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An investigation into the fructose block association with the brewing process

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AN INVESTIGATION INTO THE FRUCTOSE BLOCK ASSOCIATED WITH THE BREWING PROCESS

David Timothy Cason

A dissertation submitted to the faculty of Science
University of the Witwatersrand, Johannesburg
in the fulfilment of the requirements for the degree,
Doctor of Philosophy.

Johannesburg 1986

ABSTRACT

The uptake of fructose in Saccharomyces cerevisiae 2036 is via a biphasic transport system, in which the first component is a high affinity, Km = 0.3 mM, low capacity, V_{max} = 0.6 mmoles/h/g dry weight, proton symport which does not transport glucose and is independant of the maltose proton symport. The presence of glucose has no effect on the uptake of fructose via the symport. The two independent symports compete for protons to drive their respective transport systems. The stoichiometry of uptake is one proton per molecule of fructose. Maltose and ethanol non-competitively inhibit fructose uptake via the proton symport with Ki = 21.3 mM and k = 0.74 L/mol repectively. The second component is a lower affinity, higher capacity facilitated diffusion system which transports both glucose and fructose. Glucose uptake is monophasic and has the highest affinity, Km = 1.3 mM, of all sugars for this transport system. Glucose competitively inhibits fructose uptake with Ki = 1.15 mM. Fructose competitively inhibits glucoses uptake to a lesser degree with Ki = 36.0 mM. Ethanol non-competitively inhibits glucose uptake of the facilitated diffusion system with k = 0.96 L/mol. The fermentation data closely correlates with the parameters of membrane transport kinetics for glucose and fructose uptake. In the fermentations containing glucose and fructose together, glucose competitively inhibits fructose uptake causing preferential utilization of glucose over fructose. When glucose and fructose are fermented separately glucose is utilized faster than fructose as it has a higher affinity for the facilitated diffusion system than fructose has for its biphasic transport. A consequence of slower fructose utilization results in residual fructose concentrations remaining at the end of fermentation when sucrose adjuncts are used, hence causing the "fructose block". A mathematical model, based on the increase in biomass up to

flocculation and its subsequent decrease thereafter, plus all the parameters of membrane transport kinetics which affect fructose utilization, accurately predicts fructose utilization in laboratory scale fermentations using normal and high gravity sucrose adjunct worts.

Amelioration of the "fructose block" is multifaceted. The residual fructose concentrations in wort for the last three days of fermentation are inversely proportional to the pitching rate. An increase in the pitching rate will result in decreased fructose concentrations. The additions of growth stimulants such as "nutromix", a commercial preparation of nitrogen and vitamin sources, or pulses of oxygen during the initial stages of fermentation which are directly used in the production of sterols, chiefly ergosterol (9,99), both cause decreased fructose concentrations at the end of fermentation. Extension of the fermentation time from 240 to 336 hours also reduces residual fructose concentrations. The above methods may be used singly or in combination to bring about the reduction in residual fructose concentrations to levels which are below the fructose taste threshold for bottled beer samples.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for my degree or examination in any other university.

David T Cason

DAVID TIMOTHY CASON

27th DAY OF OCTOBER 1986

To my Mother.

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My thanks go to:-

Dr. Graham Reid for his guidance and supervision of this project.

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PREFACE

South African Breweries (SAB) observed that significant concentrations of fructose may be present in Carling Black Label, bottled beer samples. This phenomenon only occurs when the beer is produced using a sucrose adjunct in the wort, and has been termed the "fructose block". The S.A.Breweries in Cape Town and Bloemfontein both produce Carling Black Label beer using sucrose as an adjunct in the wort and the accompanying bar charts show the fructose concentrations from in-bottle samples over a period of time. Fructose is a very sweet sugar, and on a mole per mole basis is 50% sweeter than sucrose. Therefore only very small concentrations would be needed to taint the beer with a sweet off-flavor. This situation may cause consumer reaction and affect the sales of Carling Black Label beer, or any beer in which sucrose is used as a adjunct in the fermentation process. This project was undertaken in order to gain a full understanding of the fructose block, and to this end, the project was divided into three main areas:

1. To establish the fructose block in a laboratory fermentation system and observe the rates of fructose and glucose utilization under conditions of differing growth media and sugar concentrations.
2. Manipulation and alteration of fermentation procedures in order to reduce the concentrations of fructose at the end of fermentation.
3. To find the cause of the fructose block in terms of the constituents of the wort and the metabolic pathways of the brewing yeast, Saccharomyces cerevisiae 2036, which is used in the brewing of Carling Black Label beer by SAB.

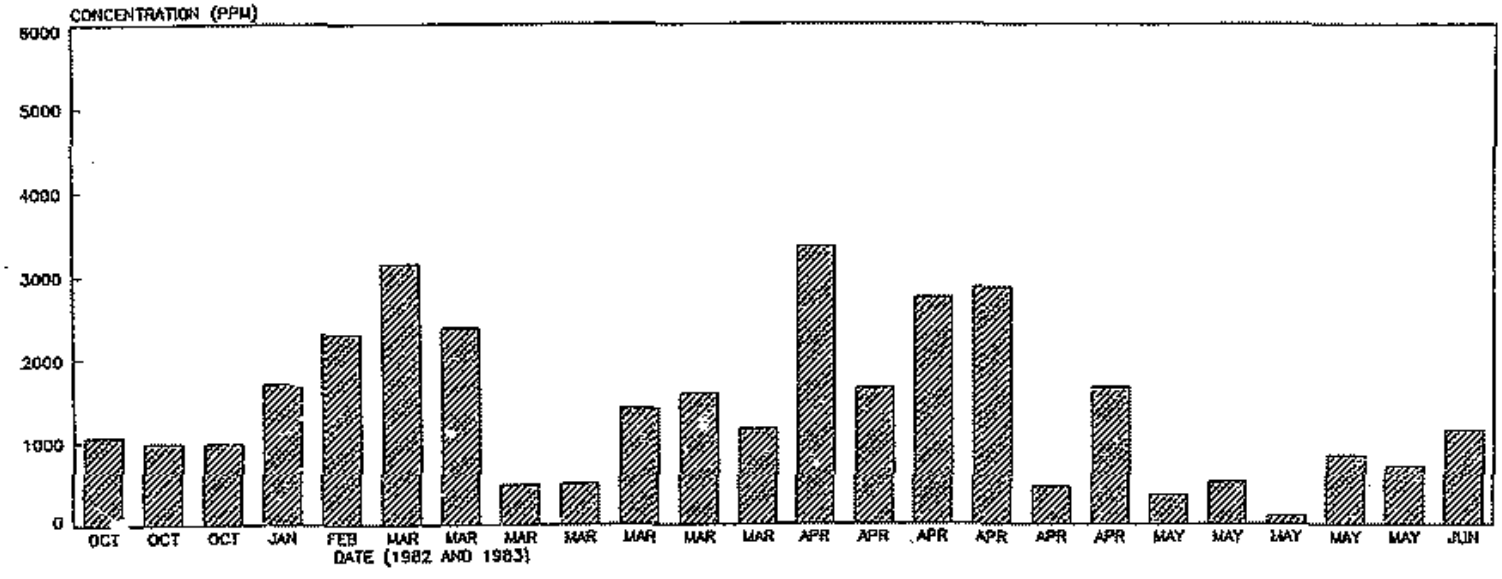


Figure 1 Residual fructose concentrations from bottled beer samples from the Ohlssons Brewery in Cape Town, for the period, October 1982 to June 1983.

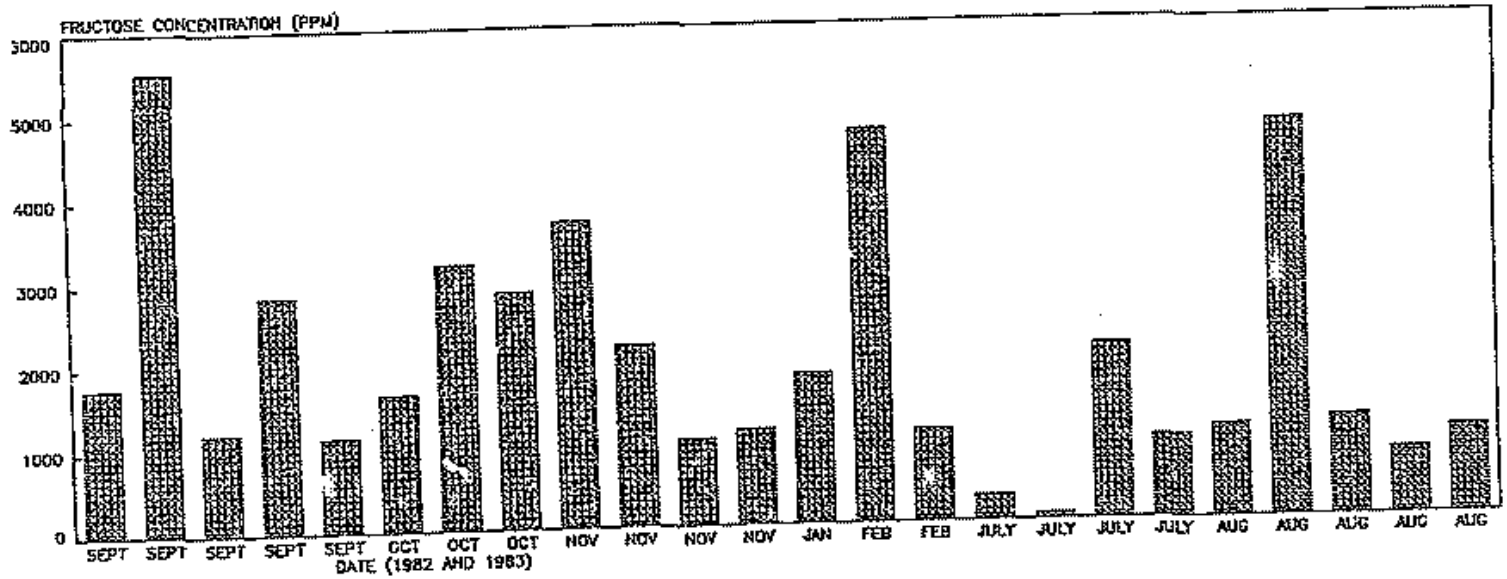


Figure 2 Residual fructose concentrations from bottled beer samples, from the SAB brewery in Bloemfontein, for the period September 1982 to August 1983.

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CHAPTER 1.

INTRODUCTION

1.1. The Role of Fructose and Glucose in Fermentation

Wort is a complex mixture of monosaccharides, disaccharides, trisaccharides, dextrins, amino acids, peptides, proteins, vitamins, ions, nucleic acids and other trace constituents (49,68,134). It is the growth medium used by brewery yeast strains in which alcoholic fermentation occurs and from which beer is finally produced. Thus the manner in which wort sugars are utilized plays a crucial role in the final quality of the beer.

The major wort sugars, in order of decreasing concentration are:- maltose, (sucrose), glucose, fructose, maltotriose and maltotetraose (28). Dextrins are present but are rarely utilized by brewing yeasts (166,168). The high concentrations of glucose and fructose in wort, approximately 2,85 g/100ml for each sugar, are caused by the use of sucrose as a carbohydrate adjunct in wort production.

A brewing adjunct may be described as any carbohydrate source other than malted barley which contributes sugar to the wort (121,163). Adjuncts come in many different forms. Historically numerous materials have been used. Some of these include coriander seeds, capsicum, orange powder, caraway seeds, salts of tartar and oyster shells. If a material gave a desirable flavour to the beer, there was a possibility that it could be used as an adjunct. At present, adjuncts used are divided into i) those which are added to the mash tun, (they may or may not need to be cooked) and ii) those added to the copper. The mash tun adjuncts include raw whole grains such as barley, wheat, rye and Torticale, (a hybrid of

wheat and rye). These cereals may be in the form of roasted or torrified grains. Other adjuncts used in the mash tun are flaked whole grains of barley, wheat, and oats, partially refined grain fractions which need cooking such as maize, sorghum, or rice grits or pre-cooked flaked maize or flaked rice (81). Interest has been shown in the use of soyflour and cassava starch (121). Sucrose is the major adjunct which is added to the copper. It may be in liquid or solid form. In South Africa sucrose is produced exclusively from sugar cane.

The sucrose adjunct acts as a non-malt, carbohydrate source with no enzymic activity and no protein present. It does not act as a flavouring agent to the beer. The required amylolytic power of the wort must be provided by the malt (94). If the malt does not have sufficient amylolytic power, then enzymes may need to be added in order to obtain acceptable extract yield and final attenuation. Sucrose adjuncts are an essential ingredient in the brewing of the lighter, more drinkable beers which have recently increased in popularity in South Africa (121). Sucrose is an adjunct which is used in the brewing industry world-wide. In Australia sucrose is regarded as the traditional brewing adjunct (172).

Impurities are always present in commercial sugar and the concentration will depend on the grade of sugar. Sugar chemists classify these impurities as:-

i) Reducing sugars
ii) Ash
iii) Other organic matter

Among group i) i.e. the plant-derived components, are polyphenols such as flavanoids and breakdown products of

lignin hemicellulose. Group iii) refers to derivatives of waxes, gums, and pigments. Included in this group are melanoidins, which are produced as a result of reaction between reducing sugars and amino acids, as well as alkaline degradation products of reducing sugars and caramels (172).

Sucrose, or cane sugar is a dissaccharide (112). It consists of two monosaccharides, glucose and fructose bonded together by an α 1⟶2 glucosidic bond. The systematic name of sucrose is O-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside. It is very abundant in the plant kingdom and is found in households as table sugar. Unlike most disaccharides, and oligosaccharides, sucrose contains no free anomeric carbon atom; the anomeric carbon atoms of the two hexoses are linked together. For this reason it is more easily hydrolysed than other disaccharides.

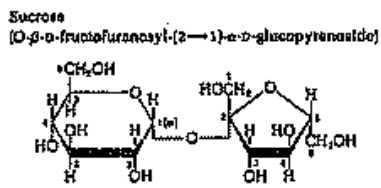


Figure 3. The Structure of Sucrose (112).

The glucose molecule of sucrose is in the form of a 6-membered-carbon ring and is called α-D-glucopyranose. The fructose molecule is found as a 5-membered-carbon ring called β-D-fructofuranose. Both monosaccharides are optically active. Glucose is foundin nature in a dextrorotatory form $[\alpha]_D^{20} = +52,7°$, whilst the usual form of fructose is laevorotatory, $[\alpha]_D^{20} = -92,4°$. Glucose in its crystalline form exists as a

straight chain structure. It is called an aldohexose due to a carbonyl oxygen at the end of the open chain form. D-glucose may exist in two different isomeric forms, α-D-glucose and β-D-glucose both of which differ in specific optical rotation. For α-D-glucose $[\alpha]_d^{20}$= 112,2° and for β-D-glucose $[\alpha]_d^{20}$= 18,7°. The two forms, become apparent in aqueous solution, where the optical rotation of each changes with time. The open chain form becomes an intermediate in the interconversion between the α- and β- glucose isomers. It is present only in minute quantities. The equilibrium finally rests at $[\alpha]_d^{20}$= 52,7°. This change, called mutarotation, is due to the formation of an equilibrium mixture of the two isomers. The and B isomers are both 6 membered ring forms called pyranoses (61,69).

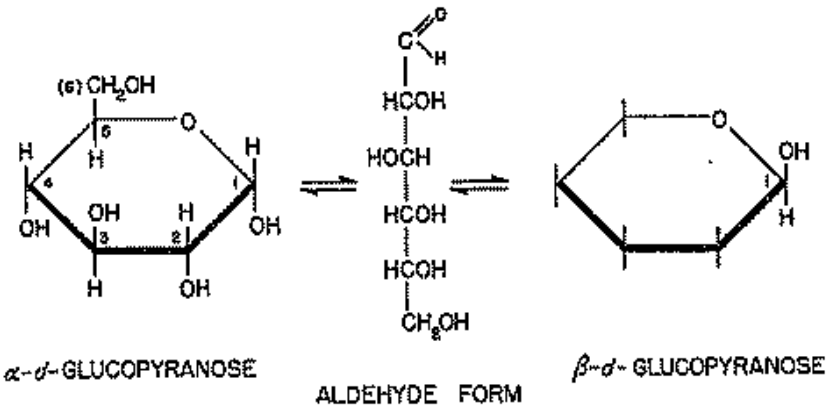
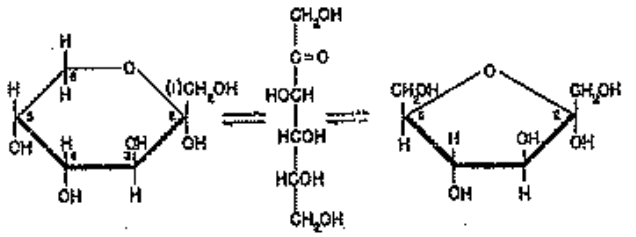


Figure 4. The different structural forms of glucose (61).

Aldohexoses may exist in furanose forms, but as six membered aldo pyranose rings they are more stable than 5 membered keto furanose rings, and thus the pyranose form predominates in aldohexose solutions.

D-fructose or levulose or fruit sugar (14,139), is found

abundantly in nature. In its free state it is found in many fruits. L-fructose does not occur naturally. Fructose is called a ketohexose due to the presence of a carbonyl oxygen in the middle of the straight chain. In this case it is at C_2. The crystalline form of fructose, is exclusively the 6-membered pyranose ring form. There are two other isomers of fructose. These are a straight chai structure and a 5-membered furanose ring form. As with glucose, the fructopyranose ring is more stable than the fructofuranose ring form. On addition of crystalline fructose to an aqueous solution, all three isomers become present (49,112). After a period of time, an equilibrium is established between the



Temperature				
0°C	88%	≪1%	12%	(56)
25°C	69%	≪1%	31%	(4)

Figure 5. The different structural forms of fructose.

5-membered fructopyranose, with the straight chain aldehyde form being present as an intermediate between the two ring forms. The aldehyde form is irrelevant to this discussion because it is present in minute quantities of much less than 1% of the total amount of fructose present (86). At 0°C there is only 12% D-fructofuranose, the remaining 88% fructose is in the pyranose form (56). As the temperature increases so the

equilibrium shifts slightly more toward the furanose form. At 25°C there is 31% β-D-fructofuranose present in the equilibrium mixture (4).

Invert sucrose, sucrose which has been hydrolysed to glucose and fructose, may also be used as another form of sucrose adjunct. In sugar refineries this process is usually carried out using dilute sulphuric acid, although it can also be performed using the enzyme invertase. The hydrolysis results in an equimolar mixture of glucose and fructose. Glucose (dextrose) is

$$C_{12}H_{22}O_{11} + H_2O \longrightarrow C_6H_{12}O_6 + C_6H_{12}O_6$$

	sucrose	glucose	fructose
	20		
$[\alpha]_D$	+ 66,53°	+ 52,7°	- 92,4°

-39,7°

Invert sugar (49)

dextrotatory, but fructose (levulose) is strongly laevorotatory, so the equimolar mixture has a negative rotation. It is therefore known as invert sucrose. The production of invert sucrose results in impurities such as methyl furfural and laevulinic acid which are produced from side reactions with fructose. Both glucose and fructose may react with nitrogenous material to produce melanoidin-like condensation products. The impurities present in both invert sucrose and sucrose may give "character" or flavour to the beer and under certain circumstances, may possibly affect yeast metabolism (23).

The wort under investigation in this project is of the high gravity type produced normally at 15.1 - 15.3 °P. A sucrose adjunct is used in its production and it is usually added in the ratio of 65% malt : 35% adjunct (7). Thus for every 15.1 g of total wort sugar, there

are 5.29 g of sucrose present.

The sequence in which the wort sugars are utilized starts with glucose and fructose. They are utilized simultaneously, but the rate of glucose utilization is faster than that of fructose utilization. Once these two sugars have reached low concentrations in the partially-fermented wort, then maltose and maltotriose are utilized. Sucrose maybe taken-up in the form of its constituent moieties glucose and fructose because it is cleaved into the two sugars, by the yeast enzyme B-fructosidase (invertase) outside the cell. The molecular weight of invertase is approximately 270 000. It is a glycoprotein which contains about 50% carbohydrate - predominately mannan with a small percentage of glucosamine - and is located in the cell wall (131,170). In S. cerevisiae there is a excess of hydrolysing capacity of sucrose over fermentation capability of 50 -100 fold. Yeast cells very quickly become flooded with the excess hexoses liberated from sucrose owing to the large concentrations of invertase produced by the yeast (51).

The first mention of glucose being preferentially utilized over fructose occurred in 1843 when Soubeiran observed that during the fermentation of invert sugar by yeast, the optical rotation of the growth medium diminished but remained laevo-rotatory. At that time the composition of invert sugar was unknown and so Soubeiran was unable to deduce what was happening to the two sugars .

Hopkins (74,75,76,77) and others (87,158) found that glucose was utilized faster than fructose in fermentations containing a mixture of the two sugars when using S. cerevisiae. He showed that this selective fermentation phenomenon was independant of the

concentration of total sugars between the limits 3.6% and 19%, between temperatures of 12°C and 26°C , and between the range pH 4.0 – 7.5. Variations in selective fermentations occurred with different yeast species and under different conditions of nutrition. Glucose was preferentially utilized over fructose in defined growth promoting medium (DGPM), in fermentations using ale and lager brewing strains (167). Yeast cells subjected to an increase in osmotic pressure (by addition of increasing amounts of sorbitol, a non-metabolizable substrate, to the media), resulted in a decrease in the difference between the utilization rates of the two sugars, until the rate of glucose utilization equalled the rate of fructose utilization (135,137). Comparisons of DGPM and defined minimal media (DMM) with sucrose as the sole carbon source showed a reduced preferential utilization of glucose over fructose in the DGPM compared to DMM (137). Conditioning of the yeast by growing it either in glucose, fructose or maltose, and then transferring it to a DGPM with sucrose had no effect on the preferential utilization of glucose over fructose (74,165). In all cases glucose was preferentially utilized over fructose. This phenomenon does vary slightly from strain to strain. Using a different brewing strain, Stewart (165) showed that glucose grown cells produced slightly higher utilization rates for both glucose and fructose when compared to fructose grown cells. Maltose grown cells showed the same preferential utilization of glucose over fructose, but at a reduced rate when compared to glucose grown and fructose grown cells. Stewart stated that in DGPM with either glucose or fructose as the sole carbon source, the rate of glucose utilization equalled the rate of fructose utilization (137). Hopkins observed that glucose and fructose were utilized at approximately the same rates when they were in separate fermentations, with both sugars above 1% concentration. Glucose was utilized faster than

fructose below 1% (169). When Saccharomyces bailii, the Sauternes wine yeast, was pitched into a fermentation containing an equal mixture of glucose and fructose, fructose was utilized faster than glucose (59,160).

Hopkins (78) postulated that fructose was utilized slower than glucose in S. cerevisiae because the yeast is only able to utilize the β-D-fructofuranose form of fructose, which accounts for a maximum of only 31% of the total fructose present at 25°C in aqueous solution (4). He also postulated that if the concentration of total sugar is low enough, a limiting factor controlling the rate of fermentation would be the concentration of the fermentable component and thus the rate of fermentation would be limited by the rate of muta-rotation of the pyranose to the furanose form (78). Hopkins and Roberts later showed that both the α- and β-pyranose forms of glucose were utilized at the same rate (79). Hopkins and Roberts also showed there was a direct relationship between yeast pitching rate and the rate of fermentation with a fixed concentration of fructose. As the pitching rate was increased so there was a corresponding increase in the rate of fermentation (76).

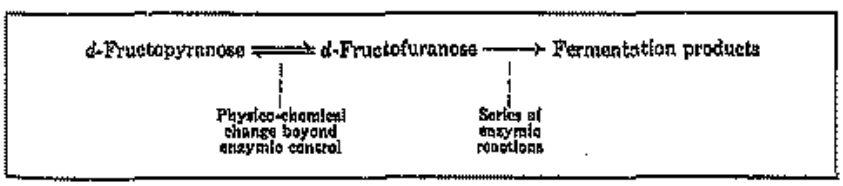


Figure 6. Gottschalk's scheme of fructose interconversions prior to utilization (61).

Gottschalk stated that he had experimentally verified Hopkins hypothesis. He found β-D-fructopyranose to be unfermentable by yeast and only the fructofuranose form of fructose could be utilized by brewing yeast (55,57,60).

Gottschalks method involved a comparison of the utilization rates of fructopyranose, equilibrium fructose, and fructofuranose using a brewing yeast. At 0°C and pH 4,6 he observed that fructofuranose and glucose were utilized at approximately equal rates, whilst at 25°C he found that glucose was utilized faster than fructose, and from this observation he implied that only fructofuranose was being utilized, and fructopyranose was unable to be fermented by yeast. He postulated that the lack of an OH group at carbon atom six rendered the β-pyranose form unfermentable. (56). However, this result was questioned by Heredia et al (72), who ascribed a definite although rather small affinity to β-D-fructopyranose for the transport system. They concluded that the Km for the hexose transport system is likely to be about 0,3M some two orders of magnitude greater than that for fructofuranose. Suomalainen and Toivinen state (60,169) that fructofuranose is fermented more rapidly than glucose (60,169). They postulated that in the fermentation of sucrose by yeast, sucrose was inverted much faster than the resultent moieties glucose and fructose could be utilized by the yeast. Therefore fructofuranose, the fructose form in the sucrose molecule, would be present in excess concentrations in the immediate vicinity of the yeast cells. Insufficient quantity of the furanose form, then, should not be a limiting factor in the fermentation of fructose which under these conditions should be utilized faster than glucose (169).

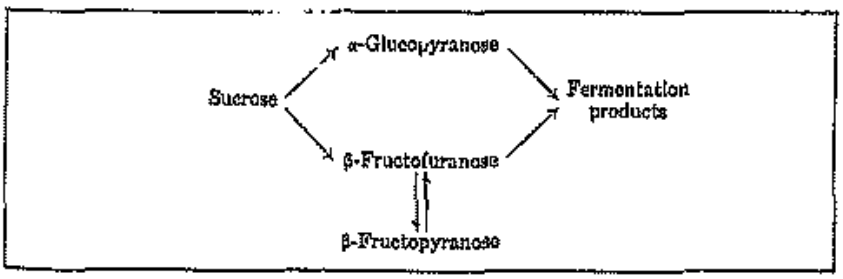


Figure 7. Gottschalks scheme of sucrose utilization (58).

The rate of fructose utilization in beer fermentations is important because fructose is a very sweet sugar (107). On a mole per mole basis, fructose is 50% sweeter than sucrose (167) and therefore if fructose remains at the end of fermentation in even residual concentrations it may affect the flavour of the beer by rendering it tainted with a sweet taste. It has been judged by the brewery that there are two threshold levels of fructose in beer, (123) 1) 300 mg/litre - the level at which educated tasters in the brewery are able to detect the sugar and 2) 1000 mg/litre - the level at which the man in the street is able to notice the beer is tainted with a sweet off-flavor.

1.2. The role of Oxygen and Sterols in Wort Fermentations

Wort fermentations are anaerobic in nature, but despite this, oxygen is required by the brewing yeast in order to ferment the wort to the required degree (95). The wort is supplied with dissolved oxygen by bubbling either compressed air or pure oxygen through it. Oxygen may serve (120) two functions in yeast growth. It may act as a final electron acceptor of electrons from the respiratory chain, or it may act as a growth factor. Owing to the minute concentrations of oxygen available to the yeast (93) and the rapidity with which it is absorbed, it is certain that very little aerobic growth occurs. Rather, oxygen is needed by the yeast cells for the production of lipids such as sterols and unsaturated fatty acids, without which the yeast cells cannot grow and reproduce. The yeast strain used is particularly important as the oxygen requirement is not standard. Examination of large numbers of yeast strains (96) shows that the need for oxygen varies greatly. They may be grouped into 4 classes on the basis of their oxygen need to completely satisfy their metabolic requirements :-

Class O1	Yeasts whose requirement is satisfied if wort is half saturated with air
Class O2	Yeasts whose requirement is satisfied by air saturated wort.
Class O3	Yeasts whose requirement is satisfied by oxygen-saturated wort.
Class O4	Yeasts whose requirement is not satisfied by oxygen-saturated wort.

(99)

The precise oxygen requirement varies from as little as 2 mg/litre (93) to more than 30 mg/litre, the latter being the approximate concentration of dissolved oxygen in oxygen saturated wort. Yeast activity can be regulated when the oxygen requirement of the yeast is relatively high. When the oxygen requirement is relatively low, accurate control of minute concentrations of oxygen is much more difficult. The concentration of dissolved oxygen in saturated wort decreases as the temperature increases, and also decreases as the wort concentration, i.e. specific gravity, increases (93). Air saturated wort of 1,040 and 1,060 specific gravity at 20°C contains approximately 8,5 mg/litre and 8,0 mg/litre of oxygen, respectively (11).

It appears that the varying oxygen requirements of brewing yeasts reflect differences in sterol metabolism (93). An interesting correlation exists between the magnitude of oxygen demand and the nature of sterols synthesized aerobically. Among these ergosterol and dehydro-ergosterol are particularly prevalent. It has been found that strains which synthesize dehydro-ergosterol in preference to ergosterol have a relatively low oxygen requirement after anaerobic growth. The reasons for this are not clear. Dehydro-ergosterol disappears during anaerobic growth and cells whose further growth is inhibited by a lack of oxygen contain only ergosterol of the principal sterols.

Fundamentally oxygen determines growth, and thus, all other parameters related to growth will also be affected. The rate of fermentation is determined by the extent of new cell production in the reproduction period at the beginning of fermentation.

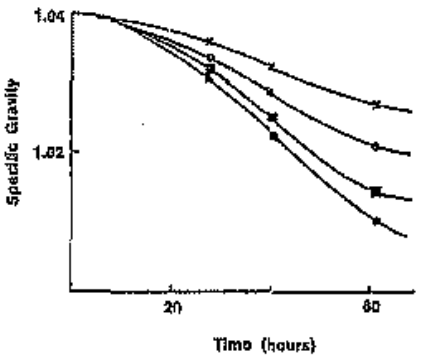


Figure 8. The response of S. cerevisiae NCYC 1062 to different levels of wort aeration:- X, deaerated wort; ○ wort 50% saturated with air; ■ wort air saturated; ● wort oxygen saturated (95).

An oxygen deficiency causes yeast growth to be restricted. The fermentative activity of the new cells which are produced, is not substantially affected, but as the total number of new cells is reduced, so the total activity is reduced as seen in figure 8. If oxygen was supplied in optimum quantities, then the number of new cells would be increased, resulting in an increase in the total activity, and so fermentation would proceed more rapidly, than in a fermentation with an oxygen deficient yeast (93). Using a strain of S. cerevisiae, Markham (120) showed that the rate of fermentation (°P/h) increases as the degree of oxygen saturation is increased from approximately 5% up to 20%. Above 20% oxygen saturation the increase in the rate of fermentations falls off (figure 9). Wort completely saturated with air corresponds to approximately 20% oxygen saturation.

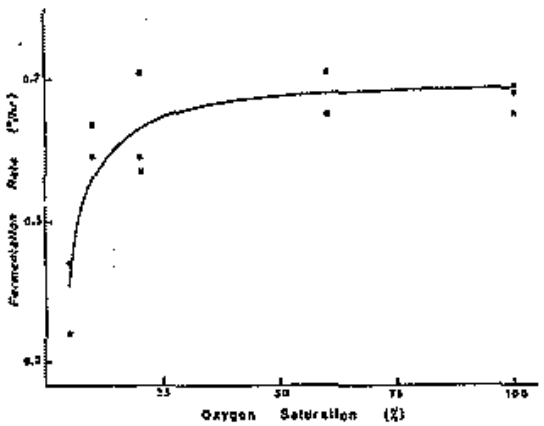


Figure 9. The relationship between the fermentation rate and the initial percentage oxygen saturation of wort (120).

A similar relationship was found between the yeast yield (g/litre) and oxygen saturation. As the oxygen saturation (%) increases to approximately 20% so there is a resultant increase in yeast yield, whereafter it remains approximately constant when the oxygen saturation increases (120).

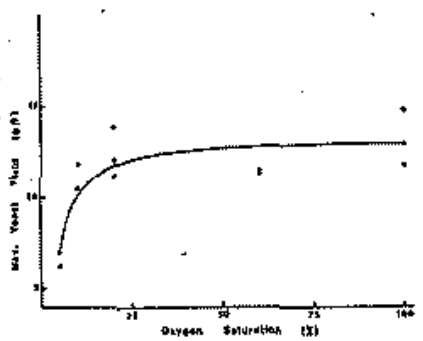


Figure 10. The relationship between the initial percent of oxygen saturation of brewers wort and the maximum yield of yeast obtained (120).

A decline in yeast viability may result from an oxygen deficiency associated with sub-minimal concentrations of

sterols and unsaturated fatty acids (120). The percentage of dead cells at the end of fermentation increases as the initial oxygen saturation of the wort decreases (120).

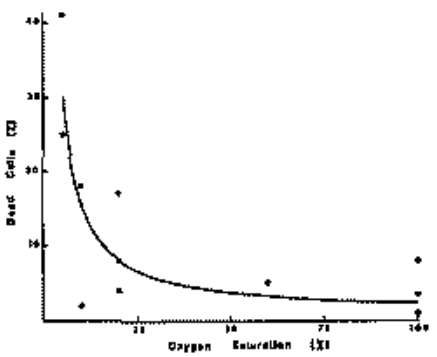


Figure 11. The relationship between the percentage dead cells at the end of fermentation and the initial percentage oxygen saturation of the wort (120).

Yeast collected at the end of a wort fermentation contains very little sterol, i.e. approximately 1 mg/g yeast on a dry weight basis (99). However, it contains considerable quantities of squalene, the immediate sterol precursor, which has accumulated during the previous fermentation. When the yeast is added to wort which contains dissolved oxygen, sterols are then rapidly synthesized from the squalene and may reach 5 mg/g of yeast in 20 minutes. Sterol synthesis does not continue at this rate indefinitely even in the presence of oxygen and the production rate slows down considerably. Once sterol concentrations reach 10 mg/g of yeast (10), production then either stops or becomes very slow. Only once new yeast mass is produced by growth and the sterol concentration is diluted (71), will sterol synthesis begin again, and it is approximately proportional to the growth rate. The synthesis of sterols is dependent on the presence of oxygen in the wort (90). Replication of cells ceases

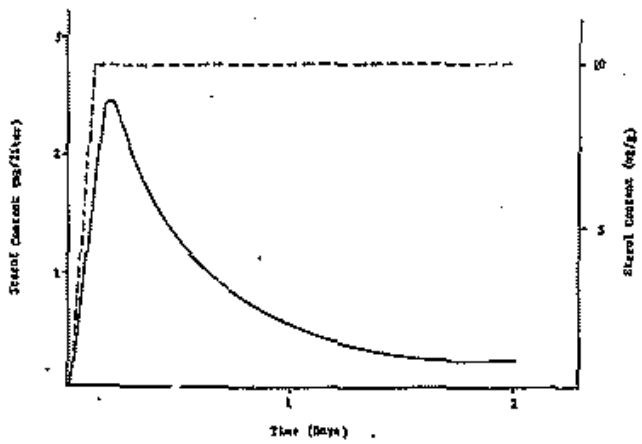


Figure 12. Pattern of synthesis of sterol by the yeast S. cerevisiae NCYC 240 as total sterol in yeast in one litre of wort ---- ; as total sterol per gram of dry yeast —— (98).

when a limiting value of approximately 1 mg sterol/g of yeast is reached in the medium.

When S. cerevisiae is grown aerobically, in order to produce a suitable inoculum size for pitching, unsaturated lipids such as sterols and fatty acids are produced in excess of their optimun concentrations needed in the cells. The yeast can be pitched into deaerated wort and a normal fermentation produced (41). The lipids present are "diluted" with subsequent growth of the yeast, but they remain present in sufficient concentrations to support adequate yeast growth. If the yeast from the fermentation is harvested and used to pitch another fermentation, then aerated wort must be used as there are insufficient reserves of the necessary lipids. They have to be synthesized in the initial stages of fermentation in the presence of oxygen (99). Alternatively, yeast pitched into deaerated wort will produce a correctly attenuated fermentation, if sterols and unsaturated fatty acids are added directly to the wort (95). Malt wort contains approximately 2% lipid and the procedures of wort preparation lead to the

retention of much of this in the spent grains i.e. it becomes lost to the wort. However, small quantities of lipid do remain in the wort. This results in a restricted amount of growth occurring in malt wort when an oxygen-requiring yeast is used to ferment wort to which no dissolved oxygen has been added (93). The addition of spent grain lipids, which contain sterols,

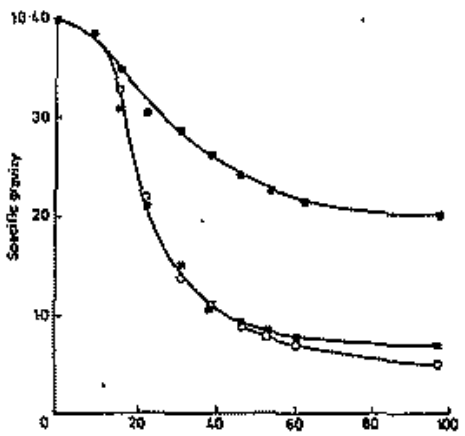


Figure 13. The effect of spent grain lipids on fermentation of de-oxygenated wort by yeast S. cerevisiae NCYC 1134 , ■ aerated wort control, ● deoxygenated wort, ○ deoxygenated wort and lipids (171).

can remove the need for oxygen to be present in the wort used for fermentation (171). The lipids also stimulate yeast growth and fermentation when yeasts with high oxygen requirement are added to wort containing less than adequate concentrations of dissolved oxygen

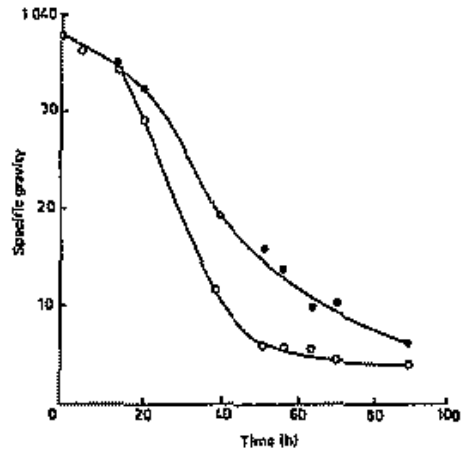


Figure 14. Effect of spent grains on fermentation of air-saturated wort by yeast S. cerevisiae NCYC 0854. ● no addition, ○ lipid addition (171).

(Figure 14). The limiting lower concentration for sterols as a class is in the order of 1 mg/g dry yeast. The sterol content does not increase beyond the level of about 10 mg/g (9), even if oxygen is continuously present. It has been suggested that catabolite repression is responsible for this phenomenon, as much higher levels of sterols are formed when the concentration of glucose is reduced (143). The practical significance of these sterol levels is that the upper sterol limit in the cell is relatively low and can be quickly reached when oxygen is present.

