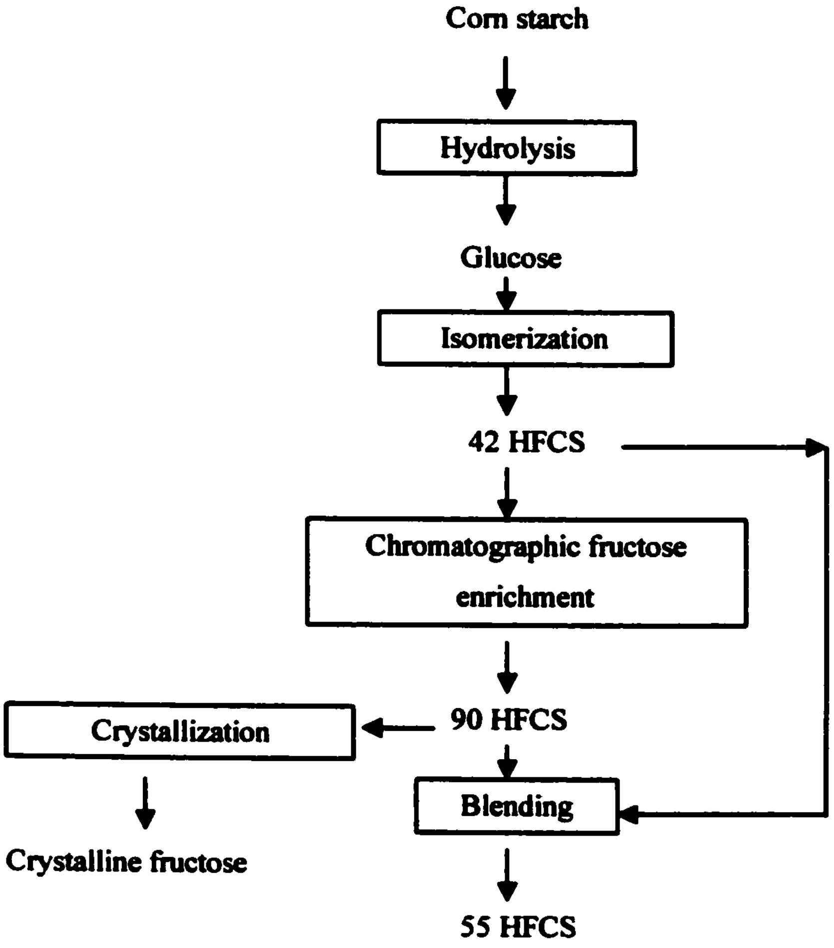

Figure 2.1 A typical process for the production of HFCS from corn starch.

Once corn starch is liquefied, glucoamylase is added to break the remaining starch bonds, the temperature is maintained at 60°C and the mixture is agitated for 48-96 hours until a maximum glucose concentration is attained. The glucoamylase action is stopped, by heat inactivation or removal using ion exchange resins, to minimize yield loss due to re-polymerization of the glucose to the disaccharide isomaltose (Luenser, 1987). At this point, the glucose concentration is about 95% of the total carbohydrate in the hydrolysate. This is later filtered to remove suspended matters, treated with activated carbon and ion exchange resins to remove colourants and ash materials, and then concentrated to 40-50% solids before isomerization. In the isomerization step, the glucose liquor is preheated then magnesium, which acts as a co-factor, is added before flowing through a bed of immobilized glucose isomerase that is kept at a temperature of 60-65°C (Coker and Venkatasubramanian, 1987; Luenser, 1987).

Glucose isomerase converts glucose into fructose. The equilibrium of the glucose/fructose reaction is established at a concentration of about 55% of fructose and 45% glucose (dry basis). As with all enzymatic reactions, the rate of reaction slows progressively as the equilibrium is approached. Therefore, to attain maximum productivity, most manufacturers produce a mixture containing 42% fructose (Bucke, 1979; Coker and Venkatasubramanian, 1987). The final product, after additional purification with activated carbon and ion exchange resins, contains 42% fructose, 54% glucose and 4% higher saccharides (42 HFCS). The mixture is then concentrated to 71% solids. Since fructose is sweeter than glucose, the sweetness of this syrup is comparable to sucrose, which makes the substitution possible (Coker and Venkatasubramanian, 1987).

Commercial chromatographic separation techniques are used to produce an enriched fructose syrup containing 90% fructose (90 HFCS) from the 42 HFCS. Manufacturers usually blend the 90 HFCS with the 42 HFCS to produce a syrup containing 55% fructose (55 HFCS). The composition and properties of various HFCS's are given in Table 2.1 (Tilburg, 1985; Coker and Venkatasubramanian, 1987; Long, 1991; Osberger, 1991).

Table 2.1 Composition and properties of High Fructose Corn Syrups.

	42 HFCS	55 HFCS	90 HFCS
Fructose (%)	42	55	90
Glucose (%)	54	42	9
Higher saccharides (%)	4	3	1
Solids (%)	71	77	80
Sweetness (sucrose = 100)	90-95	95-100	120-160

It can be seen from Table 2.1 that the sweetness of corn syrups, relative to sucrose increases as its fructose content increases. Since the 55 HFCS has a sweetness comparable to sucrose, it is used as a substitute in many foods and beverages. This product is also potentially useful in low calorie and dietetic products. However, manufacturers are interested in higher fructose syrups (90 HFCS) due to its high relative sweetness and the possibility of using it to produce crystalline fructose. Crystalline fructose is about 1.6 times sweeter than sucrose (Blanchard and Geiger, 1984). Furthermore, crystalline fructose is advantageous for making new products due to its rapid solubility and metabolic superiority (Osberger, 1991).

The production of pure, solid fructose is carried out by crystallization of syrups containing 90-95% fructose. The use of currently available syrups (i.e. 42 HFCS and 55 HFCS) for the production of pure fructose requires a substantial reduction in their glucose content. The existing industrial methods employ difficult and costly chromatographic techniques to produce 90 HFCS (Coker and Venkatasubramanian, 1987). Therefore, it is highly desirable to produce syrups with high fructose concentrations using other techniques (Visuri and Klibanov, 1987). This would be possible if either the isomerization reaction could provide higher fructose yields or a less expensive and effective separation technique could be developed.

The addition of certain ions to the glucose liquor was found to shift the isomerization reaction in favour of fructose by forming a complex with that sugar. Studies of this complexation method with borate (Takasaki, 1971) and germanate oxyanions (Barker et al., 1983) resulted in the production of syrups containing 90 and 94% fructose, respectively. However, the toxicity of these anions prevented their use in the production of food grade syrups.

Another method to increase the fructose yield in the isomerization reaction is by carrying it out at higher temperatures. Existing industrial isomerization of glucose to fructose is carried out at 60-65°C to produce 42 HFCS (Coker and Venkatasubramanian, 1987; Luenser, 1987). Tewari and Goldberg (1985) produced 55 HFCS by carrying out the reaction at 100°C. Unfortunately, glucose isomerase activity decreases significantly at higher temperatures, which makes it unreliable for commercial operations. In another study, Visuri and Klibanov (1987) found that glucose isomerase could produce 55 HFCS when the process was conducted at ambient temperature in 85-90% ethanol. Unfortunately, the high ethanol concentration reduced the enzymatic activity by up to 75% and only dilute sugar solutions could be treated this way due to their low solubilities. Other methods to provide high fructose yield, using effective and less expensive separation techniques, will be discussed in the next section.

2.2 Glucose-fructose separation

Many methods for chemical separation of glucose and fructose from carbohydrate solutions have been developed based on precipitation, complex formation and chromatographic procedures. The classical one is precipitation, which involves precipitation of fructose as calcium fructosate using excess calcium hydroxide (Kieboom and van Bekkum, 1985). The precipitate is then treated with carbon dioxide to produce calcium carbonate and an aqueous solution of fructose. A similar type of separation involves the precipitation of sodium chloride-glucose double salt from solutions containing fructose, glucose and sodium chloride (Tatuki, 1972). This process requires maintaining the pH of the solution at 9.0, which results in some fructose loss due to alkaline degradation (Barker and Petch, 1985).

Liquid membranes have also been used for the separation of fructose from glucose/fructose mixtures (Paugam et al., 1996; Di Luccio et al., 2000). This technology combines solvent extraction and stripping in a single step. In this process, solute partition from the aqueous to the membrane (organic) phase occurs followed by its complexation with the carrier inside the membrane phase. The solute-complex then diffuses through the membrane and decomplexation and partitioning of the solute into the aqueous phase at the permeate side takes place. Paugam et al. (1996) used a flat sheet of polymer-supported liquid membrane containing boronic acid carrier to separate fructose from glucose/fructose mixtures. When immobilized glucose isomerase was added to a solution of glucose in the source compartment of a liquid membrane transport cell, the receiving phase mixture was greater than 80% fructose. The idea was to remove fructose continuously from a glucose-fructose isomerization reaction and alter the isomerization equilibrium towards more complete fructose production. The fructose flux rate and fructose/glucose selectivity were 55×10^{-8} mole/m^2 s and 19, respectively. The sugar mixture used contained 27 g/L of each glucose and fructose. The fructose flux rate and fructose/glucose selectivity decreased by 42 and 35%, respectively, when the membrane was washed and reused. Di Luccio et al. (2000) also used a polymer-supported liquid membrane containing a boronic acid carrier to separate fructose from glucose/fructose mixtures with higher sugar concentrations. The fructose/glucose selectivity and fructose flux rate were about 14 and 42×10^{-8} mole/m^2 s, respectively, and were obtained using a hollow fiber liquid membrane with a glucose/fructose mixture (45 g/L of each sugar). The major drawbacks of using liquid membrane separation are the instability of the membranes, low fructose flux rates and the low sugar concentrations.

The most common used technique for the separation of glucose and fructose is chromatography. Much of the work in this area originated from Jones and Wall (1960), who discovered that monosaccharides have different affinities to ion exchange resins. Therefore, when a mixture of glucose and fructose is passed through a column containing Ba^{+2} resin, glucose appears first in the effluent followed by fructose. Different types of resins were tested, however, calcium-based resins are predominantly used due to its lack of toxicity. Batch and continuous separation processes can be used for the production of 90 HFCS from the 42 HFCS (Teague and Arnold, 1983; Blanchard and Geiger, 1984). Batch separation systems are suitable for the production of small quantities of enriched fructose syrups. Manufacturers use continuous separation techniques for large-scale production of 90 HFCS. In one of the continuous separation schemes, the feed sugar mixture (42 HFCS) enters the process through a rotary valve and is directed to one of the compartment where the adsorbent is placed (Blanchard and Geiger, 1984). As the feed mixture flows down over the resin, fructose is retained while glucose passes through to the next compartment. After the first compartment is saturated, the valve is altered so that the operation is moved one compartment downstream. Then, the elution of the fructose from the first compartment takes place. Syrup containing 90% fructose is produced by such chromatographic technique. Since chromatographic techniques are both expensive and difficult, manufacturers treat small quantities of the 42 HFCS this way. The rest is either sold as 42 HFCS or blended with 90 HFCS to produce 55 HFCS. This means that only very small amounts of 90 HFCS remain for crystallization, and therefore only a very small quantity of crystalline fructose is obtained at the end of the process. To eliminate this difficult and expensive chromatographic process, novel methods of separating glucose from fructose for the production of enriched high fructose syrups have been studied. These methods involve a microbial, selective conversion of glucose to another substance such as ethanol, which is more easily removed from the glucose/fructose mixture. Different microorganisms that have this selective conversion characteristic were studied and are discussed in the next section.

2.3 Selective conversion of glucose

Novel fermentation methods that involve converting glucose into a substance more easily separated from fructose for the production of HFS have been studied. For instance, the microbial conversion of glucose to ethanol, a valuable byproduct, would be an advantage in terms of the overall profitability of the process. This is due to the decreased separation costs and the increase in income generated from selling ethanol. Many researchers have studied this microbial selective conversion process.

Among the first researchers to propose the selective conversion process for the production of HFS were Reusser et al. (1960). In their study, *Tricholoma nudum* was used in synthetic sucrose and molasses media to produce fructose. The microbe hydrolyzed sucrose to glucose and fructose and then preferentially consumed glucose to form cells, which could be easily separated by filtration. Some fructose was consumed by the microbe but at lower rates than glucose. The microbe consumed as much as 30% of the fructose in a synthetic medium with 80 g/L sucrose. Lower fructose yields were obtained in molasses media.

Ueng et al. (1982) studied the ability of two mycelial fungal systems, *Mucor sp.* M105 and *Fusarium sp.* F5, to preferentially utilize glucose to produce ethanol. *Fusarium sp.* F5 was able to utilize sucrose directly to produce fructose and ethanol. Results showed that fructose consumption was nearly insignificant in the presence of glucose. However, in glucose/fructose mixtures, both fungal systems consumed glucose faster than fructose. When the initial concentrations of both sugars were equal, only 54% of the initial fructose was left unconverted after all the glucose was fermented to ethanol by *Mucor sp.* M105.

The bacterium *Zymomonas mobilis* was thoroughly studied for the production of fructose syrups. This bacterium uses glucokinase and fructokinase for glucose and fructose consumption, respectively (Doelle, 1982a). It was found that fructokinase activity was strongly inhibited by the presence of glucose, which resulted in controlling the uptake of fructose (Doelle, 1982b). Other researchers have suggested that competition for fructose transport plays an important role in preferential catabolism of glucose from sugar mixtures (Parker et al., 1997). *Z. mobilis* hydrolyzes sucrose faster than either glucose or fructose can be consumed, and therefore, glucose and fructose accumulate in the medium. When both sugars were present in the media, this bacterium started consuming fructose to produce sorbitol, which resulted in significant reductions in ethanol yields (Viikari, 1984; Leigh et al., 1984). It was found that sorbitol was produced in the media containing both sugars when the glucose concentration was above 7 g/L (Leigh et al., 1984).

In order to reduce fructose consumption, mutants of *Z. mobilis* (ATCC 39676 and ATCC 53432), which have higher glucose consumption rates, have been developed (Doelle and Greenfield, 1985a, b; Suntinanalert et al., 1986). The use of these mutants resulted in less fructose consumption. The consumption of fructose for sorbitol production was reduced significantly with pH control at 6.0 in media with 414 g/L sucrose using *Z. mobilis* ATCC 39676, but this reduced the extent of sucrose hydrolysis to 64% and increased the residual glucose concentration in the medium (Doelle and Greenfield, 1985b). A lower ethanol yield (about 65%) and a higher sorbitol concentration were obtained with *Z. mobilis* ATCC 53432 in a medium with 390 g/L sucrose (Suntinanalert et al., 1986). Bringer-Meyer et al. (1985) studied another mutant of *Z. mobilis* (DSM 3126) which lacks fructokinase activity and thus could not use fructose for ethanol production. When glucose and fructose were present in the medium, the mutant consumed glucose faster than fructose. The fructose that was consumed was converted to sorbitol. The amount of sorbitol produced increased as the initial sugar concentration increased. Therefore, Bringer and Sahm (1988) recommended substrate concentrations of 75 to 100 g/L of each glucose and fructose for an efficient production of fructose and ethanol. In these media, the fructose and ethanol yields were below 74% of the theoretical values and about 25 g/L of sorbitol was produced.

Edye et al. (1989) used *Z. mobilis* ATCC 53431 in fed-batch fermentations with cane syrups and pure sucrose media to reduce the amount of sorbitol formation. Sorbitol was formed largely during late exponential growth corresponding to an increase in glucose levels in the medium due to rapid sucrose hydrolysis. Once glucose levels decreased, sorbitol formation was negligible, despite high concentration of fructose. In the fed-batch approach, the initial sugar concentration in the process was kept low to prevent the accumulation of high glucose concentrations in the medium during sucrose hydrolysis. The amount of sorbitol decreased to 0.5 g/L in a medium containing a total sucrose concentration of 201 g/L from cane syrup with a fructose yield of 70%. However when the total sucrose concentration in cane syrup medium was increased to 350 g/L, a substantial increase in sorbitol concentration was observed (35 g/L). This also resulted in a decrease in the fructose yield to 54%. A higher fructose yield (66%) and a lower sorbitol formation (6.5 g/L) were obtained in a medium of cane syrup when either salts and complex organic supplements or pure sucrose were added. Although the used mutant *Z. mobilis* ATCC 53431 was fructokinase negative, the decrease in fructose yield at high sucrose concentrations was related to the conversion of fructose to ethanol. It was reported that fructose can be phosphorylated by glucokinase when the fructose concentration is higher than 18 g/L (Edye et al., 1990).

Doelle and Doelle (1991) also showed that the presence of mineral salts or molasses in the fermentation medium could effectively reduce sorbitol production. When sugar cane syrup was mixed with the molasses in a ratio of 1:2.8, the ethanol yield was 94%. An increase in the ratio to 1:16.8 resulted in a 36% decrease in ethanol yield. Fructose yield slightly decreased from 99 to 96%. Invertase was added to the fermentation medium to accelerate hydrolysis of both the sucrose and fructo-oligosaccharides formed. When HFCS was used as substrate to form a medium of about 96 g/L each of glucose and fructose, a loss of 34% in fructose yield as well as 23 g/L sorbitol were obtained.

Kirk and Doelle (1994) studied the production of fructose and ethanol from sucrose using *Z. mobilis* ATCC 53431, which was co-immobilized with invertase in alginate beads. In batch fermentations with semi-defined media, when the sucrose concentration was increased from 102 to 300 g/L, the fructose and ethanol yields decreased from 94 to 81% and 94 to 75%, respectively. The lower yields at high sucrose concentration are due to the increase in byproducts formation (sorbitol and fructo-oligosaccharides). The fermentation times were from 3 to 6 hours due to co-immobilization, which resulted in high ethanol productivity. In similar experiments with sugar cane syrup media in which the sucrose concentrations were between 102 and 314 g/L, the fructose yields were considerably reduced due to high sorbitol production.

Another microorganism that preferentially ferments glucose and has been used for the production of fructose is *Pullularia pullulans* (Fan, 1988). This microbe selectively converts glucose to a biopolymer that can be separated from the broth by filtration when used in media with either sucrose or a glucose/fructose mixture. In this process up to 50% of the fructose was consumed.

Mutants of *Saccharomyces cerevisiae* have also been shown to have selective fermentation capabilities. Wild yeast, *S. cerevisiae*, has at least three enzymes for catalyzing hexose phosphorylation, which is the first chemical transformation that sugars undergo inside cells. These enzymes are hexokinase A, which has a ratio of phosphorylation of fructose to glucose of approximately 2.5:1 (Gancedo et al., 1977), hexokinase B whose ratio is 1.3:1 (Colowick, 1973) and glucokinase that exhibits no activity on fructose (Maitra, 1975). A mutant lacking hexokinase A and B, and thus lacking fructose phosphorylating activity, has been obtained by treating the wild strain with N-methyl-N$'$-nitro-N-nitrosoguanidine (Maitra, 1970). The mutant *S. cerevisiae* ATCC 36859 was obtained by this method. Another mutant *S. cerevisiae* ATCC 36858, which lacks hexokinase A and B, was obtained by crossing a wild strain with a mutant lacking pyruvate kinase, an enzyme that plays a crucial role in the glycolytic breakdown of sugars (Lobo and Maitra, 1977).

Tests carried out with the mutant *S. cerevisiae* ATCC 36859 showed that media containing glucose and fructose in concentrations of up to 200 g/L each could be used to produce ethanol and fructose (Lamarche, 1988). In other experiments, the yeast cells were immobilized in Ca-alginate beads and used in a vertical packed bed reactor to produce fructose and ethanol continuously from glucose/fructose mixtures and from sucrose media. In the sucrose medium, two continuous processes were used in series: sucrose hydrolysis into glucose and fructose by invertase followed by conversion of glucose to ethanol using *S. cerevisiae* ATCC 36859, which cannot utilize sucrose directly. Using a feed containing sucrose (300 g/L), the fructose and ethanol yields were 68 and 72%, respectively. In addition, some sucrose (24 g/L) and glucose (30 g/L) accumulated at the end of the process.

Koren (1991) studied the kinetic behavior and the selective fermentation of glucose using *S. cerevisiae* ATCC 36859. In batch fermentations, no significant amounts of fructose were consumed and no unwanted byproducts were produced. Various types of media were used for the production of fructose and ethanol such as hydrolyzed Jerusalem artichoke juice (Duvnjak and Koren, 1987), HFCS supplemented with Jerusalem artichoke juice (Koren and Duvnjak, 1990) and glucose/fructose mixtures (Koren and Duvnjak, 1992).

It was found that glucose and fructose at high concentrations equally inhibited the growth of *S. cerevisiae* ATCC 36859, when grown on separate medium of either of these two sugars (Koren, 1991). The mutant showed the ability to produce and consume fructose and sorbitol.

When the mutant was pre-grown on fructose and then transferred to a sorbitol medium, ethanol and fructose were produced (Duvnjak et al., 1991a). Sorbitol production with this mutant started after all the glucose was consumed from either glucose/fructose mixtures or from Jerusalem artichoke extracts (Duvnjak et al., 1991b).

Continuous systems with immobilized *S. cerevisiae* ATCC 36859 cells for the production of fructose and ethanol from glucose/fructose mixtures (Guénette and Duvnjak, 1996) and from hydrolyzed Jerusalem artichoke (Koren, 1991) were also studied. This technique substantially enhanced ethanol productivity. A maximum ethanol productivity of 21.9 g/L h was attained using a feed containing 100 g/L glucose and 100 g/L fructose. Only 62% of the glucose was consumed with ethanol and fructose yields of 96 and 99% at a dilution rate of 0.74 h^{-1}, respectively. The addition of oleic acid to the fermentation medium was found to increase the ethanol productivity by 13% at the same dilution rate (Guénette and Duvnjak, 1995).

Koren and Duvnjak (1992) used fed-batch fermentation to produce ethanol and fructose from various glucose/fructose mixtures by *S. cerevisiae* ATCC 36859. Applying this technique, fructose syrups with over 250 g/L of that sugar were obtained using HFCS and hydrolyzed Jerusalem artichoke extract. The HFCS was added continuously to the reactor after inoculation. The initial glucose and fructose concentrations inside the reactor were 41 and 133 g/L, respectively. Ethanol concentration inside the reactor was kept below 50 g/L by high aeration rate to reduce inhibition by that product. The gases then passed through a condenser to recover ethanol. The final ethanol concentration would be about 100 g/L if the stripped ethanol from the reactor was included.

Remize et al. (1998) studied the construction of a flocculent form of *S. cerevisiae* ATCC 36859 by integration of the cloned FLO 1 flocculation gene and its utilization for the production of fructose and ethanol. The use of flocculating yeast is an alternative to other systems such as entrapped or adsorbed biomass or membrane reactors in which high cell concentrations can be maintained. Hydrolyzed Jerusalem artichoke juice was used in a gas-lift reactor with internal biomass recycle. The advantage of such system is that it has a high biomass hold-up, which increases the productivity of the reactor. The introduction of the flocculation gene into the yeast did not influence its fermentation potential for simultaneous production of fructose and ethanol, when tested with either synthetic glucose/fructose or hydrolyzed Jerusalem artichoke juice. In a test using a gas-lift reactor with a feed of hydrolyzed Jerusalem artichoke juice containing a total sugar concentration of 60 g/L, the fructose level remained unchanged, and the ethanol productivity and yield were 7.0 g/L h and 77%, respectively. In a batch test with a synthetic medium containing 100 g/L glucose and 100 g/L fructose, about 6% of the initial fructose was consumed, while the ethanol productivity and yield were 0.45 g/L h and 84%, respectively. In a similar test using a hydrolyzed Jerusalem artichoke juice medium containing only 30% of the previous total sugar concentration, the ethanol productivity was lower (0.1 g/L h), while the ethanol yield was higher (88%).

A comparison of the different microorganisms discussed above is presented in Table 2.2. High fructose and ethanol yields are important factors when comparing these microbes. Based on these criteria, *T. nudum, M. sp.* M105 and *P. pullulans* were poor in this respect. Although, the strain *Z. mobilis* ATCC 39676 showed both high fructose and ethanol yields when pH was controlled at 6.0, about 36% of the initial sucrose was not hydrolyzed and a huge amount of glucose was left unconverted at the end of the process. *F. sp.* F5 selectively converted glucose to ethanol from sucrose media with high fructose yield, however, ethanol yield and productivity were low.

The strain *Z. mobilis* ATCC 53432 was able to grow in a high sucrose medium (390 g/L) of which 93% of this sugar was used. Fructose and ethanol yields were 90 and 65% of the theoretical values, respectively. The low ethanol yield was due to sorbitol formation (about 35 g/L). The other strain *Z. mobilis* ATCC 53431 showed occasionally high fructose yields in media with low sugar concentrations. Sorbitol still accumulated as a byproduct especially in glucose/fructose media.

The mutant *S. cerevisiae* ATCC 36859 provided the best results among those reported. This yeast has the capability of utilizing high substrate concentrations with high fructose and ethanol yields without producing sorbitol or other byproducts. However, the main disadvantage of this strain is its inability to utilize sucrose in media, such as molasses, which contain this sugar. This has limited the use of *S. cerevisiae* ATCC 36859 to media with glucose/fructose mixtures or media that have these sugars.

The present study reports on the use of the mutant *Saccharomyces cerevisiae* ATCC 36858, which has the ability to selectively ferment glucose from different sugar mixtures. A

Table 2.2 Microorganisms used in the production of fructose by the selective conversion of glucose into ethanol and/or biomass.

Microbe	Feed[a] (g/L)	Product[a] (g/L)	Fructose[b] yield (%)	Ethanol[b] yield (%)	Productivity[c] (g/L h)	Reference
T. nudum	S-80	F-30	71	—	—	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) pH = 6.0	S-414	S-148 F-132 G-50 E-52	94	100	0.87	Doelle and Greenfield (1985b)
Z. mobilis (ATCC 53432)	S-390	S-27 F-172 E-69 So-35	90	65	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)
Z. mobilis (ATCC 53431) pH = 6.0	S-135[d] F-26	F-94 E-25 So-8	96	69	0.69	Doelle and Doelle (1991)
Z. mobilis (ATCC 53431) pH = 5.0-5.5	S-300[e]	F-127 E-72 So-32	81	75	7.2	Kirk and Doelle (1994)
P. pullulans	S-40 F-3.7 G-5.9	S-1.5 F-11.7 G-2.5	49	—	—	Fan (1988)
S. cerevisiae (ATCC 36859)	F-129 G-63	F-124 E-32	96	91	2.8	Koren (1991)
S. cerevisiae (ATCC 36859)	F-265[f] G-230	F-257 E-103	97	85	2.2	Koren and Duvnjak (1992)
S. cerevisiae[g] (ATCC 36859)	F-100 G-100	F-92 E-43	92	84	0.45	Remize et al. (1998)

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

[b] % of the theoretical values

[c] Ethanol production rate

[d] Cane syrup and cane molasses ratio (1:16.8) were the source of sucrose; invertase was added

[e] Semi-defined sucrose media; *Z. mobilis* was co-immobilized with invertase

[f] Hydrolyzed Jerusalem artichoke juice and HFCS with supplements were the source of glucose and fructose in fed-batch fermentation

[g] Flocculent strain

literature review did not show that such a mutant of *S. cerevisiae* has been studied previously for the production of fructose and/or ethanol. Preliminary tests showed that this mutant is able to hydrolyze sucrose into glucose and fructose and then to selectively convert glucose into ethanol. This makes the strain very useful in fermentations with various raw materials consisting of either glucose/fructose mixtures or sucrose, such as molasses. Ethanol, a valuable byproduct, was also produced in the process and its production should be optimized. Some of the techniques for ethanol production are summarized in the next section.

2.4 Ethanol production

There are several reasons why yeasts are the most important group of microorganisms that are commercially used for the production of ethanol. They have high ethanol productivity and can consume a variety of carbohydrates. Many organisms are of primary interest to industrial fermentation processes such as *Saccharomyces cerevisiae, S. uvarum* (*carlsbergensis*), *Schizosaccharomyces pombe* and *Kluyveromyces* species (Kosaric et al., 1983). Although other microorganisms can produce ethanol, these tend to yield significant quantities of undesirable byproducts and are either less tolerant to high ethanol concentration or ferment a limited number of sugars (D`Amore et al., 1990).

The yeast *S. cerevisiae* has the ability to consume and ferment a wide range of sugars, such as glucose, fructose, galactose, sucrose and maltose. The first step in the utilization of any sugar by yeast is usually either its passage intact across the cell membrane or the initial hydrolysis of higher saccharides outside the cell membrane, followed by entry of the hydrolysis products into the cell (Stewart and Russell, 1987).

Fermentation is a general term describing the anaerobic degradation of sugars into various products to obtain energy for biosynthesis and maintenance of the yeast cells. Glucose is converted to pyruvate in a sequence of 10 enzyme-catalyzed reactions in a pathway that is called either glycolysis or the Embden-Meyerhof-Parnas pathway (Figure 2.2). Glucose and fructose enter glycolysis directly, while galactose is converted first to glucose 1-phosphate, then to glucose 6-phosphate (Johnston and Carlson, 1992).

The glycolysis reactions can be arranged into three major parts. The first part is a series of preparatory rearrangement reactions that do not involve oxidation-reduction and results in the production of glyceraldehyde 3-phosphate. An oxidation-reduction reaction takes place in the

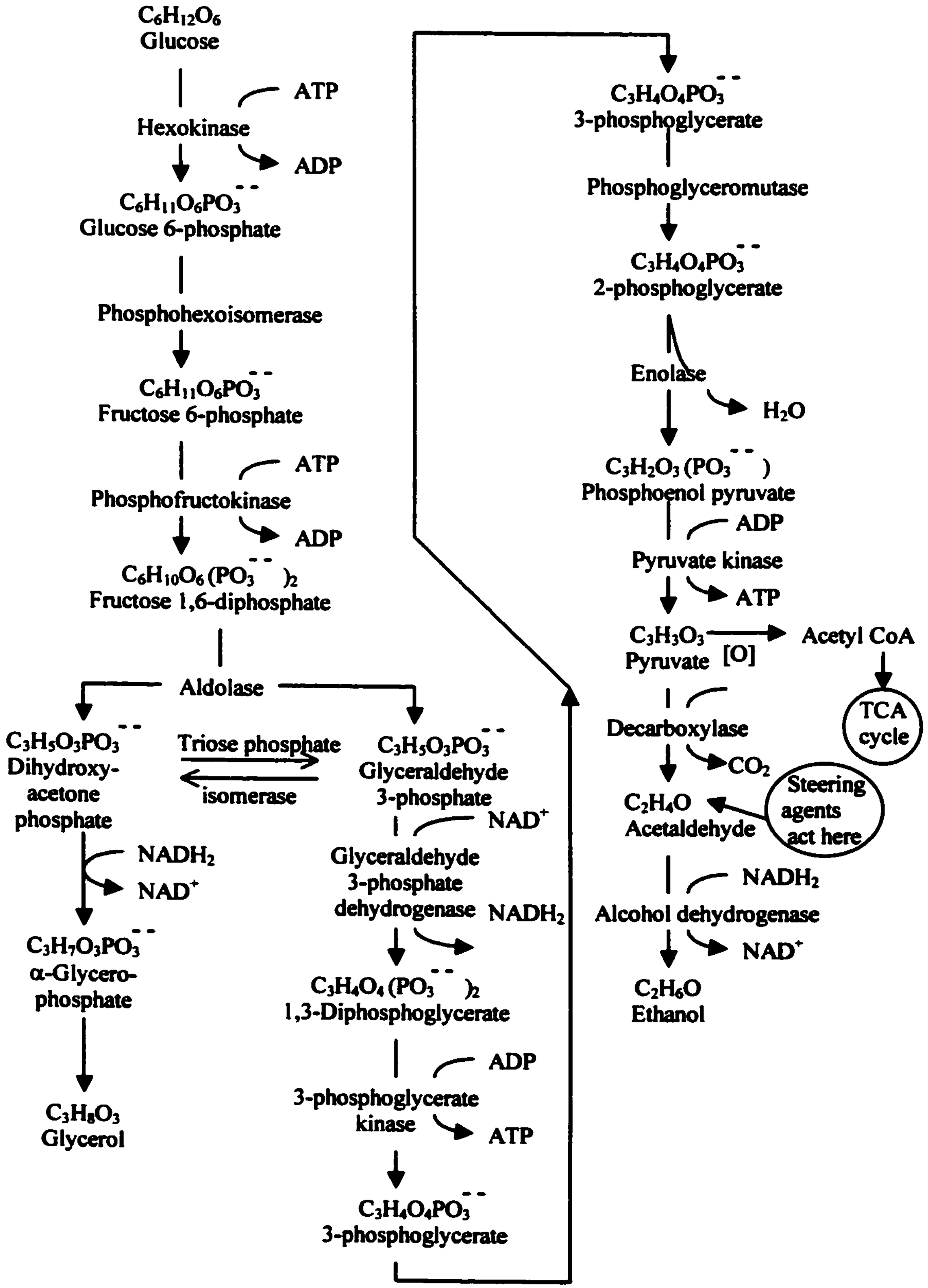

Figure 2.2 Embden-Meyerhof-Parnas pathway for the fermentation of glucose by *S. cerevisiae* (Agarwal, 1990).

second part to form pyruvate. In the third part, the pyruvate formed can proceed by two routes in the presence or absence of oxygen. Aerobically to form the acetyl-coenzyme A, in which the acetyl group is then oxidized completely to CO_2 and H_2O by the citric acid cycle (TCA). The other major pathway leads to the production of ethanol and CO_2. The pyruvate loses its carboxyl group by the action of pyruvate decarboxylase to form acetaldehyde. The acetaldehyde is then reduced to ethanol through the action of alcohol dehydrogenase.

The overall stoichiometry of ethanol production is as follows:

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2 + \text{energy} \qquad (2.1)$$

According to this equation, 1.00 g of glucose will theoretically yield 0.51 g ethanol. However, ethanol yields attained in practical fermentations do not usually exceed 90-95% of the theoretical values (Kosaric et al., 1983). This is due to the requirement of some nutrients to be utilized for cell growth and maintenance. Depending on the substrate and the fermentation conditions, various byproducts are also produced. Some byproducts that may be found in the medium after fermentation are glycerol, lactic acid, acetic acid, succinate, acetaldehyde and fusel oils (Oura, 1977). Up to 4-5% of the total substrate can be consumed for the formation of glycerol and succinate. Glycerol production under anaerobic conditions is usually explained by the necessity to consume the NADH produced in the formation of biomass (Nordstorm, 1966), as well as in the formation of acetic and succinic acids (Oura, 1977). Glycerol is also produced under aerobic conditions, but normally at much lower yields than under anaerobic conditions (Bjorkqvist et al., 1997). When sulfites are added to the fermentation medium, a substantial overproduction of glycerol from monosaccharides can be achieved by yeast forming an acetaldehyde-sulfite complex that limits ethanol production and promotes reoxidation of glycolytically formed NADH by glycerol synthesis (Rehm, 1988; Agarwal, 1990). Alternatively, glycerol production can also be increased by growing at pH values around 7 or above and by using osmotolerant yeasts (Rehm, 1988; Agarwal, 1990).

There are three main industrial uses of ethanol: as a solvent, a chemical intermediate or as a fuel. Ethanol produced by fermentation can compete directly with petroleum derived ethanol. Therefore, opportunities for increased alcohol production depend on process improvement and the use of inexpensive raw materials.

Various types of raw materials have been used for the production of ethanol: sugar, starch and cellulosic materials. When starch or cellulosic materials are used, preliminary acid or enzyme hydrolysis is required to break down the long chain polysaccharides to form simple sugars, which adds to the cost of the raw material. Since 55-75% of the final ethanol sale price depends on the raw materials cost, selection of cheap and suitable raw materials is critical (Kosaric et al., 1983; Maiorella et al., 1984a). However, environmental parameters such as pH, temperature, osmotic pressure, and CO_2 levels may directly affect yeast growth and ethanol productivity (Nagashima, 1990).

Different fermentation schemes have been developed for the production of ethanol. Conventional batch, continuous, cell recycle, and immobilized cell processes, as well as membrane, extraction and vacuum processes which selectively remove ethanol from the fermentation medium as it is formed were compared on identical bases using a consistent model for yeast metabolism (Maiorella et al., 1984a). The continuous flow stirred tank fermentor (CSTR) with cell recycle, tower and plug flow fermentors showed similar cost savings compared to batch fermentation. Cells recycle increases cell density inside the fermentor, which is important in reducing fermentation cost. Selective ethanol removal fermentation processes offer even greater savings as more concentrated feeds and products can be handled. In our laboratory in the production of ethanol and fructose from sucrose media, the selective removal of ethanol by a pervaporation unit coupled to a bioreactor enhanced the ethanol productivity by 66% (Gagné et al., 2002).

In the present study, ethanol and fructose production by *S. cerevisiae* ATCC 36858 in media with various carbohydrates was carried out using conventional batch fermentation processes. Mathematical models that describe the production of ethanol and fructose using this mutant were proposed. The use of mathematical models to describe the kinetics of growth of microorganisms and product formation is discussed in the next chapter.

Chapter 3

Mathematical Modelling

3.1 Introduction

Mathematical modelling, which provides a generalized description of the kinetics of the process, is an essential tool for fermentation research and development. Mathematical models can be used as experimental planning tools for the determination of the limiting experimental conditions and to assist in the interpolation and the extrapolation of the results.

Models can have varying complexities, depending on their purposes. For example, it would be possible to model every single reaction during fermentation and write rate equations for each, which is not practical. For technological purposes, models that take into account components that are easy to measure would be sufficient, although they show little about the biological mechanisms involved. These simplified models, known as unstructured models, will be used in this study.

3.2 Growth kinetics

Microorganisms can be regarded as a single reacting species that consumes substrates for its reproduction and for the production of metabolic products. Many kinetic models for growth and inhibition by substrate or product are based on those formulated for enzyme kinetics. This is because the cell is a biochemical reactor in which complex networks of enzyme reaction pathways are occurring. A relationship between the specific growth rate of a microorganism and the concentration of a limiting medium constituent has been proposed (Monod, 1949). The form of the equation, which is similar to Langmuir adsorption isotherm and the standard rate equation for enzyme-catalyzed reactions with a limiting substrate, states that:

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

where μ is the specific growth rate, S is the concentration of substrate, μ_{max} is the maximum specific growth rate and K_s is the saturation constant (i.e. the value of the limiting substrate concentration at which μ is equal to half μ_{max}).

The number of cells and their mass after the lag phase doubles regularly with time. The microorganism at this stage is undergoing exponential growth and 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 results in an expression for the growth rate:

$$\frac{dX}{dt} = \mu_{\max}\left(\frac{S}{K_S + S}\right)X \tag{3.3}$$

Although the above model is an oversimplification of the growth process, it reasonably expresses the interrelationship between biomass and substrate concentrations with time in most instances. Other equations for specific growth rate have been reported (Bailey and Ollis, 1986):

Tessier model: $\mu = \mu_{\max}\left(1 - e^{-S/K_s}\right)$ (3.4)

Moser model: $\mu = \mu_{\max}\ (1 + K_S\ S^{-\lambda})^{-1}$ (3.5)

Contois model: $\mu = \mu_{\max}\left(\frac{S}{K\,X + S}\right)$ (3.6)

where K and λ are empirical constants and X is the biomass concentration. In some cases, these equations may give better fits of experimental data than the Monod equation but they are still empirical models (Dhurjati and Leipold, 1990). Although many models have been proposed, the Monod model is the most widely used for microbial growth kinetics, primarily because it is simple and mathematical tractable (Dhurjati and Leipold, 1990). Medium constituents such as substrate or product may inhibit the growth rate. Many models for the specific growth rate with substrate and product inhibition have been reported (Bailey and Ollis, 1986).

3.2.1 Substrate inhibition

The μ expressions in the previous section predict that the specific growth rate will approach its asymptote value (μ_{max}) at high substrate concentrations. In practice, the growth rate declines at high substrate concentrations as the metabolic activities of cells are reduced. Edwards (1970) reported some of the possible causes of substrate inhibition on microbial kinetics. These include modification of the chemical potential of substrates, intermediates or products, alteration of the cell's permeability, change in the activity of one or more enzymes and the dissociation of one or more enzymes or metabolic aggregates. It has also been reported that growth inhibition is related to osmotic effects in which plasmolysis of the cells starts to occur at high substrate concentrations (Jones et al., 1981). The substrate concentration at which plasmolysis occurs is strain dependent. In most cases, equations for inhibition kinetics are derived from theories of the inhibition of a single enzyme.

The following models for substrate inhibition have been used by various authors attempting to contract a best fit for their experimental data:

Edwards (1970): $$\mu = \mu_{\max} \frac{S}{(K_S + S)\left(1 + \frac{S}{K_i}\right)} \tag{3.7}$$

Aiba et al. (1968): $$\mu = \mu_{\max} \frac{S}{K_S + S} \cdot e^{-S/K_i} \tag{3.8}$$

Moser (1985): $$\mu = \mu_{\max}(e^{-S/K_i} - e^{-S/K_S}) \tag{3.9}$$

where K_i is an empirical constant related to inhibition effects.

3.2.2 Product inhibition

Growth and metabolic activity of microbial cells may be inhibited by product. Ethanol fermentation is an example on such inhibition. It was reported that ethanol has possible effects on key glycolytic enzymes, such as hexokinase and alcohol dehydrogenase as well as cell constituents and membrane, which affects its properties for substrate uptake and ethanol excretion (Jones et al., 1981; Kosaric et al., 1983; Nagashima, 1990). Growth rate expressions with product inhibition can be described by equations similar to those proposed for substrate inhibition, if common mechanisms for inhibition are assumed (Edwards, 1970). Some of the relationships that take into consideration product inhibition on specific growth rate are summarized below:

Holzberg et al. (1967): $$\mu = \mu_{\max} - K(P - P_{\min}) \tag{3.10}$$

Aiba et al. (1968): $$\mu = \mu_{\max} \frac{S}{K_S + S} \cdot e^{-K_1 . P} \tag{3.11}$$

Ghose and Tyagi (1979b): $$\mu = \mu_{\max}\left(1 - \frac{P}{P_{\max}}\right) \tag{3.12}$$

where P is the ethanol concentration, P_{min} is the ethanol concentration above which inhibition becomes measurable, P_{max} is the lowest ethanol concentration above which growth is not measurable and K is an empirical constant.

There are no explicit equations that can be universally applied for growth inhibition by substrate or ethanol. It was found that the inhibition effect of ethanol on growth and fermentation vary with strain, the concentration of ethanol, the chemical and physical conditions of the fermentation and the physiological state of the cells (van Uden, 1985). Therefore, the choice between these expressions for growth inhibition by substrate and product is partially a matter of convenience (one equation is easier to manipulate than another) as long as the model has sufficient accuracy with respect to experimental results (Sinclair and Kristiansen, 1987).

3.3 Ethanol fermentation models

The formulation of a fermentation model involves the determination of an expression for the growth rate. In many cases this involves the use of the Monod model with additional terms to take into account substrate and product inhibition. Kinetic models for substrate consumption and product formation rates are then formulated using yields coefficients. In ethanol fermentation, product formation kinetics is growth associated (Bailey and Ollis, 1986). The simplest types of product formation kinetics arise when there are simple connections between product formation and substrate utilization or cell growth. Therefore, the model for product formation can be written as follows:

$$\frac{dP}{dt} = -Y_{P/S}\left(\frac{dS}{dt}\right) = \frac{Y_{P/S}}{Y_{X/S}}\left(\frac{dX}{dt}\right) \qquad (3.13)$$

where P is the ethanol concentration, $Y_{P/S}$ is the ethanol yield coefficient and $Y_{X/S}$ is the cell yield coefficient.

Aiba et al. (1968) studied ethanol fermentation in batch and continuous reactors using Baker's yeast. The proposed model, which includes the effects of product inhibition, is described by the following equations:

$$\frac{dX}{dt} = \mu_{\max} X \frac{S}{K_S + S} \cdot e^{-K_1 . P} \qquad (3.14)$$

$$\frac{dS}{dt} = -\frac{1}{Y_{P/S}}\left(\frac{dP}{dt}\right) = -\frac{1}{Y_{X/S}}\left(\frac{dX}{dt}\right) \tag{3.15}$$

$$\frac{dP}{dt} = v_{\max} X \frac{S}{K_S^{'} + S} \cdot e^{-K_2 .P} \tag{3.16}$$

where μ_{max} and v_{max} are the maximum specific growth and ethanol formation rates, which were 0.4 and 1.0 h^{-1}, respectively. The parameters K_S and K'_S are the saturation constants for growth and product formation, which were 0.22 and 0.44 g/L, respectively. The coefficients $Y_{P/S}$ and $Y_{X/S}$ are the ethanol and biomass yields, which were 0.35 and 0.10 g/g, respectively. The parameters K_1 and K_2 are empirical constants with values of 0.028 and 0.015 L/g, respectively.

Ghose and Tyagi (1979b) proposed another model for ethanol fermentation in batch and continuous reactors using *S. cerevisiae*. In their model, the combined effects of the inhibition imposed by ethanol and substrate were described by the following equations:

$$\frac{dX}{dt} = \mu_{\max} X \left(\frac{S}{S + K_S + S^2 / K_S \omega} \right) \left(1 - \frac{P}{P_{\max}} \right) \tag{3.17}$$

$$\frac{dS}{dt} = -\frac{1}{Y_{P/S}}\left(\frac{dP}{dt}\right) = -\frac{1}{Y_{X/S}}\left(\frac{dX}{dt}\right) \tag{3.18}$$

$$\frac{dP}{dt} = v_{\max} X \left(\frac{S}{S + K_S^{'} + S^2 / K_S^{'} \omega^{'}} \right) \left(1 - \frac{P}{P_{\max}^{'}} \right) \tag{3.19}$$

where μ_{max} and v_{max} were 0.4 and 1.4 h^{-1}, respectively. The parameters K_S and K'_S were 0.48 and 0.67 g/L, respectively. The coefficients $Y_{P/S}$ and $Y_{X/S}$ were 0.47 and 0.09 g/g, respectively. The parameter ω is the degree of growth inhibition by substrate and $\omega^{'}$ is the degree of ethanol formation inhibition by substrate. The values obtained for ω and $\omega^{'}$ were 428 and 455, respectively, which are nearly equivalent and indicate a similar inhibitory effect of glucose on the growth and ethanol production rates. The parameter P_{max} represents the lowest ethanol concentration above which growth is not measurable (87 g/L), while P'_{max} is the lowest ethanol concentration above which cells no longer produce ethanol (114 g/L). These values showed that the growth was more strongly inhibited by ethanol than the glucose consumption and ethanol production rates. This was also reported to be the case with other microbes (van Uden, 1985).

Koren (1991) proposed a model for ethanol fermentation in batch reactors using *S. cerevisiae* ATCC 36859. In his model, inhibition by substrate was neglected but expressions similar to those used by Ghose and Tyagi (1979b) for inhibition by ethanol were employed as described by the following equations:

$$\frac{dX}{dt} = \mu_{\max} X \left(\frac{S_G}{K_S + S_G} \right) \left(1 - \frac{P}{P_{\max}} \right) \tag{3.20}$$

$$\frac{dS_G}{dt} = -\mu_{\max} X \left(\frac{S_G}{K_S + S_G} \right) \left(1 - \frac{P}{P'_{\max}} \right) \frac{1}{Y_{X/S}} \tag{3.21}$$

$$\frac{dP}{dt} = \mu_{\max} X \left(\frac{S_G}{K_S + S_G} \right) \left(1 - \frac{P}{P'_{\max}} \right) \frac{Y_{P/S}}{Y_{X/S}} \tag{3.22}$$

where S_G is the concentration of glucose. The values of μ_{max} and K_S were 0.24 h^{-1} and 10.2 g/L, respectively. The coefficients $Y_{P/S}$ and $Y_{X/S}$ were 0.46 and 0.16 g/g, respectively. The parameter P_{max}, the lowest ethanol concentration above which growth is not measurable, was 73 g/L, while P'_{max}, the lowest ethanol concentration above which glucose is no longer consumed and ethanol no longer produced, was 162 g/L. Koren (1991) obtained different values for these parameters than those reported by other researchers, which was due to the differences in the characteristics of the strains used (Aiba et al., 1968; Ghose and Tyagi, 1979b).

3.4 Ethanol fermentation and fructose production models

3.4.1 Modelling of batch processes in media with glucose/fructose mixtures

Koren and Duvnjak (1993) proposed a kinetic model for the production of ethanol and fructose from glucose/fructose mixtures using *S. cerevisiae* ATCC 36859. Since this mutant selectively converts glucose to ethanol in media with glucose/fructose mixtures, while fructose accumulated in the media, they extended the previous model (Koren, 1991) by the addition of terms to take into account substrate inhibition and an equation to describe the kinetics of fructose production, as described by the following equations:

$$\frac{dX}{dt} = \mu_{\max} X \left(\frac{S_G}{K_S + S_G} \right) \left(1 - \frac{P}{P_{\max}} \right) \left(1 - \frac{S_{TC}}{S_{\max}} \right) \tag{3.23}$$

$$\frac{dS_G}{dt} = -\mu_{\max} X \left(\frac{S_G}{K_S + S_G} \right) \left(1 - \frac{P}{P'_{\max}} \right) \left(1 - \frac{S_{TC}}{S'_{\max}} \right) \frac{1}{Y_{X/S}} \tag{3.24}$$

$$\frac{dP}{dt} = \mu_{\max} X \left(\frac{S_G}{K_S + S_G} \right) \left(1 - \frac{P}{P'_{\max}} \right) \left(1 - \frac{S_{TC}}{S'_{\max}} \right) \frac{Y_{P/S}}{Y_{X/S}} \tag{3.25}$$

$$\frac{dS_F}{dt} = -\mu_{\max} X \left(\frac{S_G}{K_S + S_G} \right) \left(1 - \frac{P}{P'_{\max}} \right) \left(1 - \frac{S_{TC}}{S'_{\max}} \right) \frac{1}{Y_{X/S} \cdot R} \tag{3.26}$$

where S_G and S_F are the concentrations of glucose and fructose, respectively and S_{TC} is the total concentration of both sugars. Experimental data from batch tests in media containing glucose/fructose mixtures (222 to 413 g/L) were used for parameter estimation (Koren and Duvnjak, 1993). The parameters μ_{max} and K_S were 0.24 h^{-1} and 4.56 g/L, respectively. The coefficients $Y_{P/S}$ and $Y_{X/S}$ were 0.47 and 0.14 g/g, respectively. The parameters P_{max} and S_{max}, the lowest ethanol and total carbohydrate concentrations above which growth is not measurable, were 62 and 488 g/L, respectively, while P'_{max} and S'_{max}, the lowest ethanol and total carbohydrate concentrations above which glucose is no longer consumed and ethanol no longer produced, were 152 and 789 g/L, respectively. The parameter R, the ratio of glucose to fructose consumption rate, was 23. When this model was used to fit the experimental data, the model underpredicted biomass concentrations in the media with total sugar concentrations below 290 g/L (Koren and Duvnjak, 1993). The predictions of finial biomass concentration were within 12% of the experimental values when the total sugar concentration was less than 310 g/L. A deviation of up to 21% was obtained above this concentration. The predictions of final fructose and ethanol concentrations were within 12% of the experimental values.

3.4.2 Modelling of batch processes in media with sucrose

The mutant, *S. cerevisiae* ATCC 36858, used in this study is different from those previously reported. This mutant has the ability to simultaneously hydrolyze sucrose into glucose and fructose and then to selectively convert glucose into ethanol and biomass.

$$\text{Sucrose} + \text{Biomass} \longrightarrow \text{Glucose} + \text{Fructose} + \text{Biomass}$$

$$\text{Glucose} + \text{Biomass} \longrightarrow \text{Ethanol} + \text{Biomass}$$

A new model is formulated to describe the kinetics of the production of biomass, ethanol and fructose from sucrose media. In this model, the rate of biomass formation is related to glucose concentration using the Monod expression:

$$\frac{dX}{dt} = \mu_{\max} X \left(\frac{S_G}{K_S + S_G} \right) \tag{3.27}$$

where X and S_G are the biomass and glucose concentrations, respectively; μ_{max} is the maximum specific growth rate, and K_S is the saturation constant for growth.

The rate of sucrose uptake is related to the sucrose and biomass concentrations in a Monod type expression:

$$\frac{dS_{SUC}}{dt} = -\left(\frac{h_S \, X \, S_{SUC}}{K_{SS} + S_{SUC}} \right) \tag{3.28}$$

where S_{SUC} is the concentration of sucrose. The parameters h_S and K_{SS} are the specific sucrose uptake rate and the saturation constant for sucrose uptake, respectively.

The rate of glucose utilization is composed of two terms: the first term is related to the rate of sucrose uptake and the second part is proportional to biomass formation:

$$\frac{dS_G}{dt} = -\left(\frac{dS_{SUC}}{dt} \right) Y_{G/SUC} - \left(\frac{dX}{dt} \right) \frac{1}{Y_{X/S}} \tag{3.29}$$

where $Y_{G/SUC}$ and $Y_{X/S}$ are the glucose and biomass yield coefficients, respectively.

The rate of ethanol production, which is growth associated, is proportional to the rate of biomass formation:

$$\frac{dP}{dt} = \left(\frac{dX}{dt} \right) \frac{Y_{P/S}}{Y_{X/S}} \tag{3.30}$$

where P is the concentration of ethanol and $Y_{P/S}$ is ethanol yield coefficient.

The rate of fructose production is related to the rate of sucrose uptake:

$$\frac{dS_F}{dt} = -\left(\frac{dS_{SUC}}{dt} \right) Y_{G/SUC} \tag{3.31}$$

where S_F is the concentration of fructose and $Y_{G/SUC}$ is the yield coefficient of fructose and is equal to that of glucose.

3.4.3 Modelling of batch processes in media with raffinose

The mutant *S. cerevisiae* ATCC 36858 was also able to produce ethanol and fructose from raffinose media. The mutant hydrolyzes raffinose into fructose and melibiose. The melibiose is then hydrolyzed into glucose and galactose, which are selectively converted into ethanol and biomass. The formulated model describes the kinetics of biomass, ethanol and fructose production in raffinose media. In the model, it is assumed that the production of biomass and ethanol is related to melibiose consumption.

$$\text{Raffinose} + \text{Biomass} \longrightarrow \text{Melibiose} + \text{Fructose} + \text{Biomass}$$

$$\text{Melibiose} + \text{Biomass} \longrightarrow \text{Ethanol} + \text{Biomass}$$

The rate of biomass formation is related to melibiose concentration using the Monod expression:

$$\frac{dX}{dt} = \mu_{\max} X\left(\frac{S_M}{K_S + S_M}\right) \qquad (3.32)$$

where X and S_M are the biomass and melibiose concentrations, respectively; μ_{max} is the maximum specific growth rate, and K_S is the saturation constant for growth.

The rate of raffinose uptake is related to the raffinose and biomass concentrations in a Monod type expression:

$$\frac{dS_R}{dt} = -\left(\frac{h_R\, X\, S_R}{K_R + S_R}\right) \qquad (3.33)$$

where S_R is the concentration of raffinose. The parameters h_R and K_R are the specific raffinose uptake rate and the saturation constant for raffinose uptake, respectively.

The rate of melibiose utilization is composed of two terms: the first term is related to the rate of raffinose uptake and the second term is proportional to biomass formation:

$$\frac{dS_M}{dt} = -\left(\frac{dS_R}{dt}\right) Y_{M/R} - \left(\frac{dX}{dt}\right)\frac{1}{Y_{X/M}} \qquad (3.34)$$

where $Y_{M/R}$ and $Y_{X/M}$ are the yield coefficients of melibiose and biomass, respectively.

The rate of ethanol production is proportional to the rate of biomass formation:

$$\frac{dP}{dt} = \left(\frac{dX}{dt}\right)\frac{Y_{P/M}}{Y_{X/M}} \qquad (3.35)$$

where P is the concentration of ethanol and $Y_{P/M}$ is ethanol yield coefficient.

The rate of fructose production is related to the rate of raffinose uptake:

$$\frac{dS_F}{dt} = -\left(\frac{dS_R}{dt}\right) Y_{F/R} \tag{3.36}$$

where S_F is the concentration of fructose and $Y_{F/R}$ is the yield coefficient of fructose. The proposed models for the production of fructose and ethanol using *S. cerevisiae* ATCC 36858 in media with sucrose or raffinose were tested to predict the experimental results obtained in this study.

Chapter 4

Materials and Methods

4.1 Microorganisms, media and culture conditions

The yeast *Saccharomyces cerevisiae* ATCC 36858, obtained by mutation by Lobo and Maitra (1977), and a wild strain of *S. cerevisiae* were kept on malt agar slants medium I (Table 4.1). The medium was sterilized at 115°C for 15 min, inoculated after cooling and incubated at 30°C for 5 days. After the fifth day, the slants were stored at 4°C until needed. The yeasts were transferred to fresh slants every two to three months. Inoculum medium was prepared by aseptically transferring a loopful of cells from test tube slants into a sterilized liquid medium II (Table 4.1). Growth was carried out in 500 mL Erlenmeyer flasks containing 100 mL of medium. The flasks were incubated in a rotary shaker (Lab-Line Instrument Inc, Illinois, USA) at 160 rpm and 33°C for 24-30 h. This time was adequate to ensure that the cells were in the exponential growth phase. The cells were concentrated by decanting the supernatant before their use in the production media.

Table 4.1 Composition of the various synthetic media used in this study

	Medium I	Medium II	Medium III
Bacto malt agar, g	45.0	----	----
Glucose, g	----	10.0	variable
Fructose, g	----	----	variable
Galactose, g	----	----	variable
Sucrose, g	----	----	variable
Raffinose, g	----	----	variable
Melibiose, g	----	----	variable
Yeast extract, g	----	30.0	variable
Peptone, g	----	3.5	3.5
KH_2PO_4, g	----	2.0	2.0
$MgSO_4 \cdot 7H_2O$, g	----	1.0	1.0
$(NH_4)_2SO_4$, g	----	1.0	1.0
Deionized water, L	1.0	1.0	1.0

4.2 Production media

4.2.1 Synthetic medium

The production medium used in this study is medium III (Table 4.1). Various sugars were used to study the characteristics of the mutant *S. cerevisiae* ATCC 36858. Various concentrations of sucrose, raffinose or glucose/fructose mixtures were used to produce fructose and ethanol. In addition, various concentrations of glucose, fructose, galactose or melibiose were used for the production of ethanol.

4.2.2 Complex media

Sugar cane and sugar beet molasses, which were obtained from Lallemand Inc, Montreal, were used in the production of fructose and ethanol. The molasses contained a total carbohydrate concentration of 50 to 60% (w/v), minerals and other nutrients that supported the growth of yeast. The composition of the sugar cane and sugar beet molasses used is shown in Table 4.2.

The sugar cane and sugar beet molasses media were prepared by diluting the molasses with deionized water to a specific sugar concentration. This was followed by removal of suspended solids by centrifugation at 17,000 g for 15 min. The initial pH of some of the sugar beet molasses media was adjusted to values between 5.0 and 5.1 with HCl.

Table 4.2 Composition of sugar cane and sugar beet molasses

	Sugar cane molasses	Sugar beet molasses
Glucose, g/L	63.7	---
Fructose, g/L	85.1	9.5
Sucrose, g/L	529.1	637.8
Raffinose, g/L	21.7	40.4
Suspended solids, g/L	20.5	5.2
Water, % (w/w)	28.0	22.6
Other matters	----	not quantified

Preliminary tests have shown that the use of molasses media to produce fructose and ethanol does not require additional growth factors or mineral salt supplementation. In addition, no change in the fermentation capacity of the strain *S. cerevisiae* ATCC 36858 was noticed in the presence or absence of suspended solids from the molasses media. However, suspended solids interfered with the measurement of biomass concentration in the fermentation media. Therefore, suspended solids were removed as described above.

The tests, with both synthetic and complex media, were carried out in 500 mL Erlenmeyer flasks containing 100 mL of medium. However, tests with raffinose and melibiose synthetic media were done in 125 mL Erlenmeyer flasks containing 25 mL of medium. All media were sterilized at 115°C for 15 min. After sterilization and cooling, media in the flasks were inoculated with approximately 10% (v/v) of a concentrated inoculum preparation and were placed in a rotary shaker (Lab-Line Instrument Inc, Illinois, USA) at 160 rpm and 33°C. Samples were taken aseptically at various times to determine biomass, sugar and ethanol concentrations and pH.

4.3 Analyses

4.3.1 Biomass

Biomass concentration was determined by the dry weight method or spectrophotometrically. The spectrophotometric method was used only for synthetic media, while the other technique was used for both complex and synthetic media. In the dry weight method, a known volume of sample was centrifuged at 17,000 g for 15 min; the supernatant was collected and analyzed for sugar and ethanol content while the biomass was washed with deionized water and re-centrifuged. The biomass was then transferred to a pre-weighed aluminum dish and dried at 105°C overnight. The dish and dried biomass were then reweighed and the biomass concentration was expressed in g/L. In the spectrophotometric method, samples were diluted to contain up to 0.2 g/L biomass. The absorbance was then measured at 600 nm and 25°C against deionized water as reference using a Beckman DU 640 spectrophotometer equipped with a temperature controller (Beckman, California, USA). Biomass yields were calculated based on the total carbohydrates consumed. The biomass standard curve is shown in Appendix A. The percentage of error of biomass measurement was between 1 and 4%.

4.3.2 Sugars

Glucose, fructose, galactose, sucrose, melibiose, lactose, fructo-oligosaccharides, raffinose, stachyose, glycerol and sorbitol were measured using a 600E system controller Water's high performance liquid chromatograph (HPLC) with a Water's 410 differential refractometer as the detector. A Sugar-Pak™ I column (Waters, Massachusetts, USA) operated at 75°C, with deionized water containing approximately 50 mg/L EDTA-disodium-calcium salt as the mobile phase flowing at 0.5 mL/min, was used. About 20 μL of various solutions containing between 1.0 and 4.0 g/L of the sugars, glycerol or sorbitol were injected into the HPLC to prepare standard curves. Standard curves and chromatograms for these compounds are shown in Appendix A. Standard sugar solutions were run from time to time to verify the accuracy of the calibration curves. Samples analyzed were diluted to contain concentrations of sugars below 4.0 g/L. About

20 µL of each sample was injected into the HPLC for analysis. The acquired chromatographic data were analyzed using the Millennium™ Chromatography Manager System version 2.1. The percentages of error of sugar measurements were in the range of 1 to 3%.

4.3.3 Ethanol

Ethanol concentration was determined enzymatically using alcohol dehydrogenase (Bernt and Gutmann, 1974). This method involves oxidation of ethanol to acetaldehyde in the presence of nicotinamide adenine dinucleotide (NAD) by alcohol dehydrogenase (ADH) according to the following equation:

$$\text{Ethanol} + \text{NAD}^+ \xleftrightarrow{\text{ADH}} \text{Acetaldehyde} + \text{NADH} + \text{H}^+ \quad (4.1)$$

The concentration of NADH, which is proportional to the amount of ethanol present, was measured spectrophotometrically at 340 nm. The equilibrium is pushed completely to the right at a pH equal to 8.7. The reagents required for this analysis are buffer solution, NAD and ADH. The buffer solution consisted of: 10 g $Na_4P_2O_7 \cdot 10H_2O$, 2.5 g semicarbazide hydrochloride, 0.5 g glycine and deionized water (up to 250 mL). The pH of the buffer solution was then adjusted to 8.7 with 4N KOH and diluted to 300 mL. The nicotinamide adenine dinucleotide was prepared by addition of 50 mg NAD into 3 mL deionized water. The alcohol dehydrogenase consisted of 5 mg ADH in 1 mL deionized water.

Samples for analysis were diluted to contain up to 0.115 g/L ethanol. The following components were then added into the test tubes: 3 mL buffer, 0.10 mL NAD, 0.20 mL sample and 0.02 mL ADH. The tubes were thoroughly mixed and then incubated at 37°C for 25 min. They were mixed once after 10 min of incubation. The absorbance of the solution was measured against a reference at 340 nm and 25°C, using a Beckman DU 640 spectrophotometer equipped with a temperature controller (Beckman, California, USA). The reference was prepared by replacing the 0.20 mL sample with 0.20 mL deionized water. Ethanol yields were calculated on the basis of the total sugars that were consumed at the maximum ethanol concentration. The standard curve for ethanol is shown in Appendix A. The percentage of error of ethanol measurement was between 1 and 5%.

4.3.4 pH determination

The pH measurements were conducted using an Orion Research digital ionalyzer 501 (Orion Research Inc, Massachusetts, USA). The pH meter was calibrated with pH reference buffer solutions of 4.00 and 7.00 prior to each measurement.

4.3.5 Recovery and identification of the fructo-oligosaccharides

During the production of fructose and ethanol from sucrose media, an unknown product was produced at the beginning of fermentation that had the same retention time as raffinose when analyzed using Sugar-Pak^{TM} I column. To verify if the unknown product was raffinose, the same sample was injected into a 0.39 × 30 cm-Carbohydrate Analysis column (Waters, Massachusetts, USA). The column was maintained at 40°C with an acetonitrile/water mixture (80:20) as the mobile phase flowing at 1.0 mL/min. The unknown product eluted at a retention time different from raffinose. The unknown product was recovered from the Carbohydrate Analysis column by collecting the effluent corresponding to its retention time. The unknown product was then recovered from the acetonitrile/water mixture. Acetonitrile was removed by vacuum evaporation and water was removed by freeze drying.

The unknown product was quantified and identified using an HPLC-AS6 carbohydrate column (inert styrene-divinylbenzene polymer) in a Dionex Series 4000 ion chromatograph with pulsed amperometric detection as described by Chatterton et al. (1989). Kestose separation was accomplished using 150 mM NaOH as the mobile phase flowing at 1.0 mL/min. The unknown product was identified as a mixture of fructo-oligosaccharides. It was composed of 6-kestose (61.5%), neokestose (29.7%) and 1-kestose (8.8%). The identification and quantification of these fructo-oligosaccharides was conducted with the assistance of Dr. Jerry N. Chatterton (U.S.D.A.-Agricultural Research Service, Utah State University, U.S.A).

4.4 Purification of fructose syrups

The fructose syrup produced after fermentation of sugar cane molasses media was purified by the removal of biomass, ethanol, colour and minerals from the fermentation broth.

4.4.1 Biomass removal

Biomass was removed from the fermentation broth by centrifugation at 17,000 g for 15 min. The supernatant was collected and used for further treatment.

4.4.2 Ethanol removal

Ethanol was removed from the biomass-free broth after fermentation using a vacuum evaporator (Buchi RE121 Rotavapor, Switzerland). A water aspirator was used to create vacuum. The temperature of the water bath was 45°C and the temperature of the circulating cooling liquid through the condenser was −14°C. Vacuum evaporation was carried out using about 250 mL of the solution in 500 mL round flask rotating at 120 rpm for 30 min. The remaining broth and the collected condensate were analyzed for sugar and ethanol concentrations as described in section 4.3.

4.4.3 Colour removal

After fermentation, the decolourization of the biomass-free broth, containing either fructose and ethanol as main products or only fructose after ethanol removal, was carried out using activated carbon. In addition, biomass-free broth containing only fructose was used in decolourization tests using membranes followed by activated carbon.

4.4.3.1 Decolourization using activated carbon

4.4.3.1.1 Biomass-free broth containing fructose and ethanol

In the first test, 16.5 g of activated carbon (Darco G-60) was placed in an Erlenmeyer flask and 50 mL of the biomass-free broth, containing fructose and ethanol, was added and the flask was placed for 10 min in a rotary shaker (Lab-Line Instrument Inc, Illinois, USA) at 33°C and 200 rpm. The carbon was removed from the broth by centrifugation at 17,000 g for 15 min. The supernatant was analyzed for sugar concentration, pH, conductivity and colour. It was noticed that only about 14 mL of supernatant was obtained after centrifugation. The rest of the broth remained in the carbon. In the second test, 36 mL of deionized water was added to 16.5 g of activated carbon and mixed for 5 min. Then, 50 mL of the biomass-free broth containing fructose and ethanol was added to the flask containing the wetted carbon and mixed for 10 min and analyzed as described above.

4.4.3.1.2 Treatment of biomass- and ethanol-free broth containing fructose with various amounts of activated carbon

In one test, various amounts of the broth containing fructose, after biomass and ethanol removal, were mixed in Erlenmeyer flasks with 11, 22 and 33% of activated carbon in the slurry and placed in a rotary shaker at 33°C and 200 rpm for 20 min. The carbon was then removed from the broth by centrifugation at 17,000 g for 15 min. The supernatant was analyzed for sugar concentration, pH, conductivity and colour. In another test, about 20 mL of the same broth was mixed with 11% of carbon in the slurry for 20 min, centrifuged and the supernatant was analyzed. This was repeated another two times using the same supernatant and fresh carbon.

4.4.3.1.3 Treatment of biomass- and ethanol-free broth containing fructose for various time intervals

In one test, 8.25 g of activated carbon was placed in each of five Erlenmeyer flasks and 25 mL of the broth containing fructose was then added in each flask to obtain slurry with 33% carbon. The flasks were placed in a rotary shaker for various time periods (between 10 and 120 min) at 200 rpm and 33°C. The carbon was then removed from the broth by centrifugation and the supernatant was analyzed for sugar concentration, pH, conductivity and colour. In another test, 18 mL of deionized water was added to 8.25 g of activated carbon and mixed for 5 min. Then, 25 mL of the broth used above was added to the flask and mixed with the carbon for 20 min and analyzed as described above.

4.4.3.1.4 Colour removal by activated carbon followed by washing the carbon to recover fructose

In this test, 33 g of activated carbon was placed in an Erlenmeyer flask and 100 mL of the broth containing fructose, after biomass and ethanol removal, was added and the flask was then placed in a rotary shaker for 20 min at 200 rpm and 33°C. The percentage of carbon used in the slurry was 33%. The carbon was removed from the broth by centrifugation and the supernatant (i.e. treated broth) was analyzed for sugar concentration, pH, conductivity and colour. The spent carbon was washed four times to recover the sugars, each time with 72 mL of fresh deionized water for 5 min. After each washing, the carbon was removed from the liquid by centrifugation and the supernatant was analyzed as above. The four washouts were combined and added to the treated broth. The mixture was then passed through five columns containing ion exchange resins to remove minerals as described in section 4.4.4.

4.4.3.2 Decolourization using membranes followed by activated carbon

Two separation techniques were used for the removal of colourants from the broth containing fructose after biomass and ethanol removal. Ultrafiltration followed by adsorption by activated carbon were used in order to have high colour removal from the broth.

Two ultrafiltration membranes used in this study were provided by the Industrial Membrane Research Institute (IMRI) at the University of Ottawa. The first membrane was made of polyethersulfone (PES) and is commercially known as PES-HO51 (Osmonics Inc, USA). This membrane has a molecular weight cut off (MWCO) of 10,000 Daltons. The second membrane was a thin film composite (TFC) membrane. This membrane, which was prepared by IMRI, has a thin, selective layer coating over a thicker support layer. The thin layer was made of brominated sulfonated poly (2,6-dimethyl-1,4-phenylene oxide) (SPPOBr) and the supportive layer was PES-HO51. This membrane has a MWCO of 1,000 Daltons and was negatively charged. These membranes were used to remove colourants using the setup described in Figure 4.1. After biomass and ethanol removal, the broth containing fructose was filtered through a 0.45 µm surfactant free, nitrocellulose membrane (Millipore Corporation, Massachusetts, USA) to remove

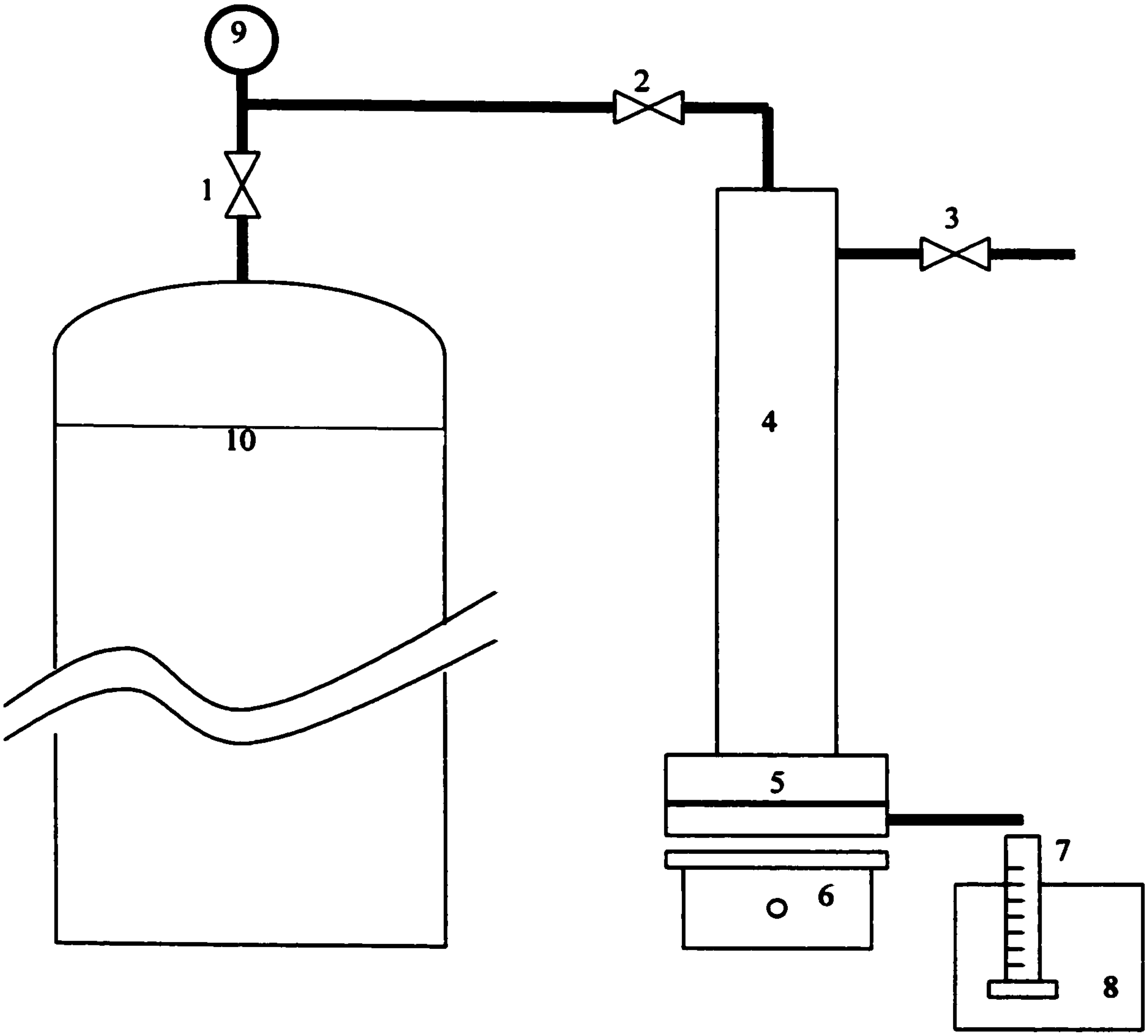

Figure 4.1 Schematic diagram of the decolourization process using a membrane system: (1-2) valves; (3) pressure relief valve; (4) permeation cell; (5) membrane; (6) magnetic stirrer; (7) permeate collector; (8) ice bath; (9) pressure gauge; (10) nitrogen cylinder.

any suspended solids. The permeation cell containing the membrane to be tested was filled with 100 mL of the filtered broth. The permeation cell was placed on a magnetic stirrer and connected to a compressed nitrogen cylinder as shown in Figure 4.1. The ultrafiltration experiments were carried out at 200 psig and room temperature (23°C). The permeate was collected in a graduated cylinder that was placed in an ice bath to reduce the possibility of sugar consumption.

The flux rate of the permeate was calculated according to the following equation:

$$F = \frac{V_P}{t_P\, A} \tag{4.2}$$

where F is the flux rate of the permeate, V_P is the permeation volume, t_p is the permeation time, and A is the effective membrane area. The effective area of the membranes used was 9.73×10^{-4} m^2.

The permeate and the retentate of the PES and TFC membranes were analyzed for pH, conductivity, sugar contents and colour. The permeate of the PES membrane was further treated with activated carbon to remove residual colour. This was carried out in three consecutive stages. The first two stages were carried out using 3% activated carbon in the slurry and the third stage with 1.5% activated carbon. The permeate of the TFC membrane was also treated once with 3% activated carbon in the slurry. The permeate after each carbon treatment stage was analyzed as described above.

4.4.4 Minerals removal

The broth, after colour removal by carbon and recovery of sugars by washing the spent carbon with fresh water as described in section 4.4.3.1.4, was deionized using a mixture of cation and anion exchange resins (Rexyn I-300) that were packed into a plexiglass column. About 286 mL of the diluted broth containing 44.5 g/L fructose was used, which was prepared by mixing 25 mL of the treated broth with activated carbon, 63 mL from the first washout, 67 mL from the second washout, 69 mL from the third washout and 69 mL from the fourth washout. The diluted broth was passed through five columns of resins at a rate of approximately 8.5 mL/min using a peristaltic pump. Each column was packed with 28 mL of resins. The broth collected after each column treatment was analyzed for pH, conductivity, sugar contents and colour.

4.4.5 Conductivity determination

The broth conductivity, in millimhos, was measured using a VWR model 2052 conductivity meter (Mississauga, Ontario).

4.4.6 Colour determination

Colour was determined spectrophotometrically using the absorbance of the broth at 420 nm as recommended by the International Commission for Uniform Methods of Sugar Analysis (ICUMSA) method 4 (Meads, 1977). The broth was filtered through a 0.45 µm surfactant free, nitrocellulose membrane (Millipore Corporation, Massachusetts, USA). The absorbance of the broth was measured against deionized water as a reference at 420 nm and 25°C, using a Beckman DU 640 spectrophotometer. In addition, samples were diluted before absorbance measurement to be in transmittance range between 20 and 80%. The pH of the samples was adjusted to 7.0 ± 0.2 with HCl or NaOH.

In the measurement of coloured sugar solutions, the light absorbing components are a complex mixture of colourants whose concentration is difficult to quantify in terms of g/L. Therefore, as an approximation, their concentrations are assumed to be proportional to the density of sugar solution (Chou, 1993). The colour of the sugar solution is calculated according to the following equation:

$$IU = \frac{A_{420}}{b\,c} \times 1000 = \frac{-\log T_s}{b\,c} \times 1000 \qquad (4.3)$$

where IU is the ICUMSA colour units, A_{420} is the absorbance at 420 nm, T_s is the transmittance at 420 nm, b is the path length of light (cm) and c is the solids content (g/mL). The path length of the light (i.e. cell width) was 1 cm. In the ICUMSA method 4, the solids content is determined refractometrically and converted to density units using standard tables (Meads, 1977). However, the determination of solids content refractometrically has its limitations, especially for low-grade materials such as molasses due to the presence of a significant proportion of salts and non-sugars, which are of high specific gravity compared to the sugar in the solution (Chen, 1993c). The results from the refractometer measurements are approximating those that could be obtained by drying. Even with the former method, Chen (1993c) reported that the determination of total solids is one of the most unsatisfactory tests in practical control work. This is related to the tendency of several constituents to decompose or to occlude moisture causing the total solids determination to yield uncertain results. Therefore, in this study, the solids content in the broth was determined by HPLC (section 4.3.2) as the summation of the concentrations in g/mL of raffinose, sucrose, glucose, fructose and glycerol. Minerals and non-sugars were not included in the solids content of the broth. This was the only modification done to the ICUMSA method 4 used in this study. It should also be pointed out that absorbance should be used in Eq. 4.3 for calculating decolourization efficiency and comparison of the results. The use of transmittance (T_s) should be avoided because of the nonlinear relationship between transmittance and concentration (Chou, 1993). The absorbance and transmittance as a function of total solids present in the broth used in the present study are shown in Appendix B (Figure B.1).

