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**OPTIMISATION OF INDUSTRIAL WHEY ETHANOL
FERMENTATION PROCESS**

by

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ABSTRACT

Ethanol is produced from whey at four distilleries in New Zealand. The Anchor Ethanol Co. distillery at Tirau was established in the 1981 and employs a continuous fermentation process. The aim of this work was to characterise the production yeast employed at this plant and to evaluate possible methods by which this commercial fermentation could be optimised.

Fermentation and assimilation tests confirmed the production yeast (strain Fi) as a strain of *K. marxianus*. The kinetics of ethanol fermentation of lactic acid casein whey serum were examined in continuous culture. The data were best fitted with a Langmuir plot and gave μ_{\max} of 0.21 h^{-1} and K_s of 4.94 g/l . A maximum ethanol productivity of 1.27 g/l.h was achieved at a dilution rate of 0.10 h^{-1} . Experiments were conducted in shake flask cultures using semi-synthetic media and sulphuric acid casein whey permeate to investigate the effect of lactate on the fermentation performance of the yeast. Lactic acid is present naturally in lactic acid casein whey at a concentration of about 7 g/l and may be present at concentrations up to 30 g/l , if there is gross bacterial contamination or if the whey were concentrated. Lactate added up to 30 g/l in the presence of 50 or 100 g/l lactose had no effect on the ethanol production rate or yield, although the biomass yield was slightly reduced.

The yeast strain Fi was grown aerobically on the slops, the liquid remaining after ethanol distillation, which contains 7 g/l lactic acid as the major component. The biomass produced was used as an inoculum for the whey ethanolic fermentation and performed as well in this role as an inoculum pre-grown on lactic whey. Continuous culture of the yeast grown aerobically on slops was again best fitted with Langmuir plot to give μ_{\max} of 0.30 h^{-1} and K_s of 0.32 g/l .

The yeast by-product from whey ethanolic fermentation was autolysed in a batch or continuous systems. On the basis of α -aminonitrogen release, yeast grown aerobically autolysed more readily than yeast grown anaerobically. The optimum autolysis condition of 55°C and pH 5.5 for anaerobically grown yeast was established on the basis of the α -aminonitrogen released after 6 h and the stimulatory effect of the lysate on the ethanol fermentation. Continuous autolysis was conducted successfully and the autolysate produced at dilution rate of 0.10 h⁻¹ gave the highest stimulatory effect on the ethanol fermentation. Improvements in the ethanol productivity and production rate of 10-20 % were observed following the addition of autolysate. A direct relationship between the α -AN utilized and the ethanol volumetric productivity was established.

Overall this work has identified three potential areas for process intensification:

1. An increase in the operating dilution rate from the current value of 0.07 h⁻¹ to the optimum of 0.10 h⁻¹ will lead to an approximately 30% increase in throughput.
2. The use of distillation slops containing lactic acid as a growth medium for inoculum production. This will enable an extra of 62,000 kg of ethanol per year to be produced from the lactose currently used for growth of the inoculum.
3. The addition of a continuously produced autolysate to the ethanolic fermentation will improve the fermentation rate and allow the whey throughput to be increased further.

Each of these options could be implemented at the commercial plant with a payback period of one year or less.

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ABBREVIATIONS

α -AN	α -aminonitrogen
$^{\circ}$ C	Degree Celcius
D	Dilution rate
EtOH	Ethanol
g	Gram(s)
h	Hour(s)
K_s	Saturation constant
l	Litre(s)
LACWP	Lactic acid casein whey permeate
LACWS	Lactic acid casein whey permeate
M	Molar
ml	Millitre(s)
nm	Nanometre
q_s	Specific substrate uptake rate
rpm	Revolutions per minute
SACWP	Sulphuric acid casein whey permeate
S	Residual substrate
μ l	Microlitre(s)
μ m	Micrometre(s)
μ_{max}	Maximum specific growth rate
% (w/v)	Percentage weight by volume
% (v/v)	Percentage volume by volume
% (w/w)	Percentage weight by weight
YEP	Yeast Extract Peptone
YEPL	Yeast Extract Peptone Lactose
YEPLL	Yeast Extract Peptone Lactose Lactate

I dedicate this thesis to my beloved mother and my uncle Jacob

CHAPTER 1

INTRODUCTION

Whey is produced during the manufacture of cheese and casein and the major constituents of whey are lactose, minerals and protein. The large mass of whey produced (typically 7 to 9 tonnes and 25 tonnes per tonne of cheese and casein, respectively) and its high biological oxygen demand (BOD), from 35,000 to 60,000 mg/l, make it necessary for dairy companies either to process whey or to dispose of it in some environmentally acceptable manner (Hobman, 1984). Increasingly, the dairy industry is recognising whey as a by-product rather than as a waste product. The extraction of soluble proteins from whey using ultrafiltration is now well established but has only a minor effect on the volume and BOD of the whey. The disposal of deproteinated whey thus can still present a considerable environmental problem and cost to the dairy industry.

The lactose content of the whey ultrafiltrate (40-50 g/l) may be regarded as a potential fermentation substrate. The options of producing compounds, such as lactic acid, propionic acid, citric acid, butanol, enzymes, and vitamins by fermentation have been proposed to the industry. Despite these many options, production of ethanol has developed as the only important fermentation process for whey utilization. Ethanol is an important industrial solvent and can also be used in internal combustion engines as a substitute fuel or as an octane booster (Coombs 1984). Potable grade ethanol is used for spirit manufacture and wine fortification.

The whey to ethanol fermentation has two factors mitigating against it. First, due to the dilute nature of the substrate, and hence dilute ethanol solution obtained, the recovery of ethanol by distillation is energy intensive, and these energy costs

are the major component of the production cost (Mawson 1990). Secondly the choice of the yeast to be used is limited. There are only a few genera of yeasts able to ferment lactose, the most appropriate being *Kluyveromyces*. However many of these strains lack desirable properties for ethanol production. In particular most strains appear non flocculent and many are inhibited at relatively low ethanol concentrations, particularly in the presence of high lactose or salts when the whey is concentrated.

Despite these shortcomings, ethanol production from deproteinated cheese and casein whey is established on a commercial basis in several countries. The first whey-ethanol fermentation plant was established in late 1978 at Carbery, Ireland. In New Zealand, there are four whey distilleries operating which supply the entire local market and limited export markets for both potable and industrial ethanol. The distillery at Tirau is the only whey distillery in the world known to employ a continuous fermentation system. The capacity of the plant is nominally 1.5 million litres of deproteinated lactic acid whey per day to produce approximately 32,000 litres of industrial ethanol. The plant was built in the 1980's, but without an extensive research base. Thus it was not designed optimally. The company is continually refining its processes to develop a more effective fermentation technology. Currently two areas have been identified where development could lead to an improvement of the overall plant efficiency.

The first arises from the nature of the whey substrate used, i.e. lactic acid casein whey. Lactic acid is a normal constituent of this raw material, at a concentration of approximately 7 g/l. It remains unused during the anaerobic fermentation and the subsequent distillation process. Thus, the stillage (slops) contains lactic acid, and requires addition of lime to adjust the pH value to pH 7.0, prior to further treatment in the waste pond and subsequent final discharge into a natural water way. The disposal of the slops has added considerable cost to the company's operations. Lactic acid is considered as a good carbon source for many microorganism, and its potential of as a carbon source for SCP production or propionic acid production has been evaluated by several authors (El-hagarawy

et al. 1956; Lembke *et al.* 1975; Ruiz *et al.* 1978). For propionic acid production using *Propionibacterium*, lactic acid is a preferred carbon source over lactose. However, the option of producing propionic acid from the slops is not commercially viable due to the dilute nature of the slops. Furthermore as a bulk chemical, lactic acid is worth more than propionate. The production of SCP using yeasts is a possible option. However, although this operation would solve the problem of disposing of the slops, the process is expensive and the solid yeast produced would also require disposal or to be utilized. *Kluyveromyces* yeasts are known to assimilate lactate oxidatively (Barnett *et al.* 1990). Thus, it is possible that the slops can be used to grow the production yeast as an inoculum for the subsequent whey fermentation. At present, the yeast inoculum is grown on lactose in sterile whey, and this is continuously fed to the fermenter.

Secondly, during the recovery of ethanol, yeast is produced as a by-product. This spent yeast, a valuable source of nitrogen, vitamins and minerals, is currently not utilized, but simply pumped to the waste treatment ponds. This adds extra cost to the process. Yeast extract produced from the autolysis of yeast has been used widely on a laboratory scale to enrich media for fermentations. In general, it is accepted that whey requires additional nutrients for optimal fermentation, and the addition of yeast extract to whey has been reported to improve the fermentation rate (Vienne and von Stockar 1983). It appears that addition of yeast extract alone is sufficient to overcome any nutrient deficiency in whey. However, in a large scale industrial fermentation process, producing a high-volume, low-value product such as ethanol, the cost of adding a large amount of yeast extract to increase the fermentation rate would not be economically viable. But if yeast extract could be produced cheaply, using the yeast by-product on site, the option might be feasible.

Initially the present project was set up to investigate potential uses of the lactic acid in the whey, but it subsequently expanded to a more general optimisation of the ethanol fermentation process. Initially, characterization studies on the yeast were undertaken. Then, the use of slops for the production of a yeast

inoculum in a batch or continuous system was examined. Finally, experiments were undertaken to examine the possibility of autolysing the spent yeast in a batch or in continuous system, and the effects of this autolysate on the rate of anaerobic whey fermentation.

CHAPTER 2

LITERATURE REVIEW

The literature review is divided into three parts :

- Part I Ethanol production from whey
- Part II Utilization of lactic acid in whey
- Part III Yeast autolysis

PART I ETHANOL PRODUCTION FROM WHEY

2.1.1 Introduction

Whey is produced during the manufacture of cheese, and rennet and acid casein, and each process gives rise to a characteristic whey. The whey contains most of the lactose, minerals, and whey proteins that were present originally in the milk. Sweet whey, pH > 5.6, is derived from the manufacture of cheese and rennet casein, and is produced at a rate of 7-9 kg per kilogram of cheese. Acid whey, pH < 5.0, is obtained when acid is either added to the process or produced in the process to facilitate the coagulation of casein. Typical processes are the manufacture of lactic or sulphuric casein, and some 25 kg of whey is produced per kilogram of casein (Larsen and Maddox, 1987). The typical composition of three types of whey produced in New Zealand is shown in Table 2.1 (Clark, 1988).

The disposal of whey poses a considerable environmental threat due to its high biochemical oxygen demand, ranging from 35,000 mg/l to 60,000 mg/l, and also the enormous volume of whey that is being generated every year (Cunningham, 1980; Lyons and Cunningham, 1980; Sandbach, 1981a,b; Rajagopalan and Kosikowski, 1982; Maiorella and Castillo, 1984; Walker *et al.* 1985). In the USA alone, about 22 million tonnes of whey are generated annually and only 60% is

Table 2.1 Typical analyses of lactic acid casein, rennet casein and cheddar cheese whey (Clark, 1988).

	Lactic acid casein whey	Rennet casein whey	Cheddar cheese whey
Total solids (g/l)	57.6	60.0	58.0
Total nitrogen (g/l)	1.40	1.40	1.38
Non-protein nitrogen (g/l)	0.49	0.39	0.49
Protein (g/l)	5.8	6.4	5.7
Lactose (g/l)	39.5	46.0	41.2
Ash (g/l)	6.8	4.3	5.2
Lactate (g/l)	7.5	0.2	0.8
Sodium (mg/l)	450	370	370
Potassium (mg/l)	1520	1505	1500
Magnesium (mg/l)	105	74	80
Calcium (mg/l)	1163	330	430
Phosphate (mg/l)	1790	950	960
Chloride (mg/l)	980	895	920
Sulphur (mg/l)	140	140	135
Zinc (mg/l)	2.6	0.11	0.14
Iron (mg/l)	0.2	0.14	0.36
Copper (mg/l)	0.03	0.01	0.03
Manganese (mg/l)	<0.01	<0.01	<0.01
Molybdenum (mg/l)	<0.03	<0.03	<0.03
Boron (mg/l)	0.30	0.26	0.10
Cobalt (mg/l)	<3	<4	<1
Selenium (mg/l)	1.5	1.5	1.25
Nickel (mg/l)	<0.05	<0.05	<0.05
pH	4.6	6.7	6.0

utilized while the remainder is discarded (Zall *et al.* 1979; Mehaia *et al.* 1985; Walker *et al.* 1985). In Canada approximately 1 million tonnes of whey are produced per year, only 56% being utilized. Only a few countries such as Denmark, Holland, Poland, France, and USSR utilize up to 80% of their whey, for a variety of purposes (Cunningham, 1980).

In New Zealand in the 1992/1993 season dated to January 1993, approximately 3.1 million tonnes of whey were produced and an average 78% was utilized as shown in Table 2.2.

Table 2.2 Whey production in New Zealand 1992/93 season (Bell, personal communication 1993)

1992/93	Acid whey	Sweet whey
Whey production (m ³ /y)	1,540,000	1,605,000
% Processed	64%	62%
Serum/Permeate production (m ³ /y)	985,000	370,000
% Processed	87%	100%

Considerable progress has been made during the past fifty years in developing novel approaches for utilization of surplus whey. In particular the lactose content of whey of 40-50 g/l may be regarded as a valuable resource which can be further processed to maximize economic returns. Processes such as recovery of protein by ultrafiltration, recovery of whey fat, manufacture of crystalline lactose monohydrate, production of nonalcoholic beverages and a variety of fermentation processes have been reported (Hobman, 1984; Kravchenko, 1988). Fermentation products which have been described include single cell protein, enzymes, solvents, methane, food acids, microbial oil, vitamins, amino acids, antibiotics and food polysaccharides (Friend and Shahani, 1979; Hobman, 1984; Maddox and Archer, 1984; Zadow, 1988). Ethanolic fermentation produces a range of alcoholic products such as whey vodka, whey wine, and whey beer

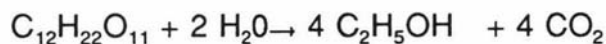
range of alcoholic products such as whey vodka, whey wine, and whey beer (Rogosa *et al.* 1947; Roland and Alm, 1975; Kosikowski and Wzorek, 1977; Friend and Shahani, 1979; Mann, 1980; Maiorella and Castillo, 1984). The production of ethanol from whey has been further stimulated by its potential use as a gasoline replacement. These products, coupled with the waste treatment problem, have been a major factor in the development of whey utilization by fermentation. The traditional fermentation process for the production of ethanol was established prior to World War 2. A preliminary report by Browne (1941) described the use of whey as a substrate, while further details were reported by Rogosa *et al.* (1947) and Rogosa (1948). Since then, more than 70 papers have been published concerning whey ethanol fermentation.

A major disadvantage of utilising whey as substrate for alcohol production is the relatively low concentration of sugar substrate (approximately 45 g/l lactose) compared with other substrates such as molasses, which would generally be fermented with a sugar content in the order of 100-180 g/l. Due to the dilute nature of the whey, and hence dilute fermented whey beer produced, a whey alcohol plant requires larger fermentation and distillation equipment and a greater energy input to distillation. Overall, this leads to higher operating and capital costs compared to the processes using more concentrated substrates.

There only a few genera of yeasts which can ferment lactose. Among them is the genus *Kluyveromyces*, of which the most widely used species is that now designated *Kluyveromyces marxianus*. This has been variously classified as *Torula cremoris*, *Candida pseudotropicalis*, *Kluyveromyces fragilis*, and *Saccharomyces fragilis* (Barnett *et al.* 1990). However, the original literature designations of this yeast will be retained throughout the discussion. This yeast is widely found in man, other mammals and dairy products, and is described as producing white cream butyrous colonies, while the cells are subglobose, spheroidal or ellipsoidal to cylindrical. Typically the cell size is in the range of 1 to 7 μm ; it reproduces by budding, and occurs singly or in pairs. Conjugation usually immediately precedes ascus formation, or diploid, vegetative cells may

be directly transformed into asci. The asci are evanescent and one to four smooth, oval, round or reniform ascospores are formed per ascus (Kreger-van Rij, 1984; Barnett *et al.* 1990).

An illustration of the typical batch fermentation kinetics to produce ethanol from whey is shown in Figure 2.1 (Vienne and von Stockar, 1983). The stoichiometry of ethanol formation from lactose according to the Gay Lussac equation is as follows:



This corresponds to a maximum theoretical yield of 0.538 g ethanol/g lactose. Hence the use of the term "% of theoretical yield", which is the ratio of the actual ethanol concentration to the maximum theoretical ethanol concentration based on the lactose consumed. Another term which is used when describing this fermentation process is "ethanol productivity". Ethanol productivity in the batch fermentation process is defined as the overall rate of production on a volumetric basis, and has units of g/l.h. In continuous culture, it is the product of dilution rate and ethanol concentration. Specific ethanol productivity is the ethanol productivity expressed per unit mass of biomass, and has the units g/g.h. These volumetric and specific productivities may be calculated at a given time (e.g. when the values are maximal), or averaged (e.g. over the duration of the fermentation). Typical batch fermentation of whey gives an ethanol productivity in the order of 1-5 g/l.h and 85-93% ethanol yield of theoretical.

2.1.2 Factors affecting the fermentation

Factors which influence the fermentation process include temperature, pH, addition of nutrients, aeration (oxygen) and sterols, inoculum size, salt concentration, osmotic pressure, and ethanol and substrate concentrations. By optimizing these fermentation parameters, a significant improvement of the fermentation process can be expected.

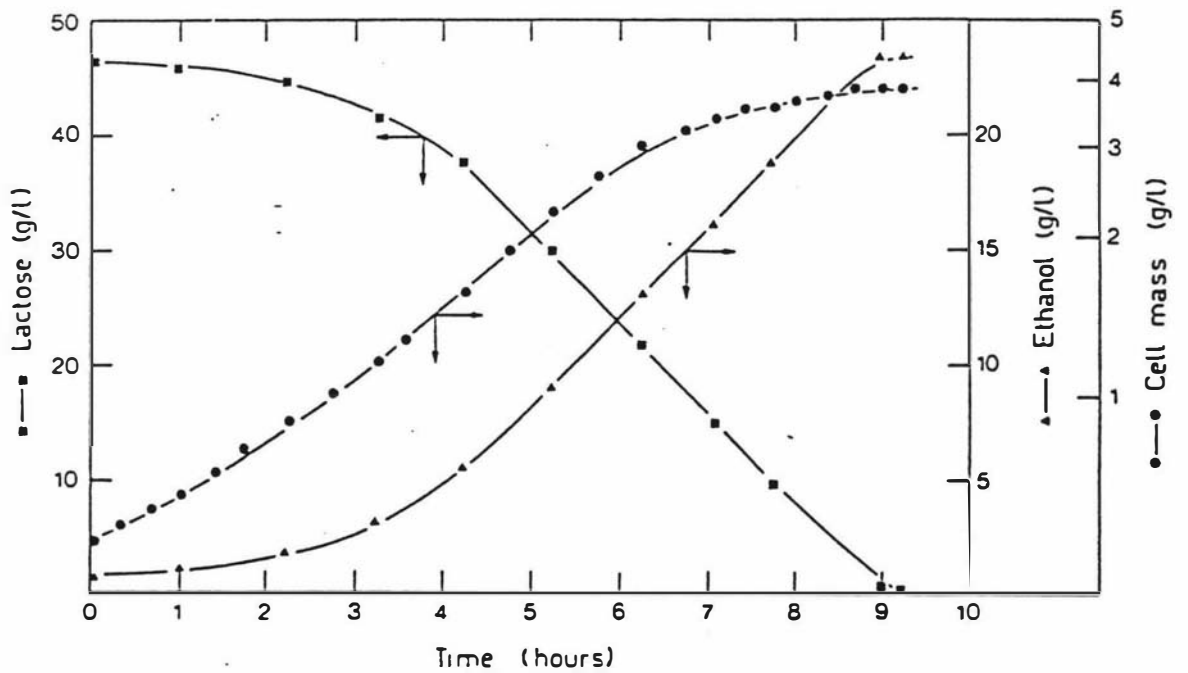


Figure 2.1 Batch culture of *K. fragilis* NRRL 665 on whey permeate supplemented with 3.75 g/l yeast extract. Temperature 38°C and pH 4.0 (Vienne and von Stockar, 1983).

1.2.1 Temperature

The effect of temperature has been investigated by Rogosa *et al.* (1947), Burgess and Kelly (1979), Demmott *et al.* (1981), Chen and Zall (1982), Castillo *et al.* (1982), Marwaha and Kennedy (1984), Vienne and von Stockar (1983), Tu *et al.* (1985), and Zayed and Foley (1987). The general conclusion is that the optimum temperature is in the range 30° to 40°C, with most reports identifying 35°C as the peak for ethanol production. However, there does seem to be some variation depending on the strain of yeast studied.

1.2.2 pH

The pH of the fermentation medium affects the fermentation patterns, mainly due to its effect on the transport of compounds across the cell membrane and on the activities of enzymes. Several workers have reported an optimum pH range between 4.0-6.0, with some variation depending on the strain of the yeast and the type of whey used (Rogosa *et al.* 1947; Castillo *et al.* 1982; Marwaha and Kennedy, 1984; Vienne and von Stockar, 1985b; Tu *et al.* 1985). Others have concluded that an initial pH in the range 4.6 to 5.6 has no significant effect on the rate of lactose fermentation and pH control is unnecessary because of the buffering capacity of the whey (Burgess and Kelly, 1979; Chen and Zall, 1982; Friend *et al.* 1982; Linko *et al.* 1984).

It is now generally accepted that the optimum pH range is in the range 4.0 to 6.0 depending on the strain of the yeast and the type of whey used, acid whey at the lower range of pH 4.0-5.0 and cheese whey at the upper range of pH 5.0-6.0.

1.2.3 Supplementary nutrients

2.3.1 Yeast extract

Moulin and Galzy (1980a) observed that when *K. fragilis* CBS 397 and *C.*

pseudotropicalis IP 513 were grown in complex synthetic medium containing all the necessary nutrients, or in whey permeate, identical biomass and ethanol yields were obtained. Based on these results, they suggested that whey contains all the necessary nutrients and that there is no need for any supplementation of the whey permeate. However the data these authors presented during growth on whey permeate show an obvious uncoupling between growth and alcohol production; growth ceased after 20 h, while ethanol production continued for more than 50 h until the lactose was completely utilized. This indicates a nutritional limitation of their unsupplemented whey permeate medium.

Castillo *et al.* (1982) reported that addition of 0.1 g/l yeast extract to whey medium had no effect on the yield of ethanol. But one must question the value of this finding since the yeast extract concentration added may have been too low to show any pronounced effect. Marwaha and Kennedy (1984) also claimed that whey permeate has all the required nutrients for alcoholic fermentation because they were able to grow their immobilized yeast cells successfully in this medium. However they did not investigate the effect of addition of any nutrients. Vienne and von Stockar (1983) disputed the conclusion of Castillo *et al.* (1982). They carried out an elemental analysis of both the permeate and the dry yeast cells, and found a possible stoichiometric limitation by nitrogen. This was confirmed when addition of nitrogen source (1.7 g/l ammonium sulfate) to the whey increased the biomass yield by 26 % compared to the control, but had no substantial effect on the growth rate. However, addition of 1 g/l of yeast extract had an effect on both values as shown in Table 2.3; the specific growth rate and biomass yield were increased by 44% and 65%, respectively, compared to the control. This result indicated that addition of yeast extract would overcome the nitrogen and growth factor limitation of whey permeate medium. In all cases the ethanol yield remained constant.

Vienne and von Stockar (1983) further investigated the characteristics of the *C. pseudotropicalis* IP 513 strain used by Moulin *et al.* (1980a) and observed the results noted above, i.e. an uncoupling between growth and product formation

during growth in a nonsupplemented whey permeate. After 12 h, growth ceased while ethanol production continued for more than 24 h until the lactose was completely utilized. When the medium was richly supplemented this uncoupling disappeared and was replaced by continuous exponential growth. In addition, the fermentation time was reduced to almost half of that of the unsupplemented medium. The data are summarised in Table 2.4. Vienne and von Stockar (1983) established a ratio of 3.75 g/l to 50 g/l lactose to be the minimum yeast extract concentration to avoid nutrient limitation in whey permeate. In a continuous culture of *K. fragilis* NRRL 665, a two-fold increase of ethanol productivity was observed when the whey was supplemented with 3.75 g/l yeast extract, in comparison to the nonsupplemented whey.

Other studies have demonstrated the stimulatory effect of yeast extract. Chen and Zall (1982) established an optimum requirement of 7 g/l yeast extract for their 100 g/l lactose whey medium. Burgess and Kelly (1982) also observed higher ethanol production and cell biomass compared to the control with the addition of 0.1 g/l yeast extract and 0.5 g/l urea to their whey medium containing 150 g/l of lactose. A similar finding was reported by Zayed and Foley (1987) who observed that addition of 4 g/l yeast extract to whey permeate containing 110 g/l lactose, increased the % of theoretical yield of ethanol from 75 to 83%. Addition of both 4 g/l yeast extract and 10 g/l urea increased the % of theoretical yield to 84% compared to that of nonsupplemented permeate.

2.3.2 Other nutrients

Castillo *et al.* (1982) reported that the addition of phosphorous salt, nitrogen salt and corn steep liquor to a whey medium had little or no effect on the ethanol yield.

Mahmoud and Kosikowski (1982) reported that addition of 1% ammonium sulfate to concentrated demineralized whey containing 250-270 g/l lactose had no effect on ethanol production, but addition of 1% of urea and peptone decreased the

Table 2.3 Kinetic parameters for growth of *K. fragilis* NRRL 665 on whey permeate with different supplementation: Temperature 38°C and pH 4.0 (Vienne and von Stockar,1983).

Type of medium supplementation	μ_{\max} (h ⁻¹)	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (g/g)
0.1% yeast extract	0.236	0.056	0.4933
0.17% (NH ₄) ₂ SO ₄	0.177	0.043	0.4726
None	0.164	0.034	0.4836

Table 2.4 Kinetic parameters for growth of *C. pseudotropicalis* IP 513 on whey permeate with different supplementation: Temperature 32°C and pH 5.0 (Vienne and von Stockar,1983).

Type of medium supplementation	μ_{\max} (h ⁻¹)	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (g/g)
0.1% yeast extract	0.205	0.065	0.4834
0.17% (NH ₄) ₂ SO ₄	0.165	0.065	0.4823
None	0.132	0.03	0.4884

ethanol yield by 36% and 56%, respectively, compared to the control. No explanation of the effects was offered by the authors.

Vienne and von Stockar (1985a) studied the effect of addition of various mineral ions, vitamins and amino-acids on the performance of *K. fragilis* NRRL 665. The basal medium (reconstituted whey containing 90 g/l lactose, supplemented with 8.25 g/l yeast extract) to which the various supplements were added, was used as control. They observed that neither the specific growth rate nor the biomass yield could be improved by the addition of various mineral ions, vitamins or combinations of both. However a slightly higher biomass yield was obtained following the addition of some amino acids. There was no mention of the effect of these nutrients on the ethanol yield.

Walker *et al.* (1990) reported a positive effect of added magnesium ions on the fermentation of cheese whey by *K. marxianus* NRRL Y-2415. They observed that when 0.05 g/l of magnesium chloride was added to the medium, the lactose utilization rate was faster and a 92% ethanol yield of theoretical was achieved as opposed to 77% in the control. The authors suggested that magnesium ions play an important role in cell division and carbohydrate metabolism.

In conclusion, it appears that the nutrient limitation of whey media can be overcome by the single addition of yeast extract. Yeast extract provides a source of nitrogen and many growth promoting factors such as vitamins and trace minerals which are used in biosynthesis by yeast, and therefore result in faster fermentation rates but not necessarily higher ethanol yields.

1.2.4 Oxygen and sterols

A minimum level of oxygen is required in ethanol fermentation to permit the biosynthesis of unsaturated fatty acids and sterols which are essential for the cell membrane structure of yeast. By adding sterols to the media, the need for any aeration may be eliminated.

Chen and Zall (1982) and Janssens *et al.* (1983) reported that addition of ergosterol and unsaturated fatty acids to the whey medium resulted in the increase of ethanol yield and reduced fermentation time. In contrast, Vienne and von Stockar (1985a) observed that addition of ergosterol (20 mg/l) did not significantly improve either the specific growth rate or the biomass yield when compared to the control containing 8.25 g/l yeast extract. However, it was possible that the yeast extract in the control medium provided sufficient ergosterol to meet the yeast requirement, but this point was not considered by the authors.

Linko *et al.* (1984) reported that in continuous fermentation using immobilized *K. fragilis* cells, the stability of ethanol production depended on aeration. They observed that at a dilution rate of 0.25h^{-1} with whey containing 50 g/l lactose, ethanol production was stable for at least two months (approx. 78% ethanol yield of theoretical) when it was continuously aerated at 0.1 bed vol./min. In the unaerated culture, however, the ethanol concentration began to decrease after three weeks of fermentation falling to around 55% ethanol yield of theoretical. No explanation for this effect was offered, although it is more likely that the initial sterol provided by the inoculum could have been depleted in the long term fermentation without aeration.

Vienne and von Stockar (1985b) studied the effect of aeration on continuous fermentation of concentrated whey containing 89 g/l lactose at a dilution rate of 0.205h^{-1} . They observed that increasing the air flow rate above 0.06 vvm induced a shift from ethanol production towards biomass production and found no maximum alcohol production rate as a function of aeration rate. In contrast, Zayed and Foley (1987) established an optimum air flow rate of 0.15 vvm for *K. fragilis* grown in whey containing 110 g/l lactose. At this optimum air flow rate, an increase of % ethanol yield of theoretical from 63% to 80% and of ethanol productivity from 0.38 g/l.h to 1.1 g/l.h were observed compared to cultures without aeration. However, increasing the air flow rate beyond 0.25 vvm resulted in a higher biomass, but lower ethanol yield.

1.2.5 Inoculum size

It is well known that high cell density results in rapid fermentation rates. It has been established that a yeast inoculum between 1 and 2% (dry weight yeast / weight of lactose) is sufficient to ensure satisfactory rate of fermentation for both concentrated and dilute whey (Rogosa *et al.* 1947; Castillo *et al.* 1982; Rajagopalan and Kosikowski, 1982; Maiorella and Castillo, 1984).

1.2.6 Salt concentration

The effect of salt concentration on the performance of *K. lactis* CU 10689 was studied by Gawel and Kosikowski (1978). They observed that when whey medium was supplemented with either NaCl or CaCl₂ at 1.5% (the units were not specified) there was an inhibition of the fermentation rate. At 3.5%, the inhibition effect was more pronounced. It was suggested that the high ash concentration induced a high osmotic pressure and this in turn affected the lactose uptake by the cells.

A similar observation was reported by Mahmoud and Kosikowski (1982) where five *Kluyveromyces* strains were grown anaerobically in deproteinized concentrated whey (30-32% total solids) containing three different ash concentrations: 3.5%, 1.80% and 0.77%. *K. fragilis* NRRL Y-2415 showed an improvement in productivity from 0.21 g/l.h to 0.60 g/l.h, and in % of theoretical yield from 18% to 52%, when the ash concentration was reduced from 3.5% to 0.77%. On average the five yeasts showed an approximately three-fold increase in productivity and % of theoretical yield, when the ash content was reduced from 3.5% to 0.77%.

Linko *et al.* (1981) observed that a high salt concentration in ultrafiltrated whey resulted in a declining trend of ethanol production by immobilized *K. fragilis* compared to demineralized whey. Marwaha and Kennedy (1984) also reported that a higher concentration of calcium chloride (11 g/l) used to form the beads of

immobilized cells resulted in lower ethanol yield, as compared to the free cells and beads formed by using low concentrations of calcium chloride (0.1-0.5 g/l).

The general conclusion is that many of lactose-fermenting yeast strains are affected by moderate concentrations of salt (above 1% w/v), possibly due to the high osmotic pressure exerted.

1.2.7 Osmotic pressure

Harbison *et al.* (1983) studied the effect of mannitol-induced osmotic pressure on the lactose fermentation by supplementing a semi-synthetic medium containing 130 g/l lactose with 124 and 234 g mannitol/l. The control used was semisynthetic medium containing 100 g/l lactose. The authors observed that after 24 h of fermentation, only 83% and 45% of the lactose had been consumed in the 124 and 234 g mannitol/litre supplemented media, respectively, compared to the control, where the lactose was exhausted. The % of theoretical yield for ethanol was 78% for the two mannitol-supplemented media after 72 h compared to 90% for the control. It is not understood why the lactose concentration in the control was only 100 g/l compared to 130 g/l in the mannitol test media.

Grubb (1991) investigated the effect of maltose-induced osmotic pressure on lactose fermentation by *K.marxianus* Y-113. A range of concentrations of maltose (100 to 250 g/l) were added to the semi-synthetic medium containing 100 g/l lactose. The author reported that in the presence of maltose, the maximum specific growth rate was reduced approximately by half compared to the control. However the effect on the ethanol specific productivity was less significant.

1.2.8 Lactose and ethanol concentration

The two effects are discussed jointly as it is difficult to raise the lactose level without resulting in a increase of ethanol concentration. Moulin *et al.* (1980b) studied the effect of added lactose and ethanol on the fermentation rate by

exposing cells to various lactose and ethanol concentrations for 5 h. They found that ethanol was inhibitory to *C. pseudotropicalis* IP 513, even at concentrations as low as 16 g/l. The inhibition increased markedly as the ethanol concentration increased. Furthermore they found no evidence of inhibition of the fermentation rate by lactose up to 250 g/l when no ethanol was present. There was, however a synergistic inhibitory effect when the ethanol and lactose concentration exceeded 64 g/l and 175 g/l, respectively. It is possible that different effects might be observed in the normal batch fermentation process, where the yeast cells are exposed to lactose and endogenous, rather than exogenous ethanol, for longer periods of time.

Bajpai and Margaritis (1982) studied the ethanol inhibition kinetics of *K. marxianus* UCD(FST) grown on Jerusalem artichoke juice. They found a linear relationship between μ_{\max} and the added ethanol concentration and reported that growth ceased completely ($\mu_{\max} = 0$) when the ethanol added reached a concentration of 95 g/l. The authors suggested that the ethanol tolerance level of this strain of *K. marxianus* is about the same as the ethanol tolerance level for *Saccharomyces cerevisiae*. Marwaha and Kennedy (1984) reported that under optimum temperature conditions, immobilized and free cells of *K. marxianus* NCYC 179 showed no end product inhibition up to 60 g/l of extracellular ethanol.

Vienne and von Stockar (1985a) studied the inhibition effect of ethanol occurring during the continuous fermentation of whey. They showed that ethanol inhibition of growth was non-competitive (Figure 2.2) and that the maximum concentration of ethanol tolerated by *K. fragilis* NRRL 665 (where $\mu/\mu_{\max} = 0$) was 45 g/l.

Ruggeri *et al.* (1987) modelled the kinetics of lactose fermentation by *K. fragilis* CBS 5795 and also observed that yeast growth ceased when the ethanol concentration reached 43 g/l, regardless whether the substrate concentration was 25 g/l or 126.5 g/l.

Bothast *et al.* (1986) evaluated the performance of 107 lactose-fermenting yeast strains grown on complex medium containing 50, 100 and 200 g/l lactose. The

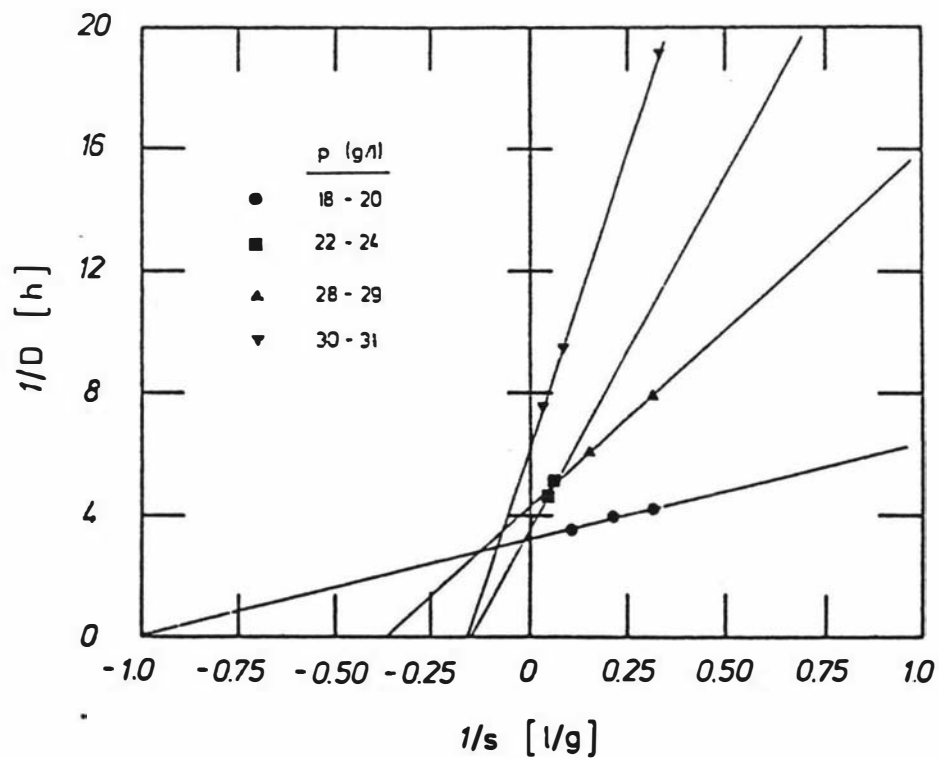


Figure 2.2 Lineweaver-Burk plot of the continuous culture of *K. fragilis* NRRL 665 on permeate supplemented with 3.75 g/l yeast extract and with various amounts of ethanol added to the medium. S_0 :46 g/l. Parameter: steady state ethanol concentration.

authors reported that the specific ethanol productivity averaged 0.43 g/g.h among the 107 strains on 50 g/l lactose with 13 strains producing > 0.69 g/g.h. On 100 g/l lactose, the specific production rate decreased to an average of 0.31 g/g.h with 10 strains producing > 0.45 g/g.h. Increasing the lactose concentration further to 200 g/l resulted in a decreased specific production rate to an average of 0.18 g/g.h with 5 strains producing > 0.31 g/g.h. Eight strains of *K. marxianus* (NRRL Y-1174, Y-1175, Y-1179, Y-1194, Y-1195, Y-1196, Y-1200, and Y-2498) demonstrated good production rate (>0.30 g/g.h) averaged over the two high lactose concentrations. *K. marxianus* NRRL Y-1195 was selected as the best strain with a production rate of 0.35 g/g.h averaged over the two high lactose concentrations.

The interaction of substrate and product inhibition effect is complex, although most studies considered that product inhibition is more dominant. It appears that there may be two groups of *K. marxianus*. One group, which is ethanol sensitive, can only tolerate ethanol up to 45 g/l, and could include strains CBS 5795 and NRRL 665. The other group, which has a higher ethanol tolerance of up to 95 g/l, could include strains UCD, NCYC 179 and *C. pseudotropicalis* strains.

2.1.3 New technological approaches

1.3.1 Hydrolysed whey

A major obstacle in using whey as a fermentation substrate is that relatively few organisms are able to ferment lactose. Yeasts of the genus *Kluyveromyces* have been found to be the most efficient lactose-fermenting microorganisms, but this is offset by the problem that many strains are alcohol-sensitive compared with *S. cerevisiae*. If the lactose in whey could be hydrolysed into its constituent monosaccharides, glucose and galactose, highly fermentative strains of *S. cerevisiae*, which can tolerate higher concentrations of alcohol, could be used for ethanol production (O'Leary *et al.* 1977 a,b; Friend and Shahani, 1979; Bailey *et al.* 1982).

Roland and Alm (1975) and O'Leary *et al.* (1977 a,b) reported that the hydrolysed whey permeate, *S. cerevisiae* was subject to catabolite repression by glucose and because of that, long fermentation times and high solids contents were required to produce the amount of alcohol equivalent to that obtained in typical molasses-based industrial fermentations.

Bailey *et al.* (1982) and Terrel *et al.* (1984) were able to overcome this by using catabolite repression-resistant mutant strains, derived from a *S. cerevisiae* yeast, which were capable of fermenting mixtures of glucose and galactose rapidly and completely. The best, spontaneously isolated, mutant was capable of fermenting a mixture of 100 g/l glucose and 100 g/l galactose to completion in less than 37 h, and produced about 90 g/l ethanol, with an ethanol productivity of 2.4 g/l.h. Both these reports dealt with mutant strains of *S. cerevisiae* grown on semi-synthetic media and not authentic hydrolysed whey. Fermentation of concentrated hydrolysed whey by mutant *S. cerevisiae* may be an improvement to the conventional direct whey fermentation, but the questions of the long term stability of mutants and whether or not the reduction in capital and operating costs would compensate for the added cost for hydrolysis, are unclear.

1.3.2 Concentrated whey

As stated earlier, the fermentation of whey to ethanol suffers a serious setback due to the low ethanol concentration obtained. This makes the process energy intensive, and energy costs are the major component of the production cost (Mawson, 1990). To improve the economics of whey alcohol production, the lactose concentration could be increased by means of reverse osmosis up to three or four times that of normal whey. The low solubility of lactose in water prevents the concentration of whey to more than about 20% total solids, or approximately 160 g/l lactose, after which it begins to precipitate at room temperature (Sanderson and Reed, 1985). The main benefit of the use of concentrated whey will be energy savings gained primarily from the ethanol recovery and purification process. Concentrated whey has a high lactose and

mineral content and many of the lactose-fermenting yeast strains tolerate moderate concentrations of sugar, ethanol and salt (refer to Section 1.2.8). Due to the combined effects of these and osmotic pressure, fermentation is usually slow requiring up to two weeks to complete, and also lower ethanol yields have been observed (Gawel and Kosikowski, 1978; Burgess and Kelly, 1979; Moulin *et al.* 1980a; Castillo *et al.* 1982; Izaguirre and Castillo, 1982; Mawson and Taylor, 1989). The results obtained by these authors are summarized in Table 2.5.

As noted before, in general, it appears that there are two distinct groups of *Kluyveromyces*. One group performs poorly in concentrated whey, probably due to a low ethanol tolerance (Section 2.8), and includes such strains as *K. fragilis*, Y-1109, Y-113, *K. lactis* NCYC 1368, and *C. pseudotropicalis* NCYC 143. The other group, which performs well in concentrated whey, possibly due to the fact that they have a higher ethanol tolerance (Section 2.8), includes most strains of *C. pseudotropicalis* (IP 513, ATCC 8628, NCYC 744), and *K. fragilis* strains CBS 397, CBS 5795, and NRRL 1156.

1.3.3 Continuous culture

Conventional batch fermentation suffers the disadvantages of being inefficient owing to the high fermenter volume requirements resulting in high capital costs. Additionally, the continual start-up and shut-down makes it difficult to automate and results in high operating costs. With continuous cultures some of these problems can be overcome. The potential of continuous culture for achieving a high productivity can be improved by coupling it with cell recycle or cell immobilization. Consequently many studies have considered these process options and very limited data are available on the continuous culture with free cells.

TABLE 2.5 Summary of concentrated whey fermentation data

Organism	Lactose Concentration (g/l)	Productivity (g/l.h)	% ethanol yield of theoretical	Refs	Comment
<i>K. fragilis</i> NRRL Y 1109	100-250	?	91-30	1	Productivity data not available
<i>K. fragilis</i> CBS 5975	100-150	1.38-1.95	84-92	2	In 150 g/l lactose, only two third of the substrate was utilized
<i>C. pseudotropicalis</i> NCYC 744	100-150	1.76-2.20	83-86	2	
<i>K. fragilis</i> NRRL Y-1109	100-150	2.64-2.15	88-30	2	
<i>C. pseudotropicalis</i> IP513	200-300	2.4 -?	85-50	3	Lactose concentration in the range of 100,150,200,250,and 300 g/l.
<i>K. fragilis</i> CBS 397	100-300	?	90-50		
<i>C. pseudotropicalis</i> NCYC 143	100-250	0.21-0.11	73-17	4	Lactose concentration in the range of 100, 150, 200 and 250 g/l were used.
<i>C. pseudotropicalis</i> ATCC 8619	100-250	0.32-0.15	86-24	4	
<i>C. pseudotropicalis</i> ATCC 8628	100-200	0.15-0.29	91-58	4	
<i>K. fragilis</i> NCYC 151	150-250	0.29-0.12	76-20	4	
<i>C. pseudotropicalis</i> ATCC 8619	175	3.8	88	5	
<i>C. pseudotropicalis</i> IP 513	90-135	1.54-1.16	89	6	Lactose concentration of 90 and 135 g/l were used.
<i>K. fragilis</i> CBS 397	90-135	1.61-1.11	97-86	6	
<i>K. fragilis</i> NRRL Y1109	90-135	1.17-0.89	87	6	
<i>K. fragilis</i> NRRL 1156	90-135	1.61-1.49	86-93	6	
<i>K. fragilis</i> NRRL Y 665	90-150	1.77-2.10	91	6	
<i>K. fragilis</i>	110-150	1.10-0.85	78-44	7	Lactose concentration of 100 and 150 g/l were used.
<i>K. lactis</i> NCYC 1368	110-150	0.44-0.36	54-30	7	
<i>K. marxianus</i> Y113	100-150	0.77-0.83	74-71	8	

1 O'Leary, *et al* 1977
5 Castillo *et al*, 1982

2 Burgess & Kelly, 1979
6 Vienne & von Stockar, 1983

3 Moulin *et al*, 1980a
7 Zayed&Foley, 1987

4 Izaguirre & Castillo, 1982
8 Mawson & Taylor, 1989

3.3.1 Continuous culture with free cells

King and Zall (1983) studied the continuous culture of free cells of *K. fragi* on concentrated whey containing 100 g/l lactose concentration. The authors reported a maximum ethanol productivity of 1.9 g/l.h at a dilution rate of 0.16 h⁻¹.

Vienne and von Stockar (1983) studied the continuous culture of *K. fragilis* NRRL 665 on a whey permeate supplemented with 3.75 g/l yeast extract. They reported two possible μ_{\max} and K_s values from their Lineweaver-Burk plot. It appeared that the points were not aligned on a single straight line as would be expected for Monod kinetics, but that two distinct straight lines could be drawn. At low residual substrate concentrations, μ_{\max} of 0.216 h⁻¹ and K_s of 0.014 g/l were obtained. At high concentrations, μ_{\max} of 0.310 h⁻¹ and K_s of 0.971 were obtained. The authors suggested that two systems for lactose transport systems through the membrane might exist. At low concentrations an active transport mechanism was involved, resulting in a high affinity for the substrate, which would be reflected by a low saturation constant. At high concentrations, the transport mechanism might be passive with a lower affinity. Barnett and Sims (1982) reported that aerobically, lactose uptake occurred through active transport in *K. fragilis*. Anaerobically, however, lactose uptake appeared to be by facilitated diffusion.

Vienne and von Stockar (1985b) also studied the continuous culture of *K. fragilis* NRRL 665 on sterilized or non-sterilized permeate, and in sterilized concentrated whey. They observed no apparent difference on the ethanol productivity when the yeast was grown in sterile or non-sterile unsupplemented whey media. In a supplemented whey permeate containing 46 g/l lactose, a maximum productivity of 5.23 g/l.h and 91 % of ethanol of theoretical were obtained. With 86 g/l lactose and 8.15 g/l yeast extract, a maximum productivity of 4.6 g/l.h was obtained but with a residual lactose concentration of 45 g/l. Increasing the lactose concentration further to 150 g/l resulted in a maximum productivity of approximately 3 g/l.h, with a residual lactose concentration of 115 g/l. This

compared poorly with batch fermentation at 100g/l lactose where the same yeast utilized the lactose completely in 14 h to achieve 91% of the theoretical ethanol yield.

3.3.2 Continuous culture with cell recycle

In general the productivity of a fermentation process can be improved by incorporating a cell recycle system. Janssens *et al.* (1984) evaluated a single stage continuous fermentation process with cell recycle. At a dilution rate of 0.15h^{-1} , they reported a steady state productivity of 7.1 g/l.h and 88% ethanol yield of theoretical when *K. fragilis* CBS 397 was maintained on a feed of cheese whey containing 100 g/l lactose with a biomass concentration of 20-25 g/l. Attempts to increase the dilution rate and/or to increase the lactose concentration resulted in decreased ethanol productivities and yields. The results obtained using this system represent a significant improvement on the batch fermentation process using the same yeast as reported by Vienne and von Stockar (1983) and Moulin *et al.* (1980a).

Vienne and von Stockar (1985b) studied a cell recycle system using the flocculant strain *K. lactis* NCYC 179 on whey permeate containing 45 and 95 g/l lactose. They reported that at 45 g/l lactose, with a biomass concentration of 21 g/l and dilution rate of 0.48h^{-1} , lactose was completely fermented to give an ethanol productivity of 10.2 g/l.h, and 90% ethanol yield of theoretical. At 95 g/l lactose concentration with a biomass concentration of 21 g/l, an ethanol productivity of 9.3 g/l.h and 89% ethanol yield of theoretical were achieved. An increase of almost two-fold in productivity was achieved with recycle compared with continuous cultivation with free cells.

1.3.3.3 Continuous culture with immobilized cells

Processes based on immobilized living microbial cells have several advantages compared with the traditional free cell fermentation process, such as : higher

fermentation rate due to higher cell packing, high specific product yield, and the possibility of operating continuously at high dilution rates without washout (King and Zall 1983). Methods of cell immobilization, and some results obtained for lactose fermentation, are summarized in Table 2.6. In general, cell immobilization resulted in marked improvement of the ethanol productivities, but often at the expense of a high residual lactose concentration and low ethanol yield.

2.1.4 New biological approaches

1.4.1 Strain adaptation

Many of the lactose-fermenting yeast strains are limited by their ability to ferment high lactose concentrations. However, it is possible to overcome this limitation by adapting the yeast to a high lactose environment. Kosikowski and Wzorek (1977), Gawel and Kosikowski (1978) and Rajagopalan and Kosikowski (1982) periodically transferred the yeast strains into successively higher lactose concentration to enable the yeasts to adapt to high concentration. They reported that the adapted strain gave higher ethanol productivity and yield compared to the non-adapted culture. The best result was obtained by Rajagopalan and Kosikowski (1982) using the adapted strain of *K. fragilis* NRRL Y-2415, where 240 g/l lactose whey was fermented to give 84.3% ethanol yield of theoretical and a batch ethanol productivity of 3.2 g/l.h.

1.4.2 Genetic manipulation

The technique of genetic manipulation is commonly used to produce a superior microorganism. For example, protoplast fusion can be used to combine the desirable properties of parental strains, i.e, to produce a yeast strain which is highly ethanol tolerant and capable of fermenting lactose. Recombinant DNA technology can also be used to introduce a β -galactosidase gene into *S. cerevisiae*.

Table 2.6 Summary of cell immobilization methods and fermenter performance for ethanol production from whey

Lactose concentration (g/l)	Organism	Immobilization method	Reactor	Dilution rate (h ⁻¹)	Productivity (g/l.h)	% ethanol yield of theoretical	Reference
50	<i>K.fragilis jorgensen</i> B-1-5	Calcium alginate entrapment	PB	0.26	5.1	73	Linko <i>et al.</i> 1981
100	<i>S.fragi</i>		AFEB	1.1	6.9	12	Chen & Zall 1982
150*	<i>K.marxianus</i> NRLL Y-2415	Hollow fibre cartridge	MRF	6	240	60	Cheryan & Mehaia 1983
200	<i>K.fragi</i>	Polyacrimide entrapment	PB	0.8	7.8	18	King & Zall 1983
100	<i>K. fragi</i>	Kappa-carrageenan entrapment	PB	0.68	13.3	36	King & Zall 1983
100	<i>K.fragi</i>	Calcium alginate entrapment	PB	0.69	6.3	17	King & Zall 1983
100	<i>K.marxianus</i> NCYC 179	Calcium alginate entrapment	PB	0.15	6.5	83	Marwaha & Kennedy 1984
45	<i>K.fragilis</i> NRRL Y-2415	Hollow fibre cartridge	MRF	1.3-2.7	30-34	95-52	Mehaia & Cheryan 1984
45	<i>K.fragilis</i> NRRL Y-2415	Calcium alginate	PB	0.09	1.1	50	Hans-Hagerdal 1984
100	<i>S.cerevisiae</i> Y1	Coimmobilized with - galactosidase in calcium alginate	PB	0.09	4.5	93	Hans-Hagerdal 1984
50	<i>K.fragilis</i> NRRL Y2415 and <i>K.fragilis</i> TKK 3021	Calcium alginate entrapment	PB	0.25	5	80-90	Linko <i>et al.</i> 1984
150	<i>K.fragilis</i> CBS 5795	Adhesion to activated charcoal	Tubular	0.96	17.2	22	Gianetto 1986
100-200	<i>K.marxianus</i> NCYC 179	Calcium alginate entrapment	PB	0.42	13.8-28.2	61-62	Marwaha <i>et al.</i> 1988

PB = Packed bed AFEB = Attached Film expanded bed MRF = Membrane recycle fermenter * = Synthetic medium

Taya *et al.* (1984) and Farahnak *et al.* (1986) studied fusants of *K. fragilis* and *S. cerevisiae* grown in semi-synthetic media and reported that the fusants were more tolerant to ethanol than was the parent strain although further work must be carried out particularly in evaluating the long term stability of the fusant performance in whey.

