NHIBITION OF THE LACTOSE TO ETHANOL FERMENTATION OF *KLUYVEROMYCES MARXIANUS* Y113 AND ATTEMPTS AT ITS ALLEVIATION THROUGH MEDIA IMPROVEMENT.

BY

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ABSTRACT

Inhibition of the lactose to ethanol fermentation of *K.marxianus* Y113 was investigated. The use of initial lactose concentrations of 150 g/litre or greater resulted in less biomass accumulation, lower ethanol productivity and incomplete substrate utilisation.

Keeping the initial lactose concentration at 100 g/litre but increasing the medium osmolality by up to 5 times via the addition of non-utilised salt or maltose resulted in substantially reduced biomass accumulation and slightly lower ethanol productivity. This suggested that high medium osmolality inhibits the yeast in a non-specific way by increasing the energy required for cell maintenance at the expense of biomass production.

Keeping the intial lactose concentration at 100 g/litre but adding up to 5% (by weight) ethanol reduced the amount and rate of biomass accumulation and led to incomplete substrate utilisation, as well as dramatically lowering the amount of ethanol produced by the yeast itself. The detrimental effects of added ethanol became significant only when more than 2 to 3% (by weight) was added.

A maximum alcohol concentration of 4 to 5% (by weight) was observed in all cases, irrespective of the concentration of ethanol added initially. These results suggested that the ethanol inhibited the energy metabolism of the cell in some specific way and did not merely increase the requirement for cell maintenance energy.

In the concentrations tried supplementation of the medium with yeast extract, magnesium, calcium and chitin all failed to produce any change in the performance of the fermentation. Supplementation with still bottoms was found to be quite strongly inhibitory to the fermentation.

Demineralisation of the whey permeate medium reduced that the performance of the fermentation compared to that carried out on standard whey permeate medium. *K.marxianus* Y113 was able to ferment a medium of defined composition but the biomass growth, ethanol productivity and lactose utilisation were not as good as those achieved using complex media such as whey or lactose broth. increasing the concentration of nutrients in the defined medium was of small benefit but the performance was still well below that seen on complex media.

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1. INTRODUCTION

The conversion of wheys and whey ultrafiltrate permeates to ethanol has been well established as a commercial operation in New Zealand for more than a decade (Mawson 1987). On a worldwide basis this fermentation is not a large source of ethanol and its importance in New Zealand has arisen as a result of particular economic factors of importance here.

New Zealand's dairy industry is relatively large and modern and the processing plants tend to be large and centralised. This means that significant quantities of wheys are produced at single points and their treatment and disposal by conventional means is expensive.

Whey is quite a valuable end product and can be converted to a number of high value food grade products (see chapter 2.2.4) but many of these have markets of limited size. Ethanol is a commodity chemical that is produced in large quantities throughout the world. The usual raw materials are grains and other plants such as beets or sugarcane but in New Zealand pastoral farming is dominant and large quantities of these crops are not available for fermentation. These factors led to the production of ethanol from whey becoming an economic process here.

The Biotechnology Department at Massey University has conducted research into the use of whey as a raw material for commercial fermentations for some years. As well as ethanol (Mawson and Taylor 1989; Mawson 1990, Boontanjai 1983), citric acid, (Hossain, *et al*, 1984, Hossain, *et al*, 1985, Maddox, *et al*, 1986), butanol, acetone and ethanol (Qureshi and Maddox, 1987) and 2,3-butane diol (Lee and Maddox 1984), have all been investigated as potential products of whey fermentations.

The whey (permeate) - to - ethanol fermentation is interesting in terms of research because potentially it's use could become much more widespread than it is at present if the productivity and concentration of ethanol produced were increased.

Commercial operations use unconcentrated whey or whey permeate, containing around 45 g/litre lactose, to produced beers with ethanol contents of about 2 to 2.5% (w/w). This is low compared to the ethanol concentrations resulting from around 10% (w/w). This lower alcohol concentration in whey fermentations leads to higher distillation costs and makes the process less attractive economically.

It has long been known that ethanol is inhibitory to the metabolisms of many cells and it is thought that ethanol inhibition is responsible for the inability of lactose fermenting organisms to produce very high concentrations of this alcohol. The object of this thesis was to confirm this and to get some idea of the magnitude of inhibition experienced by *Kluyveromces marxianus* Y113.

Another aim was to investigate the role of low water activity in the inhibition of this yeast, as it has been suggested by Jones (1987) that this may be the chief limiting factor for some yeast strains used to produce ethanol.

These investigations involved using *K.marxianus* Y113 to ferment lactose media to produce ethanol. After some experiments with varying the initial lactose concentration (section 4.2) fermentations were carried out using media containing 100 g/litre lactose along with varying concentrations of maltose - a dissacchaside which is not utilised by the yeast but which by it's presence, lowered the water activity (section 4.3). The biomass, ethanol and lactose concentrations were monitored and the results gave an indication of the inhibition caused by substrate dissolved in the media.

In the next experiments different amounts of ethanol were added to the media prior to fermentation (section 4.4). The inhibition resulting the relative contributions of both inhibitory effects could be compared.

Numerous media additives have been suggested which are claimed to reduce the inhibitory effects of high alcohol concentration (see section 2.2.5.7). It was thought that investigating these claims would be of interest and as nutritional studies are best carried out using media of defined compositions it was decided to try and develop one suitable for *K.marxianus* Y113.

The recipes for Difco yeast media (Difco Manual, 1953), were used as a basis and preliminary investigations involved leaving out certain ingredients such as amino acids and vitamins to see which ones were vital to the growth of the yeast (section 5.6). The medium developed from this work never produced a fermentative performance equal to non-defined media such as lactose broths or whey permeates however, so the latter type were chosen as base media for further studies with additives.

In some of the initial experiments (see section 4.2) the yeast extract ratio was varied in proportion to the amount of lactose initially present in the media. An experiment was carried out to find out if this was necessary (section 5.2). In this the initial lactose concentration was kept at 200 g/litre and the amount of yeast extract added was varied between 3 g/litre and 12 g/litre. The results of these fermentations showed that the yeast extract could be kept as low as 3 g/litre when concentrations of lactose up to 200 g/litre were used, without detrimental effects.

With the detrimental effects of high media osmolality suggested by the results of previous experiments (see section 4.2) it was

thought that reducing the ash content of the whey permeate media may have been beneficial to the fermentation. This was tested by using demineralised whey permeate as a fermentation medium. However, it did not produce fermentation results as good as the whey permeate medium, which suggested that some vital nutrients' concentration was lowered during demineralisation, and that this neutralised any advantage which may have been gained by the lowered medium osmolality.

Still bottoms contain the remains of biomass that was formed during the fermentation process and are rich in complex organic compounds. It was thought that they would be a good supplement for the whey permeate fermentation under study and this was tried in the experiments described in section 5.4.

However, it was found that still bottoms led to lowered biomass and ethanol production and reduced lactose consumption. The results suggested that the still bottoms contained an inhibitory compound which prevented any benefit being gained by the addition of the other nutrients it contained.

A number of other supplements have been suggested by various workers for the improvement of ethanol fermentations, and experiments were conducted to test these with *K.marxianus* Y113. (Nobais, *et al*, 1988, Patil and Patil, 1989, Pament and Desri, 1990) Chitin, Calcium Chloride and Magnesium Chloride were all tried (see section 5.5).

The results showed that none of them were of use in fermentations using either lactose broth or whey permeate media and neither biomass nor ethanol production were increased by their addition.

Overall, the results of these studies confirmed that high ethanol concentration is the main inhibitory factor in the *K.marxianus* Y113 lactose - to - ethanol fermentation. They

also showed that medium osmolality is of some importance. None of the supplements tried did anything to improve the fermentations performance (in the concentrations used for these experiments), and demineralisation of the whey permeate by electrodialysis was detrimental.

2. ETHANOL PRODUCTION FROM WHEY - LITERATURE REVIEW

2.1 GENERAL ASPECTS OF ETHANOL PRODUCTION

2.1. 1. Introduction

Ethanol serves a multitude of uses in the modern world. In industry it's low cost and relatively non-toxic nature have led to ethanol being favoured over other solvents with similar chemical or physical properties.

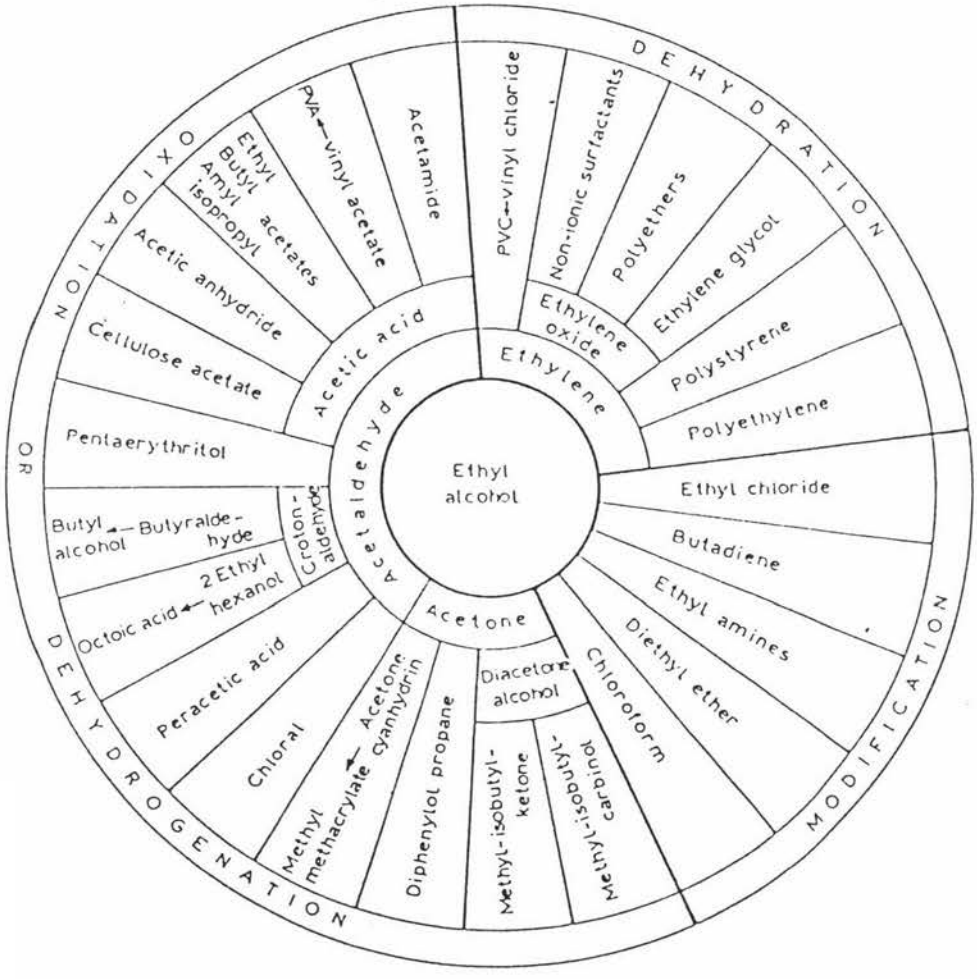
It's longest and most popular use has been as a beverage. Evidence of the production of alcoholic drinks goes back several thousand years in some Middle Eastern areas and the practice itself undoubtably dates from somewhat earlier still. Today the consumption of alcohol lays an important part in most western cultures.

Ethanol is an important chemical intermediate used in the manufacture of fats, waxes, olefins, ethers, detergents, plastics, lacquers, lubricants, plasticizers, emulsifiers and numerous other substances (Maiorella, 1985). (See figure 2.1 for compounds which can be made from ethanol).

Prior to the development of the petrochemical industry ethanol was produced by the fermentation of carbohydrates by yeasts. From the 1930's however, ethanol produced from the hydration of ethane became considerably cheaper and was soon the predominant source of industrial ethanol. The demand for potable, fermentation - derived alcohol for the food industry remained reasonably steady however (except in periods of prohibition). (Maiorella, 1985)

FIGURE 2.1 CHEMICAL DERIVATIVES FROM ETHANOL

(PATURAN, 1989)



During the past century the use of ethanol as a fuel for motor vehicles has been considered periodically. The last such occasion has been the years since 1974, when the price of crude oil increased considerably and pushed the price of petrol up with it. The interest stimulated in alternative fuels manufactured from renewable resources continued at a reasonable level, even after the price of oil dropped again, as people realised that supplies could not be guaranteed in the long term.

Research and development of ethanol fermentations has been aided also by the commitment of countries such as the U.S.A. and Brazil to supplement part or all of their vehicle fuels with ethanol. Under present economic circumstances government subsidies or favourable tax regimes are required to make alcoholic fuels cost-competitive with gasoline.

In the United States gasohol - a petrol blend containing 10% ethanol - is produced and sold at a lower price than standard fuel. Brazil's program is based on the fermentation of sugar cane, cassava and other starchy crops (Pimentel, 1980; Liquid Fuels Trust Board, 1984).

However, the Brazilian program has foundered in recent years due to the low price of oil and the high price that sugar cane is currently fetching on world markets. Growers are directing their crops away from ethanol production creating a shortage which must be filled using a petrol/methanol blend. Many car owners have been converted back to straight petrol fuel in response (Vanvolsem, 1990).

Economic factors, especially the relatively low cost of oil at present, mean that projects as ambitious as Brazil's are unlikely to be instigated elsewhere for a number of years. However, it is extremely unwise to rely totally on oil fuels and it is therefore vital that research into the production of ethanol by fermentation is continued in preparation for the time when such technology is needed to ensure a smooth transition from petrochemical to fermentation - derived fuels.

Growing scientific and public awareness of the need to use renewable resources and to recycle materials formerly regarded as waste (such as whey) is an important factor also in encouraging research in this area.

2.1 2. Biochemistry

The formation of ethanol from carbohydrates takes place via the Embden-Meyerhof-Parnas gylcolytic pathway (figure 2.2). The major substrate is glucose but other saccharides such as Xylose are also fermented by various organisms (Kosaric, *et al*, 1990). The use of substrates other than glucose requires the fermenting organism to possess the correct enzyme or enzymes to convert them to glucose 6-phosphate, fructose 6-phosphate or some other intermediate which can then enter the EMP pathway.

Glycolysis produces pyruvate as an end product. This compound generally is metabolised by one of two major metabolic pathways, depending on a number of variables. Most important among these are oxygen and sugar concentrations.

The reactions of glycolysis yield ATP (adenosine triphosphate) via substrate level phosphorylation, that is, ATP is formed from ADP through direct participation in the glycolysis reactions and not via some intermediate as occurs in oxidative phosphorylation.

These energy-yielding reactions are oxidative and require an electron carrier to accept the electrons they release. This carrier is NAD (Nicotinamide Aderine Dinucleotide), a co-factor of limited concentration in the cell. As the NAD concentration is reduced by glycolysis it must be regenerated at an equal rate to allow metabolism to continue.

Under conditions allowing the TCA cycle to operate the reduced NADH is re-oxidised to NAD via oxidative phosphorylation, during which oxygen is reduced.

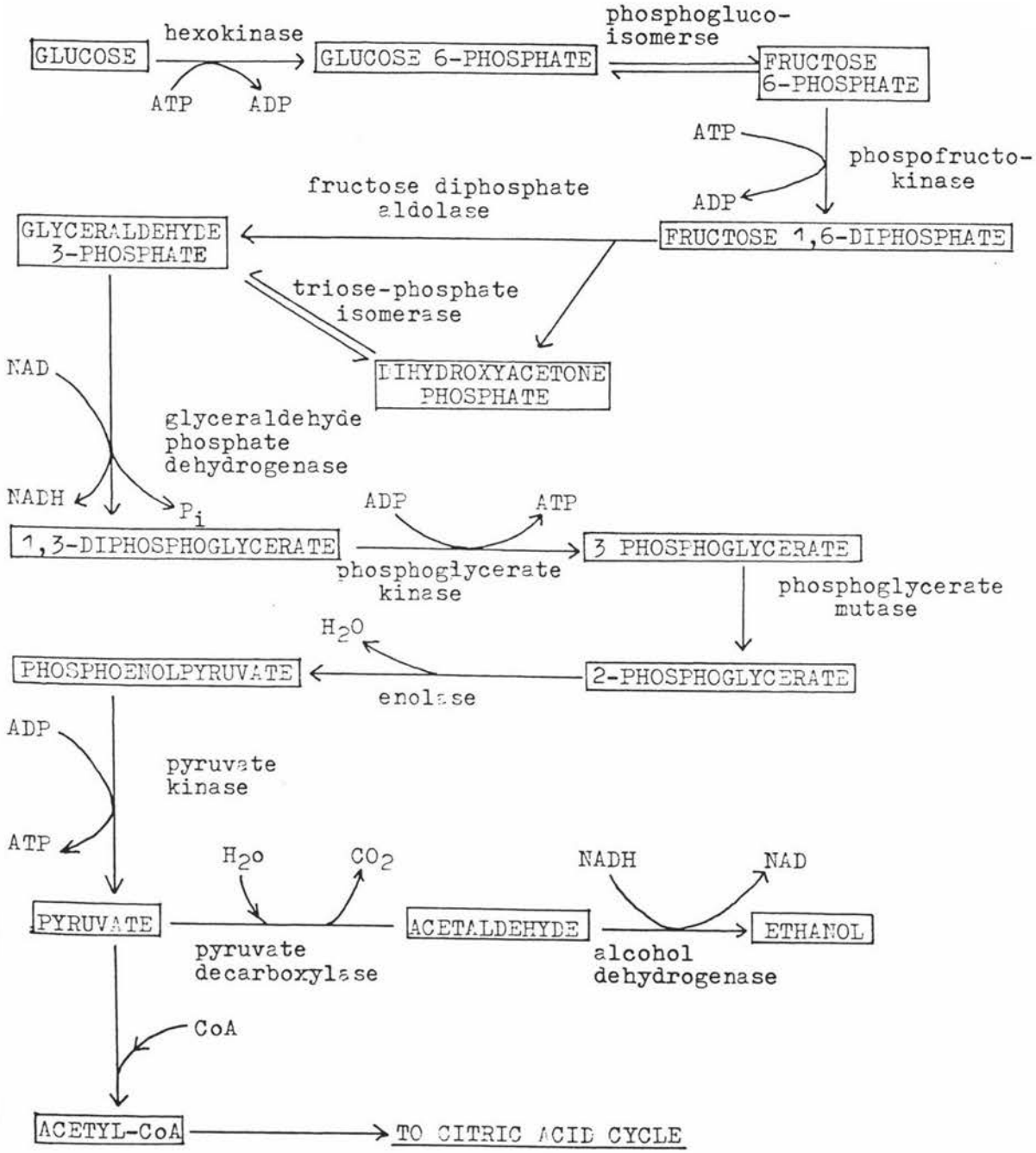
In an absence of oxygen the compound reduced is pyruvate. In yeasts it is decarboxylated to form acetaldehyde which is then reduced to ethanol (see figure 2.2) (Stryer 1981).

The stoichiometry for the oxidation of glucose to ethanol is given by the Gay-Lussac equation:

$C_6H_{12}O_6$ ---------- $2\ C_2H_5OH + 2\ CO_2$

Glucose Ethanol Carbon Dioxide

FIGURE 2.2 EMBDEN-MEYERHOF-PARNAS GLYCOLYTIC PATHWAY.



The maximum yield is 51.1% (w/w) but in practice this is never achieved due to the conversion of some of the glucose to cell biomass along with other by-products such as glycerol, higher alcohols (fusel oils) and succinate. These compounds often are important contributors of flavour and aroma in alcoholic beverages (Harrison and Graham, 1970).

.1 3. Microbiology and Substrates

The microbiology of ethanol production depends very much on the substrates used and the two cannot really be considered separately.

The most widely used organisms for ethanol production are species of the yeast genus *saccharomyces* and more especially *S.cerevisiae S.ellypsoideus* and *S.carlsbergensis*, along with *Schizosaccharomyces pombe* (Maiorella, 1985). While these strains can ferment most hexose sugars and combinations of them such as sucrose or maltose, they are unable to utilise other significant saccharides including lactose and pentoses such as arabinose and xylose (Lodder, 1970).

The *Saccharomyces* strains also cannot ferment starch directly and this polymer - a common source of sugars for ethanol production, found in numerous grains, potatoes, jerusalein artichoke, cassava, etc - must first be hydrolysed using either malt diastase or microbial amylase enzymes from species such as *Aspergillus oryzae, A.niger* or *Endomycopsis bisporia* (Maiorella, 1985).

Cellulosic materials are extremely common waste products but as yet there is no successful industrial scale use of them to produce ethanol. Cellulases and hemicellulases derived from *Trichoderma*, *Phanerochaeta* or *Fusarium* fungi are available but can be prohibitively expensive and often cannot act effectively on the cellulose because it is contained within a lignin sheath (Maiorella, 1985). However, techniques like steam explosion have shown some promise of overcoming this problem by removing the lignin physically.

Organisms that can ferment the sugars present in hydrolysed cellulose to produce ethanol include *Pachysolen tannophilus, Candida tropicalis, Fusarium spp, C.guilliermandii, C.terebra, Pichia guilliermandii, P.stipitis* and a number of other yeasts also. However, the production of ethanol generally is very low (Jeffries, 1983; Kurtzman, 1983).

Thermophilic, ethanol-producing organisms have attracted some interest due to a number of advantages that come with fermentations operated at increased temperatures. These include greater catabolic activity (and therefore higher productivity) lower oxygen solubility in the medium, increased substrate and nutrient solubilities, lower medium viscosity (therefore less energy required for agitation), enhanced ethanol recoverability from the gas phase and a decreased requirement for aseptic conditions (Kosaric *et al*, 1983).

One thermophile investigated for ethanol production is *Thermoanaerobacter ethanolicus*. It has been

reported to have an optimum growth temperature of 69 C and to have achieved yield coefficients of 0.14 (Yxs) and 0.46 (Yps) when grown on media containing 50 g/litre glucose plus yeast extract (Wiegel and Ljungdahl, 1981; Sonnleitner, 1983).

Problems with ethanol sensitivity in wild type strains have been overcome by selection, although there are still unwanted by-products (especially acetate) formed by some strains (Sonnleitner, 1983).

Zymomanas mobilis is a gram negative bacterium that has been actively investigated as an ethanol producer for a number of years. It can produce ethanol at significantly higher rates than yeasts and with greater yields. (Sen, 1989).

The bacterium uses the Entner-Duodorff Pathway to metabolise carbohydrates which means that it forms the same amount of ethanol but only half as much ATP as yeasts which use the Embden-Meyerhof-Parnas Pathway. Thus it forms less biomass which has the effect of increasing the ethanol yield (Sen, 1989)

Large scale pilot plant trials have been carried out with this organism and it will almost certainly become commercially important in the next few years (Doelle, *et al*, 1989; Millichip and Doelle, 1989).

2.2 WHEY AS A POTENTIAL SUBSTRATE FOR ETHANOL PRODUCTION

2.2 1. Introduction

Whey is a liquid by-product of the dairy industry. It contains sufficient concentrations of lactose and

protein to make it either a significant resource or a significant waste disposal problem. These days the emphasis is firmly on utilisation and a large proportion of whey - especially cheese whey - is now used to produce valuable products (see section 2.2.4).

2.2 2. Types and Compositions of Whey

Whey is the liquid obtained following the separation of the solids from coagulated cream, milk or skim milk. It is formed during the production of rennet and acid caseins and cheese. The wheys produced from rennet casein and cheese manufacture have pH values greater than 5.5 and are referred to as sweet wheys. Acid whey, produced during the manufacture of cottage cheese, lactic casein or sulphuric acid casein has a pH value less than five (Short 1978).

The compositions of all wheys varies slightly between individual areas and plants, and also changes over the production season (Matthews, 1978). A typical composition is that given in table 2.1.

The protein in whey comprises approximately 20% of the original protein present in milk and mainly comprises of - lactalbumin (21% of the protein), 3 - lactoglobulin (46%), serum albumin (5%), protease-peptone (19%) and immunoglobulin (9%). (Irvine and Hill, 1985).

The vitamin content of sweet and acid wheys has been analysed by Glass and Hendrick (1977). They found that the concentrations of the various vitamins varied quite a lot between sources and attributed this to differences in processing and storage

treatments between plants. Their mean results are given in Table 2.2.

TABLE 2.1 Typical Chemical Analysis of Sulphuric Casein Whey and Cheese Whey of Commercial Origin (from Radford, 1986; Short and Doughty, 1977).

	Sulphuric Casein Whey	Cheese Whey
Total Solids (%)	6.23	5.87
Lactose (%)	4.56	3.94
Protein (%)	0.53	0.57
Ash (%)	0.72	0.81
Nitrogen (%)	0.12	0.14
Inorganic Phosphorus (g/kg)	0.60	0.33
Total Phosphorus (g/kg)	0.63	0.39
Potassium (g/kg)	1.51	1.48
Sulphur (g/kg)	0.88	0.14
Calcium (g/kg)	1.04	0.47
Magnesium (g/kg)	0.10	0.09
Chlorine (g/kg)	1.03	1.19
Sodium (g/kg)	0.37	0.56
Boron (ug/kg)	0.35	0.11
Copper (ug/kg)	0.01	0.07
Iron (ug/kg)	0.14	0.36
Manganese (ug/kg)	<0.01	<0.01
Molybdenum (ug/kg)	<0.03	<0.03
Zinc (ug/kg)	2.06	0.14
Cobalt (ng/kg)	2-7	<4.00
Selenium (ng/kg)	1.00	1.30

TABLE 2.2 Analysis of Vitamin Content of Acid and Sweet Wheys (Glass and Hendrick, 1977).

Vitamin *	Sweet Whey	Acid Whey
Vitamin A (IU/100g)	136	107
Vitamin C (mg/100g)	1.41	0.33
Vitamin B_6 (mg/100g)	0.59	0.62
Vitamin B_{12} (ug/100g)	2.40	2.50
Tocopherol (mg/100g)	0.063	0.071
Thiamin (mg/100g)	0.510	0.49
Riboflavin (mg/100g)	2.14	1.85
Pantothenic Acid (mg/100g)	11.14	11.14
Biotin (ug/100g)	43.00	35.00
Niacin (mg/100g)	1.30	1.16
Folacin (mg/100g)	0.116	0.0332
Choline (mg/100g)	104	101

*Concentrations given per 100g dried whey.

Forsum and Hambaeus (1977) examined the nutritional qualities of wheys and various whey derivatives such as whey powders and concluded that they were a potentially valuable food supplement. They commented on their high digestibilities and their retention of good biological value even after undergoing heat treatment.

2.2 3. Whey and Whey Derivative Production

The quantity of whey produced in New Zealand has been increasing steadily over the past few decades (see Figure 2.3). For every tonne of casein manufactured approximately 25 tonnes of whey are produced and above 7 tonnes of whey results from every tonne of cheese made. Whey production is seasonal and reaches a peak around October in New Zealand.

Production of whey derived products in New Zealand during the 1988/89 season is shown in Table 2.3. It is reported (Anon, 1989a) that the New Zealand Dairy Board supplies up to 60% of the world whey protein market through it's New Zealand operations and it's American joint venture company, Calpro.

.2 4. Whey Utilisation

.2 4.1 INTRODUCTION

Whey can be used/disposed of in a number of ways ranging from simple operations like spray irrigation to more complex procedures such as separation of the protein and lactose components for more specialised applications.

Unprocessed whey can be fed to livestock or applied to agricultural land as a fertiliser substitute (Radford, 1986). Spray irrigation is an important disposal method for acid wheys in New Zealand and Australia.

Most of the sweet wheys produced in New Zealand are used either for the production of lactose powder by crystallisation or for direct incorporation into baby food (Marshall, 1978). Dried whey powder is produced in large quantities and is used in animal feeds, ice cream, cheese, confections and as a skim milk replacement or extender (Irvine and Hill, 1985).

A study of whey utilisation in the Netherlands which has a relatively advanced whey utilisation industry, found that of the whey produced about 38% was used for calf milk replacement, 35% for lactose, 11% for dried demineralised lactose-free whey and 8% for simple, dried whey. The remainder was used in liquid form for such uses as beverage manufacture (Hoogstraten 1987).

FIGURE 2.3 NEW ZEALAND ANNUAL WHEY PRODUCTION 1975 TO 1989

(BOONTANJAI, 1983; ANON 1989b)

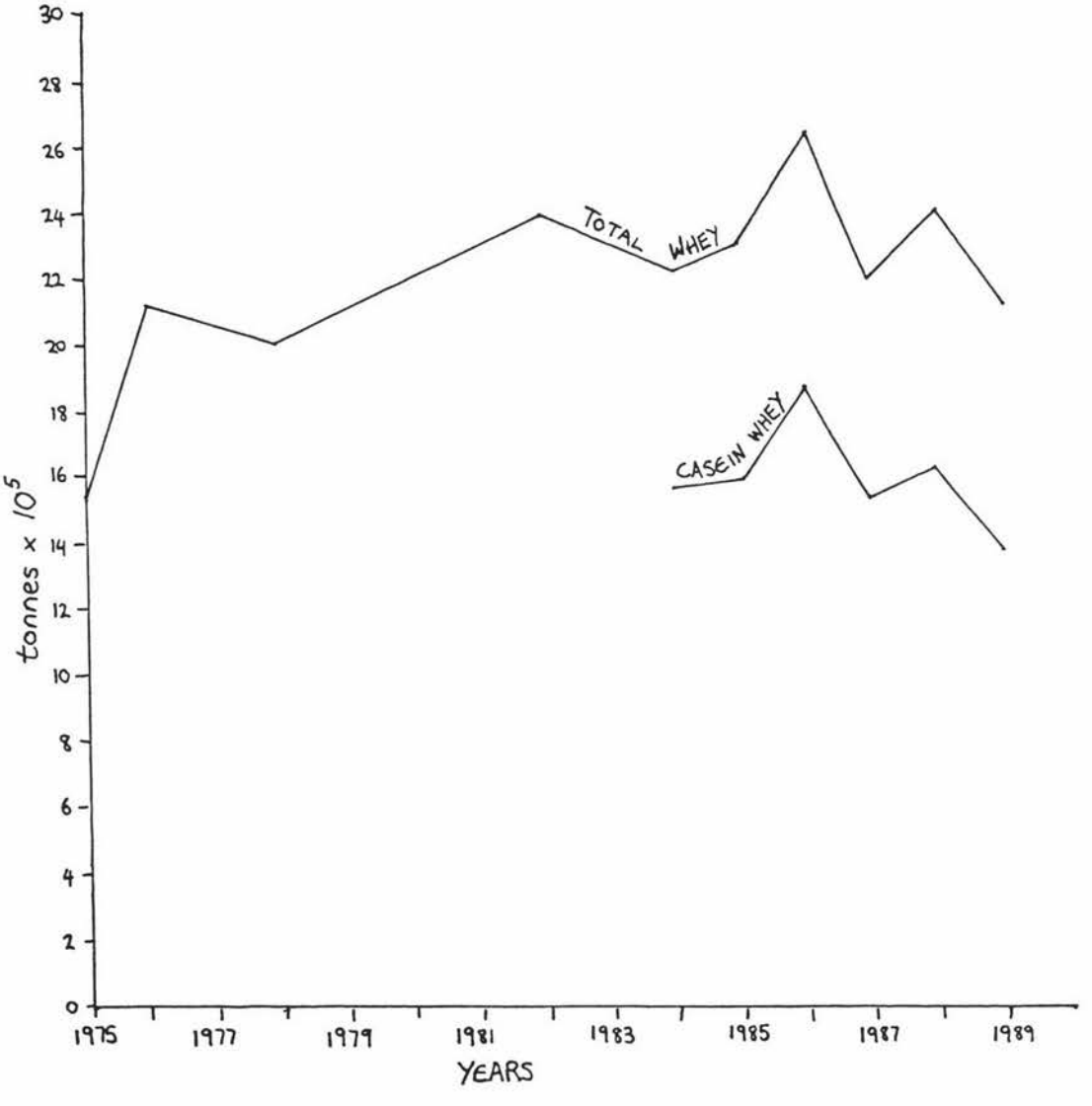


TABLE 2.3: Production of Whey-Derived Products in New Zealand during the 1988/89 season (Anon 1989a, 1989b)

PRODUCT	TONNES PRODUCED
Lactose	17853
Whey Powder	13000
Whey Protein	5000
Whey Butter	1800

Australia, by contrast, used 56% of it's whey for pig food or spray irrigation and utilised only 44% for manufacturing processes (Zadow, 1987).

2.2 4.2 PROTEIN RECOVERY AND USE

The proteins present in whey can be separated by a number of processes including ultrafiltration, heat-acid precipitation, co-precipitation chemical precipitation, ion exchange and gel filtration (Irvine and Hill, 1985). Of these heat-acid precipitation and UF (Ultrafiltration) are the most important and have found widespread commercial application.

The conditions used for heat-acid precipitation vary but it has been found that denaturing of proteins for 10 to 30 minutes at pH 6 to 7 and temperatures greater than 90 C, followed by precipitation at pH 4.5 to 5.5 leads to greatest protein recovery. The maximum recovery of crude protein by this method is 55 to 65% and commercial processes generally recover greater than 50% (Irvine and Hill, 1985).

Membranes used in the UF process normally have a cut off of 3000 to 15000 MW (molecular weight) so that they retain fats and proteins but allow lactose and soluble salts to pass through in the permeate. Ultrafiltration produces WPC - concentrates which can be dried to powders containing up to 75% whey protein (Irvine and Hill, 1985). The composition of a typical permeate is given in Table 2.4.

2.2 4.3 LACTOSE RECOVERY AND USE

The lactose in whey can be recovered by crystallisation after heating deproteinated whey at 40 to 44 C and evaporating it to 55 to 65% total solids. Alternatively RO (reverse osmosis) can be used to partially concentrate the deproteinated whey. This process is similar to ultrafiltration except the membranes used allow fewer chemical species to pass into the permeate, retaining up to 99% of the salt content of the feed. The practical maximum level of concentration by reverse osmosis is only about three times (up to 18 to 24% total solids), so some evaporation must still be carried out to attain concentrations high enough to allow crystallisation (Irvine and Hill, 1985).

TABLE 2.4: Typical Dried Whey Permeate Compositions
(Ennis, B.M., NZDRI, Private Communication)

Component (% (w/w))	Lactic Permeate	Sulphuric Permeate
Moisture	4.08	2.51
Total Nitrogen	0.98	0.70
Non-Protein Nitrogen	0.81	0.51
Ash	13.40	13.10
Lactose	69.60	80.30

Lactose is used as a food additive where it gives body and viscosity without producing excessive sweetness. It also stabilises and absorbs flavours and aromas in food and beverages. Reacted with urea to form lactosylurea it is used in stock feeds as a nitrogen supplement (Irvine and Hill, 1985).

The ash content of lactose produced by the methods described above is too high for some applications and the deproteinated whey may be demineralised using electrodialysis or ion exchange processes. High-grade, demineralised lactose is used by the pharmaceuticals industry as an inert filler for capsules and pills and as a substrate for antibiotic and B-galactosidase production (Irvine and Hill, 1985).

2.2 4.4 FERMENTATION PROCESSES

Whey and deproteinated whey can be fermented to produce a variety of products. Ethanol production will be discussed in detail in later sections. Besides ethanol production a few other processes utilising whey as a fermentation substrate have been commercialised and a number of others have been investigated on a laboratory scale.

Following conversion of the lactose in whey to ethanol the yeast can be harvested and the ethanol oxidised to acetic acid following inoculation with *Acetobacter aceti* bacteria. this is a commercial operation in France, Switzerland and the United States (Irvine and Hill, 1985) where large-scale packed-column reactors are used to provide sufficiently aerobic conditions for the process (Short, 1978).

Commercial penicillin production has been carried out since 1941 and originally used *Penicillium notatum.* However, since the discovery that certain strains of *P.chrysogenum* during the 1940's this has been the organism used. The fermentation is a fed batch process. Lactose is present in the medium initially and when much of that has been used by the culture for biomass production, glucose and a nitrogen source are fed in at a controlled rate, optimised for penicillin production. (Swartz 1985).

The technology of producing lactic acid from whey or deproteinated whey supplemented with yeast extract or corn steep liquor has been established for some time. Lactic bacteria such as *Lactobacillus bulgaricus* are used and the pH of the culture medium is kept at 5.5 by addition of calcium hydroxide or carbonate, resulting in almost complete conversion of lactose to calcium lactate in 14 to 24 hours (Friend and Shahani, 1972).

The lactate is recovered as calcium lactate crystals which are harvested and converted to lactic acid by addition of sulphuric acid. Removal of the lactic acid during the fermentation itself through ultrafiltration is another possibility, and also avoids the build-up of product in the medium leading to inhibition (Short, 1978).

A number of whey beverages utilising unfermented or fermented whey or deproteinated whey have been formulated and some have been successfully marketed. Unfermented and non-alcoholic, fermented products have been the most successful. Whey combines well with citrus juices whose flavours mask the whey taste (Lang and Lang, 1979). The New Zealand Dairy Board markets a whey/fruit juice product in europe called Reviva (Anon 1989b).

Single cell protein can easily be produced from whey, or deproteinated whey supplemented with a nitrogen source and perhaps yeast extract or corn steep liquor.

Yields of biomass up to 1.8% by weight of whey can be obtained in 6 to 10 hours (Irvine and Hill, 1985). In some areas single cell protein production from whey has been carried out commercially for long periods. Moulin, *et al,* (1983) conducted a study on the yeast flora in a reactor in France which had been fermenting continuously for 20 years. They found a stable community of 3 species of yeasts - *K.fragilis, T.spherica* and *T.bovina* - interacting in a non-competitive manner. The *T.spherica* consumed only the lactic acid in the medium, while the *T.bovina* existed on the ethanol produced by the *K.fragilis,* which utilised the lactose present.

Yeast biomass is rich in protein (45 to 50% (w/w) on a dry basis) and ash (12 to 16% (w/w)) (Bernstein and Plantz, 1977).

Other products produced from the fermentation of whey or deproteinated whey on a laboratory scale include citric acid (Hossain, *et al,* 1984), butanol, acetone and ethanol (Quereshi and Maddox, 1987); butylene glycol (Speckman and Collin, 1982) and gibberellic acid (Maddox and Richert, 1977).

2 5. Ethanol Production from Whey

2 5.1 MICROBIOLOGY

Investigations of ethanol production from lactose have been carried out for some time, with some of the earlier publications dating from the 1940's. Browne (1941) and Rogara, *et al* (1947), found (what is now known as)

Candida pseudotropicalis to be the most successful of the lactose fermenting yeast strains, which included *Kluyveromyces lactis* and *K.marxianus.*

Since then however, most work has concentrated on strains of *Kluyveromyces* species. An examination of 107 different strains of *K.marxianus, K.lactis, C.pseudotropicalis, C.verstilis, Brettanomyces anomalus, B.claussenii* and *Trichosporon milibiosacem* by Bothast, *et al* (1986) found eight strains of *K.marixianus* (NRRL Y-1174, Y-1175, Y-1179, Y-1194, Y-1195, Y-1196, Y-1200 and Y-2498) to be uniformly superior to the other organisms tested, over the range of lactose concentrations tried (5, 10 and 20% (w/v)). It was found that *B.anomalus, B.claussenii, C.verstilis* and *T.melibiosacem* strains produced only little ethanol from lactose.

A number of other *Kluyveromyces* strains have been tried by various workers but in examining the literature care should be taken to take note of the various taxonomic changes that have taken place in this group of organisms in recent decades. It is now accepted that *K.fragilis* is a synonym for *K.marxianus* and that *C.pseudotropicalis* (apparent synonym of *C.kefyr*) is the anamorph, or asexual stage, of the *Kluyveromyces* species (Bothast, *et al,* 1986).

It was also suggested that the thermophilic bacterium *Thermoanaerobacter ethanolicus* JW200 be used to ferment lactose at high temperatures. This would allow ethanol to be evaporated off and collected during the fermentation reducing recovery casts. However, this system appeared to have no significant advantages over traditional methods (Schaeffer, *et al,* 1985).

In recent years several workers have attempted to develop

whey-fermenting systems using *Saccharomyces* strains. Members of this genus do not naturally possess genes enabling them to utilise lactose and enzymes must be supplied which hydrolyse the disaccharide (Hahn-Hagerdal, 1985).

Also the genes coding for B-galactosidase and lactose permeate enzymes (which facilitate the uptake and consumption of lactose) have been transferred to *Saccharomyces* species to enable them to ferment whey directly (Brunt 1986).

It is likely the *Kluyveromyces* strains will continue to be the most important group of micro-organisms used for the whey-to-ethanol fermentation in the forseeable future however, as their characteristics are well known and the technology required by them is simple. For this reason the remainder of this review will concentrate on the *Kluyveromyces* fermentation of lactose to ethanol.