Chapter 5

Results and Discussion

5.1 Production of ethanol from synthetic glucose, fructose and galactose media

The production of ethanol using *Saccharomyces cerevisiae* ATCC 36858 in synthetic media that contained either glucose, fructose or galactose is discussed in this section. In addition, the effects of yeast extract on the fermentation process in glucose media is also examined.

5.1.1 Effect of various carbohydrates

The ability of *S. cerevisiae* ATCC 36858 to utilize various sugars as a carbon source for the production of ethanol is shown in Figures 5.1 to 5.3. The concentration of yeast extract in the glucose and fructose media was 30.0 g/L, while it was 10.0 g/L in the galactose medium. From Figure 5.1, it is seen that the yeast completely consumed all of the glucose (90 g/L) to produce ethanol and biomass within 16 h from the beginning of the process. The biomass concentration increased from 2.14 to 13.2 g/L, which represents a biomass yield of 0.123 g/g of glucose consumed. The ethanol concentration was 36 g/L, which represents a yield of 78% of the theoretical value based on total glucose consumed. When the carbon source was fructose, it took 22 h to consume the same amount of this sugar (Figure 5.2). During this time, the biomass concentration increased from 2.13 to 14.8 g/L, which represents a biomass yield of 0.141 g/g of fructose consumed. The ethanol concentration was 38 g/L, which represents a yield of 82% of the theoretical value based on total fructose used. The results showed that *S. cerevisiae* ATCC 36858 consumed glucose to produce ethanol and biomass at a faster rate than fructose. Ethanol productivity and specific growth rate were higher when glucose was used (Table 5.1). However, a higher biomass yield was obtained using the fructose medium. 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 sugar concentration was increased above 20.0 g/L. In general, the difference in fermentation rates in media containing either glucose or fructose might be attributed to their transport systems into the yeast cells.

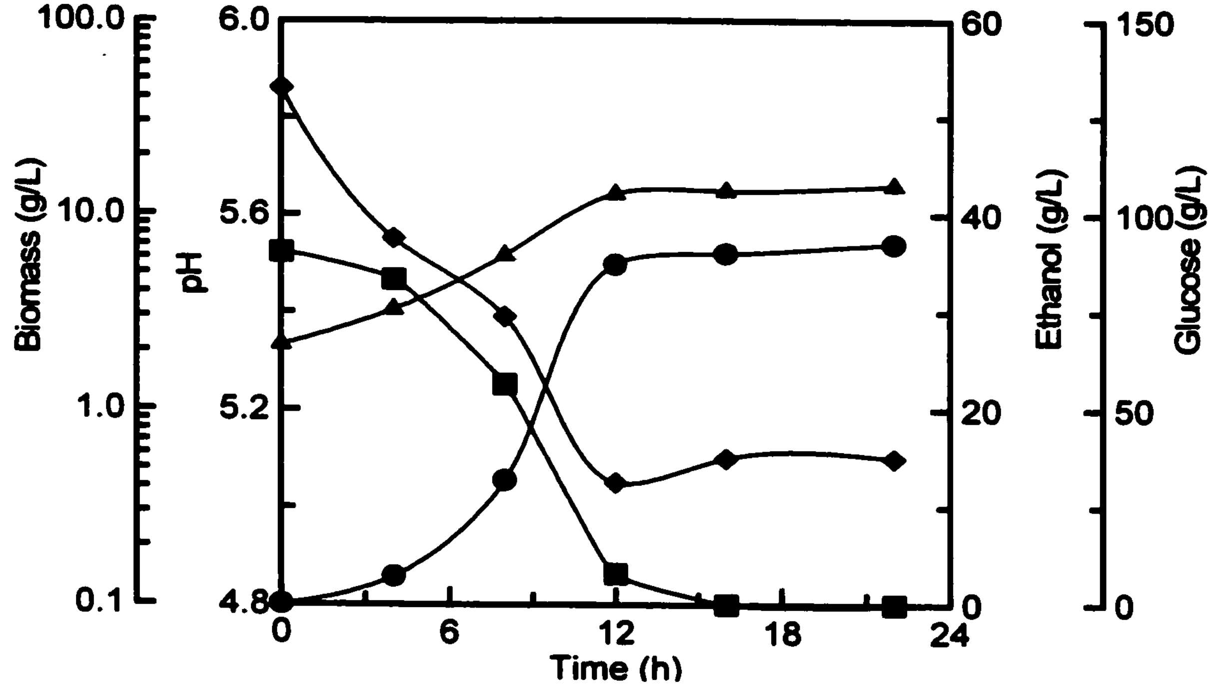

Figure 5.1 Kinetics of growth of *S. cerevisiae* ATCC 36858 and ethanol production in a medium containing 90 g/L glucose: (▲) biomass; (◆) pH; (●) ethanol; (■) glucose.

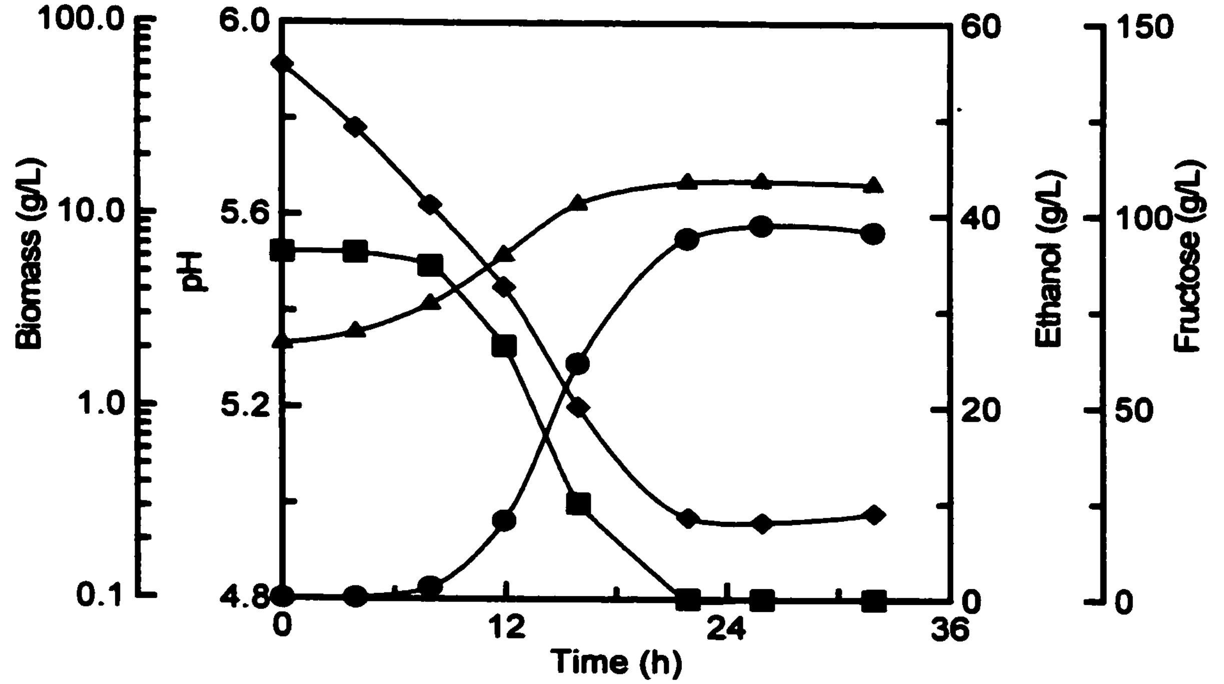

Figure 5.2 Kinetics of growth of *S. cerevisiae* ATCC 36858 and ethanol production in a medium containing 90 g/L fructose: (▲) biomass; (◆) pH; (●) ethanol; (■) fructose.

Table 5.1 Ethanol and biomass yields, specific growth rates and specific initial rates of sugar utilization and ethanol productivities for media with various sugar concentrations using *S. cerevisiae* ATCC 36858.

Sugar (g/L)	Initial biomass (g/L)	Specific growth rate, μ (h^{-1})	Biomass yield[a] (g/g)	Ethanol productivity[a] (g/L h)	Specific initial rate of sugar utilization[d] (g/g h)	Ethanol yield[a] (%)
90.2 glucose[b]	2.14	0.181	0.123	2.25	1.23	78.3
139.1 glucose[b]	1.96	0.192	0.104	3.69	1.34	83.0
90.2 fructose[b]	2.13	0.146	0.141	1.71	0.11	81.6
134.0 fructose[b]	2.02	0.153	0.127	2.16	0.24	81.6
143.4 glucose[c]	1.69	0.170	0.083	2.26	2.24	74.0
150.7 galactose[c]	1.79	0.127	0.085	1.79	0.19	74.5

[a] Values are calculated at maximum biomass and ethanol concentrations

[b] Medium contains 30 g/L yeast extract

[c] Medium contains 10 g/L yeast extract

[d] Values are calculated after 5 h of fermentation

Barford et al. (1992a, b) suggested two separate transport systems for glucose and fructose. The first uptake system was characterized by equal uptake of glucose and fructose, while the second by preferential uptake of glucose to fructose. However, Cirillo (1962, 1968) proposed that both sugars are transferred by the same membrane transport system, namely a carrier mediated facilitated diffusion, and metabolized by the yeast in similar manner.

Koren (1991) found a slight difference between fermentations carried out in media containing glucose and those carried out with fructose when a wild strain of *S. cerevisiae* was used. The wild strain was able to consume about 95 g/L of either glucose or fructose in 4 h with ethanol yields above 94% of the theoretical values. Fermentation of similar concentrations of either glucose or fructose by the mutant *S. cerevisiae* ATCC 36859 was completed in 14.5 and 58 h, respectively. Ethanol yields dropped to 89 and 61% of the theoretical values in glucose and fructose media, respectively. The initial biomass concentrations of the wild and mutant strains used were between 2.2 and 2.9 g/L. Comparing *S. cerevisiae* ATCC 36858 that was used in the present study with *S. cerevisiae* ATCC 36859 used by Koren (1991), both strains have similar characteristics when grown on glucose media with a slightly lower ethanol yield with the *S. cerevisiae* ATCC 36858 strain. However, *S. cerevisiae* ATCC 36858 showed higher performance when fructose was the only carbohydrate in the medium. The fructose consumption rate and ethanol productivity were 2.2 and 3.8 times higher for *S. cerevisiae* ATCC 36858. Furthermore, ethanol yield was 34% higher. About 2.0 g/L of sorbitol was produced in the medium that contained 134 g/L fructose, while no sorbitol was detected in the medium with 90 g/L fructose. In the former medium, *S. cerevisiae* ATCC 36859 produced 23 g/L sorbitol (Koren, 1991).

When galactose was used as a carbon source with *S. cerevisiae* ATCC 36858 to produce ethanol and biomass, a complete consumption of this sugar was noticed in 32 h (Figure 5.3). The biomass increased from 1.79 to 14.6 g/L, which represents a biomass yield of 0.085 g/g of galactose consumed. The ethanol concentration was 57 g/L, which represents a yield of 75% of the theoretical value.

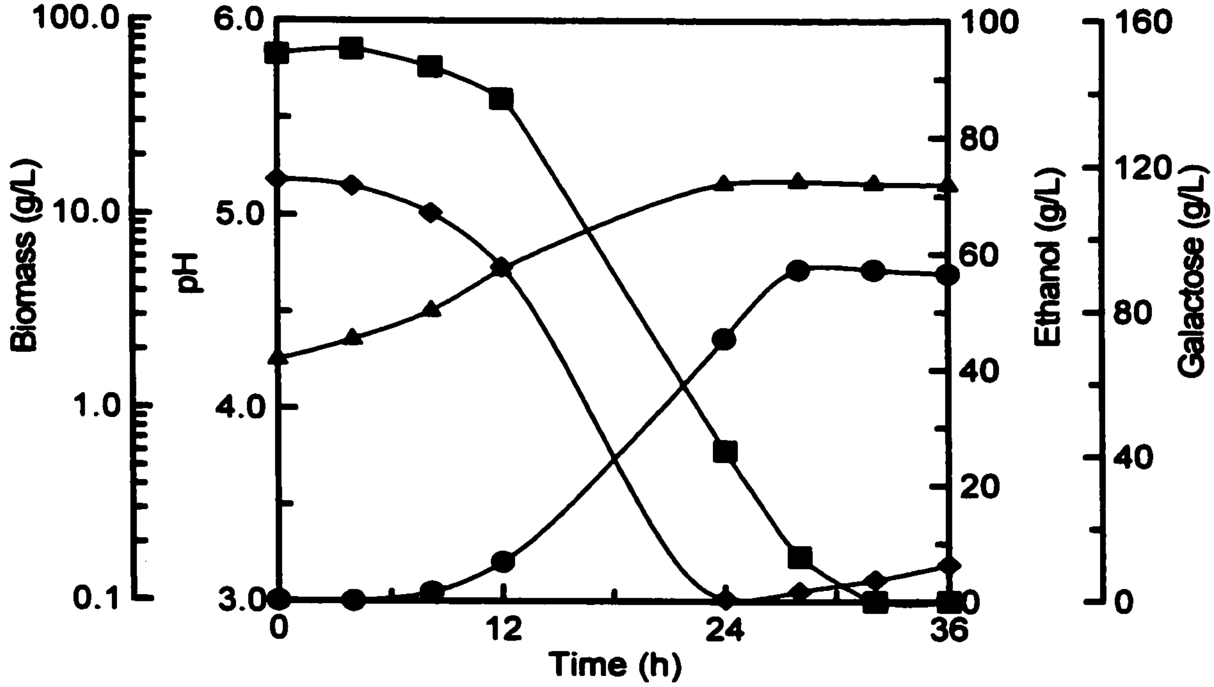

Figure 5.3 Kinetics of growth of *S. cerevisiae* ATCC 36858 and ethanol production in a medium containing 151 g/L galactose: (▲) biomass; (◆) pH; (●) ethanol; (■) galactose.

The kinetics of biomass production by *S. cerevisiae* ATCC 36858 and pH changes in media containing various carbohydrates are shown in Figure 5.4. A maximum biomass concentration of 20 g/L was attained in the medium with a concentration of fructose of 134 g/L (Figure 5.4a). In addition, biomass yield in the media with fructose was the highest when compared with other carbohydrates (Table 5.1). However, the specific growth rate of the yeast was higher in glucose media than in either fructose or galactose media. In addition, the biomass yield and specific growth rate of the yeast were also higher in glucose media with higher yeast extract concentration. The pH of the fermentation medium with the various sugars decreased with time (Figure 5.4b). In general, the pH decrease in fermentation processes is due to the production of acids such as lactic, acetic and propionic acids (Stewart and Russell, 1987). In addition, acidification is an important indicator of metabolic activity and vitality of yeasts. This process may be considered as a series of reactions in which substrate is converted by enzymes to acid metabolites, accompanied with the expelling of excess protons from the cell. It is assumed that the extent of the pH decrease is directly proportional to the metabolic activity of some wine yeast strains (Malik et al., 1996). However, it is not always true that the strain displaying the best acidification properties will have the best technological properties.

A comparison of sugar consumption and ethanol production by *S. cerevisiae* ATCC 36858 in media containing various carbohydrates is shown in Figure 5.5. The yeast consumed 139 g/L of glucose in 16 h (Figure 5.5a) with a specific initial rate of sugar consumption of 1.34 g/g h (Table 5.1). This is more than 5.5 and 7.0 times higher than the specific initial rates of fructose and galactose utilization, respectively. A lag period in ethanol production was noticed in the media with fructose and galactose (Figure 5.5b). This is because the inoculum was grown in a glucose medium, and therefore, some time was necessary for the yeast to adjust to the new environment in which either fructose or galactose was the only carbohydrate. The ethanol production rate was the highest in the glucose medium (3.69 g/L h) containing an initial yeast extract concentration of 30 g/L (Table 5.1). Ethanol yields were between 74 and 83% of the theoretical values in media with the three sugars studied.

The mutant *S. cerevisiae* ATCC 36858 showed no activity when tested in media containing lactose. The results showed that the strain was able to ferment various types of sugars, with the exception of lactose. The results also showed that yeast extract had an effect on the specific growth rate of this yeast and biomass yield. Therefore, the effect of yeast extract concentration on the fermentation process using this yeast will be further discussed in the next section.

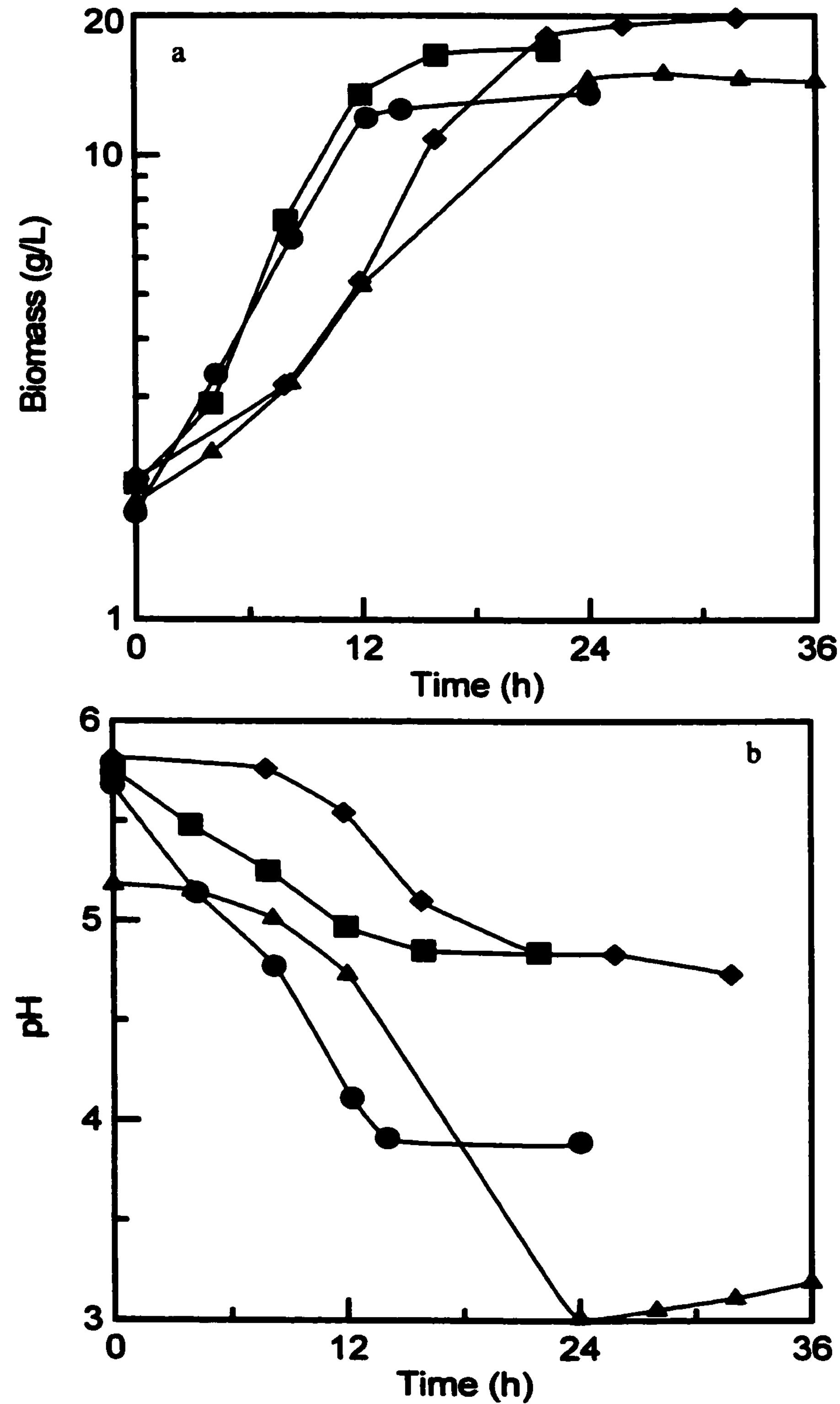

Figure 5.4 Kinetics of a) biomass production by *S. cerevisiae* ATCC 36858 and b) pH changes in media containing: (■) 139 g/L glucose and 30 g/L yeast extract; (◆) 134 g/L fructose and 30 g/L yeast extract; (▲) 151 g/L galactose and 10 g/L yeast extract; (●) 143 g/L glucose and 10 g/L yeast extract.

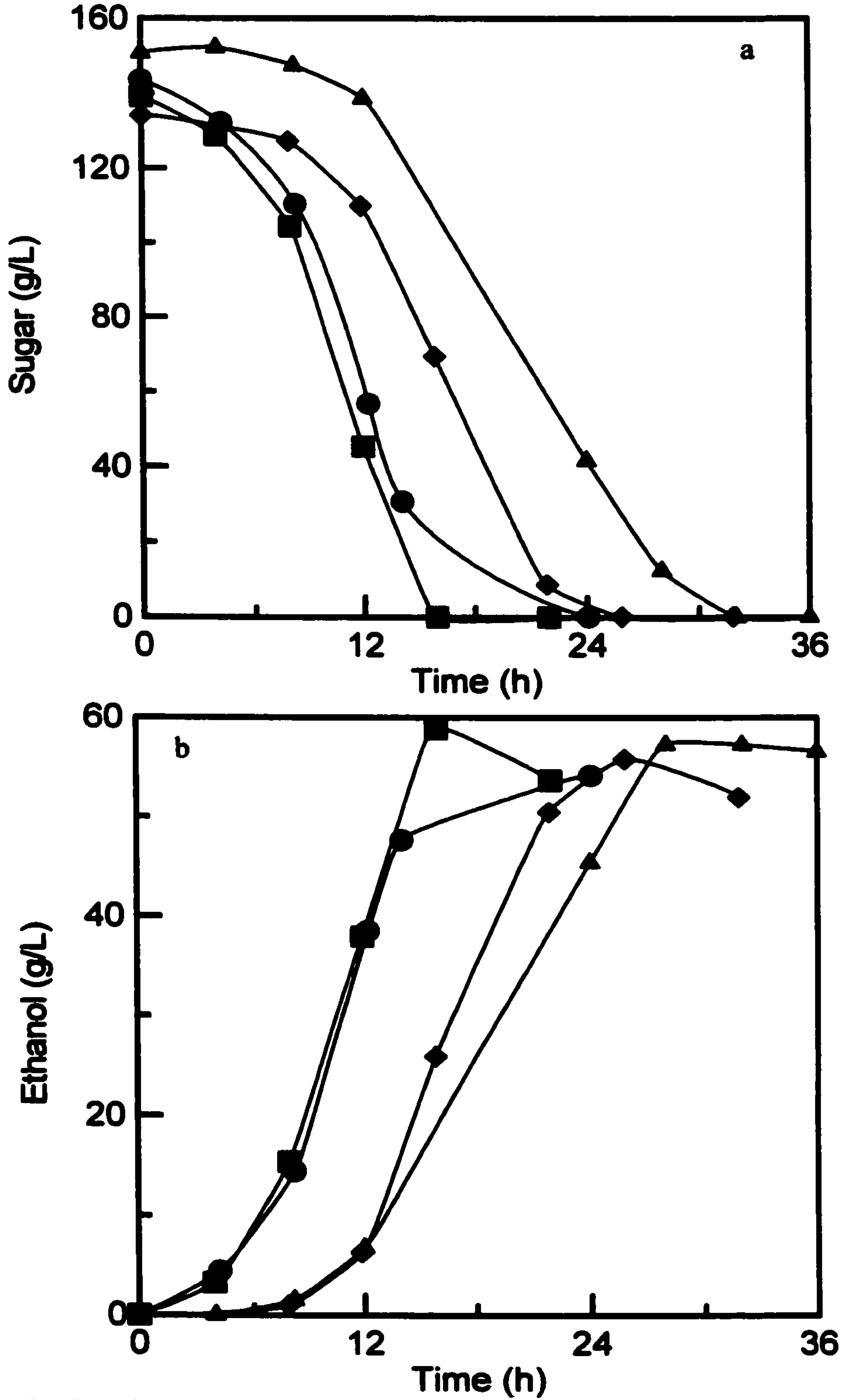

Figure 5.5 Kinetics of a) sugar consumption and b) ethanol production by *S. cerevisiae* ATCC 36858 in media containing: (■) 139 g/L glucose and 30 g/L yeast extract; (◆) 134 g/L fructose and 30 g/L yeast extract; (▲) 151 g/L galactose and 10 g/L yeast extract; (●) 143 g/L glucose and 10 g/L yeast extract.

5.1.2 Effect of yeast extract

In this section the effect of yeast extract on the growth and production of ethanol by *S. cerevisiae* ATCC 36858 will be discussed. As with all microorganisms, in order to grow, yeasts must obtain from their environment all the substances required for the synthesis of their cellular materials. The culture medium must contain all the essential elements for growth, in proportions similar to those occurring in the yeast cells. The main structural units of microbes including yeast cells are hydrogen, oxygen, nitrogen, and carbon. A dry microbial cell typically contains by weight approximately 50% carbon and 8-12% nitrogen (Cooney, 1981; Berry and Brown, 1987). The most widely utilized carbon sources are sugars, which also provide hydrogen and oxygen. Nitrogen can be supplied from many sources such as inorganic salts containing nitrogen or amino acids. Phosphorous, sulphur, magnesium and other elements should also be supplied for yeast growth (Berry and Brown, 1987). Yeast extract is a complex material, which serves as a source of vitamins, growth factors and nitrogen, and is often used to enhance the growth and fermentation by yeasts (Cooney, 1981; Jones et al., 1981).

Tests were carried out to study the effect of yeast extract concentrations on biomass growth, glucose consumption and ethanol production in media containing about 145 g/L glucose. The results showed that an increase in yeast extract concentration from 1.0 to 30.0 g/L resulted in an increase in biomass concentration (Figure 5.6a). The amount of biomass obtained in the glucose medium with 40 g/L yeast extract was comparable to that with 30 g/L. The specific growth rate increased from 0.135 to 0.187 h^{-1} when the yeast extract concentration was increased from 1.0 to 40.0 g/L (Table 5.2). This was expected since yeast extract is a very rich raw material in vitamins, growth factors and nitrogen. The pH of fermentation media with various yeast extract concentrations decreased with time (Figure 5.6b). The pH change in media with higher initial yeast extract concentrations was considerably lower than in media with low yeast extract concentrations (Figure 5.6b). This could be due to the buffering capacity of yeast extract at high concentrations. It has also been reported that the addition of a complex nitrogen source, such as distillery waste liquors, provides nutrients and increases the buffering capacity of the fermentation medium (Jones et al., 1981).

The yeast extract concentration can also affect the rate of glucose consumption by the yeast. The specific initial glucose consumption rate increased by 63% when yeast extract

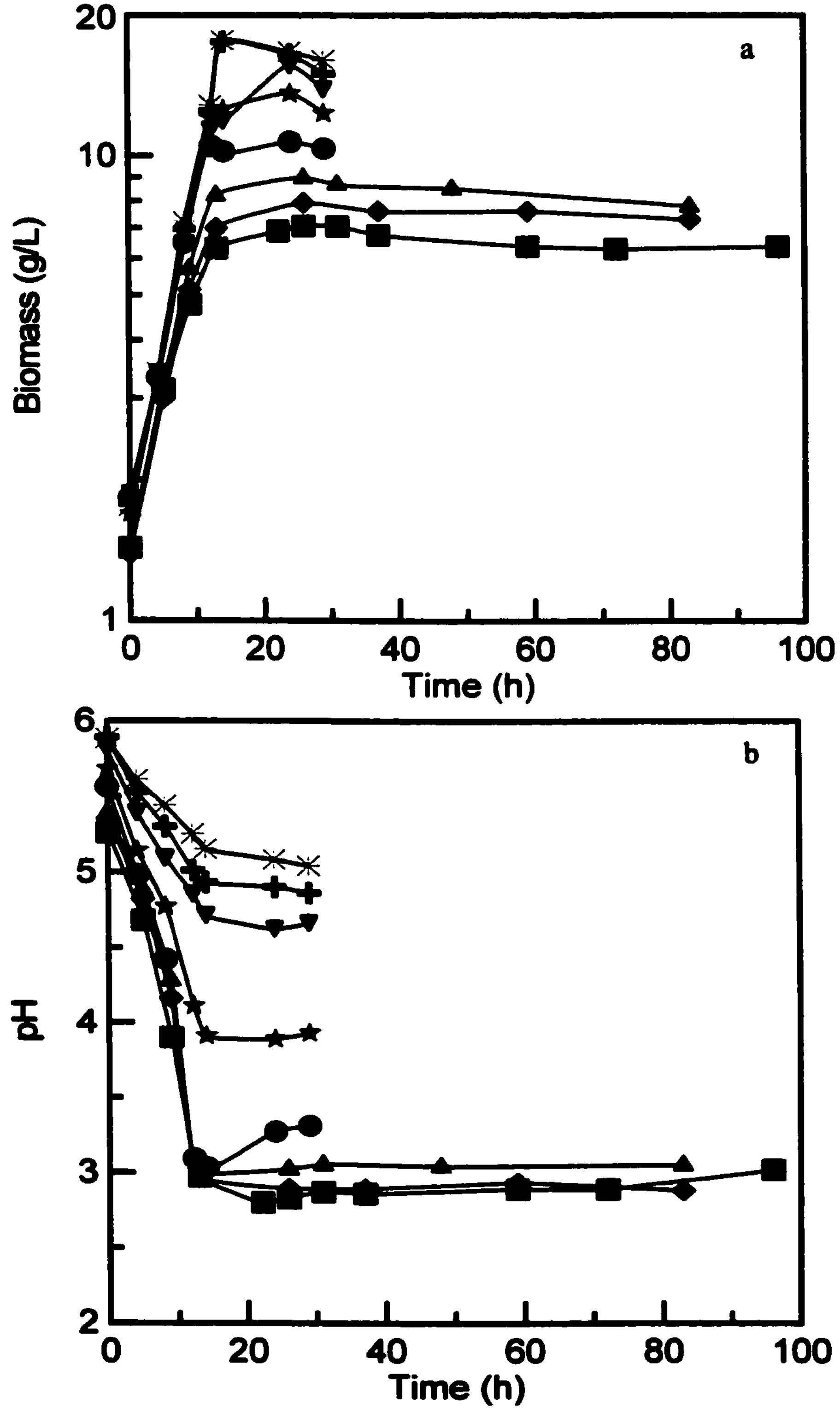

Figure 5.6 Kinetics of a) biomass production by *S. cerevisiae* ATCC 36858 and b) pH changes in media containing about 145 g/L glucose and various yeast extract concentrations of: (■) 1.0 g/L; (◆) 2.0 g/L; (▲) 3.0 g/L; (●) 5.0 g/L; (★) 10.0 g/L; (▼) 20.0 g/L; (✚) 30.0 g/L; (✱) 40.0 g/L.

concentration was increased from 1.0 to 5.0 g/L (Table 5.2). However, a further increase in yeast extract concentration had little effect on the glucose consumption rate. Glucose was completely consumed in the first 24 h of the fermentation process when yeast extract concentration was above 3.0 g/L (Figure 5.7a). However, some glucose was not consumed in media with lower yeast extract concentrations.

Table 5.2 Ethanol and biomass yields, specific growth rates and specific initial rates of glucose utilization and ethanol productivity for media with various yeast extract concentrations using *S. cerevisiae* ATCC 36858.

Yeast extract (g/L)	Glucose (g/L)	Initial biomass (g/L)	Specific growth rate, μ (h^{-1})	Biomass yield[a] (g/g)	Ethanol productivity[a] (g/L h)	Specific initial rate of sugar utilization[b] (g/g h)	Ethanol yield[a] (%)
1.0	147.4	1.43	0.135	0.058	1.34	1.25	77.7
2.0	147.5	1.38	0.146	0.061	1.79	1.40	84.7
3.0	147.5	1.43	0.154	0.059	1.85	2.17	74.0
5.0	144.8	1.83	0.167	0.061	2.25	2.04	73.1
10.0	143.4	1.69	0.170	0.083	2.26	2.24	74.0
20.0	146.0	1.86	0.176	0.094	2.45	2.14	78.8
30.0	143.7	1.78	0.184	0.102	2.33	2.10	76.3
40.0	139.2	1.75	0.187	0.107	2.39	1.77	80.8

[a] Values are calculated at maximum biomass and ethanol concentrations

[b] Values are calculated after 5 h of fermentation

The kinetics of ethanol production in media containing various yeast extract concentrations is shown in Figure 5.7b. Rapid ethanol production was noticed in media with yeast extract concentrations above 5.0 g/L. Ethanol productivity increased as yeast extract concentration was increased from 1.0 to 5.0 g/L (Table 5.2). However, the ethanol productivity was about 2.3 g/L when yeast extract concentration was above 5.0 g/L. Ethanol yield varied between 73 and 85% of the theoretical values with no specific trend in the range of yeast extract concentrations studied.

Koren (1991) obtained similar results on the effects of yeast extract on growth and ethanol production in media with glucose concentrations of about 93 g/L using a wild *S. cerevisiae* and the mutant *S. cerevisiae* ATCC 36859. Ethanol yields with those yeast strains (Koren, 1991) were about 10% higher than with the strain *S. cerevisiae* ATCC 36858 used in this study.

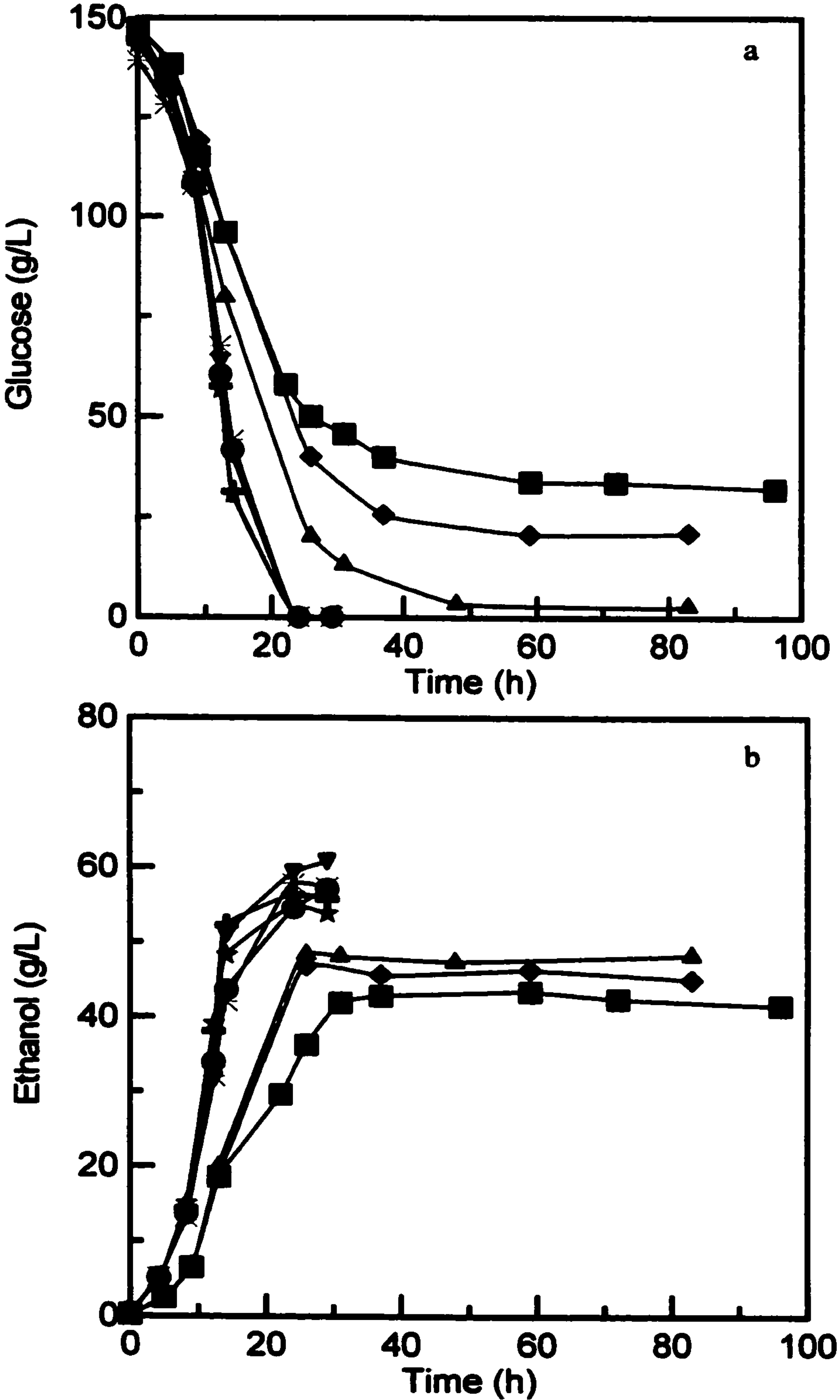

Figure 5.7 Kinetics of a) glucose consumption and b) ethanol production by *S. cerevisiae* ATCC 36858 in media containing about 145 g/L glucose and various yeast extract concentrations of: (■) 1.0 g/L; (◆) 2.0 g/L; (▲) 3.0 g/L; (●) 5.0 g/L; (★) 10.0 g/L; (▼) 20.0 g/L; (✚) 30.0 g/L; (✱) 40.0 g/L.

In the present study, the glucose consumption and ethanol production rates were not significantly affected by an increase in yeast extract concentration in the tested media above 5.0 g/L. This indicates that these media had sufficient vitamins and growth factors required by *S. cerevisiae* ATCC 36858. However, Koren (1991) found that optimum yeast extract concentrations for the wild *S. cerevisiae* and mutant *S. cerevisiae* ATCC 36859 were 15.0 and 20.0 g/L, respectively. This indicates that *S. cerevisiae* ATCC 36858 used in this study requires a lower level of vitamins and growth factors than the previously reported strains.

In a study using an industrial distillery yeast strain *S. cerevisiae* Pz 90 in media with 300 g/L glucose, the rate of glucose consumption increased by 2.8 times when the yeast extract concentration was increased from 3 to 12 g/L (Bafrncova et al., 1999). The percentage of glucose utilization also increased from 79 to 95% in the 54 h of the fermentation process. In addition, about 20% more ethanol was produced as a result of the increase in yeast extract concentration. However, the ethanol yield was about 68% of the theoretical value, which was not affected by the increase in yeast extract concentration. In this study with *S. cerevisiae* ATCC 36858, ethanol yield was also not significantly affected by increasing yeast extract concentrations (Table 5.2). It was reported that supplying growth promoting factors, such as those found in yeast extract, to the fermentation medium relieves the cell of synthesizing these compounds (Jones et al., 1981). This could divert the substrate and energy to other maintenance functions and enhance ethanol yield.

The results of this study demonstrated the ability of the mutant *S. cerevisiae* ATCC 36858 to produce ethanol in glucose media with all tested initial yeast extract concentrations. A slower fermentation and lower ethanol concentrations were noticed in media with yeast extract concentrations below 5.0 g/L. The ethanol productivity drastically increased as yeast extract concentration was increased from 1.0 to 5.0 g/L. However, it was about 2.3 g/L h when the initial yeast extract concentration was above 5.0 g/L. Ethanol yields were between 73 and 85% of the theoretical values. The specific growth rate and biomass yield increased with increasing yeast extract concentrations. A yeast extract concentration of 5.0 g/L or higher in media with about 145 g/L glucose is required for *S. cerevisiae* ATCC 36858 to completely ferment this sugar.

Considering that this yeast strain can use a variety of sugars for the production of ethanol as shown in section 5.1, the production of fructose and ethanol using media with various carbohydrates will be discussed in the next section.

5.2 Production of fructose and ethanol from various carbohydrate media

The production of fructose and ethanol using *S. cerevisiae* ATCC 36858 from various carbohydrate media is discussed in this section. The effects of initial sugar, biomass, yeast extract and ethanol concentrations, pH and media sterilization are also examined.

5.2.1 Production of fructose and ethanol from synthetic glucose/fructose mixtures

The effect of various glucose/fructose concentrations in media containing 10 g/L yeast extract on growth and production of ethanol and fructose by *S. cerevisiae* ATCC 36858 was studied. The kinetics of the selective fermentation of glucose to ethanol from synthetic medium containing 99 g/L glucose and 103 g/L fructose is displayed in Figure 5.8.

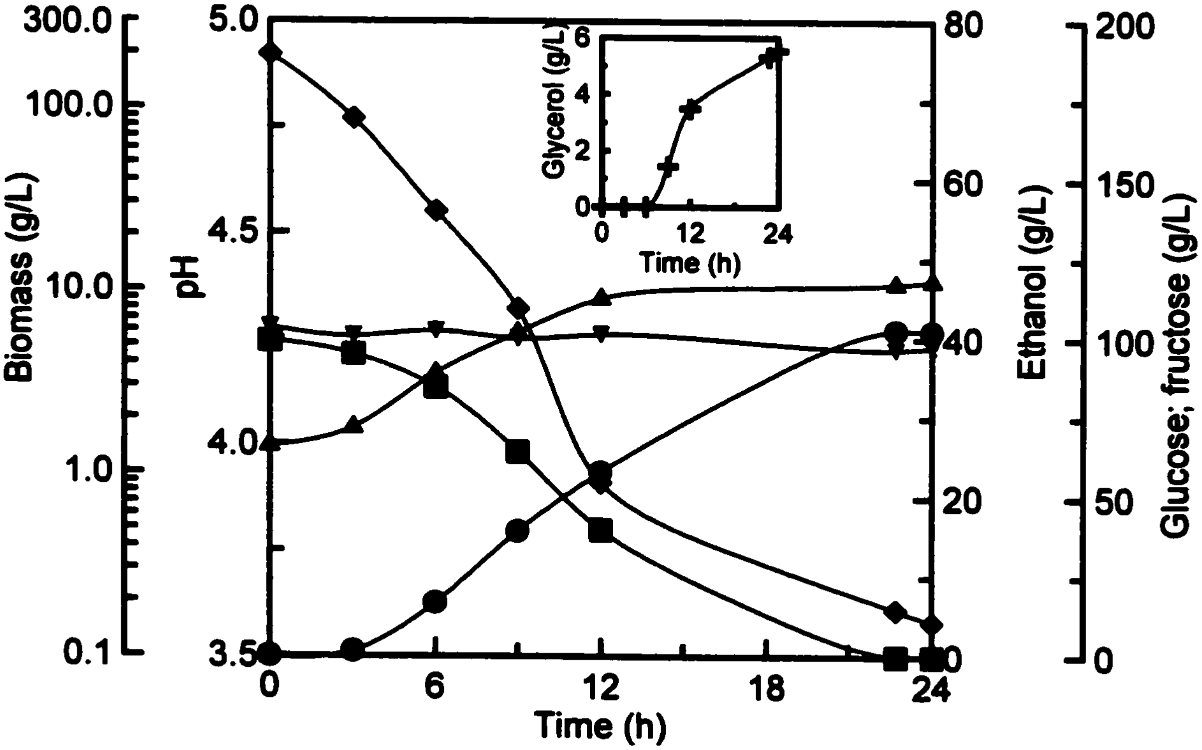

Figure 5.8 Kinetics of growth of *S. cerevisiae* ATCC 36858 and fructose and ethanol production in a medium containing 99 g/L glucose and 103 g/L fructose: (▲) biomass; (◆) pH; (●) ethanol; (■) glucose; (▼) fructose; (✚) glycerol.

The results of this fermentation showed that this yeast is able to selectively convert glucose to ethanol and biomass. Glucose was completely consumed within 22 h of the fermentation process, while ethanol accumulated in the medium. Fructose was only slightly consumed by the yeast. The fructose and ethanol yields were 94 and 76% of the theoretical values, respectively (Table 5.3). The calculation of the ethanol yield was based on glucose and fructose consumed in the fermentation process. The biomass increased from 1.4 to 11 g/L, which represents a biomass yield of 0.090 g/g of glucose and fructose consumed. Some glycerol was produced and accumulated during the fermentation process.

Table 5.3 Fructose, ethanol and biomass yields, specific growth rates and specific initial rates of glucose utilization and ethanol productivity for media with various concentrations of glucose/fructose mixtures using *S. cerevisiae* ATCC 36858.

Glucose-Fructose (g/L)	Initial biomass (g/L)	Specific growth rate, μ (h^{-1})	Biomass yield[a] (g/g)	Ethanol productivity[a] (g/L h)	Specific initial rate of glucose utilization[b] (g/g h)	Fructose yield (%)	Ethanol yield[a] (%)
99.1-103.4	1.40	0.201	0.090	1.81	1.53	93.5	76.2
139.2-134.8	1.54	0.140	0.055	1.82	1.19	98.2	76.1
149.5-150.3	1.34	0.157	0.058	1.86	1.16	98.4	76.6

[a] Values are calculated at maximum biomass and ethanol concentrations
[b] Rates are calculated after 5 h of fermentation

The results of the production of fructose and ethanol in glucose/fructose media with total initial concentrations of glucose and fructose of 274 and 300 g/L were very similar (Figures 5.9 and 5.10). Glucose was completely consumed in both media within the first 36 h of the process. This time is 64% longer than the time required to completely utilize glucose in the medium when the total initial concentration of glucose and fructose was 202 g/L (Figure 5.8). The specific growth rate and biomass yield decreased as a result of increasing the total initial sugar concentration from 202 to 300 g/L (Table 5.3). This was expected because of growth inhibition by high substrate concentrations. Generally, the growth inhibition is related to osmotic effects in which plasmolysis of the cells starts to occur at high substrate concentrations (Jones et al., 1981). In this study, although the yeast partially consumed fructose, the presence of this sugar in the fermentation medium affected the yeast growth.

According to Stewart et al. (1983), an increase in the osmotic pressure in the medium due to the presence of a non-metabolized sugar results in an increase in the intracellular ethanol

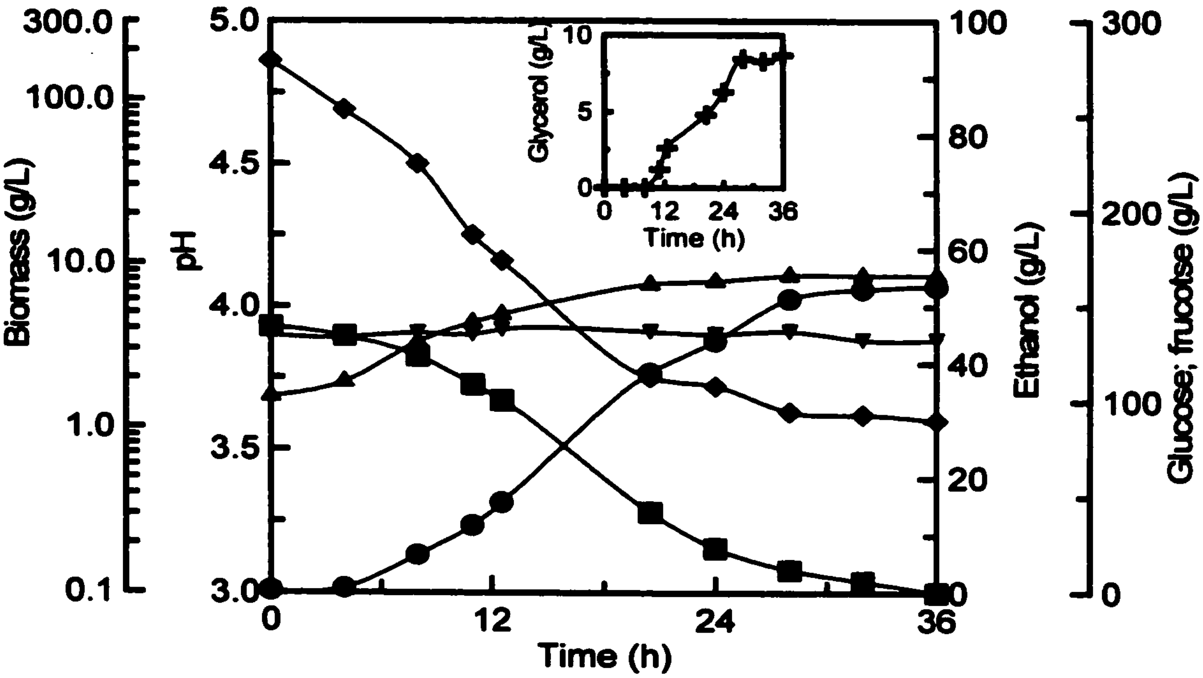

Figure 5.9 Kinetics of growth of *S. cerevisiae* ATCC 36858 and fructose and ethanol production in a medium containing 139 g/L glucose and 135 g/L fructose: (▲) biomass; (◆) pH; (●) ethanol; (■) glucose; (▼) fructose; (✚) glycerol.

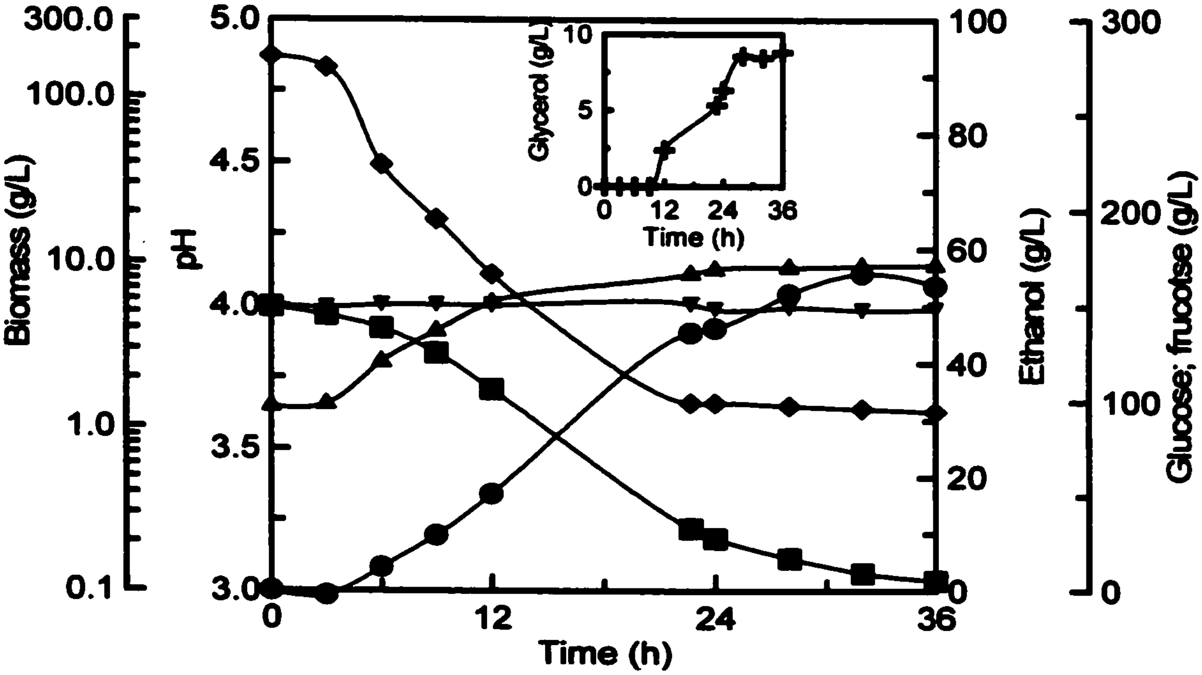

Figure 5.10 Kinetics of growth of *S. cerevisiae* ATCC 36858 and fructose and ethanol production in a medium containing 150 g/L glucose and 150 g/L fructose: (▲) biomass; (◆) pH; (●) ethanol; (■) glucose; (▼) fructose; (✚) glycerol.

concentration. This has a more damaging effect on the cell than extracellular ethanol and results in a reduction in the growth rate. In the present study, when the initial total sugar concentration was increased, an increase in the fructose yield was noticed without significant changes in the ethanol yield (Table 5.3). It was also noticed that the growth rates, biomass yields and glucose consumption rates were more strongly inhibited by increasing the total sugar concentration than ethanol productivities and yields. This was expected because the growth rate is generally more sensitive to inhibitory compounds than the fermentation rate (van Uden, 1985). This has also been reported in glucose/fructose mixtures when *S. cerevisiae* ATCC 36859 was used (Koren and Duvnjak, 1992).

Koren and Duvnjak (1992) reported similar fructose yields with approximately 13% higher ethanol yields when using *S. cerevisiae* ATCC 36859 in glucose/fructose mixtures having total sugar concentrations of 215 and 357 g/L. However, their strain had a slightly lower growth rate than *S. cerevisiae* ATCC 36858 despite the fact that their medium was supplemented with three times more yeast extract.

When *Mucor sp.* M105 was used in a medium with concentrations of glucose and fructose of 70 g/L each, fructose and ethanol yields were 54 and 81% of the theoretical values, respectively (Ueng et al., 1982). Fructose yields between 66 and 70% were reported when *Z. mobilis* ATCC 53431 was used in glucose/fructose media with total glucose/fructose concentrations between 130 and 192 g/L (Doelle and Doelle, 1991). In addition, about 22 g/L of sorbitol was produced with *Z. mobilis* ATCC 53431. Contrary to the process with *Z. mobilis*, sorbitol was not detected when glucose was present in the medium with *S. cerevisiae* ATCC 36858. However, *S. cerevisiae* ATCC 36858 started to produce sorbitol when the process was continued after all the glucose from the medium was consumed. The production of sorbitol was very slow. After 22 h of the process (Figure 5.8), at which point all the glucose has been consumed in the medium that initially contained 99 g/L glucose and 103 g/L fructose, only 2% of the fructose was utilized between 22 and 72 h of fermentation. The amount of sorbitol produced during that period was 2.2 g/L. Similar results were obtained with other glucose/fructose mixtures.

In the case of *Z. mobilis*, sorbitol is produced when both glucose and fructose are present in the medium (Bringer-Meyer et al., 1985; Doelle and Greenfield, 1985b; Edye et al., 1989;

Doelle and Doelle, 1991). This bacterium produced up to 60 g/L sorbitol in glucose/fructose media (Bringer-Meyer et al., 1985). No sorbitol production was reported in media with glucose/fructose mixtures when *S. cerevisiae* ATCC 36859 was used (Koren and Duvnjak, 1992). However, about 23 g/L of sorbitol was produced by *S. cerevisiae* ATCC 36859 in a fructose medium with a concentration of 90 g/L of this sugar (Koren, 1991). In the present study, sorbitol was not detected in a similar medium with the same fructose concentration using *S. cerevisiae* ATCC 36858.

Glycerol is another fermentation byproduct, which was produced in concentrations below 10 g/L by *S. cerevisiae* ATCC 36858 (Figures 5.8-5.10). More glycerol was produced in the fermentation media with increasing glucose/fructose concentrations. In general, the formation of glycerol depends on the yeast strain and composition of the fermentation medium (Radler and Schutz, 1982). Furthermore, glycerol affects ethanol yield (Oura, 1977; Radler and Schutz, 1982).

The results showed that *S. cerevisiae* ATCC 36858 has the ability to selectively convert glucose from glucose/fructose mixtures into ethanol with almost no fructose consumption and with an ethanol yield of 76% of the theoretical value. The mutant *S. cerevisiae* ATCC 36859 (Koren and Duvnjak, 1992) was also able to selectively convert glucose to ethanol but with higher ethanol yields. However, this strain could not utilize sucrose, which limits its use to media with glucose/fructose mixtures. There has been no *S. cerevisiae* strain reported in the literature concerning the production of fructose and ethanol from sucrose media. Since sucrose is a widely available sugar, the production of fructose and ethanol from sucrose media by the new mutant *S. cerevisiae* ATCC 36858 is discussed in the next section.

5.2.2 Production of fructose and ethanol from synthetic sucrose media

Tests were carried out to study the ability of *S. cerevisiae* ATCC 36858 to produce ethanol and fructose in media with sucrose. The effects of initial sucrose, yeast extract, biomass and ethanol concentrations, and initial pH and media sterilization on the fermentation process were studied.

5.2.2.1 Effect of sucrose concentration with 30 g/L yeast extract

The effect of various sucrose concentrations on the ability of *S. cerevisiae* ATCC 36858 to produce fructose and ethanol from media with 30 g/L yeast extract was studied. The kinetics of the growth of *S. cerevisiae* ATCC 36858 and fructose and ethanol production in a synthetic medium containing 216 g/L sucrose is displayed in Figure 5.11. The results show the ability of this yeast to hydrolyze sucrose and then to selectively convert glucose into ethanol and biomass (Atiyeh and Duvnjak, 2001a). A fast hydrolysis of sucrose was observed, which resulted in the accumulation of fructose and ethanol. In the beginning of the process, glucose also started to accumulate since its production by hydrolysis of sucrose was faster than its consumption. After reaching a peak of 86 g/L, its concentration started to decrease and it was completely consumed by the end of the process. The biomass increased from 2.1 to 12.9 g/L, which represents a biomass yield of 0.09 g/g of glucose and fructose consumed (Table 5.4). Fructose and ethanol yields were 97 and 79% of the theoretical values, respectively. The calculation of ethanol yield was based on the amount of glucose and fructose consumed in the fermentation process. It was noticed that a small amount of fructo-oligosaccharides was produced in the beginning of the process and were entirely consumed by the end of the fermentation. Glycerol was also produced and accumulated during the fermentation process.

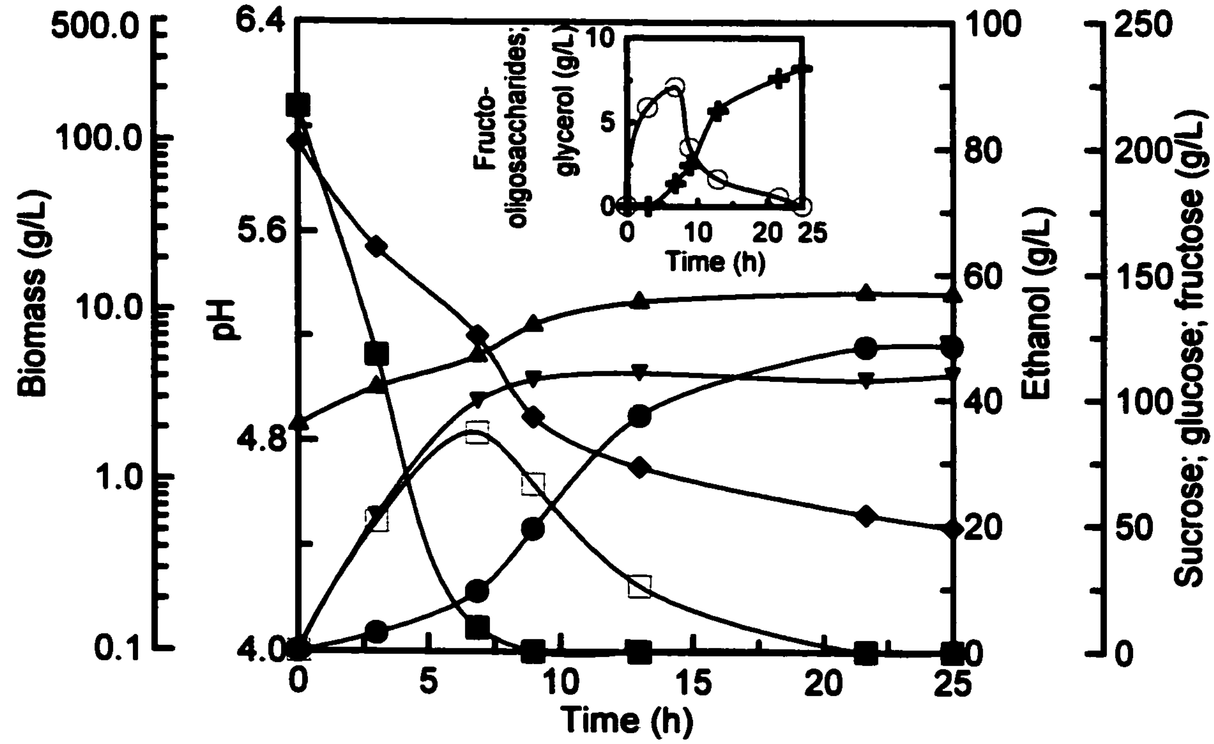

Figure 5.11 Kinetics of growth of *S. cerevisiae* ATCC 36858 and fructose and ethanol production in a medium containing 216 g/L sucrose: (▲) biomass; (◆) pH; (○) fructo-oligosaccharides; (✚) glycerol; (●) ethanol; (■) sucrose; (□) glucose; (▼) fructose.

Table 5.4 Fructose, ethanol and biomass yields, specific growth rates and specific initial rates of sucrose hydrolysis and ethanol productivities for media with various sucrose concentrations using *S. cerevisiae* ATCC 36858.

Sucrose (g/L)	Initial biomass (g/L)	Specific growth rate, μ (h^{-1})	Biomass yield[a] (g/g)	Ethanol productivity[b] (g/L h)	Specific initial rate of sucrose hydrolysis (g/g h)	Fructose yield[b] (%)	Ethanol yield[b] (%)
34.6	2.06	0.260	0.217	1.59	5.72	100.0	95.3
80.6	2.05	0.218	0.160	2.05	13.11	91.0	75.2
121.6	2.28	0.198	0.126	2.36	14.73	100.0	79.7
168.3	2.29	0.190	0.111	2.79	19.87	89.6	71.0
169.3	2.21	0.188	0.107	2.73	18.16	91.8	72.5
216.4	2.10	0.146	0.090	2.23	15.85	97.0	79.2
257.4	2.06	0.128	0.075	2.35	15.80	96.0	84.6

[a] Values are calculated at maximum biomass concentrations

[b] Values are calculated at maximum ethanol and fructose concentrations

The kinetics of biomass production by *S. cerevisiae* ATCC 36858 and the changes in pH in media containing various sucrose concentrations are shown in Figure 5.12. A maximum biomass concentration of 13.7 g/L was attained in the medium with a sucrose concentration of 169 g/L (Figure 5.12a). The biomass concentration decreased with an increase in sucrose concentration above 169 g/L. The specific growth rate of the yeast exhibited a two fold decrease when the sucrose concentration increased from 35 to 257 g/L (Table 5.4). The biomass yield in these media decreased from 0.217 to 0.075 g/g of glucose and fructose consumed. This decrease in growth rates and biomass yields occurred because of growth inhibition due to a high substrate concentration. The pH of the fermentation medium with all tested concentrations decreased with time (Figure 5.12b).

The kinetics of sucrose hydrolysis and glucose production and consumption by *S. cerevisiae* ATCC 36858 in media containing various sucrose concentrations are shown in Figure 5.13. Complete sucrose hydrolysis within the first 10 h of the fermentation process was observed with all tested concentrations (Figure 5.13a). The specific initial rates of sucrose hydrolysis increased from 5.72 to 19.87 g sucrose/g biomass h with an increase in sucrose concentration from 35 to 168 g/L, respectively (Table 5.4). However, when sucrose concentration was above 168 g/L, the specific rate of sucrose hydrolysis decreased by 20%. Although glucose was used from the very beginning, its uptake by the yeast at the beginning of the fermentation was

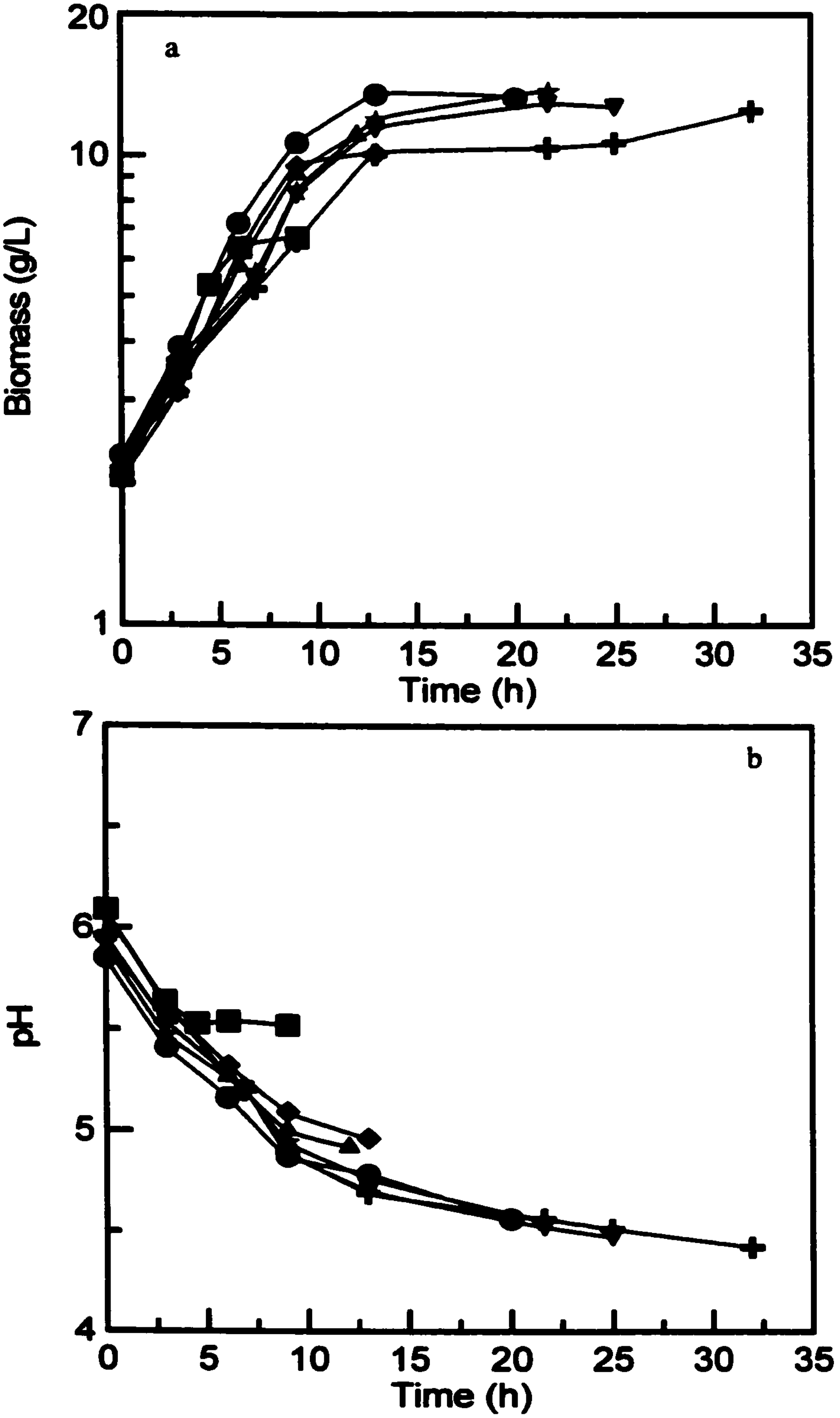

Figure 5.12 Kinetics of a) biomass production by *S. cerevisiae* ATCC 36858 and b) pH changes in sucrose media containing: (■) 35 g/L; (◆) 81 g/L; (▲) 122 g/L; (●) 168 g/L; (★) 169 g/L; (▼) 216 g/L; (✚) 257 g/L.

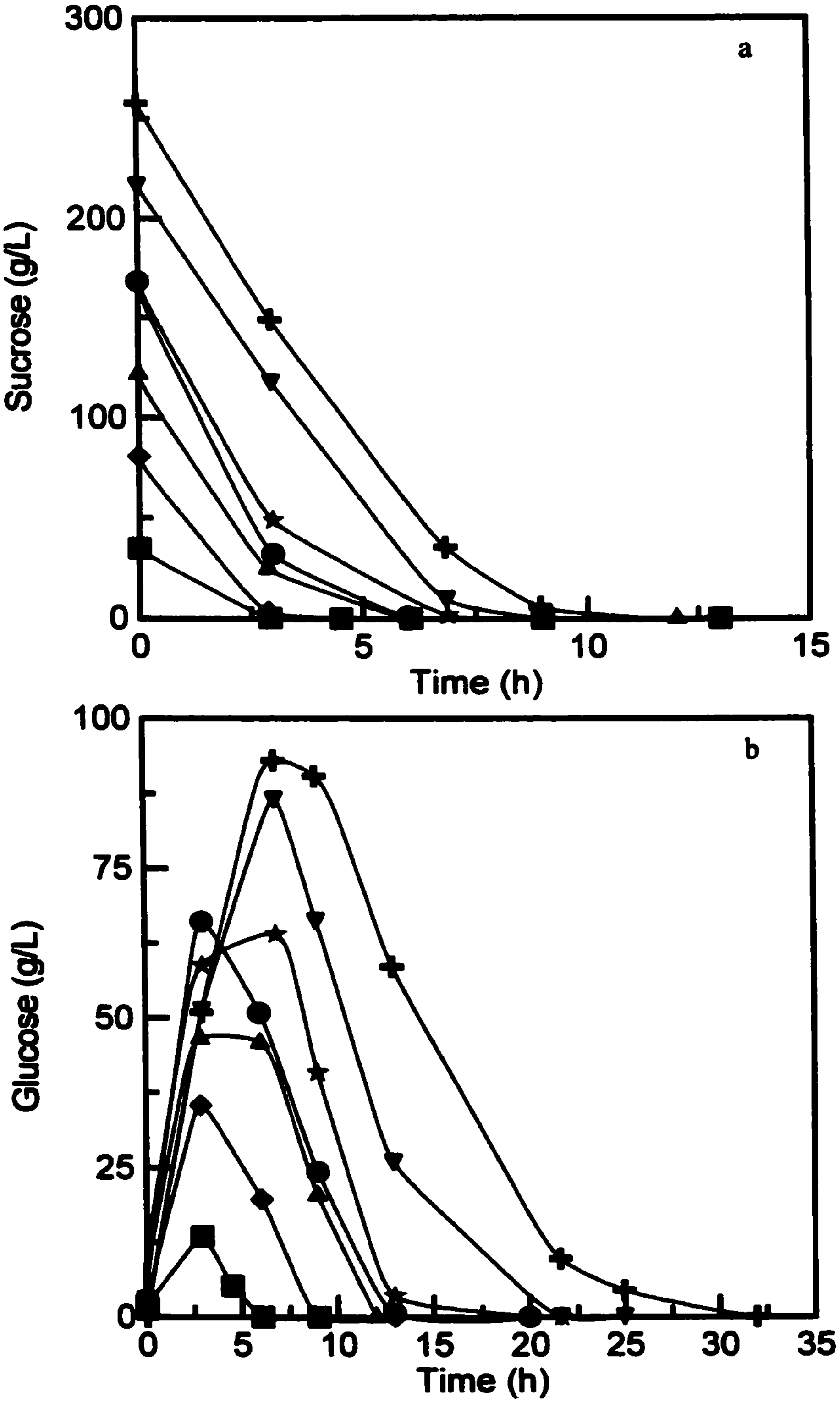

Figure 5.13 Kinetics of a) sucrose hydrolysis and b) glucose production and consumption by *S. cerevisiae* ATCC 36858 in sucrose media containing: (■) 35 g/L; (◆) 81 g/L; (▲) 122 g/L; (●) 168 g/L; (★) 169 g/L; (▼) 216 g/L; (✚) 257 g/L.

slower than the hydrolysis rate of sucrose. This explains the accumulation of glucose during that phase of the fermentation process (Figure 5.13b). However, at the end of the fermentation, glucose was completely consumed in all the sucrose media studied.

The kinetics of fructose and ethanol production by *S. cerevisiae* ATCC 36858 in media containing various sucrose concentrations are shown in Figure 5.14. The maximum concentrations of fructose and ethanol in the medium with a sucrose concentration of 257 g/L were 132 and 59 g/L, respectively. The fructose yield was above 90% of the theoretical value (Table 5.4). Ethanol yields and productivities were from 71 to 85% of the theoretical values and between 2.1 and 2.8 g/L h for the media containing between 81 and 257 g/L sucrose, respectively.

The fructose yield was 100% of the theoretical value in the medium with a sucrose concentration of 122 g/L (Table 5.4). A high fructose yield was also obtained using *Fusarium sp.* F5 (Ueng et al., 1982) in a medium that contained 100 g/L sucrose, however, the ethanol productivity and yield were six fold and 14% lower, respectively, than with *S. cerevisiae* ATCC 36858. Fructose and ethanol yields, using *S. cerevisiae* ATCC 36858, increased from 90 to 96% and 71 to 85% of the theoretical values, respectively, when the sucrose concentration was increased from 168 to 257 g/L (Table 5.4). When *Z. mobilis* ATCC 53431 was used in media in which the sucrose concentration increased from 152 to 249 g/L, fructose and ethanol yields decreased from 93 to 80% and 87 to 74% of the theoretical values, respectively (Kirk and Doelle, 1994). It is appropriate to mention that, at the end of the process, neither sorbitol nor residual sucrose were detected in the tested sucrose media when *S. cerevisiae* ATCC 36858 was used, while more than 5% of the initial sucrose (203 g/L) was not consumed and sorbitol (18 g/L) was also produced as a byproduct in a batch process with *Z. mobilis* ATCC 53432 (Suntinananalert et al., 1986). In that process, relatively high fructose and ethanol yields, 82 and 86%, respectively were reported. The amount of sorbitol produced decreased to 0.5 g/L in a fed batch process with a medium that contained 201 g/L sucrose using *Z. mobilis* ATCC 53431 (Edye et al., 1989). However, the fed batch fermentation contributed to a drastic reduction in fructose yield, which dropped to 70% of the theoretical value. In an attempt to increase fructose yields in batch fermentations, free invertase was either added to the sucrose medium (Doelle and Doelle, 1991) or co-immobilized with the same strain of *Z. mobilis* in alginate beads (Kirk and Doelle, 1994) to accelerate sucrose hydrolysis and to hydrolyze the fructo-oligosaccharides that accumulated

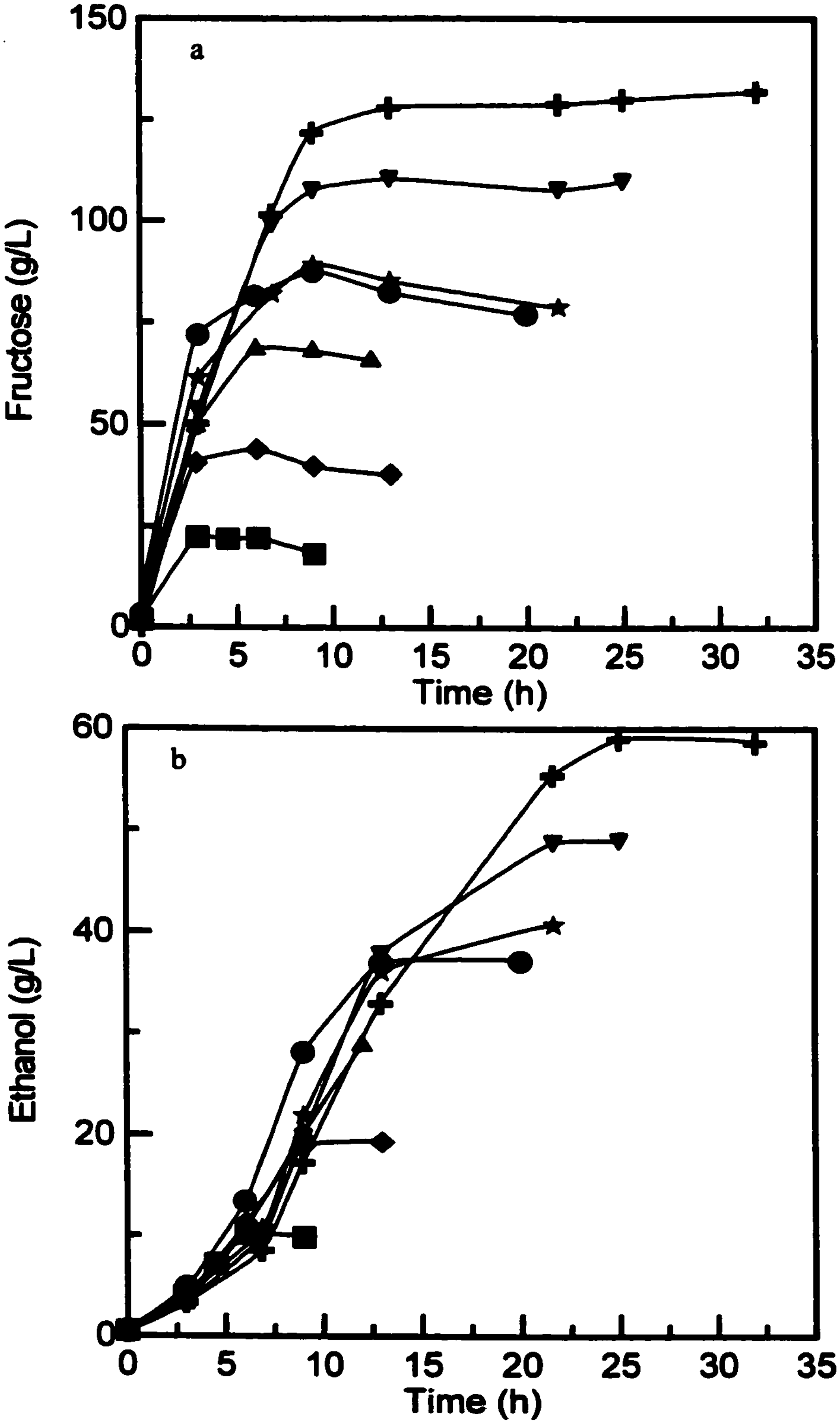

Figure 5.14 Kinetics of a) fructose and b) ethanol production by *S. cerevisiae* ATCC 36858 in sucrose media containing: (■) 35 g/L; (◆) 81 g/L; (▲) 122 g/L; (●) 168 g/L; (★) 169 g/L; (▼) 216 g/L; (✚) 257 g/L.

during the process. A higher fructose yield was obtained but more sorbitol was also produced than in the fed batch fermentation process.

In the present study, only small amounts of oligosaccharides and glycerol were produced using *S. cerevisiae* ATCC 36858 (Table 5.5). The maximum concentration of fructo-oligosaccharides (up to 9 g/L) was attained in the 257 g/L sucrose medium at the beginning of the fermentation. They started to hydrolyze to fructose and glucose, in a ratio of 2:1, when the concentration of sucrose in the media dropped below 30% of its initial value. The fructo-oligosaccharides were completely hydrolyzed to glucose and fructose by the end of the fermentation process in all media. The analysis indicated that these fructo-oligosaccharides were a mixture of 6-kestose (61.5%), neokestose (29.7%) and 1-kestose (8.8%).

Table 5.5 Maximum fructo-oligosaccharides and final glycerol concentrations in the fermentation broth from media with various sucrose concentrations using *S. cerevisiae* ATCC 36858.