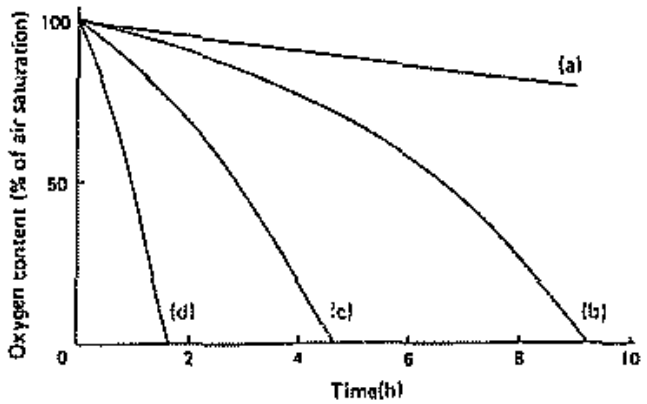


Figure 15. Oxygen uptake from air-saturated wort containing (a) no yeast; or S. cerevisiae NCYC 240 pitched at 0,25; (c) 0,5 and (d) 2,5 g wet weight/litre (8).

Oxygen consumption continues in the absence of sterol formation (figure 15), indicating that not all the oxygen is consumed by the cells for sterol synthesis. Calculations show that only 5 - 10% of oxygen available in air saturated wort is used in this way, and that the synthesis of unsaturated fatty acids does not account for much larger amounts (8). Two consequences follow from the rapid and limited synthesis of sterols; (a) the quantity of sterols produced varies directly with the inoculum size (8) and (b) the pattern of fermentation is dependant on the inoculum, and the sterols determine the extent of yeast growth (2,3). This is particularly true when high gravity wort is used (97).

Far more efficient use can be made of oxygen if it is supplied in increments rather than one batch. If the *wasted oxygen is made available* once yeast growth had started, then new sterol could be produced to supplement the sterol diluted by growth. This explains why a fermenter filled with wort in increments over a period of 8 hours results in greater growth, faster fermentation and a greater attenuation potential than a fermenter which is rapidly filled with one batch of wort (99).

Sterols are a heterogeneous group, of which ergosterol, episterol and lanosterol are the most important. In the biosynthesis of sterols in S. cerevisiae, the initial step involves the cyclization of squalene to

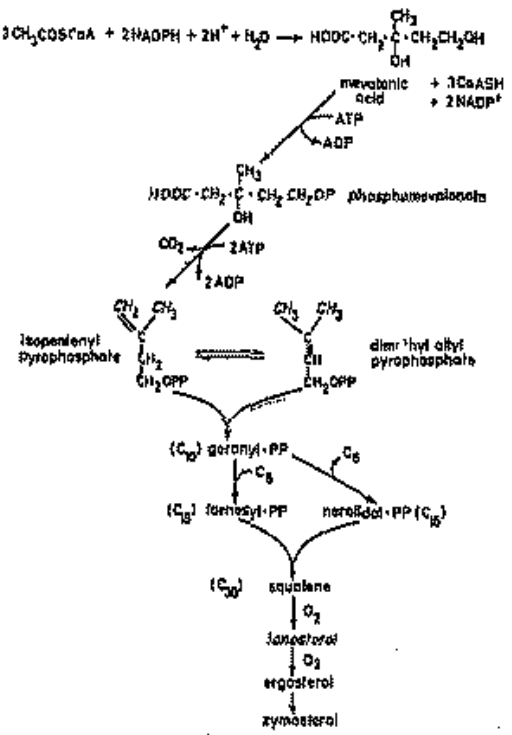


Figure 16. The biosynthesis of sterols in S. cerevisiae (82)

form lanosterol the first compound with a sterol nucleus in the pathway. Oxygen is essential for this conversion. The subsequent conversion of lanosterol to ergosterol via demethylation and desaturation reactions also requires oxygen.

Sterols are involved in the physiological function of membrane systems and they appear to add stability to the membrane structure (140). In model membrane systems sterols such as cholesterol determine the fluidity of the bulk lipid in the membrane, by preventing membrane lipids from undergoing major phase transitions (132).

1.3. The Nitrogen Sources in Wort Fermentations

The nitrogen sources in wort, which are used for yeast growth, are obtained from barley. In the production of malt from barley, germination occurs and amino acids are produced by proteolytic enzyme activity (90). Approximately half of the amino acid content is produced during malt production, and the other half results from proteolysis during mashing (99). The amino acid composition of the wort depends primarily on the free amino acids found in malt (90). There is a large degree of consistency of composition of barley proteins and proteolytic enzymes in various types of malting barley, and the proteolytic enzyme activities show general similarity. Hence there are marked qualitative similarities in the nitrogenous compounds of various worts, even if the total nitrogen and amino nitrogen contents differ substantially (99). Only traces of true protein occur in wort, as most proteins have been degraded to peptides and amino acids (85). The amount of protein within the wort is largely irrelevant as brewers yeast is unable to utilize protein as a nitrogen source (85). There is a complete profile of amino acids in wort (89). Sequential uptake occurs, and

Group A Most Readily Assimilated	Group B Taken Up More Slowly	Group C Taken Up Less Quickly	Group D Only Slowly Utilized
Glutamic acid	Than Group A	Than Groups A and B	Proline
Aspartic acid	Valine	Glycine	
Asparagine	Methionine	Phenylalanine	
Glutamine	Leucine	Tryptophan	
Serine	Isoleucine	Tyrosine	
Threonine	Histidine	Alanine	
Lysine		Ammonia	
Arginine			

Table 1. Grouping of amino acids based on their role of assimilation by brewers yeast (85).

they can be separated into four groups (85), based on the rate at which the particular amino acid is assimilated. This order is dependant on the affinity of the various amino acid permeases for their particular amino acid substrates. Transamination is the mechanism of utilization of most amino acids as they are not directly incorporated into new protein (90). Ammonia appears not to be a preferred nitrogen source, as it is absorbed after the majority of amino acids. Proline is not normally utilized in the anaerobic conditions of beer fermentations.

The changes in climatic factors from year to year may cause the quantitative difference in the total nitrogen content of malt, and this is inevitably reflected in the total assimilable nitrogen in wort which may vary between 100 - 250 mg/litre in 10% wort (99). Mailgaard (122) states that yeast growth and fermentation time may be seriously affected if the free amino nitrogen content of wort, as measured by the ninhydrin method, is below 150 mg/litre. The fermentation of all-malt worts does not produce major changes in the patterns of fermentation which parallel the changes in content of assimilable nitrogen (99). Thus it appears that there are excess concentrations of assimilable nitrogen in such wort. However the situation changes when carbohydrate adjuncts are used in substantial proportions in the preparation of adjunct wort (97). The total amount of nitrogen present is reduced when carbohydrate adjuncts are added to wort because the adjunct functions only as a carbon and energy source and does not supply any nitrogen material. An adjunct wort composed of malt wort and a carbohydrate adjunct in equal proportions will have the total nitrogens reduced by 50% compared to the original malt wort. The growth of yeast in this adjunct wort is slow and inadequate in comparison to the growth of the yeast in the malt wort

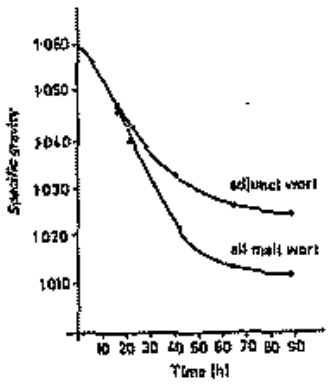


Figure 17. Comparison of the fermentations of adjunct wort and all malt wort (97).

(97). Nitrogen nutrients are almost entirely absorbed from the wort during the exponential phase of growth and removal of these and other nutrients is accompanied by new cell growth (97). *Ingledew* (85) points out that in lager fermentation, once yeast growth ceases, although a substantial amount of nitrogen material remains unused in the wort, wort appears to be deficient in utilizable nitrogen. A nitrogen deficiency triggers the utilization of the internal carbohydrates glycogen and trehalose within the yeast cell (138).

Experiments carried out in three litre EBC tall-tube fermenters at 18°C, showed the addition of serine as a readily utilizable amino acid to an aerated adjunct wort, adjunct 50% : malt wort 50%, brought about a slight increase in the rate of attenuation, but it still did not equal that of an aerated malt wort (66). The joint treatment of oxygenation and added serine to adjunct wort produced attenuation patterns which approached, but did not equal that produced by

adjunct wort produced attenuation patterns which approached, but did not equal that produced by oxygenated malt wort. There was a synergistic effect between the two treatments, with the effect of the joint treatments being greater than the sum of the two separate treatments (99). Hsu et al (83) showed that the addition of a growth stimulant composed of amino acids, lipoproteins, trace minerals and vitamins to an adjunct wort of 60% malt wort : 40% adjunct at 15,3°P at a rate of 40 mg/litre produced shorter fermentation times than a control adjunct wort without the growth stimulant. In stirred fermentations (91) the addition of growth factors such as yeast extract and casein hydrolysate produced more rapid fermentations than an untreated control. Further experiments showed that the rate of fermentation in an adjunct wort composed of equal proportions of malt wort and corn starch hydrolysate was proportional to the values of amino nitrogen implying that nitrogen exhaustion was the limiting factor of growth. The extent of exponential growth in the early stages of fermentation was proportional of the amount of amino nitrogen added to the fermentation in adjunct wort. The extent of exponential growth in adjunct wort with the addition of 150 mg/litre of serine, equalled that of malt wort (91). The growth of S. cerevisiae appears to be reduced when the vitamins biotin, pantothenate and inositol are each singly omitted from a growth medium (1,91). The results of Kirsop (77), indicate that limited growth caused by the omission of a single nutrient, only occurs in simple media, and does not apply to a complex medium such as wort. In order to improve fermentation it may be necessary to alter more than one parameter at once e.g. an increase of the levels of dissolved oxygen and the amino nitrogen content in the wort can act synergistically to produce more rapid attenuation in the fermentation.

1.4. Sugar Utilization Related to Yeast Growth in Wort Fermentations

The rate of fermentation will depend on the rate and extent of yeast growth. Non-growing yeast ferments slowly, while growing yeast ferments rapidly. The rate of a fermentation which contains appreciable numbers of yeast cells in suspension may become very slow before the desired level of attenuation is reached i.e. it "sticks" or "hangs". This phenomenon is primarily caused by yeast growth ceasing too early in the fermentation. In order to get rapid and full attenuation it is necessary to somehow extend the growth phase (97). Thus if satisfactory fermentation is to take place then most of the attenuation must occur within the first few days of fermentation (98,111).

There is no stoichiometric relationship between yeast growth and sugar use. However, the sugar utilisation in wort fermentations must be seen as occurring primarily in association with yeast growth, rather than a manifestation of the activity of the yeast population once growth has ceased. Growth and attenuation must be considered to be two aspects of the same phenomenon (97).

The specific rate of fermentation in stirred vessels i.e. the rate of sugar utilisation per gram of yeast present, continually declines from the start of fermentation, until a low and essentially constant level is reached after a period of ca. 45 hours in malt wort and adjunct wort composed of 50% malt wort : 50% corn starch hydrolysate. The overall rate of fermentation only declines at a later stage as the increase in cell mass masks the reduction in the specific rate of sugar utilisation. Once cell mass ceases to increase then the overall rate of fermentation also ceases to increase and remains essentially constant.

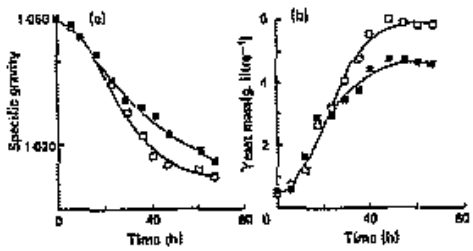


Figure 18. Patterns of attenuation (a) and yeast growth (b) when S. cerevisiae NCYC 1236 was used to ferment malt wort □, and adjunct wort ■, (152).

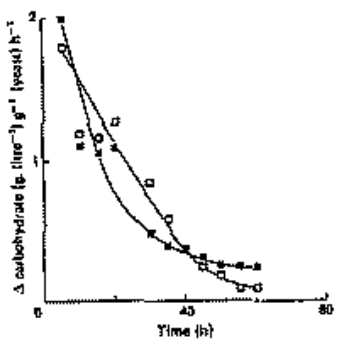


Figure 19. Changes in the specific rate of carbohydrate utilisation with time when S. cerevisiae NCYC 1236 was used to ferment malt wort □, and adjunct wort ■, (152).

The application of treatments to the adjunct wort, such as i) the addition of ergosterol and Tween 80 ii) alteration of the pitching rate, iii) the addition of air to the fermenter for a brief period and iv) the use of yeast propagated in a shake flask rather than yeast harvested from a fermenter, all cause the overall rate of fermentation to be increased.

In all cases, once yeast growth has terminated a low but essentially constant specific rate of fermentation is observed. This period is termed the "maintenance phase" of yeast activity because it indicates the amount of activity necessary to maintain a non-growing yeast population (152). The specific rate of carbohydrate utilization is substantially higher in growing yeast cells compared to non-growing cells. When new cell growth ceases then the rate of fermentation decreases considerably. It may be as much as 33 fold (30). Yeast mass continues to increase once cell growth has stopped due to the accumulation of internal carbohydrates, trehalose and glycogen plus other reserves and cell wall polymers. The mass of yeast produced in the post exponential phase depends on several factors, and it changes from strain to strain (25). Once the wort sugars have been fully utilised, then the total yeast mass declines due to the catabolism

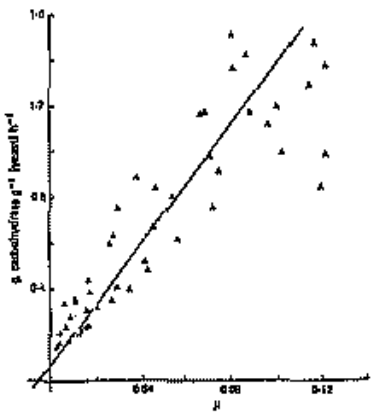


Figure 20. Relationship between the specific rate of carbohydrate utilization and specific growth rate, u, using data from a range of fermentations with S. cerevisiae NCYC 1236. Calculation of the regression line of carbohydrate utilization on u for the 49 values gave the following values, r^2 = 0,76336, m (slope) = 0,0748, intercept on y axis = 0,0044 (152).

of the internal carbohydrate, glycogen, which the cells use as a carbon and energy source (92). The relationship between the specific growth rate, u, and the specific rate of carbohydrate utilisation is apparently linear. Thus where u approaches zero in the "maintenance phase", the corresponding rate of sugar utilization is also very low. Most of the utilization of sugars is associated with growth, and the rate of utilization which is not associated with growth is so low that it will only contribute significantly to fermentation attenuation if the fermentation times are extended. The wort composition or the strains of yeast used do not significantly affect the yield of dry yeast mass per ml. of hexose utilised when the cells are growing in exponential phase (25). In batch fermentations it appears that yeast growth and sugar utilisation are tightly coupled, and an increase in yeast growth results in an increase in the rate of sugar utilisation. An increase in the pitching rate produces a corresponding increase in the amount of yeast mass produced (98).

Pitching rate and yeast growth

Yeast (NCYC)	Pitching Rate (g wet yeast/litre)	Yeast Mass Produced (g dry yeast/litre)
240	5.0	4.0
	2.5	3.1
	1.25	2.6
1216	5.0	3.8
	2.5	3.3
	1.25	2.8
1062	2.5	2.7
	0.5	1.8
1245	2.5	3.9
	0.5	2.8

Table 2. Alteration of the pitching rate and the corresponding yeast growth produced (98).

The yield co-efficient relating yeast growth to sugar utilisation alters with various conditions. The use of synthetic media, worts of various compositions, and different yeast strains, may all produce different yield co-efficients. In order to obtain fermentations with full attenuation, yeast growth must continue to the point where little, if any, attenuation is required to be carried out in the maintenance phase. The presence of adverse conditions such as the premature removal of actively growing yeast cells from the wort by flocculation (98) or premature chilling of the partially fermented wort may also result in incomplete attenuated fermentations (152). Thus the overall rate of fermentation is dependant on the rate and extent of the exponential growth phase in the early stages of fermentation (25).

1.5. High Gravity Wort Fermentations

High gravity brewing, in which high gravity wort is fermented, and concentrated beer is produced and then diluted at the final stage of production, has become standard practice in many breweries due to the inherent economic advantages of the process. An increase of ca. 25 - 30% in beer production can be obtained using existing brewhouse and fermentation plant, manpower and energy (65,183). However, there are disadvantages to the process and the use of high gravity worts in the range of 19,8 - 39°P causes loss of yeast viability within the first 24 hours of fermentation (29,42). This phenomenon becomes increasingly severe with the increase in wort gravity. The loss of yeast viability is large enough to cause the pitching rate to be effectively reduced due to the large numbers of dead cells. Each yeast cell is intrinsically able to bring about fermentation at the same rate as the surrounding cells in the medium, provided that the conditions are constant. So the rate of fermentation of a given volume of fermentation medium should be able to be increased if the yeast concentration is increased (141). Thus an increase in pitching rate is able to counteract the loss of viability (29). In worts of 18°P and above the osmotic pressure generated is substantial and may distort cell metabolism and also play a role in decreased yeast viability (133).

The maximum level of ethanol tolerance by brewing yeasts in fermentation is reported (42) to be in the range of 7-9% (v/v). Ethanol toxicity effects at higher concentrations were thought to be the primary cause of cell death and therefore low yeast viability, causing fermentations to become either sluggish or stuck (30). However, the addition of 40 ppm ergosterol 0,4% Tween 80, with 1% yeast extract to a 29° Plato wort produces good attenuation patterns in which complete fermentation

occurs in 5 days compared to 14 days for the unsupplemented control (31). The final ethanol concentration reached is 14,2% (v/v). In 31° Plato wort, concentrations of 16,2% (v/v) are obtained. The yeast viability at the end of fermentation is high. The yeast can be repitched 5 successive times into wort of the same concentration producing normal attenuation patterns in each case (31). These experiments show that the inability of yeasts to ferment high gravity worts i.e. 18°P and above, is due primarily to a nutrient deficiency in wort and not to ethanol toxicity effects. Thus brewers yeast is able to *tolerate ethanol concentrations* of 16% (v/v) without genetic manipulation (31), which is similar to the tolerance levels found in distillers and sake yeast (31)

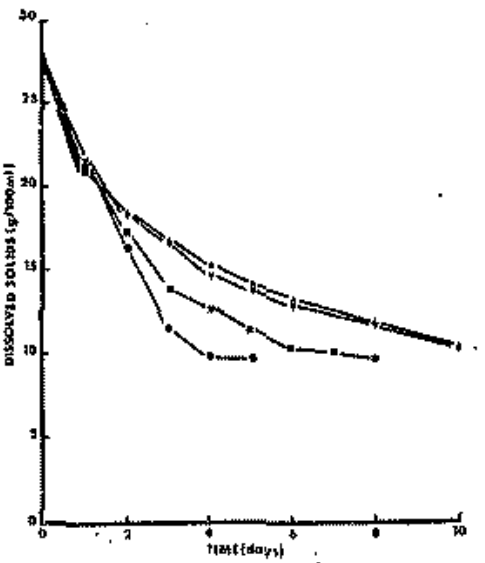


Figure 21. Dissolved solids versus time during fermentation of high gravity worts which were; unsupplemented ▲ ; 1% yeast extract supplemented ■ ; 40 ppm ergosterol and 0,4% Tween 80 supplemented ▼ ; or 1% yeast extract with 40 ppm ergosterol and 0,4% Tween 80 supplemented ●, (31).

1.6. Characteristics of the Glucose Membrane Carrier

The yeast cell membrane acts as a protective barrier to the cell, and controls the entry and exit of solutes (72). For a sugar to be utilized by a yeast cell, it must first be transported across the cell membrane, by means of a carrier associated with the membrane. These carriers have several similarities to enzymes.

1. Where investigated the carriers have been shown to be proteins.

2. The carriers form complexes with their substrates.

3. The carriers have varying degrees of substrate specificity.

4. They show saturation kinetics.

5. Many are inducible and repressible.

The general principle by which the carriers function involves the binding of the sugar to the carrier on the outside of the membrane, it is translocated across the membrane, and the sugar is released inside the cell. Such movement is described as either i) facilitated diffusion, when the movement requires no metabolic energy or ii) active transport, which involves the expenditure of metabolic energy. A sugar entering a yeast cell by active transport may be accumulated inside the cell to several hundred times the concentration of the sugar outside the cell (13,103,105).

Glucose is taken up by a membrane carrier which is reported to be common to glucose, fructose and a wide range of metabolizable and non-metabolizable sugars in S. cerevisiae (35).

Kotyk and Horak (80) have isolated a 35 000 molecular weight glucose binding lipoprotein from the cell membrane of S. cerevisiae. It has the specificity of the typical glucose carrier by binding to D-glucose, D-xylose, D-arabinose and L-sorbose but not to D-galactose and L-arabinose. They estimate there are a minimum of $3x10^6$ glucose binding sites per cell.

The basic structural requirement for transport to be performed is reported to be a pyranose ring for glucose (36,37) and a furanose ring for fructose (72). With compounds that can be regarded as structurally related to D-glucopyranose, there is a broad tolerance for modification (72). The relative importance of single substitutions at each of the carbon positions, with glucose in the C1 glucopyranose conformation, decreases in the following order C1 > C3 > C4 > C5 (36,72,104,106, 109). The hydroxyl group at carbon 2 is not necessary for uptake, as 2-deoxy-glucose has the same affinity for uptake as D-glucose. Cirillo (36) points out that in all cases of single substitutions, there is a decrease of sugar affinity, but it is not significant. The exception is the methylation (or glycosylation) of the anomeric hydroxyl group, which results in abolition of binding. However, marked reduction in affinity results from multiple changes of the D-glucopyranose form. In all cases the combination of more than one change has an effect greater than the effect of each change alone. Cirillo states that the pyranose ring is the active structure for transport since D-glucose shows the highest affinity for the membrane transport carrier and is known to occur almost exclusively in the pyranose conformation. To support his argument he notes that 1,5 anhydroglucitol, which is a permanent pyranose, has a relatively high uptake rate. This finding with respect to fructose contrasts with the results of Heredia et al (72) and Gottschalk (55,60) who both state that the furanose form of fructose is necessary for uptake.

L-fucose when compared to C1 D-glucopyranose, has changes at carbon 1,2, and 5 and shows a very low membrane carrier affinity. This supports Cirillo's finding that multiple substitutions cause significant changes in membrane carrier affinity. However, when D-fructose is compared to the pyranose conformation, it also has multiple substitutions at carbons 1,2 and 5 and yet it has a relatively high affinity when compared to D-glucose and one several hundred times higher than L-fucose. Cirillo states the high affinity of fructose is an enigma (36), because it does not fit the pattern of the other aldoses listed and he notes that in solution at 25°C, 31% of fructose is in the furanose form (4). The issue of the relative importance of the pyranose and furanose forms in fructose uptake appears ambiguous.

Several authors have examined the affinity of fructose and glucose for the glucose transport system in *S. cerevisiae* and the various Km values determined are listed in Table 3.:-

AUTHOR		Km (mM)	
		FRUCTOSE	GLUCOSE
Kotyk	(109)	17	4-6
Cirillo	(36)	25	5-9
Bisson and Fraenkel	(21)	ca. 6	ca. 1
Becker and Betz	(16)	7.7	4
" "	"	10.0	0.6
" "	"	7.0	0.4

Table 3. Km values for glucose and fructose in *S. cerevisiae*

The differences reported by the various workers for each sugar in the table may be due to the different strains of S. cerevisiae and different techniques used for the determinations. The Km for glucose is approximately five times lower than the Km for fructose and consequently glucose has a higher affinity for the membrane carrier than fructose. Kotyk and Kleinzeller found that the Michaelis constant for glucose transport is higher aerobically (17.4mM) than anaerobically (6.7mM) and that the difference may be attributed to the Pasteur effect (106). The maximum rates of transport for glucose and fructose i.e. Vmax are approximately equal (16). The intracellular levels of free fructose are approximately ten times higher than free glucose. This difference is caused by hexokinase's affinity for fructose (16) being approximately ten times lower than its affinity for glucose with Km's of c.a. 1×10^{-3} mM and 1×10^{-4} mM respectively (17,18,22,150,157).

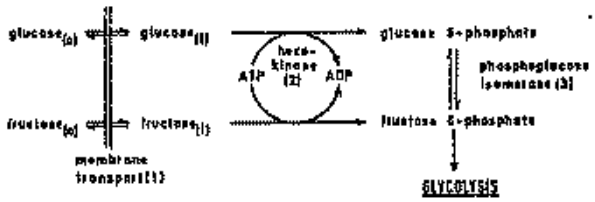


Figure 22. The initial reactions of glycolysis involving glucose and fructose (16).

1.7. Facilitated Diffusion Verses the Transport Phosphorylation Theory

The nature of the glucose transport system of S. cerevisiae has not been unequivocally resolved despite more than two decades of study. The controversy centre around the question of whether glucose is transported by a carrier across the cell membrane by facilitated diffusion, and then phosphorylated by the action of hexokinase after it enters the cell, or whether it is transported via a group translocation system in which it is phosphorylated during transport, as in the vectorial-phosphorylation transport systems found in bacteria (147). The transport associated phosphorylation hypothesis, advanced in 1954 by Rothstein (148) was based on observations that i) intracellular free glucose was not detected in actively metabolizing cells ii) yeast cells were reportedly impermeable to non-metabolizable sugars and iii) there was an apparent co-relation between substrate specificity for sugar transport and sugar phosphorylation. Experimental evidence appears to be weighted toward the facilitated diffusion model and consequently it has gained wider acceptance. Cirillo (36) has shown that the non-metabolizable sugars L-sorbose, L-fucose, D-xylose, L-glucose and many others of S. cerevisiae are all taken up, but not concentrated, by the hexose transport system, and all these sugars competitively inhibit D-glucose transport to varying degrees.

Cells inhibited with iodoacetic acid under nitrogen, in order to completely block metabolism, exhibited normal glucose uptake with free glucose detected inside the cells (36). These results indicate metabolism is not involved in transport i.e. the first step in the glycolytic pathway, after transport, involves the phosphorylation of glucose and or fructose, using one

ATP molecule per molecule of substrate, and results in the formation of glucose-6-phosphate and fructose-6-phosphate. Iodoacetic acid completely depletes the cell of ATP, and the presence of nitrogen inhibits metabolic activity. The lack of ATP implies that the phosphorylation reaction of the two sugars cannot be performed. Hence if uptake occurs under the above conditions it should be due to the action of the membrane carrier alone and not phosphorylation i.e. the uptake is by facilitated diffusion and not active transport. Glucose-6-phosphate and fructose-6-phosphate act as regulators of facilitated diffusion using feed back inhibition (16,48).

A variation of the original phosphorylation theory of sugar uptake was revived by van Steveninck (178), in the form of a hybrid carrier-phosphorylation model based on metal binding studies. The proposed uptake system had two parts i) facilitated diffusion where glucose has a low affinity uptake in poisoned iodoacetic acid cells, and so have non-metabolizable sugars in normal cells. Under these conditions, where facilitated diffusion occurs the binding of Ni^{2+} ions to the cell surface did not inhibit glucose uptake (175). UO_2^{2+} ions inhibited facilitated diffusion at relatively high concentrations ii) active transport, in which uptake is directly connected to metabolism. Here high affinity uptake occurs via polyphosphate groups at the cell surface (176). Glucose is supposedly transported in normal cells in this mode. The argument was that Ni^{2+} ions partially inhibit glucose binding at the cell surface and therefore inhibit transport. The binding sites were said to be phosphoryl groups and Tijssen and van Steveninck (174) postulated that they were polyphosphate in nature. By implication these polyphosphate groups are part of a carrier system, where the phosphorylation reaction plays an integral role perhaps by chemically altering the carrier at the outer surface. UO_2^{2+} ions

completely inhibited transport at relatively low concentrations. The difference in inhibition concentrations of UO_2^{2+} for facilitated diffusion and phosphorylation is due to the different sites of its action. Van Steveninck states UO_2^{2+} causes inhibition in facilitated diffusion by interacting with the carboxyl groups in the cell surface whereas in metabolically active phosphorylation UO_2^{2+} ions inhibit phosphoryl groups. In facilitated diffusion Ni^{2+} did not affect glucose transport, as the Ni^{2+} ions did in active transport phosphorylation, due to the different sites of action for the two systems. Van Steveninck (176,177) and van Steveninck and Jaspers (88) supported the above model by performing experiments using 2-deoxy-D-glucose. This is a partially metabolized analog of D-glucose with a high affinity for the D-glucose transport system, and can be phosphorylated by hexokinase. In pulse-labelling experiments with radioactive 2-deoxy-glucose conducted in order to determine the time sequence of the appearance of 2-deoxy-glucose in intracellular pools, they reported that intracellular 2-deoxy-glucose first appeared in its phosphorylated form. They also reported that intracellular concentrations of 2-deoxy-glucose phosphate reach higher concentrations than external 2-deoxy-glucose. Meredith and Romano (124) also found that 2-deoxy-glucose phosphate is the first species to be detected intracellularly in pulse-label experiments. However they did not find that 2-deoxy-glucose exceeded the extracellular concentrations. This implies transport is by facilitated diffusion and not active transport phosphorylation. This result was confirmed by Franzusoff and Cirillo (50). Using single-kinase mutant strains containing only one of the two hexokinases or glucokinase, they showed that if a phosphorylation-associated transport mechanism is present, then it does not depend on a specific kinase to operate. However, they urged caution in interpreting the results of pool

labelling experiments with whole cells, in which problems of compartmentalization and multiple pools are difficult to access. Becker and Betz (16), and Romano (146) point out that the Km values of hexokinase and glucokinase are one and two orders of magnitude lower than the glucose Km value for transport. This difference causes membrane transport to be tightly coupled to hexokinase activity and would result in immediate phosphorylation of 2-deoxy-glucose as it enters the cell. Thus initial free sugar pools may be too small to be detected by the methods used (16,37,146).

It was found by Fuhrmann and Rothstein (46) that the effects of Ni^{2+} on glucose uptake, which was formally attributed to the association of Ni^{2+} with superficial polyphosphate involved in glucose transport, is actually due to its adsorption into the yeast cell, where it probably inhibits alcohol dehydrogenase. Also, it has been shown that uranyl ions also inhibit transport of amino acids and various other solutes and the existance of this effect need not imply that polyphosphate is involved in all or any of these processes (46).

Further evidence against the phosphorylation theory comes from Romano (146), who showed that 6-deoxy-D-glucose, a structural homomorph of D-glucose which lacks a hydroxyl group at carbon 6 and thus cannot be phosphorylated by *S. cerevisiae*, is transported by means of facilitated diffusion. It has the same affinity for membrane transport as does glucose. This result negates one of the principal tenets of the phosphorylation theory of transport, namely, that phosphorylation is necessary for the carrier to operate in the high affinity mode (146). The previously observed low affinity uptake of the transport system for glucose analogs could be due to their reduced structural incompatibility with the stereospecific characteristics of the carrier. Kotyk and Michaljanicova (108) observed

that D-glucose is taken up without phosphorylation, and at a high affinity rate by facilitated diffusion.

The glucose transport system in S. cerevisiae has two uptake components i) a high affinity uptake with Km ca. 1 mM for glucose and Km ca. 6 mM for fructose; and a low affinity uptake with values of Km ca. 20 mM and Km ca.50 mM respectively (19). The enyzmes hexokinase A (P-I), hexokinase B (P-II) and glucokinase are linked to the functioning of the two uptake systems. The high Km - i.e. low affinity uptake - for glucose only exists when all three kinases are absent, whereas for fructose, the high Km system requires the loss of the two hexokinases. Evidence points against a direct connection between entry and metabolism, where a transient phosphorylation occurs during entry, or where formation of the first metabolic intermediate hexose-6-phosphate, would occur by an obligatory vectorial phosphorylation (as with the bacterial phosphotransferase system (19)). Experiments with 6-deoxy-glucose, also show the low affinity uptake when all three kinases are absent, and high affinity uptake when any one of the kinases is present (21). Thus high affinity uptake appears to depend on the presence of any one of the kinases, but not in their phosphorylation role. Bisson and Fraenkel (19) propose a model where the kinases form a functional part of the hexose uptake system i.e. they are involved in transport, but not in a phosphorylating capacity. Thus the kinases should be membrane bound enzymes. However, they are characterised as soluble enzymes. It is possible that a small fraction are tightly membrane bound, or that there is a loose association with some membrane components that perform the primary recognition of the external sugar (19). In yeast the two hexokinases act to phosphorylate fructose in the 6-position (161), while all three kinases are able to phosphorylate glucose. The roles of the three kinases for glucose are not well understood even with respect to

metabolism (20). Hexokinase A (P-I) has a ratio of phosphorylation of fructose to glucose of approximately 2:5 and hexokinase B (P-II) has a fructose to glucose ratio of approximately 1:3. Glucokinase has no activity for fructose (52,119). Although any one of the kinases is adequate for growth on glucose (52,102,113,114) in the wild-type strain hexokinase B is predominant in growth on glucose and hexokinase A seems to be a repressible enzyme that appears when glucose has been used up. Fructose mediated growth requires at least one of the two hexokinases to be present (52). The activity of the two hexokinases appears to be adaptive and depends on conditions in the growth medium. While glucokinase action is constitutive and independent of the carbon source present. Cells grown on 3% ethanol showed predominant hexokinase A and little hexokinase B. When grown on 6% glucose the pattern was reversed with hexokinase B being the major enzyme present. When cells grown on the 3% ethanol were incubated on medium containing 6% glucose, hexokinase A was repressed and hexokinase B was induced (127). According to Bisson and Fraenkel (19), any one of the three kinases (for glucose) or either of the two hexokinases (for fructose) is adequate for low Km i.e. high affinity uptake. Both the high affinity uptake and low affinity uptake operate by facilitated diffusion. Low affinity uptake is present in growth in high concentrations of glucose (100 mM) whereas high affinity uptake occurs in growth in low concentrations of glucose (0,5 mM) and in growth in normal concentrations of ethanol, galactose or lactate plus glycerol. A change from growth in 100 mM glucose to 0,5 mM glucose results in a ten-fold increase in the level of high affinity uptake in 90 minutes. Either protein synthesis or energy metabolism or both is required (21). Re-imposition of the high glucose concentration caused the cells to revert to the low affinity uptake. Depression of high affinity uptake is not correlated with the levels of the kinases

themselves. Bisson and Fraenkel (21) propose that low and high affinity uptake should be seen in a physiological context, where the cells can obtain most benefit from the prevailing growth conditions i.e. in low glucose concentrations high affinity uptake occurs and vice-versa. They also observe that the low and high affinity components of their model may go someway to explaining the low affinity uptake and high affinity uptake of van Steveninck's phosphorylation theory of sugar transport.

Not all yeasts utilize glucose faster than fructose. S. bailii (59,69,144,159,160) and Torulopsis stellata (137) preferentially utilize fructose over glucose, whilst Kloeckera apiculata (136) uses both glucose and fructose at equal rates.

Gottschalk (59) thoroughly ground dried preparations of S. bailii, causing the cells to rupture. He found the ground cells utilized glucose faster than fructose. He proposed that the differences in the rates of uptake for the two sugars was due to the preferential permeability of the cell membrane for fructose. Sols had similar findings (160) and stated fructose was taken-up faster than glucose owing to its greater affinity for the common hexose transport carrier.

1.8. Direct Sucrose Transport

A proton symport for carbohydrates was demonstrated in yeast using a strain of S. cerevisiae, which, after adaptation to the sugar, absorbed protons with maltose, sucrose and α-methylglucoside but not with 2-deoxy-glucose, galactose and glucose (43,45,108,151).

A specific system exists in S. cerevisiae for the transport of sucrose across the cell membrane as an intact molecule i.e. without hydrolysis occurring outside the cell prior to uptake. Sucrose uptake does not involve active transport. Cells pretreated with *azide, an agent* which inhibits glycolysis and ; substrate phosphorylation and causes complete de... ...n of ATP in the cell, carried out sucrose transport normally. The energy requirement for sucrose transport is provided directly by μH^+, the electrochemical gradient of protons *across the yeast cell* membrane. It is known as a proton symport in which the initial rate of uptake shows a proton/sucrose stoichiometry of 1:1. The K^+ cation appears to act as a counter ion to the protons entering the cell, in order to maintain electroneutrality. The sucrose transport system shows similarities with the transport system for maltose uptake (151), in that,

1. Both systems are proton symports.
2. NaF exerts an inhibitory effect on both systems.
3. Both systems are stimulated by Na^+ ions, the *sucrose symport more so than the maltose symport*

1.9. Proton Symports with Particular Emphasis on the Maltose Transport System of S. cerevisiae.

Proton symports are transport systems located within the cell membrane of yeasts and bacteria (43,46) and function to translocate solutes across the cell membrane into the cell. They operate via secondary active transport in which the movement of the solute across the cell membrane (46) is coupled by a purely physical process to the movement of some other specific particle. Here the particles are protons. This system is in contrast to primary active transport, where the flow of solute across the cell membrane is coupled to an exergonic chemical reaction, where the increase in free energy of the solute is derived from changes in chemical bond energy associated with the reaction e.g. the bacterial phosphotransferase system (68). In its simplest form one proton is taken up with one molecule of solute. This number may vary and the ratio could be 2:1 or 3:1. The membrane potential required to just balance the proton current through the proton pump is about -0.4 V. The total energy available from the hydrolysis of ATP in vivo is estimated to be approximately -0.5 V. This indicates that the voltage generator across the cell membrane is fuelled by ATP, and infers that the pump ejects one proton for each ATP molecule hydrolysed. The proton stoichiometry appears to be variable and can rise in certain circumstances of energy restriction (46). The cell membrane Mg^{2+} ATPase is an integral membrane protein, which is able to expel charged species such as protons from the cell. This indicates that ATPase is probably the electrogenic proton pump (46). It functions to maintain electroneutrality across the cell membrane after solute uptake has occurred.

Maltose is a disaccharide composed of two molecules bound together by an alpha 1-4 linkage (68). It is

transported directly into the cell, and in the process of catabolism two glucose units are produced. This is in comparison to sucrose which, although it is transported directly into the cell via a proton symport, may also be hydrolysed into its constituent moieties glucose and fructose by the enzyme invertase outside the cell membrane prior to uptake (71). In S. cerevisiae the transport of maltose into the cell is dependant on the proton gradient of the cell membrane (24,154). When the proton gradient is removed by the presence of uncouplers, maltose transport cannot take place. One molecule of maltose is co-transported with one proton. Electroneutrality during maltose transport is maintained by the efflux of cations such as K^+ ions from the cells (154). The simultaneous exit of K^+ ions and the influx of protons into the cell during maltose transport has a stoichiometry of 1:1. This occurs prior to the activation of the proton pump, which begins to eject protons from within the cell approximately 0.5 minutes after induction with maltose, and adds to the electroneutrality effect.

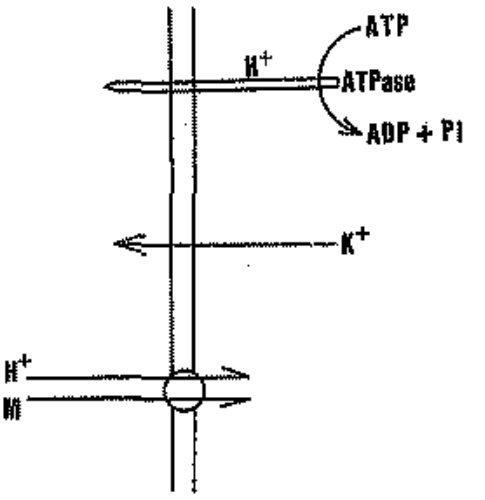


Figure 23. The operation of the maltose proton symport including the mechanism of charge neutralization (46).