Jeong *et al.* (1991) developed a recombinant strain of *S. cerevisiae* which contained an integrated plasmid and autonomously replicating plasmid. These plasmids contained lactose utilization genes from *K. lactis*. It was observed that 76% of the cells retained the plasmid after 8 days fermentation.

1.4.3 Use of mixed cultures

Catabolite repression by monosaccharides (glucose and galactose) present in small quantities in whey have been suggested to result in a less optimal fermentation rate. So, creating a sink for these monosaccharides using a coculture could improve the fermentation rates (Tu *et al.* 1985; Wang *et al.* 1987).

Mixed cultures of *K. fragilis* NRRL 665 and the bacteria *Zymomonas mobilis* NRRL 1960 and 4286 were studied by Kamini and Gunasekaran (1989). *Z. mobilis* can ferment glucose but not lactose. These authors reported that a coculture of *K. fragilis* and *Z. mobilis* 4286 in a complete medium containing 200 g/l lactose, gave an ethanol yield of theoretical of 56 % and a productivity of 1.26 g/l.h compared to a 45% ethanol yield of theoretical and a productivity of 1.02 g/l.h using a monoculture of *K. fragilis*. At 250 g/l lactose, however, loss of yield was observed, with the coculture giving 46% of theoretical yield and a productivity of 1.28 g/l.h, compared to the pure yeast culture which gave 39% theoretical yield and a productivity of 1.1 g/l.h. The significant improvement of ethanol yield when the coculture process was employed may have been due to the rapid use of glucose by *Z. mobilis* and galactose by *K. fragilis*. However, the yields obtained are very poor in comparison to the performance of single cultures under high lactose concentrations reported by others (Section 1.3.2). The authors suggested

that product and substrate inhibition were the reasons for the low ethanol yields.

Friend *et al.* (1982) found that there was no advantage in using mixed cultures of *K. fragilis* FST and *S. cerevisiae* over a mono culture of *K. fragilis* when grown in sweet whey permeate containing 51 g/l lactose at pH 5.0. Both techniques gave a productivity of 0.83 g/l.h and a 73% ethanol yield of theoretical within 24 h of fermentation. However, during further experiments with a mixture of whey and hydrolysed grain (both lactose and glucose present), they observed that the mixed cultures produced slightly more alcohol than the pure cultures of either *K. fragilis* or *S. cerevisiae*.

Tu *et al.* (1985) investigated the performance of a coculture of *K. marxianus* CBS 397 and *S. cerevisiae*. They pre-fermented mozzarella whey containing 160 g/l lactose, 10.8 g/l galactose, and 2.7 g/l glucose using *S. cerevisiae* then followed this by a *K. marxianus* fermentation. The results show a 20% faster lactose utilization rate compared to the monoculture of *K. marxianus*. They suggested there was an interaction between the different yeasts whereby any monosaccharide which accumulated was utilized by *S. cerevisiae* resulting in an improvement of the fermentation rate. They observed that when *S. cerevisiae* was inoculated at the same time as *K. marxianus* at initial cell concentrations of 7 g/l and 0.2 g/l, respectively, it resulted in an approximately 20% faster lactose utilization and slightly higher ethanol yield, in comparison with monoculture of *K. marxianus*. No reason was given for the use of a high initial cell concentration of *S. cerevisiae* inoculum with respect to *K. marxianus* cell concentration. There is a possibility that given such a high initial cell concentration of *S. cerevisiae* and a low nutrient concentration, starvation could occur causing a higher rate of death. The lysed cells could then provide a source of nitrogen to *K. marxianus* which could explain the faster rate of fermentation. Wang *et al.* (1987) investigated the effect of glucose and galactose on the whey fermentation by *K. marxianus* CBS 397. They reported that glucose at concentrations of 10-20 g/l is associated with strong inhibition of lactose uptake in a cheese whey containing either 20 g/l or 160 g/l lactose. The effect of glucose on lactose uptake was

much stronger than that of galactose. No explanation was offered as to why a lower concentration of glucose was not investigated as it is unlikely such a monosaccharide concentration would arise in practice.

The evidence for improved fermentation performance using mixed cultures of yeast and yeast, or yeast and bacteria, is conflicting and could be investigated further. A mixed culture process was briefly adopted in industrial fermentation at Golden Cheese Co, Corona, California, USA. With mixed cultures of *C. tropicalis* and *K. marxianus* it was reported that the frequency of contamination was reduced and an increased ethanol efficiency was obtained in comparison to the normal pure culture fermentation. However, the process was discarded due to the pathogenicity of the *Candida* strain used in the culture (Mawson, personal communication 1990).

2.1.5 Feasibility assessment of ethanol production from whey

Feasibility studies of the whey-based alcoholic fermentation process have been performed by Reesen (1978), Reesen and Strube (1978), Moulin and Galzy (1981), Rajagopalan and Kosikowski (1982), Singh *et al.* (1983), Maiorella and Castillo (1984), and Walker *et al.* (1985). Most confirm the process is viable but regardless of the outcome of these theoretical studies, the success of the whey to ethanol fermentation is demonstrated by the establishment of commercial plants in several countries. In the 1980's the economic feasibility of whey ethanolic fermentation plant was calculated with the assumption that whey has a very low cost or even a negative cost owing to the necessary cost of waste treatment. However, in the 1990's the price of whey is no longer set by waste treatment costs but by other uses, especially lactose manufacturing, for which the market is rapidly increasing. This factor needs to be considered if a new whey-ethanol plant is to be built.

2.1.6 Industrial whey-based ethanol fermentation

The whey ethanol fermentation process has been studied since the 1940's. However, it was not until in the late 1970's that the first commercial-sized whey ethanol plant was established at Carbery, Republic of Ireland. Currently, there are at least nine commercial whey ethanol fermentation plants. Four of these are located in New Zealand, three in USA, one in Ireland and possibly one in Poland. Nevertheless, the volume of whey being processed is only a small fraction of the world's total whey production. The commercial plants' operation will be discussed briefly on the basis of existing information available from the literature and personal communication.

The world's first commercial whey-based ethanol plant was established in late 1978 at Carbery, by Carbery Milk Products Ltd, Ireland (Cunningham 1980; Lyons and Cunningham 1980; Sandbach 1981a,b; Barry 1982; Sanderson and Reed 1985). A flow diagram of the Carbery process is shown in Figure 2.3. Permeate from the Abcor UF plant containing about 45 g/l lactose is pumped via a balance tank to one of the six fermenter vessels, each of 25,000 gallons capacity. The throughput of the UF plant is approximately 110,000 Imperial gallons/day and hence four full fermenter volumes are generated during a 24 h period. The fermentation process takes approximately 20 h and therefore at any one time there are normally three vessels fermenting, one being filled, one feeding the still and one being cleaned prior to receiving the next charge. At the end of fermentation, the beer, containing about 22 g/l alcohol, is passed through a centrifuge where the yeast cream is recovered and the clean beer is then fed to the still to achieve a product containing 96.5%(v/v) ethanol. The effluent generated from the washing plant is then treated in a large anaerobic digester to reduce the BOD₅ further and also to reduce the phosphate level. A fresh yeast culture is propagated for each fermentation to ensure high productivity and to minimise contamination. The procedure is from 25 l laboratory culture flasks to the 6000 gallon preparation tank prior to the inoculation of the six 35000 gallon fermenters. It has been reported that an average 85% theoretical yield has been

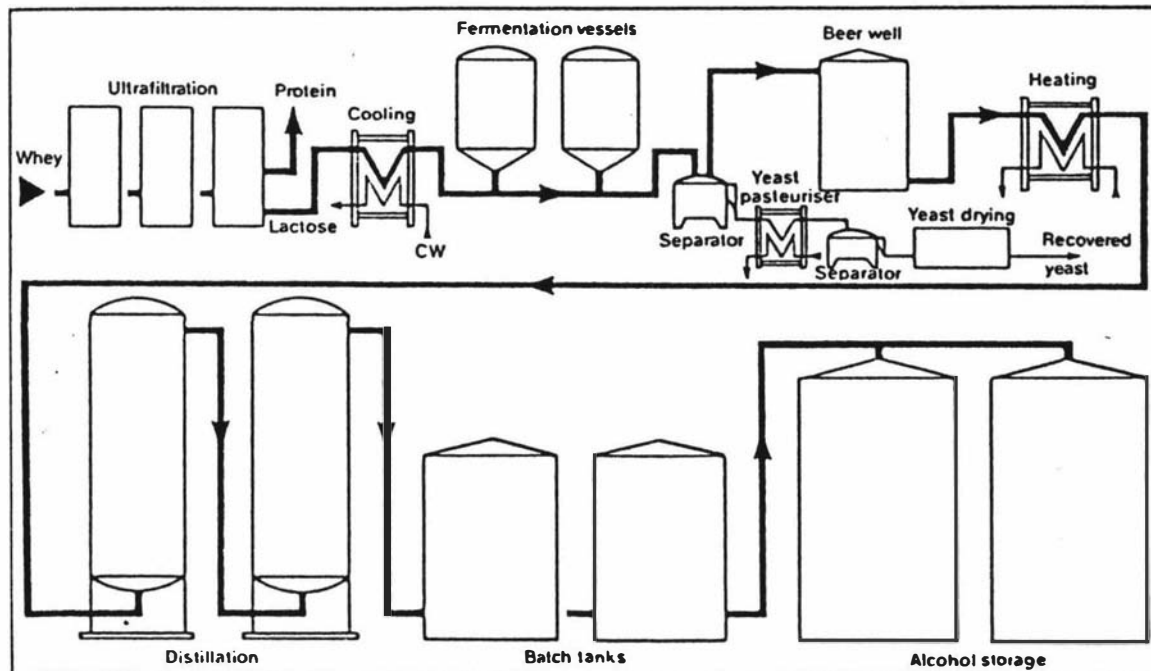


Figure 2.3 Flow diagram of Carbery process (Lyons and Cunningham, 1980).

achieved since the commencement of the fermentation process. The capital cost of the plant was around US\$5 million, including the building and the effluent treatment plant. Production costs, including depreciation, royalties etc are about US\$0.8/Imperial gallon. Total production of the plant is 1.27 million proof gallons per year at an average price of US\$1.40/per proof gallon; the project earns about 28% on the capital investment (Sandbach, 1981a,b).

In New Zealand, the first distillery was commissioned by the New Zealand Co-operative Dairy Co.Ltd (N.Z.C.D.C) at Reporoa in September 1980 (Anon 1980; Howell, 1981; Mawson, 1987). There are now four distilleries in production. These are at Reporoa and Tirau operated by the Achor Ethanol Company (a joint venture between N.Z.C.D.C and the Whey Products Corporation, an arm of the New Zealand Dairy Board), the Clandeboye distillery of Chemical Technology Ltd, and the New Zealand Distillery Co. Ltd plant at Edgecumbe. The operation of these plants is summarised in Table 2.7. Together they supply the New Zealand market for both industrial and potable ethanol and part of the total output is exported. The export market is growing as the domestic market is now saturated. The combined annual production of the distilleries exceeds 10 million litres absolute ethanol (personal communication, Mawson 1990).

The Reporoa plant uses technology under license from Carbery Milk Products Ltd. Initially 550,000 l of deproteinized sulphuric and lactic whey were used to produce about 12,000 l per day of 96% (v/v) potable alcohol or anhydrous alcohol for the New Zealand industrial market and alcohol beverages but production has increased in recent years (Table 2.7). The production process is similar to Carbery plant.

The second distillery was commissioned by Anchor Ethanol in September 1981 at Tirau. The fermentation system used here is a three stage continuous process, with some of the yeast being recycled if required, although normally the spent yeast is discarded to the waste pond for further treatment. The initial plant capacity was 1.5 million litres of deproteinized lactic whey per day to produce

Table 2.7 Whey alcohol production in New Zealand

Distillery	Reporoa	Tirau	Clandeboye	Edgecumbe
Date commissioned	September 1980	September 1981	September 1982	October 1982
Substrates processed	Deproteinated lactic whey & Sulphuric	Deproteinated lactic whey	Cheese whey	Sulphuric acid whey permeate Maize
Design production of whey alcohol (x10 ⁶ litres absolute)	2.5	4.1	0.5	2.5
Actual production 1989/90 season (x10 ⁶ litres absolute)	4.4	4.6	?	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)

about 32,000 l of ethanol. Fermentation is completed within 15 h to produce a beer containing approximately 20 g/l ethanol. The lactic acid present originally in the whey remains untreated during the entire anaerobic fermentation and the subsequent distillation process. The stillage (slops) from the distillation process contains lactic acid, and requires pH adjustment prior to discharge into the waste pond for further treatment. The distillation unit at Tirau is less complex than at Reporoa but has much greater capacity, the beer still being 2.5 metres in diameter. The Tirau plant produces only industrial alcohol and operates for 4-5 months per year (Howell, 1981; Mawson, 1987).

The third distillery was commissioned in September 1982 by Chemical

Technology Ltd at Clandeboye. The whey is supplied by a neighbouring cheese factory 100m away, through an overhead piping system. The plant capacity is reported to be only 136,000 l of whey per day to produce about 2000 l of raw alcohol or 1000 l of refined alcohol (Gooding, 1982; Mawson, 1987).

The fourth distillery was commissioned in October 1982 at Edgecumbe. The distillery is unique amongst the four New Zealand plants in its ability to produce alcohol from another substrate, maize, during the winter months when whey permeate is not available. The production process is similar to other whey ethanol plants (Mawson, 1987).

In the USA, there are four whey based ethanol plants: Golden Cheese Co. of Corona in California, Universal Foods Corporation, Amber Laboratories Division plant in Juneau, Wisconsin, Kraft plant located in Melrose, Minnesota (Sanderson and Reed, 1985) and Dairyman's Association in Tulare (Elliot, 1989).

The Amber Laboratories Division plant, Wisconsin, developed the Milbrew whey fermentation process whereby lactose is converted to single cell protein and alcohol (Bernstein *et al.* 1977; Cunningham, 1980; Lyons and Cunningham, 1980). Selected strains of *K. fragilis* are sequentially propagated in shake flasks, a 14 l fermenter and 300-500 US gal seed tanks which would then be used as inocula for 3000 to 10,000 US gal, and then 15,000 US gal fermenters. Acid or sweet whey, obtained in concentrated form (45-50% solids) from cheese manufacturers, is diluted with water, raw whey or condensate water to 10-15% solids. Some nutrient addition and pH adjustment are performed, and the resulting medium is then pasteurised at 88°C for 45 min and then cooled. Fermentation is carried out in a 15,000 US gal stainless steel deep tank fermenter which is fully aerated and jacketed. An aeration rate of 1 vvm permits maximum biomass production. After fermentation the yeast cream is recovered and spray dried. Ethanol is collected at levels of up to 3-9%(v/v) from the evaporator and subsequently distilled. The primary objective of this process is to obtain SCP with a minimum effluent or waste discharge. However by

switching the conditions of the fermentation by introducing a limited amount of aeration initially (0.1-0.3 vvm), or incremental addition of fresh condensed whey, the ethanol level can be increased to 7%(w/v) alcohol with more than 90% of the available lactose being consumed. The Milbrew plant apparently has an annual production rate of 2000 tonnes of yeast solids from 50,000 tonnes of raw whey. No figure was mentioned for ethanol production (Bernstein *et al.* 1977).

The Corona plant was commissioned during 1986, and employs technology similar to the Carbery plant (Stein and Morris, 1986; Anon, 1989). Concentrated whey (9.1% w/w) is fermented batchwise in 8 fermentation towers each holding 48,000 US gal. Fermentation is generally completed in 18 to 30 h to yield 5.6% (v/v) ethanol. It has the capacity to process about 190 million litres of cheese whey per annum to produce 8-10 million litres absolute alcohol per year (Hansen, 1986).

There are also reports of Kraft in Melrose operating a whey ethanol plant for production of 100% fuel grade ethanol (Sanderson and Reed, 1985). The Dairyman's Association in Tulare, USA, uses a process from Kraft with a production capacity of approximately 4 million litres of alcohol per annum (Elliot, 1989).

2.1.7 Discussion

Overall, an optimum ethanol whey-based fermentation can be achieved by operating at a temperature in the range 30-40°C, pH in the range of 4.0-6.0 (where no pH control is necessary), with an inoculum size of 1-2%(w/w), supplementation with yeast extract (4-8 g/l) and with microaeration. Supplementing with yeast extract alone will probably be sufficient to overcome any nutrient limitation of whey. However, there is no report that the industrial plants supplement with yeast extract or other nutrients to improve their fermentation processes. The high cost of adding extra nutrients possibly prohibits such supplementation.

The scientific approaches used to improve or overcome the limitation of the ethanol whey-based fermentation process have met with mixed success. Fermentation using hydrolysed whey, with the aim of using a more ethanol-tolerant yeast such as *S. cerevisiae*, has been hindered by the catabolite repression effect of glucose on the yeast. The use of catabolite repression-resistant mutant strains of *S. cerevisiae* has shown positive results, but there is still a question of the long term stability of such mutants. Together, the cost of adding lactase enzyme and the stability of the yeast strain have prevented this process being adopted by the industry. The application of other approaches such as genetic manipulation of the yeast strain, is still confined to the laboratory scale. To overcome the high cost of the ethanol recovery process, the use of concentrated whey has been explored. However, the fermentation of concentrated whey suffers some drawbacks. The fermentation rate is generally slower, due to the combination of effects exerted by high lactose, salt and ethanol concentrations. The approach of using concentrated whey containing a lactose concentration higher than 100 g/l depends whether the whey is processed on site or at centralized facility. It may be economical if whey is concentrated centrally. However, the costs of preconcentration, membrane replacement and possibly the yeast strain used, limit the maximum lactose concentration to about 100 g/l (Mawson ,1990). Fermentation at this concentration has been successfully commercialized at the Carbery, Corona, and, latterly the Edgumbe plants.

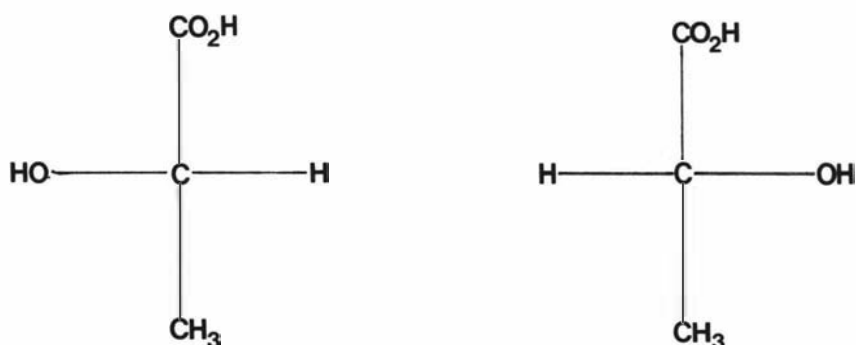
The cell immobilization and membrane bioreactor approaches have good potential, but have not yet been adopted by the industry. Although high productivity can be achieved with these methods, this is often at the expense of higher residual lactose and lower ethanol yield. The cost of treating further the residual sugar in the effluent and the cost associated with product recovery are not economically justified and so limit the extent of productivity improvement possible. However, in the future, it is likely that options of nutrient supplementation and cell immobilization methods, to improve the whey ethanolic fermentation process, will be considered by the industry .

Part II UTILIZATION OF LACTIC ACID IN WHEY

2.II.1 Introduction

Lactic acid is an organic hydroxy acid which occurs widely in animals, plants and microorganisms. It was first discovered in 1780 by the Swedish chemist Scheele in sour milk. Following that, Blondeau in 1847 recognized lactic acid as the final product of a fermentation process (Vickroy, 1980; Buchta, 1984). Lactic acid exists in two optically active isomeric forms:

L(+)-lactic acid and D(-)-lactic acid.



The L(+) isomer is present in animal, including human, cells, although both L(+) and D(-) isomers are found in biological systems (Vickroy, 1980; Buchta, 1984).

The important microbial producers of lactic acid belong to the family *Lactobacillaceae* and are differentiated into four genera: *Lactococcus*, *Pediococcus*, *Lactobacillus* and *Leuconostoc*. These bacteria may be classified as homofermentative, producing mainly lactic acid, or heterofermentative, producing lactic acid and other byproducts such as acetic acid, carbon-dioxide, ethanol and glycerol. The metabolic pathways are shown in Figure 2.4.

Sucrose from cane and beet sugar, lactose from whey, and maltose and glucose from hydrolysed starch are currently used commercially as substrates for the lactic acid fermentation process. In casein manufacture, lactic acid bacteria

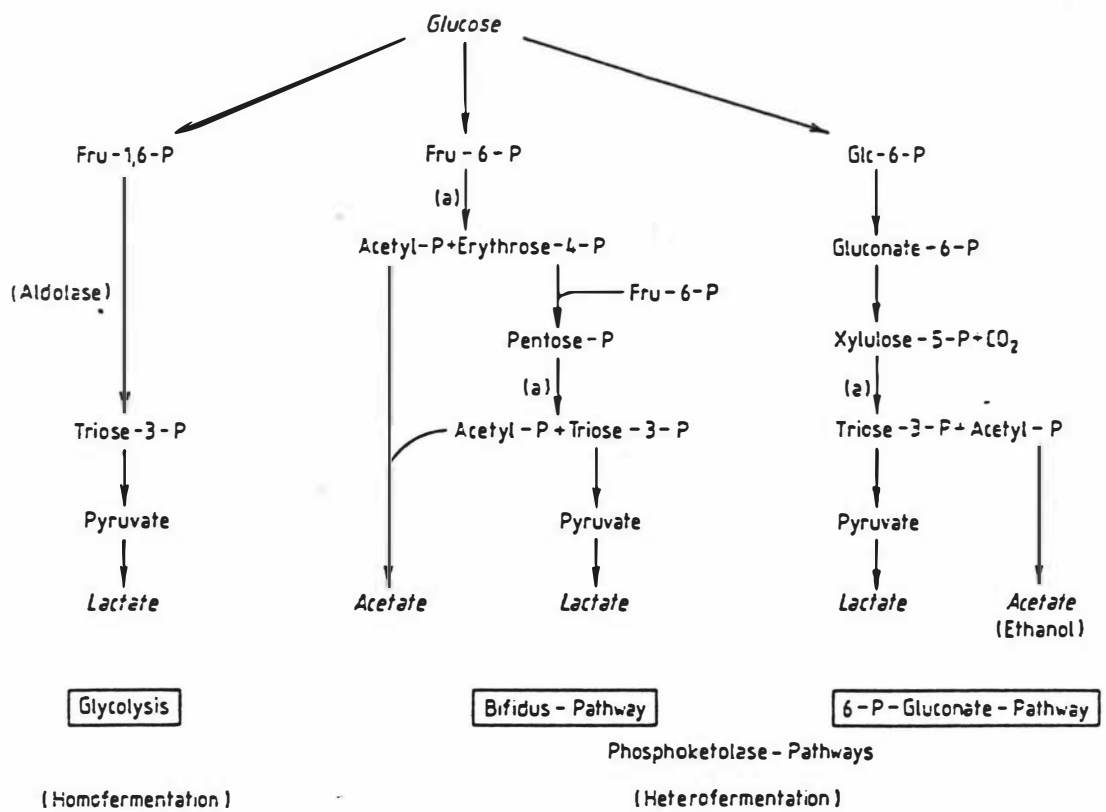


Figure 2.4 The different metabolic pathways of glucose in lactic acid bacteria (Buchta, 1984).

(starter culture) are used to produce lactic acid from whey. The lactic acid produced (approximately 7 g/l) facilitates the coagulation in the whey of casein; hence the term lactic acid casein whey.

Today, the fermentation process makes up half of the world's total production of lactic acid, with the remaining half chemically synthesized. More than half of the world's total lactic acid production, both synthetic and fermentation-derived acid goes to food uses as an acidulant and a preservative. The production of stearoyl-2-lactylates, which are used in the baking industry, consumes another 20%. The remainder is used by the pharmaceutical industry or in numerous industrial applications such as leather tanning and textiles (Vickroy, 1980; Buchta, 1984).

2.II.2 Effect of lactic acid on the yeast alcoholic fermentation process

The presence of this acid in the yeast alcoholic fermentation process can have a detrimental effect. It is usually associated with spoilage of the beer by contaminating lactic acid bacteria which can be difficult to eliminate. Several authors have reported the inhibitory effect of lactic acid on the alcoholic fermentation by *S. cerevisiae*, resulting in a lower ethanol production rate. However the mechanism of the inhibition has not been clearly understood. In lactic acid casein whey, it is a normal constituent of the raw material, at a concentration of 7 g/l. No reports are available on the effect of lactic acid on lactose-fermenting yeasts.

Samson *et al.* (1954) investigated the effects of acetate and other short chain fatty acids on *S. cerevisiae* metabolism. They observed that the presence of acetate at a low concentration of 1.2 g/l enhanced phosphate uptake by the yeast cell, while at a higher concentration, 12 g/l, the phosphate uptake was completely inhibited regardless of whether the yeast was grown aerobically or anaerobically. Their preliminary data gave no evidence that lactic acid produces such an inhibition, although no detail was supplied of the lactate concentration used in the study.

Inoue *et al.* (1962) investigated the tolerance of Sake yeast to lactic acid. They observed that there was a strong correlation between the effect of lactic acid and the culture temperature. The higher the temperature, the stronger the effect on growth of the yeast exerted by lactic acid. They also found that the maximum concentration of lactic acid in which Sake yeast could grow was in the range of 15 g/l to 28 g/l depending on the yeast strain. In a further study Tani *et al.* (1963) attributed the effect of lactic acid to the flocculation and increase in the number of dead cells of *Saccharomyces sake*.

Maiorella *et al.* (1983) studied inhibition by lactic acid in continuous fermentation of *S. cerevisiae*. A reduction in cell population was observed at a lactic acid concentration of 10 g/l, and at a concentration of 40 g/l, an 80% reduction in the final population occurred. As the cell density decreased, the specific ethanol productivity increased (from 0.55 to 0.80 g/g.h). Based on this, the authors suggested that the mechanism of lactic acid inhibition is some form of chemical interference with cell maintenance functions requiring increased ATP expenditure.

Essia Ngang *et al.* (1989) studied the effect of lactic acid on *S. cerevisiae* growing on beet molasses wort, especially the impact of high osmotic pressure on the observed inhibition. They observed that for normal strength wort, with 70 g/l initial sugar concentration, the acid added at up to 30 g/l did not affect the alcohol yield. However the fermentation rates were reduced significantly (by some 40%) at 30 g/l added lactic acid compared to the control. Doubling the initial sugar concentration to 140 g/l gave a more pronounced inhibitory effect, whereby an added lactic acid concentration of 15 g/l affected the alcohol yield significantly. The inhibition constants for the specific growth rate of the yeast, where $\mu = 1/2 \mu_{\max}$ were reported to be 14.5 g/l and 10.3 g/l for normal strength wort and double strength wort, respectively. For double strength wort the maximum specific rate of alcohol production was observed at a lactic acid concentration of 2 g/l, with strong inhibition at higher concentrations. The authors concluded that the observed toxicity of lactic acid depends strictly on its concentration and the inhibitory action is enhanced by the osmotic pressure of

the sugar substrate.

In the following year, in the continuance of their study, Essia Ngang *et al.* (1990) studied the inhibition of *S. cerevisiae* metabolism by a homofermentative *Lactobacillus* during beet molasses fermentation. They reported that stronger inhibition was linked to the bacterial metabolite than to pure added lactic acid. To achieve 50% inhibition of growth, about 10 g/l pure lactic acid needed to be added to the medium, whereas only 2.5 g/l of lactic acid produced by contaminating bacteria had the same inhibition effect. It was concluded that the inhibition is linked to unknown interactions between viable yeast cells and *Lactobacillus*.

Makanjuola *et al.* (1992) also studied the effect of lactic acid bacteria on a beer wort alcoholic fermentation using *S. cerevisiae*. They observed that ethanol yield, yeast numbers and substrate utilization were adversely affected in the presence of *L. plantarum*, *L. brevis* and *Leuconostoc sp.* In one case ethanol yield was reduced by 21% in the presence of a massive bacterial inoculum of 1.8×10^8 cfu/ml. The authors considered three factors which can contribute to the inhibition phenomenon. First, it is due in part to the inability of the yeast to make full use of the carbohydrate in the presence of bacteria. Thus it was observed that residual carbohydrate was increased in the presence of bacteria. Secondly, the flocculation of yeast cells by the bacteria might be an important mechanism of inhibition. However, the authors observed that when lactic acid was added to the yeast suspended in wort, it did not affect yeast flocculation. Also when the yeast was suspended in buffer at pH values ranging from 3.45 to 5.60, no flocculation was detected. Thus the flocculation effect was not due to lowering of the wort pH nor to a specific effect of lactic acid. Finally, the product of the contaminant bacteria, mainly lactic acid, might directly and indirectly affect the cells' metabolism.

2.II.3 Use of lactic acid as a microbial carbon source

Lactic acid has been considered as a good carbon source for growth of many organisms. Of all the 500 yeasts species which are currently known, about 250 species are capable of using DL- lactic acid as a carbon and energy source (Leao and van Uden, 1986). Some bacteria such as *Propionibacterium* have been known to utilize lactate to produce other organic acids, ie propionic acid, acetic acid (El-hagarawy *et al.* 1956; Crow, 1987; Champagne *et al.* 1989; Marcoux *et al.* 1992). Production of propionate by this bacterium is the only known anaerobic conversion of lactate, whereas many microorganisms use lactate as a carbon source for growth using respiratory metabolism.

II.3.1 Lactate in whey as a substrate for biomass production

There are several reports on the feasibility of production of single cell protein (SCP) using lactic acid as substrate. Lembke *et al.* (1975) studied two-stage and mixed culture fermentation processes using bacteria and yeast to produce SCP from whey through intermediary lactic acid. They described three processes (Meyrath and Bayer 1979):

- (a) Repeated fed batch lactic fermentation followed by repeated fed batch yeast production.
 - (i) Initially whey was inoculated heavily with *Lactobacillus bulgaricus*. The anaerobic fermentation lasted 7-11 h during which time temperature and pH were maintained at 44°C and pH 4.0-4.5, respectively. Upon completion of the lactic fermentation, half of the fermenter volume was transferred to the second fermenter to commence yeast production, while fresh whey was added to the first.
 - (ii) In the second stage, *Candida krusei*, a lactose-negative and lactate-positive yeast, was grown aerobically with lactate as carbon source to produce biomass in a semi-continuous fashion. This was carried out by repeated removal of some 62% of the fermented broth and replacement with fresh substrate from the first fermenter. The aerobic fermentation

lasted about 7 h, temperature was maintained at 40 to 45°C and pH was controlled at 4.0-5.5. About 16.2 kg of dry weight yeast biomass/tonne of whey was usually obtained at the end of the fermentation.

- (b) Continuous lactic fermentation followed by continuous yeast production.
- (i) Lactic fermentation by *L. bulgaricus* was carried out in the first fermenter, continuously, under gentle aeration with addition of ammonia to control pH at 4.8. Partial yeast development also took place.
- (ii) The effluent from the first fermenter was continuously fed to the second fermenter to serve as substrate for the production of yeast biomass. The technical data for this two-stage process are shown in Table 2.8.

Table 2.8 Two-stage continuous culture of *L. bulgaricus* and *C. krusei* on lactic acid whey

	1st stage (<i>L. bulgaricus</i>)	2nd stage (<i>C. krusei</i>)
Temperature (°C)	44	44
pH value	4.8	6.2
Dilution rate(h ⁻¹)	0.07	0.14
Aeration rate(vvm)	0.12	0.47
Dry weight biomass(g/l)	11	21

- (c) Single stage continuous mixed culture process. Both *L. bulgaricus* and *C. krusei* were grown together under the following conditions:

Temperature	44°C
pH value	5.5
Dilution rate	0.12h ⁻¹
Aeration rate	0.35vvm
Biomass dry weight	22 g/l

A biomass productivity of 2.64 g/l.h was obtained from this process.

Moulin *et al.* (1983) analyzed the microbial flora of an industrial airlift fermenter which was used to produce yeast biomass from whey (the Bel process). They identified three dominant species: *K. fragilis*, *Torulopsis sphaerica* and *T. bovina*. Evaluation of growth of these three yeasts on different carbon sources (lactose, lactic acid, ethanol and glucose) showed growth rates on lactic acid of 0.25h⁻¹, 0.32h⁻¹ and 0.27h⁻¹, respectively. The complete data are shown in Table 2.9.

Table 2.9 Growth rate (μ , h⁻¹) of yeast species isolated from the Bel yeast SCP process (Moulin *et al.* 1983).

Yeast	Glucose	Lactose	DL-lactic acid	Ethanol
<i>K. fragilis</i>	0.32	0.32	0.25	0.09
<i>T. bovina</i>	0.32	-	0.32	0.32
<i>T. sphaerica</i>	0.27	0.17	0.27	0.17

Ruiz *et al.* (1978) reported the removal of lactic acid from lactic acid casein whey using *Candida ingens*. This yeast was reported to assimilate lactate but not lactose. They observed that *C. ingens* consumed more than 98% of the available lactic acid during the aerobic fermentation and approximately 40% of the non protein-nitrogen. Ash reduction of up to 45% also resulted following precipitation of calcium apatite due to the increase in pH caused by the removal of lactic acid. Biomass yield was approximately 0.52 g/g after 48 h fermentation. The authors claimed that this process had two potential uses. Firstly, it produced yeast biomass as a possible SCP and secondly, the removal of lactic acid and ash from whey appeared to improve the ultrafiltration during the subsequent protein recovery process. The drawback of this *C. ingens* treatment process, however, is the culturing time, requiring 48 h to complete. No explanation was offered as

to why this yeast was selected to remove the lactic acid. There are other yeasts which are also known to assimilate lactate aerobically, but not lactose. Bakers' yeast is one to consider as it is widely used in the food industries and has been known to assimilate lactate (Cassio *et al.* 1987).

Champagne *et al.* (1990) studied the production of bakers' yeast *S. cerevisiae* using fermented cheese whey ultrafiltrate containing galactose and lactic acid. Cheese whey ultrafiltrate was fermented by lactic acid bacteria, *S. thermophilus* to produce galactose and lactic acid prior to aerobic growth of bakers' yeast, since it was observed that lactic acid at high concentration (50 g/l) was inhibitory to yeast respiration. The authors observed that in the presence of both carbon sources, galactose was assimilated rapidly by the yeast to completion and lactate subsequently. Champagne *et al.* (1989) reported that the quality of the bakers' yeast grown on fermented cheese whey ultrafiltrate on the basis of the leavening activity exhibited by the yeast, was comparable to the molasses-grown yeast.

II.3.2 Lactate as a substrate for propionic acid production

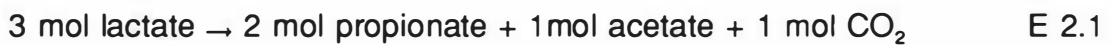
Propionibacteria can assimilate various carbon substrates but are traditionally grown on lactose and lactate-based media (El-Hagarawy *et al.* 1956; Champagne *et al.* 1989; Marcoux *et al.* 1992).

El-Hagarawy *et al.* (1956) evaluated the use of various carbon sources for the production of propionate by *P. shermanii*. They found a faster fermentation rate using sodium lactate, reaching a maximum propionate concentration in two days as compared to eight days when using lactose. Champagne *et al.* (1989) studied the propionic fermentation using immobilized cells, and observed that *P. shermanii* grew faster on lactate than on lactose, the fermentation time being reduced from 7 days to 1-2 days.

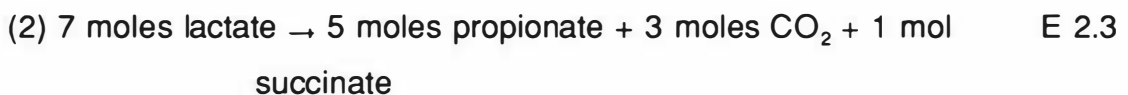
Marcoux *et al.* (1992) studied the production of *P. shermanii* in whey-based media. Again, they reported that this organism grew faster on lactate than on

lactose, and also observed diauxic growth. The organism used lactic acid preferentially to lactose, but neither temporary growth cessation nor two distinct growth phases were observed. This particular type of diauxic growth was also reported by Lee *et al.* (1974) who observed that *P. shermanii* used lactate completely prior to glucose, without growth cessation. This property is thought to be important in the ecology of Swiss cheese manufacture.

Although lactate is the preferred carbon source over lactose, comparison of the product yields from the two carbon sources are difficult as the yield varies with the bacterial strain used. The stoichiometry of conversion of lactate via the generally accepted pathway is as follows (Crow, 1987; Champagne *et al.* 1989):

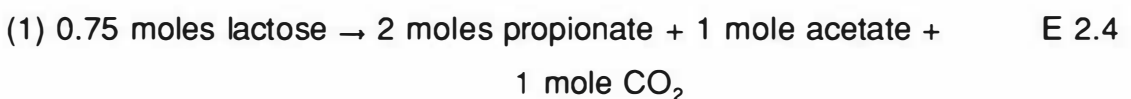


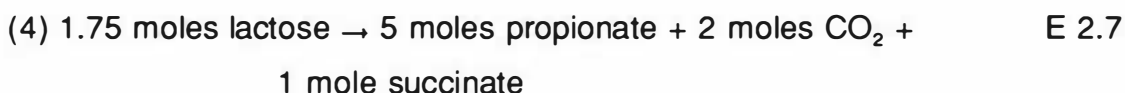
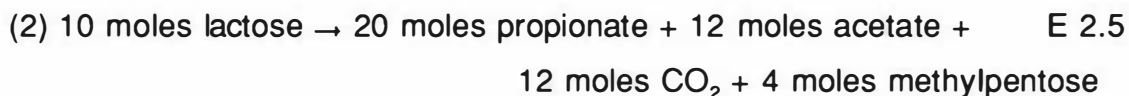
Crow (1987), however, reported that lactate can also be fermented under certain conditions to give different product ratios. The other possible pathways are:



The accepted pathway will give a theoretical yield of 0.54 g/g whereas the alternative pathways give theoretical yields of 0.74 g/g and 0.59 g/g.

The stoichiometry of lactose conversion to propionate also operates in four possible pathways which are strain-dependent (Crow and Clark, 1990).





These four pathways give maximum theoretical yields of 0.58, 0.43, 0.74 and 0.62 g/g, respectively.

2.II.4 Discussion

Although the mechanism of the inhibitory effect of lactic acid on the yeast alcoholic fermentation process has not been clearly understood, in general it is accepted that the presence of a lactic acid concentration above 15 g/l will affect the cell population, which in turn might affect the fermentation rate and ethanol yield. The inhibitory effect of lactic acid has been mainly observed with *S. cerevisiae*; so far there has been no report on the effect of lactate on other alcohol producing yeasts such as *Kluyveromyces*.

Lactic acid has been considered as a good carbon source for many microorganisms, and many yeasts can assimilate lactic acid via oxidative metabolism. However the application of lactic acid utilization by yeast has been limited to the production of single cell protein using strains of *Candida*. In the industrial ethanol-whey based fermentation using lactic acid casein whey as substrate, lactic acid is not being utilized by *Kluyveromyces* during the anaerobic fermentation process. Consequently the effluent containing approximately 7 g/l lactic acid must be treated prior to discharge into the receiving water with added expense to the manufacturer. The process of lactate utilisation for SCP production or propionic acid production outlined in this part II of this literature

review could serve as an option for the whey-based alcohol manufacturer.

Part III YEAST AUTOLYSIS

2.III.1 Introduction

The term "autolysis" was first introduced into the biological literature by Salkowski in 1889 (Vosti and Joslin, 1954a; Farrer, 1955; and Babayan *et al.* 1985). Ever since, the term has been used to refer to self-digestion of cells under the action of their own intracellular enzymes. It is a well-recognized phenomenon, and of those who are concerned with yeast autolysis, the brewer, the baker, and the manufacturer of bakers' and other food yeasts wish to prevent it, while the manufacturer of yeast extract wishes to promote it. Autolysis of yeast in the brewery or winery can lead to unwanted 'off-flavours' in the finished beer or wine, and to an increased susceptibility to bacterial contamination. Autolysis of bakers' yeast can lead to liquefaction and loss in leavening power (Farrer, 1955; Arnold, 1981).

Traditionally the yeast autolysis process is used mainly for the production of yeast extract; however there has also been considerable interest in using this low cost process for the production of various enzymes and coenzymes and flavour compound (Reed and Pepler, 1973, Anon, 1992). Yeast extract, approved by the Food and Drug Administration as a natural flavouring, has long been used as an additive in food products such as meat pastes, meat pie fillings, soups, gravies, sauces, cocktail snacks, and savory spreads (Acraman, 1966; Maddox and Hough, 1970; Pepler, 1982). As reliable and economical sources of peptides, amino acids, trace minerals and the vitamin B-complex group, yeast extracts are nutritional additives in health food formulations, baby foods, and feed supplements, and are also used for enrichment of growth and production media for microorganisms in fermentation processes.

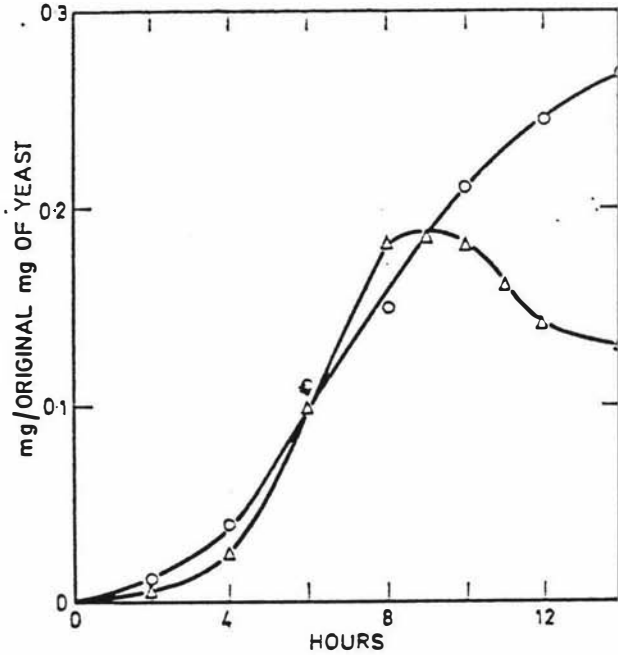
Biomass for the manufacture of yeast extract is obtained primarily from breweries as surplus brewers' yeast, and occasionally from other sources such as molasses-grown *S. cerevisiae*, whey-grown *K. marxianus*, and wood sugar- and ethanol-grown *C. utilis*. However, although the autolysis of brewers' or bakers' yeast (*S. cerevisiae*) has been studied extensively, only a few studies are available describing the autolysis of other yeasts.

2.III.2 Factors affecting autolysis

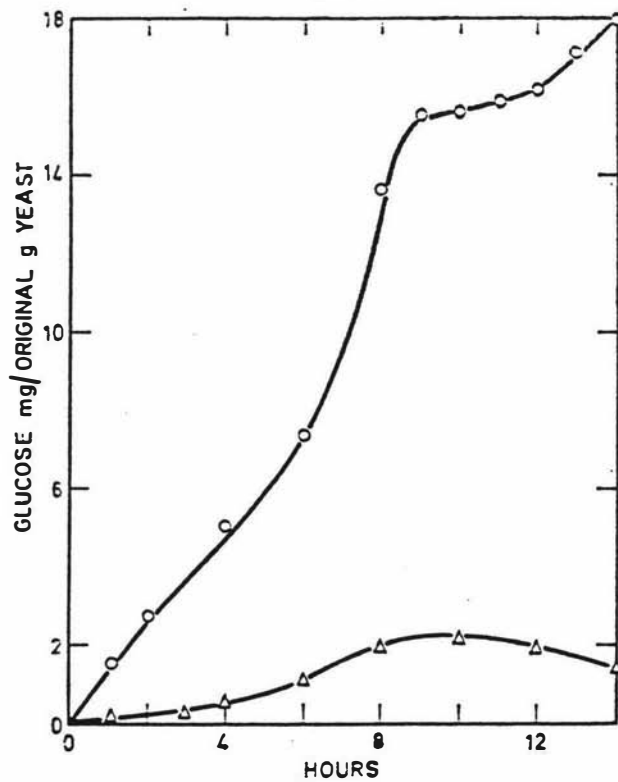
During autolysis cell macromolecules are hydrolysed to smaller molecules principally by carbohydrases, nucleases, and proteases. The products of autolysis are then released into the surrounding medium (extract). The yeast cell wall remains essentially intact, so that living and autolysed cells cannot readily be distinguished by light microscopy. However it has been established that during autolysis, the porosity of the cell wall is increased by endogenous β -glucanase action (Arnold, 1981). The release of carbohydrate into the extract is small compared to that of protein and nucleic acid. A typical time course of autolysis is shown in Figure 2.5 (Hough and Maddox, 1970). It is evident from the changes in amino-acid concentrations that fairly extensive proteolysis occurs outside the cell. Due to the different criteria used by different authors to measure autolysis, the basis for the determination of optimum autolysis conditions is difficult. However the release of protein and amino-acids is generally considered the most important aspect of yeast autolysis. The rate of release of protein and amino-acids and the activity of proteolytic enzymes are influenced by several factors, including temperature, method of growth, pH, and the presence of various chemicals.

III.2.1 Temperature

Although autolysis can occur over a wide range of temperatures, even under refrigeration, the rate of autolysis does vary. At elevated temperatures, the rate of autolysis is faster and is attributed to an increased activity of the proteolytic



(a)



(b)

Figure 2.5 (a) Change in protein and amino-acids levels in extract during autolysis (Δ protein; o amino-acids) and (b) Changes in carbohydrate levels in extract during autolysis (o total sugar; Δ protein bound sugar) (Hough and Maddox, 1970).

enzymes. The effects of temperature are well studied and many authors have established the optimum temperature for autolysis (Vosti and Joslin, 1954a; Farrer, 1955; Orberg *et al.* 1984).

The effect of temperature on the autolysis of bakers' yeast has been clearly shown by Vosti and Joslin (1954a). They demonstrated that little proteolysis took place at 35°C, but extensive proteolysis occurred at 45°C and 55°C with respect to nitrogenous material liberated. No reason was given why such a wide range of temperatures were used nor why higher temperatures were not investigated.

Trevelyan (1976) reported that when bakers' yeast suspended in 0.1M succinate buffer was autolysed at 60°C, the intracellular ribonucleases and proteinases were rapidly activated causing substantial release of purines and total nitrogen within 2 h of autolysis; subsequently the rate of protein breakdown slowed, presumably due to thermal inactivation. At 50°C, there was a lag period where only a slight release of protein occurred in the first 4 h, but the total nitrogen released after 24 h was much higher compared to that at 60°C. The results obtained at 55°C were intermediate between those for 50°C and 60°C. If a faster rate of total nitrogen release is the criterion for optimum conditions, then 60°C will be the optimum temperature, however, if the absolute amount of total nitrogen released is the criterion, then 50°C will be the optimum temperature. Presumably 55°C will be the compromise optimum condition for both high concentration and rate of total nitrogen release. The author also investigated the effect of heat shock prior to autolysis. A heat shock for 1 minute at 70°C was sufficient to eliminate the lag period during the subsequent autolysis at 50°C. The author suggested that the heat shock treatment had the effect of disrupting the plasmalemma and allowing coenzymes to diffuse out of the cell.

Orberg *et al.* (1984) reported an optimum temperature of 55°C for the autolysis of *K. fragilis* Y-601. Autolysis was carried out for 12 h at 40, 45, 51, 55 and 60°C in distilled water, with a starting pH of 6.5 and an initial solids content of 15%. When autolysate (7.5 g dry wt./l) was added to a medium in which lactic

streptococci were cultivated, it was observed that the medium containing the autolysate produced at 55°C gave the highest acid production compared to the others. The stimulatory effect of autolysate produced at 55°C also compared favourably with a commercially produced yeast extract. No attempt was made to identify and measure the nutrients in the autolysate which stimulated the acid production of the starter culture.

Maddox and Hough (1969, 1970) isolated four proteolytic enzymes from *S. carlsbergensis* N.C.Y.C 74S. They observed that the optimum temperatures for activity of each enzyme were 30-35°C, 50°C, 50°C, and 60°C for enzymes A, B, C, and D, respectively. However, enzyme A, which was an exopeptidase, was unstable as the temperature increased and was completely inactivated at 50°C, whereas enzymes B, C, and D, which were endopeptidases, were stable at temperatures up to about 55°C. Details of the data are shown in Table 2.10. The implication of this finding is that in the yeast extract manufacturing process, it is possible to control the quality of the product by manipulating the autolysis temperature. Hough and Maddox (1970) also compared the optimum temperatures of the four proteolytic enzymes from three different autolysing yeasts : bakers' yeast (*S. cerevisiae*), top fermenting brewers' yeast (*S. cerevisiae*) and bottom fermenting brewers' yeast (*S. carlsbergensis*). The optimum temperatures of the four enzymes varied between the brewers' and bakers' strains of *S. cerevisiae* but were identical between the two different brewery yeast species. The data are shown in Table 2.11.

III.2.2 Growth conditions

The growth condition of the yeast may affect the subsequent autolysis process but the reason for this is unclear. Vosti and Joslyn (1954b), using *S. carlsbergensis*, observed that yeast cells grown under aerated conditions autolysed easily, but when the yeast was grown without aeration its autolysis was less, and the optimum pH was shifted from 5.0 to 4.7 with regard to the amount

Table 2.10 Characteristics of the four proteolytic enzymes released during yeast autolysis (Hough and Maddox, 1970).

Enzyme	Optimum temperature for activity (°C)	Maximum temperature for 100% stability (°C)	Optimum pH for activity	Optimum pH for stability
A	35	0	7.5	6.0-6.2
B	50	55	6.2	6.0-6.2
C	50	55	6.2	6.0-6.2
D	50	65	3.5	6.0-6.2

Table 2.11 Comparison of proteolytic enzymes from three different autolysing yeasts (Hough and Maddox, 1970)

	Enzymes	Baker's yeast <i>S. cerevisiae</i>	Top-fermenting brewer's yeast <i>S. cerevisiae</i>	Bottom-fermenting brewer's yeast <i>S. carlbergensis</i>
pH optimum	A B C D	6.8 4.9 4.5 4.3	7.3 4.5 6.6 6.3	7.5 6.2 6.2 3.5
Temperature optimum (°C)	A B C D	37 51 59 66	35 50 50 60	35 50 50 60

of nitrogen and phosphate liberated. At the optimum pH, the aerated culture produced 18% more soluble nitrogen, as a percent of total nitrogen, compared to the unaerated culture. Further experiments showed that yeast grown at the optimum pH autolysed less rapidly on the basis of soluble nitrogen released than that grown at unfavourable pH and temperature. The data are shown in Table 2.12. The pH optimum for growth of *S. carlsbergensis* under unaerated conditions was pH 3.0, while under aerated conditions it was pH 3.8.

Table 2.12(a) Effect of growth condition of unaerated *S.carlsbergensis* on the subsequent autolysis (Vosti and Joslyn, 1954b).