Sucrose (g/L)	Fructo-oligosaccharides (g/L)	Glycerol (g/L)
34.6	0.0	1.9
80.6	1.1	5.6
121.6	2.9	6.0
168.3	5.2	8.8
169.3	5.9	8.7
216.4	7.1	8.2
257.4	9.1	8.3

The production of fructo-oligosaccharides by *S. cerevisiae* ATCC 36858 in the fermentation media containing sucrose could be due to the transfructosylating capability of invertase. Based on our literature search, the formation of fructo-oligosaccharides in fermentation media using the yeast *S. cerevisiae* has not been previously reported. However, the production of trisaccharides was first reported using an invertase preparation in sucrose solutions (Bacon and Edelman, 1950; Blanchard and Albon, 1950). These trisaccharides were identified as 1-kestose, 6-kestose and neokestose (Albon et al., 1953; Gross et al., 1954). During the hydrolysis of sucrose by invertase, which is also responsible for fructosyl transfer to the primary hydroxyl groups of sucrose, the production of small amounts of these fructo-oligosaccharides occurred and their formation should not be neglected in solutions containing between 100 and 800 g/L of sucrose (Gross et al., 1954; Bacon, 1954; Andersen et al., 1969). In the present study, these oligosaccharides were detected in media with sucrose concentrations above 34.6 g/L and their concentration increased with an increase in the sucrose concentration (Table 5.5). It is also possible that a small amount of fructo-oligosaccharides was produced even in the medium with 34.6 g/L sucrose but it may be that the method used for their analysis was not sensitive enough to detect them. Bacon (1954) reported that oligosaccharides formation was difficult to detect in the reaction mixture at low sucrose concentrations with the chromatographic method used. However, their formation in the reaction mixture with a sucrose concentration of 10 g/L was detected after concentrating the solution ten times before chromatographic analysis. In the present study, the obtained results showed that the concentration of 6-kestose in the fermentation media was two times and seven times higher than the concentration of neokestose and 1-kestose, respectively. It has also been previously observed in sucrose solutions that 1-kestose and neokestose were present at a concentration far below that of 6-kestose (Gross et al., 1954; Straathof et al., 1986; Farine et al., 2001).

In the present study, glycerol was produced by *S. cerevisiae* ATCC 36858 in the fermentation media in concentrations below 9 g/L (Table 5.5). However, more glycerol was formed in the media as the initial sugar concentration increased. This is similar to the results obtained in the media with glucose/fructose mixtures (section 5.2.1).

These results showed the ability of *S. cerevisiae* ATCC 36858 to produce fructose and ethanol from sucrose in media with sucrose concentrations between 35 and 257 g/L. Complete sucrose hydrolysis and high fructose yields (above 90%) were observed in all the media tested. Ethanol yields and productivities were from 71 to 85% of the theoretical values and between 2.1 and 2.8 g/L h for media between 81 and 257 g/L sucrose, respectively. Syrups with a carbohydrate content of 100% fructose can be produced from all the media tested.

5.2.2.2 Effect of high sucrose concentration with 10 g/L yeast extract

The production of fructose and ethanol in high concentrations using microorganisms would reduce the separation costs (i.e. distillation and evaporation) of syrups. The production of such syrups requires carrying out fermentations in media with high concentration of sucrose. However, the capability of most microorganisms to ferment under such conditions is drastically reduced due to the inhibition effect of high substrate concentrations (Jones et al., 1981). In this section, the capability of *S. cerevisiae* ATCC 36858 to produce fructose and ethanol in media with high sucrose concentrations is discussed.

The kinetics of the fermentation process in a synthetic medium containing 360 g/L sucrose and 10 g/L yeast extract is shown in Figure 5.15. The results show the ability of the yeast to hydrolyze sucrose and then to convert the glucose mainly to ethanol and biomass (Atiyeh and Duvnjak, 2001b). A fast and almost complete hydrolysis of sucrose was observed in the first 12 h of the process. This resulted in the accumulation of fructose. In the beginning of the process, glucose also started to accumulate since its production by hydrolysis of sucrose was faster than its consumption. The biomass concentration increased from 7.5 to 12.4 g/L, which represents a biomass yield of 0.03 g/g of glucose and fructose consumed (Table 5.6). Fructose and ethanol yields were 94 and 82% of the theoretical values, respectively. It was noticed that some glycerol (9.3 g/L) was produced. In addition, a small amount of fructo-oligosaccharides was produced in the beginning of the process and simultaneously consumed by the end of fermentation.

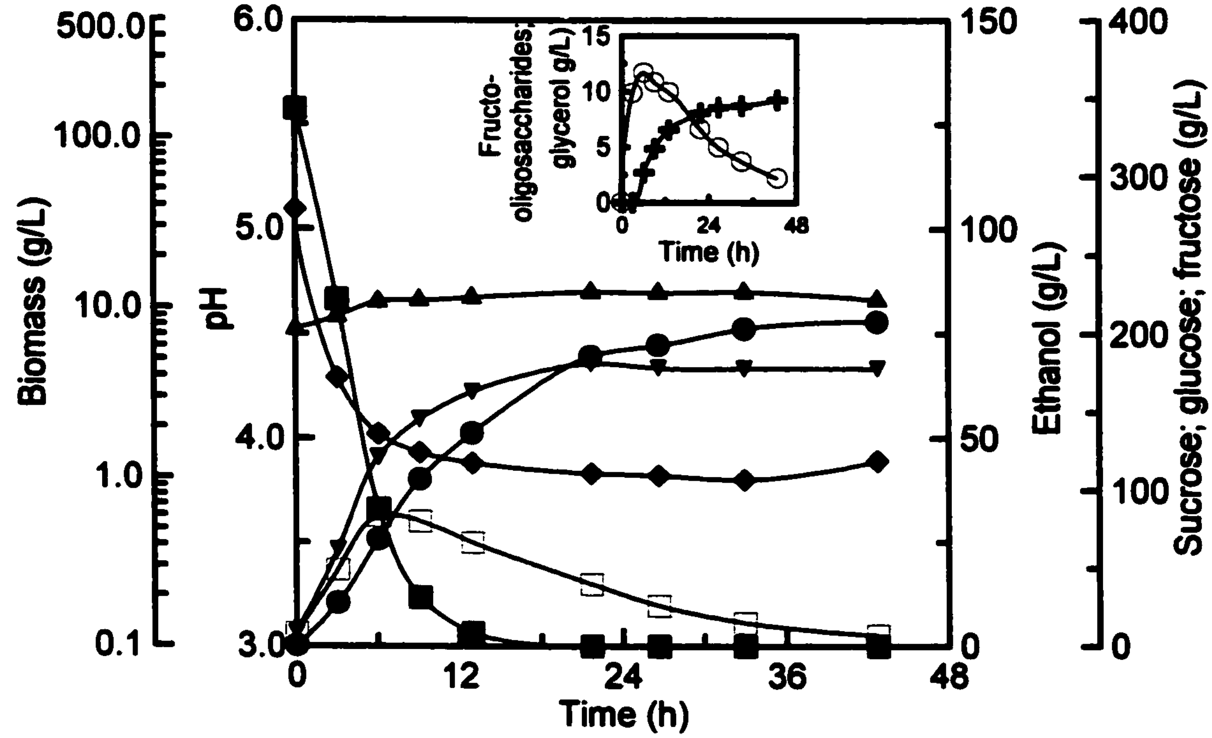

Figure 5.15 Kinetics of growth of *S. cerevisiae* ATCC 36858 and fructose and ethanol production in a medium containing 360 g/L sucrose: (▲) biomass; (◆) pH; (○) fructo-oligosaccharides; (✚) glycerol; (●) ethanol; (■) sucrose; (□) glucose; (▼) fructose.

Table 5.6 Fructose, ethanol and biomass yields, specific growth rates and specific initial rates of sucrose hydrolysis and ethanol productivities for media with various sucrose concentrations using *S. cerevisiae* ATCC 36858.

Sucrose	Initial biomass	Specific growth rate, μ	Biomass yield[a]	Ethanol productivity[b]	Specific initial rate of sucrose hydrolysis	Fructose yield[b]	Ethanol yield[b]
(g/L)	(g/L)	(h^{-1})	(g/g)	(g/L h)	(g/g h)	(%)	(%)
180.1	6.74	0.101	0.084	5.22	8.43	95.1	82.8
270.8	7.20	0.085	0.046	4.22	8.35	95.1	82.4
315.2	7.20	0.078	0.033	3.17	6.90	94.9	82.2
360.0	7.52	0.062	0.027	2.71	5.29	94.0	82.0
451.3	7.78	0.038	0.027	2.11	4.25	96.2	82.6
539.1	11.77	0.011	0.010	1.21	3.50	99.2	47.0
574.3	11.71	0.008	0.010	1.02	3.23	96.1	61.2
629.4	11.36	0.004	0.009	0.79	3.13	97.1	46.7
664.0	10.41	0.002	0.008	0.31	1.61	98.0	60.6
726.4	10.30	0.000	0.000	0.00	1.58	92.4	0.00

[a] Values are calculated at maximum biomass concentrations

[b] Values are calculated at maximum ethanol and fructose concentrations

The kinetics of biomass production and pH changes in media containing various sucrose concentrations are shown in Figure 5.16. The maximum biomass concentration of 15.2 g/L was attained in the medium with a sucrose concentration of 180 g/L. The biomass concentration decreased with an increase in the sucrose concentration above 180 g/L (Figure 5.16a). The biomass yield and specific growth rate of the yeast decreased by more than eight fold when the sucrose concentration was increased from 180 to 539 g/L (Table 5.6). This drastic decrease in the growth rate and biomass yield occurred because of the growth inhibition by a high substrate concentration. A slight growth was noticed even at sucrose concentrations above 539 g/L (Figure 5.16a) with biomass yields below 0.010 g/g of glucose and fructose consumed (Table 5.6). This indicates the osmophilic characteristic of this mutant. The pH of the fermentation medium with all concentrations decreased with time (Figure 5.16b).

The kinetics of sucrose hydrolysis and glucose production and consumption in media containing various sucrose concentrations are shown in Figure 5.17. A near complete hydrolysis of sucrose by the end of fermentation was noticed when its concentration was below 629 g/L (Figure 5.17a). The specific initial rate of sucrose hydrolysis decreased by more than five times with an increase in sucrose concentration from 180 to 726 g/L, respectively (Table 5.6). The

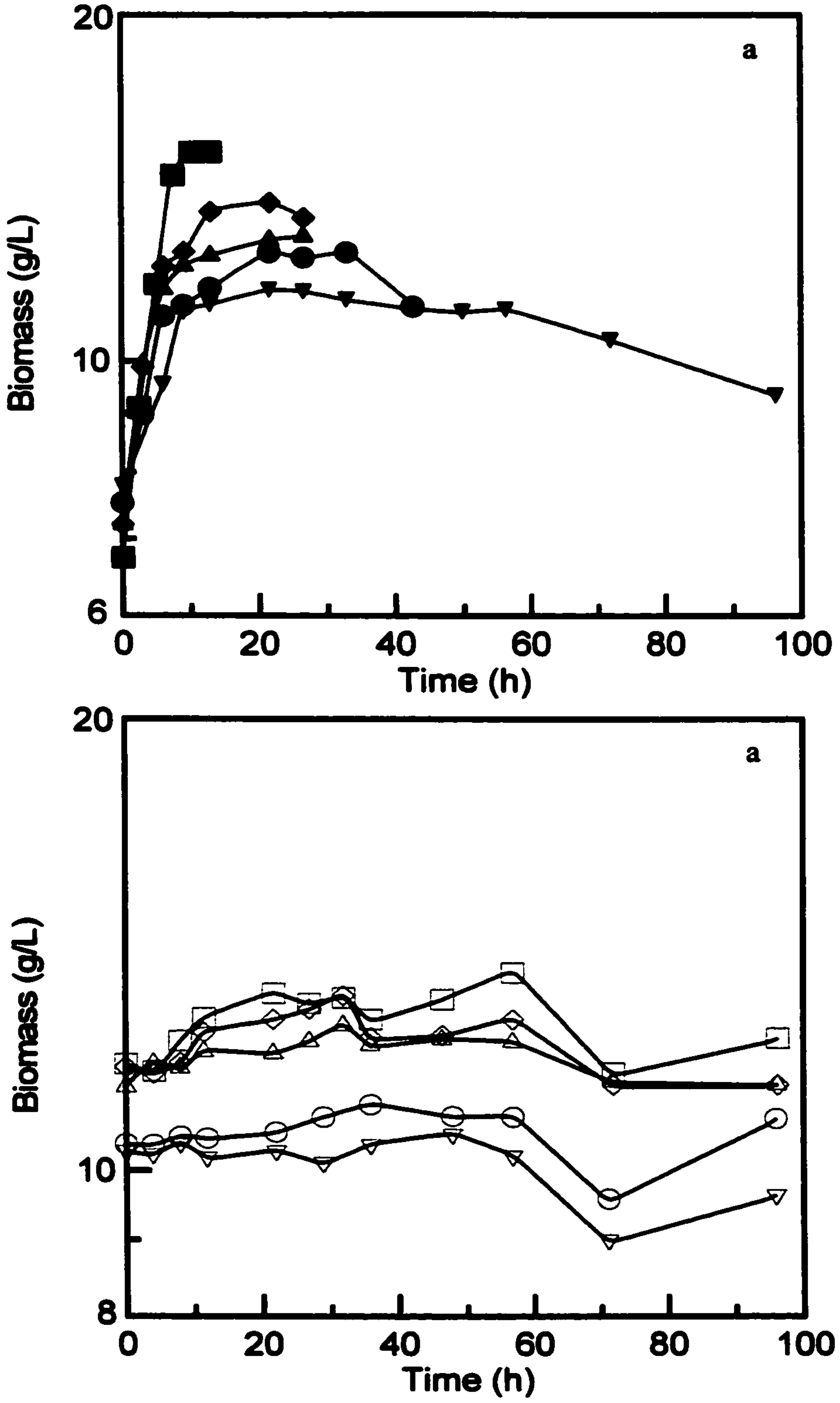

Figure 5.16 Kinetics of a) biomass production by *S. cerevisiae* ATCC 36858 and b) pH changes in sucrose media containing: (■) 180 g/L; (◆) 271 g/L; (▲) 315 g/L; (●) 360 g/L; (▼) 451 g/L; (□) 539 g/L; (◇) 574 g/L; (△) 629 g/L; (○) 664 g/L; (▽) 726 g/L.

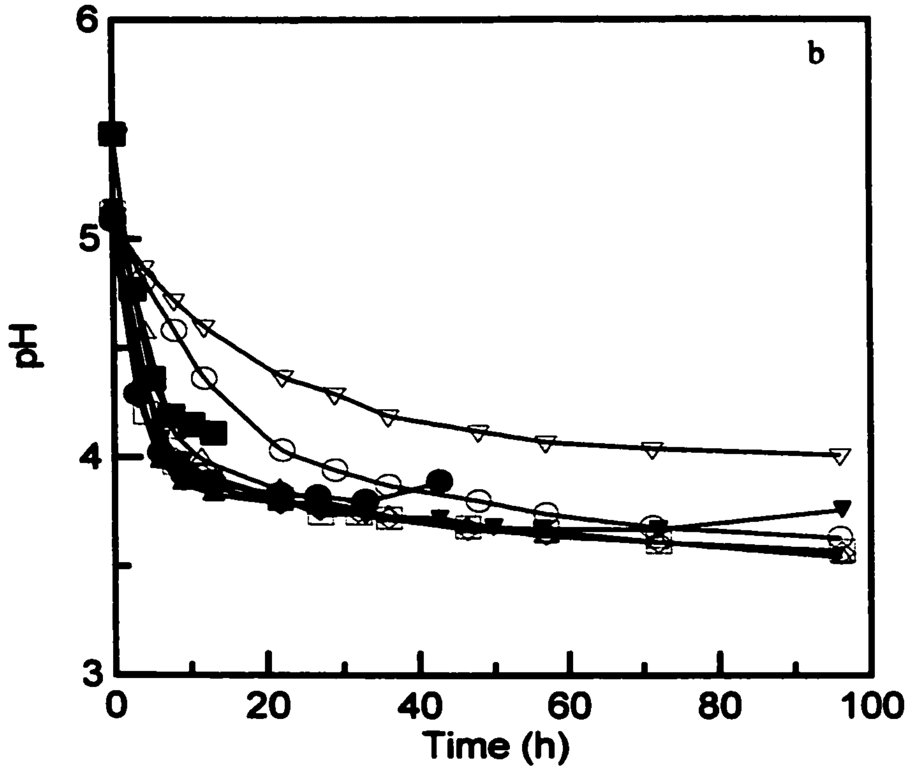

Figure 5.16 (continued)

uptake of glucose by the yeast at the beginning of fermentation was slower than the hydrolysis rate of sucrose (Figure 5.17b). This explains the accumulation of glucose in the early phase of the process. However, the glucose was completely consumed at the end of fermentation in media with sucrose concentration not higher than 360 g/L.

The kinetics of fructose and ethanol production are shown in Figure 5.18. A maximum fructose concentration of 316 g/L was attained in the medium with a sucrose concentration of 664 g/L (Figure 5.18a). In the same medium, the concentration of ethanol was only 11 g/L (Figure 5.18b). However, the maximum amount of ethanol was produced (79 g/L) in the medium with a sucrose concentration of 360 g/L. The concentration of fructose in the same medium was 178 g/L. The fructose yield was above 92% of the theoretical value in all media, (Table 5.6). The ethanol yield was about 82% of the theoretical value for all media up to 451 g/L sucrose. The ethanol productivity decreased substantially when the sucrose concentration was increased from 180 to 451 g/L, respectively. The drop in ethanol productivity and yield was much higher when the sucrose concentrations in the media were above 451 g/L.

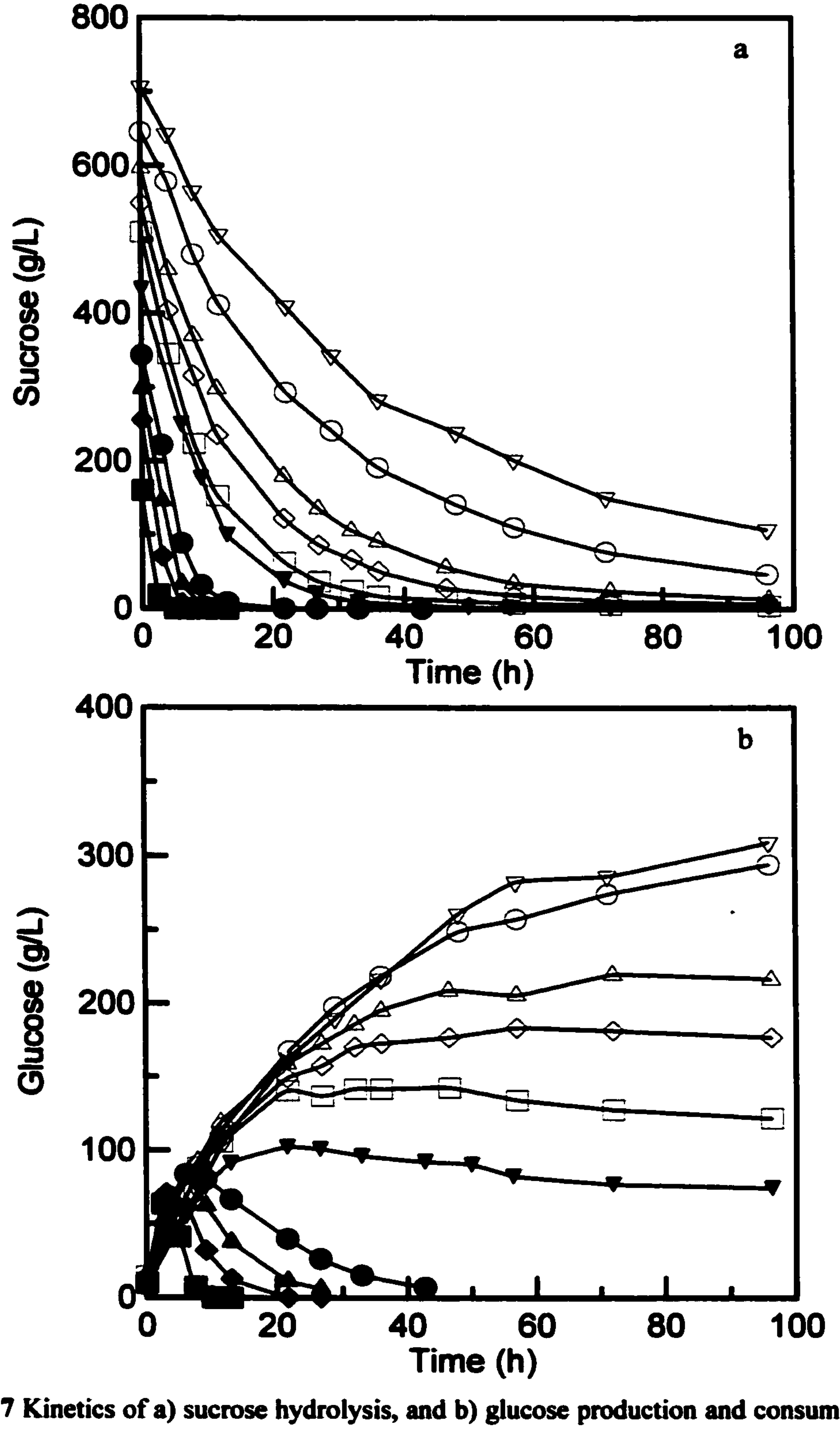

Figure 5.17 Kinetics of a) sucrose hydrolysis, and b) glucose production and consumption by *S. cerevisiae* ATCC 36858 in sucrose media containing: (■) 180 g/L; (◆) 271 g/L; (▲) 315 g/L; (●) 360 g/L; (▼) 451 g/L; (□) 539 g/L; (◇) 574 g/L; (△) 629 g/L; (○) 664 g/L; (▽) 726 g/L.

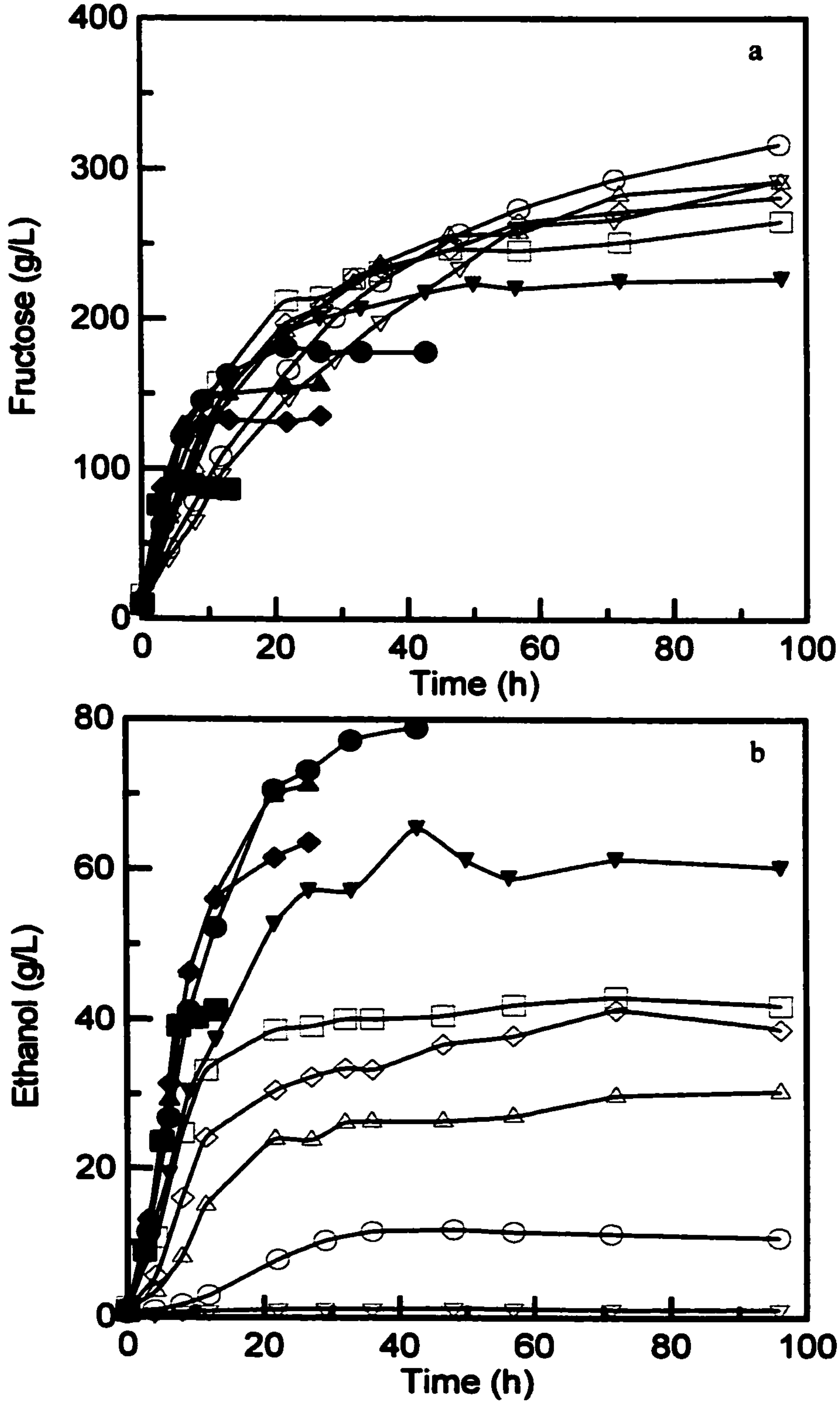

Figure 5.18 Kinetics of a) fructose production and b) ethanol production by *S. cerevisiae* ATCC 36858 in sucrose media containing: (■) 180 g/L; (◆) 271 g/L; (▲) 315 g/L; (●) 360 g/L; (▼) 451 g/L; (□) 539 g/L; (◇) 574 g/L; (△) 629 g/L; (○) 664 g/L; (▽) 726 g/L.

The results with *S. cerevisiae* ATCC 36858 demonstrate that syrups with high concentrations of fructose and ethanol can be produced using media with high sucrose concentrations. This yeast has some advantages over other microbes used for the production of fructose and ethanol from media with high substrate concentrations. These advantages include high sucrose hydrolysis efficiency, the ability to ferment in media with high sugar concentrations and high fructose and ethanol yields.

The sucrose hydrolysis efficiency with *S. cerevisiae* ATCC 36858 was above 98% in media with sucrose concentrations up to 629 g/L (Table 5.7). It decreased to 85% when the sucrose concentration increased to 726 g/L. However, the sucrose hydrolysis efficiency of *Z. mobilis* was between 73 and 94% in media that contained sucrose in concentrations between 350 and 462 g/L (Doelle and Greenfield, 1985b; Suntinanalert et al., 1986; Edye et al., 1989). It is important to mention that *S. cerevisiae* ATCC 36858 can stand much higher carbon source concentrations than other microbes used for the production of fructose and ethanol. The highest sucrose concentration studied in batch process with *Z. mobilis* was 462 g/L (Doelle and Greenfield, 1985b). When *S. cerevisiae* ATCC 36859 was used, the highest concentration of total sugar (glucose/fructose) was 415 g/L (Koren and Duvnjak, 1992). No other studies concerning fructose production by microorganisms in media with sugar concentrations above 462 g/L were found in the literature. In the present study, fructose and ethanol yields were above 94 and 82% of the theoretical values, respectively, using *S. cerevisiae* ATCC 36858 in media that contained between 180 and 451 g/L sucrose (Table 5.6). A slight decrease in fructose yield was noticed when the sucrose concentration was 726 g/L. However, the ethanol yield dropped below 62% when the sucrose concentration was above 451 g/L. These results showed that higher fructose and ethanol yields were obtained in the present study than with *Z. mobilis* when the sucrose concentration was above 250 g/L (Doelle and Greenfield, 1985b; Suntinanalert et al., 1986; Edye et al., 1989; Kirk and Doelle, 1994). Sorbitol, as much as 86 g/L, was also produced in the process with *Z. mobilis*. In order to increase fructose and ethanol yields, Doelle and Greenfield (1985b) controlled the pH of the fermentation medium, which resulted in fructose and ethanol yields above 94% of the theoretical values. However, a drawback of the pH-controlled process was that more than one third of the initial sucrose as well as one third of the produced glucose remained in the broth at the end of the process. In another study using *Z. mobilis* ATCC 53432 in a medium with a sucrose concentration of 390 g/L, a high fructose yield (90%) and a relatively high ethanol yield (65%) were reported (Suntinanalert et al., 1986). However, 35 g/L sorbitol was also produced in this process.

Table 5.7 Maximum and residual fructo-oligosaccharides, final glycerol concentrations in the fermentation broth, fructose contents in the produced syrups and sucrose hydrolysis efficiency of *S. cerevisiae* ATCC 36858 in media with various sucrose concentrations.

Sucrose (g/L)	Maximum fructo-oligosaccharides (g/L)	Residual fructo-oligosaccharides (g/L)	Glycerol (g/L)	Fructose contents in syrups produced[a] %	Sucrose hydrolysis efficiency %
180.1	5.2	0.0	6.5	100	100
270.8	8.8	0.0	7.2	100	100
315.2	10.0	0.0	8.7	100	100
360.0	11.7	0.0	9.3	97	100
451.3	15.3	3.6	11.1	74	99
539.1	20.0	6.6	11.1	67	99
574.3	20.0	10.9	10.2	59	99
629.4	20.8	15.7	9.5	54	98
664.0	25.5	20.0	5.4	47	93
726.4	23.4	19.3	0.0	40	85

[a] Values are calculated based on the final total sugar concentrations

It is important to mention that in the present study with *S. cerevisiae* ATCC 36858, sorbitol was not detected in any of the sucrose media and the addition of invertase was not required because the mutant was able to produce it. The mutant *S. cerevisiae* ATCC 36859 was used previously in several studies for the production of high fructose syrups, however, that mutant was not able to hydrolyze sucrose due to lack of invertase (Duvnjak and Koren, 1987; Koren and Duvnjak, 1989; Koren and Duvnjak, 1992; Koren and Duvnjak, 1993; Remize et al., 1998). This limited its use to media that contained glucose/fructose mixtures. Comparing these two mutants, it can be said that similar fructose and a slightly higher ethanol yields were obtained with *S. cerevisiae* ATCC 36859. However, this strain required three times more yeast extract for the production of fructose and ethanol than *S. cerevisiae* ATCC 36858.

The mutant *S. cerevisiae* ATCC 36858 was also able to ferment in media with sugar concentrations above 462 g/L, which corresponds to the highest concentration tested with *S. cerevisiae* ATCC 36859. However, the specific growth rate and biomass yield decreased with increasing sucrose concentration due to inhibition by substrate (Table 5.6). Even in a medium with a sucrose concentration of 664 g/L, a slight growth activity of *S. cerevisiae* ATCC 36858 was noticed. In that medium, the final fructose and ethanol concentrations were 316 and 11 g/L, respectively (Figure 5.18a and 5.18b). This represents fructose and ethanol yields of 98 and 61% of the theoretical values, respectively. This ethanol yield was higher than that obtained in a medium that contained 629 g/L sucrose. This, however, could be attributed to a lower glucose consumption for maintenance since it is likely that a fraction of the cells did not function (i.e. were inhibited completely). Neither growth activity nor ethanol production were noticed when the sucrose concentration was increased to 726 g/L, however, 85% of the initial sucrose was hydrolyzed after 96 h. In this case, the bioreactor behaved as a reactor with a single enzyme activity.

Syrups the carbohydrate content of which was more than 97% fructose based on the final total sugar concentrations were produced from the media with sucrose concentrations below 360 g/L (Table 5.7). However, a further increase in sucrose concentrations resulted in a significant decrease of the fructose fraction in the produced syrups due to a slow uptake of glucose and its accumulation in the fermentation media.

Some fructo-oligosaccharides and glycerol were produced by *S. cerevisiae* ATCC 36858 in all the tested media (Table 5.7). A maximum concentration of fructo-oligosaccharides was attained in the medium that contained 664 g/L sucrose. These compounds were produced during the initial phase of fermentation (Figure 15.5). The fructo-oligosaccharides were entirely hydrolyzed to glucose and fructose in a ratio of 1:2 when the sucrose concentration in the medium was below 451 g/L (Table 5.7). However, up to 20 g/L residual fructo-oligosaccharides were noticed in the medium with sucrose concentration of 664 g/L.

The results also illustrate that the concentration of fructo-oligosaccharides increased with sucrose concentration (Table 5.7). The production of these sugars could be due to the transfructosylating capability of invertase as mentioned in section 5.2.2.1. However, the formation of fructo-oligosaccharides by *Z. mobilis* in sucrose media was a result of transfructosylating capability of levansucrase (Crittenden and Doelle, 1994). The major oligosaccharide, 98% of total oligomers, produced from sucrose by *Z. mobilis* was identified as 1-kestose (Crittenden and Doelle, 1993). The fructo-oligosaccharides that were produced were not hydrolyzed by levansucrase. Invertase was added to the fermentation media to hydrolyze these oligomers, i.e. to increase fructose yield (Doelle and Doelle, 1991; Kirk and Doelle, 1994).

Glycerol is another byproduct which was produced in low concentrations by *S. cerevisiae* ATCC 36858 (Table 5.7). The glycerol concentration increased from 6.5 to 11.1 g/L with an increase in the initial sucrose concentration in the medium from 180 to 539 g/L, respectively. A smaller amount of glycerol was produced with further increases in sucrose concentration probably due to a smaller quantity of glucose consumed in the process. It could be supposed that this triol plays a role in the osmoregulation of the mutant (Maiorella et al., 1984b; Myers et al., 1997).

The results showed that *S. cerevisiae* ATCC 36858 possesses osmophilic characteristics in media with sucrose for the production of fructose and ethanol. High fructose yields (above 92%) were obtained in all media. In addition, ethanol yields were above 82% of the theoretical values in media with sucrose concentrations of 451 g/L or lower. Complete hydrolysis of sucrose was noticed in the media with concentrations of this sugar below 629 g/L. Syrups with more than 97% fructose based on the final total sugar concentrations can be produced from media with sucrose concentrations below 360 g/L. Even at a sucrose concentration of 574 g/L, the produced syrup contained 59% fructose and thus, it was richer in fructose than the ordinary 55% HFCS.

5.2.2.3 Effect of initial biomass concentration

The use of a high initial biomass concentration in the fermentation medium can increase the productivity of the process and reduce the risk of contamination. However, the use of rapid fermentation has some disadvantages related to nutritional and environmental limitations. These include high death rate of cells due to the rapid depletion of nutrients and the quick accumulation of products such as ethanol that inhibit fermentation (Ghose and Tyagi, 1979a, b). In addition, heat transfer limitations and high concentrations of CO_2 in the fermentation media are also disadvantages for the rapid fermentation (Jones et al., 1981; Jones and Greenfield, 1982; Kosaric et al., 1983). In this section, the effect of initial biomass concentration on the production of fructose and ethanol in media with sucrose concentrations of approximately 180 and 279 g/L was studied. These media contained 10 g/L yeast extract.

The results of biomass production and pH changes in media containing about 180 and 279 g/L sucrose and various initial biomass concentrations are shown in Figures 5.19 to 5.20. The yeast grew faster in the media with lower initial biomass concentrations (Figures 5.19a and

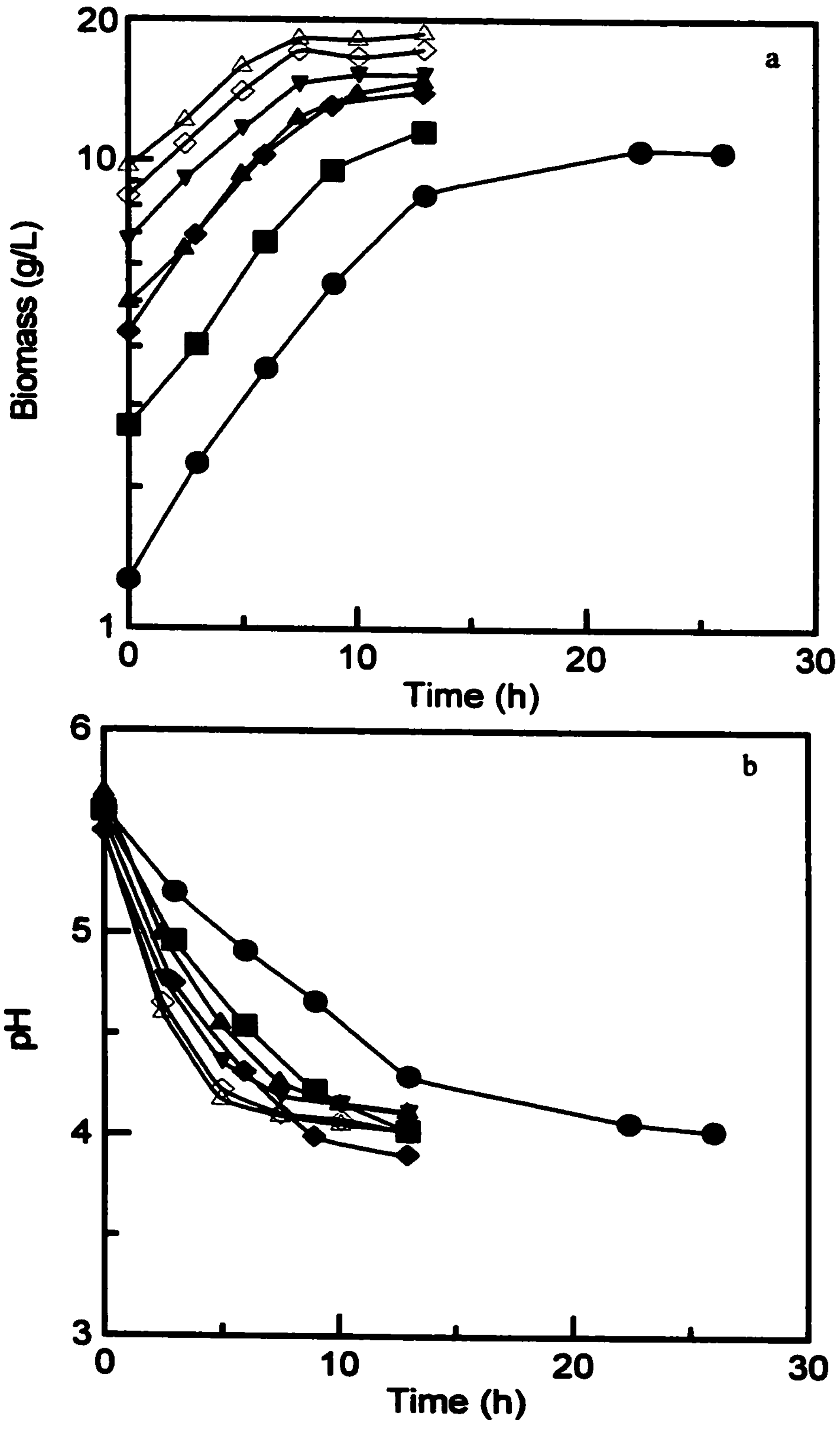

Figure 5.19 Kinetics of a) biomass production by *S. cerevisiae* ATCC 36858 and b) pH changes in media containing about 180 g/L sucrose and various initial biomass concentrations: (●) 1.27 g/L; (■) 2.71 g/L; (◆) 4.29 g/L; (▲) 4.97 g/L; (▼) 6.74 g/L; (◇) 8.36 g/L; (△) 9.68 g/L.

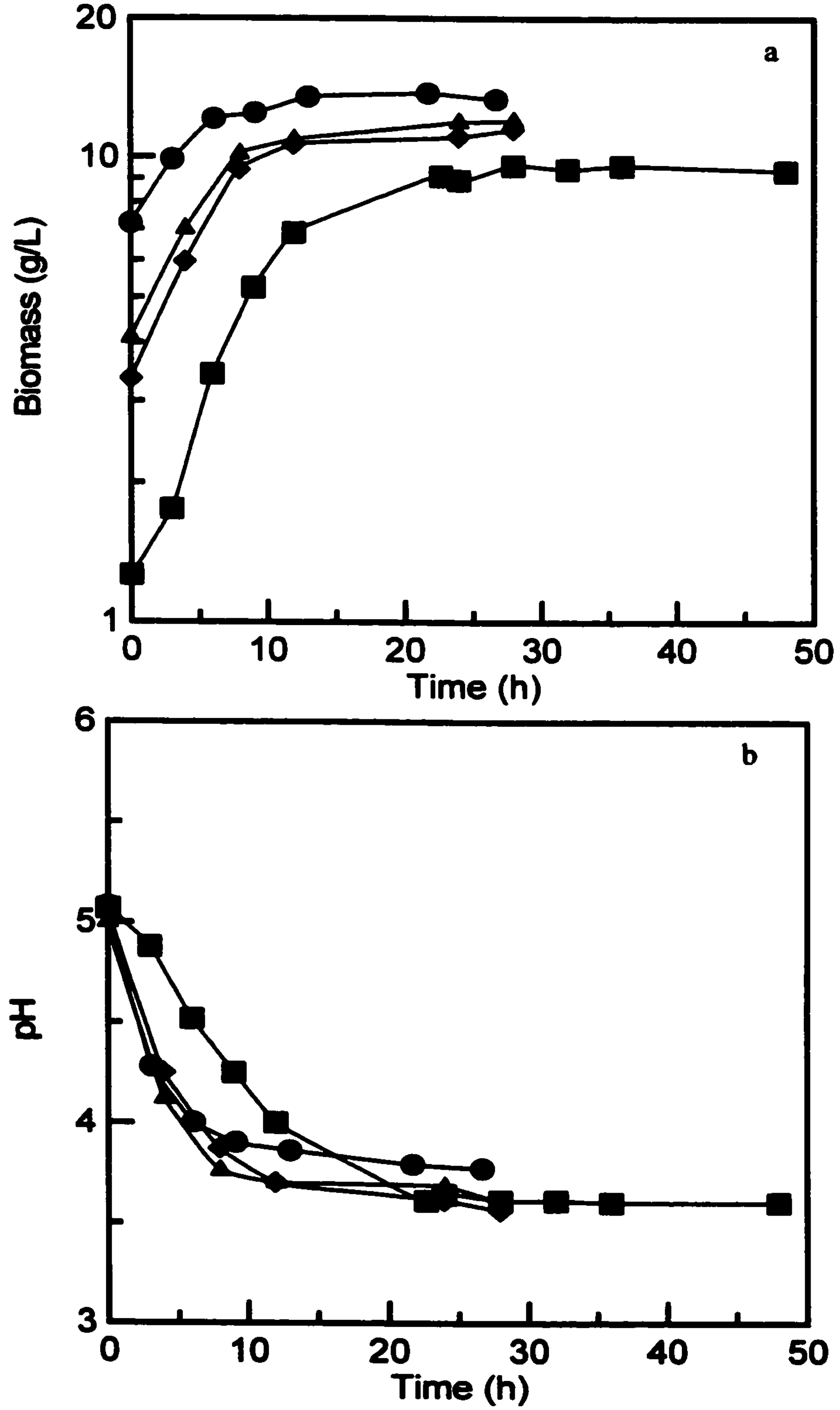

Figure 5.20 Kinetics of a) biomass production by *S. cerevisiae* ATCC 36858 and b) pH changes in media containing about 279 g/L sucrose and various initial biomass concentrations: (■) 1.26 g/L; (◆) 3.35 g/L; (▲) 4.09 g/L; (●) 7.20 g/L.

5.20a). The specific growth rates of the yeast substantially decreased with increasing initial biomass concentrations due to the fast production of ethanol that inhibited the yeast activity (Tables 5.8). However, the biomass yield was not greatly affected by the initial biomass concentration. Lower specific growth rates and biomass yields were obtained in the media that contained 279 g/L sucrose than in the media with about 180 g/L sucrose. This could be due to inhibition by high substrate and product concentrations (Jones et al., 1981). When a wild strain of *S. cerevisiae* was used to produce ethanol from a bagasse hydrolysate medium containing 220 g/L glucose, the biomass yield decreased from 0.041 to 0.025 g/g when the initial biomass concentration was increased from about 2 to 11 g/L (Ghose and Tyagi, 1979a). In their study, no growth was noticed in media with initial biomass concentration above 21 g/L due to the use of the available nutrients for the increased maintenance requirements at such high initial cell concentrations. In the present study, the pH of the fermentation medium decreased with time (Figures 5.19b and 5.20b). The trends in the pH changes in these media were very similar to those discussed in previous sections.

Table 5.8 Fructose, ethanol and biomass yields, specific growth rates and specific initial rates of sucrose hydrolysis and ethanol productivities for sucrose media with various inoculum concentrations using *S. cerevisiae* ATCC 36858.

Sucrose (g/L)	Initial biomass (g/L)	Specific growth rate, μ (h^{-1})	Biomass yield[a] (g/g)	Ethanol productivity[b] (g/L h)	Specific initial rate of sucrose hydrolysis (g/g h)	Fructose yield[b] (%)	Ethanol yield[b] (%)
187.9	1.27	0.162	0.083	1.92	23.68	89.2	76.9
175.5	2.71	0.152	0.094	3.07	17.06	95.0	83.2
175.8	4.29	0.147	0.099	4.31	12.33	96.7	80.1
178.2	4.97	0.146	0.096	4.38	10.15	92.6	80.1
180.1	6.74	0.101	0.084	5.22	8.43	95.1	82.8
173.8	8.36	0.096	0.084	5.37	6.85	93.7	82.3
185.7	9.68	0.087	0.085	5.60	6.17	96.4	81.1
287.1	1.26	0.151	0.058	2.30	17.15	98.9	82.9
273.3	3.35	0.146	0.057	2.38	16.42	99.5	80.8
284.1	4.09	0.134	0.051	2.49	13.70	96.3	78.6
270.8	7.20	0.085	0.046	4.22	8.35	95.1	82.4

[a] Values are calculated at maximum biomass concentrations

[b] Values are calculated at maximum ethanol and fructose concentrations

The kinetics of sucrose hydrolysis and glucose production and consumption in media containing about 180 and 279 g/L sucrose and various initial biomass concentrations are shown in Figures 5.21 and 5.22. A complete hydrolysis of sucrose was noticed in all media (Figure 5.21a and 5.22a). The time required for sucrose hydrolysis sharply decreased as a result of increasing the initial biomass concentration. However, the specific initial rate of sucrose hydrolysis in media with about 180 g/L sucrose decreased by more than three times with an increase in the initial biomass concentration from 1.27 to 9.68 g/L (Table 5.8). The specific initial rate of sucrose hydrolysis in media with about 279 g/L sucrose decreased by more than two times with an increase in the initial biomass concentration from 1.26 to 7.20 g/L. The uptake of glucose by the yeast at the beginning of fermentation was slower than the hydrolysis rate of sucrose (Figures 5.21b and 5.22b). However, all of the glucose was completely consumed by the end of fermentation in all media. The fermentation times, in media with sucrose concentrations of about 180 and 279 g/L, decreased by more than 40% when the initial biomass concentration increased from 1.27 to 2.71 g/L and from 1.26 to 3.35 g/L, respectively. A further increase in the initial biomass concentration resulted in a further decrease in the fermentation time.

The kinetics of the production of fructose and ethanol are shown in Figures 5.23 and 5.24. In general, the rate of fructose production increased with increasing initial biomass concentration (Figures 5.23a and 5.24a). This was due to the higher specific initial rates of sucrose hydrolysis in media with higher initial biomass concentrations (Tables 5.8). The rate of ethanol production also increased with increasing initial biomass concentration (Figures 5.23b and 5.24b). The fast production of ethanol inhibited yeast growth and resulted in decreasing the specific growth rates (Tables 5.8). The ethanol productivity in media with sucrose concentrations of about 180 g/L increased by more than two fold when the initial biomass concentration increased from 1.27 to 4.29 g/L. A further increase in the initial biomass concentration resulted in additional increase in ethanol productivity. The ethanol productivity in media with sucrose concentrations of about 279 g/L increased by about 83% when the initial biomass concentration increased from 1.26 to 7.20 g/L (Table 5.8). Fructose and ethanol yields in all tested media were above 89 and 77% of the theoretical values, respectively. Generally, ethanol and fructose yields were not affected by increasing the initial biomass concentrations. Previous researchers have found that when a wild strain of *S. cerevisiae* with an initial biomass concentration of 23.6 g/L was used in a medium containing 220 g/L glucose, the ethanol yield and productivity were 97% and 9.07 g/L h, respectively (Ghose and Tyagi, 1979a).

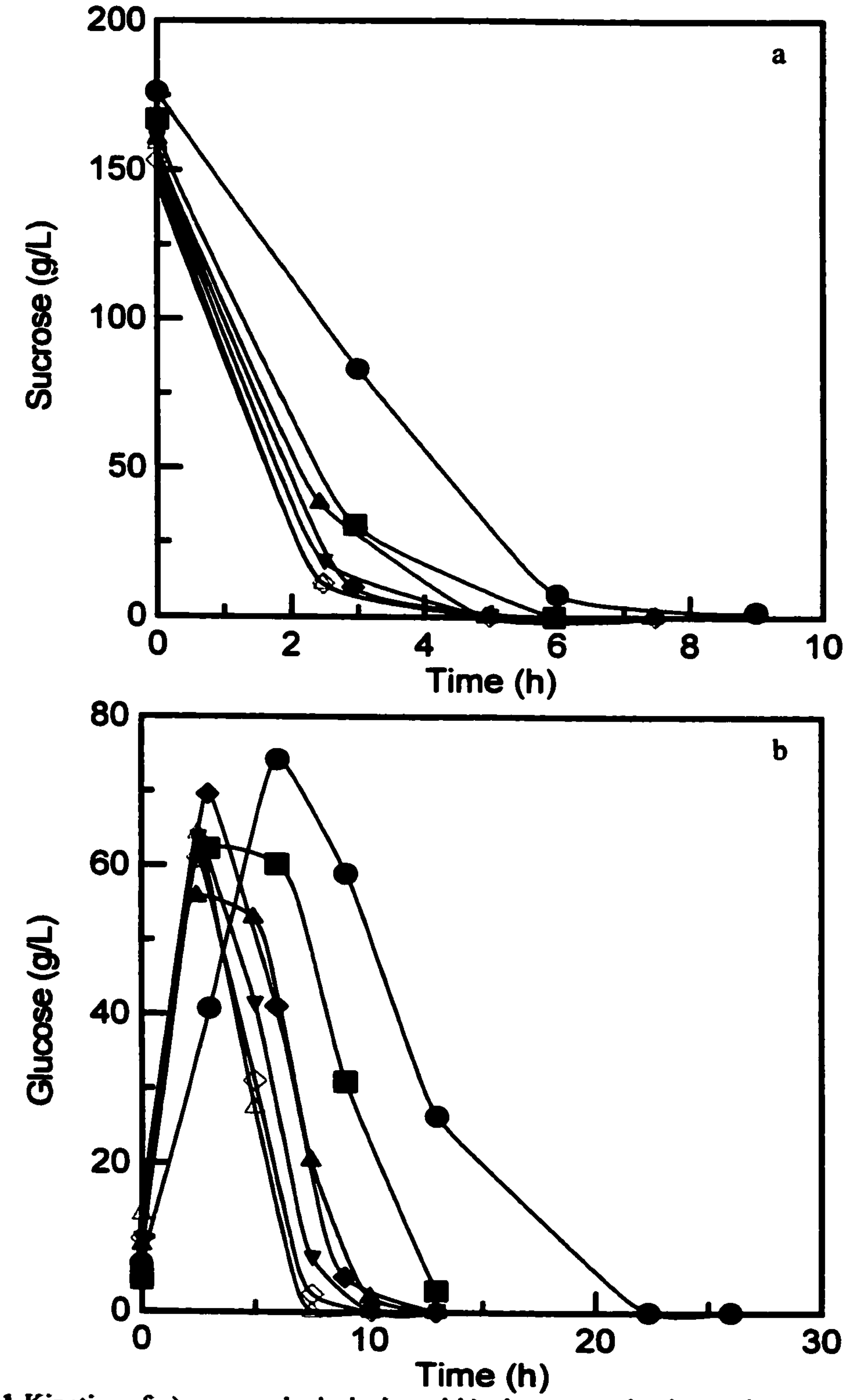

Figure 5.21 Kinetics of a) sucrose hydrolysis and b) glucose production and consumption by *S. cerevisiae* ATCC 36858 in media containing about 180 g/L sucrose and various initial biomass concentrations: (●) 1.27 g/L; (■) 2.71 g/L; (◆) 4.29 g/L; (▲) 4.97 g/L; (▼) 6.74 g/L; (◇) 8.36 g/L; (Δ) 9.68 g/L.

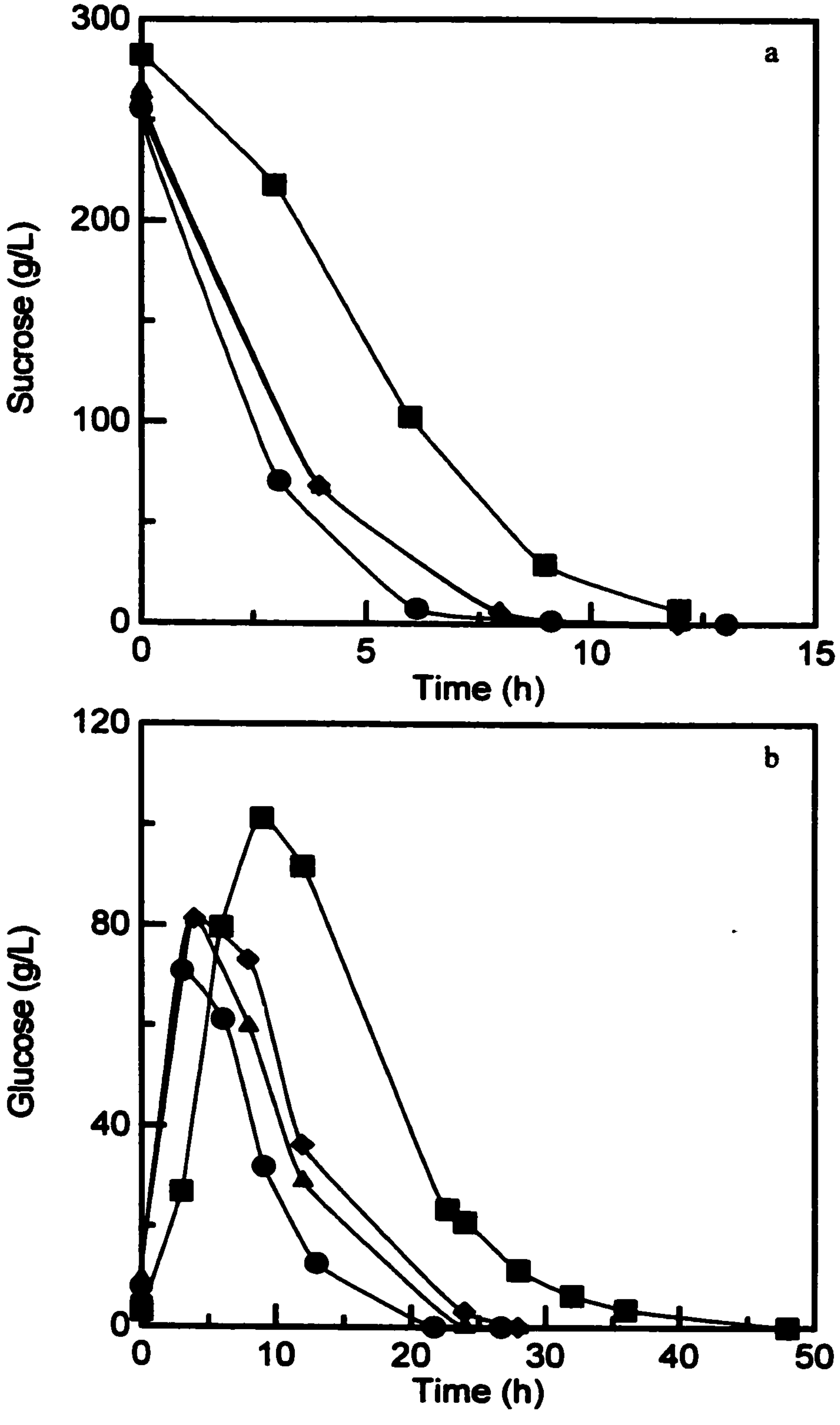

Figure 5.22 Kinetics of a) sucrose hydrolysis and b) glucose production and consumption by *S. cerevisiae* ATCC 36858 in media containing about 279 g/L sucrose and various initial biomass concentrations: (■) 1.26 g/L; (◆) 3.35 g/L; (▲) 4.09 g/L; (●) 7.20 g/L.

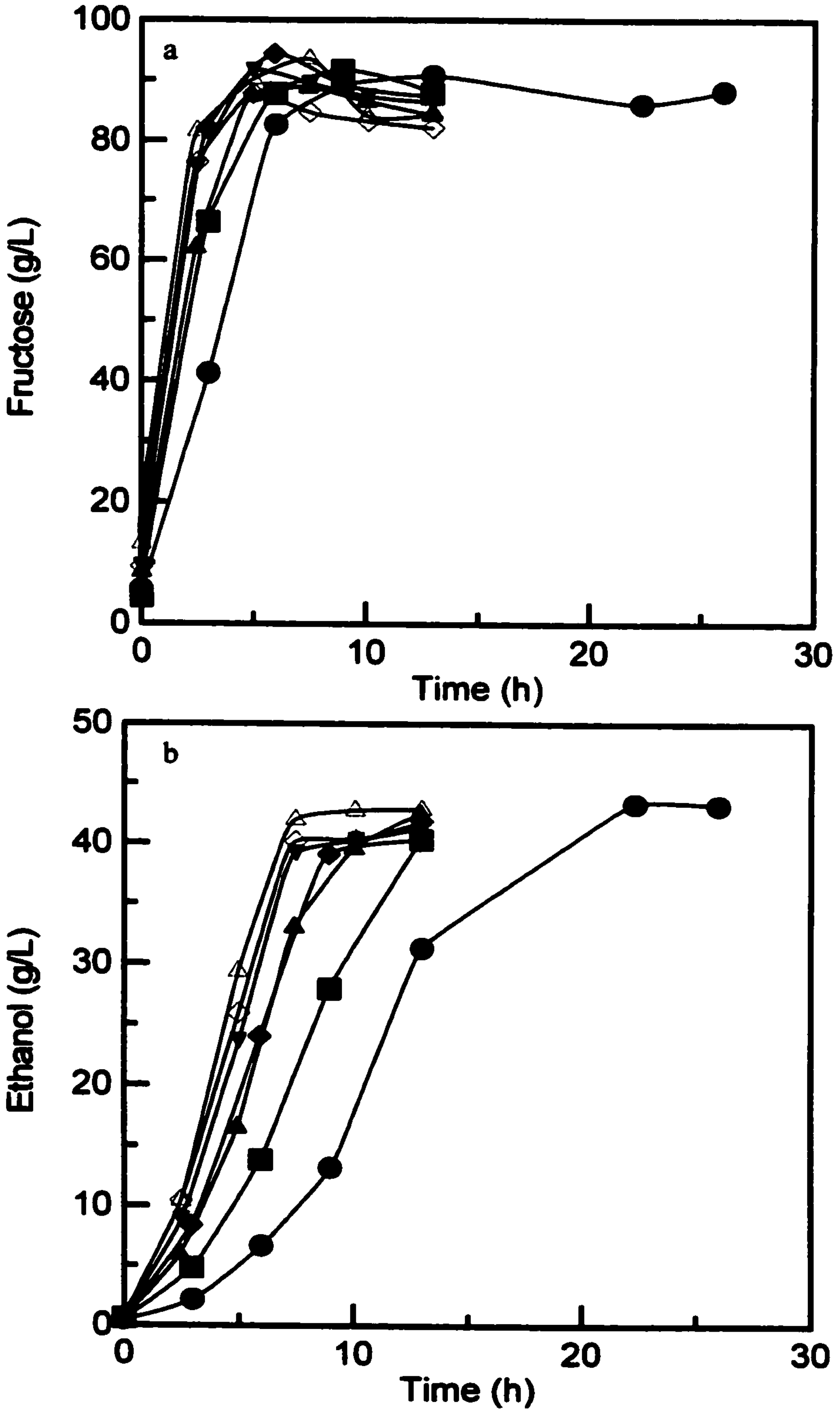

Figure 5.23 Kinetics of a) fructose and b) ethanol production by *S. cerevisiae* ATCC 36858 in media containing about 180 g/L sucrose and various initial biomass concentrations: (●) 1.27 g/L; (■) 2.71 g/L; (◆) 4.29 g/L; (▲) 4.97 g/L; (▼) 6.74 g/L; (◇) 8.36 g/L; (Δ) 9.68 g/L.

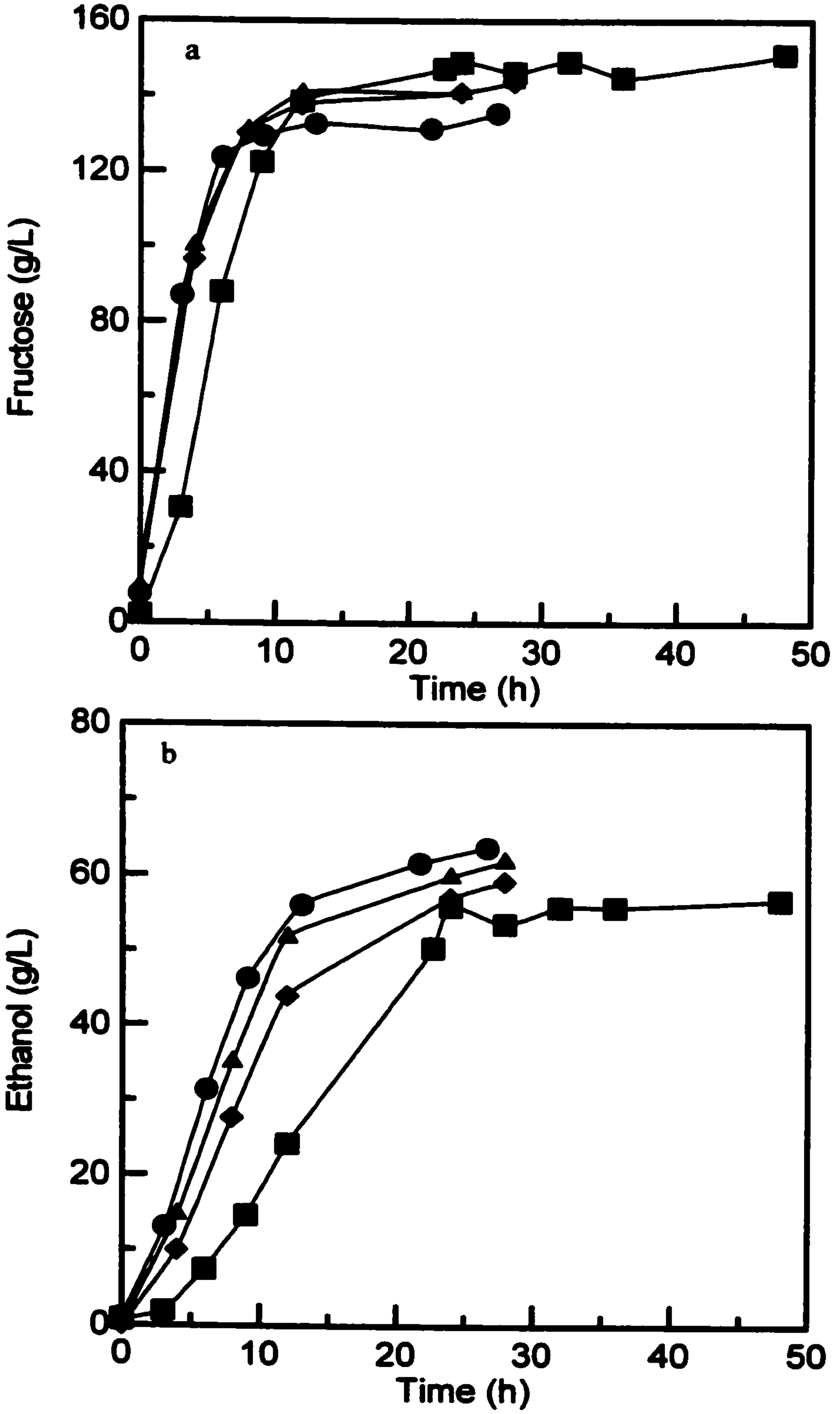

Figure 5.24 Kinetics of a) fructose and b) ethanol production by *S. cerevisiae* ATCC 36858 in media containing about 279 g/L sucrose and various initial biomass concentrations: (■) 1.26 g/L; (◆) 3.35 g/L; (▲) 4.09 g/L; (●) 7.20 g/L.

In the present study, small amounts of fructo-oligosaccharides and glycerol were produced (Tables 5.9). There were no clear trends in their production as a result of increasing the initial biomass concentrations in the fermentation media. The amount of fructo-oligosaccharides that were produced in the media with about 279 g/L sucrose was almost double of what was formed in the media with about 180 g/L sucrose. The fructo-oligosaccharides were produced at the beginning of the fermentation. However, they were completely hydrolyzed to glucose and fructose by the yeast by the end of the process, which is consistent with the results discussed in section 5.2.2.1.

Table 5.9 Maximum fructo-oligosaccharides and final glycerol concentrations in the fermentation broth for sucrose media with various inoculum concentrations using *S. cerevisiae* ATCC 36858.

Sucrose (g/L)	Initial biomass (g/L)	Fructo-oligosaccharides (g/L)	Glycerol (g/L)
187.9	1.27	4.7	6.1
175.5	2.71	4.7	6.7
175.8	4.29	3.9	8.5
178.2	4.97	5.0	7.8
180.1	6.74	5.2	6.5
173.8	8.36	4.6	8.0
185.7	9.68	4.3	9.3
287.1	1.26	10.1	7.4
273.3	3.35	8.7	6.8
284.1	4.09	9.7	8.5
270.8	7.20	8.8	7.2

The results demonstrated that the initial biomass concentration had a considerable effect on specific growth rate, ethanol productivity and specific initial rate of sucrose hydrolysis, and no effect on fructose and ethanol yields. The increase in the initial biomass concentration in the fermentation media resulted in a considerable decrease in the specific growth rate and initial rate of sucrose hydrolysis but increased ethanol productivity. Ethanol and fructose yields in all media were above 77 and 89% of the theoretical values, respectively.

5.2.2.4 Effect of initial ethanol concentration

The production of high concentrations of fructose and ethanol is advantageous in reducing the distillation and evaporation costs when producing syrups. However, this requires carrying out fermentations in media with high sucrose concentrations. The use of media with high sucrose concentrations resulted in the inhibition of growth and ethanol production by *S. cerevisiae* ATCC 36858 as discussed in section 5.2.2.2. Ethanol is a product that has been shown to inhibit growth, viability and fermentation capability of many organisms to different extents (Ghose and Tyagi, 1979b; Jones et al., 1981; Kosaric et al., 1983; Stewart et al., 1983; D'Amore et al., 1990). It has been reported that ethanol has inhibitory effects on key glycolytic enzymes such as hexokinase and alcohol dehydrogenase as well as on cell constituents and membrane, which affects the cells properties for substrate uptake and ethanol excretion (Jones et al., 1981; Kosaric et al., 1983; Nagashima, 1990). It is therefore important to study the effect of ethanol on growth and fermentation capabilities of *S. cerevisiae* ATCC 36858. The effect of the addition of ethanol to media with sucrose concentrations of about 220 g/L on the production of fructose and ethanol is discussed in this section. The fermentation media contained 30 g/L yeast extract.

The kinetics of the fermentation process using media containing various initial ethanol concentrations are shown in Figures 5.25 to 5.27. The results showed that the final biomass concentration and yield were not affected, however, the specific growth rate decreased by 22% when the initial ethanol concentration was 28.0 g/L (Figure 5.25a and Table 5.10). Only slight growth of *S. cerevisiae* ATCC 36858 was noticed when the initial ethanol concentration in the fermentation medium was 67.3 g/L. The specific growth rate and biomass yield decreased by about four fold when the initial ethanol concentration was increased from 0.4 to 67.3 g/L. These results can be compared with those of *S. cerevisiae* ATCC 36859 in media with about 87 g/L glucose (Koren, 1991). In his study, the specific growth rate and biomass yield decreased by more than three fold when the initial ethanol concentration was increased from 1 to 64 g/L. He also reported no growth of *S. cerevisiae* ATCC 36859 and no production of ethanol when the initial ethanol concentration was 79 g/L. In another study, Ghose and Tyagi (1979b) noticed a decrease in the specific growth rate of a wild strain of *S. cerevisiae* in a medium with about 10 g/L glucose by more than four fold when 66 g/L of ethanol was initially added to the fermentation medium. There was no growth when the initial ethanol concentration was about 87 g/L.

It has been reported that ethanol added to the medium during rapid yeast fermentation had a less damaging effect on the cells than the same or smaller quantities of ethanol produced by the yeast (Nagodawithana and Steinkraus, 1976). The addition of 120 g/L ethanol to a medium containing 25% honey resulted in a 43% decrease in yeast viability. However, the loss of yeast

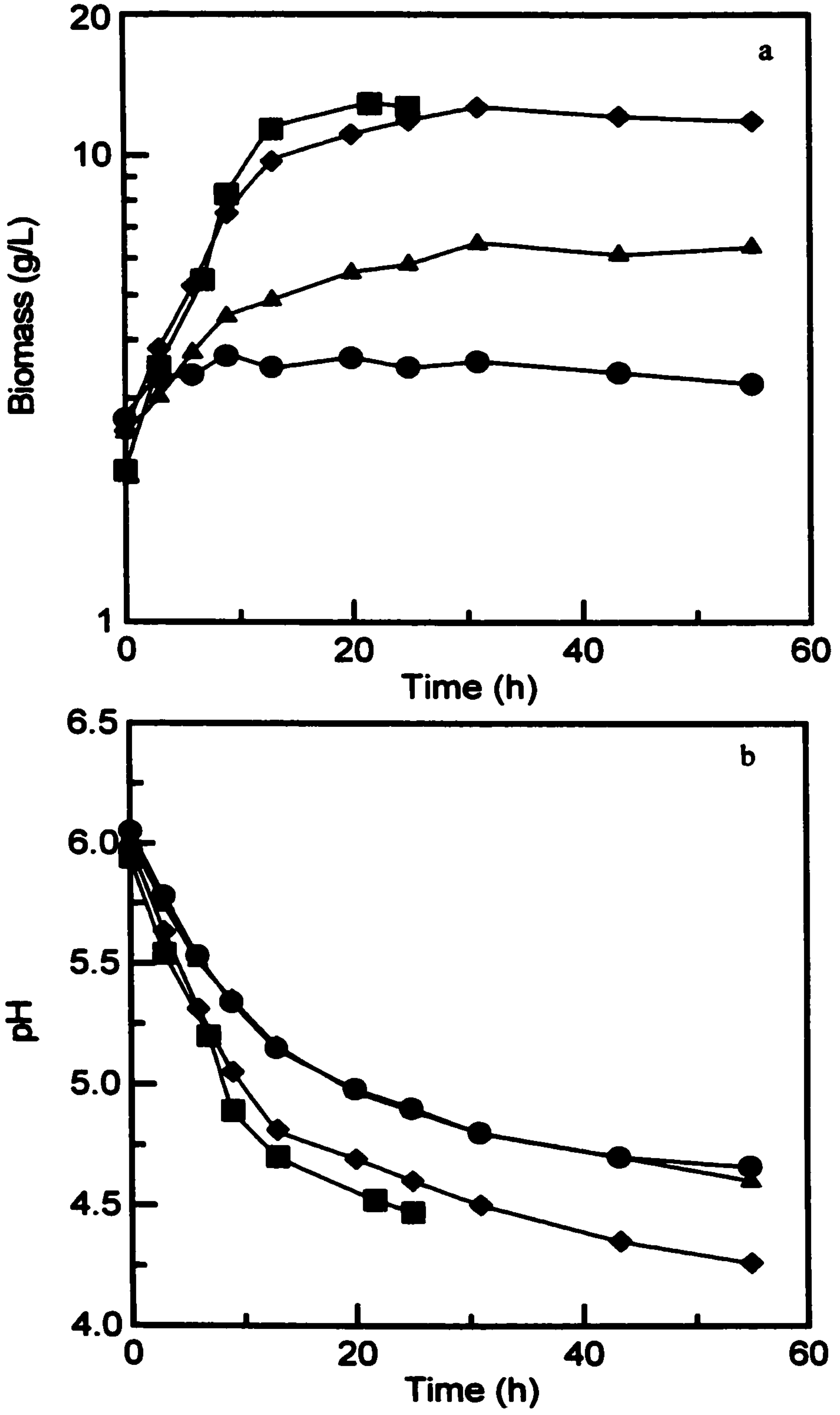

Figure 5.25 Kinetics of a) biomass production by *S. cerevisiae* ATCC 36858 and b) pH changes in media containing about 220 g/L sucrose and various initial ethanol concentrations: (■) 0.4 g/L; (◆) 28.0 g/L; (▲) 43.5 g/L; (●) 67.3 g/L.

Table 5.10 Fructose, ethanol and biomass yields, specific growth rates and specific initial rates of sucrose hydrolysis and ethanol productivities for media with about 220 g/L sucrose and various initial ethanol concentrations using *S. cerevisiae* ATCC 36858.

Initial ethanol	Sucrose	Initial biomass	Specific growth rate, μ	Biomass yield[a]	Ethanol productivity[b]	Specific initial rate of sucrose hydrolysis	Fructose yield[b]	Ethanol yield[b]
(g/L)	(g/L)	(g/L)	(h^{-1})	(g/g)	(g/L h)	(g/g h)	(%)	(%)
0.4	216.4	2.10	0.146	0.090	2.23	15.85	97.0	79.2
28.0	194.0	2.55	0.120	0.093	1.73	7.28	95.6	67.2
43.5	238.6	2.53	0.067	0.037	0.64	3.26	94.1	57.0
67.3	231.0	2.71	0.037	0.021	0.00	2.75	85.6	0.0

[a] Values are calculated at maximum biomass concentrations
[b] Values are calculated at maximum ethanol and fructose concentrations

viability in a similar medium was about 85% when ethanol (94 g/L) was produced. They also found that ethanol was produced by yeast cells at much faster rate than the cells could excrete it. The ratio of the intracellular to extracellular ethanol was 2.7. Other researchers reported that the ratio of the intracellular to extracellular ethanol was as high as 25 during the early stages of fermentation in a medium with 300 g/L glucose (D'Amore et al., 1990). However, this ratio was three times lower in a medium with 100 g/L glucose. In addition, this ratio decreased during the fermentation until the intracellular and extracellular concentrations were similar. They reported that the intracellular and extracellular ethanol concentration difference occurs under certain fermentation conditions such as in rapid fermentation, high osmotic pressure and high temperature. It was concluded that the intracellular ethanol produced during the early stages of fermentation, as well as high osmotic pressure, may contribute to the decrease in the growth and fermentation activities of the yeast (D'Amore et al., 1990). However, it was also suggested that factors such as the physiological state of the cells and the production of toxic compounds other than ethanol resulted in a greater apparent toxicity of the intracellular ethanol than ethanol added to the medium (van Uden, 1985).

In the present study, the pH of the fermentation media decreased with time (Figure 5.25b). This is similar to the trends noticed previously in media with various sucrose concentrations.

The kinetics of sucrose hydrolysis and glucose production and consumption in media containing various initial ethanol concentrations are shown in Figure 5.26. A complete hydrolysis

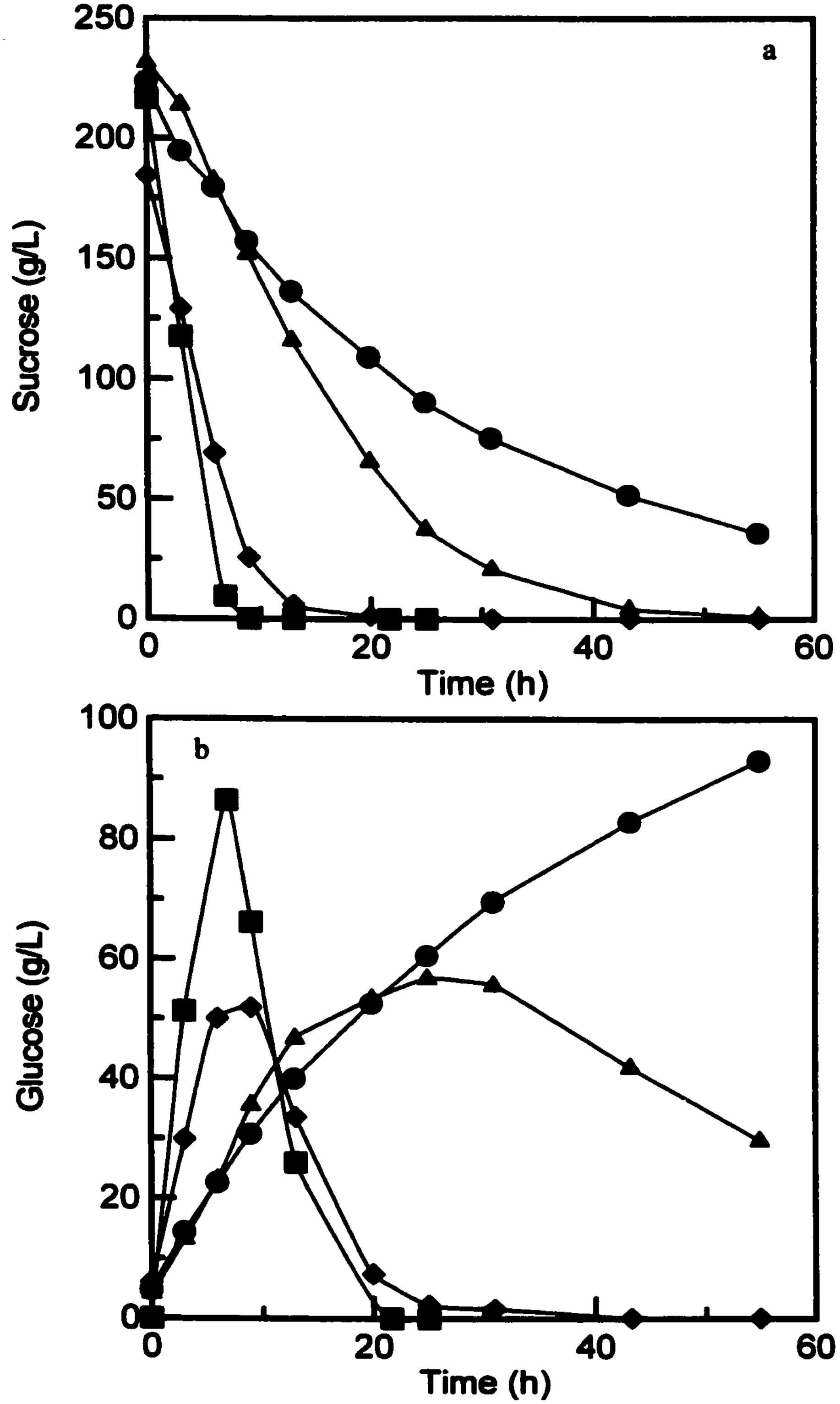

Figure 5.26 Kinetics of a) sucrose hydrolysis and b) glucose production and consumption by *S. cerevisiae* ATCC 36858 in media containing about 220 g/L sucrose and various initial ethanol concentrations: (■) 0.4 g/L; (◆) 28.0 g/L; (▲) 43.5 g/L; (●) 67.3 g/L.

of sucrose was noticed in media with initial ethanol concentrations below 67.3 g/L (Figure 5.26a). However, about 16% of the initial sucrose was not hydrolyzed in the medium with 67.3 g/L ethanol after 55 h of the process (Figure 5.26a and Table 5.11). The specific initial rate of sucrose hydrolysis decreased by more than five fold with an increase in the initial ethanol concentration from 0.4 to 67.3 g/L (Table 5.10). The uptake of glucose by the yeast at the beginning of fermentation was slower than the rate of sucrose hydrolysis, which explains the accumulation of this monosaccharide during that phase of the process (Figures 5.26b). Glucose was completely consumed by the end of fermentation when the initial ethanol concentration was below 43.5 g/L. However, about 76 and 24% of the glucose was consumed in media with initial ethanol concentrations of 43.5 and 67.3 g/L, respectively, due to the inhibition of ethanol on yeast cells. Similar effects of ethanol inhibition on glucose uptake by yeast cells have been reported in a medium with 87 g/L glucose (Koren, 1991). He found that about 72 and 17% of the glucose was consumed in media with initial ethanol concentrations of 48 and 64 g/L, respectively.