The Km of maltose transport increases from 5.2 mM at pH 5.8 to 38.0 mM at pH 7.8 in S. cerevisiae. For the same pH increase the V_{max} is halved. External pH affects the specific growth of a petite mutant of S. cerevisiae in the range pH 5 - 7, while the cells showed no such effect when grown on glucose or galactose. This pH effect is probably related to the transport step. The presence of K^+ ions has an inhibitory effect on transport. At values above pH 5.0, the V_{max} is decreased and the Km is increased. At pH 4.0 and below the presence of K^+ ions has a stimulatory effect (117). The addition of zinc, magnesium and ammonium ions to S. cerevisiae grown in metal-free buffers produced stimulatory effects on maltose utilization (182). In S. cerevisiae intracellular maltase has a relatively low affinity, Km = 10mM, for maltose. Thus proton symports may have been developed in order to maintain high intracellular concentrations of maltose, which drives the reaction catalysed by maltase, to keep a good rate of maltose hydrolysis (117).

Ethanol has a non-competitive effect on the maltose transport system in S. cerevisiae. The V_{max} for maltose transport is decreased compared to untreated cells but the Km remains constant. This decrease is probably caused by an alteration of the lipid environment of the maltose transport system in the cell membrane (116).

1.10. Catabolite Repression of Maltose Transport

The transport of maltose across the cell membrane in S. cerevisiae is via a membrane transport protein α-glucoside permease. Once maltose enters the cell, it is catabolized into two glucose units by the enzyme α-glucosidase (156). Maltose is needed to induce the maltose transport system in S. cerevisiae, whereas it may be repressed in the presence of glucose and fructose (118).

A brewing yeast S. cerevisiae NCYC 240 showed delayed utilization of maltose in a laboratory fermentation system, using brewers wort, when the inoculation yeast was grown up in a glucose rich MYPG medium. When the yeast is inoculated into the fermentations, repression of maltose utilization occurs whilst glucose is present in the wort. Once the glucose concentrations in the wort reach low levels, maltose utilization begins (27). A similar phenomenon was shown in synthetic media. Cells grown in synthetic media containing 2% (w/v) maltose and 2% glucose, exhibited diauxic growth (64). Only glucose was utilized initially, with maltose utilization completely repressed. When glucose reached a cut-off concentration of 0.4% (w/v), derepression occurred and maltose utilization commenced. Glucose causes the inhibition of transcription as well as translation of α-glucoside permease. Hence there is repression of de novo synthesis of the α-glucoside permease (155). Maltose grown cells incubated in glucose over a 90 minute period show almost complete inactivation of the maltose transport system due to inactivation of α-glucoside permease. This occurs under aerobic as well as anaerobic conditions. During this period the Km for maltose transport increases from 4 mM at time zero to 13 mM at 45 minutes and at 90 minutes is about 50 mM. Recovery of the maltose transport system occurs

within one hour with the Km decreasing to its original value, once glucose reaches its cut-off concentration. The maltose in the medium induces the synthesis of α-glucoside permease (156). The levels of α-glucosidase remain approximately constant in the presence of both glucose and maltose. This implies that α-glucosidase does not have a regulatory role in maltose uptake (54,63). Maltose grown cells show slight maltose utilization when inoculated into a synthetic medium containing 2% (w/v) glucose and 2% maltose, after which maltose utilization commences. The α-glucoside permease is present in the maltose grown cells and is able to function for a short while and hence utilize small concentrations of maltose before it is inactivated by the presence of glucose in the new medium. This effect does not occur in glucose grown cells. cAMP exerts a positive control mechanism over catabolite repression in S. cerevisiae similar to the system existing in E. coli, with the concentration of intracellular cAMP being inversely proportional to the glucose concentration in the medium (43).

1.11. The Effect of Ethanol on the Glucose Transport System

Ethanol inhibits the rate of fermentation and the growth of S. cerevisiae (43,110). Ethanol has the effect of decreasing T max (the maximum temperature for growth), increasing T min (the minimum temperature for growth) and decreasing T op. (the optimum temperature for growth). A temperature profile of maximum ethanol tolerance shows an increase in ethanol tolerance from 3°C to 14°C, then a plateau of ethanol tolerance at 11% v/v from 13 - 27 C and thereafter from 28°C to 42°C the ethanol tolerance drops to zero (149,181).

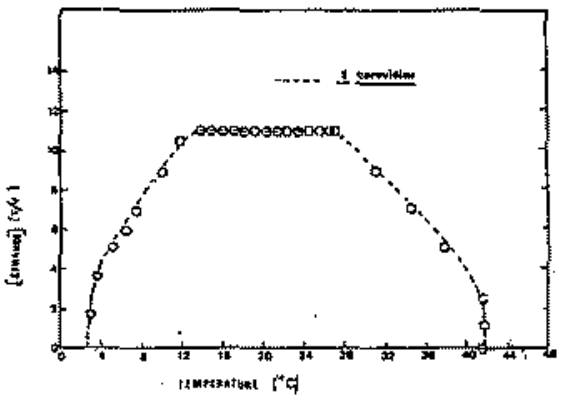


Figure 24. Temperature profile of maximum ethanol tolerance of S. cerevisiae. Experimental points indicate the concentrations of ethanol above which growth could not be detected (149).

In lower temperature fermentations, i.e. those before the plateau (below 13°C), the yeast is more ethanol sensitive with respect to yeast growth, than in fermentations at intermediate temperatures (180). This situation is especially relevant to the beer industry where the trend is toward the use of high gravity fermentations.

Existing plant can produce more beer by using concentrated wort and an increased pitching rate. A consequence of this phenomenon is the production of higher alcohol beer. If the ethanol concentration in the beer fermentation exceeds the maximum ethanol tolerance level shown in the graph, then the inhibitory effect of ethanol on yeast growth and viability may cause the fermentation to terminate prematurely.

Ethanol affects the glucose transport system in S. cerevisiae causing an inhibition of uptake (110). This inhibition is of a non-competitive type i.e. ethanol affects the maximum uptake rate of the system i.e. Vmax, but not the affinity of the system expressed by Km, the Michaelis constant (180). Some authors have reported that the capacity for inhibition is a hyperbolic function of the ethanol concentration, while others fitted linear, exponential or other formulae to their experimental data (110). Aiba and Shoda (180) managed to fit both hyperbolic and exponential relations to their data, when using a Bakers yeast. Romano (146) used a strain of Schizosaccharomyces pombe in the ethanol inhibition of D-xylulose fermentation and found his results fitted linear, hyperbolic and exponential results equally well. All these apparently equivocal relations for ethanol inhibition imply that several different mechanisms are involved and the overall kinetics of ethanol inhibition is composite (180).

The relative weights of the different kinetic contributions may vary with the strain, the concentration, the physiological state of the cells and other chemical and physical factors. Leao and Van Uden (110) studied the effects of several different alkanols i.e. ethanol, isopropanol, propanol and butanol on the transport of glucose across the cytoplasmic membrane in S. cerevisiae. Each of the alkanols inhibited the

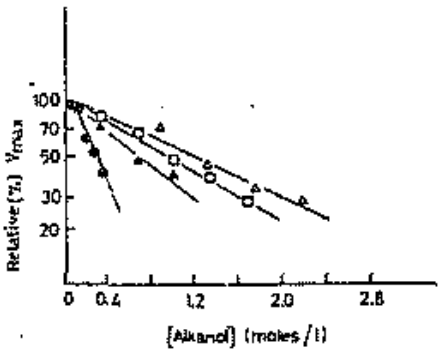


Figure 25. Semi-log plots of the relative (%) maximum initial uptake rates of d-xylose by S. cerevisiae as a function of alkanol concentration. ● Ethanol; ▲ isopropanol; □ propanol; and △ butanol (110).

uptake of D-xylose by the glucose transport system and the inhibition effect increased with their lipid solubility. Also, the molecular volume of each alkanol played a role in the inhibition mechanism there being an inverse relationship between the molecular volume and degree of inhibition. The alkanols only decreased the Vmax, and did not affect the Km. Each obeyed exponential kinetics. The effect of the alkanols on the glucose transport system could be expressed in terms of the equation (110):

$$v = V_{max} \cdot e^{-kx} \frac{S}{Km + S}$$

Where S is the concentration of glucose, Km is the Michaelis constant, v is the initial uptake rate without alkanol under the same conditions, x is the ethanol concentration, and k is the inhibition constant characteristic for the alkanol. Thus using a determined

value for k in S. cerevisiae 2036, this equation can be applied specifically to ethanol inhibition of growth and fermentation using a particular sugar as the carbon source. Thus the ethanol inhibition effect on the glucose transport system in S. cerevisiae, which is an electroneutral uniport, may be explained exclusively by its action on the hydrophobic areas of the membrane (173,180).

The above equation exhibits exponential kinetics of ethanol inhibition as long as the transport step determines the overall rate of growth and fermentation. When other metabolic steps show significant inhibition by ethanol, compared to membrane uptake, then their own inhibition kinetics will contribute to the overall kinetics which may then no longer conform to a simple model (110).

As ethanol is able to exert an inhibitory effect on the membrane carrier protein, it may also possibly exhibit a similar effect on soluble enzymes within the cell involved in the subsequent metabolic steps to membrane transport (180).

The degree of inhibition of glycolytic enzymes by ethanol shows large variation from strain to strain. In bakers yeast, ethanol showed no inhibitory effects at 6.3% (v/v) on twelve glycolytic enzymes and only at concentrations between 21.5% (v/v) and more than 44.3% (v/v), at 30°C, did ethanol irreversibly reduce the activities of these enzymes. Using S. cerevisiae, Nagodawithana (130) showed that ethanol inhibits hexokinase and aldolase activity at concentrations of 19% v/v, while key glycolytic enzymes in Kloeckera apiculata exhibited irreversible denaturation in vivo in the presence of 10% ethanol. In contrast, 10% ethanol showed insignificant effects on the same enzymes in

Saccharomyces oviformis. In high gravity beer fermentations, there is approximately 7% (v/v) alcohol at the end of fermentation (42). At this concentration, the ethanol inhibitory effect on the glycolytic pathway enzymes in S. cerevisiae is negligible, the assumption being that the ethanol concentration within the yeast cell is the same as that in the surrounding medium. Becker and Betz (16) showed that membrane transport is the rate limiting step in the glycolytic flux of S. cerevisiae. Miller et al (125), using a Saccharomyces strain showed that even if the hexokinase capacity is reduced by 50%, the glycolytic flux would not drop noticeably due to the excess capacity of the enzyme as compared with the glucose membrane transport system. Thus, although ethanol does have an inhibitory effect on the action of certain enzymes of the glycolytic pathway in S. cerevisiae (130), this effect is insignificant when compared to the rate of glucose uptake across the membrane which is the rate limiting step in the glycolytic flux. If one assumes that the concentration of ethanol within the yeast cell is much higher than that in the surrounding medium, then ethanol should have a much greater inhibitory effect on the glycolytic flux than the experimental data presented above supposes. This situation also implies that the inhibition of glycolytic enzymes may be significant and it is possible that the uptake step is not the pacemaker of the glycolytic pathway. This assumption is probably false, as will be shown below.

Several authors (15,180) have reported that intracellular levels of ethanol in batch fermentations may be much higher than its concentration in the extracellular medium. Nagodawithana and Steinkraus (128,129) and others (126) reported that externally added ethanol was less toxic for S. cerevisiae than

ethanol measured at similar external concentrations which was endogenously produced. Their explanation for this phenomenon was that the rate of net outflow of ethanol from the cell was slower than the rate of ethanol production within the cell, and consequently, there was an increase in the concentrations of intracellular labelled ethanol. Loureiro and Ferreiro (115) showed that in S. cerevisiae equilibrium between extra- and intracellular ethanol is established in 25 seconds, and the maximum ethanol gradient to be expected is less than 0.1 M.

Beavan et al (15) showed that when the fermenting yeast is washed with water or buffer, ethanol is very rapidly released. Their findings confirm that ethanol diffuses rapidly through the membrane which is unlikely to constitute a barrier against ethanol accumulation in the cell. In batch fermentations, they found that the intracellular ethanol concentration was higher than the extracellular concentration, and in the earliest stages the ethanol gradient was approximately 1.0 M.

In checking the technique for determining the intracellular ethanol levels, Dasari et al (40) found that fermentation continued during processing of the yeast sample. By precooling samples to 4°C and reducing the centrifugation time, they found that the intracellular ethanol levels were significantly reduced. Further, during the early stages of fermentation when the ethanol concentrations are relatively low, the intracellular concentration was higher than the extracellular conconcentration, with a maximum ethanol gradient in the order of 0.2 M, while in the later stages of fermentation, when the ethanol concentrations approach the maximum ethanol tolerance limit of 11% (v/v) in the case of S. cerevisiae, the ethanol gradient was reversed so that the extracellular ethanol concentration was higher than the

intracellular concentration, with the gradient exceeding 0.3 M in the opposite direction.

Thus if the glycolytic enzymes of S. cerevisiae are not inhibited at the external maximum ethanol tolerance concentration of 11% (v/v) in vitro, then this effect should be similar in vivo as the results above show that the intracellular ethanol concentrations are not higher than the extracellular concentrations at the upper limits of ethanol tolerance in S. cerevisiae. Indeed the glycolytic enzymes of S. cerevisiae are not inhibited at concentrations of 11% (v/v) in vitro. Hence the ethanol effects on intracellular components such as the glycolytic enzymes are not likely to play a significant role in the overall ethanol inhibition kinetics (180).

1.12. General Classes of Mathematical Models

Biological systems may be quantified and predicted through the use of mathematical models. The more complex the system the more likely mathematical models are to be used. They allow quantification of systems where it is not possible to measure the complex underlying mechanisms and interactions which allow the particular system to operate.

The first general type of mathematical models are of the empirical form (12). They rely on the identification of the most significant factors affecting the behaviour of the system. Through observation and intuitive ideas these factors are conceptualized into the form of a mathematical model, where the model correlates the performance of the biological system. This type of model is rarely predictive as it does not significantly examine the underlying processes which determine the overall system behaviour ie. it is a "black box" approach, where the intricacies of the system are either unnecessary or too complex to be included in the model (101). The model will only operate for a specific set of conditions, and it can not be manipulated to predict the operation of the system, when the conditions of the system are altered. Therefore, an empirical model is not able to add to our fundamental knowledge of the system under question.

The mechanistic model (12) is the second general type of mathematical model. It is much more sophisticated than the empirical model in that it attempts to incorporate the postulates of how the system variables interact to form an integrated whole. This type of model requires much greater scientific understanding and precision as the underlying mechanisms of the system are set out explicitly in a mathematical form within the model. Once all the basic premises of the system have been

established, they are correlated and a mathematical statement is developed from them. Provided all the underlying assumptions within the model are correct, and they have been rigorously developed into a mathematical form, then the model should accurately predict the performance of the system. Thus the model should be able to add a greater insight into the operation of the system as it is able to predict within the system what happens under different conditions. As far as possible the assumptions within the model must be capable of experimental verification. Also, all the assumptions within the model must be clearly stated, and the model must not contain any hidden or inherent assumptions. When mechanistic models are successful in predicting a particular system they become useful scientific tools and add to our progressive understanding of the system under investigation.

Obviously a mathematical model which could be used by the brewing industry to predict the concentrations of residual sugars in fermentations when using various adjunct types would be of use. Hence the aim of the present study was to develop a mathematical model which would predict the rate of fructose utilization during sucrose adjunct fermentations and the residual concentrations of fructose in the fermented wort at the end of fermentation.

CHAPTER 2.

The Establishment of a Laboratory Fermentation System

2.1. Introduction

A laboratory scale fermentation system needs to produce data which, ideally, is identical to data that is obtained from full scale plant fermentation vessels when both types of fermentation vessels are operated under exactly the same conditions. In practice, this may not always be achieved, with small differences existing between the two systems. Therefore, a laboratory system which produces data which correlates as closely as possible with data from full scale plant fermentation vessels is required. Hulse (84) had previously made use of 500 ml Kartell plastic volumetric cylinders as laboratory scale fermentation vessels. He found good correlation existed between data produced by such fermentation vessels, and data produced by full scale plant fermentation vessels, when both systems were run under identical conditions. The parameters he monitored during the course of the fermentation were yeast count, and specific gravity. The same laboratory fermentation vessels were chosen for this project. New parameters, of fructose, glucose and in some cases maltose concentration would also be monitored. In order to check the correlation between the two systems when monitored for these new parameters and recheck the correlations for yeast count and specific gravity, the two systems were set up using identical conditions of growth and temperature. Both types of fermentation vessels were monitored daily and comparison was made of the subsequent data.

It was important to know the number of repetitions of fermentations needed to produce valid data. Six

laboratory fermentations were set up, using exactly the same yeast and wort, and each fermentation was incubated at the same temperature. Each fermentation was monitored daily for yeast count, specific gravity and glucose and fructose concentration. Analysis was carried out on the data using statistical methods.

2.2. Methods

2.2.1. The Comparison of a Laboratory Fermentation System with a Full Scale Plant Fermentation Vessel.

A three litre sample of pitched wort was taken from a plant fermentation vessel in which a Carling Black Label (CBL) fermentation had been set up several hours previously. This fermentation contained the wort excluding adjunct, i.e. CBL all-malt wort plus a glucose adjunct as opposed to a sucrose adjunct, which when fermented under normal full-scale plant conditions, produced the fructose block. As each laboratory fermentation vessel held 500 ml, this allowed six identical fermentations to be established. The sample was stirred, to effect homogeneity and then poured into *six 500 ml Kartell plastic volumetric cylinders*, up to volume. They were incubated at 11°C for the duration of the fermentation. This was the temperature used in all other fermentations establishedfor this research, unless otherwise stated. A Lauda K4R water bath set at 11 C, which was connected to an external bath, in which the six laboratory fermentation vessels were placed, maintained temperature control. The plant fermentation vessel and the six laboratory fermentations were monitored every 24 hours, for the duration of the fermentation i.e. 216 hours (9 days). The sample from the plant fermentation vessel was taken aseptically from its sample cock, and the samples from the laboratory fermentation vessels were taken using sterile 10 ml pipettes. Thereafter, each sample was analysed for the parameters :

a) Yeast count
b) Specific gravity
c) Glucose concentration
d) Fructose concentration

a) Yeast Count

The number of yeast cells per ml of wort was determined either by using a Coulter Counter Industrial D cell counter (Florida, U.S.A.), or by determining the relative yeast count using a spectrophotometric method by reaching the absorbance of the sample at 800 nm against a wort blank.

b) Specific Gravity

The partially fermented wort sample was filtered using a 0.45 um membrane filter to remove the yeast from the sample. The specific gravity was measured by injecting approximately 2 ml of sample into an Anton Paar DM20 calculating density meter (Graz, Austria). The reading was converted to degrees Plato (°P) using standard tables (5,53).

c) and d) The Determination of the Glucose and Fructose Concentration

Sugar analysis was performed using a high performance liquid chromatography (HPLC) system (26,39), which consisted of a Micromeritics (Georgia, U.S.A.) 750 autoinjector, a 750 solvent delivery system operated in the constant flow mode at 0.5 ml/minute of solvent and a 731 column compartment set at 85°C in which an Aminex HPX - 87 carbohydrate HPLC column (Bio-Rad Laboratories, California, U.S.A.) was placed. Sugar detection was by a Knauer differential refractometer (Taunis, G.F.R.) set at a sensitivity of 32. Sugar concentrations were

recorded and calculated using a Hewlett-Packard 3390 A recording integrator (California, U.S.A). The eluent used was double distilled de-ionized water filtered through a 0.45 um membrane filter. The HPLC system was operated using external standards of glucose 1.000 g, fructose 0.500 g, maltose 1.000 g, and sucrose 1.000 g (Analytical grade, Merck, Darmstadt, G.F.R. for each sugar). All fermentation samples were filtered through 0.45 um membrane filters prior to HPLC analysis. Two drops of a bacteriostatic solution of pentachlorophenol (BDH, Poole, U.K.) were added to the samples which were stored in McCartney bottles at ~20ºC until ready for use (6).

The detector response was linear up to a concentration of 2 g per 100 ml of solute. All samples had to be diluted so that this upper limit was not exceeded. As the concentrations of glucose and fructose exceeded this upper limit at the beginning of fermentation, the samples were diluted 1:3 with filtered distilled water. The sugar standards were also diluted to the same concentration. The first sample to run through the HPLC flushed the column. The next three samples were glucose/fructose standards which were run to determine response factors for the integrator. The fermentation samples followed, and interspaced, at regular intervals by water, standard, and water samples. At the end of every run two aliquots of 30% acetonitrile were used to clean the column and remove unwanted waste substances.

2.2.2. Statistical Test Fermentations

Carling Black Label wort was collected from the paraflow i.e. CBL all-malt wort plus a glucose adjunct as opposed to a sucrose adjunct. When CBL all-malt wort with a sucrose adjunct is fermented under normal full-scale plant conditions, the fructose block is produced. The collected wort was oxygenated to 14 mg/litre using medical grade oxygen (Afrox, Johannesburg, R.S.A.) and a

Yellow Springs Instrument Oxygen meter, model 54, ARC (Ohio, U.S.A.). Approximately 50 g of the brewing yeast Saccharomyces cerevisiae 2036, formerly named Saccharomyces uvarum 2036, was collected aseptically, from the yeast collecting vessels. The yeast was centrifuged at 3000 rpm for 10 minutes on a Beckman TJ-6 centrifuge (Galway, Ireland). The supernatant was discarded. Six, 6 g samples of the compressed yeast pellet were weighed out and used to establish six identical fermentations each containing 500 ml of wort. The yeast and the wort were thoroughly mixed in order to effect homogeneity. The length of the fermentations was 170 hours and they were monitored every 24 hours for specific gravity, yeast count, and glucose concentration.

The number of fermentations set up was arbitarily chosen to be six. Each time a parameter was monitored, six values were obtained, and 10% of the mean of these values was determined. This procedure was carried out for each parameter monitored, on each day of fermentation. The standard deviation and standard error were calculated each time a parameter was monitored (see Table 6). Next a standard error was calculated which presumed there were only two identical fermentations instead of six. If this standard error value was less than 10% of the mean, then the data would show fermentations produced in duplicate would produce valid data. If this standard error value was greater than 10% of the mean, then the number of fermentation repetitions would have had to be increased until this standard error was equal to, or less than, 10% of the mean.

The variability of the HPLC assay for glucose and fructose was tested using statistical methods. Each sample was assayed twice, to determine the glucose and fructose concentrations present and the standard deviation (S.D.) of each assay for a particular sample was calculated. These standard deviation values were

then determined as a percentage of the the mean of the two assay values. This procedure was carried out on fifty samples assayed for glucose concentration and fifty samples assayed for fructose concentration.

2.3. Results

Overall good correlation was found between the data for the laboratory fermentations and the plant fermentation vessel with respect to yeast count, specific gravity, and glucose and fructose concentration (Figure 26.). Small variations between the two systems did occur. The specific gravity plots both showed similar trends in gross terms with an initial slow decrease up to 120 hours and finally a very gradual decrease until 196 hours (Figure 26B.). In each of these three stages, the plant fermentation showed a slightly greater decrease than the laboratory fermentations. The glucose and fructose concentration plots (Figure 26A.) reflected the specific gravity results (Figure 26A. and 26B.). Here the trend of decrease in the glucose concentration was the same for both systems but the plant fermentation showed a slightly greater decrease than the laboratory fermentations. This difference was most prominent at the end of the fermentations. The yeast count plots both showed (Figure. 26C) an increase in biomass until flocculation, and thereafter a gradual precipitation of yeast. The plant system showed a greater number of cells at flocculation and a more rapid increase up to this point than the laboratory fermentations. Flocculation occurred slightly earlier in the plant fermentation vessel. The rate of precipitation of cells out of suspension after flocculation was very similar in both cases until the end of fermentation where the laboratory fermentations, showed a slower decrease than the plant fermentation vessel.

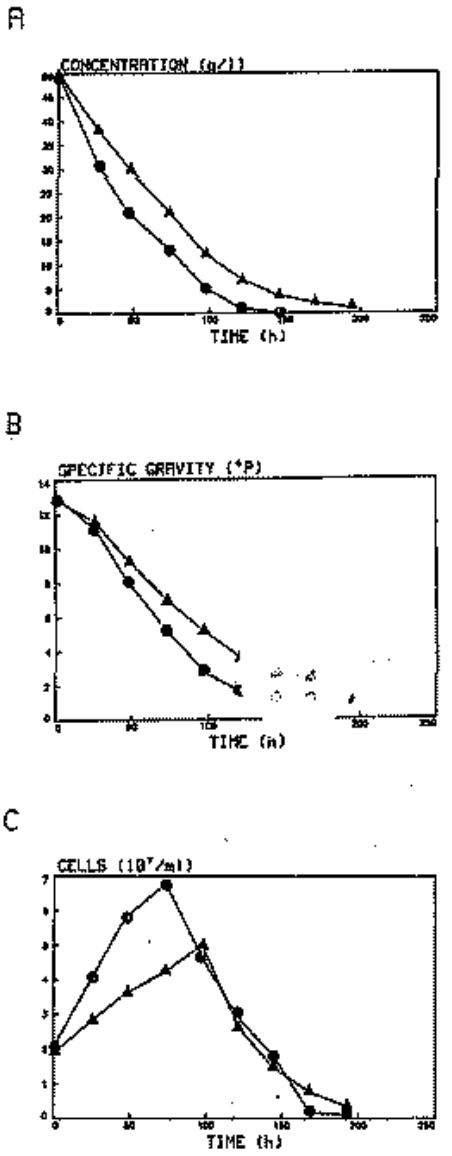


Figure 26. A comparison of the laboratory fermentation system with a full scale plant fermenter, for the parameters:- A, glucose and fructose concentration. B, specific gravity and, C, cell count. ▲ indicates the laboratory system; ● the plant fermenter. The plots of experimental data from the laboratory fermentation system in A,B, and C were drawn from mean values obtained from the 6 identical fermentations.

SAMPLE TIME	GLUCOSE CONCENTRATION (mg/ litre)		SPECIFIC GRAVITY (RP)		CELL COUNT (number of cells)	
(hours)	Standard Error(S.E) *Two* Samples	10% of *mean*	S.E. *Two* Samples	10% of *mean*	S.E. *Two* Samples	10% of *mean*
0	0.05	0.521	0.186	1.464	0	0.09
24	0.055	0.459	0.642	1.142	0.028	0.122
48	0.033	0.345	0.361	0.577	0	0.140
72	0.117	0.254	0.072	0.315	0.025	0.126
96	0.084	0.234	0.041	0.227	0.030	0.108
120	0.055	0.146	0.037	0.176	0.040	0.088
144	0.042	0.084	0.039	0.155	0.023	0.059
168	0.012	0.035	0.044	0.149	0.025	0.410

Table 6. Results from the statistical tests showing a comparison of the values for 10% of the mean and the standard error for two samples monitored. For each set of data compared, the standard error values were always less than the value for 10% of the mean.

The standard error calculated for the two repetitions of the fermentations was less than 10% of the mean for each of the three parameters tested in the sample taken from the fermentations, each time the fermentations were monitored. Thus fermentations may be set up in duplicate and the subsequent data obtained from them may be considered valid.

The variability of the HPLC assay showed that for glucose the mean S.D. value for the fifty values was 1.003 % of the mean assay value. For fructose the mean S.D. value was 1.302% of the assay mean. Hence in all further analysis each fermentation sample was analysed twice in order to obtain a value for the sugar concentration.

2.4. Discussion

The statistical tests showed that only two replicates of each fermentation under investigation were needed in order for the fermentations to produce representative data. Thus throughout the project, all fermentations were set up in duplicate, unless otherwise stated. The results of the comparison between the laboratory fermentation system and the full scale plant fermentation vessel with regard to yeast count, specific gravity and fructose and glucose concentration, showed good overall correlation. Thus the results from experiments set up in the laboratory fermentation system should be, under conditions of identical growth media, temperature and pitching rate, approximately equal to those obtained from full scale fermentation vessels used in the brewery.

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CHAPTER 3.

An Investigation into the Rates of Glucose and Fructose Utilization in Different Fermentation Media.

3.1. Introduction

Fermentations were set up in the laboratory fermentation system, established as satisfactory by data presented in chapter 2, using brewery wort with a sucrose adjunct, to see if the fructose block which occurred in full-scale plant fermentation vessels could be reproduced in the laboratory fermentation system. Also the utilization rates of fructose and glucose were examined when fructose, glucose and equal mixtures of fructose and glucose were used as adjuncts in a variety of growth media, at several different concentrations. Panchal and Stewart (137) found that the relative rate of glucose utilization, when both sugars were present in the same fermentation, altered with different physiological conditions. In a defined growth promoting medium, with equal mixtures of glucose and fructose, glucose was utilized faster than fructose (167). In a defined minimal medium with equal mixtures of glucose and fructose, the preferential utilization of glucose over fructose was reduced (137). Also an increase in the osmotic pressure exerted on the yeast, by the addition of sorbitol to the growth medium, resulted in a decrease in the preferential utilization of glucose over fructose (137). Here a series of wort fermentations were performed using malt wort as a base, and then separately adding fructose and glucose as adjuncts at four different concentrations; 10 g/litre, 20 g/litre, 40 g/litre, and 80 g/litre for each sugar. In the brewery, fermentations are performed using high gravity (HG) sucrose adjunct wort - 15.2 ºP - which has approximately 40 g/litre glucose and 32 g/litre fructose present, and

normal gravity (NG) sucrose adjunct wort which has approximately 24 g/litre glucose and 16 g/litre fructose. There is therefore more fructose present in HG wort than in NG wort. The trends in the rates of glucose and fructose utilization in laboratory fermentations with the four different adjunct concentrations can be extended to brewery fermentations with NG and HG wort as the glucose and fructose concentrations are similar. Experiments were performed to assess utilization rates of the two sugars using a much higher adjunct concentration of 80 g/litre for glucose; fructose; and 160 g/litre of an equal mixture of glucose and fructose were also performed.

Impurities are present in commercial sucrose and the grade of sucrose depends on the concentration of the impurities present. Here the aim was to establish whether the impurities from the sucrose adjunct exert a significant inhibitory effect in the rate of fructose and glucose utilization, by using several different grades of sucrose as adjuncts in wort fermentations. A set of fermentations using six different grades of sucrose adjuncts was set up. The sugars used were:

1. Analytical grade sucrose.
2. Laboratory grade sucrose.
3. Commercial grade invert sucrose (produced by acid hydrolysis)
4. Liquid sucrose.
5. Invert sucrose (hydrolysed by the enzyme invertase).
6. Brewery grade sucrose.
7. Equal mixtures of laboratory grade glucose and fructose.

A further test was established to examine whether the rates of glucose and fructose utilization were

adversely affected by trace elements in wort. Defined media fermentations with maltose at the same concentration found in wort, and four different adjuncts: glucose; fructose; an equal mixture of glucose and fructose; and sucrose, each at 40 g/litre concentration were compared with wort fermentations with the same adjuncts and at the same concentrations.

To examine the rates of fructose utilization and glucose utilization as separate adjuncts, defined media fermentations were set up with maltose at the same concentration as wort fermentations and either glucose or fructose at several different concentrations. Also glucose and fructose were added to separate defined media fermentations, at several concentrations, as the only carbohydrate source present.

Aerobic growth studies of maltose and glucose or fructose utilization were conducted in order to determine whether S. cerevisiae 2036 exhibits diauxic growth patterns when grown on these combinations of sugars i.e. does glucose or fructose exert catabolite repression on maltose uptake or its subsequent catabolism to glucose?

3.2. Methods

3.2.1. Wort Fermentations Set Up with Several Different Grades of Sucrose as Separate Adjuncts.

Eight litres of Carling Black Label malt wort was collected from the brewhouse of Castle Brewery, Isando. The wort was cooled and filtered using kieselguhr and folded filters. The specific gravity of the wort was adjusted to 7.36 °P with water. This is the Plato value of normal gravity Carling Black Label wort

prior to the addition of the carbohydrate adjunct. The adjunct was added in the ratio of 35% adjunct: 65% malt wort. The final specific gravity of the wort was 11.2 °P. The specific gravity of the malt wort was increased from 7.36 °P to 11.2 °P using laboratory grade sucrose as the adjunct. Hops were added at the rate of 2g/litre. The wort was boiled for 50 minutes to sterilize it, and then allowed to cool. The dissolved oxygen content in the wort was oxygenated to 14 mg/litre using medical oxygen and a Yellow Springs Instrument Oxygen Meter, model 54 ARC. Approximately 50g of yeast was collected from the yeast collecting vessel. The yeast was centrifuged at 3000 rpm for 10 minutes and the supernatant was then discarded. Six grams of the pelleted compressed yeast was weighed out and added to 1 litre of wort and mixed until a homogeneous mixture of yeast and wort was formed. The mixture was equally divided into two laboratory fermentation vessels. The technique described above was used to produce 1 litre of adjunct wort with the adjuncts below.

i. Analytical grade sucrose (Merck, Darmstadt, G.F.R.)
ii. Laboratory grade sucrose (Saarchem, Muldersdrift, R.S.A).
iii. Acid hydrolysed sucrose (Hewletts refineries Ltd, Durban, R.S.A.).
iv. Liquid sucrose (Hewletts refineries Ltd Durban, R.S.A.).
v. Brewery grade sucrose (Hewletts refineries Ltd, Durban, R.S.A.).
vi. Enzyme hydrolysed sucrose. A wort with a laboratory grade sucrose adjunct was produced. Twenty four hours prior to pitching 160 g of invertase enzyme, Invertase concentrate from yeast (BDH, Poole, U.K.) was added to wort. This was sufficient concentration to invert all the sucrose present in the wort as the enzyme has an activity of 340 EU per ml.

vii. Equal ratios of glucose and fructose (laboratory grade, Saarchem, Muldersdrift, R.S.A.)

Each of the above adjunct worts were pitched separately and fermentations were set up in duplicate and monitored using the techniques stated in 2.2.1.

3.2.2. Wort Fermentations Set Up Using Different Concentrations of Glucose; Fructose; and Equal Mixtures of Glucose and Fructose as Adjuncts.

A set of adjunct worts were prepared using the methods stated in 3.2.1. Three types of adjuncts were used. These were fructose, glucose and equal ratios of glucose and fructose. The concentrations used were 10 g/litre, 20 g/litre, 40 g/litre, and 80 g/litre. In the case of the adjuncts composed of equal mixtures of glucose and fructose, the concentrations above specified the amount of each sugar present e.g. at 10 g/litre, the adjunct was made up of 10 g/litre glucose and 10 g/litre fructose. The fermentations were performed in duplicate, and pitched and monitored for maltose, glucose and fructose concentrations. Each fermentation sample was analysed for glucose and fructose concentration using HPLC techniques (2.2.1.). The length of the fermentations was 336 hours (14 days). The pooled estimate of variance (s^2_{pooled}) was calculated for the plots of glucose and fructose utilization in the fermentations containing equal mixtures of glucose and fructose as adjuncts. The utilization curves of glucose and fructose resulting from the fermentations using separate adjuncts of either glucose or fructose, were drawn using polynomial regression methods with 95% confidence limits using a statistical package designed for use on an Hewlett-Packard 9845 B computer. The pooled estimate of variance (s^2_{pooled}) was also calculated for the maltose utilization plots in these fermentations.

3.2.3. Fermentations Set Up Using Defined Growth Promoting Media with Fructose; Glucose; Sucrose; and an Equal Mixture of Fructose and Glucose as Adjuncts.

A growth promoting medium was prepared according to the formula (137) below:

Maltose (Saarchem, Muldersdrift, R.S.A.)	60 g
Yeast Nitrogen Base (Difco, Detroit, U.S.A.)	6.7 g
Citric Acid (Saarchem, R.S.A.)	3.3 g
Sodium Citrate (Saarchem, R.S.A.)	5.2 g
Distilled water to	1 litre
Adjust pH to	5.0

The above formula represented the medium without the carbohydrate adjunct. To each 1 litre batch of medium was separately added the adjuncts below:

i. Fructose	40 g
ii. Glucose	40 g
iii. Sucrose	40 g
iv. Fructose	20 g
Glucose	20 g

The fermentations were performed in duplicate and oxygenated, pitched, and monitored using the same technique as described in 3.2.1. Each fermentation sample was analysed twice for glucose and fructose concentration (2.2.1.). The length of fermentation was 336 hours (14 days). The plots resulting from glucose and fructose utilization were drawn using the statistical methods stated in 3.2.2.

3.2.4. Fermentations Set Up Using Defined Growth Promoting Media with either Glucose or Fructose at Several Different Concentrations.

The growth promoting medium was prepared, using the

formula as in 3.2.3. Glucose and fructose were used as separate adjuncts at seven different concentrations for each sugar. The concentrations were 1 g/litre; 3 g/litre; 6 g/litre; 10 g/litre; 20 g/litre; 40 g/litre; and 80 g/litre. The fermentations were performed in duplicate and oxygenated, pitched and monitored using the techniques stated in 3.2.1. Each fermentation sample was analysed twice for glucose and fructose concentration (2.2.1.). The length of fermentation was 240 hours (10 days). The plots of glucose and fructose utilization were drawn using the statistical methods stated in 3.2.2.

3.2.5. Defined Media Fermentations Set Up Using either Glucose or Fructose as the only Carbohydrate Source

The growth promoting medium was prepared as 3.2.3. with the exclusion of maltose. Glucose or fructose were added to separate fermentations at the rate of i) 5 g/litre, ii) 10 g/litre, and iii) 20 g/litre. The fermentations were oxygenated to 20 mg/litre of dissolved oxygen, pitched at 6 g/litre of yeast, and monitored for glucose and fructose concentrations every 24 hours for a period of 240 hours using the techniques described in 3.2.1. Each fermentation sample was analysed twice for glucose and fructose concentration and the resultant utilization plots were drawn using the statistical methods stated in 3.2.2.

3.2.6. The Relationship Between Maltose and Fructose, and Maltose and Glucose Utilization Under Aerobic Growth Conditions

A defined growth medium was prepared using the formula in 3.2.3. except maltose, fructose and glucose were added at 2% (w/v). Two separate media were prepared. One contained maltose and fructose, the other maltose and

glucose. Each medium was inoculated with 6 g/litre of yeast and then incubated in an orbital shaker (Gallenkamp, U.K.) at 110 rev/min at 20°C. Samples were taken at regular intervals, and analysed twice for fructose and glucose concentration as well as yeast count.

3.3. Results

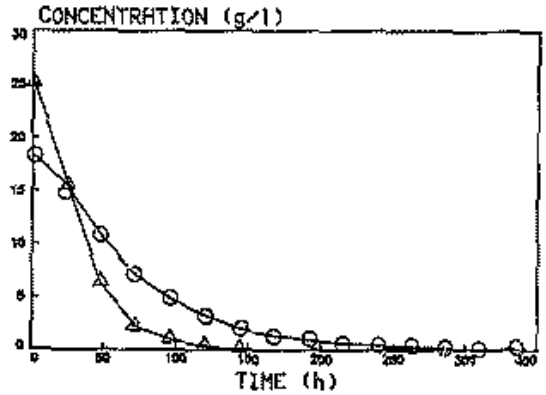
3.3.1. Wort Fermentations Set Up with Several Different Grades of Sucrose as Separate Adjuncts.