2.2 5.2 BIOCHEMISTRY

The metabolism of lactose is essentially the same as that for glucose except for the initial steps. A specific, inducible enzyme system transports lactose across the cell membrane, after which hydrolysis of the disaccharide into glucose and galactose is carried out by the B-galactosidase enzyme. Glucose is fed directly into the glycolytic pathway while the galactose undergoes three intermediate steps before conversion to D-glucose-6-phosphate and entry into glycolysis (see Figure 2.4) (Stryer 1981).

2.2 5.3 EFFECT OF TEMPERATURE

The ability of yeasts to tolerate both high and low

temperatures varies according to the concentration of ethanol in the medium and vice versa. Thus, as the ethanol concentration is increased the yeast will tolerate a narrower range of temperatures *K.fragilis* was found to exhibit measurable growth between 5 and 34 C when the alcohol concentration was 4% (2/v) but at 6% (w/v) the thermal limits were 10 and 28 C. Tolerance of thermal/ethanol extremes could be increased by growing cultures in 4.8% (w/v) ethanol at 32 C (Sa-Correia and Van Uden, 1982, 1983).

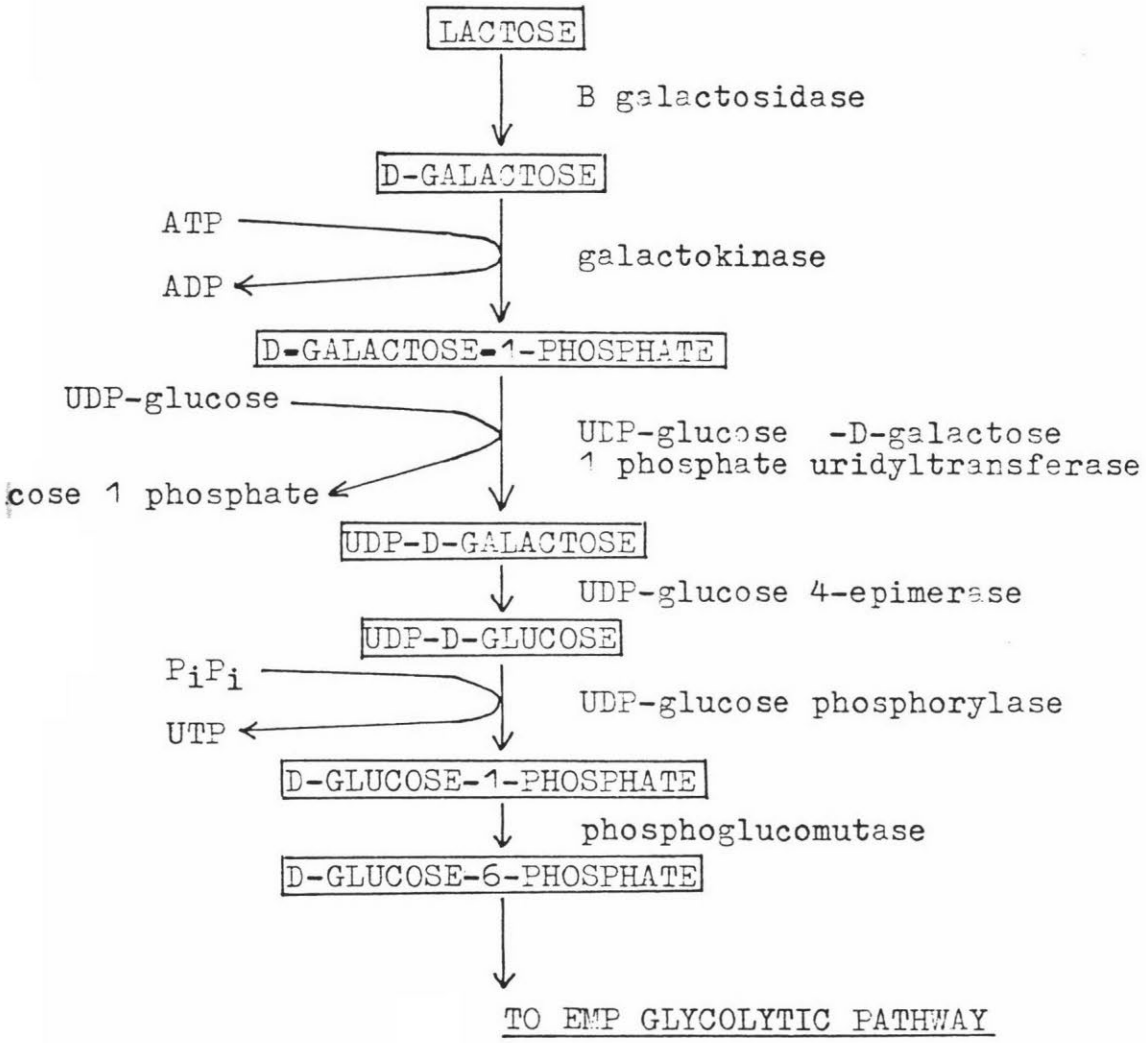
Lipid supplements, notably oleic acid and ergosterol can increase the limits of thermal tolerance as well, suggesting that membrane disruption of some sort is responsible (Anderson, *et al*,1986).

2 5.4 EFFECT OF pH

In their experiments using *K.fragilis* NRRL Y2415 and concentrated whey permeate (TS 30-32%) Mahmoud and Kosikowski (1982) found that the optimum pH for ethanol production was around four under aerobic or anaerobic conditions. Product concentrations dropped away sharply below pH4 but only gradually at pH values up to 7. Biomass production was greatest at around pH5.

Giec and Kosikowski (1982) found that pH had little effect on lactose fermentation rate and biomass yield if it was kept between 4.5 and 5.6. they used cheese whey permeate and skim milk permeate containing approximately 47 g/litre lactose for their experiments and found similar results (i.e, a lack of pH effects) for 10 different lactose-fermenting yeasts.

FIGURE 2.4 METABOLISM OF LACTOSE BEFORE ENTRY INTO THE E.M.P. GLYCOLYTIC PATHWAY.



2.2 5.5 EFFECT OF ETHANOL CONCENTRATION

Ethanol inhibition of lactose-fermenting strains has traditionally (Jones, 1989, Irvine and Hill, 1985) been blamed for limiting the lactose to ethanol fermentation to low substrate and product concentrations. Ethanol has been found to inhibit several vital functions in a wide variety of cells including fungi, bacteria (gram positive and gram negative), protosoa, blue-green algae, eukaryatic algae and any other eukaryatic plant and animal cells (Jones, 1989).

Many of the inhibited functions, such as mitosis, are known to be associated with membranes or membrane-bound proteins and this has led to the hypothesis that ethanol interacts with the hydrophobic interior of lipid bilayer membranes (see Figure 2.6). This view is supported by the observations that a number of the inhibitory effects are intensified when ethanol is substituted by longer chain alcohols such as butanol or pentanol (Van Uden, 1985).

Jones (1989) completed an extensive review of ethanol inhibition and suggested that ethanol *per se* may not be the cause of some of the effects observed. Acetaldehyde is an intermediate in ethanol metabolism (see Figure 2.2) and under certain circumstances can accumulate within the cell to concentrations of 0.75 x [ethanol (g/litre)] mM. Thus if the ethanol concentration is 10 g/litre the intracellular acetaldehyde concentration could be expected to be in the order of 7.5 mM.

Jones (1989) cites the results of studies that have shown inhibition of cellular functions to begin at around 500 uM acetaldehyde. At that concentration an increase in

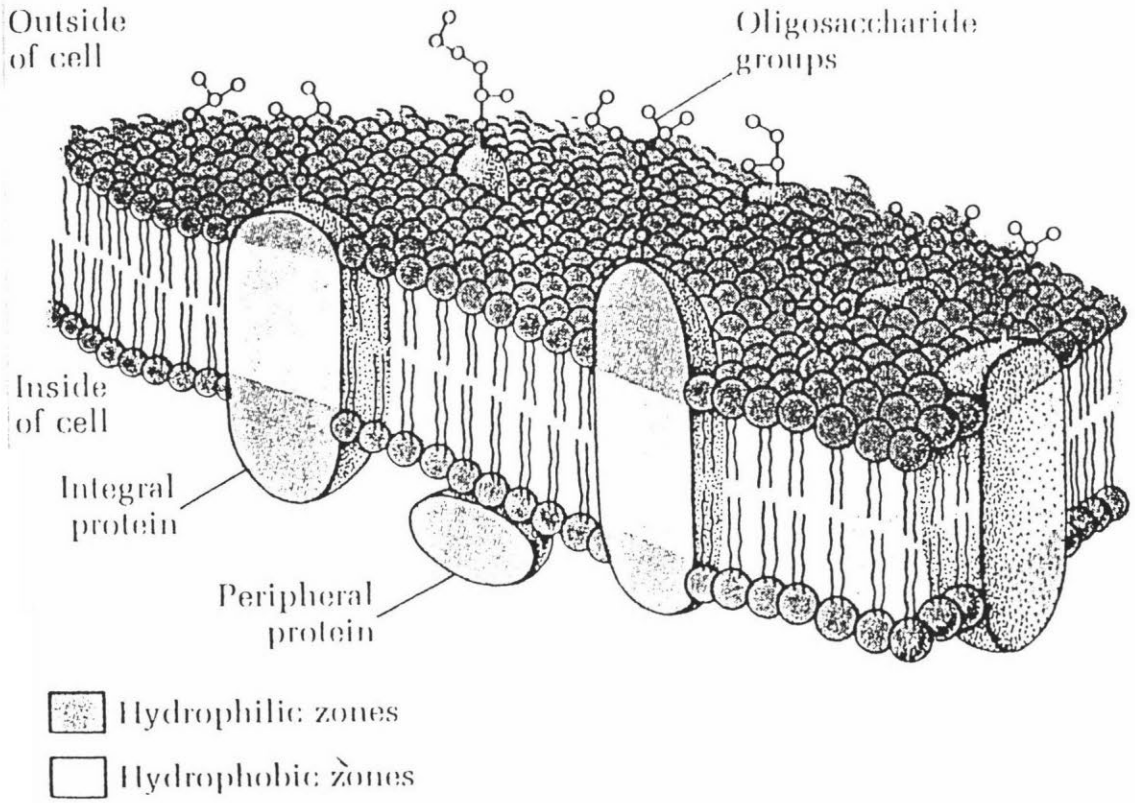
mutagenic effects become detectable. At concentrations up to 10 mM various other effects have been detected including reductions in RNA and protein synthesis, inhibition of TCA cycle activity and increased cell doubling time.

Acetaldehyde is toxic to cells because the aldehyde group reacts with amino groups present on cellular proteins, nucleic acid bases and other compounds such as catecholamines and tryptamines, via the formation of Schiff bases. The toxic effects of acetaldehyde often occur at concentrations between one and five orders of magnitude less that ethanol (Jones, 1989).

Whichever compound is actually responsible one of the most common effects observed as a consequence of inhibition is the slowing or cessation of cell division and growth. Generally speaking significant inhibition of glycolytic enzymes or other specific metabolic enzymes occurs only in a few particularly ethanol-sensitive species and mostly the fermentation rate is reduced due to the inhibition of enzymes transporting carbohydrates, amino acids, ammonium and other nutrients across the plasma membrane (Van Uden, 1985).

A number of mechanisms for ethanol inhibition of membrane-bound processes have been suggested. Ethanol molecules are more hydrophobic than water but are still substantially polar and hydrophilic in behaviour. They have a 35 - times preference for water over benzene and a 300 times preference for it over totally non-polar, non-hydrogen-bonding environments such as the interior of phospholipid membranes (Jones, 1989).

FIGURE 2.5 STRUCTURE OF LIPID BILAYER MEMBRANE
(LENINGER, 1982)



It has been noticed by many workers (eg. Thomas and Rose, 1979; Beavan, *et al*, 1982; Watson, 1982; Janssens, *et al*, 1983; Ginova-Stojanova and Janeva, 1985) that the composition of the phospholipids in the yeasts cell membranes is an important factor in ethanol tolerance and that supplementation of low tolerance strains with certain lipids such as ergosterol or linoleic acid can lead to enhancement of performance.

Given their hydrophilic nature it is most likely that ethanol molecules compete with water molecules associated with the polar heads of the membrane phospholipids. Geometric changes in the packing of phospholipid groups require changes in the compositions of the lipid tails - including changes in unsaturation - and this may be the cause of changing lipid requirements during ethanol inhibition (Jones, 1989).

The increase in permeability of biological membranes to hydrogen ions during ethanol-induced stress is a protein-regulated process (Jones, 1989). It results in a reduction in the potential difference across such membranes and therefore an increase in cell maintenance energy. This is because the cell needs to maintain the proton gradients to allow processes such as active transport of metal ions, amino acids and sugars, ATP generation and NADPH synthesis to continue (Styer, 1981).

2.2 5.6 EFFECT OF MEDIA TONICITY

Many studies have found that increasing the lactose concentration in the fermentation medium above a level of around 50 to 100 g/litre leads to decreased productivity and ethanol yield and increased fermentation times (Burgess and Kelly, 1979; Janssens, *et al*, 1984; Vienne

and Von Stockar, 1985a, 1985b; Bothast, *et al* 1986, Mawson and Taylor, 1989).

In a typical study, using a continuous culture of *K.fragilis*, an increase in substrate concentration in the feed from 86 g/litre lactose to 150 g/litre resulted in a 10% decrease in product yield (Yps), a 21% decrease in specific substrate utilisation rate (qs), a 29% decrease in product (ethanol) concentration and a 30% decrease in volumetric ethanol productivity (rp) (Vienne and Von Stockar, 1985b). In batch fermentations growth and ethanol production are often observed to cease even when a substantial concentration of lactose remains in the medium (Harbison, *et al*, 1984).

The cause of this inhibition has been thought to be either ethanol (product) or lactose (substrate) or a combination of both. While ethanol (and an associated metabolic intermediate, acetaldehyde) is known to inhibit many key functions in the cell metabolism (Thomas and Rose, 1979, Jones, 1989), along with lactose it also contributes significantly to the tonicity of the medium (Jones and Greenfield, 1986).

The effect of a solute on the activity of a solution is given by the osmolality. This is defined as 'that mass of solute which, when dissolved in 1 kg of solvent, will exert an osmotic pressure equal to that exerted by a gram-molecular weight of an ideal, un-ionised substance' (Jones, 1987). It is similar to molarity but takes account of non-ideal solute behaviour where molarity doesn't.

It should be noted that the osmolality of the medium increases throughout the fermentation as lactose is converted into ethanol. Not only are four molecules of

ethanol produced for every molecule of lactose consumed (theoretically) according to the equation:

$C_{12}H_{22}O_{11} + H_2O$ ---------- $4C_2H_5OH + 4CO_2$

Lactose Water Ethanol Carbon Dioxide

....but the ethanol molecule contributes more to the osmolality on a per mass basis than does the lactose molecule (for example, 10 grams per litre of ethanol in aqueous solution has the same osmolality as a solution of 67 grams per litre lactose) (Wolf, *et al*,1989).

It has long been known that low water activity inhibits microbial growth and this has been exploited by man for many years in the preservation of foodstuffs such as jams, where the water activity is low due to large amounts of dissolved sucrose; and salted butter or meat where the high concentrations of dissolved sodium chloride reduce the water activity.

Lowering the water activity of the fermentation increases the amount of energy required for cell maintenance and this leaves less available for growth (Pirt, 1975). Watson (1970) found that the maintenance energy required by *Saccharomyces cerevisiae* growing in anaerobic conditions was increased ten-fold by the addition of one mole per litre sodium chloride, up to 0.36g glucose/g biomass hour.

Glycerol is a by-product of the ethanol fermentation of yeasts which tends to be formed in greater quantities during unfavourable environmental conditions. This was the basis for some commercial glycerol-producing operations as long ago as the First World War, when sulphite was added to the yeast fermentation to stimulate

glycerol production. (Prescott and Dunn, 1940).

It's production by *S.cerevisiae* was also increased in media of high dextrose content. Brumn and Hebeda (1988) suggested that rather than being a result of high medium tonicity this was caused by the high rate of glucose uptake leading to an excess of NADH and it's oxidation to NAD.

Glycerol production by *K.fragilis* was observed to be a function of pH by Vienne and Von Stockar (1985b) with concentrations up to 16.7 g/litre being produced in continuous fermentation of whey permeate at pH 6.35. At pH 4.8 the glycerol concentration in the chemostat was reduced to only 1.3 g/litre.

Wheys have rather high ash (dissolved ions) concentrations. Because they are charged, ions in solution reduce the water activity significantly more than lactose or ethanol. Compared to lactose only 11.2% as much dissolved sodium chloride produces the same osmolality (Wolf, *et al*, 1989). Thus in a typical ultrafiltered whey permeate containing 4.7% lactose and 0.5% ash (see Table 2.4) the osmolality contributed by the ash could be around the same as that contributed by the lactose.

The manufacture of certain cheeses such as domiati, where salt is added prior to renneting, produces wheys with as much as 60 to 100 g/litre salt. El-Samragy and Zall (1988) investigated nine different strains of lactose-fermenting yeasts on whey permeates supplemented with 0, 3, 6 and 9% (w/v) salt and found two which could completely utilise the lactose present in the 0, 3 and 6% salted permeates to produce biomass in aerobic culture. This demonstrated that some lactose-utilising yeasts at

least, are relatively osmotolerant although it must be remembered that aerobic metabolism is substantially different from fermentation and yields considerably more energy for the same amount of carbohydrate consumed.

Studying the effects of sodium chloride on cultures of *Candida tropicalis*, Furyaeva, *et al*, (1985) found results similar to El-Samragy and Zall (1988) with specific growth rate dropping by 75% as the salt concentration was raised to 110 g/litre. The strain used by Furyaeva, *et al*, (1985) developed a certain amount of resistance, however, which was correlated with, amongst other things a doubling of the cells trehalose and glucan content, a lessening of the cell wall's permeability to nucleotide compounds and a drop in the intracellular concentration of acid metabolites. As a result of this adaption to a high salt medium the growth rate in 11% (w/v) sodium chloride was 30% higher than unadapted cultures.

Increased medium osmolality was found to inhibit the fermentation of lactose by *Kluyveromyces fragilis* (strain LAB/105) by Harbison and co-workers (Harbison, 1984; Harbison, *et al* 1984). The water activity was lowered by the addition of mannitol, a carbohydrate which is not utilised by that yeast.

Supplementation of fermentations containing initially 130 g/litre lactose, with either 24 g/litre or 234 g/litre mannitol resulted in significant decreases in lactose uptake rate and lactose-ethanol conversion efficiencies compared to cultures supplied only with 100 g/litre lactose. In the case of 234 g/litre mannitol supplementation the ethanol production rate also was decreased to approximately 50% of that achieved by the unsupplemented and 124 g/litre mannitol fermentations.

The ash content of whey can be reduced by demineralisation techniques such as ion exchange chromatography or electrodialysis and this is often carried out commercially where lactose powder is to be produced from the whey. Electrodialysis tends to remove sodium and potassium ions preferentially and some workers, such as Mahmoud and Kosikowski (1982) have found that the performance of yeasts in demineralised whey media is better than that observed in standard, deproteinated wheys.

Mahmoud and Kosikowski (1982) found that reducing the ash concentration in ultrafiltered whey permeate media from an initial level of 3.5% (w/v) down to 0.77% (w/v) increased the specific growth rate of all the *Kluyveromyces* strains they tested. The most dramatic improvement was a tripling of growth rate by a strain of *K.lactis* (CU10689). Also, the peak ethanol concentration produced by this strain more than doubled, from 2.6% (v/v) to 5.9% (v/v) when the ash concentration was reduced to 0.77% (w/v). The initial lactose concentration in all these experiments was 24.4% (w/v).

In their work on the effects of demineralisation of concentrated whey permeate media Giec and Kosikowski (1982) found that reduction of the ash content to about 0.5% had little effect on fermentation performance if the lactose concentration was less than 150 g/litre. However, if an initial lactose concentration of 200 g/litre was used, the reduction of ash content to 0.5% resulted in a doubling of substrate utilisation rate in two of the three strains of *Kluyveromyces* tested, and a significant increase in the other strain.

Jones and Greenfield (1986) suggested that medium tonicity is the most significant controlling factor in

fermentations by yeasts which are quite ethanol tolerant, such as some strains of *Saccharomyces*. In evidence they demonstrated that with such strains the growth rate slowed to zero at certain specific osmolalities whereas in fermentations using strains more susceptible to ethanol inhibition the cessation of growth correlated either with a distinctive ethanol concentration and osmolality.

2.2 5.7 EFFECTS OF OTHER INHIBITORS AND PROMOTERS

Where ethanol and osmolality have been unable to account for the inhibition observed in some ethanol fermentations, other compounds have been blamed by several authors. Continuous processes with cell recycle or selective removal of ethanol (by vacuum or membrane, etc) have been noted as being susceptible to such inhibition which is thought to result from a build-up of non-metabolised compounds in the medium.

Shin, *et al*, (1983) and Maiorella, *et al* (1984) both suspected salts as causing inhibitory effects. Although Shin *et al* (1983) did not identify specific salts the latter group tested a number of compounds and found inhibition decrease in the order: Calcium Chloride, Ammonium Sulphate, Sodium Chloride, Ammonium Chloride, Potassium Dihydrogen Phosphate, Magnesium Chloride, Magnesium Sulphate and Potassium Chloride.

Maiorella, *et al* (1984) reported that 0.23 M calcium caused an 80% decline in biomass production. In contrast Nabais, *et al* (1988) have found that calcium chloride added at concentrations between 0.75 and 2 mM led to the rapid production of higher concentrations of ethanol and increased growth rate in fermentations using *S.cervisiae*, *S.bayanus* and *K.marxianus*.

Magnesium also has been found to promote an increased rate of lactose utilisation and increase yields in the fermentation of cheese whey by *K.marxianus*, if added at a concentration of around 0.5 mM (Walker, *et al*, 1990). The concentration of magnesium in cheese whey is around 3.7 mM (see Table 2.1) normally but as the authors point out, the level of free magnesium ions may be significantly reduced in such substrates due to the presence of chelating agents such as proteins.

Proteins themselves were blamed in part for inhibition by Shin, *et al* (1983) who found that autoclaving spent medium before re-use resulted in less inhibition being observed than when the spent medium was not autoclaved. Unlike inorganic ions, proteins generally are heat labile and will become denatured if autoclaved and this was the basis for these workers' suspicions.

Octanoic and decanoic acids are by-products of ethanol production and have also been suggested as inhibitors (Viegas, *et al*, 1989). However, their effect is pH-dependant and at the concentrations produced in *Kluyveromyces* fermentations (of jerusalem artichoke juice) the pH does not drop enough to result in significant reduction of the growth rate.

Chitin, xylan, acacia gum and other polysaccharides were found to increase markedly the rate of ethanol production by *S.cerevisiae* and *S.uvarum* when added at concentrations of 0.2% (w/v) by Patil and Patil (1989). The addition of chitin reduced the time required to ferment a molasses medium containing 180 g/litre reducing sugars from 72 hours down to 30 hours.

Various compounds known to increase the activity of alcohol dehydrogenase (ADH) enzymes have been suggested

as potential promoters of ethanol fermentations (Jones, 1989). ADH is responsible for converting acetaldehyde to ethanol during fermentations and enhancement of its activity would help alleviate increases in acetaldehyde concentration (see section 2.2.5.5). An example of one of these compounds is B-indoliloacetic acid. This was found to enhance growth rate by 15% and sugar uptake by 50% when added to fermentation media in concentrations between 1 and 10 mg/litre. ADH activity also was significantly activated (Jones, 1989).

2.2 5.8 COMMERCIAL ASPECTS

In New Zealand there are four distilleries which use whey or deproteinated whey for ethanol production (see Table 2.5). The ethanol produced at these sites supplies the New Zealand market, plus a limited export market with several grades of industrial and potable alcohol (Mawson, 1987).

The processes use unconcentrated, unsupplemented whey or whey permeate in batchwise fashion except for Tirau which uses a 3-stage continuous, stirred tank reactor. At Tirau and Rèparoa the yeast is separated and recycled for continued culture while at Edgecombe a fresh inoculum is usually grown for each batch (Howell 1981; Mawson, 1987).

The lactose content of the whey permeate varies between 43 and 46 g/litre throughout the season giving a maximum possible yield of between 23.1 and 24.7 g/litre ethanol. At Tirau ethanol production reduces the BOD (biological oxygen demand) of the whey permeate by 85% and the resulting still bottoms are treated anaerobically (yielding methane gas which is collected) and discharged. Edgecombe sprays it's still bottoms, along with any unused whey permeate from the adjacent dairy factory, onto

local dairy farms using a fleet of tankers (Howell, 1981; Mawson, 1987).

TABLE 2.5 Commercial Ethanol Production From Whey in New Zealand (Mawson, 1987; Mawson, Private Communication)

	DISTILLERY			
	Reparoa	Tirau	Clandboye	Edgecombe
Date Commisioned	Sept 1980	Sept 1981	Sept 1982	Oct 1982
Substrates Processed	Sulphuric Whey Permeate	Deproteinated Lactic Whey	Cheese Whey	Sulphuric Whey Permeate Maize
Nominal Annual Ethanol Production (millions litres absolute)	4.0	3.5	0.5	4.0
Grades of Alcohol Produced	Industrial: 95% (v/v) 99.5% + % (v/v) Potable: 96.5% (v/v)	Industrial: 95% (v/v)	Industrial: 95% (v/v) 99.5% + (v/v) Potable: 96.5% (v/v)	Potable 96.5% (v/v)

The energy requirements for the overall process of fermentation and distillation at Edgecombe is estimated at 22 MJ/litre ethanol which is good compared with similar plants elsewhere (Mawson, 1987).

The inability of lactose-fermenting yeasts to satisfactorily ferment solutions containing greater than around 100 to 150 g/litre lactose results in the

production of beers of low ethanol content (54 to 81 g/litre ethanol maximum possible yield). The energy required for ethanol distillation (up to 70% of total energy input) falls rapidly as the alcohol content of the beer increases to 100 g/litre and continues falling at a slower rate as the beer gets more concentrated. An increase in ethanol concentration in the feed from 7 to 10% (w/v) saves between 20 and 35% of the energy required for distillation (Kosaric, *et al*, 1983) (see Figure 2.6).

However, the cost of increasing the concentration of substrate in whey (permeate) media is a major limiting factor. Mawson (1990) calculated that on a purely energetic basis the economic limit for whey concentration is around 12% (w/v) total solids (8-10% (w/v) sugar). Concentration above this level requires more energy than that saved through the distillation of a more concentrated feedstock.

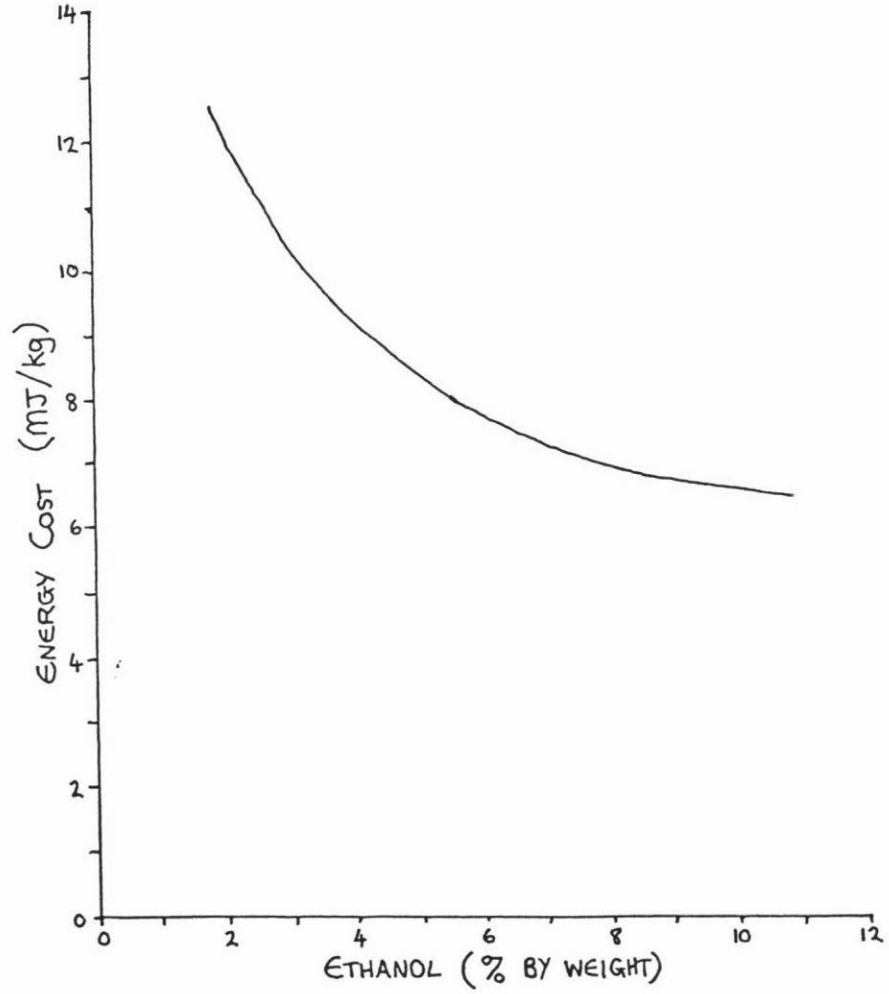
At Edgecombe distillery the savings in steam would be in the order of NZ$125,000.00 per annum if concentrated whey (100 g/litre lactose) was used. However, the cost of an RO (reverse osmosis) plant would be more than NZ$1.5 million, with on-going costs of at least NZ$80,000 per annum for maintenance, membrane replacement, etc (Mawson 1990).

Thus the opportunities for improving the economics of the whey-to-ethanol fermentation through whey concentration are limited at the present time but with technological improvements in the area of RO and related technologies this may change.

FIGURE 2.6 COMPARISON OF DISTILLATION COSTS AND ETHANOL CONCENTRATION

(PARISI, 1983)

(COST IS THAT REQUIRED TO PRODUCE 95% ETHANOL)



2.3 SUMMARY

There is a need for continued research into the whey to ethanol fermentation. In New Zealand especially it is an important industrial process, not just from an economic viewpoint but also because it demonstrates the practical utilisation of what was once considered a waste product. The need for all industries to re-think their priorities with regard to by-products and effluent has achieved a prominent position in the public's mind and although a great deal of research has been carried out, over the last few decades in particular, the majority of waste products produced by industry are still under-utilised or simply not utilised at all.

The dairy industry is an example where significant progress has already been made but like any industry it will only adopt a process if it has proven economic potential. The production of ethanol from whey presently is profitable only under certain conditions.

These include an absence of strong competition form ethanol derived from other sources such as grain or petrochemicals, and also the availability of large quantities of whey for significant periods of the year. Thus it is not a viable option in many places overseas where grain or petrochemical ethanol is cheap and readily available. Also the need to process large quantities of whey excludes many small dairy plants.

These restrictions can be reduced by improving the efficiency or productivity of the fermentation, something that may only be achieved through increased knowledge of the variables affecting them.

Currently the amount of information accumulated about the whey to ethanol fermentation is still relatively small. Some of the research carried out with non-lactose fermenting yeasts such as *Saccharomyces* is relevant but much of it is not. In the areas of trace element requirements and media supplements this is especially true.

The research described in this thesis was intended to expand our knowledge of the nature and extent of inhibition in the fermentation using *K.marxianus* Y113. This would provide a data base from which models of the yeast's behaviour could be developed and using these models the results of subsequent research could be evaluated more rapidly and reliably.

It was also felt that there was a need to test some of the suggestions for methods of improving the fermentation's performance made by other researchers. A number of interesting claims have been made and it is important that their validity and applicability to different strains be tested.

3. MATERIALS AND METHODS

3.1 3.1 MATERIALS

3.1 1. Organisms

The organism used in this study was *Kluyveromyces marxianus* Y113, a lactose-fermenting yeast from the collection at the Biotechnology Department, Massey University. This was derived from the strain used by New Zealand Distillery Co Limited to ferment whey permeate to ethanol at their plant in Edgecombe, NZ.

3.1 2. Media

The two media used most commonly in these studies were LYB Agar and LYB Broth. The former is a solid medium used for culture maintenance and in the initial stages of propagation. LYB broth is a liquid substrate used during propagation of the yeast under aerobic conditions for inoculum preparation.

LYB Agar = Lactose 10g/litre
Yeast Extract 3g/litre
Bactopeptone 5g/litre
Agar 15g/litre
Distilled Water .....to 1000ml

LYB Broth = Lactose .....50 g/litre
Yeast Extract .....3 g/litre
Bactopeptone .....5 g/litre
Distilled Water .....to 1000ml

Other media used will be described where appropriate in the relevant chapters. All media were adjusted to pH 4.5

prior to sterilisation. (See section 3.2.3).

3.1 3. CHEMICALS

Chemicals used in this study were obtained from major supply companies and generally were of laboratory grade. Those used in experiments investigating specific nutritional requirements were of analytical (AR) grade.

Sulphuric casein whey permeate powder and demineralised sulphuric casein whey permeate powder were obtained from the NZ Dairy Research Institute (Palmerston North, NZ).

Distilled water was used throughout this work.

3.2 METHODS

3.2 1. Biological Methods

3.2 1.1 CULTUREL MAINTENANCE

The yeast was maintained on slopes of LYB agar at 4 C. These were produced by spreading a loopful of cells on each slope and incubating at 30 C for three days. Sub-culturing onto fresh slopes was carried out every six months.

3.2 1.2 PROPAGATION AND INOCULATION

An inoculum of the yeast was prepared by sub-culturing onto a fresh LYB slope using a wire loop and incubating this at 30 C for 3 days. The slope was then washed with 5ml of Sterile LYB broth and the washings poured off into 50ml of sterile LYB broth in a 250ml conical flask. This was incubated at 30 C on a rotary shaker (Gallenkamp,

England; Labline Instruments Inc., USA), operated at 200 to 250 r.p.m.

After 24 hours the biomass concentration was between 5 and 10 gD.W./litre and the culture was in the late exponential phase of growth. To inoculate the flasks for an experiment, 20ml of this aerobic culture was pipetted into 180ml of the sterile medium to be tested. The ingredients in the test media were in amounts such that their correct concentrations were only achieved after addition of the 10% inoculum.

3.2 1.3 INCUBATION AND SAMPLING OF EXPERIMENTS

After inoculation flasks containing test media were placed on shelves in a temperature - controlled room and left to incubate at 30 C for a period of up to 120 hours. Periodically 1ml samples were removed with a sterile pipette and stored at - 20 C in 1.5ml plastic Eppendorf tubes until analysed.

3.2 1.4 STERILISATION OF MEDIA AND EQUIPMENT

A variety of equipment required sterilisation at one time or another during this work. This included flasks, filtering apparatus, pipettes, wire loops, etc. Glassware was autoclaved at 121 C for 15 minutes in most cases. The exceptions were pipettes which were sterilised in metal containers by heating them to 105 C overnight in an oven. Loops and such like were flamed in a burner.

Media generally were autoclaved, but in experiments involving whey permeate, they were filter-sterilised through 0.45 um cellulose nitrate discs (Millipore, Bedford, USA) into sterile Buchner flasks. Following

filtration the sterilised media were aseptically transferred into sterile 250ml conical flasks for inoculation and incubation.

In Run 9 chitin was used as a culture additive. This was weighed into the conical flasks and sterilised dry; filter-sterilised media was then added to this.

3.2 2. Analytical Methods

3.2 2.1 CLEANING OF GLASSWARE

All glassware, except that used in biomass and lactose assays, was washed in hot Labcleen (Labware Supplies, National Dairy Association, Palmerston North, NZ) solution, rinsed in tap water and dried by hot air.

Test tubes and spectrophotometer cells used in assays were usually rinsed several times after use, in tap water and then in distilled water. They were also soaked regularly for two hours in hot chromic acid solution, rinsed in distilled water and then dried in hot air.

3.2 2.2 pH MEASUREMENT

This was carried out using an electronic pH meter (Orion Research, USA) which was regularly calibrated against a standard buffer solution.

3.2 2.3 BIOMASS MEASUREMENT

The biomass concentration in the samples was measured by absorbance at 620nm wavelength using a spectrophotometer (Philips PU8625 UV/VIS, Cambridge, UK). The samples were thawed and agitated to make certain any lactose which may

have crystallised out of solution during freezing was redissolved. An aliquot of 0.5ml was withdrawn from each sample and put into a clean test tube. To each aliquot was added 0.5ml of a 10% (w/w) solution of propan-2-ol, which acted as the internal standard for the ethanol assay to be carried out later.

The test tubes were then centrifuged to collect the yeast cells (7000g; Clandon T52 England, UK). Meanwhile the labelled Eppendorf tubes were washed and dried to remove any residual sample fluid.

The supernatant in the test tubes was poured back into the correct Eppendorf tubes and returned to the freezer. The harvested cells were resuspended in either 5 or 10ml distilled water to obtain an absorbance reading below 1.00. The absorbance was measured and the dry weight determined from a standard curve (see appendix B).

3.2 2.4 LACTOSE MEASUREMENT

Lactose concentration was estimated using the phenol/sulphuric acid assay (New Zealand Ministry of Agriculture and Fisheries - Dairy Division, 1984).

The frozen samples which had had the yeast cells removed and propan-2-ol added (see section 3.2.2.3) were thawed and agitated to redissolve any lactose which may have crystallised out of solution during freezing.

From each sample 100ul was pipetted into a test tube and diluted to 10ml with distilled water. From this a further 100ul was transferred to a fresh test tube and diluted to 1ml. This gave a final solution which was one-thousandth the concentration of the initial sample.

The assay was only accurate when measuring solutions containing less than 100 g/litre lactose so any sample which was suspected of containing more than that was diluted by a half in addition to the 1000-fold dilution described above. (It should be remembered that the addition of the propan-2-ol solution initially had already reduced most samples to below the 100 g/litre limit).

To the 1ml in the test tube was added 1ml of 5% (w/v) phenol solution and this was agitated vigorously on a vortex mixer. While still being held on the mixer, to these tubes was added 5ml concentrated sulphuric acid (specific gravity 1.86). An orange-brown colour developed if carbohydrates were present, and the intensity of this was measured at 490nm using a spectrophotometer (Philips PU8625 UV/VIS, Cambridge, UK).

Care had to be taken to ensure that the dilution steps were carried out accurately and that no dust or foreign particles fell into the test tubes as these reacted along with the lactose and caused an inaccurate reading. Despite extensive precautions problems occurred surprisingly frequently and the extreme sensitivity of the assay led to 5 to 10% of the results being discarded.

The use of High Performance Liquid Chromatography (HPLC) to estimate lactose concentration was tried at one stage during this work in an effort to improve accuracy. A Waters Associates (Model ALC/GPC 244, Milford USA) chromatograph was used with a Sugar Pak-1 column (Waters Association). The moving phase was 0.0001 M calcium acetate solution in deionised water and the flowrate was 0.5ml/minute, with a column temperature of 90 C. Aliquots of 40ul were injected. A differential refractometer (Model R401, Waters Associates) was used as

the detector and the data was quantified by a Waters Associates Model 740 Data Module.

It was found that the accuracy of the HPLC method was only slightly better than that achieved using the phenol-sulphuric acid assay. The longer time required to analyse the numerous samples by HPLC made it less suitable so consequently its use was discontinued.

3.2 2.5 ETHANOL MEASUREMENT

The measurement of ethanol concentration was carried out using a Shimadzu (Kyoto, Japan) model GC-8A gas chromatograph. The column was operated at 190 C and was packed with Poropak Q (Applied Science laboratories Inc., State College, Pennsylvania, USA). The injector temperature was 220 C and 2ul aliquots were used. Detection was by flame ionisation and the data was quantified by a Varian integrator.

The samples measured were the same as those used in the lactose assay (section 3.2.2.4), that is the supernatant left after separation of the yeast cells by centrifugation. An internal standard of propan-2-ol had been added prior to centrifugation and the concentration of ethanol was determined by comparing the size of it's peak with that of the standard (see Appendix B).

THE EFFECTS OF OSMOLALITY, LACTOSE, SALT AND ETHANOL CONCENTRATION ON ETHANOL PRODUCTION FROM LACTOSE

.1 INTRODUCTION

An apparently simple method of increasing the final alcohol concentration in the fermentation medium is to increase the initial lactose concentration but as explained in the introduction (Section 2.3.5.3) this procedure leads to decreased yield, incomplete lactose utilisation and lowered productivity which negate it's possible advantages. The aim of the experiments described in this chapter was to investigate the extent of these effects and to collect data with which to assess their significance.

The parameters measured were biomass ethanol and lactose concentration and from these the others of value, such as yields, growth rates and rates of ethanol production could be calculated (see Appendix A for details of calculation methods).