Growth temperature and pH	% soluble nitrogen/Total nitrogen
26°C and pH 3.00	75.6%
26°C and pH 4.55	78.3%
31°C and pH 3.00	68.1%
31°C and pH 4.55	73.3%

Table 2.12(b) Effect of growth pH of aerated *S.carlsbergensis* on the subsequent autolysis process (Vosti and Joslyn,1954b)

pH of growth	% soluble nitrogen / Total Nitrogen		
	8h autolysis	24h autolysis	48h autolysis
2.95	62%	87%	-
3.47	21%	68%	84%
3.80	17%	55%	84%
4.30	26%	71%	91%
4.85	30%	68%	92%

Hough and Maddox (1970) suggested that because proteolysis plays a major role in the phenomenon of autolysis, during its growth the yeast must possess its full complement of proteolytic enzymes. They grew *S. carlsbergensis* in synthetic

medium with various nitrogen sources. The yeast was then autolysed with chloroform and the proteolytic activity was measured. The authors reported that proteolytic activity was present when protein was used as the nitrogen source in the growth medium, but activity was lacking when amino-acids or ammonium salts alone were used. Even the presence of only small amounts of protein produced a positive effect.

III.2.3 pH

The optimum pH for autolysis is not constant but can vary with the physiological state of the yeast, its genetic constitution and the conditions of culture (Vosti and Joslin, 1954b; Farrer, 1955; Hough and Maddox, 1970).

Vosti and Joslyn (1954a), studied the effect of pH on the autolysis of bakers' yeast. They demonstrated that the pH value had a marked influence not only on the degree of autolysis, but also on the nature of the products formed. Although the optimum pH for the formation of amino nitrogen was near pH 4.0, the proportion of amino nitrogen in the total soluble nitrogen showed a 50% increase at pH 7.0. They suggested that proteinases and peptidases enzymes were responsible for the complete degradation of protein to amino acids. There was no mention of pH control during the autolysis process.

Maddox and Hough (1969, 1970) studied the proteolytic enzymes of autolysed *S. carlsbergensis*. They reported that the four enzymes have different pH optima for activity and stability. Although the optimum pH values for activity varied from one enzyme to another, the optimum pH values for stability were the same, i.e. pH 6.0-6.2. The results are shown in Table 2.10. Hough and Maddox (1970) in their subsequent paper compared the pH optima of the four proteolytic enzymes from three different yeasts. These data are shown in Table 2.11. There were marked differences among the three yeasts. The authors attributed the differences between the two brewery yeasts to the species differences, but those between the brewers' and bakers' strains of *S. cerevisiae* were probably due to

the different growth conditions.

Orberg *et al.*(1984) autolysed *K. fragilis* which had been previously grown on deproteinised cheese whey, aerated and agitated at 1vvm and 200 rpm, respectively. They observed that autolysate produced at initial pH of 5.5 had the highest stimulatory effect on the lactic acid production when added back to growth medium for the lactic acid fermentation using *Lactococci*. No pH adjustment was performed during the entire 12 h autolysis process nor during the original cultivation of yeast culture. On the completion of the yeast biomass cultivation process, the pH of the medium dropped dramatically to pH 2.5 from an initial pH of 4.5. The authors speculated that this low pH condition had contributed to their low biomass yield, but they did not consider the possibility that some sugar might also have been used to produce ethanol. The combination of low pH and the ethanol formed during the biomass cultivation could possibly affect the subsequent autolysis process. These factors were not considered by the authors.

III.2.4 Effect of Chemical agents.

The aim of adding chemical agents to the autolysis process is primarily to increase the rate of release and yield of lysis products. The effects of various chemicals such as salts, organic reagents, solvents, and detergents upon the autolysis of yeast have been investigated by numerous workers.

2.4.1 Salt and/or ethanol

Farrer (1955), in his review of the literature, suggested that since autolysis was essentially an endocellular process, at least in the early stages, it would not be expected that salts would have any positive effect. It was reported that addition of 0.1M and 0.2M solutions of sodium chloride, potassium bromide, sodium fluoride, potassium sulphate, sodium nitrate and mixed phosphates had virtually no effect on autolysis.

Sugimoto (1974) investigated the synergistic effect of ethanol and sodium chloride as plasmolysers on the autolysis of bakers' yeast. Three intracellular proteinases (A, B, and C) were extracted from bakers' yeast and subjected to treatment with ethanol and sodium chloride at various combinations of concentrations. In no case were the intracellular proteinases ever activated by these agents. This finding on the effect of salt agreed with that of Farrer (1955). When the yeast was autolysed at 40°C for 24 h in the presence of added plasmolysers (ethanol and NaCl), the autolysate containing intra- and extracellular enzymes, showed a considerable decrease in proteolytic activities, with the exception of proteinase C which remained highly active in the case where the autolytic mixture contained 5% (v/v) ethanol. Further experiments with proteinase C showed that during 8 days of autolysis at 40°C, its activity reached a maximum at day 2. In comparison with the initial activity, the maximum activity obtained in the presence of 5% (v/v) ethanol, and 5% (v/v) ethanol plus 5% (w/v) NaCl, reached 128% and 157% respectively. The significance of proteinase C in the autolysis process was not explained, however. The author also observed that the released of Kjeldahl nitrogen was maximum in the presence of 5% (v/v) ethanol and 5% (w/v) NaCl. Compared to the control, there was an approximately three-fold increase in Kjeldahl nitrogen when operating at the optimum condition. Ethanol concentrations above 5% gave little further advantage, nor was any significant difference in the recovery of Kjeldahl nitrogen observed in the 0-9% (w/v) range of sodium chloride concentration. The autolysis at 40°C was completed after 4 days. Given the long time of autolysis, it is possible that loss of ethanol due to evaporation might have occurred, therefore the stated optimum concentration of ethanol could have altered.

Kollar *et al.* (1991) also studied the effect of addition of salt and ethanol independently on the autolysis of bakers' yeast at 50°C. They reported that addition of 5% (w/w) sodium chloride and 5% (w/w) ethanol resulted in total nitrogen release of 46% and 51% respectively, after 24 hours of autolysis, compared to that of 26% in the control. No reason was given for the choice of autolysis temperature nor for the concentration of the plasmolysers used. Again,

the final ethanol concentration on completion of the autolysis process was apparently not checked.

Trevelyan (1977) investigated the effect of ethanol on the total nitrogen release during the autolytic degradation of RNA of bakers' yeast. The autolysis of pressed bakers' yeast was carried out at 50°C in succinate buffer, pH 5.0, for 4.5 h, with various ethanol concentrations added. A slight increase in the total nitrogen released from the cells was observed when ethanol was present at 2.5% (v/v), but then the rate of release increased rapidly as the ethanol concentration was increased to 7.5% (v/v). After 4.5 h of autolysis, the amounts of total nitrogen released were 24%, 69%, and 72.5% with 2.5%, 5% and 7.5% (v/v) added ethanol, respectively, compared to total nitrogen release of 8% with the control. It was also reported that without alcohol, no RNase action was detected even after incubation as long as 4 h. It is not understood why the autolysis was carried out for such a short period of time. The author suggested that although the rate of autolysis was much faster in the presence of 7.5% (v/v) ethanol, the proteinases were slowly but progressively inactivated. The same author (Trevelyan, 1976) also studied the plasmolysis of bakers' yeast in the presence of ethyl acetate followed by autolysis at 50°C. It was observed that after 2 h almost 70% of the total nitrogen was released compared to only 5% in the control.

2.4.2 Effect of solvents and other chemical

Breddam and Beenfeldt (1991) investigated the effect of a range of solvents on yeast autolysis for the production of intracellular enzymes. They reported that when bakers' yeast was treated with 10% and 25% (w/v) ether for 1 h at room temperature, followed by 24 h pH-stat autolysis, there was a complete release of the enzyme carboxypeptidase Y. The authors also screened other solvents and concluded that straight chain alcohols of medium chain length, i.e. C₆-C₉, appeared to be optimal in amounts of only 1.2% (v/w), whereas 2.5% - 10% (v/w) of trichloroethane, chloroform and ether were required to achieve the same

fractional release of enzyme.

Shetty and Kinsella (1978) studied the effect of thiol reagents on the extractability of protein from freeze-dried brewers' yeast. They observed that yeast cells treated with monothioglycerol, mercaptoethanol or dithiothreitol for 16 h at 37°C exhibited over two-fold improvement in the extractability of protein compared with the control. Also, the increased presence of low molecular weight proteins suggested that the intracellular proteolytic enzymes were being activated by the thiol reagents. It is not clear whether the effect of the thiol reagents might have been compounded by the freeze-dried condition of the yeast.

Breddam and Beenfelt (1991) also investigated the effect of detergents on the release of enzyme during yeast autolysis. A range of non-ionic, anionic, cationic and zwitterionic detergents were tested for their ability to accelerate autolysis. In the presence of a minimum 5% (w/w) NaCl, the presence of non-ionic Triton X-100 and anionic N-lauroylsarcosine detergents resulted in the highest enzyme release after 24 h of autolysis. The authors suggested that these detergents were the most promising for large scale yeast autolysis due to their low price and toxicity.

Kollar *et al.* (1991) reported a novel approach of accelerating autolysis, by adding some of the fresh autolysate back to the process. Adding back 15% (v/v) of fresh autolysate to the autolysis of *C. utilis*, *S. cerevisiae*, *S. carlsbergensis* and *K. marxianus*, resulted in an improvement of total nitrogen release of 85%, 46%, 178%, and 37%, respectively, compared to the control. The autolysis process was carried out at 50°C, with 10% dry weight yeast/w for 25 h. The authors also compared the effectiveness of returned yeast autolysate with other inductors (NaCl and ethanol) for the autolysis of bakers' yeast. After 24 h, the yeast autolysate performed as effectively as 5% (w/w) NaCl and 5% (w/w) ethanol, with almost double the total nitrogen released compared to the control. The combination of 15% (v/v) autolysate, 5% (w/w) NaCl and 5% (w/w) ethanol had the greatest effect, resulting in a three-fold increase in total nitrogen release

compared to the control. No explanation for the effect was offered by the authors.

2.III.3 Other lysis methods

Many techniques are available for breakage of microorganisms, both mechanical and non-mechanical. The types of mechanical cell disruption in current use include ultrasonics, blenders, shakers and homogenisers, grinding, and shearing (Hughes press and French pressure cell). The non-mechanical types include drying, chemical lysis and enzymatic lysis (Wiseman 1969). Each process has its particular drawbacks. Physical breakage methods such as homogenization are commonly used for yeast disruption and can deliver good disintegration of yeast cells, but require harsh conditions and, render the downstream processing difficult. Enzymic lysis, the second most popular yeast disruption technique, while excellent for laboratory scale use, is far too expensive for large scale application (Wiseman 1969; Breddam and Beenfeldt, 1991). Comparison of the efficiency of these techniques is difficult due to inconsistencies in the product criteria.

Numerous workers have studied the lysis of yeast cells using different sources of lytic enzymes. Kobayashi *et al.*(1982) evaluated kitalases, an enzyme of fungal (*Rhizopus solani*) origin, on *S. cerevisiae*. They reported that kitalase induced the release of invertase from bakers' yeast by 80%. Rowley and Bull (1977) evaluated the production of bacterial lytic enzymes from *Arthrobacter* in batch and continuous using *S. fragilis* and *S. cerevisiae* yeasts as substrate, while Knorr *et al.* (1979) evaluated commercially produced Zymolase and lysozyme on brewers' yeast and reported that enzyme treatment followed by extraction at pH 9.0 resulted in a yield of more than 80% of the total nitrogen of the yeast cell.

Chemical lysis, the third most common yeast lysis technique, suffers from problems of potential toxicity, chemical recovery and high costs. Several workers have studied the extraction of protein using chemical agents. Fenton (1982)

investigated the release of β -galactosidase from whey-grown *Kluyveromyces* yeast cells using a combination of solvents and buffer treatment. It was reported that 90% of the enzyme was obtained with ethanol and isopropanol at concentrations of 80-90% (w/v). Solvent treatment times of approximately 5 min and extraction times of 12-17 h in phosphate buffer were found to give good yields. Joshi *et al.* (1989) studied the permeabilization of *K. fragilis* NRRL Y-1196 to lactose using digitonin, a detergent. When the yeast pre-grown in lactose medium was treated with 0.1% (w/v) of the surfactant digitonin in 0.1 M potassium phosphate buffer at 24°C for 30 min, they observed that the intracellular β -galactosidase activity was nearly 500-fold greater than that in untreated cells. Siso *et al.* (1992) also studied permeabilization of *K. lactis* for milk whey saccharification with various combinations of ethanol and toluene. They reported that 40-fold increased of β -galactosidase activity was obtained when the aerobically whey-grown *K. lactis* NRRL-Y-1140 was treated with 4 : 96 toluene:ethanol or with 70 % (w/v) ethanol for 3 min at 25°C, compared to the untreated cells. The permeabilized cells hydrolysed 90% of the lactose in whey (40g/l) in 10 minutes whereas to the control required 30 minutes.

2.III.4 The use of yeast autolysate as a nutrient source in commercial fermentation processes.

It was pointed out earlier that yeast extract provides a readily assimilable source of nitrogens and nutrients for fermentation processes. Thus, yeast extract is widely used as a rich nutrient source in laboratory scale fermentation processes. However, in large scale industrial fermentation processes where large amount of yeast extract are required, the cost of adding this nutrient source will be tremendous, rendering it uneconomical. This is certainly true for those fermentation processes producing low value and high volume products such as the ethanol fermentation of whey. Thus, some workers have proposed the inexpensive production of yeast autolysate from spent yeast, which later can be added back to the fermentation process.

Lembke *et al.* (1975) prepared the autolysate by mixing 10 kg of spent yeast of *C. krusei* (20% dry mass) with 10 kg of ammonium sulphate, which was supposedly a plasmolyser, and allowing it to autolyse for 24 h at 49°C. The whole autolysate was then added to 4 tonnes of lactic whey medium for the cultivation of mixed bacteria and yeast. The authors reported that the fermentation was improved after the addition of the autolysate, but no detailed information was supplied.

Lam and GrootWasink (1990) proposed a process whereby *K. fragilis*, a waste yeast from the inulase extraction process, was used as growth substrate for the production of the lytic enzyme complex by *Arthrobacter* sp. They observed that the yeast lytic enzyme activity from *Arthrobacter* sp. is induced by whole yeast cells. The lytic enzyme produced was then used to lyse more yeast to generate nutrients for the subsequent inulase production. The stimulatory effect of the autolysate on the production of inulase compared favourably with that of added 10 g/l commercial yeast extract. However, the authors made no attempt to quantify the amount of protein or nitrogen in the autolysate, hence the basis for comparison of the effectiveness of the autolysate-based medium and the standard yeast extract-based medium is unclear. The choice of the control was also puzzling. The authors used the autolysate solution as the control, to compare with the autolysate solution with 20 g/l added sucrose and with a 10 g/l yeast extract solution with 20 g/l added sucrose. They concluded that the autolysate supported only very poor yeast cell growth, indicating a requirement for additional carbon source. It seems that the authors sent a conflicting statement, since the aim of their work was never to produce autolysate as a carbon source, rather to provide a nitrogen, vitamins and minerals source. Nevertheless this enzymic lysis process looks promising and deserves further investigation, particularly with regard to scaling up to an industrial process.

Orberg *et al.* (1984) investigated the potential of an autolysate of whey-grown yeast as a substitute for yeast extract in dairy starter culture media. The autolysate (0.63 dry wt %) was added to the starter culture medium and

compared with 0.5 (dry wt%) yeast extract added as control. On the basis of acid production, the quality of the autolysate compared favourably with that of Amberex, the commercial yeast extract added. While Amberex was a true soluble extract, the whole autolysate contained a large proportion of inert material (cell debris). The authors suggested that high quality extract could be obtained by removing the cell debris, although no comparison was made between the whole autolysate and the true soluble autolysate. The authors also observed that little benefit was gained by further increasing the autolysate usage above 0.63%. Steven (1985) reported that the autolysate prepared from the aerobically grown *K. fragilis*, when added to culture media had significantly improved the lactic culture growth. Unfortunately, no further details were available.

Reader and Kennedy (1992) investigated the use of commercially-produced bakers' yeast autolysate as a substitute for yeast extract during the cultivation of an oil-accumulating fungi. They reported that when a confectionery waste was supplemented with the autolysate, and compared with the standard powdered yeast extract as a control, an increase of 28% in productivity (mg gamma linoleic acid /l.h) for *Mucor hiemalis* IPD 51 over the yeast extract was obtained. For *M. javanicus* IPD 155 there was a 46% increase in productivity. The autolysate was in the form of wet paste containing 9-10% salt. It is unclear whether the superiority of the autolysate over the standard yeast extract was due to the effect of salt contained in the autolysate or just the difference in the amount of yeast extract added.

2.III.5 Discussion

Due to the different criteria of autolysis used by different authors (e.g. total nitrogen, amino-nitrogen, stimulatory effect on a dairy starter culture), the basis for determination of optimum autolysis conditions is difficult. In general, it is accepted that the optimum temperature and pH for yeast autolysis are 45°- 55°C and pH 5.0-6.0, respectively, for the formation of amino-acids and low molecular peptides. However experience in the yeast extract industry indicates that the

ratio of amino nitrogen to total nitrogen decreases above 45°C. Overall the chemical agents used as inductors for yeast autolysis process can increase the total nitrogen release. They normally accelerate the release of products from the cells, possibly by altering the cell membrane permeability, thus allowing more molecules to leak out rather than activating the intracellular proteolytic enzymes. Many of the chemical inductors, while effective, will be unsuitable for the preparation of yeast extract for the food and fermentation industries, with the exception of sodium chloride and ethanol. A high concentration of sodium chloride in the yeast autolysate may also be undesirable. The positive effect of the technique of adding back yeast autolysate, suggests that it is amenable to continuous operation.

A fermentation process could benefit from the application of yeast autolysate as a means to supply additional nutrients. The main advantages of the autolytic method are that it is a simple process, easy to scale up and does not require sophisticated equipment. Industrial ethanol fermentations from whey, which produces large amounts of yeast as a by-product, could benefit from this since it has been established that addition of yeast extract to whey media improves the fermentation rate. However the economics of adding commercial yeast extract to the large scale whey fermentation process cannot be justified. Thus, the autolysis process could be applied to produce autolysate from the spent yeast, which at present, is largely wasted, to provide a source of nitrogens, minerals and vitamins to the fermentation process.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Microbiological Media

3.1.1.1 Whey Media

Spray dried sulphuric acid casein whey permeate (SACWP) and lactic acid casein whey permeate (LACWP) were obtained from the New Zealand Dairy Research Institute (NZDRI, Palmerston North) and were used for anaerobic fermentation by yeast to produce ethanol. The media were prepared by dissolving whey permeate powder in distilled water to give a final lactose concentration of 50 g/l. A typical composition of each powder is shown in Table 3.1. Lactic acid casein whey serum (LACWS) was obtained from Anchor Ethanol Co., Tirau and liquid LACWP was obtained from Tui Milk Products Ltd, Longburn, Palmerston North. The liquid whey permeate and serum were also used for alcohol production. The composition is expected to be similar to the SACWP powder as shown in Table 3.1, except that serum will contain more protein. In some experiments, 62 g/l SACWP powder was reconstituted in distilled water to give a lactose concentration of 50 g/l and supplemented with various concentrations of yeast extract (0.5 to 4 g/l) or sodium lactate (7 to 30 g/l).

Table 3.1 Typical composition of lactic acid casein whey permeate and sulphuric acid casein whey permeate

		Lactic acid Casein Whey Permeate ^a	Sulphuric Acid Casein Whey Permeate ^a
Total solids	(g/kg)	55.0	56.4
Total nitrogen	(g/kg)	0.98	0.70
Non-protein Nitrogen	(g/kg)	0.81	0.51
Lactose	(g/kg)	40.0	46.0
Lactate	(g/l)	6.2 ^b	-
Ash	(g/kg)	7.0	7.0
Calcium	(g/kg)	1.2	1.1
Phosphate	(g/kg)	2.0	1.9
Sulphate	(g/kg)	-	2.2
α -aminonitrogen	(g/l) ^c	0.23	0.11
pH		4.0-4.5	4.5-4.7

a Personal communication; Dr B. Ennis, (NZDRI)

b Hobman, 1984.

c when dissolved in distilled water, assay method was described in Section 3.3.7

3.1.1.2 Slops

The slops (stillage) was obtained from Anchor Ethanol Co., Tirau, and used for aerobic lactic acid fermentation. The composition of the slops is shown in Table 3.2.

3.1.1.3 Semi-synthetic media

The three liquid media used commonly in these studies were Yeast Extract Peptone Lactose (YEPL), Yeast Extract Peptone Lactose Lactate (YEPLL) and Yeast Extract Peptone Lactate broths. Yeast extract and proteose peptone were obtained from Difco Laboratories (Detroit, Michigan, USA). The composition of YEPL is shown in Tables 3.3. The composition of YEPLL is the same as to YEPL except that sodium lactate was added in the range of 3-30 g/l. Yeast Extract Peptone Lactate was also based on YEPL except that lactose was replaced by sodium lactate as the carbon source at 7 g/l.

3.1.1.4 Agar slopes and plates

The agar slant used for culture maintenance and inoculum development was YM agar obtained from Difco Laboratories. WL(Wallerstein) nutrient agar used for colony counting was obtained from Oxoid (Basingstoke, Hampshire, England).

3.1.2 Chemicals

All chemicals used for fermentation media and analytical work were of analytical grade. They were obtained from various sources: BDH Chemicals Ltd (Palmerston North, New Zealand); Ajax Chemicals (Sydney, Australia); Sigma Chemical Co. (St Louis, Missouri, USA); Serva (Heilderberg, Germany); Rhone-

Table 3.2 Typical composition of Tirau slops for aerobic batch and continuous yeast cultivation

Component	Concentration (g/l)
suspended solids	0.22
Lactose	< 0.1
Ethanol	< 0.1
Total nitrogen	0.0504
Lactic acid	6.0-7.0
Ash	0.088
pH	4.0-4.5

Table 3.3 Yeast Extract Peptone Lactose (YEPL) used for anaerobic batch fermentation

Component	Concentration (g/l)
Lactose	50
Proteose peptone	3
Yeast extract	5
$(\text{NH}_4)_2\text{SO}_4$	5
K_2HPO_4	5

Poulenc (Victoria, Australia); Riedel-De Haen AG (Hannover, Germany); May&Baker Ltd (Dagenham, England); Merck-Schuchardt (Munich, Germany); J.T Baker chemical Co. (Phillipsburg, New-Jersey, USA).

Pyroneg detergent was supplied by Diversey-Wallace Ltd (Papatoetoe, New Zealand). Dow-Corning antifoam A.F (food grade) was supplied by Swift Consolidated Ltd (Wellington, New Zealand).

3.1.3 Other materials

Some of the yeast cream used for the autolysis work was obtained from Anchor Ethanol Co., Tirau.

3.1.4 Organisms

The stock culture of *Kluyveromyces marxianus* Fi yeast was obtained originally from Anchor Ethanol Co., Tirau. It was kept at 4°C and maintained by regular subculture (every 2 months) onto a fresh YM agar slope and incubation at 30°C for 48 h. This stock culture was used for inoculum preparation throughout the duration of this study. Other strains of *K. marxianus* (previously *Saccharomyces fragilis*, *K. fragilis*, *Candida pseudotropicalis*; Barnett *et al.* 1990) used in this study were held in the collection of Biotechnology Department, Massey University, Palmerston North, New Zealand. These were strains Y-113, NRRL Y-2415, TC2, 1607, 1496, and DSIR. The strain of *C. tropicalis* was obtained from Anchor Ethanol Ltd and originated from the ethanol production facility at Golden Cheese Co., Corona, California, USA. The unknown yeast designated as strain 19 was isolated from the ethanol production plant at Anchor Ethanol Co., Tirau.

3.2 CLEANING AND STERILIZATION PROCEDURES

3.2.1 Cleaning of glassware

All glassware was washed in hot Pyroneg detergent, rinsed in tap water, and then in distilled water, and hot air dried.

3.2.2 Media Sterilization

All microbiological media were sterilized by autoclaving at 121°C. All fermentation media of volumes less than 10 litres were sterilized by autoclaving at 121°C for 15 or 20 minutes and for volumes greater than 10 litres at 121°C for 25 minutes. All media for inoculum preparation and batch flask fermentations were autoclaved immediately prior to use. For some experiments, LACWP was pasteurised at 85°C for 25 seconds by standing in a hot water bath.

3.2.2 Equipment Sterilization

Glass wool gas filters and some glassware were sterilized in a hot air oven at 160°C for 4 h. Membrane air filter units were sterilized at 121°C for 20 minutes. Pipette tips were sterilized at 121°C for 15 minutes. pH electrodes for insertion into fermenters were sterilized in 50% (v/v) ethanol solution overnight and rinsed with hot sterile distilled water (approx 70°C) immediately before use.

3.3 ANALYTICAL METHODS

3.3.1 pH Measurement

Routine pH measurements were made using a Metrohm pH meter (Metrohm AG,

Herisau, Switzerland, Model E 520) which was calibrated with pH 4.0 and 7.0 buffers immediately prior to use.

3.3.2 Determination of Biomass Dry Weight

3.3.2.1 Spectrophotometric Method

A standard curve of absorbance versus biomass dry weight was generated by the following procedure: yeast was cultivated aerobically on semi-synthetic or whey media in a shake flask culture at 30°C and agitated at 250 rpm. Four 25 ml volumes of culture were centrifuged at 7000 rpm for 15 minutes in a clinical centrifuge (International Equipment Co., USA). The supernatant liquid was discarded and the cell pellet was washed twice with distilled water. The washing water was decanted and the cells were resuspended in distilled water to the original volume. The suspension was transferred to pre-weighed moisture dishes and dried in a hot air oven at 105°C overnight. The dishes were then reweighed and the biomass was calculated. The remaining culture was diluted appropriately with fresh growth medium and the absorbance was measured at 650 nm using a Philips spectrophotometer (Philips Scientific, Cambridge, Great Britain; Model PU 8625). The standard biomass dry weight versus absorbance curve was then generated. During shake flask fermentation experiments, 5 ml samples were collected at regular intervals. Appropriate dilution with fresh media was made to bring the absorbance reading into the range of 0.1-0.4, and the biomass concentration was then calculated using the regression equation for the standard curve.

3.3.2.2 Dry weight method

During continuous fermentation experiments, a known volume (generally 25 ml) of culture was collected at regular intervals and centrifuged at 7000 rpm for 15 min in a clinical centrifuge. The supernatant liquid was discarded and the cell

pellet was washed twice with distilled water or citrate buffer. For experiments involving lactate utilisation, the citrate buffer pH 4.0 was used to wash out the calcium precipitate formed during the process, and so obtain the true yeast biomass. The washing water was discarded and the cells were resuspended in distilled water to the original volume. The suspension was transferred to a pre-weighed moisture dish and dried in a hot air oven at 105°C overnight. The dish was then reweighed and the biomass was calculated.

The selection of citrate buffer pH 4.0 was based on the optimum solubilization test. The procedure was as followed: citrate buffer was made up with three different pH values : 3.0, 4.0, 4.6. The pH of the clear slops was adjusted to 7.0 from 4.5 using 4M NaOH, to form the calcium precipitate. The turbid slops (1 ml) was then added to the 9 ml buffers, or distilled water as the control, to give a ten fold dilution. The absorbance was then read at 650 nm, with the pH 3.0, 4.0, 4.6 buffer and the control giving values of 0.009, 0.001, 0.007 and 0.124, respectively.

The ensure that the pH 4.0 citrate buffer washed out all the calcium precipitate, the yeast solids plus calcium precipitate was harvested from slops after 20 h growth (final pH 7.0). A control was the yeast grown on YEP lactate in which no precipitate formed. Some of the yeast harvested from the slops was washed with the buffer once and or twice and ashed at 800 °C for 3 h. The remaining yeast solids from the slops and YEP lactate were also ashed after twice washing with distilled water. The ash content of the samples are shown below:

	Ash content (% w/w)
Yeast from slops washed with buffer once	8.5
Yeast from slops washed with buffer twice	4.0
Yeast from slops washed with distilled H ₂ O	43
Yeast from YEP lactate washed with distilled H ₂ O	3.9

These results indicated washing twice with pH 4.0 buffer removed all the mineral precipitate and thus gave an ash value the same as the yeast solids alone.

3.3.3 Determination of Colony Forming Units

Colony forming units (cfu) were determined using the pour plate or spread plate methods in Wallerstein(WL) agar. Dilutions were prepared using peptone water (5g/l). The agar plates were incubated at 30°C for 48 h, and the colonies were counted using a Colony Counter (Suntex Instruments.Co Ltd, Taipei, Taiwan, Model 560).

3.3.4 Ethanol Analysis

Two gas chromatographs fitted with flame ionization detector were used : a Shimadzu gas chromatograph (Shimadzu Corporation, Kyoto, Japan; Model GC-APF) and a Carlo Erba gas chromatograph (Carlo Erba strumentazione, Milan, Italy; Model GC 6000 vega series 2). The methods were very similar for each but operating conditions varied. Isopropyl alcohol at 20 g/l was used as an internal standard. Standard ethanol solutions including the internal standard were prepared in the ranges of 5 g/l to 25 g/l in steps of 5 g/l. The sample volume injected was 2 μ l. For the Shimadzu, a 1m \times 0.15cm ID column containing Porapak Q was used at a carrier gas (nitrogen) flow rate of 60 ml/min and a column temperature of 200°C. The detector and injector temperatures were 220°C. Quantitation of the sample ethanol concentration was accomplished using a Varian integrator data system III C (Varian, California, USA) to measure the peak areas. The peak spectrum was also recorded using a Seconic SS 250 G recorder (Japan). Ethanol concentration was calculated by measuring the relative area of the ethanol and isopropyl alcohol peaks and comparing this with the standard curve prepared from values for the known standard solutions.

For the Carlo Erba, a 2m \times 0.4cm ID column was used at a carrier gas (nitrogen) flow rate of 40 ml/min and the same column temperatures. Quantitation of the sample ethanol concentration was achieved using a Hitachi Chromato-integrator (Tokyo, Japan, Model D-2500) as for the procedure above.

3.3.5 Lactose Analysis

3.3.5.1 Modified DNS method

Lactose was analysed using a modified dinitrosalicylic acid (DNS) method based on that of Miller (1959). The procedure used was : A modified Miller reagent was prepared comprising 2 g DNS and 0.4 g phenol dissolved in 100 ml of 20 g/l sodium hydroxide. Solution A was then prepared by mixing 100 ml of modified Miller solution with 100 ml of 1 g/l sodium sulphite. The sample containing lactose was diluted as appropriate, and 3 ml was mixed with 3 ml of solution A and heated in a boiling water bath for 15 min. After heating, 1 ml of 400 g/l Rochelle salt (potassium-sodium tartrate) solution was added and the resulting mixture was cooled to ambient temperature under running tap water. The absorbance of the cooled solution was measured at 575 nm using a Philips spectrophotometer. A standard curve was prepared using lactose solutions of concentration 1-5 g/l.

3.3.5.2 Enzymatic method

Lactose was also analysed using an enzymatic method. The procedure was : Buffer A was prepared comprising potassium phosphate buffer (0.1M, pH 7.0), 0.77 mM 4-aminoantipyrene, 9300 units/l glucose oxidase (β -D-glucose:oxygen 1-oxido-reductase; EC 1.1.3.4. Type X-S, Sigma Co.), and 1500 units/l peroxidase (EC 1.11.1.7, Type VI-A; Sigma Co.). Solution B was then prepared by adding 0.5 ml of 1M magnesium chloride, 2.5 ml of 11 mM phenol and 1 ml of β -galactosidase (Maxilact LX-5000, Gist Brocades Ltd, Delft, The Netherlands) to every 100 ml of freshly-made buffer A. The sample containing lactose was diluted as appropriate to give a lactose concentration in the range 0 to 1 g/l. The diluted sample (0.2 ml) was mixed with 2 ml of solution B and incubated at 30°C for 4 h. The absorbance of the solution was then measured at 510 nm using a Philips spectrophotometer. A standard curve was prepared using lactose solutions in the concentration range 0 to 1 g/l.

To check the recovery of lactose using this method, the set of standard lactose solutions of known concentration was made up in duplicate (0 to 1.0 g/l). A lactose standard solution (0.4 g/l) was prepared. Lactic whey permeate powder was dissolved in distilled water to give a lactose concentration of 50 g/l and diluted 100 and 200 times. The lactic whey permeate solution was then supplemented with 40 g/l analytical grade lactose and diluted 100 and 200 times. The analytical procedure was then conducted. The results of this analysis are shown in Table 3.4 and confirm acceptable recovery of the added lactose

Table 3.4 Lactose recovery

Dilution	Lactose concentration (g/l)		% recovery
	Serum	Serum + 40 g/l lactose	
100	53.7 ± 3.4	93.8 ± 8.7	100 ± 14
200	54.5 ± 3.9	95.7 ± 6.9	103 ± 11

3.3.6 Lactate Analysis

D and L-lactate were determined enzymatically (Gawehn and Bergmeyer, 1974; Gutmann and Wahlefeld, 1974).

3.3.6.1 L(+)- lactate

The procedure used was : Buffer A was prepared by dissolving 11.4 g glycine in 200 ml of deionised water and mixing with 25 ml of 24% (w/v) hydrazine hydrate.

The pH was checked and adjusted to 9.0. The solution was then made up to 300 ml with deionised water and stored at 4°C for up to 3 months. Solution B was prepared by dissolving 0.03 g β -NAD in 1 ml distilled water and was stored at -20°C. Solution C was L(+) lactate dehydrogenase at 5000 units/ml (Sigma L2625). On the day of use, the three reagents were mixed in the following proportions to form solution D

Buffer A	0.895 ml	}
Solution B	0.100 ml	} \times number of sample assays + 7
Solution C	0.0005 ml	} (hold 0°C)

The sample containing L-lactate was diluted as appropriate to give a concentration less than 0.12 g/l. The diluted sample (0.1 ml) was mixed with 1 ml of solution D and incubated at 25°C for 1 h. After incubation, the absorbance of the solution was measured at 340 nm using a Philips spectrophotometer. A standard curve was prepared using L(+)-lactic acid solutions in the concentration range 0 to 0.12 g/l.

3.3.6.2 D(-)-lactate

Buffer A and Solution B were prepared as described in Section 3.3.6.1. Solution C was D(-)- lactate dehydrogenase at 1500 units/ml (Sigma L9636). Solution D was prepared by mixing the reagents in the following proportions:

Buffer A	0.890 ml	}
Solution B	0.100 ml	} \times number of sample assays + 7
Solution C	0.010 ml	} (hold 0°C)

The remaining procedure was as described in Section 3.3.6.1 with the exception that the incubation time was 190 minutes and the standard curve was prepared using D(-)- lactic acid solutions in the concentration range 0 to 0.12 g/l.

3.3.7 Amino-nitrogen Analysis

α -amino-nitrogen was determined using the spectrophotometric method developed by Church *et al.* (1983). The reagent was prepared by combining the following solutions and diluting to a final volume of 50 ml using deionised water: 25 ml of 100 mM sodium tetraborate; 2.5 ml of 20% (w/w) sodium dodecyl sulphate; 40 mg of *o*-phthalaldehyde, OPA (Sigma Co.) dissolved in 1 ml of methanol; and 100 μ l of β -mercaptoethanol. This reagent was prepared daily. The centrifuged sample containing α -amino-nitrogen was diluted as appropriate to give a concentration less than 0.2 g/l, and 0.1 ml of this diluted sample was added to 2.9 ml of the OPA reagent. This was mixed and incubated for exactly 2 minutes at ambient temperature and the absorbance was measured at 340 nm using a Varian spectrophotometer (Varian, California, USA; Model 634 UV). A standard curve was prepared using a glycine solution of concentration in the range of 0 to 0.2 g/l.

3.3.8 Protein Analysis

The protein method is based on the binding of Coomassie Brilliant Blue to the protein and was developed by Bradford (1976). The procedure was : A concentrated Bradford reagent was prepared by mixing 50 ml ethanol (95% v/v), 100 ml phosphoric acid (85% w/w) and 100 mg of Coomassie Blue G-250. This reagent was kept at 4°C. On the day of use, the concentrated reagent was diluted 1.5: 10 with distilled water. Sample containing protein was diluted as appropriate and 0.1 ml was added to 5 ml of diluted Bradford reagent. This was mixed and incubated for 2 minutes at ambient temperature and the absorbance was measured at 595 nm on a Philips spectrophotometer. A standard curve was prepared using a bovine serum albumin solution in the concentration range 0 to 1 g/l.

3.3.9 Total Nitrogen

Total nitrogen was determined using the standard Kjeldahl digestion and titration method.

3.3.10 Glucose analysis

Analysis was carried out using a YSI fixed enzyme sugar analyzer (Yellow Springs Instruments Co., USA; model 27). A 2 g/l glucose was used as standard and the sample volume injected was 25 μ l.

3.4 FERMENTATION PROTOCOLS

3.4.1 Shake Flask Cultures

(a) Aerobic condition

50 ml volumes of medium in 250 ml conical flasks with cotton wool as a stopper were incubated at 30°C on an Environ shaker (Lab line Instrument Inc., Illinois, USA, Model 3597) and agitated at 250 rpm. The medium was inoculated by direct transfer of yeast from agar slants using a loop. This procedure was normally used for inoculum development for the subsequent fermentation process in either shake flask or fermentation culture.

(b) Anaerobic condition

200 ml volumes of medium in 250 ml conical flasks with cotton wool as a stopper were incubated at 30°C on an Environ shaker and agitated at 50 rpm. They were inoculated aseptically with a 5% (v/v) inoculum (24 h

incubation), prepared as described in (a) above. Samples (5 ml) were withdrawn from the flasks using sterile pipettes at 8 h intervals initially, and every 4 h afterward, until the completion of the fermentation process. Prior to sampling, a thorough mixing by hand was carried out to obtain a homogenous sample.

3.4.2 Batch Fermentation Culture

3.4.2.1 2-litre Fermentation Apparatus

The fermenter used was a Multigen F2000 Benchtop culture apparatus (New Brunswick Scientific Co., New Jersey, USA) equipped with a 2-litre pyrex glass vessel. Continuous pH measurement and one way control of pH were performed using a Kent pH electrode (Kent Industrial Measurements Ltd, Gloucestershire, England; Model 1117) connected to a Horizon pH controller (Ecology Co., Oak Park Avenue, Chicago, Illinois, USA; Model 5997-20). Where necessary either 4 M HCl or 4 M NaOH was used for pH control and this was delivered using a Masterflex peristaltic pump (Cole Palmer Instrument Co., Illinois, USA). A membrane air filter (0.2 μm) was used in conjunction with a glass wool filter to obtain sterile aeration. They were supplied by Sartorius (Gottingen, Germany) and Millipore (Bedford, Massachusetts, USA, Millex - FG50). Prior to each fermentation, the pH electrodes were calibrated using pH 4.0 and pH 7.0 buffer solutions.

3.4.2.2 14-litre Fermentation Apparatus

The fermentation apparatus was constructed in the Biotechnology Department, Massey University. The fermenter used was a 14-litre pyrex glass vessel (New Brunswick Scientific Co.) with a stainless steel head containing ports for various applications. Continuous pH measurement was as described in Section 3.4.2.1.

3.4.2.3 Batch Fermenter Operation

The Multigen F2000 Benchtop culture apparatus was used with a 2-litre vessel of 1 litre working volume. The 2-litre vessel containing medium was removed from the autoclave and attached to the fermentation apparatus while still hot (85-90°C). After cooling, aseptic inoculation was carried out. For experiments involving lactate utilization, the fermenter was inoculated (10 % v/v) and the temperature was maintained at 30°C by means of a heating element. A thermometer inserted in a water-filled well was used as a additional visual check on temperature. Agitation was maintained at 600 rpm and was provided by a single 6-bladed disc turbine mounted 3 cm above the vessel base. Aeration was maintained at 1 vvm and was sparged through a fine porous stainless steel air filter mounted 2 cm above the vessel base. The culture pH was measured using a Kent pH probe connected to a Horizon pH meter (section 3.4.2.1). Antifoam emulsion (20% w/v, Dow Corning Antifoam AF) was added to the culture using an automatic foam control system to suppress severe foaming in the culture. When foam contacted the stainless steel sensor, the completed foam controller circuit (Biotechnology Department, Massey University) activated a Masterflex peristaltic pump to add a small dose of sterile antifoam solution to the fermenter. Foam collapse caused the circuit to be broken and the pump to stop. A mechanical foam breaker, constructed from a plastic tie, mounted on the agitator shaft was used in combination with the antifoam solution. Samples were taken at various times during the fermentation and stored at -20°C for further analysis.

3.4.2.4 Continuous Fermenter Operation

Continuous fermentation were commenced in batch mode, as outlined in Section 3.4.2.3, prior to the commencement of continuous feed of substrate:

- (a) For experiments involving lactic acid utilization, the Multigen F2000 Benchtop culture apparatus was used with a 2- litre vessel and a culture volume of 1 l. After batch fermentation for 8 h, sterile fresh medium was

fed continuously to the top of the fermenter using a Masterflex peristaltic pump at a set flow rate. The culture volume was controlled using a fixed level stainless tube connected to a constantly running Masterflex peristaltic pump. Antifoam was added automatically as described in Section 3.4.2.3. pH was continually measured but no pH control was carried out. Fermentation samples were removed via the effluent line, every residence time, and more frequently after three residence times, to establish that a steady state condition had been attained.

- (b) For continuous anaerobic whey fermentation, both the 2-litre and 14-litre fermenter vessels were used, with working volumes of 1 l and 10 l, respectively. After inoculation (5% v/v inoculum) the temperature was maintained at 30°C. For the 2 litre vessel, agitation was maintained at 200 rpm, while for the 14-litre vessel, agitation was also at 200 rpm, but using 2 four-bladed impellers mounted 3 cm and 20 cm above the vessel base. No antifoam addition or aeration were required for these anaerobic fermentations. pH was continually measured but no pH control was carried out. The sampling procedure was the same as described in (a) above.
- (c) Coupling of continuous aerobic yeast production from lactate and anaerobic ethanol production from whey were carried out using the 2-litre vessel and 14-litre vessel in series. Initially the two fermentations were run independently. The aerobic slops fermentation was conducted in the 2-litre fermenter, as described in (a), while the anaerobic whey fermentation was conducted in the 14-litre fermenter as described in (b). When both fermentations had reached steady state, 5% of the effluent from the aerobic slops fermentation was fed continuously to the anaerobic whey fermentation to serve as a yeast inoculum. The other 95% went to waste. Figure 3.1 shows a photo of the coupled fermentation processes.

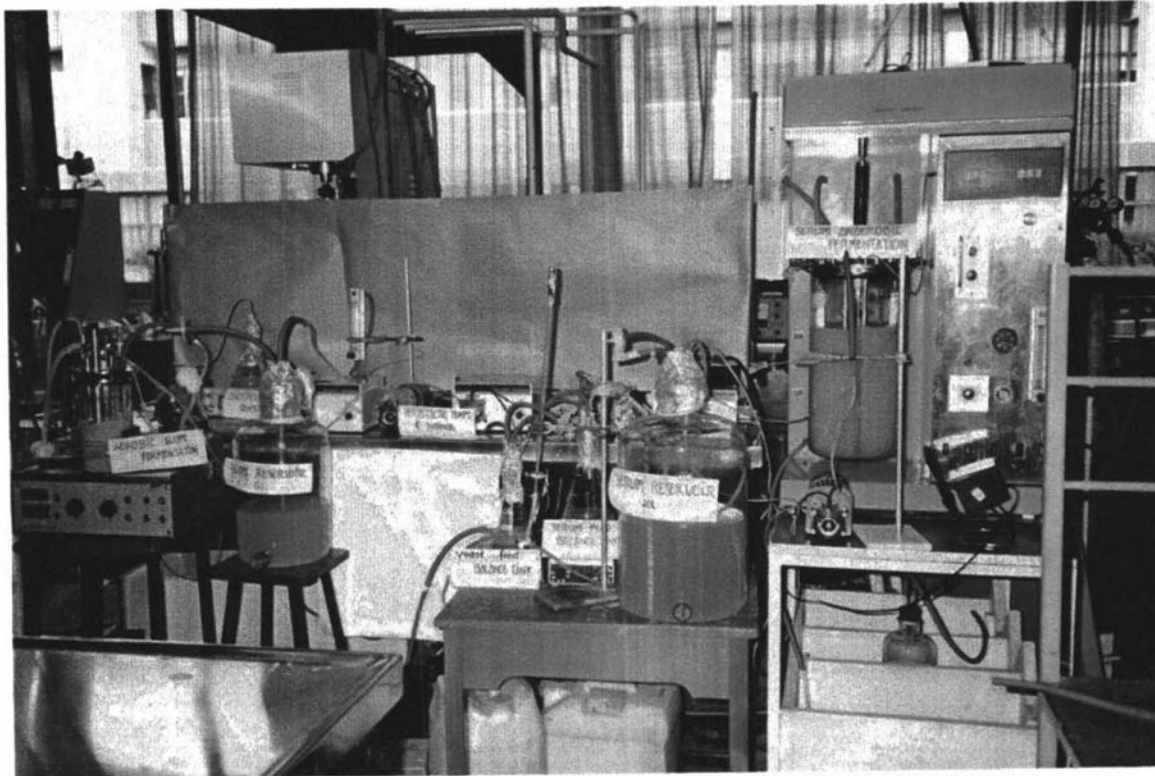


Figure 3.1 Coupling of aerobic yeast growth on slops (shown in the left side of the photo) and whey serum fermentation (shown in right hand side, with larger vessel).

3.4.3 Pump Calibration

The continuous fermentations were run at various dilution rates. These were achieved by varying the feed flow rate to the fermenter which operated at constant working volume. The Masterflex peristaltic pump was calibrated at the appropriate flowrate prior to the fermentation. In order to maintain accurate control of feed rate, frequent flow rate checks were necessary, since tubing in the pump head became worn over long periods of operation, resulting in variable flow rates. For determining the feed flow rates during aseptic fermentations, a sterile in-line flow meter, constructed from a 25 ml burette, was installed. Under normal working conditions the tubing to the flow meter was closed with a gate clip. To calibrate the pump, the clip was opened and the burette allowed to fill. The flow rate was determined by measuring the volume in the burette over a given time interval. The pump could then be adjusted accordingly. This ensured that continuous fermentations were operated at the desired dilution rate.

3.5 AUTOLYSIS PROTOCOL

3.5.1 Yeast Cream Production

Yeast was grown aerobically or anaerobically, as required in semi-synthetic medium and whey medium. A single 14-litre vessel with a working volume of 10 l was used to grow the yeast aerobically in batch culture for 16 h. Culture conditions were 30°C, with agitation and aeration rates of 600 rpm and 1vvm, respectively. Antifoam was added automatically as described in section 3.4.2.3, while pH was controlled at 4.5 by automatic addition of 25% (v/v) ammonia solution via a peristaltic pump. After 16 h, when the yeast was in exponential phase, it was harvested by centrifugation at 9000 rpm for 15 min, at 4°C, using a Sorvall RC-5C centrifuge (Dupont Company, USA). The cell pellet was washed twice with sterile distilled water. The washing water was discarded and the cells were resuspended in sterile distilled water to give a yeast biomass dry weight

concentration of 65-70 g/l.

Two 14-litre vessels were used to grow yeast anaerobically in batch culture for 24 h. The cultures were held at 30°C and agitated at 200 rpm without aeration. The culture pH was maintained at 4.5 using 25% (v/v) ammonia solution. After 24 h, when the yeast had reached stationary phase, it was harvested by centrifugation as described above for the aerobically-grown cells.

3.5.2 Batch Autolysis

3.5.2.1 Flasks

100 ml of yeast cream (65-70 g dry weight/l) in 250 ml conical flasks with cotton wool or a rubber bung as a stopper were incubated without agitation in a Grant water bath (Grant instruments, Cambridge Ltd, Barrington, Cambridge, England; Model W38) set at the desired temperature (50, 55 or 60°C). Samples (5 ml) were withdrawn from the flasks every 2 h for the first 12 h and every 12 h afterward until the completion of autolysis (48 h). At the same time, the pH of the autolysing cell suspension was measured and adjusted manually using 4M NaOH or 4M HCl. Prior to sampling, a thorough mixing was carried out by hand to obtain a homogenous sample.

3.5.2.2 2-litre Vessel

The vessel used was the Microferm laboratory Fermenter (New Brunswick) equipped with a 2-litre pyrex glass vessel of 1 l working volume. The autolysis start-up procedure was as follows : The empty 2-litre glass vessel was sterilized (121°C, 15 min), cooled and filled with fresh yeast cream (1 l) of concentration 70-80 dry weight g/l, obtained from Anchor Ethanol Co. A Grant water bath was used to maintain the temperature of the autolysis process at 55°C by

continuously circulating hot water through the heating baffle of the vessel. Agitation was maintained at 150 rpm. A Horizon pH controller was used for pH control (pH 5.5) in conjunction with a Masterflex peristaltic pump for 4M NaOH delivery and a Kent combination pH electrode.

3.5.3 Continuous Autolysis

The Microferm Laboratory Fermenter equipped with a 2- litre pyrex glass vessel of 1 l working volume was used. Initially the vessel was filled with 1 l of fresh yeast cream obtained from Anchor Ethanol Co.; and autolysis was allowed to proceed for 6 h in batch mode. The Grant water bath was used to maintain the temperature at 55°C. After 6 h of batching, fresh yeast cream was continuously fed to the autolysis vessel. Masterflex peristaltic pumps were used to control the level of the fermenter by regulating the rate of inflow and outflow. Agitation was maintained at 150 rpm, while 4 M NaOH was delivered automatically via a peristaltic pump to maintain the pH at 5.5. A condenser was fitted on the head of the vessel to minimise the loss of water due to evaporation. A 14-litre vessel was used as a yeast cream reservoir to feed the continuous autolysis process. The yeast cream feed was stirred continuously at 100 rpm to maintain a homogenous suspension, and was kept at 4°C by using a refrigerated water bath (Julabo labortechnik GMBH, West- Germany, Model F10) which continuously circulated the -2°C water-glycol mixture through the vessel baffles. Autolysis samples were removed via the effluent line every retention time and frequently after three retention times to determine the establishment of steady state conditions. Figure 3.2 shows a photo of the continuous autolysis process.

3.6 DATA ANALYSIS

Data from continuous culture of whey fermentation was analyzed using four different plots i.e the Lineweaver-Burk, Eadie and Hofstee, and Langmuir plots

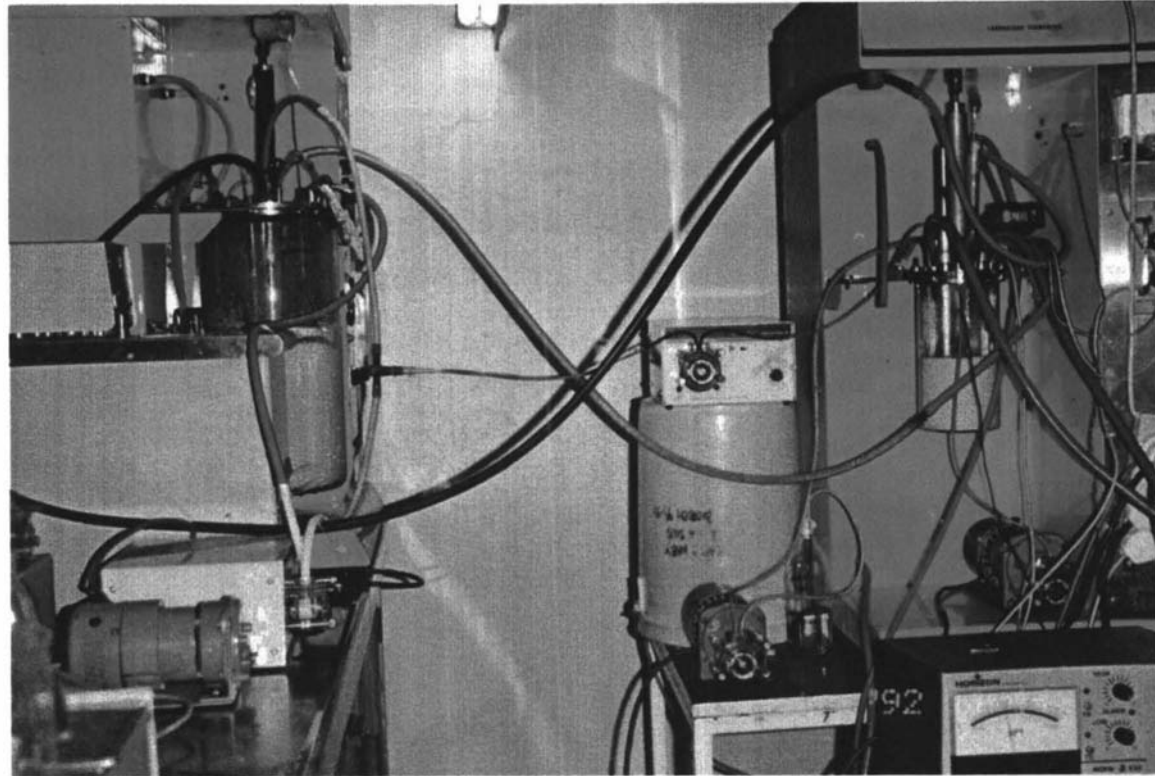


Figure 3.2 Continuous autolysis process with the yeast storage tank shown in the left side of the photo and the autolysis vessel in the right side.

described in standard texts (e.g. Moser, 1988) and a Heijnen plot (1992) which is based on the Lineweaver-Burk plot but uses q_s (specific substrate uptake rate) rather than μ . The description of the four plots are shown in Figure 3.3.