A comparison of the rates of glucose and fructose utilization in each fermentation with a different grade of sucrose adjunct, showed that the rate of glucose utilization was much faster than the rate of fructose utilization throughout the entire fermentation period. This difference in the utilization rates of the two sugars, resulted in a fructose block occurring at approximately 168 hours (7 days) and 240 hours (10 days) in all samples (Table 7.). A comparison of the residual concentrations of glucose and fructose at the end of fermentation, of the various grades of sucrose versus the reference sucrose, (Figure 27.) showed almost identical correlation for all fermentations except those containing the adjunct composed of an equal mixture of glucose and fructose. Here the adjunct concentration was approximately 3 g/litre higher than the reference fermentation containing the laboratory grade adjunct, against which it was compared. The difference in sugar concentration at the beginning of fermentation would account for the difference in the rate of glucose and fructose utilization between the two fermentations as the rate of utilization of the two sugars is related to the concentrations of sugar present.

3.3.2. Wort Fermentations Set Up Using Different Concentrations of Glucose, Fructose, and Equal Mixtures of Glucose and Fructose, as Adjuncts.

The utilization rates of fructose and glucose were examined in terms of the slopes of the linear sections of the graphs for each adjunct type, at each sugar concentration, from the initial stages of the fermentation. This allowed comparisons to be made of the rates of sugar utilization amongst the fermentations. The fermentations containing either glucose or fructose as adjuncts showed that the utilization rate of glucose and fructose increased with increasing sugar concentration (Table 8.). This trend was most marked for fructose from 10 g/litre to 40 g/litre, whereas from 40 g/litre to 80 g/litre the increase in the rate decreased . There was no fructose block at 240 hours for the fermentations containing a fructose adjunct of 10 g/litre and 20 g/litre. At 240 hours there was residual fructose of 1310 mg/litre in the fermentations with fructose at 40 g/litre concentration and at 80 g/litre, 7480 mg/litre fructose remained after 240 hours. These two values constitute serious fructose blocks, especially the residual fructose from the 80 g/litre fructose adjunct fermentation (Table 9.). There was a decrease in the residual fructose concentration from 168 hours (7 days) to 336 hours (14 days) for each fructose adjunct concentration (Table 9.). The slopes of the graphs of the fermentations using equal mixtures of glucose and fructose as adjuncts showed the utilization rate of fructose increased from 10 g/litre to 20 g/litre; it stayed approximately constant from 20 g/litre to 40 g/litre, and from 40 g/litre to 80 g/litre it decreased (Table 10.). With the increasing concentration of fructose in the adjuncts, there was a corresponding increase in residual fructose toward the end of fermentation (Table 11.). From 10 g/litre to 20 g/litre there was a decrease from 405 mg/litre to 335

A



B

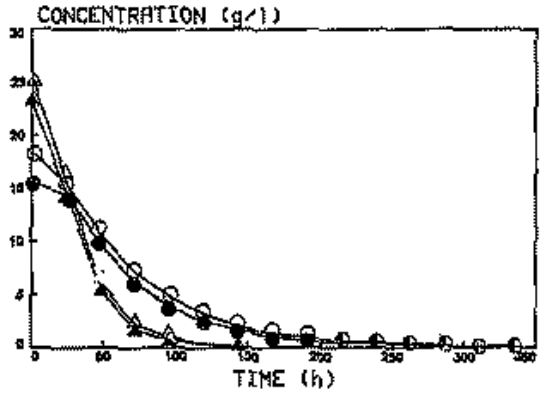
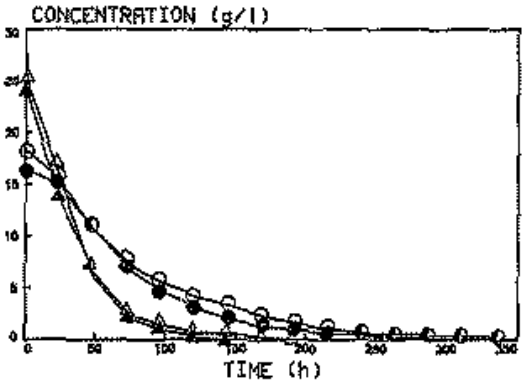


Figure 27. A comparison of the rates of fructose and glucose utilization when using various grades of sucrose *adjuncts*. The glucose △, and fructose ○, components of the laboratory sucrose adjunct ,A, are used as a *reference* (s^2_{pooled} = 0.665) against which all other grades of sucrose are separately compared :- B, *analytical grade sucrose* (s^2_{pooled} = 0.359); C, liquid sucrose (s^2_{pooled} =0.404); D, brewery grade sucrose (s^2_{pooled} = 0.369); E, *acid* hydrolysed sucrose (s^2_{pooled} = 0.359); F, enzyme hydrolysed sucrose (s^2_{pooled} = 0.481) and G, *equal ratios of* glucose and fructose (s^2_{pooled} = 0.459). The constituent moieties for each of the above grades of sucrose are shown by glucose ▲, and fructose ●. Each fermentation was performed in duplicate. Each sample was assayed twice. (See following pages)

C



D

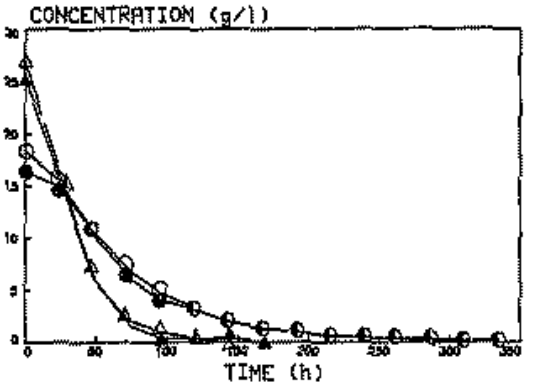
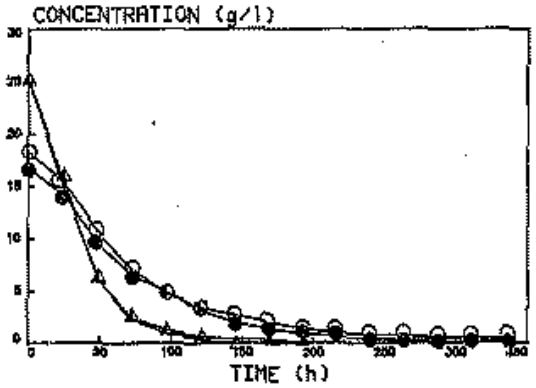
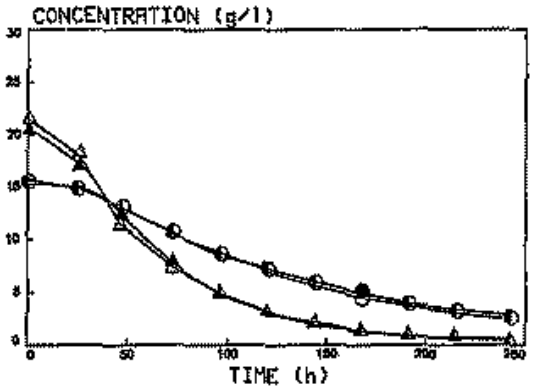


Figure 27. (continued).

E



F



G

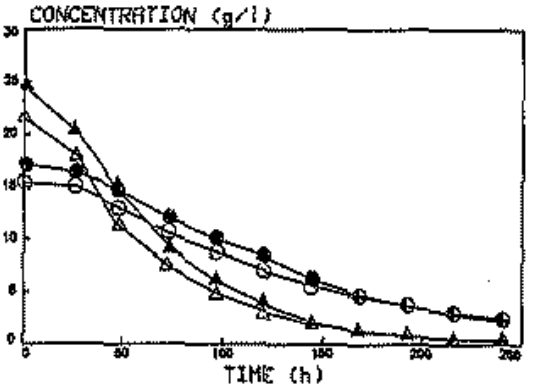


Figure 27. (continued)

GRADE OF SUCROSE USED IN THE FERMENTATION	RESIDUAL FRUCTOSE (mg / litre) 168 HOURS (7 DAYS)	240 HOURS (10 DAYS)	336 HOURS (14 DAYS)
Brewery sucrose	1160	415	100
Invert sucrose	1985	785	335
Liquid sucrose	2280	640	320
Analytical sucrose	975	400	165
Laboratory sucrose	1305	420	80
Autoclaved sucrose	1980	440	180
Laboratory sucrose	4540	2380	fermentations
Enzyme inverted sucrose	4975	2785	terminated
Equal concentrations of fructose and glucose	4795	2135	after 240 hours

Table 7. Residual concentrations of fructose at various times during fermentation, when fermentations were set up using various different grades of sucrose as adjuncts.

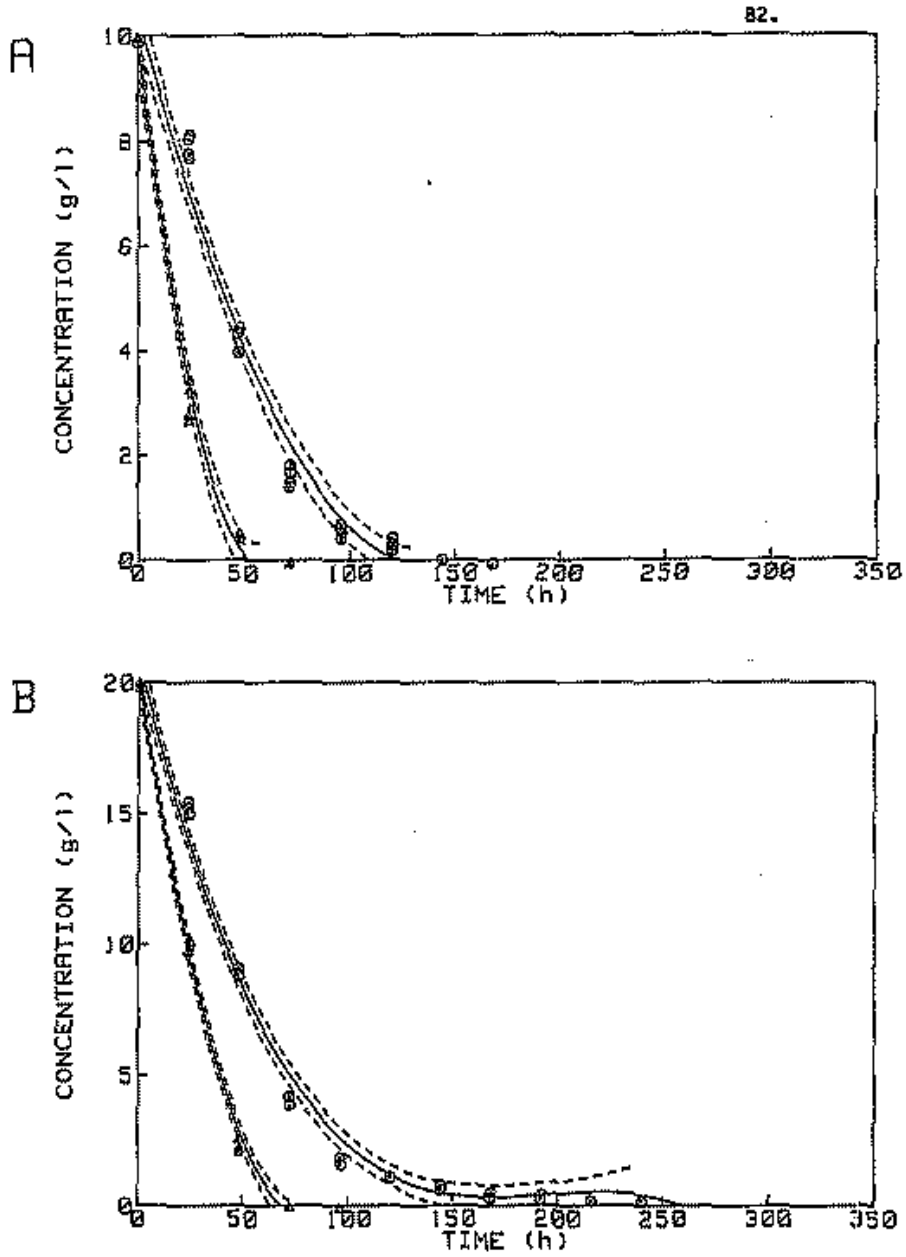
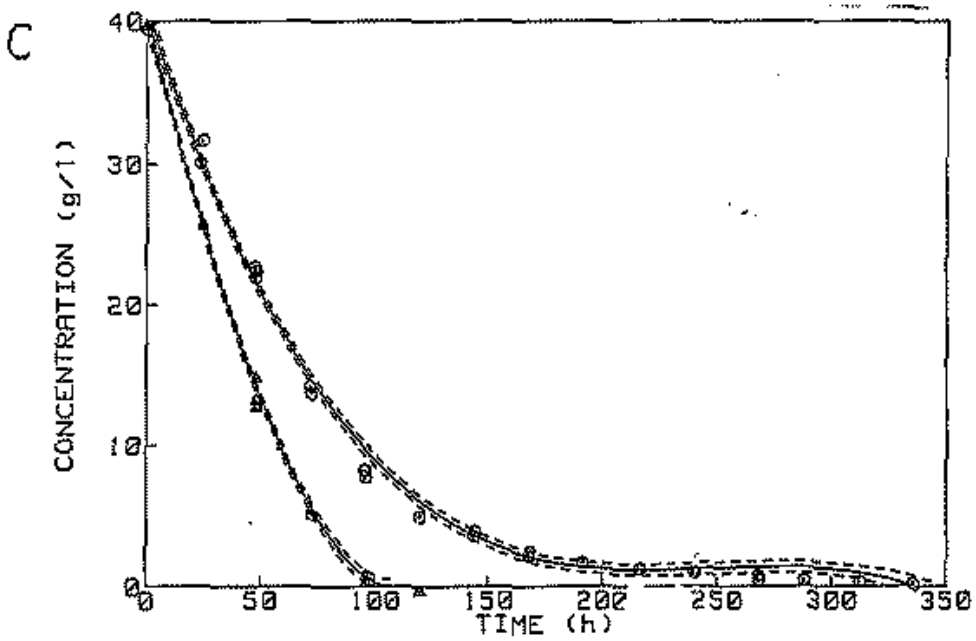


Figure 28. A comparison of the glucose △, and fructose ⊙, utilization rates in wort fermentations using each sugar as a separate adjunct at:- A 10g/litre; B 20g /litre; C 40g/litre; and D 80g/litre. The dashed lines represent 95% confidence limits for each of the curves of fructose and glucose utilization. Each fermentation was performed in duplicate. Each sample was assayed twice.



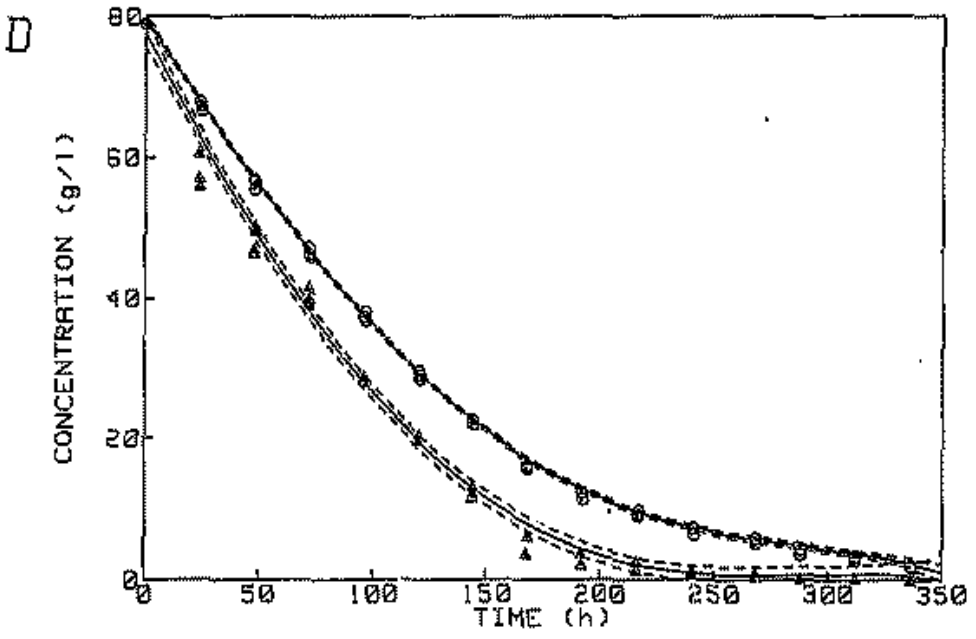
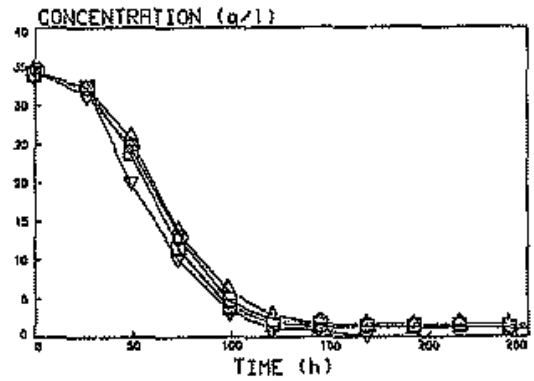


Figure 28. (continued)

A



B

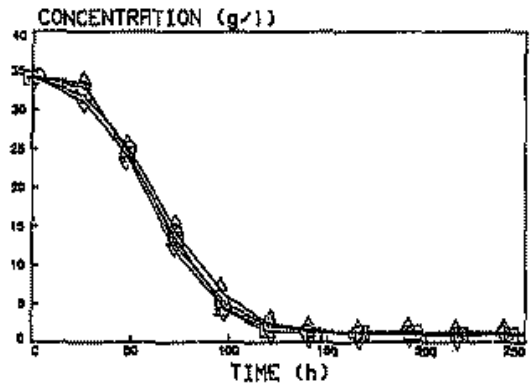
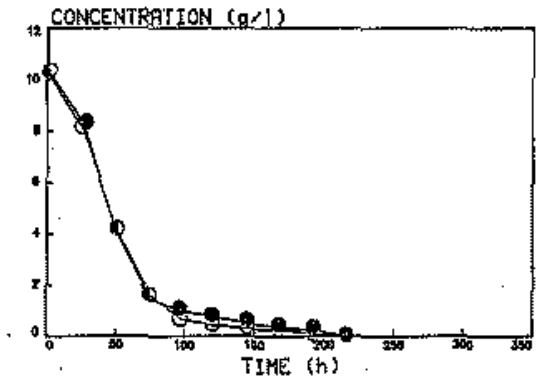


Figure 29. The rate of maltose utilization in wort fermentations using adjuncts of glucose: A (s^2_{pooled} = 0.373); and fructose: B (s^2_{pooled} = 0.246); at:- △ 10 g/litre; ○, 20 g/litre; □ 40 g/litre; ▽ 80 g/litre. Each fermentation was performed in duplicate. Each sample was assayed twice.

A



B

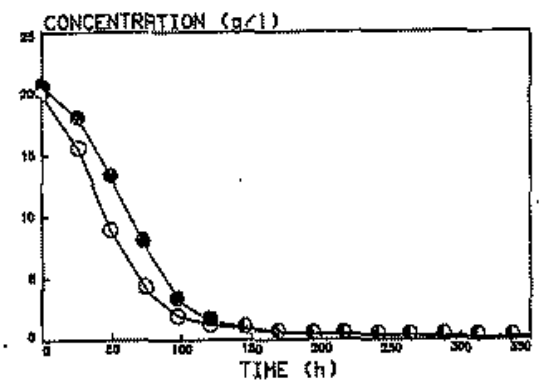
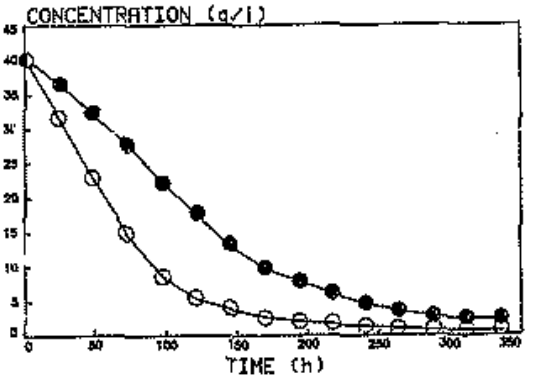


Figure 30. A comparison of the rates of fructose utilization from the fructose adjunct fermentations ○, and the fructose component ●, of the fermentations with equal mixtures of glucose and fructose at adjunct concentrations of A, 10 g/litre (s^2_{pooled} = 0.044); B, 20 g/litre (s^2_{pooled} = 0.220); C, 40 g/litre (s^2_{pooled} = 0.309) and D, 80 g/litre (s^2_{pooled} = 0.498). Each fermentation was performed in duplicate. Each sample was assayed twice, (continued overleaf).

C



D

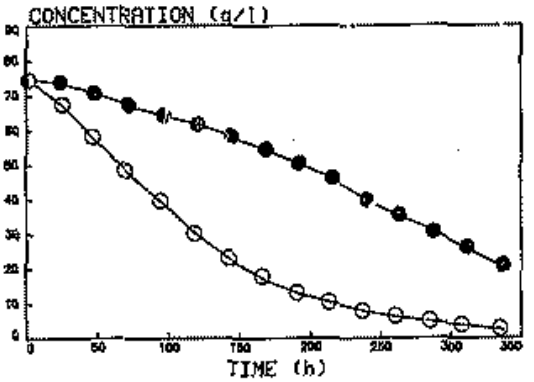


Figure 30. (continued)

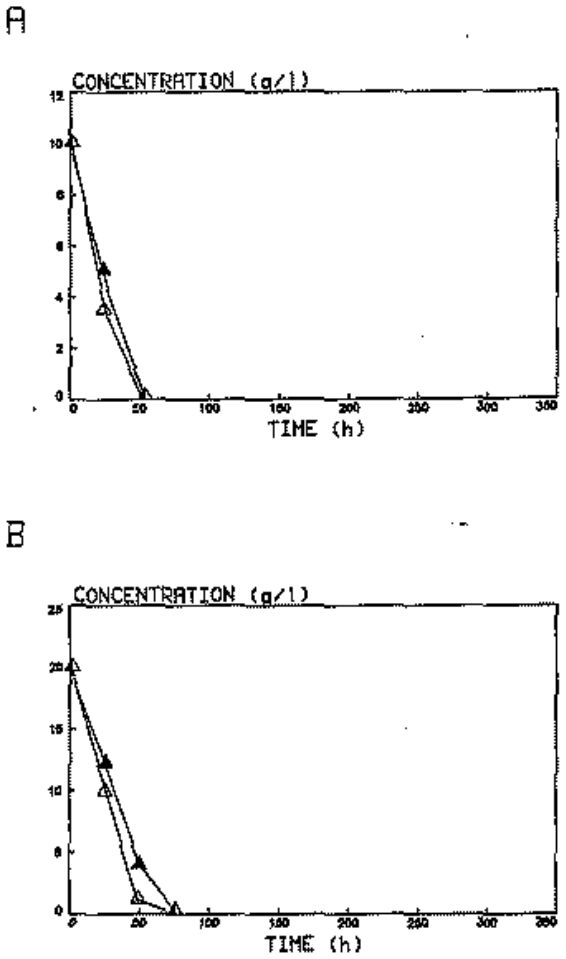
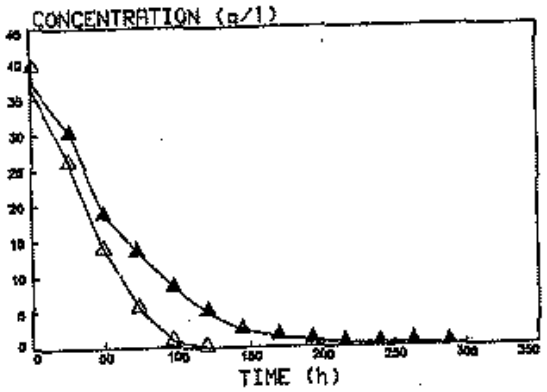


Figure 31. A comparison of the rates of glucose utilization from the glucose adjunct fermentations △, and the glucose component ▲, of the fermentations with equal mixtures of glucose and fructose at adjunct concentrations of A, 10 g/litre (s^2_{pooled} = 0.074); B, 20 g/litre (s^2_{pooled} = 0.188); C, 40 g/litre (s^2_{pooled} = 0.623) and D, 80 g/litre (s^2_{pooled} = 0.853). Each fermentation was performed in duplicate. Each sample was assayed twice, (continued overleaf).

C



D

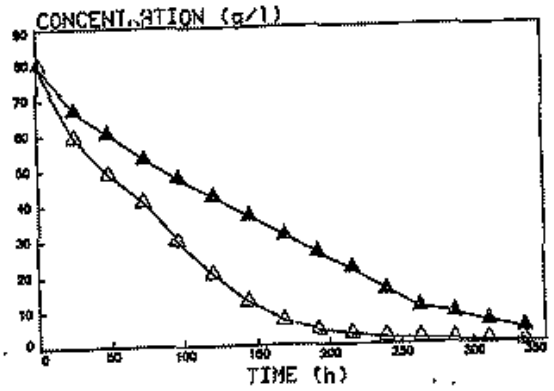


Figure 31. (continued).

ADJUNCT CONCENTRATION (g/litre)	ADJUNCT TYPE: FRUCTOSE slope of linear section of plot (mg/litre fructose utilized/h)	ADJUNCT TYPE: GLUCOSE slope of linear section of plot (mg/litre glucose utilized/h)
10	121	192
20	220	341
40	388	413
80	399	447

Table 8. The rates of glucose and fructose utilization in wort fermentations containing either glucose or fructose as adjuncts.

FRUCTOSE ADJUNCT CONCENTRATION (g/litre)	RESIDUAL FRUCTOSE (mg/litre): 168 HOURS (7 DAYS)	240 HOURS (10 DAYS)	336 HOURS (14 DAYS)
10	250	0	0
20	610	0	0
40	2605	1310	500
80	16280	7480	2335

Table 9. The concentration of residual fructose at various times during fermentation, in fructose adjunct fermentations at several concentrations of fructose.

	EQUAL MIXTURES OF GLUCOSE AND FRUCTOSE	
ADJUNCT CONCENTRATION (g/litre for each sugar)	FRUCTOSE slope of linear section of plot (mg/litre fructose utilized/h)	GLUCOSE slope of linear section of plot (mg/litre glucose utilized/h)
10	115	187
20	208	314
40	200	364
80	119	282

Table 10. The rate of glucose and fructose utilization in fermentations containing equal mixtures of glucose and fructose as adjuncts.

	RESIDUAL FRUCTOSE (mg/litre)		
EQUAL MIXTURES OF GLUCOSE AND FRUCTOSE (g/litre for each sugar)	168 HOURS (7 DAYS)	240 HOURS (10 DAYS)	336 HOURS (14 DAYS)
10	605	405	95
20	705	335	185
40	9550	4465	1730
80	53685	39080	20190

Table 11. Residual fructose concentration at various times during fermentation, in fermentations containing equal mixtures of glucose and fructose as adjuncts at various concentrations.

mg/litre of residual fructose, at 240 hours (10 days). From 40 g/litre to 80 g/litre there was an increase from 4465 mg/litre to 39080 mg/litre of residual fructose (Table 11.). The residual fructose concentrations decreased from 168 hours (7 days) to 336 hours (14 days) for all four adjunct concentrations.

3.3.3. Fermentations Set Up using Defined Growth Promoting Media with Fructose, Glucose, Sucrose, and an Equal Mixture of Glucose and Fructose as Adjuncts.

The fermentations which contained either glucose or fructose as adjuncts, showed that glucose was utilized slightly faster than fructose (Figure 32.). The difference in the utilization rates became apparent after 24 hours and continued until the end of fermentation. The fermentations which contained sucrose, and an equal mixture of glucose and fructose as adjuncts, both exhibited similar trends (Figure. 32A and 32B). In each case glucose was preferentially utilized over fructose, with the rate of glucose utilization being significantly faster than the rate of fructose utilization. This difference between the utilization rates of the two sugars when they were together, was greater than the difference in the utilization rates of glucose and fructose when they were separately fermented.

3.3.4. Fermentations Set Up Using Defined Growth Promoting Media with either Glucose or Fructose at Several Different Concentrations.

At all concentrations of fructose and glucose tested, glucose was utilized faster than fructose. The difference was most marked from 1 g/litre to 10 g/litre, where glucose was utilized significantly faster than fructose. In fermentations containing 20 g/litre and 40

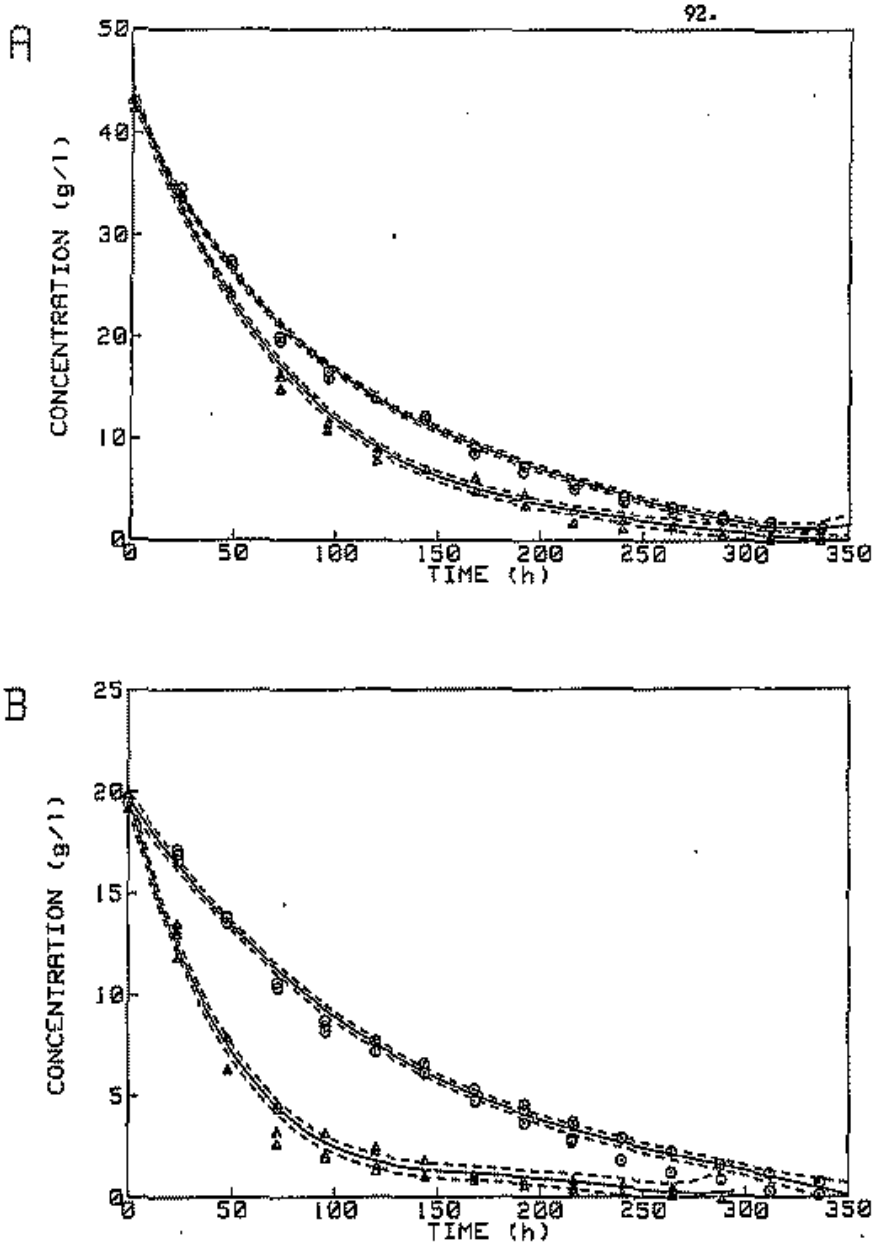


Figure 32. The rates of glucose △ , and fructose ⊙ , utilization in defined medium fermentations using, A, either glucose or fructose, B, an equal mixture of glucose and fructose and C, sucrose. The dashed lines represent 95% confidence limits for each utilization curve. Each fermentation was performed in duplicate. Each sample was assayed twice. (continued overleaf).

C

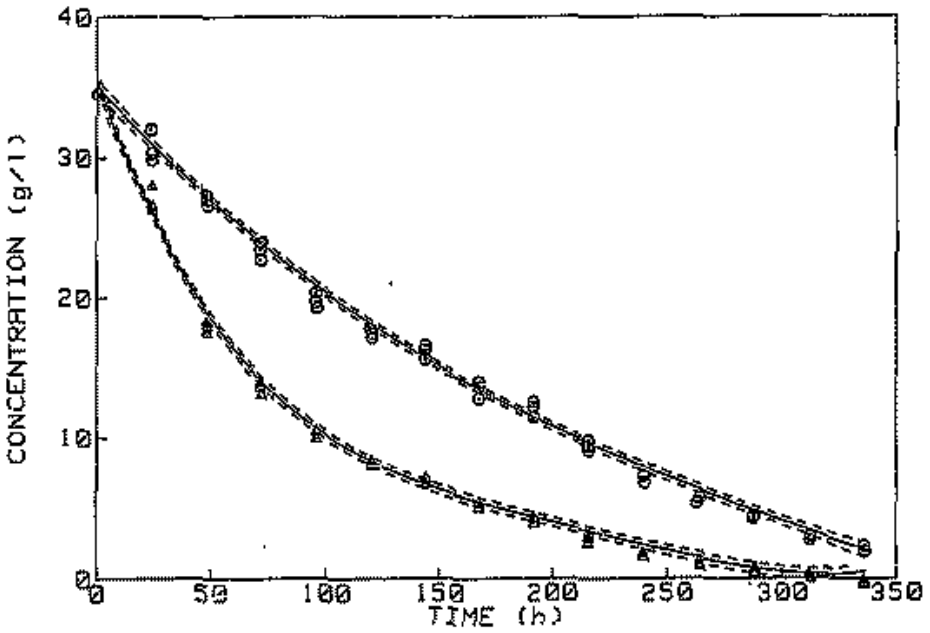


Figure 32. (continued).

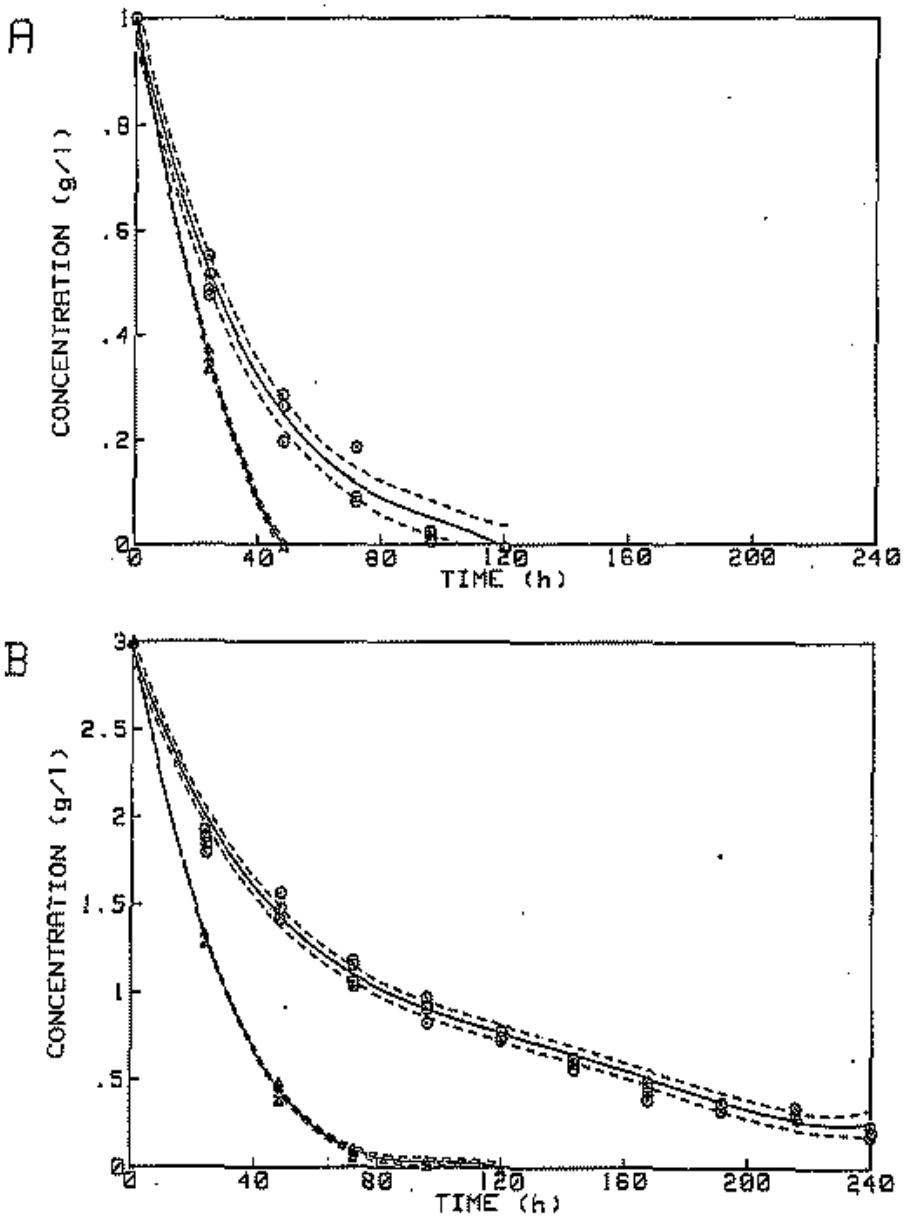
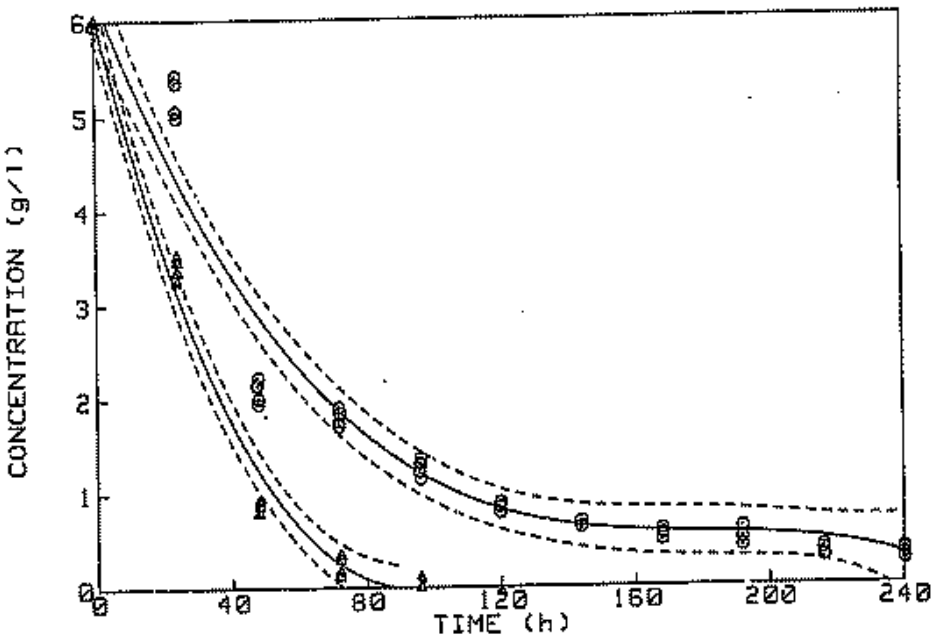


Figure 33. A comparison of the rates of glucose and fructose utilization in defined medium fermentations containing maltose with either glucose △ , or fructose ⊙ , at concentrations of:- A 1.0 g/litre; B 3.0 g/litre; C 6 g/litre; D 10 g/litre; E 20 g/litre; F 40 g/litre; and G 80 g/litre. The dashed lines represent 95% confidence limits for each utilization curve. Each fermentation was performed in duplicate. Each sample was assayed twice, (see following pages).