.2 LACTOSE CONCENTRATION

.2 1. Introduction

There are certain economic factors that are important when considering the pre-concentration of whey permeate for use in fermentations. If transportation of the whey from the dairy plant to the distillery is not required, it has been calculated by Mawson (1990) that on a purely energetic basis the economic limit of whey concentration is to around 10% (w/v) carbohydrate. (This assumed that the fermentation performance was unaffected by medium

composition).

Above that level the cost of electricity required for concentration by reverse osmosis exceeds that of the steam saved during the distillation step.

However, if transportation to a different site is required, concentration up to about 20% (w/v) would be carried out to reduce transport costs and then the permeate would be fermented at the higher concentration to reduce distillation steam costs. (Mawson, 1990).

Another reason why it was necessary to use concentrations of lactose up to 20% (w/v) in these experiments was that they were required so that sufficient data could be gathered to assess quantitatively, the biological effects they produced.

The ingredients in the media used in the experiments reported in this section are detailed in the appropriate tables (Tables 4.1, 4.2, and 4.3). All cultures were grown 250ml in shake-flasks at 30 C, after receiving a 10% (v/v) inoculum (see Section 3.2.2 for preparation of inoculum).

4.2 2. Results and Discussion

The results of Run one can be seen in Table 4.1 and figures 4.1, 4.2 and 4.3. The initial lactose concentrations were 50, 100, 150, and 200 g/litre and the yeast extract concentration was increased in proportion, giving 3, 6, 9 and 12 g/litre respectively for cultures A, B, C and D.

TABLE 4.1 Summary of Results; Run 1

Media all contained 5 g/litre bactopeptone and various concentrations of lactose and yeast extract:

A = 50 g/litre lactose
3 g/litre yeast extract
B = 100 g/litre lactose
6 g/litre yeast extract
C = 150 g/litre lactose
9 g/litre yeast extract
D = 200 g/litre lactose
12 g/litre yeast extract

1. Initial Biomass = 0.8 g/litre

2. Maximum Biomass

A = 4.5 g/litre (60 hours)
B = 4.2 g/litre (55 hours)
C = 3.4 g/litre (55 hours)
D = 2.7 g/litre (55 hours)

3. Ethanol Production (maximum)	Rate (g EtOH/g bio.h)
A = 26 g/litre (30 hours)	.32
B = 40 g/litre (55 hours)	.29
C = 49 g/litre (75 hours)	.31
D = 43 g/litre (75 hours)	.33

4. Growth Rate (u max)

A = .085 h^{-1}
B = .078 h^{-1}
C = .070 h^{-1}

D = .060 h^{-1}

5. Lactose used (to attain maximum ethanol concn)

A = 94%
B = 89%
C = 88%
D = 60%

6. Yields

(i) Yxs	(ii) Yps	(iii) Ypx
A = .074	A = .52	A = 7
B = .037	B = .45	B = 12
C = .022	C = .37	C = 17
D = .017	D = .36	D = 21

It can be seen that the yeast behaved in a fashion similar to that described previously (section 2.3.5.3) The biomass production, lactose utilisation and yields were all lowered when higher initial substrate concentrations were used, but it is interesting to note that the specific rate of ethanol production stayed about the same as did the specific lactose utilisation rate, and the yield coefficient Ypx actually increased with greater substrate concentration.

This suggested that the growth of the yeast was the metabolic function inhibited by increased medium tonicity and/or ethanol concentration and that ethanol production, which is substantially correlated with growth, slowed only as a result of decreased growth rate (initially at least). This is in agreement with results reported in the literature which are discussed in the introduction (Section 2.3.5.3).

Fig. 4.1: Biomass Concn Vs Time; Run 1

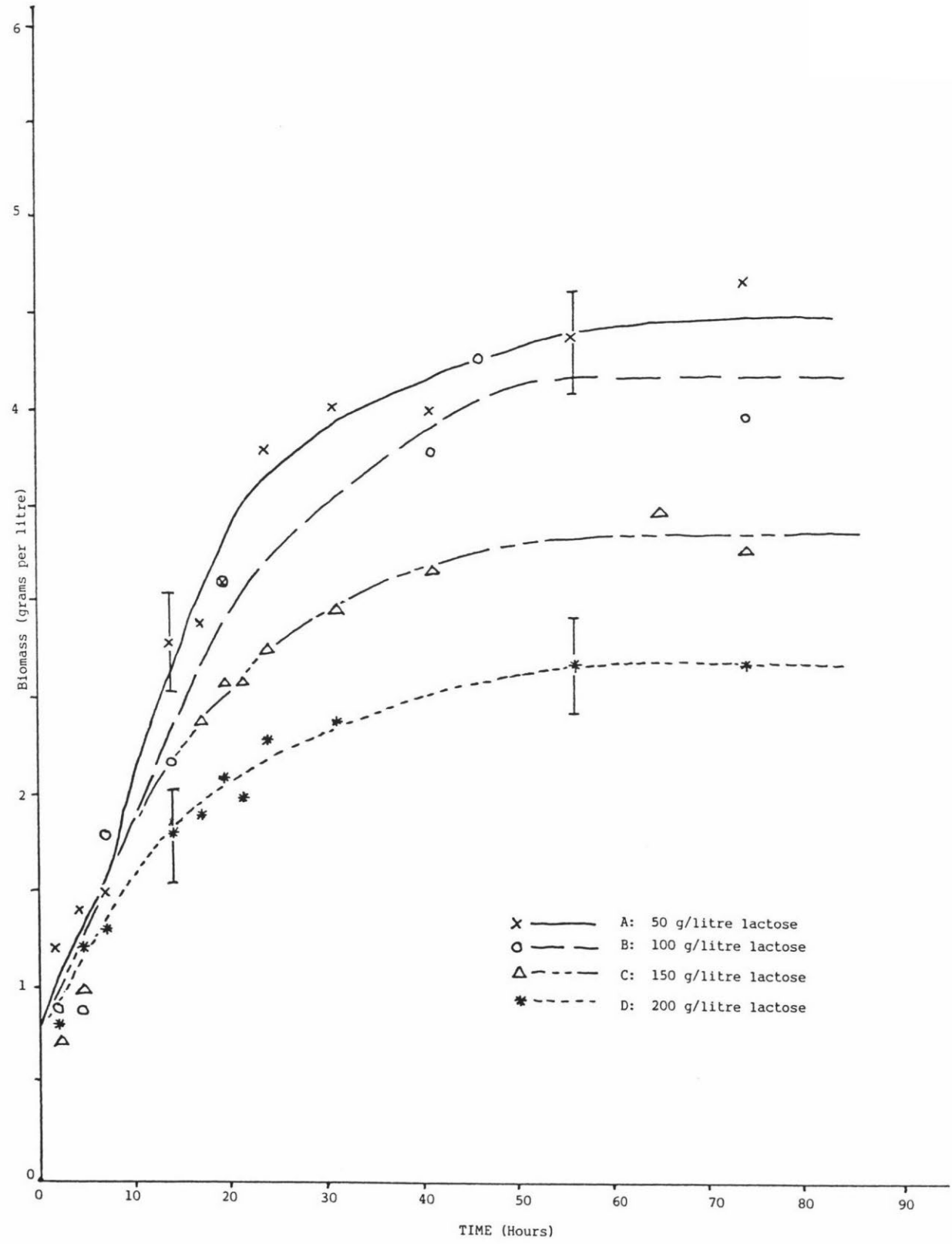


Fig. 4.2: Ethanol Concn Vs Time; Run 1

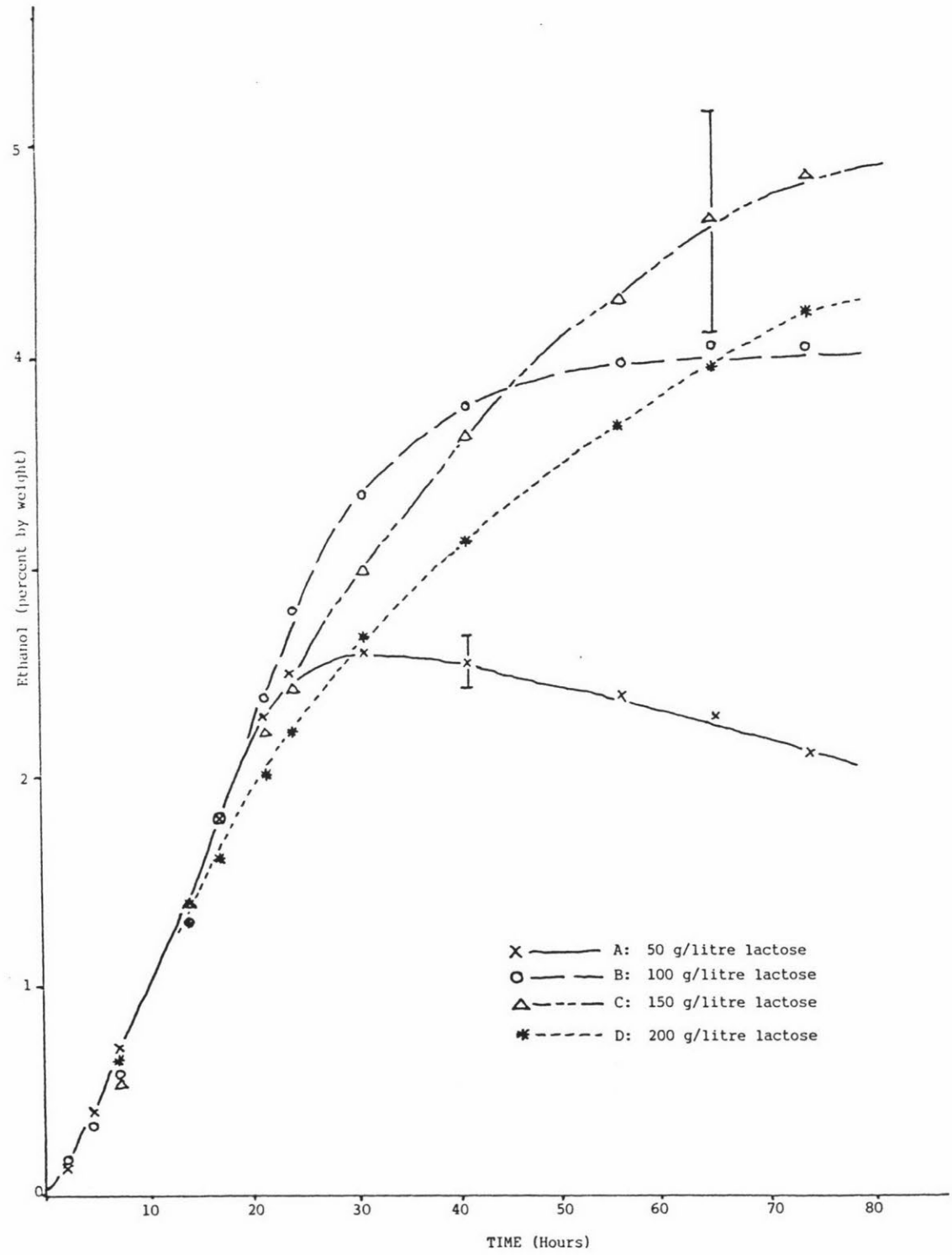


Fig. 4.3: Lactose Concen Vs Time; Run 1

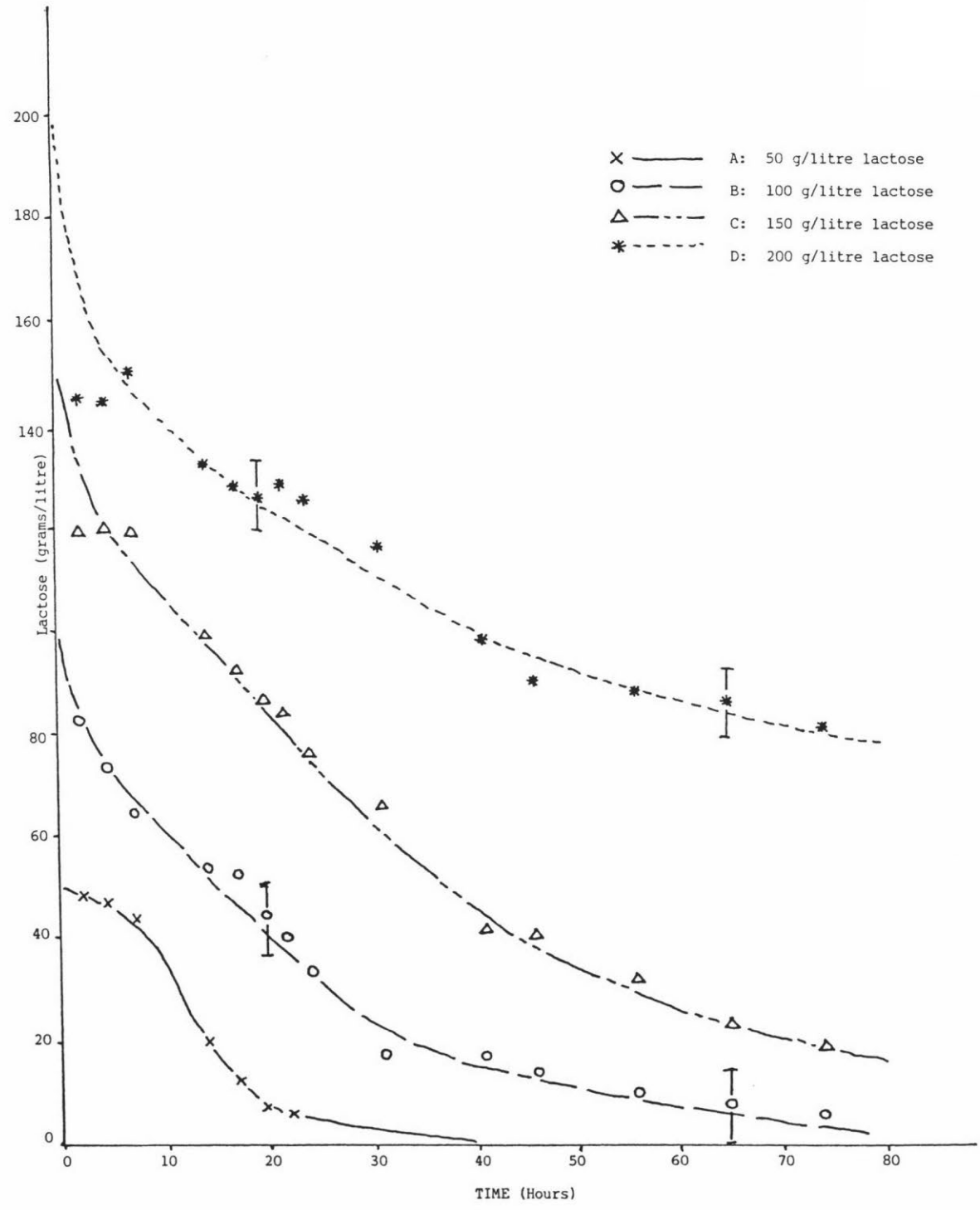


TABLE 4.2 Summary of Results; Run 2

All media contained 5 g/litre bactopeptone and 3 g/litre yeast extract plus:

A = 50 g/litre lactose
B = 100 g/litre lactose
C = 150 g/litre lactose

1. Inital Biomass = 1 g/litre

2. Max Biomass:

A = 2.7 g/litre (55 hours)
B = 2.6 g/litre (55 hours)
C = 2.3 g/litre (50 hours)

3. Growth Rate (u max)

A = .041 h^{-1}
B = .032 h^{-1}
C = .030 h^{-1}

4. Ethanol Production (maximum) Rate (gEtOH/g bio.h)

A = 26 g/litre (50 hours)	.28
B = 44 g/litre (75 hours)	.33
C = 44 g/litre (75 hours)	.36

5. Lactose used (to attain maximum ethanol concn)

A = 100%
B = 92%
C = 62%

6. Yields:

(i) Yxs	(ii) Yps	(iii) Ypx
A = .036	A = .52	A = 14
B = .020	B = .48	B = 24
C = .016	C = .48	C = 30

As a result of experiments whose results are reported in Section 5.2 the level of yeast extract supplementation was reduced to a base concentration of 3 g/litre, regardless of lactose concentration in following experiments. Bactopeptone supplementation remained at 5 g/litre throughout.

Results for Run 2 can be seen in table 4.2 and figures 4.4, 4.5 and 4.6. Lactose concentrations were 50, 100 and 150 g/litre for cultures A, B and C respectively and one can once again see the reduction in growth rate, lactose utilisation and biomass yield observed previously.

The growth rates in Run 2 were noticeably lower than those in Run 1 and this illustrated the variability of small scale batch experiments. Relatively small changes, such as the state of the inoculum when transferred, the temperature of incubation (which may vary if doors are left open, etc.) and even the degree of media aeration resulting from agitation prior to sampling, may affect the fermentations to varying degrees. The relatively uniform behaviour of the yeast within each run demonstrates that the yeast itself is not unreliably variable and that it is merely responding to environmental variables unaccounted for in the experimental plan.

Fig. 4.4: Biomass Concn Vs Time; Run 2

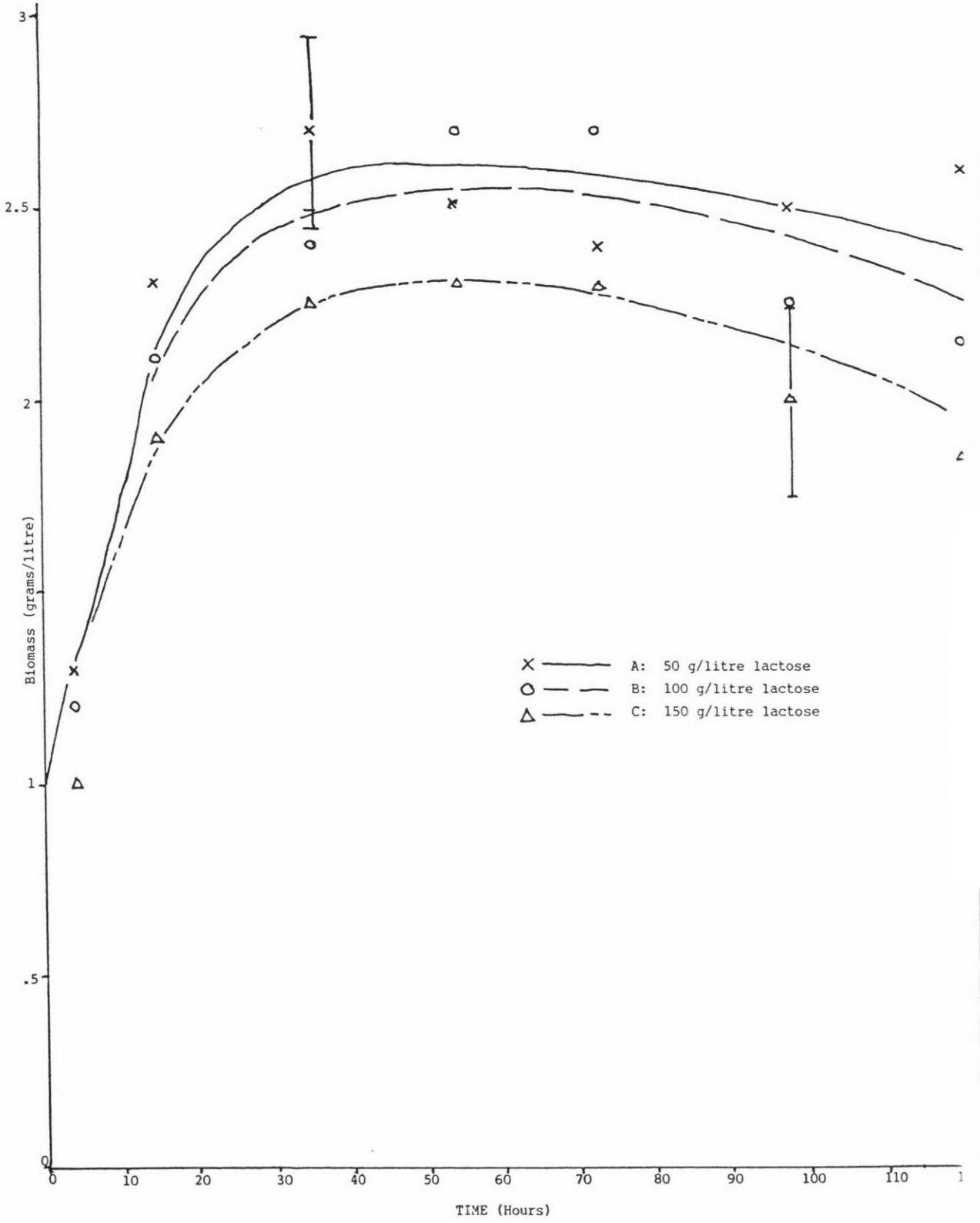


Fig. 4.5: Ethanol Concn Vs Time; Run 2

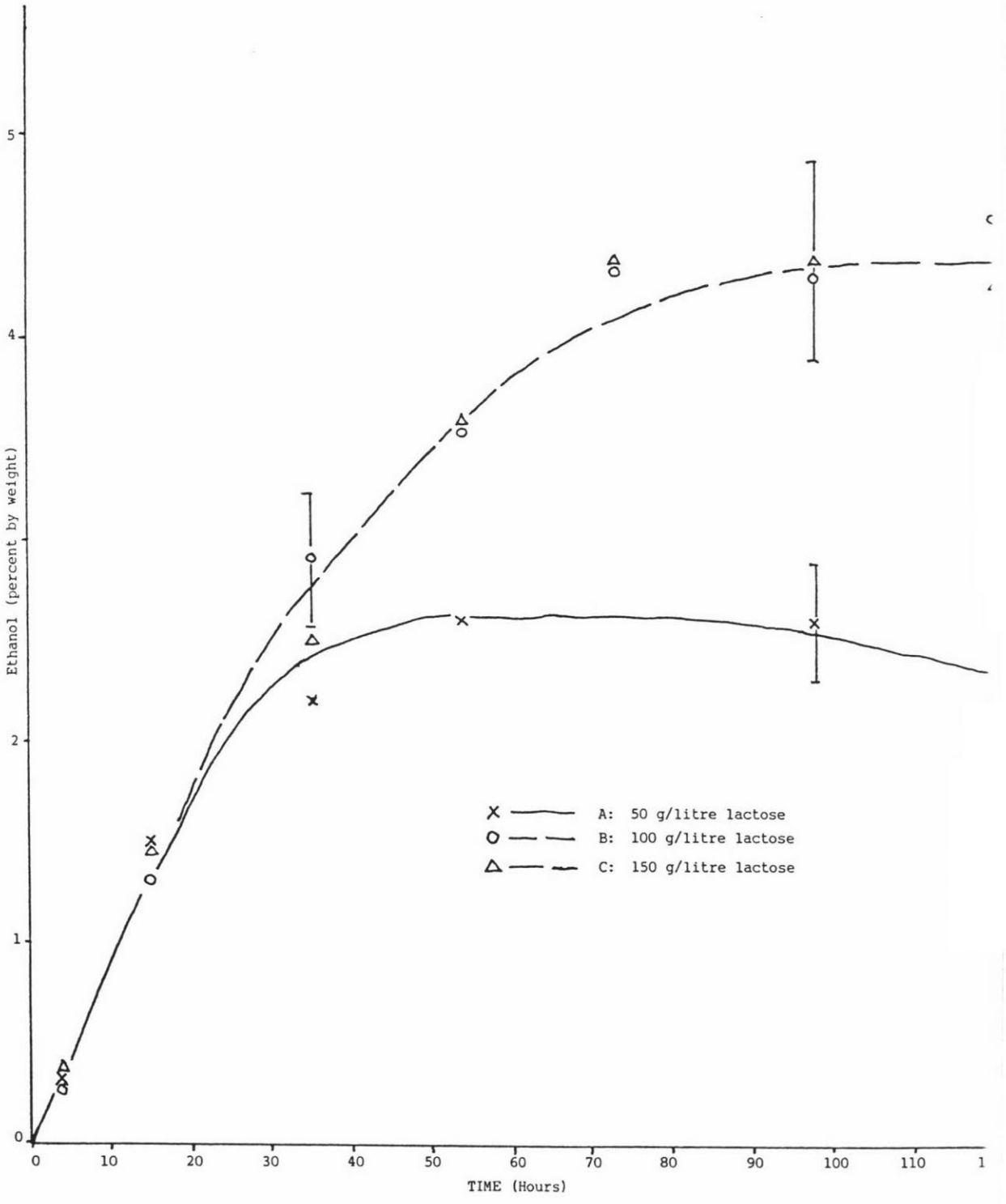


Fig. 4.6: Lactose Concn Vs Time; Run 2

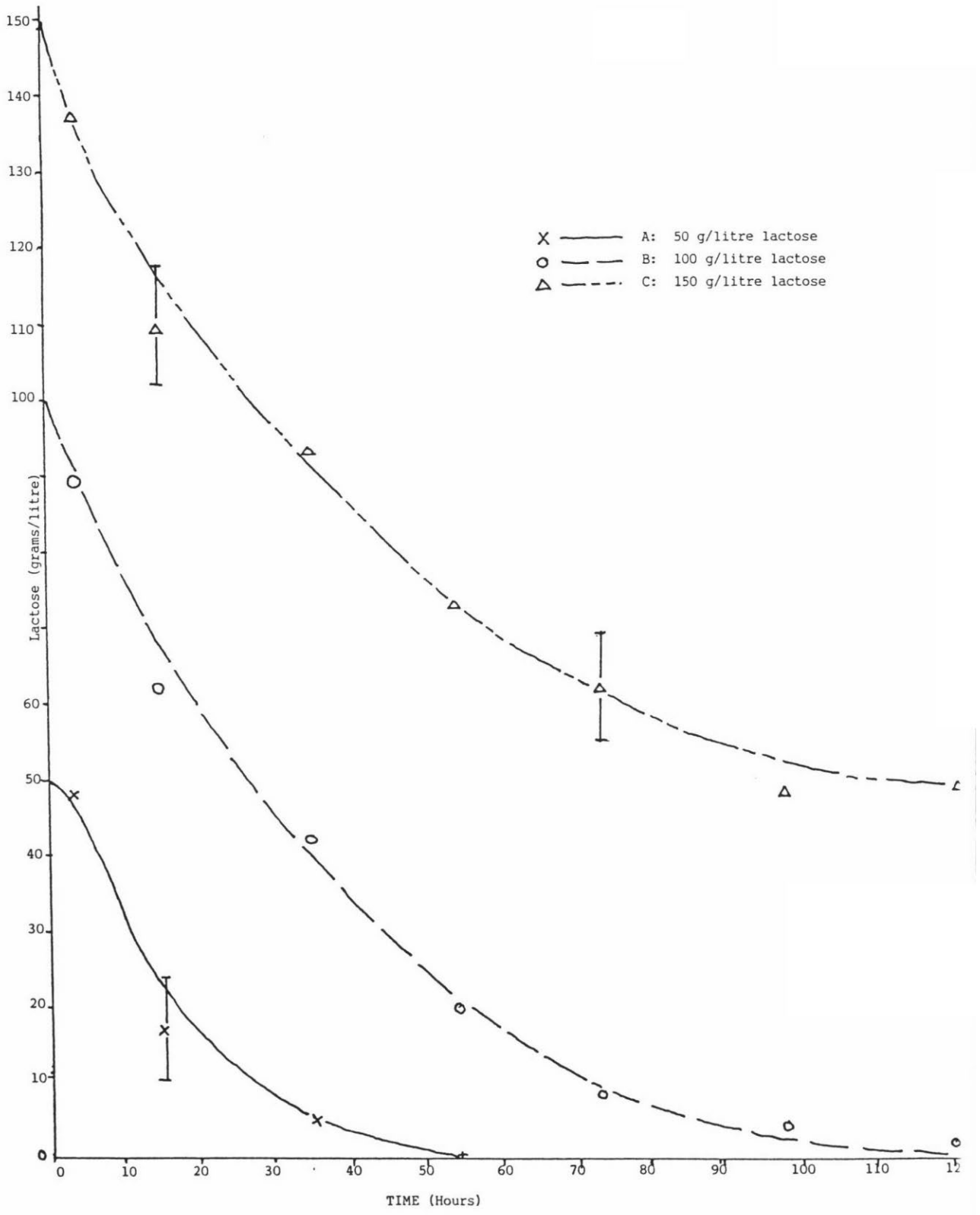


TABLE 4.3 Summary of Results; Run 3

Flask A: 100 g/litre lactose, 3 g/litre Yeast Extract, 5 g/litre Bactopeptone

Flask B: 150 g/litre lactose, 3 g/litre Yeast Extract, 5 g/litre Bactopeptone

Flask C: 150 g/litre lactose, 3 g/litre Yeast Extract, 5 g/litre Bactopeptone

Flask D: 200 g/litre lactose, 3 g/litre Yeast Extract, 5 g/litre Bactopeptone

RESULTS:

1. Initial Biomass = 1.25 g/litre

2. Maximum Biomass

A = 2.5 g/litre (40 hours)
B = 2.7 g/litre (40 hours)
C = 2.5 g/litre (40 hours)
D = 2.4 g/litre (40 hours)

3. Growth Rate Maximum (u max)

A = 0.044 h^{-1}
B = 0.044 h^{-1}
C = 0.044 h^{-1}
D = 0.035 h^{-1}

4. Ethanol Production (maximum) Rate

A = 40 g/litre (50 hours)	.43 (g EtOH/gBio.h)
B = 46 g/litre (60 hours)	.39 " " " "
C = 46 g/litre (60 hours)	.41 " " " "
D = 45 g/litre (70 hours)	.35 " " " "

5. Lactose Used (to attain maximum ethanol concn)

A = 85%

B = 83%

C = 82%

D = 60 %

6. Yields

A/ Yxs	B/ Yps	C/ Ypx
A = .016	A = .45	A = 28
B = .014	B = .38	B = 27
C = .012	C = .38	C = 32
D = .012	D = .38	D = 32

Fig. 4.7: Biomass Concn Vs Time; Run 3

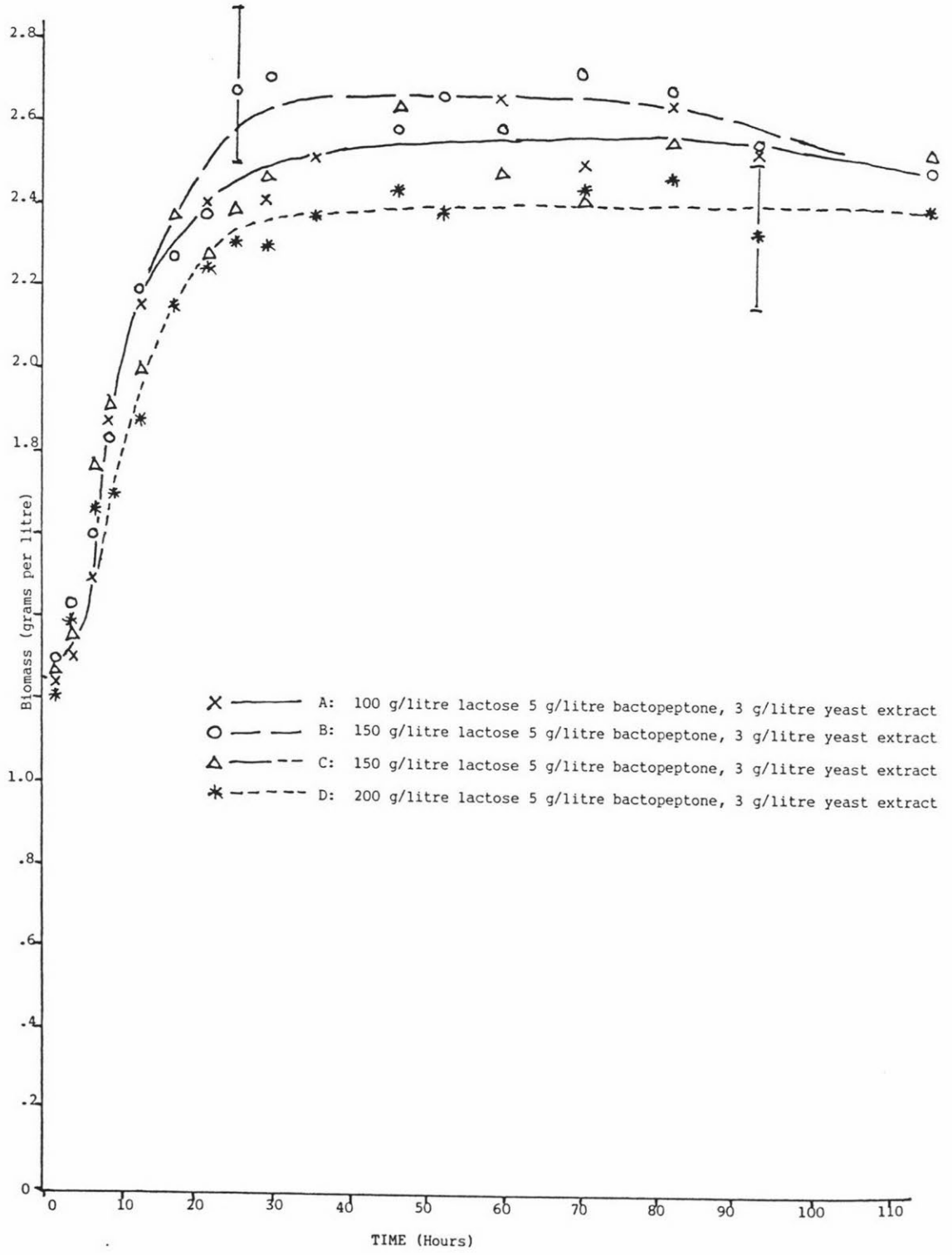


Fig. 4.8: Ethanol Concn Vs Time; Run 3

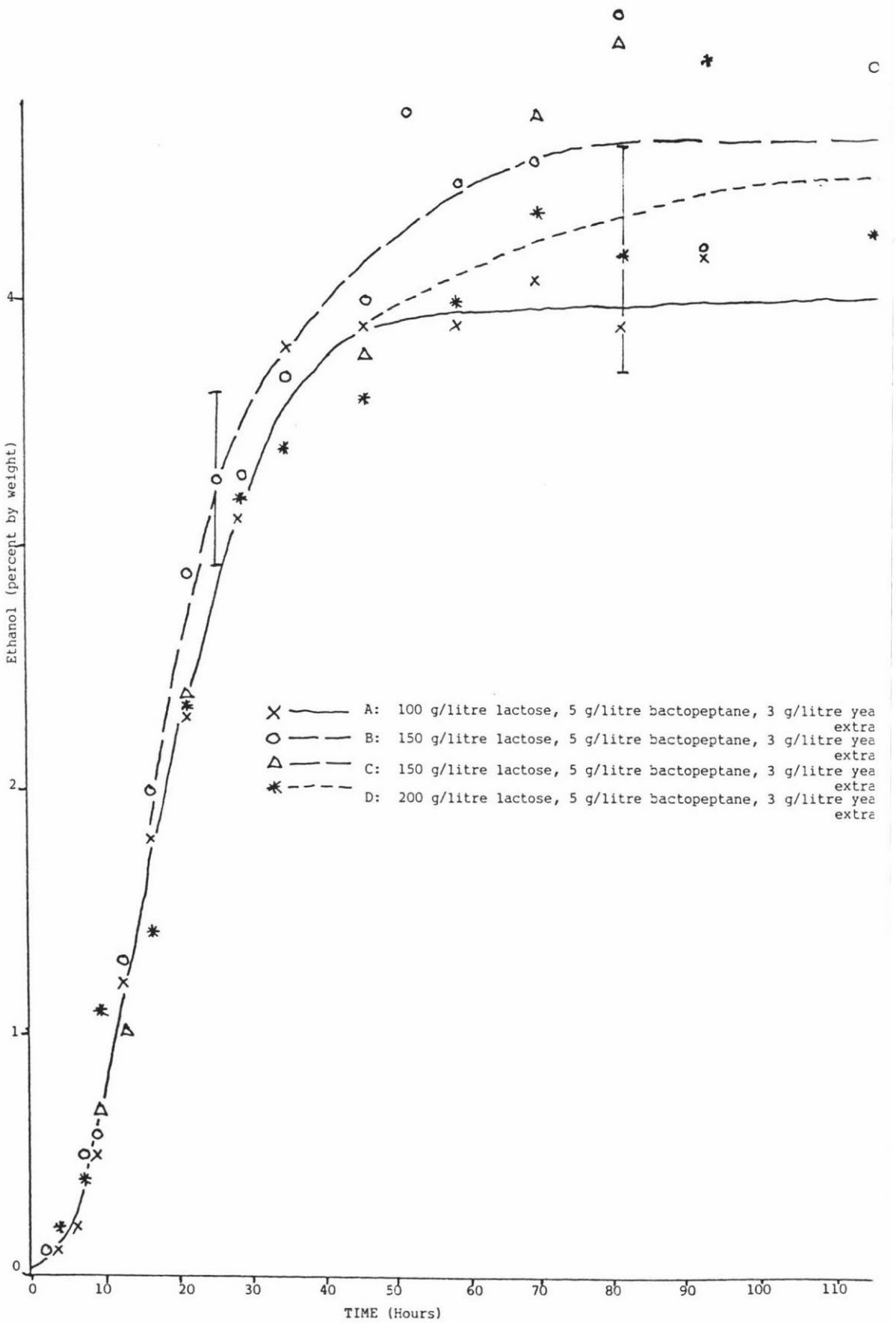
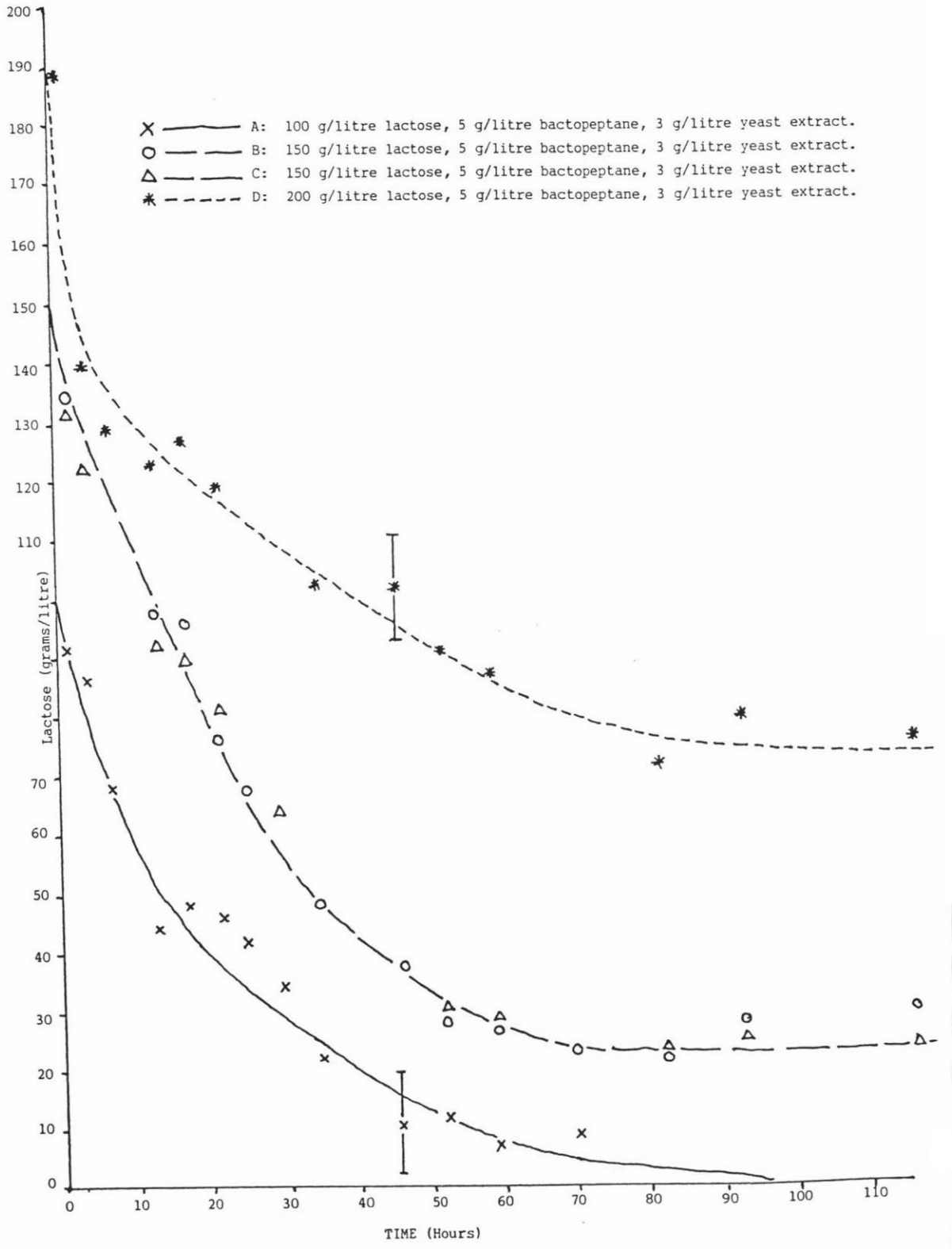


Fig. 4.9: Lactose Concn Vs Time; Run 3



In any case it can be seen that the ethanol concentration, yield (Yps) and rate of production are similar between the two runs suggesting that, once again, it is the growth that is more sensitive to environmental changes than the formation of ethanol.