3.7 DISCUSSION OF METHOD

For lactose analysis, initially the modified DNS colorimetric method was used. This worked reasonably well when using synthetic medium, but not so well when using whey media. Furthermore the accuracy, of this method was reduced when only a small lactose concentration was present (<0.5 g/l). Therefore the enzymatic method was explored. This enzymatic method performed well when using whey media, as judged by the reproducibility and the accuracy of the data obtained for lactose recovery (spike) tests.

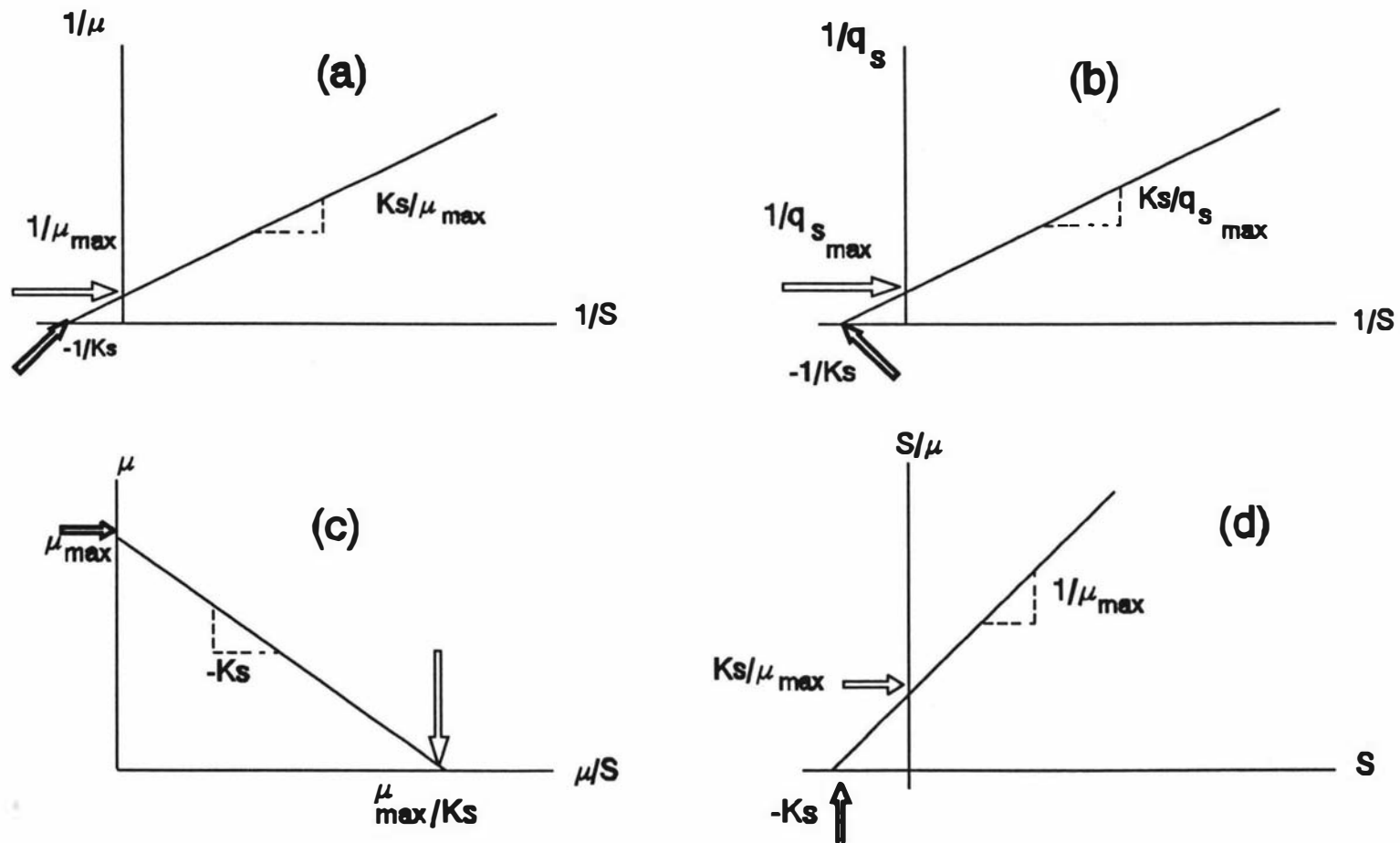


Figure 3.3 Four different plots used for the analysis of continuous culture data (a) Lineweaver-Burk (b) Heijnen (c) Eadie and Hofstee and (d) Langmuir.

CHAPTER 4

CHARACTERISATION OF ETHANOL PRODUCTION BY INDUSTRIAL KLUYVEROMYCES YEAST

4.1 INTRODUCTION

New Zealand's second whey distillery was commissioned in September 1981 by the New Zealand Co-operative Dairy Co. at their Tirau site, near Hamilton. The fermentation process was designed by the company engineers and is the only known whey distillery in the world which operates a continuous fermentation system. It was established as a downstream process of the major operation of protein recovery in the dairy factory. From the dairy company's viewpoint, ethanol production both partially solves the waste disposal problem and provides some financial return. However no systematic studies of the fermentation were done prior to design of the plant and although batch ethanol production from whey has been studied extensively, there are only three reports on the continuous fermentation using free cells (Vienne and von Stockar, 1983, 1985b; King and Zall, 1985). No report (batch or continuous) specifically addressed lactic acid casein whey fermentation so consequently it is likely there is still potential for further improvement in the Tirau process to maximise rates of ethanol production and the yield of ethanol.

The primary objective of the work described in this chapter was to study the fermentation characteristics of the Tirau process yeast strain, *Kluyveromyces marxianus* strain Fi, in batch and continuous culture. Additionally, the interactions of this production strain and other yeasts in mixed culture, and the effect of lactic acid on lactose fermentation were also investigated.

4.2 MATERIALS AND METHODS

4.2.1 Organisms

The production yeast *K. marxianus* strain Fi, obtained originally from Anchor Ethanol Co., was used together with *K. marxianus* strains: Y-113, NRRL Y-2415, TC2, 1607, 1496 and DSIR. A strain of *C. tropicalis* obtained originally from Golden Cheese Co, Corona, California, USA, and yeast 19, which was isolated from the Tirau distillery, were used for mixed culture experiments (Section 3.1.4).

4.2.2 Yeast differentiation media

Media to test for sugar fermentation, or assimilation of carbon and nitrogen compounds, were prepared according to van der Walt (1970). These were used to confirm the classification of *K. marxianus* strain Fi.

4.2.3 Fermentation media

The fermentation media used in this work were: sulphuric acid casein whey permeate (SACWP), SACWP supplemented with 3-30 g/l sodium lactate and the semi-synthetic media YEPL, YEPLL, and YEP with lactate. The composition of these media are described in Section 3.1.1.

4.2.4 Fermentation apparatus and operation

Shake flask cultures of 250 ml, or 2-litre and 14-litre stirred anaerobic fermentations were conducted as described in Section 3.4. A fermentation temperature of 30°C was used for this work since this temperature has been used in the industrial process with this organism. An inoculum size of 5%(v/v) was used throughout this work for batch and continuous fermentations. The inoculum was developed as described in Section 3.4.1. Samples (5ml) were withdrawn from the flasks using sterile pipettes. For continuous cultures, 25 ml

samples were removed via the effluent line as described

4.2.5 Chemical analysis

Ethanol, lactose, glucose and lactate were analyzed as outlined in Section 3.3.4, 3.3.5, 3.3.10, and 3.3.6, respectively. Biomass was determined by both the spectrophotometric and dry weight methods as described in Section 3.3.2. pH was measured with a Metrohm pH meter.

4.2.6 Data analysis

Batch culture data were analyzed using the following conventions:

- (a) Fermentation time, t_f , is the time including any lag phase, when the ethanol concentration was 90% of the maximum concentration observed. This was determined from the concentration profiles of each individual fermentation. Sugar utilization, biomass yield, specific product yield, the % ethanol yield of theoretical, and overall volumetric ethanol productivity values were determined based on t_f .
- (b) Biomass yield (Y_{xs}). This yield was calculated as ethanol concentration divided by sugar utilized (g/g).
- (c) Specific product yield (Y_{px}). This was calculated as ethanol produced divided by biomass produced (g/g).
- (d) % ethanol yield of theoretical. This yield coefficient was calculated from the ratio of ethanol formed at t_f to the maximum theoretical ethanol concentration based on the lactose consumed (0.583 g/g, based on Guy-Lussac equation described in Section 2.1.1).
- (e) The overall volumetric ethanol productivity was calculated as : ethanol concentration/ t_f (g/l.h).

For continuous culture data, four approaches were used to determine μ_{max} and K_s values. They were Eadie and Hofstee, Langmuir, and Lineweaver-Burk plots as described in Moser (1988), and Heijnen plot (1992). Details of the plots are

described in Section 3.6. The data were fitted by linear regression using Minitab (Minitab Inc., Enterprise drive, State College PA, USA).

4.3 RESULTS

4.3.1 Yeast identification and differentiation.

For fermentation tests, the sugar solutions were examined for acid and gas production after incubation at 30°C for 3, 7 and 21 days. If acid was produced the colour of the sugar solution with added bromothymol blue changed from green to yellow. Gas production was observed by bubbles trapped inside the Durham tubes and positive fermentation was indicated by both acid and gas production. A positive result for assimilation of carbon (oxidative utilization of carbon compounds) or assimilation of nitrogen compounds was based on increased turbidity of the solution. These readings were also taken after incubation at 30°C for 3, 7 and 21 days. Two serial inoculations in fresh test media were carried out with nitrogen assimilation tests to ensure that growth was not due to soluble nitrogenous compounds excreted by the cells during the first incubation. The results of all tests are shown in Table 4.1. The characteristics of the production strain match the description of *K. marxianus* given by Barnett *et al.* (1990).

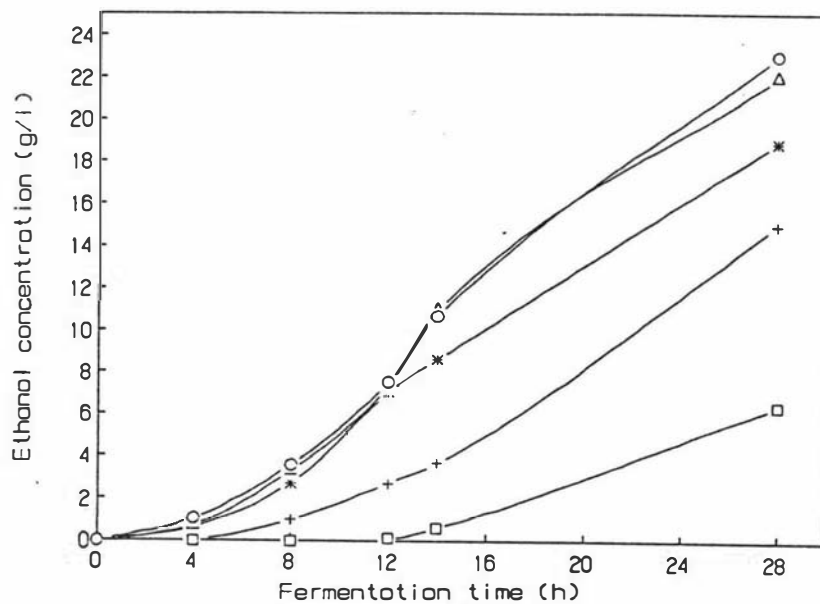
4.3.2 Batch fermentation

4.3.2.1 Comparison of yeast performance

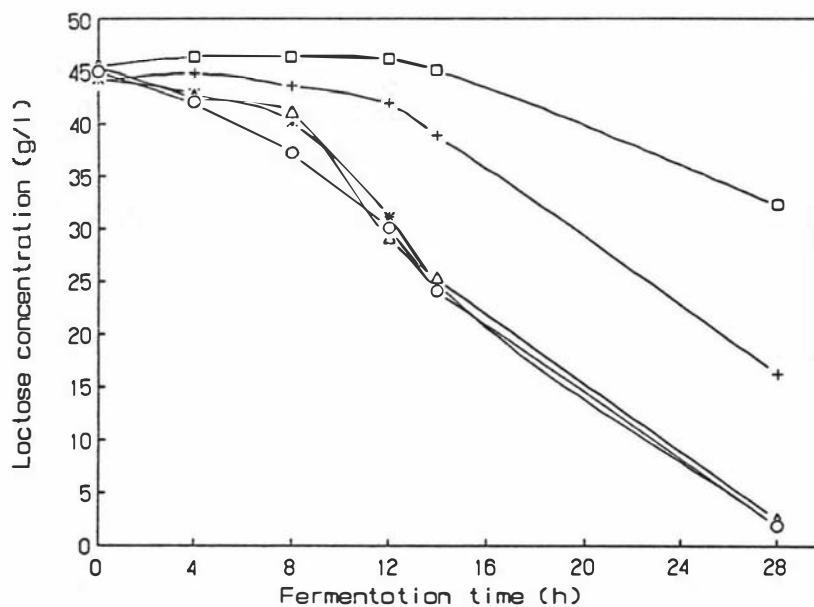
Ethanol production by five different strains of *Kluyveromyces* yeast was compared. The yeast strains were grown anaerobically in YEPL in batch mode and the fermentation profiles are shown in Figure 4.1. The fermentation was essentially completed after 28 h for strains Fi, TC2, and *S. fragilis* based on

Table 4.1: Characterisation of the industrial production yeast *K. marxianus* strain Fi

<u>Fermentation:</u>					
Glucose	+	Maltose	-	Trehalose	-
Galactose	+	Sucrose	+		
Lactose	+	Raffinose	+		
<u>Assimilation of Carbon Compounds:</u>					
Glucose	+	Maltose	-	L-Sorbose	+
Galactose	+	Melizitose	-	Sucrose	+
L-Arabinose	+	Raffinose	+	D-ribose	+
Lactose	+	Ribitol	+	Trehalose	-
				Xylose	+
				Starch	-
<u>Assimilation of Nitrogen Compounds:</u>					
Nitrate	-				
Nitrite	-				
Ethylamine	+				
<u>Miscellaneous</u>					
Vitamins-free medium					-
Growth on 50% w/w glucose yeast extract agar					+
Growth in the presence of 100 ppm cycloheximide					+
Splitting arbutin					+



(a)



(b)

Figure 4.1 Comparison of the fermentation performance of five different yeast strains. (a) Ethanol production and (b) lactose utilization : o Fi; * TC2; Δ S. fragilis; + 1201; □ 1496.

lactose utilization (Figure 4.1b). The other two strains (1201 and 1496) exhibited long lag periods and only about 60% and 20% of lactose was utilized, respectively, after 28 h. Overall the production strain Fi performed as well as the best of the other yeasts (*S. fragilis* and TC2) giving an ethanol yield of theoretical of 93%.

4.3.2.2 Mixed culture

A co-culture of *C. tropicalis* and *K. marxianus* strains (not strain Fi) had been evaluated on a production scale by Golden Cheese Co., Corona, USA. However only anecdotal data were available to confirm its potential. The distillery claimed a reduction in acidity (possibly due in part to lactate utilization) and an increase in fermentation efficiency of nearly 2% over the use of *K. marxianus* alone (personal communication, Mawson 1990). However due to the pathogenicity of the *Candida* strain the mixed culture process has since been discontinued. The objective of this part of work was to evaluate if a mixed culture of strain Fi and another yeast could provide an increased ethanol yield with reduced acidity after fermentation of acid whey.

The performance of the mixed culture was studied in anaerobic shake flask cultures. As lactic whey medium was not available throughout the experimental program, a semi-synthetic medium (YEPLL) as described in Section 3.1.1.3 was used in the first trial using strain Fi and *C. tropicalis*. A pure culture of strain Fi growing under the same conditions was run as a control. The ethanol, lactose, and lactic acid profiles are shown in Figure 4.2. Figure 4.2a shows that ethanol production by the mixed culture and monoculture were not significantly different. Lactose utilization rates were the same and an 85% ethanol yield of theoretical was achieved by both systems. Figure 4.2b shows that neither L- nor D-lactic acid were utilised to a marked extent, although during the first 4 hours of fermentation a slight decrease of L-lactic acid concentration was noted. The extracellular glucose formed during fermentation was almost insignificant at 0.20

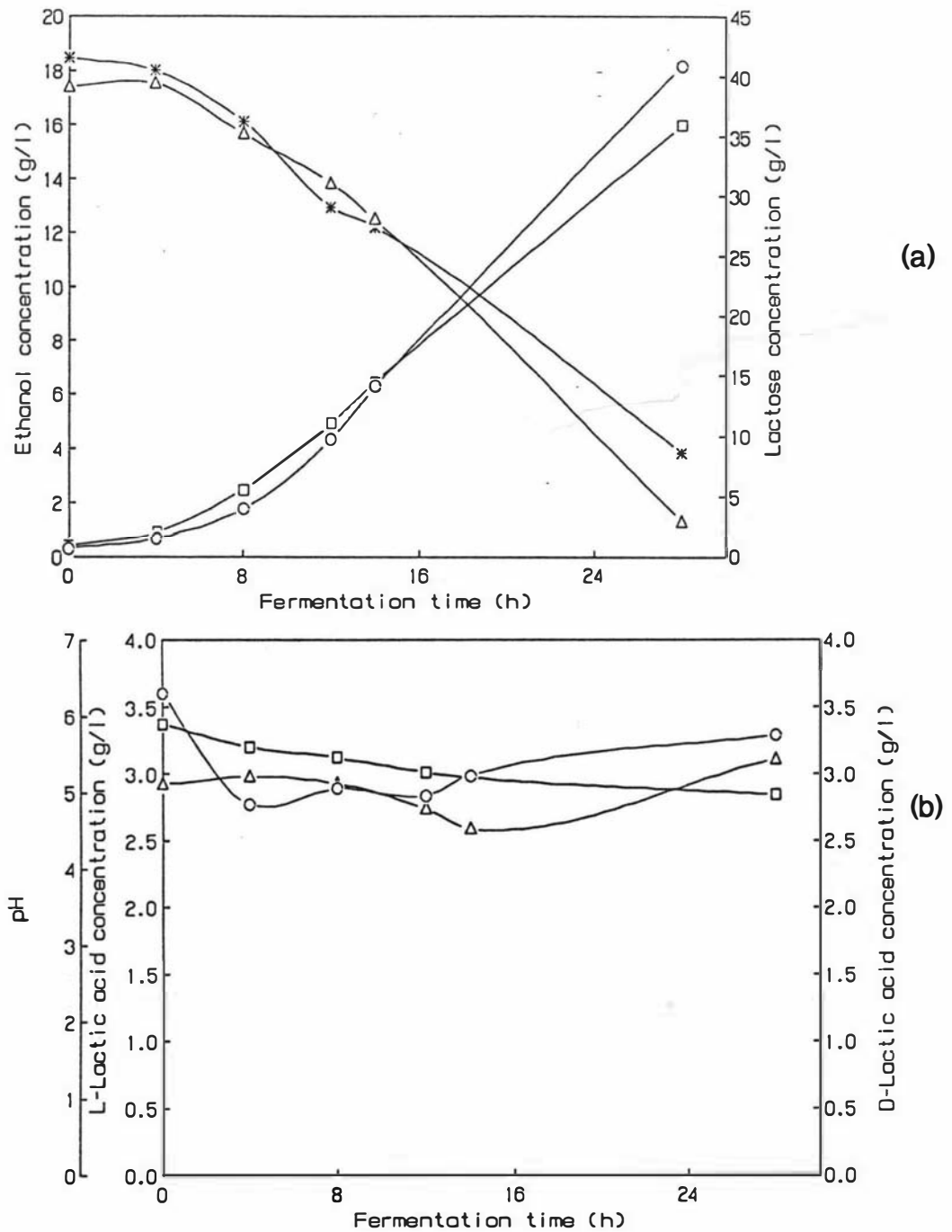


Figure 4.2 The fermentation performance of pure culture and a mixed culture (with *C. tropicalis*) of the production yeast strain Fi. (a) Ethanol and lactose profiles : ○ Fi ethanol; Δ Fi lactose; □ Fi & C.t ethanol; * Fi & C.t lactose. (b) Lactic acid and pH profile : ○ L-lactic acid; Δ D-Lactic acid; □ pH.

g/l and this had disappeared after 28 h.

A yeast isolated from the Anchor Ethanol distillery and designated as strain 19 was also grown in mixed culture on lactic whey serum with strain Fi. Of several strains isolated from the distillery, strain 19 was the best in terms of lactic acid assimilation under aerobic conditions, and it can also assimilate glucose. Strain 19 was tentatively identified as *Torula delbruckii*, based on the sugar fermentation and assimilation of carbon and nitrogen compounds tests. Frozen lactic serum was thawed and pasteurised at 85°C for 25 seconds prior to inoculation. The results of the anaerobic fermentation are shown in Figure 4.3. Again, there was no significant difference between the monoculture and the mixed culture processes in terms of the ethanol yield and fermentation rate.

4.3.2.3 Effect of lactic acid

It is known that ethanol production from molasses using *S. cerevisiae* is inhibited by lactic acid. Therefore the effect of lactic acid on the ethanolic fermentation by *K. marxianus* Fi was investigated using semi-synthetic (YEPLL) and sulphuric acid casein whey permeate (SACWP) media. These media were chosen to simulate the effect of lactate on the lactic whey serum fermentation because they contain no lactate. Thus, the concentration of lactate could be varied independently of the concentration of lactose and other medium constituents.

Initially, lactate concentrations of 0, 3 and 7 g/l in YEPLL were studied. Lactic acid at a concentration of approximately 7 g/l is the normal constituent of lactic acid casein whey (Table 3.1). There were no significant differences in ethanol production and lactose utilization among the three lactate concentrations (Figure 4.4). The initial pH of the media was 6.8 and it gradually decreased to 5.5 after 28 h.

YEPLL medium was then used to study the effect of higher lactate concentrations, up to 30 g/l, on the fermentation. In the extreme event, when the

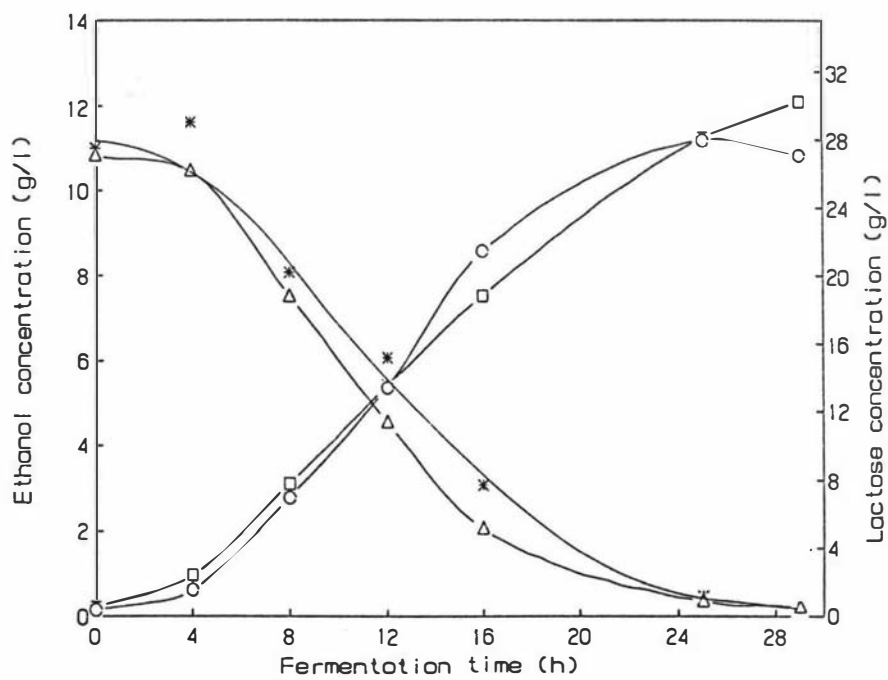
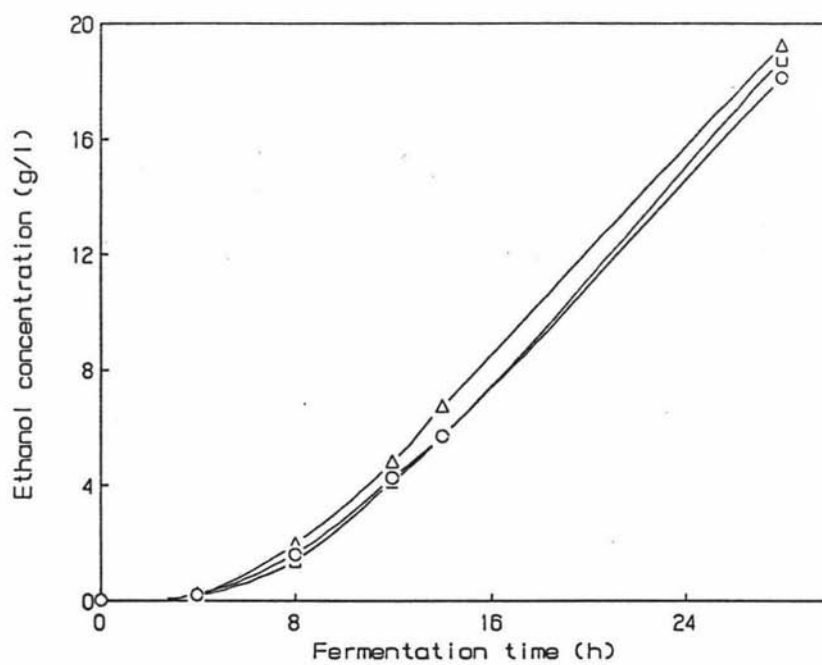
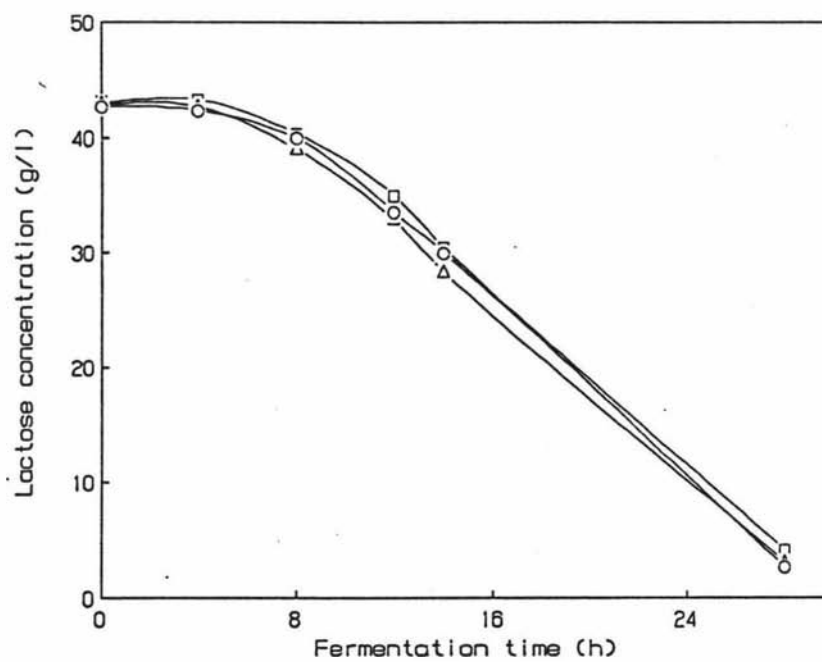


Figure 4.3 The fermentation performance of a pure culture of strain Fi and a mixed culture of strain Fi and yeast 19 : \circ Fi ethanol; Δ Fi lactose; \square Fi & 19 ethanol; $*$ Fi & 19 lactose.



(a)



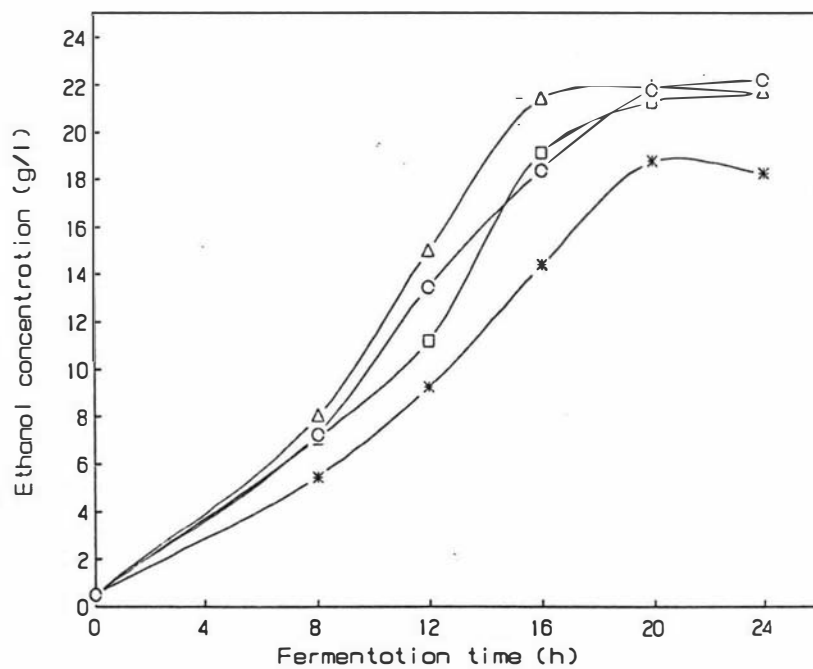
(b)

Figure 4.4 The effect of lactate on the fermentation performance of strain Fi.
(a) Ethanol production and (b) lactose utilization : \circ 0 g/l lactate; Δ 3 g/l lactate;
 \square 7 g/l lactate.

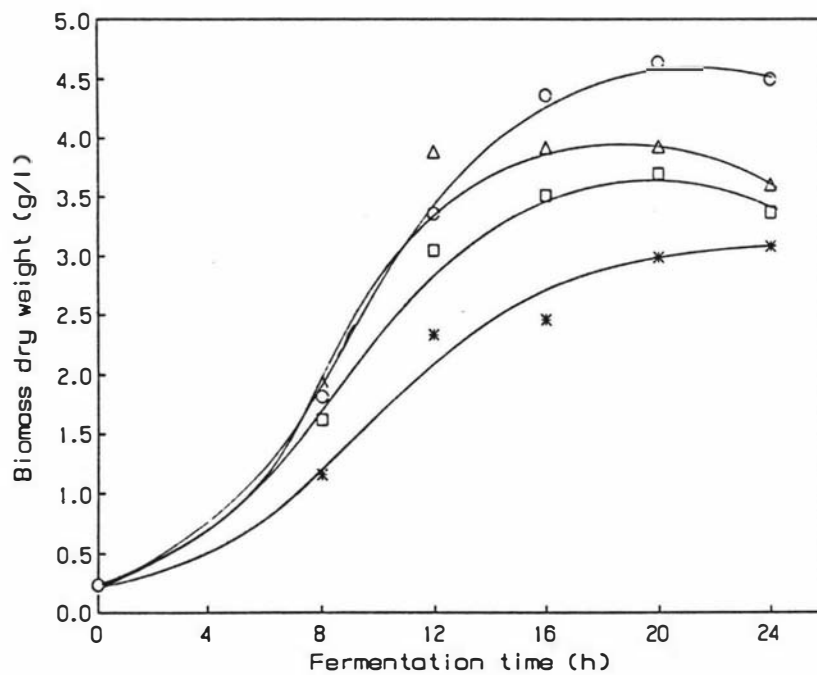
ethanolic fermentations are heavily contaminated by lactic acid bacteria, up to 15-30 g/l of lactic acid may be produced (Essia Ngang *et al.* 1989). Thus, this experiment aimed to simulate the effects of lactate at normal and extreme concentrations on whey fermentation. The pH of each medium was adjusted to 4.5 prior to autoclaving, as this is the normal pH of lactic whey serum. Fermentation profiles for this experiment are shown in Figures 4.5 and 4.6. Results of further analysis of the data are summarised in Table 4.2.

Figure 4.5a shows the ethanol concentration peaked after 20 h for all conditions. There were no significant differences in ethanol productivity when lactate was present at 7 and 15 g/l concentration compared to the control, however a lower ethanol productivity was observed when 30 g/l lactate was present. The % ethanol yield of theoretical was slightly higher in the presence of lactate (Table 4.2). Figure 4.5b shows that in the presence of lactate, growth ceased earlier, at 16 h, compared to the control, while ethanol continued to be produced (Fig 4.5a). The inhibitory effect of lactate appeared to be confined to the biomass yield. When lactate was present at a concentration of 7 g/l or higher, there was a lower biomass yield compared to the control. However, as the cell concentration decreased, the specific product yield increased and was approximately 40% higher in the presence of 30 g/l lactate (Table 4.2). Lactose was utilised completely after 20 h, for the 0, 7, and 15 g/l lactate concentrations, and after 24 h for the 30 g/l lactate (Figure 4.6a). During the fermentation the pH value changed only slightly in media with added lactate due to the buffering capacity of lactate, whereas a more dramatic drop of pH to 3.8 was observed with the control (Figure 4.6b).

To investigate the effect of lactate on the fermentation of concentrated whey, the lactose concentration of YEPLL medium was doubled to 100 g/l and the lactate concentration was varied from 0, 7, and 15 g/l. Fermentation profiles are shown in Figure 4.7 and 4.8, while summarized data are given in Table 4.3. The same trends were observed as previously, indicating no interaction between the higher lactose and lactate concentrations. There was further evidence of the uncoupling



(a)



(b)

Figure 4.5 The effect of lactate on (a) ethanol and (b) biomass production by strain Fi on YEPLL medium : ○ 0 g/l lactate; △ 7 g/l lactate; □ 15 g/l lactate; * 30 g/l lactate.

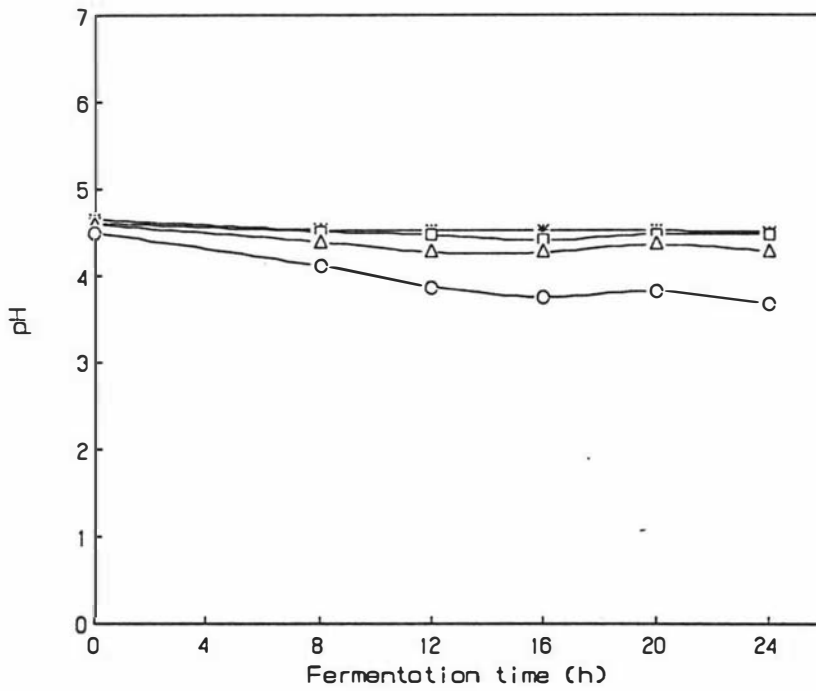
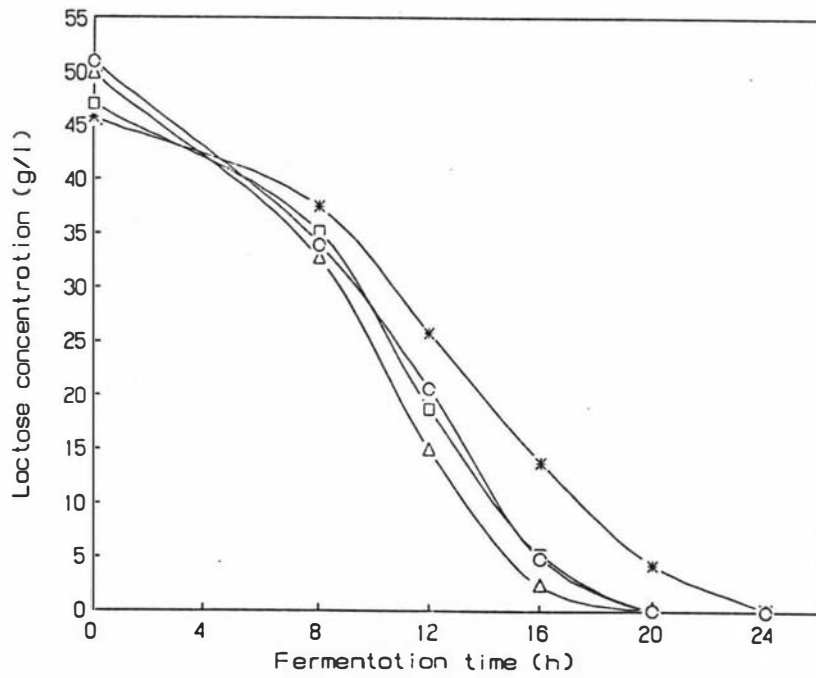
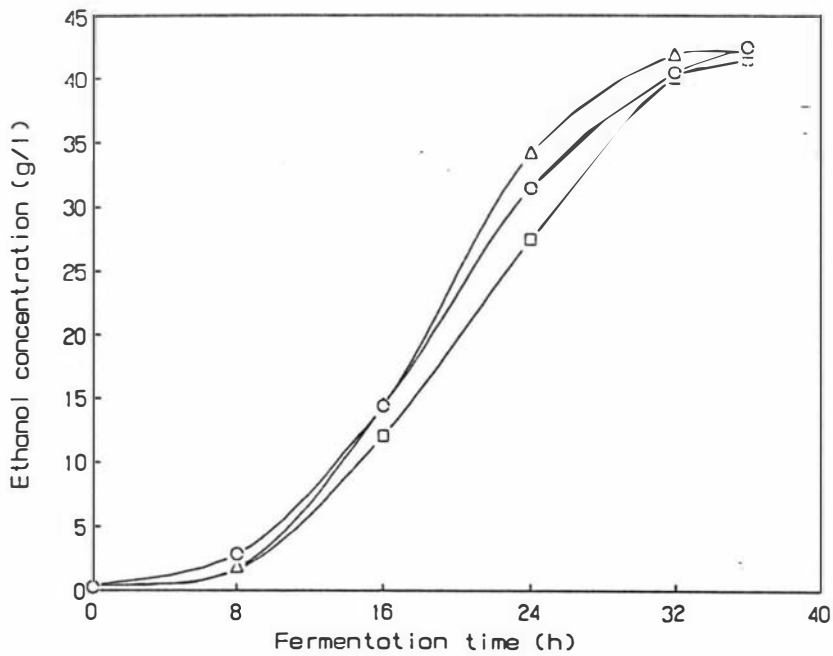


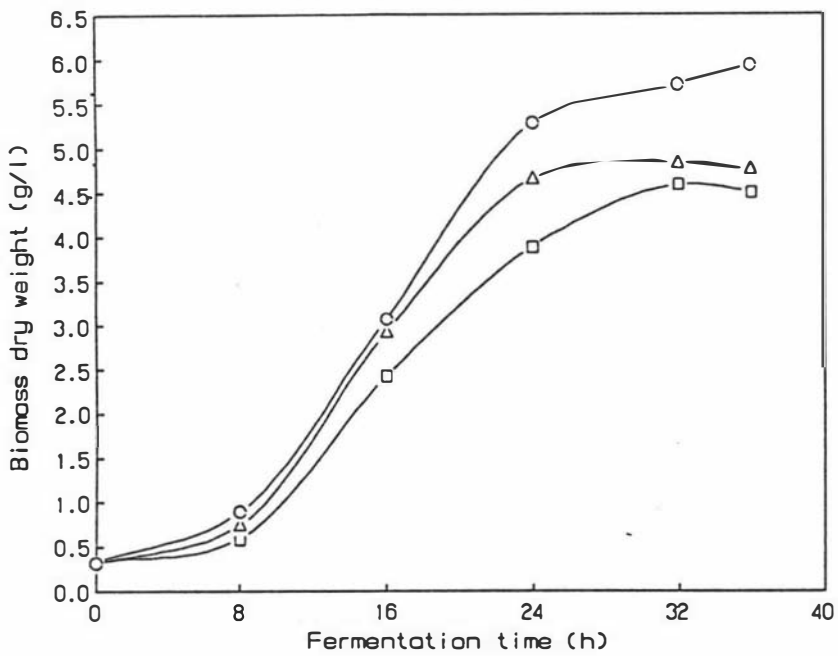
Figure 4.6 The effect of lactate during growth on YEPLL. (a) Lactose consumption and (b) culture pH (symbols as figure 4.5).

Table 4.2 : Effect of lactate on the ethanolic fermentation using YEPLL containing 50 g/l lactose.

	Lactate concentration (g/l)			
	0	7	15	30
Fermentation time; t_f (h)	17.5	14.6	16.3	18.0
Ethanol (g/l)	20	19.7	19.4	16.9
Residual lactose (g/l)	2.3	4.6	5.4	8.3
Biomass dry weight (g/l)	4.5	3.9	3.5	2.8
Biomass yield Y_{xs} (g/g)	0.09	0.08	0.08	0.07
Specific product yield Y_{px} (g/g)	4.6	5.3	5.9	6.6
Overall volumetric ethanol productivity (g/l.h)	1.1	1.3	1.2	0.9
% Ethanol yield of theoretical	76	81	87	84

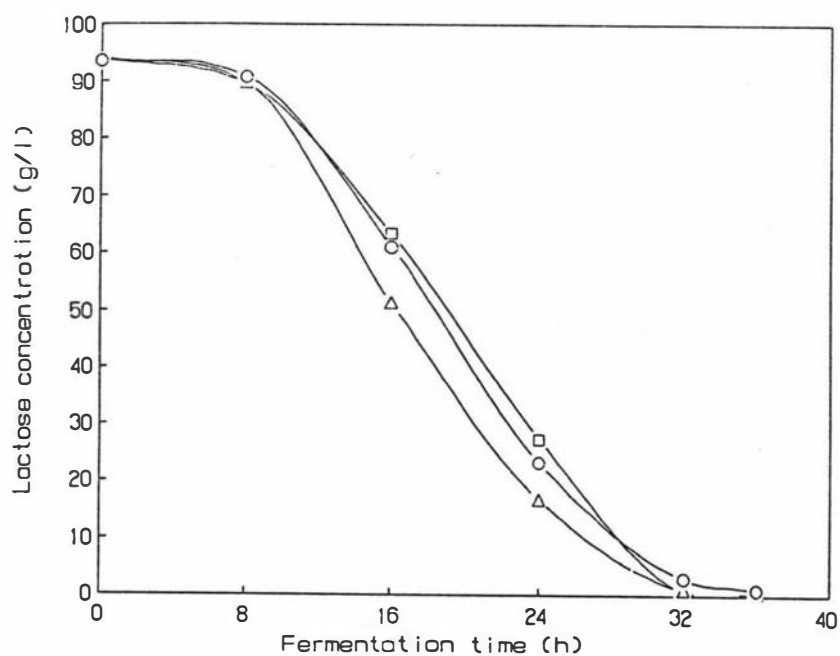


(a)

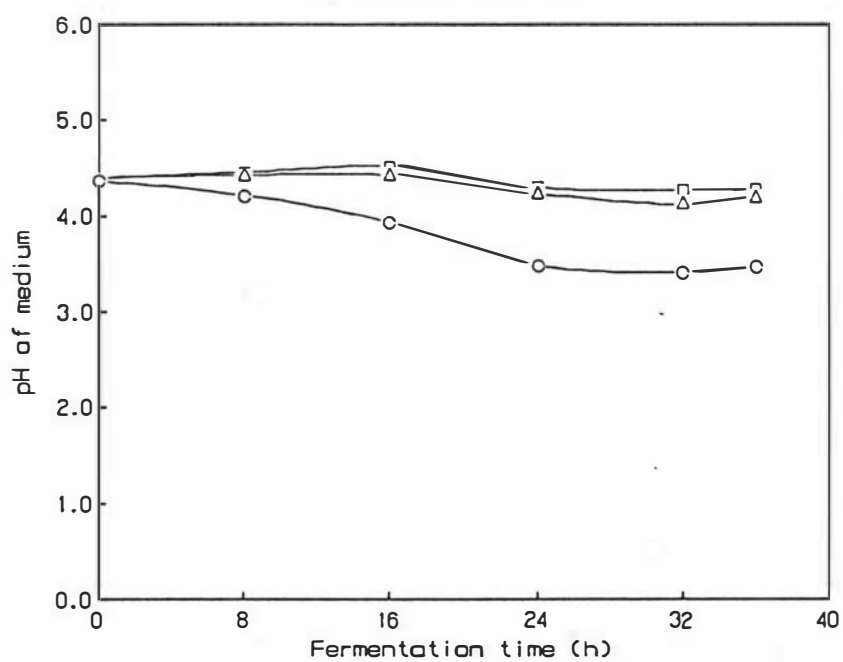


(b)

Figure 4.7 The effect of lactate on fermentation of YEPLL at a higher lactose concentration (100 g/l). (a) Ethanol production and (b) biomass concentration (symbols as in Figure 4.5)



(a)



(b)

Figure 4.8 The effect of lactate on (a) lactose utilization and (b) culture pH at a lactose concentration of 100 g/l (symbols as Figure 4.5).

Table 4.3 : Effect of lactate on the ethanolic fermentation using YEPLL containing 100 g/l lactose

	Lactate concentration (g/l)		
	0	7	15
Fermentation time; t_f (h)	29.5	26.8	29.8
Ethanol (g/l)	38.2	37.9	37.4
Residual lactose (g/l)	7.2	4.8	6.4
Biomass dry weight (g/l)	5.6	4.8	4.5
Biomass yield Y_{xs} (g/g)	0.06	0.05	0.05
Specific product yield Y_{px} (g/g)	7.1	8.3	8.9
Overall volumetric ethanol productivity (g/l.h)	1.3	1.4	1.2
% Ethanol yield of theoretical	82	79	79

of growth and ethanol formation observed in the previous experiment using 50 g/l lactose. The biomass yield coefficients were reduced compared to the fermentation of 50 g/l lactose, and this was also indicated by the increased specific product yields, Y_{px} (Table 4.3).

The effect of lactate on the fermentation of whey was investigated using SACWP powder. The powder was reconstituted in distilled water to give a final lactose concentration of 50 g/l and sodium lactate was added to give concentrations of 0, 15, and 30 g/l. Fermentation profiles are shown in Figure 4.9, and further data are shown in Table 4.4. Regardless of whether lactate was present or absent, growth ceased after 24 h although ethanol continued being produced at a slow rate until 32 h of fermentation (Figure 4.9). The time required to complete the fermentation was considerably longer with whey media compared to the semi-synthetic media and consequently the overall volumetric ethanol productivity was almost half of that using semi-synthetic media (Tables 4.2 and 4.4). The uncoupling effect was clear as was the decrease in yield coefficient due to lactate. The decreased biomass yield was reflected in an increased specific product yield but otherwise there was no marked difference in whey fermentation in the presence of added acid.

When the lactose concentration was doubled to 100 g/l in the presence of 15 g/l lactate, the ethanol production curve lagged behind that of the control (Figure 4.10a) and a lower overall volumetric ethanol productivity was observed. However the % ethanol yield of theoretical remained unchanged (Table 4.5) and this was also reflected by the slower lactose utilization compared to the control (Figure 4.10a). There was a marked uncoupling between growth and ethanol production in both cultures, where growth ceased after 32 h but ethanol continued being produced until 48 h (Figure 4.10).

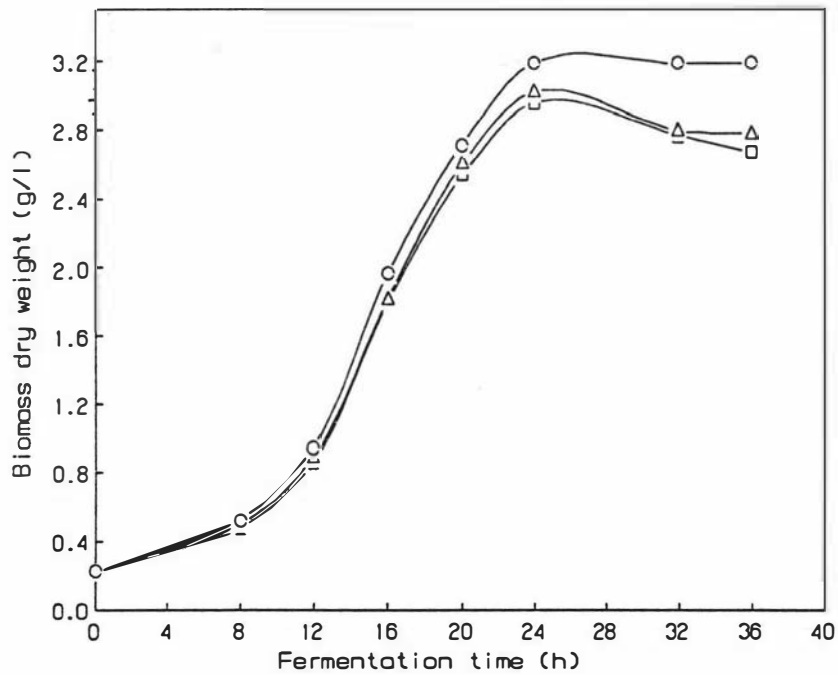
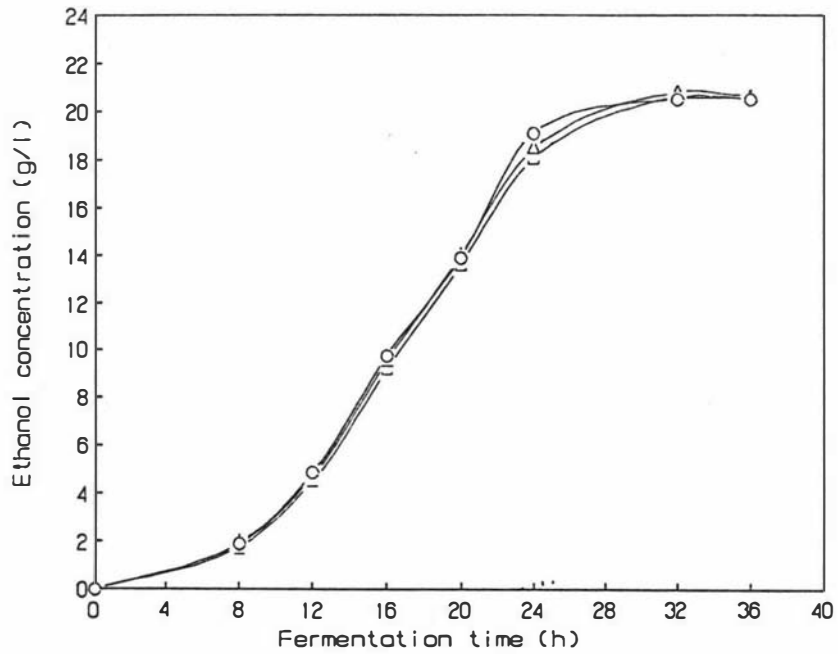
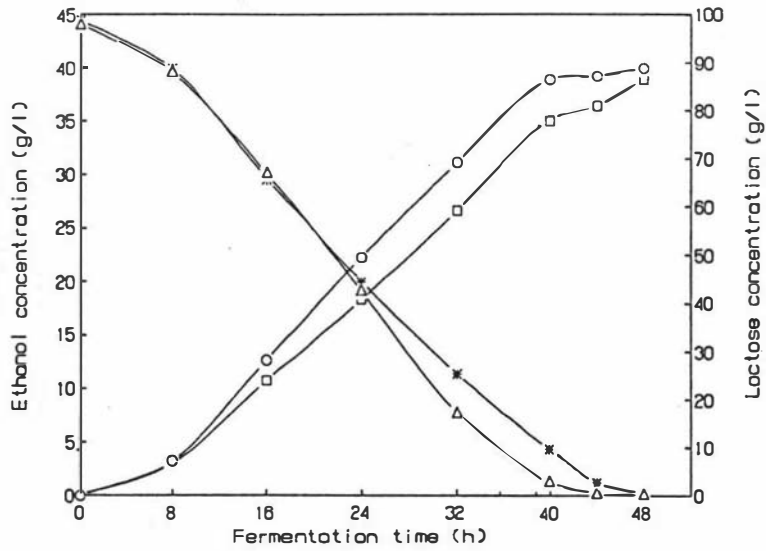


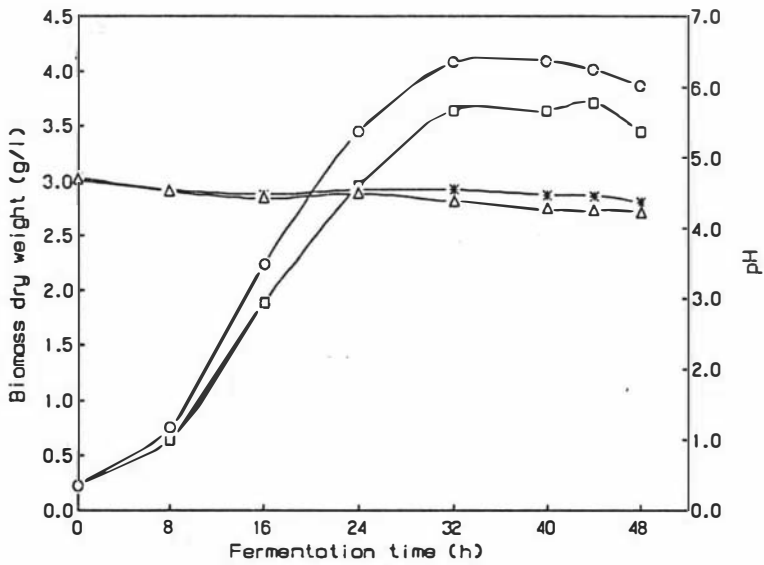
Figure 4.9 The effect of lactate in SACWP on (a) ethanol and (b) biomass production : \circ 0 g/l lactate; Δ 15 g/l lactate; \square 30 g/l lactate.