C



D

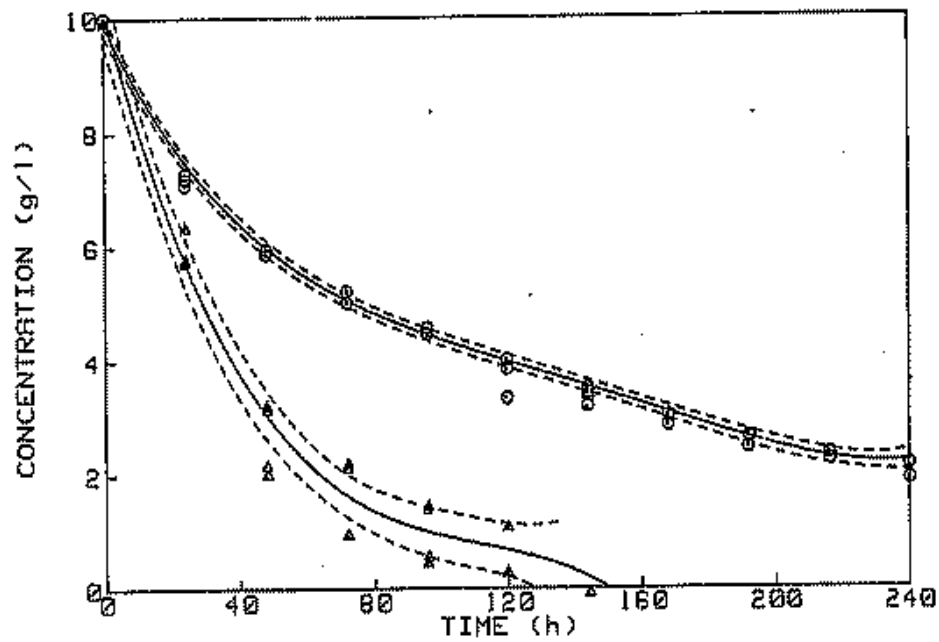


Figure 33. (continued).

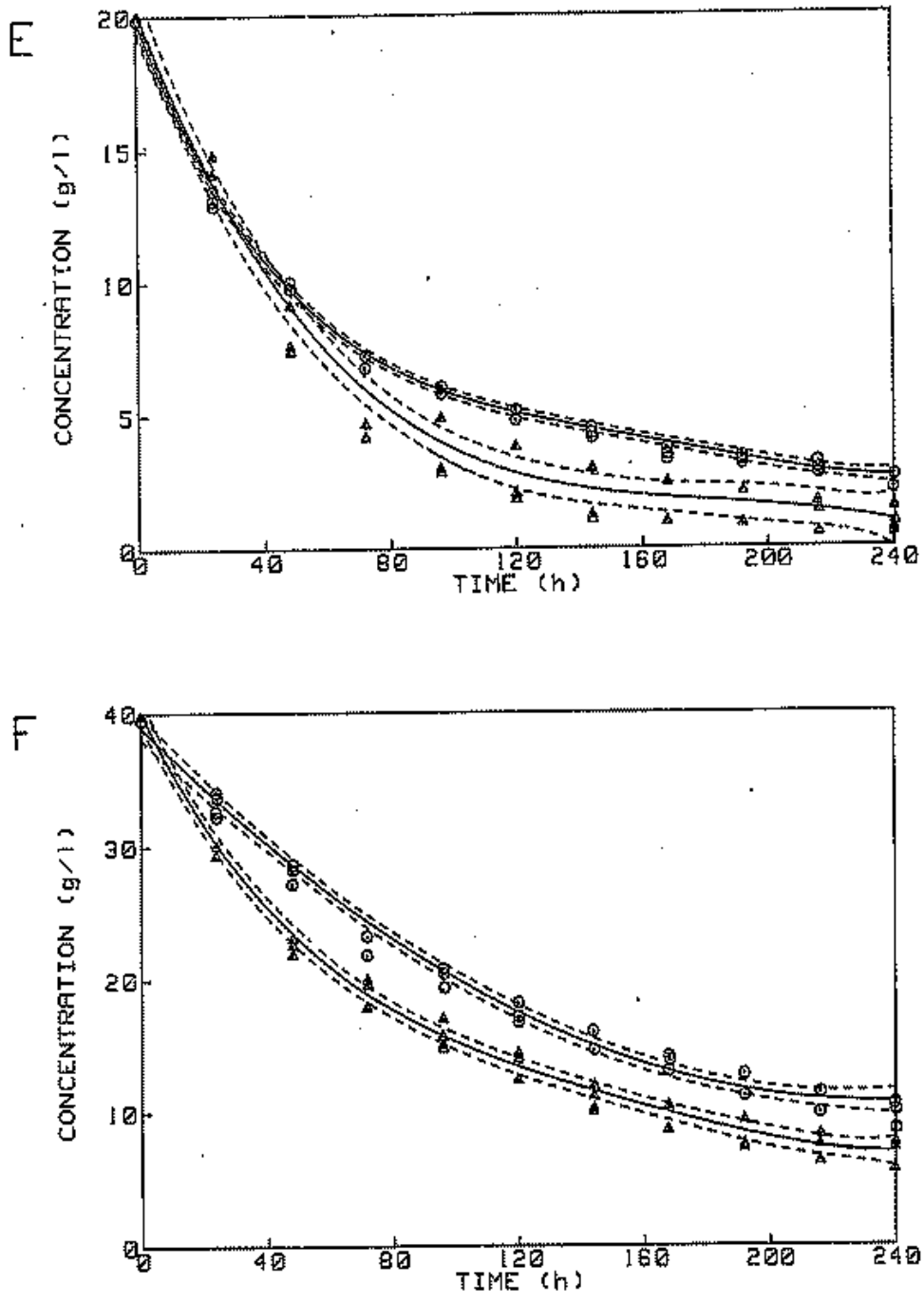


Figure 33. (continued)

G

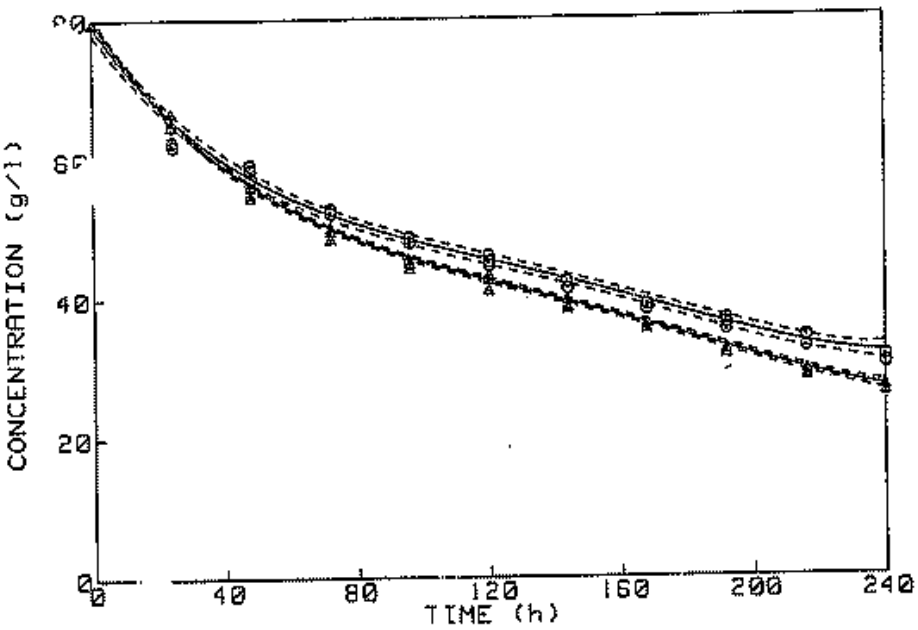


Figure 33. (continued)

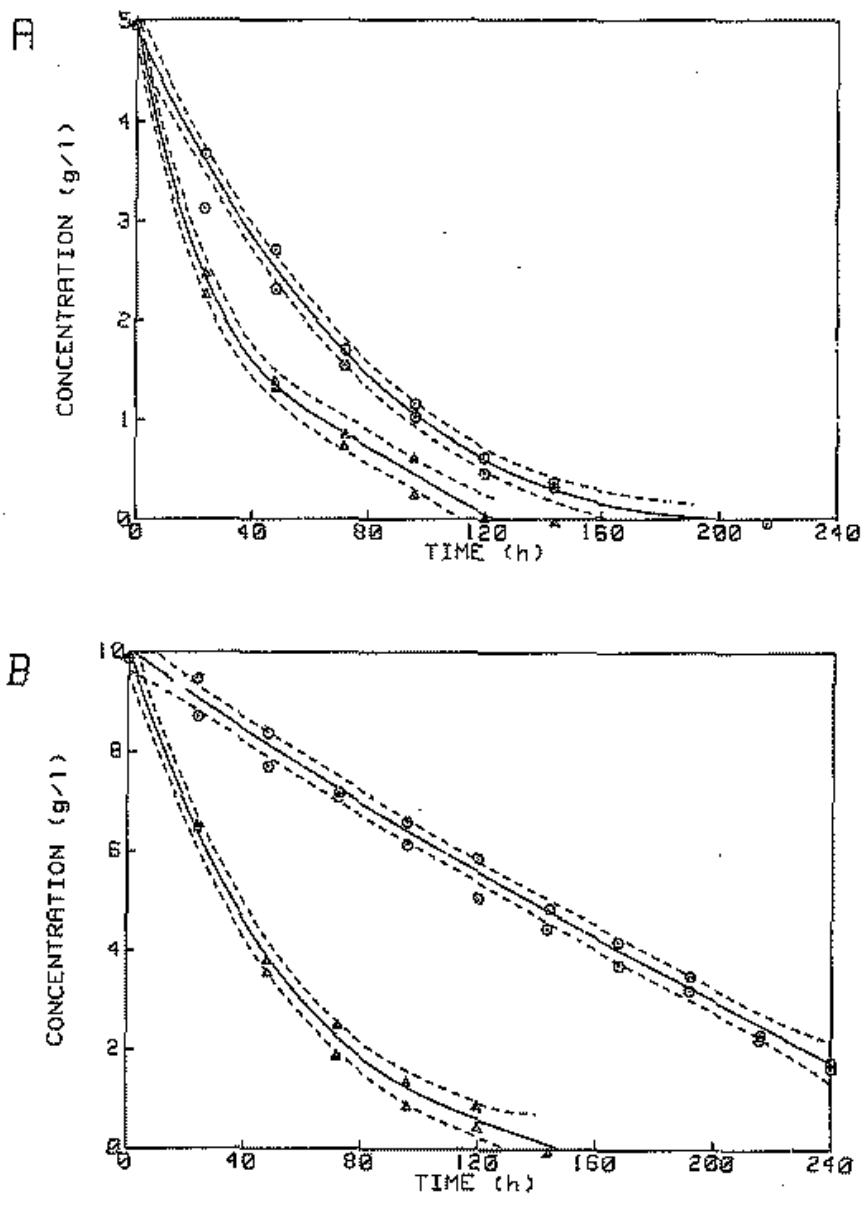
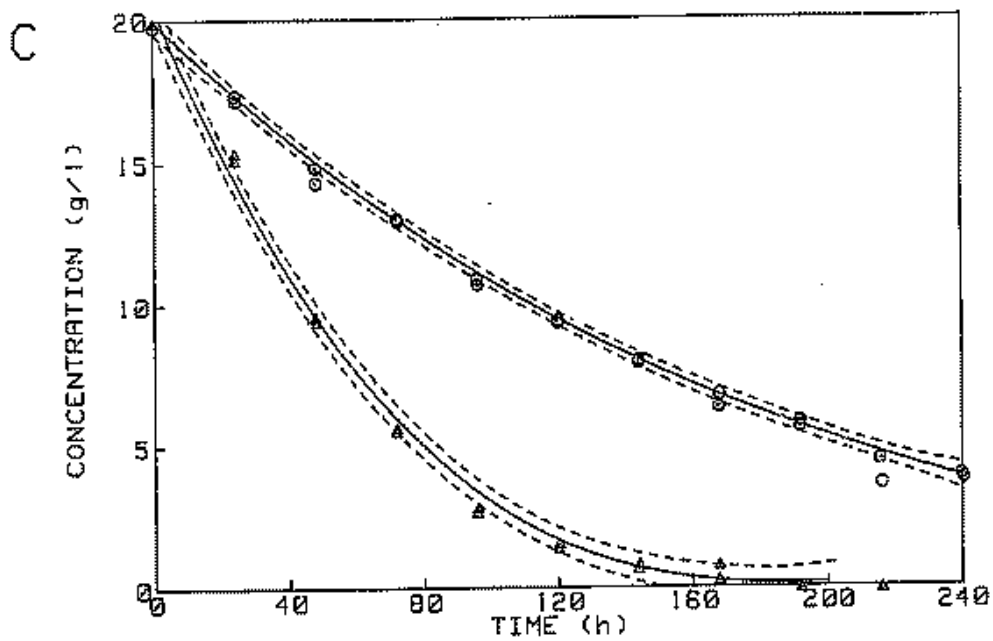


Figure 34. Defined medium fermentations using either glucose △, or fructose ⊙, as the only carbon source at concentrations of:- A 5 g/litre; B 10 g/litre; C 20g/litre; and D 40 g/litre. The dashed lines represent 95% confidence limits for each of the utilization curves. Each fermentation was performed in duplicate.



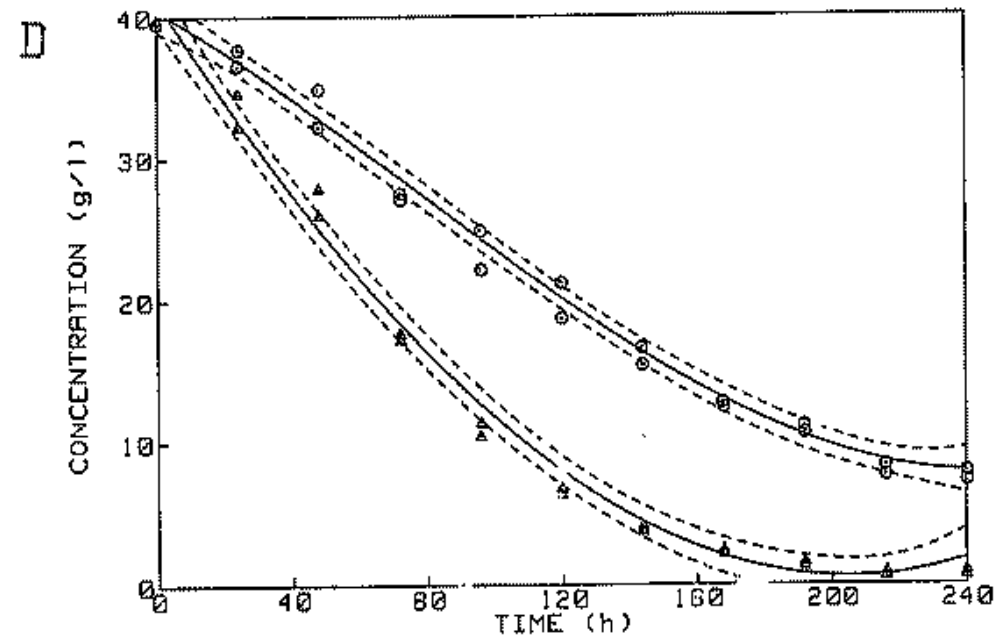
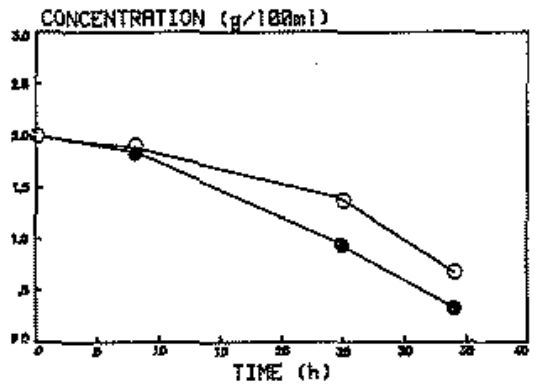


Figure 34. (continued)

A



B

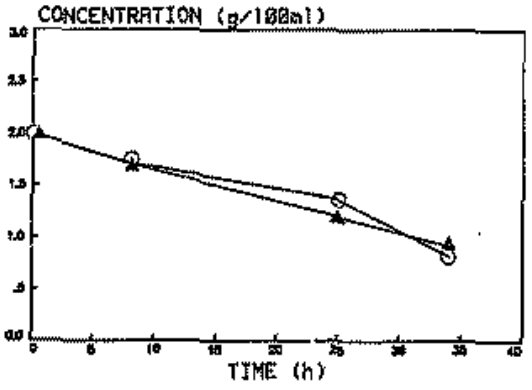


Figure 35. The rates of utilization of ,A, maltose ○ , and glucose ● , and, B, maltose ○ , and fructose ▲ , under aerobic growth conditions in a defined medium.

g/litre of either sugar, the glucose was utilized only slightly faster than fructose (Figure 33). The difference in the rates of utilization of the two sugars decreased as the concentrations were increased to 80 g/litre. At this concentration glucose was utilized only marginally faster than fructose.

3.3.5. Defined Media Fermentations Set Up Using either Glucose or Fructose as the only Carbohydrate Source

In the concentration range tested from 5 g/litre to 40 g/litre for glucose and fructose, glucose is utilized at a faster rate than fructose in all cases (see figure 34.). The difference in utilization rates is smallest at 5 g/litre. From 10 g/litre to 40 g/litre, the difference becomes more marked. At all concentrations, bar the lowest one, residual fructose remains in the medium at the end of fermentation.

3.3.6. The Relationship Between Maltose and Fructose, and Maltose and Glucose Utilization Under Aerobic Growth Conditions.

The growth of S. cerevisiae 2036, in both the maltose/fructose and maltose/glucose defined media, does not exhibit a characteristic diauxic growth curve. Plots of sugar utilization for the media, (see figure 35.) show that maltose is utilized simultaneously with glucose and fructose from the time of inoculation. Maltose and fructose are utilized at approximately equal rates, while glucose is utilized faster than maltose. Comparison of the rates of glucose and fructose utilization reveal that glucose is utilized faster than fructose.

3.4. Discussion

The laboratory fermentation system produced residual

fructose concentrations above the fructose taste *threshold for beer*, when sucrose adjunct wort fermentations were performed. These fermentations were reproducible, (Figure 27 and Table 7.).

There were no marked differences amongst the fermentations set up with various *grades of sucrose* adjuncts. If differences were present in the *fermentations* then they would have had to be attributed to the grades of sucrose used, as this factor was the only variable present. The results from these fermentations indicated that the impurities present in the various grades of sucrose used, do not markedly affect *glucose and fructose* utilization in sucrose adjunct wort fermentations. The impurities do not "block" fructose uptake, or contribute an inhibitory effect such that fructose utilization is greatly reduced.

Defined media fermentations containing maltose, with *either glucose or fructose* at several concentrations revealed that glucose was utilized faster than fructose at all concentrations tested (Figure 33.) (149). The defined media fermentations using only glucose or fructose as carbohydrate sources, also showed similar trends (Figure 34.) (32) *and in the wort fermentations* with either glucose or fructose as adjuncts, glucose was utilized faster than fructose at all concentrations tested. With increasing fructose concentrations, there was an increase in the rate of fructose utilization, but it was not sufficient to maintain or reduce the residual fructose concentrations at the end of fermentation at a *constant level and consequently* with each increase in the fructose concentration there was a corresponding increase in the residual fructose concentrations at the end of fermentation (Figure 28. and Table 9.). This phenomenon was particularly evident at initial fructose

concentrations of 40 g/litre and 80 g/litre. Hence much lower adjunct concentrations of fructose compared to glucose would have to be used if these two sugars were required to be completely fermented. Based on the fermentation parameters of a temperature of 11°C, and pitching rate of 6 g/litre of yeast, the use of fructose adjuncts at 60 g/litre and above (32) would produce problems of residual fructose concentrations above its taste threshold. However the use of glucose adjunct concentrations of up to 80 g/litre would not present problems of residual sugar.

In the fermentations using equal mixtures of glucose and fructose as adjuncts, the rate of glucose utilization increased from 10 g/litre to 40 g/litre and decreased from 40 g/litre to 80 g/litre. Fructose utilization increased from 10 g/litre to 20 g/litre, appeared approximately constant from 20 g/litre to 40 g/litre, and dropped significantly from 40 g/litre to 80 g/litre It would appear that this slower increase in the rate of fructose utilization, compared to the increase in the rate of glucose utilization (compare Figure. 30. and 31.), could be due to glucose competitively inhibiting the uptake of fructose, particularly at higher sugar concentrations where it appears that the rate-limiting factor is the total number of membrane transport carriers present. One would expect the competitive inhibition to be much lower at lower sugar concentrations, as there are more available membrane transport carriers for the sugars, if a constant inoculum of yeast is used for each adjunct concentration. A consequence of the slower utilization rates of fructose, when glucose is present, is that the fructose concentrations at the end of fermentation are higher than in the fructose adjunct fermentations (compare Table 9. and 11.) and this is particularly evident at the higher adjunct concentrations. For 40

g/litre and 80 g/litre of both glucose and fructose, there was 1730 mg/litre and 20190 mg/litre respectively, of fructose at the end of fermentation. These results are quite relevant to the brewery particularly as the trend is toward the use of higher gravity worts, so that more beer can be brewed in existing plant. The indication is that at 80 g/litre glucose and fructose, which is equivalent to a wort of 19.78ºP - a poor fermentation would result using the present temperature (11ºC) and pitching rate of 6 g/litre of yeast for high gravity wort.

Under aerobic growth conditions S. cerevisiae 2036 ...es maltose simultaneously with either glucose or ...e which shows that the strain is de-repressed. It ...not exhibit typical diauxic growth patterns with either combination of sugars. Thus glucose or fructose does not appear to exert catabolite repression maltose uptake or its subsequent utilization. Maltose utilization plots for the fermentations containing either fructose or glucose adjuncts ranging from 10 g/litre to 80 g/litre reveal that maltose utilization is virtually identical in all fermentations. If glucose and fructose are able to exert catabolite repression on the maltose membrane transport system or any of the subsequent steps of maltose catabolism to glucose, then maltose would only begin to be utilized when glucose or fructose levels fall to a cut-off point below which catabolite repression does not occur (32). A concentration of 0.4% (w/v) has been reported for glucose (64). If catabolite repression did occur in S. cerevisiae 2036, then the lag period before maltose utilization begins would become longer as the adjunct concentration is increased. However, results obtained do not exhibit this trend as the maltose utilization patterns are approximately equal irrespective of the adjunct concentration.

CHAPTER 4.

The Development of Methods to Overcome the Fructose Block

4.1. Introduction

Having established that the fructose block could be obtained in the laboratory fermentation system, various procedures to ameliorate this effect were investigated. Panchal and Stewart pointed out (137) that the relative rates of utilization of glucose and fructose are dependant on the physiological condition of the yeast. Increasing concentrations of sorbitol, caused an increase in the osmotic pressure of the growth media, and this resulted in a decrease of the preferential utilization of glucose over fructose. He also reported, the preferential utilization of glucose over fructose decreased in a minimal media, compared to the preferential utilization of glucose over fructose in a growth promoting media.

Conditioning of S. cerevisiae by subculturing the yeast on either glucose or fructose as the sole carbon source, may cause one or both sugars to be utilized at a faster rate in a subsequent wort fermentation. Stewart et al (167) observed the conditioning effect varied slightly from strain to strain. In this set of experiments, S. cerevisiae 2036 was grown up from a slope culture, through several steps, until a 3.5 litre stage with either glucose or fructose as the sole carbon source. Then both types of propagated yeast were pitched into two separate wort fermentations, each with a fructose adjunct and allowed to ferment over a 240 hour (10 day) period. The aim was to see if conditioning had any effect on reducing the residual fructose concentrations at the end of fermentation. In the brewery, yeast is never repitched through a series of successive

fermentations containing relatively high concentrations of fructose, because it is thought that the residual concentrations of fructose may increase with each subsequent fermentation. The normal practice is either to discard the yeast once it has been used in a fermentation with a sucrose adjunct wort, or repitch the yeast into a fermentation with another non-sucrose adjunct *e.g.* a glucose adjunct. In this study, a fermentation containing wort with 40g/litre fructose adjunct was established and the yeast used was repitched through eight successive generations, each time using the same type of wort as above. The trends in the rates of fructose utilization were then observed over the course of the eight fermentations.

Of all the main parameters in wort fermentations, the one which is likely to exhibit most variability is the pitching rate. The measurement of specific gravity (ºP) by saccharometers in breweries is remarkably accurate. Cross-checks with HPLC analysis revealed fluctuations of only 200-300 mg/litre per 0.1 ºP. Dissolved oxygen concentrations in the wort are kept within close tolerances. The free amino nitrogen content of the malt remains within the narrow range of 160 - 200 mg/litre, using the the ninhydrin method (2). A preliminary examination of data from full scale fermenters indicated that up to 8 fold variations in the pitching rate occur. Laboratory fermentations were performed with various pitching rates in order to determine their relationship to residual fructose concentrations.

In order to bring about increased attenuation of fermentation and therefore greater fructose utilization in fermentations, the addition of various growth supplements to the wort was investigated. Various substances have been shown to stimulate yeast growth in fermentation (9,83,99). The growth supplements

investigated were nitrogen sources in the form of amino acids e.g. serine; salts, e.g. ammonium sulphate; and yeast nitrogen base, which were added to fermentations as well as the general growth stimulants yeast extract and nutromix, (Chemserve, Johannesburg, R.S.A.) a commercial preparation of vitamins, trace elements and nitrogen sources. Also short bursts of oxygen were added to deoxygenated wort during the initial stages of fermentation, and near oxygen saturated wort was used at pitching.

4.2. Methods

4.2.1. Propagation of Yeast in either Glucose or Fructose in order to Determine the Subsequent Fermentation Patterns

A batch of 5.5 litres of defined growth promoting media was prepared according to the formula:

Yeast Nitrogen Base	6.7 g
Citric Acid	3.3 g
Sodium Citrate	5.2 g
Fructose	40 g
Distilled water to	1 litre
Adjust pH to	5.0

The media was oxygenated to 14 mg/litre of oxygen using medical oxygen, and 10 ml of the media was inoculated with S. cerevisiae 2036 and incubated overnight at 20°C and then centrifuged at 3000 rpm for 10 minutes. The pelleted yeast was resuspended in 5ml of water and transferred into 200 ml of oxygenated media, incubated overnight at 20°C, and again centrifuged, as before. The yeast pellet was resuspended in 20 ml of water. The yeast was then propagated in 1.5 litres and subsequently 3.5 litres of media. Each stage was incubated overnight at 20°C, with continuous oxygenation. Finally, the media

was centrifuged, and the yeast was collected as before, and six grams were weighed out. The procedure above was used to produce 6 g of glucose-propagated yeast, using a growth promoting media, which had the 40g of fructose, substituted with 40 g of glucose.

Concurrently with the above procedure, 2.5 litres of Black label wort with a 40g/litre fructose adjunct was prepared using the technique described in 3.2.1. The fructose-propagated and glucose-propagated yeast were separately pitched into the above wort and two sets of duplicate fermentations were set up. The fermentations were monitored as before for a fermentation period of 336 hours (14 days). Each fermentation sample was analysed twice for fructose concentration.

4.2.2. Repitching of the Same Yeast into Successive Fermentations containing Fructose Adjuncts

A batch of 2.0 litres of Black Label wort with a 40 g/litre fructose adjunct was prepared, and four separate identical fermentations were set up using a pitching rate of 6g/litre of yeast and the techniques described in 3.2.1. The fermentations were monitored for fructose concentration over a period of 240 hours (10 days) as described in 2.2.1. Each fermentation sample was analysed twice for fructose concentration. At the end of the fermentation period, the yeast from each fermentation vessel was collected and stored in 20 ml of the fermented wort for 24 hours, at 4°C. A batch of wort of the same type used in the first set of fermentations was prepared, and another set of four fermentations was set up, using the same pitching rate, and monitoring procedure as above. At the end of the fermentation period of 240 hours (10 days) the yeast from the fermentations was collected, and stored as before, and then repitched into another batch of the same Black

Label wort as above, and so setting up another set of four fermentations. This technique was repeated over a period of eight successive fermentations.

4.2.3. Fermentations Set Up Using Various Pitching Rates

A normal gravity wort containing a sucrose adjunct in the ratio of 65% malt wort: 35% sucrose was prepared using the technique described in 3.2.1. The wort was oxygenated to 13.8 mg/litre of dissolved oxygen. The fermentations were performed in duplicate pitched at varying pitching rates of i) 1.5 g/litre, ii) 3.0 g/litre, iii) 6.0 g/litre, iv) 12.0 g/litre, and 24.0 g/litre of yeast and were monitored every 24 hours for a period of 240 hours for glucose and fructose concentration using the methods stated in 2.2.1.

4.2.4 High Gravity Fermentations Set Up With the Addition of *Various Supplements*

A high gravity wort containing an adjunct of equal ratios of glucose and fructose, in the ratio of 60% malt wort: 40% adjunct was prepared using the technique described in 3.2.1. The wort *was oxygenated to* 14 mg/litre of dissolved oxygen. The supplements added to *the fermentations* were i) yeast nitrogen base 2.5 g/litre, ii) yeast extract 2.5 g/litre, iii) serine (Merck, Darmstadt, G.F.R.) 200 mg/litre, iv) magnesium stearate (AR, Riedel-de Haen, Seelze, G.F.R.) 2 mg/litre, v) ammonium sulphate (Saarchem, R.S.A.) 160 mg/litre, *vi) zinc sulphate* (Saarchem, R.S.A.) 160 mg/litre vii) Nutromix at 2.5 g/litre and viii) 8 mg/litre, a commercially available growth supplement which, according to independant laboratory analysis, is composed of the following components:-

Total soluble nitrogens	9.4%
Inositol	260 mg/litre

Biotin	0.73 mg/litre
Choline	2.25 g/litre
Niacin	60 mg/litre
Pantothenate	15 mg/litre
Riboflavin	4 mg/litre
Thiamin	10 mg/litre
Zinc	7.0 g/litre
Sodium	2.45 g/litre
Potassium	5.8 g/litre
Manganese	1.35 g/litre
Lead	5 mg/litre
Iron	110 mg/litre
Copper	10 mg/litre
Calcium	2.0 g/litre
Arsenic	1 mg/litre

and ix) the addition of dissolved oxygen to the wort to reach concentations approaching oxygen saturation. The fermentations were pitched at 6 g/litre of yeast monitored every 24 hours for a period of 240 hours for fructose concentration using the methods stated in 2.2.1. An additional fermentation was set up with the high gravity adjunct wort as described above using the same pitching rate. The wort was not oxygenated before pitching. Once the fermentation had commenced medical oxygen was bubbled through the fermentation for a three hour period at a rate of 25,8 ccpm for each of the first three days of fermentation. The length of fermentation was 240 hours and was monitored as described in 2.2.1. All of the fermentations were performed in duplicate.

4.3. Results

4.3.1. Propagation of Yeast in either Glucose or Fructose in order to determine the Subsequent Fermentation Patterns.

The fructose-propagated yeast appeared to utilize

fructose at a marginally slower rate than the glucose-propagated yeast (figure 36A). The difference between the two fermentations showed itself from the start of fermentation and continued right up to the end of fermentation at 336 hours (14 days). The fructose concentrations at various times during the fermentation are listed in Table 12.

TYPE OF FERMENTATION	168 HOURS (7 DAYS)	240 HOURS (10 DAYS)	336 HOURS (14 DAYS)
GLUCOSE PROPAGATED YEAST	6865	3202	1245
FRUCTOSE PROPAGATED YEAST	9065	4665	1495

Table 12. Fructose concentrations (mg/litre) at various times during the fermentations

The fructose concentrations at 240 hours (10 days) in the fermentations with both the fructose-propagated and glucose-propagated yeast constituted serious fructose blocks. After an additional 96 hours (4 days) of fermentation for each yeast, the fructose concentrations in the two fermentations decreased to 1245 mg/litre and 1495 mg/litre respectively. These concentrations were about the taste threshold for fructose in beer.

4.3.2. Repitching of the Same Yeast into Successive Fermentations containing Fructose Adjuncts

When the same yeast was repitched in eight successive fermentations, the rate of fructose utilization decreased progressively with each fermentation (figure

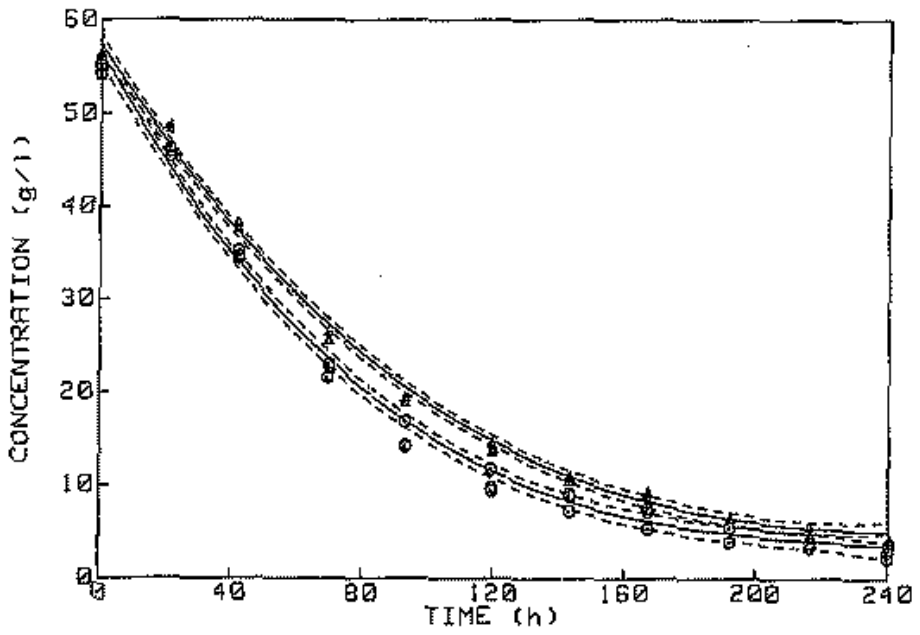


Figure 36. Fructose utilization in wort fermentations using fructose adjuncts pitched with either:- △ glucose propagated yeast; or ⊙ fructose propagated yeast. The dashed lines represent 95% confidence limits. Each fermentation was performed in duplicate. Each sample was assayed twice.

37.). The plots of the rates of fructose utilization for the eight successive fermentations show the first fermentation to have the fastest rate of fructose utilization. With each successive fermentation the rate of fructose utilization decreased. The only exception is the fourth generation fermentation which showed a slower rate of fructose utilization than all the other fermentations. A consequence of this decrease in the rate of fructose utilization with successive fermentations was that the residual concentrations of fructose which remained in the beer at the end of fermentation increased with each successive fermentation (figure 38.).

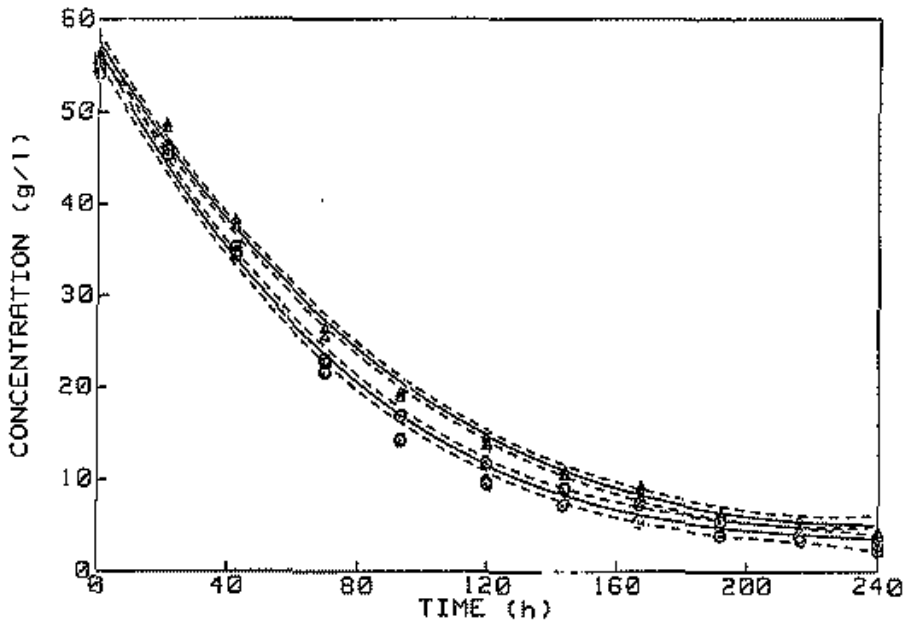


Figure 36. Fructose utilization in wort fermentations using fructose adjuncts pitched with either:- △ glucose propagated yeast; or ⊙ fructose propagated yeast. The dashed lines represent 95% confidence limits. Each fermentation was performed in duplicate. Each sample was assayed twice.

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4.3.3. Fermentations Set Up using Various Pitching Rates

There is a direct relationship between the pitching rate and the rate of fructose utilization. As the pitching rate is increased, so there is a corresponding increase in the rate of fructose utilization during fermentation (figure 39.). Consequently the residual fructose concentrations at the end of fermentation decrease with increasing pitching rates. Plots reveal that the pitching rate is inversely proportional to the residual fructose concentrations for the last 3 days of fermentation (figure 41.). Samples taken at 240 hours contained 2.7 g/litre of residual fructose when the fermentation was pitched using 1.5 g/litre of yeast. A five fold decrease of residual fructose was found in the fermentation pitched with 24 g/litre of yeast, which contained 0.54 g/litre of fructose. When the normal pitching rate of 6 g/litre of yeast was doubled or halved it resulted in the residual fructose concentration either decreasing by 1.35 fold or increasing by 1.30 fold respectively.

4.3.4. High Gravity Fermentations Set up with the Addition of Various Supplements

The treatment of all fermentations, bar the addition of zinc sulphate, by the addition of various supplements, had a positive effect causing the concentrations of residual fructose at the end of fermentation to be reduced when compared to an untreated control. Treatments containing nitrogen sources of various types i.e. serine, a readily utilizable amino acid; ammonium sulphate, an inorganic nitrogen salt, and yeast nitrogen base all exhibited modest decreases in residual fructose concentrations with serine causing the largest decrease of the three treatments (figure 42.). The addition of general growth stimulants i.e. nutromix and yeast

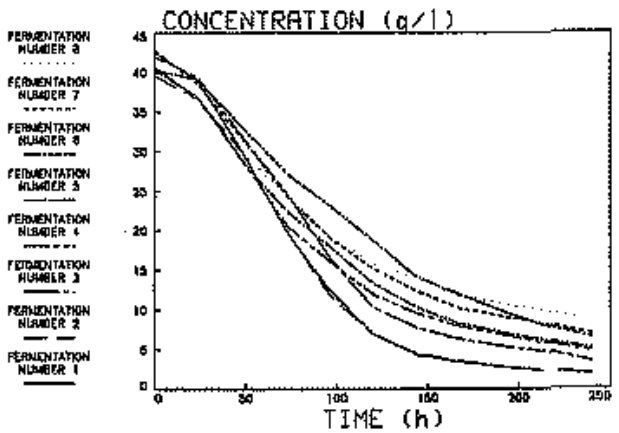


Figure 37. The utilization rate of fructose for eight successive fermentations with each fermentation pitched using the same yeast, (s^2_{pooled} = 0.809). Each fermentation was performed in quadruplicate. Each sample was assayed twice.