Table 5.11 Maximum and residual fructo-oligosaccharides, final glycerol concentrations in the fermentation broth, fructose contents in the produced syrups and sucrose hydrolysis efficiency of *S. cerevisiae* ATCC 36858 in media with about 220 g/L sucrose and various initial ethanol concentrations.

Initial ethanol (g/L)	Sucrose (g/L)	Maximum fructo-oligosaccharides (g/L)	Residual fructo-oligosaccharides (g/L)	Fructose contents in syrups produced[a] %	Sucrose hydrolysis efficiency %	Glycerol (g/L)
0.4	216.4	7.1	0.0	100	100	8.2
28.0	194.0	4.1	1.1	100	100	4.0
43.5	238.6	5.2	2.7	78	100	2.2
67.3	231.0	3.6	3.4	40	84	0.0

[a] Values are calculated based on the final total sugar concentrations

The kinetics of fructose and ethanol production in media with various initial ethanol concentrations are shown in Figure 5.27. The fructose production rate decreased with increasing initial ethanol concentration (Figure 5.27a). This was due to a decrease in the sucrose hydrolysis rate as a result of increasing the initial ethanol concentration in the fermentation medium (Figure 5.26a). Ethanol production and productivity decreased with increasing initial ethanol concentration (Figures 5.27b and Table 5.10). More ethanol was produced in the medium in

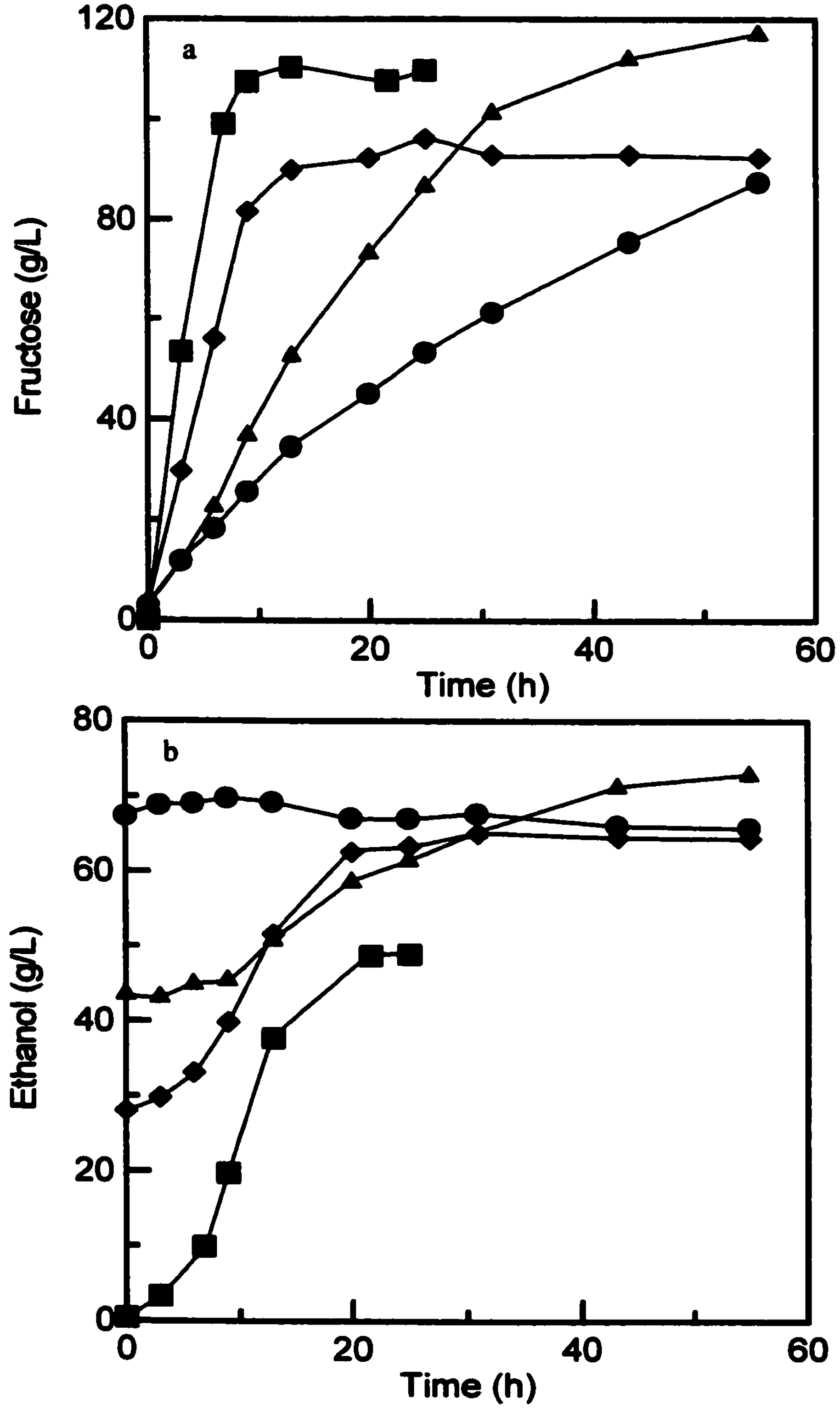

Figure 5.27 Kinetics of a) fructose and b) ethanol production by *S. cerevisiae* ATCC 36858 in media containing about 220 g/L sucrose and various initial ethanol concentrations: (■) 0.4 g/L; (◆) 28.0 g/L; (▲) 43.5 g/L; (●) 67.3 g/L.

which ethanol was not added. The ethanol yield in that medium was 79% of the theoretical value (Table 5.10). A maximum ethanol concentration of 73 g/L (added and produced) was obtained in the medium with an initial ethanol concentration of 43.5 g/L. The ethanol yield was 57% of the theoretical value. The fructose yield also decreased with an increase in the initial ethanol concentration (Table 5.10). The fructose yield in the medium with an initial ethanol concentration of 67.3 g/L was 14% lower than the theoretical value. It was found that the decrease in ethanol yields was more severe than the decrease in fructose yields. This could suggest that the addition of ethanol to the fermentation medium has higher inhibitory effects on the glycolytic enzymes responsible for ethanol formation than invertase, which is responsible for sucrose hydrolysis and therefore fructose production. In addition, the high cell maintenance requirements at higher initial ethanol concentrations contributed to the lower ethanol yields. Koren (1991) found that the ethanol yield was only 26% of the theoretical value in a medium with an initial ethanol concentration of 64 g/L and 87 g/L glucose.

In the present study, ethanol was not produced in the medium with an initial ethanol concentration of 67.3 g/L (Figures 5.27b). However, a slight yeast growth was noticed in this medium (Figure 5.25a). This could suggest that the growth of *S. cerevisiae* ATCC 36858 was less inhibited by this high initial ethanol concentration than its fermentation capacity. However, when the effect of increasing the initial ethanol concentration in the fermentation medium on yeast growth and ethanol production was examined, it was noticed that yeast growth was more affected than ethanol production (Table 5.10). Other researchers have also reported similar results. In a study by Ghose and Tyagi (1979b) using a wild strain of *S. cerevisiae*, neither growth nor ethanol production were noticed when the concentration of the initially added ethanol to the fermentation medium was 114 g/L. However, there was no growth but some ethanol was still produced when the initially added ethanol concentration was 87 g/L. Koren (1991) reported no growth of *S. cerevisiae* ATCC 36859 and no ethanol production when the initial ethanol concentration was 79 g/L. He also found that below this ethanol concentration, yeast growth was more inhibited than ethanol production. It has also been reported that for most microbes, cell growth and cell viability were more inhibited by ethanol than their fermentation capacity due to the differences in the effect of ethanol on the various cellular functions (van Uden, 1985; Berry and Brown, 1987).

In the present study, the production of fructo-oligosaccharides and glycerol decreased with increasing initial ethanol concentration in the fermentation medium (Table 5.11). The yeast cells did not produce glycerol but did produce 3.6 g/L of fructo-oligosaccharides when the initial ethanol concentration was 67.3 g/L. The fructo-oligosaccharides were entirely hydrolyzed to glucose and fructose in the medium in which ethanol was not added (Table 5.11). Some fructo-oligosaccharides accumulated in the medium when the initial ethanol concentration was above 28.0 g/L. Syrups with more than 78% fructose based on the final total sugar concentrations were produced from media with initial ethanol concentrations below 43.5 g/L. The fructose content in the syrup produced from the medium with an initial ethanol concentration of 67.3 g/L was 40%. This was due to the inhibition of the yeast cells by ethanol, which prevented them from consuming all the glucose from the medium.

The results concerning the effects of initially added ethanol on the production of fructose and ethanol by *S. cerevisiae* ATCC 36858 in sucrose media demonstrated that an increase in the initial ethanol concentration resulted in a considerable decrease in yeast growth and fermentation ability. Fructose and ethanol yields and productivities decreased as a result of increasing the initial ethanol concentrations. Although neither ethanol nor glycerol were produced in the medium with initial ethanol concentration of 67.3 g/L, fructo-oligosaccharides were formed. This showed that the initially added ethanol had different effects on the various cellular functions.

5.2.2.5 Effect of pH

The pH, a measure of hydrogen ion concentration, is in general a significant factor in industrial fermentations and has been reported to have effects on yeast growth, fermentation rates, byproduct formation and controlling bacterial contaminations (Jones et al., 1981). The limits of pH values for growth of most strains of *S. cerevisiae* were between 2.4 and 8.6, with an optimum for growth between 4 and 5 (Jones et al., 1981, Cooney, 1981). Yeast fermentation rates are relatively insensitive to pH values between 3.5 and 6.0 (Jones et al., 1981). They also reported that the fermentation of sucrose is expected to be more pH sensitive than the fermentation of glucose because yeast invertase activity is more affected by low pH values than is its fermentative ability. Therefore, the effect of pH on growth and production of ethanol and fructose by *S. cerevisiae* ATCC 36858 in media containing about 268 g/L sucrose and 10 g/L yeast extract was examined and the results are discussed in this section.

The kinetics of biomass production and changes in pH during fermentation are shown in Figure 5.28. Growth and biomass production were relatively insensitive to the initial pH values

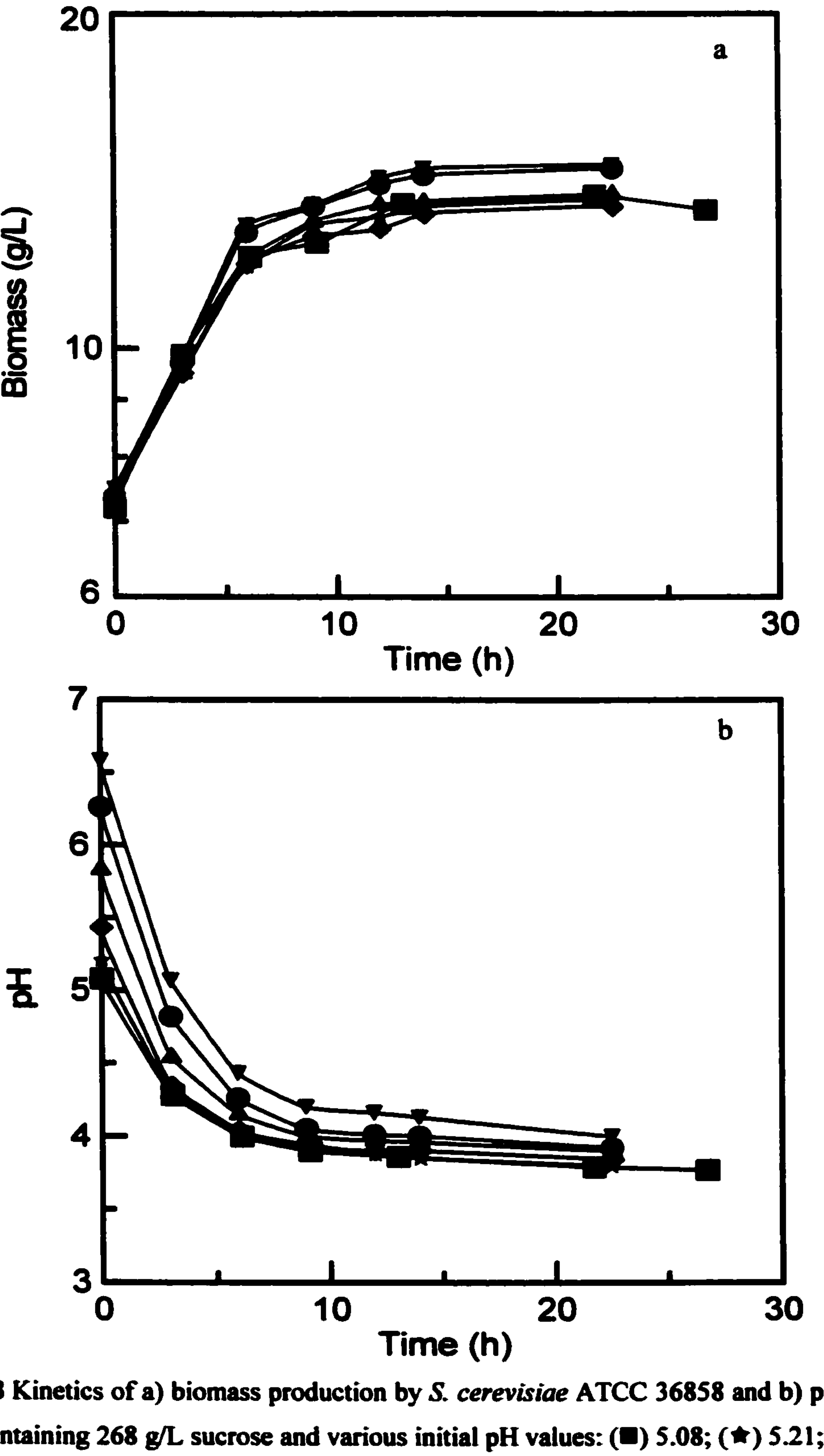

Figure 5.28 Kinetics of a) biomass production by *S. cerevisiae* ATCC 36858 and b) pH changes in media containing 268 g/L sucrose and various initial pH values: (■) 5.08; (★) 5.21; (◆) 5.43; (▲) 5.83; (●) 6.26; (▼) 6.58.

(Figure 5.28a). The final biomass concentrations were about 13.7 g/L when the initial pH in the media was below 6.26. Slightly more biomass was produced in the media with pH 6.26 and 6.58. The specific growth rate and biomass yield were about 0.085 h^{-1} and 0.045 g/g, respectively (Table 5.12). Verduyn et al. (1990) reported that the maximum amount of biomass produced in a chemostat culture of *S. cerevisiae* was obtained with an initial pH between 5.0 and 5.5. They also found that the biomass concentration more than doubled when the initial pH was increased from 2.7 to 5.5, however there was about 20% decrease from the maximum biomass concentration when the initial pH was increased to 7.0. In the present study, the pH of all tested media decreased with time to about 4.0 (Figure 5.28b). As previously mentioned the pH drop in the fermentation medium is generally due to the production of acids such as lactic, acetic and propionic (Stewart and Russell, 1987).

Table 5.12 Fructose, ethanol and biomass yields, specific growth rates and specific initial rates of sucrose hydrolysis and ethanol productivities for media with about 268 g/L sucrose and various initial pH values using *S. cerevisiae* ATCC 36858.

Sucrose (g/L)	pH	Initial biomass (g/L)	Specific growth rate, μ (h^{-1})	Biomass yield[a] (g/g)	Ethanol productivity[b] (g/L h)	Specific initial rate of sucrose hydrolysis (g/g h)	Fructose yield[b] (%)	Ethanol yield[b] (%)
270.8	5.08	7.20	0.085	0.046	4.22	8.35	95.1	82.4
262.2	5.21	7.37	0.079	0.042	4.14	8.82	95.1	80.7
261.6	5.43	7.41	0.079	0.043	4.01	10.86	96.3	82.6
272.4	5.83	7.36	0.082	0.043	4.22	11.53	94.4	80.7
268.2	6.26	7.32	0.092	0.049	4.20	11.60	95.7	80.5
265.4	6.58	7.49	0.091	0.049	4.22	11.39	95.0	81.1

[a] Values are calculated at maximum biomass concentrations

[b] Values are calculated at maximum ethanol and fructose concentrations

The results from this study concerning sucrose hydrolysis and glucose production and consumption in media with various initial pH values are shown in Figure 5.29. A complete hydrolysis of sucrose was noticed in all media within the first 6 h of the process (Figure 5.29a). The specific initial rate of sucrose hydrolysis increased by 36% with an increase in the initial pH from 5.08 to 6.58 (Table 5.12). Glucose accumulated at the beginning of the fermentation since its uptake by the yeast was slower than its formation by hydrolysis of sucrose (Figure 5.29b). However, the glucose was completely consumed by the end of fermentation in all media.

The kinetics of fructose and ethanol production in the fermentation media are shown in

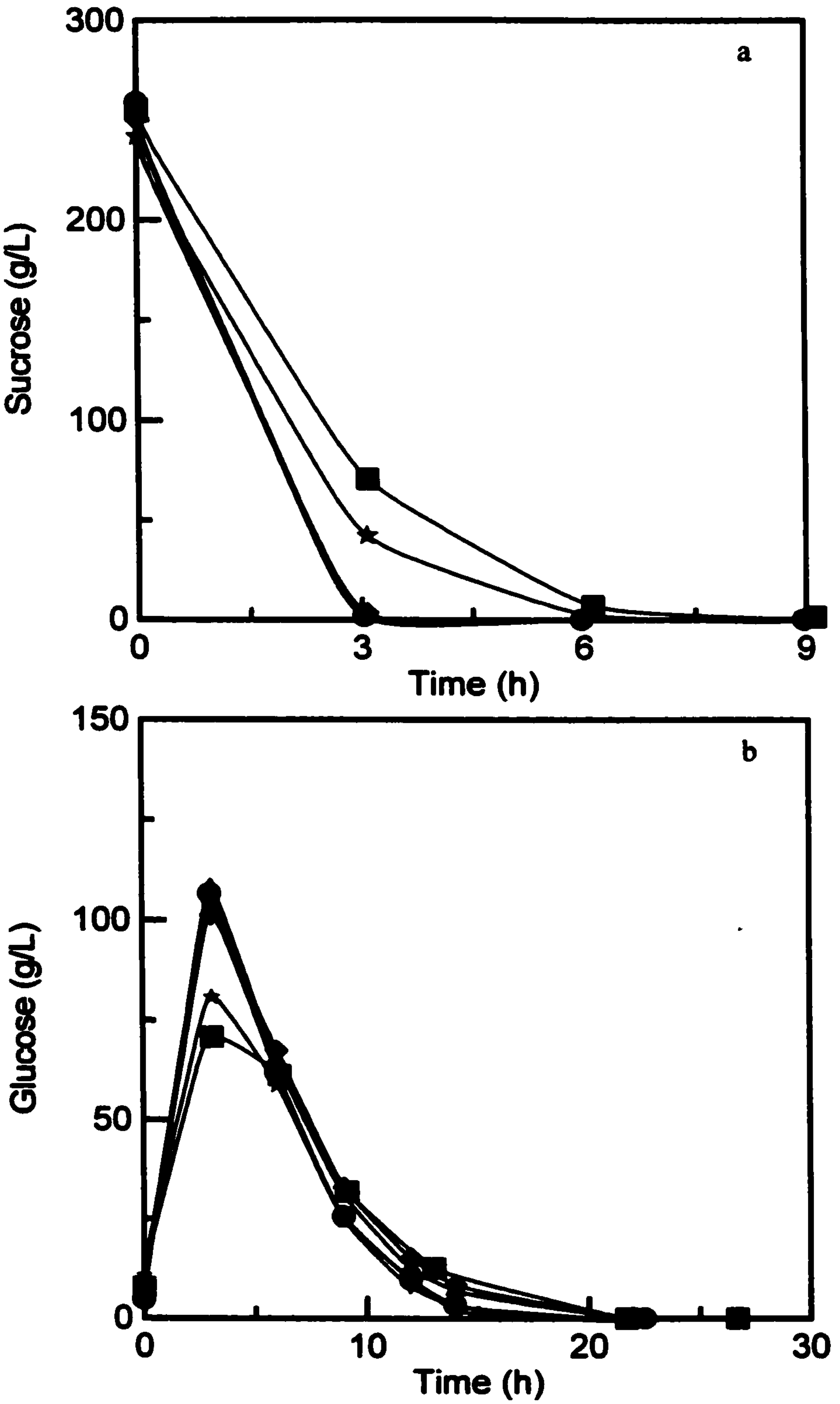

Figure 5.29 Kinetics of a) sucrose hydrolysis and b) glucose production and consumption by *S. cerevisiae* ATCC 36858 in media containing 268 g/L sucrose and various initial pH values: (■) 5.08; (★) 5.21; (◆) 5.43; (▲) 5.83; (●) 6.26; (▼) 6.58.

Figure 5.30. The rate of fructose production when the initial pH was 5.21 or less was lower than in the media with higher pH values, due to slower hydrolysis of sucrose (Figures 5.30a and 5.29a). Similar amounts of ethanol were produced in all tested media (Figure 5.30b). The ethanol productivities were above 4.0 g/L h in all media (Table 5.12). The fructose and ethanol yields were above 94 and 81% of the theoretical values, respectively. Initial pH values in the fermentation media between 5.08 and 6.58 had insignificant effects on fructose and ethanol yields or ethanol productivity.

Glycerol and oligosaccharides were produced in all media (Tables 5.13). The glycerol concentration in the fermentation broth increased from 7.2 to 8.9 g/L with an increase in the initial pH from 5.08 to 6.58. In a study by Verduyn et al. (1990), glycerol concentrations increased from 1.4 to 1.9 g/L in a chemostat culture of *S. cerevisiae* when the initial pH was increased from 2.7 to 5.5. In addition, when the pH was further increased to 7.0, the glycerol concentration increased by another 85%. The capability of *S. cerevisiae* strains to produce glycerol is well known especially at higher pH values (Rehm, 1988; Wang et al., 2001).

Table 5.13 Maximum fructo-oligosaccharides and final glycerol concentrations in the fermentation broth for media with about 268 g/L sucrose with various initial pH values using *S. cerevisiae* ATCC 36858.

Sucrose (g/L)	pH	Fructo-oligosaccharides (g/L)	Glycerol (g/L)
270.8	5.08	8.8	7.2
262.2	5.21	8.5	7.7
261.6	5.43	5.5	7.8
272.4	5.83	5.2	8.3
268.2	6.26	5.4	8.4
265.4	6.58	4.8	8.9

In the present study, more fructo-oligosaccharides were produced in media with lower initial pH values (Table 5.13). This could be due to the slightly lower sucrose hydrolysis rates in these media (Figure 5.29a and Table 5.12). As discussed in section 5.2.2.1, the production of fructo-oligosaccharides in the fermentation medium could be due to the transfructosylating capability of invertase. The production of fructo-oligosaccharides from sucrose solutions with invertase occurs at all pH values in which invertase is active (Blanchard and Albon, 1950; Bacon

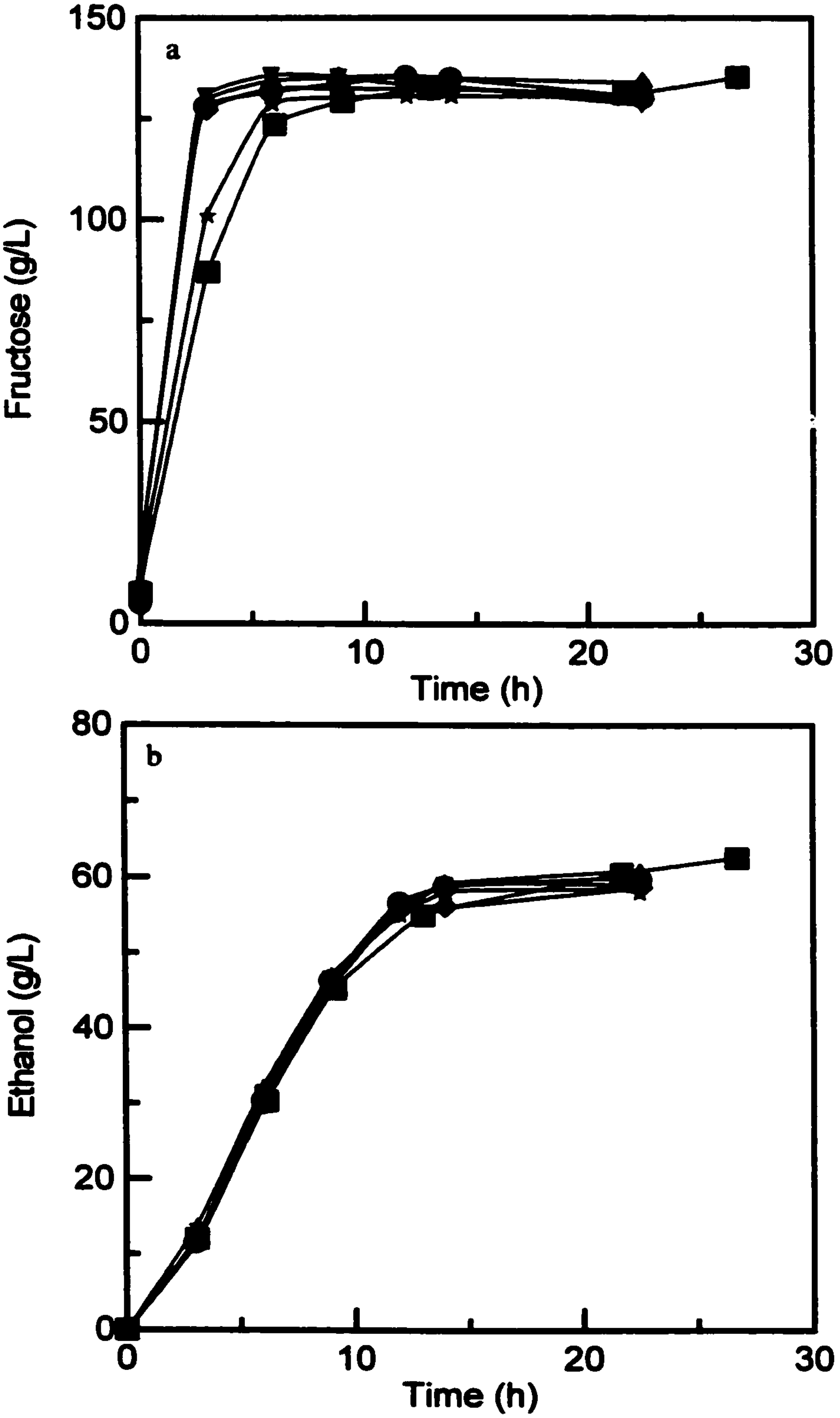

Figure 5.30 Kinetics of a) fructose and b) ethanol production by *S. cerevisiae* ATCC 36858 in media containing 268 g/L sucrose and various initial pH values: (■) 5.08; (★) 5.21; (◆) 5.43; (▲) 5.83; (●) 6.26; (▼) 6.58.

and Edelman, 1950; Bacon, 1954). However, these researchers did not show the effect of pH on the amount of oligosaccharides formed. It was reported that the optimum pH for yeast invertase activity was between 3.5 and 5.5 (Gascon et al., 1968). Other researchers reported the optimum activity of yeast invertase at pH about 4.5 (Samoshina et al., 1981; Combes and Monsan, 1983). Ghosh (1990) found that at pH about 4.5 the invertase activity of intact yeast cells was optimum. However, Arnold (1987) noticed a pH range, between 4.5 and 5.5, for maximum invertase activity of intact yeast cells. Most of the studies on the production of oligosaccharides from sucrose solutions using enzyme preparations were carried out at a pH between 4.5 and 5.0 (Blanchard and Albon, 1950; Bacon and Edelman, 1950; Albon et al., 1953; Bacon, 1954; Gross et al., 1954; Andersen et al., 1969; Straathof et al., 1986; Farine et al., 2001). Although invertase activity was low at pH 10, oligosaccharides were still found in the reaction mixture (Blanchard and Albon, 1950).

In the present study, the fructo-oligosaccharides produced by *S. cerevisiae* ATCC 36858 were completely hydrolyzed to glucose and fructose by the end of the fermentation process in all media. In addition, no residual glucose was noticed in all fermentation media studied. The complete hydrolysis of fructo-oligosaccharides and utilization of glucose was also noticed in media with sucrose concentrations below 360 g/L as discussed in sections 5.2.2.1 and 5.2.2.2.

In the case of *Z. mobilis*, levan, fructo-oligosaccharides and sorbitol were formed as byproducts in the production of ethanol and fructose from sucrose media (Doelle and Greenfield, 1985b; Suntinananalert et al., 1986; Edye et al., 1989; Doelle and Doelle, 1991; Kirk and Doelle, 1994). The production of levan and fructo-oligosaccharides by *Z. mobilis* was due to the transfructosylating capability of levansucrase (Doelle et al., 1993; Crittenden and Doelle, 1994). This enzyme was also responsible for the hydrolysis of sucrose for the production of fructose and ethanol by *Z. mobilis* strains. It was reported that the optimum pH for levan production by a *Z. mobilis* culture was around 5.0, with only trace quantities of levan produced at pH 6.5 (Doelle and Greenfield, 1985a, b; Doelle et al., 1993). In addition, increasing the fermentation temperature to 35°C inhibited levan formation (Doelle and Greenfield, 1985a, b; Crittenden and Doelle, 1994). Crittenden and Doelle (1994) also found that the optimum pH for levansucrase activity from *Z. mobilis* was 5.5 for sucrose hydrolysis, and for fructo-oligosaccharides and levan formation. However, the optimum temperature for this enzyme activity for levan formation was

15°C, while it was 45°C for both sucrose hydrolysis and fructo-oligosaccharides formation. The sucrose hydrolysis efficiency with *Z. mobilis* dropped by 20% when the pH of the fermentation medium was controlled to 6.4 instead of 5.0 (Doelle and Greenfield, 1985b). The production of fructo-oligosaccharides by *Z. mobilis* was reported to increase at pH values above 6.0 as well as with increasing sucrose concentrations (Doelle et al., 1993). The fructo-oligosaccharides produced using *Z. mobilis* were not hydrolyzed by levansucrase. Therefore, invertase was added to the fermentation media to hydrolyze these oligomers, i.e. to increase fructose yield (Doelle and Doelle, 1991; Kirk and Doelle, 1994).

The production of sorbitol by *Z. mobilis* was suggested to be due to a glucose/fructose transhydrogenase that was involved in the oxidation of glucose to gluconate with the simultaneous reduction of fructose to sorbitol (Leigh et al., 1984). The optimum pH for this enzyme activity was 5.5. Zachariou and Scopes (1986) reported that glucose/fructose oxidoreductase in *Z. mobilis* is responsible for the reduction of fructose to sorbitol in conjunction with the oxidation of glucose to gluconate. The optimum pH for this enzyme was 6.2. Sorbitol, as much as 86 g/L, was produced and fructose and ethanol yields were below 30 and 77%, respectively, in the process with *Z. mobilis* when the pH of the fermentation media was controlled at 5.0 (Doelle and Greenfield, 1985b). In order to increase fructose and ethanol yields and to reduce sorbitol production, they controlled the pH of the fermentation media at 6.4, which resulted in fructose and ethanol yields above 94% of the theoretical values and only traces of sorbitol. However, a major drawback of this controlled process was the accumulation of more than one third of the initial sucrose and produced glucose in the broth at the end of the process.

The results of the present study showed that the specific growth rate, biomass, fructose and ethanol yields and productivities by *S. cerevisiae* ATCC 36858 in sucrose media were not significantly affected by an increase in the initial pH from 5.08 to 6.58. However, the specific initial rate of sucrose hydrolysis increased by more than 36% when the initial pH was increased above 5.43. In addition, more glycerol and less fructo-oligosaccharides were produced in the media with higher initial pH values.

5.2.2.6 Effect of media sterilization

Medium sterilization is one of the most important unit operations required for a successful fermentation. Its objective is to remove or destroy any of the microorganisms that could adversely affect the bioconversion process or medium storage (Cooney, 1985). Some of the methods used for medium sterilization are filtration, chemical treatment and thermal treatment. The latter is more commonly used in biochemical processes (Corbett, 1985; Cooney, 1985). The choice of a sterilization method depends on several factors such as its effectiveness in achieving acceptable levels of sterility, cost and positive or negative effects on medium quality (Corbett, 1985; Cooney, 1985). During the thermal sterilization and killing of microbial spores and cells, there is the possibility of destroying ingredients in the medium by caramelizing sugars, denaturing proteins and inactivating vitamins (Corbett, 1985; Wallhausser, 1985). The thermal destruction of such ingredients would reduce the nutritive quality of the medium, which may adversely affect the fermentation process by lowering yields of desired products. Sterilization costs of the medium and the reactor contribute significantly to the total production cost for many microbial processes (Maiorella et al., 1984a). A significant reduction in the production cost can be achieved if fermentation can be done without media sterilization. In this section, the effects of media sterilization on the production of fructose and ethanol by *S. cerevisiae* ATCC 36858 in media with various sucrose, yeast extract and biomass concentrations are discussed.

The kinetics of the fermentation process in un-sterilized media containing various sucrose concentrations and 10 g/L yeast extract are shown in Figures 5.31 to 5.33. Although the initial biomass and/or yeast extract concentrations in the non-sterile media were different from sterile media (sections 5.2.2.1 and 5.2.2.2), the kinetic patterns of the fermentation process were similar. A maximum biomass concentration of 11.4 g/L was attained in the un-sterilized medium with a sucrose concentration of 282 g/L (Figure 5.31a). The biomass yields and specific growth rates of the yeast decreased as the sucrose concentration increased (Table 5.14). This is similar to the trends observed in sterilized sucrose media (Tables 5.4 and 5.6). However, the biomass yields and the specific growth rates obtained in un-sterilized media were different from those obtained in the sterilized media due to the differences in the initial biomass and yeast extract concentrations used in these media. The pH of the broth with all tested concentrations decreased with time to the same finial level (Figure 5.31b).

The results of sucrose hydrolysis and glucose production and consumption are shown in Figure 5.32. A complete hydrolysis of sucrose by the end of fermentation was noticed in all tested media (Figure 5.32a). The uptake of glucose by the yeast at the beginning of fermentation was

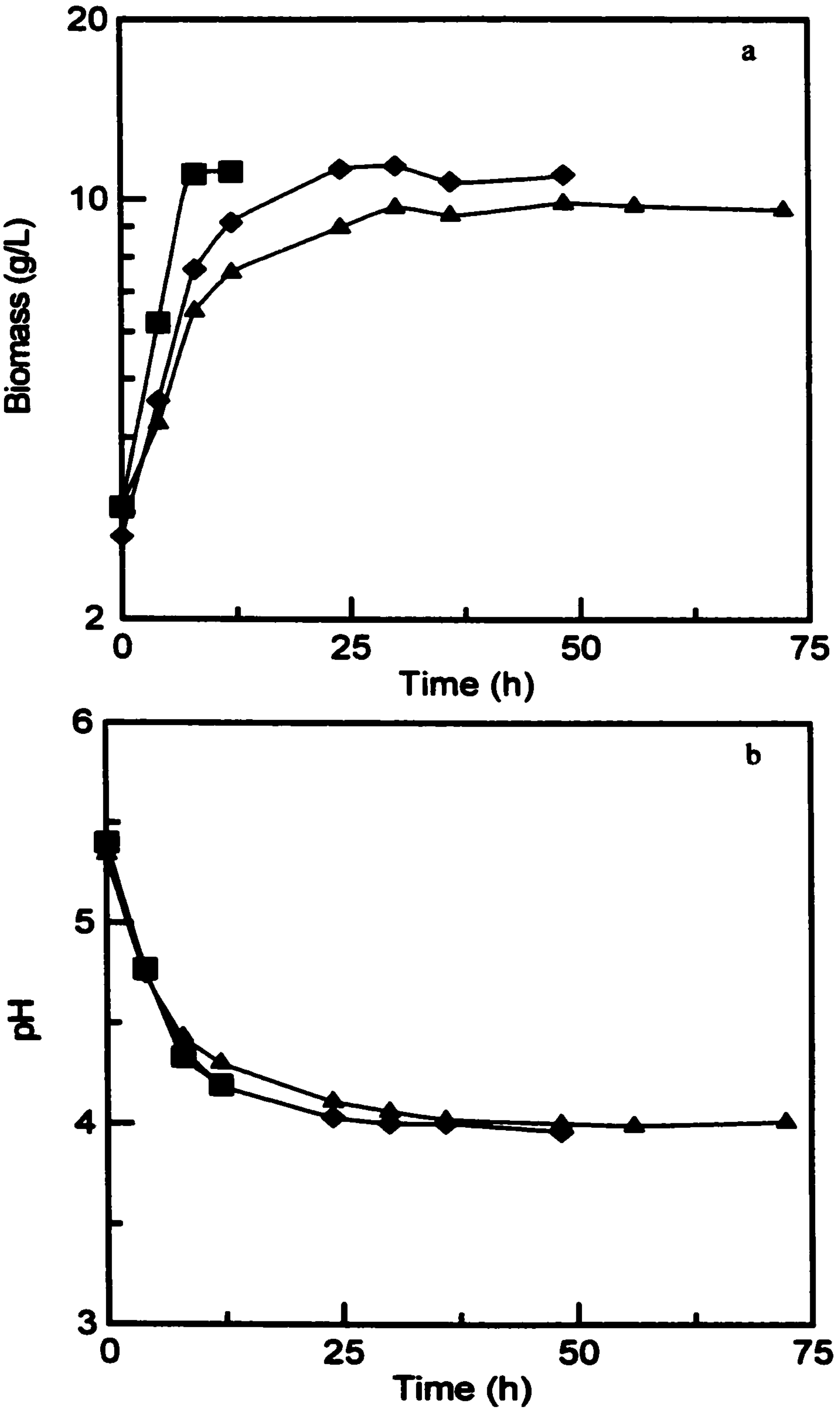

Figure 5.31 Kinetics of a) biomass production by *S. cerevisiae* ATCC 36858 and b) pH changes in un-sterilized media containing 10 g/L yeast extract and various concentrations of sucrose: (■) 95 g/L; (◆) 282 g/L; (▲) 378 g/L.

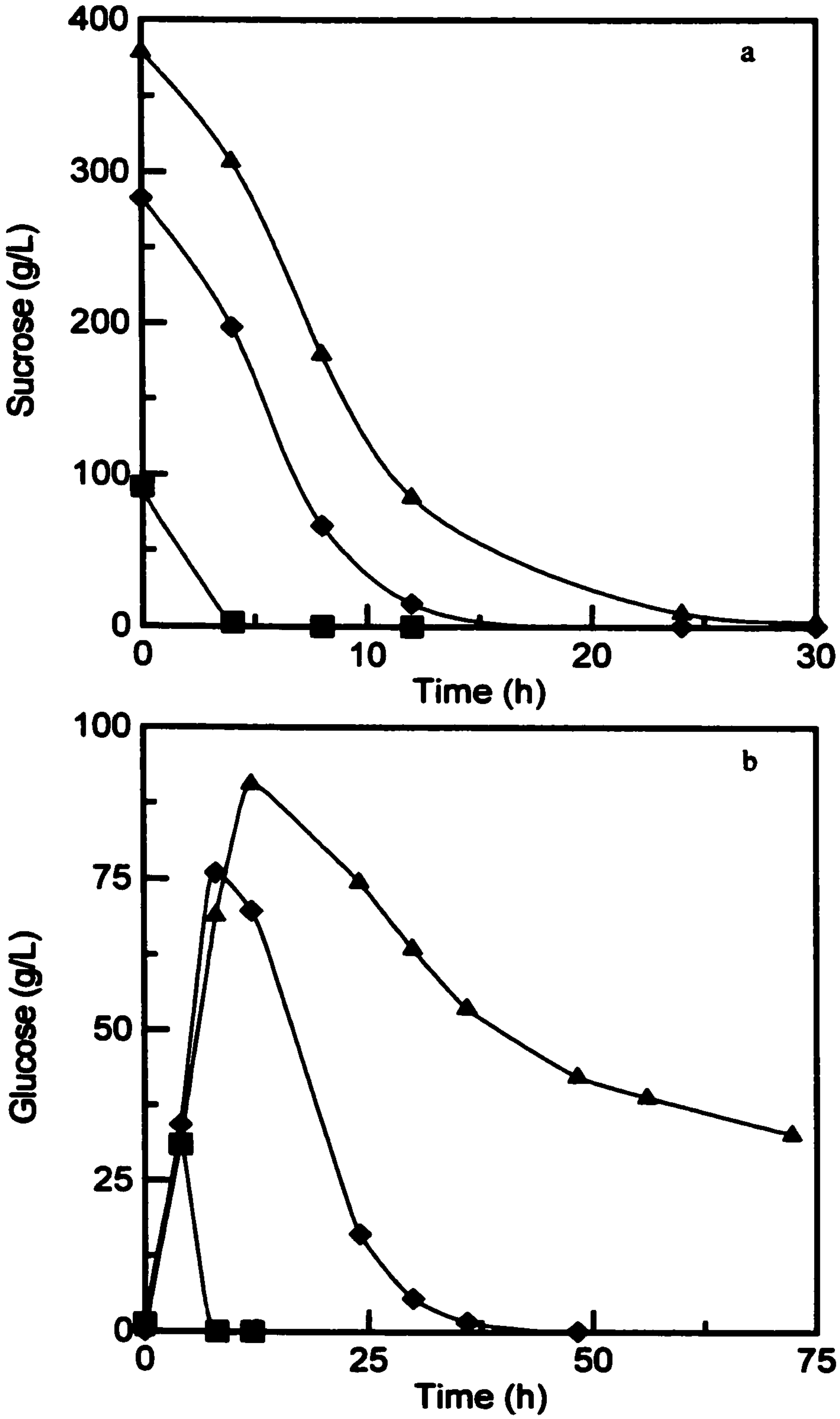

Figure 5.32 Kinetics of a) sucrose hydrolysis and b) glucose production and consumption by *S. cerevisiae* ATCC 36858 in un-sterilized media containing 10 g/L yeast extract and various concentrations of sucrose: (■) 95 g/L; (◆) 282 g/L; (▲) 378 g/L.

slower than the hydrolysis rate of sucrose (Figure 5.32b). However, glucose was not completely consumed by the end of fermentation in the un-sterilized medium with a sucrose concentration of 378 g/L. This is similar to the results discussed in section 5.2.2.2 in which glucose was not completely consumed in sterilized media with sucrose concentrations higher than 360 g/L (Figure 5.17b). However, the glucose consumption rates in the sterilized media were higher due to the higher initial biomass used.

Table 5.14 Fructose, ethanol and biomass yields, specific growth rates and specific initial rates of sucrose hydrolysis and ethanol productivities for sterilized and un-sterilized media with various sucrose and yeast extract concentrations using *S. cerevisiae* ATCC 36858.

Sucrose (g/L)	Yeast extract (g/L)	Initial biomass (g/L)	Specific growth rate, μ (h^{-1})	Biomass yield[a] (g/g)	Ethanol productivity[b] (g/L h)	Specific initial rate of sucrose hydrolysis (g/g h)	Fructose yield[b] (%)	Ethanol yield[b] (%)
94.5	10.0[c]	3.06	0.160	0.149	2.73	7.37	91.8	80.1
282.2	10.0[c]	2.74	0.128	0.060	2.35	7.70	99.5	85.1
378.3	10.0[c]	3.08	0.093	0.047	1.93	5.66	98.8	78.6
319.2	3.0[c]	3.53	0.125	0.046	2.26	7.80	100	78.2
328.9	3.0	3.69	0.123	0.044	2.28	7.09	98.4	76.8
326.2	5.0[c]	3.44	0.145	0.048	2.49	7.17	98.8	77.2
334.1	5.0	3.52	0.147	0.046	2.49	6.81	97.7	76.4
333.2	10.0[c]	3.67	0.157	0.057	2.62	5.70	98.7	76.8
330.7	10.0	3.67	0.154	0.054	2.66	4.72	97.2	78.5
315.7	10.0[c]	7.17	0.091	0.052	3.10	5.25	96.4	77.9
313.5	10.0	7.30	0.093	0.053	3.17	4.61	98.1	82.6

[a] Values are calculated at maximum biomass concentrations
[b] Values are calculated at maximum ethanol and fructose concentrations
[c] Medium is not sterilized

The kinetics of fructose and ethanol production in un-sterilized media containing various sucrose concentrations and 10 g/L yeast extract are shown in Figure 5.33. A maximum fructose concentration of 208 g/L was attained in the medium with a sucrose concentration of 378 g/L (Figure 5.33a). In the same medium, the concentration of ethanol was 57 g/L, which was slightly lower than that obtained with a sucrose concentration of 282 g/L (Figure 5.33b). The incomplete glucose consumption by the yeast in the medium with a sucrose concentration of 378 g/L resulted in a lower ethanol production (Figure 5.32b). Fructose and ethanol yields were above 92 and 79% of the theoretical values, respectively (Table 5.14). Similar fructose and ethanol yields were obtained in sterilized media with sucrose concentrations below 451 g/L (Tables 5.4 and 5.6).

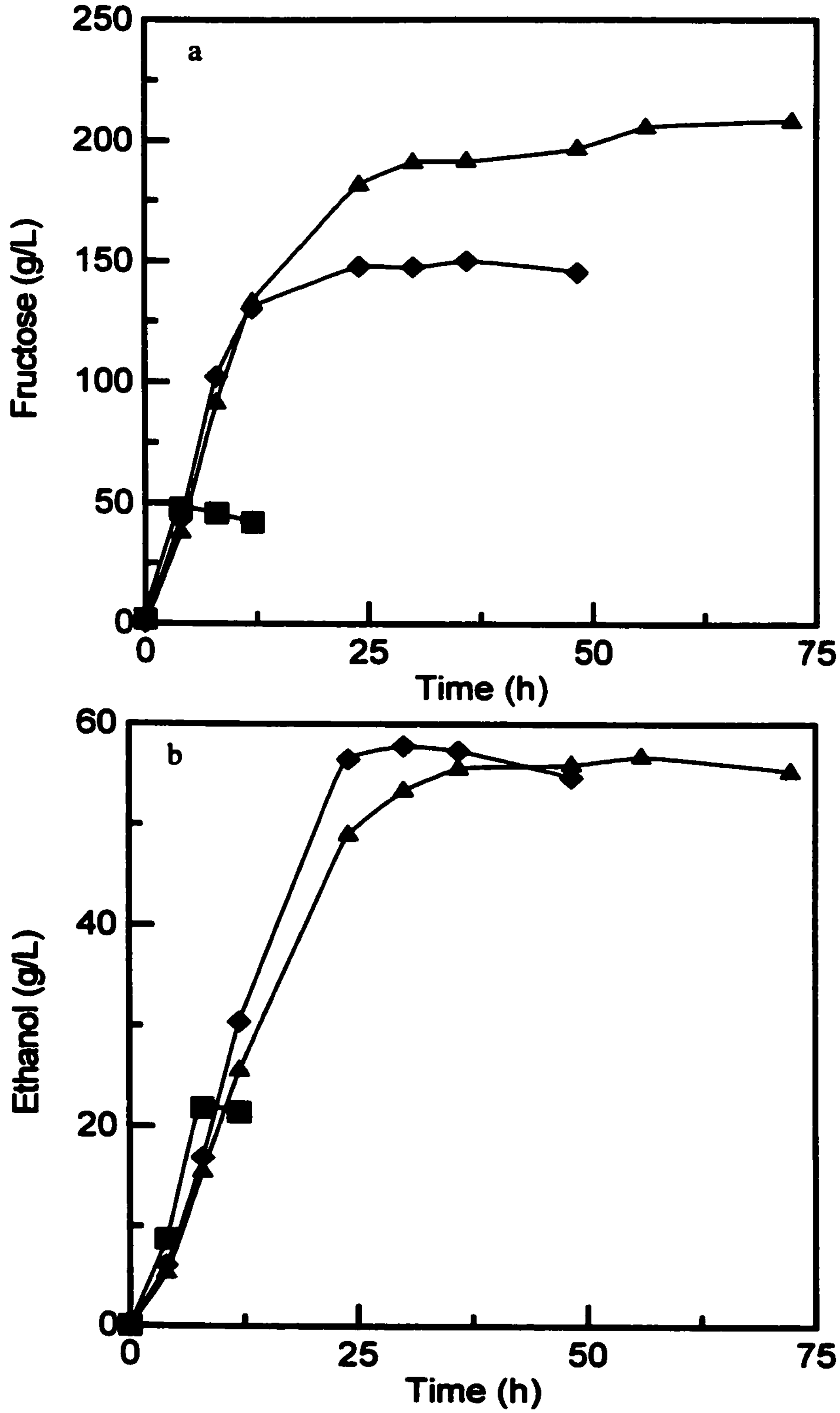

Figure 5.33 Kinetics of a) fructose and b) ethanol production by *S. cerevisiae* ATCC 36858 in un-sterilized media containing 10 g/L yeast extract and various concentrations of sucrose: (■) 95 g/L; (◆) 282 g/L; (▲) 378 g/L.

The results of the fermentation process in sterilized and un-sterilized media containing about 325 g/L sucrose and various initial yeast extract and biomass concentrations are shown in Figures 5.34 to 5.36. The kinetics of biomass production and pH changes in sterile and non-sterile media with the same concentrations of yeast extract and biomass were similar (Figure 5.34). Production of biomass increased with increasing yeast extract concentration in both sterile and non-sterile media (Figure 5.34a). In addition, there were no significant differences in the specific growth rates and biomass yields when the fermentation was carried out in either sterile or non-sterile media with similar concentrations of yeast extract and biomass (Table 5.14). The pH of the fermentation media decreased to the same level when two different initial biomass concentrations and 10 g/L yeast extract were used (Figure 5.34b). However, a faster decline of pH in media with higher biomass concentrations was noticed due to higher fermentation activity in these media.

The kinetics of sucrose hydrolysis and glucose production and consumption by the yeast were also similar in the sterile and non-sterile media with the same yeast extract and biomass concentrations (Figure 5.35). A complete hydrolysis of sucrose by the end of fermentation was noticed in all media (Figure 5.35a). The specific initial rate of sucrose hydrolysis decreased by about 30% when the initial yeast extract concentration was increased from 3.0 to 10.0 g/L in either the sterile or the non-sterile media (Table 5.14). Slightly lower specific initial rates of sucrose hydrolysis were obtained in sterile media. The uptake of glucose by the yeast at the beginning of fermentation was slower than the hydrolysis rate of sucrose (Figure 5.35b). Glucose was completely consumed by the end of the fermentation in the media with a yeast extract concentration of 10 g/L. Some glucose remained in the media with yeast extract concentrations of 5 g/L or lower after 72 h. The incomplete utilization of glucose in these media was due to the low initial yeast extract concentrations used.

Similar kinetics for fructose and ethanol production by the yeast in sterile and non-sterile media with the same yeast extract and biomass concentrations can be seen in Figure 5.36. Slightly higher initial fructose production rates were noticed in the media with high biomass or low yeast extract concentrations, because of higher invertase activities under these conditions (Figure 5.36a). The amounts of ethanol produced during the first 12 h of the process were identical in the media with similar initial biomass concentrations (Figure 5.36b). However, more ethanol was produced in the media with yeast extract concentration above 5.0 g/L as the process proceeded.

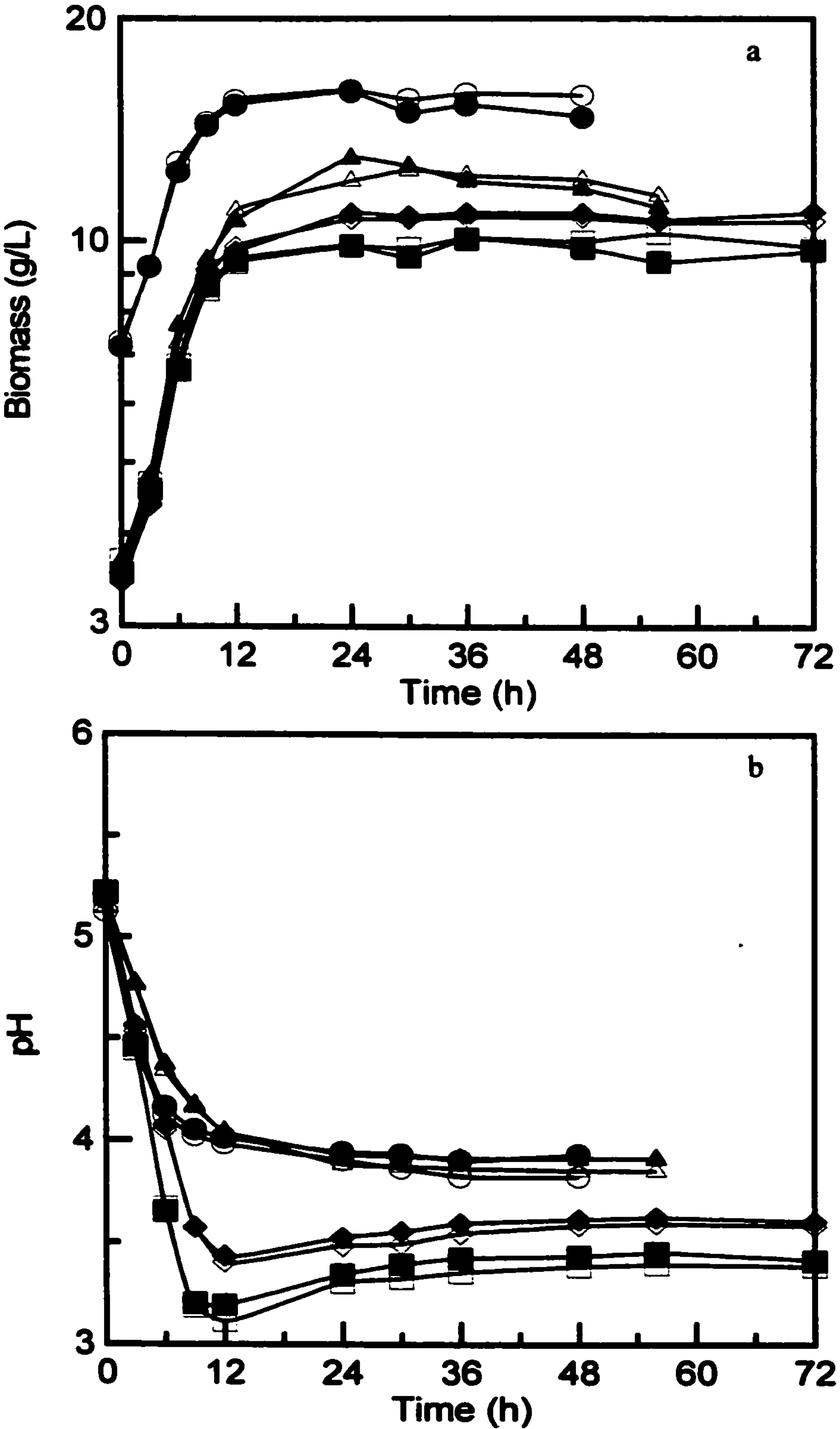

Figure 5.34 Kinetics of a) biomass production by *S. cerevisiae* ATCC 36858 and b) pH changes in media containing about 325 g/L sucrose and 3.6 g/L biomass and various concentrations of yeast extract: (■,□) 3.0 g/L; (◆,◇) 5.0 g/L; (▲,△) 10.0 g/L; (●,○) 10.0 g/L yeast extract and about 7.2 g/L biomass; solid and opened symbols are for un-sterilized and sterilized media, respectively.

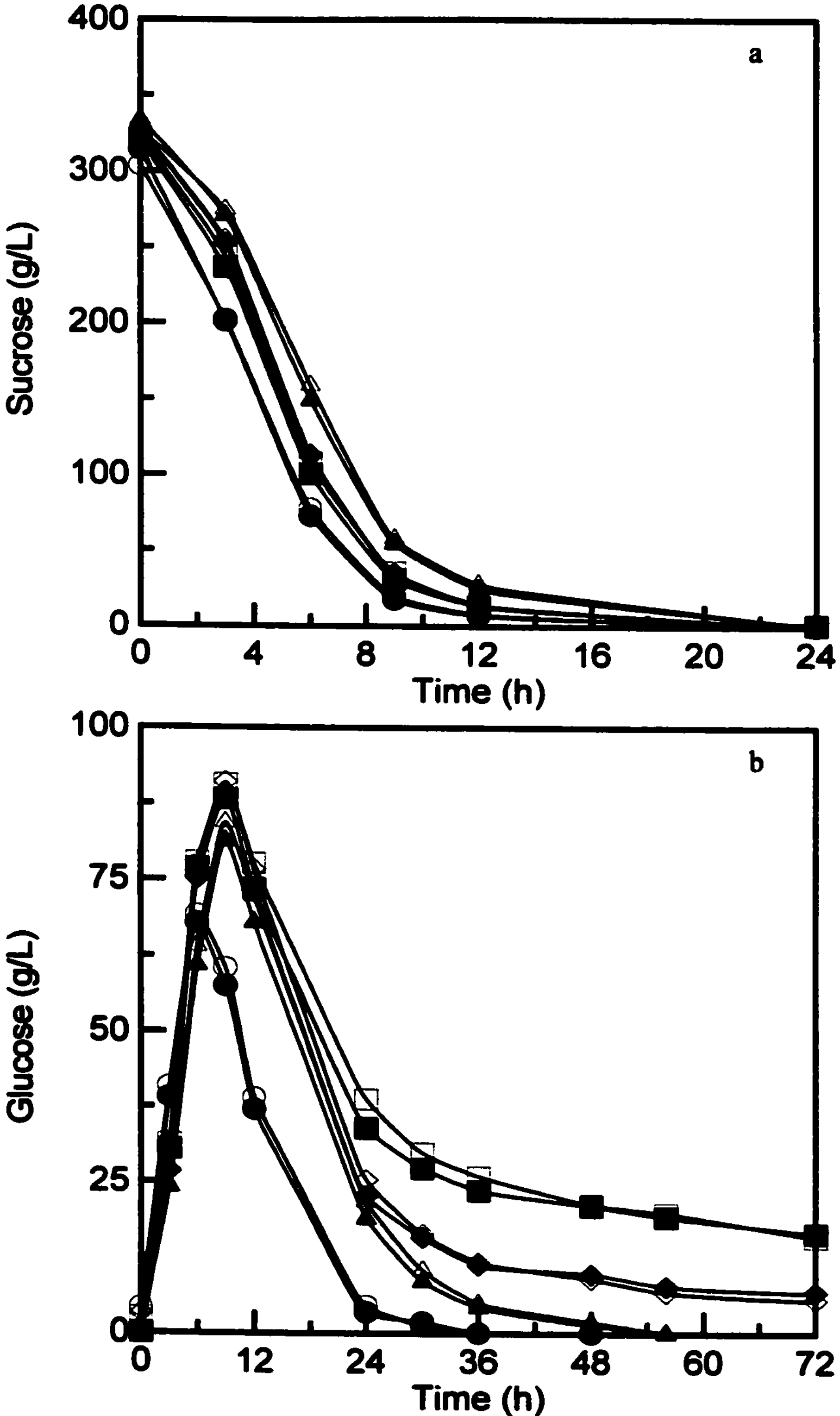

Figure 5.35 Kinetics of a) sucrose hydrolysis and b) glucose production and consumption by *S. cerevisiae* ATCC 36858 in media containing about 325 g/L sucrose and 3.6 g/L biomass and various concentrations of yeast extract: (■,□) 3.0 g/L; (◆,◇) 5.0 g/L; (▲,△) 10.0 g/L; (●,○) 10.0 g/L yeast extract and about 7.2 g/L biomass; solid and opened symbols are for un-sterilized and sterilized media, respectively.

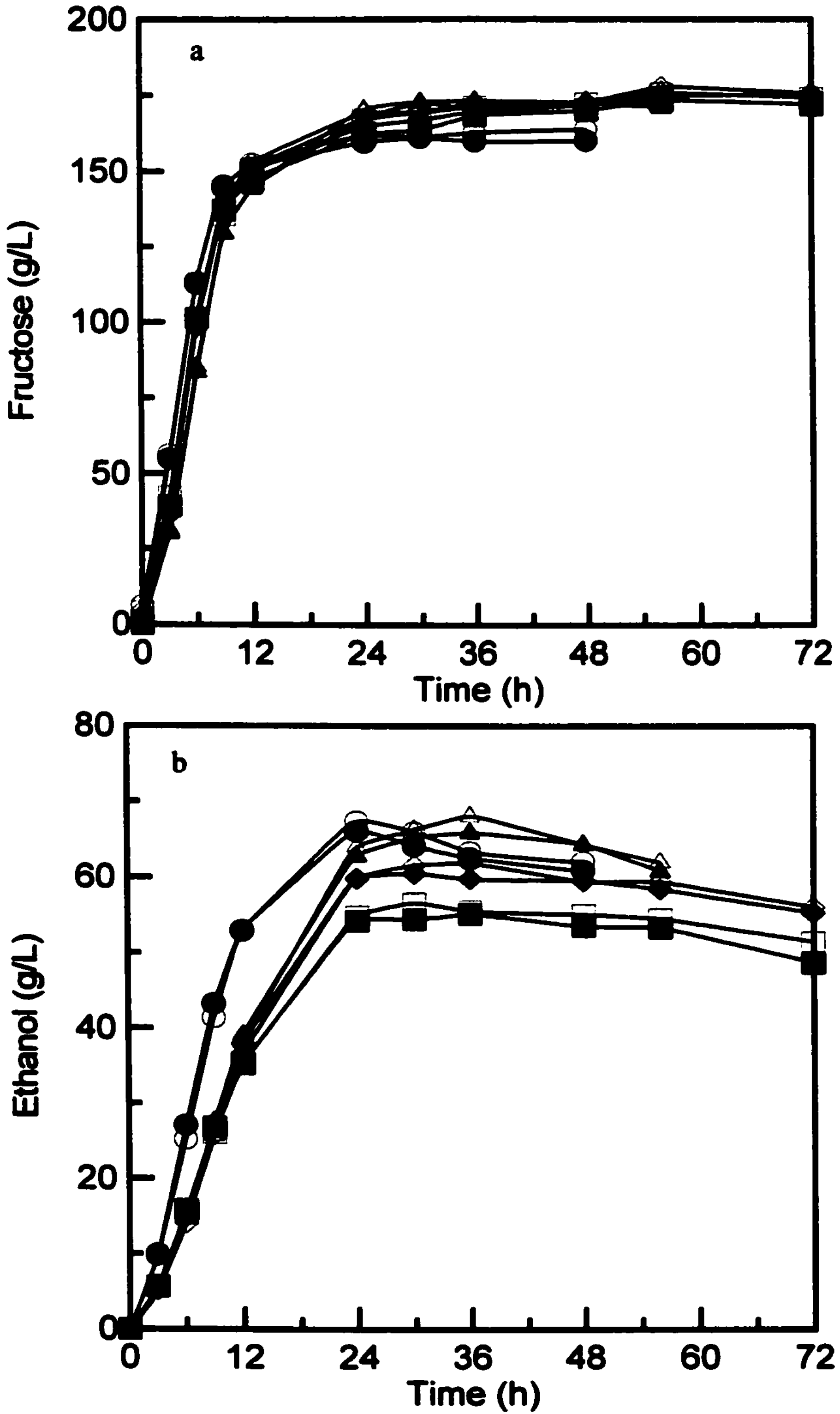

Figure 5.36 Kinetics of a) fructose and b) ethanol production by *S. cerevisiae* ATCC 36858 in media containing about 325 g/L sucrose and 3.6 g/L biomass and various concentrations of yeast extract: (■,□) 3.0 g/L; (◆,◇) 5.0 g/L; (▲,△) 10.0 g/L; (●,○) 10.0 g/L yeast extract and about 7.2 g/L biomass; solid and opened symbols are for un-sterilized and sterilized media, respectively.

This was due to the complete glucose consumption by the yeast in these media (Figure 5.35b). The yields of fructose and ethanol in media with about 325 g/L sucrose and various initial yeast extract and biomass concentrations were above 96 and 76% of the theoretical values, respectively (Table 5.14). These yields were not significantly different in media with and without sterilization or by increasing either initial yeast extract or biomass concentrations.

Fructo-oligosaccharides and glycerol were also produced in the sterilized and un-sterilized media (Table 5.15). Media sterilization did not have a significant effect on the amount of glycerol and fructo-oligosaccharides produced during the fermentation process. In addition, the amounts of these byproducts in un-sterilized media with various sucrose concentrations were similar to those produced in sterilized media (Tables 5.5 and 5.7). The fructo-oligosaccharides, which were produced during the initial phase of the fermentation, were entirely hydrolyzed by the end of the process in all tested media except at a sucrose concentration of 378 g/L (Table 5.15). Glycerol was steadily produced during the process and accumulated in all the media tested. Syrups with a carbohydrate content of 100% fructose were produced from the media with yeast extract concentrations above 5.0 g/L and sucrose concentrations below 282 g/L. However, an increase in sucrose concentration to 378 g/L resulted in a considerable decrease of the fructose

Table 5.15 Maximum and residual fructo-oligosaccharides, final glycerol concentrations in the fermentation broth and fructose contents in the produced syrups using *S. cerevisiae* ATCC 36858 in sterilized and un-sterilized media with various sucrose and yeast extract concentrations.

Sucrose (g/L)	Yeast extract (g/L)	Initial biomass (g/L)	Maximum fructo-oligosaccharides (g/L)	Residual fructo-oligosaccharides (g/L)	Glycerol (g/L)	Fructose contents in syrups produced[b] %
94.5	10.0[a]	3.06	1.3	0.0	7.0	100
282.2	10.0[a]	2.74	10.6	0.0	6.0	100
378.3	10.0[a]	3.08	15.2	1.8	9.6	86
319.2	3.0[a]	3.53	10.3	0.0	7.1	91
328.9	3.0	3.69	10.3	0.0	7.4	92
326.2	5.0[a]	3.44	10.7	0.0	7.4	96
334.1	5.0	3.52	10.4	0.0	7.8	97
333.2	10.0[a]	3.67	11.7	0.0	7.6	100
330.7	10.0	3.67	11.5	0.0	7.9	100
315.7	10.0[a]	7.17	11.2	0.0	6.9	100
313.5	10.0	7.30	11.2	0.0	7.2	100

[a] Medium is not sterilized

[b] Values are calculated based on the final total sugar concentrations

fraction in the syrup due to an incomplete uptake of glucose. This is similar to what was noticed in sterilized media with various sucrose concentrations (Table 5.7).

The results showed that media sterilization did not affect the kinetics of the fermentation process or the product yields. A complete hydrolysis of sucrose was noticed in all tested media. Fructose and ethanol yields were above 92 and 76%, respectively. Syrups containing more than 91% fructose based on the final total sugar concentrations can be produced from media with yeast extract concentrations of 3.0 g/L or higher and sucrose concentrations below 378 g/L. Non-sterile media can be used for the production of fructose and ethanol by *S. cerevisiae* ATCC 36858. This can result in a significant reduction in the production costs associated with energy and time requirements for the sterilization process.

Considering that raffinose is found in molasses in the amount of about 2-5% based on total sugars and since most strains of *S. cerevisiae* cannot utilize the melibiose part of raffinose, the capability of *S. cerevisiae* ATCC 36858 to produce fructose and ethanol from synthetic raffinose media is discussed in the next section.

5.2.3 Production of fructose and ethanol from synthetic raffinose media

Most strains of *S. cerevisiae* can utilize the fructose moiety of raffinose by hydrolyzing it with invertase, however, they do not have melibiase which is required to degrade the remaining melibiose part (Gasent-Ramirez et al., 1995). The yeast *S. cerevisiae* utilizes a variety of sugars such as glucose, fructose and galactose as carbon and energy sources. Yeast can also obtain glucose, fructose and galactose from di- and trisaccharides such as sucrose, melibiose and raffinose. After sucrose, raffinose is the most abundant sugar found in nature. It is widely found in plants (soy, corn) and in molasses. The di- and trisaccharides are cleaved into their monosaccharide components by specific glycosidases. Sucrose is hydrolyzed by β-fructosidase (invertase) and melibiose by α-galactosidase (melibiase). Raffinose is hydrolyzed by a combination of glycosidases. The glucose-fructose bond is cleaved by invertase, and the galactose-glucose bond is cleaved by melibiase (Johnston and Carlson, 1992). The latter enzyme is important in many applications such as the hydrolysis of raffinose, which inhibits the crystallization of beet sugars (Kobayashi and Suzuki, 1972). In addition, it is also used for the hydrolysis of raffinose and stachyose present in various soya products (Khare et al., 1994; Rehms and Barz, 1995) and chickpea flour (Mulimani and Ramalingam, 1997), since these sugars can cause intestinal discomfort and flatulence (Cristofaro et al., 1974).

Various strains of *S. cerevisiae* differ in their ability to utilize carbohydrates due to their extensive genotype variability. The synthesis of yeast β-fructosidase is encoded by the SUC genes, while MEL genes encode α-galactosidase production. Yeast strains commonly used in bread or fuel ethanol production are MEL$^-$ (i.e. unable to completely metabolize raffinose) (Liljestrom-Suominen et al., 1988). MEL$^+$ yeast strains have many advantages when molasses is used as a raw material, such as increased biomass and ethanol yields and a lower biochemical oxygen demand of the factory effluent. These facts were taken into consideration for the development of recombinant strains of baker's yeasts that produce α-galactosidase (Liljestrom-Suominen et al., 1988; Casey et al., 1988; Gasent-Ramirez et al., 1995; Vincent et al., 1999). Recombinant bacteria strains that ferment raffinose, such as *Zymomonas mobilis* (Yanase et al., 1990), *Escherichia coli*, *Klebsiella oxytoca* and *Erwinia chrysanthemi* (Moniruzzaman et al., 1997) were developed for ethanol production. However, it was reported that raffinose was not completely utilized and low ethanol yields were obtained with these microorganisms.

In the previous sections, the use of *S. cerevisiae* ATCC 36858 to selectively ferment glucose to ethanol from synthetic media with sucrose for enriched fructose syrups production was discussed. In this section, the ability of this mutant to utilize raffinose and melibiose from synthetic media is discussed. The fermentation media used in these tests contained 10 g/L yeast extract.

The kinetics of a typical fermentation of raffinose by *S. cerevisiae* ATCC 36858 is displayed in Figure 5.37. The results show the ability of this mutant to hydrolyze raffinose to fructose and melibiose by invertase (Atiyeh and Duvnjak, accepted for publishing). Melibiose was then hydrolyzed by melibiase to glucose and galactose. Glucose and galactose were then selectively converted to ethanol and biomass, while fructose accumulated in the medium. It was noticed that glucose consumption was slightly faster than the utilization of galactose. In the beginning of the process melibiose started to accumulate since its production was faster than its consumption, then a decrease in the concentration of this carbohydrate followed. However, when the concentration of biomass increased to 4.7 g/L, 8 h after the beginning of the process, the hydrolysis rate of raffinose increased, which resulted in a significant increase of the concentration of melibiose in the medium. Neither raffinose nor melibiose were detected in the medium after 24 h. The biomass increased from 1.2 to 11.4 g/L, which represents a biomass yield of 0.173 g/g of the monosaccharides consumed (Table 5.16). Fructose and ethanol yields were 85 and 74% of the theoretical values, respectively. The calculation of the ethanol yield was based on the monosaccharides consumed in the fermentation process. It was also noticed that some glycerol and stachyose were produced but, by the end of the process, the latter compound was entirely consumed.

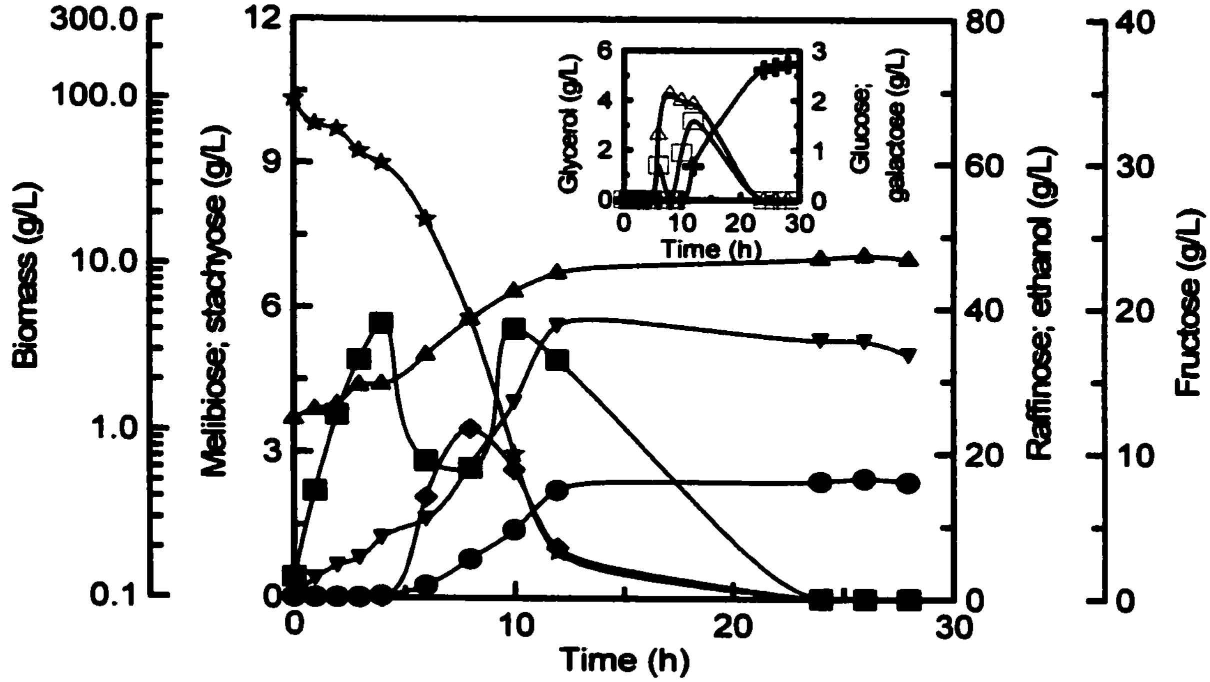

Figure 5.37 Kinetics of growth of *S. cerevisiae* ATCC 36858 and fructose and ethanol production in a medium containing 70 g/L raffinose: (▲) biomass; (■) melibiose; (◆) stachyose; (★) raffinose; (●) ethanol; (▼) fructose; (✚) glycerol; (□) glucose; (Δ) galactose.

The kinetics of the growth and ethanol production by a wild strain of *S. cerevisiae* in a medium with an initial raffinose concentration of 77 g/L is presented in Figure 5.38. An initial raffinose concentration of 27 g/L is shown in the figure because of the instantaneous hydrolysis of raffinose to melibiose and fructose upon inoculation. The wild *S. cerevisiae* hydrolyzed the whole amount of raffinose in less than 4 h to fructose and melibiose. The fructose was consumed, while melibiose accumulated in the fermentation broth. The biomass concentration increased from 1.3 to 6.4 g/L, which represents a biomass yield of 0.137 g/g of the fructose consumed (Table 5.16). An ethanol yield of 85% of the theoretical value was attained. In the process with the wild strain, neither glycerol nor stachyose were produced.

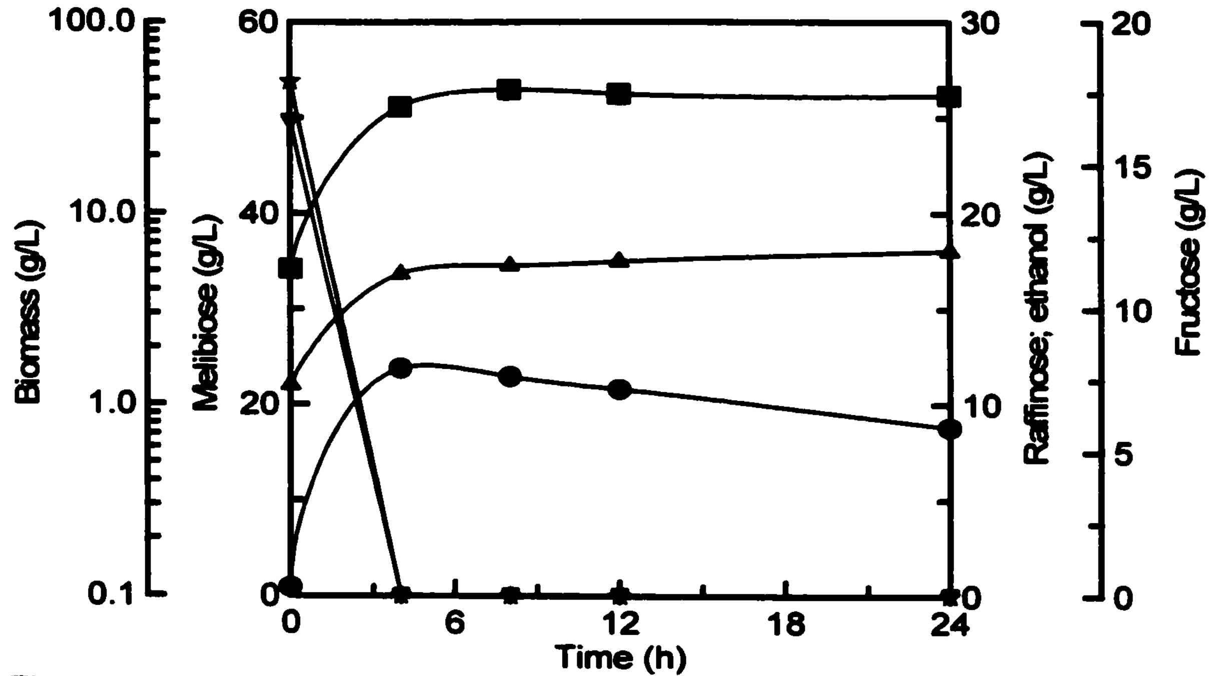

Figure 5.38 Kinetics of growth of wild *S. cerevisiae* and ethanol production in a medium containing 77 g/L raffinose: (▲) biomass; (■) melibiose; (★) raffinose; (●) ethanol; (▼) fructose.

Table 5.16 Fructose, ethanol and biomass yields, specific growth rate and specific initial rate of sugar hydrolysis and ethanol productivity in media with raffinose, melibiose and sucrose using *S. cerevisiae* ATCC 36858.

Initial sugar[a] (g/L)	Initial biomass (g/L)	Specific growth rate, μ (h^{-1})	Biomass yield[b] (g/g)	Ethanol productivity[c] (g/L h)	Specific initial rate of sugar hydrolysis[d] (g/g h)	Fructose yield[c] (%)	Ethanol yield[c] (%)
R- 29.7	1.33	0.166	0.290	0.48	0.78	96.5	66.5
R- 50.2	1.20	0.230	0.214	0.94	1.36	84.4	68.9
R- 69.5	1.16	0.221	0.173	1.24	1.94	85.1	74.3
R- 80.7	1.20	0.209	0.157	1.60	2.14	81.6	73.8
R- 131.5	1.27	0.206	0.117	1.58	2.33	88.8	75.8
R- 189.1	1.37	0.197	0.088	2.04	4.97	90.8	73.0
R- 242.0	1.27	0.184	0.073	1.72	5.51	95.9	70.1
R- 77.3[e]	1.26	0.335	0.137	2.97	15.28	----	84.6
M- 51.8	1.48	0.315	0.202	2.23	3.38	----	82.3
S- 187.9	1.27	0.162	0.083	1.92	23.68	89.2	76.9
S- 157.1[e]	1.55	0.323	0.089	2.66	25.32	----	82.5

[a] R = raffinose, M = melibiose and S = sucrose
[b] Values are calculated at maximum biomass concentration
[c] Values are calculated at maximum ethanol and fructose concentrations
[d] Values are calculated after 4 h of hydrolysis
[e] Wild strain of *S. cerevisiae*

The kinetics of the growth of *S. cerevisiae* ATCC 36858 and ethanol production in a medium with melibiose is shown in Figure 5.39. The melibiose was hydrolyzed by α-galactosidase to glucose and galactose in the first 7 h of the process. Subsequently, these two monosaccharides were converted to ethanol and biomass. Glucose was consumed at a slightly higher rate than galactose. A biomass yield of 0.202 g/g of the glucose and galactose consumed and an ethanol yield of 82% of the theoretical value were obtained in this process (Table 5.16). No glycerol was produced. However, raffinose and stachyose were detected in the beginning of the process and were consumed by the end of fermentation.