Table 4.4 : Effect of lactate on fermentation of SACWP at 50 g/l lactose

	Lactate concentration (g/l)		
	0	15	30
Fermentation time; t_f (h)	23.4	24.5	25.0
Ethanol (g/l)	18.5	18.7	18.5
Residual lactose (g/l)	4.6	4.0	4.0
Biomass dry weight (g/l)	3.2	3.0	3.0
Biomass yield Y_{xs} (g/g)	0.07	0.06	0.06
Specific product yield Y_{px} (g/g)	6.3	6.7	6.8
Overall volumetric ethanol productivity (g/l.h)	0.8	0.8	0.7
% ethanol yield of theoretical	78	78	78



(a)



(b)

Figure 4.10 The fermentation performance of concentrated SACWP supplemented with lactate. (a) Ethanol and lactose profiles : o 0 g/l ethanol; Δ, 0 g/l lactose; □ 15 g/l ethanol; * 15 g/l lactose. (b) Biomass and pH profiles : o 0 g/l biomass; Δ 0 g/l pH; □ 15 g/l biomass; * 15 g/l pH.

Table 4.5 : Effect of lactate on fermentation of SACWP at 100 g/l lactose concentration

	Lactate concentration (g/l)	
	0	15
Fermentation time; t_f (h)	35.6	38.9
Ethanol (g/l)	35.0	34.3
Residual lactose (g/l)	4.29	5.36
Biomass dry weight (g/l)	4.1	3.7
Biomass yield Y_{xs} (g/g)	0.04	0.04
Specific product yield Y_{px} (g/g)	6.8	7.7
Overall volumetric ethanol productivity (g/l.h)	1.0	0.9
% ethanol yield of theoretical	69	68

4.3.3 Continuous fermentation

4.3.3.1 The effect of dilution rate on the fermentation of LACWP and LACWS

An initial attempt to evaluate the maximum growth rate of the production strain for ethanol production was made using spray dried LACWP powder. Dilution rates of 0.05 h^{-1} , 0.10 h^{-1} , 0.15 h^{-1} and 0.20 h^{-1} were used. The steady state data, except those for a dilution rate of 0.20 h^{-1} , are summarised in Table 4.6. A steady state condition was normally assumed after three residence times when the biomass dry weight, ethanol, and lactose concentrations remained constant ($\pm 10\%$). Frequent sampling was carried out after three residence times to ensure that the steady state condition had been achieved. Fermentations were generally completed after four residence times. The results are shown in Table 4.6. Increasing the dilution rate from 0.05 h^{-1} to 0.15 h^{-1} resulted in only half of the lactose being utilized, indicating that washout was imminent. The data at a dilution rate of 0.2 h^{-1} are not included in Table 4.6 as steady state was not obtained. Thus, the results from these four runs indicated that the maximum specific growth rate (μ_{max}) of the production yeast strain Fi is between 0.15 h^{-1} and 0.20 h^{-1} .

These runs were repeated using liquid lactic acid whey serum obtained from Anchor Ethanol Co., Tirau. Five dilution rates were used : 0.05 h^{-1} , 0.075 h^{-1} , 0.10 h^{-1} , 0.15 h^{-1} , and 0.20 h^{-1} . The steady state results of these five runs are summarized in Figure 4.11 and Table 4.6. Figure 4.11 shows that as the dilution rate increased, the biomass and ethanol concentrations decreased steadily and residual lactose increased. The ethanol productivity initially increased with increasing dilution rate and exhibited a maximum value at $D= 0.10\text{ h}^{-1}$. The biomass concentration was low compared to that obtained using LACWP powder. Due to the precipitation of calcium salts, and probably protein, after autoclaving, the biomass dry weight was calculated taking account of these precipitates. Thus, accurate estimates of the biomass may not have been obtained.

Table 4.6: Summarized steady state parameters from continuous cultures of *K. marxianus* strain Fi in various whey media

Dilution rate (h ⁻¹)	lactose utilized (g/l)	Ethanol concentration (g/l)	Biomass dry weight (g/l)	% Ethanol yield theoretical	Productivity (g/l.h)	Biomass yield (g/g)	Influent lactose (g/l)
0.05	45.8	18.0	1.71	73	0.9	0.04	46 ^a
0.10	43.1	17.9	1.94	77	1.79	0.045	46 ^a
0.15	24.8	11.5	1.47	86	1.73	0.05	46 ^a
0.05	37.1	16.1	0.64	81	0.8	0.017	38 ^b
0.075	34.9	16.2	0.78	86	1.22	0.022	38 ^b
0.10	33.4	12.7	0.42	71	1.27	0.013	38 ^b
0.15	15.2	7.8	0.29	89	1.17	0.019	38 ^b
0.20	7.2	3.3	0.10	85	0.66	0.014	38 ^b

^a LACWP powder

^b LACWS

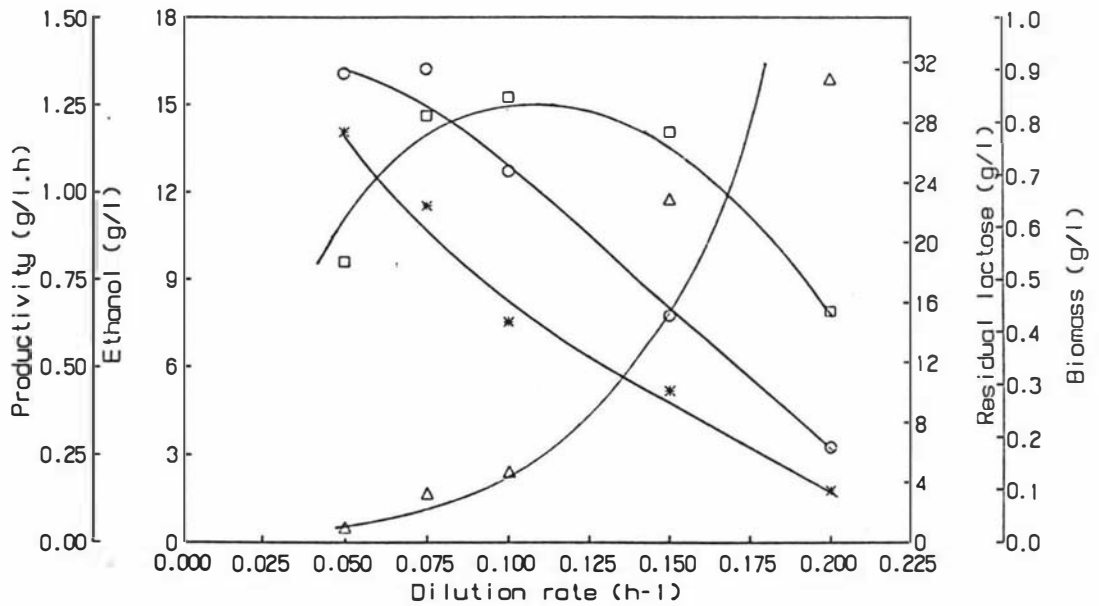
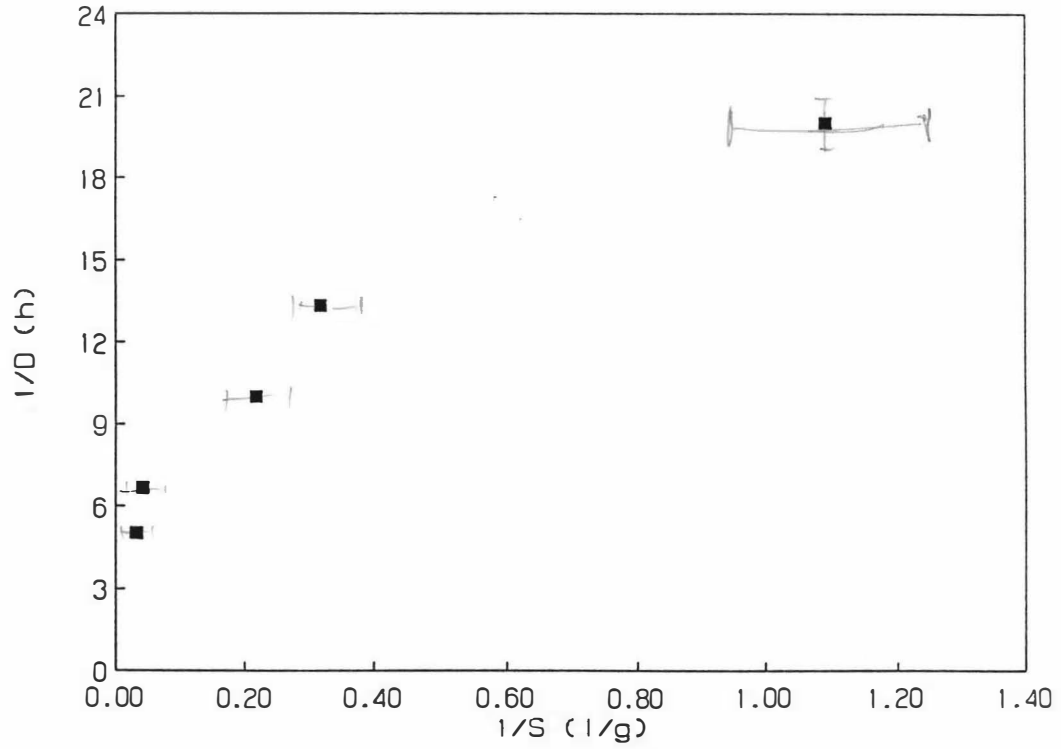


Figure 4.11 Steady state parameters for continuous culture of *K. marxianus* Fi on LACWS : \circ ethanol; Δ lactose; \square productivity; $*$ biomass.

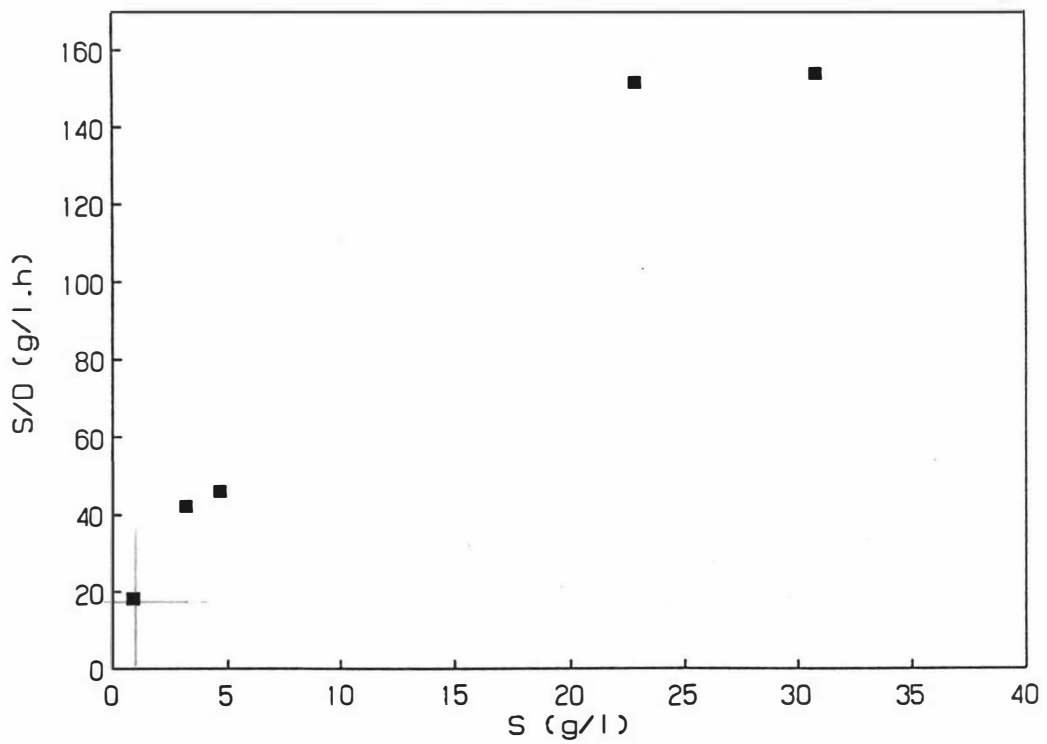
Nevertheless, the yeast biomass concentration was similar to that obtained at Tirau for the fermentation process operating at a dilution rate of 0.07 h^{-1} using the same medium. From these data, a double-reciprocal plot was constructed (Figure 4.12a). A μ_{\max} value of 0.15 h^{-1} and K_s of 1.92 g/l were estimated by linear regression. The Langmuir plot (Figure 4.12b) gave the best fit compared to the other approaches, with a μ_{\max} value of 0.21 h^{-1} and K_s value of 4.94 g/l (Table 4.7). When the irregular data point at the lowest dilution rate ($D= 0.05 \text{ h}^{-1}$) was omitted, an improved fit was obtained for both Lineweaver-Burk and Eadie and Hofstee plots, and the μ_{\max} and K_s values were very similar to those obtained for the full data using the Langmuir approach. Deleting the 0.05 h^{-1} point resulted only in slight changes in the Langmuir parameters (Table 4.8). The Heijnen approach gave a poor fit to the data in each case as indicated by the low R square value. Although the trend towards cell washout became more apparent as the dilution rate was increased further, a steady state was obtained at a dilution rate of 0.20 h^{-1} and was maintained for eight residence times. Therefore the maximum specific growth rate will be slightly higher than 0.20 h^{-1} ; this is in agreement with the value estimated by the Langmuir approach.

4.3.3.2 The effect of different types of whey substrate

The effect of two different types of substrate, SACWP and LACWP, on the continuous fermentation of the production yeast strain Fi was investigated. A dilution rate of 0.10 h^{-1} was used because maximum ethanol productivity occurred at this dilution rate for the production strain Fi when grown in lactic whey permeate and serum. A dilution rate of 0.10 h^{-1} was repeated with a new batch of LACWP powder. This was necessary to ensure that the yeast behaved similarly with the new batch of powder. A 78% ethanol yield of theoretical and an ethanol productivity of 1.41 g/l.h were obtained, compared to 77% ethanol yield of theoretical and an ethanol productivity of 1.79 g/l.h using the old powder under identical conditions (Table 4.6 and 4.9). It is unclear whether the difference was due to poor reproducibility of the data or due to different nutrient



(a)



(b)

Figure 4.12 The plots of the continuous culture data on LACWS (a) Lineweaver-Burk and (b) Langmuir.

Table 4.7 : Determination of μ_{\max} and K_s from continuous lactic whey serum fermentation with full data :

Plot	μ_{\max} (h^{-1})	K_s (g/l)	R_{square} (%)
Monod	0.15	1.92	91
Eadie and Hofstee	0.17	2.56	72
Langmuir	0.21	4.94	96
Heijnen	0.15	1.94	71

Table 4.8 : Determination of μ_{\max} and K_s from continuous lactic whey serum fermentation without data from dilution rate of $0.05 h^{-1}$.

Plot	μ_{\max} (h^{-1})	K_s (g/l)	R_{square} (%)
Monod	0.21	5.55	97
Eadie and Hofstee	0.21	5.48	86
Langmuir	0.22	6.61	95
Heijnen	0.27	9.40	75

levels.

It also appeared that ethanol production was poorer in SACWP than in LACWP. The ethanol yield of theoretical was only 65%, and ethanol productivity was 1.16 g/l.h, compared to 72-77% ethanol yield and productivity of 1.41-1.79 g/l.h in LACWP (new and old powder, respectively) at the same dilution rate (Table 4.6 and 4.9).

4.3.3.3 Comparison with other yeast strains

The performance of *K. marxianus* NRRL Y-2415 was compared with that of the production strain at a dilution rate of 0.10 h⁻¹, using the new LACWP powder. This yeast has been used in other studies of whey ethanol production (Vienne and von Stockar 1983, Walker *et al.* 1990). An ethanol productivity of 1.52 g/l.h and a 77% ethanol yield of theoretical were obtained with this yeast, compared to a productivity of 1.41 g/l.h and a 72% ethanol yield of theoretical, with the production strain under the identical dilution rate. Therefore this indicated the performance of *K. marxianus* NRRL Y-2415 was slightly better than that of the production strain yeast.

The performance of *K. marxianus* Y-113 was also compared with the production strain, also at a dilution rate of 0.10 h⁻¹. This yeast is used in at the N.Z Distillery Co, Ltd, Edgecumbe and has been used in other studies of whey ethanol production (Mawson and Taylor, 1989). An ethanol productivity of 1.17 g/l.h and 76% ethanol yield of theoretical were achieved with this yeast (Table 4.9). The high residual lactose levels suggested that this yeast may have a lower specific growth rate than that of *K. marxianus* strain Fi, the Tirau production yeast strain.

Table 4.9: Summarized steady state parameters from continuous cultures.

Dilution rate (h ⁻¹)	Lactose utilized (g/l)	Ethanol concentration (g/l)	Biomass dry weight (g/l)	% Ethanol yield theoretical	Productivity (g/l.h)	Biomass yield (g/g)	Comment
0.10	33.3 ^a	11.6	1.41	65	1.16	0.04	SACWP
0.10	36.6 ^b	14.1	1.40	72	1.41	0.039	LACWP
0.10	36.7 ^a	15.2	1.78	77	1.52	0.041	Yeast 2415
0.10	28.6 ^a	11.7	1.17	76	1.17	0.04	yeast 113

^a Influent lactose 50 g/l

^b Influent lactose 43 g/l

4.4 DISCUSSION AND CONCLUSIONS

It was considered important to characterize strain Fi, the Tirau production yeast, for two reasons. Firstly, so that comparisons of performance with other strains of the same species could be made. The history of the culture is unclear but the organism was believed to be a strain of *K. fragilis*, since classified as *K. marxianus*. Secondly, this yeast has been used throughout many years and possible physiological changes by mutation could not be ruled out. The results from the carbon fermentation, carbon assimilation and nitrogen assimilation tests on the production yeast strain Fi matched the description of *K. marxianus* yeast by Barnett *et al.* (1990). These tests are specific enough to distinguish any yeast to species level. The lactose fermentation test alone narrowed down the approximately 500 species of yeasts to 10 possible species. Five species of *Candida* yeast, one species of *Dekkera* genus, one species of *Pichia* genus and three species of *Kluyveromyces* genus are able to ferment lactose. Among the 15 species of *Kluyveromyces*, only three are able to ferment lactose : *K. cellobiovorus*, *K. lactis* and *K. marxianus*. The main difference between *K. lactis* and *K. marxianus* is that the former is able to assimilate maltose, α - α -trehalose, and melizitose as carbon sources for growth whereas the latter species is unable to do so. The main difference between *K. cellobiovorus* and *K. marxianus* is that the former is able to assimilate maltose and α - α -trehalose but not melizitose, while it is unable to ferment raffinose, which the latter is able to (Barnett *et al.* 1990). The tests have confirmed that the production strain yeast is a strain of *K. marxianus*.

The use of mixed cultures of different yeasts or of yeast and bacteria for whey fermentation to improve the fermentation rate has been studied by several authors (Tu *et al.* 1985; Friend *et al.* 1987; Kamini and Gunasekaran, 1989). Tu *et al.* (1985) reported that a coculture of *K. marxianus* CBS 397 and *S. cerevisiae* gave approximately 20% faster lactose utilization and a slightly higher ethanol yield, compared to a monoculture of *K. marxianus*. The authors suggested that the presence of low concentration of monosaccharides (glucose and galactose

at 3 and 11 g/l, respectively) in cheese whey could affect lactose utilization by *K. marxianus*. By using a coculture of *K. marxianus* and *S. cerevisiae*, the monosaccharides which are present initially could be utilized by *S. cerevisiae*, resulting in an improvement of the fermentation rate. Kamini and Gunasekaran (1989) studied a mixed culture of *K. fragilis* NRRL 665 and the bacterial strains *Zymomonas mobilis* NRRL 1960 and 4286 in a complex medium containing 200 g/l lactose. They reported that higher productivity and ethanol yield were achieved with the mixed cultures compared to the monoculture of *K. fragilis*. *Z. mobilis* metabolized glucose but not lactose. They suggested that the positive effect observed was due to disappearance of the glucose which accumulated during the fermentation, although evidence to support this was not given. Wang *et al.* (1987) reported that lactose fermentation performance was strongly influenced by the presence of glucose and galactose. The inhibitory effect of glucose (10-20 g/l) on lactose uptake was much stronger than that of galactose at the same concentration.

This work has shown none of the advantages of using a mixed culture process as claimed by earlier studies. When mixed cultures of strain Fi and either *C. tropicalis* or yeast 19 were grown in semi-synthetic medium or lactic whey serum, no significant differences in the ethanol yield or fermentation rate were observed compared to the control. Both *C. tropicalis* and yeast 19 can ferment glucose but not lactose. Overall, lactic acid was not utilized anaerobically, thus no decreased acidity was observed as claimed by Corona distillery. A very low concentration of glucose (0.20 g/l) was formed and utilized during the pure culture fermentation. A similar result was observed by Ravesteijn and Mawson (1991 unpublished data); when using semi-synthetic medium containing 50 g/l lactose at pH 6.5, with *K. marxianus* Y-113 and *K. marxianus* NRRL Y-2415, only 0.25 g/l and 0.12 g/l extracellular glucose were formed, respectively. It appears that the presence of a small quantity of glucose has no effect on the fermentation performance. No clear role for glucose or glucose utilization could be identified, and it is possible that the effect of glucose only becomes significant when its concentration is in the order of 3-20 g/l as reported by Wang *et al.* (1987) and Tu *et al.* (1985). Friend

et al. (1982) also reported that there was no advantage in using mixed cultures of *K. fragilis* and *S. cerevisiae* in comparison to a monoculture of *K. fragilis*.

The inhibitory effects of lactic acid on the ethanolic fermentation by *S. cerevisiae*, which result in lower ethanol production and inhibition of growth, have been studied by several authors (Inoue *et al.* 1962, Tani *et al.* 1963; Maoirella *et al.* 1983; Essia Ngang *et al.* 1989, 1990; and Makanjuola *et al.* 1992). Although the mechanism of the inhibition is not clearly understood, it is generally accepted that the presence of lactic acid at concentrations of 10-40 g/l has a pronounced effect on the fermentation performance of *S. cerevisiae*. However, no reports are available on the effect of lactic acid on lactose-fermenting yeasts, and this deficiency was addressed in this work. It was observed that in semi-synthetic media and SACWP, the presence of lactate up to a concentration of 30 g/l has minimal effect on the overall volumetric ethanol productivity or ethanol yield of *K. marxianus* strain Fi. However, the presence of lactate at 7 g/l and higher reduced the biomass yield coefficient (Y_{xs}) and growth ceased earlier compared to the control. The specific product yield (Y_{px}) was increased as a consequence of these effects and was 40% higher in the presence of 30 g/l lactate in YEPLL than in the control. The same effect was observed by Maoirella *et al.* (1983) during continuous fermentation by *S. cerevisiae* in semi-synthetic medium. The cell density decreased with increasing lactic acid concentration, and at 40 g/l lactic acid the specific ethanol productivity was increased by 45% over the control. The authors suggested that the mechanism of lactic acid inhibition is some form of chemical interference with cell maintenance functions, requiring increased ATP expenditure.

Essia Ngang *et al.* (1989) reported that the observed toxicity of lactic acid on the ethanolic fermentation from wort by *S. cerevisiae* was enhanced by the osmotic pressure of the sugar substrate. They observed that the inhibition constants (K_i) for growth were 14.5 and 10.3 g/l for normal and doubled sugar concentrations, respectively. However in this work with strain Fi, doubling of the lactose concentration to 100 g/l, produced no further adverse effects on the whey

fermentation.

Overall, it appears that a lactate concentration up to 30 g/l has little effect on the fermentation performance of *Kluyveromyces* Fi yeast. Warth (1977) observed that the resistance of *S. bailii* to benzoic, sorbic and acetic acids, used as food preservatives, was the result of an inducible, energy-requiring system which transported preservative from the cells. Stimulation of the fermentation, based on carbon dioxide evolution, and reduction in growth yield in the presence of the preservatives, were consequences of the energy demand of the preservative pump. It was reported that the preservative pump was not present in *S. cerevisiae* and consequently this yeast was sensitive to these preservatives. The authors also observed that growth of the organism in the presence of moderate amounts of preservative greatly increased the subsequent resistance to higher concentrations. Lactic acid was not studied by these authors although it is also generally used as food preservative. It is possible that a similar mechanism may be present in *Kluyveromyces* strain Fi. It is also possible that the resistance of strain Fi to lactic acid was due to a long period of adaptation to moderate concentrations of lactic acid (approximately 7 g/l) in LACWS.

Lactic acid is generally viewed as less inhibitory than other preservatives such as acetic acid, propionic acid and sorbic acid (Eklund, 1989). Thus not much research effort has been directed towards the mechanism of action of lactic acid. The inhibition effect of the lactate may depend on the pH value of the culture, where at low pH the undissociated acid predominantly influences fermentation performance as it is transported by simple diffusion into the cells. However Moon (1983) reported that growth inhibition of the yeasts studied (*Geotrichum candidum*, *Hansenula canadensis*, *Endomycopsis burtonii* and *Saccharomyces uvarum*) was not due entirely to the undissociated lactic acid. The author did not offer a further explanation.

Uncoupling of growth and ethanol production was observed in SACWP medium containing 50 or 100 g/l lactose without lactate. In the SACWP containing 50 g/l

lactose, growth ceased after 24 h, whereas ethanol production continued up to 32 h. In the same medium containing 100 g/l lactose, growth ceased after 32 h but ethanol continued to increase up to 48 h. Vienne and von Stockar (1983) attributed this uncoupling to a nitrogen limitation of the whey medium. They observed that when yeast extract was added to the whey medium, the uncoupling effect disappeared and exponential growth was observed until the completion of the fermentation. Furthermore the fermentation time was reduced by half with yeast extract supplementation. Comparison of the performance in the richly supplemented YEPLL and SACWP media, with no lactate present, shows that the overall volumetric ethanol productivity obtained using the rich medium was almost 30 to 38% higher than in SACWP, at normal and double lactose concentrations, respectively. The % ethanol yield of theoretical was unaffected at the normal lactose concentration of 50 g/l, however at 100 g/l lactose concentration, a 19% improvement in the ethanol yield was achieved with the rich medium compared to SACWP. This suggested that the nutritional status of whey permeate could be improved by the addition of nutrients such as yeast extract, peptone or ammonium sulphate.

When the data for continuous lactic whey serum fermentations were analyzed using a Lineweaver-Burk plot, the points were not aligned on a straight line. Vienne and von Stockar (1983) observed the same effect for continuous fermentation of supplemented whey permeate. They estimated μ_{\max} and K_s to be 0.216 h^{-1} and 0.014 g/l , respectively, for low dilution rate data ($D < 0.220 \text{ h}^{-1}$) and to be 0.31 h^{-1} and 0.971 g/l , respectively, at higher dilution rates. The data calculated from the steady state values of continuous fermentation of unsupplemented whey permeate obtained by Vienne and von Stockar (1985b), when analyzed using a Lineweaver-Burk plot, also indicated a deviation at low dilution rate. Vienne and von Stockar (1983) hypothesized that two types of lactose transport systems through the membrane might exist. At low concentrations (corresponding to low D), an active transport mechanism might be involved, with a high affinity for the substrate reflected by a low saturation constant ($K_s = 0.014 \text{ g/l}$). At high concentrations, the transport mechanism might

be passive and with a lower substrate affinity ($K_s = 0.97$ g/l). This was supported by the finding that the biomass yield decreased at the low lactose concentration at which the postulated active transport mechanism was predominant. An active transport requires more energy than passive transport. The lower biomass yield observed at low dilution rate is consistent with increased substrate utilization for maintenance (of which substrate transport may be a component). However, the hypothesis of two possible lactose transport mechanisms in *Kluyveromyces* is not supported by the literature. Barnett and Sims (1982) studied the lactose transport in *K. fragilis*, and reported that, aerobically, lactose uptake was by active transport but anaerobically was by facilitated diffusion. Dickson and Barr (1983), and Moulin and Galzy (1987), also reported that lactose uptake in *K. lactis* was by a single active transport mechanism, presumably under aerobic conditions, although this was not specifically stated by these authors. This work also reported a lower biomass concentration at low dilution rate ($D = 0.05$ h⁻¹) compared to one step higher dilution rate ($D = 0.075$ h⁻¹), partly supporting the hypothesis of alternate transport system at low dilution rate and low lactose concentration.

Analysis of several methods of estimating the kinetic constants revealed that the Langmuir approach gave better fits to the data with acceptable μ_{\max} values. This was true for both the results obtained in this work and for the data of Vienne and von Stockar. This is shown in Tables 4.7 and 4.8 (from Results section) and in Table 4.10. It is unclear whether the deviation of the Lineweaver-Burk plot at low dilution rate is due to the alternative lactose transport systems suggested by Vienne and von Stockar (1983), or because the yeast was more sensitive to any fluctuation of the dilution rate during the fermentation, or if it is only an artificial effect which can be smoothed out with the Langmuir approach. Further work is required to investigate the behaviour of the yeast and the lactose transport system at low dilution rates.

The half saturation constant (K_s) value obtained in this work (4.94 g/l) using the Langmuir plot was considerably higher than the values reported by Vienne and

Table 4.10 Summary of μ_{\max} and K_s values obtained from Lineweaver-Burk and Langmuir plots

Authors	Plot	μ_{\max} (h^{-1})	K_s (g/l)	R square(%)
Vienne and von Stockar (1983)	Lineweaver-Burk	0.31 ^a	0.97	99
	Langmuir	0.29	0.29	100
Vienne and von Stockar (1985b)	Lineweaver-Burk	0.13	0.23	95
	Langmuir	0.14	0.51	100
This work (1993)	Lineweaver-Burk	0.15	1.92	91
	Langmuir	0.21	4.94	96

^a The value obtained by these authors with half of the points (three) omitted at $D= 0.176, 0.200,$ and 0.220 h^{-1} .

von Stockar (1983 and 1985b). However Ruggeri *et al.* (1987) also reported higher K_s values of 9-10 g/l from batch whey fermentation using *K. fragilis* CBS 5795. The difficulty of accurately estimating K_s value is widely recognised.

The Heijnen approach is based on q_s (specific substrate uptake) rather than μ and gave a poor fit in all cases.

On the basis of the μ_{\max} value obtained from continuous culture, the fermentation performance of strain Fi in unsupplemented whey is better than that of *K. fragilis* NRRL Y-665 used by Vienne and von Stockar (1985b) also grown on unsupplemented whey. Currently, at the Tirau distillery, a dilution rate of 0.07 h^{-1} is used. The distillery might instead opt for the dilution rate where ethanol productivity is maximum, i.e $D = 0.10 \text{ h}^{-1}$. A 30% increase of throughput could be expected when operating at a dilution rate of 0.10 h^{-1} .

In conclusion, it has been demonstrated that the production yeast is a strain of *K. marxianus*. The fermentation performance is as good or better than many other *K. marxianus* strains, with a μ_{\max} value of 0.21 h^{-1} in unsupplemented lactic whey serum. Mixed cultures have been shown to offer no advantage and the lactic acid present naturally in the whey has no effect on the fermentation performance. High lactate concentrations from gross bacterial contamination or concentration of the whey (with concomitantly higher lactose concentrations) also do not affect the fermentation, in contrast to the results observed with *S. cerevisiae* in other ethanolic fermentations. The organism is thus well suited to its application but, clearly, fermentation performance can be improved by nutrient supplementation of the whey.

CHAPTER 5

AEROBIC YEAST CULTIVATION ON STILLAGE

5.1 INTRODUCTION

Lactic acid is a normal constituent of the lactic acid casein whey processed at the Tirau site of Anchor Ethanol Co. The acid is present at a concentration of approximately 7 g/l and is not used during the fermentation process. The liquid effluent, called stillage or slops, remaining after distillation of ethanol from the fermented beer presents a significant disposal problem for the distillery. It is acidic in nature, in part due to its lactic acid content, with a typical pH of 4.0-4.5, and requires addition of lime to adjust the pH value to 6.5-7.0 prior to further treatment in the waste ponds and subsequent final discharge into an adjacent stream.

The use of stillage as a substrate for microbial biomass production is a promising disposal method. The stillage from alcohol production from Jerusalem artichokes, containing mainly fructose and protein, has been used for production of *S. cerevisiae* biomass (Kosaric *et al.* 1989). Stillage originating from alcohol production from cane molasses has also been used for bacterial biomass manufacture (Kumar and Viswanathan, 1991). Lactic acid is considered a good carbon source for many microorganisms and its potential as a carbon source for yeast SCP production has been evaluated by several authors (Lembke *et al.* 1975; Ruiz *et al.* 1978; Champagne *et al.* 1989, 1990). However the market for yeast biomass in New Zealand is limited, therefore an alternative use for the lactate in whey distillation slops, such as growing the yeast inoculum for the whey ethanolic fermentation, is more appropriate.

The objective of this work was to examine the aerobic growth of the Tirau production yeast strain on slops, in batch and continuous culture, and to evaluate this yeast as an inoculum for the subsequent anaerobic whey fermentation.

5.2 MATERIALS AND METHODS

5.2.1 Organism

The production yeast *K. marxianus*, strain Fi, was used throughout the study.

5.2.2 Fermentation and Growth Media

Lactic acid casein whey permeate (LACWP) powder, LACWS, YEP lactate and YEPLL media were as described in Section 3.1.1.1 and 3.3.1.3, respectively. The slops were obtained mainly from Anchor Ethanol Co., Tirau, with a composition as shown in Table 3.2. Slops were also produced in small quantities by the following procedure: two litres of autoclaved LACWS were fermented by *K. marxianus* Fi yeast in shake flasks. The fermented beer was distilled in a pilot scale packed column within the Biotechnology Department to recover the stillage or slops, followed by rotary evaporation to remove further ethanol. The slops produced contained little ethanol (approximately 0.5 g/l), about 8 g/l lactose and 9 g/l lactic acid.

5.2.3 Maintenance Media

Stock cultures of Fi yeast were grown on YEP lactose agar and YEP lactate agar slants previously autoclaved at 121°C for 15 minutes. The composition of YEP lactose agar medium was as follows: yeast extract 5 g/l, proteose peptone 3 g/l, lactose 50 g/l, and agar 20 g/l. The composition of YEP lactate agar was similar to YEP lactose agar except that lactose was replaced by 7 g/l sodium lactate.

5.2.4 Chemical Analysis

Analysis of ethanol, lactose and lactate were performed as described in Section 3.3.4, 3.3.5, and 3.3.6, respectively. Biomass concentration was determined using the oven-drying method described in Section 3.3.2.2

5.2.5 Fermentation operation

Batch, shake flask, aerobic fermentations were conducted as described in Section 3.4.1. Continuous growth experiments and some batch culture experiments were run in the 2-litre benchtop fermenter following the procedure outlined in Section 3.4.2. This unit was also coupled to the 14-litre fermenter (10 l working volume; Section 3.4.2.4) in a dual-stage fermentation comprising aerobic yeast growth and anaerobic ethanol production. All fermentations were conducted at 30°C. No pH control was carried out but the value was measured.

5.2.6 Data analysis

Modified Gompertz and logistic equations were fitted to the biomass data for batch experiments to determine the maximum specific growth rate (μ_{\max}) of the production yeast strain grown on lactate. The Gompertz and logistic equations describe a sigmoidal growth curve and are usually written in terms of mathematical parameters (a,b,c,...) rather than as parameters with a biological meaning. Zwietering *et al.* (1990) reparameterised the Gompertz and logistic equations to include the biological parameters A, μ_{\max} , and λ . The term A is found from the ratio of final and initial population size ($A = \ln(N_{\infty}/N_0)$) and λ is the lag time. The modified Gompertz and logistic equations are shown in equations 5.1 and 5.2, respectively.

$$y = A \exp \left\{ - \exp \left[\frac{\mu_{\max} \cdot e}{A} (\lambda - t) + 1 \right] \right\} \quad \text{Equation 5.1}$$

$$y = \frac{A}{1 + \exp \left[\frac{4\mu_{\max}}{A} (\lambda - t) + 2 \right]} \quad \text{Equation 5.2}$$

where $e = \exp(1)$

The values (A , μ_{\max} and λ) were obtained by non-linear regression using the LEASTSQUARE subroutine of MATLAB (The Mathworks Inc., South Natick, MA, USA). The program uses a Gauss-Newton method with a cubic polynomial search algorithm and is contained within the MATLAB Optimization Toolbox.

Continuous culture data were plotted as outlined in Section 3.6 and kinetics constants determined by linear regression using Minitab.

5.3 RESULTS

5.3.1 Aerobic growth of *Fi* yeast on lactate in batch culture

5.3.1.1 Growth in semi-synthetic medium.

The production yeast strain was grown aerobically in YEP lactate medium containing 7 g/l of a mixture of D- and L- lactate. The medium was inoculated by direct transfer of yeast from either YEP lactose or YEP lactate agar slants using a loop. Figure 5.1 shows that L-lactate was exhausted by the yeast after

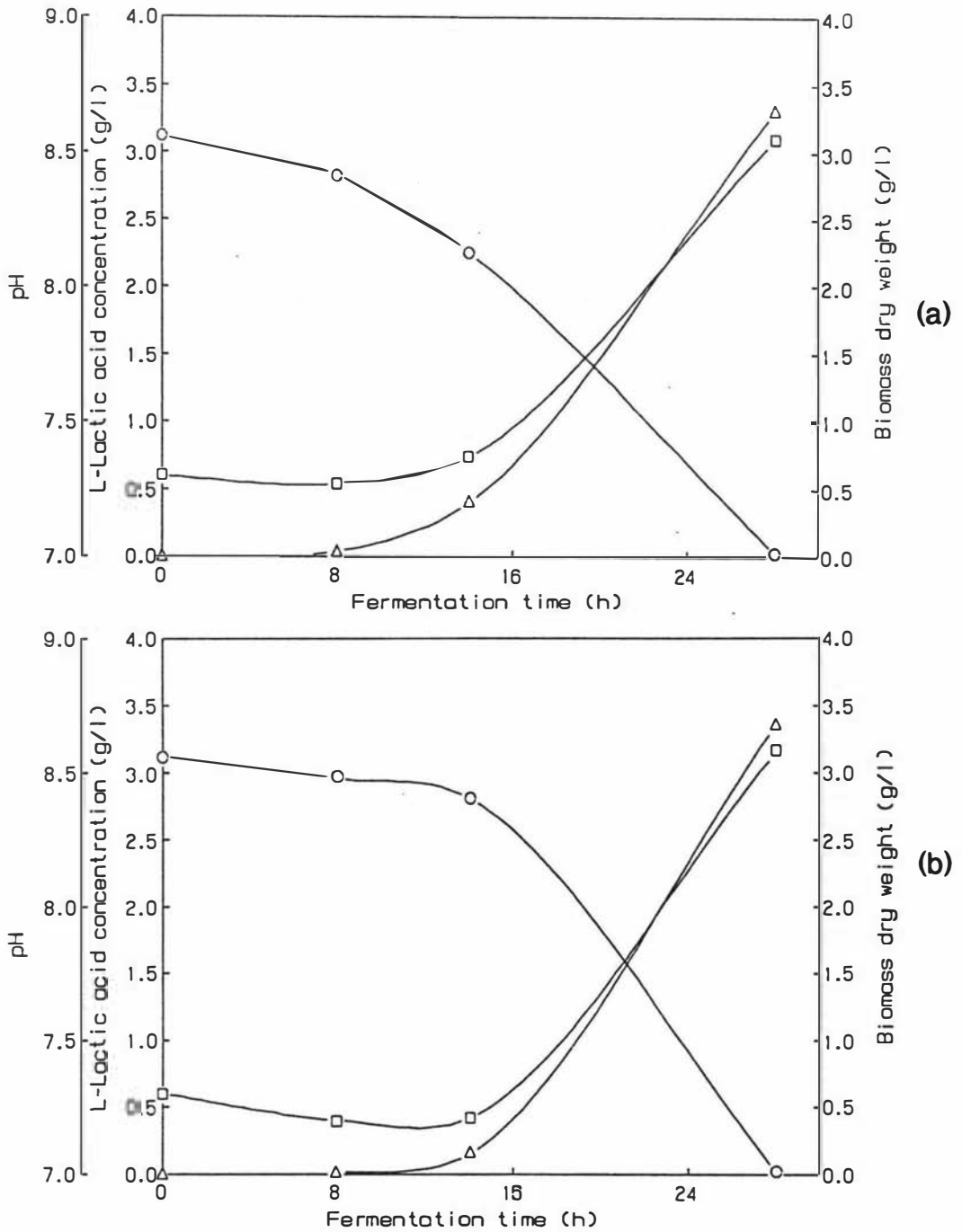


Figure 5.1 Aerobic growth of strain *Fi* on YEP lactate with (a) lactose-pregrown yeast and (b) lactate-pregrown yeast : ○ L-lactic acid; △ biomass; □ pH.

28 h of cultivation. There was no apparent difference in the lactate utilization between the lactose- and lactate-pregrown yeast. The biomass concentration after 28 h was 3.3 g/l, giving a yield coefficient (Y_{xs}) of 0.48 g/g. D-lactate was not measured in this experiment as initial samples of lactic whey serum obtained from the ethanol production plant contained predominantly L-lactic acid.

When lactose at 50 g/l was incorporated into the medium, it was observed that the lactose-pregrown yeast utilized lactose completely, but only approximately 40% of the L-lactate was utilized during the 28 h of the growth process. A biomass concentration of 5.8 g/l was obtained (Figure 5.2a). In comparison, using lactate-pregrown yeast, lactose was also utilized almost completely but only 14% of the L-lactate was utilised. A slightly lower biomass concentration of 5.5 g/l was obtained (Figure 5.2b). It appeared that in both cases, the yeast used lactose preferentially to lactic acid, but two distinct growth phases were not observed.

5.3.1.2 Growth on slops supplemented with yeast extract

A slops sample obtained from Anchor Ethanol Co. was used to grow strain Fi aerobically in shake flasks. The composition of slops indicated that there was a possible nitrogen limitation in the medium (Section 3.1). Therefore, addition of yeast extract at 1 and 2 g/l, in successive experiments, was investigated. When yeast extract was supplemented at 1 g/l, lactate was utilized completely after 14 h of cultivation, compared to 16 h using the unsupplemented slops (Figure 5.3a). A similar effect was observed for supplementation with 2 g/l yeast extract (Figure 5.3b). Biomass could not be determined accurately due to the presence of a calcium salt precipitate. At pH values above 5.5, a precipitate, presumably calcium apatite, formed in the medium and this appeared to increase the absorbance readings which led to overestimation of biomass concentrations.

Experiments were also conducted using a second batch of slops obtained from

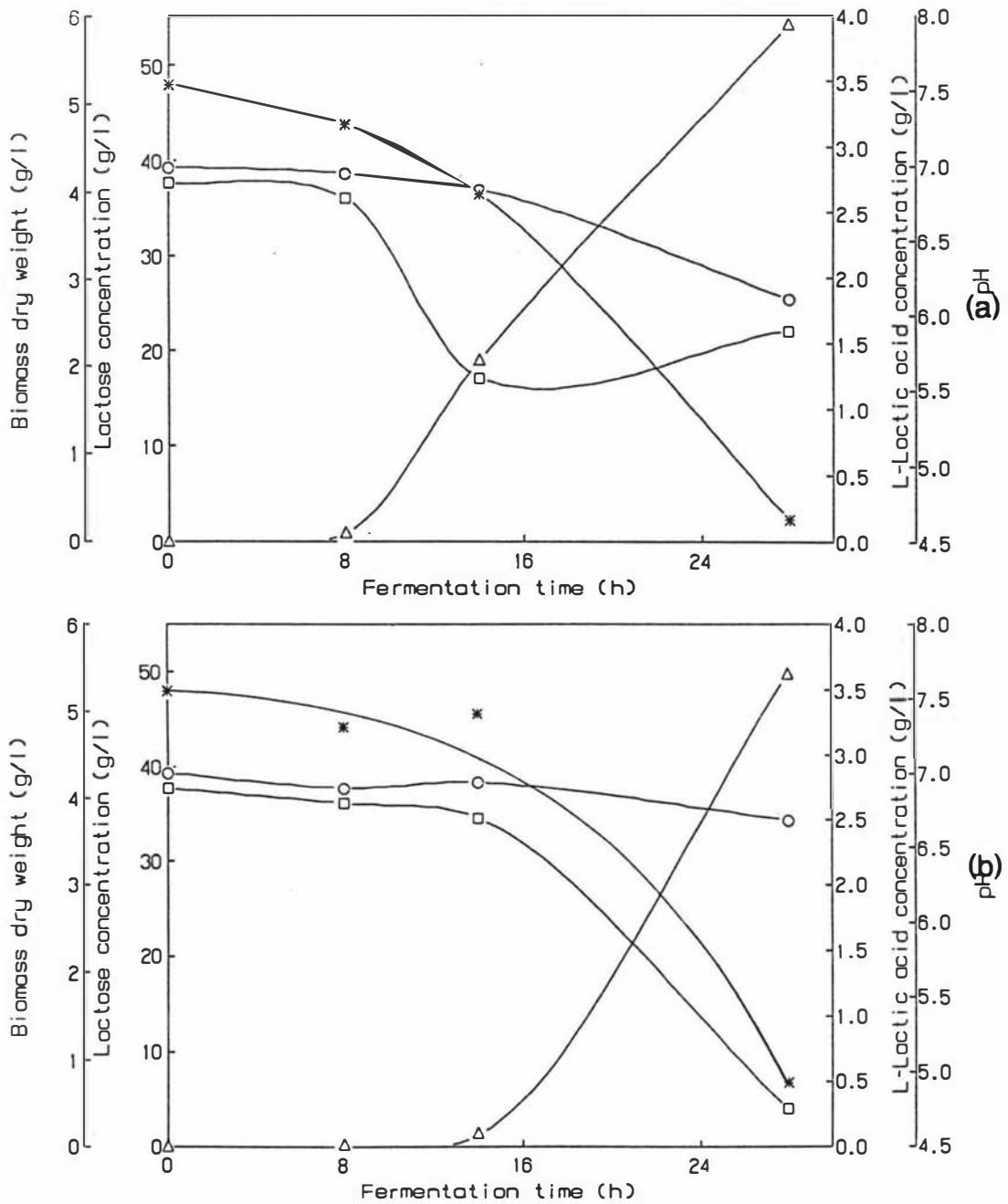


Figure 5.2 Aerobic growth of strain Fi on YEPLL with (a) lactose-pregrown yeast and (b) lactate-pregrown yeast : \circ L-lactic acid; Δ biomass; \square pH; $*$ lactose.