Run 3 showed similar results to Run 2 with observed growth and alcohol production rates being only slightly higher than in the latter experiment. The yields in both runs were also similar. (See table 4.3 and figure 4.7, 4.8 and 4.9).

The data collected from these experiments did little to separate ethanol inhibition from inhibitory effects caused by medium tonicity however, so a further experiment was devised to gauge the effects of increasing the tonicity of this medium alone without altering the potential ethanol concentration produced.

The results of these experiments are very similar to those recorded by Mawson and Taylor (1989). Those workers used *K.marxianus* Y113 also. Figures 4.18, 4.19, 4.20 and 4.21 show the ethanol and lactose results for fermentations with initial lactose concentrations of 100 g/litre and 150 g/litre respectively. Along with those of Mawson and Taylor (1989) are plotted results from equivalent fermentations from this work.

Like this work, the earlier results demonstrated that maximum attainable ethanol concentration for this yeast lies around 40 to 50 g/litre when it is grown on simple lactose broth media. Once this level was attained fermentation ceased, even if residual lactose was present.

However, the data collected from these experiments did little to separate ethanol toxicity from inhibitory effects caused by medium tonicity. Jones (1987) demonstrated that for some yeasts high medium osmolality is the limiting factor, rather than ethanol concentration. To investigate this effect with *K.marxianus* Y113 a further experiment was devised where the osmolality was increased without altering the lactose concentration and hence the potential ethanol concentration produced.

4.3 MEDIUM TONICITY

4.3 1. Introduction

Maltose is a disaccharide which is not able to be utilised by *K.marxianus* Y113 (Mawson, Personal Communication, 1989) and could therefore be used to increase the osmolality of the fermentation medium without resulting in greater amounts of biomass or ethanol formation. In a parallel experiment sodium chloride was used to increase the osmolality. It has been suggested (Vienne and von Stockar, 1985a, Mahmoud and Kosikowski, 1982) that demineralisation of whey can lead to better fermentation performance and as electrodialytic demineralisation removes ions such as potassium and sodium preferentially (Mahmoud and Kosikowski, 1982) it was considered important to test the effects of increasing the concentrations of such ions and to see whether their ionic nature meant that they were more toxic to the yeast than uncharged species such as maltose, lactose or ethanol.

Concentrations of maltose were added to give levels where growth and ethanol production would have been largely

prevented were tonicity the prime controlling factor (see Iso-osmolality plot; figure 4.22). From tabulated data (Wolf, *et al*, 1989) the amounts of sodium chloride required to produce similar osmolalities were calculated and these were added to parallel cultures. (Unfortunately the calculations contained some inaccuracies, meaning that the cultures were less comparable at higher salt concentrations. See Appendix for details).

4.3 2. Results and Discussion

The results of these experiments are shown in Tables 4.4 and 4.5 and Figures 4.10, 4.11, 4.12, 4.13, and 4.14. It was found that maltose and lactose could not be accurately quantified together in solution using either the phenol, sulphuric acid colorimetric assay or HPLC analysis. This meant that for these experiments comparisons were made only on the basis of ethanol and biomass accumulation and production rates.

Although a higher medium osmolality led to significant decreases in growth rate (refer to Tables 4.4 and 4.5) ethanol production and lactose utilisation were less affected. It can be seen that in the most extreme case (Culture I, containing 28 g/litre sodium chloride) the maximum growth rate was reduced by 70% compared to the culture without added salt. By comparison, the maximum ethanol concentration was reduced by 22% and the lactose utilisation by 18%. The yield coefficients, Yxs and Yps were reduced slightly (12% and 4% respectively for culture I) and surprisingly the reductions were greater for the cultures with less salt added.

TABLE 4.4 Summary of Results; Run 4, Maltose Cultures

All media contained 100 g/litre lactose, 5 g/litre bactopeptone and 3 g/litre yeast extract. Maltose, an non-utilised carbohydrate, was also added:

J = No maltose
B = 100 g/litre maltose
C = 150 g/litre maltose
D = 200 g/litre maltose
E = 250 g/litre maltose

1. Initial Biomass = .95 g/litre

2. Maximum Biomass

J = 2.9 g/litre (55 hours)
B = 2.2 g/litre (50 hours)
C = 2.1 g/litre (50 hours)
D = 1.8 g/litre (50 hours)
E = 1.8 g/litre (50 hours)

3. Ethanol Production (maximum)

	Rate (g ETOH.g Bio.h)
J = 37 g/litre (50 hours)	.38
B = 32 g/litre (50 hours)	.41
C = 31 g/litre (60 hours)	.34
D = 30 g/litre (60 hours)	.36
E = 28 g/litre (60 hours)	.34

4. Growth Rate

(i) Umax J = .040 h^{-1}
B = .028 h^{-1}
C = .019 h^{-1}
D = .019 h^{-1}
E = .019 h^{-1}

These yield results contrast with the results for Runs 1, 3 and 6 where increased initial lactose concentrations led to substantially reduced Yxs yield coefficients.

Harbison (1984) conducted experiments using the lactose-fermenting strain *K.fragilis* LAB 105 and mannitol to increase the medium osmolality. His results were very similar to those reported above, with the maximum growth rate being the most affected property and the other coefficients being reduced by lesser amounts.

Obviously the medium osmolality did have some influence on growth and ethanol production but not enough to provide an explanation for the inhibition observed during fermentation of concentrated lactose media. The next logical step was to add ethanol to the medium initially and to observe the effect on the yeast's performance.

Fig. 4.10: Biomass Concn Vs Time; Run 4

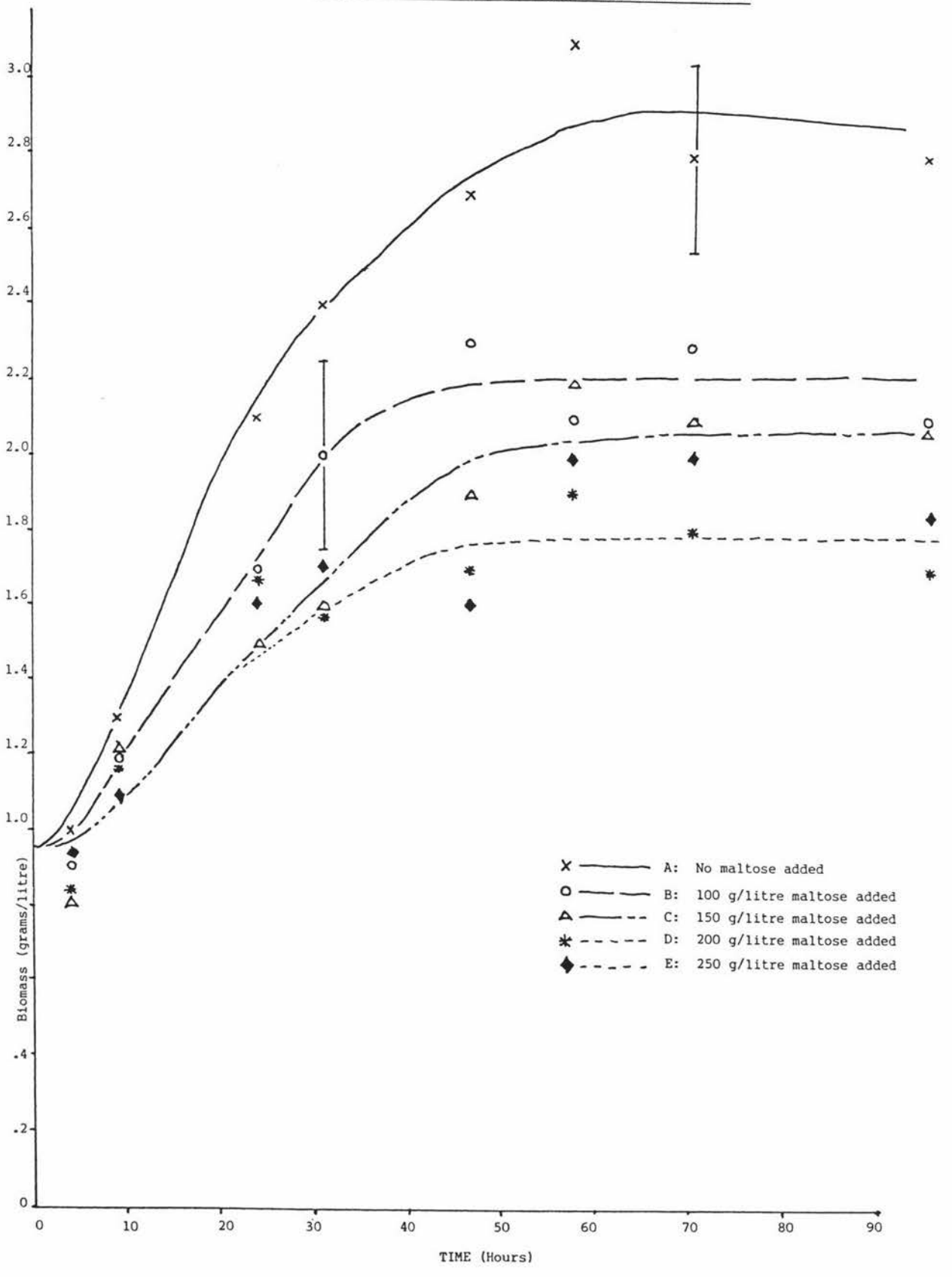


Fig. 4.11: Ethanol Concn Vs Time; Run 4

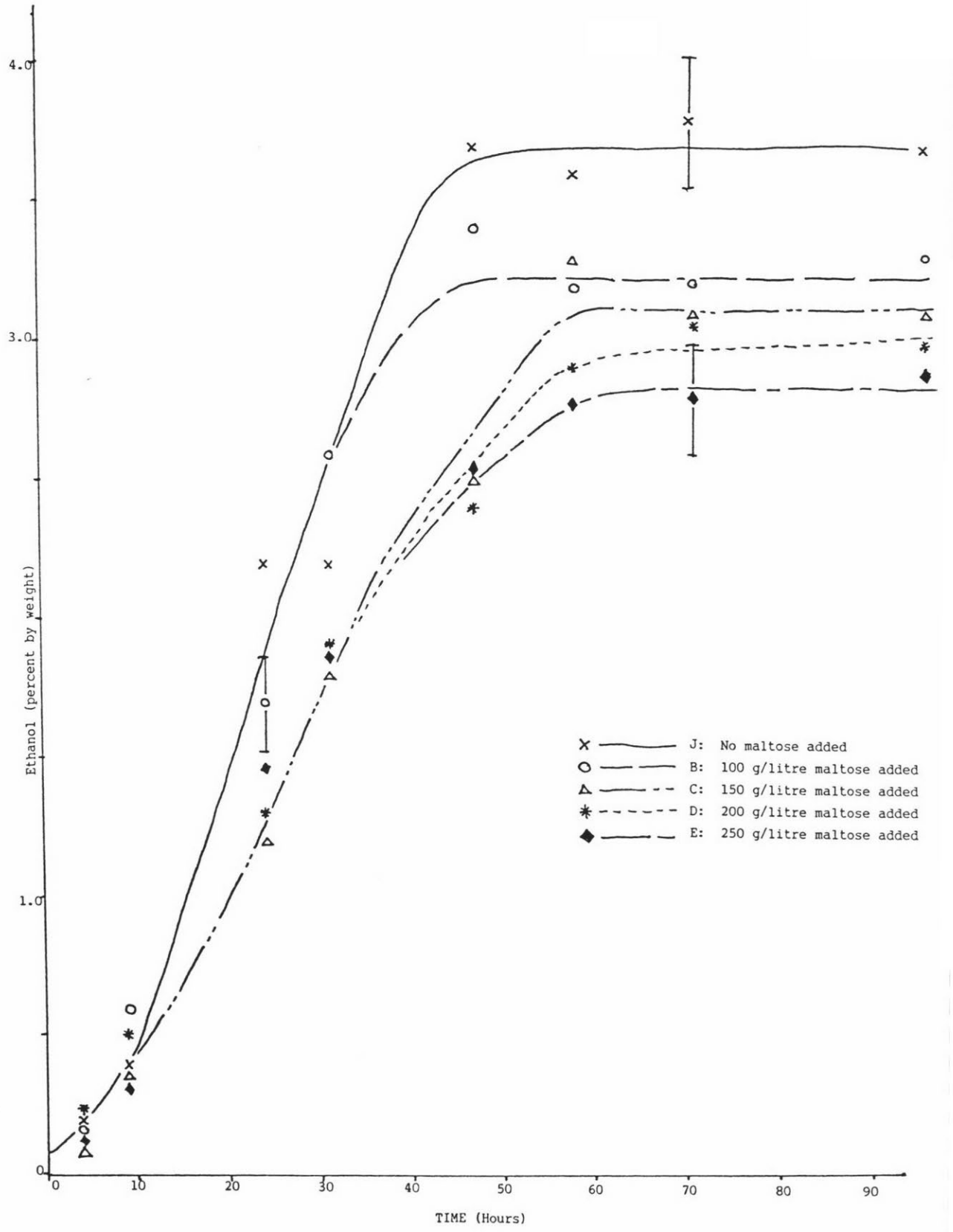


TABLE 4.5 Summary of Results; Run 4, Salted Cultures

All media contained 100 g/litre lactose, 5 g/litre bactopeptone and 3 g/litre yeast extract. Sodium Chloride was also added:

J = No salt added
F = 11.2 g/litre
G = 16.8 g/litre
H = 22.4 g/litre
I = 28.0 g/litre

1. Initial Biomass = .95 g/litre

2. Maximum Biomass

J = 2.9 g/litre (55 hours)
F = 2.1 g/litre (55 hours)
G = 2.1 g/litre (55 hours)
H = 1.9 g/litre (55 hours)
I = 1.7 g/litre (55 hours)

3. Ethanol Production (maximum) Rate (g ETOH/g Bio.h)

J = 37 g/litre (50 hours)	.38
F = 36 g/litre (70 hours)	.34
G = 32 g/litre (70 hours)	.30
H = 29 g/litre (70 hours)	.31

4. Growth Rate

(i) Umax J = .040 h^{-1}
F = .026 h^{-1}
G = .026 h^{-1}
H = .021 h^{-1}
I = .012 h^{-1}

5. Lactose Used (to attain maximum ethanol concn)

J = 78%
F = 95%
G = 89%
H = 76%
I = 64%

6. Yields

(i) Yxs	(ii) Yps	(iii) Ypx
J = .035	J = .47	J = 13
F = .025	F = .38	F = 15
G = .027	G = .36	G = 13
H = .030	H = .42	H = 14
I = .031	I = .45	I = 15

Fig. 4.12: Biomass Concn Vs Time; Run 4

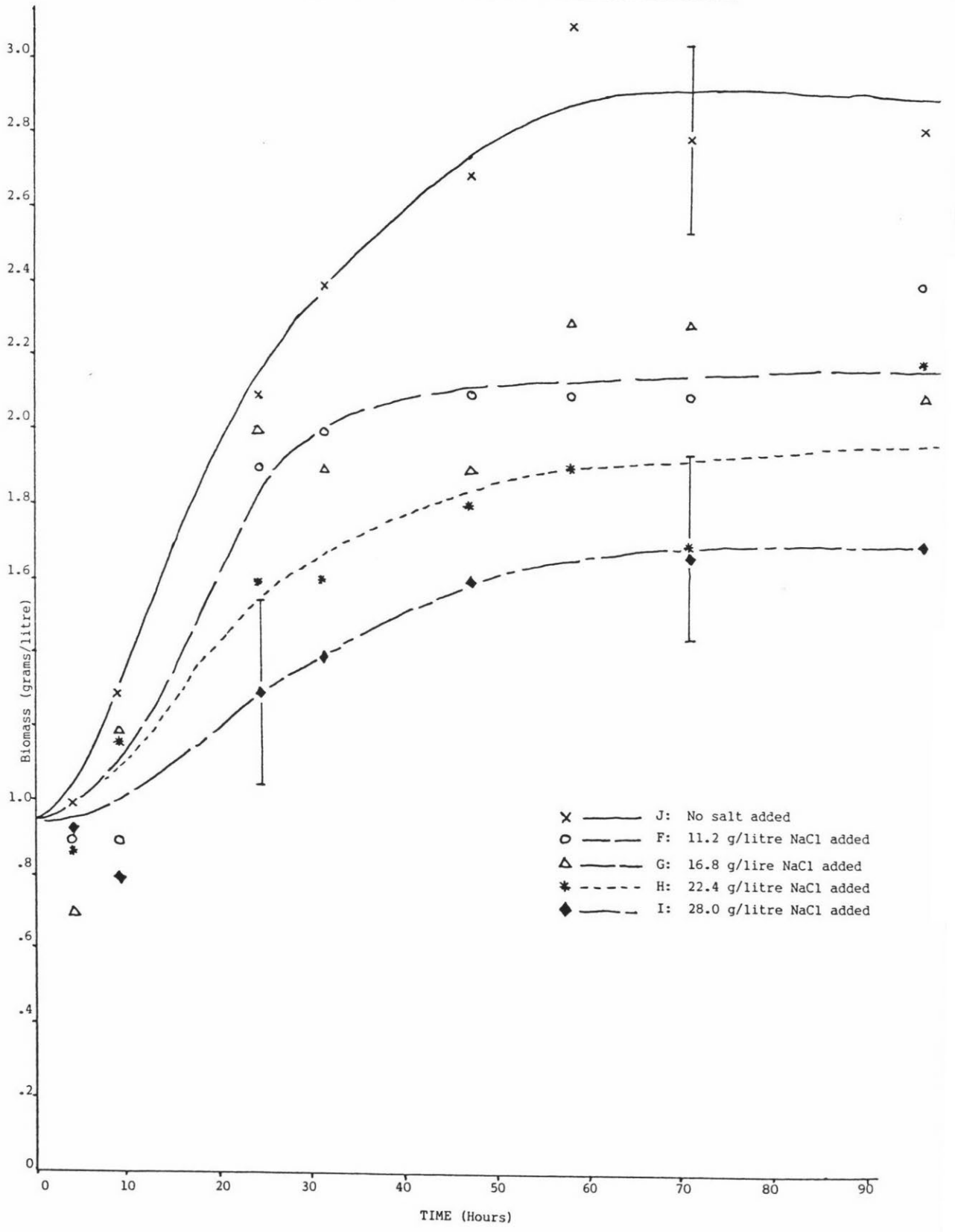


Fig. 4.13: Ethanol Concn Vs Time; Run 4

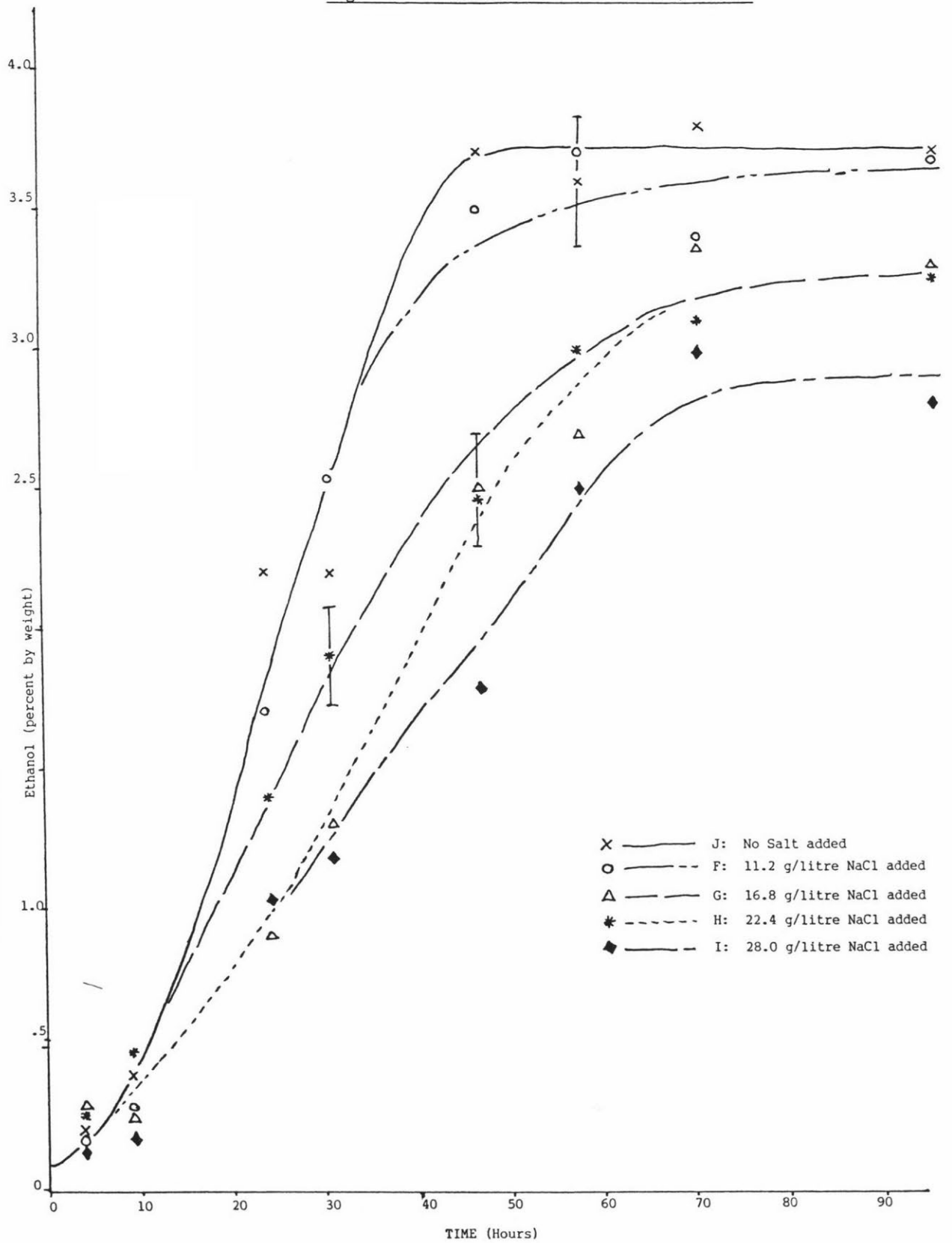
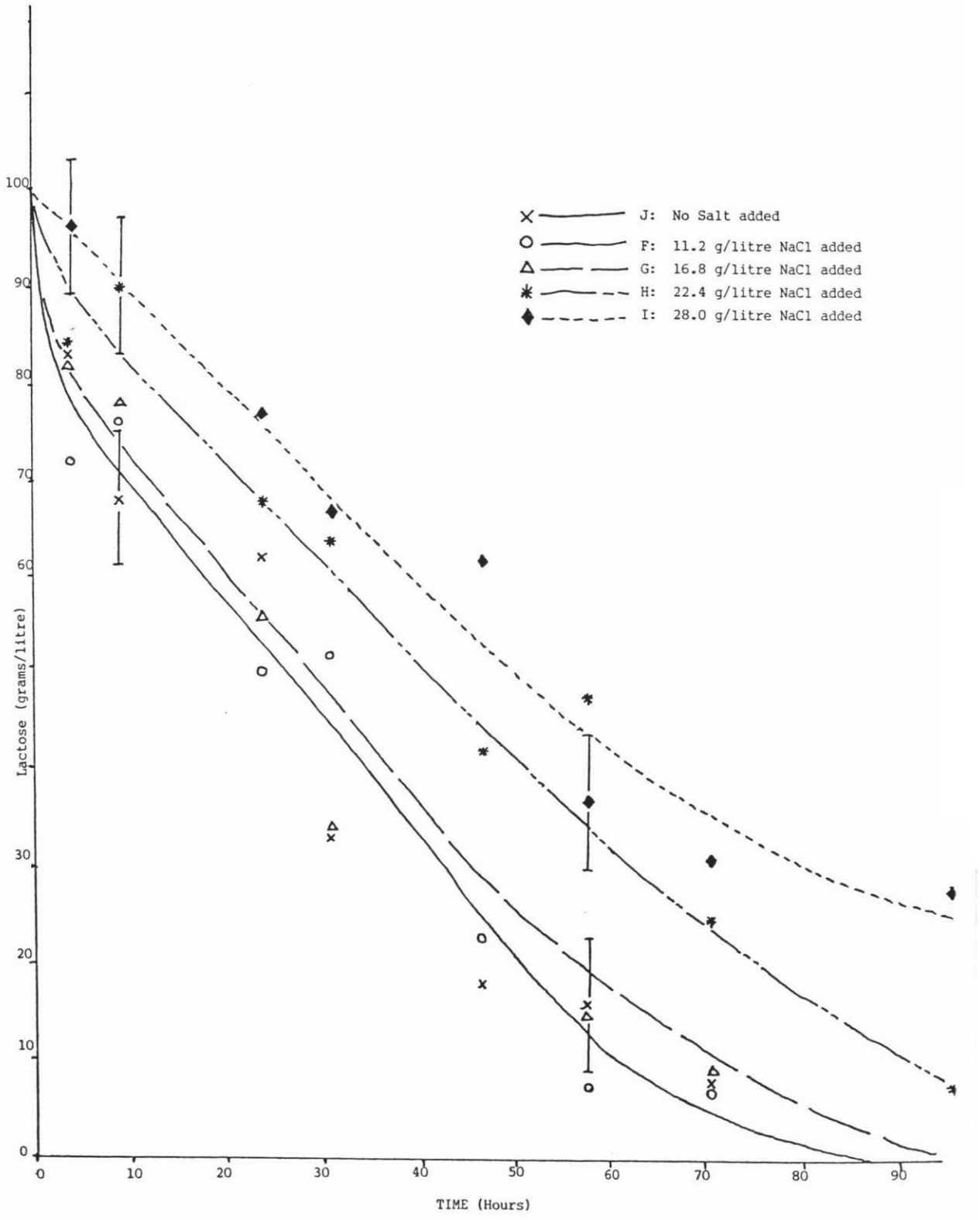


Fig. 4.14: Lactose Concn Vs Time; Run 4



4.4 ETHANOL CONCENTRATION

4.4 1. Introduction

The effects of ethanol on yeasts were discussed in Chapter 2 (Section 2.3.5.4). Many *Kluyveromyces* strains have been shown to be sensitive to ethanol (Vienne and von Stockar, 1985a, Harbison, 1984; Mawson and Taylor, 1989) and it appeared likely that ethanol concentration was the most important controlling factor in the fermentation under scrutiny in these studies. From the runs carried out up to this point it appeared that the maximum concentration able to be produced by this strain of *K.marxianus* lay between 4 and 5% (by weight) so absolute ethanol was added (after filter sterilisation) in concentrations up to 50 g/litre.

If ethanol was inhibitory it was expected that the growth and alcohol production would be successively lower as the concentration neared 40 to 50 g/litre.

4.4 2. Results and Discussion

The results to this experiment (Run 5) are presented in table 4.6 and figures 4.15, 4.16 and 4.17 and confirm that the inhibitory effects of ethanol is more important than those of tonicity. Higher initial alcohol concentrations led to lowered growth rate and less biomass formation, incomplete lactose utilisation and lowered product yields (Yps, Ypx).

TABLE 4.6 Summary of Results; Run 5

All media contained 100 g/litre lactose, 3 g/litre yeast extract and 5 g/litre bactopeptone. Ethanol was added as follows:

A = No ethanol added
B = 10 g/litre added
C = 20 g/litre added
D = 30 g/litre added
E = 40 g/litre added
F = 50 g/litre added

1. Initial Biomass = 1.2 g/litre

2. Maximum Biomass

A = 2.9 g/litre (50 hours)
B = 3.0 g/litre (35 hours)
C = 2.8 g/litre (50 hours)
D = 2.4 g/litre (35 hours)
E = 2.0 g/litre (35 hours)
F = 1.8 g/litre (35 hours)

3. Ethanol Production (maximum)	Rate (g ETOH/g Bio.h)
A = 38 g/litre (55 hours)	0.34
B = 45 g/litre (55 hours)	0.32
C = 51 g/litre (55 hours)	0.35
D = 51 g/litre (55 hours)	0.32
E = 51 g/litre (55 hours)	0.25
F = 51 g/litre (55 hours)	0.03

4. Growth Rate (umax)

A = .048 h^{-1}
B = .048 h^{-1}
C = .048 h^{-1}
D = .026 h^{-1}
E = .024 h^{-1}
F = .018 h^{-1}

5. Lactose used (to attain maximum ethanol concn)

A = 95%
B = 95%
C = 85%
D = 68%
E = 53%
F = 37%

6. Yields

A/ Yxs	B/ Yps	C/ Ypx
A = .018	A = .40	A = 22.2
B = .023	B = .37	B = 16.1
C = .019	C = .36	C = 18.9
D = .020	D = .31	D = 15.5
E = .017	E = .21	E = 12.4
F = .021	F = .03	F = 1.4

Fig. 4.15: Biomass Conc Vs Time; Run 5

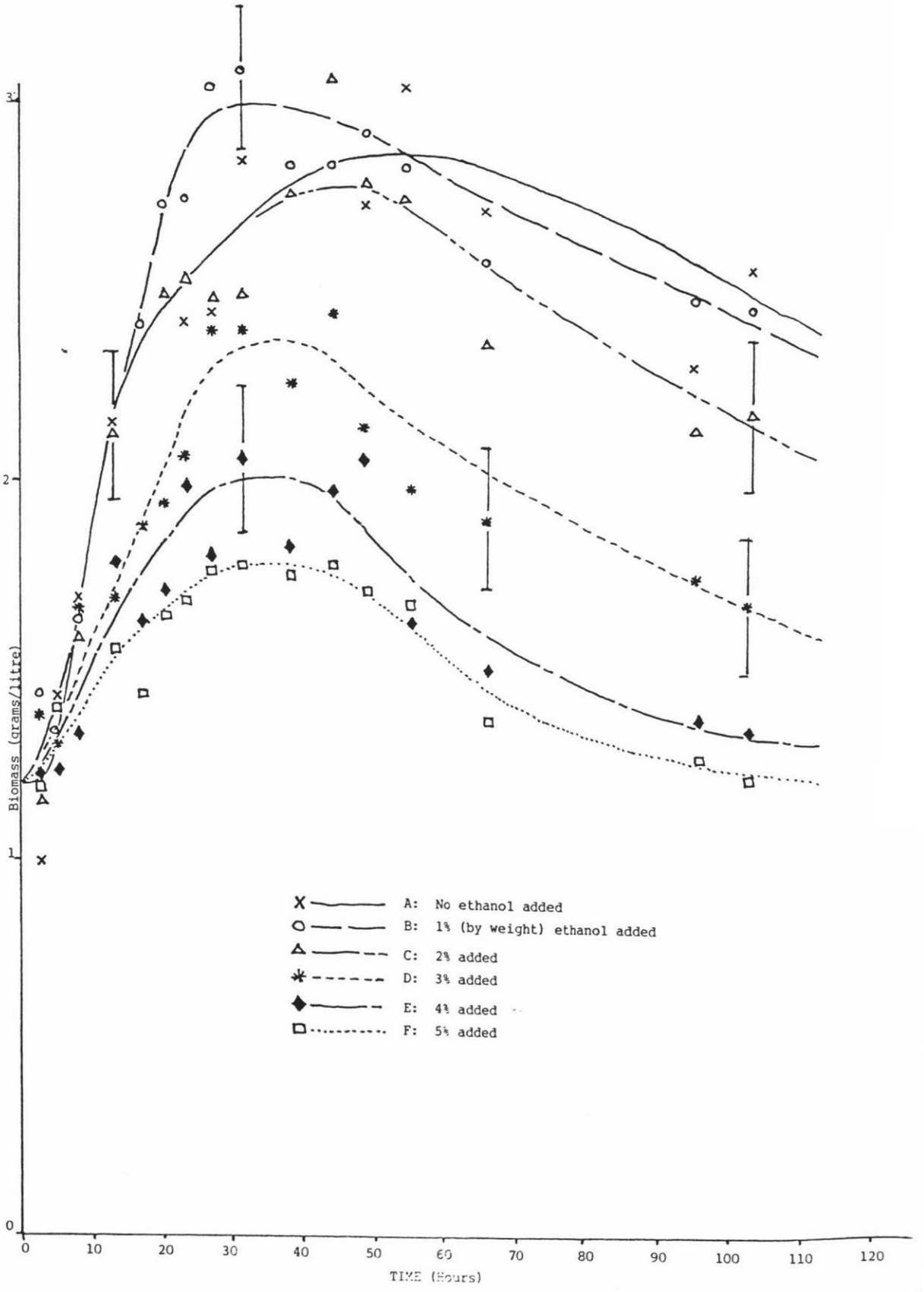


Fig. 4.16: Ethanol Concn Vs Time; Run 5

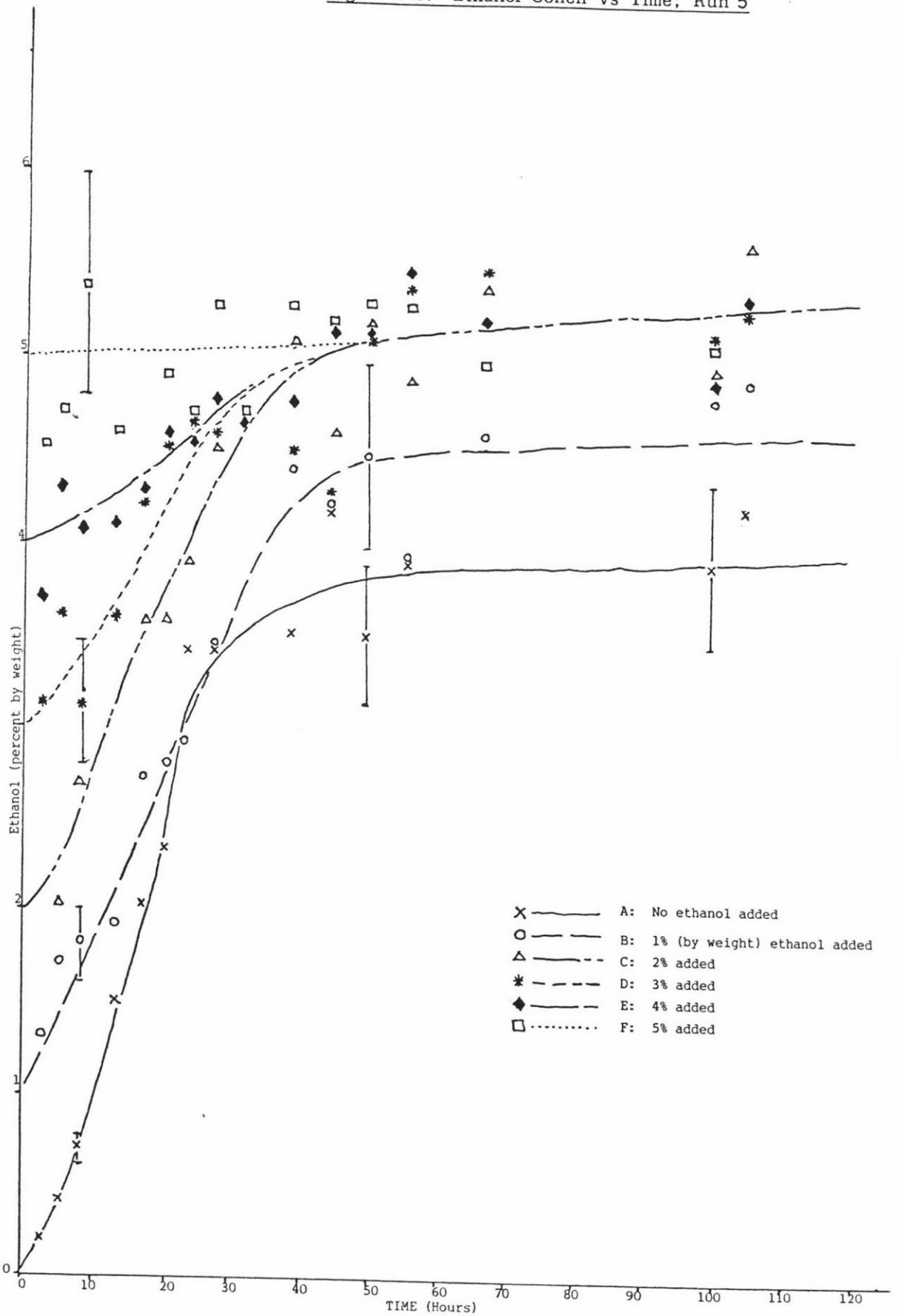
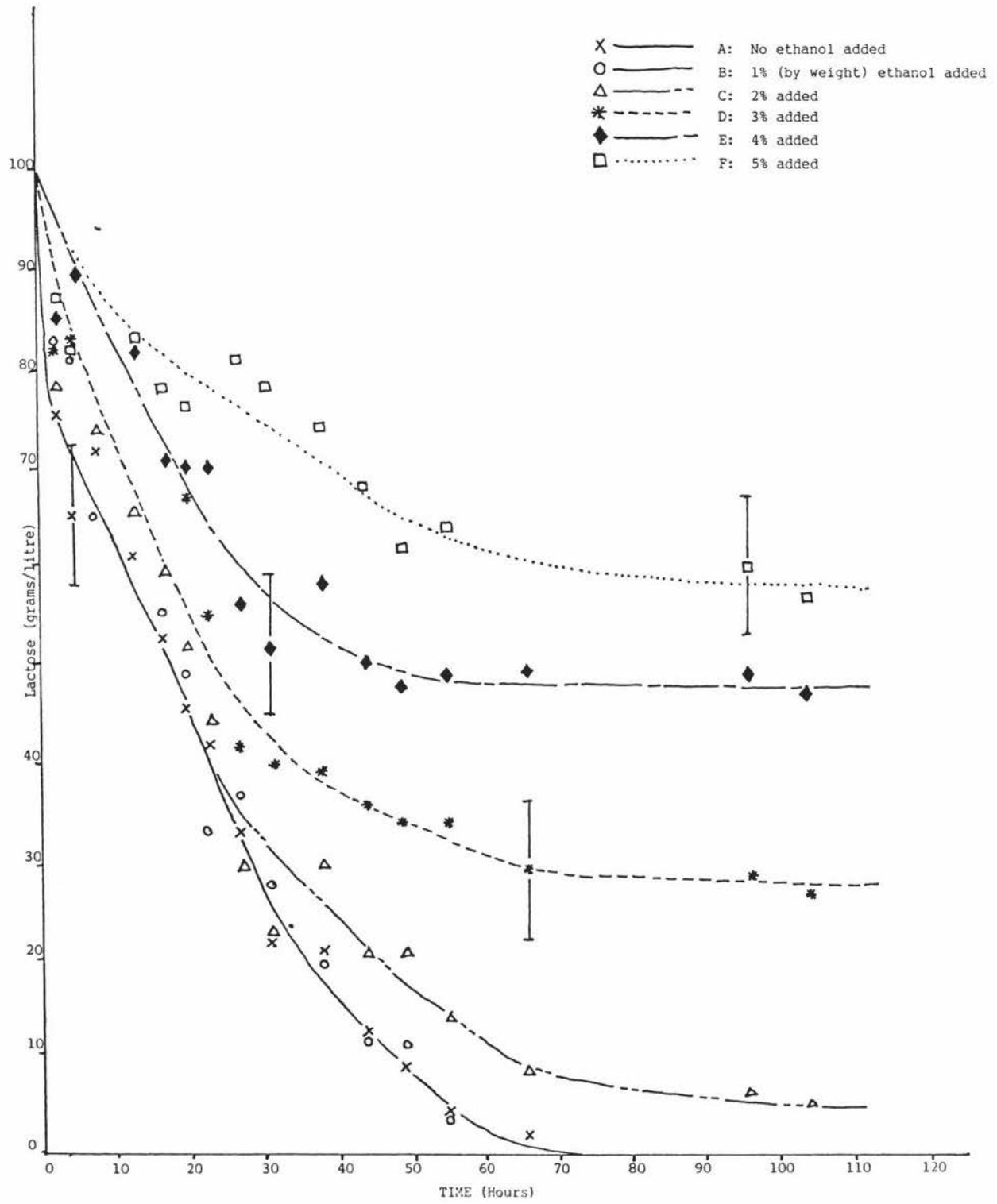


Fig. 4.17: Lactose Concn Vs Time; Run 5



It is noticeable however, that these inhibitory effects were really only significant when the initial concentration of ethanol was greater than around 20 to 30 g/litre. Below that concentration those parameters were virtually unchanged. Also it is interesting to note that the biomass yield (Yxs) remained almost unchanged at around .02 g biomass/g lactose, over the entire range of alcohol concentrations tested.