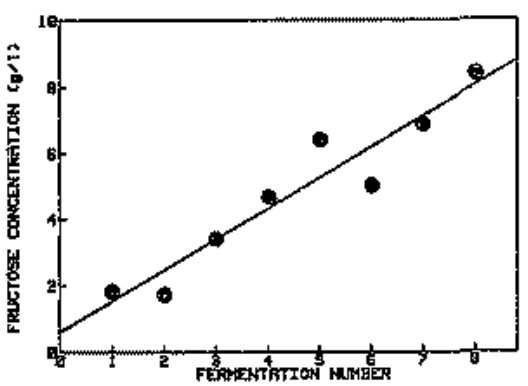


Figure 38. The residual fructose concentration at the end of eight successive fermentations, with each fermentation pitched using the same yeast, (s^2_{pooled} = 0.352).

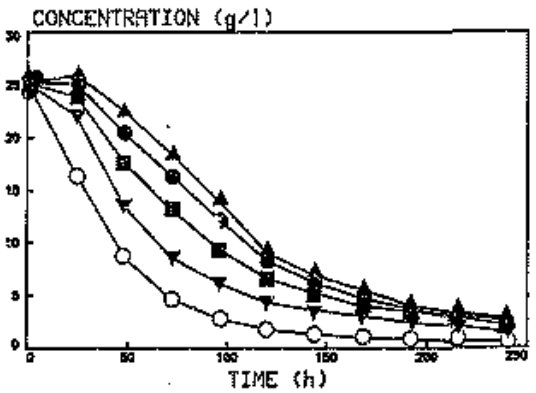


Figure 39. The rate of fructose utilization during fermentation when using pitching rates of ▲ 1.5 g/litre; ● 3.0 g/litre; ■ 6.0 g/litre; ▼ 12.0 g/litre; and ○ 24 g/litre of yeast, (s^2_{pooled} = 0.321). Each fermentation was performed in duplicate.

extract, which contains a general array of vitamins, trace elements, minerals, and nitrogen sources, showed larger decreases than the nitrogen sources used above. Nutromix at the higher concentration of 2.5 g/litre showed the largest decrease within this group, with an approximate 2.75 fold decrease when compared to the control. Nutromix was used at concentrations of 8 mg/litre and 2.5 g/litre, and although there is a several hundred fold differencein the concentrations added to the fermentations the differences in their effect were marginal with the larger and smaller concentrations reducing fructose concentrations to 1.710 g/litre and 1.947 g/litre respectively (figure 42.). Further treatments involved the addition of oxygen to the wort in near saturating concentrations prior to pitching. Also oxygen was supplied by slowly bubbling it through the deoxygenated wort for 3 hour periods during the first three days of fermentation. Oxygen is a

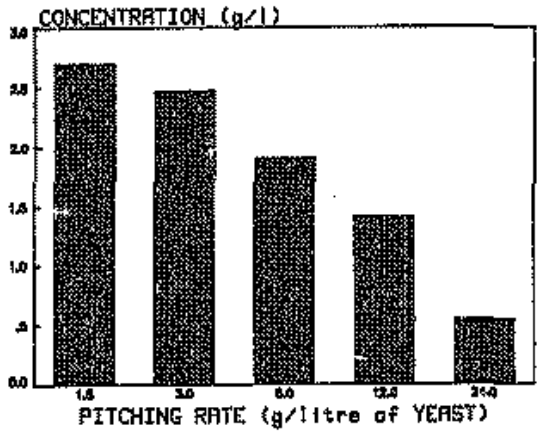


Figure 40. Residual fructose concentrations in wort at 240 hours when the pitching rates used ranged from 1.5 to 24.0 g/litre of yeast. Each fermentation was performed in duplicate.

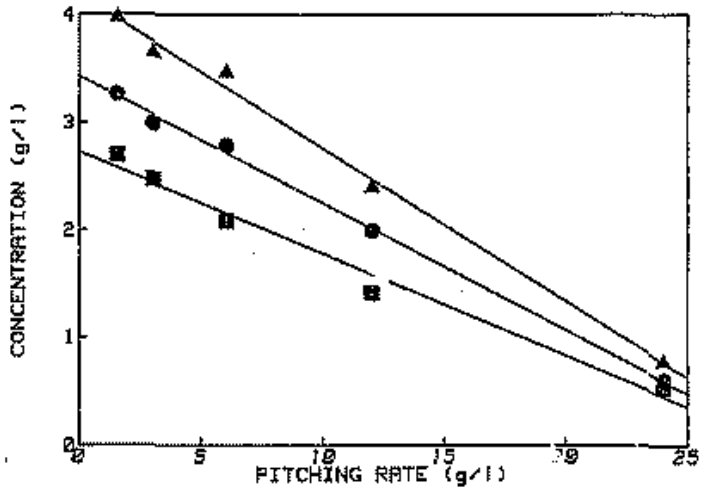


Figure 37. The relationship betw[illegible] rate and residual fructose concentration in[illegible] at ▲ 192 h; ● 216 h; and ■ 240 h, (s^2_{pooled} = 0.103). Each fermentation was performed in duplicate.

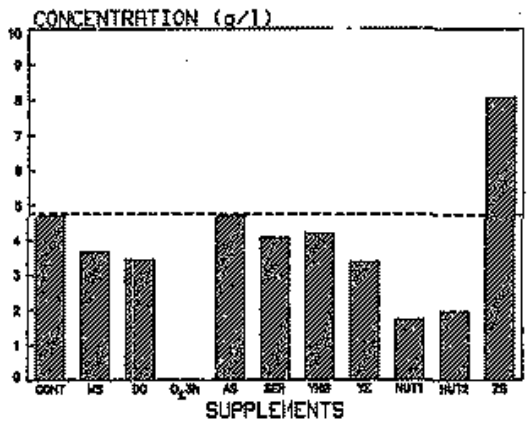


Figure 42. The residual fructose concentrations at the end of fermentation after addition of various supplements to the wort at the time of pitching :- CONT = control; MS = magnesium stearate 2 mg/litre; DO = dissolved oxygen at concentrations near saturation concentration; O_2 3h = oxygenation of the wort for 3 hour periods for each of the first three days of fermentation; AS = ammonium sulphate 200 mg/litre; YNB = yeast nitrogen base 2.5 g/litre. YE = yeast extract 2.5 g/litre; NUT 1 = nutromix 2.5 g/litre; NUT 2 = nutromix 8 mg/litre; ZS = zinc sulphate 160 mg/litre. Each fermentation was performed in duplicate.

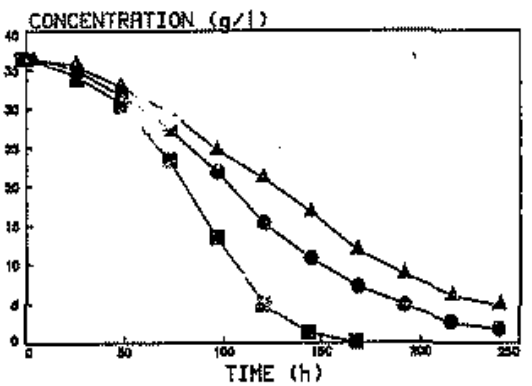


Figure 43. Fructose utilization in fermentations with additions of ■ oxygen bubbled through the wort for 3 hours for each of the first 3 days of fermentation, ● 2.5 g/litre nutromix and, ▲ untreated control, (s^2_{pooled} = 0.294). Each fermentation was performed in duplicate.

necessary precursor in the production of sterols, primarily ergosterol, and therefore the addition of oxygen concentrations to the wort probably causes increased levels of ergosterol to be present. Both oxygen treatments produced reduced fructose concentrations. The oxygen added prior to pitching caused a modest decrease of approximately 1.4 fold in the residual fructose concentration (figure 42.), whilst the oxygen added to the initial stages of fermentation showed the most dramatic effect of all the treatments with no fructose remaining in the fermentation (figure 42.). In fact the plot of fructose utilization for this particular treatment (figure 42.), reveals that all the fructose in the wort was utilised by 168 hours. The addition of magnesium stearate, a salt of a C18 unsaturated fatty acid, also produced a modest decrease in fructose concentration (figure 42.). Zinc sulphate was added at a concentration of 160 mg/litre and it appeared to have an inhibitory effect on fructose utilization as the residual fructose concentration in this fermentation was approximately 1.75 fold higher than the control (figure 42.).

4.4. Discussion

A primary factor which regulates the residual fructose concentrations in wort is the pitching rate. During the last three days of fermentation the pitching rate is inversely proportional to the fructose concentration. Thus under pitched fermentations will produce residual fructose concentrations above the taste threshold (figure 3.) and taint the beer with a *sweet off-flavor. Conversely the use of higher than* normal pitching rates will reduce residual fructose and keep the concentrations within acceptable limits. Pitching rates above the present value of 6 g/litre of yeast would appear to be the inoculum size needed. However, as not all breweries use identical fermenters,

trials would have to be run to determine the ideal pitching rate for any particular full scale fermenter, when using high gravity sucrose adjunct wort.

Kirsop (97) has shown that dilution of malt wort with a carbohydrate adjunct to give an adjunct : malt wort ratio of 50% : 50% produces longer fermentation times and decreased attenuation, owing to the reduction in the nitrogen sources by 50% caused by the carbohydrate addition. The wort used for this set of experiments had a malt wort : adjunct ratio of 60% : 40%. The dilution of the nitrogen sources due to the adjunct did not appear to have any effect on fermentation attenuation as supplementation of the wort with separate nitrogen sources, i.e. serine, ammonium sulphate, and yeast nitrogen base, did not cause dramatic increases in maltose, fructose and glucose utilization (figure 42.). The addition of growth stimulants such as nutromix, a commercial preparation of nitrogen sources, vitamins, and trace elements, brought about a dramatic reduction in residual fructose concentration at the end of fermentation (figure 42.). It appears the elements within this preparation act in a synergistic fashion in order to bring about increased yeast growth and hence increased fructose utilization. It is unlikely that the nitrogen sources within nutromix have a large effect on yeast growth because, as mentioned above, the additon of single nitrogen sources to fermentations only brought about modest reductions in residual fructose concentrations. The list of contents within this preparation does not appear to be an exhaustive one and, as it is produced from yeast, the possibility exists that it contains a lipid fraction, which along with the other elements, stimulates yeast growth. Short bursts of oxygen to wort during fermentation caused dramatic reductions in fructose concentrations with fructose being completely utilized 3 days prior to the end of

fermentation. The attenuation patterns for the control compared to the above treatment (see figure 43.) are not dramatically different while the fermentations were being oxygenated up to 72 hours. However the differences become enlarged once the treatment had stopped. This was probably due to a carry over effect of the increased sterol concentrations present in the yeast, which were produced as a result of the oxygenation, being passed on to subsequent generations which thus aids further growth, and in turn causes increased concentrations of fructose to be utilized. The common factor in all of the above treatments is that the concentration of yeast in the fermenter is increased, either by the addition of substances which stimulate yeast growth, or by using more yeast at the beginning of fermentation. Increased yeast inoculum sizes utilize greater concentrations of fructose and this results in a reduction of the residual fructose concentrations in the wort at the end of fermentation.

The conditioning experiment, showed the yeast which was propagated in the medium with glucose as the sole carbon source, utilized fructose in the subsequent fermentation at a marginally faster rate than the yeast which was propagated in a medium containing fructose as the only carbon source. Stewart (102) reported similar findings. He noted that glucose-propagated cells utilized both glucose and fructose at slightly faster rates than fructose-propagated cells. This technique would not significantly reduce the fructose concentrations at the end of fermentation, as it only brings about a marginal increase in the rate of fructose utilization.

The data presented here has shown that there are several methods of ameliorating the "fructose block" problem. If past records reveal that fructose concentrations of in bottle beer samples exhibit large variations, then it

will be highly likely that pitching rates are not being kept constant at breweries using sucrose adjunct wort fermentations, and tighter controls would be appropriate. This assumes that all other parameters in the brewing process have been kept constant. If fructose concentrations from bottled beer samples are consistantly above the recommended taste threshold levels, then the problem may be ameliorated by a) increasing the pitching rate, b) adding nutromix at concentrations above 8 mg/litre, c) giving short bursts of oxygen to the wort during the initial 72 hours of fermentation, d) extending the fermentation times by up to 96 hours. Care must be taken to closely monitor fructose concentrations in the wort, as the "fructose block" problem becomes exacerbated with an increase in fermentation number if the fermentations are consecutively pitched with the same yeast (figure 38.). Experimental data shows that residual fructose concentrations at the end of fermentation rose from 1.851 g/litre for the first fermentation to 8.38 g/litre at the end of the 8^{th} fermentation. e) a combination of some or all of the above treatments may be used depending on the individual requirements of the particular brewery.

CHAPTER 5.

The Relationship between Glucose, Fructose, Maltose and Ethanol and the Glucose Membrane Transport System in S. cerevisiae 2036.

5.1. Introduction

The fermentation studies suggested that glucose is taken up considerable faster than fructose in S. cerevisiae 2036 and and this situation occurs for other strains of S. cerevisiae (74,75). The glucose transport system in S. cerevisiae is a constitutive electroneutral uniport, operating by facilitated diffusion, with a broad specificity base of various metabolizable sugars. e.g. D-glucose; D-fructose and D-mannose, and several non-metabolizable sugars (36) e.g. 6-deoxy-D-glucose; 2-deoxy-D-glucose; L-sorbose and D-xylose. Glucose has the highest affinity for the membrane carrier. All the other sugars transported by this system show lesser degrees of affinity, and their affinity depends on how similar their structure is to the glucose pyranose conformation, which is the form of glucose taken up (36). The relative affinity of a particular sugar for the membrane carrier is described by the Michaelis constant, Km, at which the initial reaction velocity is

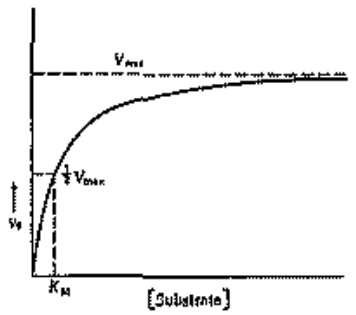


Figure 44. The Relationship between Km and the Velocity of an Enzyme Catalysed Reaction (112).

half maximal. It is independant of the enzyme concentration (112).

If the two sugars, which use the glucose transport system, are present in a growth medium, with yeast cells, then both sugars will compete for binding, and, therefore, uptake, via the same membrane carrier. The transport of the sugar with the lower affinity for the membrane carrier should be competitively inhibited by the sugar with the higher affinity (38).

Membrane transport studies were performed using S. cerevisiae 2036 to see if the membrane kinetics of fructose and glucose uptake correlated with glucose and fructose utilization data obtained under various fermentation conditions. These experiments aimed to determine whether fructose transport is inhibited by the presence of glucose, and vice versa. Maltose is present in wort at the highest concentration of all the wort sugars, and although it is taken up by a proton symport, a transport mechanism separate from the glucose transport system, it was thought important to determine if maltose had any effect on glucose transport.

Leao and van Uden (110) demonstrated that ethanol adversely affects the operation of the glucose transport system by inhibiting the rate of glucose uptake. The inhibition by ethanol of the glucose transport system in S. cerevisiae 2036 exhibits exponential kinetics, which allows a simple equation to express the inhibition relationship (110).

$$v = V_{max} \cdot e^{-kx} \cdot \frac{S}{Km + S}$$

The inhibition constant k enables a value to be placed

on the degree of ethanol inhibition, with respect to the transport of a particular sugar e.g. glucose in a particular yeast strain. Using the yeast S. cerevisiae IGC 3507, Leao and van Uden (110) calculated the inhibition constant k, for glucose uptake in the presence of ethanol, to be 0.616 L/mol. In this set of experiments k was calculated for glucose and fructose uptake in the presence of ethanol, using the yeast strain S. cerevisiae 2036.

5.2. Methods

5.2.1. The Determination of the Km for Glucose Transport

Growth media was prepared according the formula:

Yeast Nitrogen Base	3.4 g
Citric Acid	[illegible] g
Sodium Citrate	[illegible] g
Glucose	[illegible]
Fructose	[illegible] g
Distilled Water to	500 ml
Adjust pH to	5.0

The growth medium was inoculated with S. cerevisiae 2036 and incubated at 25ºC overnight on a Gallenkamp orbital shaker set at 150 rpm. The medium/yeast mixture was centrifuged at 10 000 rpm for 10 minutes. The supernatant was discarded. The yeast was washed twice in water by centrifugation at 10000 rpm and then stored on ice until required. An aliquot of 100ul of the yeast suspension was placed on a piece of filter paper and allowed to dry in an oven at 70ºC overnight, and then weighed to determine the dry weight of the yeast. All uptake experiments were carried out in conical centrifuge tubes. To each tube was added 20 ul of 6% (dry weight/volume) cell suspension and 20 ul of 100mM

tris-citrate buffer pH 5.0. The tubes were allowed to equilibrate for 5 minutes in a 25°C water bath. Final glucose concentrations of 0.5 mM, 1.0 mM, 2.0 mM, and 20 mM were prepared, with D-[^{3}H]-glucose (Amersham, U.K.) in each concentration at 50 uci/ml. For each concentration, four uptake experiments were performed in duplicate. These were at time intervals of 0s, 5s, 10s, and 15s. Each uptake experiment was started by adding the radioactive labelled-glucose into the conical tube and was stopped by the addition of 5 ml of ice cold water into the tube after the required time interval. For 0s, the labelled-glucose was added after the ice cold water. The contents of each conical tube were filterd through a glass fiber filter using vacuum apparatus. The filter was then washed with 10 ml of ice cold water, and transferred into a scintillation vial containing 10 ml of scintillation fluid, which was composed of 10% (w/v) naphthalene, 0.7% (w/v) 2,5-diphenyloxazole (PPO) and 0.03% (w/v) 1,4-bis-2(5-phenyloxazolyl) benzene (POPOP) in 1,4-dioxane. An aliquot of 10 ul of each labelled-glucose solution was directly added to the scintillation fluid to determine the specific activity of the labelled-glucose at each concentration. The scintillation vials were placed into a Beckman LS 8100 scintillation counter, and the counts from each vial were recorded.

5.2.2. The Determination of the Fructose Ki for Glucose Uptake.

A suspension of S. cerevisiae 2036 yeast and its dry weight, was obtained using the techniques outlined in 5.2.1. The labelled-glucose concentrations used in this experiment were the same as those used in 5.2.1. Separate uptake experiments were performed for each glucose concentration in the presence of three fructose concentrations at time intervals of 0s and 15s. Each time interval was performed in duplicate. For each uptake experiment, an aliquot of 20 ul of fructose and

	RADIOACTIVE-LABELLED GLUCOSE CONCENTRATIONS			
	0.5 mM	1.0 mM	2 mM	20 mM
FRUCTOSE CONCENTRATIONS	4 mM 8 mM 16 mM	4 mM 8 mM 16 mM	4 mM 8 mM 16 mM	4 mM 8 mM 16 mM

Table 13. Concentrations of fructose and radioactive labelled glucose used to determine the fructose Ki for glucose uptake.

10 ul of labelled-glucose were mixed together in a test tube, and the uptake experiment was started by adding 20 ul of yeast suspension to the tube. The reaction was stopped with ice cold water, as before and the samples were processed as outlined in 5.2.1.

5.2.3. The Determination of the Glucose Ki for Fructose Uptake.

The methods for this experiment follow those of 5.2.2. except the concentrations of labelled solutions and inhibitors were changed. Four final concentrations of 2 mM, 5 mM, 10 mM, and 50 mM, radioactive-labelled D-[^{14}C]-fructose (Amersham, U.K.) were used. The concentrations of glucose used were 0.5mM, 2.0 mM, and 5.0 mM.

5.2.4. The Determination of the Ethanol Inhibition k for Glucose Uptake.

The yeast was prepared as stated in 5.2.1. The methods used were essentially the same as those described in 5.2.1. with the following modifications:- i) the 20 ul of tris-citrate buffer was replaced with separate 20 ul 3%, 5%, 7%, (w/v) final concentrations of ethanol in tris-citrate buffer. The mixture was vortexed, and

	RADIOACTIVE-LABELLED GLUCOSE CONCENTRATION			
	0.5 mM	1.0 mM	2 mM	20 mM
ETHANOL CONCENTRATIONS (% w/v)	0	0	0	0
	3	3	3	3
	5	5	5	5
	7	7	7	7

Table 14. Concentrations of ethanol used to determine the ethanol inhibition constant k

allowed to stand for 5 minutes prior to the addition of labelled glucose. iii) the time intervals used were 0s and 15s. Each time interval was performed in duplicate. iv) the control for the uptake experiments was the redetermination of the glucose Km, as this was effectively 0% ethanol. v) the determination of k for glucose uptake was repeated, but the ethanol concentrations used were 4%, 6%, 8%, (w/v).

5.2.5. The Determination of the Ethanol Inhibition Constant k for Fructose Uptake.

The methods for this experiment follow those stated in 5.2.4. The same ethanol concentrations were used as in 5.2.4. and the labelled-fructose concentrations used were 2mM, 5 mM, 10 mM, and 50 mM.

5.2.6. The Determination of the Maltose Ki for Glucose Transport.

The method used for this experiment followed that outlined in 5.2.2. The concentrations of maltose used were 10 mM, 20 mM, and 50 mM. The concentrations of labelled-glucose used were the same as those in 5.2.2.

5.2.7. Determination of the Km for Maltose Transport

A growth medium was prepared according to the formula:-

Yeast Nitrogen Base	6.6g
Citric Acid	3.3g
Sodium citrate	5.2g
Maltose	20g
Distilled water	1 litre
adjust pH to	5.0

The growth medium was inoculated with S. cerevisiae 2036, and grown overnight at 25°C on a mechanical shaker set at 150 rev/min. The cells were harvested in exponential phase and washed as described previously in 5.2.1. Water was added to the yeast so that a concentration of approximately 60 mg dry weight per ml was obtained. The dry weight of the cells was determined as in 5.2.1. The cells were kept on ice prior to use. The equipment used consisted of a Radiometer PHM 82 standard pH meter, (Copenhagen, Denmark) with its pH electrode immersed in a 10 ml capacity water-jacketed chamber set at 25°C. A magnetic stirrer provided an homogeneous mix of yeast and water. Connected to the pH meter was a Perkins-Elmer R100 flat-bed recorder (Connecticut, U.S.A.). To the chamber was added 3.7 ml of water, and 0.5 ml of yeast suspension. The pH was adjusted to 5.0 using 10 mM hydrochloric acid. After a baseline was obtained, final maltose concentrations of 1.19 mM; 3.6 mM; 5,95 mM; 8.3 mM and 11,9 mM were separately added. The subsequent alkalization of the yeast/water mixture was followed with the recorder which was set at a sensitivity of 100 mV and a chart speed of 60 mm/min. Calibration was performed by adjusting the pH of a yeast/water mixture in the chamber to pH 5.5. Several aliquots of 100 ul, 1 mM hydrochloric acid were sequentially added to the chamber and the

resulting drop on pH in each case was noted. From this, the rate of proton uptake was calculated.

5.2.8. Determination of the Ethanol Inhibition Constant, k, for Maltose Uptake

S. cerevisiae 2036 was grown, harvested, washed and suspended as described 5.2.1. The pH meter/recorder apparatus (5.2.7.) was used to perform the experiment. Yeast suspension and water were added in the same quantities as before (5.2.7.). Separate ethanol solutions of final concentrations 4% (w/v); 5%; 6%; 7%; 8%; and 10% were then added to the chamber and the resultant mixture was allowed to stand for 5 minutes. A maltose solution of 23.8 mM final concentration was then added to the mixture and the subsequent alkalization of the mixture was traced by the recorder. Calibration was performed as before (5.2.7.).

5.3. Results

5.3.1 The Determination of the Km for Glucose Transport.

The specific activity of each of the glucose concentrations was determined using the equation:

$$\text{Specific activity} = \frac{\text{cpm}}{\text{n moles of D-glucose}}$$

The rate of uptake of D-glucose (n moles/sec) was calculated by plotting a mean value of cpm/specific activity versus time, and determining the slopes of the

$$\text{n moles of D-glucose} = \frac{\text{cpm}}{\frac{\text{cpm}}{\text{n moles of glucose}}}$$

graphs for each glucose concentration. The initial uptake was converted from (n moles/sec) to (m moles/g.h) using the yeast dry weight values. The reciprocal of the initial uptake rate of D-glucose against the reciprocal of the D-glucose concentration was plotted i.e. a Lineweaver-Burk plot (figure 45.). Manipulation of the Michaelis equation:

$$v = V_{max} \cdot \frac{S}{Km + S}$$

produced an equation for a straight line on the double reciprocal plot.

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{Km \cdot 1}{V_{max} \cdot S}$$

Dry weight of yeast = 1.1 mg

S (mM)	1/S	v (n mole/s)	v (m moles/g.h)	1/v
20	0.05	0.384	1.521	0.658
2	0.5	0.104	0.412	2.436
1	1	0.067	0.265	3.769
0.5	2	0.042	0.166	6.013

Table 15. Experimental data resulting from the uptake of labelled-glucose.

when S=0 then $\frac{1}{v} = \frac{1}{V_{max}}$ and $\frac{1}{V_{max}} = 0.843$

V_{max} = 1.2 m moles/h.g dry weight.

Slope of the plot $= \frac{Km}{V_{max}} = 2.675$

The correlation co-efficient (r) for the plot is 0.992
Manipulation of the equation $\frac{1}{v} = \frac{1}{V_{max}} + \frac{Km \cdot S}{V_{max} \cdot S}$ results

in $Km = -S$ when $v = 0$. Using the data in Table 15 :-

$$0 = 0.843 + \frac{2.675 \cdot 1}{S} \quad \text{and} \quad \frac{-1}{S} = \frac{0.843}{2.675}$$

$-S = 3.17$ and $Km = 3.2$ mM.

A mean value of $Km = 2.6$ mM for glucose affinity of the facilitated diffusion system was calculated from 10 determinations. From the experimental data, resulting from labelled-glucose uptake, linear regression analysis was used for the straight line fit on the Lineweaver-Burk plot. Also, all subsequent straight line plots in this chapter and the following ones were fitted using this technique.

5.3.2. *The Determination of the Fructose Ki for Glucose Uptake*

The specific activity and initial uptake rates were determined using the methods stated in 5.2.1. Lineweaver-Burk plots of each fructose inhibitor concentration including the control, were produced, and the slopes of the plots at each inhibitor concentration were calculated. Manipulation of the equation (114):

$$K_{app} = Km + \frac{Km \cdot I}{Ki}$$

where K_{app} is the apparent Km value in the presence of

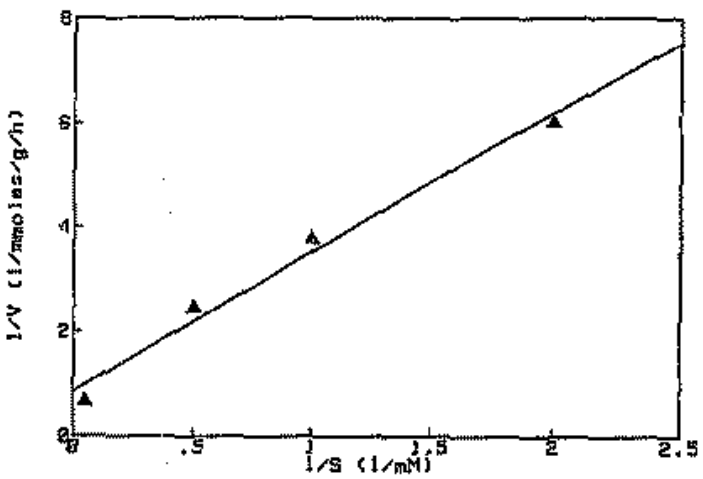


Figure 45. A Lineweaver-Burk plot of labelled-glucose uptake using 4 concentrations.

FRUCTOSE CONCENTRATION	SLOPE	CORRELATION CO-EFFICIENT r
0	2.536	0.992
4 mM	3.418	0.995
8 mM	3.552	0.999
16 mM	3.978	0.981

Table 16. The slopes of the Lineweaver-Burk plots of labelled glucose uptake, alone and, in the presence of 4 mM, 8 mM, and 16 mM fructose.

an inhibitor concentration I, Ki is the inhibitor constant, and the Km is the Michaelis constant, produces the derivative equation:

$$\frac{K_{app}}{V_{max}} = \frac{Km + Km.I}{Ki \quad V_{max}}$$

$$\frac{K_{app}}{V_{max}} = \frac{Km}{V_{max}} + \frac{Km \quad .I}{V_{max}.Ki}$$

The equation in this form, may be plotted as a straight line (y = mx + c). Information from the graph i.e. the slope and the intercept were used to calculate Ki.

When I=0, then Y intercept = $\frac{Km}{V_{max}}$ = 2.804 (r=0.914)

Slope of the plot = $\frac{Km}{V_{max}.Ki}$ = 0.0811

$$\frac{\text{Y int.}}{\text{slope of plot}} = \frac{\frac{Km}{Vmax}}{\frac{Km}{Vmax.Ki}} = Ki$$

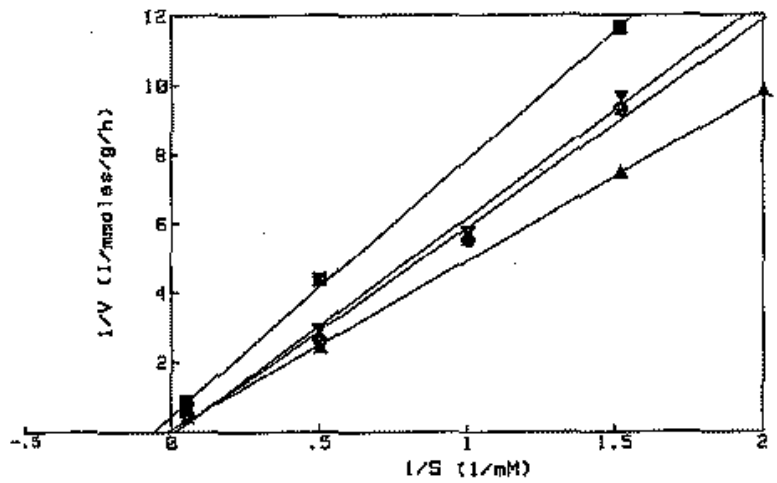


Figure 46. Lineweaver-Burk plots of labelled-glucose uptake; ▲ alone; in the presence of ● 4 mM; ▼ 8 mM; and ■ 16 mM, fructose.

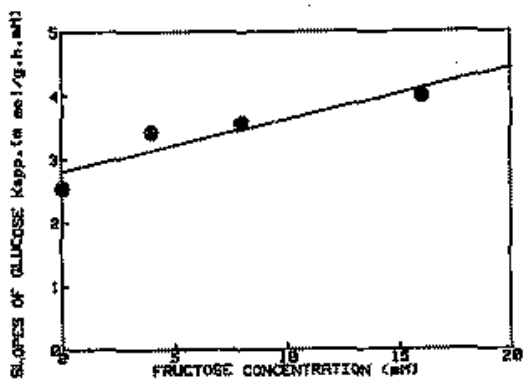


Figure 47. Slopes of the Lineweaver-Burk plots in figure 46. above, versus the fructose concentrations.

therefore $K_i = \frac{2.804}{0.0811} = 34.6$ mM

The fructose Ki determination was repeated twice and Ki values of 34.6 mM and 37.3 mM were obtained, with the mean value being Ki = 36.0 mM.

5.3.3. The Determination of the Glucose Ki for Fructose Uptake

The specific activity and initial uptake rates were *calculated using the same method* as outlined in 5.2.1. The Km and Vmax for the control were determined.

GLUCOSE CONCENTRATION	K_{app}	CORRELATION CO-EFFICIENT r
0	7.3 mM	0.961
0.5 mM	22.8 mM	0.999
2 mM	23.4 mM	0.998
5 mM	56.1 mM	0.999

Table 17. The K_{app}'s of labelled-fructose uptake, alone and, in the presence of 0.5 mM, 2 mM, and 5 mM glucose.

Likewise the K_{app} and V_{max} for each inhibitor concentration of glucose, was determined, using Lineweaver-Burk plots and the same calculation methods as stated in 5.2.1. The glucose Ki was calculated by drawing a plot (figure 38.) of K_{app} versus glucose inhibitor concentrations. From the equation below the Ki was determined (114).

$$K_{app} = Km + \frac{Km.I}{Ki}$$

when I=0, then Kapp = Km and Km = 11.29 mM.

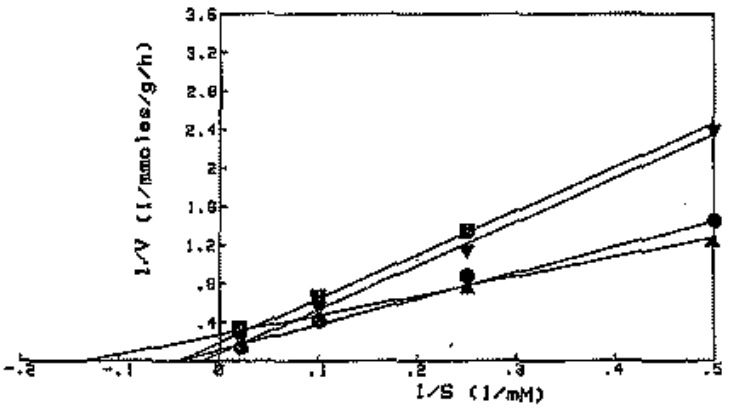


Figure 48. Lineweaver-Burk plots of labelled-fructose uptake; ▲ alone; in the presence of ● 0.5 mM, ▼ 2 mM and ■ 5 mM, glucose.

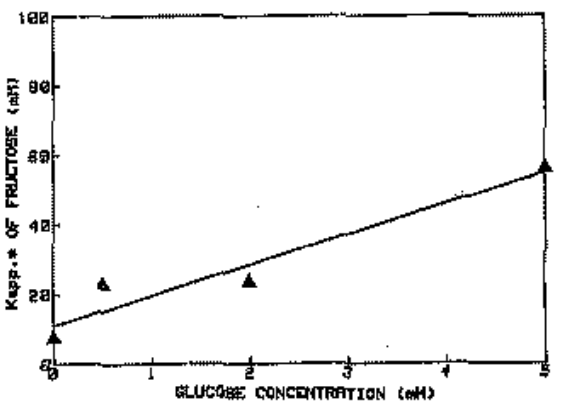


Figure 49. The K_{app}'s for labelled-fructose uptake in the presence of glucose determined from figure 48. above, versus the glucose concentrations.

slope = $\frac{Km}{Ki}$ = 8.682 (r = 0.961)

$Ki = \frac{11.29}{8.682}$ = 1.3 mM

The glucose Ki determination was repeated twice and Ki values of 1.0 mM and 1.3 mM were obtained, with the mean value being Ki = 1.15 mM.

5.3.4. The Determination of the Ethanol Inhibition Constant k for Glucose Uptake

The specific activity and initial uptake rates as well as the V_{max} for each ethanol concentration was determined using Lineweaver-Burk plots (figure 50.) and the methods stated in 5.2.1. The natural logarithm of V_{max} against the ethanol concentration was plotted (figure 51.) and the inhibition constant k was calculated using the equation (179):

$$\ln V^{x}_{max} = \ln V^{o}_{max} - kx$$

where x is the ethanol concentration.

slope = -k (r=0.992) therefore k= 0.84 L/mol

The data as depicted in figures 50 and 51 and Table 17 were used to determine the ethanol constant for glucose uptake. The glucose k determination was repeated twice and values of 0.84 L/mol and 1.07 L/mol were obtained, with the mean value being k = 0.96 L/mol.

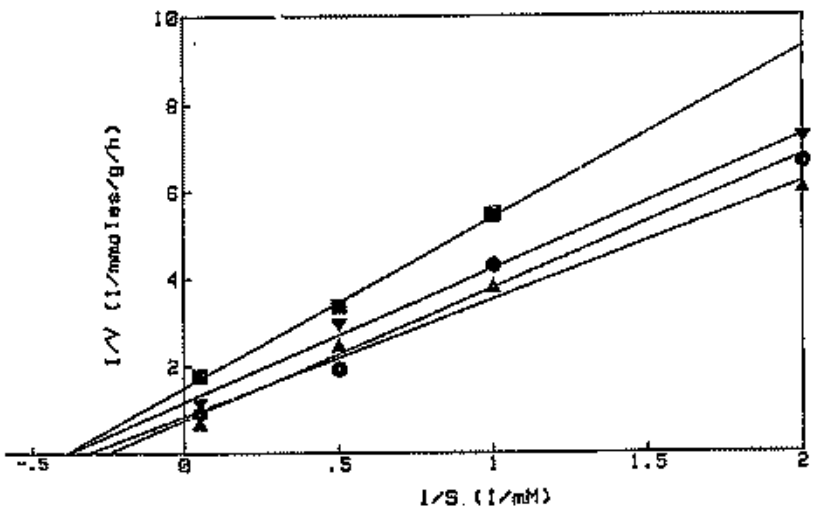


Figure 50. Lineweaver-Burk plots of labelled-glucose uptake; ▲ alone; in the presence of ● 4 % (w/v); ▼ 6 %; and ■ 8% ethanol.

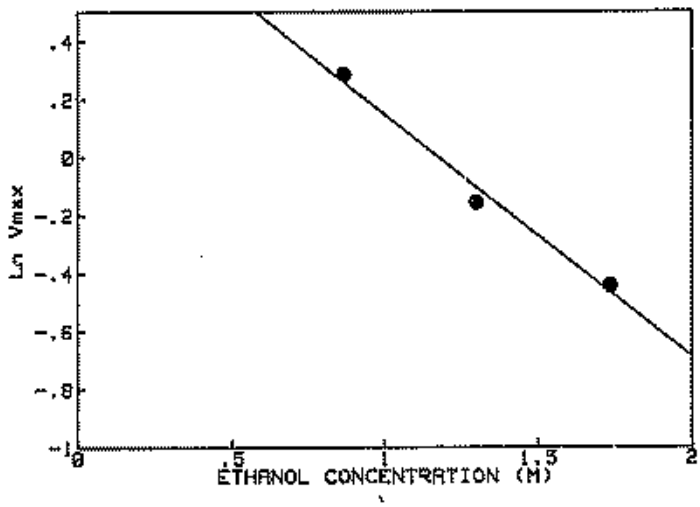


Figure 51. A semi-log plot of the ln V_{max} for labelled glucose uptake in the presence of 4 % (w/v); 6 %; and 8 % ethanol versus the ethanol concentration.

ETHANOL CONCENTRATION				
% (w/v)	M	V_{max}	ln V_{max}	r
0	0	1.186	0.171	0.992
4	0.868	1.330	0.285	0.990
6	1.302	0.853	-0.159	0.997
8	1.736	0.643	-0.442	0.999

Table 17. the V_{max}'s of labelled-glucose uptake in the presence of ethanol concentrations ranging from 0 to 8 % (w/v).