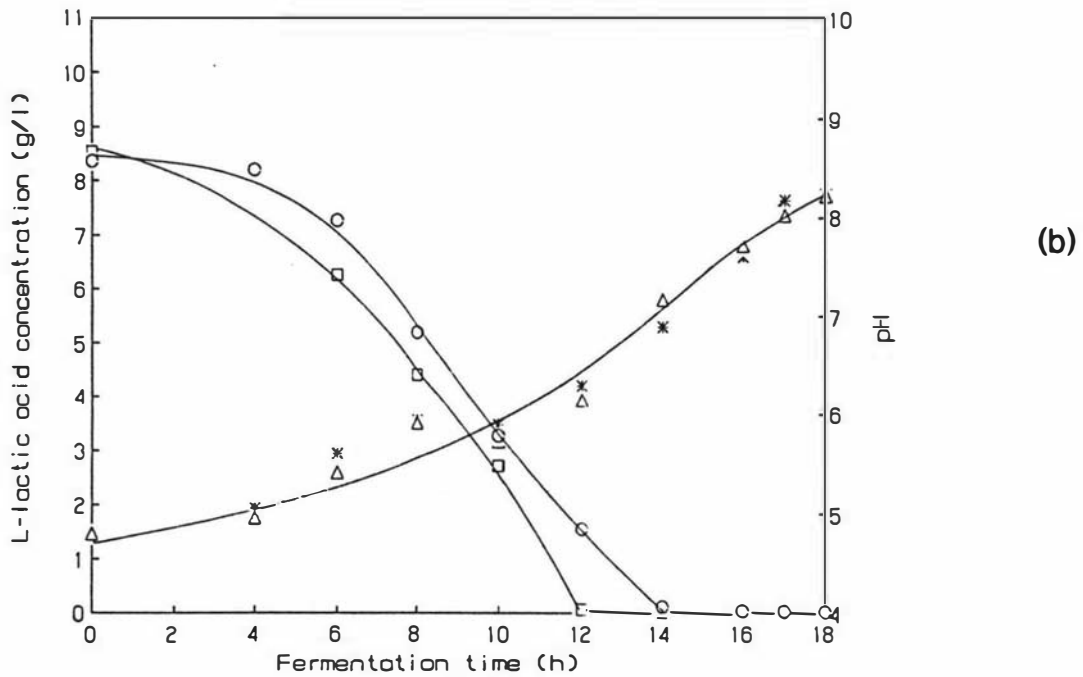
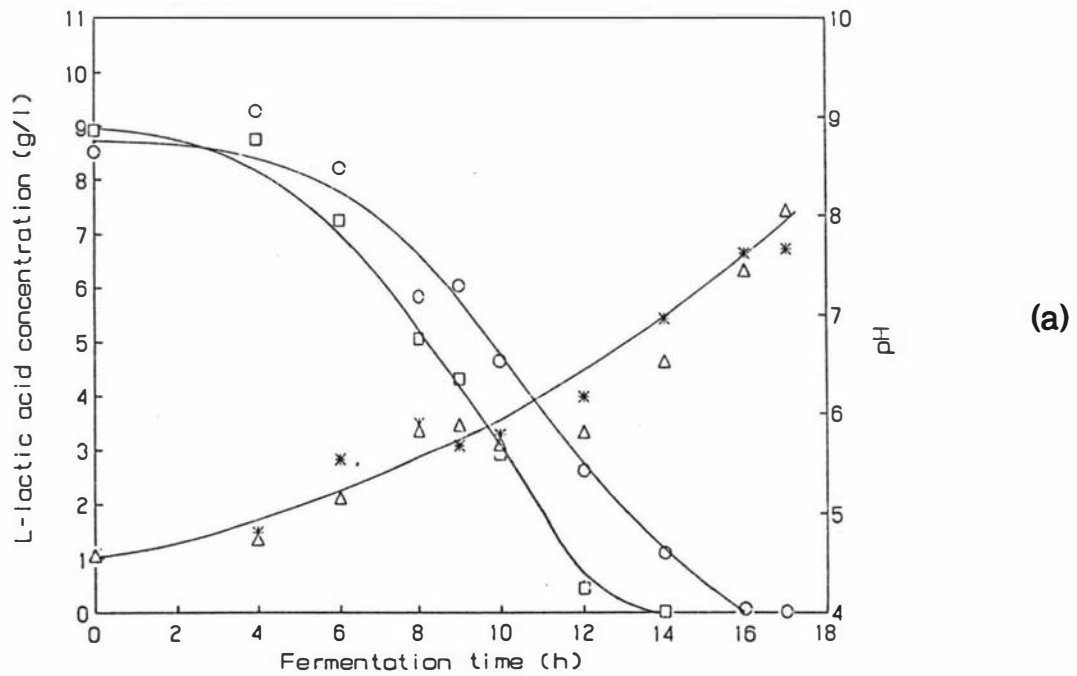
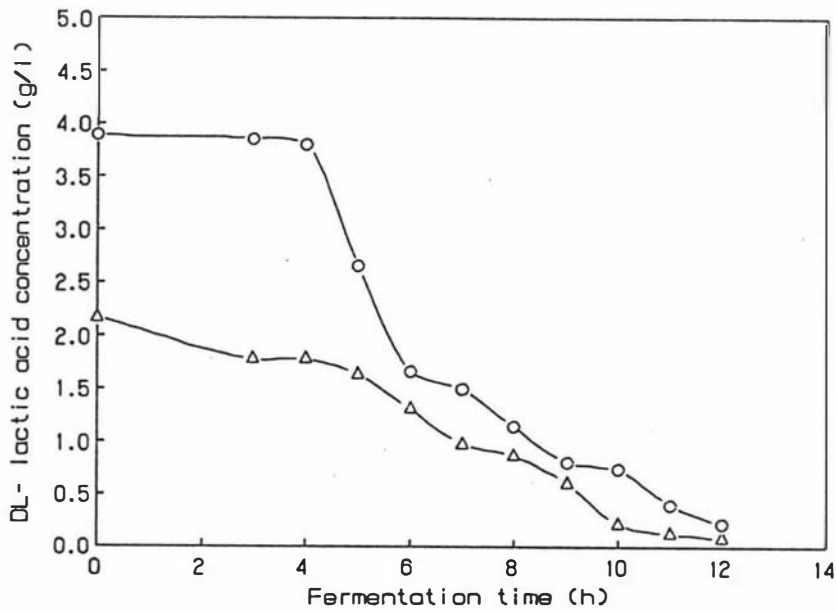


Figure 5.3 Effect of addition of yeast extract on the growth of *Fi* yeast on slops.
 (a) 1 g/l YE : o control lactate; Δ control pH; □ 1 g/l YE lactate; * 1g/l YE pH)
 (b) 2 g/l YE : o control lactate; Δ control pH; □ 2 g/l YE lactate; * 2 g/l YE pH).

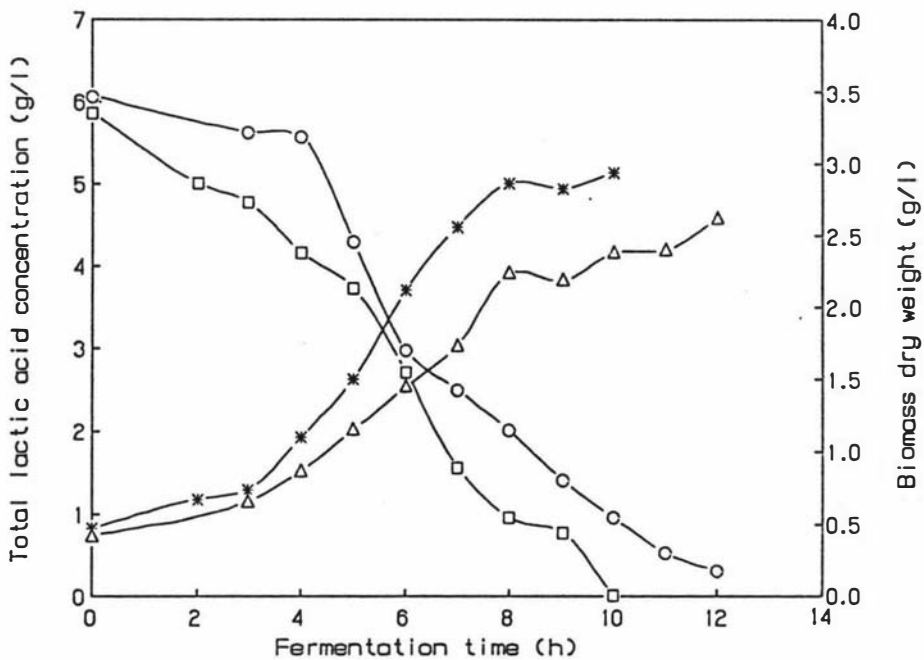
Anchor Ethanol Co. In the previous batch, lactic acid was present predominantly as the L-isomer. However, a different mixed starter culture had been used to prepare the casein from which the second batch of slops was derived, and this produced both D- and L-lactic acids. The 2-litre fermenter was used in this experiment and the effect of yeast extract at a concentration of 1 g/l on the growth of the yeast was again examined. For accurate biomass determinations, the cells were separated from calcium apatite by washing with pH 4.0 citrate buffer. For growth on unsupplemented slops, after 12 h of cultivation, a final biomass dry weight of 2.62 g/l was obtained and the pH was 8.15. Almost all the lactic acid (both D- and L-isomers) was consumed to give a biomass yield of 0.4 g/g. L-lactate was utilized faster than D-lactate during the first 6 h of growth (Figure 5.4a). The biomass yield compared favourably with the value of 0.48 g/g using YEP lactate, assuming that D-lactate was also utilised completely. A faster rate of fermentation was observed when the slops were supplemented with yeast extract at 1 g/l (Figure 5.4b). No residual lactic acid was detected after 10 h of growth and a biomass yield of 0.54 g/g was obtained. For growth on unsupplemented slops, the modified Gompertz and logistic equations both estimated a μ_{\max} value of 0.29 h⁻¹. For growth on yeast extract-supplemented slops, a μ_{\max} of approximately 0.36 h⁻¹ was obtained (Table 5.1).

Table 5.1 Estimated μ_{\max} values for strain Fi grown on supplemented and unsupplemented slops

	Gompertz equation	Logistic equation
Unsupplemented slops	0.29 h ⁻¹	0.29 h ⁻¹
Supplemented slops (1 g/l yeast extract)	0.35 h ⁻¹	0.37 h ⁻¹



(a)



(b)

Figure 5.4 (a) The rate of L- and D-lactate utilization in the unsupplemented slops : o L-lactate; Δ D-lactate (b) The effect of supplementation of yeast extract on the growth of yeast aerobically on slops : o control lactate; Δ control biomass; \square 1 g/l YE lactate; * 1 g/l YE biomass.

5.3.2 Aerobic growth of *Fi* yeast on slops in continuous culture

5.3.2.1 The effect of dilution rate and yeast extract

Slops were obtained from Anchor Ethanol Co. and frozen for storage. The frozen slops were thawed prior to autoclaving at 121°C. In this series of runs, the dilution rate was varied in an attempt to evaluate the kinetic parameters of the yeast for biomass production. Four dilution rates were used : $D= 0.15 \text{ h}^{-1}$, 0.20 h^{-1} , 0.25 h^{-1} and 0.30 h^{-1} which corresponded to residence times of 6.7 h, 5 h, 4 h and 3.3 h. A steady state condition was normally assumed after three residence times, when the biomass dry weight, residual lactic acid and culture pH remained constant ($\pm 10\%$). Fermentations were generally completed after four to five residence times. The steady state results of runs at the four dilution rates are summarised in Table 5.2 and plotted in Figure 5.5.

The trend of the steady state plot shows that biomass concentration decreased with increased dilution rate. The residual lactic acid concentration increased with increasing dilution rate and this was reflected by the decreased pH of these cultures. However, the biomass productivity values were similar for all dilution rates, indicating that the yeast can assimilate the substrate at higher dilution rates as efficiently as at lower dilution rates. This suggests a possible nutrient limitation.

The results from this set of continuous runs were analyzed using a Lineweaver-Burk plot (Figure 5.6). Linear regression gave values for μ_{\max} of 0.266 h^{-1} and K_s of 0.14 g/l . Other approaches such as those of Eadie and Hofstee, Langmuir and Heijnen were also used to determine the μ_{\max} and K_s values and the data are shown in Table 5.3. The apparent best fit of μ_{\max} was obtained using the Langmuir approach. This confirms the finding of the previous chapter that the continuous culture data are best fitted using the Langmuir approach. The estimated μ_{\max} value of 0.30 h^{-1} was slightly lower than expected, since in continuous culture it was possible to operate at $D= 0.30 \text{ h}^{-1}$. Hence, the

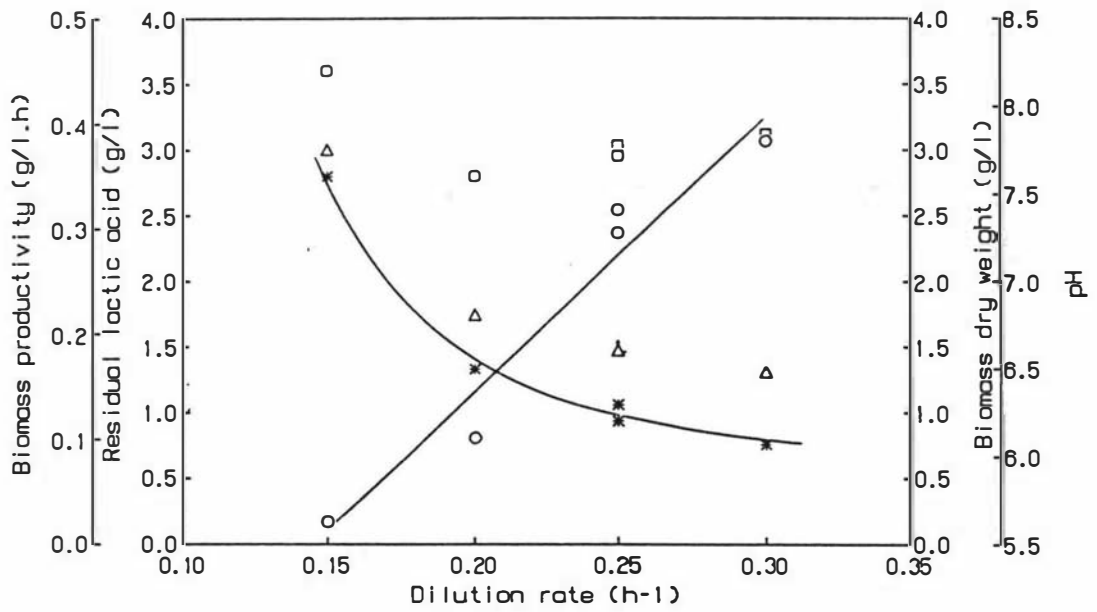


Figure 5.5 Steady state parameters of the continuous runs of aerobic yeast growth on unsupplemented slopes : \circ Lactic acid; Δ biomass; \square productivity; $*$ pH.

Table 5.2 : Summary of steady state parameters for continuous aerobic yeast growth of *K. marxianus* strain Fi on slops.

Dilution Rate (h ⁻¹)	Influent Lactic acid (g/l)	Residual lactic acid (g/l)	Biomass dry weight (g/l)	Biomass productivity (g/l.h)	Biomass yield (g/g)	pH	Comment
0.15	7.0	0.17	3.00	0.45	0.44	7.6	
0.20	6.3	0.81	1.74	0.35	0.32	6.5	
0.25	6.5	2.54	1.47	0.37	0.37	6.3	
0.25	6.3	2.36	1.50	0.38	0.38	6.2	repeated run at the same dilution rate
0.25	8.4	2.08	2.10	0.53	0.33	6.0	Coupling to the whey fermentation
0.25	6.3	0.14	2.38	0.60	0.39	6.7	Supplemented with 1 g/l yeast extract
0.30	6.5	3.07	1.30	0.39	0.38	6.07	

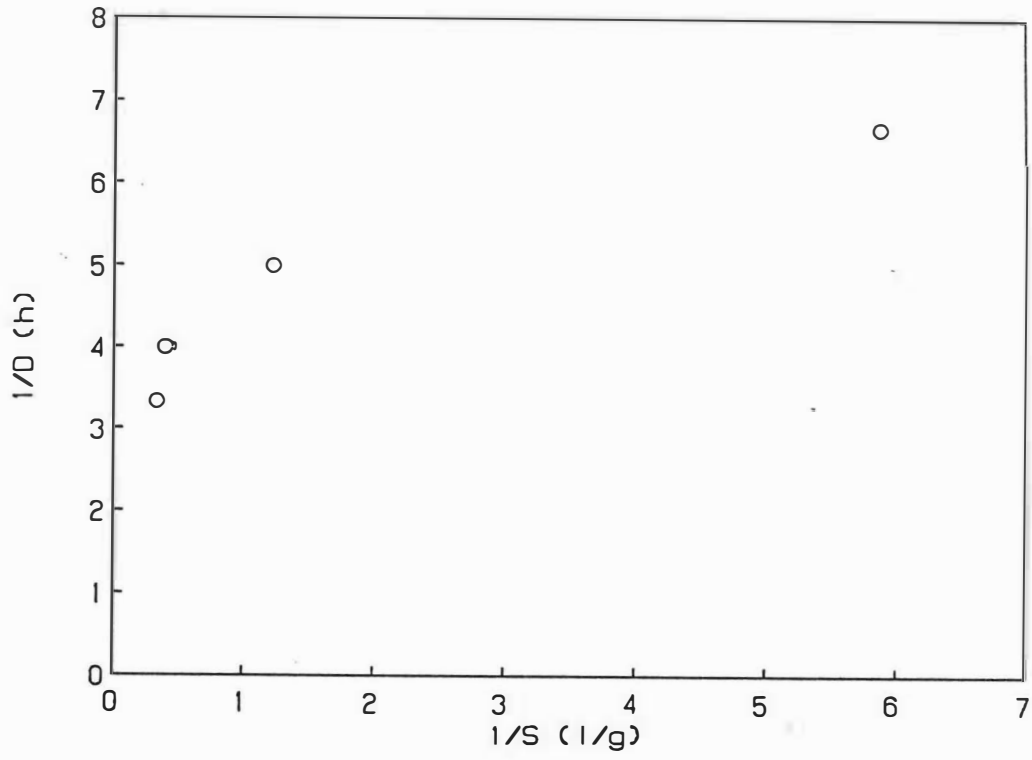


Figure 5.6 Lineweaver-Burk plot of continuous aerobic yeast growth on slops

maximum specific growth rate must be greater than 0.30 h^{-1} . However, given the maximum specific growth rate of 0.29 h^{-1} obtained from the batch culture, the true μ_{\max} of the continuous culture is probably very close to this value. Removing the outlying point at $D = 0.15 \text{ h}^{-1}$ resulted in an increase of μ_{\max} values to 0.31 h^{-1} and 0.34 h^{-1} using Lineweaver-Burk and Langmuir plots, respectively.

Table 5.3 : Determination of μ_{\max} from continuous growth data on slops using four different approaches

Plot	$\mu_{\max} (\text{h}^{-1})$	$K_s (\text{g/l})$	R_{square}
Monod	0.266	0.14	89%
Eadie and Hofstee	0.272	0.15	75.2%
Langmuir	0.301	0.32	97%
Heijnen	0.29	0.21	97.8%

The effect of addition of yeast extract to the slops for the continuous production of yeast biomass was investigated. Yeast extract at 1 g/l was added to the slops, and the culture was operated at $D = 0.25 \text{ h}^{-1}$. Supplementation with yeast extract resulted in complete utilization of lactic acid and the biomass productivity of 0.6 g/l.h was almost double that observed using unsupplemented slops at the same dilution rate (Table 5.2). The culture pH was 6.7 compared to $6.2-6.3$ during the runs using unsupplemented slops.

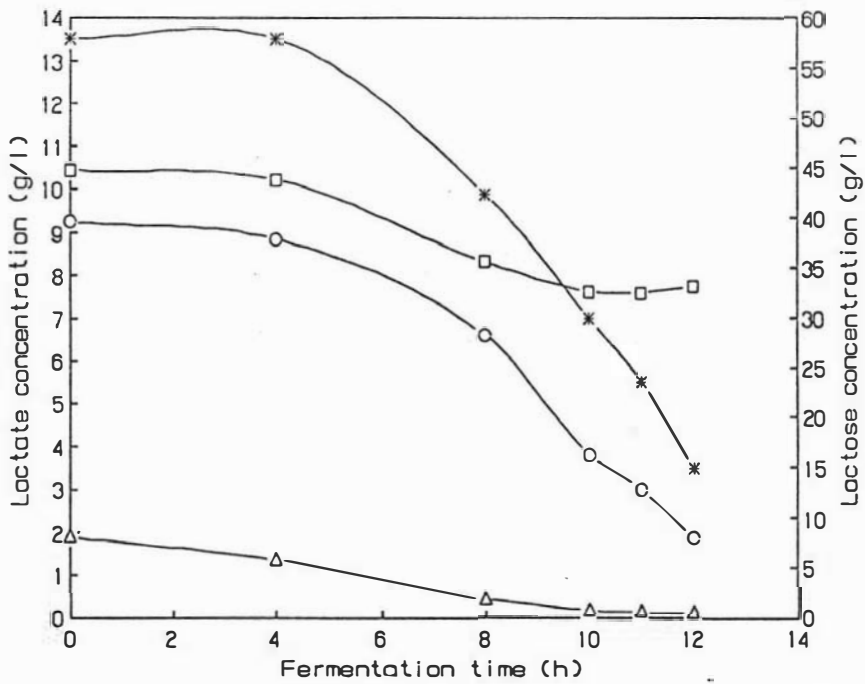
5.3.3 Growth of Fi yeast on slops as an inoculum for serum ethanolic fermentation

5.3.3.1 Slops-grown inoculum for batch fermentations

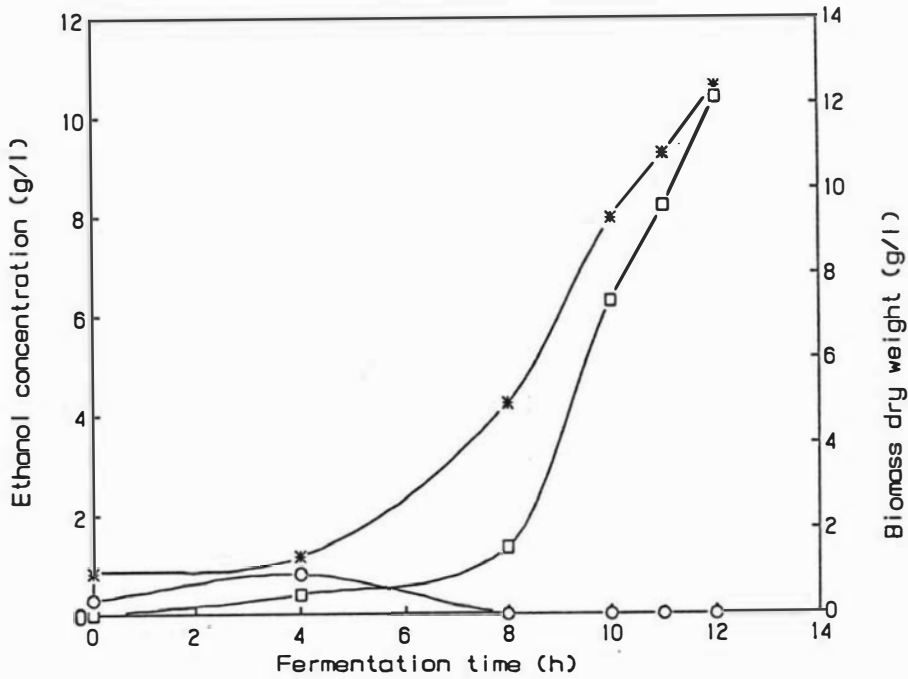
Strain Fi yeast was grown aerobically on slops, prepared as described in Section

5.2.1. In a parallel experiment, *Fi* yeast was also grown aerobically on lactic serum to provide a comparison of inoculum sources. Both the slops and lactic serum media were pasteurised at 85°C for 25 seconds prior to inoculation. During growth on slops approximately 80% of the L-lactate available was metabolized by the yeast after 12 h of cultivation, compared to only 26% in the lactic serum. The lactate available in both media was predominantly L-lactate and less than 0.3 g/l of D-lactate was present. L-lactate utilization ceased in the lactic serum medium after 10 h, but lactose consumption continued, so that 74% was utilized after 12 h of growth (Figure 5.7a). When both lactose and L-lactate were present at low concentration (8 g/l and 9 g/l respectively) in slops, lactose was metabolized rapidly and simultaneously with lactate (Figure 5.7a). During the aerobic cultivation a small amount of ethanol accumulated in the slops, but disappeared after 8 h, presumably being consumed by the cells. As lactate was utilized in the slops the culture pH increased to 6.8 from an initial value of pH 4.6. This compared to a final pH value of 5.2 for growth of the yeast in lactic serum (Figure 5.7b). Also, as the pH of the medium increased, there was formation of a calcium salt precipitate, and the biomass could not be determined accurately (the technique of acid washing reported in the previous section was being evaluated at this time).

After 12 h of aerobic growth, when the yeast cells were still actively growing, the cultures were used to inoculate anaerobic serum fermentations. In an attempt to ensure that the fermentations commenced with a similar initial biomass concentration, as estimated from the absorbance curve, a 7.5%(v/v) inoculum from the slops culture was used, compared to a 5%(v/v) inoculum from the serum culture. The 81% ethanol yield of theoretical obtained using the inoculum pregrown on serum, was similar to the value of 84% obtained with the inoculum pregrown on slops. However, the cultures pregrown on serum exhibited slightly faster ethanol production throughout the fermentation (Figure 5.8a). A possible explanation for this is that a smaller yeast inoculum was obtained from the slops culture, due to difficulties of estimating the biomass concentration. Overall, however, there appeared to be no major difference in ethanol production

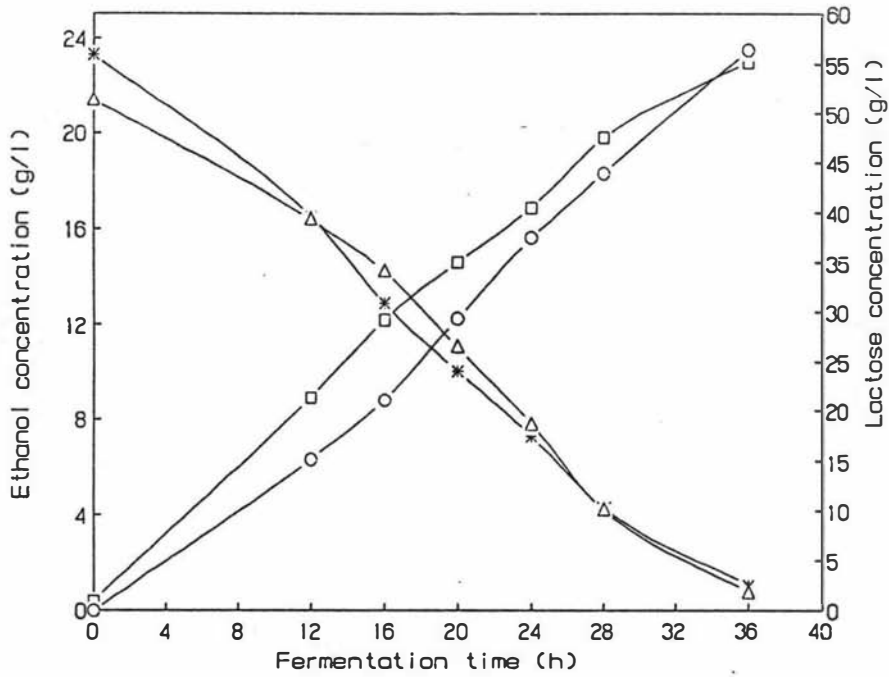


(a)

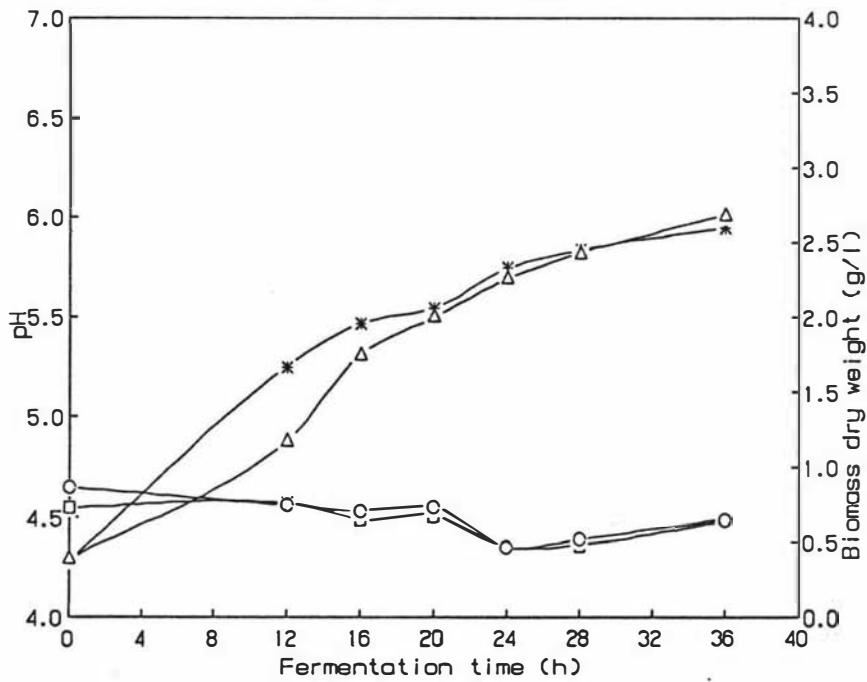


(b)

Figure 5.7 Aerobic growth of strain Fi on slops and LACWS. (a) Lactate and lactose profiles : o slops lactate; Δ slops lactose; □ serum lactate; * serum lactose (b) Ethanol and biomass profiles : o slops ethanol; □ serum ethanol; * serum biomass.



(a)



(b)

Figure 5.8 Fermentation of LACWS with yeast inoculum pregrown on slops and serum. (a) Ethanol and lactose profiles : \circ ethanol ex slops; Δ lactose ex slops; \square ethanol ex serum; $*$ lactose ex serum. (b) Biomass and pH profiles : \circ pH ex slops; Δ biomass ex slops; \square pH ex serum; $*$ biomass ex serum.

capability between the inoculum yeast pregrown on lactose or lactate sources. The culture pH during the anaerobic fermentation did not differ markedly, and the biomass concentration obtained in both systems was 2.6 g/l after 36 h of fermentation (Figure 5.8b).

5.3.3.2 Slops-grown inoculum for continuous whey fermentations

The objective of this work was to confirm that yeast pregrown on lactic acid in slops behaved in a similar manner in continuous culture to a lactose-pregrown yeast. Continuous whey fermentation was conducted at $D = 0.10 \text{ h}^{-1}$ in LACWP medium. A 79% ethanol yield of theoretical and an ethanol productivity of 1.51 g/l.h were achieved in this experiment compared to a yield of 72% and a productivity of 1.41 g/l.h, with the lactose-pregrown yeast grown at the same dilution rate on the same medium (Table 5.4).

5.3.4 Continuous inoculum growth on slops coupled with continuous whey fermentation.

A dual stage fermentation process was conducted as shown in Figure 3.1 in Section 3.4. In the first stage, yeast biomass was grown aerobically on slops and 5% of the effluent was fed to the second fermenter for ethanol production from lactic whey serum. The remainder of the yeast effluent was discarded. Prior to entering the second fermenter, the yeast suspension merged with the fresh serum feed. These flow conditions duplicated the real industrial environment at the Anchor Ethanol distillery, Tirau. The lactic whey serum fermentation was run at $D = 0.10 \text{ h}^{-1}$, with or without yeast feeding, and the results are plotted in Figure 5.9 and tabulated in Table 5.4. The ethanol fermentation was run independently until it stabilized. After three residence times, all the major parameters (biomass dry weight, residual lactose and ethanol concentration) were stable and a 78% ethanol yield of theoretical, and an ethanol productivity of 1.65 g/l.h, were

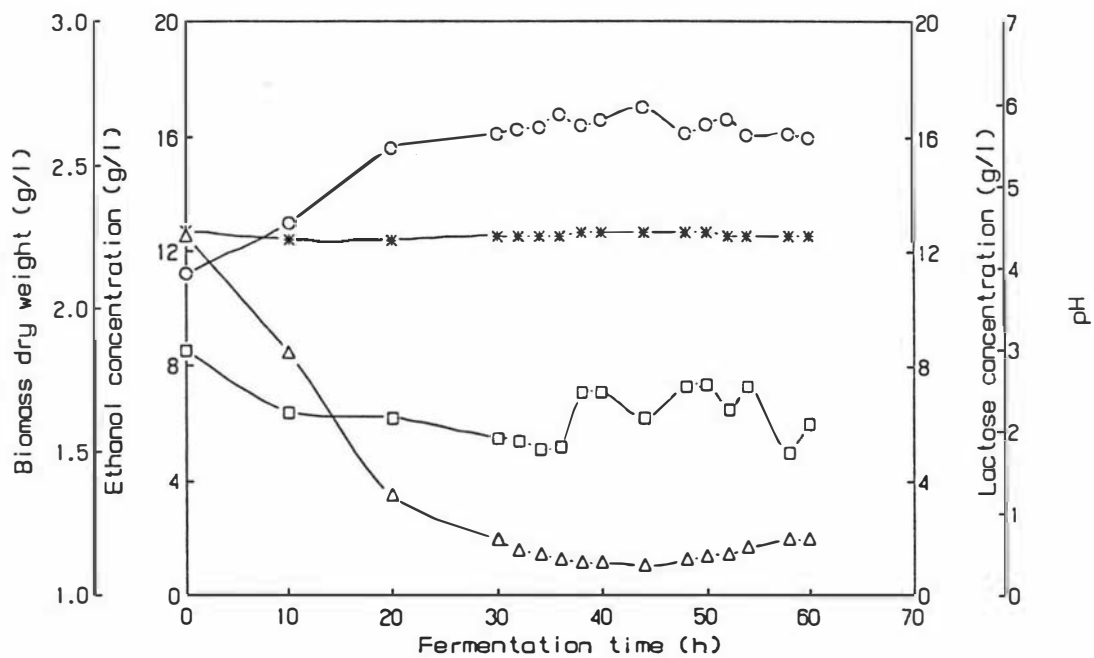


Figure 5.9 Continuous LACWS fermentation at $D = 0.10 \text{ h}^{-1}$ coupled with the aerobic yeast growth on slops : \circ ethanol; Δ lactose; \square biomass; $*$ pH.

Table 5.4 : Summarized steady state parameters from continuous lactic whey fermentations at $D = 0.1\text{h}^{-1}$

Medium	Inoculum	Influent lactose (g/l)	Lactose utilized (g/l)	Ethanol concentration (g/l)	Biomass dry weight (g/l)	% ethanol yield of theoretical	Productivity (g/l.h)
LACWP	Lactose-grown	43	36.6	14.1	1.40	72	1.41
LACWP	Lactate-grown	46	35.5	15.1	1.66	79	1.51
LACWS	Lactose-grown	40	38.6	16.5	1.59	78	1.65
LACWS	Coupling with aerobic yeast growth on slops	40	38.5	16.3	1.63	78	1.63

obtained. In the aerobic fermenter, operating independently at a dilution rate of 0.25 h^{-1} , the biomass concentration stabilised after three residence times at 2.1 g/l . This batch of slops had a higher influent lactic acid concentration value compared to the previous batches used in the continuous culture (refer Table 5.2). After 26 h of running independently, the yeast grown in the first vessel was fed to the ethanol fermenter which had then been run for 40 h. For two residence times (20 h), the serum fermentation was monitored closely to observe any changes in performance. The previous ethanol yield and productivity were maintained, and the culture pH was also unchanged. It appeared that coupling of aerobic growth on slops and anaerobic serum fermentation was successful.

It was apparent during these experiments that the use of different batches of sera and powders influenced the continuous fermentation behaviour of the yeast. At the same dilution rate of 0.10 h^{-1} , but using a different batch of lactic whey serum, an ethanol productivity of 1.27 g/l.h was obtained (Chapter 4) compared to 1.63 g/l.h obtained in this section.

5.4 DISCUSSION AND CONCLUSIONS

There is some evidence to suggest that the relative concentrations of the carbon sources may have an influence on the pattern of the carbon uptake. In the present work it was observed that in the presence of two carbon sources i.e. lactose and lactate at concentrations of 50 g/l and 7 g/l , respectively, lactate assimilation was repressed to some extent. However, when equal proportions of lactose and lactate were present (9 g/l and 8 g/l , respectively), the carbon sources were utilized simultaneously, although lactose was assimilated more rapidly. Champagne *et al.* (1990) reported that when *S. cerevisiae* was cultivated in the presence of two substrates, galactose and lactate, at concentrations of 18 g/l and 25 g/l , respectively, galactose was assimilated much more rapidly than lactate. Cassio *et al.* (1987) reported that when *S. cerevisiae* was grown in a lactic acid medium, lactate was transported by active transport and was subject

to glucose (catabolite) repression. Thus, growth in a medium containing glucose and DL-lactic acid at concentrations of 2 g/l and 5 g/l, respectively, was diauxic. Glucose was metabolized rapidly, and, upon its exhaustion, lactic acid was metabolized. Hence two distinct growth phases were observed. It is presumed that the lactate transport system in *Kluyveromyces* yeast operates similarly, and there is evidence to show that it is subject to catabolite (lactose) repression.

Although the μ_{\max} obtained from the Lineweaver-Burk plot based on Monod kinetics, indicated a value of 0.266 h^{-1} , the linear regression analysis did not show a good fit. The use of other approaches improved the statistical fit and increased the μ_{\max} value to 0.30 h^{-1} . This confirms that the Langmuir plot gives a better fit than the Lineweaver-Burk plot, as shown in the previous chapter, although in this case the Lineweaver-Burk plot using q_s data (Heijnen approach) was more successful than before. For continuous culture, a μ_{\max} value of 0.3 h^{-1} still appeared low since stable operation without washout could be maintained at this dilution rate. However, the value was in agreement with that obtained from batch fermentation data. Ignoring the outlying point at the lowest dilution rate (0.15 h^{-1}) resulted in an increased μ_{\max} value. The reason for this outlying point at low dilution rate was presumably due to sample handling. Samples were generally collected from the effluent line and if substrate continued to be utilized, a small change in the residual substrate concentration would alter the presentation of data dramatically, particularly in the Lineweaver-Burk plot. Moulin *et al.* (1983) reported that a *K. fragilis* strain isolated from the Bel yeast biomass plant at Verdun, France, could grow on lactic acid at growth rates up to 0.25 h^{-1} .

Over the range of dilution rates evaluated, there appeared to be no clear maximum biomass productivity. Therefore a wide range of dilution rates can be used. To achieve a high throughput, a dilution rate closer to μ_{\max} should be used but this would be at the expense of high residual lactic acid and lower biomass yield. Dilution rates of $0.20\text{-}0.25 \text{ h}^{-1}$ are the options to consider.

There is a possibility that the different operating pH values of the continuous culture experiments might have affected the transport of lactate. For all

experiments with yeast growing on slops, in batch or continuous culture, the pH was not controlled. It is undesirable to attempt to control pH unless it is demonstrated to be essential for the yeast performance in the ethanolic fermentation. As this does not appear to be so, economic and practical reasons favour no adjustment.

The effect of pH on lactate transport in *Kluyveromyces* has not been reported but some data are available for *Saccharomyces*. Cassio *et al.* (1987) have reported that undissociated lactic acid enters the cells of *S. cerevisiae* by passive diffusion and that the permeability of the plasma membrane for undissociated lactic acid increases exponentially with the pH value. Thus the diffusion constant increased 40-fold when the pH was increased from 3.0 to 6.0. However, as the permeability of the membrane increased exponentially, so the undissociated acid concentration decreased exponentially due to the rising pH value. The overall net driving force will thus be determined by which factor prevails, although the authors did not detail which this would be. Champagne *et al.* (1990) found that lactic acid respiration of *S. cerevisiae* in the pH range of 5.0 to 6.0 was higher than that at lower pH values (3.0 to 4.0), which perhaps suggests that the membrane permeability was the dominant factor. It is unclear whether *Kluyveromyces* yeast behaves in the same way.

A factor which needs to be considered in this work follows from a report by Maitra and Lobo (1971). During growth of a hybrid of *S. fragilis* and *S. dobzhanskii* (which is now designated as *K. marxianus*) on media containing non-fermentable substrates such as acetate, ethanol, pyruvate and glycerol, a reduced level of activity of all glycolytic enzymes except alcohol dehydrogenase and aldehyde dehydrogenase was observed, compared to when the yeast was grown on glucose. Champagne *et al.* (1989) also reported that when lactic acid was used as a single carbon source for the production of bakers' yeast, *S. cerevisiae*, the leavening activity of the yeast was reduced compared to the molasses-grown bakers' yeast. However, in the presence of two carbon sources, galactose and lactic acid, the leavening activity of the yeast obtained was comparable with that

of the yeast grown in molasses. They suggested that the presence of a fermentable substrate such as galactose induced a higher production of glycolytic enzymes. The present work, however, shows that yeast pre-grown in lactate or lactose has no effect on the ethanol production capability of the yeast. Thus there is no evidence to support the suggestion that the activities of glycolytic enzyme levels were reduced when the yeast was grown on a non-fermentable substrate such as lactate. Surprisingly, the present work showed a lower lactate utilization when the yeast was pregrown in a lactate-agar based medium compared to the lactose pre-grown yeast. The reason for this is unclear.

The use of stillage as a substrate for microbial biomass production has been studied by Kosaric *et al.* (1989) and Kumar and Viswanathan (1991). The stillage used by these authors originated from alcoholic Jerusalem artichoke and molasses fermentations, respectively, and contained mainly carbohydrate (glucose or fructose) which contributed to the high BOD content of the effluent.

In whey distilleries, the composition of the stillage varies depending on the type of the whey substrate and the downstream processing methods used. It may contain a small amount of residual lactose, lactic acid arising from controlled or uncontrolled (contamination) fermentation by lactic acid bacteria, or yeast biomass. For example, at the N.Z Distillery Co Ltd., Edgecumbe, the fermented beer is distilled directly without prior yeast biomass separation. Thus the remains of yeast biomass might contribute to the high BOD of the stillage when discharged directly to the receiving water. Consequently, it is generally disposed of by spray irrigation. Grubb (1991) investigated the use of this stillage as a supplement for the whey permeate fermentation but found this resulted in lower biomass and ethanol yields, and reduced lactose consumption, compared to the control. It was suggested that the stillage may contain an inhibitory compound, probably formed by chemical reactions within the distillation columns.

At the Anchor Ethanol Co., Tirau, production plant, the stillage contains mainly lactic acid and requires a different strategy for disposal. There were four options

available to dispose of the lactic acid, before or after the anaerobic ethanol fermentation:

1. Continuous growth of the production yeast strain aerobically on lactic whey serum, as an inoculum for the subsequent ethanolic fermentation.
2. Aerobic growth of a non-lactose assimilating yeast, such as *Saccharomyces* or *Candida* on the incoming lactic whey serum. This would remove the lactate, but not the lactose.
3. Aerobic growth of another microorganism on the slops, again for SCP production.
4. Growth of the production yeast strain, aerobically, on slops in continuous culture as an inoculum for the whey ethanolic fermentation.

Option 1, which is the current process, could solve the problem of disposing of the lactic acid and at the same time producing a yeast inoculum. Although, in batch culture, lactic acid was utilised to only a limited extent in the presence of lactose, it is possible that in continuous culture, both carbon sources will be utilised simultaneously. A drawback, however, is that some lactose will always be consumed to produce biomass rather than ethanol, so this reduces the maximum attainable ethanol production from the plant. Option 2, using another yeast such as *Saccharomyces* or *Candida*, which does not metabolize lactose, suffers two serious drawbacks. Firstly, utilization of lactic acid aerobically by the yeast will increase the pH of the lactic whey serum which may affect the fermentation performance during the subsequent anaerobic whey fermentation. Also, a higher pH will make the fermentation more susceptible to bacterial contamination, and pH control for a large scale process will be costly. Thirdly, the market for yeast biomass in New Zealand is limited, thus the additional biomass formed creates another disposal problem, rather than another product for the company. This is also the drawback of option 3. Therefore, the best option available was the last, i.e. to grow the production yeast aerobically on slops to produce an inoculum for the subsequent ethanolic fermentation.

The fermentation system employed at Anchor Ethanol production plant at Tirau

is a continuous system. There are two fermentation trains operating simultaneously, each consisting of three 250,000-litre stainless steel fermenters. Figure 5.10 shows the existing process. Currently two propagators operate continuously at a dilution rate of 0.25-0.30 h⁻¹ to produce the yeast as an inoculum for the two trains. Normally, approximately 1 g biomass dry weight/l is obtained from the aerobic growth on lactose, which is very low compared to results in the literature; e.g. Moresi *et al.* (1990) reported a biomass concentration of 10.6 g/l, using whey containing 45 g/l lactose, at a dilution rate of 0.40 h⁻¹. Whey serum for the preparation of sterile inoculum is passed through a UHT (Ultra High Temperature) system operating at 140°C for 20 seconds prior to growth in the propagator (Figure 5.10). Normally the heating system of the UHT requires cleaning with nitric acid and caustic soda every three hours to keep it operating successfully.

In continuous culture, the yeast will be stably maintained in the fermenter when operating below the critical dilution rate, where cell washout occurs. Therefore, there should be no need for any yeast addition. The reasons for the continuous inoculation with fresh yeast are:

- a) To prevent the fermentation from being overrun by contaminants (mainly lactic acid bacteria), pure yeast culture must be continuously supplied. In the past, where continuous pure culture was not supplied, the continuous whey fermentation had to be shut down frequently. Thus the feeding of fresh yeast is a preventative measure.
- b) If, in the worst case, restart of the fermentation is required, due to heavy contamination, yeast inoculum is readily available.

This study has demonstrated that the use of slops as a growth medium for the production yeast strain is feasible in batch and continuous cultures. Its application as a yeast propagation method for the subsequent whey serum fermentation has some advantages and disadvantages if it is to be implemented commercially. Figure 5.11 shows the proposed new fermentation process incorporating growth on slops. The advantages of the improved process are :

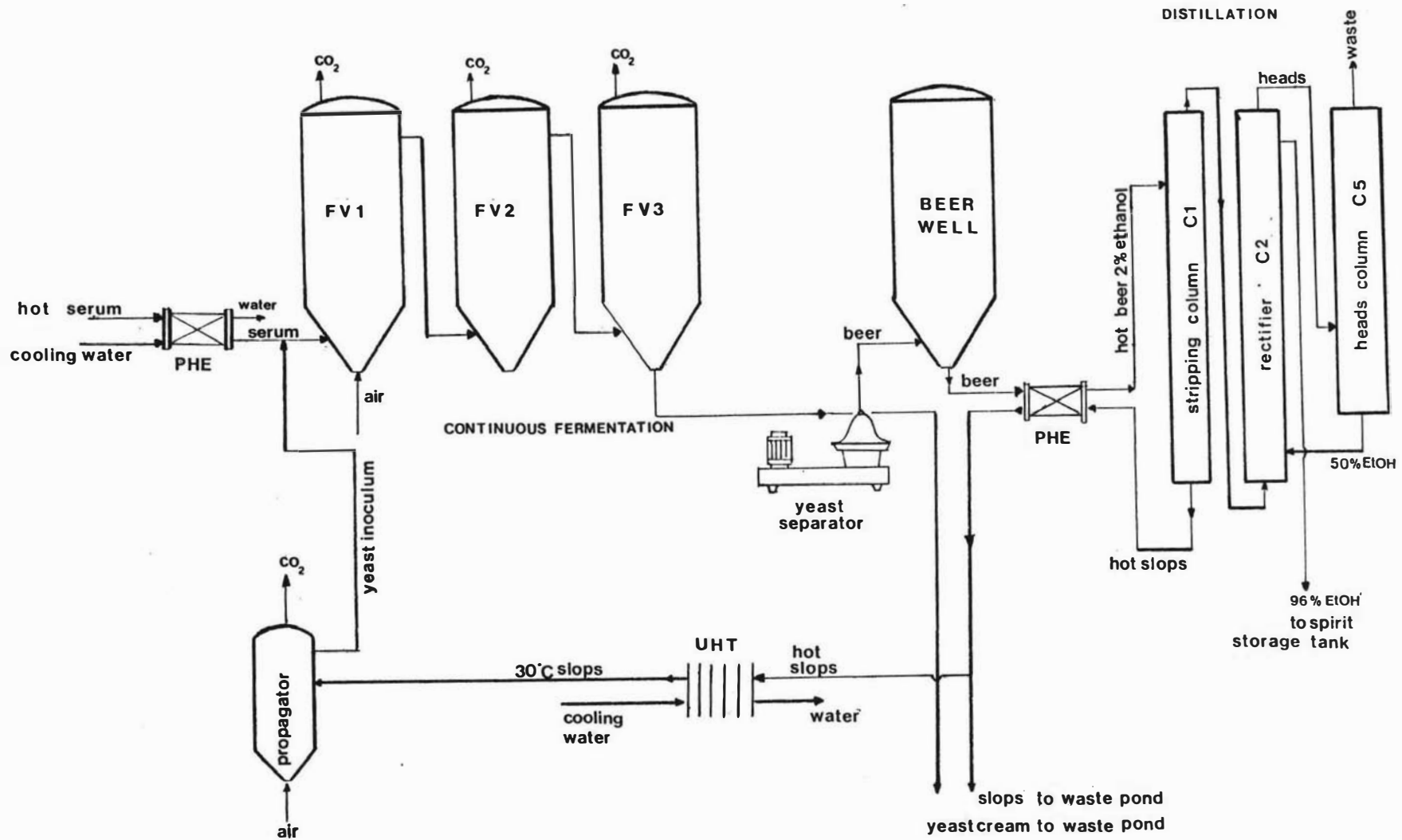


Figure 5.10 Fermentation process at Anchor Ethanol Co., Tirau

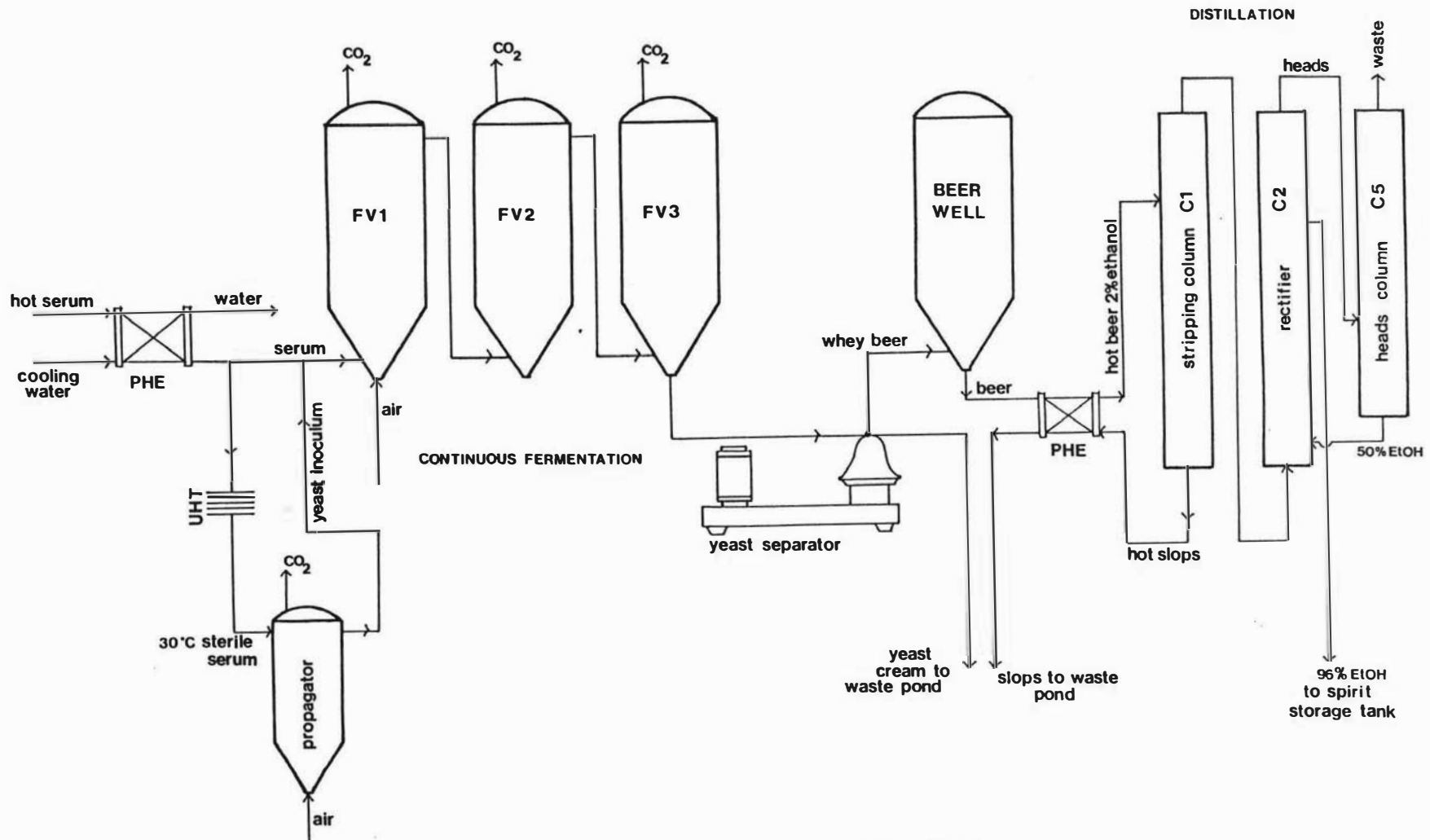


Figure 5.11 The proposed modification of the fermentation process

- (a) Total ethanol production will increase, as all lactose can be diverted to ethanol production rather than some being used for biomass production as currently practised. Approximately 100,000 litres of whey serum per day could be diverted to ethanol production with the improved new process.
- (b) With the proposed system, the UHT will be used to cool the hot slops at approximately 100°C directly from the stripping column to 30°C prior to addition to the propagators (Figure 5.11). The saving made from the elimination of the heating system of the UHT could be substantial in terms of chemical cleaning, power and labour costs. The microbiological quality of the slops should present no problems and indeed no microorganisms were detected in a 1 ml sample grown in BHI (Brain Heart Infusion) agar. During start-up the heating system of the UHT can be used to sterilise the serum prior to feeding into the propagator.
- (c) The BOD of the slops used for propagation will be reduced, thus slightly lowering the load on the waste treatment ponds.