The fact that ethanol concentrations over 50 g/litre were achieved by the culture which started with more alcohol, suggested a certain build-up of tolerance that did not take place in the other cultures. Culturing yeasts in successively more alcoholic media has been used successfully by other workers to isolate ethanol-tolerant strains of *Kluyveromyces* (Boontanjai, 1983) and it is possible that this occurred to a smaller extent in the experiments reported here.

It is difficult to explain the drop in biomass concentrations observed in all the cultures in the latter stages of the fermentations. It is probable that cell lysis was responsible but the reasons for that taking place are not clear. As the method of biomass measurement was spectrophotometry it is possible that agglutination or flocculation could give the impression of biomass concentration reduction but *K.marxianus* Y113 is a non-flocculating strain so this explanation would seem less likely.

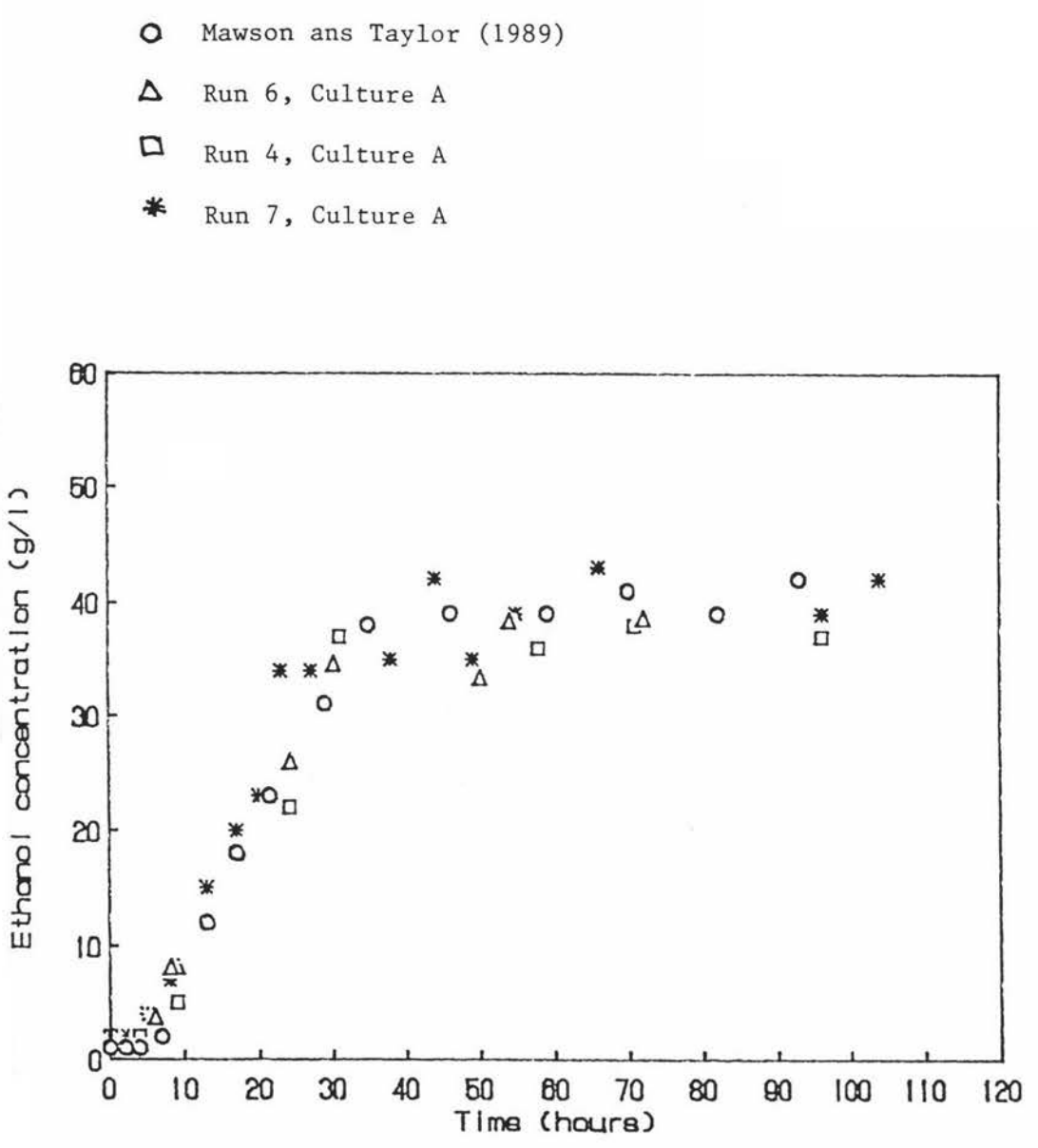


Fig 4.18: COMPARISON OF ETHANOL PRODUCTION IN 100 g/LITRE LACTOSE BROTH

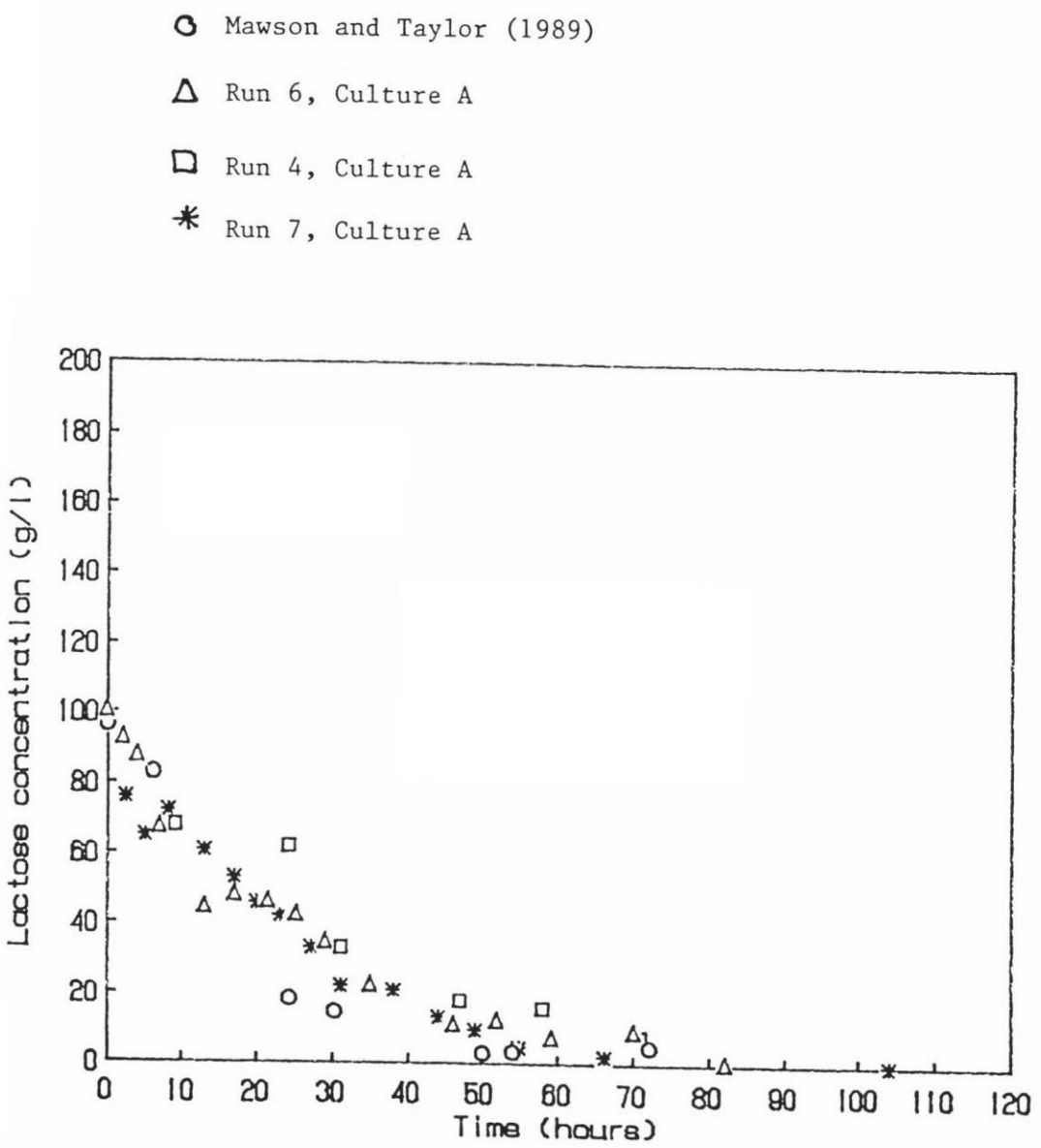


Fig 4.19: COMPARISON OF LACTOSE CONSUMPTION IN 100 g/LITRE LACTOSE BROTHS

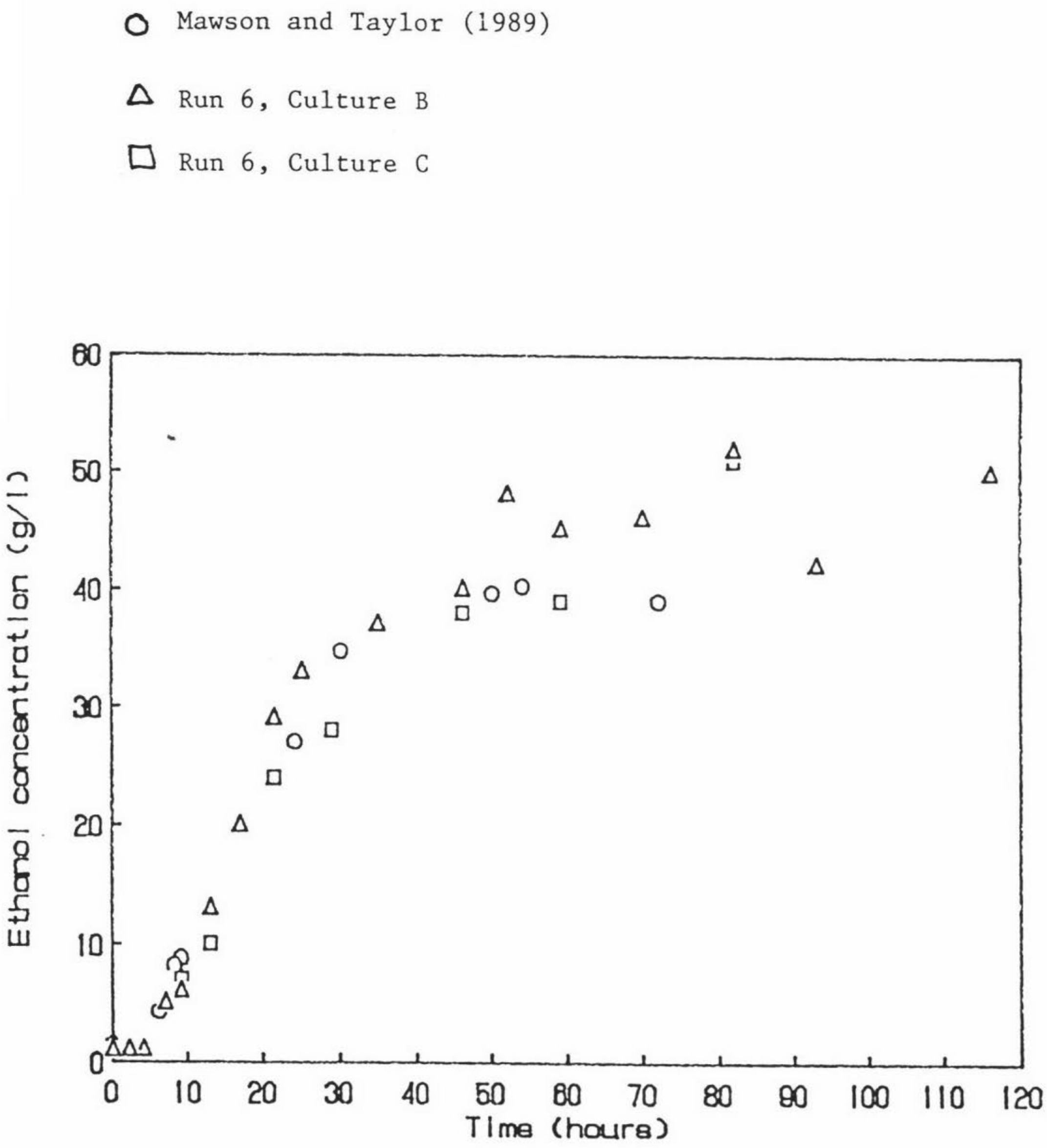


Fig 4.20: COMPARISON OF ETHANOL PRODUCTION IN 150 g/LITRE LACTOSE BROTHS

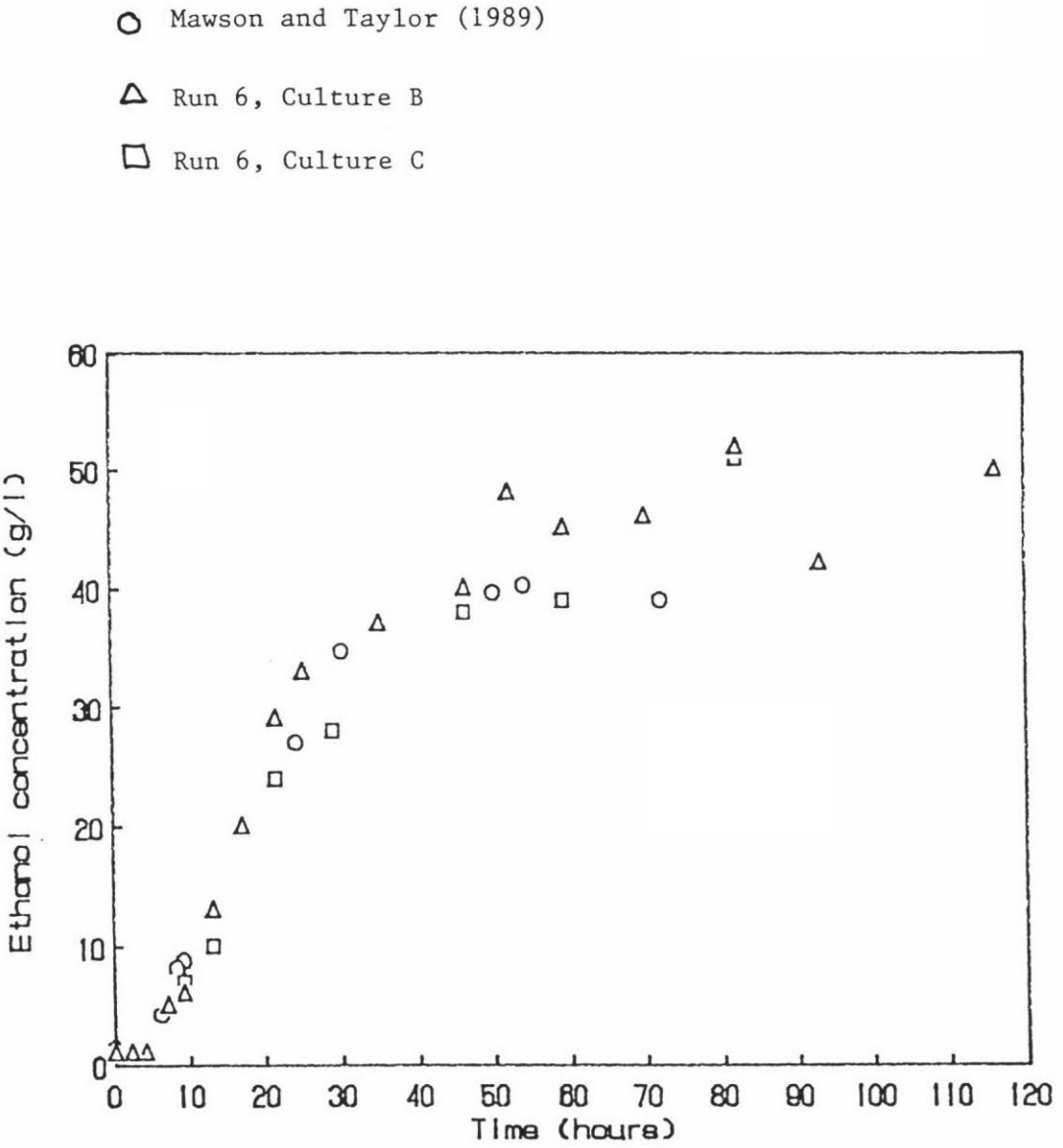


Fig 4.20: COMPARISON OF ETHANOL PRODUCTION IN 150 g/LITRE LACTOSE BROTHS

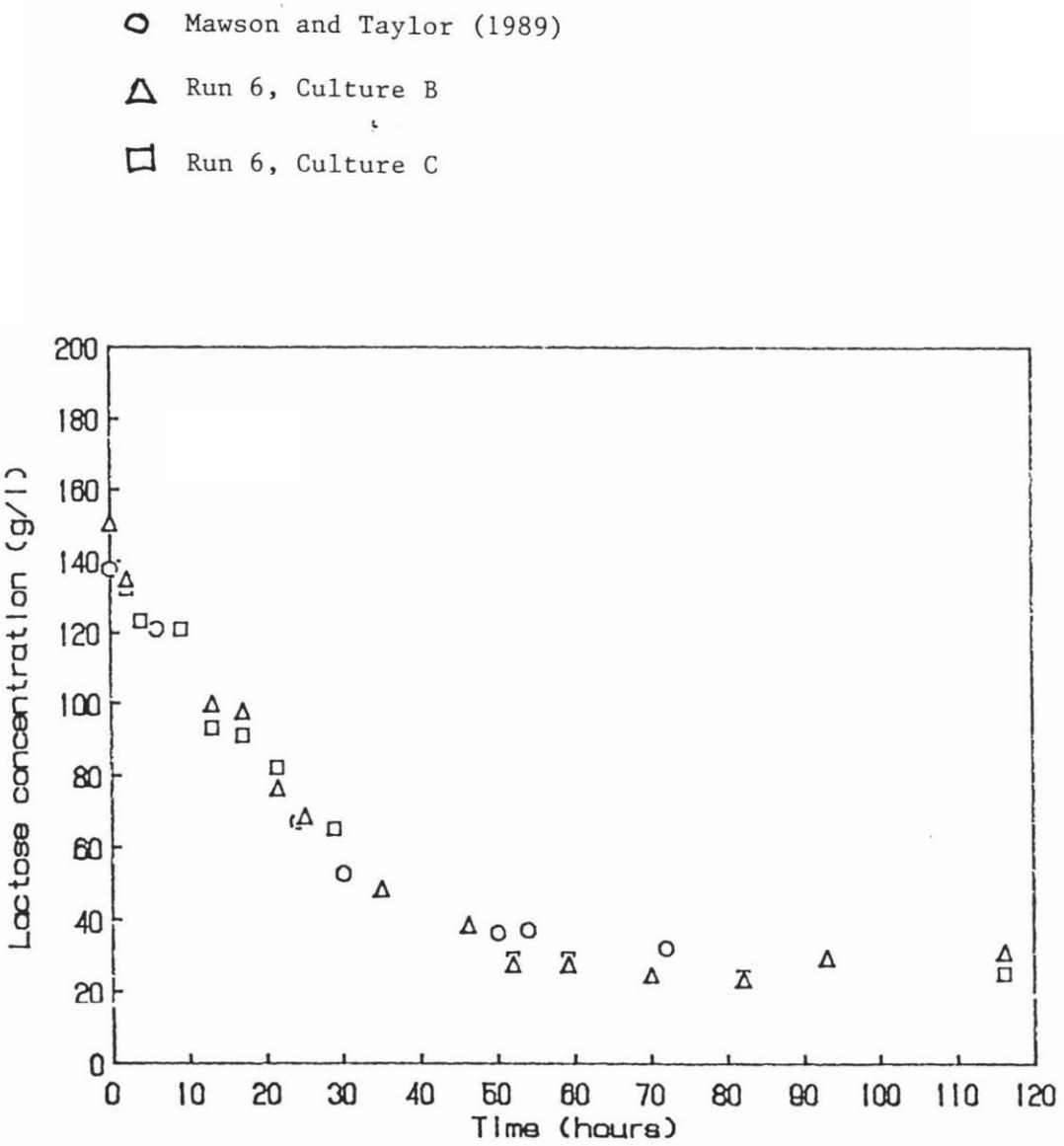
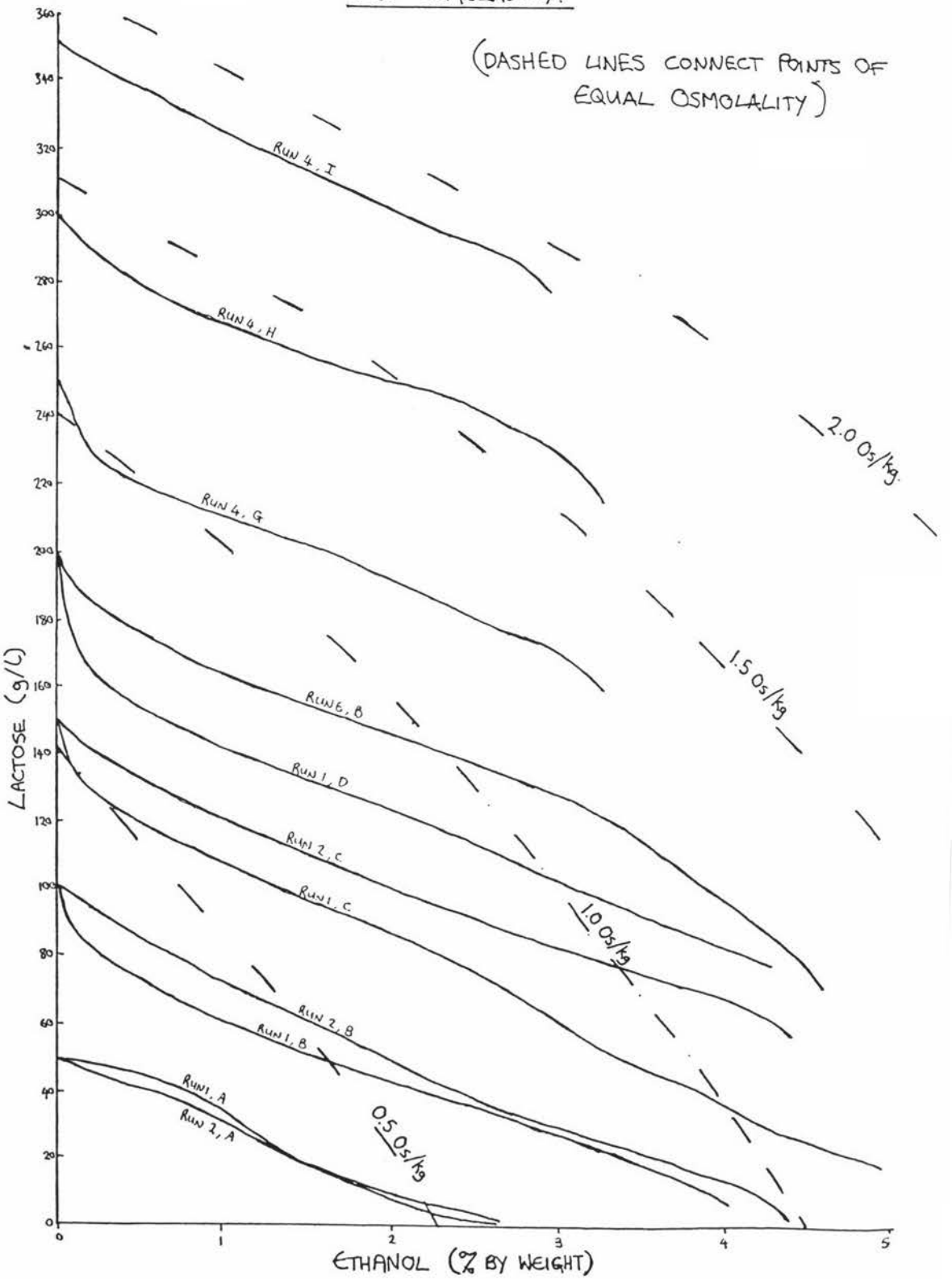


Fig 4.21: COMPARISON OF LACTOSE CONSUMPTION IN 150 g/LITRE LACTOSE BROTHS.

FIG. 4.22 PLOT OF LACTOSE CONCENTRATION AGAINST ETHANOL CONCENTRATION SHOWING LINES OF ISO-OSMOLALITY.



4.5 SUMMARY AND CONCLUSIONS

The work presented in this chapter demonstrated that the fermentative performance of *K.marxianus* Y113 on lactose substrate declined when higher initial lactose concentrations were used. Specifically the maximum growth rate and biomass concentration achieved the lactose utilisation and the yield coefficients, Yxs and Yps were all reduced. This confirmed previous work carried out with this strain (Mawson and Taylor, 1989) as well as other yeast strains (Burgess and Kelly, 1979, Janssens, *et al*, 1984, Vienne and von Stockar, 1985a, 1985b; Bothast, 1986).

It was found that the addition of non-fermentable substances to the fermentation medium to raise the osmolality was detrimental to the accumulation of biomass, ethanol production and lactose utilisation. The yield coefficients, Yxs and Yps were reduced slightly but the coefficient, Ypx was unchanged. Very similar results were found by Harbison (1984) using *K.fragilis* LAB105, another lactose-fermenting strain.

Ethanol added at the start of the fermentation reduced the maximum growth rate and biomass concentration formed, as well as lowering the ethanol production rate, lactose consumption and the yield coefficients Yps and Ypx. It did not change the coefficient Yxs however.

The detrimental effects of added ethanol were not significant below 20 to 30 g/litre. Above this concentration the reduction in performance was large with maximum growth rate reduced by 62% by the addition of 50 g/litre ethanol to the initial fermentation medium.

However, the effects of added ethanol were not as great as expected after observing the detrimental effects of ethanol produced by the yeast itself. This phenomenon has been seen before and has been the subject of a certain amount of debate (Jones, 1990; Pamment and Desri; 1990). Various reasons have been suggested including magnesium deficiency and acetaldehyde build-up in the cell but the problem has yet to be solved satisfactorily.

Overall these experiments confirmed for *K.marxianus* Y113 behaviour that had been observed in other yeast strains previously. It showed that there were two inhibitory mechanisms operating to reduce the yeast's performance during the fermentation of concentrated lactose media:high osmolality and ethanol toxicity. While the ethanol effects were difficult to measure precisely due to the differences in toxicity between added and generated ethanol, it appeared that they were the most important.

5. THE EFFECTS OF MEDIUM NUTRITIONAL STATUS ON ETHANOL PRODUCTION FROM LACTOSE

5.1 INTRODUCTION

The intolerance of *K.marxianus* Y113 to ethanol and high medium osmolality were shown to be important in limiting the production of ethanol from lactose by this strain (see Chapter 4). Work on solving these problems in other yeast strains has been carried out for several years and much of it has concerned the addition of nutrient supplements to the fermentation broth.

This chapter describes work carried out by the author on *K.marxianus* Y113 in which the effects of medium nutritional status on the fermentative performance were investigated.

Section 5.2 describes experiments in which the importance of yeast extract concentration was tested. High concentrations of lactose were fermented with varying levels of yeast extract supplementation.

The importance to the yeast's performance of nutrients contained in whey permeate was tested in the following section, where demineralised whey permeate was compared with unaltered permeate and lactose broth. The concentrations used were set so that the initial lactose concentrations were the same and the only differences between the media were their nutrient contents.

Yeast extract is an expensive supplement which makes it inappropriate to use in industrial ethanol production. Still bottoms are a by-product of ethanol distillation which are cheap and available in large quantities. It

was thought that they could be used as a substitute for yeast extract as they contain the remains of boiled yeast biomass and may have a similar nutrient content. Their use as a supplement was tested in the experiments described in section 5.4, in which they were added at concentrations between 2 and 20% to whey permeate media containing 100 g/litre lactose.

The suggestions by some researchers that Magnesium (Pamment and Desri, 1990, Walker, *et al*, 1990), Calcium (Nobais, *et al*, 1988) or chitin (Patil and Patil, 1989) were beneficial to ethanol production by yeasts were investigated for *K.marxianus* Y113 in section 5.5 Pamment and Desri (1990) claimed that magnesium acts as a survival factor, increasing yeast's ability to survive and metabolize during exposure to high concentrations of ethanol.

The supplementation of yeast ethanol-producing fermentations with polysaccharides such as chitin or calcium ions was claimed by Patil and Patil (1989) and Nabais (1988) respectively to increase productivity and in the case of calcium, to stimulate the production of higher concentrations of ethanol.

Finally, section 5.6 describes attempts by the author to use a defined medium to ferment lactose with *K.marxianus* Y113. This was tried because supplementation of whey permeate and lactose broth media had not produced improved results. The demineralisation of whey permeate had produced a medium deficient in nutrients and supplementation with still bottoms had introduced some toxic substance or substances which were detrimental to the fermentation.

The unidentified nature of the nutrients and toxins meant that further work to overcome these problems would have been difficult, so it was thought that a medium containing constituents of known identity and concentration would be a useful place to begin further experiments.

5.2 EFFECTS OF YEAST EXTRACT CONCENTRATION

5.2 1. Introduction

In the first run, as mentioned before (sections 4.2.1) the concentrations of yeast extract supplied to the cultures at the start of the fermentations was increased with the lactose concentration to maintain a ratio of 6 g/litre yeast extract per 100 g/litre lactose. This was considered necessary at the time due to reports in the literature that yeast extract and/or a nutrient-rich medium was important in lessening the effects of ethanol and high medium tonicity inhibition (Jones 1987: Pamment and Desri 1990).

Jones (1987) reported work carried out with *S.cerevisiae* strains which exhibited distinct medium osmolality inhibition during fermentations of 10 to 20% (w/v) glucose media. Increasing the yeast extract supplementation for 1.7 g/litre up to 17 g/litre appeared to alleviate this inhibition. He suggested that the biotin in the extract was responsible for this effect.

Pamment and Desari (1990) investigated the reasons for apparent toxicity differences between added and generated ethanol in yeasts and found that supplementation of the fermentation media with 80 g/litre yeast extract reduced

this difference. They suggested that magnesium in the yeast extract acted as a survival factor which reduced ethanol inhibition (see section 5.5).

To investigate the relevance of these findings for *K.marxianus* Y113 and test the necessity of increasing the yeast extract concentration in proportion to the lactose concentration an experiment was conducted in which lactose broth was made up with 200 g/litre lactose, 5 g/litre bactopeptone and yeast extract concentrations between 3 and 12 g/litre. At such a high lactose concentration any advantages of yeast extract addition should have been evident.

The possibility of the formation of toxic compounds between the lactose and nitrogenous compounds in the bactopeptone and yeast extract supplements due to Maillard reactions during autoclaving was also investigated in this experiment. Two of the cultures contained 12 g/litre yeast extract but one had the lactose autoclaved separately while the other did not.

5.2 2. Results and Discussion

The results to this experiment (Run 6) can be seen in table 5.1 and figures 5.1, 5.2, and 5.3. It appeared that fermentation performance was independent of yeast extract concentration between 3 and 12 g/litre, and that autoclaving the nitrogenous supplements separately from the lactose made no difference either.

TABLE 5.1 Summary of Results; Run 6

Media all contained 200 g/litre lactose and 5 g/litre bactopeptone, plus:

A = 3 g/litre yeast extract
B = 6 g/litre yeast extract
C = 12 g/litre yeast extract
D = 12 g/litre yeast extract

Medium D had the bactopeptone and yeast extract autoclaved separately from the lactose while the other three media had all their constituents autoclaved together.

1. Initial Biomass = .65 g/litre

2. Maximum Biomass

A = 3.2 g/litre (55 hours)
B = 3.2 g/litre (55 hours)
C = 3.2 g/litre (55 hours)
D = 2.8 g/litre (40 hours)

3. Ethanol Production (maximum)	Rate (gEtOH/g bio.h)
A = 47 g/litre (90 hours)	.27
B = 46 g/litre (90 hours)	.27
C = 44 g/litre (90 hours)	.25
D = 45 g/litre (90 hours)	.29

4. Growth Rate (umax)

A, B, C, D = .068 h^{-1}

5. Lactose Used (to attain maximum ethanol concn)

A, B, C, D = 63%

6. Yields

(i) Yxs A,B,C = .029	(ii) Yps A = .38	(iii) Ypx A = 13
D = .030	B = .37	B = 13
	C = .35	C = 12
	D = .36	D = 12

Although this result seemed to contradict some of the reports in the literature, mentioned in section 5.2.1, it is quite possible that the mechanisms under scrutiny here are different from those studied by other workers who used different yeast strains such as *Saccharomyces* strains. Generally speaking the strains studied elsewhere have had higher alcohol tolerances than *Kluyveromyces* so it is probable that different enzymes or metabolic systems are inhibited in each organism and in the case of *K.marxianus* Y113 yeast extract cannot supply the correct nutrients to relieve the inhibition significantly.

Fig. 5.1: Biomass Conc Vs Time; Run 6

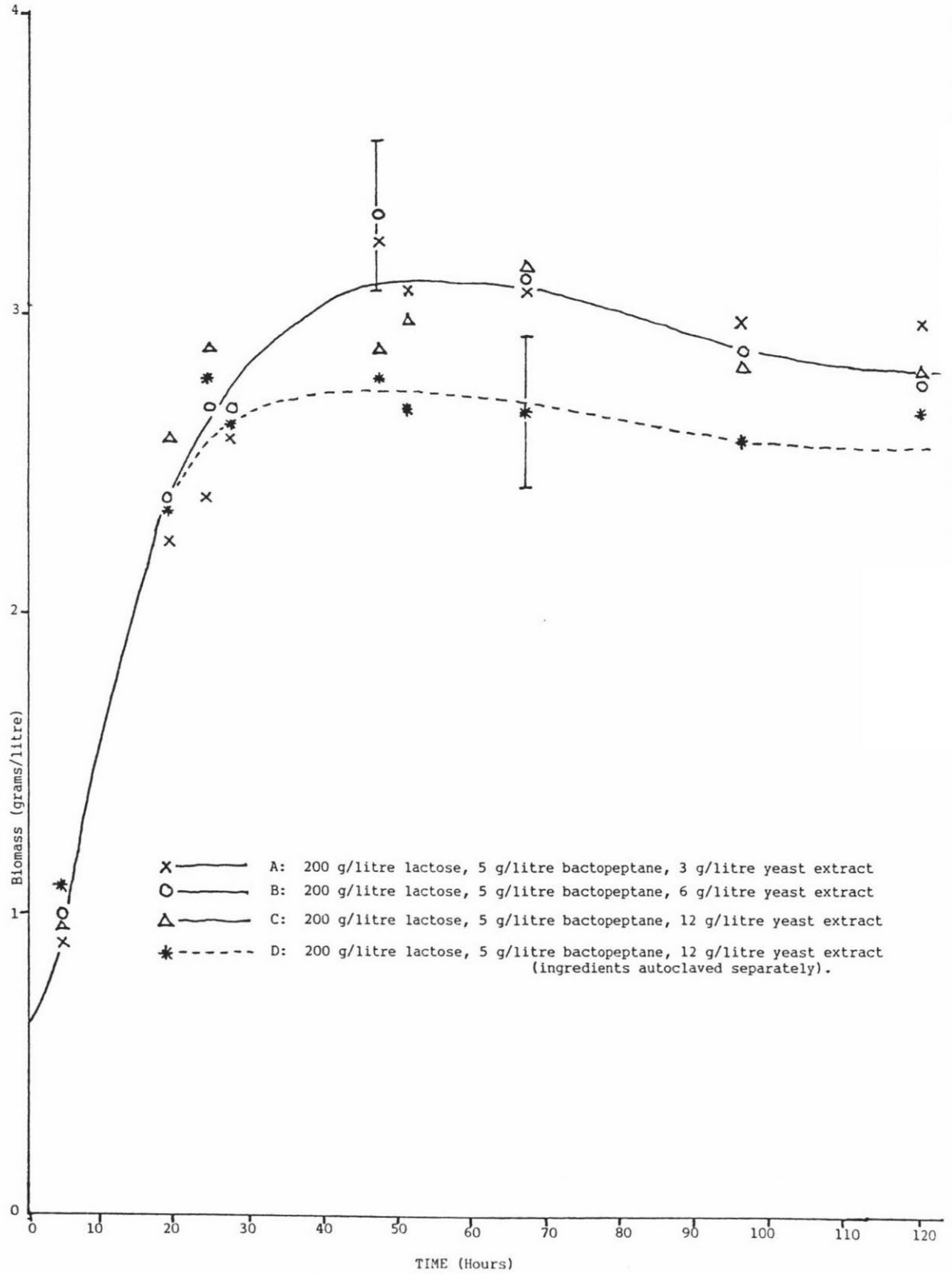


Fig. 5.2: Ethanol Concn Vs Time; Run 6

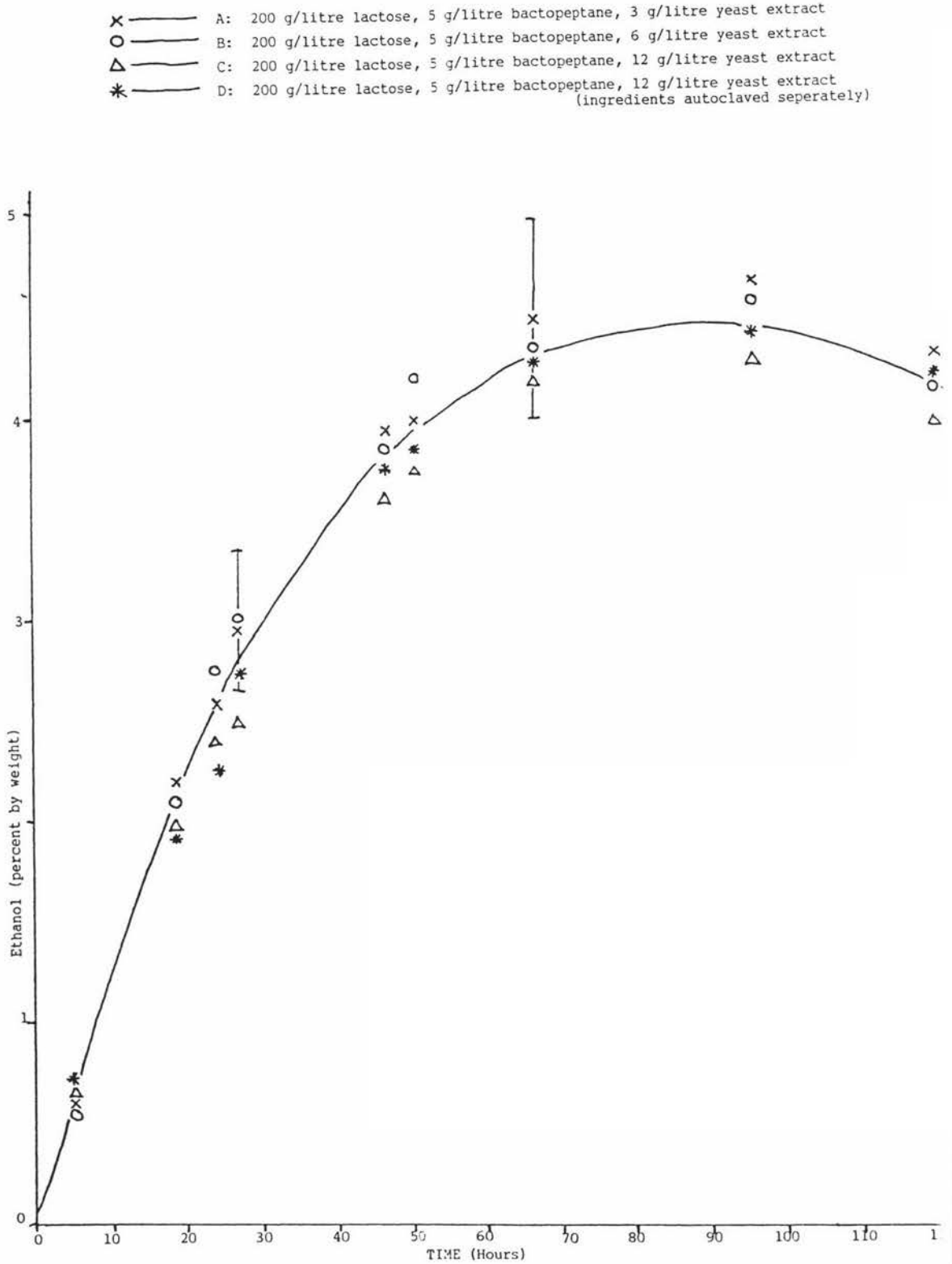
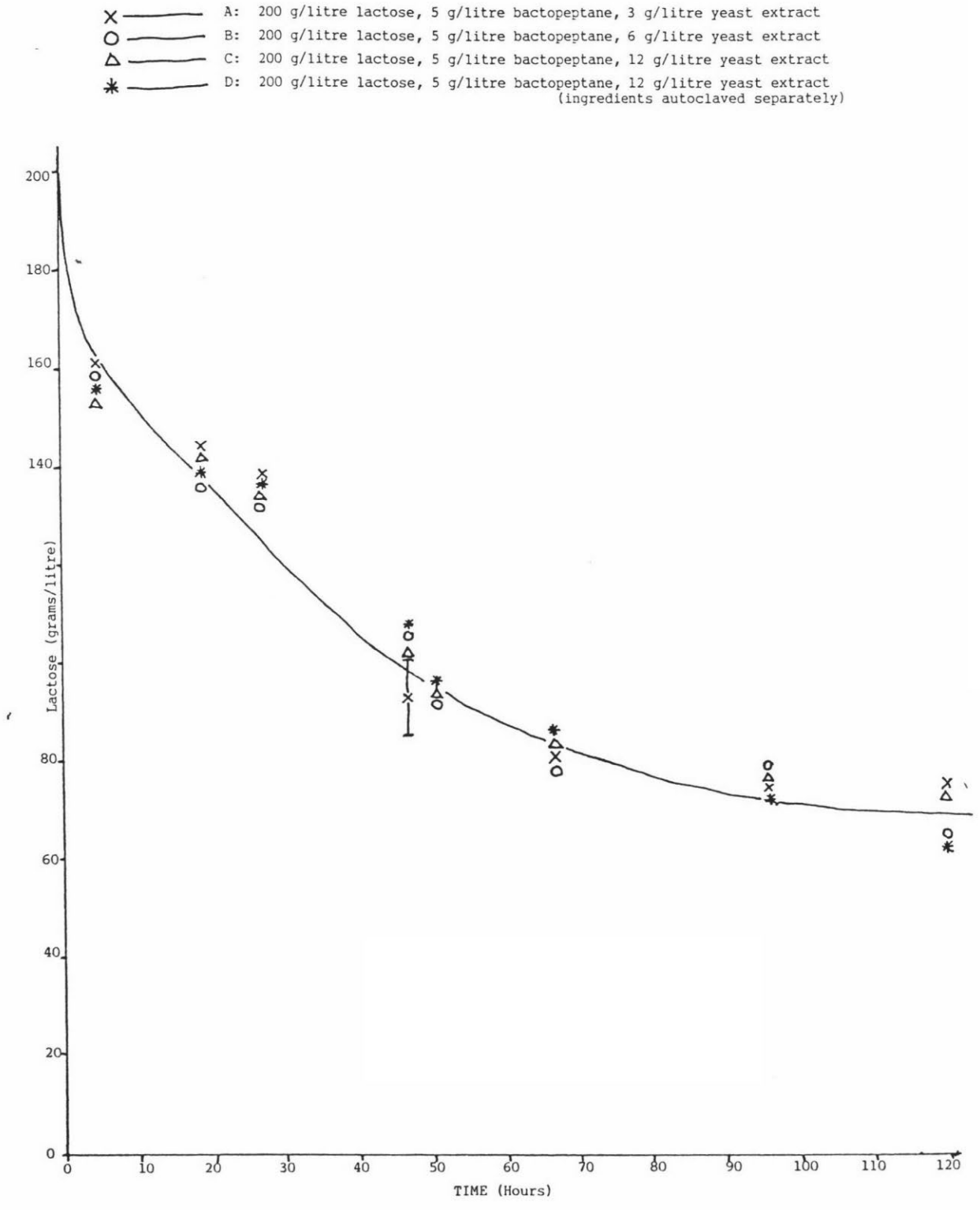


Fig. 5.3: Lactose Concn Vs Time; Run 6



5.3 COMPARISON OF WHEY PERMEATE AND DEMINERALISED WHEY FOR THE PRODUCTION OF ETHANOL

5.3 1. Introduction

It has been established by the experiments reported in this thesis that high medium osmolalities inhibitory to the fermentation of whey permeate and several authors have named salts as being either specific inhibitors or inhibitory due to their lowering of the water activity (El-Samragy and Zall, 1988; Maiorella, *et al*, 1984; Mahmoud and Kosikowski, 1982, Shin, *et al*, 1983).