5.3.5. The Determination of the Ethanol Inhibition Constant k for Fructose Uptake

The k glucose method (5.2.4.) was used to calculate k for fructose uptake using the data presented in Table 18 and analyses of the plots in figures 52 and 53.

ETHANOL CONCENTRATION				
% (w/v)	M	V_{max}	ln V_{max}	r
0	0	1.381	0.322	0.951
4	0.868	0.788	-0.238	0.999
6	1.302	0.618	-0.481	0.949
8	1.736	0.422	-0.863	0.999

Table 18. The V_{max}'s of labelled-fructose uptake in the presence of ethanol concentrations ranging from 0 to 8% (w/v).

$$\ln V^{x}_{max} = \ln V^{\circ}_{max} - kx$$

slope = -k and k = 0.73 L/mol (r=0.993)

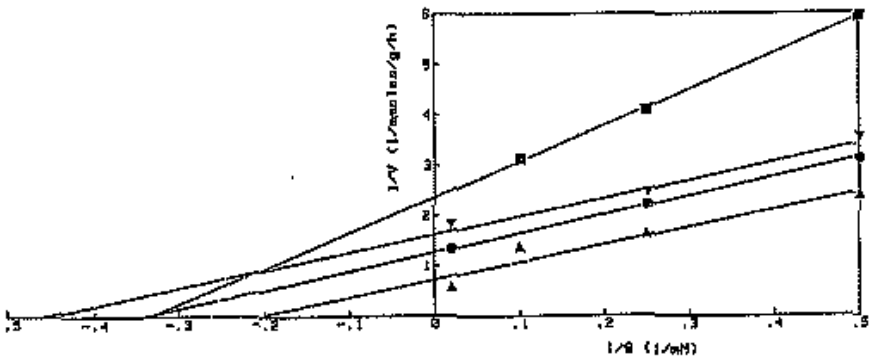


Figure 52. Lineweaver-Burk plots of labelled-fructose uptake; ▲ alone; in the presence of ● 4 % (w/v); ▼ 6 %; and ■ 8%; ethanol.

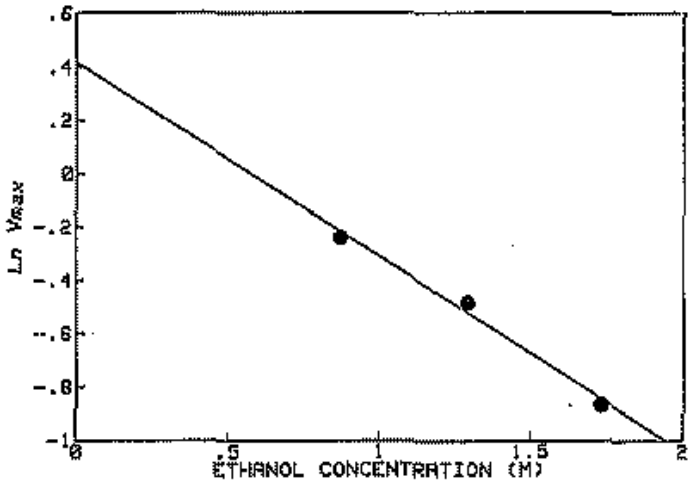


Figure 53. A semi-log plot of the ln V_{max} for labelled-fructose uptake in the presence of 4 % (w/v); 6 %; and 8 % ethanol versus the ethanol concentration.

The fructose k determination was repeated twice and values of 0.73 L/mol and 1.05 L/mol were obtained, with the mean value being k = 0.89 L/mol.

5.3.6. The determination of Maltose Ki for Glucose Uptake

The analyses of these results were performed using the same methods as stated in 5.2.2. The slopes of the plots (Table 19.) were calculated for each concentration of maltose as well as the control, using Lineweaver-Burk plots.

MALTOSE CONCENTRATION	SLOPE OF PLOTS
0	1.595
10 mM	1.414
20 mM	1.398
50 mM	1.278

Table 19. Slopes of the plots of labelled-glucose uptake, alone and, in the presence of maltose concentrations ranging from 0 to 50 mM.

Using the equation:

$$\frac{K_{app}}{V_{max}} = \frac{K_m}{V_{max}} + \frac{K_m}{V_{max}.K_i}.I$$

When I=0 then Y intercept $= \frac{K_m}{V_{max}} = 1.458$

slope of the plot $= \frac{K_m}{V_{max}.K_i} = -0.0036$

$K_i = \frac{\text{Y int.}}{\text{slope of plot}}$ *and therefore* $K_i = \frac{1.458}{-0.0036}$

K_i = -405.0 mM

This value is theoretically impossible. The inhibition constant must be a positive value if there is any inhibitory effect present. Therefore *maltose has no* inhibitory effect on glucose uptake.

5.3.7. Determination of the Km for Maltose Transport

The tangents of the slopes, which result from the alkalization of the medium on maltose addition, were *calculated for each maltose concentration*, figure 54 being a representative curve.



Figure 54. An example of alkalization on maltose addition.

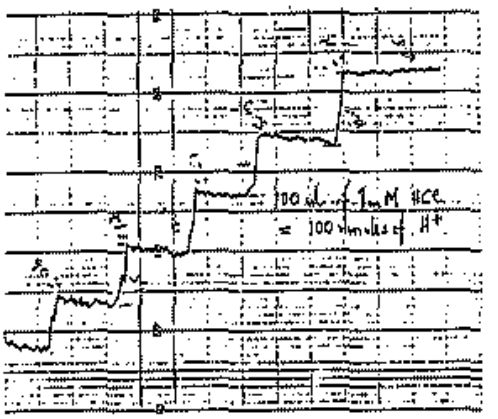


Figure 55. Calibration.

Using the calibration, (figure 55.) the rate of proton uptake for each maltose concentration can be calculated. These values were converted to units of m moles H^+/g.h using yeast dry weight values, i.e. 27 mg of yeast/ 0.5 ml of yeast suspension and the results presented in Table 20 are used to draw a Lineweaver-Burk plot of maltose uptake (figure 56).

S (mM)	1/S	v (m moles H^+/g.h)	1/v
11.9	0.084	3.565	0.280
8.3	0.120	2.565	0.399
5.95	0.168	2.347	0.426
3.60	0.278	1.217	0.822
1.19	0.840	0.521	1.918

Table 20. The experimental data of maltose uptake used to draw the Lineweaver-Burk plots in figure 56.

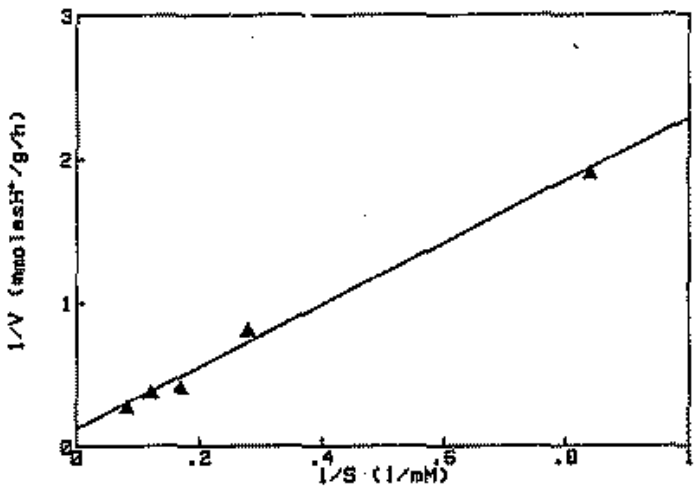


Figure 56. A Lineweaver-Burk plot of maltose uptake via the maltose proton symport.

Calculations from the plot reveal Km = 17.4 mM and V_{max} = 8.7 mmoles/h.g. dry weight for maltose uptake.

5.3.8. Determination of the Ethanol Inhibition Constant, k, for Maltose Uptake

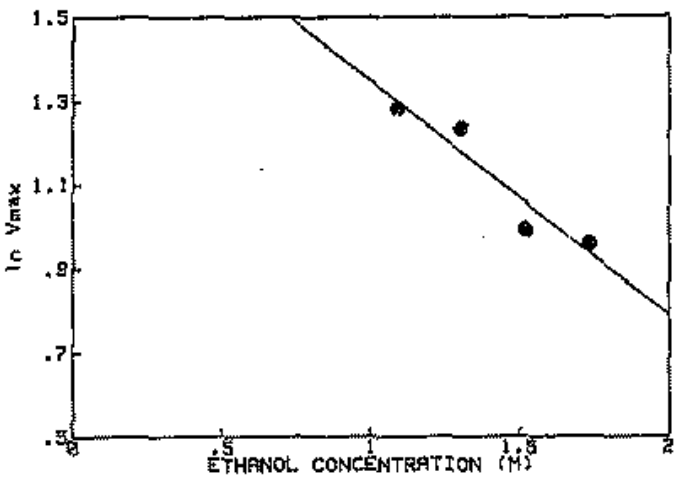


Figure 57. A semi-log plot of V_{max} of maltose uptake in the presence of several ethanol concentrations.

A semi-log plot (figure 57.) of ethanol concentration verses *ln* V_{max} *was drawn using* the methods stated in 5.3.4. Calculations from the plot reveal that the ethanol inhibition constant for maltose uptake is k = 0.56 l./mol.

5.4. Discussion

The glucose affinity for the *facilitated diffusion* system has a Km of 2.6 mM, in S. cerevisiae 2036. This value is *within the same order* of magnitude for the Km determined by Cirillo and others (21,36,109) for various strains of S. cerevisiae. The values are closest to those of Becker and Betz (16) who calculated in three

separate trials, glucose Km values of 4 mM, 0.6 mM, and 0.4 mM. The differences in the Km values determined by the various authors may be attributed to the use of different experimental methods and different strains of S. cerevisiae. The Lineweaver-Burk plots used in the calculation of Ki for glucose and fructose in S. cerevisiae, show a pattern which is characteristic of competitive inhibition i.e. glucose competitively inhibits the uptake of fructose, and fructose competitively inhibits glucose uptake. Here, the Km for the sugar being taken up, increased with a corresponding increase in the inhibitor concentration although the V_{max} for uptake with each inhibitor concentration remained approximately constant (figure 46 and 48). This situation implies that both sugars use the same membrane transport system. The rate of sugar transport across the cell membrane in the presence of a second sugar as a competitive inhibitor, is described by the equation:

$$v = V_{max} \cdot \frac{S}{Km + \frac{Km.I}{Ki} + S}$$

where v is the velocity of uptake, S is the concentration of the sugar, Km is its affinity for the membrane transport system, V_{max} is the maximum velocity of uptake, I is the inhibitor concentration, and Ki is the inhibition constant. From the equation it can be seen that a small Ki value implies a large " Km.I/Ki" term, and consequently a decreased v value. Thus a small value for Ki implies a large degree of competitive inhibition, and vice versa. For glucose inhibiting fructose uptake, the Ki = 1.15 mM, while the value for fructose inhibiting glucose uptake, is much larger at Ki = 36.0 mM. Glucose inhibits fructose uptake much more strongly than fructose inhibits glucose uptake. In a growth medium with both glucose and fructose present,

glucose would be expected to be taken up faster than fructose and hence the rate of glucose utilization should be faster than the rate of fructose utilization. This assumes that the rate limiting step in glycolysis is the transport of sugars across the cell membrane via the membrane transport systems. The Km value for sugar uptake, in the presence of another sugar acting as a competitive inhibitor, is described by the term " K apparent" (Kapp). The equation below shows the relationship between Kapp, the Km, the competitive inhibitor concentration I, and the competitive inhibition constant Ki (114).

$$K_{app} = Km + \frac{Km.I}{Ki}$$

The K_{app} for glucose in the presence of maltose is the same as the glucose Km. Therefore maltose exerts no effect on the uptake of glucose (5.3.6). This is not surprising as glucose has the highest affinity of all sugars for the glucose transport system, and maltose is taken up by a separate transport system to glucose, which operates as a proton symport (117). Maltose has an affinity of Km = 17.4 mM for the maltose proton symport (5.3.7.), and ethanol non-competitively inhibits maltose transport with k = 0.56 L/mol (5.3.8.).

The ethanol inhibitory constant k for glucose transport is 0.96 L/mol (5.3.4.), and k for fructose transport is 0.89 L/mol (5.3.5.). Leao and van Uden (64) determined k = 0.62 L/mol for glucose uptake in a strain of S. cerevisiae. This value is in the same order of magnitude as k for glucose and fructose uptake in S. cerevisiae 2036. Although not identical, these two values for S. cerevisiae 2036 imply that the preferential affinity for uptake which glucose has over fructose is not compounded by an ethanol effect, when both sugars are

present in the growth medium, as ethanol inhibits the uptake of the two sugars at approximately equal rates.

CHAPTER 6.

The Existence of a Proton Symport which Transports Fructose in S. cerevisiae 2036

6.1. Introduction

Many different solutes are transported by proton symports in a variety of yeast (46). These include the uptake of the amino acids L-methionine, L-lysine, L-phenyl-alanine, and L-leucine in Saccharomyces spp. (153) via the general amino acid permease system with an uptake ratio of protons to amino acid molecules of 2:1. The specific amino acid permeases for L-lysine, L-methionine, L-proline and glutamate are proton symports (46) as well as the transport systems for phosphate, sulphate, and hypoxanthine. Several sugars are taken up via proton symports and they include glucose in Neurospora spp.; 6-deoxyglucose, 1-deoxyglucose and D-glucose in Chorella spp.; D-xylose, D-galactose, 2-deoxyglucose and xylitol in Rhodotorula spp. (73); and D-glucose and D-xylose in Candida spp. In Saccharomyces spp. sucrose (151), maltose (154) and 6-methylglucose (24) have all been reported to be transported via independant proton symports with an uptake rate of protons to sugar molecules of 1:1. A strain of Saccharomyces fragilis absorbed protons with lactose uptake but not with glucose uptake. Beak (43) tested seven strains of S. cerevisiae grown in two different media, each containing glucose, one at 2% (w/v), and the other at 4%, for the existance of proton symports when induced with glucose. In all cases the results were negative. The strains were not tested for fructose. Here, S. cerevisiae 2036 was tested for the possible existance of a fructose proton symport. This chapter documents, for the first time, the occurrence of such a proton symport in S. cerevisiae.

6.2. Methods

6.2.1. The Kinetics of Fructose Transport using Radioactive Labelled Fructose

S. cerevisiae 2036 was inoculated onto a peptone, fructose (2% w/v) agar slant and grown overnight at 25°C. The yeast was then inoculated into a defined medium (5.2.1.) which contained 2% (w/v) fructose, (Analar, BDH, Poole, U.K.) as the only carbohydrate source present, and grown overnight on an orbital shaker at 150 rev/min at 25°C. The yeast was harvested in exponential phase and washed twice as described in 5.2.1. Nine concentrations of radioactive labelled-[^{14}C]-fructose were used for the uptake experiment. The final concentrations used were:- 0.2 mM; 0.275 mM; 0.4 mM; 1 mM; 2 mM; 2.75 mM; 4 mM; 10 mM; and 50 mM. The protocol for the experiment follows that described in 5.2.1.

6.2.2. Determination of the Membrane Kinetics of the Fructose Proton Symport

S. cerevisiae 2036 was grown in a 2% (w/v) fructose defined medium, (5.2.1.) harvested, washed and suspended in water, and the dry weight calculated as described in 5.2.1. The protocol for the experiment follows that described for the maltose Km determination (5.2.7.).Six final concentrations of fructose were used :- 0.125 mM; 0.25 mM; 0.5 mM; 1 mM; 5 mM; 25 mM.

6.2.3. The Kinetics of Radioactive Labelled Fructose Transport in the Presence of 5 mM and 50 mM glucose

S. cerevisiae 2036 was grown overnight in a 2% (w/v) fructose defined medium (5.2.1.). The protocol of the experiment follows that described in 5.2.2. except the concentrations of the labelled and unlabelled solutions were changed. The final concentrations of radioactive

radioactive labelled fructose used were :- 0.2 mM; 0.275 mM; 0.4 mM; 1 mM; 2 mM; 2.75 mM; 4 mM; 10 mM; and 50 mM. The concentrations of glucose used were 5 mM and 50 mM. The experiment was repeated using cells grown in a 2% (w/v) glucose (Analar, BDH, Poole, U.K.) defined medium (5.2.1.).

6.2.4. The Kinetics of Glucose Transport using Radioactive Labelled Glucose

S. cerevisiae 2036 was grown on peptone fructose agar slants and transfered into 2% (w/v) fructose defined medium and grown overnight as described in 5.2.1. The final concentrations of radioactive labelled glucose solutions used were :- 0.1 mM; 0.15 mM; 0.3 mM; 1 mM; 1.33 mM; 2 mM; 4 mM; and 10 mM. The protocol for the experiment follows that described in 5.2.1.

6.2.5. Determination of the Ethanol Inhibition Constant, k, for the Fructose Proton Symport

S. cerevisiae 2036 was grown overnight in a 2% (w/v) fructose defined medium (5.2.1.). The protocol for the experiment follows that described in 5.2.8. except the final radioactive labelled fructose concentration used was 0.04 mM, and the ethanol concentrations used were :- 1% (w/v); 2%; 3%; 4%; 5%; 6%; 7%; and 8%.

6.2.6. The Kinetics of Labelled Fructose Transport via the Proton Symport in the Presence of 1 mM, 10 mM, and 100 mM Maltose.

S. cerevisiae 2036 was grown overnight in a 2% (w/v) fructose defined medium (5.2.1.). The protocol for the experiment follows that described in 5.2.2. The concentrations of labelled-fructose used were 0.2 mM, 0.275 mM, 0.4 mM, 1 mM, and 2 mM. The concentrations of maltose used were 1 mM, 10 mM and 100 mM.

6.2.7. The Kinetics of Fructose Transport in the Presence of 1mM and 10 mM L-Sorbose

S. cerevisiae 2036 was grown overnight in a 2% (w/v) *fructose defined medium* (5.2.1.). The protocol for the experiment follows that described in 5.2.2. The concentrations of labelled-fructose were used were the same as in 6.2.1. The concentrations of L-sorbose used were 1mM and 10 mM.

6.2.8. Accumulation Experiments Performed with the Non-Metabolizable Sugar L-Sorbose

S. cerevisiae 2036 was grown up in a 2% (w/v) fructose defined medium, harvested, suspended, and the dry weight determined as stated in 5.2.1. The experiments were performed using conical centrifuge tubes. Into each tube was added 80 µl tris citrate buffer pH 5.0, 80 µl of the yeast suspension and 20 µl of radioactive labelled L-[^{14}C]-sorbose (Amersham, U.K.), with a final concentration of 1 mM. *Agitation was supplied* by a magnetic bar. The tubes were placed in a water bath set at 25°C. Samples of 10 µl were taken at times :- 0; 2; 5; 10; 30; 60; 65; 90; and 120 minutes, placed on microglass filters, washed with 10 ul of water and then put in scintillation vials containing scintillation cocktail and read on a Beckman LS 8100 scintillation counter. The experiment was repeated with slightly altered protocols in that i) 250 mM glucose was added after 60 minutes and *samples were subsequently taken* at 61; 65; 90 and 120 minutes. ii) 250 mM fructose was *added at* 60 minutes and samples were taken as in i). iii) CCCP, carbonyl cyanide m-chlorophenylhydrazone (Sigma, Missouri, U.S.A.), a protonophore, was added after 30 minutes, and samples were subsequently taken at 31;35; and 60 minutes. iv) CCCP was added at time zero. v) 250 mM sorbose was added after 60 minutes and then sampled as in i).

6.2.8. Investigation into the Possible Existence of Fructose Symports Occurring in Strains of S. cerevisiae other than S. cerevisiae 2036

Six different strains of S. cerevisiae :- S. cerevisiae Rh, S. cerevisiae 2332, S. cerevisiae IGC 3507, S. cerevisiae MB-8, S. cerevisiae Hb and S. cerevisiae AMS were grown overnight in a 2% (w/v) fructose defined medium (5.2.1.), and harvested, washed, and suspended in water as described in 5.2.1. The pH meter/recorder apparatus (5.2.7.) was used to determine whether fructose proton symports existed in any of the above strains. The protocol used followed that described in 5.2.7. One fructose concentration of 25 mM was used.

6.3. Results

6.3.1. The Kinetics of Fructose Transport using Radioactive Labelled Fructose

A Lineweaver-Burk and Eadie-Hofstee plots were drawn from the experimental data, using the methods stated in 5.3.1. Both plots show that fructose transport is composed of a low and a high affinity component (Table 21).

	Km (mM)	Vmax (mmoles/h.g dry weight)	
Low affinity component	7.0	3.0	(r=0.994)
High affinity component	0.4	0.77	(r=0.975)

Table 21. The Km's and V_{max}'s of the two components of biphasic fructose transport.

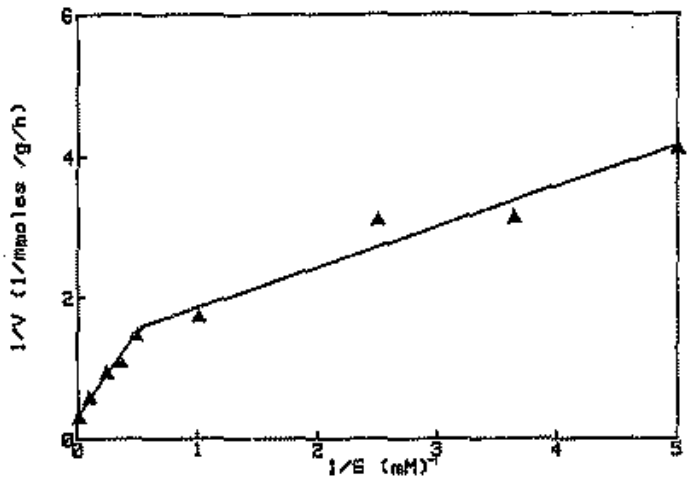


Figure 59. A Lineweaver-Burk plot of labelled-fructose transport using labelled-fructose concentrations ranging from 0.2 mM to 50 mM.

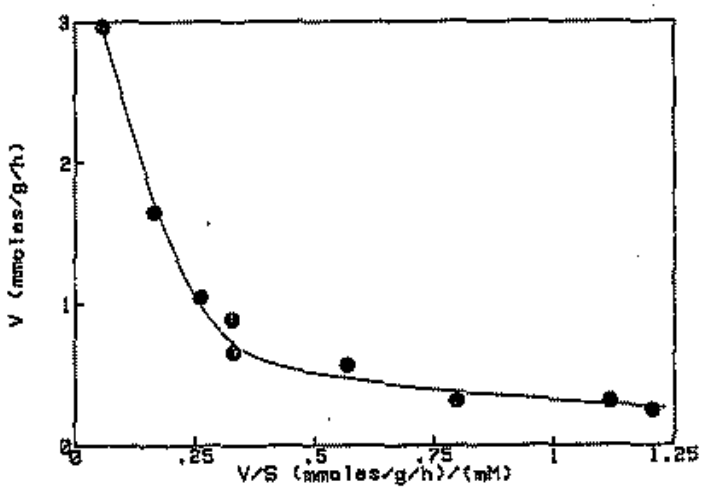


Figure 60. An Eadie-Hofstee plot of labelled-fructose transport using the same concentrations as in figure 59.

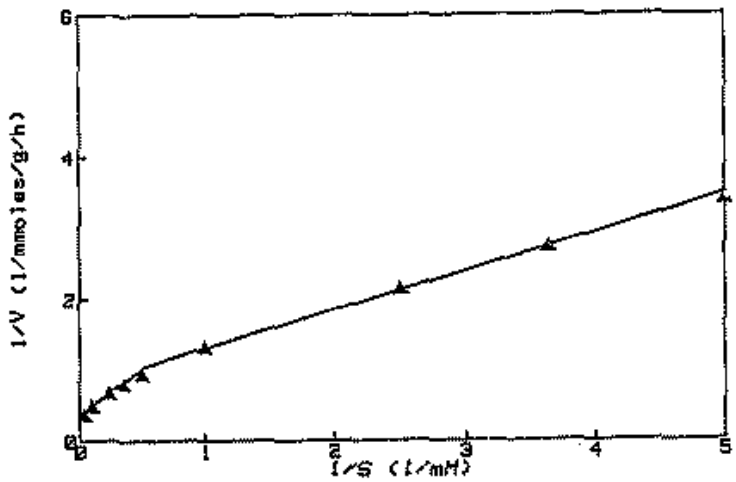


Figure 61. A plot resulting from a mathematical model which predicts the biphasic nature of fructose transport.

The high affinity component has a low maximum capacity with a V_{max} approximately 4 fold lower than the V_{max} of the low affinity component.

A theoretical plot is drawn from data produced by a mathematical model which predicts the biphasic nature of fructose transport. The model is based on the parameters of Km and V_{max} determined experimentally. The theoretical plots shows fructose transport to be composed of a low and high affinity component which exhibits close correlation with the experimental plots, further verifying the experimental results.

$$v = V^1_{max} \frac{S}{Km^f_1 + S} + V^2_{max} \frac{S}{Km^f_2 + S}$$

Figure 58. A mathematical model which predicts the biphasic nature of fructose transport.

V^1_{max} = 2.4 mmoles/h.g dry weight $\quad Km^f_1$ = 7.0 mM
V^2_{max} = 0.77 mmoles/h.g dry weight $\quad Km^f_2$ = 0.4 mM

Where S is the fructose concentration, V^1_{max} and V^2_{max} the maximum velocities of the low and high affinity components, and Km^f_1 and Km^f_2 the Michaelis constants of the low and high affinity components respectively.

6.3.2. Determination of the Membrane Kinetics of the Fructose Proton Symport

A Lineweaver-Burk plot was drawn using the methods stated in 5.3.7. The cells were harvested at O.D. = 1.05. From the plot (figure 62) the following data were obtained :-

Km = 0.3 mM and V_{max} = 0.6 mmoles/h.g dry weight
(r = 0.994)

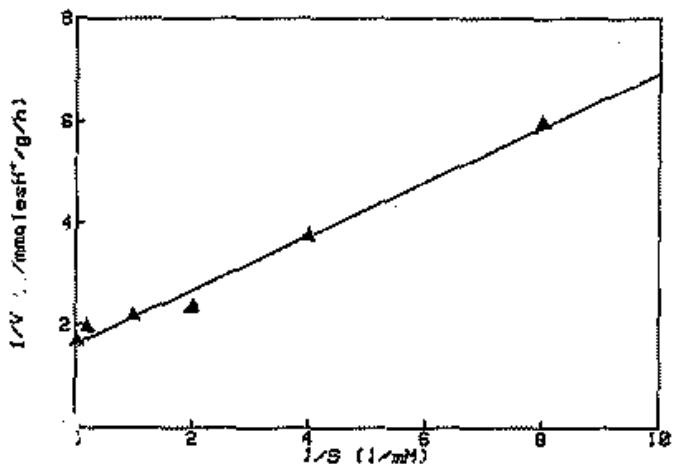


Figure 62. A Lineweaver-Burk plot of the membrane kinetics of the fructose proton symport in *S. cerevisiae* 2036.

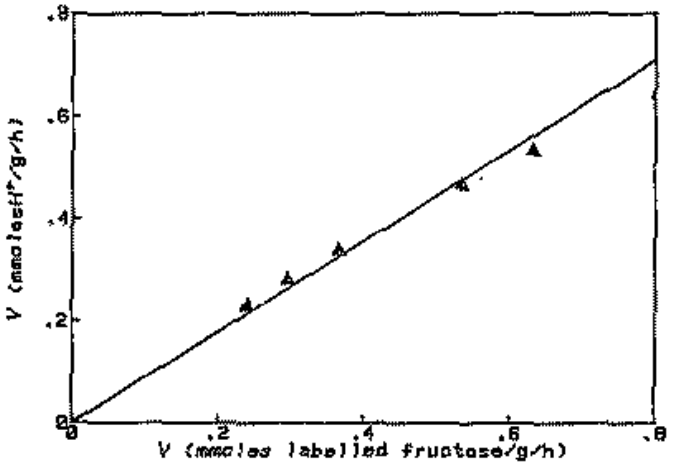


Figure 63. A plot of the rate of proton uptake by the fructose symport versus the rate of labelled-fructose transport.

The stoichiometry of the uptake of protons : fructose molecules was determined from figure 63. Calculation from the plot indicates that the stoichiometry is 1 : 1.3 (protons : fructose molecules).

6.3.3. The Kinetics of Radioactive Labelled Fructose Transport in the Presence of 5 mM and 50 mM Glucose

Lineweaver-Burk plots were drawn from experimental data using the methods stated in 5.3.3. The plots (figure 65) show that competitive inhibition by glucose occurs in the low affinity component, but does not exist in the high affinity component, when the cells are grown in a 2% (w/v) fructose defined medium. The glucose grown cells show similar results. Further verification of these results was obtained from a theoretical plot, derived from a mathematical model (figure 64) which proposes that glucose competitively inhibits fructose uptake of the low affinity component, but has no effect on fructose transport of the high affinity component. Theoretical plots correspond well with the results of the experimental plots.

$$v = V^1_{max} \frac{S}{Km^f_1 + \frac{Km^f_1 . I}{Ki^g} + S} + V^2_{max} \frac{S}{Km^f_2 + S}$$

V^1_{max} = 0.6 mmole/h.g dry weight $\quad Km^f_1$ = 7.0 mM

V^2_{max} = 0.6 mmole/h.g dry weight $\quad Km^f_2$ = 0.3 mM

Ki^g = 1.15 mM

Figure 64. A mathematical model which predicts labelled fructose transport in the presence of glucose.

I, is the glucose inhibitory concentration, and Ki^g the constant for glucose inhibiting fructose transport.

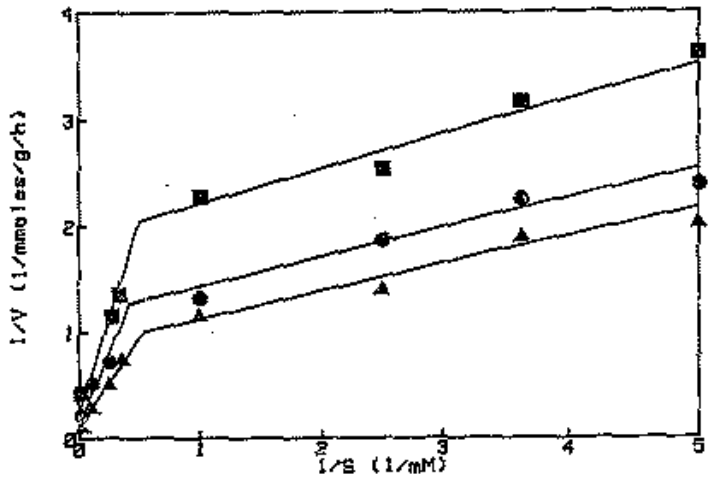


Figure 65. A Lineweaver-Burk plot of labelled-fructose transport ▲ alone; and in the presence of ● 5 mM and ■ 50 mM glucose.

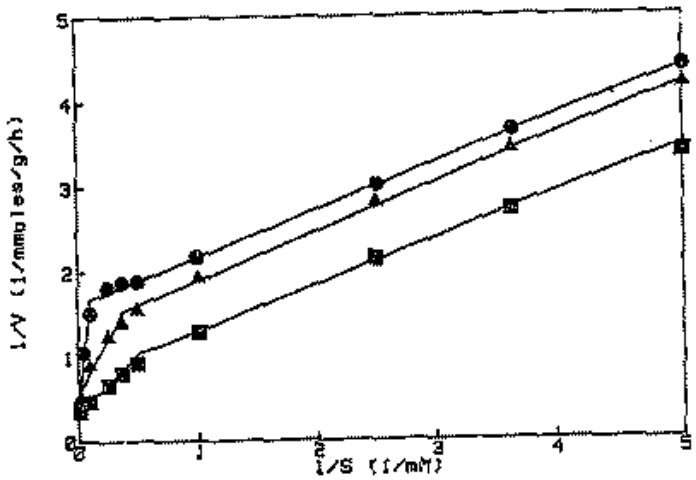


Figure 66. Lineweaver-Burk plots resulting from a mathematical model which predicts glucose competitively inhibits the low affinity component of fructose transport, but has no effect on the high affinity component. Fructose transport ■ alone; and in the presence of ▲ 5 mM and 50 mM ● glucose.

6.3.4. The Kinetics of Glucose Transport using Radioactive Labelled Glucose

A Lineweaver-Burk plot was drawn from experimental data, (figure 67) using the methods stated in 5.3.1. The plot shows glucose uptake to be monophasic with Km = 1.3 mM and V_{max} = 2.5 mmoles/h.g dry weight. This experiment, using more concentrations of substrate, confirms the results of the one performed in the previous chapter 5.3.1. In both cases glucose transport is seen to be monophasic.

6.3.5. Determination of the Ethanol Inhibition Constant, k, for the Fructose Proton Symport

A semi-log plot of ethanol concentration versus ln V_{max} was drawn using the methods stated in 5.3.4. From the plot, (figure 68) the ethanol inhibition constant for fructose uptake via the fructose symport was determined to be $k = 0.74$ L/mol.

6.3.6. The Determination of the Maltose Ki for Fructose Uptake

The analyses of these results were performed using the same methods as those for the determination of the fructose Ki for glucose uptake (5.3.6.). The slopes of

MALTOSE CONCENTRATION	SLOPE OF PLOTS	r
0	3.133	0.995
1 mM	4.209	0.992
10 mM	5.187	0.999

Table 22. The slopes of Lineweaver-Burk plots of labelled-fructose transport in the presence of 0, 1 mM, and 10 mM maltose.

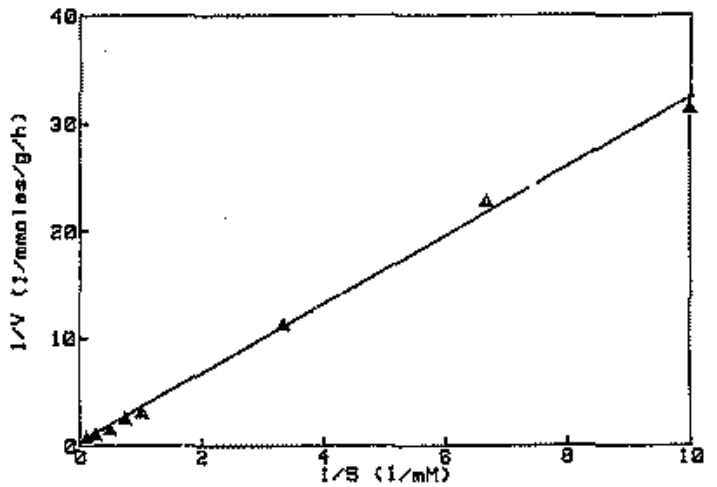


Figure 67. A Lineweaver-Burk plot of labelled-glucose transport using eight concentrations from 0.1 mM to 10 mM.

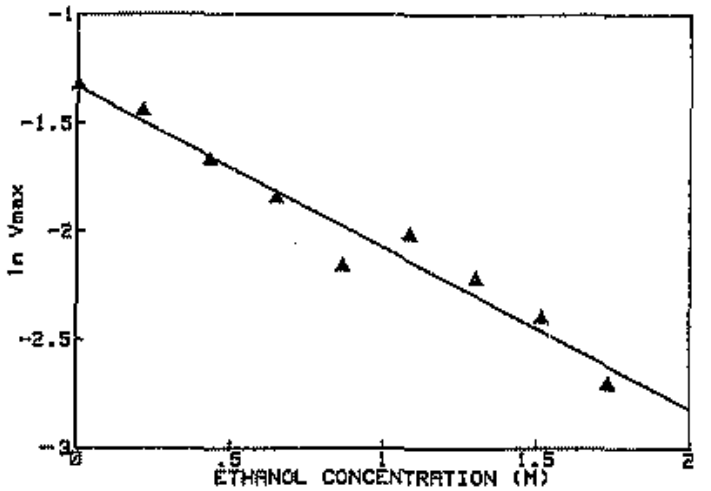


Figure 68. A semi-log plot of ln Vmax of labelled-fructose transport in the presence of several ethanol concentrations.

the straight lines (table 22) were calculated for the inhibitor concentrations of maltose as well as the control using Lineweaver-Burk plots (figure 69).

Using the equation:

$$\frac{K_{app}}{V_{max}} = \frac{Km}{V_{max}} + \frac{Km \cdot I}{V_{max} \cdot Ki}$$

a graph was plotted of the maltose inhibition concentrations versus the slopes of the Kapp plots. The slope of this straight line plot was used to determine k.

$$I=0 \quad \text{then} \quad Y \text{ intercept} = \frac{Km}{V_{max}} = 0.167$$

$$\text{slope of the plot} = \frac{Km}{V_{max} \cdot Ki} = 3.56 \qquad (r=0.896)$$

$$Ki = \frac{Y \text{ int}}{\text{slope of plot}} = 21.3 \text{ mM}$$

6.3.7. The Kinetics of Radioactive Labelled Fructose Transport in the Presence of 1 mM and 10 mM L-Sorbose

Lineweaver-Burk plots were drawn from experimental data using the methods stated in 5.3.3. Both the high and low affinity components of fructose transport are competitively inhibited by L-sorbose (figure 71), a sugar which is not metabolized by S. cerevisiae.

6.3.8. Accumulation Experiments Performed with the Non-Metabolizable Sugar L-Sorbose

L-Sorbose, a non-metabolizable sugar of S. cerevisiae accumulates to approximately 25 fold in S. cerevisiae 2036. After 60 minutes the addition of 250 mM fructose resulted in a very rapid efflux of sorbose from

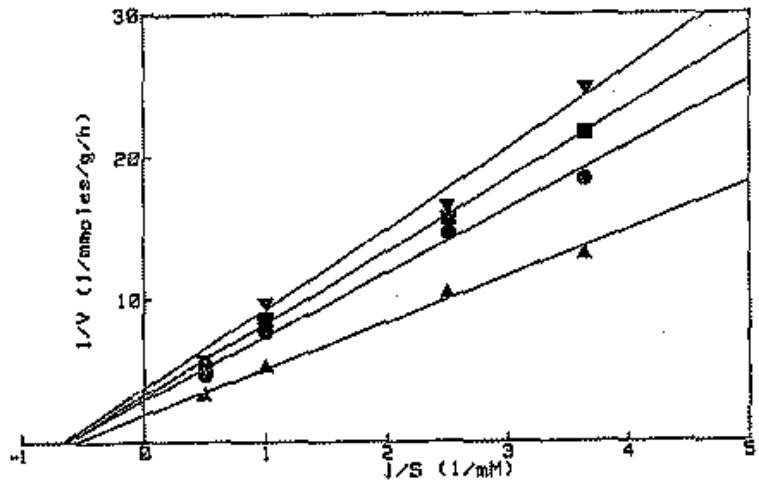


Figure 69. Lineweaver-Burk plots of labelled fructose transport ▲ alone; and in the presence of ● 1.0 mM; ■ 10 mM and ▼ 100 mM maltose.