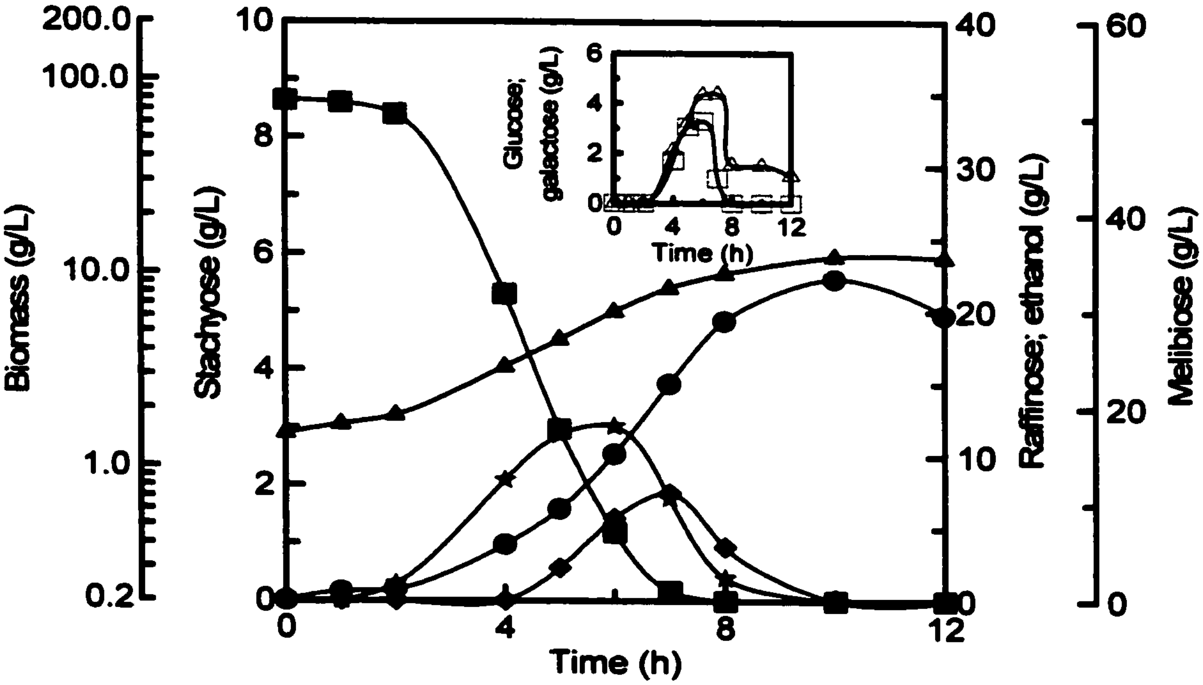

Figure 5.39 Kinetics of growth of *S. cerevisiae* ATCC 36858 and ethanol production in a medium containing 52 g/L melibiose: (▲) biomass; (◆) stachyose; (★) raffinose; (●) ethanol; (■) melibiose; (□) glucose; (Δ) galactose.

The kinetics of growth and ethanol production by a wild strain of *S. cerevisiae* in a medium with an initial sucrose concentration of 157 g/L is presented in Figure 5.40. A sucrose concentration of 80 g/L is shown in the figure because of the instantaneous hydrolysis of sucrose to glucose and fructose upon inoculation. The wild *S. cerevisiae* hydrolyzed all of the sucrose in about 4 h to fructose and glucose. These monosaccharides were consumed for biomass and ethanol production. The biomass increased from 1.6 to 12.7 g/L, which represents a biomass yield of 0.089 g/g of the glucose and fructose consumed (Table 5.16). An ethanol yield of 83% of the theoretical value was attained. In the process with the wild strain, it was noticed that a small amount of fructo-oligosaccharides was produced after inoculation and then rapidly consumed. In addition, some glycerol was produced during this process.

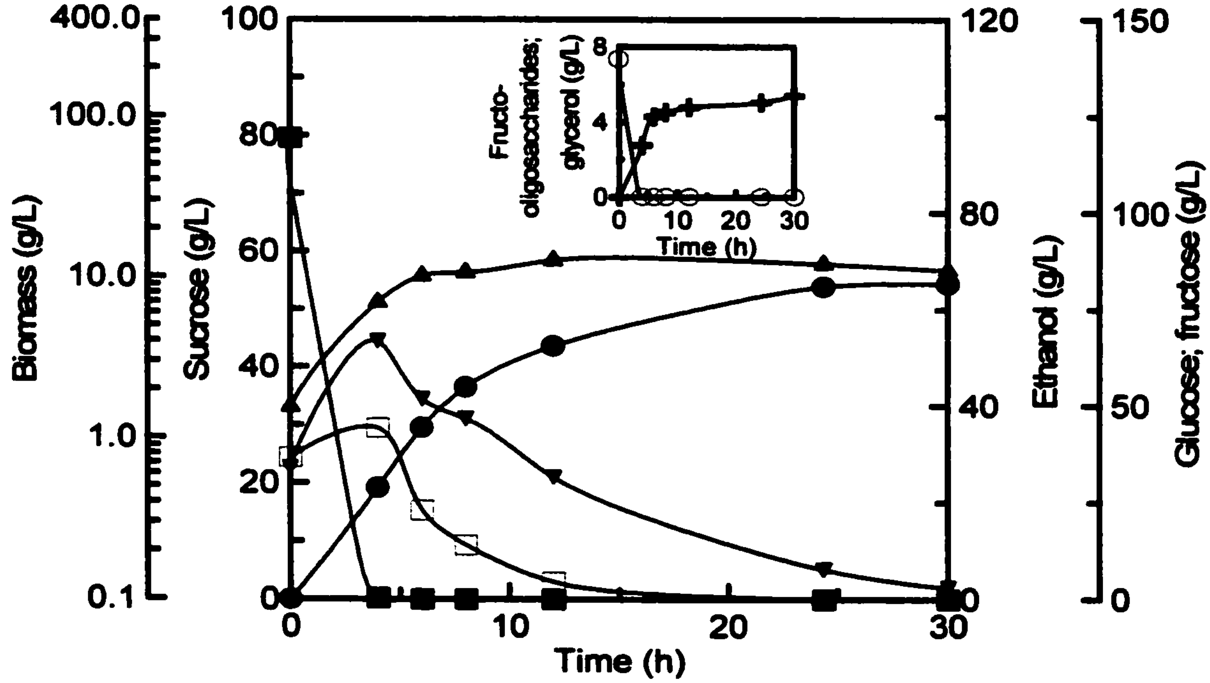

Figure 5.40 Kinetics of growth of wild *S. cerevisiae* and ethanol production in a medium containing 157 g/L sucrose: (▲) biomass; (■) sucrose; (●) ethanol; (□) glucose; (▼) fructose; (○) fructo-oligosaccharides; (✚) glycerol.

The effects of raffinose concentration on the production of fructose and ethanol by *S. cerevisiae* ATCC 36858 are summarized in Table 5.16. The biomass yield and specific growth rate of the yeast decreased when the raffinose concentration was increased from 50 to 242 g/L, while the specific initial rate of raffinose hydrolysis increased by more than four times. The yield of fructose, which accumulated in the media when the raffinose concentration was above 132 g/L, was between 89 and 96% of the theoretical value. The ethanol productivity more than doubled when the raffinose concentration was increased from 50 to 189 g/L, respectively. The ethanol yield was between 69 and 76% of the theoretical value, respectively, in media with the above raffinose concentration range.

The kinetics of raffinose hydrolysis and melibiose production and consumption are displayed in Figure 5.41. Two melibiose peaks are noticed in each raffinose medium. They appeared in the process phase when the production of this disaccharide was faster than its consumption. A maximum fructose concentration of 80 g/L was attained in the medium with raffinose concentration of 242 g/L (Figure 5.42a). In the same medium, the concentration of ethanol was about 51 g/L (Figure 5.42b). Glucose and galactose production and consumption kinetics are shown in Figure 5.43. The glucose and galactose produced were completely consumed by the end of the fermentation process when the initial raffinose concentrations were lower than 189 g/L. However, a certain amount of these sugars remained in the medium when the highest initial raffinose concentration was used. Glucose and galactose were consumed to produce ethanol and biomass. A maximum biomass concentration of 13.1 g/L was attained in the medium with a raffinose concentration of 132 g/L (Figure 5.44).

The glycerol concentration increased from 1.0 to 6.0 g/L when the initial raffinose concentration increased from 30 to 81 g/L, respectively (Table 5.17). When the raffinose concentration was further increased, the glycerol production started to decrease. The production and consumption of different oligosaccharides were noticed in all tested media. Stachyose was produced in the medium that contained raffinose, while fructo-oligosaccharides appeared in the medium with sucrose (Table 5.17). A maximum concentration of stachyose (24.2 g/L) was produced in the medium that contained 242 g/L raffinose. However, a maximum fructo-oligosaccharides concentration of 4.7 g/L was attained in the medium that contained 188 g/L sucrose. These oligosaccharides were produced during the initial phase of fermentation. They were entirely hydrolyzed to their monosaccharide constituents by the end of fermentation.

Syrups the carbohydrate content of which contains more than 96% fructose were produced from media with raffinose concentrations below 189 g/L (Table 5.17). However, a significant decrease in the fructose fraction in the produced syrup was noticed when the concentration of raffinose was 242 g/L. This drop was due to the slow uptake of both glucose and galactose by *S. cerevisiae* ATCC 36858 and their accumulation by the end of the process (Figure 5.43). In addition, syrups the carbohydrate content of which was 100% fructose were produced by *S. cerevisiae* ATCC 36858 from media with sucrose (Table 5.17).

The results demonstrated the ability of *S. cerevisiae* ATCC 36858 to hydrolyze raffinose and melibiose to their monosaccharide constituents and then to selectively convert glucose and galactose to ethanol and biomass, while fructose accumulated in the media. This is different from

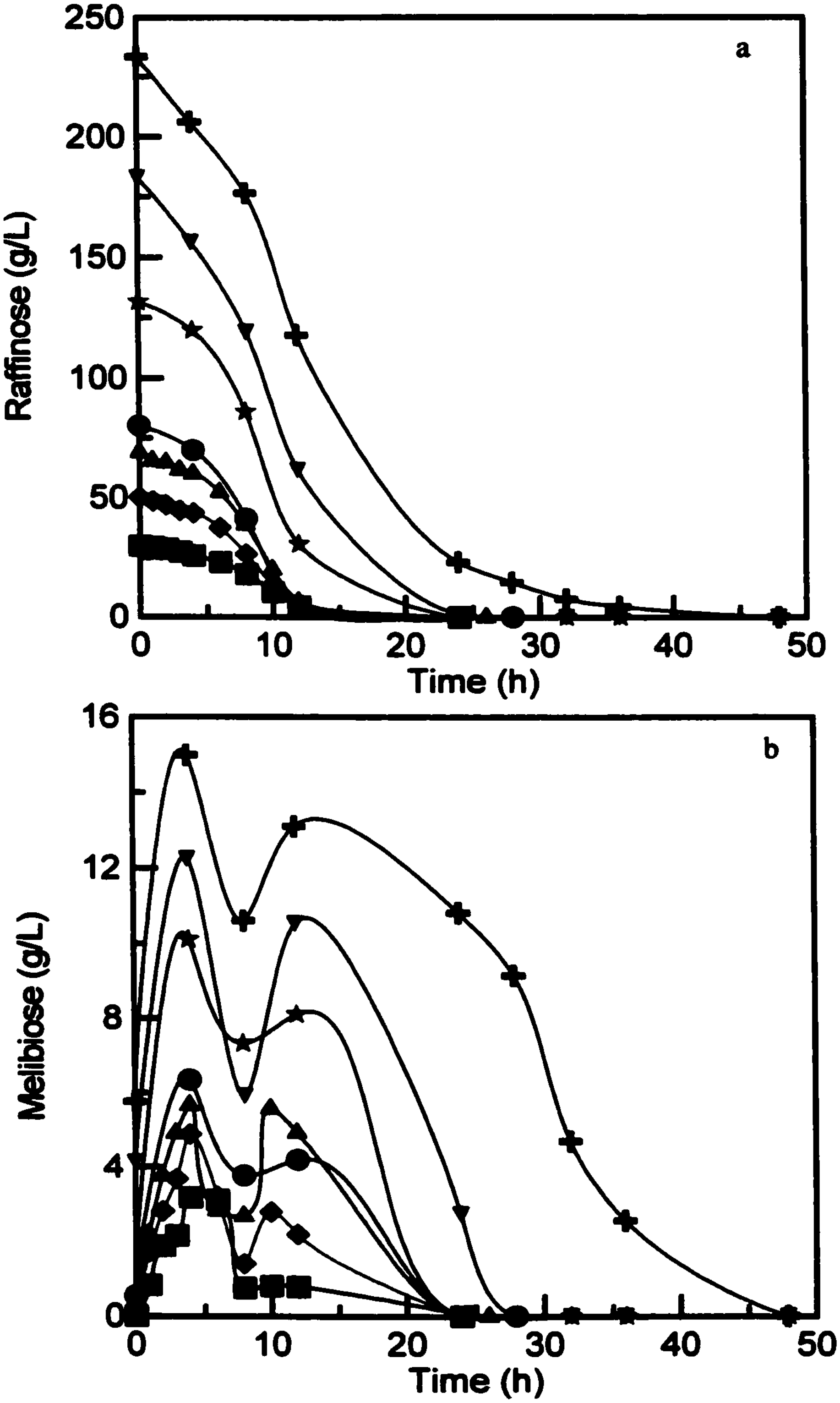

Figure 5.41 Kinetics of (a) raffinose hydrolysis, (b) melibiose production and consumption by *S. cerevisiae* ATCC 36858 in media containing: (■) 30 g/L; (◆) 50 g/L; (▲) 70 g/L; (●) 81 g/L; (★) 132 g/L; (▼) 189 g/L; (✚) 242 g/L raffinose.

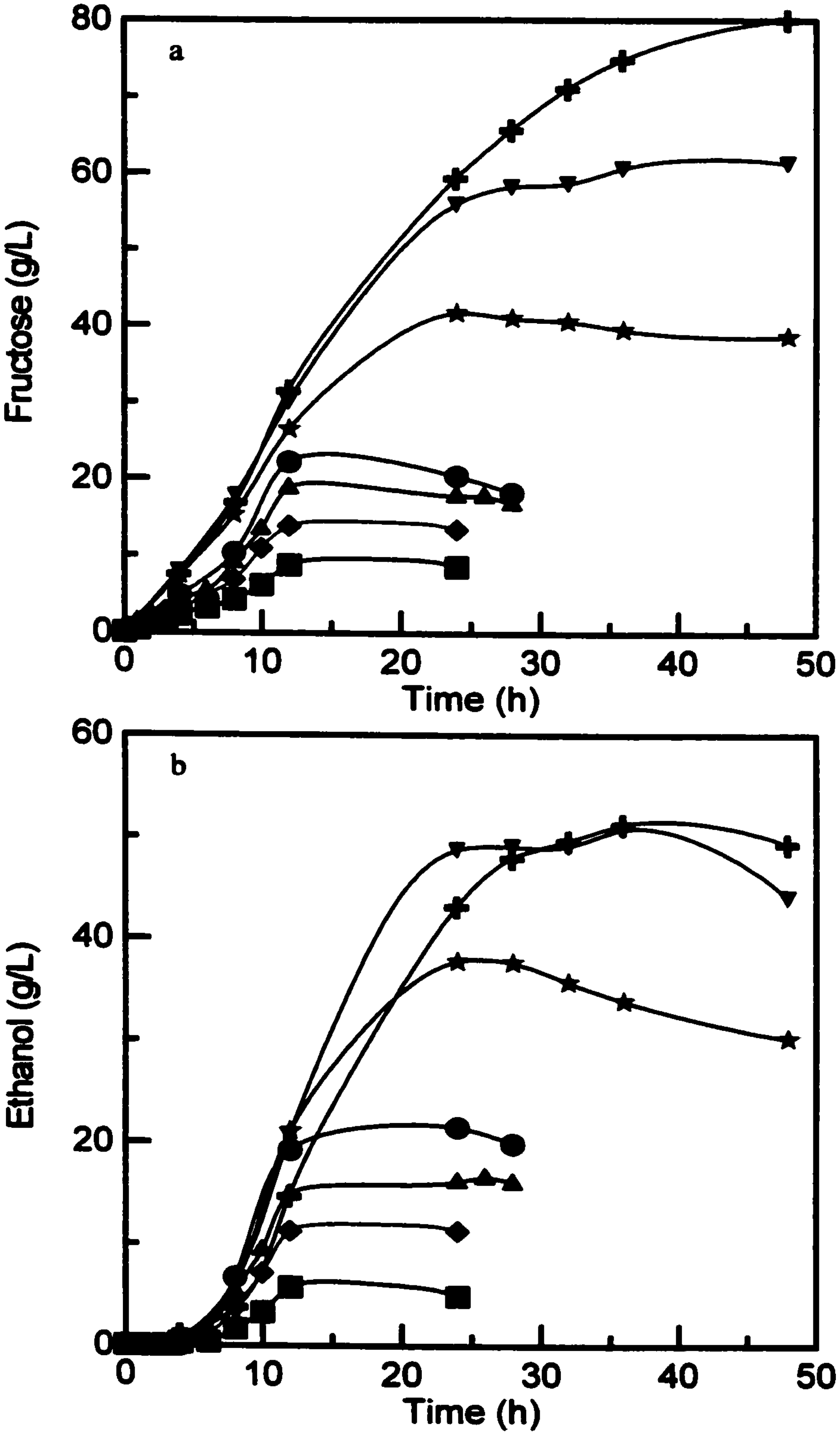

Figure 5.42 Kinetics (a) fructose and (b) ethanol production by *S. cerevisiae* ATCC 36858 in media containing: (■) 30 g/L; (◆) 50 g/L; (▲) 70 g/L; (●) 81 g/L; (★) 132 g/L; (▼) 189 g/L; (✚) 242 g/L raffinose.

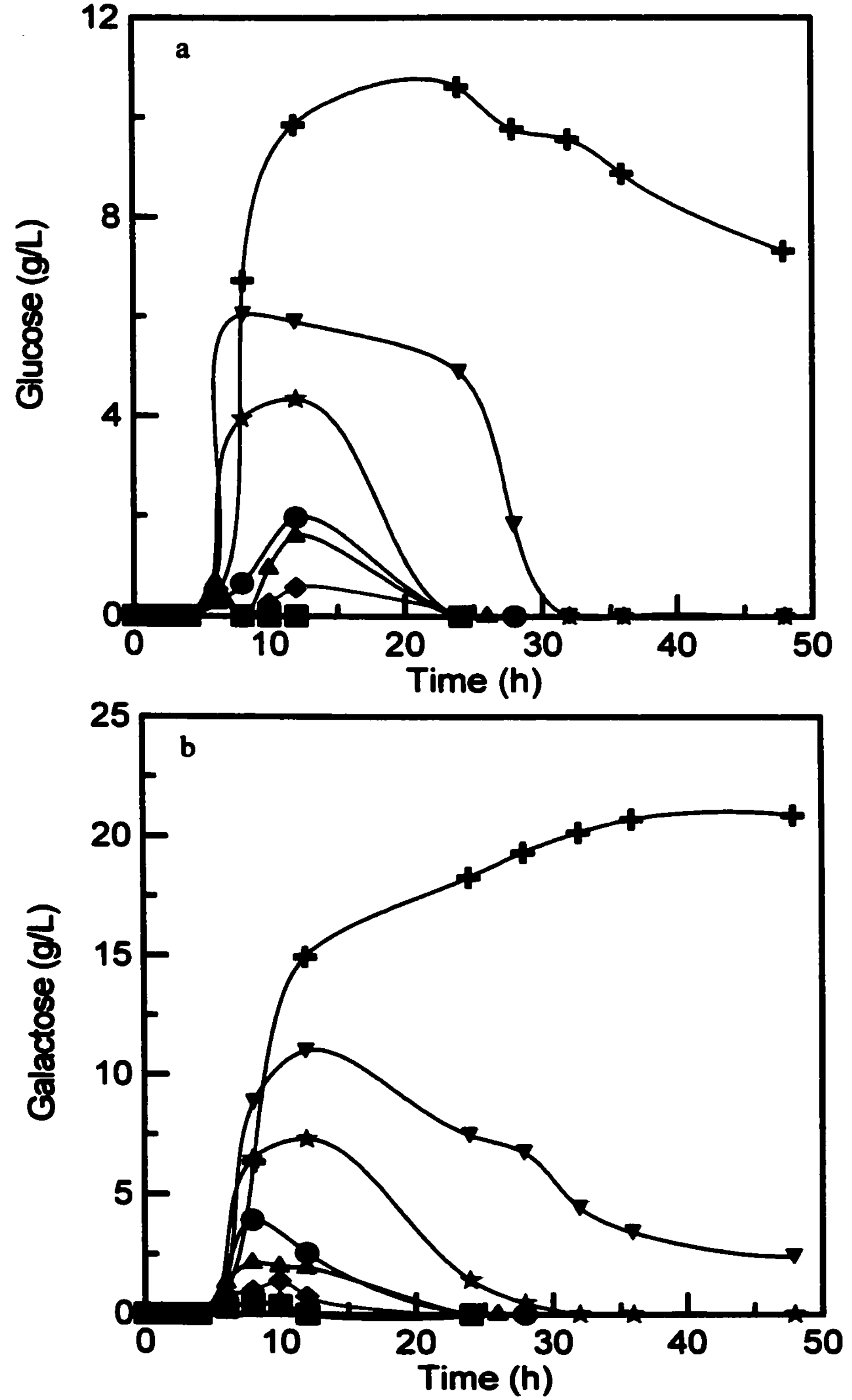

Figure 5.43 Kinetics of (a) glucose and (b) galactose production and consumption by *S. cerevisiae* ATCC 36858 in media containing: (■) 30 g/L; (◆) 50 g/L; (▲) 70 g/L; (●) 81 g/L; (★) 132 g/L; (▼) 189 g/L; (✚) 242 g/L raffinose.

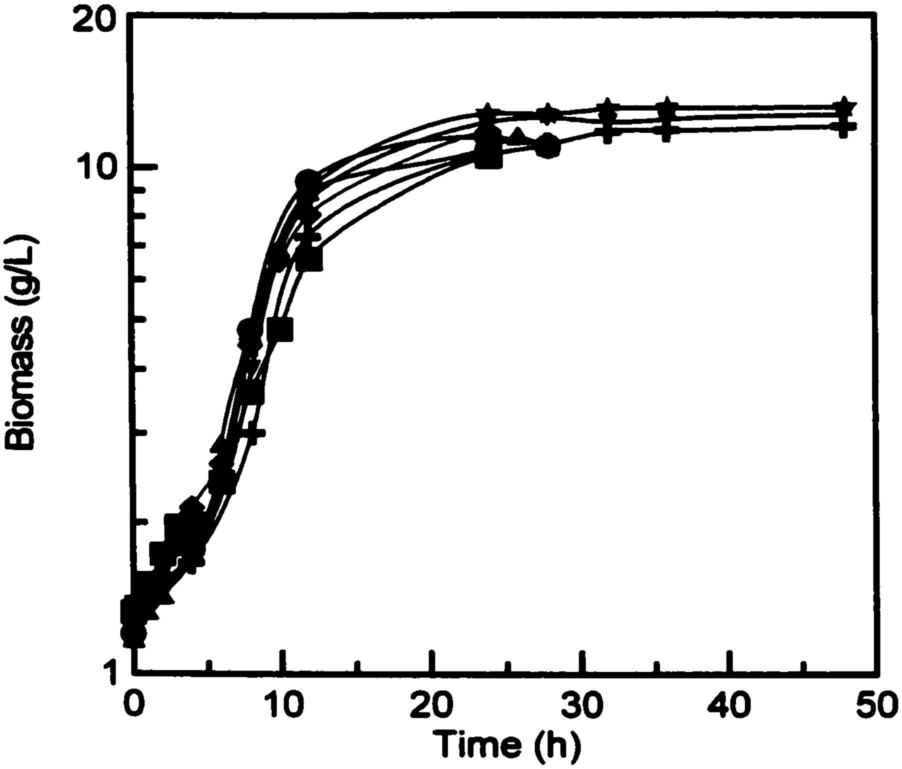

Figure 5.44 Kinetics of biomass production by *S. cerevisiae* ATCC 36858 in media containing: (■) 30 g/L; (◆) 50 g/L; (▲) 70 g/L; (●) 81 g/L; (★) 132 g/L; (▼) 189 g/L; (✚) 242 g/L raffinose.

Table 5.17 Fructose contents in the produced syrups, final glycerol and maximum oligosaccharide concentrations in the fermentation broth from media with various raffinose, melibiose and sucrose concentrations using *S. cerevisiae* ATCC 36858.

Initial sugar[a] (g/L)	Fructose contents in syrups produced[b] %	Glycerol (g/L)	Maximum oligosaccharides produced[a] (g/L)
R- 29.7	100	1.0	St- 0.5
R- 50.2	100	3.3	St- 1.7
R- 69.5	100	5.5	St- 3.5
R- 80.7	100	6.0	St- 3.9
R- 131.5	100	5.8	St- 8.4
R- 189.1	96	4.9	St- 14.1
R- 242.0	74	4.8	St- 24.2
M- 51.8	—	—	R- 12.1; St- 1.9
S- 187.9	100	6.1	FO- 4.7
S- 157.1[c]	—	5.4	FO- 7.3

[a] R = raffinose, M = melibiose, S = sucrose, St = stachyose and FO = fructo-oligosaccharides

[b] Values are calculated based on the final total sugar concentrations

[c] Wild strain of *S. cerevisiae*

the wild strain of *S. cerevisiae* used in this study, which hydrolyzed raffinose to fructose and melibiose but was not able to hydrolyze the latter disaccharide (Figure 5.38). This confirms that the wild strain did not have α-galactosidase, which is required for melibiose hydrolysis, and thus this sugar accumulated in the fermentation broth. Considering that about 65% of the initial raffinose was instantaneously hydrolyzed after inoculation (Figure 5.38), it shows that the invertase activity of the wild strain was very high. However, it was noticed that the invertase activity of *S. cerevisiae* ATCC 36858 was much lower than that of the wild strain since only a negligible amount of raffinose (less than 1%) was hydrolyzed to fructose and melibiose upon addition of the inoculum. Similar results were also noticed in sucrose media. About 50% of the initial sucrose (157 g/L) was immediately hydrolyzed after inoculation with the wild strain (Figure 5.40), while only about 6% of this substrate, the initial concentration of which was 188 g/L, was immediately hydrolyzed upon inoculating with *S. cerevisiae* ATCC 36858. This was also noticed when *S. cerevisiae* ATCC 36858 was used in media with various sucrose concentrations in sections 5.2.2.1 and 5.2.2.2 (Figures 5.13a and 5.17a). Yanase et al. (1990) used a recombinant *Z. mobilis*, in which α-galactosidase and lactose permease genes of *E. coli* were introduced, to ferment raffinose and melibiose. They found that the hydrolysis efficiencies of raffinose and melibiose after 120 h were below 25 and 50% in the media that contained 50 and 20 g/L of these sugars, respectively. In another study, Moniruzzaman et al. (1997) found that the hydrolysis efficiencies of raffinose after 24 h with *E. chrysanthemi*, *K. oxytoca* and *E. coli* were 73, 69 and 43%, respectively, in the media that contained this sugar in a concentration of 90 g/L. However, the raffinose and the melibiose that was produced were completely hydrolyzed by *E. chrysanthemi* and *K. oxytoca* after 144 h from the beginning of the process, while more than 83% of the produced galactose accumulated in the fermentation media. Ethanol yields by these microbes were about 60% of the theoretical values. A higher ethanol yield (75%) was obtained using *E. coli*, however, about 36% of the produced melibiose remained after 144 h from the beginning of the process (Moniruzzaman et al., 1997). In the present study with *S. cerevisiae* ATCC 36858, raffinose hydrolysis efficiency after 24 h in a medium that contained 242 g/L of this sugar was above 90%. Raffinose and melibiose were completely hydrolyzed in the above medium, while about 8% of glucose and 24% of galactose produced accumulated after 48 h from the beginning of the fermentation process (Figures 5.41 and 5.43). No glucose and less than 4%

of the produced galactose were detected in the media when the raffinose concentration was below 189 g/L. Ethanol yields were between 67 and 76% of the theoretical values in all the raffinose media tested (Table 5.16).

In addition to the production of ethanol, *S. cerevisiae* ATCC 36858 produced fructose in the media with raffinose (Figure 5.42). The fructose yield was above 89% of the theoretical value in the media with raffinose in concentrations above 132 g/L (Table 5.16). When the behavior of this yeast in raffinose and sucrose media is compared, in general the specific initial rates of sugar hydrolysis and fructose and ethanol yields were higher in sucrose media (Tables 5.4 and 5.6). Similar biomass yields were noticed in both media. However, the specific growth rates of the yeast were higher in raffinose media.

Some oligosaccharides were produced by *S. cerevisiae* ATCC 36858 in all of the media tested (Table 5.17). In raffinose media, the oligosaccharides concentration increased as a result of increasing initial raffinose concentrations. Stachyose was detected in the media with raffinose, while raffinose and stachyose were detected in the medium with melibiose. Fructo-oligosaccharides were produced in the medium with sucrose. These oligosaccharides were entirely hydrolyzed to their monosaccharide components by the end of the fermentation process. It was reported that the biosynthesis of raffinose and stachyose starts with the reaction catalyzed by galactinol synthetase producing galactinol from uridine diphosphate galactose (UDP-galactose) and myo-inositol. Subsequently, galactinol is used to add galactosyl residues to sucrose, raffinose and stachyose to form the corresponding higher oligosaccharides by specific transferases (Lehle and Tanner, 1972).

Glycerol is another product that was produced in low concentrations by *S. cerevisiae* ATCC 36858 (Table 5.17). Its concentration increased with increasing raffinose concentration in the media up to 132 g/L. A slight decrease in the amount of glycerol produced was noticed with further increases in raffinose concentration due to the accumulation of some glucose and/or galactose in those media (Table 5.17 and Figure 5.43). The amount of glycerol produced by the yeast in raffinose media was slightly lower than that produced in sucrose media.

The results showed the ability of *S. cerevisiae* ATCC 36858 to hydrolyze, in addition to sucrose, raffinose and melibiose and then to selectively ferment glucose and galactose to ethanol. A high fructose yield (above 89%) was obtained in the media with concentrations of either raffinose or sucrose above 132 g/L. In addition, ethanol yields were between 77 and 82% in the media with either melibiose or sucrose. Lower ethanol yields were obtained in media with raffinose. Syrups the carbohydrate content of which contains more than 96% fructose can be produced from the media with raffinose concentrations below 189 g/L. The use of *S. cerevisiae* ATCC 36858 in molasses media for high fructose syrups production could be useful since this would increase overall fructose, ethanol and biomass yields in the fermentation process.

5.2.4 Production of fructose and ethanol from sugar cane and sugar beet molasses media

Sucrose and raffinose are constituents of sugar cane and sugar beet molasses, a non-crystallizable residue after most of sucrose has been crystallized from the cane and beet juices. In addition to growth-promoting components and inhibitors, molasses contains sucrose, glucose, fructose and raffinose at a total carbohydrate concentration of 50-60% (w/v), of which more than 60% is sucrose (Jones et al., 1981; Cejka, 1985; Gough et al., 1996). Molasses has been used as animal feed as well as in baking yeast and ethanol production (Chen, 1993b). In the present study, the ability of *S. cerevisiae* ATCC 36858 to produce fructose and ethanol from sugar cane and sugar beet molasses is examined. Sugar cane and sugar beet molasses were used as complex media for the production of fructose and ethanol since they contain high concentrations of sucrose, minerals and other nutrients that support growth of yeast. The sugar beet molasses contains double the amount of raffinose and about 20% more sucrose than the sugar cane molasses. However, less fructose and glucose are present in the sugar beet molasses. The total sugar concentration in either the sugar cane or sugar beet molasses was about 694 g/L.

The kinetics of growth of *S. cerevisiae* ATCC 36858 and fructose and ethanol production in sugar cane and sugar beet molasses media containing 236 and 242 g/L total sugar, respectively, are displayed in Figures 5.45 and 5.46. In the cane molasses medium, the sugar was composed of 72.3% sucrose, 11.3% glucose, 14.1% fructose and 2.3% raffinose. The sugar composition in the beet molasses medium was 91.9% sucrose, 1.1% glucose, 2.3% fructose and 4.7% raffinose. As it was found in the previous tests with synthetic sucrose media in section 5.2.2, the pattern of the fermentation process was the same. The yeast hydrolyzed sucrose to glucose and fructose, and then selectively converted glucose to ethanol and biomass, which accumulated in both molasses media (Atiyeh and Duvnjak, 2002; Atiyeh and Duvnjak, in print). In the first 12 h of the fermentation process, more than 93% of the sucrose in the cane molasses medium was

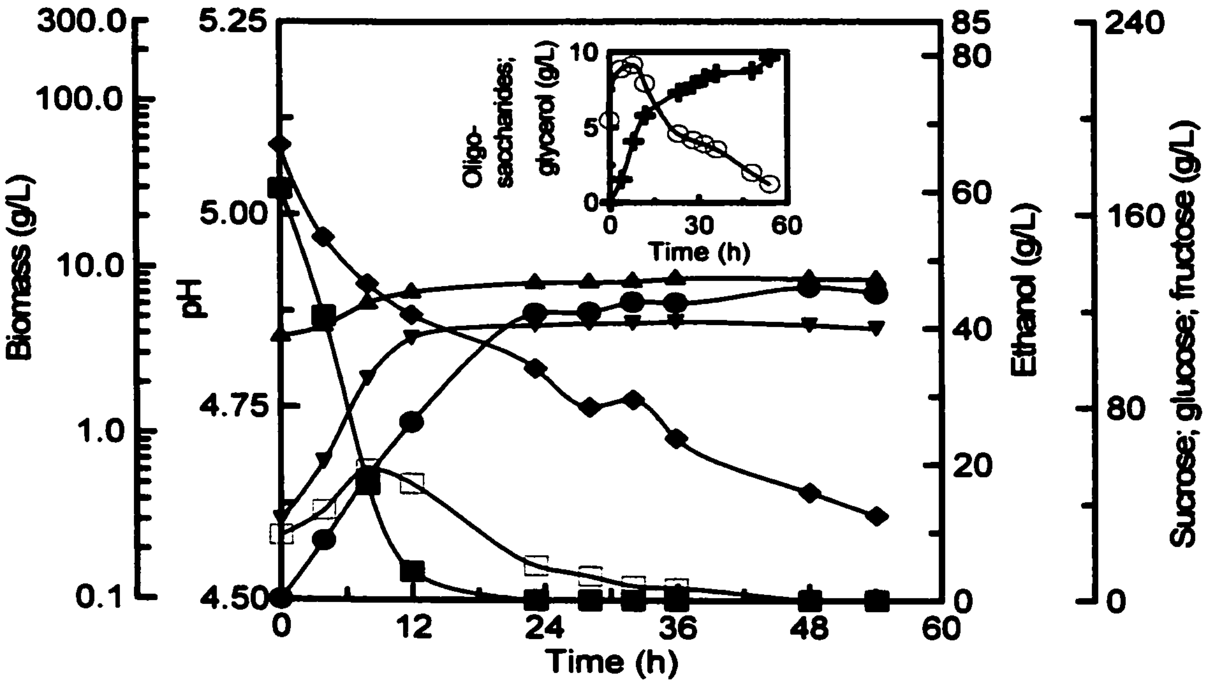

Figure 5.45 Kinetics of growth of *S. cerevisiae* ATCC 36858 and fructose and ethanol production in a sugar cane molasses medium containing 236 g/L total sugar: (▲) biomass; (◆) pH; (●) ethanol; (■) sucrose; (□) glucose; (▼) fructose; (○) oligosaccharides; (✚) glycerol.

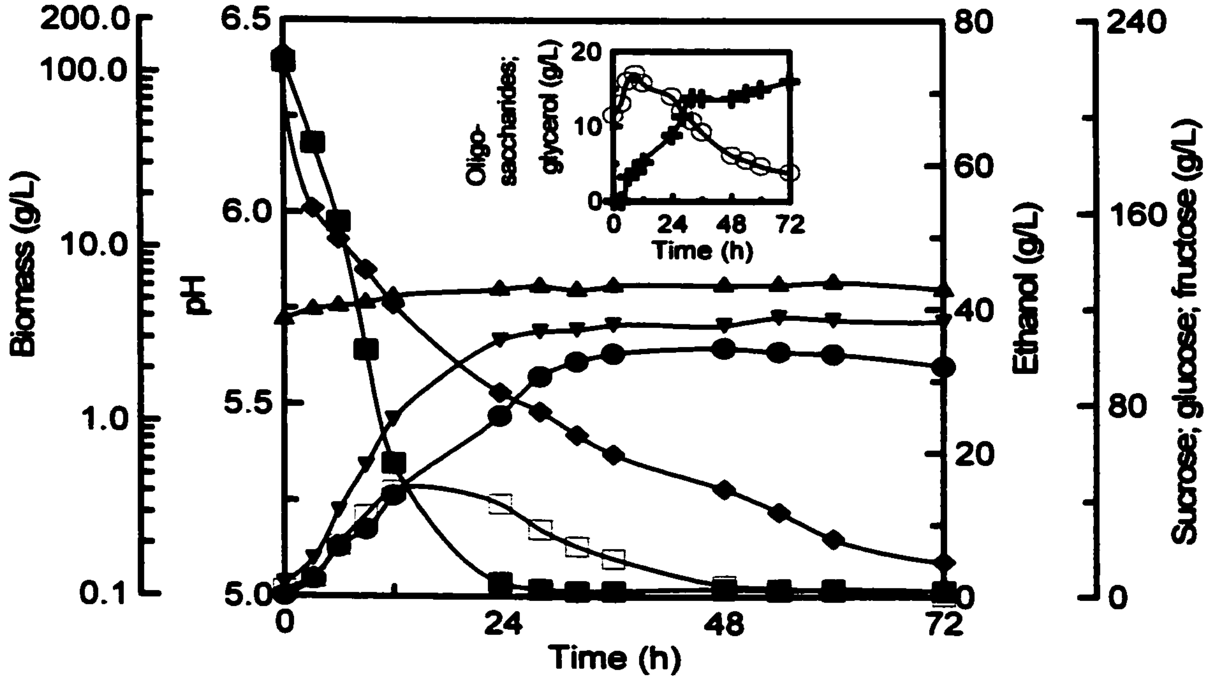

Figure 5.46 Kinetics of growth of *S. cerevisiae* ATCC 36858 and fructose and ethanol production in a sugar beet molasses medium containing 242 g/L total sugar: (▲) biomass; (◆) pH; (●) ethanol; (■) sucrose; (□) glucose; (▼) fructose; (○) oligosaccharides; (✚) glycerol.

hydrolyzed, while in the beet molasses medium, only 75% of sucrose was hydrolyzed. This is because beet molasses has initially about 20% more sucrose than in cane molasses. A small amount of fructose was consumed in both molasses media during the fermentation process. Fructose and ethanol yields in the cane molasses medium were 91 and 72% of the theoretical values, respectively (Table 5.18). A higher fructose yield was obtained in the beet molasses medium (94%) with lower ethanol yield (59%). Both sucrose and raffinose were taken into account in the calculation of fructose yield. The calculation of ethanol yield was based on glucose, galactose and fructose consumed in the fermentation process. The biomass concentration in the cane molasses medium increased from 3.82 to 8.62 g/L (Figure 5.45), which represents a biomass yield of 0.037 g/g of the monosaccharides consumed (Table 5.18). Although the same initial biomass concentration was used in the beet molasses medium, the biomass yield in this medium was about 54% lower than in the cane molasses medium. However, about 68% more glycerol was produced in the sugar beet molasses medium (Figures 5.45 and 5.46). This could

Table 5.18 Fructose, ethanol and biomass yields, specific growth rate and specific initial rate of sugar hydrolysis and ethanol productivity in sugar cane and sugar beet molasses media using *S. cerevisiae* ATCC 36858.

Total sugar[a] (g/L)	Initial biomass (g/L)	Specific growth rate, μ (h^{-1})	Biomass yield[b] (g/g)	Ethanol productivity[b] (g/L h)	Specific initial rate of sucrose hydrolysis[c] (g/g h)	Fructose yield[d] (%)	Ethanol yield[b] (%)
C- 96.7	3.76	0.147	0.108	3.15	5.58	90.6	77.0
C- 141.2	3.37	0.122	0.082	2.32	8.04	92.0	70.9
C- 191.3	3.55	0.103	0.070	1.67	5.99	93.4	76.0
C- 236.0	3.82	0.059	0.037	1.37	3.56	91.2	71.6
C- 273.8	2.93	0.034	0.027	0.88	2.07	99.3	74.8
C- 323.5	2.81	0.012	0.016	0.53	2.22	99.0	66.4
B- 94.9	4.13	0.123	0.098	2.97	6.53	94.3	76.2
B- 145.2	4.01	0.089	0.060	2.22	7.16	93.3	70.7
B- 190.1	3.88	0.069	0.035	1.38	4.05	97.6	68.2
B- 242.0	3.83	0.024	0.017	1.02	2.83	94.2	58.6
B- 276.2[e]	4.29	0.020	0.006	0.48	2.29	100	61.2
B- 312.4[e]	4.85	0.005	0.013	0.03	2.18	100	47.8

[a] C = cane molasses and B = beet molasses

[b] Values are calculated at maximum ethanol concentration

[c] Values are calculated after 3 h of hydrolysis

[d] Values are calculated at maximum fructose concentration

[e] pH was initially adjusted between 5.0 and 5.2 with HCl

indicate the presence of more growth inhibitors in the sugar beet molasses medium. The concentration of raffinose in both molasses media, shown as oligosaccharides in the previous figures, appeared to increase at the beginning of the process. As discussed in section 5.2.2.1, it is believed that this increase was due to the production of fructo-oligosaccharides that have the same retention time as raffinose when measured by HPLC. Using the same yeast strain, it was previously shown that stachyose was produced in media with raffinose, while fructose-oligosaccharides were produced in sucrose media (section 5.2.3). Considering that stachyose elutes at a shorter retention time than fructo-oligosaccharides or raffinose, it was considered that the oligosaccharides produced in both molasses media were fructo-oligosaccharides. Most of the raffinose as well as the oligosaccharides formed in the beginning of the process in both molasses media, were consumed by the end of fermentation. In addition, some glycerol was also produced in this process and it remained in the broth.

The effects of various initial sugar concentrations on biomass production and pH changes during fermentation in cane and beet molasses media using *S. cerevisiae* ATCC 36858 are shown in Figures 5.47 and 5.48. A maximum biomass concentration of 11.5 g/L was attained in the cane molasses medium with a sugar concentration of 191 g/L (Figure 5.47a). However, the maximum biomass concentration (10.1 g/L) was noticed in the beet molasses medium with a sugar concentration of 95 g/L (Figure 5.48a). No significant yeast growth occurred when the sugar concentration was above 274 g/L in the cane or beet molasses media. An increase in the concentration of either type of molasses in the fermentation media (i.e. an increase in the total sugar, minerals and other components) resulted in a significant decrease in specific growth rate of the yeast and biomass yield (Table 5.18). This decrease was expected because of growth inhibition by the high substrate and mineral concentrations. The specific growth rates and biomass yields were lower in the beet molasses than in the cane molasses media. This may indicate the presence of more inhibitors and minerals in the beet molasses. In addition, the high mineral content in both types of molasses resulted in lower growth rates and biomass yields in these media than in synthetic sucrose media with similar sugar concentrations (Tables 5.4 and 5.6). As it was noticed in previous tests using synthetic media, the pH of the fermentation medium decreased with time in all of the cane molasses media (Figure 5.47b) and in the beet molasses media with sugar concentrations below 242 g/L (Figure 5.48b). The pH of the beet

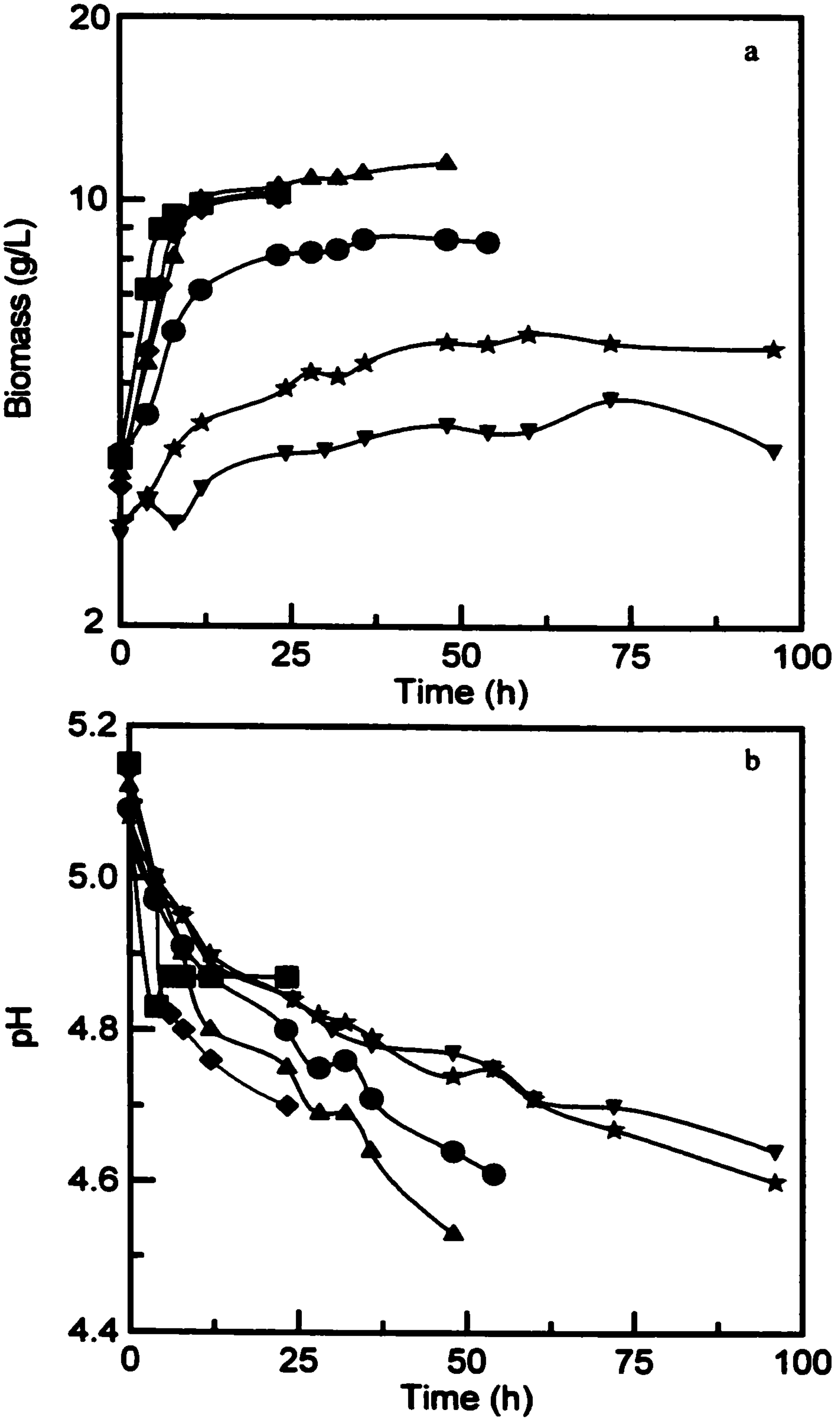

Figure 5.47 Kinetics of (a) biomass production by *S. cerevisiae* ATCC 36858 and (b) pH changes in sugar cane molasses media containing the following total sugar concentrations: (■) 97 g/L; (◆) 141 g/L; (▲) 191 g/L; (●) 236 g/L; (★) 274 g/L; (▼) 324 g/L.

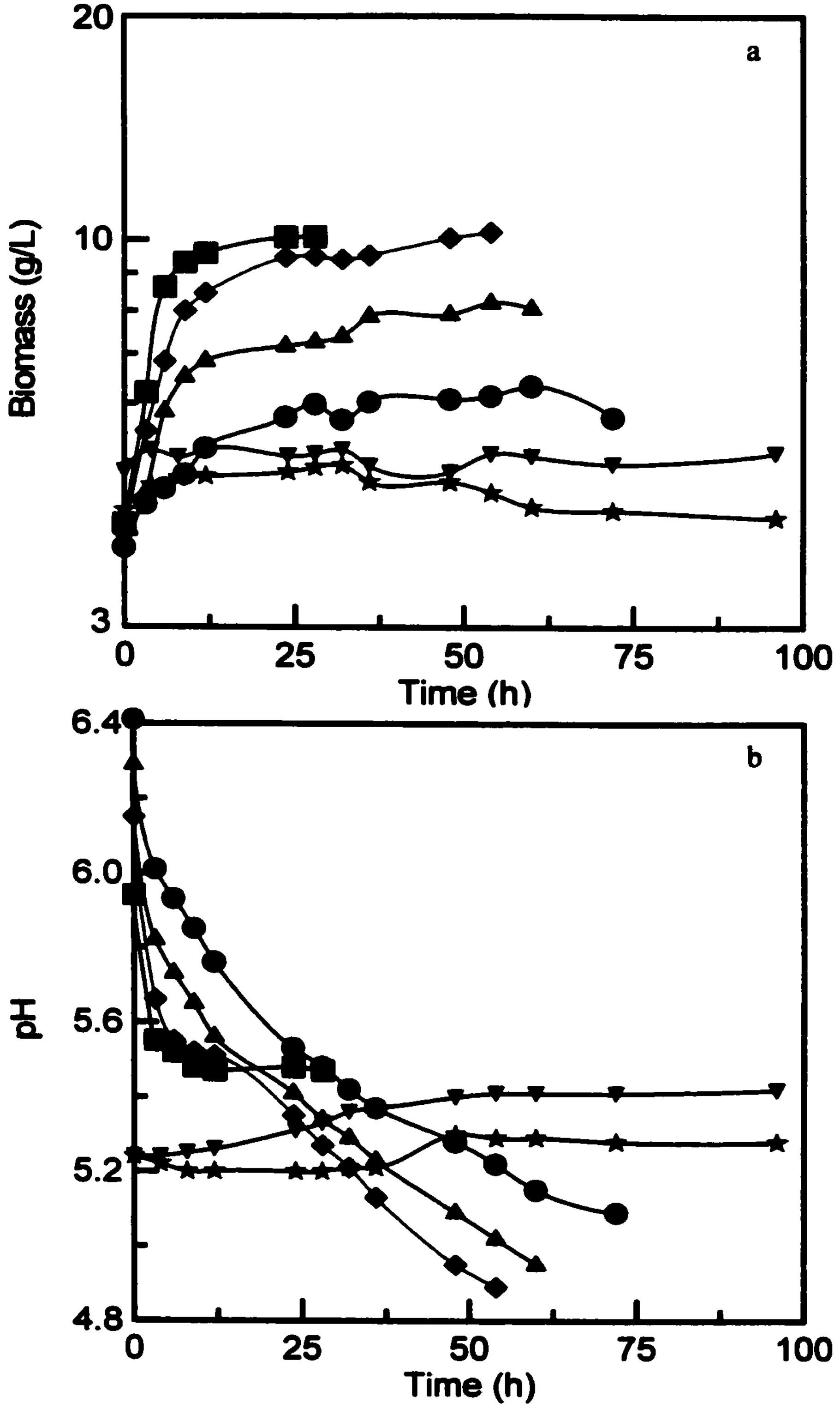

Figure 5.48 Kinetics of (a) biomass production by *S. cerevisiae* ATCC 36858 and (b) pH changes in sugar beet molasses media containing the following total sugar concentrations: (■) 95 g/L; (◆) 145 g/L; (▲) 190 g/L; (●) 242 g/L; (★) 276 g/L; (▼) 312 g/L.

molasses media with sugar concentrations of 276 g/L or higher was initially adjusted to 5.2 with HCl. The purpose of adjusting the pH in these media was to compare the fermentation with the fermentation of the synthetic sucrose media having similar initial pH values and total sugar concentrations. The pH in the beet molasses media with sugar concentrations higher than 276 g/L did not decrease. This could be due to lower yeast activity and poor growth of the yeast in these media (Figure 5.48a). However, the pH of the previously tested synthetic sucrose media having similar, or higher, sugar concentrations decreased with time (Figure 5.16b).

The kinetics of sucrose hydrolysis and glucose production and consumption in both types of molasses media are displayed in Figures 5.49 and 5.50. A complete sucrose hydrolysis in the first 24 h of the fermentation process was noticed in the molasses media when the sugar concentration was below 276 g/L (Figures 5.49a and 5.50a). Almost complete but slow hydrolysis of the sucrose occurred in the molasses media with a sugar concentration of 312 g/L after 96 h of the process. As previously shown, the sucrose was entirely hydrolyzed in the synthetic media when its concentrations were below 629 g/L (Figure 5.17a). Comparable specific initial rates of sucrose hydrolysis were noticed in both types of molasses media (Table 5.18). However, these rates were much higher in the synthetic media having similar sugar concentrations (Tables 5.4 and 5.6). The specific initial rate of sucrose hydrolysis in cane and beet molasses media increased slightly when the sugar concentration was augmented from about 95 to 145 g/L (Table 5.18). However, with a further increase in the sugar concentration, the specific initial rate of sucrose hydrolysis decreased. It has also been reported that sucrose hydrolysis rates decreased when using invertase in solutions having sucrose concentrations higher than 100 g/L (Bowski et al., 1971; Vitolo and Yassuda, 1991). This reduction was found to be due to the inhibition of invertase by a high substrate concentration (Bowski et al., 1971).

In this study, although glucose was used throughout the fermentation process, it accumulated because of the high rate of sucrose hydrolysis and, after attaining a peak, its concentration decreased (Figures 5.49b and 5.50b). A complete consumption of glucose by the end of the process was noticed in both types of molasses media when the sugar concentration was 242 g/L or lower. When the initial sugar concentration was higher than 242 g/L, the glucose uptake rate drastically decreased. The glucose consumption rates were lower in the beet molasses media than in the cane molasses media. A large amount of glucose accumulated after 96 h of

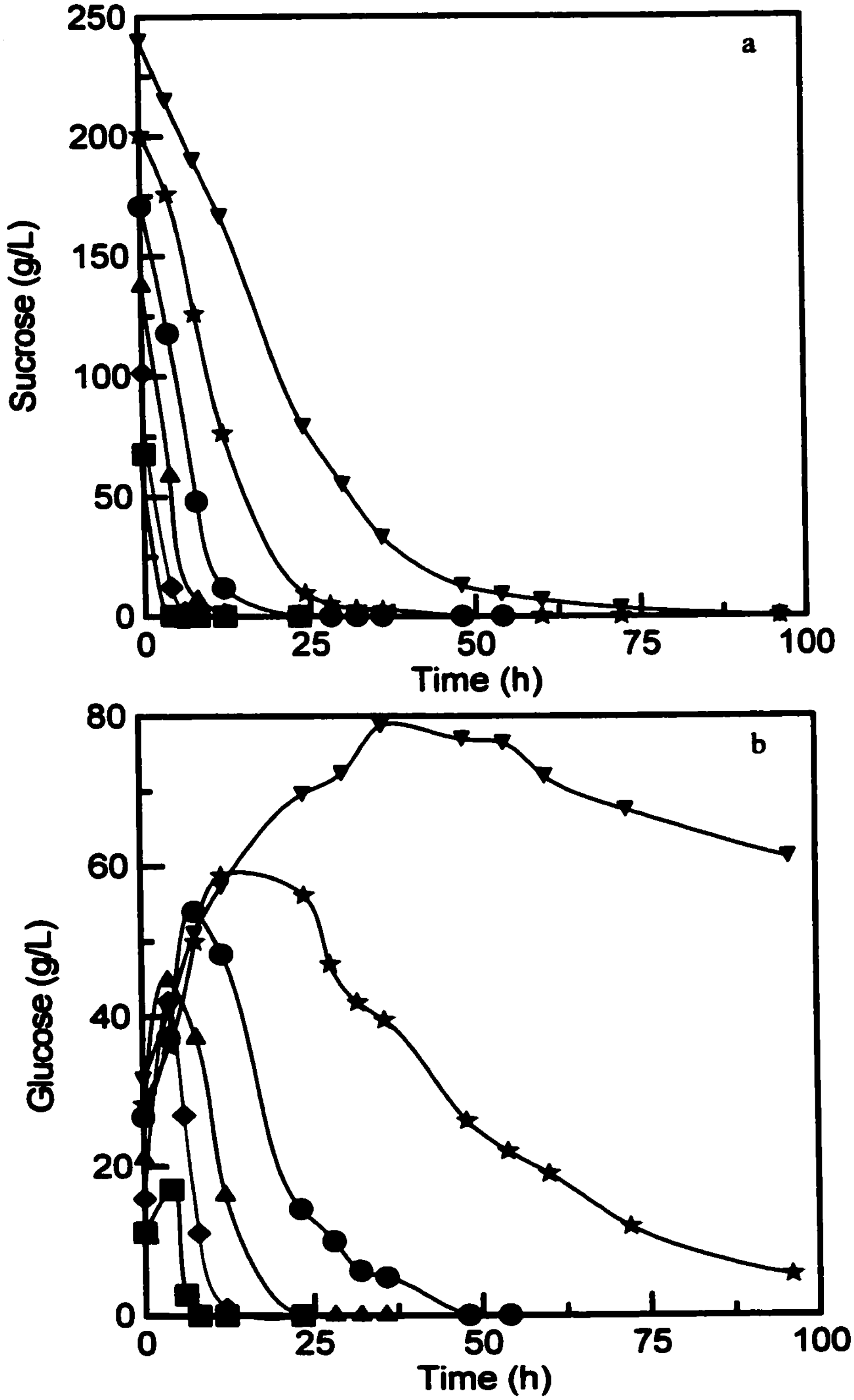

Figure 5.49 Kinetics of (a) sucrose hydrolysis and (b) glucose production and consumption by *S. cerevisiae* ATCC 36858 in sugar cane molasses media containing the following total sugar concentrations: (■) 97 g/L; (◆) 141 g/L; (▲) 191 g/L; (●) 236 g/L; (★) 274 g/L; (▼) 324 g/L.

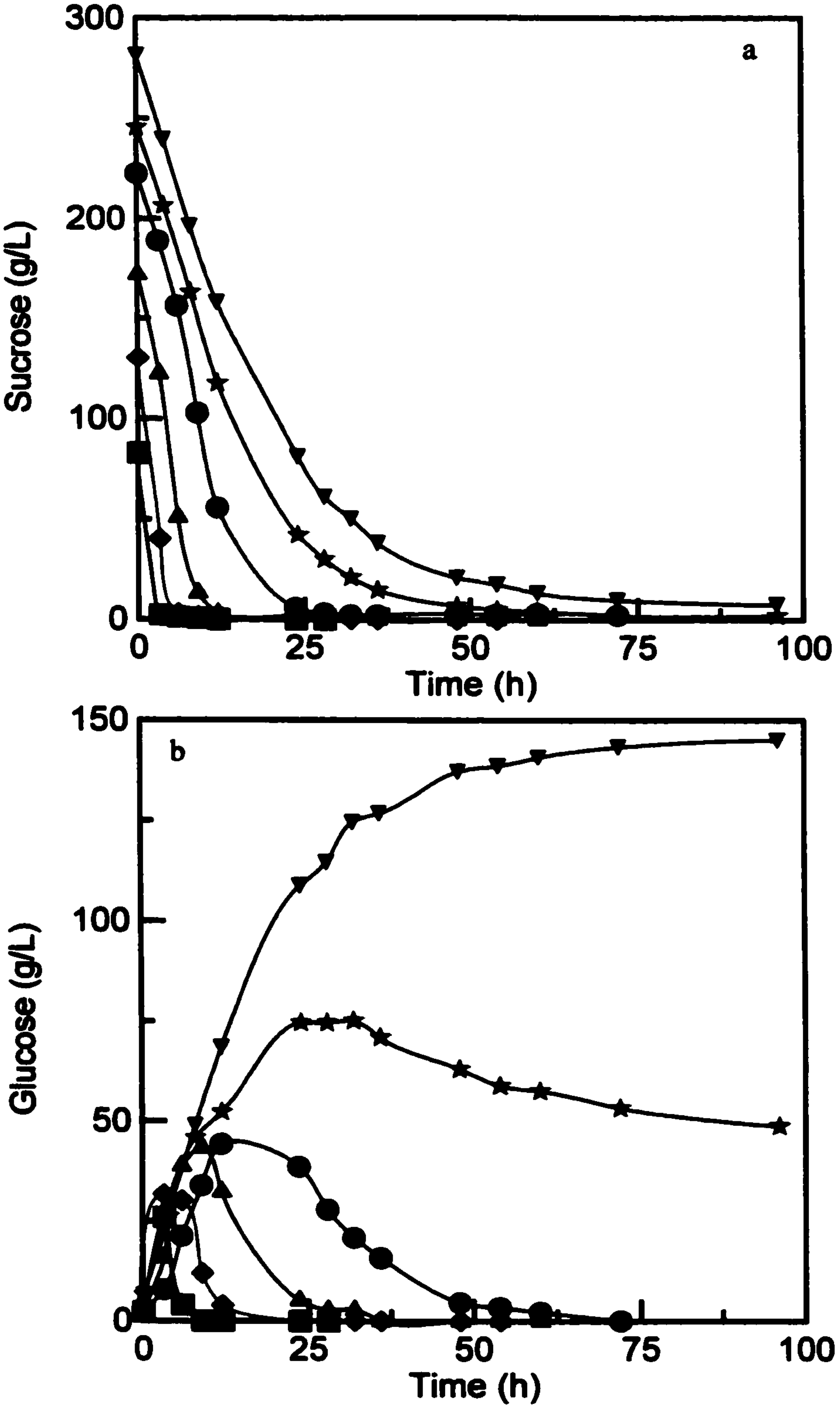

Figure 5.50 Kinetics of (a) sucrose hydrolysis and (b) glucose production and consumption by *S. cerevisiae* ATCC 36858 in sugar beet molasses media containing the following total sugar concentrations: (■) 95 g/L; (◆) 145 g/L; (▲) 190 g/L; (●) 242 g/L; (★) 276 g/L; (▼) 312 g/L.

fermentation in both types of molasses media with sugar concentrations of about 324 g/L. However in the previously tested synthetic media, glucose was completely consumed when sucrose concentrations were below 360 g/L (Figure 5.17b).

The effects of various initial sugar concentrations on fructose and ethanol production by *S. cerevisiae* ATCC 36858 are presented in Figures 5.51 and 5.52. Fructose concentrations increased with increasing initial sugar concentrations in both types of molasses media (Figure 5.51a and 5.52a). High fructose yields (91-100% of the theoretical values) were obtained in all tested media (Table 5.18). These fructose yields were comparable to the values obtained in the previously studied synthetic sucrose media (Tables 5.4 and 5.6). Maximum ethanol concentrations of 46 and 35 g/L were achieved in the cane and beet molasses media with sugar concentrations of about 242 g/L, respectively (Figures 5.51b and 5.52b). Above this initial sugar concentration, the amount of ethanol produced decreased in both molasses media due to the incomplete consumption of glucose (Figures 5.49b and 5.50b). The ethanol productivity in both types of molasses media, and ethanol yield in the beet molasses media, decreased with an increase in the total sugar concentration (Table 5.18). Comparable results of ethanol yields and productivities were obtained in both the sugar cane and sugar beet molasses media when sugar concentrations were below 145 g/L. When sugar concentrations were above 145 g/L, lower ethanol yields and productivities were obtained in the beet molasses media. Higher ethanol yields and productivities were obtained in synthetic sucrose media than in both types of molasses media especially at high initial sugar concentrations (Tables 5.4 and 5.6). Normally, microbes require the presence of some mineral components in adequate concentrations for growth and ethanol production (Akrida-Demertzi and Koutinas, 1991; Chandrasena and Walker, 1997). However, the presence of many mineral components in the molasses media in high concentrations increases the medium osmolality and lowers ethanol production (Jones and Greenfield, 1984; Doelle et al., 1990a).

In the present study, it was noticed that when glucose was reduced to low levels in the fermentation media (Figures 5.49b and 5.50b), a significant decline in ethanol concentration was observed (Figures 5.51b and 5.52b), especially, in the molasses media with a total sugar concentration of about 145 and 190 g/L. Biomass concentrations increased slightly in these media (Figures 5.47a and 5.48a), which could be due to the decrease in ethanol concentration. The

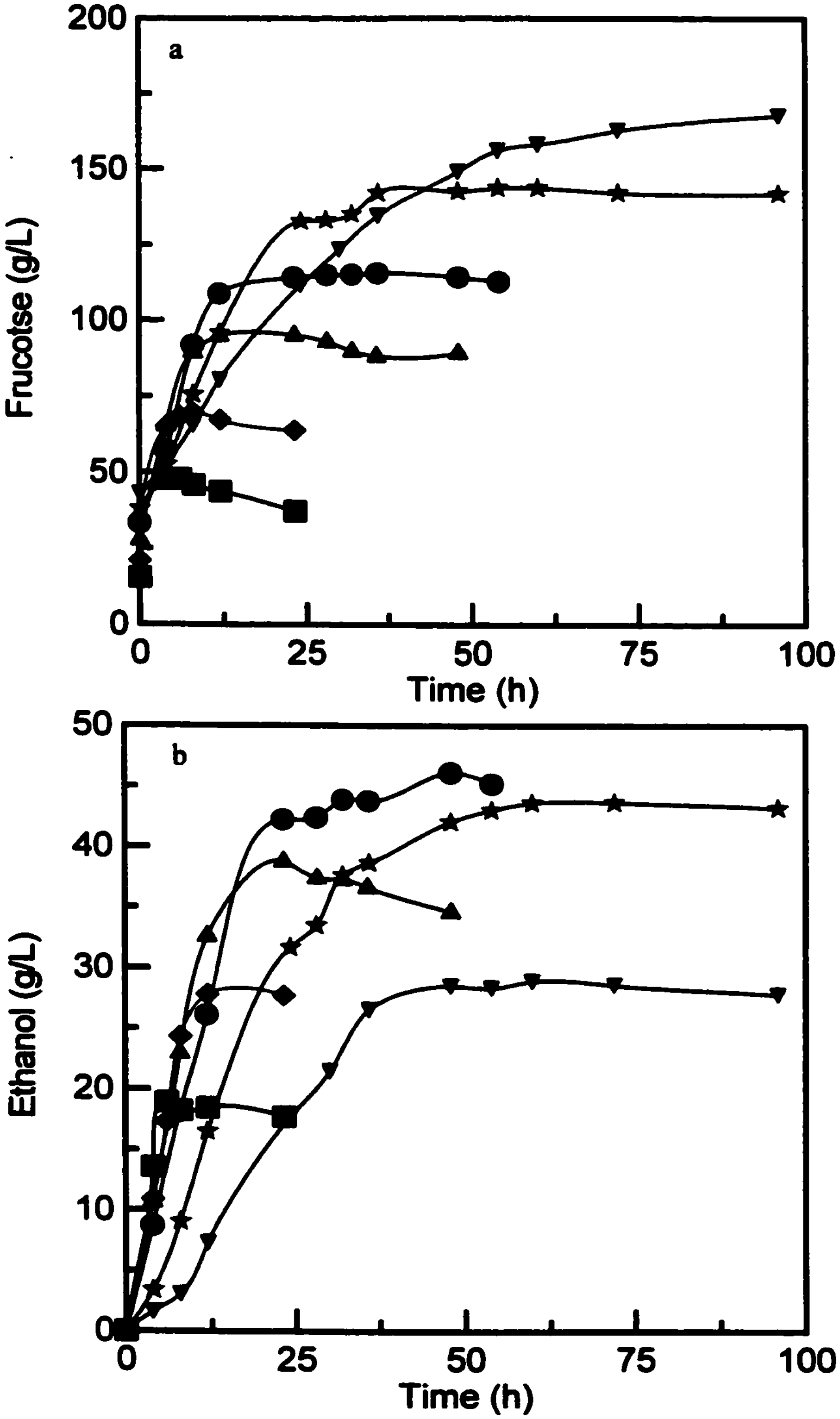

Figure 5.51 Kinetics of (a) fructose and (b) ethanol production by *S. cerevisiae* ATCC 36858 in sugar cane molasses media containing the following total sugar concentrations: (■) 97 g/L; (◆) 141 g/L; (▲) 191 g/L; (●) 236 g/L; (★) 274 g/L; (▼) 324 g/L.

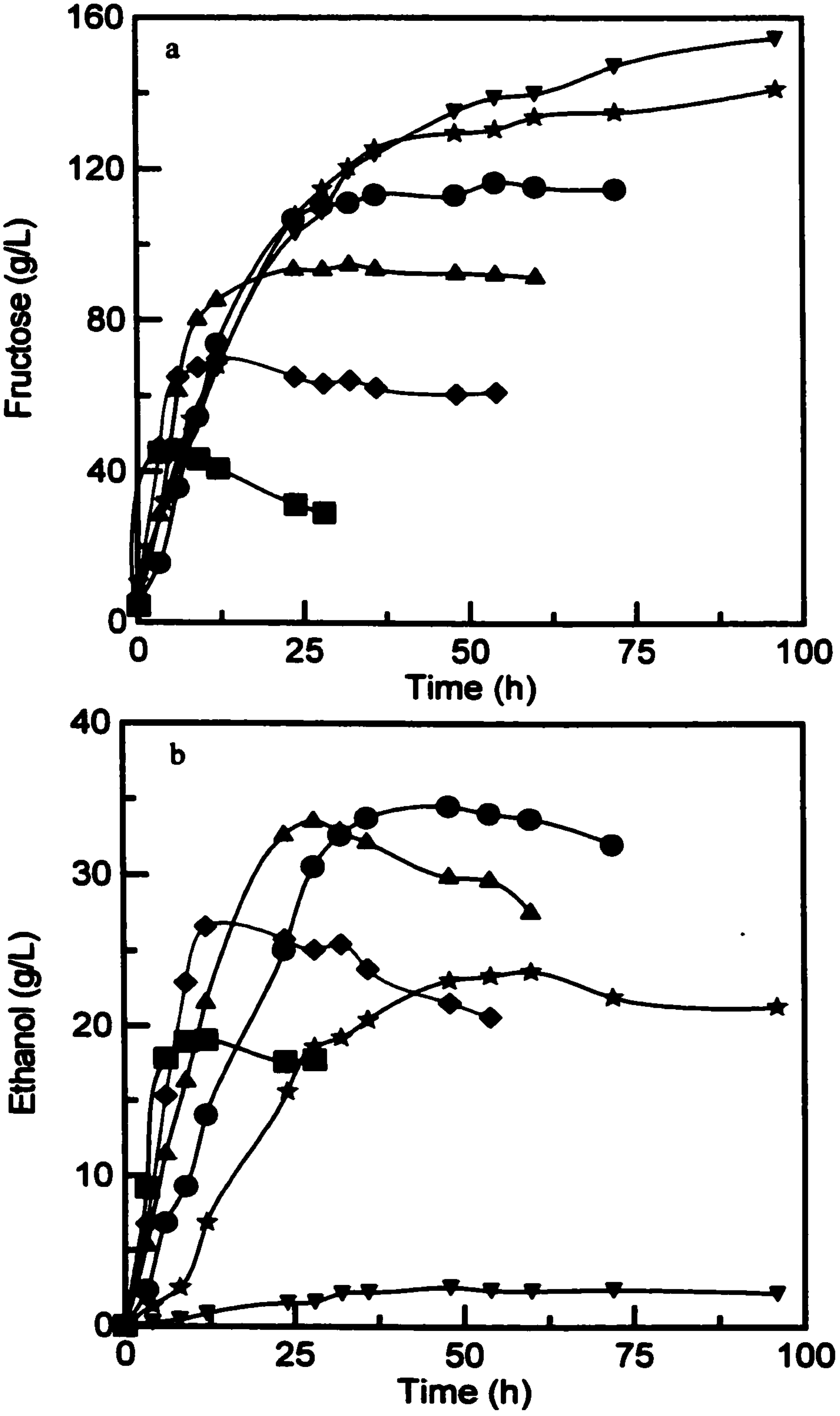

Figure 5.52 Kinetics of (a) fructose and (b) ethanol production by *S. cerevisiae* ATCC 36858 in sugar beet molasses media containing the following total sugar concentrations: (■) 95 g/L; (◆) 145 g/L; (▲) 190 g/L; (●) 242 g/L; (★) 276 g/L; (▼) 312 g/L.

consumption of ethanol when glucose is nearly exhausted is related to the oxidative metabolism of ethanol to provide energy and intermediates for biosynthesis by the tricarboxylic acid cycle (Lievense and Lim, 1982).

In the present study, the fructose yield was above 91% in all the molasses media tested (Table 5.18). A much lower fructose yield (70%) was obtained when *Tricholoma nudum* was used in molasses media with total sugar concentrations between 60 and 80 g/L (Reusser et al., 1960). Comparable fructose yields were reported in C-molasses media when *Z. mobilis* ATCC 53431 was used (Doelle and Doelle, 1991). The cane molasses produced in Australia that contains about 55-60% (w/w) total sugar is classified as C-molasses. When the concentration of C-molasses in the media was increased from 250 to 350 g/L, fructose yield increased from 93 to 95%. However, the ethanol yield dropped from 98 to 77% (Doelle and Doelle, 1991). A final fructose concentration of 86 g/L was obtained from the 350 g/L C-molasses medium. In addition, the fermentation time increased from 21 to 36 h as a result of the increase in C-molasses concentration, and up to 5 g/L sorbitol was produced. The low sorbitol production was due to salts present in the C-molasses (Doelle et al., 1990a, b; Doelle and Doelle, 1991). The presence of salts in high concentrations in C-molasses suppressed sorbitol production but stimulated oligosaccharides formation in the fermentation medium. Invertase was added to hydrolyze the formed oligosaccharides. This enzyme also increased the sucrose hydrolysis rate, which resulted in the accumulation of high concentrations of glucose in the fermentation medium. The high levels of glucose in the presence of fructose in the medium resulted in the production of more sorbitol, which was a drawback of the fermentation process (Doelle et al., 1990a, b; Doelle and Doelle, 1991; Kirk and Doelle, 1994).

Contrary to the processes with *Z. mobilis*, in the process with *S. cerevisiae* ATCC 36858, no sorbitol was detected during fermentation with either molasses or synthetic sucrose media. In the present study, a fructose concentration of 94 g/L was obtained after 24 h of the process in the cane and beet molasses media with a sugar concentration of about 191 g/L (Figures 5.51a and 5.52a). This is equivalent to using a concentration of about 385 g/L of either the cane or beet molasses. Fructose and ethanol yields were above 93 and 68% of the theoretical values, respectively (Table 5.18). The fermentation time doubled when the sugar concentration in both molasses media increased to about 242 g/L. At the end of the fermentation process, the fructose concentration was about 114 g/L in both molasses media, however, ethanol concentrations were 45 and 32 g/L in the cane and beet molasses media, respectively (Figures 5.51 and 5.52). The above results indicate that *S. cerevisiae* ATCC 36858 is able to ferment in cane and beet molasses media with higher sugar concentrations than *Z. mobilis*. In addition, higher ethanol and fructose productivities were obtained with this yeast.

In addition to fructose and ethanol, small amounts of oligosaccharides and glycerol were produced by *S. cerevisiae* ATCC 36858 in both types of molasses media (Table 5.19). During the initial phase of the fermentation process, the maximum amounts of oligosaccharides, including raffinose, in the cane and beet molasses media that contained about 324 g/L sugars were 13.4 and 21.5 g/L, respectively. These oligosaccharides, including raffinose, were entirely hydrolyzed to their monosaccharide components by the end of the process when total sugar concentrations in either molasses media were 190 g/L or lower.

The glycerol concentration increased from 6.8 to 9.9 g/L in the cane molasses medium when the initial sugar concentration was increased from 97 to 324 g/L, respectively (Table 5.19). Glycerol concentrations increased in the beet molasses media to higher levels than in the cane molasses media, when the initial sugar concentration was increased from 95 to 242 g/L. However, the glycerol concentration in the beet molasses media decreased when sugar concentrations were above 242 g/L, which was due to the accumulation of high amounts of glucose in the fermentation media (Figure 5.50b). These results are consistent with previous observations in synthetic sucrose media (Table 5.7). In this study, about 26% more glycerol was produced in the cane molasses medium than in the synthetic sucrose medium. More glycerol was also produced in the beet molasses medium than in either the synthetic sucrose or cane molasses media with sugar concentrations below 312 g/L. This could be due to a higher mineral concentration in the beet molasses media than in the cane molasses or synthetic media (Maiorella et al., 1984b).

In the present study, syrups with a carbohydrate content of more than 95% fructose were produced from the cane molasses media with sugar concentrations of 274 g/L or lower and from the beet molasses media with sugar concentrations of 242 g/L or lower (Table 5.19). When the sugar concentration in the cane and beet molasses media increased above 274 and 242 g/L, respectively, the fructose fraction of the produced syrup substantially decreased. This was also noticed in the previously tested synthetic media when the sucrose concentrations were above 360 g/L (Table 5.7). However, syrups with more than 97% fructose based on the final total sugar concentrations were produced from synthetic media with sucrose concentrations below 360 g/L.

Table 5.19 Maximum and residual oligosaccharides, final glycerol concentrations in the fermentation broth, fructose contents in the produced syrups and sucrose hydrolysis efficiency in cane and beet molasses media using *S. cerevisiae* ATCC 36858.

Total sugar[a] (g/L)	Maximum oligosaccharides (g/L)	Residual oligosaccharides (g/L)	Glycerol (g/L)	Fructose contents in syrups produced[b] %	Sucrose hydrolysis efficiency %
C- 96.7	2.4	0.0	6.8	100	100
C- 141.2	5.4	0.0	8.3	99	100
C- 191.3	8.7	0.0	8.4	99	100
C- 236.0	9.1	1.2	9.6	99	100
C- 273.8	11.0	2.2	9.7	95	100
C- 323.5	13.4	5.3	9.9	72	100
B- 94.9	5.5	0.0	6.9	100	100
B- 145.2	9.2	0.0	8.9	100	100
B- 190.1	13.3	0.0	10.5	100	100
B- 242.0	17.0	3.8	16.1	95	99
B- 276.2[c]	19.1	12.2	12.9	69	99
B- 312.4[c]	21.5	15.7	2.4	48	97

[a] C = cane molasses and B = beet molasses

[b] Values are calculated based on the final total sugar concentrations

[c] pH was initially adjusted between 5.0 and 5.2 with HCl

The results of this study showed that *S. cerevisiae* ATCC 36858 was able to produce fructose and ethanol, and to utilize raffinose, from the cane and beet molasses media. The fructose yields were above 90% of the theoretical values in all of the molasses media tested. The ethanol yields in the cane and beet molasses media with sugar concentrations below 276 g/L were above 71 and 59% of the theoretical values, respectively. Syrups with a carbohydrate content of more than 95% fructose can be produced. Even in a cane molasses medium at a total sugar concentration of 324 g/L, the supernatant contained 72% fructose and, thus, it was richer in this carbohydrate than the ordinary 55% HFCS. The syrup produced from a beet molasses medium with a total sugar concentration of 276 g/L contained 69% fructose. Some glycerol and oligosaccharides were also produced in both types of molasses media. The results from this study indicate that molasses could be considered for the industrial production of fructose syrups. However, a technique to purify such syrups has to be developed. The purification of fructose syrups is discussed in the next section.