It would be expected therefore, that lowering the concentrations of ions in the medium would affect the performance of the fermentation and could lead to improvements. This can be achieved by several available techniques but only two - ion exchange and electrodialysis - are commercially significant (Irvine and Hill, 1985).

Electrodialysis removes preferentially species such as potassium, sodium and chloride but also reduces the concentrations of ions like calcium, magnesium and phosphate (Mahmoud and Kosikowski, 1982). As a large proportion of the ash content of whey is made up of these ions electrodialysis can be used to reduce significantly the ash concentration (Radford, 1986).

It has been reported by some workers that reduction of the ash concentration in whey permeate does indeed result in improved fermentations. Demineralisation decreased the time required to ferment concentrated permeate (Vienne and von Stockar, 1985a) and improved the lactose

utilisation and ethanol production (Mahmoud and Kosikowski 1982).

The concentration of ash in dried sulphuric whey permeate powder is about 13.1% (w/w) (see Table 2.4) and from Figure C1 it can be seen that in a concentrated whey permeate medium containing 100 g/litre lactose this contributes about as much osmolality to the medium as 140 g/litre lactose (assuming the ash constituent contribute approximately the same osmolality as sodium chloride). Thus the substantial reduction of salts concentration through demineralisation could play a significant role in reducing the degree of high medium osmolality inhibition in fermentations of concentrated lactose media.

It was decided to compare the fermentation of whey permeate with that of demineralised whey permeate experimentally to see the reduction of salts concentration would improve the performance of *K.marxianus* Y113. The concentrations of permeate powders used were set so that the initial lactose content of the two media was 100 g/litre. Thus, 119 g/litre whey permeate and 102 g/litre demineralised whey permeate powders were used in the respective fermentations. No additional supplements or nutrients were added.

5.3 2. Results and Discussion

A summary of the results to this experiment is given in table 5.2 and figures 5.4, 5.5, and 5.6 and demonstrates that for this particular strain of *K.marxianus* demineralisation of the whey permeate medium did not result in an improved performance. The graphs show that the biomass concentration in the demineralised medium plateaued at a level 25% less than that in the standard permeate, that the lactose consumption was incomplete and that the ethanol concentration produced was also 25% down.

It should however, be noticed that lactose consumption and ethanol production appeared to be continuing in the demineralised culture even as the sampling was terminated and that the rates of both these processes were fairly constant in latter stages of the run. The consistency of these rates suggests that it was enzyme concentration that was the limiting factor and this is easy to reconcile with the lowered ion concentrations in the electrodialysed permeate.

Many enzymes contain a specific metal ion or ions and will not function in its absence and as the concentrations of most common ions are reduced by demineralisation it would seem probable that the rate of fermentation was limited by the low concentration of some vital element.

This hypothesis is further supported by the fact that the yields for the demineralised cultures were approximately equal to those of the standard permeate cultures, indicating that there was no significant increase in maintenance energy indicative of active inhibition in

TABLE 5.2 Summary of Results; Run 7

Medium A contained 119 g/litre whey permeae powder
Medium B contained 102 g/litre demineralised whey permeate powder
Both media were filter sterilised through nitrocellulose membranes (pore size 0.45 um) to avoid the precipitate formed by whey media during autoclaving.

1. Initial Biomass Conc. = 1.16 g/litre

2. Final Biomass Concs A = 2.4 g/litre after 80 hours
   B = 1.8 g/litre after 90 hours

3. Maximum growth rates A = 0.010 h^{-1}
   B = 0.005 h^{-1}

4. Ethanol Production maximum A = 2.4% (w/v) (80 hours)
   B = 1.8% (w/v) (120 hours)
   Overall Rate (g ETOH/g Bio.h) A = 0.017
   B = 0.010

5. Lactose used to attain maximum ethanol concn
   A = 95%
   B = 60%

6. Yields:

   (i) Yxs A = 0.0131 B = 0.0128 (ii) Yps A = 0.253 B = 0.030 (iii) Ypx A = 19.4 B = 23.4

Fig. 5.4: Biomass Concn Vs Time; Run 7

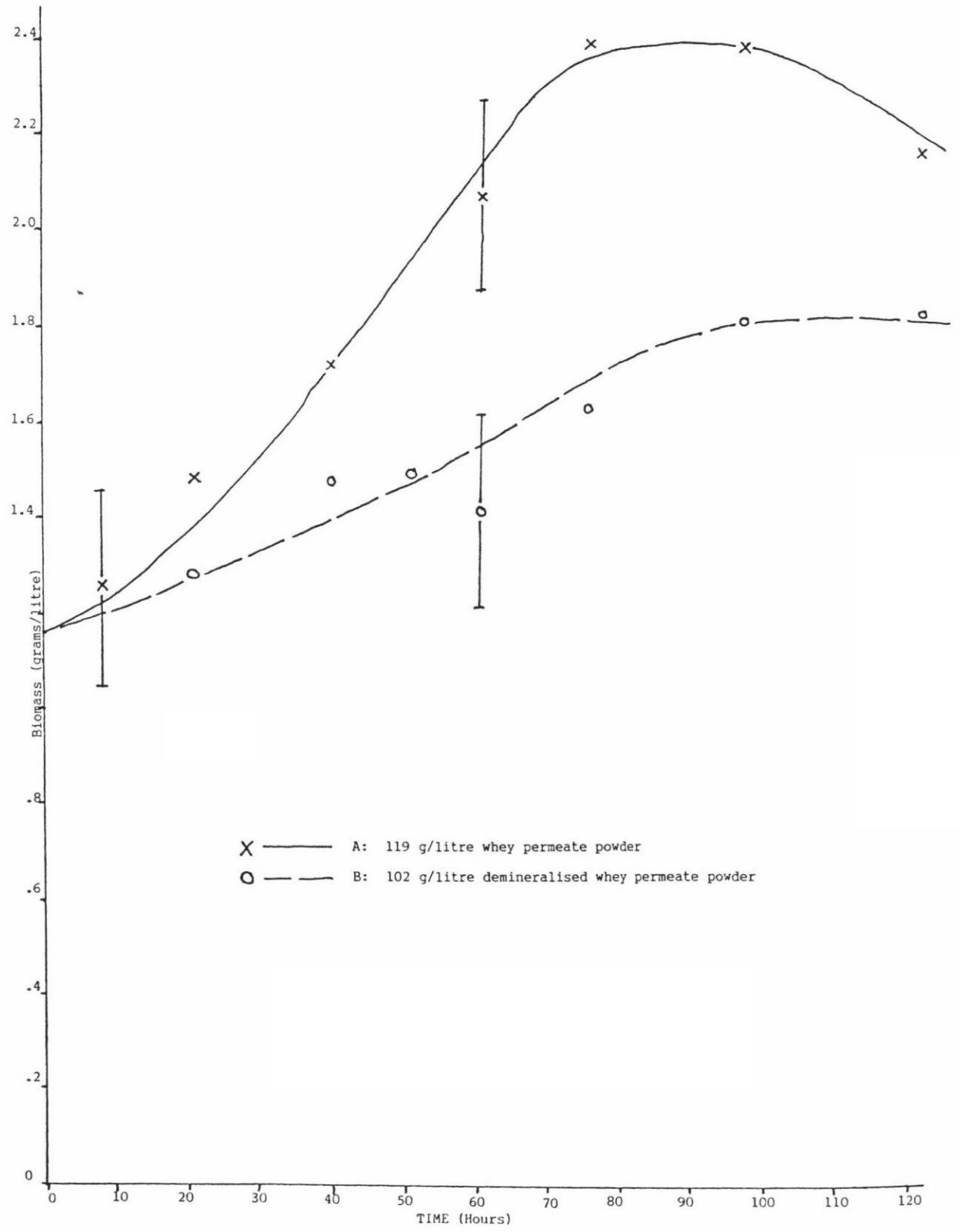


Fig. 5.5: Ethanol Concn Vs Time; Run 7

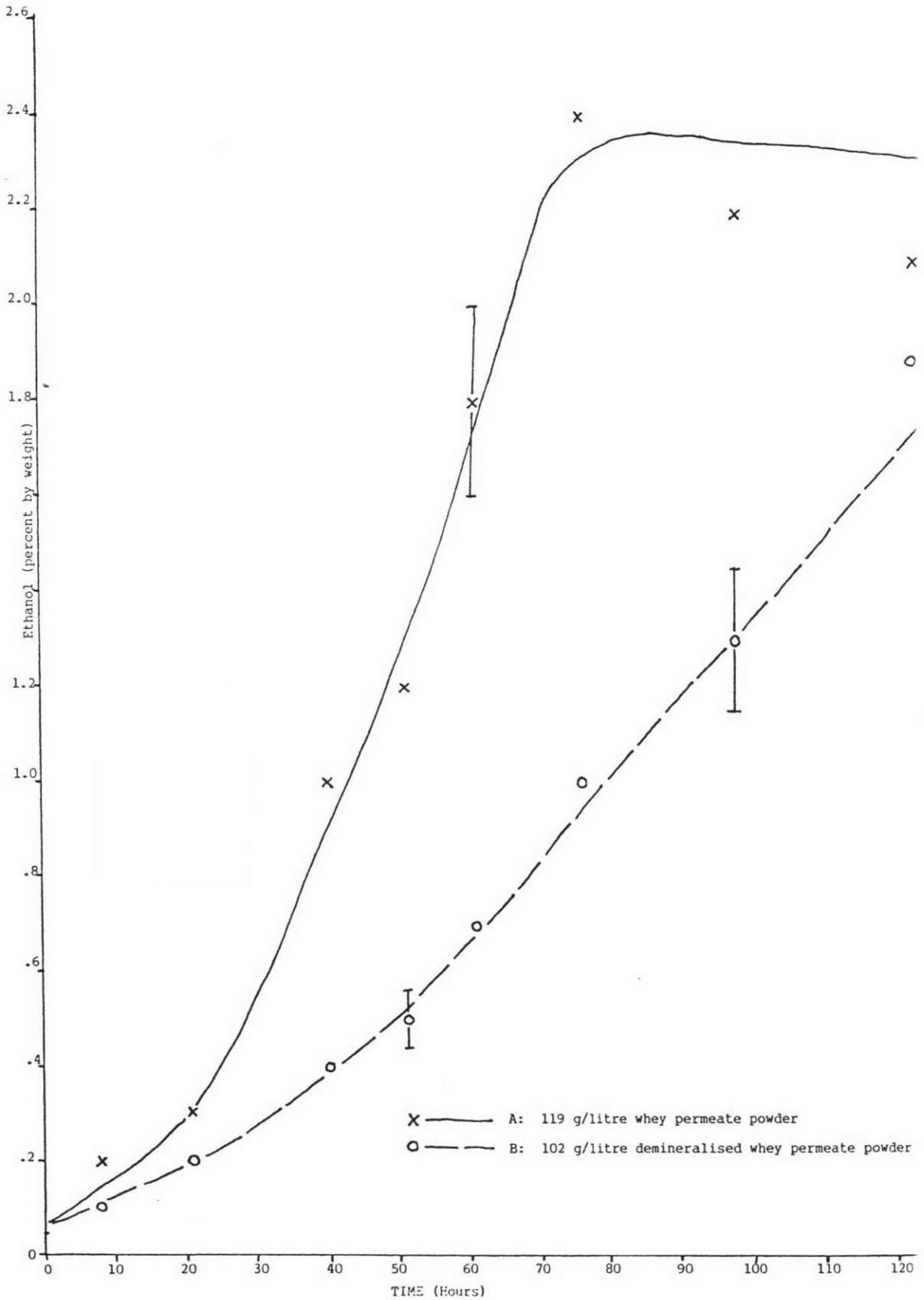
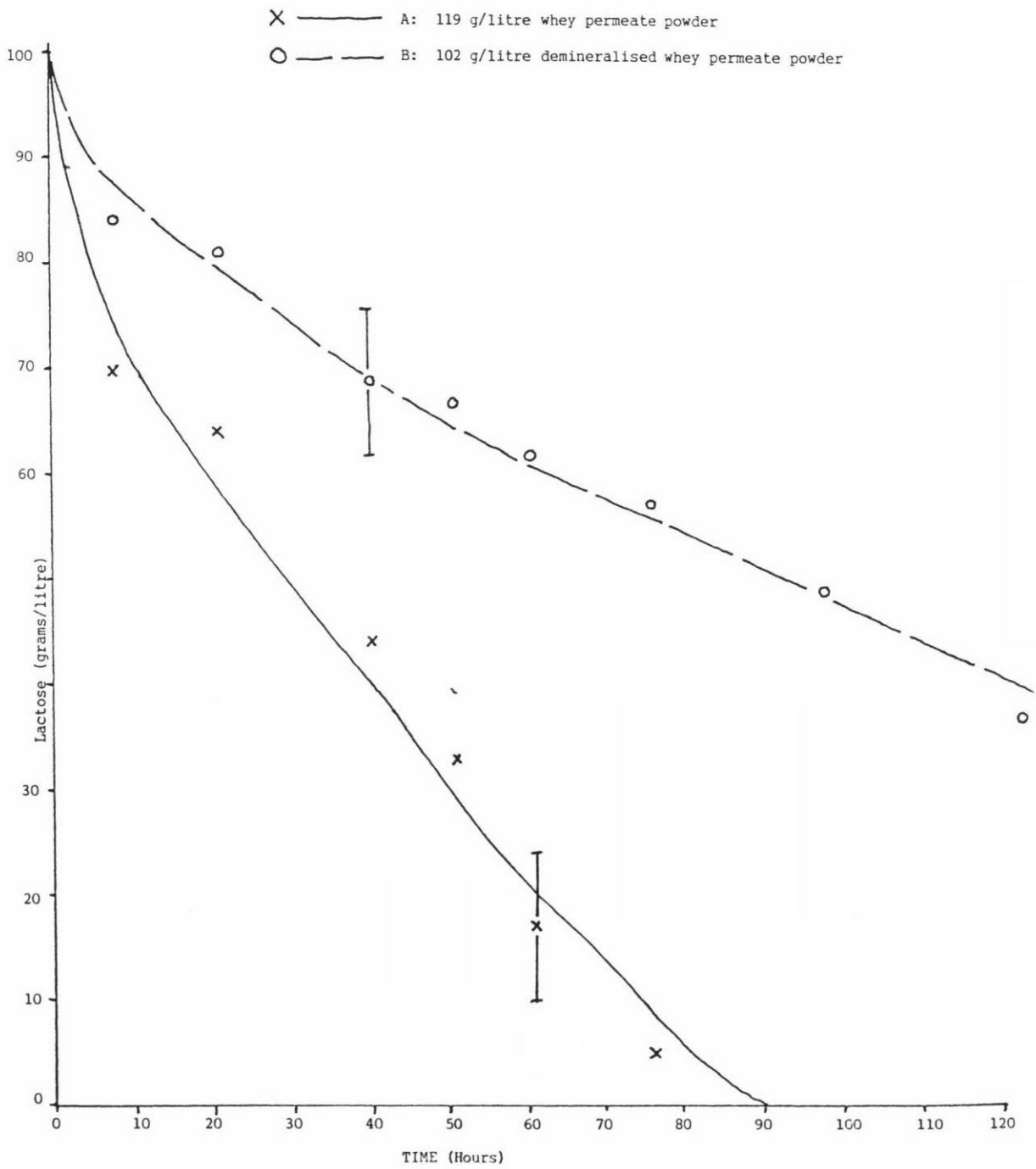


Fig. 5.6: Lactose Concn Vs Time; Run 7



either culture. Claims that reduction of the salt content of some wheys and whey permeates is benficial to their fermentation appear to be untrue for *K.marxianus* Y113 growing in sulphuric whey permeate and it can be concluded that reduction of the salts concentration is of no benefit when concentrated sulphuric whey permeate up to 100 g/litre lactose is used as a substrate, rather, it would appear to be detrimental, suggesting the possibility that supplementation of the fermentation with certain, as yet unidentified nutrients may be of benefit to the process.

5.4 EFFECTS OF SUPPLEMENTATION WITH STILL BOTTOMS

5.4 1. Introduction

Following the separation of the ethanol from the spent fermentation medium by distillation the aqueous phase is left at the base of the distillation column. This liquid contains little carbohydrates but is rich in nutrients due to the concentration of biomass present in it. During distillation the boiling lyses the yeast cells and causes the organic material to dissolve so the still bottoms that are recovered are a clear yellow-coloured liquid.

The use of still bottoms as a supplement in the whey permeate to ethanol fermentation was considered worth investigating as potentially, it was a free source of nutrients which would otherwise be too expensive to use in commercial operation.

In the experiment whey permeate powder was added to each culture (except one which contained 100 g/litre lactose broth) at a rate of 119 g/litre which gave an initial

lactose concentration of 100 g/litre. The media were made up with distilled water plus still bottoms in various proportions as follows:

A = Lactose 100 g/litre, bactopeptone 5 g/litre, yeast extract 3 g/litre.
B = Whey permeate (lactose 100 g/litre) + 2% (v/v) still bottoms
C = Whey permeate (lactose 100 g/litre) + 5% (v/v) still bottoms
D = Whey permeate (lactose 100 g/litre) + 10% (v/v) still bottoms
E = Whey permeate (lactose 100 g/litre) + 20% (v/v) still bottoms
F = Whey permeate (lactose 100 g/litre)

5.4 2. Results and Discussion

Figures 5.7, 5.8 and 5.9 and table 5.3 give the results to this experiment, and from them it can be seen that even the addition of low concentrations of still bottoms was detrimental to the fermentation. The growth of the yeast appeared to be most affected with the maximum biomass concentration being almost halved and the rate dropping to as low as one tenth of the unsupplemented rate with only 5% supplementation with still bottoms.

The production of ethanol also suffered and the final concentration achieved was halved by 2% still bottoms with the average rate being reduced similarly. Lactose consumption also was halved by 2% still bottoms but like ethanol and biomass production the further addition of this substance did not appear to inhibit the fermentation very much more with the 20% supplemented culture giving similar results to the others.

TABLE 5.3 Summary of Results; Run 8

Medium A contained 100 g/litre lactose, 5 g/litre bactopeptone and 3 g/litre yeast extract. The remaining media contained 119 g/litre whey permeate powder plus the following:

A = Lactose, 100 g/litre; bactopeptone, 5 g/litre; Yeast extract, 3 g/litre
B = 2% (v/v) still bottoms
C = 5% (v/v) still bottoms
D = 10% (v/v) still bottoms
E = 20% (v/v) still bottoms
F = Whey permeate alone

All media were filter sterilised through nitrocellulose membranes (pore size 0.45 um) to avoid precipitate formation in the whey permeate media during autoclaving.

1. Initial Biomass 0.9 g/litre

2. Maximum Biomass
   A = 2.2 g/litre (50 hours)
   B = 1.4 g/litre (80 hours)
   C = 1.2 g/litre (80 hours)
   D = 1.0 g/litre (80 hours)
   E = 1.2 g/litre (80 hours)
   F = 1.9 g/litre (50 hours)

3. Maximum Growth Rate

   A = .038 h^{-1}
   B = .004 h^{-1}
   C = .003 h^{-1}
   D = .001 h^{-1}
   E = .003 h^{-1}
   F = .026 h^{-1}

4. Ethanol Production (maximum)

(i) Maximum	(ii) Rates (gEtOH/gbio.h)
A = 42 g/litre (90 hours)	0.30
B = 20 g/litre (117 hours)	0.12
C = 11 g/litre (117 hours)	0.07
D = 10 g/litre (117 hours)	0.08
E = 16 g/litre (117 hours)	0.11
F = 39 g/litre (110 hours)	0.25

5. Lactose used to attain maximum ethanol concn.

A = 100%
B = 45%
D, E, C = 35%
F = 95%

6. Yields

(i) Yxs	(ii) Yps	(iii) Ypx
A = .017	A = 0.42	A = 25
B = .013	B = 0.44	B = 34
C = .009	C = 0.31	C = 34
D = .003	D = 0.29	D = 97
E = .009	E = 0.46	E = 51
F = .018	F = 0.43	F = 24

Fig. 5.7: Biomass Concn Vs Time; Run 8

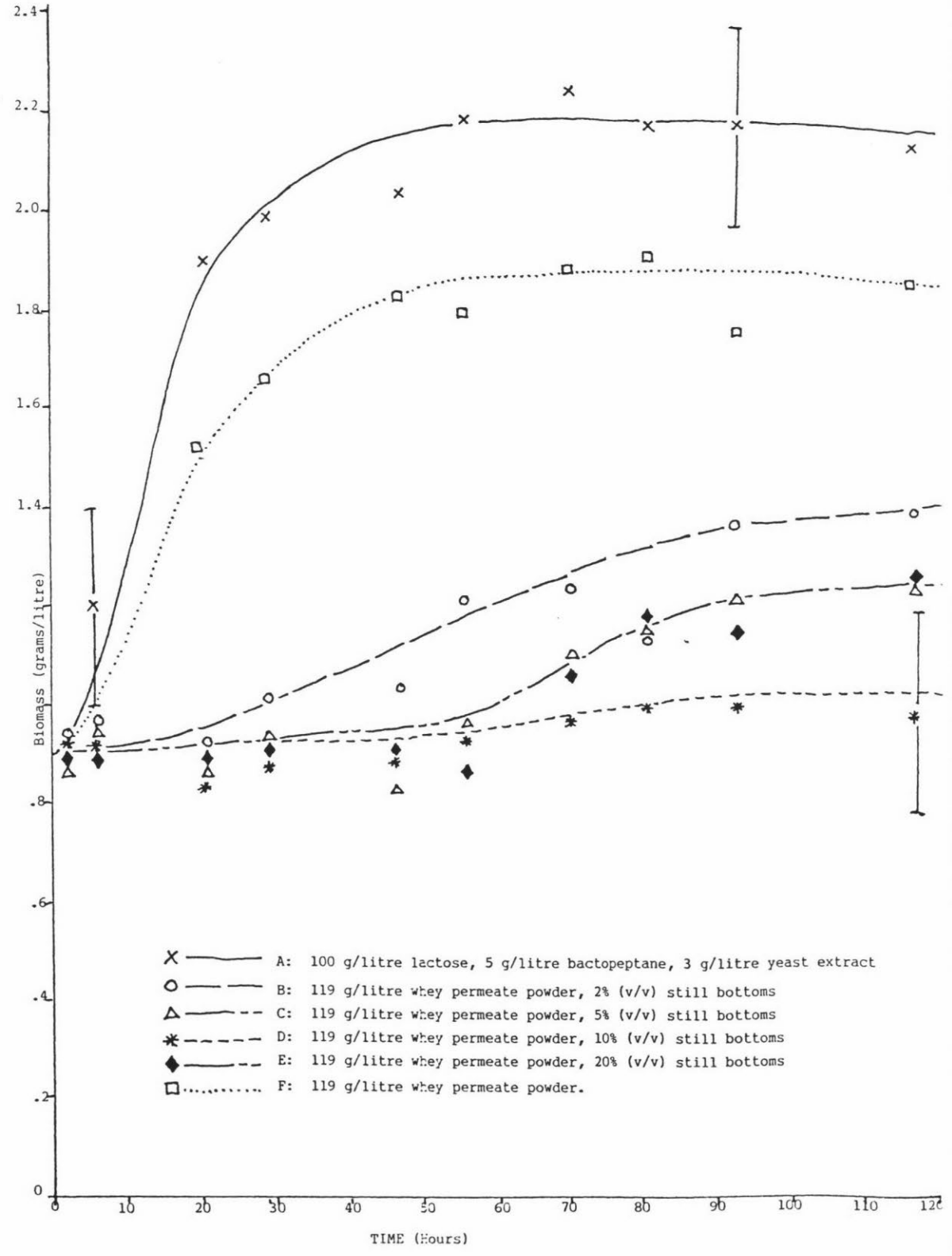


Fig. 5.8 : Ethanol Concn Vs Time; Run 8

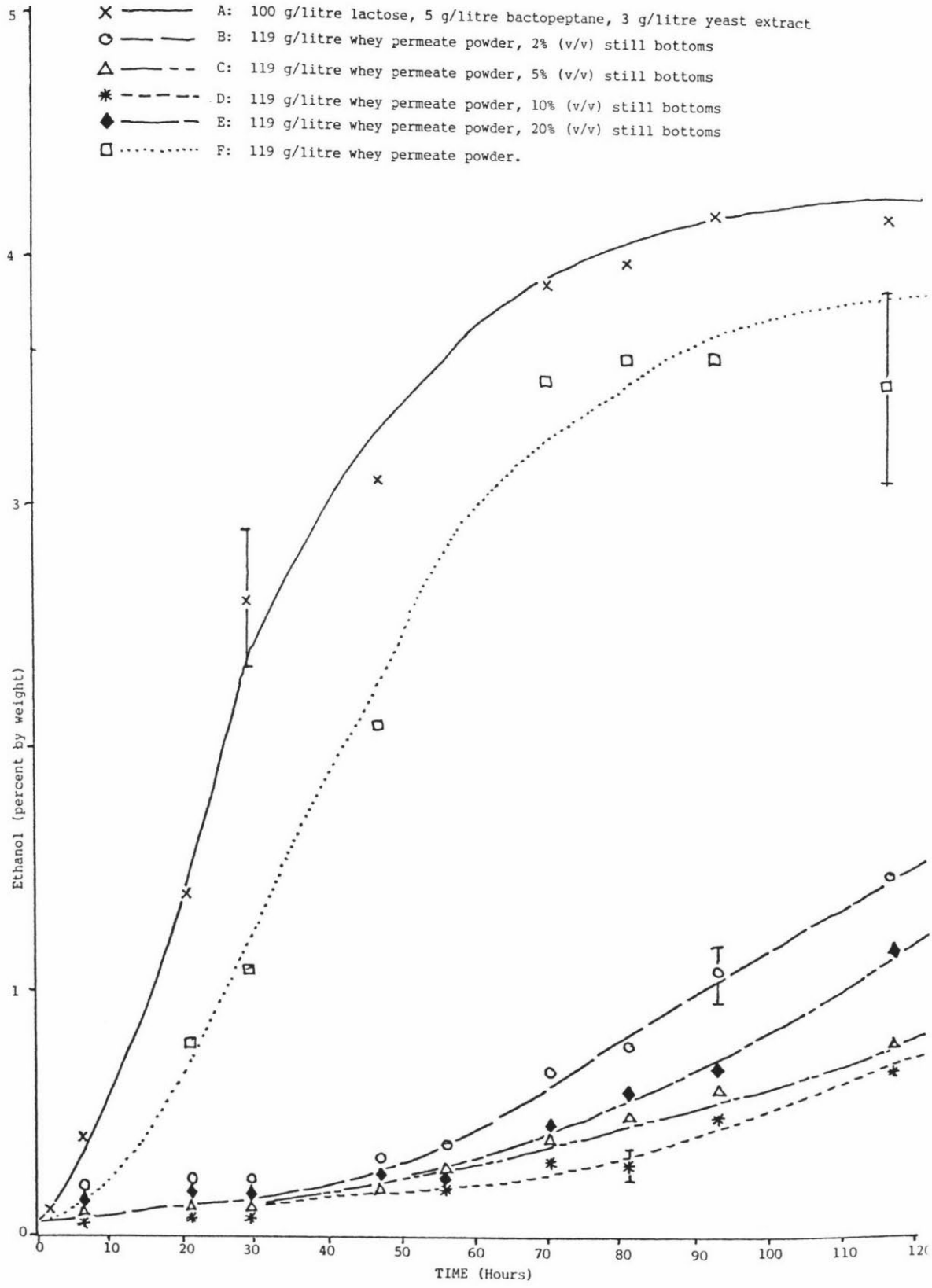
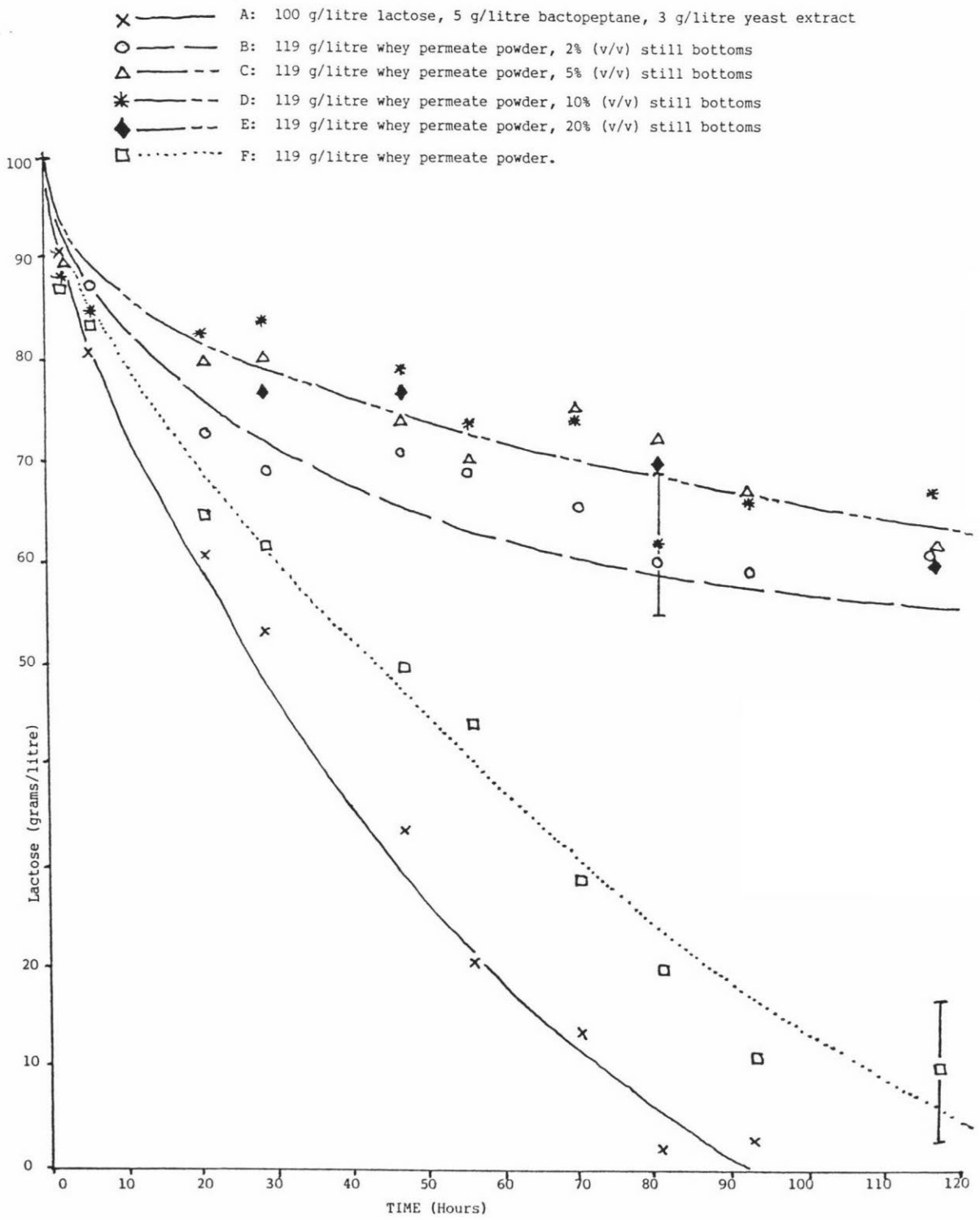


Fig. 5.9 : Lactose Concn Vs Time; Run 8



The yield for biomass production was the only yield that was reduced by the still bottoms which contrasts with the experiment where ethanol was added (section 4.4) in which the product yield was reduced but the biomass yield remained unaffected. It also contrasts with the defined media results (section 5.6) where, although the fermentations were inhibited, all the yields were unaffected. The reduction of the biomass yields in this experiment suggests that some part of the growth metabolism of the yeast was inhibited by some substance in the still bottoms which resulted in an increase in the amount of energy required for growth.

It can be concluded that the use of still bottoms to provide nutrients for the whey permeate to ethanol fermentation was not of benefit and in fact was detrimental and led to a lowered fermentation performance.

5.5 EFFECTS OF SUPPLEMENTATION WITH CHITIN CALCIUM OR MAGNESIUM

5.5 1. Introduction

The intention of this experiment was to test suggestions made by various workers that the addition of chitin, calcium chloride or magnesium chloride to culture media could result in improved fermentative performances by yeasts (Nabais, *et al*, 1988, Patil and Patil, 1989, Pamment and Desari, 1990).

Patil and Patil (1989) reported that the supplementation of fermentations of cane molasses by *Saccharomyces cerevisiae* or *S.uvarum* with polysaccharides such as chitin, xylan or acacia gum increased their rate of

ethanol production. Polysaccharide was added at a concentration of 2 g/litre to the fermentation media. The time required to ferment a molasses medium containing 18% (w/v) reducing sugars to produced 6 - 6.9% (w/v) ethanol was reduced from 72 hours to only 30 hours when polysaccharide was supplied.

Chitin was readily available so it was tried in fermentations of both lactose broth medium and whey permeate medium to see if *K.marxianus* Y113 responded as the *Saccharomyces* species were reported to have done.

Nobais, *et al*, (1988) claimed that the addition to a fermentation medium of between 0.75 and 2 mM calcium ions (as calcium chloride) led to the rapid production of higher concentrations of ethanol than usual by *S.cerevisiae*, *S.bayanus* and *K.marxianus*. Their investigations suggested that in *S.bayanus* at least, the positive effect was due to an increase in ethanol tolerance.

This section describes the results of experiments where calcium chloride dihydrate was included in lactose broth and whey permeate media at a concentration of 2 mM to see if *K.marxianus* Y113 fermentations benefitted from its inclusion.

Also reported in this section are the results of trials involving the addition of magnesium to fermentations by *K.marxianus* Y113. It has been claimed that magnesium can act as a survival factor, protecting yeasts against ethanol inhibition, thereby enhancing the concentrations of alcohol they can produce during fermentation (Pamment and Desari, 1990).

Walker, *et al* (1990) found that supplementing a

fermentation of cheese whey by *K.marxianus* with 0.5 mM magnesium chloride resulted in a marked increase in lactose utilisation rate. The time required to ferment half of an initial lactose content of 50 g/litre was reduced from almost six hours without supplementation down to only 1.3 hours when magnesium was added. They suggested that respirative metabolism was favoured over fermentation when magnesium was deficient in the medium and this led to better performances after supplementation.

5.5 2. Results and Discussion

See table 5.4 and figures 5.10, 5.11, 5.12, 5.13, 5.14 and 5.15 for details of the results to these experiments (Run 9). The whey permeate media gave slightly lower final ethanol and biomass concentrations compared to the lactose broth media. The yields (Yxs and Yps) were also lower in the cultures growing in whey permeate media.

The addition of either chitin, magnesium or calcium appeared to do little toward improving the fermentation at the concentrations tested. It should be noted that the higher biomass readings in the chitin-supplemented cultures can be explained by the presence of tiny, insoluble particles of chitin suspended in the media. In terms of lactose consumption and ethanol production those cultures containing chitin were not significantly different from the controls.

TABLE 5.4 Summary of Results; Run 9

Two different base media were used; the usual lactose broth (100 g/litre lactose, 3 g/litre yeast extract, 5 g/litre bactopeptone) and 'whey permeate' (119 g/litre whey permeate powder). The flasks were made up as follows:

A = lactose broth (as above)
B = whey permeate (as above)
C = lactose broth plus 2 g/litre chitin
D = whey permeate plus 2 g/litre chitin
E = lactose broth plus 2 mM $CaCl_2 2H_2O$
F = whey permeate plus 2 mM $CaCl_2 2H_2O$
G = lactose broth plus 2 mM $MgCl_2 6H_2O$
H = whey permeate plus 2 mM $MgCl_2 6H_2O$

A/ Initial Biomass = .73 g/litre

B/ Maximum Biomass

A = 2.9 g/litre (40 hours)
B = 4.6 g/litre (50 hours)
C = 4.1 g/litre (40 hours)
D = 3.4 g/litre (50 hours)
E = 3.2 g/litre (40 hours)
F = 2.0 g/litre (35 hours)
G = 2.9 g/litre (40 hours)
H = 2.7 g/litre (35 hours)

C/ Maximum Growth Rate (u/max)

A = .064 h^{-1}
B = .043 h^{-1}
C = .102 h^{-1}
D = .075 h^{-1}
E = .077 h^{-1}

F = .079 h^{-1}

G = .067 h^{-1}

H = .054 h^{-1}

D/ Production of Ethanol (maximum)

	Rate (gEtOH/gbio.h)
A = 44 g/litre (50 hours)	.48
B = 41 g/litre (60 hours)	.41
C = 45 g/litre (50 hours)	.37
D = 39 g/litre (60 hours)	.31
E = 45 g/litre (50 hours)	.45
F = 39 g/litre (60 hours)	.36
G = 45 g/litre (50 hours)	.49
H = 39 g/litre (60 hours)	.37

E/ Consumption of Lactose to attain ethanol concn.