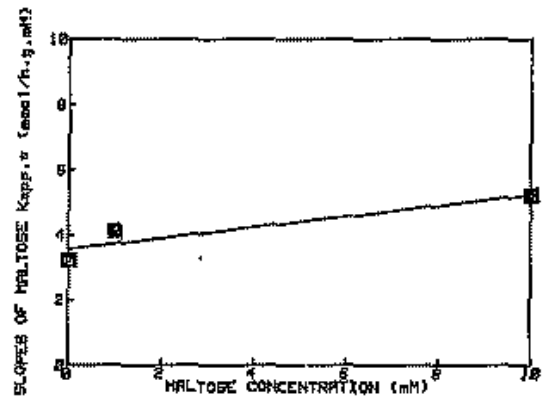


Figure 70. Slopes of the Lineweaver-Burk plots in figure 69, versus the fructose concentration.

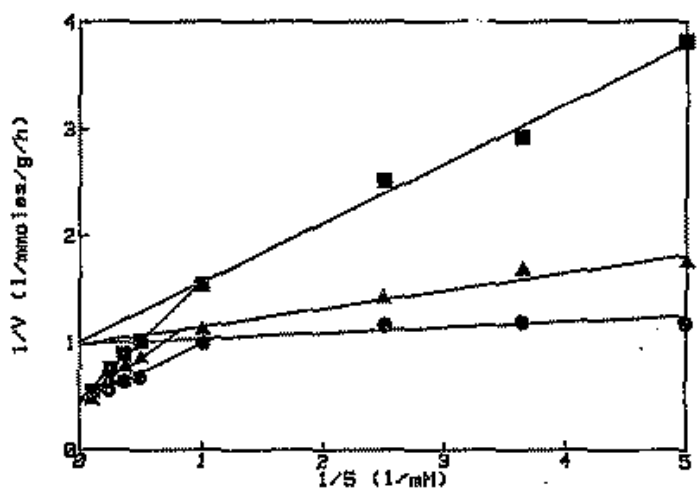


Figure 71. A Lineweaver-Burk plot of labelled-fructose transport; ● alone; and in the presence of ▲ 1 mM and ■ 10 mM L-sorbose.

the cell, thus causing a drop in the amount of accumulated sorbose (figure 72.). The addition of 250 mM glucose caused a transient increase, and then a decrease in sorbose accumulation but at a slower rate than that which occurred after fructose addition (figure 72.). The addition of 50 uM CCCP, at time zero, a protonophore which breaks down the proton gradient across the membrane and allows protons to flow freely into and out of the cell, resulted in negligible sorbose accumulation (figure 72.). When 50 uM CCCP was added after 30 minutes (figure 73.) the amount of sorbose accumulated decreased rapidly. Calculation of the sorbose concentration inside and outside the cell for accumulation studies on untreated fructose grown cells is shown below for the 2 minute sample. The same method of analysis was used for all the other samples :-

Dry weight of cells 5.0 mg/100 ul
There is 80 ul of yeast suspension in the total volume of 180 ul. The concentration of yeast in the total volume is 0.222 mg/10 ul dry weight of cells.

The internal volume of water within the cell is taken to be 2ul/mg dry weight of yeast (73).

The internal volume is 2 x 0.222 = 0.444 ul

For the 2 minute sample :-
Sorbose within the cell = 0.539 nmoles (specific activity = 4461 cpm/nmol, see 5.3.1. for method)

$$\text{Molarity} = \frac{\text{moles}}{\text{litre}} \qquad \frac{0.539 \times 10^{-9}}{0.444 \times 10^{-6}} = 1.214 \text{ mM}$$

$[S]_{inside} = 1.214$ mM

Total sorbose (nmole) - sorbose inside (nmole) = sorbose outside (nmole)

the cell, thus causing a drop in the amount of accumulated sorbose (figure 72.). The addition of 250 mM glucose caused a transient increase, and then a decrease in sorbose accumulation but at a slower rate than that which occurred after fructose addition (figure 72.). The addition of 50 uM CCCP, at time zero, a protonophore which breaks down the proton gradient across the membrane and allows protons to flow freely into and out of the cell, resulted in negligible sorbose accumulation (figure 72.). When 50 uM CCCP was added after 30 minutes (figure 73.) the amount of sorbose accumulated decreased rapidly. Calculation of the sorbose concentration inside and outside the cell for accumulation studies on untreated fructose grown cells is shown below for the 2 minute sample. The same method of analysis was used for all the other samples :-

Dry weight of cells 5.0 mg/100 ul
There is 80 ul of yeast suspension in the total volume of 180 ul. The concentration of yeast in the total volume is 0.222 mg/10 ul dry weight of cells.

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The internal volume is 2 x 0.222 = 0.444 ul

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$$\text{Molarity} = \frac{\text{moles}}{\text{litre}} \qquad \frac{0.539 \times 10^{-9}}{0.444 \times 10^{-6}} = 1.214 \text{ mM}$$

$[S]_{inside} = 1.214$ mM

Total sorbose (nmole) − sorbose inside (nmole) = sorbose outside (nmole)

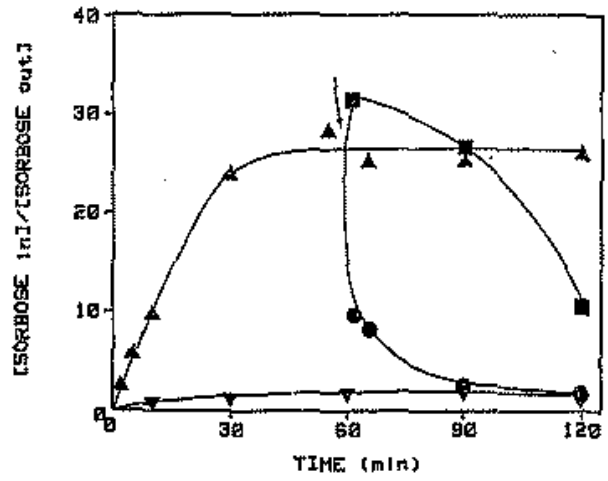


Figure 72. The uptake of labelled L-sorbose by S. cerevisiae 2036. Initial extracellular sorbose concentration is 1 mM. Uptake of sorbose ▲, by fructose grown cells. The arrow indicates the addition of ■ 250 mM glucose, and ● 250 mM fructose, at 60 minutes. 50 uM CCCP ▼, was added at time zero.

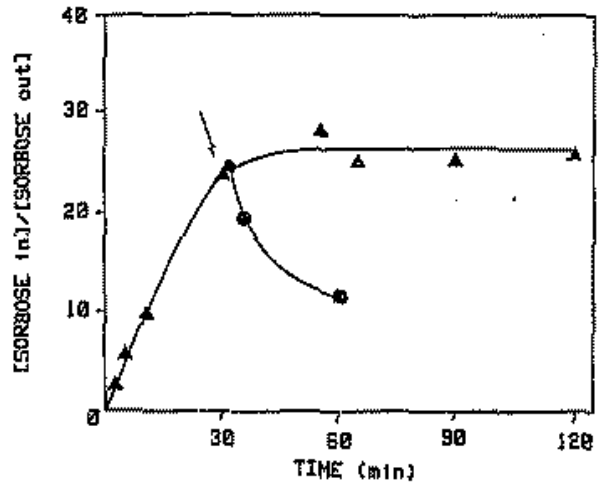


Figure 73. The uptake of labelled L-sorbose by S. cerevisiae 2036. Initial extracellular sorbose concentration is 1 mM. Uptake of sorbose ▲, by fructose grown cells. The arrow indicates the addition of 50 uM CCCP ●, after 30 minutes.

There is a total of 10 nmoles of sorbose

10 - 0.539 = 9.461 nmoles of sorbose outside

and $[S]_{outside} = \frac{9.461 \text{ nmoles}}{10 \text{ ul}} = 0.946 \text{ mM}$

$\frac{[S]_{inside}}{[S]_{outside}} = \frac{1.214 \text{ mM}}{0.946 \text{ mM}} = 1.283$

6.3.9. Investigation into the Possible Existence of Fructose Proton Symports in Strains of S. cerevisiae other than S. cerevisiae 2036

Preliminary investigations into the existence of *fructose proton symports*, reveal that they exist in more than one strain of S. cerevisiae. The results are summarized in Table 23.

Yeast strain	The existence of a fructose proton symport
S. cerevisiae Rh	YES
S. cerevisiae 2332	NO
S. cerevisiae IGC 3507	NO
S. cerevisiae MB-8	YES
S. cerevisiae Hb	YES
S. cerevisiae AMS	NO

Table 23. *Investigation into the possible existence of* fructose proton symports in 6 strains of S. cerevisiae.

6.4. Discussion

Fructose uptake by S. cerevisiae 2036 is via a biphasic transport system (33) in which the first component is a high affinity, Km = 0.3 mM, low capacity, V_{max} = 0.6 mmoles/h/g dry weight of yeast, proton symport (figure 62). The second component is a facilitated diffusion system of lower affinity and higher capacity which transports both glucose and fructose, plus other sugars which are structurally similar (36). The fructose proton symport is independant of the symport which transports maltose. Maltose grown cells showed very weak proton signals when induced with fructose but strong proton signals when induced with maltose. Also maltose non-competitively inhibits the uptake of fructose, Ki = 21.3 mM, by the proton symport. These two separate symports compete for protons in order to transport their respective sugars across the cell membrane. Two transport systems are known to exist for glucose in several different yeast species (162) e.g. Candida wickerhamii, where glucose is transported into the cell via a proton symport, but under conditions of glucose repression, the symport is not formed and glucose is taken up via a facilitated diffusion system. In S. cerevisiae 2036 glucose is not transported via the fructose proton symport, neither does its presence affect fructose transport of the symport in any way (figure 65). S. cerevisiae 2036 appears to be derepressed as both glucose and fructose grown cells exhibit very strong proton signals when induced with maltose. Ethanol non-competitively inhibits fructose uptake, with k = 0.74 L/mol, via the symport. Analysis of experimental data shows the stoichiometry of molecules of fructose transport : proton uptake is 1.3 : 1. However this value for fructose transport is a combined value for both the proton symport and the facilitated diffusion system. If the contribution from

the facilitated diffusion system is discounted then the ratio becomes 1:1. Glucose transport is monophasic (figure 67), Km = 1.3 mM and V_{max} = 2.5 mmoles/h/g dry weight of yeast, via the facilitated diffusion system which also transports fructose.

A mathematical model which postulates that glucose competitively inhibits fructose transport via the facilitated diffusion component, but has no effect on the fructose proton symport, is verified by experimental data from kinetic studies of labelled-fructose transport in the presence of glucose, using both glucose and fructose grown cells. The upward displacement of the high affinity transport components of labelled-fructose in the presence of glucose compared to the high affinity component of labelled-fructose alone, (figure 65) is due to the high maximum velocity which glucose has for the facilitated diffusion system, which in turn makes a contribution to the velocities of fructose transport via the proton symport.

Glucose and fructose grown cells produce proton signals when induced with L-sorbose. This sugar competively inhibits both components of fructose transport, indicating that it is able to utilize these two transport systems. L-sorbose has a 5-carbon furanose ring structure identical to that of β-D-fructofuranose. Fructose also exists in the 6-carbon pyranose ring form, β-D-fructopyranose, which is the same ring structure in which glucose exists. As glucose is not transported via the fructose proton symport, it is interesting to pose the question of whether the fructose proton symport transports only furanose sugars? Also does this imply that only β-D-fructofurenose is transported via the proton symport and β-D-fructo-pyranose must use the facilitated diffusion system? Perhaps further work in the future will answer these questions.

Since L-sorbose is not metabolized by S. cerevisiae it can be used to verify whether the fructose proton symport actually represents secondary active transport. Sorbose accumulates to about 25 fold within the cell compared to the extracellular sorbose concentration (figure 72) at pH 4.5. This accumulation must be via the fructose proton symport, implying that secondary active transport does occur, as the facilitated diffusion system cannot operate against a concentration gradient. Sorbose accumulation within the cell does not go above 25 times the concentration outside the cell, as the facilitated diffusion system probably acts as a leak for the intracellular sorbose. The addition of 250 mM fructose effectively stops the entry of sorbose into the cell as this very large concentration of fructose competitively inhibits the uptake of the relatively small concentration, 9 mM, of labelled-sorbose. Counterflow is induced and there is rapid efflux of sorbose from within the cell causing the concentration of accumulated sorbose to decrease. The addition of 250 mM glucose caused a transient increase (figure 72) in the sorbose accumulation ratio, probably due to ATP production, stimulation of the proton pump and increase in the membrane potential. Thereafter the glucose induced a slow efflux of the labelled sorbose probably by exchange through the facilitated diffusion system. CCCP, a protonophore, added at time zero, prevented the accumulation of sorbose, by allowing the free flow of protons into and out of the cell and thus stopping the establishment of a membrane potential across the cell membrane. Six additional strains of S. cerevisiae were tested for the existance of symports which transport fructose, and preliminary results reveal they were found to occur in three of them. Whether the existence of this symport is a relatively common phenomenon of S. cerevisiae remains to be determined.

CHAPTER 7.

A Mathematical Model Predicts Fructose Utilization in Sucrose Adjunct Wort Fermentations

7.1. Introduction

From the experiments detailed in the preceding chapters, it has become clear that several factors directly affect fructose utilization. A greater understanding of fructose utilization maybe obtained if these factors are combined together into a mathematical model which could quantify and predict the amounts of fructose remaining in the wort during the course of fermentation. For the model to have any value, all the factors which govern fructose utilization must be deduced and experimentally verified. Only then can they be incorporated into a mechanistic mathematical model which would provide greater insight into the way these factors operate, in combination, to affect fructose utilization under various fermentation conditions.

The importance of developing a mathematical model based on experimentally proven, and scientifically documented fact cannot be overemphasized. Engasser and Marc et al (47) have developed mathematical models which predict the rate of utilization of various wort sugars during fermentation. Despite the fact that these equations do predict wort sugar utilization, they are of little value as the underlying premises on which they are based do not fit experimental results. e.g. 1) Glucose inhibits maltose uptake. Glucose is transported via a facilitated diffusion system which is independant of, and not affected by, maltose transport which is via a proton symport. 2) Glucose transport is unaffected by fructose. These two sugars are transported by the same facilitated diffusion system and if they are both present in the wort, fructose will have a competitive

inhibitory effect on glucose uptake. 3) Yeast flocculation starts when glucose is completely exhausted from the wort. This statement does not fit experimental data. It would require flocculation to occur during the latter stages of fermentation. In the laboratory fermentation system used in this project, flocculation routinely occurred between 84 - 100 hours (figure 26), but glucose was only completely utilized by approximately 144 hours of a 240 hour fermentation when using a glucose adjunct wort fermentation. Similar results are observed in full-scale plant fermenters.

This chapter details the development of a mechanistic mathematical model and shows that it is able to predict fructose utilization in sucrose adjunct wort fermentations under various conditions in the laboratory scale fermentation system.

7.2. Methods

7.2.1. Fermentations Established Using Normal and High Gravity Sucrose Adjunct Wort.

A high gravity wort containing a sucrose adjunct in the ratio of 60% malt wort : 40% adjunct was prepared using the methods stated in 3.2.1. and oxygenated to 14.8 mg/litre of dissolved oxygen. Three fermentations were established with pitching rates of 3g/litre, 6g/litre and 12g/litre of yeast. The fermentations were run for 240 hours and monitored every 24 hours for specific gravity, yeast count, and glucose, fructose and maltose concentrations. Analysis of these parameters followed the methods stated in 2.2.1. The fermentation samples were assayed for ethanol using a Varian Model 3700 gas chromatogram (California U.S.A.) at oven temperature 110°C, injection temperature 150°C and detector temperature 150°C. Nitrogen was the carrier gas, used at

a flow rate of 28 - 30 ml/min while the fuel gases were hydrogen and air at flow rates of 25 ml/min and approximately 245 ml/min respectively (all gases supplied by Afrox, Johannesburg, R.S.A.). The column was composed of 10% carbowax 20m on chromosorb w in a 3mm x 4m stainless steel casing. Ethanol was used as the external standard, and butanol as the internal standard. Additional fermentations were run, using a normal gravity wort containing a sucrose adjunct, under the same conditions of wort composition, pitching rate, fermentation time and monitoring procedure as detailed above.

7.3. Development of the Mathematical Model

Mathematical equations were developed which predicted the biomass, glucose, maltose and ethanol concentrations during the course of the laboratory fermentations for the three different pitching rates. These sets of equations were derived from the experimental data obtained for the biomass, glucose, maltose and ethanol changes in the fermentations. The constants within these sets of equations are dependant on the wort composition and pitching rates used. The variable in each of these derived equations is time. They are then incorporated into a mechanistic mathematical model which predicts fructose utilization based on the kinetics of the various membrane transport systems in S. cerevisiae 2036 which are involved in fructose transport.

7.3.1. The Biomass

Growth of the yeast in the fermenter normally causes the inoculum size, or pitching rate, to increase approximately 3 - 4 fold until the time of flocculation when the yeast cells in the wort start to precipitate to the bottom of the fermenter. Pitching rates of 3g/litre,

used in normal and high gravity laboratory fermentations using sucrose adjunct wort. The specific growth rate constants for the high gravity fermentation pitched at 6g/litre of yeast, were k_1 = 0.0085 1/h and k_2 = 0.0315 1/h. The equations below predict the increase in biomass, up to flocculation and the decrease thereafter to the end of fermentation.

$$X_t = X_o.e^{k_1.t} \qquad (t<t_{c_1})$$

$$X_t = X_o.e^{k_1.t_{c_1} - k_2(t - t_{c_1})} \qquad (t>t_{c_1})$$

X_t = Biomass concentration at time t.
X_o = Biomass concentration at time zero.
k_1 = Specific growth rate over time range $t<t_{c_1}$.
k_2 = Specific flocculation rate over time range $t>t_{c_1}$.
t = Time.
t_{c_1} = Cross-over time

7.3.2. Glucose Concentration

Glucose utilization occurs at a relatively slow rate during the initial stages of fermentation up to approximately 75 hours i.e. cross-over time t_{c2}, and thereafter utilization occurs more rapidly until the end of fermentation. The specific utilization rate constants for the high gravity fermentations pitched at 6g/litre of yeast, are k_3 = 0.0076 1/h and k_4 = 0.0202 1/h. The equations below predict glucose utilization at the various pitching rates used.

$$G_t = G_o.e^{-k_3.t} \qquad (t<t_{c_2})$$

$$G_t = G_o.e^{-k_3.t_{c_2} - k_4(t - t_{c_2})} \qquad (t>t_{c_2})$$

G_t = *Glucose concentration at time t.*
G_o = Glucose concentration at time zero.
k_3 = Specific utilization rate over time range $t<t_{c_2}$
k_4 = Specific utilization rate over time range $t>t_{c_2}$
t = Time.
t_{c_2} = Cross-over time

7.3.3. Maltose Concentration

Maltose utilization follows a similar pattern to that of glucose utilization. In the initial stages of fermentation maltose utilization is relatively slow, i.e. up until the cross over time, t_{c3}, where t_{c3} = 67 hours. After this period utilizaton becomes more rapid and remains so until the end of fermentation. The *specific utilization rate constants for the high gravity* fermentation pitched at 6g/litre of yeast are k_5 = 0.00206 1/h and k_6 = 0.0294 1/h. The equations below predict maltose utilization in the laboratory fermentations.

$$M_t = M_o.e^{-k_5.t} \qquad (t<t_{c_3})$$

$$M_t = M_o.e^{-k_5.t_{c_3} - k_6(t - t_{c_3})} \qquad (t>t_{c_3})$$

M_t = Maltose concentration at time t.
M_o = Maltose concentration at time zero.
k_5 = Specific utilization rate over time range $t<t_{c_3}$
k_6 = Specific utilization rate over time range $t>t_{c_3}$
t = Time.
t_{c_3} = Cross-over time

7.3.4. Ethanol Concentration

Ethanol production occurs at a relatively rapid rate during the first half of the fermentation, i.e. until the cross over time, t_{c4} = 90 hours. Thereafter it is produced much more slowly until the end of fermentation. The final concentration in the fermented wort is dependant on the pitching rate, with the range being 5-6% (v/v) for normal gravity fermentations and 7.5 - 8.2% (v/v) for high gravity fermentations. The specific production rate constants for the high gravity fermentation pitched with 6g/litre of yeast, are k_7 = 0.0288 1/h and k_8 = 0.0031 1/h. Ethanol production under these fermentation conditions in the laboratory fermentation system is predicted by the equations below.

$$a_t = a_o.e^{k_7 t} \qquad (t<t_{c4})$$

$$a_t = a_o.e^{k_7 t_{c4}} + k_8(t - t_{c4}) \qquad (t>t_{c4})$$

a_t = Ethanol concentration at time t.
a_o = Ethanol concentration at time zero.
k_7 = Specific production rate over time range $t<t_{c4}$.
k_8 = Specific production rate over time range $t>t_{c4}$.
t = Time.
t_{c4} = Cross-over time

7.3.5. Fructose Utilization

The development of a mechanistic mathematical model for fructose utilization is based on the fact that the rate-limiting step in glycolysis is at the cell membrane. Thus the kinetics of the various membrane transport systems transporting sugars into the cell, in effect will be the pacemakers olysis (16,54,100,176,

179). If the kinetic data are determined experimentally, then one should be able to predict the rate of utilization of the particular sugar, in this case fructose, under investigation. Membrane transport studies have shown that fructose uptake is via a biphasic transport system in S. cerevisiae 2036 (33), of which the first component is a proton symport of high affinity, and low capacity with Km = 0.3 mM and V_{max} = 0.6 mmoles/h.g dry weight respectively (6.3.2.) (figure 59 and 60). The second component is a lower affinity, facilitated diffusion system which transports fructose and glucose and other structurally related sugars (36). The fructose affinity for this transport system is Km = 8.3 mM. This is a mean value calculated from several determinations. Glucose competitively inhibits fructose transport with Ki = 1.15 mM (figure 67). Maltose non-competitively inhibits fructose transport with Ki = 21.3 mM. Maltose transport is via a proton symport, independant of fructose transport and has a Km = 17.4 mM. Ethanol non-competitively inhibits fructose uptake of both the proton symport with k = 0.74 L/mol and the facilitated diffusion system with k = 0.89 L/mol. The V_{max} for fructose transport is taken to be 0.275 mmoles/h.g dry weight of yeast. The mathematical model takes the form of two expanded Michaelis-Menten equations, which describe the factors affecting fructose uptake via the facilitated diffusion system, and the fructose proton symport (figure 74). All of the above experimental parameters are combined within the model. The terms for the biomass, glucose, maltose and ethanol concentrations are obtained by substituting into the model the equations obtained which predict each one of these parameters. The model relies on the approximation that all the parameters affecting fructose utilization will be constant for a suitably small time interval. Here 2 hour intervals are used. Thus for time = 0, all the parameters are substituted into the equation (figure 74), and the change in fructose concentration,

$$-\frac{dF}{dt} = V^{f_1}_{max} X_t e^{-k_1 a_t} \left[\frac{F_t}{Km_f + \frac{Km_f G_t}{Ki_g} + F_t} \right] + V^{f_2}_{max} X_t e^{-k_2 a_t} \left[\frac{Km_m}{Ki_m + M_t} \right] \left[\frac{F_t}{Km_f + F_t} \right]$$

F_t = Fructose concentration.

G_t = Glucose concentration.

M_t = Maltose concentration.

X_t = Biomass concentration.

a_t = Ethanol concentration.

Km_f = Michaelis constant of fructose transport.

Km_g = Michaelis constant of glucose transport.

Km_m = Michaelis constant of maltose transport.

Ki_g = Inhibition constant of competitive inhibition of fructose transport by glucose.

Ki_m = Inhibition constant of non-competitive inhibition of fructose transport by maltose.

$V^{f_1}_{max}$ = Maximum velocity of fructose transport for the facilitated diffusion system.

$V^{f_2}_{max}$ = Maximum velocity of fructose transport for the fructose proton symport.

k_1 = Inhibition constant of fructose transport for the facilitated diffusion system.

k_2 = Inhibition constant of fructose transport for the fructose *proton symport.*

$$F_t = F_{t+1} - \frac{dF_t}{dt}$$

F_t = Fructose concentration at time t.

F_{t+1} = Fructose concentration at time t+1.

t = Fixed time interval.

Figure 74. A mathematical model which predicts fructose utilization in a laboratory fermentation system using normal and high gravity sucrose adjunct wort.

"-dF/dt" is determined. This value is substituted into the second equation and a fructose concentration for time = 1 is obtained, which is then substituted back into the first equation to determine "-dF/dt" for time = 1, which in turn is used to calculate the fructose concentration for time = 2. Thus the model continues in a cyclic mode until fructose concentration is predicted for the time period of the fermentation. The manipulation of all the equations was performed using a computer program, written in BASIC, on an Hewlett-Packard HP 9845 B computer. A program for the prediction of fructose utilization in a high gravity sucrose adjunct wort fermentation using a pitching rate of 6g/litre of yeast appears on the following page. Similar programs exist for the pitching rates of 3g/litre and 12g/litre as well as the normal gravity fermentations.

7.4. Results

Plots of fructose utilization in the high gravity wort fermentations at pitching rates of 3g/litre, 6g/litre, and 12g/litre of yeast, obtained from experimental data by HPLC analysis of samples show close correlation with the plots of fructose utilization predicted by the model for these fermentations. An example of this correlation is shown in figure 76 for the fermentation pitched at 6g/litre of yeast. Similar correlation exists for the normal gravity wort fermentations.

7.5. Discussion

Fructose utilization is accurately predicted in laboratory fermentations when using normal and high gravity sucrose adjunct worts, by a mechanistic mathematical model. The model can be a useful scientific tool as information concerning fructose utilization is combined into a mathematical form which is then able to

```
1      PRINT "INPUT THE INITIAL VALUE FOR F"
2      INPUT F
3      PRINT "INPUT THE TIME INTERVALS "
4      INPUT To
5      PRINTER IS 16
6      PRINT "INPUT FIRST VALUE OF T"
9      INPUT T1
10     PRINT "INPUT SECOND VALUE OF T"
20     INPUT T2
21     FOR S=T1 TO T2 STEP To
22     PRINTER IS 16
23     T=S
30     IF T<106.240 THEN GOTO 40
31     IF T>106.240 THEN GOTO 50
40     Xt1=6.0200*EXP(.0085*T)
41     GOTO 51
50     Xt1=6.0200*EXP(.0085*106.240-.0915*(T-106.240))
51     PRINT "Xt1 = ";Xt1
60     IF T<106.240 THEN GOTO 72
61     IF T>106.240 THEN GOTO 74
72     Xt2=6.0200*EXP(.0085*T)
73     GOTO 75
74     Xt2=6.0200*EXP(.0085*106.240-.0915*(T-106.240))
75     PRINT "Xt2 = ";Xt2
76     IF T<89.735 THEN GOTO 120
77     IF T>89.735 THEN GOTO 122
120    At1=EXP(-.89*(.06898*EXP(.0288*T)))
121    GOTO 130
122    At1=EXP(-.89*(.06898*EXP(.02875*89.735+.0031*(T-89.735))))
130    PRINT "At1 = ";At1
131    IF T<89.735 THEN GOTO 142
132    IF T>89.735 THEN GOTO 144
142    At2=EXP(-.74*(.06898*EXP(.0288*T)))
143    GOTO 145
144    At2=EXP(-.74*(.06898*EXP(.02875*89.735+.0031*(T-89.735))))
145    PRINT "At2 = ";At2
170    IF T<77.7319 THEN GOTO 180
175    IF T>77.7319 THEN GOTO 200
180    Gt=251.275*EXP(-.0076*T)
190    GOTO 210
200    Gt=251.275*EXP(-.0076*77.7319-.0202*(T-77.7319))
201    GOTO 210
210    PRINT "Gt = ";Gt
240    IF T<67.0409 THEN GOTO 270
250    IF T>67.0409 THEN GOTO 290
270    M=17.0/(21.3+117.897*EXP(-.00206*T))
280    GOTO 400
290    M=17.0/(21.3+117.897*EXP(-.00206*67.0409-.0294*(T-67.0409)))
300    GOTO 400
400   PRINT "M = ";M
410    Dt1=.275*Xt1*At1*F/(Gt*(8.3/1.15)+8.3+F)
411    PRINT "Dt1= ";Dt
420    Dt2=.6*Xt2*At2*M*F/(.3+F)
421    PRINT "Dt2 = ";Dt2
422    Dt=Dt1+Dt2
430    PRINTER IS 0
432    R=T/24
433    Rt=INT(R)
434    IF Rt<>R THEN GOTO 447
440    PRINT "VALUE OF dF/dt = ";Dt;" USING A VALUE OF T OF ";T
446 PRINT F,T
447    PRINTER IS 16
450    Df=F-Dt*To
500    F=Df
501    NEXT S
550    END
570    RETURN
```

Figure 75. A computer program of the mathematical model which predicts fructose utilization. Here the program predicts fructose utilization at a pitching rate of 6g per litre of yeast, using a high gravity sucrose adjunct wort.

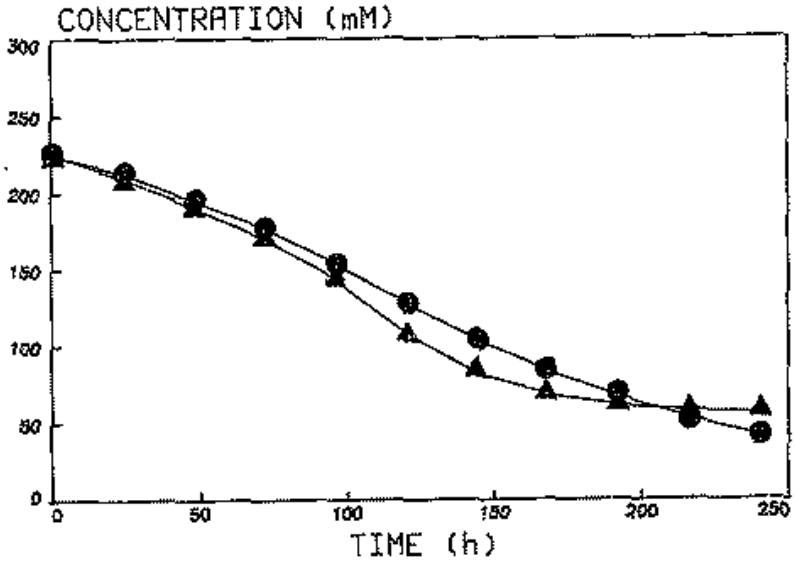


Figure 76. Plots of fructose utilization obtained from experimental data of a *high gravity* sucrose adjunct wort fermentation at a pitching rate of 6g/litre of yeast ● , compared to the utilization rate predicted by the mathematical model ▲ .

quantitatively predict fructose utilization which was not previously possible. The model may be considered a true one as the underlying assumptions from which it was developed have been verified by experimental data obtained from membrane transport studies of S. cerevisiae 2056 in the laboratory. Thus fructose utilization in laboratory scale fermentations using normal and high gravity sucrose adjunct wort, is dependant on several factors which operate in unison. These are the affinity of fructose for the fructose proton symport and the facilitated diffusion system; the competitive inhibition of fructose uptake by glucose via the facilitated diffusion system; non-competitive inhibition of fructose uptake via the symport by maltose; the non-competitive inhibition by ethanol of fructose uptake via the fructose proton symport and the facilitated diffusion system; and the increase of the biomass up to flocculation, and its subsequent decrease thereafter.

CHAPTER 8.

CONCLUSION

In all fermentations set up with equal mixtures of glucose and fructose as adjuncts, glucose was preferentially utilized over fructose. A consequence of the slower rate of fructose utilization is that at the end of fermentation not all the fructose has been fermented and residual concentrations of fructose remain in the beer above the taste threshold for fructose, 300 mg /litre, and taint the beer with a sweet off-flavor. Glucose is utilized at a faster rate than fructose and thus it is all fermented before the end of fermentation. Comparison of the fermentations using various grades of sucrose adjuncts reveal no differences amongst the rates of fructose utilization. It is highly unlikely that impurities in the sucrose somehow "block" either fructose uptake or its subsequent utilization to a significant degree. The reason for the preferential utilization of glucose over fructose is to be found at the yeast cell membrane. Several authors (16,54,100, 118,176) have observed that the rate-limiting step in glycolysis is the transport of sugars across the cell membrane by the membrane transport systems. The kinetics of these systems are effectively the pacemakers of glycolysis (16) and if all the kinetic parameters which affect fructose uptake are known, then these data can be used to predict the relative rates of fructose utilization in fermentation.

The uptake of fructose in S. cerevisiae 2036 is via a biphasic transport system in which the first component is a low capacity, V_{max} = 0.6 mmoles/h/g dry weight of yeast, high affinity, Km = 0.3 mM, proton symport. Glucose is not taken up via the symport neither does its presence affect fructose transport via the symport in

maltose grown cells exhibit very weak proton signals when induced with fructose but strong proton signals occur with maltose induction. The two independant symports compete for protons in order to transport their respective sugars across the cell membrane. Ethanol has a non-competitive inhibitory effect on fructose uptake via the symport with k = 0.74 L/mol. The stoichiometry of uptake is one proton per molecule of fructose. The second component of fructose uptake is a lower affinity higher capacity facilitated diffusion system which transports fructose, glucose and other structurally related sugars. Glucose competitively inhibits fructose uptake with Ki = 1.15 mM, while fructose exerts a decreased competitive inhibitory effect on glucose uptake of Ki = 36.0 mM. Ethanol non-competitively inhibits fructose and glucose uptake with k = 0.89 L/mol and k = 0.96 L/mol respectively. L-sorbose, a non-metabolizable sugar of S. cerevisiae is taken up via both components of fructose transport. It was used to determine whether active sugar transport exists in the fructose proton symport : sorbose accumulated approximately 25 fold within the cell at pH 4.5. Accumulation of sorbose within the cell implies secondary active transport has to be via the proton symport as facilitated diffusion is a passive system, and is not able to accumulate sugars against a concentration gradient.

The relative rates of glucose and fructose utilization in wort and defined media fermentations under various conditions show close correlation with the kinetics of the membrane transport systems. Wort and defined media fermentations which contained equal concentrations of glucose and fructose exhibited preferential utilization of glucose over fructose. This corresponds with membrane transport data which indicates that glucose competitively inhibits fructose uptake. Defined media

fermentations containing either fructose or glucose showed glucose was utilized faster than fructose at all concentations tested. Membrane kinetic data reveals the glucose affinity is approximately 4 fold greater than fructose affinity for the facilitated diffusion system. Although fructose has a higher affinity for the proton symport than glucose does for the facilitated diffusion system, the proton symport has a lower capacity and at the concentrations of glucose and fructose tested it quickly becomes saturated causing most of the fructose to be transported via the facilitated diffusion system. The difference in glucose and fructose utilization in defined media with maltose, is similar to the differences found when either glucose or fructose are the only carbohydrates present.

The kinetics of all the membrane transport parameters which affect fructose uptake are incorporated into a mathematical model which is able to predict fructose utilization in laboratory scale fermentations using normal and high gravity, sucrose adjunct wort. The model takes into account the increase in biomass up to flocculation and its subequent decrease thereafter as this parameter appears to be important in overcoming the fructose block. Glucose and maltose utilization, and ethanol production during the time course of fermentation are also included in the model. The variable in the model is time. A computer program of the model written in BASIC calculates fructose utilization for small time increments of 2 hours for the duration of fermentation. Comparison of the rates of fructose utilization predicted by the model with experimental data, reveal that a close correlation exists.

Growth of *S. cerevisiae* 2056 under aerobic conditions using maltose with either glucose or fructose do not show typical diauxic growth pattern. HPLC analysis

reveals that maltose and either glucose or fructose are utilized simultaneously indicating that catabolite repression exerted by glucose on maltose transport and its subsequent catabolism to glucose does not occur in this yeast strain. This was further confirmed by membrane transport studies in which glucose and fructose grown cells exhibited strong proton signals when induced with maltose. Analysis of fermentation data reveals that maltose utilization is not affected by the concentrations of fructose or glucose adjuncts. If catabolite repression did occur, the lag phase for maltose utilization would increase with increasing concentration of the two sugars. Only once the cut-off concentration for either glucose or fructose is reached i.e. the concentration below which catabolite repression does not exert its effect, would maltose then begin to be utilized at a much faster rate than before. However this situation does not occur in S. cerevisiae 2036. These data confirm that this strain is de-repressed. Catabolite repression by glucose of maltose transport and its subsequent catabolism to glucose appears to be a common occurrance in S. cerevisiae (67). De-repressed yeast strains have an economic advantage in a brewery as glucose and maltose would be used simultaneously, rather than sequentially as in repressed strains, which implies faster fermentation times.

Fermentations set up with glucose, fructose, and equal mixtures of glucose and fructose as adjuncts, showed that the rate of utilization of each sugar increased with an increase in the sugar adjunct concentration. However, the increased rate of utilization of the sugars did not sufficiently compensate for the higher sugar adjunct concentrations and consequently with increased sugar concentrations in wort, the fermentations took longer to attenuate to the point where glucose was completely utilized and fructose remained in the beer at

residual concentrations of below 300 mg/litre. Thus for the normal fermentation time of approximately 240 hours (10 days), the residual fructose concentrations in samples taken at this time increased with increasing adjunct concentrations. This phenomenon has important consequences for the brewery as the premise for using higher gravity wort is that more beer can be produced from existing plant. If this is not true, and the higher gravity worts take longer to ferment - as shown here using present temperatures and pitching rates - then the raison d'etre for higher gravity worts is negated. The longer fermentation times would be offset against the increased beer production from the higher gravity worts, and the net increase in the quantity of the beer produced maybe insignificant. If higher gravity worts are to be used in fermentation, then techniques will have to be devised to keep the length of fermentation to the present period of approximately 240 hours (10 days).

The problem of reducing the concentrations of residual fructose in the wort at the end of fermentation can be approached in several different ways. The pitching rate is inversely proportional to the concentrations of residual fructose for the last three days of fermentation. Increased pitching rates will result in decreased residual fructose concentrations and vice versa. The addition of growth stimulants such as nutromix, a commercial preparation of nitrogen sources plus vitamins, or pulses of oxygen during the initial stages of fermentation which is used in the production of sterols, chiefly ergosterol, both caused increased fructose utilization and consequently decreased fructose concentrations at the end of fermentation. The addition of a single nitrogen source, in the form of either a salt, ammonium sulphate, or a readily utilizable amino acid, serine, or yeast nitrogen base, exhibited only

modest reductions in residual fructose concentrations when compared to the effects produced by nutromix. It appears that the nitrogen and vitamins contained in nutromix act in a synergistic way, and it is unlikely that fructose utilization can be dramatically stimulated by a single factor irrespective of whether it be a nitrogen or vitamin source. The addition of 3 hour bursts of oxygen for the first 3 days of fermentation produced the most dramatic reductions in residual fructose. By 168 hours (day 7) of the fermentation all the fructose in the wort had been utilized. Oxygen is known to be essential in the biosynthesis of sterols (9,99), and bursts of oxygen added to the fermenting wort probably cause an increase in sterol production. Sterols are components of the yeast cell membrane and they have a physiological function in that they add fluidity to the cell membrane by preventing membrane lipids from undergoing major phase transitions, which aids in cell growth. The yeast in the wort is still metabolically active to a small degree at the end of fermentation. The extension of the fermentation time from 240 to 336 hours produces fructose utilization in this period and reduces residual fructose concentrations in the wort. The above methods may be used singly or in combination to ameliorate the "fructose block". However the economics of extended fermentation times; the application of oxygen and nutrient sources to fermention vessels and the elevation of pitching rates must be determined in each brewery system. The mathematical model developed should help to ascertain these facts as biomass is an important variable in the model.

CHAPTER 9.

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