The disadvantages of the proposed process are :

- (a) The ethanol concentration in the fermented beer will be slightly lower due to dilution by the slops. Hence, there will be a slight increase in steam consumption during distillation.
- (b) The cost of repiping to implement the new process.
- (c) The utilization of lactic acid in the slops during the aerobic yeast cultivation will result in an increase of pH which in turn will result in the formation of a calcium salt precipitate. This will need to be accounted for in the design and operation of the propagators to ensure that the vessels and pipework do not become unacceptably coated or blocked. However if a market for this calcium salt precipitate could be found, it would be an added bonus to the company. The precipitate is thought to comprise calcium phosphate which makes it useful as a fertilizer.

During the process of coupled aerobic yeast growth on slops and anaerobic serum fermentation, continuous addition of yeast suspension did not affect the

fermentation performance. Also, the pH did not change due to the buffering capacity of the serum, although the added yeast slops suspension had a pH value of 6.0. As noted, it is important that the pH of the whey serum be maintained, as an increased pH may negatively affect the fermentation due to increased risk of contamination. At the serum pH value of 4.0-4.5, many bacteria will not grow and hence the fermentation is less susceptible to contamination.

The economic feasibility of the modified process was examined using cost data available in the literature and/or supplied by Mr Colin Reid, Assistant Manager, Anchor Ethanol Co., Tirau. As noted, the existing propagator is inefficient with respect to biomass production, due to the poor design, and so lactose is also fermented to give approximately 60% ethanol yield of theoretical. Currently the fermentation process gives 78% ethanol yield of theoretical. Therefore for an extra 100,000 l of whey serum per day diverted to ethanol production with the proposed process, an extra 387.4 kg of ethanol will be produced per day. This amounts to 62,000 kg ethanol per year, assuming the ethanol production plant is running 160 days per year. Assuming the sale price of ethanol is N.Z \$ 1/kg and allowing for a 15% or 30% marginal return, additional revenue of NZ\$9300 or \$18,000 per year, respectively, are expected. The saving of the costs associated with the elimination of the heating system of the UHT (chemical and heating) is estimated to be NZ\$4000 per year, while the cost of repiping is estimated at approximately \$10,000 (Personal communication, Colin Reid, 1993). Therefore, for a 15% marginal return, the total saving made is NZ \$13,300 and the pay back time of the new capital cost will be approximately one year. However, if a 30% marginal return is used, the total saving made becomes NZ\$22,000, giving an approximate pay back time of half a year.

In conclusion, the proposed process of growing the yeast on slops as inoculum for the ethanolic fermentation has been shown to be technically and economically viable.

CHAPTER 6

YEAST AUTOLYSIS AND THE USE OF THE AUTOLYSATE AS A NUTRIENT SOURCE IN WHEY FERMENTATION

6.1 INTRODUCTION

The term 'autolysis' was first introduced into the biological literature by Salkowski in 1889 (Vosti and Joslyn, 1954; Farrer, 1955; Babayan *et al.* 1985) and refers to self digestion of cells under the action of their own intracellular enzymes. Autolysis is used commercially to produce a range of yeast extracts. The main advantages of autolysis for cell lysis are that it is a simple process, involving temperature and pH manipulation, it is easy to scale up, and it does not require sophisticated equipment. Biomass for the manufacture of yeast extract is obtained primarily from breweries as surplus brewers' yeast and occasionally from other sources, such as molasses-grown *S. cerevisiae*, whey-grown *K. marxianus*, and wood sugar- and ethanol-grown *C. utilis*.

In the commercial "whey to ethanol" fermentation, the surplus yeast is generally wasted. At Anchor Ethanol Co., Tirau, the spent yeast generated during the fermentation is treated in the waste ponds prior to discharge into natural receiving waters. An alternative would be to manufacture an autolysate for use as an additive to improve the nutritional status of whey for the ethanol fermentation. Several authors have reported that addition of yeast extract to whey can overcome a known nitrogen limitation of this medium and give faster rates of fermentation, although the ethanol yield appears not be affected (Vienne and von Stockar 1983, Chen and Zall 1985).

The objective of this work was to establish the optimum condition for autolysis, in batch and continuous systems, of the Tirau production yeast, *K. marxianus* strain Fi, and to examine the effect of the autolysates produced on the productivity of the "whey to ethanol" fermentation.

6.2 MATERIALS AND METHODS

6.2.1 Yeast cream

A concentrated suspension (cream) of *K. marxianus* strain Fi for autolysis was obtained by laboratory fermentations or from Anchor Ethanol Co. Laboratory samples were obtained by centrifuging the culture broth after growth in (10 or 20 litre) YEPL or LACWS under aerobic or anaerobic conditions (Section 3.5). A yeast cream concentration of 60-70 g dry weight/l was normally obtained. Yeast creams supplied by Anchor Ethanol Co. were harvested by centrifugation and transported via airfreight to the laboratory. Normally the yeast concentration was in the range 70-108 g dry weight/l.

6.2.2 Autolysis conditions

Batch autolysis was conducted in 250 ml flasks containing 100 ml of yeast cream. These were incubated without shaking in water baths at 50, 55 or 60°C as described in Section 3.5.1. Prior to autolysis the pH of the yeast cream was adjusted to 4.5, 5.0 or 5.5 using 4M NaOH or 4M HCl. Batch samples were autolysed for up to 48 h. Samples were removed at 2 hourly intervals for the first 12 h, and then at 12 h intervals thereafter. In some runs, ethanol was added to a final concentration of 2%, 5% or 10% (w/v) or NaCl was supplemented at 2.5%, 5% and 10% (w/v). Autolysis during these runs was conducted at 55°C and pH 5.5. To investigate the effect of cool storage on autolysis, a sample of fresh yeast cream received from Anchor Ethanol Co. was autolysed immediately at

55°C and pH 5.5, while the remaining cream was stored at 4°C. Samples were removed from storage at 12, 24 and 48 h intervals for autolysis under the same conditions.

Continuous autolysis was conducted in a 2-litre bench top vessel with a 1 l working volume with temperature and pH control as described in Section 3.5.2. The autolysate was collected after steady state had been established. This was assumed when the α -aminonitrogen concentration was stable ($\pm 10\%$) after three residence times.

6.2.3 Fermentation media

The fermentation media used in this work were: sulphuric acid casein whey permeate (SACWP), SACWP supplemented with 0.5- 4.0 g/l yeast extract, lactic acid casein whey serum (LACWS) and lactic acid casein whey permeate (LACWP) supplemented with 2 and 4 g/l yeast extract. The approximate composition of these media were as described in Section 3.1.1.

6.2.4 Fermentation operation

Batch anaerobic whey fermentations were conducted in 250 ml shake flasks in replicate. The sterile whey media were inoculated at 5% (v/v) with an aerobically-grown culture prepared as described in Section 3.4.1. When required, autolysate samples, either with the cell debris removed (clear lysate) by centrifugation (7000 rpm for 15 min), or as collected from the lysis flask (whole lysate), were pasteurised at 85°C for 25 seconds and added to the sterile whey media at 5%(v/v). Some lysate was centrifuged as above and then sterilised by filtration using 0.45 μ m filter paper prior to addition to whey medium, hence the term filter lysate.

6.2.5 Chemical analysis

Ethanol, lactose, α -aminonitrogen, protein, and total nitrogen were determined as described in Section 3.3.4, 3.3.5, 3.3.7, 3.3.8, and 3.3.9, respectively. The yeast cream concentration was determined by the oven drying method as described in Section 3.3. The biomass concentration during the whey fermentations was determined by the spectrophotometric method (Section 3.3). In some runs, plate counts were also performed as described in Section 3.3.3.

6.2.6 Data analysis

During the anaerobic batch whey fermentation, the ethanol concentration normally increases linearly with time between approximately 8 to 16-20 h. The maximum observed ethanol production rate was calculated from this region by linear regression using Minitab. The overall volumetric ethanol productivity, % ethanol yield of theoretical, and t_r were determined as described in Section 4.2.5. The maximum specific growth rate (μ_{max}) was determined by fitting the biomass data using the modified Gompertz equation as described in Section 5.3. The rate of α -aminonitrogen release was determined at 6 h and 48 h, by calculating the slope from the origin to the 6 or 48 h points.

6.3 RESULTS

6.3.1 Effects of pH and temperature

6.3.1.1 Aerobically grown yeast

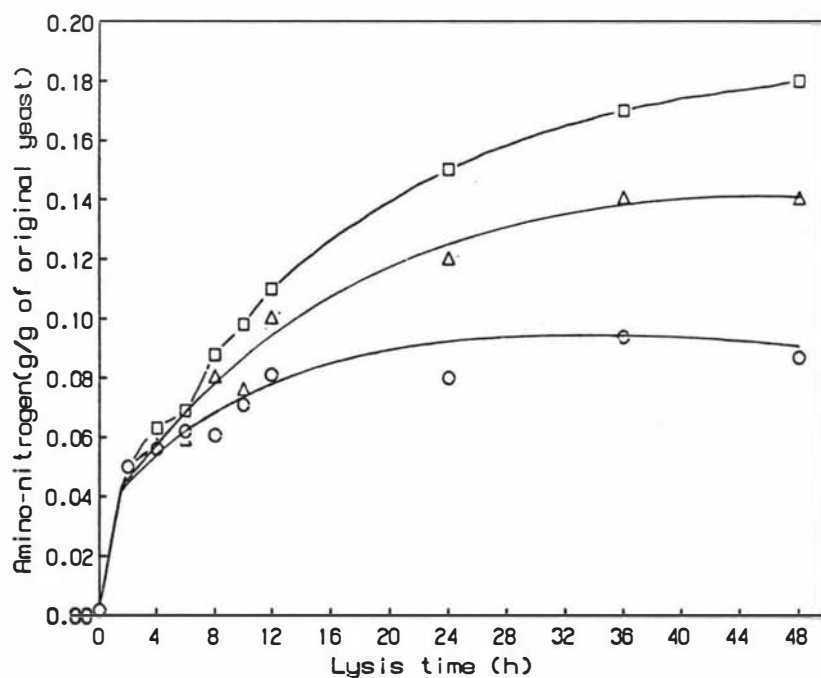
The effect of temperature and pH were initially investigated using aerobically grown yeast propagated in the laboratory on YEPL. A factorial experiments (3^2) was used with a combination of three different temperatures : 50, 55, or 60°C,

and pH 4.5, 5.0, or 5.5. Since the release of protein and amino acids is generally considered to be the most important aspect of yeast autolysis, the concentrations of these components were closely monitored.

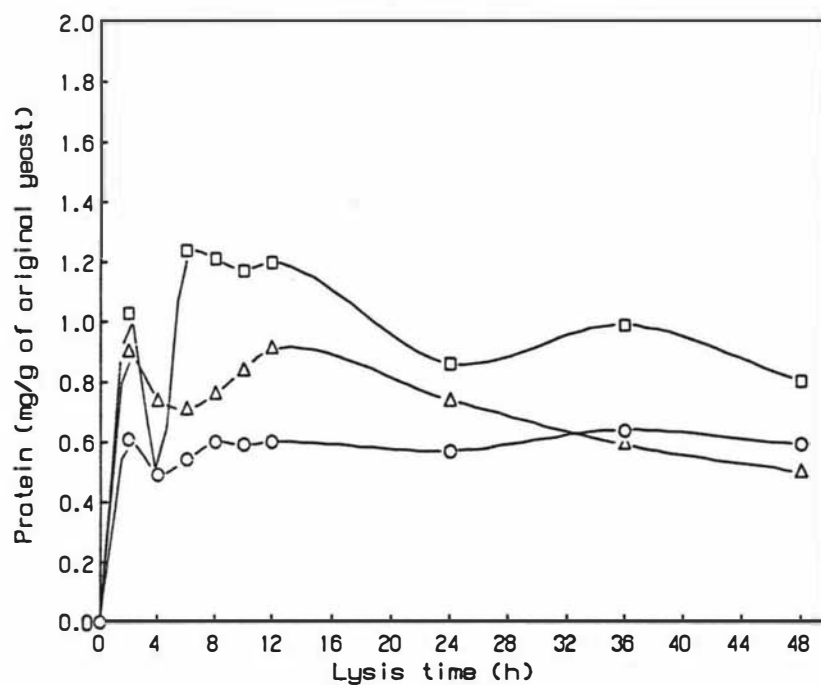
The effect of pH on the time course of autolysis at 50°C is shown in Figure 6.1. Figure 6.1a shows that at 50°C, the level of α -aminonitrogen (α -AN) increased with time for all pH values, indicating continuing hydrolysis of protein. The pH value had a marked effect on the release of α -AN and showed an optimum at pH 5.5. In contrast, the protein levels in the extract were extremely low in all cases, compared to the α -AN levels, and changed only slightly during the entire 48 h of autolysis after the initial rapid release. Protein concentration was also maximal at pH 5.5 (Figure 6.1b).

The effects of various temperatures on autolysis at pH 5.5 are shown in Figure 6.2. The level of α -AN at 50°C and 55°C increased with time; however at 60°C, the level of α -AN reach a plateau after 2 h of autolysis (Figure 6.2a). Temperature had an equally marked effect on the release of α -AN and showed an optimum at 50°C. The low amount of α -AN released at 60°C suggested that the temperature was then the limiting factor for autolysis. Protein levels were also low compared to α -AN levels and were greatest at 50°C (Figure 6.2b).

The combined effects of pH and temperature on the rate of α -AN released at 6 and 48 h are shown in Figure 6.3. The highest rate of α -AN release at 6 h was observed at 50°C and pH 5.5, and 55°C and pH 5.5 (Figure 6.3a), indicating the optimum conditions. However at 48 h, the rate of α -AN release was highest at 50°C and pH 5.5 (Figure 6.3b). In general the α -AN released at 6 h was almost half of the α -AN released at 48 h. Overall, the optimum conditions for autolysis of the aerobically-grown culture were judged to be 50°C and pH 5.5. The total nitrogen released was also measured for these conditions, and the data are shown in Table 6.1. The total nitrogen content of the yeast cream plus the supernatant prior to autolysis was determined as 5.5 g/l, therefore after 48 h, 85% of available nitrogen was released by autolysis.

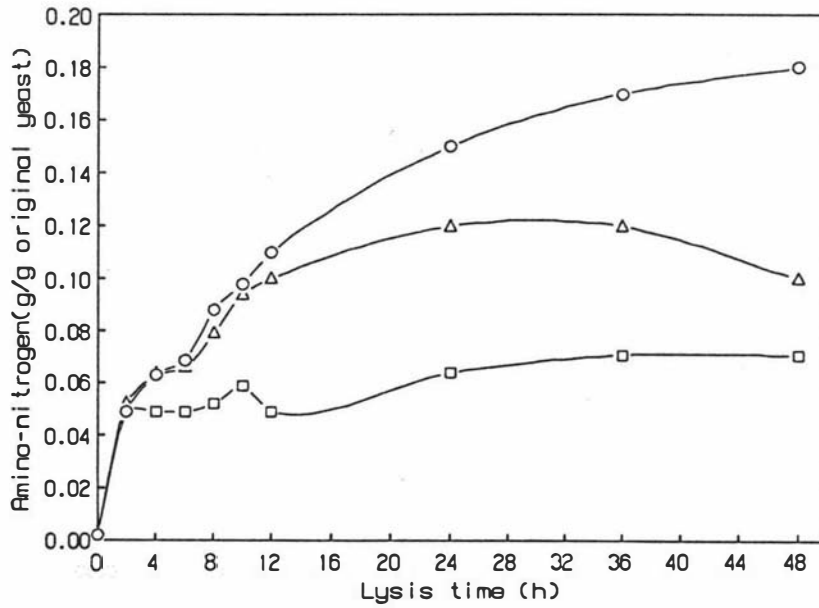


(a)

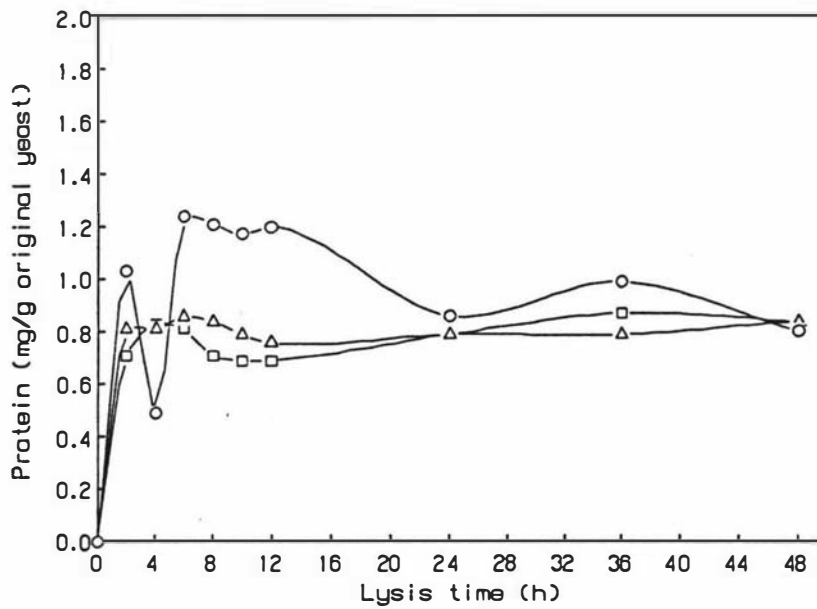


(b)

Figure 6.1 The effect of pH on the autolysis of yeast grown aerobically in YEPL showing (a) α -AN release and (b) protein release : \circ pH 4.5; Δ pH 5.0; \square pH 5.5. Yeast cream concentration was 70 g dry weight/l. Autolysis temperature was 50°C.

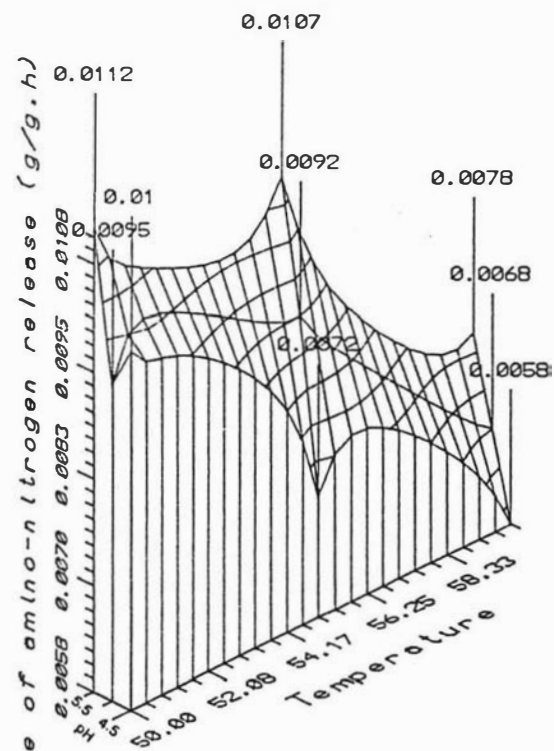


(a)

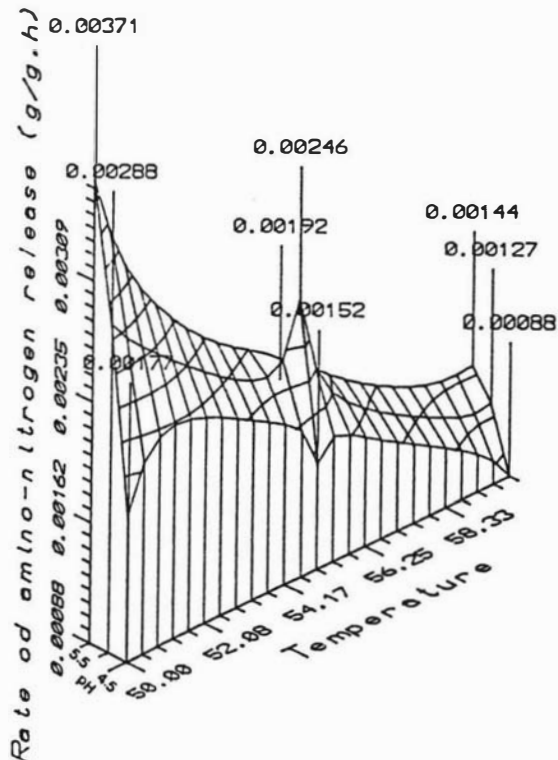


(b)

Figure 6.2 The effect of temperature on the autolysis of aerobically grown yeast at pH 5.5 (other conditions as per Figure 6.1). (a) α -AN release and (b) protein release : \circ 50°C; Δ 55°C; \square 60°C.



(a)



(b)

Figure 6.3 Three dimensional plot of the combined effects of pH and temperature on the autolysis of aerobically grown yeast (other conditions as per Figure 6.1). Rate of α -AN release (a) at 6 h (b) 48 h.

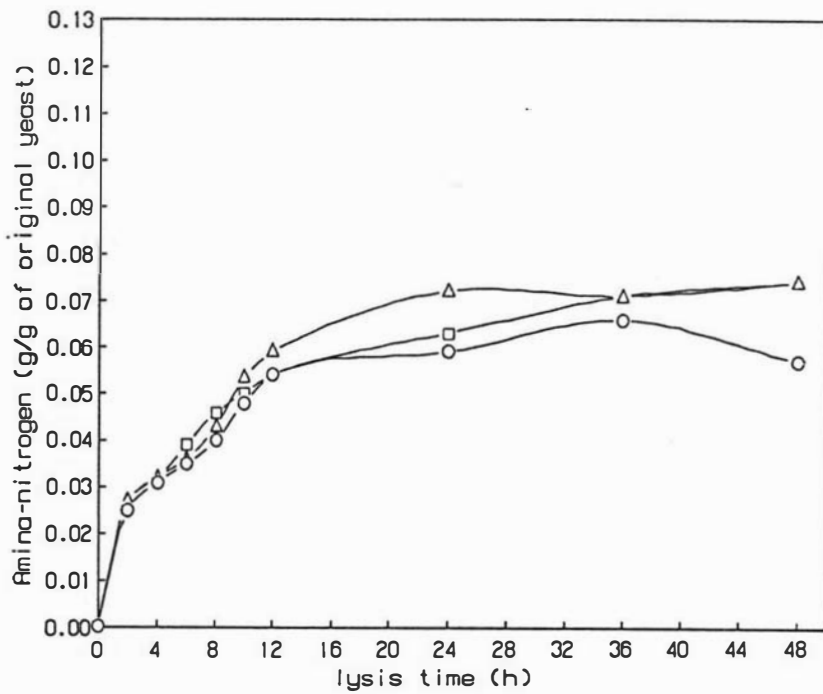
Table 6.1 Total Kjeldahl Nitrogen released during autolysis of yeast grown under different conditions

Total nitrogen release (g/l)		
Time (h)	Aerobic growth Autolysis at 50°C and pH 5.5	Anaerobic growth Autolysis at 55°C and pH 5.5
0 h	0.03 g/l	0.03 g/l
6 h	3.15 g/l	1.45 g/l
12 h	3.60 g/l	1.85 g/l
24 h	4.20 g/l	2.15 g/l
36 h	4.50 g/l	2.25 g/l
48 h	4.65 g/l	2.40 g/l

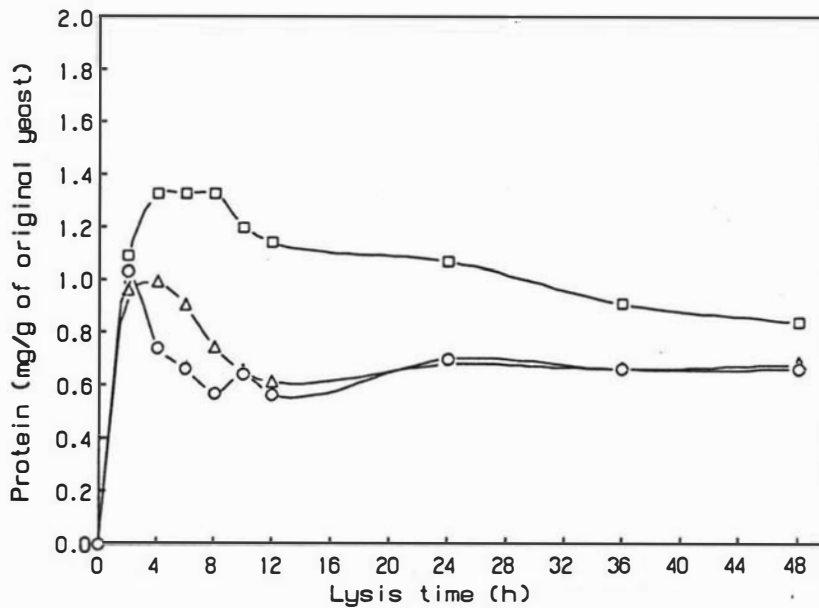
6.3.1.2 Anaerobically-grown yeast

In this experiment, yeast was grown anaerobically and autolysed under identical conditions to the aerobically-grown yeast. However because the results from the autolysis of aerobically-grown yeast indicated possible enzyme inactivation at 60°C, it was decided to include only one run, at pH 5.5, at this temperature. The effect of pH on the α -AN released at 55° was minimal and the highest concentrations were observed at pH 5.0 or 5.5 (Figure 6.4a). The protein concentration profile again indicated a limited release of protein and showed a maximum at pH 5.5 (Figure 6.4b).

The α -AN released at pH 5.5 showed marked variations among the three temperatures used. After 48 h of autolysis the α -AN released was maximal at 50°C (Figure 6.5a), while the protein concentration released was highest at 60°C (Figure 6.5b). The combined effect of pH and temperatures on the rate of α -AN release is plotted in Figure 6.6a, and shows that at 6 h, the rate of α -AN release

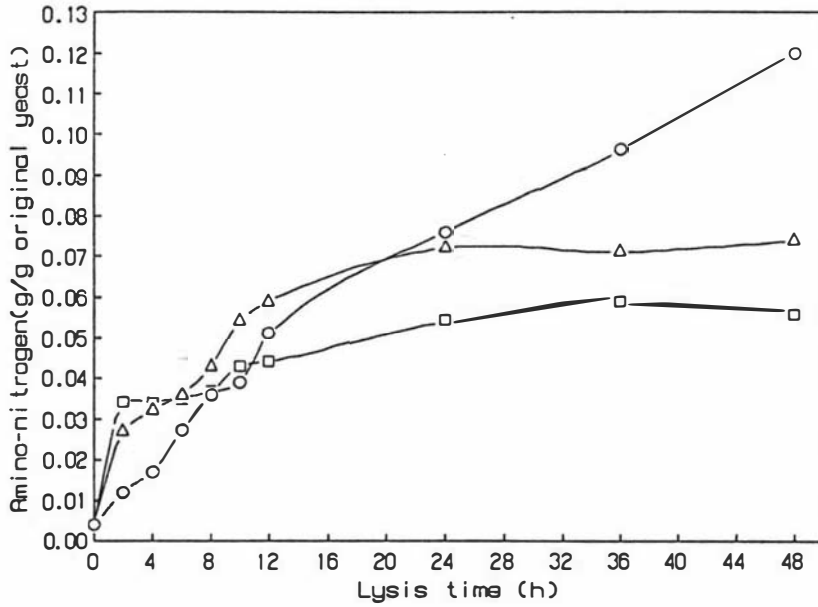


(a)

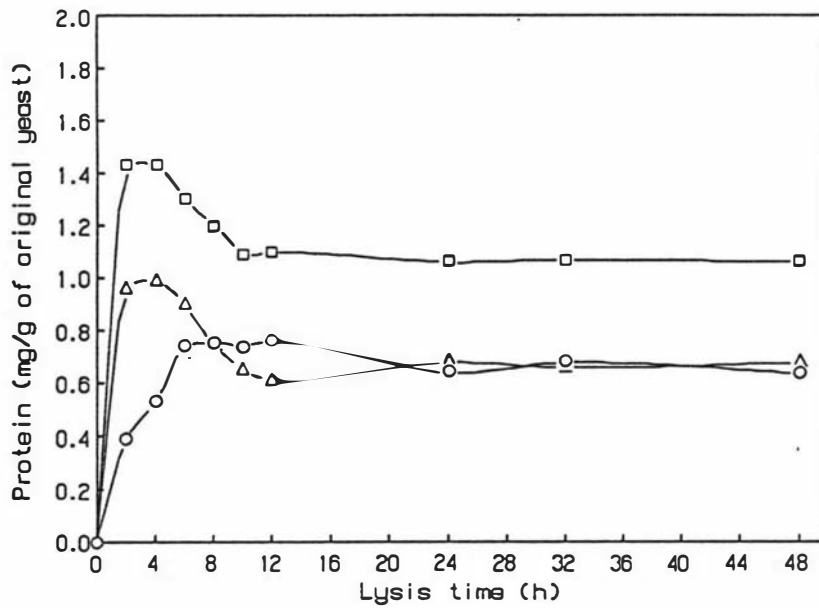


(b)

Figure 6.4 The effect of pH on the autolysis of yeast grown anaerobically in YEPL showing (a) α -AN release and (b) protein release : \circ pH 4.5; Δ pH 5.0; \square pH 5.5). Yeast cream concentration was 70 g/l. Autolysis temperature was 55°C.



(a)



(b)

Figure 6.5 The effect of temperature on the autolysis of anaerobically grown yeast at pH 5.5 (Other conditions as per Figure 6.4). (a) α -AN release and (b) protein release : \circ 50°C; Δ 55°C; \square 60°C.

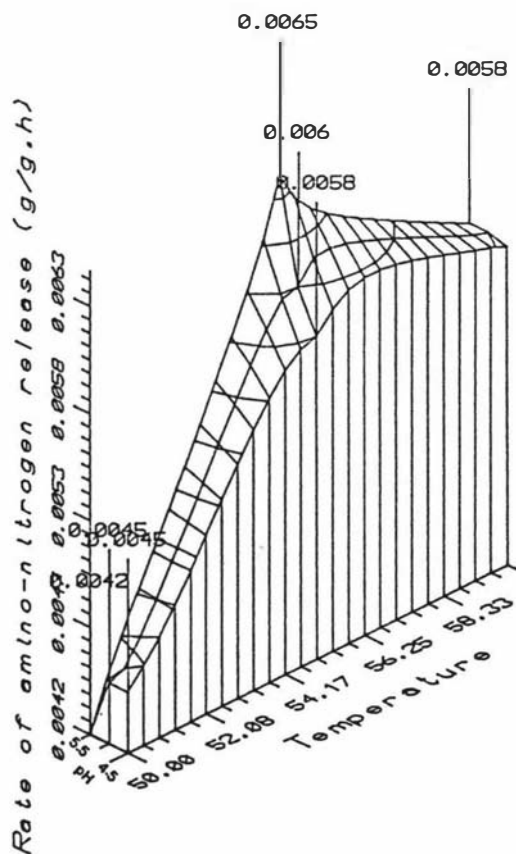
was maximum at 55°C and pH 5.5. However at 48 h, the rate of α -AN release was highest at 50°C and pH 5.0 (Figure 6.6b). The combination of 55°C and pH 5.5 was selected as optimal for the autolysis of anaerobically-grown yeast because this gave the most rapid release of the α -AN. Autolysis for 24-48 h may be too long for a commercial autolysis process. The total nitrogen content of the extract produced at 55°C and pH 5.5 was only about half of that produced by the autolysis of aerobically-grown yeast at 50°C and pH 5.5, and consequently only 43% autolysis was achieved (Table 6.1).

6.3.1.3 Effect of the growth medium and of the autolysis solution

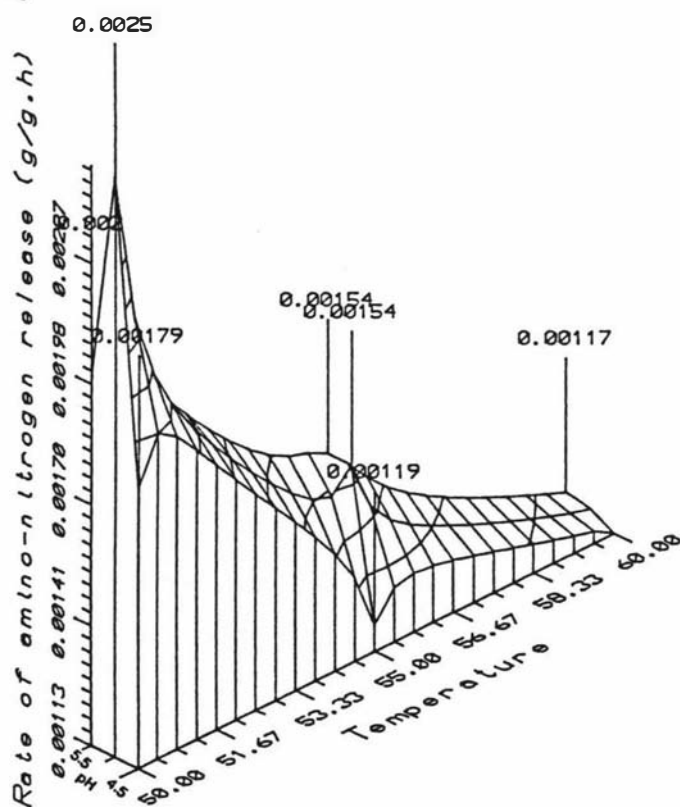
The yeast grown anaerobically in LACWS was autolysed in fermented beer at 50°C or 55°C and pH 4.5 or 5.5, and the α -AN concentration was followed closely for 48 h. The rate of α -AN released at 6 h was maximal at 55°C and pH 4.5 (Figure 6.7a). However at 48 h, the rate of α -AN release was highest at 50°C and pH 4.5 (Figure 6.7b). This result suggests that different growth and autolysis media may have a pronounced influence on the optimum conditions for autolysis.

6.3.2 Effects of salt (NaCl) or ethanol

The effects of salt or ethanol on the autolysis of anaerobically-grown yeast were investigated. The aim was to accelerate the rate of α -AN release at the previously established optimum autolysis conditions i.e. at 55°C and pH 5.5. Ethanol concentrations of 2%, 5%, and 10% (w/v) and NaCl concentrations of 2.5%, 5%, and 10% (w/v) were tested separately. The 2% (w/v) ethanol concentration was selected as this is the concentration of ethanol achieved in the commercial fermentation and consequently found in the yeast cream following centrifugation. An ethanol concentration of 5% (w/v) has previously been shown by other researchers to be the optimum concentration in terms of increasing the amount of protein release from *S. cerevisiae*. There was no marked

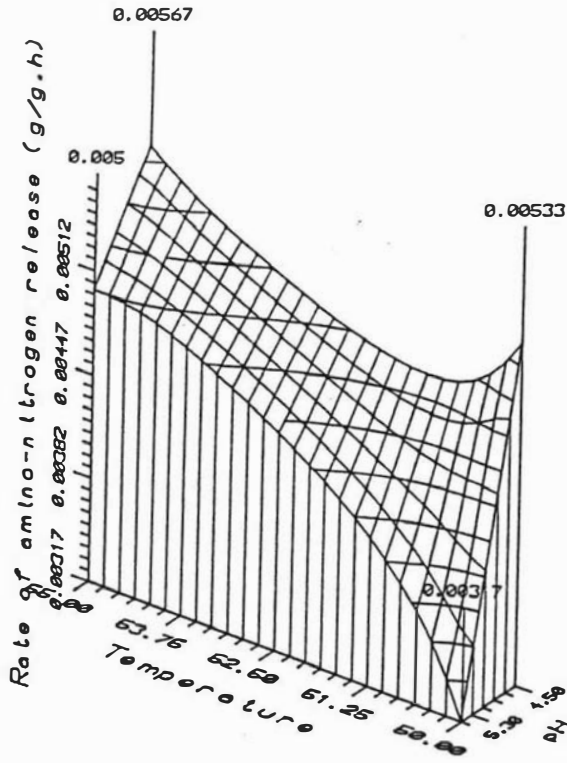


(a)

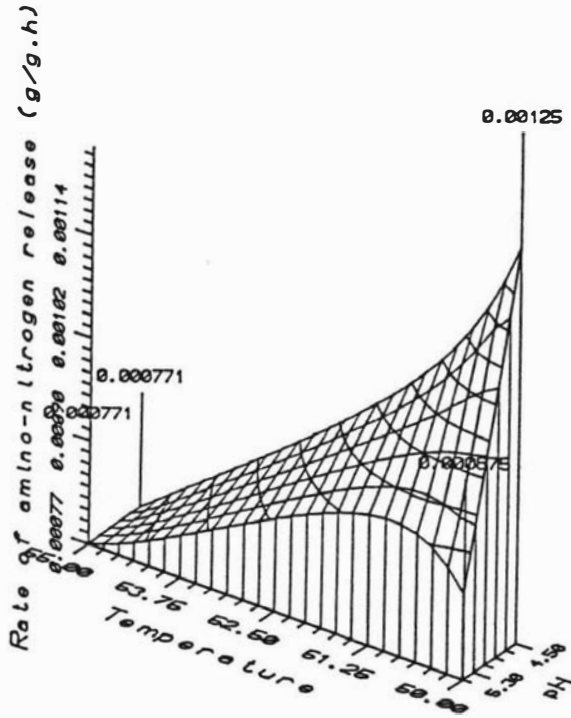


(b)

Figure 6.6 Three dimensional plot of the combined effect of pH and temperature on the autolysis of anaerobically grown yeast (other conditions as per Figure 6.4) showing (a) the rate of α -AN release at 6 h (b) 48 h.



(a)



(b)

Figure 6.7 The combined effect of pH and temperature on the autolysis of anaerobically grown yeast in LACWS on the rate of α -AN release at (a) 6 h and (b) 48 h. The yeast concentration was 65 g/l and was autolysed was in fermented beer.

improvement in the levels of α -AN released when these ethanol concentrations were added (Figure 6.8a), although a slightly higher rate of α -AN release was observed with 5% (w/v) ethanol (Table 6.2). However the protein concentration profile shows that addition of both 5% and 10% (w/v) ethanol resulted in two and three-fold improvements in the amounts of protein released, respectively compared to the control. The protein concentration peaked early at approximately 2 h after the commencement of the lysis process, and slowly degraded thereafter (Figure 6.8b). The final ethanol concentration in the lysate was also checked. During the first 12 h, negligible ethanol was lost, but after 48h, the three ethanol-containing cultures showed decreases in ethanol concentration of approximately 20%.

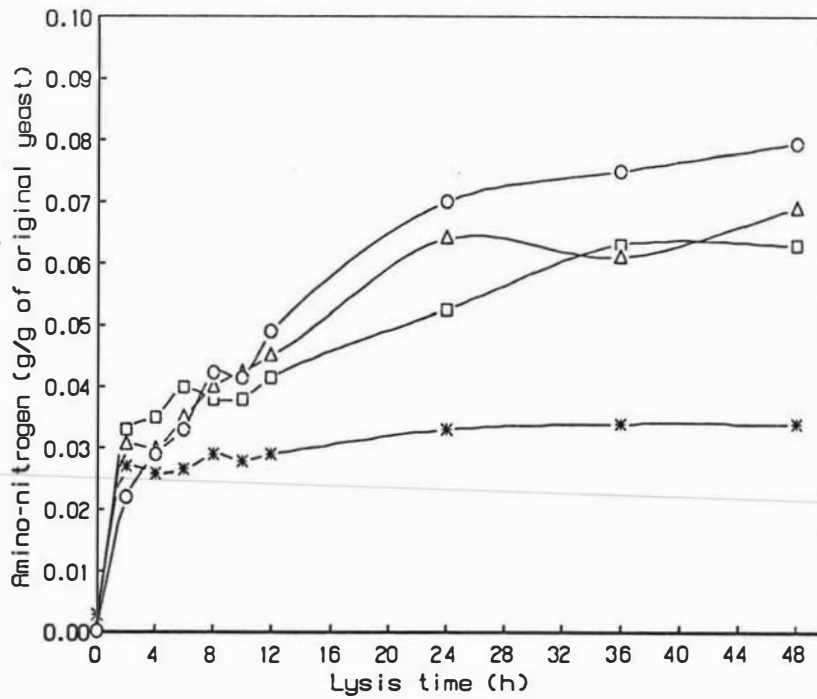
The effect of NaCl was more limited. Addition of 5% (w/v) NaCl gave the highest protein release, approximately two-fold higher (Figure 6.9), and a slightly higher rate of α -AN release, when compared to the control (Table 6.2).

Table 6.2 The effect of ethanol and NaCl on the rate of α -aminonitrogen liberation (g/g yeast.h)

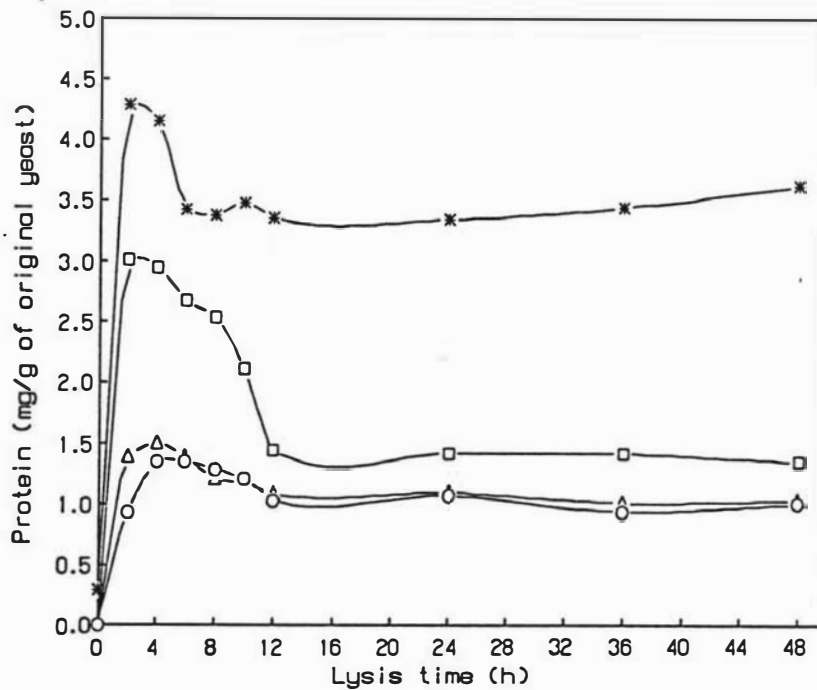
Ethanol (% w/v)	Rate of α -AN release (g/g.h)	NaCl (% w/v)	Rate of α -AN release (g/g.h)
0	5.50×10^{-3}	0	4.92×10^{-3}
2	5.83×10^{-3}	2.5	5.33×10^{-3}
5	6.67×10^{-3}	5	5.42×10^{-3}
10	4.42×10^{-3}	10	5.25×10^{-3}

6.3.3 Comparison of all autolysis trials conducted at 55°C and pH 5.5.

The α -AN release during five autolysis trials conducted at 55°C and pH 5.5 were compared. Figure 6.10 shows that the highest amount of α -AN released was achieved using the aerobically grown yeast compared to that of the remaining

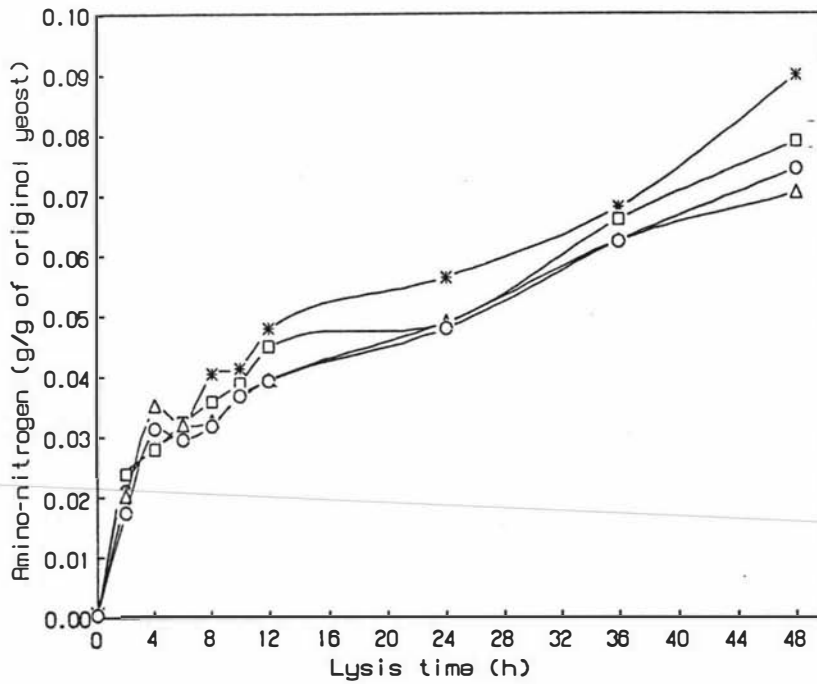


(a)

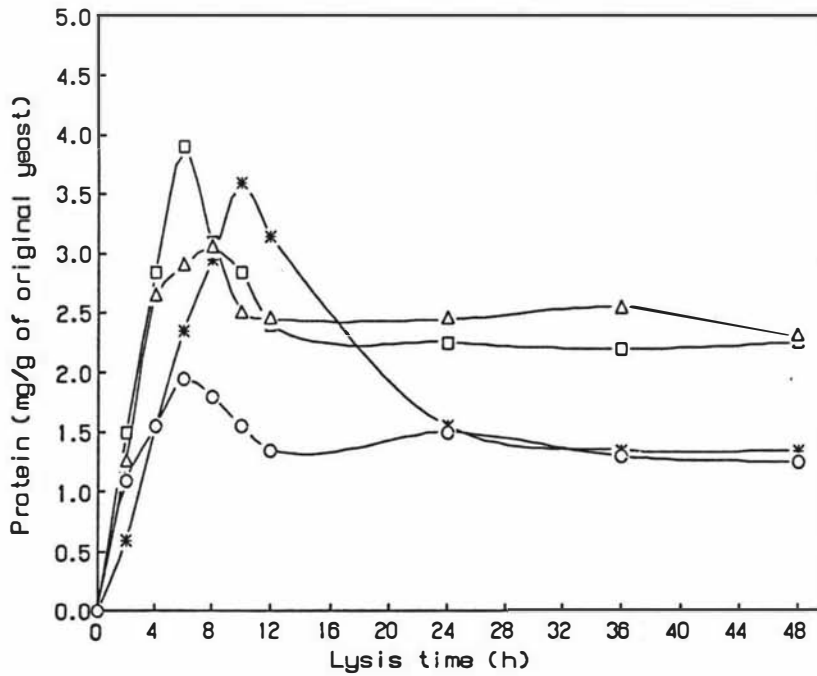


(b)

Figure 6.8 The effect of addition of ethanol on (a) α -AN release and (b) protein release, during the autolysis of anaerobically grown yeast : ○ control; Δ 2% w/v; □ 5% w/v; * 10% w/v. Autolysis condition was 55°C and pH 5.5. Yeast cream concentration was 70 g/l.



(a)



(b)

Figure 6.9 The effect of addition of NaCl for the autolysis of anaerobically grown yeast on the (a) α -AN release and (b) protein release : o 0% w/v; Δ 2.5% w/v; \square 5% w/v; * 10% w/v. Other conditions was as per Figure 6.8.

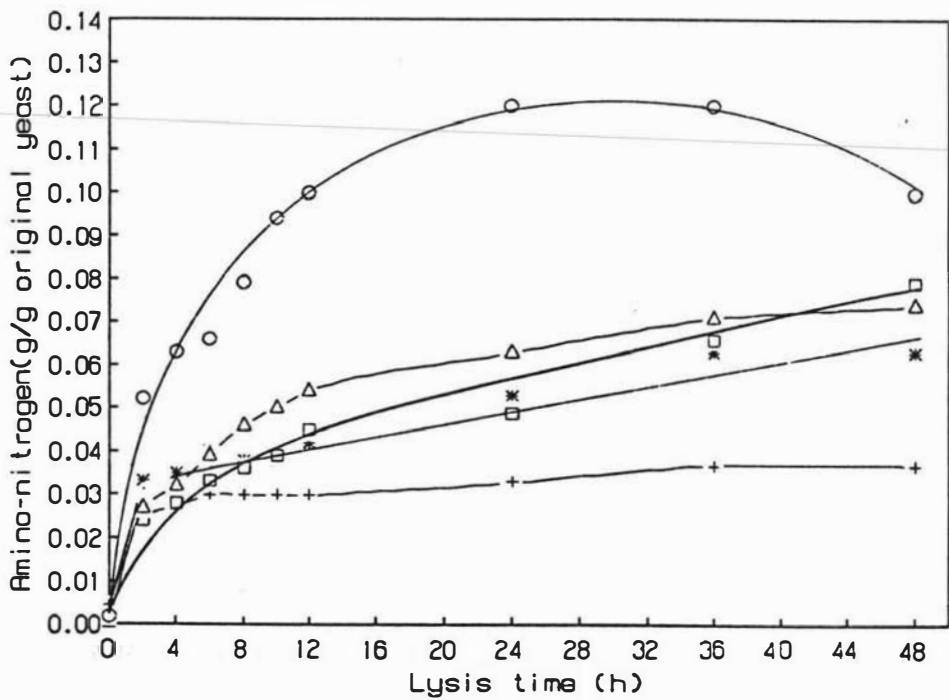


Figure 6.10 Comparison of the α -AN profiles of five autolysis trials of yeast grown aerobically : o Figure 6.2a and anaerobically : + serum; Δ Figure 6.4a; * Figure 6.8a; \square Figure 6.9a in YEPL and LACWS. The yeast cream concentration was 65-70 g/l, and autolysis was conducted at 55°C and pH 5.5.

four trials using the anaerobically grown yeast. The α -AN release of the yeast grown anaerobically on lactic whey serum was lower again.

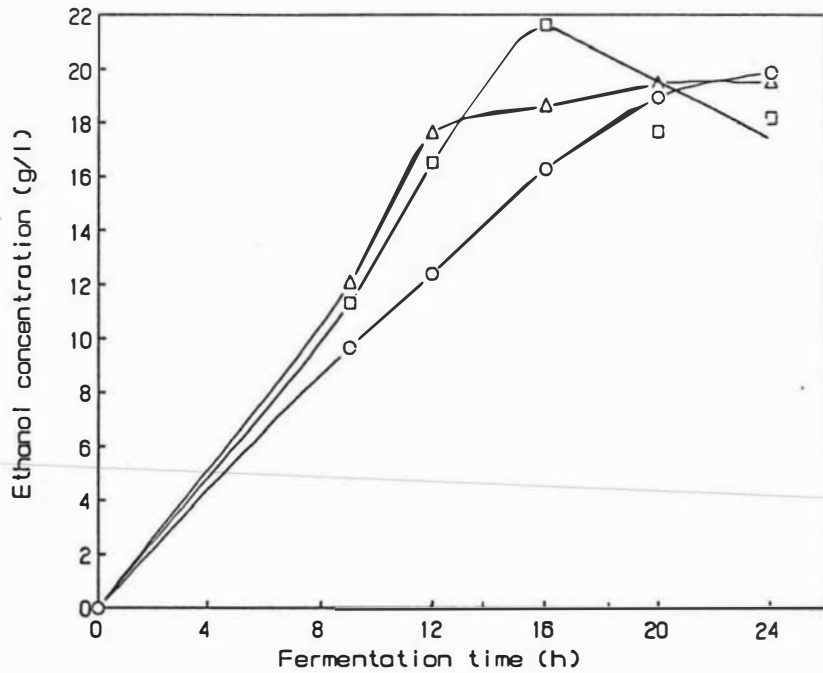
6.3.4 The use of yeast extract or yeast autolysate as a nutrient source in batch whey fermentation

6.3.4.1 Effect of yeast extract on LACWP fermentation

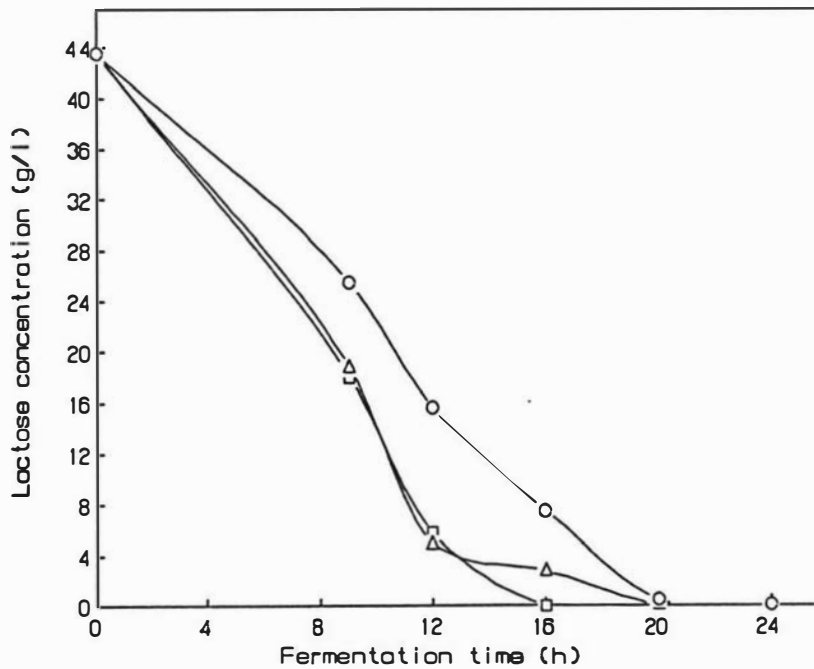
A commercial yeast extract (Difco Laboratories) as added to the LACWP at 2 and 4 g/l, and the fermentation performance was compared with the unsupplemented medium. Figure 6.11 shows the fermentation profile for ethanol and lactose concentrations. Results from analysis of the data are shown in Table 6.3. Addition of 2 g/l of yeast extract resulted in a 50% and 67% increase in overall ethanol productivity and maximum observed ethanol production rate, respectively, compared to the control. The % ethanol yield of theoretical increased slightly to 84% compared to 82% obtained with the control (Table 6.3). Addition of 4 g/l yeast extract did not further enhance the ethanol productivity or production rate, although the ethanol yield was further increased to 88% (Table 6.3).