A = 88%

B = 91%

C = 100%

D = 95%

E = 91%

F = 91%

G = 85%

H = 91%

F/ Yields

A/ Yxs	B/ Yps	C/ Ypx
A = .028	.50	18
B = .022	.45	20
C = .036	.46	13
D = .030	.41	14
E = .029	.49	17
F = .030	.43	14
G = .031	.53	17
H = .027	.43	16

Fig. 5.10: Biomass Concn Vs Time; Run 9

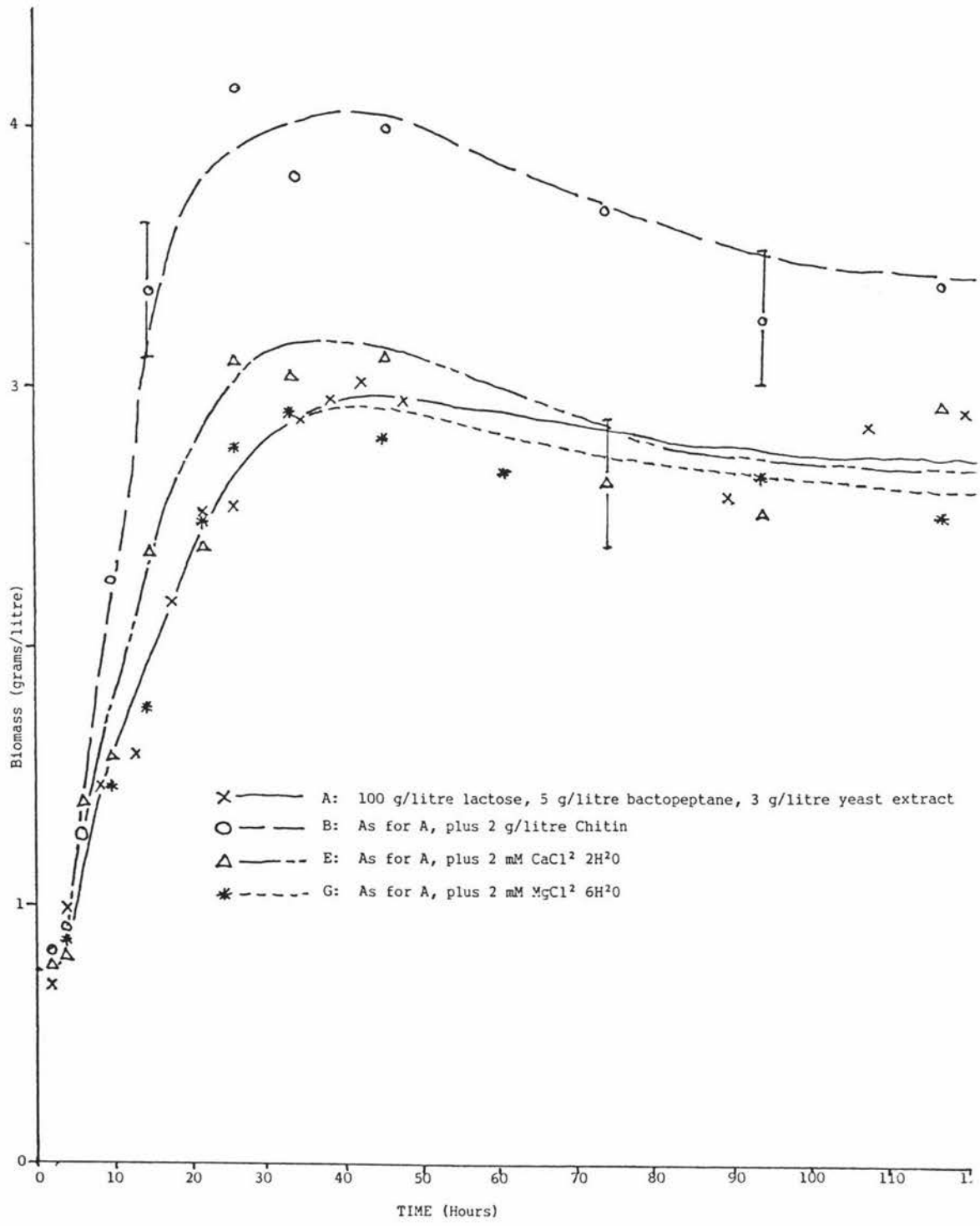


Fig. 5.11: Ethanol Concn Vs Time; Run 9

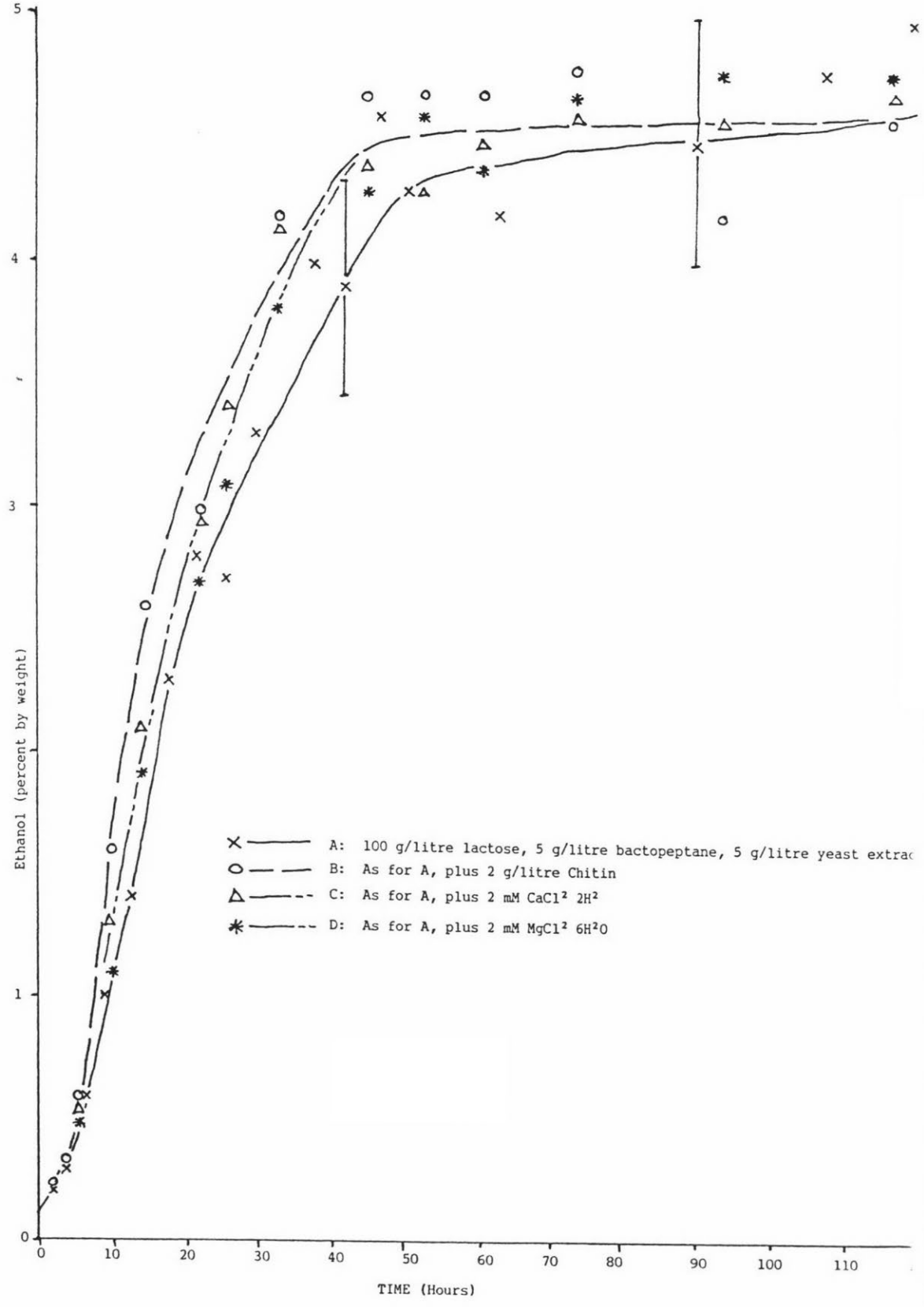


Fig. 5.12: Lactose Concn Vs Time; Run 9

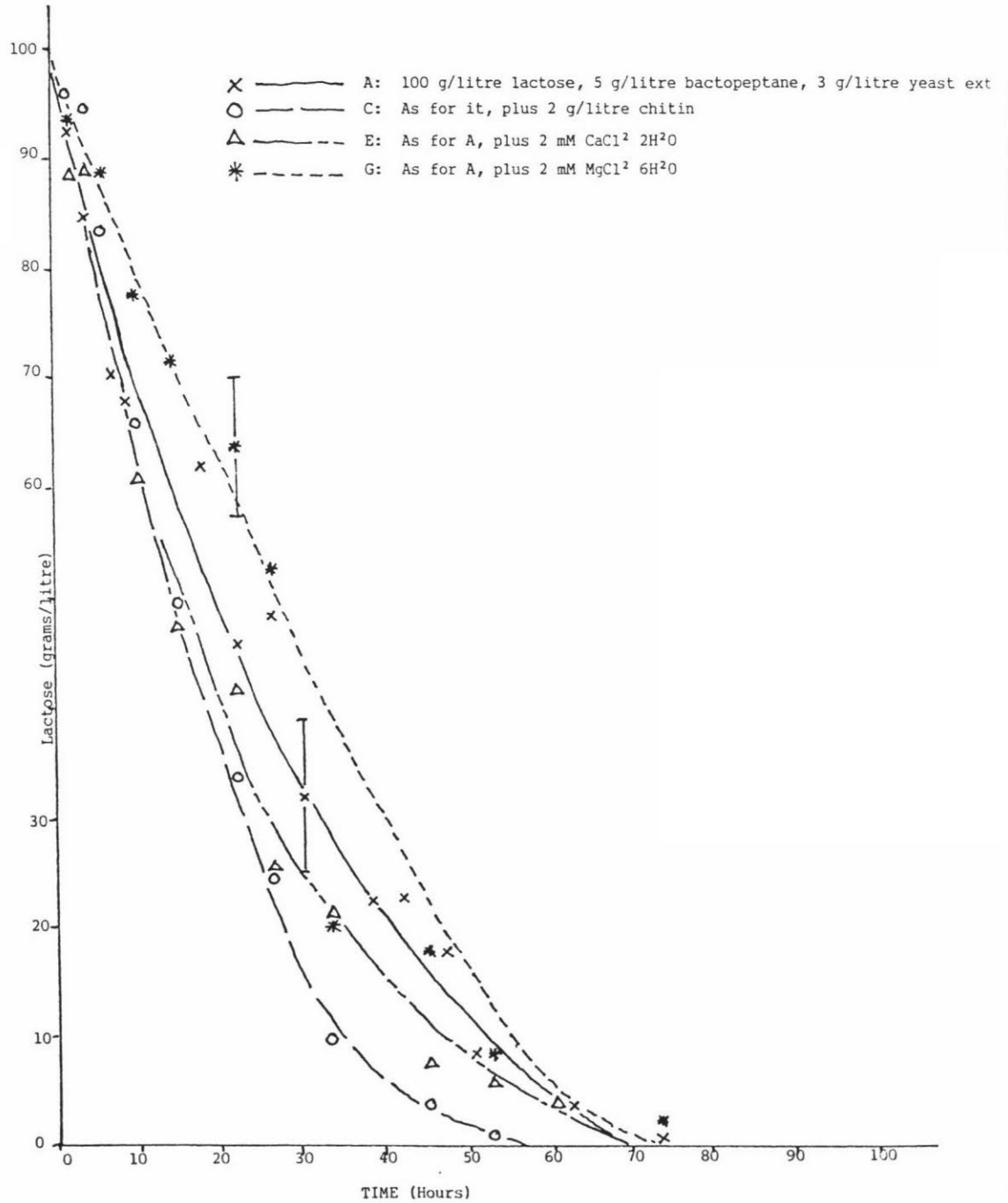


Fig. 5.13: Biomass Concn Vs Time; Run 9

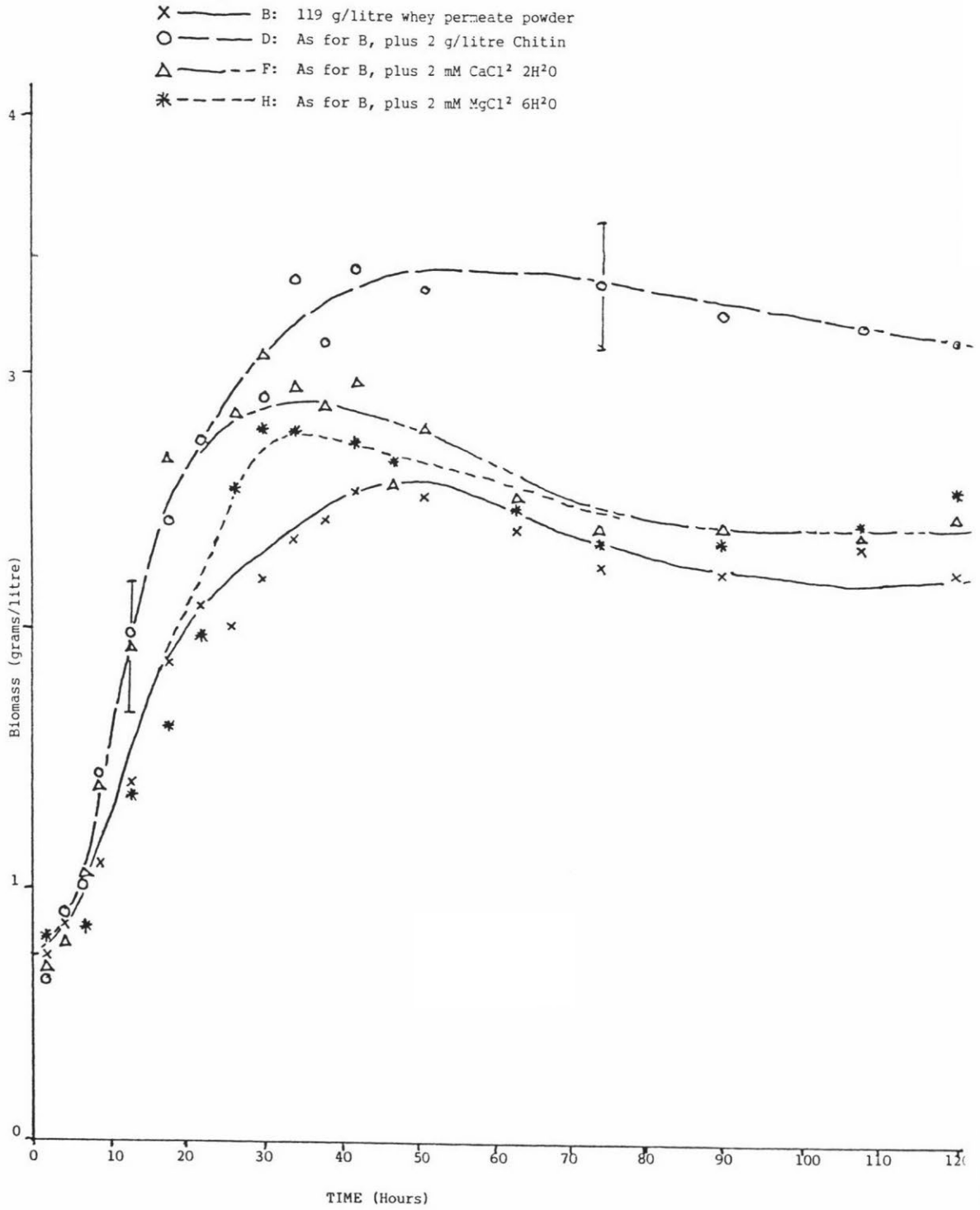


Fig. 5.14: Ethanol Concn Vs Time; Run 9

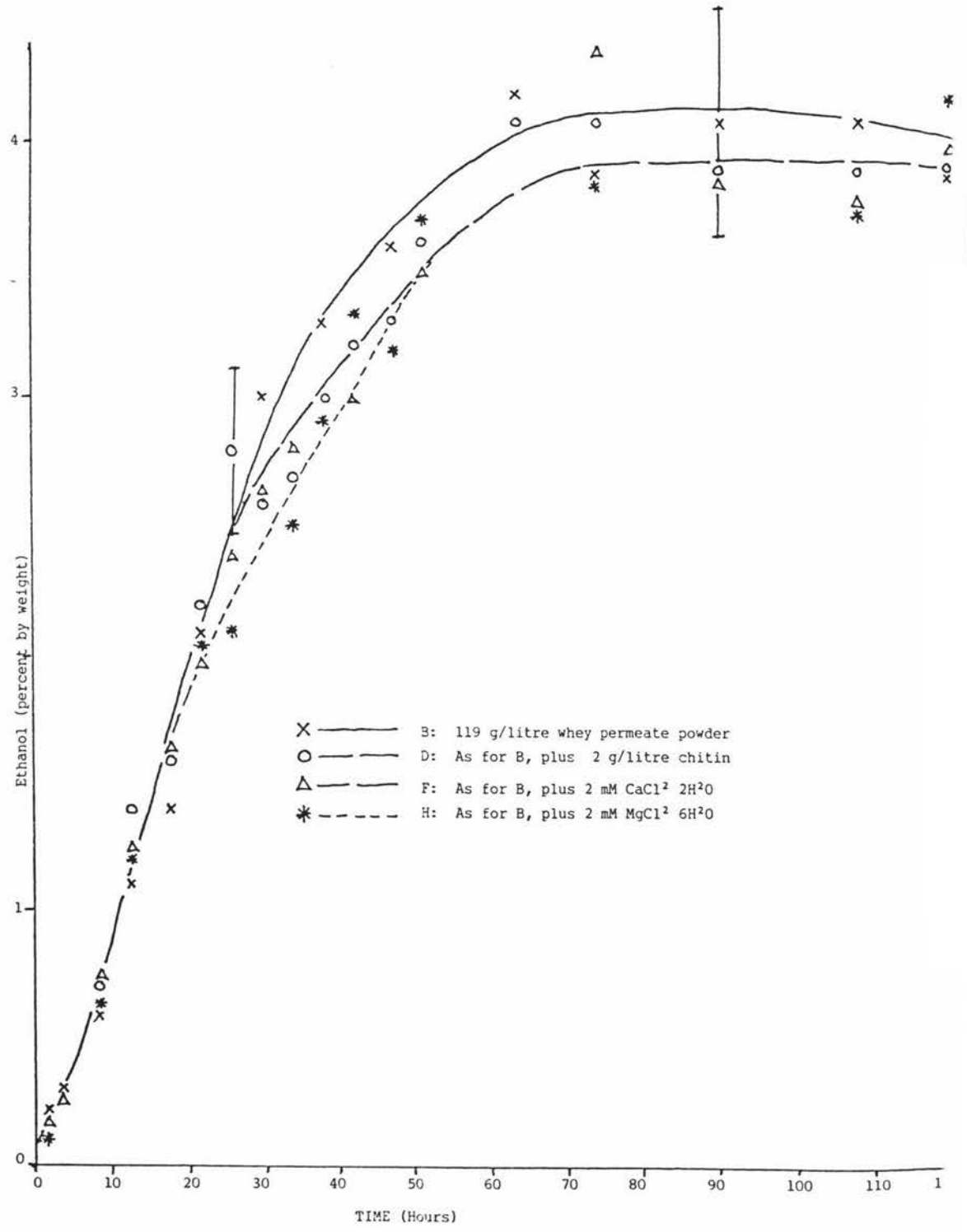
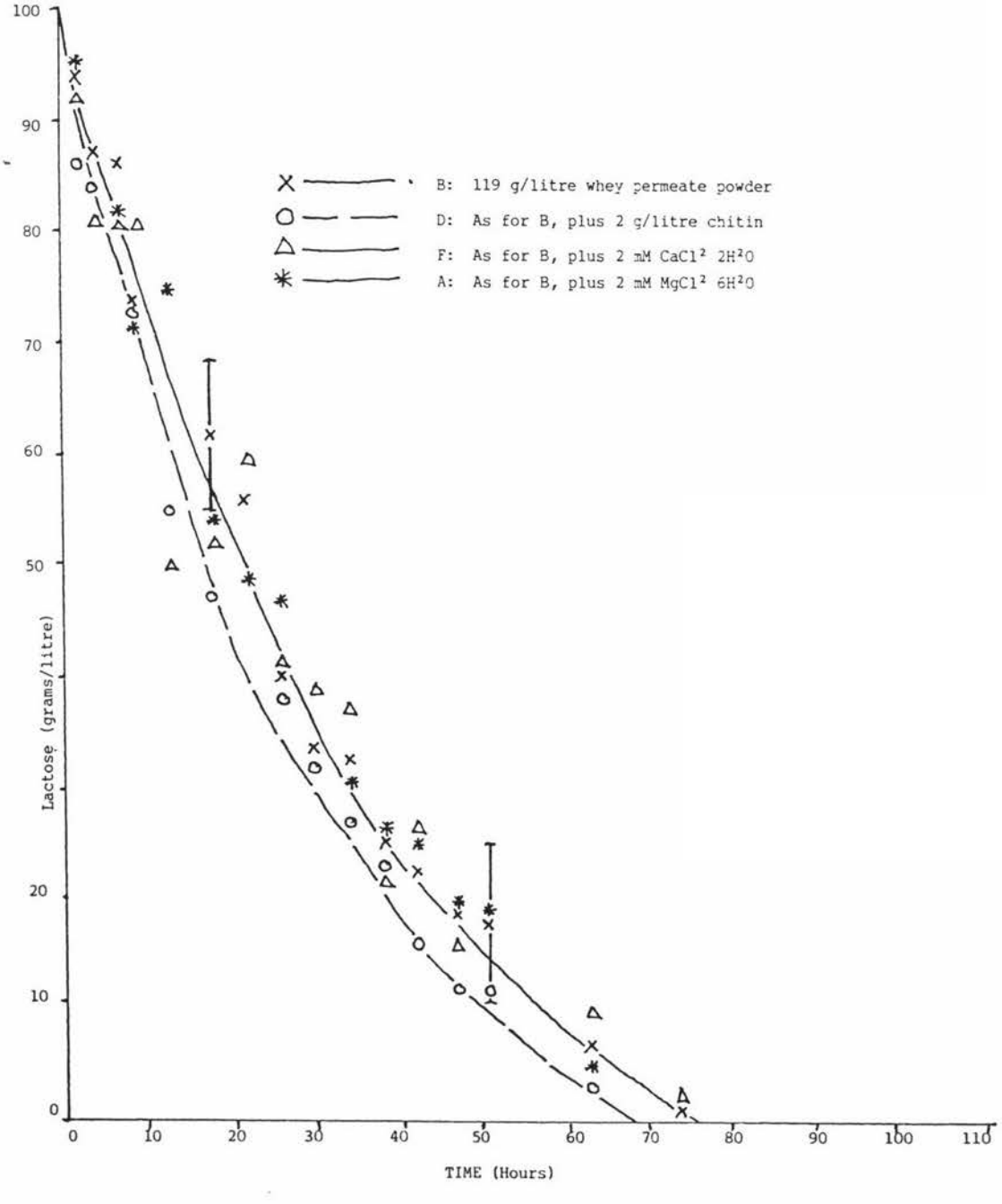


Fig. 5.15: Lactose Concn Vs Time; Run 9



The concentrations of magnesium and calcium in sulphuric acid whey are around 1 mM and 9 mM respectively, (see table 2.1), so supplementation with 2 mM of either element is quite significant, especially in the case of magnesium. The lack of positive response to the supplementation in these experiments suggests that these elements are not limiting in the case of *K.marxianus* Y113, although the situation may be different for other strains.

The other possibility is that the concentration of magnesium and calcium used was not appropriate. If the beneficial effect of their addition is not simply the relief of a nutritional deficiency there may be a threshold concentration, above 2 mM, at which the metabolism of the yeast is altered in a way that results in enhanced fermentative performance.

5.6 ATTEMPTED DEVELOPMENT OF A DEFINED MEDIUM FOR USE WITH *K.MARXIANUS* Y113

5.6 1. Introduction

The use of fully defined media is highly desirable for any work involving the investigation of microbial nutrition and the effects and peptone supplements contain many complex biological substances in ill-defined and sometimes variable concentrations, as well as numerous metal ions and trace elements. This means any supplementation testing is a matter of trial and error and is difficult to systemise and quantify.

With this in mind it was decided to attempt to produce a defined medium which could serve as a fermentation broth

in supplementation trials.

The aim was to produce a medium which contained adequate nutrients for the yeast. A medium which limited the productivity of the fermentation through nutrient deficiency would be of limited use for investigating ethanol inhibition because ethanol concentration would not be the main limiting factor. It was thought that a medium which produced a fermentative performance similar to whey permeate or lactose broth would be best because the results could more easily be compared with previous work carried out with *K.marxianus* Y113 using those media.

Difco have for some time produced a series of media of defined compositions for the purpose of nutrient requirement investigations with yeasts and it was thought that the use of these would be a sensible way to begin the investigation (Difco Manual, 1953).

The concentrations of the constituents in the Difco preparations are given in Table 5.5. It was at first thought that it would be useful to ascertain exactly which constituents were required by the yeast and an experiment investigating the amino acid requirements was successful in this respect (see results, section 5.6.2.1) but the following trial of Vitamin requirements gave rather ambiguous results and it was decided to discontinue this line of investigation and merely add the remaining constituents at the same concentrations as in the Difco media.

TABLE 5.5 Constituents of Difco Yeast Base Media

INGREDIENT	CONCENTRATION (per litre)
Nitrogen Sources:	
Ammonium Sulphate	5g
Bacto-asparagine	1.5g
Amino Acids:	
C-histidine monohydrochloride	10mg
dC-methionine	20mg
dC-tryptophan	20mg
Vitamins:	
Biotin	2ug
Calcium Pantothenate	400ug
Folic Acid	2ug
Inositol	2000ug
Niacin	400ug
p-aminobenzoic acid	200ug
Pyridoxine Hydrochloride	400ug
Riboflavin	200ug
Thiamine Hydrochloride	400ug
Trace Elements:	
Boric Acid	500ug
Copper Sulphate	40ug
Potassium Iodide	100ug
Ferric Chloride	200ug
Manganese Sulphate	400ug
Sodium Molybdate	200ug
Zinc Sulphate	400ug
Salts:	
Potassium Phosphate Monobasic	1g
Magnesium Sulphate	0.5g
Sodium Chloride	0.1g
Calcium Chloride	0.1g

5.6 2. Results and Discussion

5.6 2.1 AMINO ACID REQUIREMENTS

The amino acid requirements of the yeast were determined by growing it in Difco yeast Nitrogen Base (Difco Manual, 1953) supplemented with three of the amino acids:

asparagine	1.5 g/litre
L-histidine HCl	10 mg/litre
LD-methionine	20 mg/litre
LD-tryptophan	20 mg/litre

The experimental cultures were inoculated with a 10% (v/v), 24 hour, aerobic culture grown in standard Difco defined medium (50 g/litre lactose). Following that they were incubated for one week in test tubes containing the above media plus lactose (50 g/litre) and were than assayed for biomass growth.

A loopful of each culture was transferred to a test tube of fresh medium and incubated for a second week. This transfer ensured that there was no carry-over of nutrients from the initial inoculum which could have supplied enough essential amino acids to upset the results. The results to this experiment are given in Table 5.6 and demonstrate that none of the amino acids were essential or even stimulatory as their absence did not lead to decreased biomass accumulation in any case.

The conclusion drawn from this experiment was that *K.marxianus* Y113 can synthesis all it's amino acids from ammonium. However, these results did not show whether the rate of fermentation and growth were affected by the presence of the amino acid supplements and it is possible that supplementation would aid the productivity of the fermentation even though it did not alter the yield.

TABLE 5.6 Establishment of essential amino acid requirements for *K.marxianus* Y113

Amino Acid Not Included in Medium						
	NIL	ASPARAGINE	HISTIDINE	METHIONINE	TRYPOTOPHAN	ALL
Week 1.	*1.344	1.339	1.284	1.390	1.388	1.331
Week 2	1.158	1.049	1.152	1.158	1.179	1.115

*Figures are the absorbance of the culture at 620nm.

TABLE 5.7 Establishment of essential vitamin requirements of *K.marxianus* Y113.

Vitamins Not Included in Medium						
	NIL	THIAMINE	BIOTIN	PANTOHENIC ACID	NICOTINIC ACID	ALL
Week 1.	1.334*	1.261	1.251	1.242	.945	.972
Week 2.	1.124	.783	1.006	.477	.190	.073

*Figures are the absorbance of the culture at 620nm.

5.6 2.2 VITAMIN REQUIREMENTS

An investigation of the literature led to the conclusion that only 4 vitamins - thiamine, biotin, pantothenic acid and nicotinic acid - were likely to be essential for the growth of *K.marxianus* Y113. Research by Burkholder (1943) and Drouhet and Vieu (1957) concluded that both nicotinic acid and biotin were essential, and that pantothenic acid and thiamine were essential or stimulatory, to the growth of *K.marxianus* (This species is given the names *Candida pseudotropicalis* or

Saccharomyces fragilis in these studies but was subsequently renamed as *K.marxianus*). The other vitamins tested - riboflavin, inositol and pyridoxine - made no difference whether they were present or not but it was found that the addition of liver extract stimulated growth by over 50% above that observed with all seven vitamins present (Burkholder, 1943) indicating perhaps that there were other unidentified nutrients that were stimulatory in addition to the vitamins tested.

An experiment of similar form to that reported in table 5.6 was carried out using the 4 essential vitamins and gave results which confirmed the requirement for nicotinic acid and the benefits of pantothenic acid and thiamine supplementation (see Table 5.7). The requirement for biotin was not found to exist for this strain, but the result was ambiguous in that it's absence did lead to a small reduction in biomass and this effect was greater after the second transfer of inoculum. Biotin usually is only required in very low concentrations (2 ug/litre) and it is possible that the concentrations carried over from the inoculum medium were sufficient to give misleading results. Therefore it was decided to add all 4 vitamins to subsequent experiments with defined media.

2.3 TRIAL OF DEFINED MEDIUM FOR LACTOSE FERMENTATION BY *K.MARXIANUS* Y113

The media used for the trial run contained:

- either 150 or 200 g/litre lactose
- 5 g/litre ammonium sulphate
- 400 ug/litre thiamine
- 2 ug/litre biotin
- 400 ug/litre pantothenic acid

- 400 ug/litre nicotinic acid
- trace elements (as per Difco medium)
- salts (as per Difco medium)

The performance of this medium was compared to lactose broth and whey permeate media in Run 8 and the results are presented in Table 5.8 and figures 5.16, 5.17, 5.18, 5.19, 5.20 and 5.21. The defined medium was significantly inferior to both the lactose broth and the whey permeate media. This was true for both anaerobic and also for aerobic conditions which is demonstrated by the very low biomass concentration formed in the aerobic inoculum culture.

Table 5.8 shows that the ethanol production in the defined medium containing an initial lactose respectively concentration of 100 g/litre was 42% and 36% less respectively than the comparable lactose broth and whey permeate fermentations. Furthermore the lactose utilisation was 47% lower than both those cultures and the biomass production was 67% and 54% less respectively.

However, the maximum observed growth rates were comparable to the other media and the shapes of the graphs suggested that the cultures in the defined media were merely inhibited by the lack or low concentration of some vital nutrient, which ran out fairly soon after the start of the fermentation. The biomass concentration in both cultures C and D levelled off after about 20 hours which pointed to a halt in biomass production as being the cause of lower productivity in these media.

TABLE 5.8 Summary of Results; Run 10

The media were made up as follows:

A = 150 g/litre lactose; 3 g/litre Yeast Extract; 5 g/litre Bactopeptone

C = 150 g/litre lactose; trace elements; salts; vitamins; ammonium sulphate

D = 200 g/litre lactose; trace elements; salts; vitamins; ammonium sulphate

E = 179 g/litre whey permeate powder

F = 238 g/litre whey permeate powder

G = 153 g/litre demineralised whey permeate powder; salts; vitamins; trace elements; ammonium sulphate

H = 204 g/litre demineralised whey permeate powder; salts; vitamins; trace elements; ammonium sulphate

All the media were filter sterilised through cellulose nitrate membranes of pore size 0.45 um.

1. Initial Biomass

Inoculum grown in 50 g/litre lactose; trace elements; salts; vitamins; ammonium sulphate medium, for 24 hours with vigorous agitation (aeration). Final concentration bo biomass achieved was 1.4 g/litre.

Thus, initial biomass concentration in the test flasks was 0.14 g/litre.

2. Maximum Biomass

A = 2.0 g/litre (60 hours)
C = 0.65 g/litre (60 hours)
D = 0.55 g/litre (60 hours)
E = 1.4 g/litre (70 hours)
F = 1.0 g/litre (70 hours)
G = 1.3 g/litre (70 hours)
H = 1.65 g/litre (70 hours)

3. Growth Rate (umax)

A = 0.105 h^{-1}
C = 0.081 h^{-1}
D = 0.073 h^{-1}
E = 0.071 h^{-1}
F = 0.056 h^{-1}
G = 0.071 h^{-1}
H = 0.090 h^{-1}

4. Ethanol Production maximum

	Rate (gEtOH/gbio.h)
A = 48 g/litre (100 hours)	0.45
C = 28 g/litre (100 hours)	0.71
D = 15 g/litre (90 hours)	0.48
E = 44 g/litre (120 hours)	0.48
F = 35 g/litre (120 hours)	0.51
G = 32 g/litre (100 hours)	0.44
H = 43 g/litre (120 hours)	0.40

5. Lactose Utilisation

A = 70%
C = 31%
D = 18%
E = 69%
F = 46%
G = 58%
H = 71%

6. Yields

Yxs		Yps		Ypx	
A	= .024	A	= .46	A	= 19
C	= .015	C	= .58	C	= 39
D	= .012	D	= .43	D	= 36
E	= .018	E	= .43	E	= 24
F	= .013	F	= .38	F	= 29
G	= .016	G	= .37	G	= 23
H	= .013	H	= .30	H	= 23