5.3 Purification of fructose syrups

Purification is an important step in the production of high fructose syrups. The high fructose syrup should be colourless, have no taste other than sweetness, no undesirable odour and should be clear in appearance (Fleming and GrootWassink, 1979). The industrial purification of high fructose syrups normally includes decolourization and demineralization (Coker and Venkatasubramanian, 1987). Decolourization of high fructose syrups is generally carried out using activated carbon, whose main function is colour removal but which also helps to remove colour precursors and sugar degradation products (Blanchard and Geiger, 1984). Demineralization is commonly performed using ion exchange resins, which eliminate ionic matters from fructose syrups.

The colour of the HFCS produced commercially is 5 International Units (IU) according to the International Commission for Uniform Methods of Sugar Analysis (ICUMSA) colour method 4 (Blanchard and Geiger, 1984) or below 25 Reference Base Units (RBU) (Long, 1991). Colour specifications on sugar products for the soft drink industry in the United States are usually expressed in terms of RBU and range from 25 to 40 RBU (Chou, 1993). The ICUMSA colour units are approximately equivalent to the RBU units (Blanchard and Geiger, 1984). The calculation of ICUMSA units is based on the absorbance of sugar solution at 420 nm as shown in Eq. 4.3 (section 4.4.6). The estimation of RBU units is also based on absorbance at 420 nm but also includes a correction for turbidity by using the absorbance at 720 nm.

In this study, the fructose syrups produced from molasses media should have similar characteristics to the industrially produced HFCS. The production of fructose and ethanol using *S. cerevisiae* ATCC 36858 from molasses media was discussed in section 5.2.4. Preliminary work to explore various techniques for the purification of fructose syrups produced from a cane molasses medium was carried out. The concentrations of the ethanol and fructose produced in the fermentation broth were about 46 and 116 g/L, respectively. The produced syrup was purified by the removal of biomass, ethanol, colour and minerals.

5.3.1 Biomass and ethanol removal

Biomass was removed from the fermentation medium by centrifugation at 17,000 g for 15 min (section 4.4.1) to obtain a clear broth. The collected broth, after biomass removal, was used for further treatment to remove ethanol, colour and minerals. Ethanol was recovered from the broth by vacuum evaporation as described in section 4.2.2. The percentage of ethanol recovery from three different runs was between 94 and 98% (Table 5.20). The concentration of ethanol in the collected condensate, after vacuum evaporation, was about 184 g/L. This condensate can be further purified using different methods such as distillation, solvent extraction or membrane technology (Kosaric et al., 1983). The results of the present study demonstrate that more than 94% of the ethanol produced during the fermentation process could be recovered using vacuum evaporation. The fructose concentration in the broth increased from about 116 to 153 g/L (Table 5.20).

Table 5.20 Ethanol recovery after vacuum evaporation from a broth containing about 46 g/L ethanol and 116 g/L fructose from various runs.

	Amount (mL)	Fructose (g/L)	Fructose recovery (%)	Ethanol (g/L)	Ethanol recovery (%)
Run 1					
Original broth	220	114.7	----	45.9	----
Broth remained	165	152.5	99.8	0.0	----
Condensate collected	55	----	----	175.7	95.7
Run 2					
Original broth	780	117.1	----	44.1	----
Broth remained	610	150.1	100.2	0.0	----
Condensate collected	170	----	----	189.9	93.9
Run 3					
Original broth	250	115.5	----	47.7	----
Broth remained	187	155.2	100.6	0.0	----
Condensate collected	63	----	----	184.9	97.7

5.3.2 Colour removal

The biomass-free broth containing fructose and ethanol, which was produced after fermentation of sugar cane molasses, was dark black in colour. The broth after the ethanol was removed had a similar colour. The absorption and transmittance characteristics of the broth as a function of wavelength are shown in Figure 5.53. The absorption curve represents the summation of the optical effect of a very large number of compounds. The absorption of the sugar solution depends on the wavelength and the nature and concentration of the light-absorbing constituents (Chou, 1993). Sugar colourants are generally classified into those that occur naturally (plant pigments) and the others that are formed during sugar processing (caramel and melanoidins). Each type may consist of a highly complex mixture of compounds whose concentration is difficult to quantify in terms of g/L, therefore, their concentrations are assumed to be proportional to the density of sugar solution when colour units are calculated (section 4.4.6). In this study, the absorption and transmittance curves were similar for both broth mixtures in the visible light wavelength region (400 to 800 nm). The absorption increased with the increase of the wavelength of the incident light to about 500 nm and attained a maximum plateau in the range of 500 to 670 nm (Figure 5.53a). It then sharply decreased with an increase of the wavelength of the incident light above 670 nm. There were no clear peaks of maximum absorbance found within the visible region. This could be due to the highly complex mixture of colourants present in the broth (Chou, 1993). In the present study, the transmittance was below 8% in both broth mixtures in the visible light range (Figure 5.53b). The high absorbance and low transmittance of these broth mixtures indicated the significant intensity of colourants present. Colour removal from the broth using activated carbon is discussed in the next section.

5.3.2.1 Colour removal by activated carbon

The decolourization of HFCS at various manufacturing stages is carried out using activated carbon (Blanchard and Geiger, 1984; Coker and Venkatasubramanian, 1987). For example, it is important to have low levels of impurities such as ash, metal ions, colourants or proteins in the glucose feedstock before isomerization in order not to reduce the enzyme activity (Coker and Venkatasubramanian, 1987). In addition, after the isomerization stage, the produced HFCS is further treated with carbon to obtain syrups with a colour of 5 IU (Blanchard and Geiger, 1984; Coker and Venkatasubramanian, 1987).

The carbon treatment process in the HFCS industry is mainly used to remove colourants, proteins and sugar degradation products. For high capacity plants, this is typically done in packed columns of granular carbon. These columns are renewed by the removal of spent carbon and the addition of regenerated carbon on a regular basis. For smaller facilities, powdered carbon is the preferred means of decolourization (Blanchard and Geiger, 1984). Approximately comparable performance for a given colour removal is obtained from the same amount of granular or powdered activated carbon per unit of sugar treated. The advantage of using powdered over granulated carbon is the shorter contact time required for powdered carbon, minutes instead of

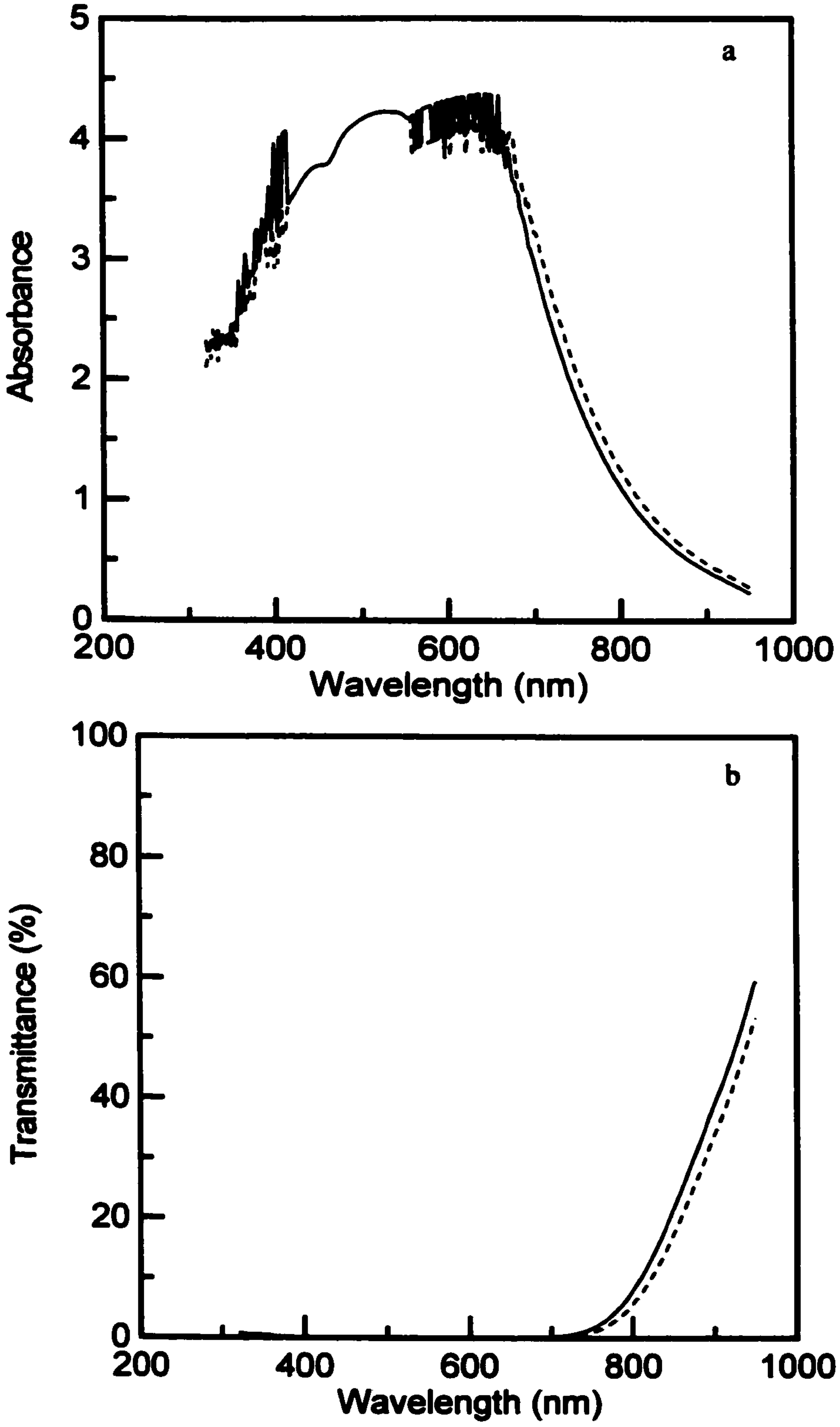

Figure 5.53 (a) Absorption and (b) transmittance curves as a function of wavelength of biomass-free broth produced from cane molasses medium containing: (————) fructose and ethanol, and (---------) fructose alone.

hours, because of a high surface to mass ratio (Riffer, 1993). However, powdered activated carbon is usually used for only two cycles and then discarded, which results in a relatively high operating cost. It was reported that about 1 g of activated carbon is required per 100 g of syrup solids for each stage of decolourization of HFCS (Blanchard and Geiger, 1984) and for decolourization of sugar liquor from a feed colour of approximately 1,200 to 120 IU (Field and Benecke, 2000). In this study, powdered activated carbon (Darco G-60) was used for colour removal from the broth.

5.3.2.1.1 Biomass-free broth containing fructose and ethanol

The decolourization of a biomass-free broth after fermentation of a cane molasses medium containing about 230 g/L total sugar was performed using powdered activated carbon. The concentrations of ethanol and fructose in the broth were 46 and 114 g/L, respectively. This broth was contacted for 10 min with 33% of activated carbon in the slurry. The results showed that about 72% of the initial broth used in the treatment remained in the carbon after centrifugation (Table 5.21). A decrease in fructose and ethanol concentrations in the remaining broth by 5 and 25%, respectively, was also noticed. This decrease was due to the redistribution of fructose and ethanol between the treated broth and the carbon. Ethanol was adsorbed more than fructose. The percentages of recovered fructose and ethanol, in the broth after carbon treatment, were 26 and 21%, respectively. The results showed that fructose and ethanol were not only lost with the broth remaining in the carbon but also due to their adsorption onto the carbon. In an attempt to reduce the amount of broth remaining in the carbon, the carbon was mixed with water prior to the addition of the broth as described in section 4.4.3.1.1. This resulted in a considerable decrease in the amount of treated broth remaining in the carbon (Table 5.21). Furthermore, the percentages of recovered fructose and ethanol in the broth after the wet carbon treatment were nearly double the values obtained without carbon wetting. However, the addition of water to the carbon prior to the treatment resulted in diluting the treated broth by 58%. Furthermore, fructose and ethanol concentrations in the treated broth were lower by 9 and 17%, respectively, than expected because of the adsorption of these compounds onto the carbon.

The conductivity of the treated broth using pre-wetted carbon slightly decreased, while it increased by 30% when the carbon was not wetted (Table 5.21). The slight decrease in the conductivity is due to the dilution effect by the water used with the wet carbon treatment.

However, the increase in the conductivity of the treated broth without prior carbon wetting could be due to the redistribution of the minerals between the treated broth and the carbon.

Table 5.21 Conductivity and pH values, fructose and ethanol concentrations and recoveries using 50 mL of biomass-free broth containing 114 g/L fructose and 46 g/L ethanol after treatment with 33% activated carbon for 10 min.

	Amount (mL)	pH	Conductivity (millimhos)	Fructose (g/L)	Fructose recovery[a] (%)	Ethanol (g/L)	Ethanol recovery[a] (%)
Untreated broth	50	4.95	18.1	113.5	——	45.8	——
Treated broth	14	5.73	23.5	107.4	26.49	34.3	20.96
Treated broth[b]	47	5.74	17.5	60.3	49.90	22.0	45.17

[a] Values are calculated based on fructose and ethanol concentrations in the broth before carbon treatment
[b] Activated carbon was mixed with 36 mL deionized water before the addition of 50 mL of untreated broth

It was reported that activated carbon has neither ash removal nor buffering capacity (Carpenter and Clarke, 1977; Riffer, 1993; Field and Benecke, 2000). Therefore, sugar liquors treated with carbon usually show a pH drop by about 1.0 unit from an initial pH value between 7.5 and 8.5 depending on the clarification method used before carbon treatment. However, in the present study, the pH of the treated broth increased after carbon treatment (Table 5.21). This could be due to higher ash content and colourants in the broth produced by fermentation of cane molasses than in raw sugar liquors. In order to test whether activated carbon contributed to the increase in the pH and conductivity of the broth after treatment, 100 mL of deionized water was contacted with 33 g of activated carbon for 20 min. The pH and conductivity of deionized water after carbon treatment increased from 7.2 to 7.8 and 6.5 to 91 μmhos, respectively. This insignificant increase in the deionized water conductivity does not explain the large increase in the broth conductivity after carbon treatment (Table 5.21).

The broth that contained fructose and ethanol used in this study has 500,475 ICUMSA colour units (Table 5.22), while raw sugar liquors before carbon treatment usually have colour in the range of 2,000 ICUMSA (Field and Benecke, 2000). This shows that there is a large difference between the broth used in this study and raw sugar liquors used in the sugar industry. The percentage of colour removed from the broth, after 10 min of contact with 33% activated carbon with and without prior wetting, was more than 99% (Table 5.22). The colour of the treated broth changed from dark black to light brown. The concentrations of sugars, ethanol and glycerol in the broth before and after carbon treatment are given in Appendix B (Table B.2). The fructose content in the treated broth, with and without prior carbon wetting, was above 95% of the total sugar concentrations. Koren and Duvnjak (1989) used 3% activated carbon (Darco G-60) in the slurry to completely decolourize a light brown syrup containing about 111 g/L fructose, 6 g/L glucose and 47 g/L ethanol without any loss of these compounds in the carbon. This syrup was produced from a mixture of diluted HFCS and Jerusalem artichoke juice by a microbial selective conversion process. The absorbance of their syrup, which was measured at 460 nm, decreased from 0.577 to 0.004 as a result of carbon treatment. In terms of colour units calculated using Eq. 4.3 with absorbance at 460 nm instead of 420 nm, their syrup before carbon treatment had 4,931 colour units. For comparison, the broth used in the present study had an initial colour of 310,349 colour units when measured at 460 nm, which is 62 times higher than the syrup used by Koren and Duvnjak (1989).

Table 5.22 Colour removal from a biomass-free broth containing 114 g/L fructose and 46 g/L ethanol after treatment with 33% activated carbon for 10 min.

	Dilution[a]	Total solids[b] (g/L)	Absorbance at 420 nm	Transmittance %	Colour IU	Colour removal[c] (%)
Untreated broth	150	126.4	0.4217	37.87	500,475	-----
Treated broth	-----	117.6	0.3630	43.35	3,088	99.4
Treated broth[d]	-----	66.4	0.1430	71.94	2,153	99.6

[a] Dilutions were made to obtain transmittance in the range of 20-80%

[b] Values are before dilution

[c] Values are calculated based on the colour of the broth before carbon treatment

[d] Activated carbon was mixed with 36 mL deionized water before the addition of 50 mL of untreated broth

This study showed that more than 99% of the colourants were removed from the broth containing fructose and ethanol after treatment with 33% activated carbon, with or without prior carbon wetting. The fructose content in the treated broth, with or without prior carbon wetting, was above 95% of the total sugar concentrations. Wetting the carbon prior to broth treatment resulted in a considerable dilution of the broth but increased the recovery of fructose and ethanol. The percentage of recovered fructose and ethanol in the broth after treatment was below 50% because a considerable amount of broth remained in the carbon. Taking into consideration that the percentage of ethanol recovery from the broth using vacuum evaporation was about 94% (Table 5.20), which is much higher than ethanol recovery after carbon treatment, it is more practical to remove ethanol from the broth before the decolourization step. Colour removal from a biomass- and ethanol-free broth containing fructose using various amounts of activated carbon is discussed next.

5.3.2.1.2 Treatment of biomass- and ethanol-free broth containing fructose with various amounts of activated carbon

The decolourization of a biomass- and ethanol-free broth after fermentation of a cane molasses medium containing about 230 g/L total sugar was performed with various amounts of activated carbon. The concentration of fructose in the broth was 154 g/L. The results showed that the amount of broth remaining in the carbon after treatment increased as a result of increasing amounts of activated carbon used (Table 5.23). Therefore, the percentage of recovered fructose in the treated broth decreased with increasing amounts of carbon used. About 72% of the broth remained in the carbon after treatment with 33% of carbon in the slurry. In addition, the fructose concentration in the treated broth decreased by 7%. This is similar to what was found in the previous section with the broth that contained both fructose and ethanol. This indicates that the presence or absence of ethanol from the broth does not have a significant effect on the decrease in fructose concentration after carbon treatment.

The pH and conductivity of the treated broth increased when carbon dosage was increased (Table 5.23). The untreated broth initially had 479,254 IU (Table 5.24). When this

Table 5.23 Conductivity and pH values and fructose concentration and recovery using a biomass- and ethanol-free broth containing 154 g/L fructose after treatment with various amounts of activated carbon.

Carbon used (%)		Amount (mL)	pH	Conductivity (millimhos)	Fructose (g/L)	Fructose recovery[a] (%)
11	Untreated broth	20.0	4.89	21.80	154.1	----
	Treated broth	14.7	5.21	24.80	146.5	69.84
22	Untreated broth	30.0	4.89	21.80	154.1	----
	Treated broth	14.5	5.48	26.80	144.8	45.40
33	Untreated broth	50.0	4.89	21.80	154.1	----
	Treated broth	14.0	5.70	28.90	143.2	26.01

[a] Values are calculated based on the fructose concentration in the broth before carbon treatment

broth was treated with various carbon dosages, the decolourization efficiency of the treated broth increased from 50 to 98% by increasing the amount of carbon in the slurry from 11 to 33%, respectively. The colour of the broth before and after treatment with various amounts of carbon is shown in Figure B.2. The colour of the treated broth with 11% carbon was black. However, it was a light brown colour after treatment with 33% carbon. Even with 33% carbon, the broth still had a considerable amount of colourants (Table 5.24).

Table 5.24 Colour removal from a biomass- and ethanol-free broth containing 154 g/L fructose after treatment with various amounts of activated carbon.

Carbon used (%)		Dilution[a]	Total solids[b] (g/L)	Absorbance at 420 nm	Transmittance %	Colour IU	Colour removal[c] (%)
-----	Untreated broth	150	180.3	0.5760	26.55	479,254	-----
11	Treated broth	100	169.6	0.4032	39.52	237,694	50.4
22	Treated broth	80	167.1	0.1225	75.42	58,655	87.8
33	Treated broth	10	163.3	0.1241	75.14	7,599	98.4

[a] Dilutions were made to obtain transmittance in the range of 20-80%

[b] Values are before dilution

[c] Values are calculated based on the colour of the broth before carbon treatment

The results showed that the fructose recovery in the treated broth with 11% carbon was more than two times higher than in the broth treated with 33% carbon. However, the decolourization efficiency was about half of that obtained with 33% carbon. Since the percentage of colour removal from the treated broth was 50% when 11% of carbon in the slurry was used, it was interesting to examine if the other 50% of the colour would be removed by treatment with a further 11% of carbon in the slurry. When the effect of multi-step treatments of the broth with 11% carbon for 20 min on fructose recovery and decolourization efficiency was tested, it was found that the percentage of broth remained in the carbon after each treatment was between 27 and 32% (Table 5.25). A slight decrease in the fructose concentration in the remaining broth after each treatment was noticed. The concentration of fructose in the broth after the third treatment with 11% carbon was slightly lower than in the broth that was treated once with 33% carbon (Table 5.23). In addition, the percentage of fructose recovery, after each treatment with 11% carbon, was between 65 and 70% (Table 5.25). The total fructose recovery after the third treatment with 11% carbon was about 30% of the initial fructose content of the untreated broth.

This is 4% higher than when one treatment with 33% carbon was done. However, more labor time was required with the multi-step colour removal process.

Table 5.25 Conductivity and pH values and fructose concentration and recovery using a biomass- and ethanol-free broth containing 154 g/L fructose after three treatments each with 11% activated carbon.

	Amount (mL)	pH	Conductivity (millimhos)	Fructose (g/L)	Fructose recovery[a] (%)
Before 1st treatment	20.0	4.80	21.80	154.1	----
After 1st treatment	14.7	5.21	24.80	146.5	69.84
Before 2nd treatment	10.0	5.21	24.80	146.5	----
After 2nd treatment	6.8	5.60	26.30	144.3	67.02
Before 3rd treatment	5.5	5.60	26.30	144.3	----
After 3rd treatment	3.7	6.20	28.30	140.0	65.28

[a] Values are calculated based on the fructose concentration in the broth before each carbon treatment

The pH and conductivity of the treated broth increased after each step of the carbon treatment (Table 5.25). The increase in conductivity after the third treatment was slightly lower than when the broth was treated once with 33% carbon (Table 5.23). However, the pH of the broth after the third treatment was 11% higher than in the broth treated with 33% carbon. The percentage of colour removal was 50% after the first treatment with 11% carbon (Table 5.26). After the second treatment with 11% carbon, another 40% of initial colourants were removed. The decolourization efficiency after the third treatment was 99% based on initial colour of untreated broth. The colour units of the treated broth after the third treatment with 11% carbon were 61% lower than in the broth treated once with 33% carbon. This could be due to the high total contact time in the multi-step treatment process.

The glycerol and sugar concentrations in the broth before and after carbon treatment are given in Appendix B (Tables B.3 and B.4). The concentrations of these constituents decreased with increasing carbon dosage and after each treatment. Similar to the broth treated with 33% carbon (Table B.3), raffinose was not detected in the broth after three treatments each with 11% carbon (Table B.4). The fructose content in the treated broth was about 91% of the total sugars, regardless of the number of treatment steps or amount of carbon used.

Table 5.26 Colour removal from a biomass- and ethanol-free broth containing 154 g/L fructose after three treatments each with 11% activated carbon.

	Dilution[a]	Total solids[b] (g/L)	Absorbance at 420 nm	Transmittance %	Colour IU	Colour removal[c] (%)
Untreated broth	150	180.3	0.5760	26.55	479,254	----
After 1st treatment	100	169.6	0.4032	39.52	237,694	50.4
After 2nd treatment	70	164.4	0.1058	78.38	45,062	90.6
After 3rd treatment	----	158.1	0.4625	34.47	2,926	99.4

[a] Dilutions were made to obtain transmittance in the range of 20-80%

[b] Values are before dilution

[c] Values are calculated based on the colour of the broth before any carbon treatment

The results showed that the decolourization efficiency was 98% when 33% of activated carbon in the slurry was used. It was 99% after three treatments each with 11% of activated carbon in the slurry. The slight increase in colour removal efficiency in the multi-step carbon treatment could be due to the increase in contact time during that process. The effect of contact time between the broth and activated carbon on colour removal is discussed next.

5.3.2.1.3 Treatment of biomass- and ethanol-free broth containing fructose for various time intervals

When broth containing 153 g/L fructose was contacted with 33% of activated carbon in the slurry for various times between 10 and 120 min, the results showed a decrease in fructose concentrations in the remaining broth by 6% (Table 5.27). The percentage of recovered fructose, in the broth after carbon treatment, was about 26% regardless of the contact time. In addition, about 72% of the initial broth remained in the carbon after treatment regardless of the contact time. To reduce the amount of broth remained in the carbon, the activated carbon was mixed with water prior to the addition of the broth. This resulted in a considerable decrease in the amount of treated broth remaining in the carbon (Table 5.27). Wetting the carbon prior to broth treatment considerably diluted the broth, however, this increased the fructose recovery in the treated broth. In addition, the fructose concentration in the treated broth using pre-wetted carbon was 8% lower than expected because of the adsorption of fructose onto the carbon. The conductivity slightly decreased in the treated broth with pre-wetted carbon, however, when the carbon was not wetted, the conductivity increased by about 29% (Table 5.27). The pH of the broth increased after carbon treatment. These results are similar to those obtained using broth containing both fructose and ethanol (section 5.3.2.1.1).

Table 5.27 Conductivity and pH values and fructose concentration and recovery using a biomass- and ethanol-free broth containing 153 g/L fructose after treatment with 33% activated carbon for various periods of time.

	Treatment time (min)	Amount (mL)	pH	Conductivity (millimhos)	Fructose (g/L)	Fructose recovery[a] (%)
Untreated broth	----	25	4.86	21.8	153.3	----
Treated broth	10	7.0	5.73	27.8	144.2	26.35
Treated broth	20	6.9	5.76	28.0	144.1	25.95
Treated broth	40	6.9	5.77	28.0	144.4	25.99
Treated broth	60	6.8	5.78	28.1	144.3	25.61
Treated broth	120	6.7	5.81	28.3	144.4	25.24
Treated broth[b]	20	24	5.78	21.6	82.0	51.36

[a] Values are calculated based on the fructose concentration in the broth before carbon treatment

[b] Activated carbon was mixed with 18 mL deionized water before the addition of 25 mL untreated broth

The decolourization efficiency was about 98% when the broth was contacted with 33% of activated carbon in the slurry for 10 min (Table 5.28). A slight increase in decolourization efficiency was noticed when the contact time increased from 10 to 120 min. Since about 98% of colour was removed in the first 20 min of treatment, instead of waiting for another 100 min to remove an additional 1% of colourants, a new treatment step for 10 or 20 min could be considered. These results show that decolourization with powdered carbon is much faster than with granular carbon, which needs about 6 h to decolourize typical Australian raw sugar liquors

Table 5.28 Colour removal from a biomass- and ethanol-free broth containing 153 g/L fructose after treatment with 33% activated carbon for various periods of time.

	Dilution[a]	Total solids[b] (g/L)	Absorbance at 420 nm	Transmittance %	Colour IU	Colour removal[c] (%)
Untreated broth	150	170.1	0.5733	26.71	505,466	----
Treated broth (10 min)	10	156.6	0.1941	63.96	12,396	97.5
Treated broth (20 min)	10	156.2	0.1438	71.81	9,209	98.2
Treated broth (40 min)	10	157.0	0.1307	74.01	8,327	98.4
Treated broth (60 min)	10	157.1	0.1019	79.09	6,486	98.7
Treated broth (120 min)	7	157.1	0.1136	76.98	5,063	99.0
Treated broth[d]	----	90.0	0.4053	39.33	4,505	99.1

[a] Dilutions were made to obtain transmittance in the range of 20-80%

[b] Values are before dilution

[c] Values are calculated based on the colour of the broth before carbon treatment

[d] Activated carbon was mixed with 18 mL deionized water before the addition of 25 mL untreated broth

by 90% (Field and Benecke, 2000). In the present study, more than 99% of the colourants were removed from the broth after treatment with 33% pre-wetted carbon (Table 5.28). The colour of the treated broth mixtures changed from dark black to light brown after treatment with 33% carbon for various times. The sugar and glycerol concentrations in the broth before and after carbon treatment are given in Appendix B (Table B.5). The fructose content in the treated broth, with or without prior carbon wetting, was about 96% of the total sugars, regardless of the contact time. Considering that the fructose recovery in the treated broth was low, it is necessary to increase the recovery by washing out fructose from the spent carbon, which will be discussed next.

5.3.2.1.4 Colour removal by activated carbon followed by washing the carbon to recover fructose

The broth, with an initial fructose concentration of 153 g/L, was contacted for 20 min with 33% of activated carbon in the slurry. The carbon was then removed from the broth by centrifugation and the spent carbon was washed four times, each with 72 mL of fresh deionized water, to recover fructose. The pH and conductivity of the deionized water used to wash the spent carbon were 7.2 and 6.5 μmhos, respectively. The results showed that the pH and conductivity of the treated broth after carbon treatment increased (Table 5.29). The conductivity of the water used for each washing step substantially increased because of minerals washed out from the spent carbon. The pH of the wash water decreased from 7.2 to about 5.8 after each carbon-washing step.

The total fructose recovered, after four washes of the spent carbon with water, was 95% (Table 5.29). The remaining 5% may have been firmly bound to the carbon. Riffer (1993) reported that there is always some retention of sucrose (2-6%) because of strong adsorption on the carbon regardless of the wash water volume. In the present study, mixing all the washouts with the treated broth resulted in diluting the broth by three times. The fructose concentration in the broth after the addition of all washouts was 46 g/L (Table 5.29).

It was found previously that the fructose concentration and recovery were 82 g/L and 51%, respectively, after decolourization of the broth using pre-wetted carbon (Table 5.27). However in this study, when the carbon was used without prior wetting but was then washed once with water, the fructose concentration and recovery in the treated broth plus the first washout

Table 5.29 Conductivity and pH values and fructose concentration and recovery using a biomass- and ethanol-free broth containing 153 g/L fructose after treatment with 33% activated carbon then washing the spent carbon with water.

	Amount (mL)	pH	Conductivity (millimhos)	Fructose (g/L)	Total fructose recovery[a] (%)	Fructose[b] (g/L)
Untreated broth	100	5.00	21.90	152.7	----	----
Treated broth	28	5.70	27.90	137.0	25.11	----
1st carbon washing	72	5.70	20.60	73.7	59.86	91.4
2nd carbon washing	72	5.81	13.38	39.9	78.67	69.9
3rd carbon washing	72	5.83	7.86	20.8	88.49	55.4
4th carbon washing	72	5.87	4.94	12.9	94.55	45.7

[a] Values are calculated based on the fructose concentration in the broth before carbon treatment

[b] Values are calculated based on the addition of all washouts to the broth after carbon treatment

were 91 g/L and 60%, respectively (Table 5.29). This showed that the treatment of broth with prior carbon wetting resulted in a lower fructose concentration and recovery.

The total solids content of the broth used in the decolourization process was 16.9% (w/v) (Table 5.30). This is lower than the total solids in syrups and liquors used in HFCS and sugar industries. In the HFCS industry, syrups containing between 40 and 45% dissolved solids are used in carbon treatment at 60°C (Blanchard and Geiger, 1984). In the sugar industry, the average concentration of the sugar liquor used in decolourization is about 65 Brix and the temperature at which the process is carried out is about 80°C (Field and Benecke, 2000). The decolourization efficiency is commonly 99% at the beginning of the cycle and 90% near the end. At the end of the decolourization cycle, carbon is washed with water to displace the sugar (Riffer, 1993; Field and Benecke, 2000). Water removes most of the sugar but little colourants since the colourants are strongly adsorbed to the carbon. Sugar removal from the column uses approximately three bed volumes of hot water. The effluent from the washed carbon, which has a sugar concentration of about 40 Brix, is added to the intermediate liquor. Intermediate liquor is used to wet fresh carbon added to the column. Once the effluent concentration is below 40 Brix, it is redirected to the sweetwater reservoir. The average concentration of sugar in the sweetwater is about 25 to 30 Brix. The sweetwater is recycled in the refinery process, usually at the sugar melt, to dissolve the sugar to 66 Brix (Pearson, 2000). After the carbon is washed with water, it is sent for regeneration.

In this study, the untreated broth had an initial colour of 518,435 IU (Table 5.30). This is considerably higher than sugar liquors, which have about 2,000 IU prior to carbon treatment (Field and Benecke, 2000). In the present study, 98% of the colour in the broth was removed (Table 5.30). Some colourants were transferred from the spent carbon to the water after each washing step. The glycerol and sugar concentrations in the broth before and after carbon treatment and in the water washouts are given in Appendix B (Table B.6). The fructose content in the treated broth and in the washouts was above 95% of the total sugar concentrations.

Table 5.30 Colour removal from a biomass- and ethanol-free broth containing 153 g/L fructose after treatment with 33% activated carbon then washing the spent carbon with water.

	Dilution[a]	Total solids[b] (g/L)	Absorbance at 420 nm	Transmittance %	Colour IU	Colour removal[c] (%)
Untreated broth	150	169.0	0.5840	26.06	518,435	----
Treated broth	10	147.2	0.1393	72.56	9,461	98.2
1st carbon washing	3	81.1	0.1081	77.97	3,998	----
2nd carbon washing	----	43.6	0.1253	74.94	2,871	----
3rd carbon washing	----	23.0	0.0403	91.14	1,754	----
4th carbon washing	----	14.2	0.0177	96.01	1,249	----

[a] Dilutions were made to obtain transmittance in the range of 20-80%
[b] Values are before dilution
[c] Values are calculated based on the colour of the broth before carbon treatment

This study showed that about 95% of the fructose was recovered in the treated broth and carbon washouts. However, the concentration of fructose in the broth after additions of washouts was 46 g/L. To concentrate this broth to contain 71% dissolved solids, as in the HFCS industry, requires a costly evaporation process. For that reason, the incorporation of a membrane technique and activated carbon could improve the economy of the decolourization process by decreasing the amount of carbon needed and producing a treated broth with a higher fructose concentration. This is discussed in the next section.

5.3.2.2 Colour removal by membranes followed by activated carbon

Membrane technology has been used in sugar industry for concentration, clarification and decolourization of sugar juices (Saska, 2000). The decolourization effect of a membrane depends on its nature, pore size (MWCO) and concentration and property of the feed and operating conditions. The nature of colourants (i.e. natural or formed during processing) and their intensity in sugar liquors also influence colour removal efficiency. Natural colourants are characterized by a low molecular weight (below 900 Daltons). However, those produced during sugar processing cover a wide range of molecular weights from below 1,000 to above 150,000 Daltons (Decloux et al., 2000). Chen (1993a) reported that the predominant colouring matter in raw sugar and molasses is represented by a molecular weight range of 3,000 to 30,000 Daltons. Membranes with MWCO between 5,000 and 300,000 Daltons have been used for decolourization of sugar liquors (Cartier et al., 1997; Saska, 2000; Decloux et al., 2000; Karode et al., 2000). The decolourization efficiency generally increased using smaller pore size membranes (smaller MWCO), however, such membranes provide low flux rates of permeate. In the sugar industry, a membrane capable of 50% colour reduction along with a steady permeate flux of 50 L/m^2 h would have an economic potential (Nielsen et al., 1982).

In the present study, two types of ultrafiltration membranes were used, a commercial polyethersulfone (PES) membrane of 10,000 MWCO and a thin film composite (TFC) with MWCO of 1,000 Daltons prepared by the Industrial Membrane Research Institute (IMRI). Prior to beginning the tests, microfiltration of the broth containing about 148 g/L fructose was carried out using a 0.45 μm nitrocellulose membrane to remove suspended solids. This would reduce the fouling effects of suspended solids on the ultrafiltration membranes. The filtered broth was then used with the PES and TFC membranes for decolourization. The results showed that the flux of the broth through the 0.45 μm nitrocellulose membrane was about 274 L/m^2 h, while the permeate flux rates of the PES and TFC membranes were 1.95 and 0.29 L/m^2 h, respectively (Tables 5.31 and 5.32). The low flux rate of the PES and TFC membranes is due to their small pore size. The concentration of fructose, conductivity and pH of the filtered broth using the 0.45 μm nitrocellulose membrane did not significantly change. Furthermore, there were no significant changes in the pH or fructose concentration in the permeate or retentate of both membranes. However, the conductivity of the permeates of both membranes was about 19% higher than that of the untreated broth, while the conductivity of the retentates was slightly lower than the untreated broth. This could be due to concentrating minerals in the permeates rather than in the retentates. After carbon treatment of the permeates of both membranes, the pH and conductivity of the treated broth increased (Tables 5.31 and 5.32). This is consistent with the results obtained when only carbon was used for colour removal from the broth (section 5.3.2.1).

Table 5.31 Flux, conductivity and pH values and fructose concentration and recovery using a biomass- and ethanol-free broth containing 148 g/L fructose after treatments with PES 10,000 MWCO membrane followed by activated carbon.

	Amount (mL)	Time collected (h)	Flux (L/m^2 h)	pH	Conductivity (millimhos)	Fructose (g/L)	Fructose recovery[a] (%)
Untreated broth	280	-----	-----	4.85	21.6	148.0	-----
Filtered broth	200	0.75	274.07	4.83	21.7	146.5	-----
Permeate	18	9.50	1.95	4.85	25.7	147.6	-----
Retentate	82	9.50	-----	4.84	21.0	142.4	-----
Permeate (1st treatment 3% carbon)	15[b]	0.33	-----	5.00	26.5	144.2	87.95
Permeate (2nd treatment 3% carbon)	12.5[b]	0.33	-----	5.16	27.2	142.5	86.94
Permeate (3rd treatment 1.5% carbon)	9.5[b]	0.33	-----	5.31	27.3	142.1	94.48

[a] Values are calculated based on the fructose concentration in the permeate before each carbon treatment

[b] Amount treated with activated carbon

Table 5.32 Flux, conductivity and pH values and fructose concentration and recovery using a biomass- and ethanol-free broth containing 148 g/L fructose after treatments with TFC 1,000 MWCO membrane followed by activated carbon.

	Amount (mL)	Time collected (h)	Flux (L/m^2 h)	pH	Conductivity (millimhos)	Fructose (g/L)	Fructose recovery[a] (%)
Untreated broth	280	-----	-----	4.85	21.6	148.0	-----
Filtered broth	200	0.75	274.07	4.83	21.7	146.5	-----
Permeate	7	25.00	0.29	4.91	26.0	154.6	-----
Retentate	43	25.00	-----	4.76	21.4	146.4	-----
Permeate (treatment 3% carbon)	5[b]	0.33	-----	5.10	26.5	150.1	85.45

[a] Values are calculated based on the fructose concentration in the permeate before carbon treatment

[b] Amount treated with activated carbon

No significant changes in the colour of the filtered broth when using the 0.45 μm nitrocellulose membrane were noticed, while the decolourization efficiencies of the PES and TFC membranes were 94 and 99%, respectively (Tables 5.33 and 5.34). The high decolourization efficiency of the TFC membrane compared to the PES membrane is due to its smaller pore size and its chemical characteristics. The TFC membrane was coated with a negatively charged material. This could have assisted in increasing colour removal by preventing negatively charged colourants from passing through the membrane. This is because large portion of sugar colourants are negatively charged (Carpenter and Clarke, 1977; Riffer, 1993).

Table 5.33 Colour removal from a biomass- and ethanol-free broth containing 148 g/L fructose after treatments with PES 10,000 MWCO membrane followed by activated carbon.

	Dilution[a]	Total solids[b] (g/L)	Absorbance at 420 nm	Transmittance %	Colour IU	Colour removal[c] (%)
Untreated broth	150	171.6	0.5579	27.68	487,590	----
Filtered broth	150	169.7	0.5679	27.05	501,944	----
Permeate	10	172.6	0.4913	32.26	28,465	94.2
Retentate	150	167.5	0.7049	19.73	631,216	----
Permeate (1st treatment 3% carbon)	10	167.7	0.1238	75.20	7,384	98.5
Permeate (2nd treatment 3% carbon)	----	165.6	0.3833	41.37	2,314	99.5
Permeate (3rd treatment 1.5% carbon)	----	164.9	0.2344	58.29	1,422	99.7

[a] Dilutions were made to obtain transmittance in the range of 20-80%
[b] Values are before dilution
[c] Values are calculated based on the colour of the broth before filtration

Table 5.34 Colour removal from a biomass- and ethanol-free broth containing 148 g/L fructose after treatments with TFC 1,000 MWCO membrane followed by activated carbon.

	Dilution[a]	Total solids[b] (g/L)	Absorbance at 420 nm	Transmittance %	Colour IU	Colour removal[c] (%)
Untreated broth	150	171.6	0.5579	27.68	487,590	----
Filtered broth	150	169.7	0.5679	27.05	501,944	----
Permeate	10	183.6	0.1186	76.10	6,460	98.7
Retentate	150	170.5	0.6799	20.90	598,047	----
Permeate (treatment 3% carbon)	----	178.0	0.0953	80.30	535	99.9

[a] Dilutions were made to obtain transmittance in the range of 20-80%
[b] Values are before dilution
[c] Values are calculated based on the colour of the broth before filtration

The flux rates of the PES 10,000 and TFC 1,000 MWCO membranes used in this study were more than three times lower than those reported by other researcher for a PES membrane of 5,000 MWCO (Karode et al., 2000). However, the decolourization efficiencies obtained in this study were 9 to 14% higher than those of the 5,000 MWCO membrane. In a study to decolourize a 50 Brix raw sugar cane solution of initial colour of 3,200 IU using mineral membranes with 15,000 and 300,000 MWCO, the decolourization efficiencies were 39 and 20%, respectively (Cartier et al., 1997). The decolourization efficiencies increased to 58 and 50%, respectively, when a flocculating agent was added to the sugar solution before membrane separation. The flux rates of permeate of the 15,000 and 300,000 MWCO membranes, with or without flocculation, were 25 and 65 L/m^2 h, respectively. The flux rate of the permeate of a mineral membrane with 300,000 MWCO was about 200 L/m^2 h when it was used to remove the turbidity of a 14 Brix sugar juice (Saska, 2000). The turbidity of the sugar juice was almost completely removed, however, the colour of the juice decreased by only 2% from 11,600 IU. Decloux and coworkers (2000) reported that the decolourization efficiency of a 30 Brix raw sugar cane syrup with a colour of 3,720 IU was 64% using a mineral ultrafiltration membrane with a permeate flux rate of 47 L/m^2 h. The differences in the flux rates and decolourization efficiencies between the various kinds of membranes are attributed to differences in the type and nature of the feed and operating conditions used with these membranes.

The colour of the broth used in this study changed from black (487,590 IU) to brown in colour (28,465 IU) after using the PES membrane and to light brown (6,460 IU) after the TFC membrane (Figure B.2). However, the colour of the treated broth using membrane separation is still considerably high, compared to the colour of HFCS, which is 5 IU (Blanchard and Geiger, 1984).

Further treatment of the permeates of both membranes was done with activated carbon to remove the remaining colourants. The permeate of the PES membrane was treated with 7.5% total carbon in the slurry in three steps, while the permeate of the TFC membrane was treated once with 3% carbon. The results showed that about 87% of the fructose was recovered after the first and second treatments of the permeate of the PES membrane with 3% carbon (Table 5.31). However, a higher fructose recovery was noticed after the third treatment with 1.5% carbon. A fructose recovery of 85% was obtained after the treatment of the permeate of the TFC membrane with 3% carbon (Table 5.32). The incomplete fructose recovery after carbon treatment was due to the loss of treated permeates in the carbon and the adsorption of fructose. After treatment with 3% carbon, the percentage of permeates of both membranes remaining in the carbon was about 11%. However, it was 5% after the third treatment with 1.5% carbon for the permeate of the PES membrane. In addition, a decrease of approximately 1 to 3% in the fructose concentration in the permeates after each carbon treatment was noticed (Tables 5.31 and 5.32).

The total fructose recovered, after the carbon treatments of the permeates of the PES and TFC membranes, were 74 and 85%, respectively. However when only carbon treatment was used, the fructose concentration of the treated broth decreased by 10%, while about 72% of the broth remained in the carbon after the treatment with 33% carbon as shown previously (Table 5.29). In addition, the total fructose recovery was only 25% prior to recovering fructose by washing the spent carbon. The washing steps diluted the treated broth.

The colour of the permeate of the PES membrane decreased from 28,465 to 1,422 IU after three treatment steps with 7.5% total carbon (Table 5.33). When the permeate of the TFC membrane was treated with 3% carbon, its colour decreased from 6,460 to 535 IU (Table 5.34). The colour removal efficiencies from the permeate of the PES membrane, after each treatment with 3% carbon, were 74 and 69%, respectively. However, when the permeate of the TFC membrane was treated with 3% carbon, the decolourization efficiency was 92%. This could be due to the differences in the nature and types of colourants that are present in the permeates of both membranes. The colour removal efficiency from the broth, after its treatment with the membranes and carbon, was above 99.7% (Tables 5.33 and 5.34). This showed that the use of membranes and carbon for colour removal results in a higher degree of decolourization than if only carbon was used. Furthermore, the use of membranes drastically decreased the amount of carbon needed for the decolourization process.

The colour of the permeate of both membranes after carbon treatment changed from brown to almost clear in colour (Figure B.2). The concentrations of the sugars and glycerol in the broth before and after membrane separation and carbon treatments are given in Appendix B (Tables B.7 and B.8). The fructose content in the broth, after membrane separation and carbon treatment, was greater than 91% of the total sugar concentrations. A considerable drop in the raffinose concentration was noticed with the use of membranes and carbon. No raffinose was noticed in the permeate of the TFC membrane (Table B.8). This could be due to the agglomeration of molasses colourants which hinders raffinose flow through the membrane, even though this sugar has a lower molecular weight than the TFC membrane MWCO. Vercellotti and coworkers (1998) reported that sugar cane molasses colourants agglomerated and formed a fouling layer on a 15,000 MWCO ceramic membrane during the processing of a 15 Brix sugar liquor. They also found that materials such as starch, dextrans and possible complexes of polysaccharide and colourants were implicated in membrane fouling. It was suggested that these materials prevented smaller molecules from passing through the membrane.

In the present study, the results showed that the decolourization efficiency increased when the smaller pore size membrane was used, but the permeate flux rate decreased. The decolourization efficiency from the PES membrane was around 94%, while it was about 5% higher from the TFC membrane. The flux rate of the PES membrane was more than six times higher than the TFC membrane. When the permeate of both membranes was further treated with activated carbon, the total percentage of colour removal was above 99.7% of the initial broth colour. The fructose concentration in the treated broth after membrane separation and carbon treatment did not change significantly. The total fructose recovered, in the treated broth using the PES and TFC membranes and carbon, were 74 and 85%, respectively. Considering that carbon removed colour from the broth but could not remove minerals, the conductivity of the broth remained high (in the order of 10^4 µmhos). The removal of minerals using a mixture of anion and cation exchange resins from the broth after colour removal is discussed below.

5.3.3 Minerals removal

The demineralization of fructose syrups and other sugar liquors can be carried out using a cation-anion multiple bed system or a combination of anionic and cationic resins in a mixed bed unit (Carpenter and Clarke, 1977; Blanchard and Geiger, 1984). The cation exchange resin replaces any cations that are present in the syrup by hydrogen ions, forming free acids. The anion exchange resin then adsorbs the acid, leaving the syrup free of ions. The use of a mixed bed system means that any hydroxide alkalinity resulted from the anion exchange resin is directly removed by the cation exchange resin. Therefore, adverse effects of hydroxide alkalinity that may cause degradation of fructose are largely eliminated. However, a careful control of temperature, residence time and the amount of anion exchange resin used are also important to avoid the degradation of fructose (Blanchard and Geiger, 1984).

In this study, mineral removal was carried out using a mixture of anion and cation exchange resins (Rexyn I-300). About 286 mL of biomass- and ethanol-free broth, after carbon treatment and addition of water washouts as shown in section 5.3.2.1.4, was passed through a 28 mL bed of Rexyn I-300 resin. The resin in the column was saturated after one passage of the broth. The broth from the first column was then passed through four other similar resin beds.

The flow rate of the broth, conductivity, pH and glucose and fructose concentrations in the broth after each bed treatment are shown in Figure 5.54. The conductivity decreased from 1.47 × 10^4 to 6 µmhos after passing the broth through the five beds. The average flow rate of the broth through all beds was about 8.5 mL/min. The pH of the broth increased from 5.8 to 10.8 after the third bed. With further treatment of the broth, the pH decreased to 7.1 after the fifth bed. This showed that, after treatment with each resin bed, the pH of broth was in the alkaline region, which could be due to the presence of more anions in the broth. In addition, optimizing the ratio of the amounts of anion to cation exchange resins is important so that the pH of sugar liquors is maintained between 7 and 8 during the demineralization process (Bourée, 2000).

The fructose concentration and recovery decreased after passing the broth through each resin bed (Figure 5.54). The fructose concentration in the broth decreased by half after the fourth bed. However, the glucose concentration was not significantly changed. The large loss in fructose

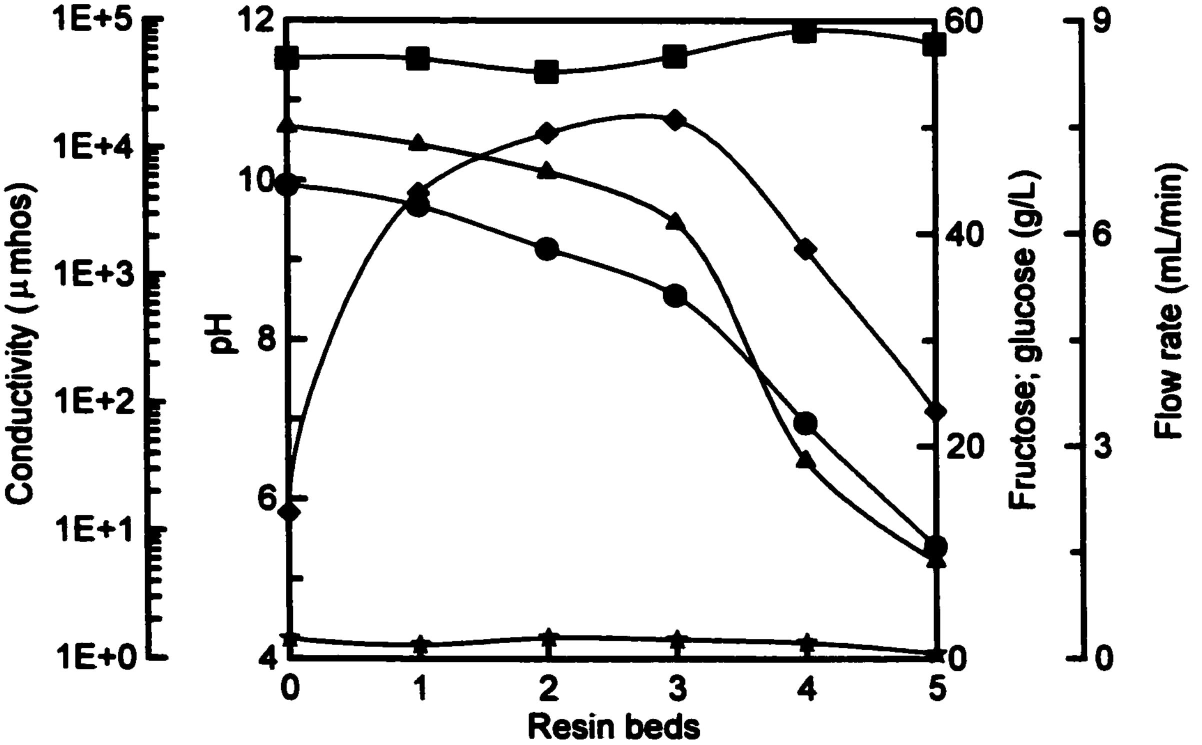

Figure 5.54 Deionization of the fructose broth with a mixture of anion and cation exchange resins: (▲) conductivity; (◆) pH; (●) fructose; (★) glucose; (■) flow rate.

may be due to its degradation at high pH values (Blanchard and Geiger, 1984; Field and Benecke, 2000). A 25% fructose loss was reported during processing of fructose syrups produced from Jerusalem artichoke, primarily during the ion exchange treatment (Fleming and GrootWassink, 1979). Koren and Duvnjak (1989) reported a loss of fructose, glucose and ethanol by 13.6, 12.5 and 25.5%, respectively, after deionization of a product containing 78 g/L fructose, 6 g/L glucose and 55 g/L ethanol produced from a mixture of HFCS and Jerusalem artichoke juice.

The broth before deionization has 5,519 IU (Table 5.35). The colour of the broth after passing through the first bed increased by 47%, which could be due to the formation of new colourants in the treated broth as a result of increasing the pH from 5.8 to 9.8 (Figure 5.54). It was reported that alkaline degradation products of fructose and to a lesser extent, glucose, particularly at pH levels above 7.5 contributes to additional colourants (Blanchard and Geiger, 1984; Field and Benecke, 2000). In the present study, the colour of the broth decreased after the second resin bed. The use of ion exchange resins not only removed minerals but also colourants.

Table 5.35 Colour removal after passing the treated broth, after the addition of washouts, through five beds of ion exchange resins.

	Dilution[a]	Total solids[b] (g/L)	Absorbance at 420 nm	Transmittance %	Colour IU	Colour removal[c] (%)
Untreated broth	150	169.0	0.5840	26.06	518,435	----
Treated broth after addition of washouts	----	49.4	0.2727	53.37	5,519	98.9
1st bed	----	46.4	0.3781	41.87	8,147	98.4
2nd bed	----	42.6	0.1292	74.27	3,032	99.4
3rd bed	----	38.5	0.0350	92.26	908	99.8
4th bed	----	26.2	0.0131	97.03	500	99.9
5th bed	----	12.8	0.0085	98.06	667	99.9

[a] Dilutions were made to obtain transmittance in the range of 20-80%
[b] Values are before dilution
[c] Values are calculated based on the colour of the untreated broth

Blanchard and Geiger (1984) reported that some colour compounds that are not adsorbed by carbon are ionized and therefore retained by ion exchange resins. They found that up to 70% of nitrogenous materials such as amino acids in the HFCS were removed by cation exchange resins. The colour of the broth in this study changed from dark black to nearly colourless after decolourization and deionization treatments (Figure B.2). The concentrations of sugars and glycerol in the broth during the demineralization process are given in Appendix B (Table B.9). The fructose content in the treated broth was above 96% of the total sugar concentrations.

The industrial HFCS has 70% total solids, a colour of 5 IU and a conductivity of 5-10 μmhos (Blanchard and Geiger, 1984; Coker and Venkatasubramanian, 1987). The results of the present study showed that the demineralization process using a mixture of anionic and cationic resins resulted in removal of most of the minerals. The conductivity of the broth after this process was in the specification range for industrial HFCS. However, a large loss of fructose (75%) in the treated broth was noticed. The colour of the broth after demineralization was 667 IU. Although the purification process is not economical, the results showed that colour and minerals could be removed from the broth. It should be noted that these results are preliminary. They may be useful for those working on the removal of colour and minerals from fructose syrups produced from cane molasses. In the next section, the modelling of the production of fructose and ethanol by *S. cerevisiae* ATCC 36858 in batch fermentations in media containing sucrose and raffinose is discussed.

5.4 Modelling of batch fermentations for the production of fructose and ethanol

The proposed models that were described in sections 3.4.2 and 3.4.3 were used to fit the experimental results obtained in the study of the production of fructose and ethanol by *S. cerevisiae* ATCC 36858 in synthetic media with sucrose and raffinose, respectively. The estimation of the model parameters and the prediction of the results were carried out using MicroMath scientist software, version 2.0 (1995). In this software, a nonlinear least squares regression using the Levenberg-Marquardt algorithm was used to find the optimal set of parameter values for fitting the model to a set of experimental data by minimizing the sum of the squared residuals between the observed data and model predictions. The models ordinary differential equations were solved numerically using the modified Adam's algorithm for stiff systems (MicroMath, 1995). The format used for model equations in the analysis of the tests carried out in sucrose and raffinose media with MicroMath scientist software is given in Appendix C.

5.4.1 Sucrose media

The model predictions for the production of fructose and ethanol in synthetic media with various initial sucrose and biomass concentrations are discussed. This was done to study the suitability of the proposed model in section 3.4.2 for a wide range of experimental conditions.

5.4.1.1 Effect of initial sucrose concentration

The data collected from experiments in synthetic media with initial sucrose concentrations between 35 and 257 g/L were used in the analysis. The experimental values of the parameters obtained in these media were previously given in Table 5.4 (section 5.2.2.1). The estimated values of the parameters in the proposed model (Eqs. 3.27-3.31), for runs with various initial sucrose concentrations, are shown in Table 5.36. The trends of the values of the estimated parameters were similar to those obtained experimentally. For example, the predicted parameters μ_{max} and $Y_{X/S}$ decreased with increasing initial sucrose concentrations (Table 5.36), which was also noticed experimentally (Table 5.4). In addition, similar values for both the experimental and the estimated parameters were obtained. In order to obtain the general parameter estimates, average sets of parameters were calculated for runs with sucrose concentrations between 35 and 81 g/L, and 122 and 257 g/L, respectively (Table 5.37).

The values of the estimated parameters obtained in this study differed from those reported by other researchers. This is partially due to the fact that no work has been performed with the yeast *S. cerevisiae* ATCC 36858, which was used in this study. In addition, no models were found in the literature that describe the simultaneous production of fructose and ethanol from sucrose media. However, Koren and Duvnjak (1993) proposed a model for the production of fructose and ethanol using *S. cerevisiae* ATCC 36859 in media with glucose/fructose mixtures with total carbohydrate concentrations between 222 and 413 g/L. In the present study, the obtained average values of μ_{max} were 0.256 and 0.167 h^{-1} in media with sucrose concentrations between 35 and 81 g/L, and 122 and 257 g/L, respectively (Table 5.37). For the former range of sucrose concentrations, the obtained μ_{max} was about 30% lower than that reported by Koren and Duvnjak (1993). Furthermore, the μ_{max} in the present study was much lower than the value of 0.4 h^{-1} that was reported for the production of ethanol by wild strains of *S. cerevisiae* in glucose media with concentrations between 10 and 20 g/L (Aiba et al., 1968), and 50 and 200 g/L (Ghose and Tyagi, 1979b). Starzak et al. (1994) obtained various values for μ_{max} (between 0.159 and

0.461 h^{-1}) when different models were used to fit their experimental results for the production of ethanol by *S. cerevisiae* in media with sucrose concentrations between 130 and 210 g/L.

Table 5.36 Parameter estimates using data collected from individual experiments with various initial sucrose concentrations.

Sucrose (g/L)	μ_{max} (h^{-1})	K_S (g/L)	h_S (g/g h)	K_{SS} (g/L)	$Y_{G/SUC}$ (g/g)	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)
34.6	0.288	2.567	4.457	0.168	0.590	0.196	0.424
80.6	0.224	3.571	15.046	9.956	0.495	0.194	0.455
121.6	0.208	11.073	16.740	23.250	0.518	0.149	0.440
169.3	0.174	11.338	20.135	27.690	0.482	0.123	0.460
216.4	0.157	11.669	18.724	49.206	0.503	0.100	0.447
257.4	0.128	16.849	18.267	51.950	0.496	0.076	0.453

Table 5.37 Average parameter estimates for experiments with a range of sucrose concentrations.

	Sucrose between 35 and 81 g/L			Sucrose between 122 and 257 g/L		
	Average	95% Confidence intervals		Average	95% Confidence intervals	
		Lower	Upper		Lower	Upper
μ_{max} (h^{-1})	0.256	0.194	0.318	0.167	0.134	0.199
K_S (g/L)	3.069	2.084	4.053	12.732	10.032	15.433
h_S (g/g h)	9.751	-0.626	20.128	18.467	17.096	19.837
K_{SS} (g/L)	5.062	-4.530	14.654	38.023	23.664	52.382
$Y_{G/SUC}$ (g/g)	0.543	0.449	0.636	0.500	0.485	0.515
$Y_{X/S}$ (g/g)	0.195	0.193	0.197	0.112	0.080	0.143
$Y_{P/S}$ (g/g)	0.440	0.409	0.471	0.450	0.442	0.459

In this study, the average values of the saturation constant K_S (3.069 and 12.732 g/L), in media with sucrose concentrations between 35 and 81 g/L, and 122 and 257 g/L, respectively, were considerably higher than those predicted by Aiba et al. (1968) (0.22 g/L) and Ghose and Tyagi (1979b) (0.48 g/L). Koren and Duvnjak (1993) reported a value for the saturation constant of 4.56 g/L, while Starzak et al. (1994) obtained values for K_S between 0.76 and 45.0 g/L.

The average values for the specific sucrose uptake rate, h_S, were 9.751 and 18.467 g/g h in media with sucrose concentrations from 35 to 81 g/L and 122 to 257 g/L, respectively (Table 5.37). For the previous ranges of sucrose concentrations, the average values of the saturation constant for sucrose uptake, K_{SS}, were 5.062 and 38.023 g/L, respectively. Barford and Hall (1981) reported values for h_S (5.95 g/g h) and K_{SS} (0.095 g/L) for their model in which a similar form for sucrose uptake, as in Eq. (3.28), was used to describe the aerobic growth of *S. cerevisiae* in media with 10 g/L sucrose. Fontana et al. (1992) also used a similar form of sucrose uptake, as in Eq. (3.28), to model sucrose hydrolysis activity of *S. cerevisiae* aggregates in media with 50 g/L of this disaccharide. They obtained values for h_S and K_{SS} of 8.3 g/g h and 12.0 g/L, respectively.

The average values of the glucose/fructose yield, $Y_{G/SUC}$, were 0.543 and 0.500 g/g in media with sucrose concentrations between 35 and 81 g/L, and 122 and 257 g/L, respectively (Table 5.37). For these ranges of sucrose concentrations, the average values of the biomass yield, $Y_{X/S}$, were 0.195 and 0.112 g/g, while those for ethanol yield, $Y_{P/S}$, were 0.440 and 0.460 g/g, respectively. This is comparable to the ethanol yield (0.466 g/g) reported by Koren and Duvnjak (1993), however, they obtained a biomass yield of 0.140 g/g. The average values of $Y_{X/S}$ and $Y_{P/S}$, which were obtained in the model used by Aiba et al. (1968), were 0.10 and 0.35 g/g, respectively. Ghose and Tyagi (1979b) reported average values of $Y_{X/S}$ and $Y_{P/S}$ of 0.09 and 0.47 g/g, respectively. Starzak et al. (1994) obtained values for $Y_{X/S}$ in the range of 0.035 to 0.092 g/g and for $Y_{P/S}$ in the range of 0.286 to 0.441 g/g. Considering that the yeast strain and the model used in the present study are not the same as those reported previously, it is expected that there will be differences in the values of the model parameter estimates.

Plots comparing the experimental and predicted results for the production of fructose and ethanol used in this analysis are shown in Figures 5.55 to 5.57. The solid lines are the predicted results using the respective estimated parameters in Tables 5.36. The dashed lines are the model predictions using the average sets of parameters that were calculated for runs with initial sucrose concentrations between 35 and 81 g/L, and 122 and 257 g/L, respectively (Table 5.37).

Figure 5.55 show the results of tests carried out in media containing 35 and 81 g/L sucrose, respectively. A very good agreement between the model predictions and experimental data was observed using the parameters in Table 5.36. In general, there was about a 5 to 10% difference between the model predictions and experimental data. When the average parameters were used (Table 5.37), the model also predicted the patterns observed in the respective experiments in both sucrose media (Figure 5.55), however, there was approximately 9 to 22% difference between the model predictions and experimental data. Taking into consideration that there is a difference between the values of individual sets of parameters, it is normal to expect

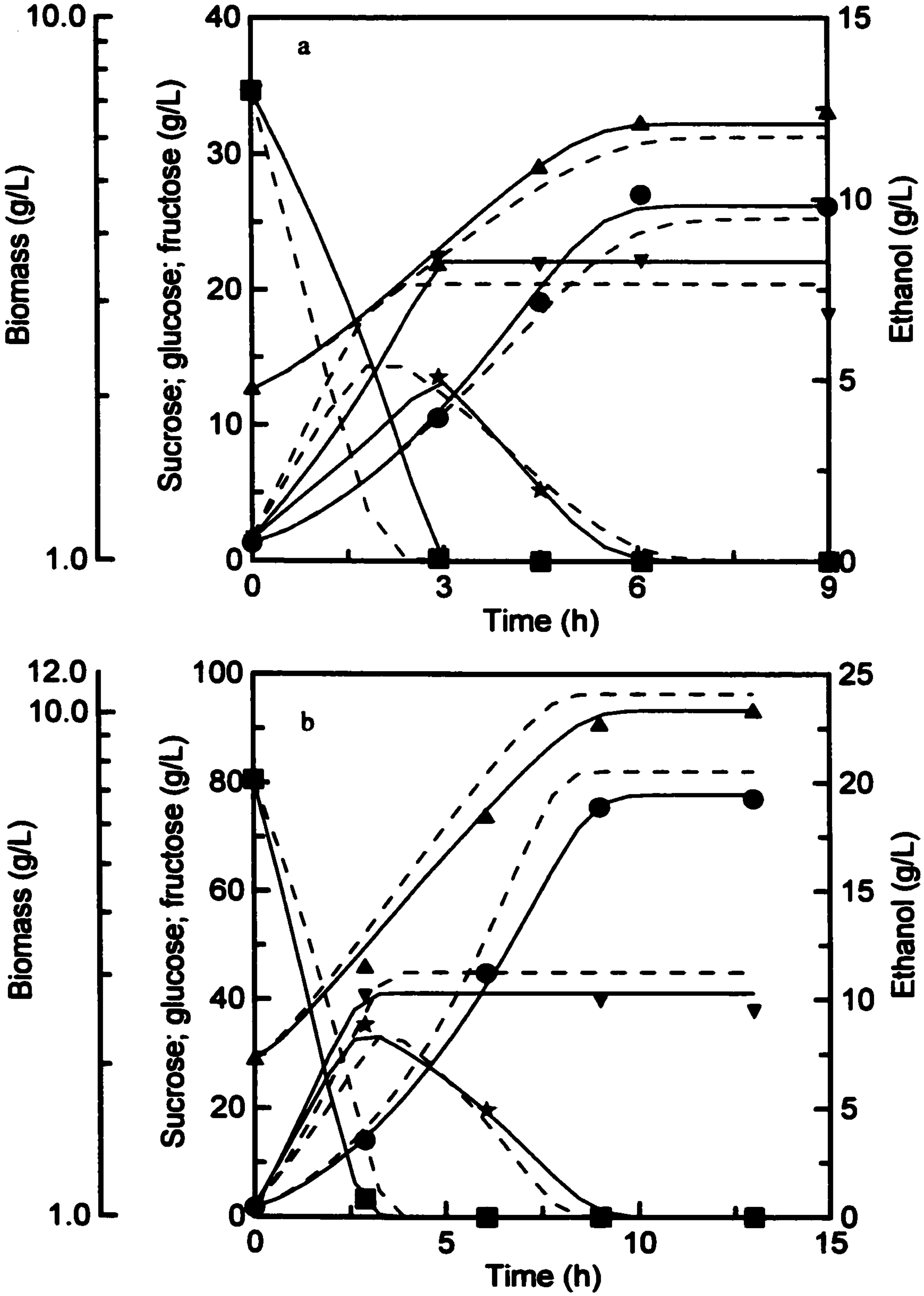

Figure 5.55 Experimental (symbols) and predicted results (———— individual; — — — average parameters in Tables 5.36-5.37) from tests carried out in media containing a) 35 g/L and b) 81 g/L sucrose: (▲) biomass; (●) ethanol; (■) sucrose; (★) glucose; (▼) fructose.

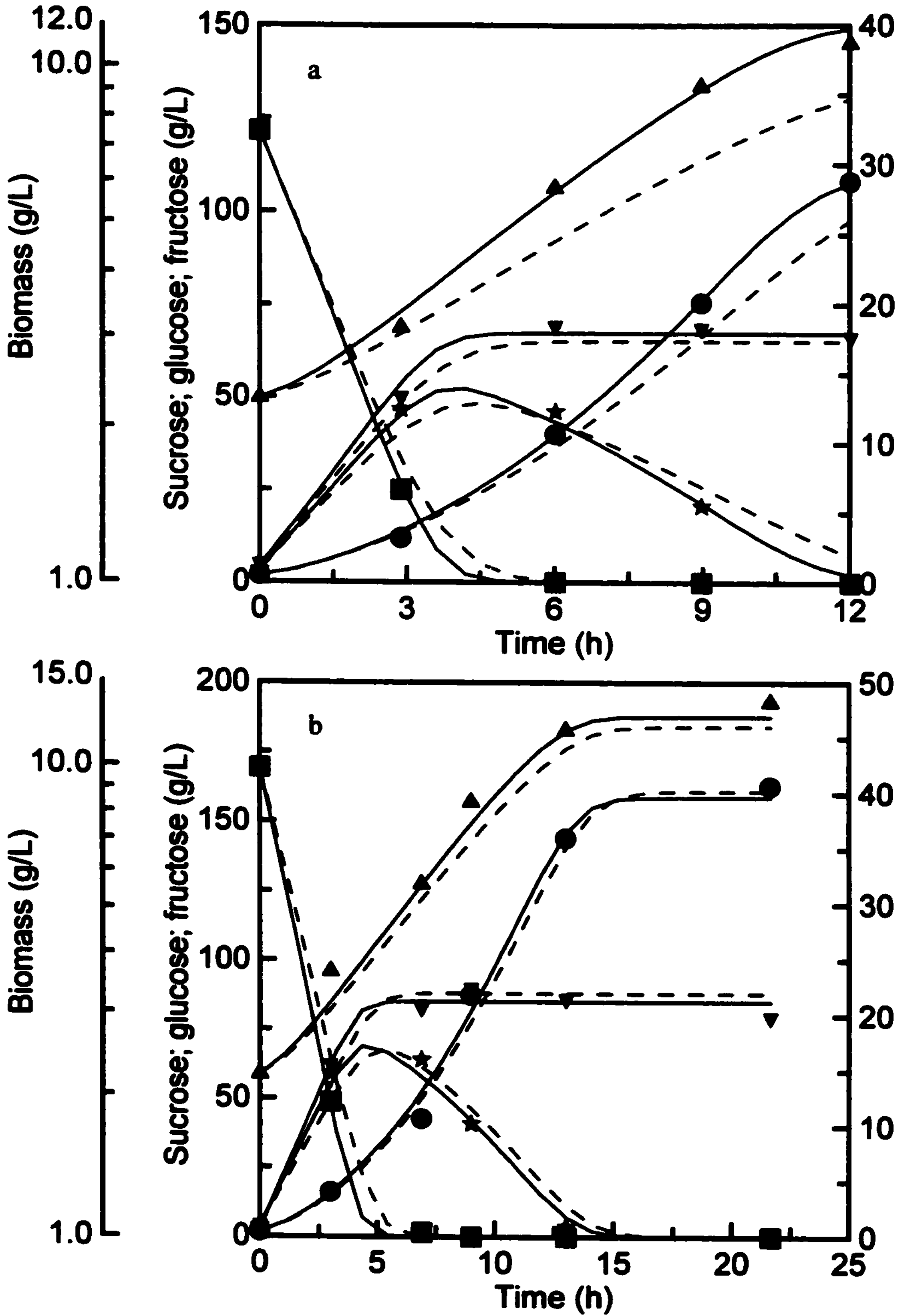

Figure 5.56 Experimental (symbols) and predicted results (———— individual; — — — average parameters in Tables 5.36-5.37) from tests carried out in media containing a) 122 g/L and b) 169 g/L sucrose: (▲) biomass; (●) ethanol; (■) sucrose; (★) glucose; (▼) fructose.

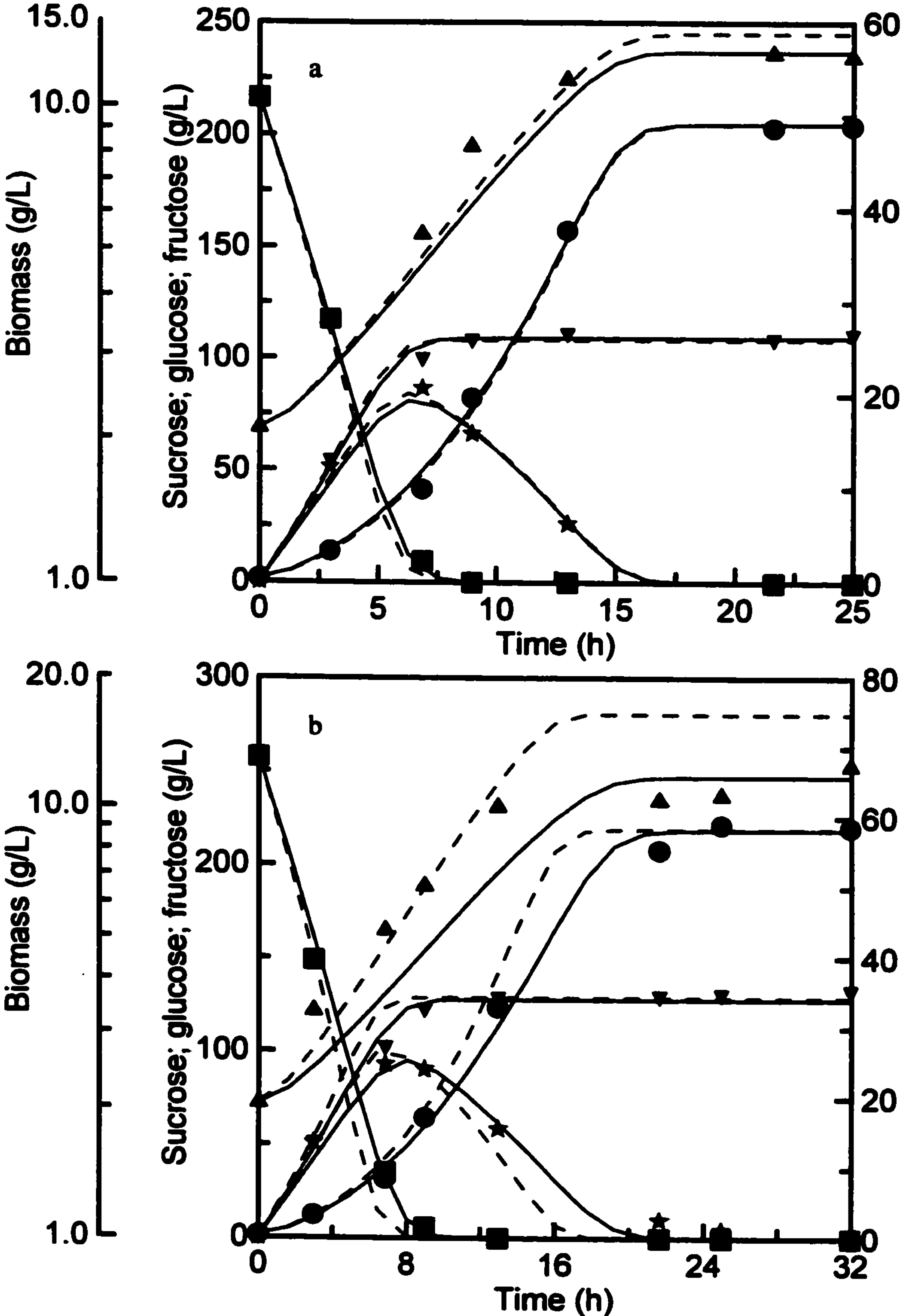

Figure 5.57 Experimental (symbols) and predicted results (———— individual; — — — average parameters in Tables 5.36-5.37) from tests carried out in media containing a) 216 g/L and b) 257 g/L sucrose: (▲) biomass; (●) ethanol; (■) sucrose; (★) glucose; (▼) fructose.

that the model predictions using the average values of the parameters is not as good as when the individual values of the parameters are used. Using the average values of the parameters, the model underpredicted biomass, ethanol, sucrose and fructose concentrations in the medium with 35 g/L sucrose, while it overpredicted these variables when the sucrose concentration was 81 g/L. These model predictions for the experimental data depend on how much the average values of the parameters differ from the individual ones.

Considering the processes with 122 and 169 g/L sucrose, there was a good agreement between the model predictions and experimental data using the individual parameters (Figure 5.56, Table 5.36). When the average values of the parameters (Table 5.37) were used, the model underpredicted biomass, ethanol and fructose, while it overpredicted sucrose and glucose concentrations in the medium with 122 g/L sucrose. However, in the medium with 169 g/L sucrose, the model underpredicted biomass concentrations but overpredicted the concentrations of the other variables. An increase in initial sucrose concentrations above 169 g/L resulted in model underpredictions of exponential growth phase data when either individual or average values of the parameters were used (Figure 5.57). However, the model adequately predicted the stationary growth phase in both media. The model predictions for the other variables (sucrose, glucose, fructose and ethanol) in media with 216 and 257 g/L sucrose were also in good agreement with the experimental values using the individual values of the parameters (Table 5.36).

The analysis of the model predictions for the production of fructose and ethanol in media with various initial sucrose concentrations showed that the model predicted the main variables that were experimentally followed very well when individual parameter estimates were used, and reasonably well when the average values of these parameters were applied. In the next section, model predictions for the process with various initial biomass concentrations in sucrose media are shown and discussed.

5.4.1.2 Effect of initial biomass concentration

The experimental data in section 5.2.2.3 for the production of fructose and ethanol in media with about 180 and 279 g/L sucrose and various initial biomass concentrations were used in parameters estimation of the previously shown model (Eqs. 3.27-3.31). This was done to examine the applicability of the model to predict the experimental data in fermentation media with various initial biomass concentrations. In addition, the reason for using media with initial sucrose concentrations of about 180 and 279 g/L was to produce syrups with about 10-15% (w/v) fructose and 4-7% (w/v) ethanol. Such initial sugar concentrations are also similar to those usually used in industrial fermentation processes.

5.4.1.2.1 Media with about 180 g/L sucrose

The experimental fructose, ethanol and biomass yields, specific growth rates and specific initial rates of sucrose hydrolysis in media with about 180 g/L sucrose and various initial biomass concentrations are shown in Table 5.8. The estimated values of the parameters in the proposed model, for each run, are shown in Table 5.38. The trends of the values of estimated parameters were similar to those obtained experimentally. The predicted parameters, $Y_{X/S}$ and $Y_{P/S}$ were higher than those obtained experimentally for the whole range of initial biomass concentrations. The estimated μ_{max} values were higher by as much as 22% than the experimental values when the initial biomass concentrations were above 6.7 g/L. The predicted h_S values were lower than those obtained experimentally. The estimated and experimental values of $Y_{G/SUC}$ were very close. The predicted K_S and K_{SS} values were between 3.395 and 8.670 g/L, and 2.367 and 10.000 g/L, respectively (Table 5.38). In order to obtain the general parameter estimates, an average set of parameters was calculated from all runs (Table 5.39).

Table 5.38 Parameter estimates using data collected from individual experiments with various initial inoculum concentrations and about 180 g/L sucrose.