Fig. 5.16: Biomass Concn Vs Time; Run 10

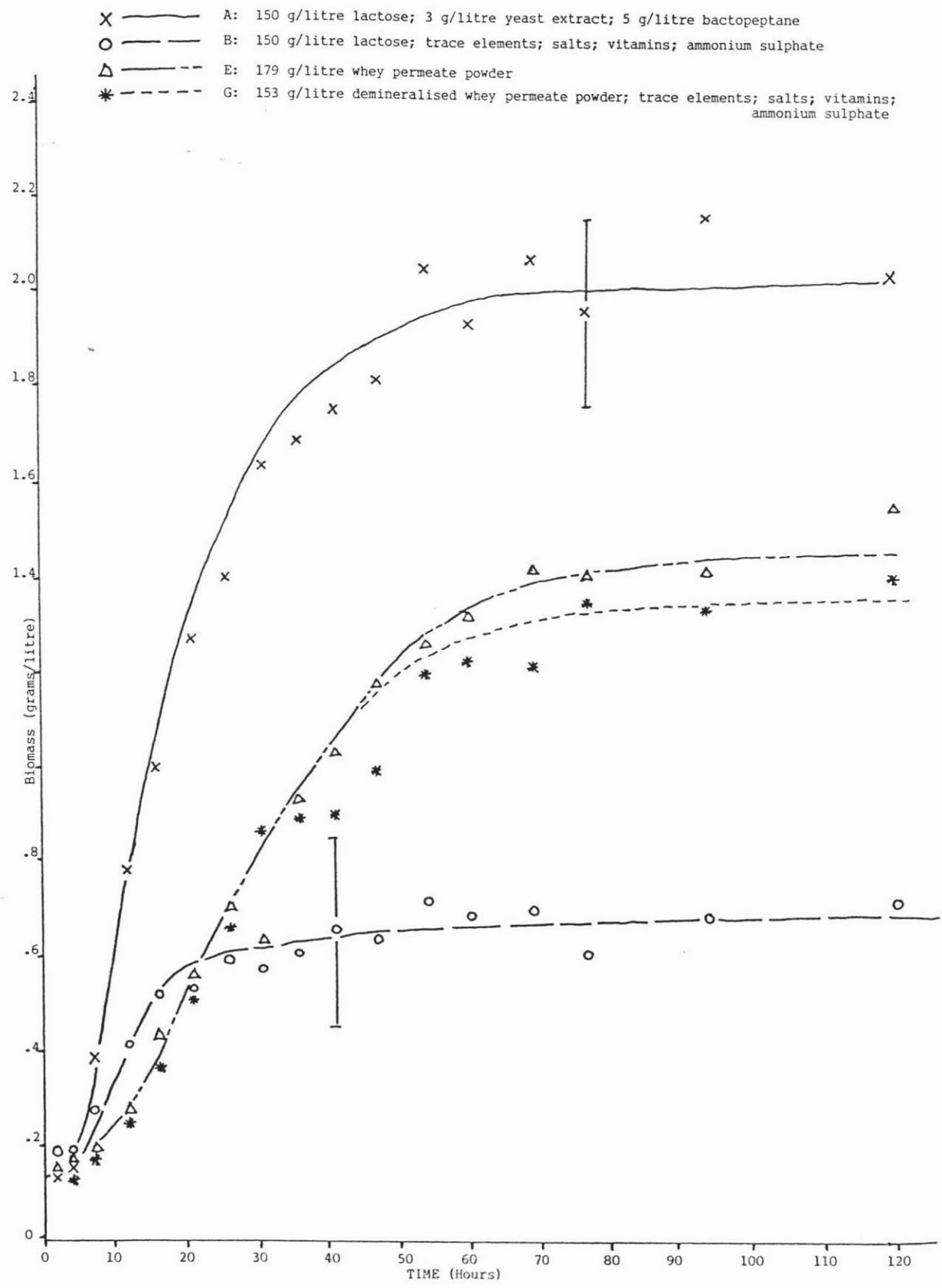


Fig. 5.17: Ethanol Concn Vs Time; Run 10

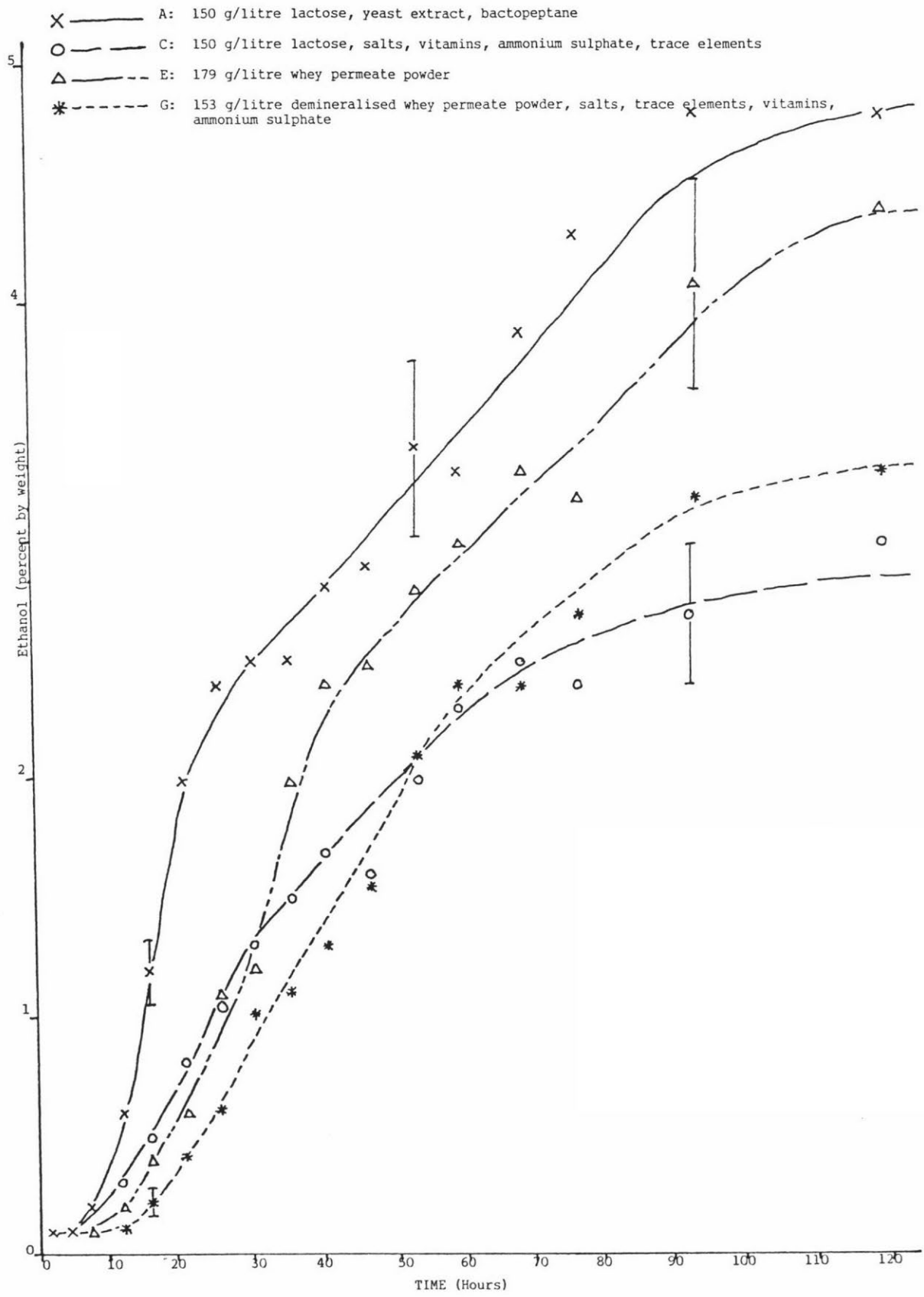


Fig. 5.18: Lactose Concn Vs Time; Run 10

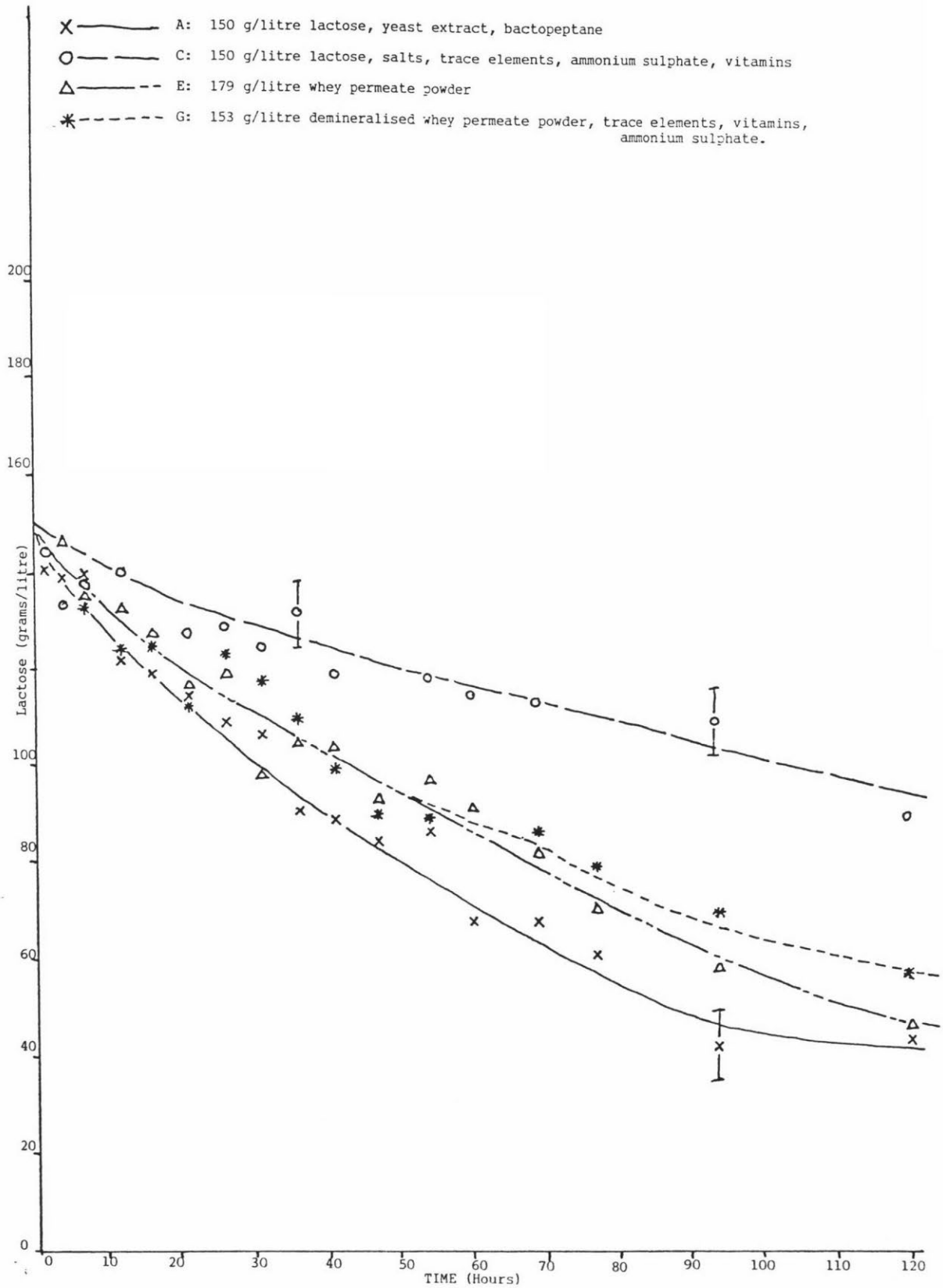


Fig. 5.19: Biomass Concn Vs Time; Run 10

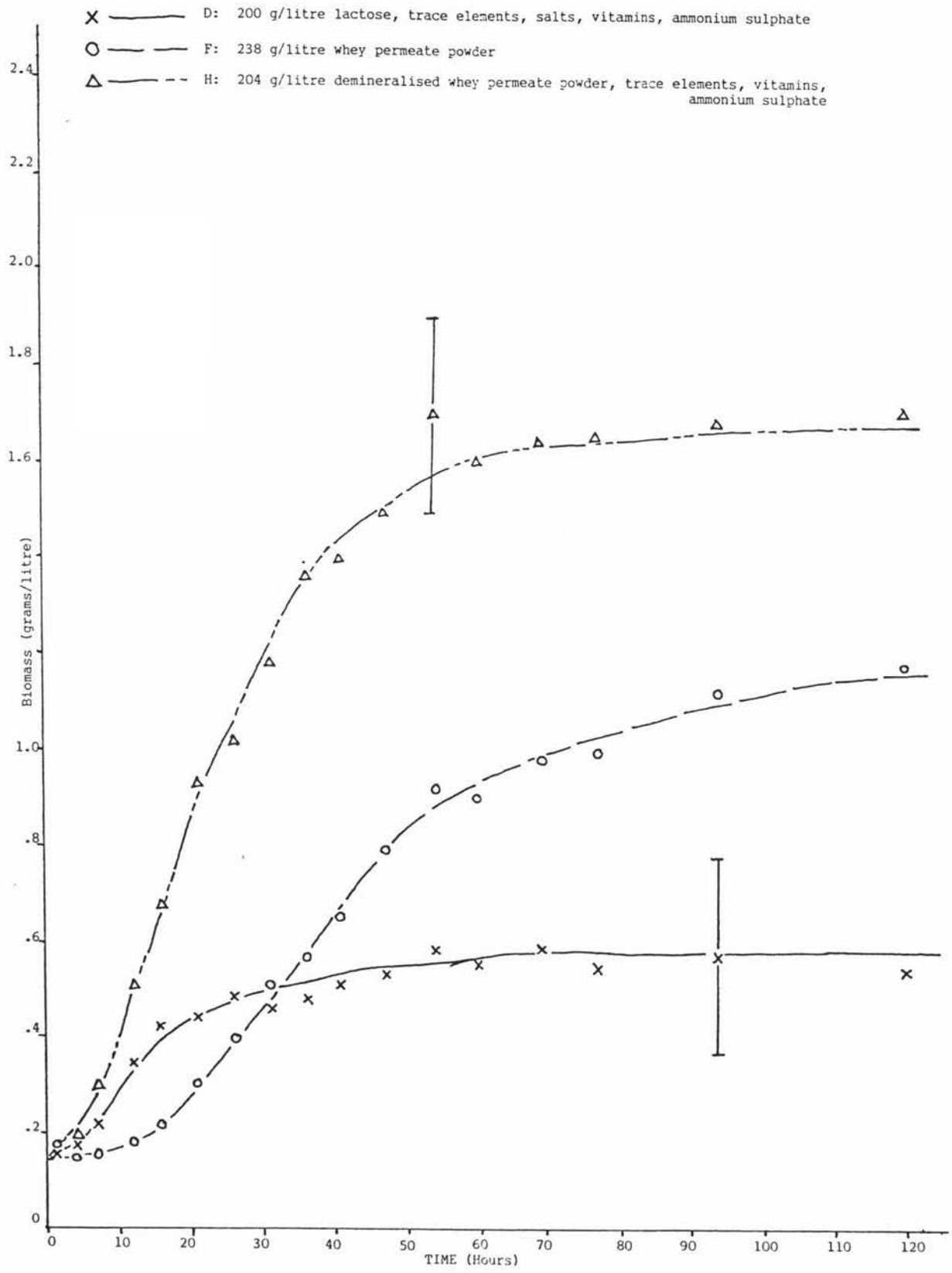


Fig. 5.20: Ethanol Concn Vs Time; Run 10

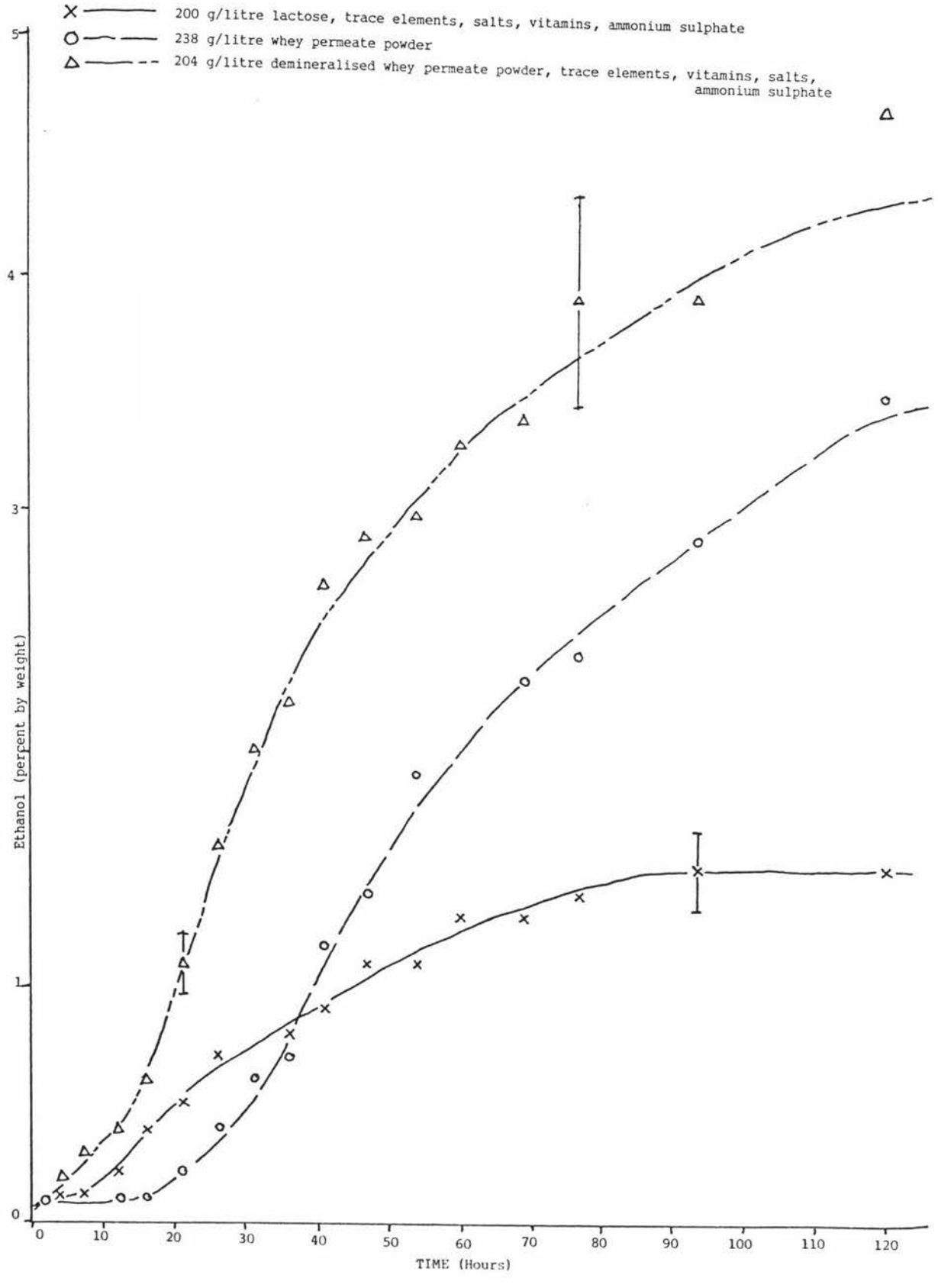
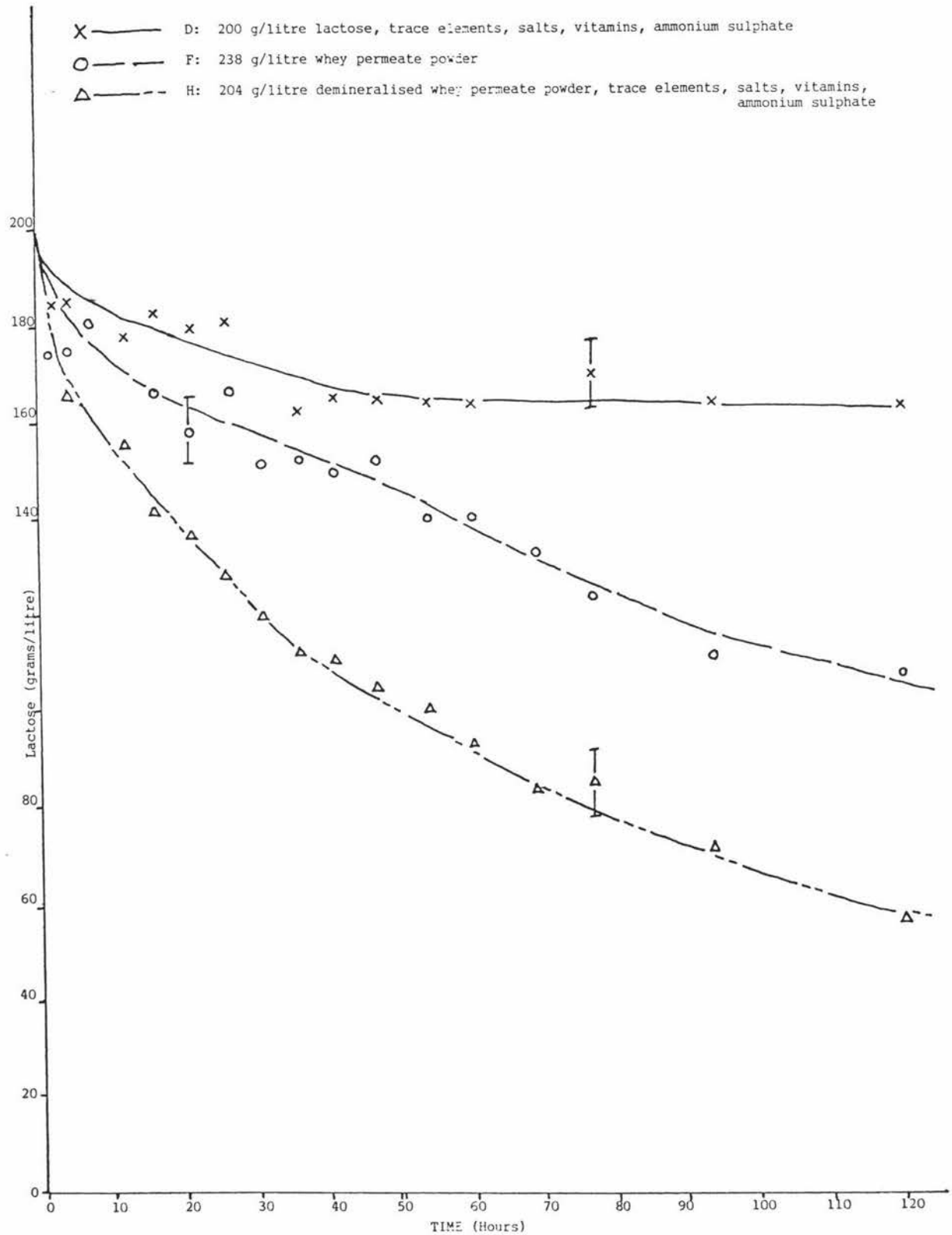


Fig. 5.21: Lactose Concn Vs Time; Run 10



This view is supported by some of the other parameters calculated from the results. The rate of ethanol production was comparable on a per unit biomass basis, as were the yield coefficients which implies that there was no increase in inhibition by alcohol or high osmolality when the defined media were used.

5.6 2.4 ATTEMPTED IMPROVEMENT OF THE DEFINED MEDIUM FOR GROWTH OF *K.MARXIANUS* Y113

As a result of that experiment another was designed to get an indication of what type of nutrient or nutrients - vitamins, trace elements, salts or nitrogen - were in short supply. Aerobic conditions were used as they led to shorter fermentation times, and the biomass concentrations only were measured.

Cultures of defined media were set up with 50 g/litre lactose and other constituents at either the usual (Difco) concentrations, or at 3 times the usual. One culture was grown in 50 g/litre lactose broth for comparison.

The results in table 5.9 tended to show a requirement for greater concentrations of salts and nitrogen (ammonium sulphate) rather than vitamins or trace elements, but even with 3 times the normal concentrations of those constituents the yeast could still not produce concentration of biomass comparable to those in the lactose broth.

A conclusion would be that the Difco medium is lacking some desirable nutrient that speeds biomass formations by *Kluyveromyces*. It seems unlikely that the nutrient is vital as some growth still occurred even without extra

supplementation but it is likely that it's absence greatly hinders the metabolism of the yeast.

TABLE 5.9 Biomass yield following modification of the defined medium

1. CONDITIONS:

CULTURE	AMMONIUM SULPHATE	SALTS	VITAMINS	TRACE ELEMENTS
A	N+	N	N	N
B	3x	N	N	N
C	N	N	3x	N
D	N	N	N	N
E	N	3x	N	N
F	3x	3x	3x	3x
G	50 g/litre lactose, 3 g/litre yeast extract, 5 g/litre bactopeptone			

2. RESULTS:

CULTURE	Biomass (g/litre) AFTER 24 HOURS	AFTER 48 HOURS
A	4.1	4.1
B	5.7	6.0
C	4.1	4.4
D	4.2	3.8
E	5.8	6.6
F	5.9	8.2
G	10.4	10.4

+N = Difco concentrations

3x = 3 times the Difco concentrations

The inoculum used was a 10% (v/v), 24 hour, aerobic culture grown in standard defined medium (50 g/litre lactose).

5.7 SUMMARY OF NUTRITIONAL STUDIES

In these studies of the effects of medium nutritional status on the fermentative performance of *K.marxianus* Y113 the results generally did not confirm the beneficial effects of the various supplements tried.

The yeast did not achieve any measureable improvement in performance when more than 3 g/litre yeast extract was used in the medium, regardless of the substrate concentration or amount of inhibition it appeared to be suffering (see section 5.2).

Furthermore the inclusion in the fermentation medium of chitin, calcium or magnesium - all reported to stimulate improved performance in other strains had no effect on any of the parameters measured in these experiments. (See section 5.5)

Demineralisation of the whey permeate medium was detrimental (see section 5.3), but supplementation with nutritionally - rich still bottoms also produced an inferior performance (seection 5.4).

The fact that so little was known about the constituents of yeast extract and still bottoms, or the nutritional effects of demineralisation led to the attempt to produce a defined medium (see section 5.6). Unfortunately the performance of the yeast on this medium never matched that which it gave on whey permeate or lactose broth media. Tripling the concentrations of any or all the main catagories of ingredients - salts, amino acids, nitrogen or vitamins - achieved small gains but they still did not compensate for the lack of complex media additives such as bactopeptone or yeast extract.

6. CONCLUSIONS

The results of these studies indicated that *K.marxianus* Y113 is inhibited by both high medium osmolality and ethanol toxicity during lactose fermentations for the production of ethanol.

These factors combined to result in a poorer fermentation performance when the substrate concentration was increased between 50 and 200 g/litre lactose (see section 4.2). Longer fermentation times, incomplete lactose utilisation, lowered maximum growth rates and reduced Yxs and Yps yield coefficients were all observed as the intial lactose concentration in the medium was increased. This suggested that both the growth and energy metabolism of the yeast were adversely affected.

The results of the experiments where initial lactose concentrations were kept at 100 g/litre but the medium osmolality was increased (section 4.3) suggested that high osmolality was more detrimental to growth than to ethanol production. The most likely reason for this is that greater amounts of energy are required for cell maintenance in media with high solute concentration so less is available for cell growth.

Addition of ethanol to the fermentation medium (section 4.4) reduced the amount and rate of biomass accumulation as well as dramatically lowering the amount of ethanol produced by the yeast itself. A maximum alcohol concentration of 4.5% (by weight) was observed in all cases, irrespective of the concentration of ethanol added initially, which suggested that there was inhibition of the energy metabolism of the cell and not just a redirection of available energy as seemed to be the case for high medium osmolality.

The fact that poorer fermentation performance was significant only when the concentration of added ethanol was greater than 2 or 3% (by weight) suggested that that was the threshold level for ethanol inhibition in this yeast strain.

Ethanol added to the medium appeared to be less toxic than that produced within the cell. This was demonstrated by the fact that maximum ethanol concentrations produced by fermentations with added ethanol were as high as 5% (by weight) compared to the 4 to 4.5% (by weight) observed throughout the studies in most fermentations without added ethanol.

In the concentrations tried supplementation of the fermentation medium with yeast extract, magnesium, calcium and chitin all failed to produce any change in the performance of the fermentation. (See sections 5.2 and 5.5). It can be concluded either that the concentrations used were inappropriate for *K.marxianus* Y113 or that the supplements were simply ineffective for this strain. It is difficult to be more specific without experimenting with different concentrations of supplements and substrates.

The fact that demineralisation had a detrimental effect on the fermentation showed that the reduction in nutrient concentrations in the medium outweighed any benefits caused by the reduction in medium osmolality. Benefits may have been seen if the degree of demineralisation had not been so great and vital nutrients' concentrations had not been depleted quite so much.

Still bottoms were quite a strong inhibitor of the fermentation (section 5.4) despite their nutrient content. The only conclusion to be drawn from that

result is that an inhibitory compound or compounds are formed right at the end of the fermentation or during distillation itself and that these are not distilled off or broken down and remain in the bottoms.

The performance of the yeast on the defined medium never matched those seen on complex media like lactose broth or whey permeate. This showed not that the defined medium was lacking some essential nutrients but that it was lacking the more complex biomolecules found in the complex media. The need to synthesise those from basic units such as amino acids or ammonium ions meant the yeast grew more slowly and required more energy in the defined medium. That would appear to be the reason why increasing the concentrations of those basic nutrients was only partially successful.

The use of media supplementation as a cure for poor fermentation performance must be considered carefully in commercial terms. While some additives, such as still bottoms, are very cheap most others are quite expensive and their cost may outweigh any savings gained through increased productivity.

Strain improvement should be considered as an alternative the medium improvement in future. Many strain improvement techniques are cheap and easy to carry out (see for example, Boontanjai, 1983) and in the long term there are no on-going costs as there would be for medium improvement.

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APPENDIX A:

CALCULATION OF FERMENTATION PARAMETERS

The results from analysis of the samples were plotted as concentration - time profiles and fermentation parameters were calculated from these. Drawing a reasonable curve through often rather variable samples is necessarily a somewhat subjective excercise so the calculated parameters are only given to two significant figures to reflect that.

A) Maximum Growth Rates

Maximum growth rates were estimated thus:

1. find period of exponential growth on biomass curve
2. plot natural logarithms of biomass concentrations during that period against time.
3. draw linear regression between points and find slope.
4. slope equals exponential growth rate.

B) Average Ethanol Production Rates

These were calculated thus:

1. estimate average biomass concentration as mid point between greatest and least concentrations.
2. Divide maximum attained ethanol concentration by hours required to reach it and average biomass concentration.

C) Yield Coefficients

The 3 yield coefficients were calculated thus:

Yxs = Biomass produced/Lactose used

Yps = Ethanol produced/Lactose used

Ypx = Yxs/Yps

APPENDIX B:

ESTIMATION OF CONCENTRATIONS AND UNCERTAINTIES

A) Biomass

The standard curve was prepared using an aerobically-grown culture containing 10 - 11 DW g/litre biomass. Ten millilitres of this was vacuum filtered through a pre-weighed 0.45 um cellulose nitrate filter to harvest the yeast cells, which were then washed 3 times with distilled water. The filter was next placed in an oven at 105 C to dry overnight. After that it was re-weighed and the biomass concentration determined.

The remainder of the culture was used to make up a series of dilutions, each of whose absorbance was recorded. Knowing the concentration from the dried sample a standard calibration curve could be constructed.

A standard curve was constructed at the start of most of the runs and it was found that the variability stayed within the limits of uncertainty throughout.

Uncertainties were calculated by sampling a standard suspension of known concentration 5 times and measuring the absorbances of the samples. From the spread of results a 95% confidence interval was determined. This procedure was repeated for samples of different concentrations to ascertain whether the confidence interval got wider or narrower at different parts of the standard curve. It was found that the interval remained at 0.25 (plus or minus) g/litre throughout the concentration range encountered in these studies.

A standard biomass curve is shown in Figure B1. It was found to be linear up to 0.5 g/litre biomass.

B) Lactose

A standard solution was made up using a weighed amount of lactose in a volumetric flask. This was used to make up a series of other standards by dilution with distilled water.

The phenol-sulphuric acid assay was used throughout all this research and in all measurements of lactose concentration in the spectrometer the blank cell contained 1ml distilled water, 1ml phenol solution and 5ml sulphuric acid (see chapter 3).

The curve was checked regularly and confidence intervals on measurements were calculated using the same method as for biomass (see section A). The linear portion of the curve fell below 100 g/litre and the uncertainty up to that point remained at (plus or minus) 7 g/litre lactose. See Figure B2 for standard curve.

C) Ethanol

Standards were made up of diluting measured weights of 99.5+% ethanol with distilled water in volumetric flasks. Gas chromatography was used to measure ethanol concentration (see chapter 3) and a standard curve was constructed in the same manner as that for biomass (Section A) or lactose (Section B). It can be seen in Figure B3.

The confidence interval was found to increase with the ethanol concentration. This can be seen in Figure B4.

FIGURE B1. STANDARD CURVE FOR BIOMASS

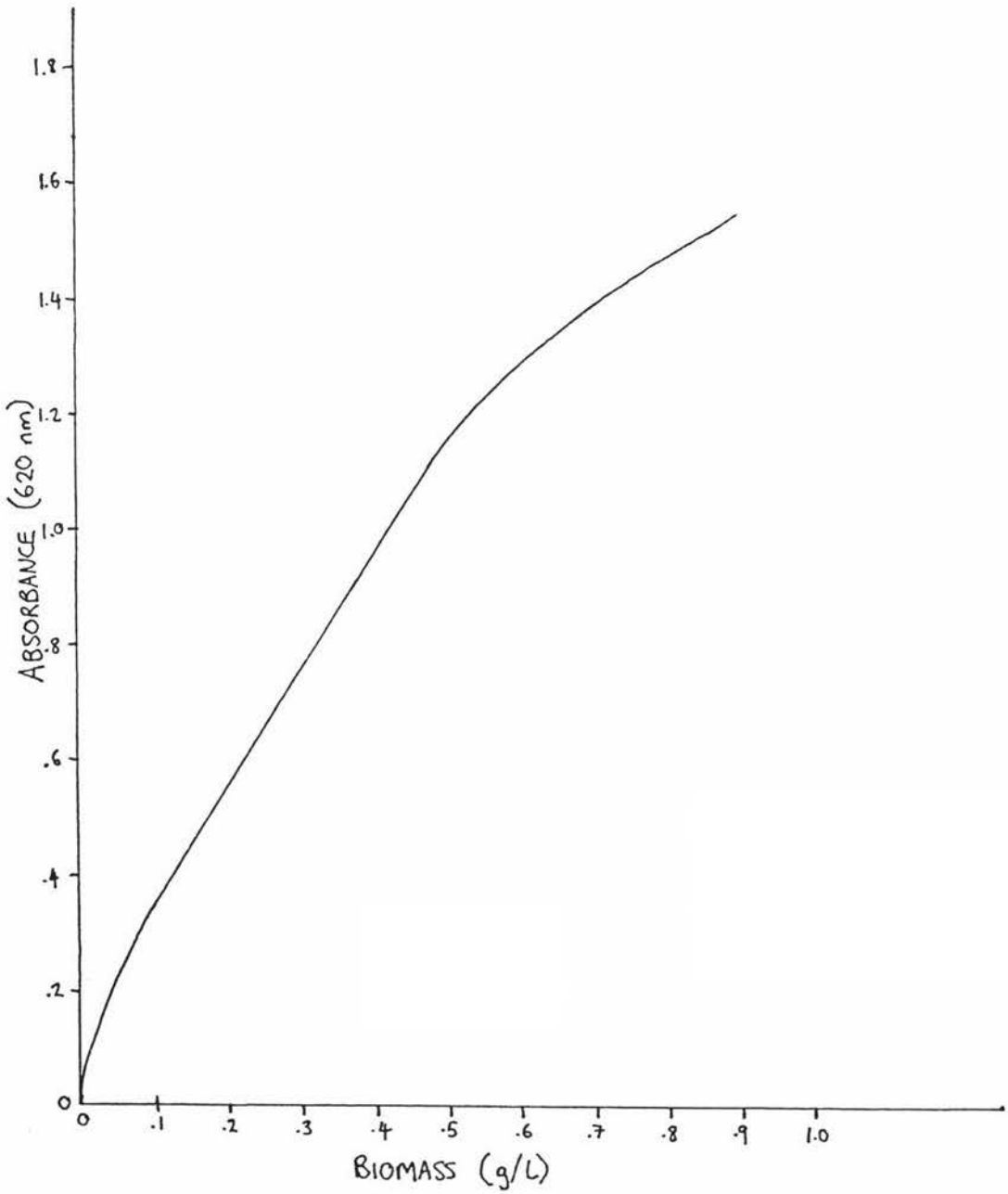


FIGURE B2. STANDARD CURVE FOR LACTOSE

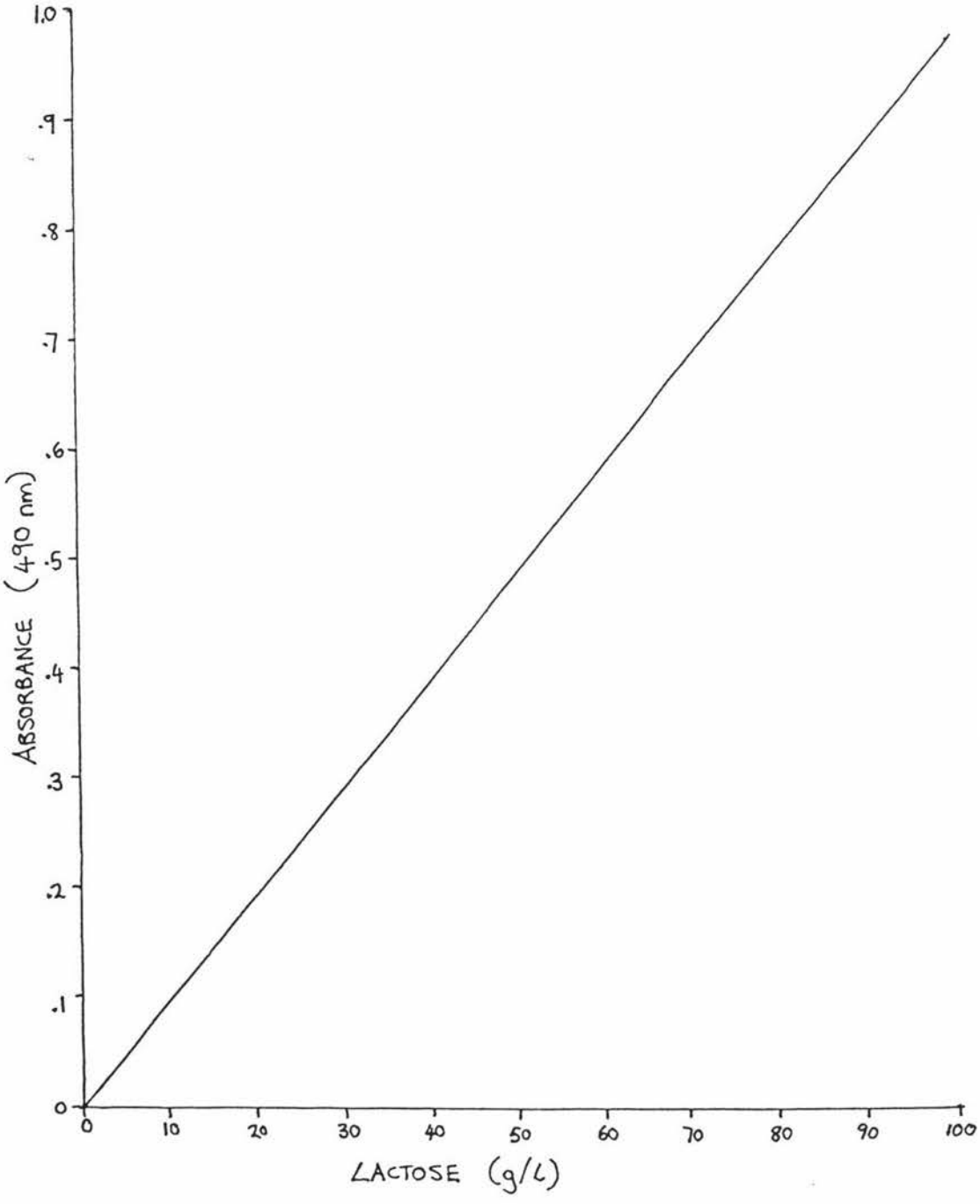


FIGURE B3. STANDARD CURVE FOR ETHANOL

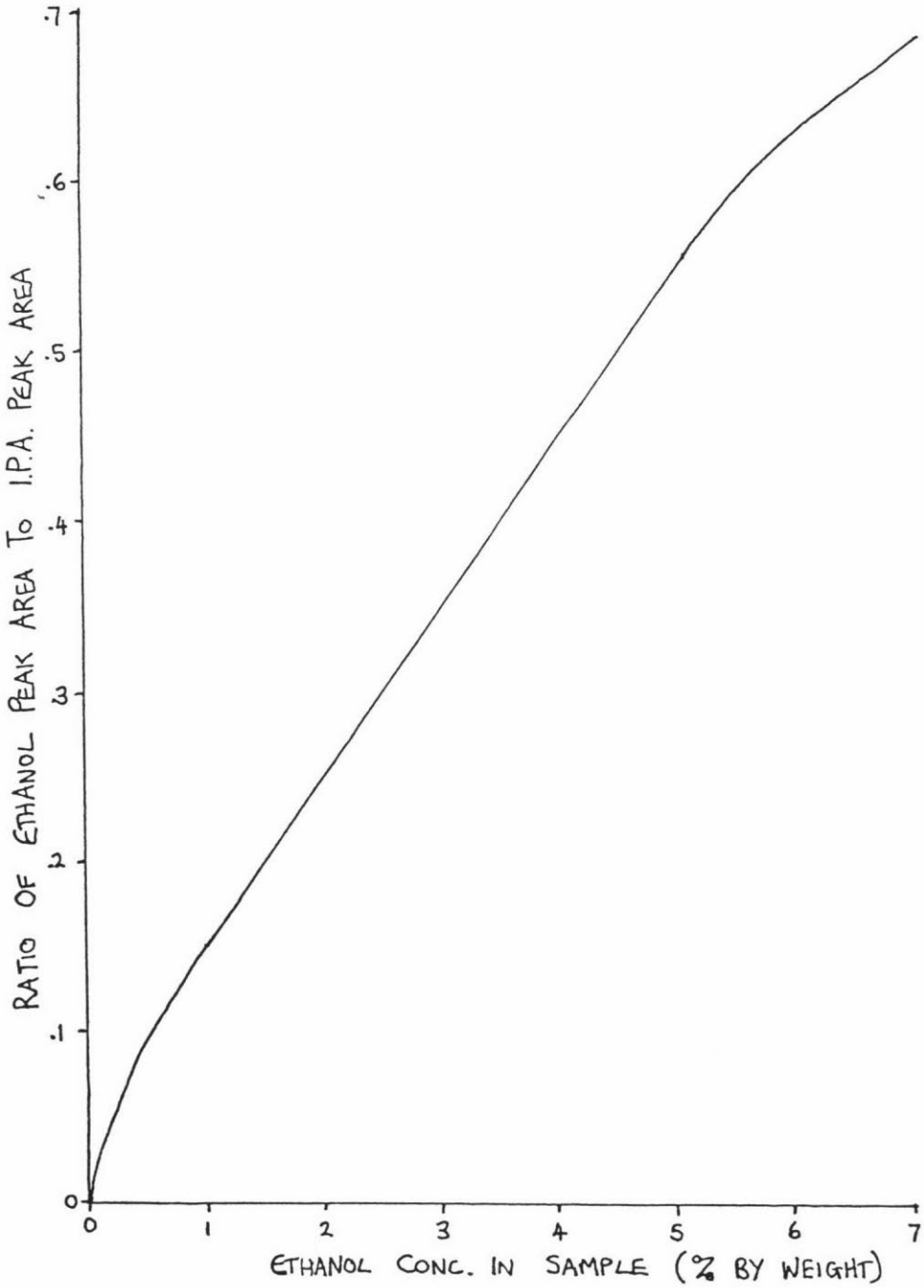
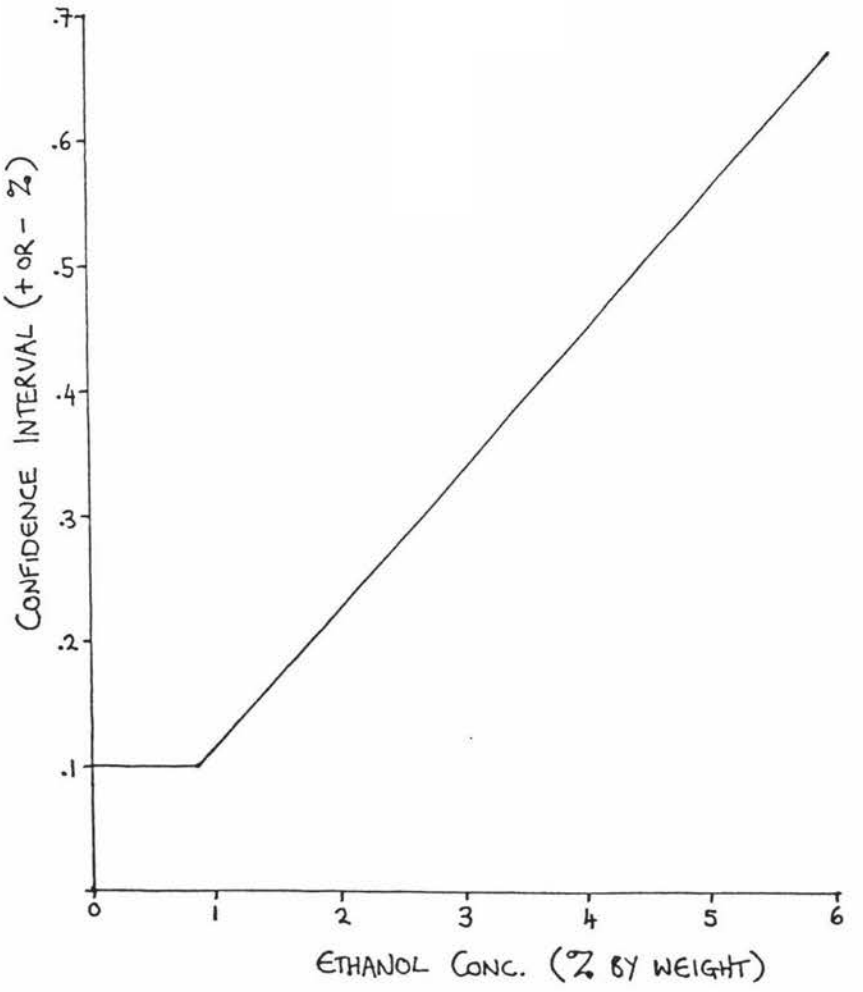


FIGURE B4. EFFECT OF ETHANOL CONCENTRATION ON CONFIDENCE INTERVAL



APPENDIX C:

RELATIONSHIP OF OSMOLALITY TO SOLUTE CONCENTRATION

The relationship between medium osmolality and the concentration of certain medium constituents is not always a linear one. This can be seen in Figure C1, which shows a plot of osmolality against solute concentration. Sodium choloride shows a linear relationship over the concentration range shown while the disaccharides sucrose and maltose have a non-linear relationship. Lactose follows a curve very close to the latter two.

Failure to recognise this difference led to the loss in comparability between cultures noted in section 4.3. Luckily the difference was not excessively great and the results were still useful.

FIGURE C1. OSMOLALITIES OF SOLUTIONS OF SALT, MALTOSE AND SUCROSE