Sucrose (g/L)	Initial biomass (g/L)	μ_{max} (h^{-1})	K_S (g/L)	h_S (g/g h)	K_{SS} (g/L)	$Y_{G/SUC}$ (g/g)	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)
187.9	1.27	0.160	4.940	20.987	10.000	0.462	0.110	0.487
175.5	2.71	0.152	7.430	15.665	7.921	0.502	0.104	0.463
175.8	4.29	0.145	4.468	12.100	9.499	0.513	0.103	0.449
178.2	4.97	0.144	8.670	9.749	8.789	0.487	0.106	0.470
180.1	6.74	0.123	5.685	7.876	3.920	0.495	0.098	0.458
173.8	8.36	0.110	3.395	6.358	2.367	0.489	0.105	0.478
185.7	9.68	0.103	4.723	6.042	4.330	0.475	0.095	0.473

Table 5.39 Average parameter estimates of the values obtained in Table 5.38.

	Average	95% Confidence intervals	
		Lower	Upper
μ_{max} (h^{-1})	0.134	0.118	0.150
K_S (g/L)	5.616	4.259	6.973
h_S (g/g h)	11.254	7.200	15.308
K_{SS} (g/L)	6.689	4.412	8.967
$Y_{G/SUC}$ (g/g)	0.489	0.477	0.501
$Y_{X/S}$ (g/g)	0.103	0.099	0.107
$Y_{P/S}$ (g/g)	0.468	0.459	0.478

The experimental and predicted results for biomass, sucrose, glucose, fructose and ethanol are shown in Figures 5.58 to 5.61. The analysis of these figures indicates that the model predictions for biomass, sucrose, glucose, fructose and ethanol were in very good agreement with the experimental results when the individual values of the parameters were used (Table 5.38). The model predictions using average parameter estimates (Table 5.39) were not as good as the individual ones, although the model still predicted fairly well the patterns observed in these experiments. When the average values of the parameters were used, the model underpredicted biomass, glucose, fructose and ethanol, while it overpredicted sucrose in media with initial biomass concentrations below 4.97 g/L (Figures 5.58-5.59a). The model predictions for biomass, glucose, fructose and ethanol were in very good agreement with the experimentally observed values, while it slightly underestimated sucrose concentrations in the medium with an initial biomass concentration of 4.97 g/L (Figure 5.59b). When the initial biomass concentrations increased above 4.97 g/L, the model underestimated sucrose concentrations but overestimated biomass, glucose, fructose and ethanol concentrations, mainly at the beginning of the fermentation (Figures 5.60-5.61).

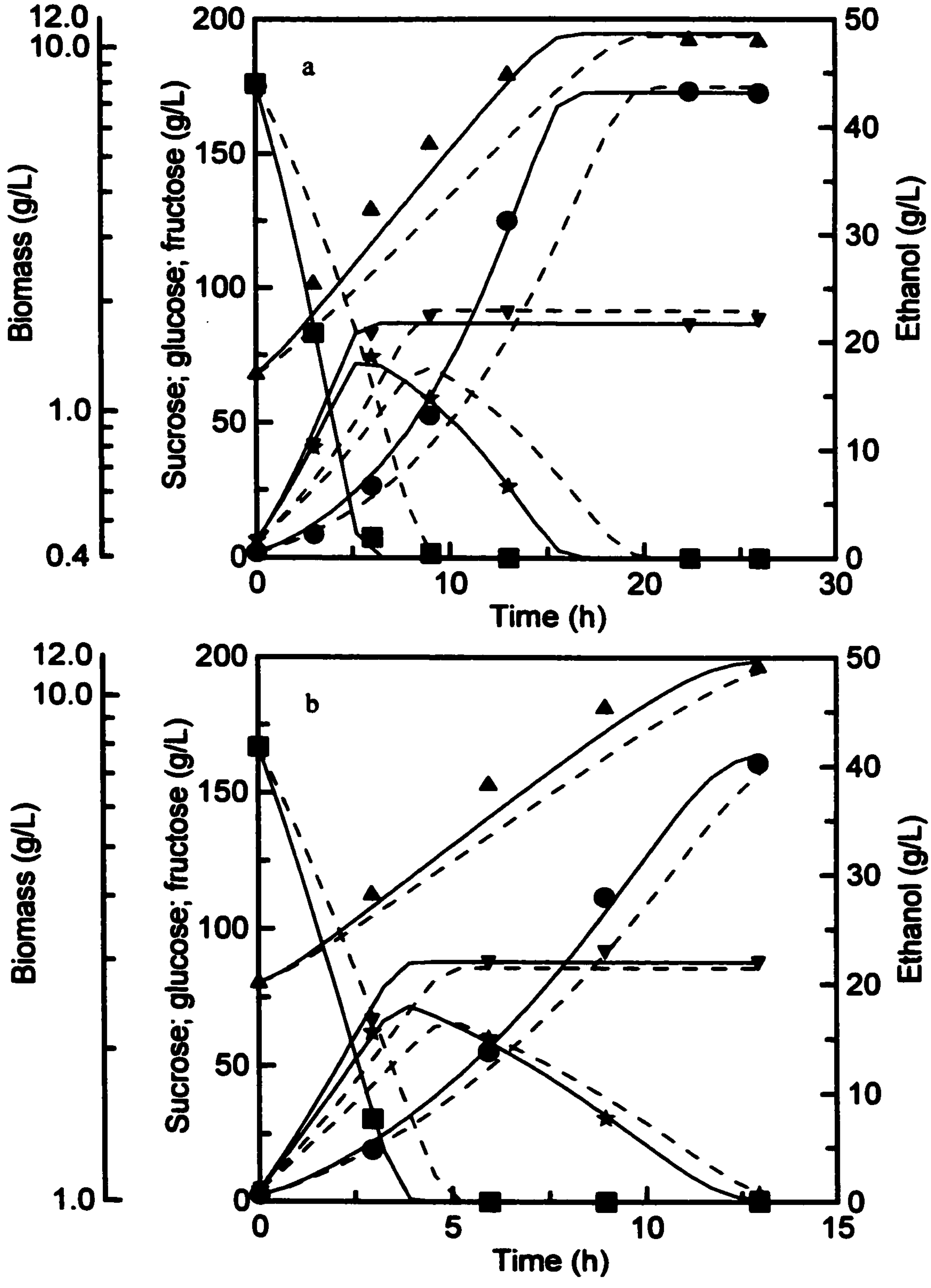

Figure 5.58 Experimental (symbols) and predicted results (——— individual; — — — average parameters in Tables 5.38-5.39) from tests carried out in media containing about 180 g/L sucrose and a) 1.27 g/L and b) 2.71 g/L biomass: (▲) biomass; (●) ethanol; (■) sucrose; (★) glucose; (▼) fructose.

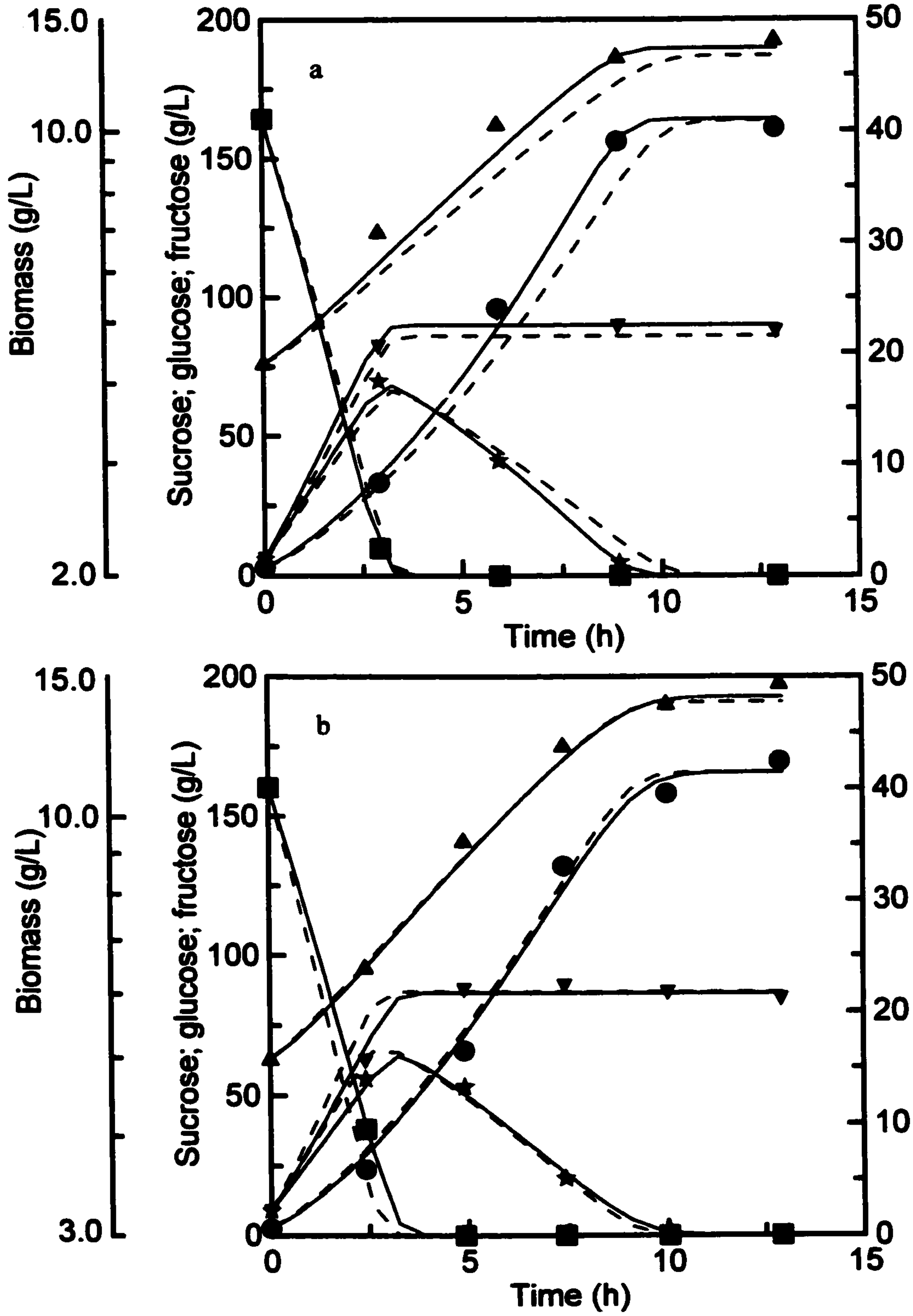

Figure 5.59 Experimental (symbols) and predicted results (——— individual; — — — average parameters in Tables 5.38-5.39) from tests carried out in media containing about 180 g/L sucrose and a) 4.29 g/L and b) 4.97 g/L biomass: (▲) biomass; (●) ethanol; (■) sucrose; (★) glucose; (▼) fructose.

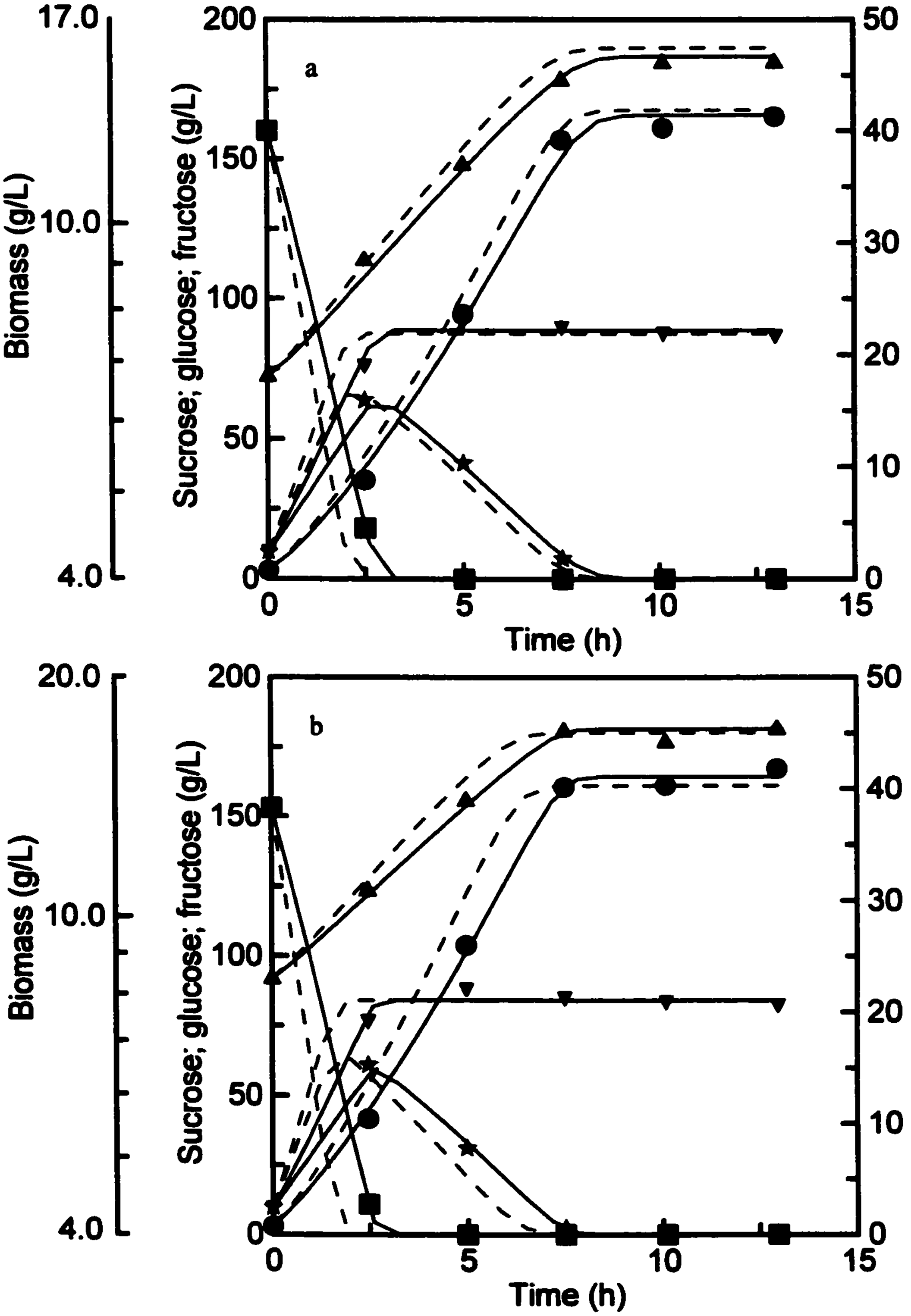

Figure 5.60 Experimental (symbols) and predicted results (——— individual; — — — average parameters in Tables 5.38-5.39) from tests carried out in media containing about 180 g/L sucrose and a) 6.74 g/L and b) 8.36 g/L biomass: (▲) biomass; (●) ethanol; (■) sucrose; (★) glucose; (▼) fructose.

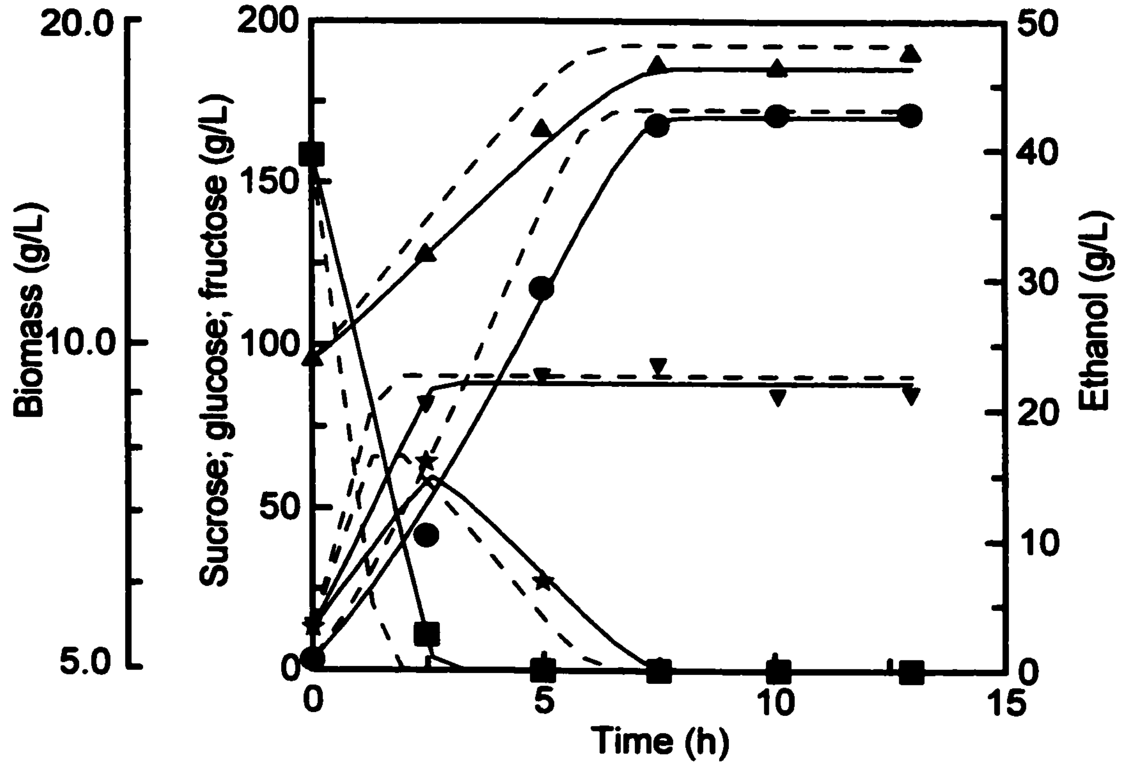

Figure 5.61 Experimental (symbols) and predicted results (——— individual; — — — average parameters in Tables 5.38-5.39) from tests carried out in a medium containing about 180 g/L sucrose and 9.68 g/L biomass: (▲) biomass; (●) ethanol; (■) sucrose; (★) glucose; (▼) fructose.

5.4.1.2.2 Media with about 279 g/L sucrose

The process parameters and their experimental values for media with about 279 g/L sucrose and various initial biomass concentrations have already been shown (Table 5.8), while the estimated values of the parameters in the proposed model (Eqs. 3.27-3.31) for each run, are provided in Table 5.40. The trends for most of the values of the estimated parameters were similar to those obtained experimentally. The predicted $Y_{X/S}$ values were slightly higher than those obtained experimentally, while those for $Y_{P/S}$ were as much as 17% higher than those obtained experimentally when the initial biomass concentration was above 3.35 g/L. The estimated μ_{max} values were lower by as much as 28% than the experimental values when initial biomass concentrations were 3.35 and 4.09 g/L, respectively. The predicted h_S values were about 15% higher than those obtained experimentally when initial biomass concentrations were 1.26 and 7.20 g/L, respectively. However, close values for the predicted and experimental h_S were obtained for the rest of initial biomass concentrations. The estimated $Y_{G/SUC}$ values were within 5% of the experimentally obtained ones. The predicted parameters K_S and K_{SS} were between 23.602 and 31.531 g/L, and 30.164 and 41.504 g/L, respectively (Table 5.40), which were much higher than those noticed in media with 180 g/L sucrose (Table 5.38). The average set of parameters was calculated from individual ones (Table 5.41), which was used in the model prediction of the experimental data.

Table 5.40 Parameter estimates using data collected from individual experiments with various initial inoculum concentrations and about 279 g/L sucrose.

Sucrose (g/L)	Initial biomass (g/L)	μ_{max} (h^{-1})	K_S (g/L)	h_S (g/g h)	K_{SS} (g/L)	$Y_{G/SUC}$ (g/g)	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)
287.1	1.26	0.157	23.602	19.682	30.164	0.510	0.057	0.390
273.3	3.35	0.130	26.471	16.188	40.704	0.496	0.060	0.434
284.1	4.09	0.126	31.531	13.612	41.504	0.482	0.055	0.451
270.8	7.20	0.091	29.805	9.627	37.694	0.476	0.050	0.464

Table 5.41 Average parameter estimates of the values obtained in Table 5.40.

	Average	95% Confidence intervals	
		Lower	Upper
μ_{max} (h^{-1})	0.126	0.100	0.152
K_S (g/L)	27.852	24.396	31.309
h_S (g/g h)	14.777	10.622	18.932
K_{SS} (g/L)	37.516	32.451	42.582
$Y_{G/SUC}$ (g/g)	0.496	0.481	0.511
$Y_{X/S}$ (g/g)	0.056	0.051	0.060
$Y_{P/S}$ (g/g)	0.435	0.403	0.466

The experimental and predicted results for biomass, sucrose, glucose, fructose and ethanol are shown in Figures 5.62 and 5.63. The analysis of these figures indicates that the model predictions for sucrose, glucose, fructose and ethanol were in very good agreement with the experimental results when the individual values of the parameters were used (Table 5.40). The biomass concentrations from the stationary growth phase were also in good agreement with the experimental observations, although the data from the exponential growth phase were poorly predicted. This was also previously noticed in the media with sucrose concentrations above 216 g/L (Figure 5.57). The poor model predictions for biomass concentrations during the exponential growth phase in media with initial sucrose concentrations above 216 g/L, could be due to the

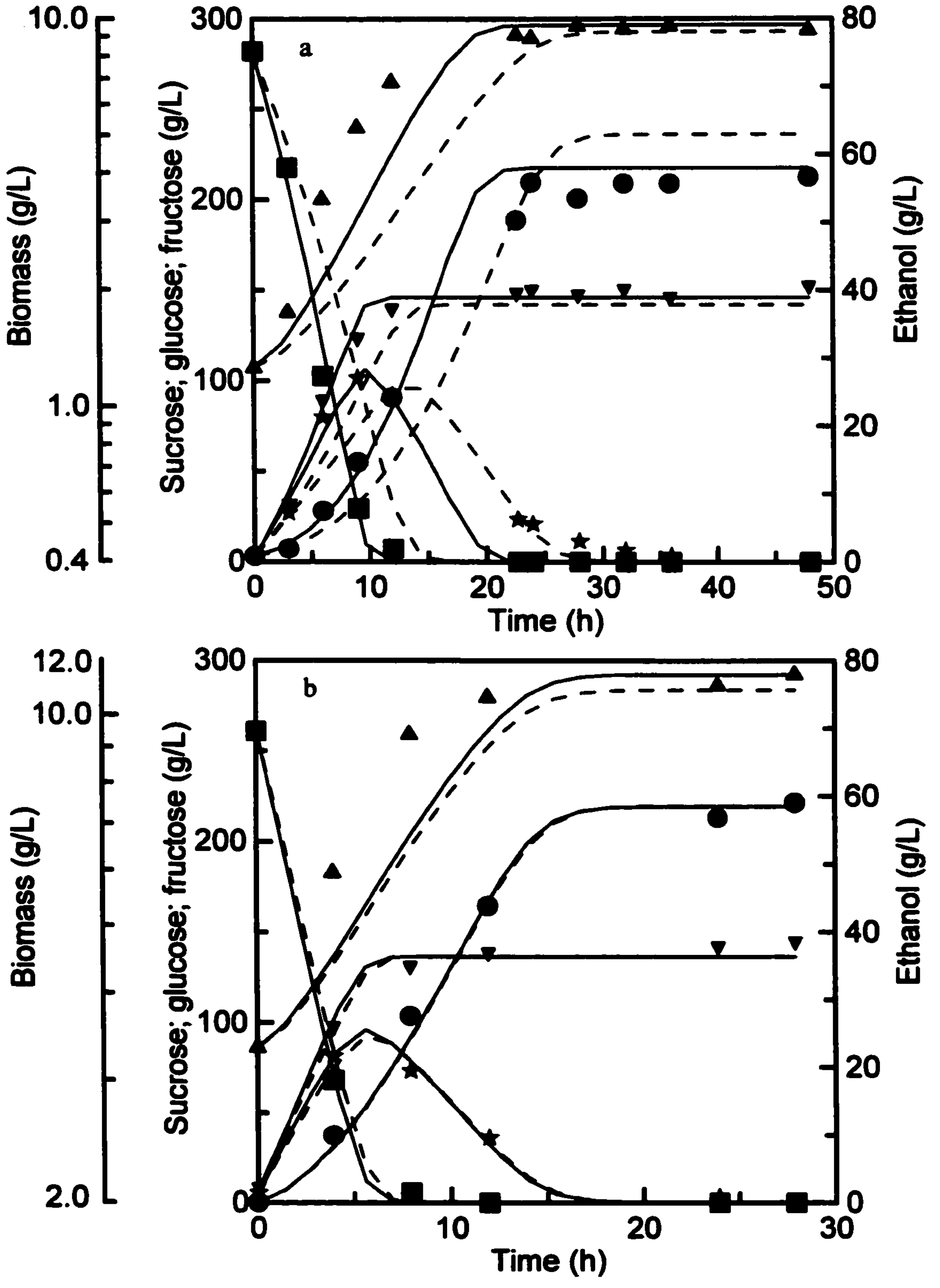

Figure 5.62 Experimental (symbols) and predicted results (——— individual; — — — average parameters in Tables 5.40-5.41) from tests carried out in media containing about 279 g/L sucrose and a) 1.26 g/L and b) 3.35 g/L biomass: (▲) biomass; (●) ethanol; (■) sucrose; (★) glucose; (▼) fructose.

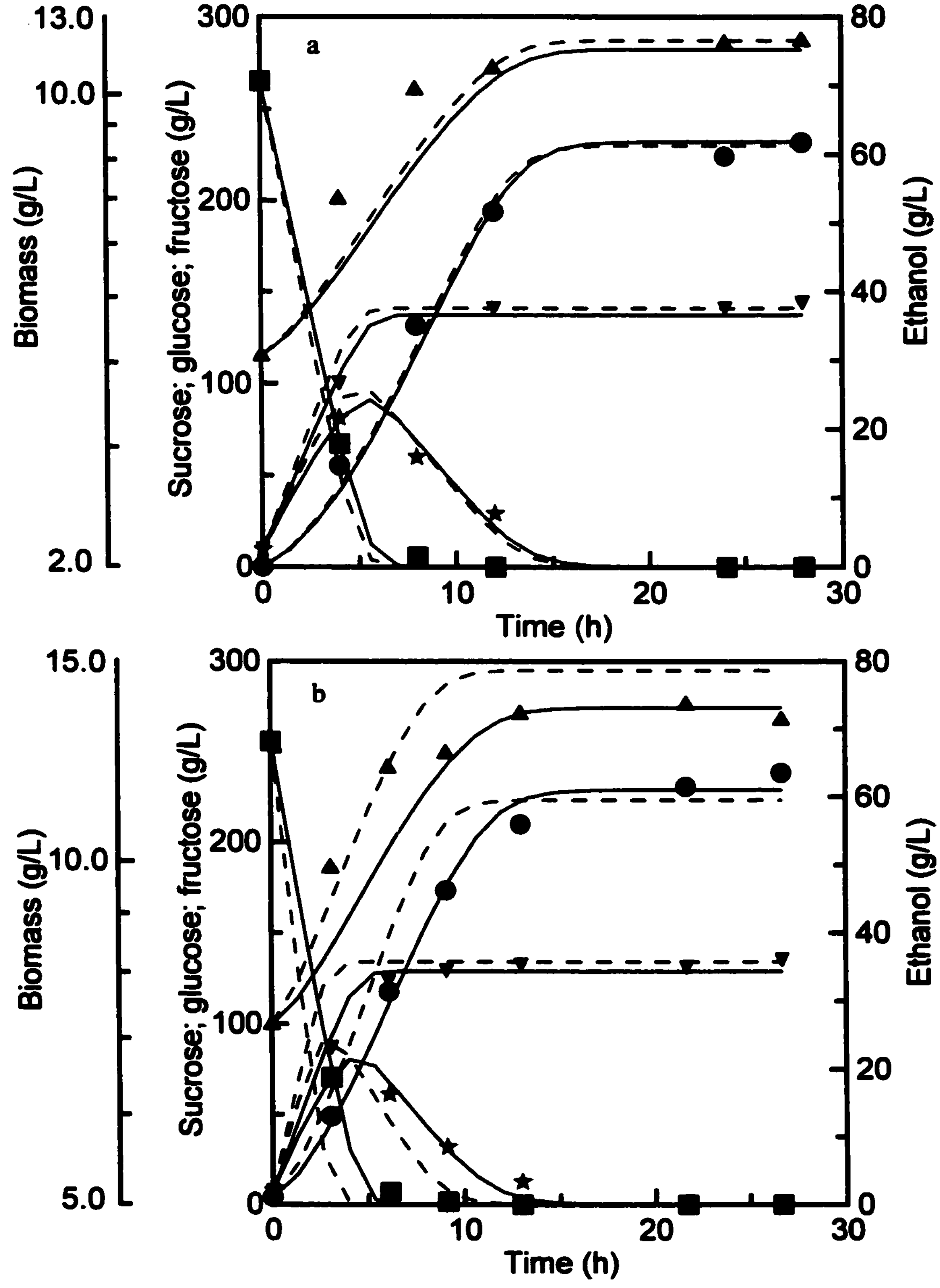

Figure 5.63 Experimental (symbols) and predicted results (——— individual; — — — average parameters in Tables 5.40-5.41) from tests carried out in media containing about 279 g/L sucrose and a) 4.09 g/L and b) 7.20 g/L biomass: (▲) biomass; (●) ethanol; (■) sucrose; (★) glucose; (▼) fructose.

simplicity of the form (Eq. 3.27) with only two parameters used to describe biomass production.

The model predictions using the average parameter estimates (Table 5.41) were not as good as with the individual values of the parameters (Table 5.40) particularly in the media with initial biomass concentrations of 1.26 and 7.20 g/L, respectively (Figures 5.62a and 5.63b). The model predictions were very close using the average and individual parameter estimates in media with initial biomass concentrations of 3.35 and 4.09 g/L, respectively. This is because the values of average parameters were close to the individual ones for these media. Model over- or under-predictions of the experimental data are normally expected, depending on the differences between the values of the average and individual parameters.

When the average values of the parameters were used (Table 5.41), the model underpredicted biomass and fructose, while it overpredicted sucrose in the medium with an initial biomass concentration of 1.26 g/L (Figures 5.62a). However, the model predictions for glucose and ethanol were lower than the experimental values during the first 10 and 22 h of the process, respectively. As the fermentation proceeded, the model predictions were in good agreement with experimental ones for glucose but overpredicted ethanol concentrations. The model also overpredicted ethanol and fructose concentrations during the first 12 h of the process when the initial biomass concentration was 7.20 g/L (Figure 5.63b). As the process continued, the model predictions were in good agreement with the remaining ethanol and fructose experimental data. Although the model predictions for biomass concentrations during the exponential growth phase were in good agreement with the experimental values, the model failed to predict the observed data in the stationary phase. The model underpredicted sucrose concentrations, which resulted in an over estimation of glucose during the first 3 h, however as the process proceeded, an under estimation of glucose concentrations was noticed.

The results showed that the model predictions for biomass, sucrose, glucose, fructose and ethanol concentrations in media with various initial biomass concentrations were very good when individual parameter estimates were used, and reasonably good when the average values of these parameters were applied. The model failed to adequately predict the exponential growth phase in the media with about 279 g/L sucrose, although it predicted quite well the stationary growth phase. Considering that *S. cerevisiae* ATCC 36858 can consume raffinose to produce fructose and ethanol, it is useful to examine the results of modelling of this process. This is shown and discussed in the next section.

5.4.2 Raffinose media

The experimental data in section 5.2.3 for the production of fructose and ethanol in media with various initial raffinose concentrations were used in parameters estimation with the previously described model (Eqs. 3.32-3.36). This was done to examine the applicability of the proposed model to describe the fermentation process. The experimental values of the fermentation parameters in raffinose media were previously shown in Table 5.16, in which the biomass and ethanol yields were calculated based on the amount of monosaccharides consumed. However in the proposed model (section 3.4.3), biomass and ethanol yields were expressed in g/g of melibiose consumed, therefore, the experimental yields were recalculated (Table 5.42).

Table 5.42 Experimental values of fructose, ethanol and biomass yields, specific growth rate and specific initial rate of raffinose hydrolysis and ethanol productivity in media with various initial raffinose concentrations.

Raffinose (g/L)	Initial biomass (g/L)	Specific growth rate, μ (h^{-1})	Biomass yield[a] $Y_{X/M}$ (g/g)	Ethanol productivity[b] (g/L h)	Specific initial rate of raffinose hydrolysis[c] (g/g h)	Fructose yield[b] $Y_{F/R}$ (g/g)	Ethanol yield[b] $Y_{P/M}$ (g/g)
29.7	1.33	0.166	0.450	0.48	0.78	0.340	0.360
50.2	1.20	0.230	0.310	0.94	1.36	0.300	0.370
69.5	1.16	0.221	0.220	1.24	1.94	0.300	0.400
80.7	1.20	0.209	0.190	1.60	2.14	0.290	0.400
131.5	1.27	0.206	0.130	1.58	2.33	0.320	0.410
189.1	1.37	0.197	0.090	2.04	4.97	0.320	0.390
242.0	1.27	0.184	0.070	1.72	5.51	0.340	0.380

[a] Values are calculated at maximum biomass concentration
[b] Values are calculated at maximum ethanol and fructose concentrations
[c] Values are calculated after 4 h of hydrolysis

The predicted values of the parameters in the media with all raffinose concentrations (Table 5.43) were generally close (within 9%) to those obtained experimentally (Table 5.42). Similar trends in the predicted parameters estimates and experimental values were noticed with increases in the initial raffinose concentration in the fermentation medium. The comparison between the predicted values of the parameters obtained in this study with those from the literature is difficult since no models were found in the literature that describe the simultaneous production of fructose and ethanol in media with raffinose. In order to obtain the general parameter estimates, average sets of parameters were calculated for runs with initial raffinose concentrations between 30 and 81 g/L, and 132 and 242 g/L, respectively (Table 5.44). The average K_S, K_R and h_R values in the media with initial raffinose concentrations between 132 and 242 g/L were higher than those when the concentrations of this trisaccharide were between 30 and 81 g/L. However, the $Y_{X/M}$ value was about three times higher in the lower range of raffinose concentrations. The values of μ_{max}, $Y_{M/R}$ and $Y_{P/M}$ were close in both ranges of raffinose concentrations.

Table 5.43 Parameter estimates using data collected from individual experiments with various initial raffinose concentrations.

Raffinose (g/L)	μ_{max} (h^{-1})	K_S (g/L)	h_R (g/g h)	K_R (g/L)	$Y_{M/R}$ (g/g)	$Y_{F/R}$ (g/g)	$Y_{X/M}$ (g/g)	$Y_{P/M}$ (g/g)
29.7	0.175	0.172	0.789	4.183	0.660	0.324	0.430	0.340
50.2	0.236	0.663	1.403	6.368	0.650	0.290	0.305	0.381
69.5	0.232	0.938	1.996	10.907	0.653	0.289	0.229	0.393
80.7	0.215	0.728	2.220	9.811	0.660	0.278	0.192	0.395
131.5	0.209	1.120	2.410	2.930	0.672	0.308	0.132	0.397
189.1	0.201	3.859	5.010	89.662	0.667	0.316	0.088	0.385
242.0	0.186	6.133	5.850	125.790	0.560	0.315	0.076	0.370

Table 5.44 Average parameter estimates for experiments with a range of raffinose concentrations.

	Raffinose between 30 and 81 g/L			Raffinose between 132 and 242 g/L		
	Average	95% Confidence intervals		Average	95% Confidence intervals	
		Lower	Upper		Lower	Upper
μ_{max} (h^{-1})	0.215	0.187	0.242	0.199	0.185	0.212
K_S (g/L)	0.625	0.308	0.943	3.704	0.863	6.545
h_R (g/g h)	1.602	0.973	2.231	4.423	2.394	6.453
K_R (g/L)	7.817	4.779	10.855	72.794	1.342	144.245
$Y_{M/R}$ (g/g)	0.656	0.651	0.661	0.633	0.561	0.705
$Y_{F/R}$ (g/g)	0.295	0.276	0.315	0.313	0.308	0.318
$Y_{X/M}$ (g/g)	0.289	0.186	0.392	0.099	0.065	0.132
$Y_{P/M}$ (g/g)	0.377	0.352	0.403	0.384	0.369	0.399

Plots comparing the experimental and predicted results for the production of fructose and ethanol in media with various initial raffinose concentrations are shown in Figures 5.64 to 5.67. A quick analysis of these figures indicates that the model predictions for ethanol, biomass, raffinose and fructose concentrations were in good agreement with the experimental results when the individual values of the parameters were used (Table 5.43). As expected, the model predictions using the average parameter estimates (Table 5.44) were not as good as with the individual values of the parameters (Table 5.43), while the model still predicted reasonably well the patterns experimentally observed. The model was unable to predict the experimental results for the production and consumption of melibiose using either the individual or the average parameter estimates.

The results of the tests carried out in media containing 30 and 50 g/L raffinose (Figure 5.64) showed good agreement between the model predictions and experimental data, except for melibiose, using the individual values of the parameters (Table 5.43). When the average values of the parameters (Table 5.44) were used, the model underpredicted raffinose, in the medium with 30 g/L of this sugar, and melibiose concentrations during the first 6 h of the process (Figure 5.64a). The model overpredicted ethanol, throughout the entire process, and fructose concentrations during the first 10 h. In the same medium, during the stationary growth phase, the model underpredicted biomass concentrations, while it overpredicted ethanol concentrations. Model over- or under-predictions of the experimental results is expected, based on the differences between the values of the average and individual parameters. The model predictions using the average values of the parameters were very similar to the individual ones in the medium with 50 g/L raffinose (Figure 5.64b). This is because the individual and average values of the parameters were close.

Considering the processes with 70 and 81 g/L raffinose (Figure 5.65), similar comments can be made using the individual values of the parameters as in media with lower raffinose concentrations. However, unlike the media with raffinose concentrations below 50 g/L, the model slightly overpredicted raffinose concentrations, while it underpredicted ethanol concentrations when the average values of the parameters were used. Biomass concentrations were also overpredicted during the stationary growth phase.

A good agreement between the model predictions and experimental data, except for melibiose, was observed using the individual values of the parameters (Table 5.43) in media containing 132 and 189 g/L raffinose (Figure 5.66). When the average parameter estimates were used (Table 5.44), the model overpredicted raffinose concentrations in the medium with 132 g/L of this sugar, while it underpredicted ethanol and biomass concentrations (Figure 5.66a). The

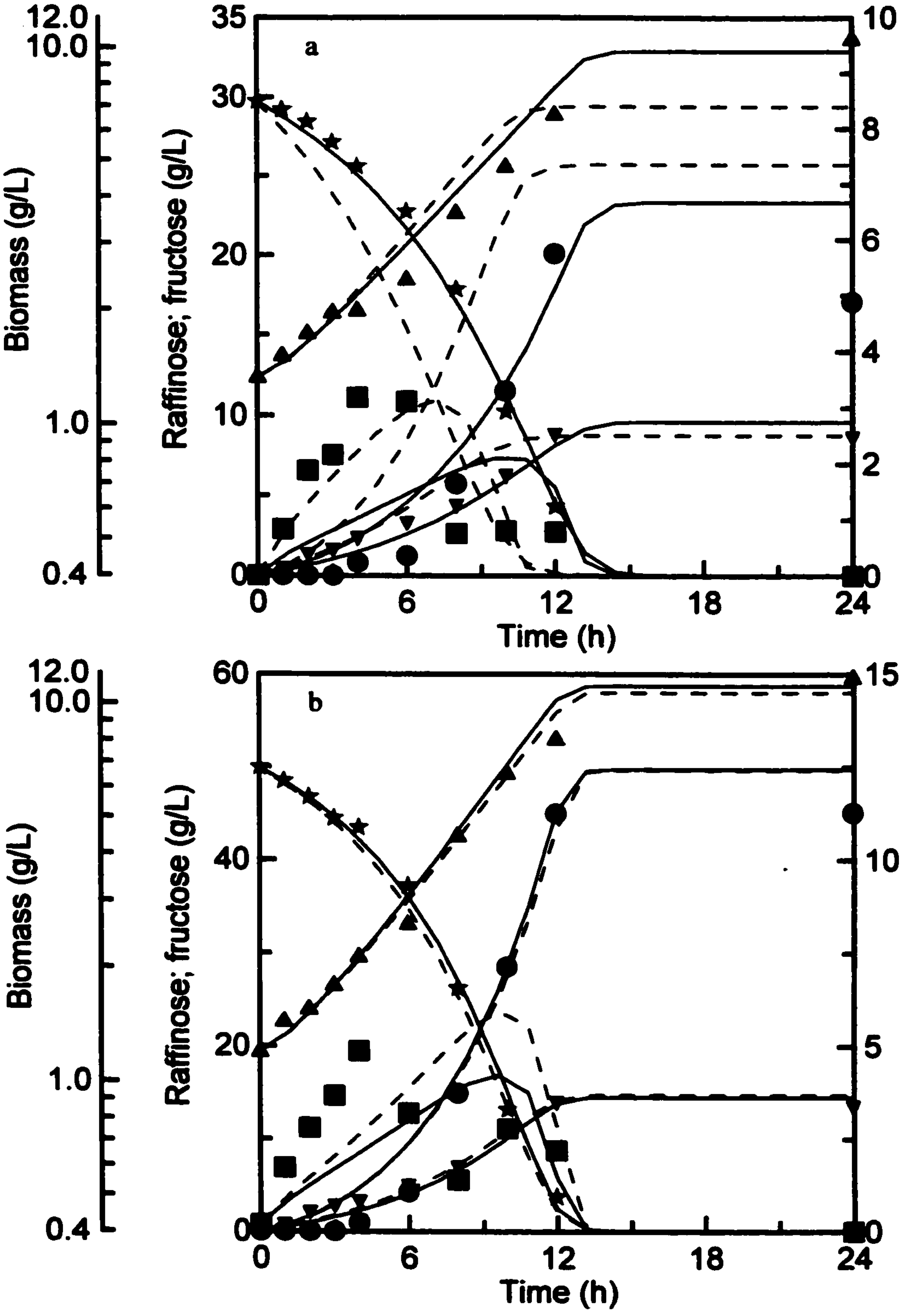

Figure 5.64 Experimental (symbols) and predicted results (———— individual; — — — average parameters in Tables 5.43-5.44) from tests carried out in media containing a) 30 g/L and b) 50 g/L raffinose: (▲) biomass; (★) raffinose; (▼) fructose; (●) ethanol; (■) melibiose.

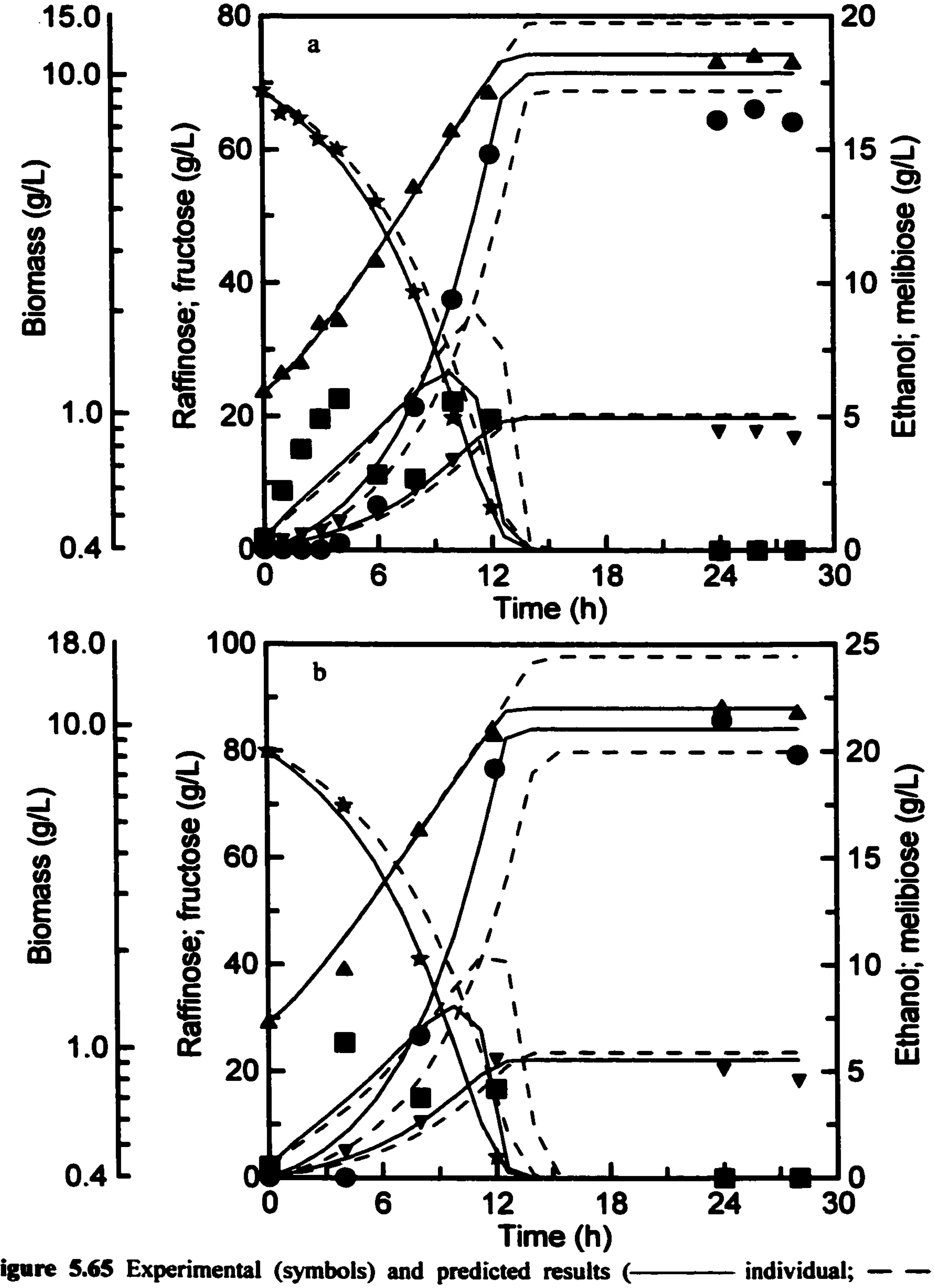

Figure 5.65 Experimental (symbols) and predicted results (———— individual; — — — average parameters in Tables 5.43-5.44) from tests carried out in media containing a) 70 g/L and b) 81 g/L raffinose: (▲) biomass; (★) raffinose; (▼) fructose; (●) ethanol; (■) melibiose.

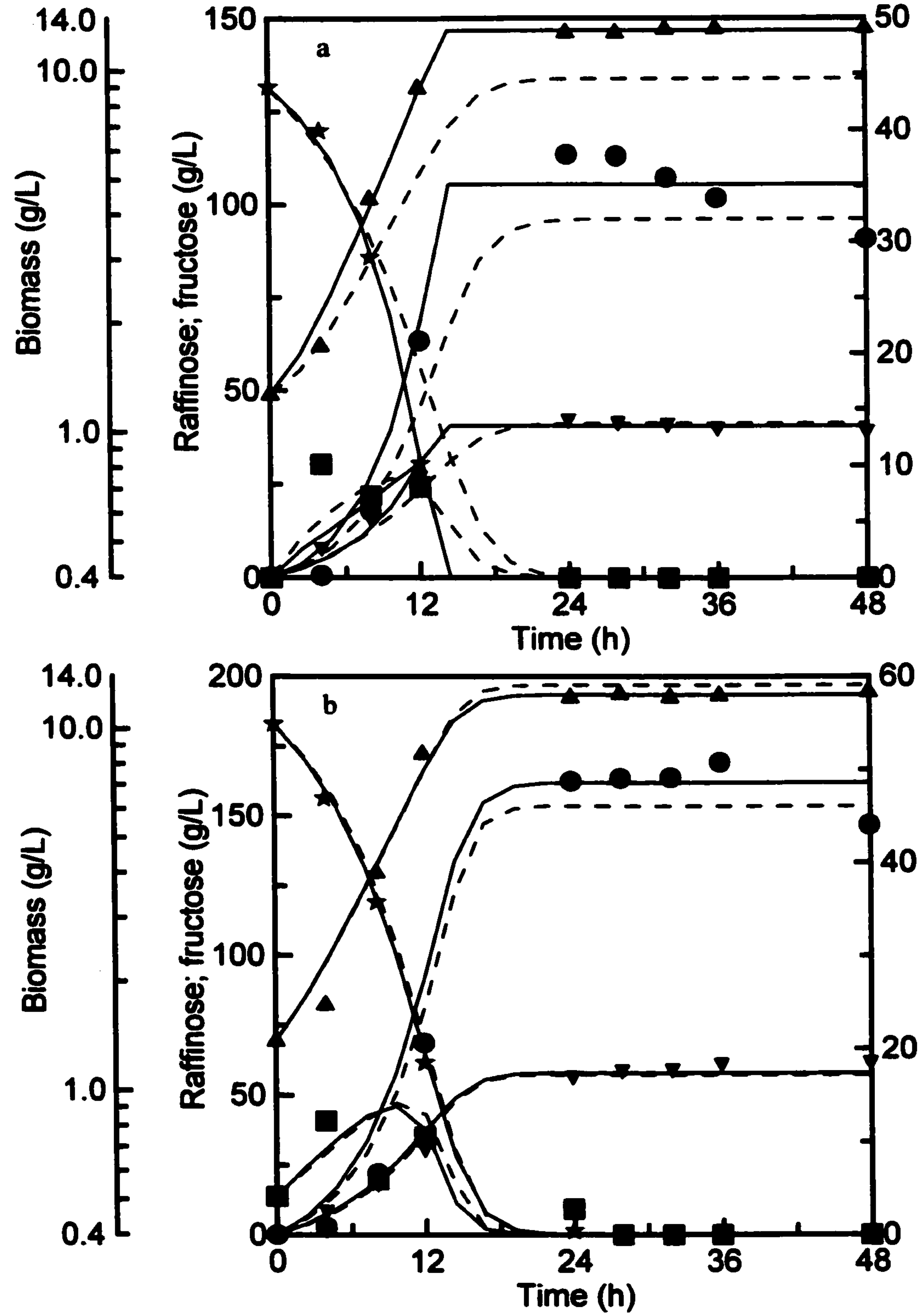

Figure 5.66 Experimental (symbols) and predicted results (———— individual; — — — average parameters in Tables 5.43-5.44) from tests carried out in media containing a) 132 g/L and b) 189 g/L raffinose: (▲) biomass; (★) raffinose; (▼) fructose; (●) ethanol; (■) melibiose.

predicted fructose concentrations agreed fairly well with the experimental data. The model predictions for raffinose and fructose concentrations were in good agreement with the experimentally observed values in the medium with 189 g/L of this trisaccharide (Figure 5.66b). However, the biomass concentrations were slightly overpredicted, while the ethanol concentrations were underpredicted during the stationary growth phase.

The test carried out in the medium containing 242 g/L raffinose (Figure 5.67) showed a fair agreement between the model predictions and the experimental data of biomass, ethanol and fructose using the individual values of the parameters (Table 5.43). However, poor model predictions for experimental raffinose concentrations, mainly after 12 h from the beginning of the process, were obtained. The model predicted total raffinose utilization in 24 h, which is 50% less than the required experimental time. In addition, poor predictions for melibiose concentrations were noticed. When the average values of the parameters (Table 5.44) were used, the model

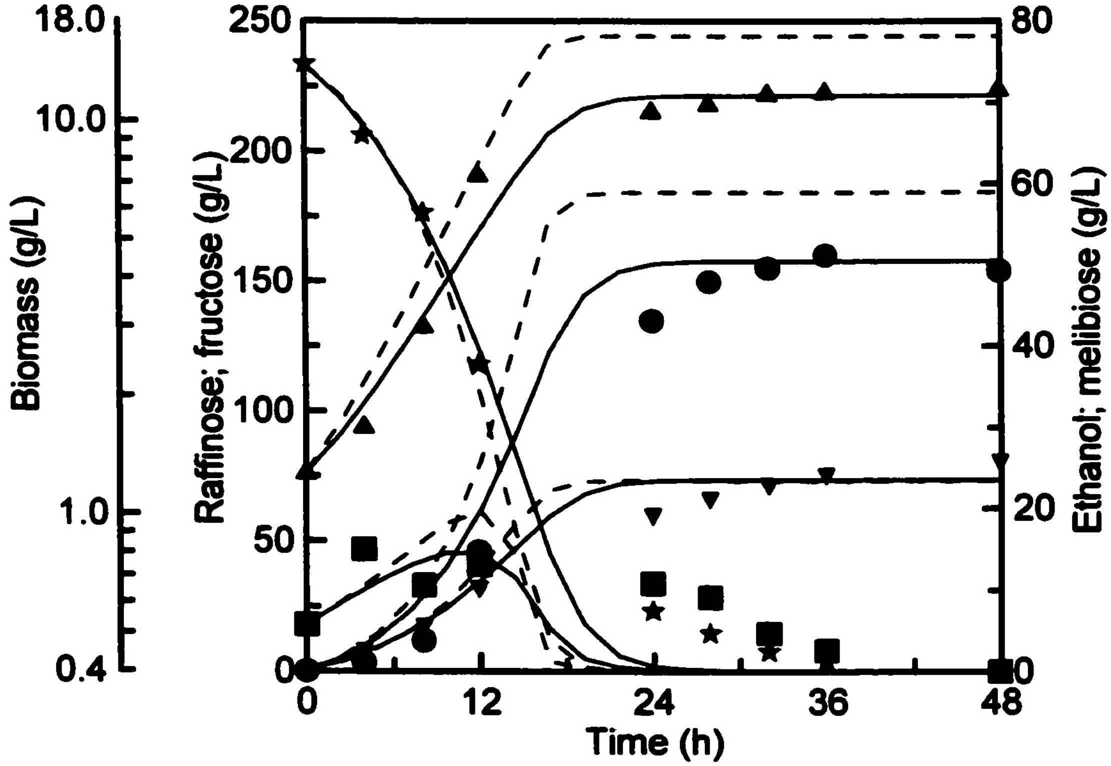

Figure 5.67 Experimental (symbols) and predicted results (———— individual; — — — average parameters in Tables 5.43-5.44) from tests carried out in a medium containing 242 g/L raffinose: (▲) biomass; (★) raffinose; (▼) fructose; (●) ethanol; (■) melibiose.

overpredicted biomass and ethanol concentrations, while it underpredicted the raffinose data. Fructose concentrations at the beginning and the end of the process were predicted reasonably well by the model.

The results showed that the model predictions for biomass, raffinose, fructose and ethanol concentrations were quite good, especially in media with initial raffinose concentrations below 242 g/L and with the individual parameter estimates. However, the proposed model was unable to predict the trends experimentally observed for the production and consumption of melibiose.

Chapter 6

Conclusions

This is a list of the conclusions that were reached in this project concerning the production of fructose and ethanol using the mutant *Saccharomyces cerevisiae* ATCC 36858 from different carbohydrate media.

- The mutant can grow on different carbohydrates such as glucose, fructose, galactose, sucrose and raffinose. However, it shows no activity when tested in media containing lactose. The mutant consumes glucose at a much faster rate than either fructose or galactose.
- The mutant can selectively convert glucose from glucose/fructose mixtures into ethanol.
- The mutant can hydrolyze sucrose to glucose and fructose and then selectively ferment glucose to ethanol with almost no fructose consumption. It shows osmophilic characteristic by growing and producing fructose and ethanol in synthetic media with very high sucrose concentrations (above 500 g/L).
- It is possible to produce a sweetener containing more than 97% fructose based on the final total sugar concentrations in a single batch fermentation process from synthetic media with sucrose concentrations below 360 g/L. Even at a sucrose concentration of 574 g/L, a syrup containing 59% fructose is produced and, thus it is richer in this carbohydrate than the ordinary 55% HFCS.
- The use of sterile or non-sterile synthetic sucrose media for the production of fructose and ethanol has no significant effect on the kinetics of the fermentation process or product yields.
- An increase in the initial biomass concentration in synthetic sucrose media decreased the fermentation time and increased the bioreactor productivity. Ethanol and fructose yields were only slightly affected.
- The biomass yield and specific growth rate increased with an increase in yeast extract concentration, while the fructose and ethanol yields were not significantly affected.
- An increase in the initial ethanol concentration in synthetic sucrose media resulted in a considerable decrease in the growth rate of the mutant, as well as in the biomass and

ethanol yields.

- An increase in the initial pH from 5.08 to 6.58 in synthetic sucrose media did not have a significant effect on the mutant's growth and fermentation capability.
- The mutant has the ability to completely hydrolyze raffinose or melibiose and then to selectively ferment the glucose and galactose into ethanol. Syrups the carbohydrate content of which contains more than 96% fructose can be produced from the media with raffinose concentrations below 189 g/L.
- The mutant is also able to utilize raffinose to produce fructose and ethanol from industrial raw materials such as sugar cane and sugar beet molasses media.
- Syrups with a carbohydrate content of more than 95% fructose are produced from the cane molasses media with sugar concentrations of 274 g/L or lower and from the beet molasses media with sugar concentrations of 242 g/L or lower.
- The ethanol yields were generally between 70 and 85% of the theoretical values, depending on the type of substrate and its concentration in the fermentation medium. Fructose yields were above 89% of the theoretical values. Biomass yields and specific growth rates decreased with increasing substrate concentrations in the fermentation medium.
- Higher biomass and ethanol yields, as well as higher specific initial rates of sucrose hydrolysis, were obtained from synthetic sucrose media compared with sugar cane or sugar beet molasses media.
- Higher biomass and ethanol yields were obtained in sugar cane molasses than in sugar beet molasses media.
- Some glycerol (about 10 g/L) was produced during the production process of fructose and ethanol. More glycerol was produced in the molasses media than in the synthetic sucrose media. In addition, more glycerol was produced in sugar beet molasses media than in sugar cane molasses media.
- Some fructo-oligosaccharides (up to 26 g/L) were produced in synthetic sucrose and molasses media. Their concentration increased with increasing sucrose concentration. The fructo-oligosaccharides were entirely hydrolyzed to glucose and fructose in a ratio of 1:2 when the sugar concentrations in the synthetic sucrose and both molasses media were

below 451 and 236 g/L, respectively.

- The decolourization of the fructose syrups produced from cane molasses media required a large amount of activated carbon due to the large amounts of colourants (about 500,000 IU) in these solutions.
- About 94% of the ethanol was recovered from the broth using vacuum evaporation prior to carbon treatment.
- The decolourization efficiency was not significantly altered by increasing the contact time between the broth and the activated carbon.
- The decolourization efficiency increased with an increase in the amount of carbon used. More than 98% of the colourants were removed from the fructose syrups after treatment with 33% of activated carbon in the slurry, while about 75% of the fructose remained in the carbon. About 95% of the original fructose was then recovered after four washes of the spent carbon with water.
- The incorporation of a membrane separation technique followed by activated carbon treatment for syrup purification improved colour removal by considerably decreasing the amount of carbon needed, if the latter was only used to obtain the same degree of decolourization (above 99%).
- The decolourization efficiency using the TFC membrane was higher than with the PES membrane due to its smaller pore size, while the permeate flux rate of the TFC membrane was lower. The total fructose recovered, after carbon treatment of the permeates of the PES and TFC membranes, was 74 and 85%, respectively.
- The fructose content in treated syrups with either activated carbon or membrane separation followed by activated carbon was more than 90% of the total sugar concentrations.
- The demineralization of fructose syrups using a mixture of anion and cation exchange resins resulted in an almost complete removal of minerals and in a large fructose loss.
- Although the purification process was not economical, it showed that colour and minerals can be removed from fructose syrups produced from cane molasses media.
- Rate equations were formulated for growth, sucrose and raffinose uptake, glucose and melibiose consumption and production, ethanol and fructose production by *S. cerevisiae*

ATCC 36858. Parameter estimates for these equations were obtained from batch experiments in sucrose and raffinose media using MicroMath scientist software.

- The model predictions for biomass, sucrose, glucose, fructose and ethanol concentrations were in quite good agreement with the experimentally observed values when the individual values of the parameters were used in media with initial sucrose concentrations below 257 g/L. The model failed to adequately predict the exponential growth phase in media with initial sucrose concentrations of 257 g/L or higher. However, model predictions for the stationary growth phase, as well as the other variables, were very good.
- In raffinose media, the model predictions for biomass, raffinose, fructose and ethanol concentrations were quite good when using individual parameter estimates, especially in media with initial raffinose concentrations below 242 g/L. However, the model failed to predict the trends experimentally observed for the production and consumption of melibiose.
- The model predictions of the fermentation process in either sucrose or raffinose media using average parameter estimates were not as good as with the individual values of the parameters, however, the model reasonably predicted the patterns experimentally observed in these media.

Chapter 7

Recommendations

Based on the results obtained in this work concerning the production of fructose and ethanol using *S. cerevisiae* ATCC 36858 with different carbohydrate media, the following recommendations are made for future studies:

- Examine continuous (free and immobilized cells) and fed batch processes for the production of fructose and ethanol using *S. cerevisiae* ATCC 36858.
- Examine the possibility of using a mixed culture of both strains *S. cerevisiae* ATCC 36858 and 36859 since the former can be used in media with sucrose and the latter has higher ethanol yields in glucose/fructose mixtures.
- Examine the possibility of reusing the produced biomass to start new fermentations. This could be done either immediately after fermentation, by adding the cells to fresh media, or by recycling cells constituents as medium ingredients, after their disruption and lysis, which could reduce the use of yeast extract.
- Explore the possibility of enhancing the production of fructose and ethanol from molasses media by prior purification (demineralization and decolourization) of the molasses. This could allow fermentation in molasses media with higher total sugar concentrations, similar to the levels in synthetic media.
- Test the effect of adding molasses in various ratios to synthetic sucrose media for the production of ethanol and fructose by *S. cerevisiae* ATCC 36858. This could reduce the amount of yeast extract, peptone and other constituents used in the process.
- Study the effect of pH and temperature on the decolourization efficiency of activated carbon when used to purify fructose syrups produced from cane molasses media.
- Study the effect of increasing membrane surface area, permeation time, pH, temperature and pressure on the performance of the PES and TFC membranes used for decolourization of fructose syrups produced from cane molasses media. In addition, test the effect of using fructose syrups without prior ethanol removal in the decolourization process. This could enhance the permeate flux rate and decolourization efficiency. The use of other types of membranes and module configurations should be explored.

- Test the effects of temperature, residence time and the type and the amount of ion exchange resins used during demineralization treatment. This could reduce fructose loss in the process and increase mineral removal. In addition, examine the effects of the demineralization of fructose syrups prior to the decolourization process.
- Study the use of other kinetic forms for the specific growth rate and sucrose and raffinose uptake by incorporating the effects of substrate and product inhibition, especially in media with high sugar concentrations.

References

Agarwal, G. P., "Glycerol", Adv. Biochem. Eng. Biotechnol. **41**, 95-128 (1990).

Aiba, S., M. Shoda and M. Nagatani, "Kinetics of Product Inhibition in Alcohol Fermentation", Biotechnol. Bioeng. **10**, 845-864 (1968).

Akrida-Demertzi, K. and A. A. Koutinas, "Optimization of Sucrose Ethanol Fermentation for K, Na, Ca and Cu Metal Contents", Appl. Biochem. Biotechnol. **30**, 1-7 (1991).

Albon, N., D. J. Bell, P. H. Blanchard, D. Gross and J. T. Rundell, "Kestose: A Trisaccharide Formed from Sucrose by Yeast Invertase", J. Chem. Soc. 24-27 (1953).

Andersen, B., N. Thiesen and P. E. Broe, "The Transferring Activity of β-Fructofuranosidase from Yeast: A Quantitative Description", Acta. Chem. Scand. **23**, 2367-2374 (1969).

Arnold, W. N., "Hydrolytic Enzymes", in "Yeast Biotechnology", D. R. Berry, I. Russell and G. G. Stewart, Eds., Allen & Unwin, London (1987), pp. 369-400.

Atiyeh, H. and Z. Duvnjak, "Study of the Production of Fructose and Ethanol from Sucrose Media by *Saccharomyces cerevisiae*", Appl. Microbiol. Biotechnol. **57**, 407-411 (2001a).

Atiyeh, H. and Z. Duvnjak, "Production of Fructose and Ethanol from Media with High Sucrose Concentration by a Mutant of *Saccharomyces cerevisiae*", J. Chem. Technol. Biotechnol. **76**, 1017-1022 (2001b).

Atiyeh, H. and Z. Duvnjak, "Production of Fructose and Ethanol from Sugar Beet Molasses Using *Saccharomyces cerevisiae* ATCC 36858", Biotechnol. Prog. **18**, 234-239 (2002).

Atiyeh, H. and Z. Duvnjak, "Production of Fructose and Ethanol from Cane Molasses Using *Saccharomyces cerevisiae* ATCC 36858", Acta. Biotechnol. In print.

Atiyeh, H. and Z. Duvnjak, "Utilization of Raffinose and Melibiose by a Mutant of *Saccharomyces cerevisiae*", J. Chem. Technol. Biotechnol. Accepted for publishing.

Bacon, J. S. D. and J. Edelman, "The Action of Invertase Preparations", Arch. Biochem. Biophys. **28**, 467-468 (1950).

Bacon, J. S. D., "The Oligosaccharides Produced by the Action of Yeast Invertase Preparations on Sucrose", Biochem. J. **57**, 320-328 (1954).

Bafrncova, P., D. Smogrovicova, I. Slavikova, J. Patkova and Z. Domeny, "Improvement of Very High Gravity Ethanol Fermentation by Media Supplementation Using *Saccharomyces cerevisiae*", Biotechnol. Lett. **21**, 337-341 (1999).

Bailey, J. E. and D. F. Ollis, "Biochemical Engineering Fundamentals", 2nd edn., McGraw-Hill, New York (1986).

Barford, J. P. and R. J. Hall, "A Mathematical Model for the Aerobic Growth of *Saccharomyces cerevisiae* with a Saturated Respiratory Capacity", Biotechnol. Bioeng. **23**, 1735-1762 (1981).

Barford, J. P., P. J. Phillips and J. H. Orlowski, "A New Model of Uptake of Multiple Sugars by *S. cerevisiae*: Part 1", Bioprocess Eng. **7**, 297-302 (1992a).

Barford, J. P., P. J. Phillips and J. H. Orlowski, "A New Model of Uptake of Multiple Sugars by

S. cerevisiae: Part 2", Bioprocess Eng. 7, 303-307 (1992b).

Barker, S. A., H. Pelmore and P. J. Somers, "Effect of Oxyanions on the D-Glucose Isomerase Catalyzed Equilibrium. 2. Effect of Germanate on the Equilibrium of D-Glucose and D-Fructose with Immobilized D-Glucose Isomerase ", Enzyme Microb. Technol. **5**, 121-124 (1983).

Barker, S. A. and G. S. Petch, "Enzymatic Processes for High-Fructose Corn Syrup", in "Enzymes and Immobilized Cells in Biotechnology", A. I. Laskin, Ed., Benjamin Cummings, California (1985), pp. 93-107.

Bernt, E. and I. Gutmann, "Ethanol Determination with Alcohol Dehydrogenase and NAD", in "Methods of Enzymatic Analysis", H. U. Bergermeyer, Ed., Academic Press, New York, (1974), pp. 1499-1502.

Berry, D. R. and C. Brown, "Physiology of Yeast Growth", in "Yeast Biotechnology", D. R. Berry, I. Russell and G. G. Stewart, Eds., Allen & Unwin, London, (1987), pp. 159-199.

Bhosale, S. H., M. B. Rao and V. V. Deshpande, "Molecular and Industrial Aspects of Glucose Isomerase", Microbiol. Rev. **60**, 280-300 (1996).

Bjorkqvist, S., R. Ansell, L. Adler and G. Liden, "Physiological Response to Anaerobicity of Glycerol-3-Phosphate Dehydrogenase Mutants of *Saccharomyces cerevisiea*", Appl. Environ. Microbiol. **63**, 128-132 (1997).

Blanchard, P. H. and N. Albon, "The Inversion of Sucrose; a Complication", Arch. Biochem. Biophys. **29**, 220-222 (1950).

Blanchard, P. H. and E. O. Geiger, "Production of High Fructose Syrups in the USA", Sugar Technol. Rev. **11**, 1-94 (1984).

Bourée, D., "Ion-Exchange Resin Process for Color and Ash Removal", in "Handbook of Sugar Refining: a Manual for the Design and Operation of Sugar Refining Facilities", C. C. Chou, Ed., John Wiley & Sons, New York (2000), pp. 135-154.

Bowski, L., R. Saini, D. Y. Ryu and W. R. Vieth, "Kinetic Modeling of the Hydrolysis of Sucrose by Invertase", Biotechnol. Bioeng. **13**, 641-656 (1971).

Bringer, S. and H. Sahm, "Fermentation Process for the Production of Fructose from Aqueous Mixtures of Fructose and Glucose and *Zymomonas mobilis* Mutants Which Can be Used for Such Fermentation", U.S. Patent 4,742,006 (1988).

Bringer-Meyer, S., M. Scollar and H. Sahm, "*Zymomonas mobilis* Mutants Blocked in Fructose Utilization", Appl. Microbiol. Biotechnol. **23**, 134-139 (1985).

Bucke, C., "Recent Development in Production and Use of Glucose and Fructose Syrups", in "Developments in Sweeteners", C. A. M. Hoough, K. J. Parker and A. J. Vlitos, Eds., Applied Science Publishers, London, (1979), pp. 43-69.

Cartier, S., L. Tatoud. M. A. Theoleyre and M. Decloux, "Sugar Refining Process by Coupling Flocculation and Crossflow Filtration", J. Food Eng. **32**, 155-166 (1997).

Casey, G. P., W. Xiao and G .H. Rank, "Construction of α-Galactosidase-Positive Strains of Industrial Baker's (*Saccharomyces cerevisiae*) Yeasts", Am. Soc. Brew. Chem. J. **46**, 67-

71 (1988).

Cason, D. T., G. C. Reed and E. M. S. Gatner, "On the Differing Rates of Fructose and Glucose Utilization", J. Inst. Brew. 93, 23-25 (1987).

Carpenter, F. G. and M. A. Clarke, "Cane Sugar Refining: II Decolorization", in "Cane Sugar Handbook: a Manual for Sugar Cane Manufacturers and their Chemists", 10th edn., G. P. Meade and J. C. P. Chen, Eds., John Wiley & Sons, New York (1977), pp. 455-493.

Cejka, A., "Preparation of Media", in "Biotechnology", Vol. 2, H. -J. Rehm, G. Reed and H. Brauer, Eds., VCH, Weinheim, Germany, (1985), pp. 629-698.

Chandrasena, G. and G. M. Walker, "Use of Response Surface to Investigate Metal Ion Interactions in Yeast Fermentations", J. Am. Soc. Brew. Chem. 55, 24-29 (1997).

Chatterton, N. J., P. A. Harrison, W. R. Thornley and J. H. Bennett, "Purification and Quantification of Kestose (Fructosylsucroses) by Gel Permeation and Anion Exchange Chromatography", Plant Physiol. Biochem. 27, 289-295 (1989).

Chen, J. C. P., "Raw Sugar Quality Criteria", in "Cane Sugar Handbook: a Manual for Cane Sugar Manufacturers and their Chemists", 12th edn., J. C. P. Chen and C. C. Chou, Eds., John Wiley & Sons, New York (1993a), pp. 343-374.

Chen, J. C. P., "By-Products of Cane Sugar Processing", in "Cane Sugar Handbook: a Manual for Cane Sugar Manufacturers and their Chemists", 12th edn., J. C. P. Chen and C. C. Chou, Eds., John Wiley & Sons, New York (1993b), pp. 375-431.

Chen, J. C. P., "Determination of Density and Total Solids", in "Cane Sugar Handbook: a Manual for Cane Sugar Manufacturers and their Chemists", 12th edn., J. C. P. Chen and C. C. Chou, Eds., John Wiley & Sons, New York (1993c), pp. 852-867.

Chou, C. C., "Determination of Color and Turbidity in Sugar Products", in "Cane Sugar Handbook: a Manual for Cane Sugar Manufacturers and their Chemists", 12th edn., J. C. P. Chen and C. C. Chou, Eds., John Wiley & Sons, New York (1993), pp. 882-903.

Cirillo, V., "Mechanism of Glucose Transport Across the Yeast Cell Membrane", J. Bact. 84, 485-491 (1962).

Cirillo, V., "Relationship Between Sugar Structure and Competition for the Sugar Transport System in Baker's Yeast", J. Bact. 95, 603-611 (1968).

Coker, L. E. and K. Venkatasubramanian, "Corn Sweeteners", in "Food Biotechnology", D. Knorr, Ed., Marcel Dekker Inc, New York, (1987), pp. 443-460.

Colowick, S. P., "The Hexokinases", in "The Enzymes", P. D. Boyer, Ed., Academic Press, New York, Vol. 9 (1973) pp, 1-48.

Combes, D. and P. Monsan, " Sucrose Hydrolysis by Invertase. Characterization of Products and Substrate Inhibition", Carbohydr. Res. 117, 215-228 (1983).

Cooney, C. L., "Growth of Microorganisms", in "Biotechnology", Vol. 1, H. -J. Rehm and G. Reed, Eds., VCH, Weinheim, Germany, (1981), pp. 73-112.

Cooney, C. L., "Media Sterilization", in "Comprehensive Biotechnology", Vol. 2, M. Moo-Young, C. L. Cooney and A. E. Humphrey, Eds., Pergamon Press, Oxford, England, (1985), pp. 287-298.

Corbett, K., "Design, Preparation and Sterilization of Fermentation Media", in "Comprehensive Biotechnology", Vol. 1, M. Moo-Young, A. Bull and H. Dalton, Eds., Pergamon Press, Oxford, England, (1985), pp. 127-139.

Cristofaro E., F. Mottu and J. J. Wuhrmann, "Involvement of the Raffinose Family of Oligosaccharides in Flatulence", in "Sugars in Nutrition", H. L. Sipple and K. W. McNutt, Eds., Academic Press, New York, (1974), pp. 313-36.

Crittenden, R. G. and H. W. Doelle, "Structural Identification of Oligosaccharides Produced by *Zymomonas mobilis* Levansucrase", Biotechnol. Lett. **15**, 1055-1060 (1993).

Crittenden, R. G. and H. W. Doelle, "Identification and Characterization of the Extracellular Sucrases of *Zymomonas mobilis* UQM 2716 (ATCC 39676)", Appl. Microbiol. Biotechnol. **41**, 302-308 (1994).

D'Amore, T., C. J. Panchal, I. Russell and G. G. Stewart, "A study of Ethanol Tolerance in Yeast", Crit. Rev. Biotechnol. **9**, 287-304 (1990).

Decloux, M., L. Tatoud and A. Mersad, "Removal of Colorants and Polysaccharides from Raw Cane Sugar Remelts by Ultrafiltration", Zuckerindustrie **125**, 106-113 (2000).

Dhurjati, P. S. and R. J. Leipold, "Biological Modeling", in "Computer Control of Fermentation Processes", D. R. Omstead, Ed., CRC Press Inc., Boca Raton, Florida, (1990), pp. 207-220.

Di Luccio, M., B. D. Smith, T. Kida, C. P. Borges and T. L. M. Alves, "Separation of Fructose from a mixture of Sugars Using Supported Liquid Membranes", J. Membr. Sci. **174**, 217-224 (2000).

Doelle, H. W., "Kinetic Characteristics and Regulatory Mechanisms of Glucokinase and Fructokinase from *Zymomonas mobilis*", Eur. J. Appl. Microbiol. Biotechnol. **14**, 241-246 (1982a).

Doelle, H. W., "The Existence of Two Separate Constitutive Enzymes for Glucose and Fructose in *Zymomonas mobilis*", Eur. J. Appl. Microbiol. Biotechnol. **15**, 20-24 (1982b).

Doelle, H. W. and P. F. Greenfield, "The Production of Ethanol from Sucrose Using *Zymomonas mobilis*", Appl. Microbiol. Biotechnol. **22**, 405-410 (1985a).

Doelle, H. W. and P. F. Greenfield, "Fermentation Pattern of *Zymomonas mobilis* at High Sucrose Concentrations", Appl. Microbiol. Biotechnol. **22**, 411-415 (1985b).

Doelle, M. B., P. F. Greenfield and H. W. Doelle, "Effect of Mineral Ions on Ethanol Formation During Sugar Cane Molasses Fermentation using *Zymomonas mobilis* ATCC 39676", Process Biochem. **25**, 151-156 (1990a).

Doelle, M. B., P. F. Greenfield and H. W. Doelle, "The Relationship Between Sucrose Hydrolysis, Sorbitol Formation and Mineral Ion Concentration during Bioethanol Formation using *Zymomonas mobilis* 2716", Appl. Microbiol. Biotechnol. **34**, 160-167 (1990b).

Doelle, M. B. and H. W. Doelle, "High Fructose Formation from Sugarcane Syrup and Molasses Using *Zymomonas mobilis* Mutants", Biotechnol. Lett. **13**, 875-878 (1991).

Doelle, H. W., L. Kirk, R. Crittenden, M. B. Doelle and H. Toh, "*Zymomonas mobilis* – Science and Industrial Application", CRC Crit. Rev. Biotechnol. **13**, 57-98 (1993).

Duvnjak, Z. and D. W. Koren, "Production of Fructose Syrup by Selective Removal of Glucose from Hydrolyzed Jerusalem Artichoke Juice", Biotechnol. Lett. **9**, 783-788 (1987).

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

Duvnjak, Z., G. Turcotte and Z. D. Duan, "Production of Sorbitol and Ethanol from Jerusalem Artichokes by *Saccharomyces cerevisiae* ATCC 36859", Appl. Microbiol. Biotechnol. **35**, 711-715 (1991b).

Edwards, V. H., "The Influence of High Substrate Concentrations on Microbial Kinetic", Biotechnol. Bioeng. **12**, 679-712 (1970).

Edye, L. A., M. R. Johns and K. N. Ewings, "Fructose Production by *Zymomonas mobilis* in Fed-Batch Culture with Minimal Sorbitol Formation", Appl. Microbiol. Biotechnol. **31**, 129-133 (1989).

Edye, L. A., Ch. Kositanont and H. W. Doelle, "Fructose Phosphorylation by *Zymomonas mobilis* Glucokinase", Acta Biotechnol. **10**, 49-53 (1990).

Fan, L., "Process for Producing Fructose", U.S. Patent 4,774,183 (1988).

Farine, S., C. Versluis, P. J. Bonnici, A. Heck, C. L'homme, A. Puigserver and A. Biagini, "Application of High Performance Anion Exchange Chromatography to Study Invertase-Catalysed Hydrolysis of Sucrose and Formation of Intermediate Fructan Products", Appl. Microbiol. Biotechnol. **55**, 55-60 (2001).

Field, P. J. and H. P. Benecke, "Granular Carbon Decolorization System", in "Handbook of Sugar Refining: a Manual for the Design and Operation of Sugar Refining Facilities", C. C. Chou, Ed., John Wiley & Sons, New York (2000), pp. 91-119.

Fleming, S. E. and J. W. D. GrootWassink, "Preparation of High-Fructose Syrup from the Tubers of Jerusalem Artichoke *Helianthus Tuberosusl*", CRC Crit. Rev. Food Sci. Nutr. **12**, 1-28 (1979).

Fontana, A., C. Bore, C. Ghommidh and J. P. Guiraud, "Structure and Sucrose Hydrolysis Activity of *Saccharomyces cerevisiae* Aggregates", Biotechnol. Bioeng. **40**, 475-482 (1992).

Gagné, I., T. Matsuura and Z. Duvnjak, "Enhanced High Fructose Syrup Production by a Hybrid Fermentation/Pervaporation System Using a Silicon Rubber Hollow Fiber Membrane Module", Sep. Sci. Technol. **37**, 2055-2075 (2002).

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

Gascon, S., P. Neumann and J. O. Lampen, "Comparative Study of the Properties of Purified Internal and External Invertases from Yeast", J. Biol. Chem. **243**, 1573-1577 (1968).

Gasent-Ramirez, J. M., A. C. Codon and T. Benitez, "Characterization of Genetically

Transformed *Saccharomyces cerevisiae* Baker's Yeasts able to Metabolize Melibiose", Appl. Environ. Microbiol. **61**, 2113-2121 (1995).

Ghose, T. K. and R. D. Tyagi, "Rapid Ethanol Fermentation of Cellulose Hydrolysate. I. Batch Versus Continuous Systems", Biotechnol. Bioeng. **21**, 1387-1400 (1979a).

Ghose, T. K. and R. D. Tyagi, "Rapid Ethanol Fermentation of Cellulose Hydrolysate. II. Product and Substrate Inhibition and Optimization of Fermentor Design", Biotechnol. Bioeng. **21**, 1401-1420 (1979b).

Ghosh, S., "Feasibility of Polyacrylamide as a Matrix for Immobilizing Yeast Cells (*Saccharomyces cerevisiae*) for Inversion of Sucrose Syrups", Biotechnol. Tech. **4**, 419-424 (1990).

Gough, S., O. Flynn, C. J. Hack and R. Marchant, "Fermentation of Molasses Using a Thermotolerant Yeast, *Kluyveromyces marxianus* IMB3: Simplex Optimization of Media Supplements", Appl. Microbiol. Biotechnol. **46**, 187-190 (1996).

Gross, D., P. H. Blanchard and D. J. Bell, "NeoKestose: A Trisaccharide Formed from Sucrose by Yeast Invertase", J. Chem. Soc. 1727-1730 (1954).

Guénette, M. and Z. Duvnjak, "Effect of Oleic Acid on the Production of Ethanol and Fructose from Glucose/Fructose Mixtures in an Immobilized Cell Reactor", Acta Biotechnol. **15**, 381-386 (1995).

Guénette, M. and Z. Duvnjak, "Wood Blocks as a Carrier for *Saccharomyces cerevisiae* Used in the Production of Ethanol and Fructose", Chem. Eng. J. **61**, 233-240 (1996).

Hanover, M. L. and J. S. White, "Manufacturing, Composition, and Applications of Fructose", Am. J. Clin. Nutr. **58** 724S-732S (1993).

Holzberg, I., R. H. Finn and K. H. Steinkraus, "A Kinetic Study of the Alcoholic Fermentation of Grape Juice", Biotechnol. Bioeng. **9**, 413-427 (1967).

Johnston, M. and M. Carlson, "Regulation of Carbon and Phosphate Utilization", in "The Molecular and Cellular Biology of the Yeast *Saccharomyces*: Gene Expression", E. W. Jones, J. R. Pringle and J. R. Broach, Eds., Cold Spring Harbor, New York, Cold Spring Harbor Laboratory (1992), pp. 193-281.

Jones, J. K. N. and R. A. Wall, "The Separation of Sugars on Ion-Exchange Resins Part II. Separation of Monosaccharides", Can. J. Chem. **38**, 2290-2294 (1960).

Jones, R. P., N. Pamment and P. F. Greenfield, "Alcohol Fermentation by Yeasts-The Effect of Environmental and Other Variable", Process Biochem. **16**, 42-49 (1981).

Jones, R. P. and P. F. Greenfield, "Effect of Carbon Dioxide on Yeast Growth and Fermentation", Enzyme Microb. Technol. **4**, 210-223 (1982).

Jones, R. P. and P. F. Greenfield, "A Review of Yeast Ionic Nutrition, I: Growth and Fermentative Requirement", Process Biochem. **19**, 48-52, 54-58, 60 (1984).

Karode, S. K., B. B. Gupta and T. Courtois, "Ultrafiltration of Raw Indian Sugar Solution Using Polymeric and Mineral Membranes", Sep. Sci. Technol. **35**, 2473-2483 (2000).

Khare, S. K., K. Jha and A. P. Gandhi, "Hydrolysis of Flatulence-Causing Galactooligosaccharides by Agarose-entrapped *Aspergillus oryzae* Cells", Food Chem. **51**, 29-31 (1994).

Kieboom, A. P. and H. vanBekkum, "Chemical Conversion of Starch-Based Glucose Syrups", in "Starch Conversion Technology", G. M. A. van Beynum and J. A. Roels, Eds., Marcel Dekker Inc., New York (1985), pp. 263-334.

Kirk, L. A. and H. W. Doelle, "Simultaneous Fructose and Ethanol Production from Sucrose Using *Zymomonas mobilis* 2864 Co-immobilized with Invertase", Biotechnol. Lett. **16**, 533-538 (1994).

Kobayashi, H. and H. Suzuki, "Studies on the Decomposition of Raffinose by α-galactosidase of Mold II: Formation of Mold Pellet and its Enzyme Activity", J. Ferment. Technol. **50**, 625-632 (1972).

Koren, D. W. and Z. Duvnjak, "Continuous Production of Very Enriched Fructose Syrup by the Conversion of Glucose to Ethanol from Glucose-Fructose Mixtures in an Immobilized Cell Reactor", Int. J. Food Sci. Technol. **24**, 429-437 (1989).

Koren, D. W. and Z. Duvnjak, "Pure Fructose Syrup and Ethanol Production from High Fructose Corn Syrup Supplemented with Jerusalem Artichoke Juice", J. Chem. Tech. Biotechnol. **47**, 117-125 (1990).

Koren, D. W., "Production of Fructose and Ethanol by Selective Fermentation of Glucose-Fructose Mixtures", Ph. D. Thesis, University of Ottawa (1991).

Koren, D. W. and Z. Duvnjak, "Fed-Batch Production of Concentrated Fructose Syrup and Ethanol Using *Saccharomyces cerevisiae* ATCC 36859", Acta. Biotechnol. **12**, 489-496 (1992).

Koren, D. W. and Z. Duvnjak, "Kinetics of the Selective Fermentation of Glucose from Glucose/Fructose Mixtures Using *Saccharomyces cerevisiae* ATCC 36859", Acta. Biotechnol. **35**, 311-322 (1993).

Kosaric, N., A. Wieczorek, G. P. Cosentino, R. J. Magee and J. E. Prenosil, "Ethanol Fermentation", in "Biotechnology", Vol. 3, H. -J. Rehm, G. Reed and H. Dellweg, Eds., VCH, Weinheim, Germany, (1983), pp. 257-385.

Kosaric, N., A. Wieczorek, G. P. Cosentino and Z. Duvnjak, "Industrial Processing and Products from the Jerusalem Artichoke", Adv. Biochem. Eng. Biotechnol. **32**, 1-24 (1985).

Lamarche, D., "Production of Fructose and Ethanol from Sucrose", M. A. Sc. Thesis, University of Ottawa (1988).

Leigh, D., R. K. Scopes and P. L. Rogers, "A Proposed Pathway for Sorbitol Production by *Zymomonas mobilis*", Appl. Microbiol. Biotechnol. **20**, 413-415 (1984).

Lehle, L. and W. Tanner, "Synthesis of Raffinose-Type Sugars", in "Methods in Enzymology: Complex Carbohydrates Part B", Vol. 28, V. Ginsburg, Ed., Academic Press, New York (1972), pp. 522-530.

Leigh, D., R. K. Scopes and P. L. Rogers, "A Proposed Pathway for Sorbitol Production by *Zymomonas mobilis*", Appl. Microbiol. Biotechnol. **20**, 413-415 (1984).

Lievense, J. C. and H. C. Lim, "The Growth and Dynamics of *Saccharomyces cerevisiae*", in "Annual Reports on Fermentation Processes", Vol. 5, G. T. Tsao, Ed., Academic Press, New York (1982), pp. 211-262.

Liljestrom-Suominen, P. L., V. Joutsjoki and M. Korhola, "Construction of a Stable α-Galactosidase-Producing Baker's Yeast Strain", Appl. Environ. Microbiol. **54**, 245-249 (1988).

Lobo, Z. and P. K. Maitra, "Genetics of Yeast Hexokinase", Genetics **86**, 727-744 (1977).

Long, J. E., "High Fructose Corn Syrup", in "Alternative Sweeteners", 2nd ed, L. O. Nabors and R. C. Gelardi, Eds., Marcel Dekker Inc, New York (1991), pp. 247-258.

Luenser, S., "Application of Microbial Enzymes to Produce High Fructose Corn Syrup and Other Corn Sweeteners", Brewer's Digest **62**, 36-39 (1987).

Maiorella, B. L., H. W. Blanch and C. R. Wilke, "Economic Evaluation of Alternative Ethanol Fermentation Processes", Biotechnol. Bioeng. **26**, 1003-1025 (1984a).

Maiorella, B. L., H. W. Blanch and C. R. Wilke, "Feed Component Inhibition in Ethanolic Fermentation by *Saccharomyces cerevisiae*", Biotechnol. Bioeng. **26**, 1155-1166 (1984b).

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

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

Malik, F., S. Sitorova, V. Vollek and K. Linczenyiova, "Some Biochemical Properties of Wine Yeasts *Saccharomyces cerevisiae* of the FV Series", Biologia. Bratislava. **51**, 635-639 (1996).

Marshall, R. O. and E. R. Kooi, "Enzymatic Conversion of D-glucose to D-fructose", Science **125**, 648-649 (1957).

Meads, P. F., "Color and Turbidity in Sugar Products", in "Cane Sugar Handbook: a Manual for Sugar Cane Manufacturers and their Chemists", 10th edn., G. P. Meade and J. C. P. Chen, Eds., John Wiley & Sons, New York (1977), pp. 613-628.

Micromath, "Scientist Handbook", MicroMath Scientist Software, Salt Lake City, (1995).

Moniruzzaman, M., X. Lai, S. W. York and L. O. Ingram, "Extracellular Melibiose and Fructose are Intermediates in Raffinose Catabolism During Fermentation to Ethanol by Engineered Enteric Bacteria", J. Bacteriol. **179**, 1880-1886 (1997).

Monod, J., "The Growth of Bacterial Cultures", Ann. Rev. Microbiol. **3**, 371-394 (1949).

Moser, A., "Kinetics of Batch Fermentations", in "Biotechnology", Vol. 2, H. -J. Rehm, G. Reed and H. Brauer, Eds., VCH, Weinheim, Germany, (1985), pp. 243-283.

Mulimani, V. H. and Ramalingam, "Short Communication: Enzymatic Degradation of Raffinose Family Sugars in Chickpea Flour", W. J. Microbiol. Biotechnol. **13**, 583-585 (1997).

Myers, D. K., D. T. M. Lawlor and P. V. Attfield, "Influence of Invertase Activity and Glycerol Synthesis and Retention on Fermentation of Media with a High Sugar Concentration by *Saccharomyces cerevisiae*", Appl. Environ. Microbiol. **63**, 145-150 (1997).

Nagashima, M., "Progress in Ethanol Production with Yeasts", in "Yeast Biotechnology and

Biocatalysis", H. Verachtert and R. De Mot, Eds., Marcel Dekker, Inc., New York (1990), pp. 57-84.

Nagodawithana, T. W. and K. H. Steinkraus, "Influence of the Rate of Ethanol Production and Accumulation on the Viability of *Saccharomyces cerevisiae* in Rapid Fermentation", Appl. Environ. Microbiol. **31**, 158-162 (1976).

Nielsen, W. K., S. Kristensen and R. F. Madsen, "Prospects and Possibilities in Application of Membrane Filtration Systems Within the Beet and Cane Sugar Industry", Sugar Technol. Rev. **9**, 59-117 (1982).

Nordstrom, K., "Yeast Growth and Glycerol Formation", Acta Chem. Scand. **20**, 1016-1025 (1966).

Osberger, T. F., "Crystalline Fructose", in "Alternative Sweeteners", 2nd ed., L. O. Nabors and R. C. Gelardi, Eds., Marcel Dekker Inc., New York (1991), pp. 219-246.

Oura, E., "Reaction Products of Yeast Fermentations", Process Biochem. **12**, 19-21, 35 (1977).

Parker, C., N. Peekhaus, X. Zhang and T. Conway, "Kinetics of Sugar Transport and Phosphorylation Influence Glucose and Fructose Cometabolism by *Zymomonas mobilis*", Appl. Environ. Microbiol. **63**, 3519-3525 (1997).

Paugam, M.-F., J. A. Riggs and B. D. Smith, "High Fructose Syrup Production Using Fructose-Selective Liquid Membranes", Chem. Commun. **22**, 2539-2540 (1996).

Pearson, T. N., "Affination", in "Handbook of Sugar Refining: a Manual for the Design and Operation of Sugar Refining Facilities", C. C. Chou, Ed., John Wiley & Sons, New York (2000), pp. 49-54.

Radler, F. and H. Schutz, "Glycerol Production of Various Strains of *Saccharomyces*", Am. J. Enol. Vitic. **33**, 36-40 (1982).

Rehm, H. –J., "Microbial Production of Glycerol and Other Polyols", in "Biotechnology", Vol. 6B, H. -J. Rehm, Ed., VCH, Verlagsgesellschaft, Weinheim, Federal Republic of Germany, (1988), pp. 51-69.

Rehms, H. and W. Barz, "Degradation of Stachyose, Raffinose, Melibiose and Sucrose by Different Tempe-Producing *Rhizopus* Fungi", Appl. Microbiol. Biotechnol. **44**, 47-52 (1995).

Remize, F., S. Schorr-Galindo, J. P. Guiraud, S. Dequin and B. Blondin, "Construction of a Flocculating Yeast for Fructose Production from Inulin", Biotechnol. Lett. **20**, 313-318 (1998).

Reusser, F., P. A. J. Gorin and J. F. T. Spenser, "The Production of Fructose as a Residue of Sucrose Fermentation by *Tricholoma nudum*", Can. J. Microbiol. **6**, 17-20 (1960).

Riffer, R., "Decolorization", in "Cane Sugar Handbook: a Manual for Cane Sugar Manufacturers and their Chemists", 12th edn., J. C. P. Chen and C. C. Chou, Eds., John Wiley & Sons, New York (1993), pp. 460-467.

Samoshina, N. M., N. I. Shustrova, I. D. Stal'naya and L. A. Nakhapetyan, "Optimum pH and Thermostability of Native and Immobilized Yeast Invertase", Appl. Biochem. Microbiol.

16, 658-660 (1981).

Saska, M., "Application of Membrane Technology in Sugar Manufacturing", in "Handbook of Sugar Refining: a Manual for the Design and Operation of Sugar Refining Facilities", C. C. Chou, Ed., John Wiley & Sons, New York (2000), pp. 335-352.

Sinclair, C. G. and B. Kristiansen, "Fermentation Kinetics and Modelling", J. D. Bu'Lock, Ed., Taylor and Francis, New York (1987).

Starzak, M., L. Krzystek, L. Nowicki and H. Michalski, "Macroapproach Kinetics of Ethanol Fermentation by *Saccharomyces cerevisiae*: Experimental Studies and Mathematical Modelling", Chem. Eng. J. **54**, 221-240 (1994).

Stewart, G. G., C. J. Panchal, I. Russell and A. M. Sills, "Biology of Ethanol Producing Microorganisms", CRC Crit. Rev. Biotechnol. **1**, 161-188 (1983).

Stewart, G. G. and I. Russell, "Biochemical and Genetic Control of Sugars and Carbohydrate Metabolism in Yeasts", in "Yeast Biotechnology", D. R. Berry, I. Russell and G. G. Stewart, Eds., Allen & Unwin, London (1987), pp. 277-310.

Straathof, A. J. J., A. P. G. Kieboom and H. van Bekkum, " Invertase-Catalysed Fructosyl Transfer in Concentrated Solutions of Sucrose", Carbohydr. Res. **146**, 154-159 (1986).

Suntinanalert, P., J. P. Pemberton and H. W. Doelle, "The Production of Ethanol Plus Fructose Sweetener Using Fructose Utilization Negative Mutant of *Zymomonas mobilis*", Biotechnol. Lett. **8**, 351-356 (1986).

Takasaki, Y., "Studies on Sugar Isomerizing Enzymes. Effect of Borate on Glucose-Fructose Isomerization Catalyzed by Glucose Isomerase ", Agr. Biol. Chem. **35**, 1371-1375 (1971).

Tatuki, R., "Method for Separating Fructose and Glucose from Sugar Solution Containing Fructose and Glucose Therein", U.S. Patent 3,671,316 (1972).

Teague, J. R. and E. C. Arnold, "UOP Technology for the Production of Fructose Sweeteners", Sugar y Azucar **78**, 18-19, 22-23 (1983).

Tewari, Y. B. and R. N. Goldberg, "Thermodynamics of the Conversion of Aqueous Glucose to Fructose", Appl. Biochem. Biotechnol. **11**, 17-24 (1985).

Tilburg, R. V., "Enzymatic Isomerization of Corn Starch-Based Glucose Syrups", in "Starch Conversion Technology", G .M. A. Van Beynum and J. A. Roels, Eds., Marcel Dekker Inc., New York (1985), pp. 175-236.

Ueng, P.P., L. D. McCracken, C. S. Gong and G. T. Tsao, "Fructose Production from Sucrose and High Fructose Syrup: A Mycelial Fungal System", Biotechnol. Lett. **4**, 353-358 (1982).

van Uden, N., "Ethanol Toxicity and Ethanol Tolerance in Yeasts", in "Annual Reports on Fermentation Processes", Vol. 8, G. T. Tsao, Ed., Academic Press, New York (1985), pp. 11-58.

Vercellotti, J. R., M. A. Clarke, M. A. Godshall, R. S. Blanco and W. S. Patout, "Chemistry of Membrane Separation in Sugar Industry Applications", Zuckerindustrie **123**, 736-745 (1998).

Verduyn, C., E. Postma, W. A. Scheffers and J. P. van Dijken, "Physiology of *Saccharomyces* *cerevisiae* in Anaerobic Glucose-Limited Chemostat Cultures", J. Gen. Microbiol. **136**, 395-403 (1990).

Viikari, L., "Formation of Levan and Sorbitol from Sucrose by *Zymomonas mobilis*", Appl. Microbiol. Biotechnol. **19**, 252-255 (1984).

Vincent, S. F., P. J. L. Bell, P. Bissinger and K. M. H. Nevalainen, "Comparison of Melibiose Utilizing Baker's Yeast Strains Produced by Genetic Engineering and Classical Breeding", Lett. Appl. Microbiol. **28**, 148-152 (1999).

Visuri, K. and A. M. Klibanov, "Enzymatic Production of High Fructose Corn Syrup (HFCS) Containing 55% Fructose in Aqueous Ethanol", Biotechnol. Bioeng. **30**, 917-920 (1987).

Vitolo, M. and M. T. Yassuda, "Effect of Sucrose Concentration on the Invertase Activity of Intact Yeast Cells (*S. cerevisiae*)", Biotechnol. Lett. **13**, 53-56 (1991).

Vuilleumier, S., "Worldwide Production of High-Fructose Syrup and Crystalline Fructose", Am. J. Clin. Nutr. **58** 733S-736S (1993).

Wallhausser, K. H., "Sterilization", in "Biotechnology", Vol. 2, H. -J. Rehm, G. Reed and H. Brauer, Eds., VCH, Verlagsgesellschaft, Weinheim, Federal Republic of Germany, (1985), pp. 699-723.

Wang, Z. –X., J. Zhuge, H. Fang and B. A. Prior, "Glycerol Production by Microbial Fermentation: a Review", Biotechnol. Adv. **19**, 201-223 (2001).

Yanase, H., M. Masuda, T. Tamaki and K. Tonomura, "Expression of the *Escherichia coli* α-Galactosidase and Lactose Permease Genes in *Zymomonas mobilis* and its Raffinose Fermentation", J. Ferment. Bioeng. **70**, 1-6 (1990).

Zachariou, M. and R. K. Scopes, "Glucose-Fructose Oxidoreductase, a New Enzyme Isolated from *Zymomonas mobilis* that is Responsible for Sorbitol Production", J. Bacteriol. **167**, 863-869 (1986).

Appendices

Appendix A: Standard curves for biomass, sugars, glycerol, sorbitol and ethanol

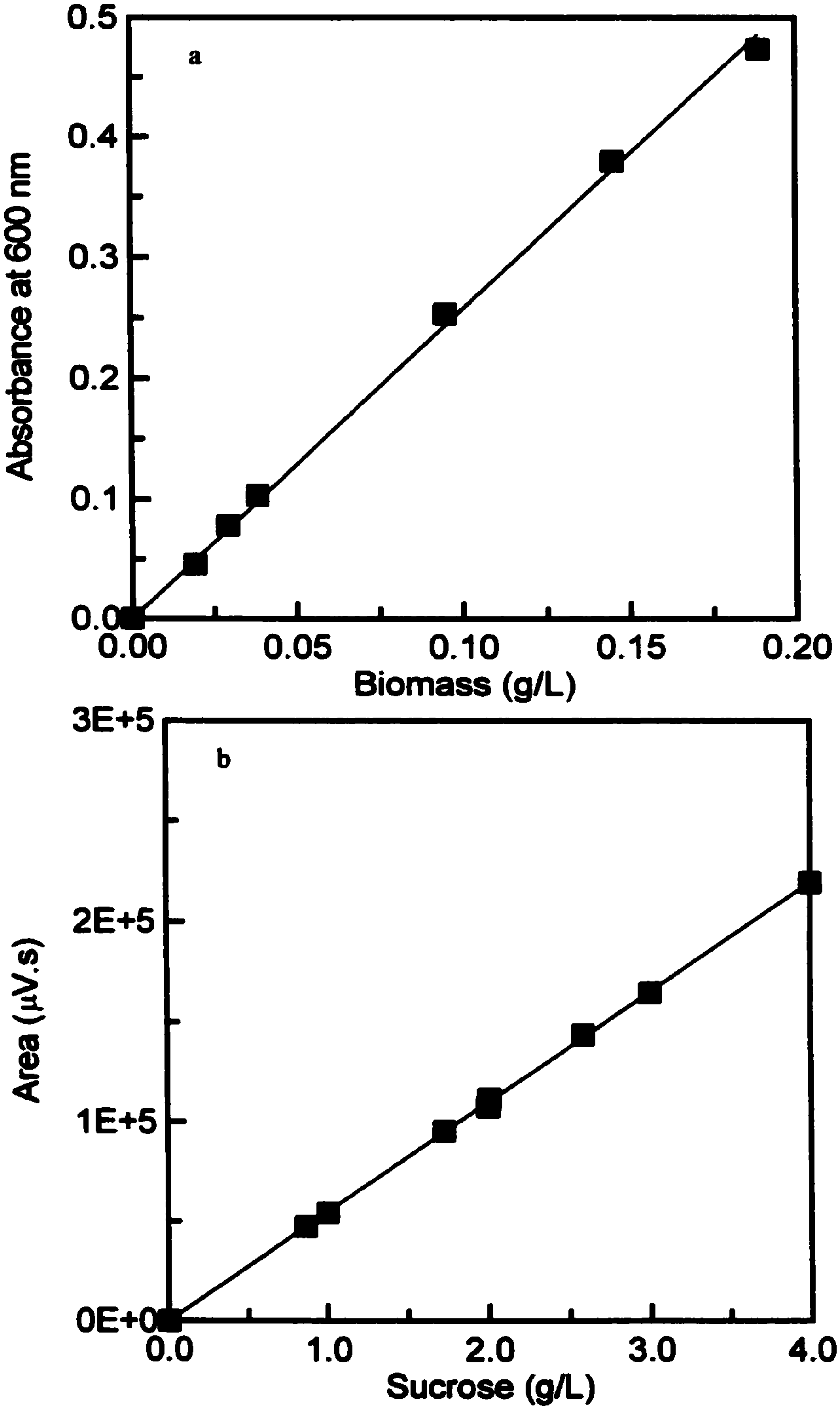

Figure A.1 Standard curves for a) biomass and b) sucrose determination.

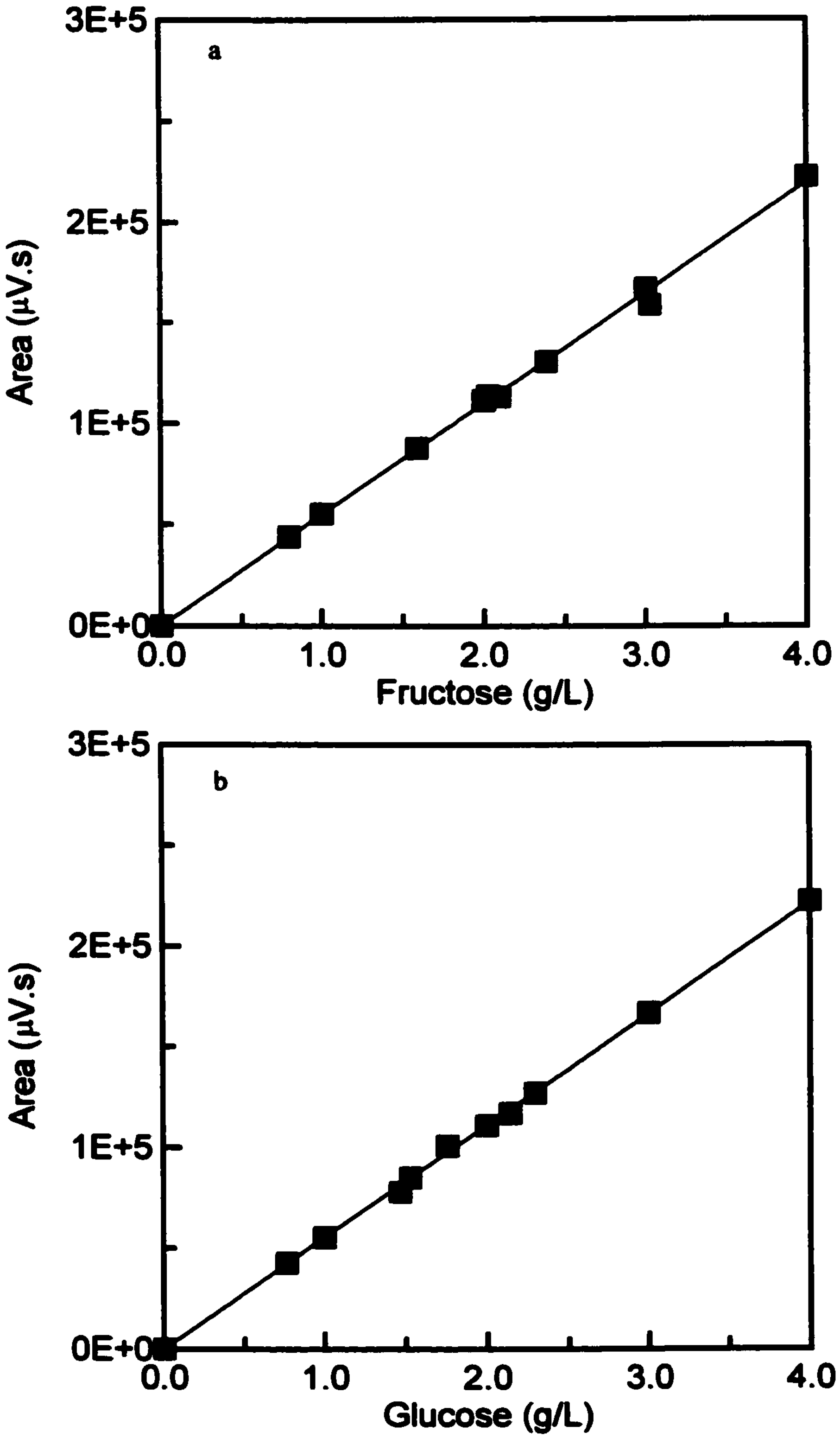

Figure A.2 Standard curves for a) fructose and b) glucose determination.

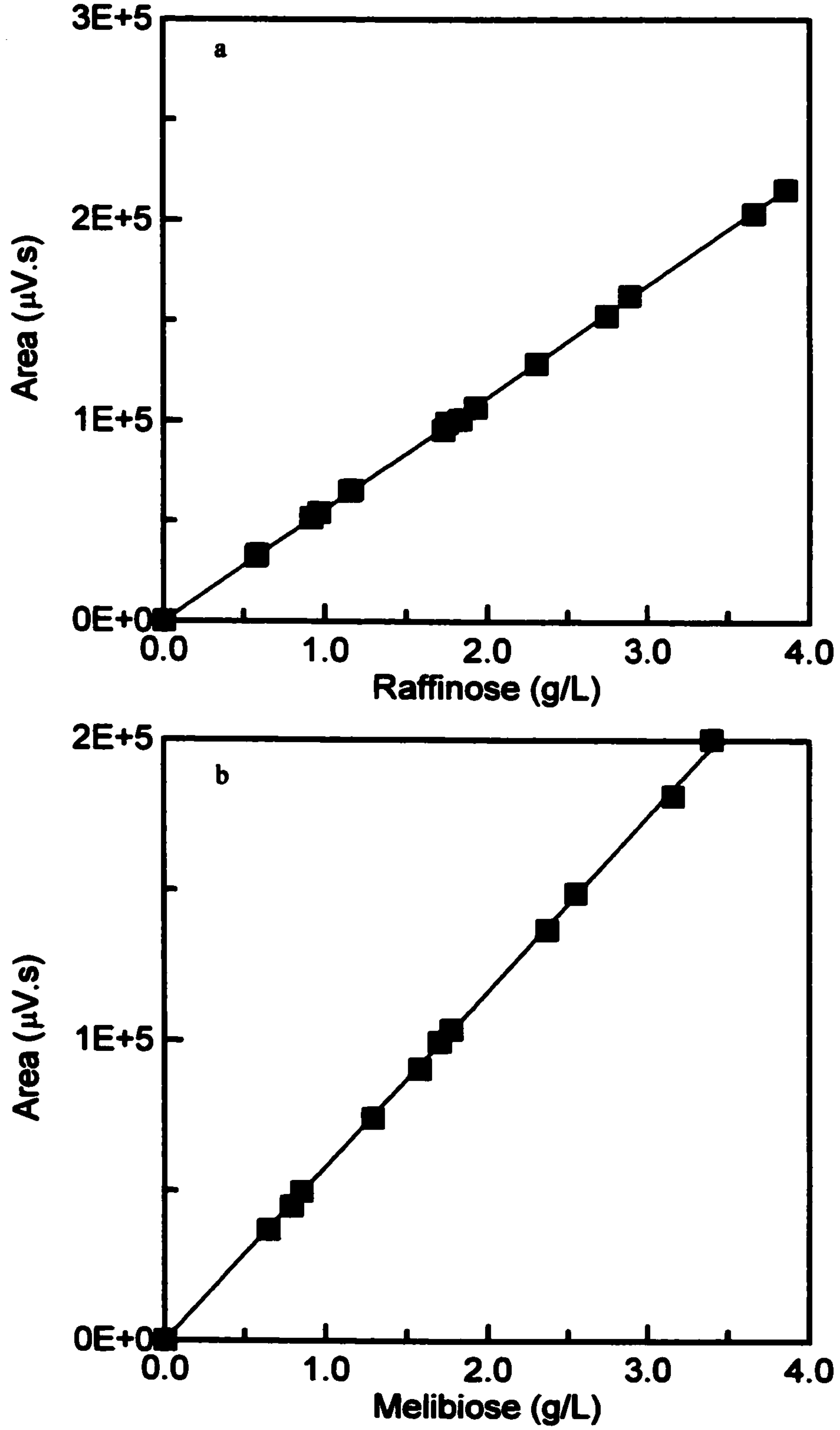

Figure A.3 Standard curves for a) raffinose and b) melibiose determination.

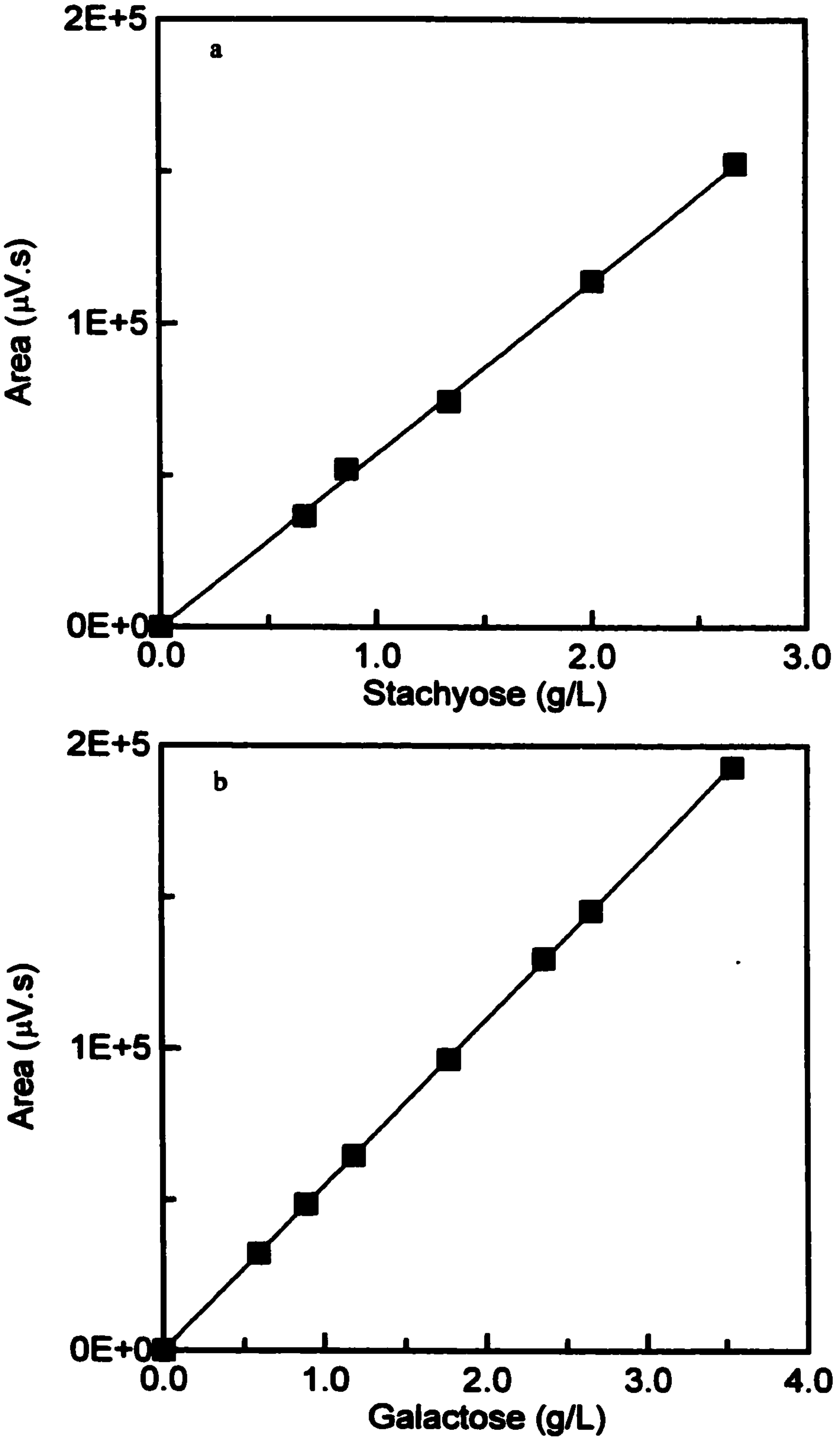

Figure A.4 Standard curves for a) stachyose and b) galactose determination.

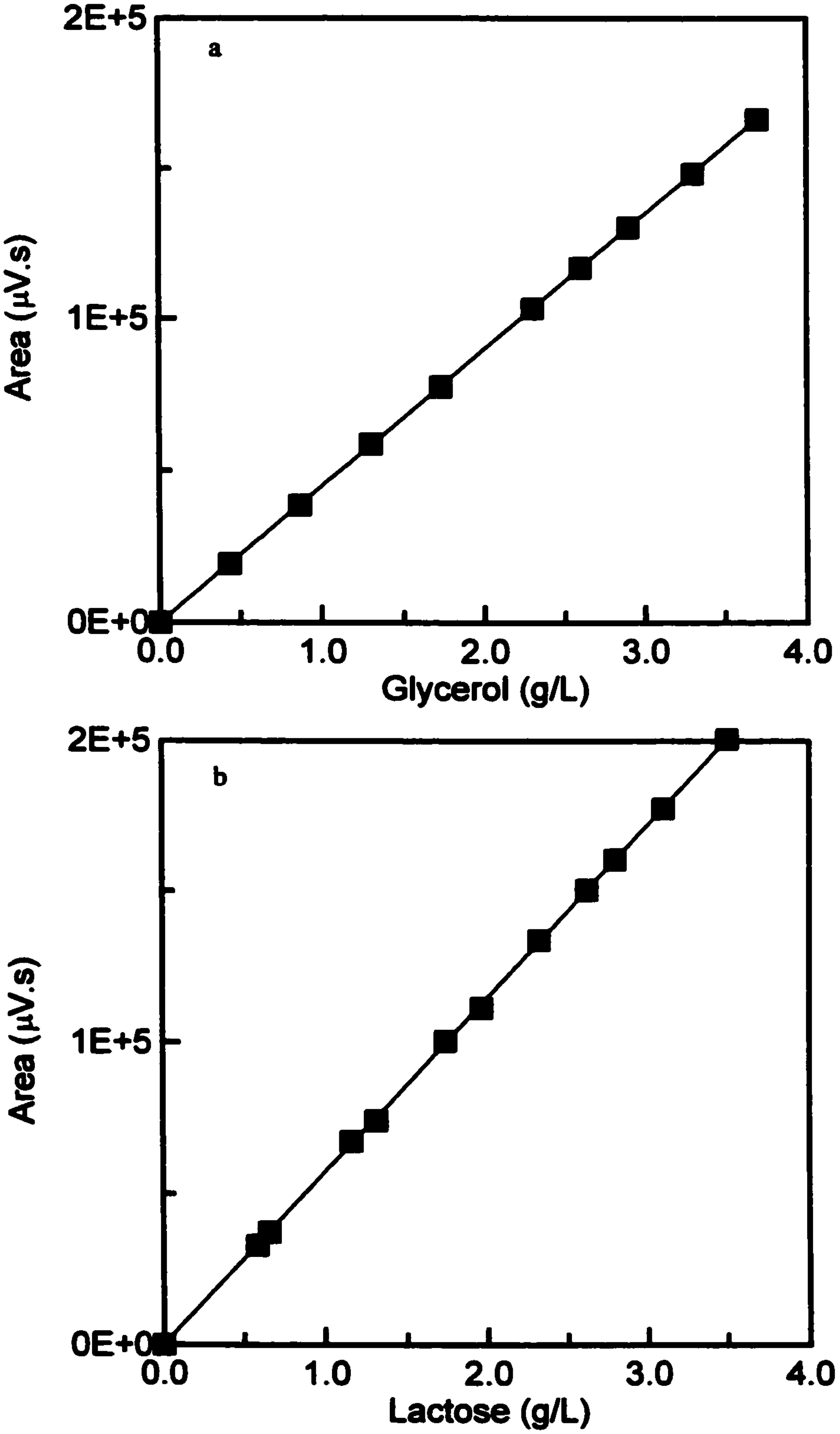

Figure A.5 Standard curves for a) glycerol and b) lactose determination.

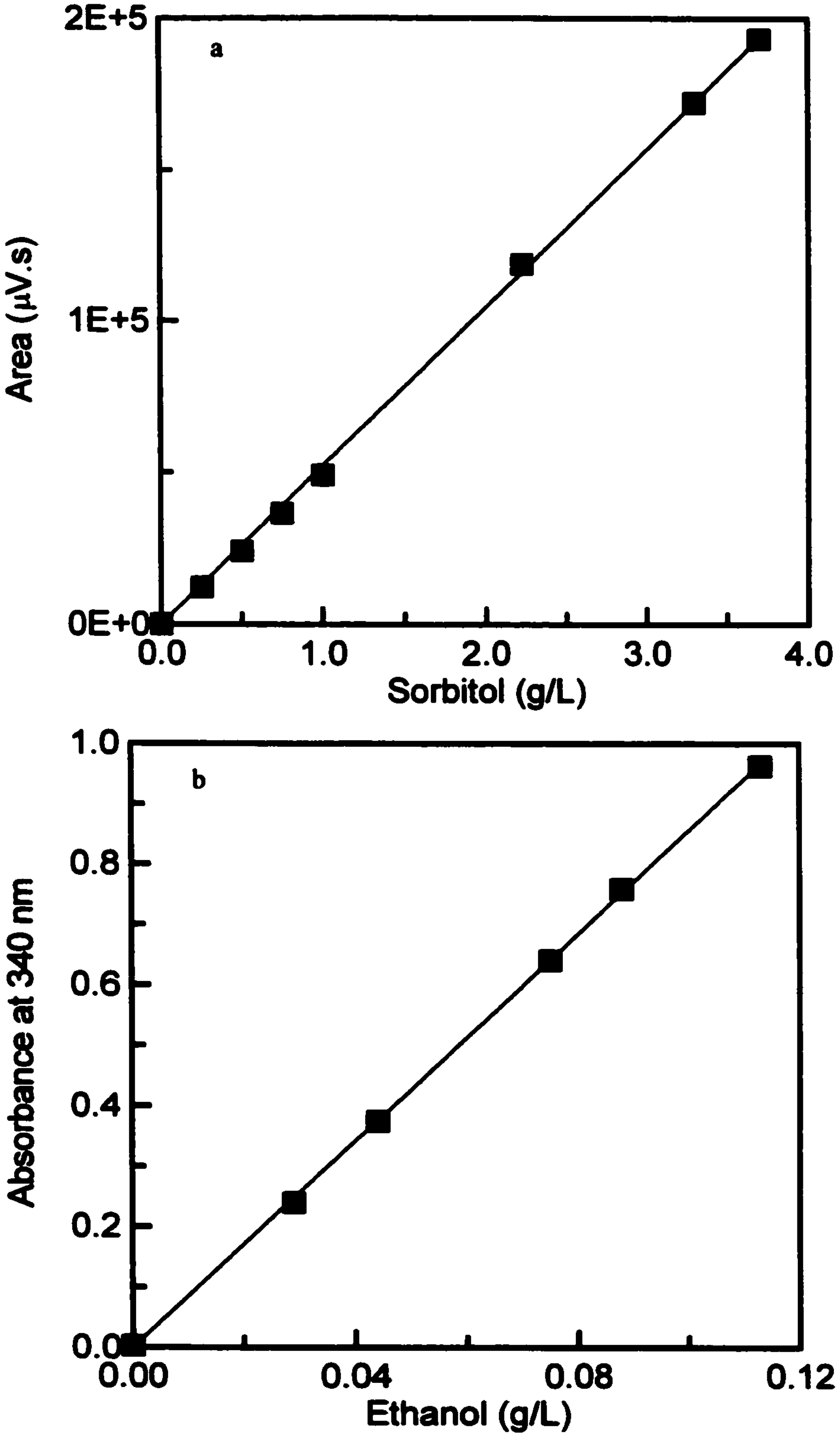

Figure A.6 Standard curves for a) sorbitol and b) ethanol determination.

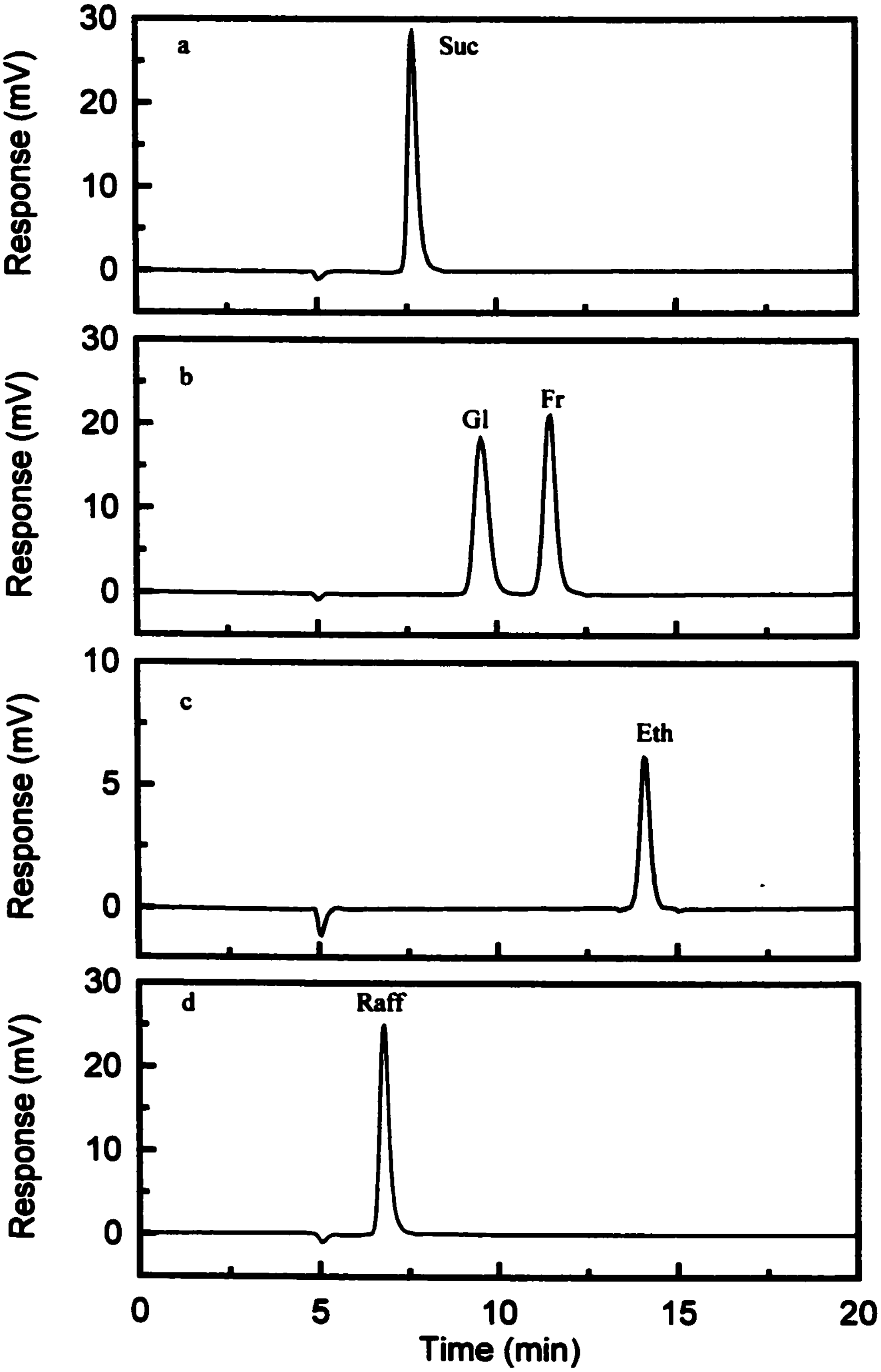

Figure A.7 Chromatograms representing: (a) sucrose; (b) glucose and fructose; (c) ethanol; (d) raffinose.

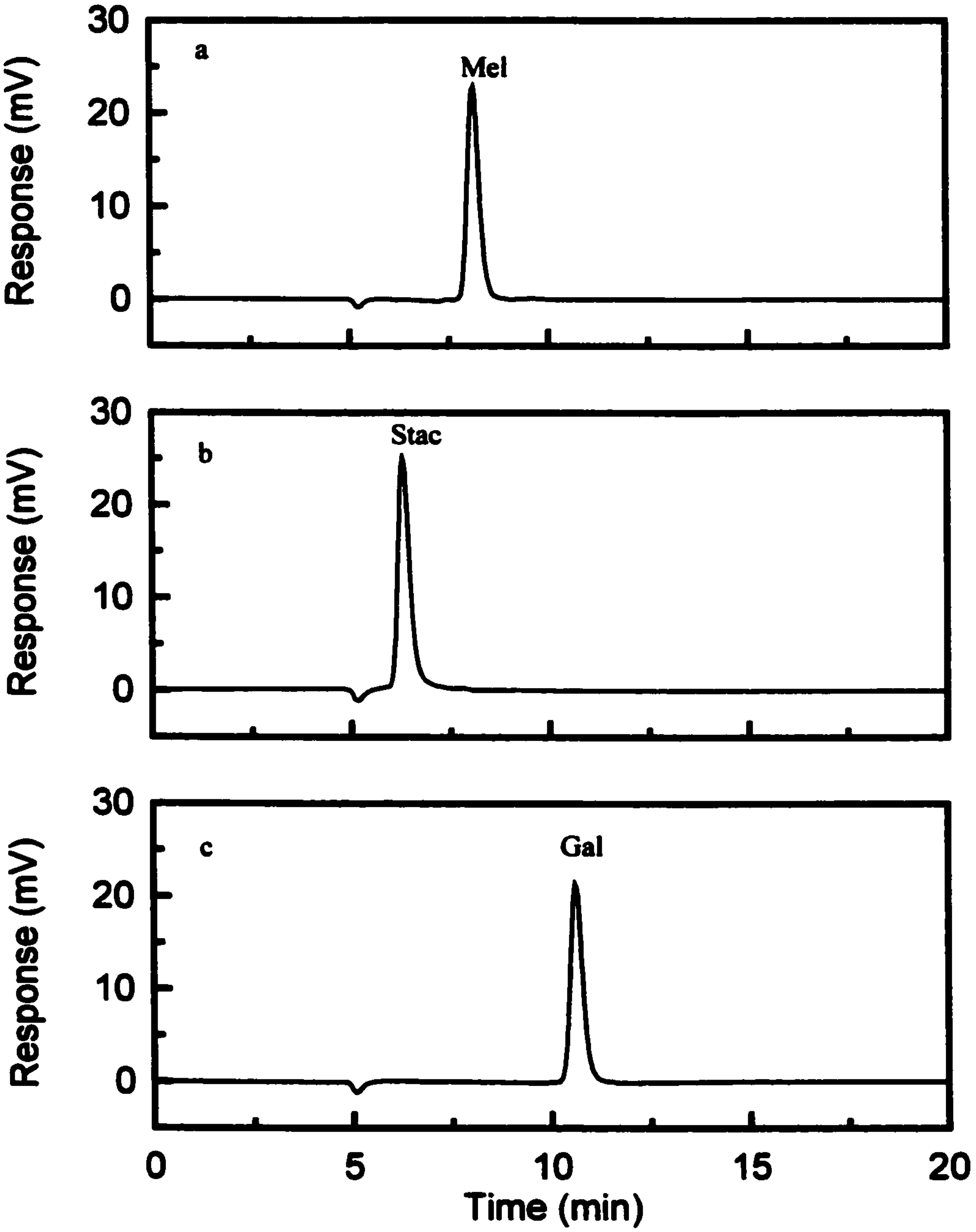

Figure A.8 Chromatograms representing: (a) melibiose; (b) stachyose; (c) galactose.

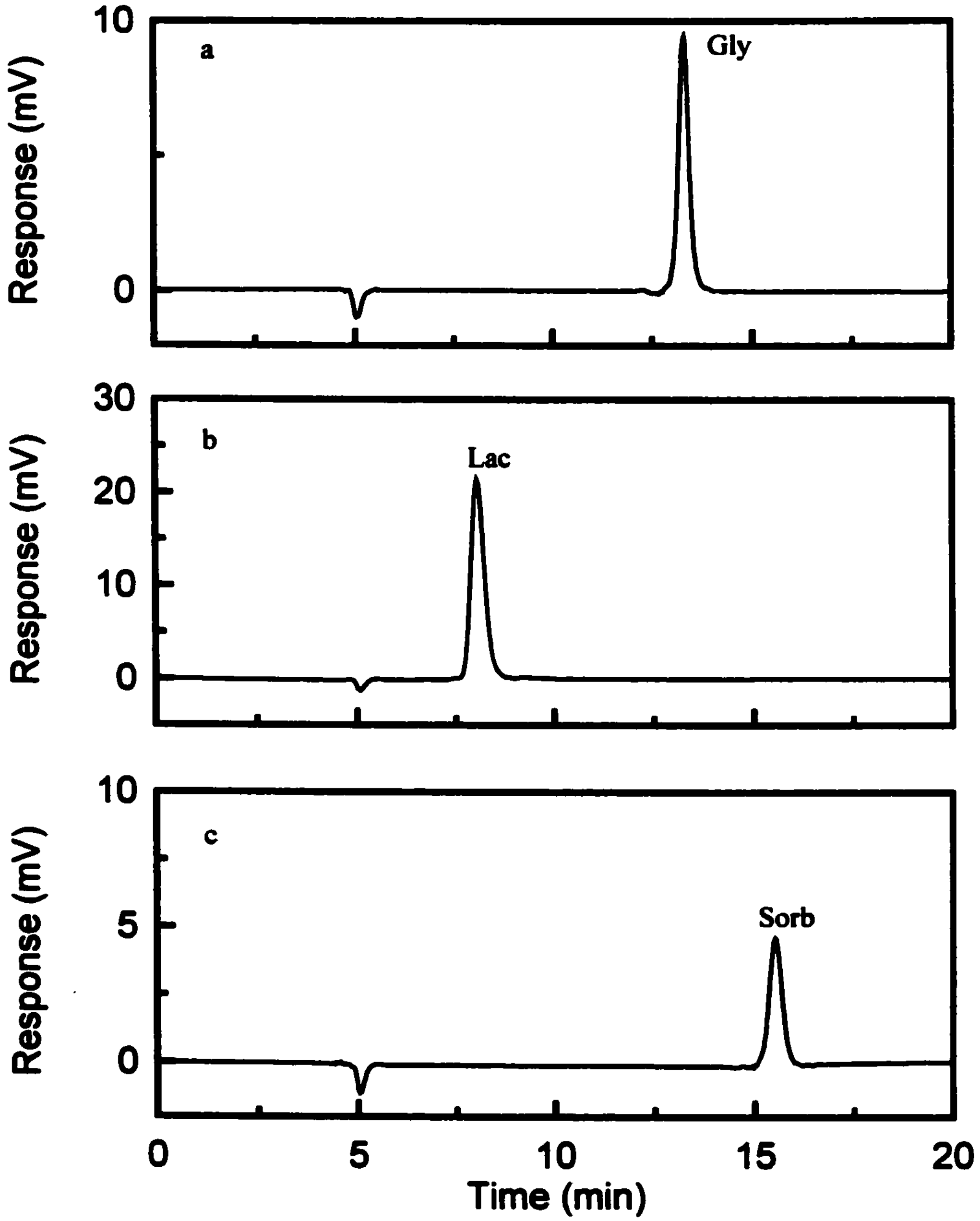

Figure A.9 Chromatograms representing: (a) glycerol; (b) lactose; (c) sorbitol.

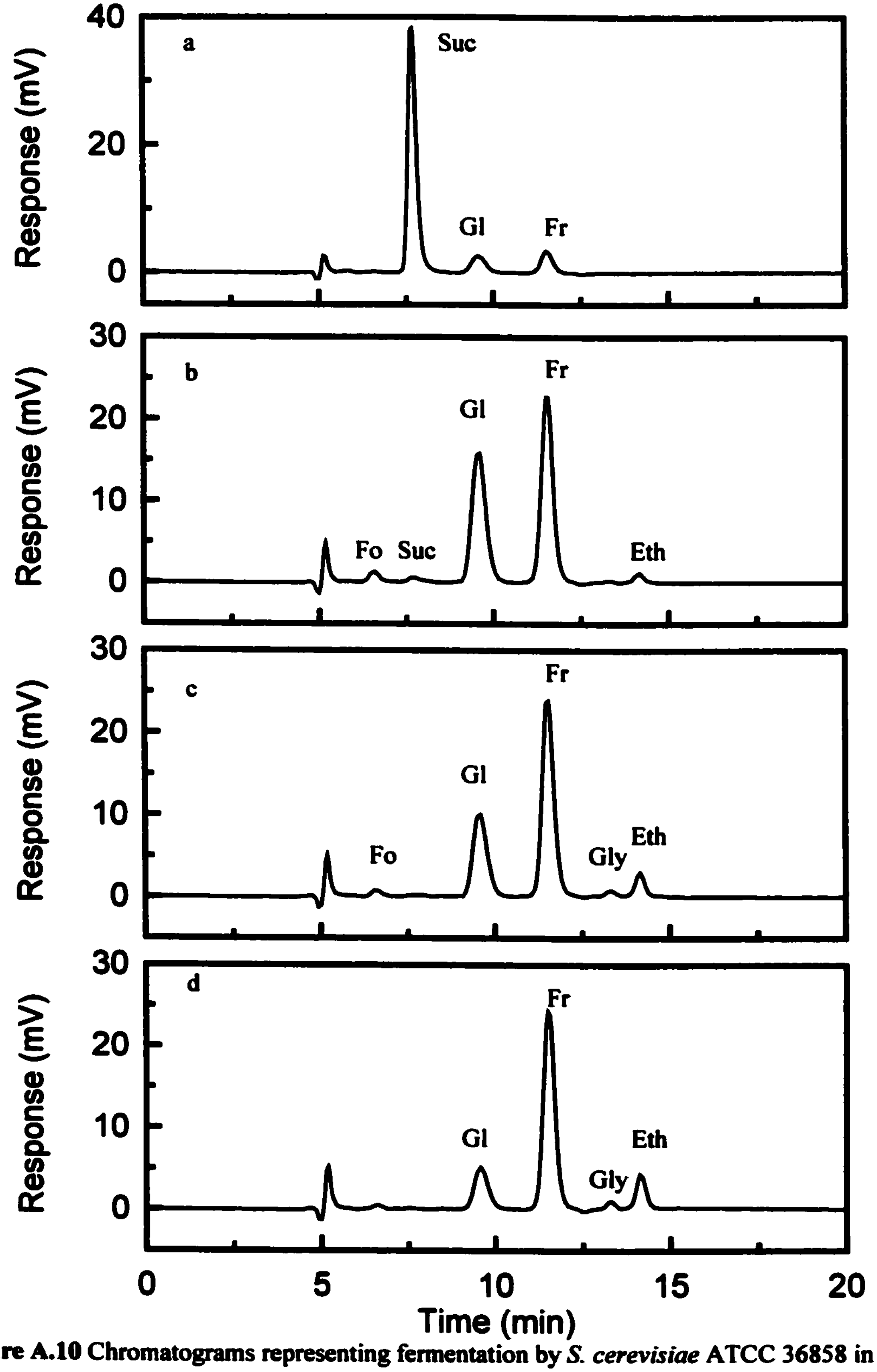

Figure A.10 Chromatograms representing fermentation by *S. cerevisiae* ATCC 36858 in a medium containing 262 g/L sucrose after: (a) 0 h; (b) 3 h; (c) 6 h; (d) 9 h. (Fo = fructo-oligosaccharides).

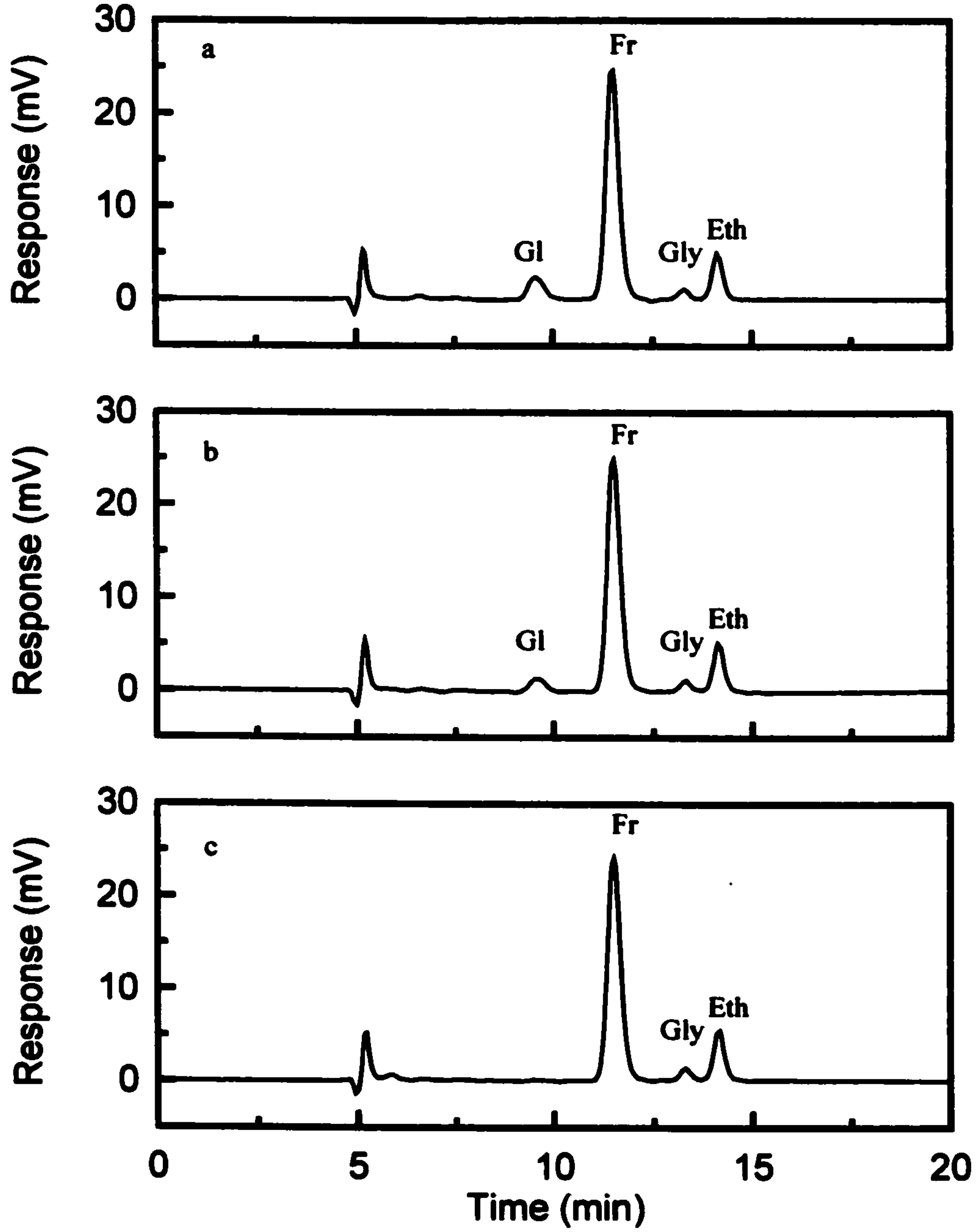

Figure A.11 Chromatograms representing fermentation by *S. cerevisiae* ATCC 36858 in a medium containing 262 g/L sucrose after: (a) 12 h; (b) 14 h; (c) 22.6 h.

Appendix B

B.1 Absorbance and transmittance changes with total solids

It should be pointed out that absorbance should be used in Eq. 4.3 in the calculation of decolourization efficiency and for the comparison of colour results. The use of transmittance (T_s) should be avoided in the calculation and comparison because of the nonlinear relationship between transmittance and concentration (Chou, 1993). This was also noticed in a biomass- and ethanol-free broth containing 150.8 g/L fructose, 13.4 g/L glucose, 2.8 g/L raffinose and 8.5 g/L glycerol (Figure B.1). The broth shown below was produced after fermentation of sugar cane molasses medium containing about 230 g/L total sugars.

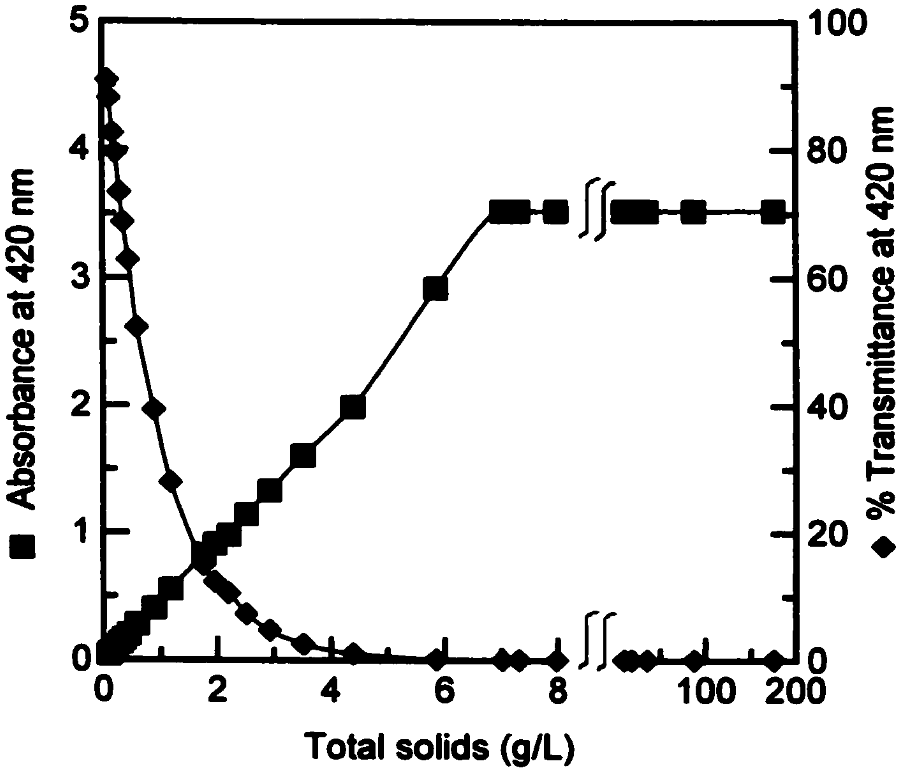

Figure B.1 Absorbance and transmittance as a function of total solids from a diluted broth originally containing about 151 g/L fructose.

The absorbance of the broth increased linearly when total solids concentration increased to 7 g/L (Figure B.1). This was obtained by diluting the broth by 27 times or more. Total solids concentration is related to the amount of colourants present in the broth. The higher the concentration of total solids in the broth, the higher the colour intensity. The absorbance of the broth at 420 nm was about 3.5, which remained constant when total solids concentrations were between 7 and 175 g/L. The transmittance of the broth at 420 nm was zero in that total solids concentration range. This showed the limitation of the spectrophotometric method, which does not differentiate colour intensity of the broth when total sugar concentrations were above 7 g/L. To overcome this problem for colour determination, the International Commission for Uniform Methods of Sugar Analysis (ICUMSA) method 4 recommended that samples should be diluted before absorbance measurement to be in the 20 to 80% transmittance range (i.e. in the 0.097 to 0.70 absorbance range).

B.2 Analysis of sugars in the broth used in the decolourization tests

Results of samples analyzed by HPLC for the broth used in the purification of fructose syrups, section 5.3 are shown in Tables B.1 to B.9.

Table B.1 HPLC analysis of the broth used in ethanol recovery after vacuum evaporation from a broth containing about 46 g/L ethanol and 116 g/L fructose from various runs (Table 5.20).

	Glucose (g/L)	Fructose (g/L)	Raffinose (g/L)	Ethanol (g/L)	Glycerol (g/L)	Fructose content[a] (%)
Run 1						
Original broth	5.34	114.65	1.50	45.9	6.62	94.4
Broth remained	6.42	152.50	3.21	----	10.40	94.1
Condensate collected	----	----	----	175.7	----	----
Run 2						
Original broth	13.65	117.10	3.80	44.1	6.95	87.0
Broth remained	15.16	150.10	4.57	----	9.57	88.4
Condensate collected	----	----	----	189.9	----	----
Run 3						
Original broth	3.91	115.46	2.56	47.7	6.15	94.7
Broth remained	5.29	155.2	3.50	----	8.50	94.6
Condensate collected	----	----	----	184.9	----	----

[a] Values are calculated based on total sugar concentrations

Table B.2 HPLC analysis of broth containing 114 g/L fructose and 46 g/L ethanol after treatment with 33% activated carbon for 10 min (Table 5.21).

	Glucose (g/L)	Fructose (g/L)	Raffinose (g/L)	Ethanol (g/L)	Glycerol (g/L)	Fructose content[b] (%)
Untreated broth	4.00	113.53	2.00	45.8	6.86	95.0
Treated broth	3.64	107.42	0.00	34.3	6.50	96.7
Treated broth[a]	2.44	60.27	0.00	22.0	3.70	96.1

[a] Activated carbon was mixed with 36 mL deionized water before the addition of 50 mL of untreated broth

[b] Values are calculated based on total sugar concentrations

Table B.3 HPLC analysis of broth containing 154 g/L fructose after treatment with various amounts of activated carbon (Table 5.23).

Carbon (%)		Glucose (g/L)	Fructose (g/L)	Raffinose (g/L)	Glycerol (g/L)	Fructose content[a] (%)
11	Untreated broth	14.22	154.13	3.03	8.90	89.9
	Treated broth	12.18	146.45	2.86	8.14	90.7
22	Untreated broth	14.22	154.13	3.03	8.90	89.9
	Treated broth	13.19	144.79	1.81	7.29	90.6
33	Untreated broth	14.22	154.13	3.03	8.90	89.9
	Treated broth	13.13	143.18	0.00	7.01	91.6

[a] Values are calculated based on total sugar concentrations

Table B.4 HPLC analysis of broth containing 154 g/L fructose after three treatments each with 11% activated carbon (Table 5.25).

	Glucose (g/L)	Fructose (g/L)	Raffinose (g/L)	Glycerol (g/L)	Fructose content[a] (%)
Before 1st treatment	14.22	154.13	3.03	8.90	89.9
After 1st treatment	12.18	146.45	2.86	8.14	90.7
Before 2nd treatment	12.18	146.45	2.86	8.14	90.7
After 2nd treatment	12.66	144.33	1.22	6.14	91.2
Before 3rd treatment	12.66	144.33	1.22	6.14	91.2
After 3rd treatment	12.20	140.05	0.00	5.80	92.0

[a] Values are calculated based on total sugar concentrations

Table B.5 HPLC analysis of broth containing 153 g/L fructose after treatment with 33% activated carbon for various periods of time (Table 5.27).

	Glucose (g/L)	Fructose (g/L)	Raffinose (g/L)	Glycerol (g/L)	Fructose content[b] (%)
Untreated broth	5.92	153.28	2.65	8.28	94.7
Treated broth (10 min)	5.54	144.24	0.00	6.80	96.3
Treated broth (20 min)	4.53	144.13	0.00	7.50	97.0
Treated broth (40 min)	5.66	144.35	0.00	6.94	96.2
Treated broth (60 min)	5.78	144.33	0.00	7.00	96.1
Treated broth (120 min)	5.81	144.37	0.00	6.89	96.1
Treated broth[a]	3.87	82.01	0.00	4.08	95.5

[a] Activated carbon was mixed with 18 mL deionized water before the addition of 25 mL untreated broth

[b] Values are calculated based on total sugar concentrations

Table B.6 HPLC analysis of broth containing 153 g/L fructose after treatment with 33% activated carbon then washing the spent carbon with water (Table 5.29).

	Glucose (g/L)	Fructose (g/L)	Raffinose (g/L)	Glycerol (g/L)	Fructose content[a] (%)
Untreated broth	4.56	152.72	2.53	9.16	95.6
Treated broth	3.92	136.97	0.00	6.34	97.2
1st carbon washing	3.04	73.70	0.00	4.37	96.0
2nd carbon washing	1.33	39.90	0.00	2.41	96.8
3rd carbon washing	0.75	20.83	0.00	1.39	96.5
4th carbon washing	0.51	12.86	0.00	0.80	96.2

[a] Values are calculated based on total sugar concentrations

Table B.7 HPLC analysis of broth containing 148 g/L fructose after treatments with PES 10,000 MWCO membrane followed by activated carbon (Table 5.31).

	Glucose (g/L)	Fructose (g/L)	Raffinose (g/L)	Glycerol (g/L)	Fructose content[a] (%)
Untreated broth	12.62	147.98	3.37	7.66	90.2
Filtered broth	12.66	146.49	3.20	7.36	90.2
Permeate	12.68	147.60	2.10	10.22	90.9
Retentate	12.98	142.41	4.06	8.06	89.3
Permeate (1st treatment 3% carbon)	12.87	144.23	1.33	9.22	91.0
Permeate (2nd treatment 3% carbon)	12.55	142.50	1.23	9.36	91.2
Permeate (3rd treatment 1.5% carbon)	12.66	142.11	1.21	8.90	91.1

[a] Values are calculated based on total sugar concentrations

Table B.8 HPLC analysis of broth containing 148 g/L fructose after treatments with TFC 1,000 MWCO membrane followed by activated carbon (Table 5.32).

	Glucose (g/L)	Fructose (g/L)	Raffinose (g/L)	Glycerol (g/L)	Fructose content[a] (%)
Untreated broth	12.62	147.98	3.37	7.66	90.2
Filtered broth	12.66	146.49	3.20	7.36	90.2
Permeate	13.23	154.60	0.00	15.76	92.1
Retentate	13.42	146.36	3.29	7.46	89.8
Permeate (treatment 3% carbon)	14.22	150.12	0.00	13.63	91.3

[a] Values are calculated based on total sugar concentrations

Table B.9 HPLC analysis of broth containing 45 g/L fructose after the addition of washouts and passing the solution through five beds of ion exchange resins (Figure 5.54).

	Glucose (g/L)	Fructose (g/L)	Raffinose (g/L)	Glycerol (g/L)	Fructose content[a] (%)
Treated broth after addition of washouts	1.84	44.52	0.00	3.05	96.0
1st bed	1.26	42.58	0.00	2.57	97.1
2nd bed	1.95	38.75	0.00	1.91	95.2
3rd bed	1.79	34.20	0.00	2.50	95.0
4th bed	1.49	22.16	0.00	2.54	93.7
5th bed	0.39	10.50	0.00	1.86	96.4

[a] Values are calculated based on total sugar concentrations

B.3 Colour of substrate and products from the purification process

The colour of samples of cane molasses and broth before and after purification processes used in this study is shown in Figure B.2. The volume of the sample in each tube is 2.8 mL. The figure shows a large difference in the colour of the broth before and after carbon and membranes treatments.

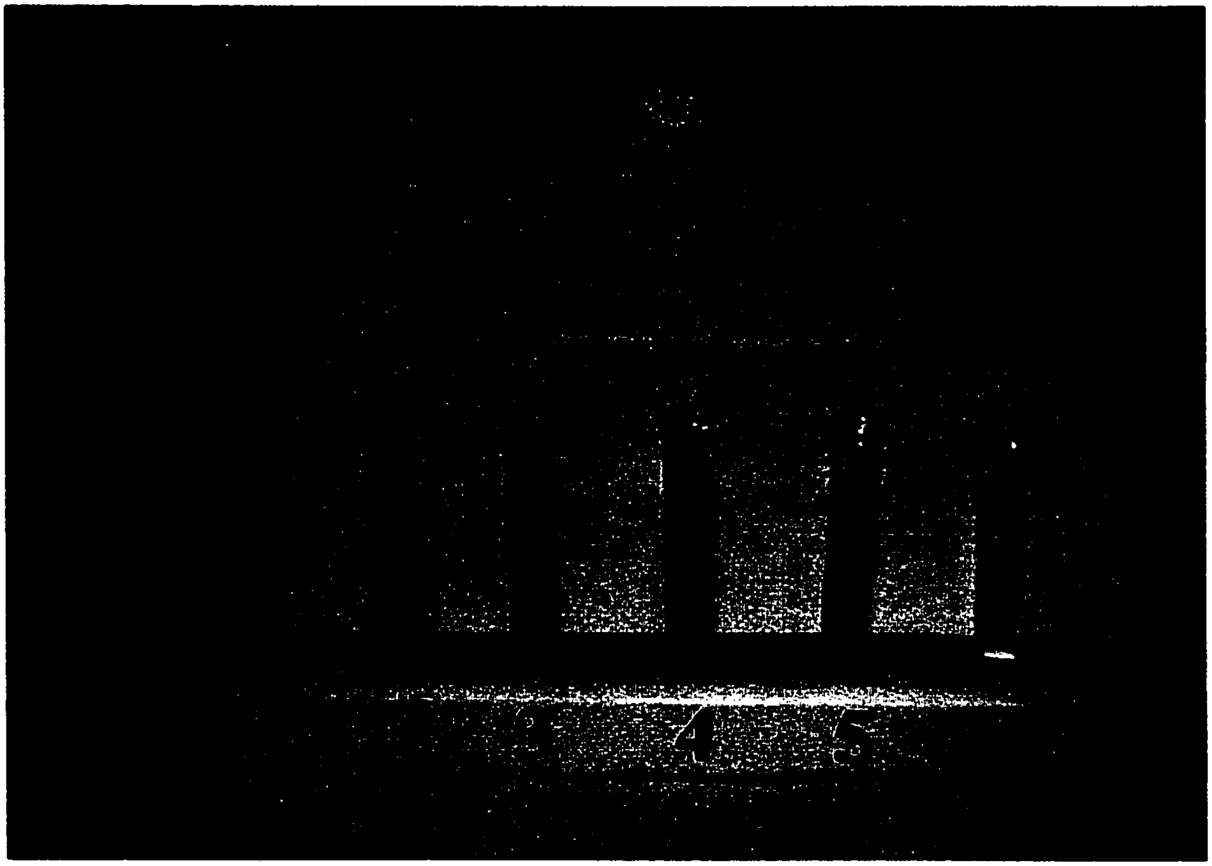

Figure B.2 Colour of (1) crude cane molasses; (2) molasses broth; (3) broth treated with 11% carbon; (4) broth treated with 22% carbon; (5) broth treated with 33% carbon; (6) broth treated with 33% carbon followed by ion exchange resins; (7) permeate of PES 10,000 MWCO membrane; (8) permeate of PES 10,000 MWCO membrane followed by treatment with 7.5% carbon; (9) permeate of TFC 1,000 MWCO membrane; (10) permeate of TFC 1,000 MWCO membrane followed by treatment with 3% carbon; (11) retentate of PES 10,000 MWCO membrane; (12) retentate of TFC 1,000 MWCO membrane.

Appendix C

C.1 Format used for model equations with MicroMath scientist software V2.0 to analyze tests carried out in sucrose media

```
// MicroMath Scientist PreCompiled Model File name: Sucros70.eqn
//Sucrose model
Independent Variable(s): t
Dependent Variable(s): X, Ssuc, Sg, P, Sf
Parameters: Ks, Ygs, Yps, Kss, Yxs, Mmax, hs
//Initial conditions
t0=0.0
X0=2.2133
Ssuc0=169.25
Sg0=3.67
P0=0.453
Sf0=3.43
//Differential Equations:
X'=Mmax*X*Sg/(Ks+Sg)
Ssuc' = -hs*X*Ssuc/(Kss+Ssuc)
Sg'=(Ygs*hs*X*Ssuc/(Kss+Ssuc))-((Mmax*X*Sg/(Ks+Sg))/Yxs)
P'=(Mmax*X*Sg/(Ks+Sg))*Yps/Yxs
Sf'=Ygs*hs*X*Ssuc/(Kss+Ssuc)
//General initial conditions:
t=t0
X=X0
Ssuc=Ssuc0
Sg=Sg0
P=P0
Sf=Sf0
//END
***
```

C.2 Format used for model equations with MicroMath scientist software V2.0 to analyze tests carried out in raffinose media

```
// MicroMath Scientist PreCompiled Model File name: Raffinose Raff18.eqn
//Raffinose model
Independent Variable(s): t
Dependent Variable(s): R, M, X, P, F
Parameters: hr, Kr, Ymr, Yfr, Mmax, Ks, Yxm, Ypm
//Initial conditions
t0=0.0
R0=68.875
M0=0.425
X0=1.158
P0=0
F0=0
//Differential Equations:
R'=-hr*R*X/(Kr+R)
M' =(-Ymr*R')-(X'/Yxm)
X'=Mmax*X*M/(Ks+M)
P'=(Ypm/Yxm)*X'
F'=-Yfr*R'
//General initial conditions:
t=t0
R=R0
M=M0
X=X0
P=P0
F=F0
//End
***
```