6.3.4.2 Effect of yeast extract on SACWP fermentation

The influence of yeast extract added at 0.5 g/l, 1 g/l, 2 g/l, or 4 g/l to SACWP is shown in Figure 6.12. Supplementation with yeast extract increased the rate of ethanol production and lactose utilization. Addition of 2 g/l yeast extract increased the overall volumetric ethanol productivity and maximum observed ethanol production rate by 50% and 42%, respectively, compared to the control. The % ethanol yield of theoretical was also improved slightly to 82% compared to 79% obtained with the control (Table 6.4). Addition of 4 g/l yeast extract further improved the ethanol production rate to give a value of 50% greater than the control (Table 6.4).



(a)



(b)

Figure 6.11 The effect of addition of commercial yeast extract (Difco Laboratories) on the fermentation performance of LACWS. (a) Ethanol profile and (b) lactose profile : \circ 0 g/l; Δ 2 g/l; \square 4 g/l.

Table 6.3 : Effect of yeast extract on lactic acid casein whey permeate fermentation

	Control	2 g/l yeast extract	4 g/l yeast extract
Fermentation time; t_f (h)	18.3	12.0	13.8
Ethanol (g/l)	17.9	17.6	19.5
Residual lactose (g/l)	3.0	4.8	2.5
Lactose utilized (g/l)	41	39	41
% ethanol yield of theoretical	82	84	88
Overall volumetric ethanol productivity (g/l.h)	1.0	1.5	1.4
Maximum observed ethanol production rate (g/l.h)	0.9	1.5	1.5

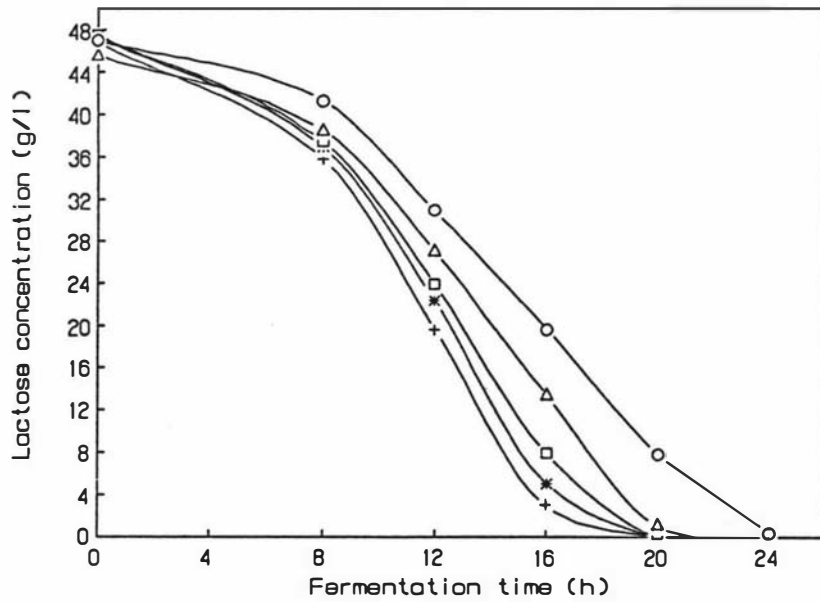
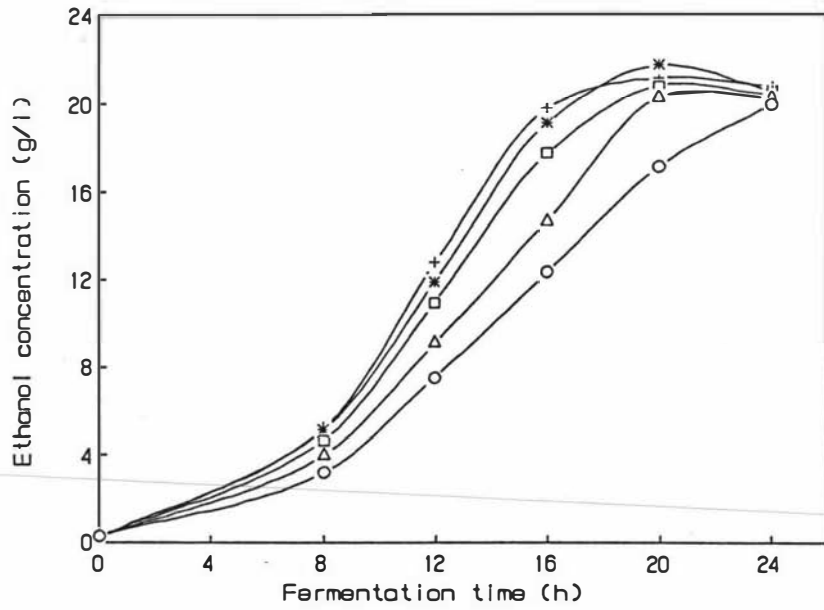


Figure 6.12 The effect of supplementation of commercial yeast extract at various concentrations on the fermentation profiles of SACWP. (a) Ethanol production and (b) lactose utilization : \circ 0 g/l; Δ 0.5 g/l; \square 1 g/l; $*$ 2 g/l; $+$ 4 g/l.

Table 6.4 : Effect of different concentrations of yeast extract supplementation on SACWP fermentation

	0 g/l yeast extract	0.5 g/l Yeast extract	1 g/l yeast extract	2 g/l yeast extract	4 g/l yeast extract
Fermentation time; t_f (h)	21.1	18.3	17.1	16.6	15.4
Ethanol (g/l)	18.0	18.2	18.7	19.6	19.0
Residual lactose (g/l)	5.6	5.2	4.7	3.7	4.3
Lactose utilized (g/l)	41	40	44	44	42
% ethanol yield of theoretical	79	83	80	82	82
Overall volumetric ethanol productivity (g/l.h)	0.8	1.0	1.1	1.2	1.2
Maximum observed ethanol production rate (g/l.h)	1.2	1.4	1.6	1.7	1.8
α -amino nitrogen utilized (g/l) ^a	0.07	0.17	0.27	0.48	0.82
Maximum specific growth rate μ_{max} (h^{-1})	0.23	0.24	0.28	0.34	0.40

^a After 24 h of fermentation

When the maximum ethanol production rate and overall volumetric ethanol productivity were plotted against the amount of α -AN utilized after 24 h of fermentation, a positive correlation was obtained (Figure 6.13). The stimulatory effect was greatest at low yeast extract supplementation levels and the data suggest that supplementation above 4 g/l would give little further improvement in rates.

Addition of increasing concentrations of yeast extract also markedly increased the maximum specific growth rate (μ_{\max}), obtained by fitting the biomass concentration data using the modified Gompertz equation. At 4 g/l yeast extract, the estimated μ_{\max} had increased 74% over the value for the unsupplemented culture.

6.3.4.3 Effect of various autolysis conditions on the quality of the autolysate

A yeast cream of concentration 108 g dry weight/l, obtained from Anchor Ethanol Co., was autolysed at 50 or 55°C and pH 4.5 or 5.5. Autolysate was collected at 6 and 48 h and was added to pasteurised LACWS to determine the effect of the lysates on whey fermentation. The ethanol and lactose profiles of the fermentation with added 6 h lysate are shown in Figure 6.14. The 6 h lysate produced at 55°C and pH 5.5 gave the highest increases in ethanol productivity and production rate, by 14% and 20%, respectively, compared to the control. The % ethanol yield of theoretical also increased slightly to 87% compared to 84% with the control (Table 6.5). Although the quality of the lysate produced at 55°C and pH 5.5 appeared to be superior, more α -AN was released at 6 h at 55°C and pH 4.5; this is identical with the result obtained previously (Section 6.3.1.3). However, more α -AN was utilized with the lysate produced at 55°C and pH 5.5

With the added 48 h lysate, the ethanol profiles of the unsupplemented and supplemented media were almost identical for the first 12 h. However after 12 h the ethanol production curve of the unsupplemented medium lagged behind

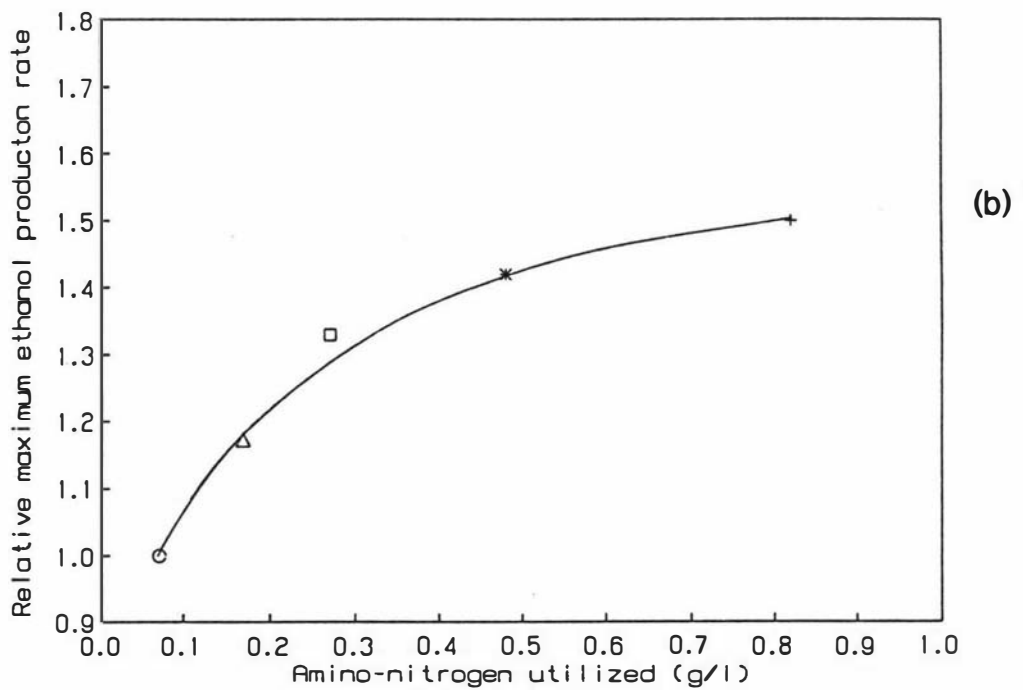
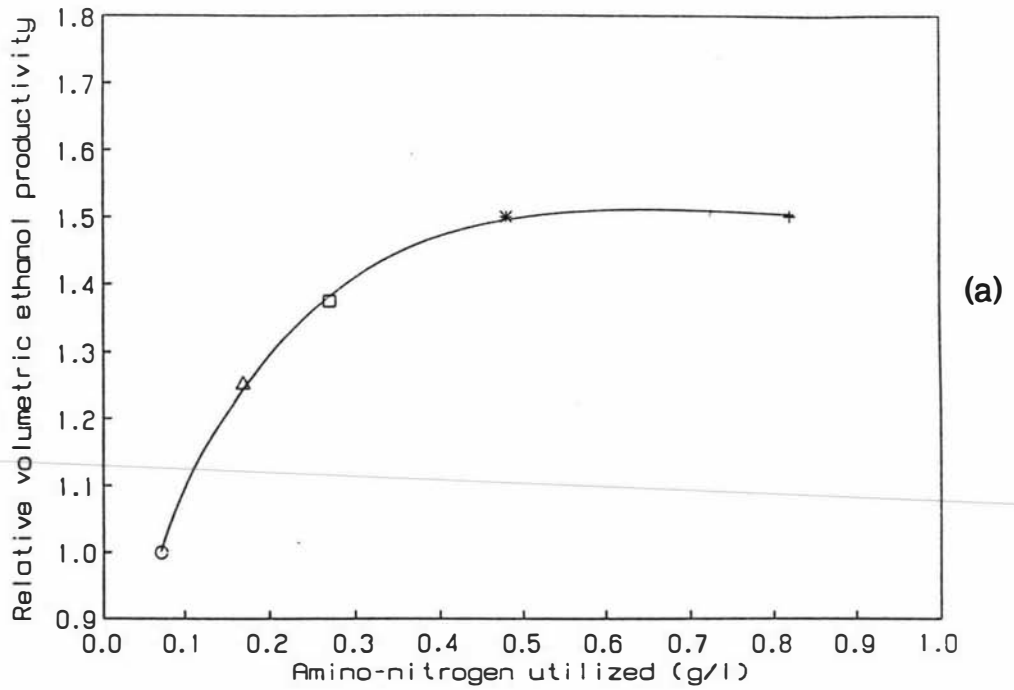


Figure 6.13 The relationship between the α -AN utilized following supplementation with yeast extract and the (a) volumetric ethanol productivity or (b) maximum ethanol production rate. Symbols as in Figure 6.12.

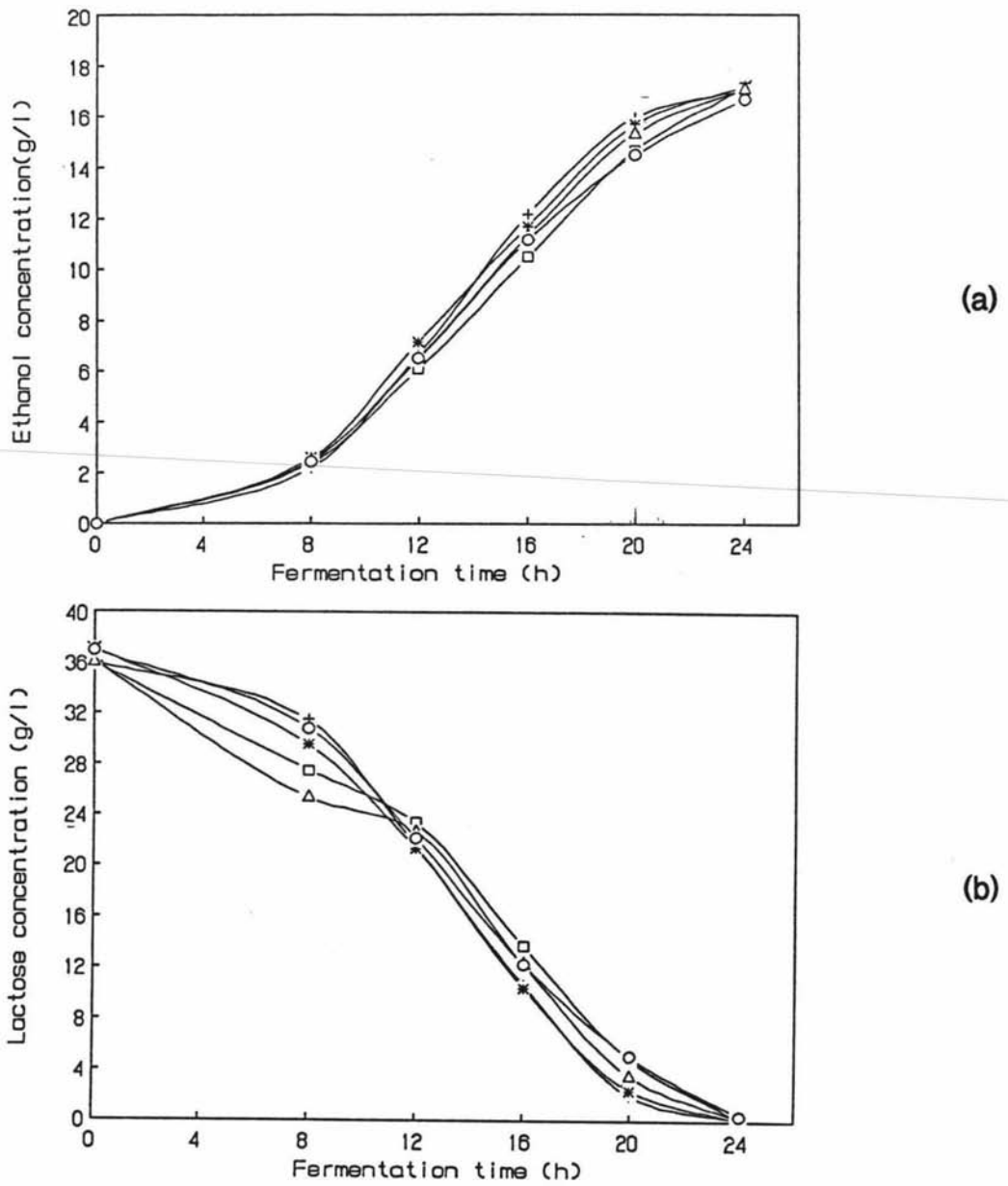


Figure 6.14 The effect of various autolysis conditions on the quality of the 6 h autolysates when added to LACWS. (a) Ethanol production and (b) lactose utilization : ○ control; △ 50°C and pH 4.5; □ 50°C and pH 5.5; * 55°C and pH 4.5; + 55°C and pH 5.5.

Table 6.5 : Effect of autolysis conditions, 6 h lysate on the LACWS fermentation

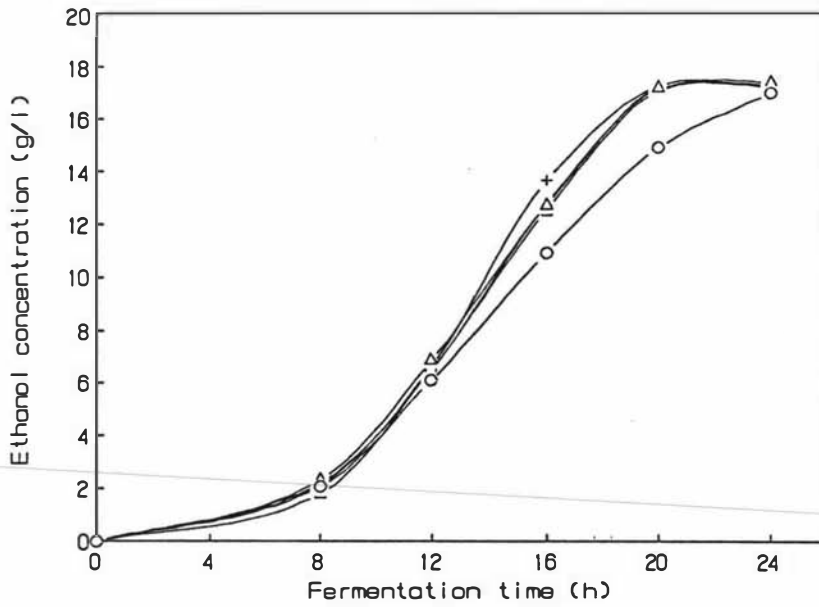
	Control	50°C pH 4.5	50°C pH 5.5	55°C pH 4.5	55°C pH 5.5
Fermentation time; t_f (h)	20.8	20.0	21.0	19.8	19.2
Ethanol (g/l)	15.0	15.4	15.4	15.5	15.4
Residual lactose (g/l)	4.0	3.6	3.6	2.9	3.3
Lactose utilized (g/l)	33	32	32	34	33
% ethanol yield of theoretical	84	88	88	84	87
Overall volumetric ethanol productivity (g/l.h)	0.7	0.8	0.7	0.8	0.8
Maximum observed ethanol production rate (g/l.h)	1.0	1.1	1.0	1.1	1.2
α -amino nitrogen utilized (g/l) ^a	0.04	0.30	0.24	0.33	0.27

^a After 24 h of fermentation

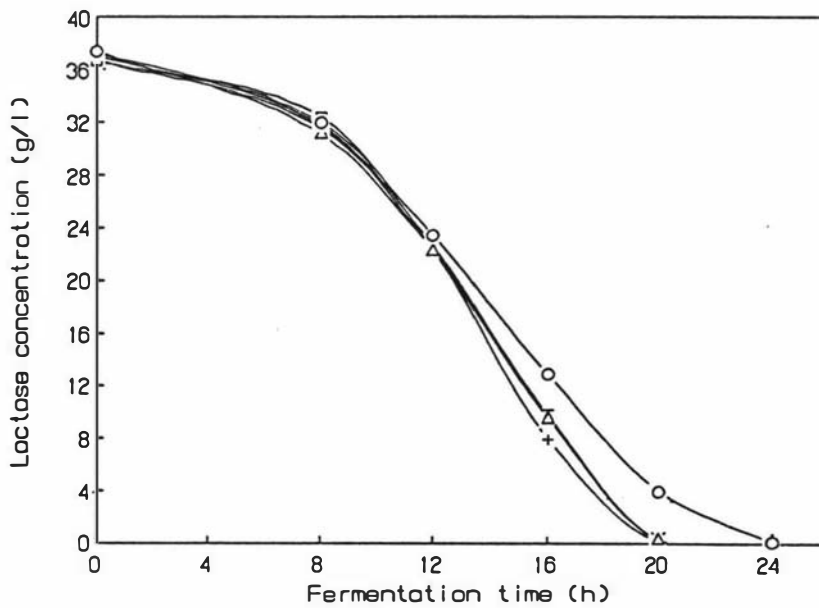
those of the supplemented media (Figure 6.15a). Lactose utilization followed a similar pattern (Figure 6.15b). Addition of all 48 h lysates to the medium resulted in the improvement of ethanol productivity and production rate by 29% and 18%, respectively, compared to the control (Table 6.6), presumably because the α -AN levels produced at all conditions were sufficiently high to meet the yeast requirements. The highest amount of α -AN release was at 50°C and pH 4.5, again similar to the result obtained in Section 6.3.1.3. The addition of all lysates resulted in a slightly increased ethanol yield to 86%-88% of theoretical compared to 83% for the control.

6.3.4.4 Effect of yeast autolysate on SACWP fermentation

The initial α -AN content of LACWS is almost two-fold higher than that of SACWP, hence the stimulatory effect of the lysate supplementation would be expected to be less (Section 3.1). Therefore, the effect of both clarified and whole lysate addition to the SACWP fermentation was investigated. Lysate samples were prepared after 6 and 48 h of autolysis at 55°C and pH 5.5, and added to sterile whey permeate for ethanolic fermentation. The amount of α -AN present in the lysate at 6 h was normally approximately half of the amount present at 48 h. Figure 6.16 shows the ethanol and lactose fermentation profiles in the presence of added 6 h lysate. An unsupplemented control and a culture with 2 g/l yeast extract were also included in this set of experiments for comparison. The addition of 6 h lysate resulted in an increase of approximately 12% and 18% for the overall volumetric ethanol productivity and the maximum ethanol production rate, respectively, compared to the unsupplemented control. Addition of 2 g/l yeast extract gave approximately double this increase in the two parameters when compared to the unsupplemented control (Table 6.7). This effect appeared to be related to the α -AN utilized. The α -AN utilized in the presence of added 2 g/l yeast extract (0.49 g/l) was almost four-fold that for the culture with the added clear lysate (0.13 g/l).



(a)



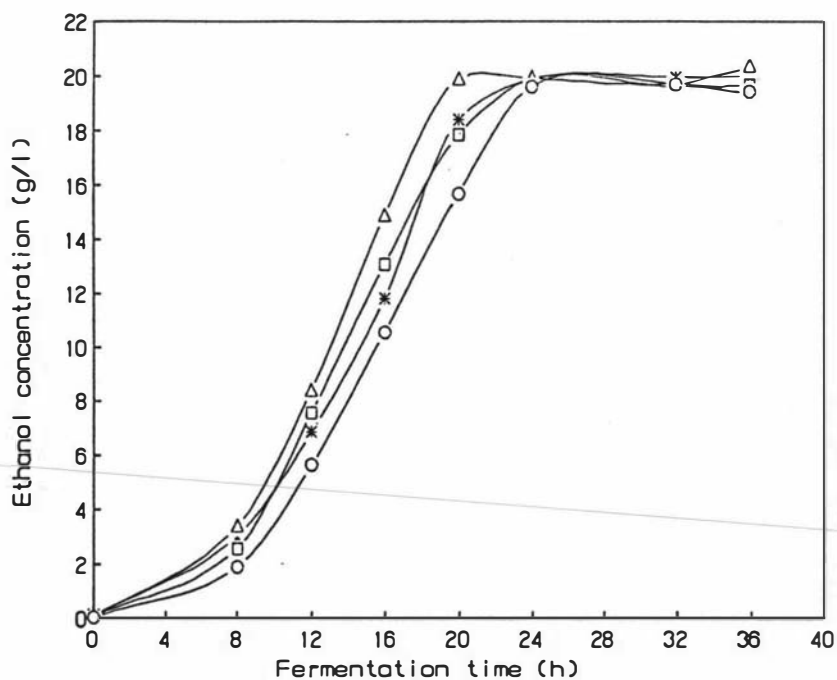
(b)

Figure 6.15 The effect of various autolysis conditions on the quality of the 48 h autolysate when added to LACWS. (a) Ethanol production and (b) lactose utilization (symbols as in Figure 6.14).

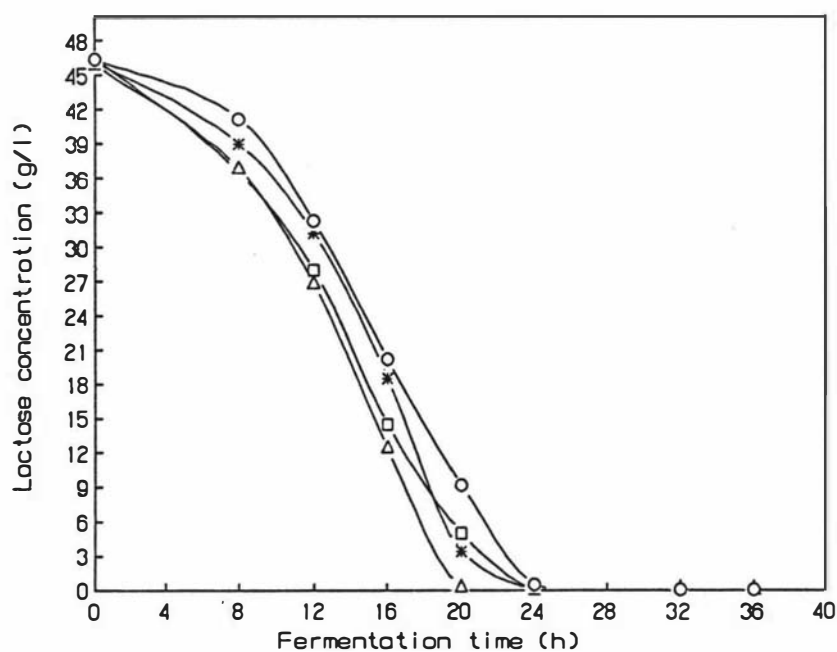
Table 6.6 : Effect of autolysis conditions, 48 h lysate, on the LACWS fermentation

	Control	50°C pH 4.5	50°C pH 5.5	55°C pH 4.5	55°C pH 5.5
Fermentation time; t_f (h)	20.6	18.3	18.3	18.3	17.7
Ethanol (g/l)	15.3	15.7	15.6	15.5	15.6
Residual lactose (g/l)	3.3	3.4	3.4	3.4	4.0
Lactose utilized (g/l)	34	34	34	34	33
% ethanol yield of theoretical	83	86	87	86	88
Overall volumetric ethanol productivity (g/l.h)	0.7	0.9	0.9	0.9	0.9
Maximum observed ethanol production rate (g/l.h)	1.1	1.3	1.3	1.3	1.3
α -amino nitrogen utilized (g/l) ^a	0.06	0.42	0.46	0.43	0.48

^a After 24 h of fermentation



(a)



(b)

Figure 6.16 The effect of 6 h lysate produced from the autolysis of anaerobically grown yeast at 55°C and pH 5.5 on the fermentation performance of SACWP. (a) Ethanol production and (b) lactose utilization : ○ control; △ 2 g/l yeast extract; □ clear lysate; * lysate.

Table 6.7 : Effect of 6 h lysate on SACWP fermentation

	Control	Lysate	Clear lysate	2 g/l yeast extract
Fermentation time; t_f (h)	21.7	19.5	20.0	18.4
Ethanol (g/l)	17.8	18.0	17.9	18.3
Residual lactose (g/l)	5.0	4.6	5	3.8
Lactose utilized (g/l)	41	42	42	42
% ethanol yield of theoretical	79	80	80	80
Overall volumetric ethanol productivity (g/l.h)	0.8	0.9	0.9	1.0
Maximum observed ethanol production rate (g/l.h)	1.1	1.3	1.3	1.4
α -amino nitrogen utilized (g/l) ^a	0.07	0.15	0.13	0.49

^a After 24 h of fermentation

Addition of 48 h clear lysate also resulted in a marked improvement in fermentation performance (Figure 6.17). Increases of 20% and 23% in ethanol productivity and production rate, respectively, were achieved compared to the control (Table 6.8). These were closer in value to the increases of 29% and 34% in the same parameters achieved with yeast extract added at 2 g/l. The amount of α -AN supplied by the 48 h lysate and subsequently utilized (0.21 g/l) was almost double that of the 6 h lysate cultures. Interestingly, when yeast debris was not removed from the lysate, the ethanol productivity was increased only slightly, by 8% compared to the control, while the ethanol production rate was hardly enhanced at all.

Preparation of a lysate by autolysing the spent yeast for 48 h might be too long for an industrial fermentation process, although the additional α -AN obtained is clearly beneficial to the ethanolic fermentation. A possible alternative would be to double the yeast concentration prior to autolysis and collect the lysate after 6 h. This should give approximately the same α -AN values as the 48 h "single strength" lysate. A yeast cream of 140 g/l dry weight yeast was used to produce "double strength" lysate, and this was used to supplement SACWP. The ethanol and lactose profiles of the subsequent fermentation are shown in Figure 6.18. Addition of 6 h double strength lysate increased the ethanol productivity and production rate only by 11% and 7% respectively, compared to the control, although the amount of α -AN utilized was similar to that for the 48 h lysate. In comparison, 22% and 29% increases in the ethanol productivity and production rate, respectively, were achieved with the addition of 2 g/l yeast extract (Table 6.9).

6.3.4.5 Effect of lysed cell material in the autolysate on whey fermentation

In the previous experiments, it was observed that the stimulatory effect of the whole lysate containing lysed yeast cells was not as pronounced as when the suspended cell material was removed, i.e. with clear lysate (Table 6.8). The

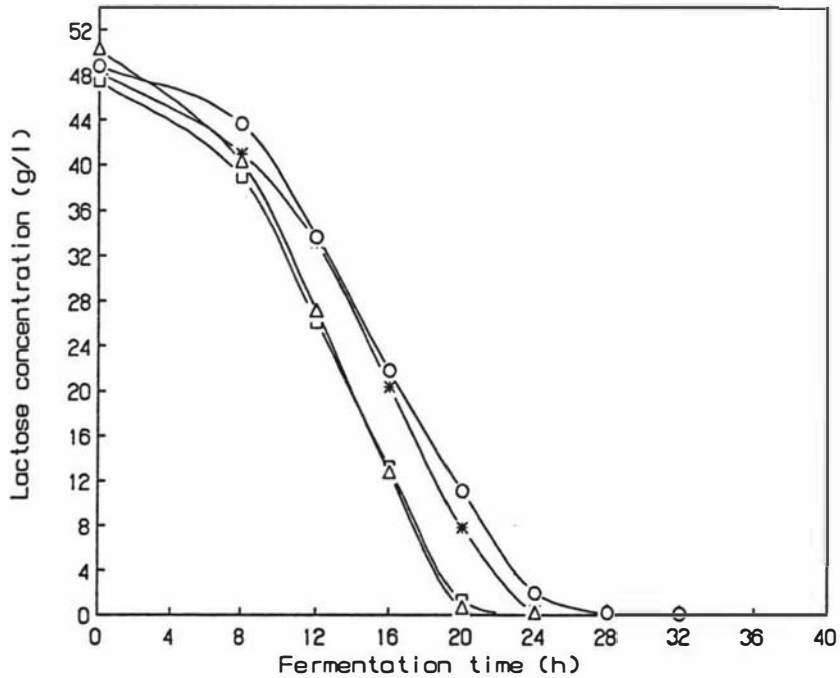
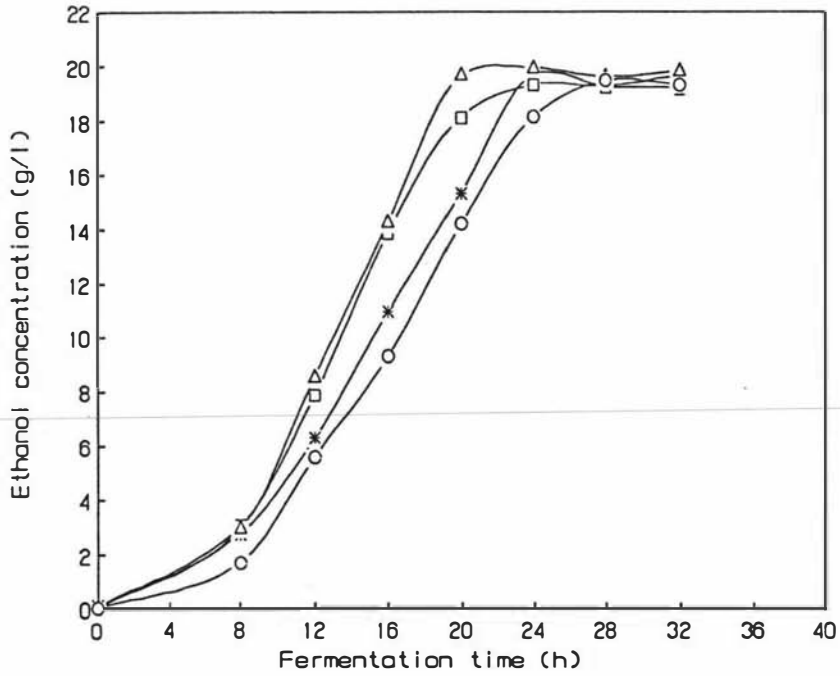
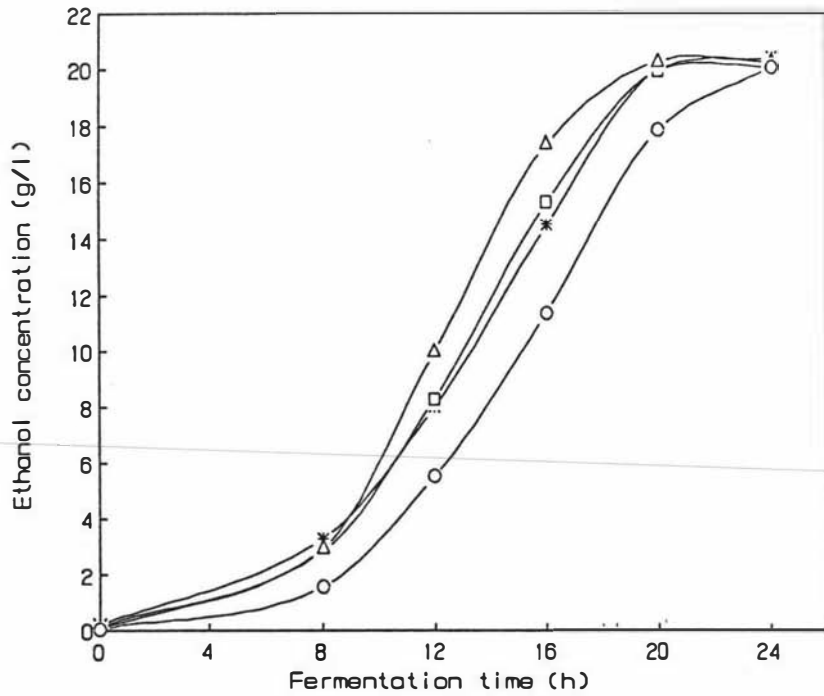


Figure 6.17 The effect of addition of 48 h lysate on the fermentation performance showing (a) ethanol production and (b) lactose utilization (symbols as in Figure 6.16).

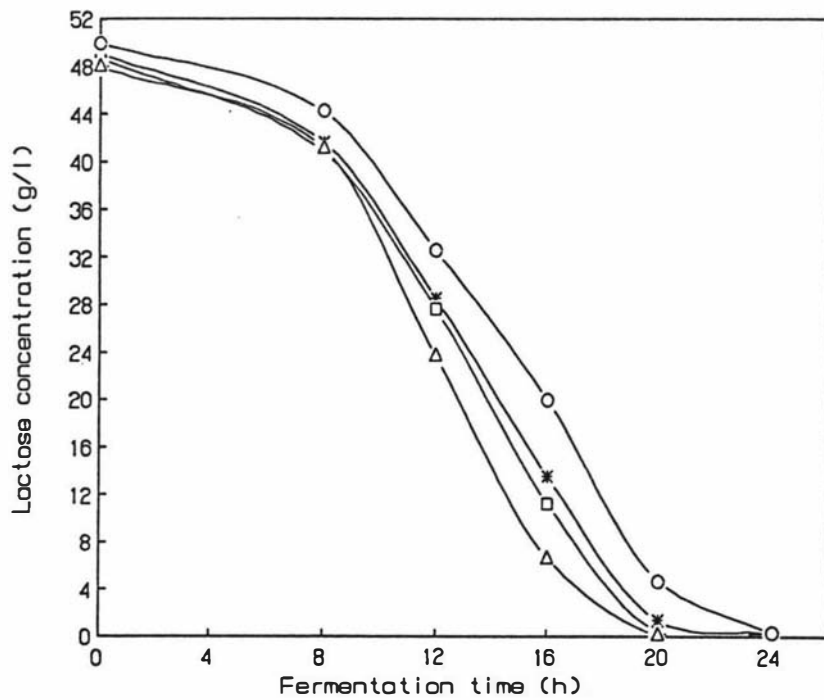
Table 6.8 : Effect of 48 h lysate on SACWP fermentation

	Control	Lysate	Clear lysate	2 g/l yeast extract
Fermentation time; t_f (h)	23	21.7	19	18.3
Ethanol (g/l)	17.5	17.7	17.4	17.9
Residual lactose (g/l)	3.6	4.0	3.4	4.9
Lactose utilized (g/l)	45	44	44	45
% ethanol yield of theoretical	72	74	73	73
Overall volumetric ethanol productivity (g/l.h)	0.8	0.8	0.9	1.0
Maximum observed ethanol production rate (g/l.h)	1.0	1.1	1.3	1.4
α -amino nitrogen utilized (g/l) ^a	0.07	0.22	0.21	0.49

^a After 24 h of fermentation



(a)



(b)

Figure 6.18 The effect of addition of 6 h "double strength" lysate on the fermentation performance showing (a) ethanol production and (b) lactose utilization (symbols as in Figure 6.14).

Table 6.9 : Effect of 6 h "double strength" lysate on the SACWP fermentation

	Control	Lysate	Clear lysate	2 g/l yeast extract
Fermentation time; t_f (h)	20.3	18.5	18	17
Ethanol (g/l)	18.0	18.4	18.1	18.2
Residual lactose (g/l)	4.0	5.0	5.0	4.6
Lactose utilized (g/l)	46	44	44	43
% ethanol yield of theoretical	73	77	75	78
Overall volumetric ethanol productivity (g/l.h)	0.9	1.0	1.0	1.1
Maximum observed ethanol production rate (g/l.h)	1.4	1.4	1.5	1.8
α -amino nitrogen utilized (g/l) ^a	0.07	0.25	0.22	0.48

^a After 24 h of fermentation

effect of the cell material on the fermentation performance was further investigated using yeast cream autolysed at 55°C and pH 5.5 for 6 h. Previously, equal volumes of whole lysate and clear lysate had been added to the fermentation, resulting in a slightly lower concentration of α -AN for the whole lysate culture due to presence of cell debris. In this experiment, the volume of whole lysate was adjusted to ensure that equal amounts of α -AN were supplied by the clear and whole lysates. A concentrated (50% w/v) suspension of cell material in distilled water was also prepared as an extra control and added at 5% (v/v). The ethanol and lactose profiles of the subsequent fermentations are shown in Figure 6.19. Addition of clear lysate resulted in 20% and 21% increases in ethanol productivity and production rate, respectively, compared to the control (Table 6.10). However addition of whole lysate, whether the volume was adjusted or not, and cell debris only, had no positive effect on the fermentation performance compared to the control. This suggests a possible negative effect associated with the cell debris.

6.3.5 Continuous autolysis

6.3.5.1 Effect of cool storage of yeast cream on autolysis

For a continuous autolysis process, continuous yeast cream feeding is required. This can be achieved by continuously producing the fresh yeast cream or by producing the yeast cream in sufficient quantity, followed by storage at low temperature to minimise any pre-autolysis. It is more convenient to use the latter method, and thus the effect of cool storage on the autolysis of yeast cream obtained from Anchor Ethanol Co., Tirau, was investigated. Yeast cream was stored at 4°C for various time periods, and then autolysed at 55°C and pH 5.5. The release of α -AN shows that there were no significant differences among the various cool storage treatment times up to 48 h (Figure 6.20). This suggested that virtually no autolysis had taken place at the temperature (4°C) used. Longer cool storage times were not investigated as it was not necessary for subsequent

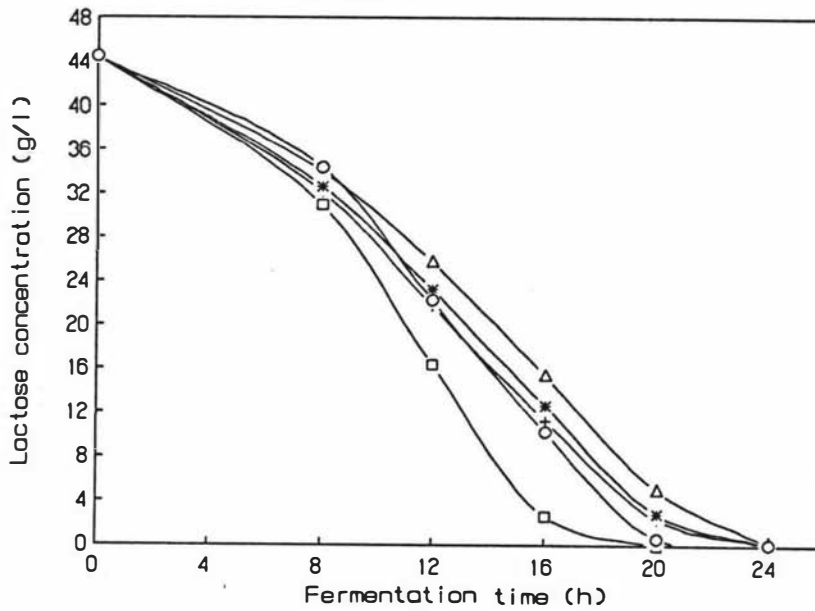
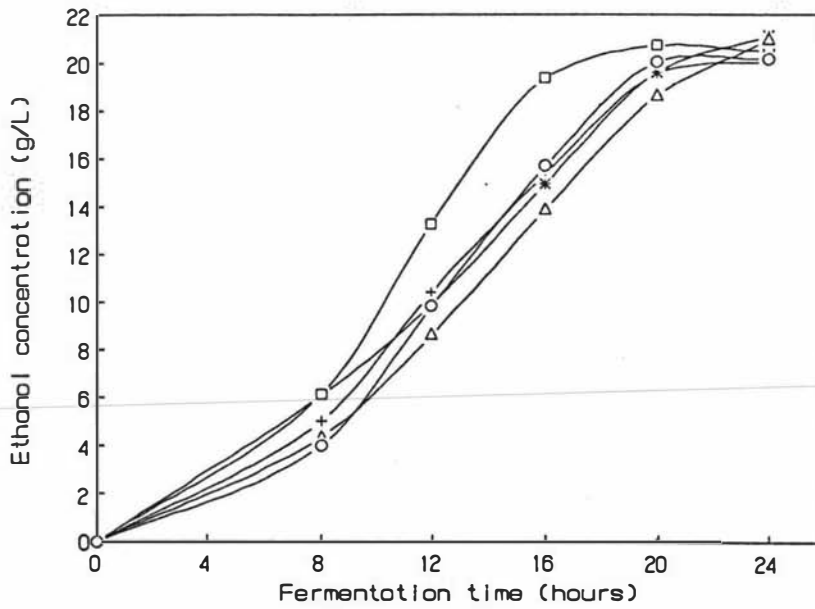


Figure 6.19 The effect of cell debris in the autolysate on the fermentation performance of SACWP showing (a) ethanol production and (b) lactose utilization : ○ control; △ cell debris; □ clear lysate; + extra lysate; * lysate.

Table 6.10 : Effect of cell debris on the batch whey fermentation performance

	Control	Lysate	Extra lysate	Clear lysate	Cell debris
Fermentation time; t_f (h)	17.9	20	19.7	15.8	20.6
Ethanol (g/l)	18.1	19.0	18.6	18.7	18.9
Residual lactose (g/l)	5.4	2.9	2.9	3.3	4.1
Lactose utilized (g/l)	39	42	42	41	40
% ethanol yield of theoretical	86	85	83	92	87
Overall volumetric ethanol productivity (g/l.h)	1.0	1.0	0.9	1.2	0.9
Maximum observed ethanol production rate (g/l.h)	1.4	1.2	1.2	1.7	1.2
α -amino nitrogen utilized (g/l) ^a	0.07	0.38	0.40	0.40	0.08

^a After 24 h of fermentation

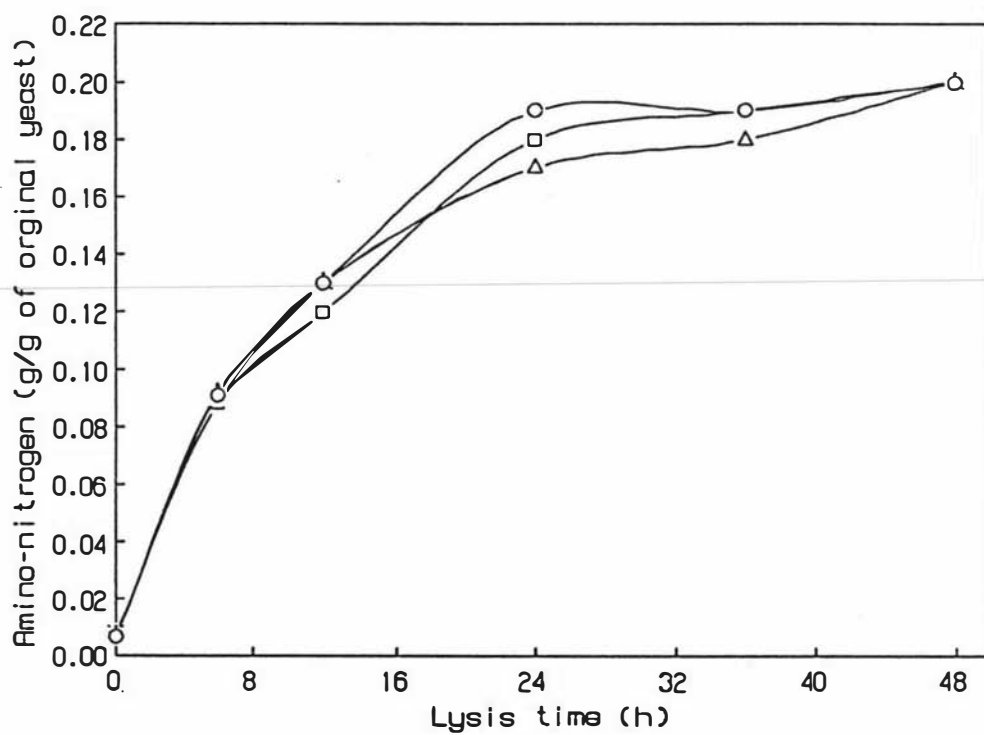


Figure 6.20 The effect of cool storage of yeast cream on the subsequent α -AN production : ○ fresh; △ 12 h storage; □ 24 h storage; ✱ 48 h storage. Autolysis conditions were at 55°C and pH 5.5.

work. This finding paved the way for continuous autolysis studies whereby the yeast cream feed was maintained at a low temperature for periods up to 48 h.

6.3.5.2 Effect of dilution rate

Dilution rates of 0.111 h^{-1} , 0.167 h^{-1} and 0.333 h^{-1} were used, which corresponded to residence times of 9 h, 6 h and 3 h, respectively, and the production of α -AN was followed. The data are shown in Table 6.11. When the dilution rate was increased, the volumetric α -AN productivity increased, although the concentration of α -AN decreased. The α -AN content of the influent yeast cream maintained at 4°C remained unchanged during the duration of the continuous autolysis process, which was less than 48 h. This was as expected from the results of the previous cool storage trials.

Table 6.11 : The effect of dilution rate on the production of α -aminonitrogen

Dilution rate (h^{-1})	Yeast cream concentration (g/l)	α -AN concentration (g/l)	α -AN released (g/g of original yeast)	volumetric α -AN productivity (g/l.h)
0.111	84	8.0	0.095	0.88
0.167	85	6.96	0.082	1.16
0.333	70	4.42	0.063	1.47

6.3.5.3 The use of continuously produced autolysate as a nutrient source in batch whey fermentation

Autolysate produced from the continuous lysis at each of the above three dilution rates was added to SACWP medium and the subsequent fermentation performance was compared to that of the unsupplemented medium. Addition of clear lysate produced at the dilution rate of 0.111 h^{-1} increased the ethanol productivity and production rate by 22% and 29%, respectively, compared to the

Table 6.12 : Effect of continuously produced autolysate ($D=0.111h^{-1}$) on whey fermentation performance

	Control	Lysate	Clear lysate
Fermentation time; t_f (h)	18.9	18.8	16.2
Ethanol (g/l)	17.6	18.6	18.4
Residual lactose (g/l)	5.8	4.3	4.5
Lactose utilized (g/l)	40	41	41
% ethanol yield of theoretical	82	84	83
Overall volumetric ethanol productivity (g/l.h)	0.9	1.0	1.1
Maximum observed ethanol production rate (g/l.h)	1.4	1.3	1.8
α -amino nitrogen utilized (g/l) ^a	0.07	0.37	0.43
Maximum specific growth rate μ_{max} (h^{-1})	0.25	0.28	0.29

^a After 24 h of fermentation

control (Table 6.12). This stimulatory effect almost matched that achieved by the addition of 2 g/l commercial yeast extract (Section 6.4.3). Utilization of α -AN was similar in both cases (0.43 g/l). The % ethanol yield of theoretical was unaffected, but the specific maximum growth rate (μ_{\max}) was improved by 16% compared to the control with no lysate addition. As before, with the addition of whole lysate, the stimulatory effect was limited, giving only 11% and 12% increases in ethanol productivity and μ_{\max} , respectively, when compared to the control.

When the autolysate produced at a dilution rate of 0.167 h^{-1} was added to the medium, a less marked improvement in the fermentation performance was observed. Improvements of 10% and 15% in ethanol productivity and production rate respectively compared to the control were achieved with the addition of clear lysate (Table 6.13). The quality of the autolysate at this dilution rate, as evidenced by the stimulatory effect on ethanol productivity and production rate was almost half of the effect achieved by the autolysate produced at $D = 0.11 \text{ h}^{-1}$. This reduced effect is probably related to the α -AN utilized.

The autolysate produced at a dilution rate of 0.33 h^{-1} resulted in no improvement in either ethanol productivity or production rate, although judging by the amount of α -AN utilized (Table 6.14), a significant improvement of ethanol productivity and production rate could have been expected. When clear filtered lysate was included, both the ethanol production and lactose utilization curves lagged behind the control (Figure 6.21).

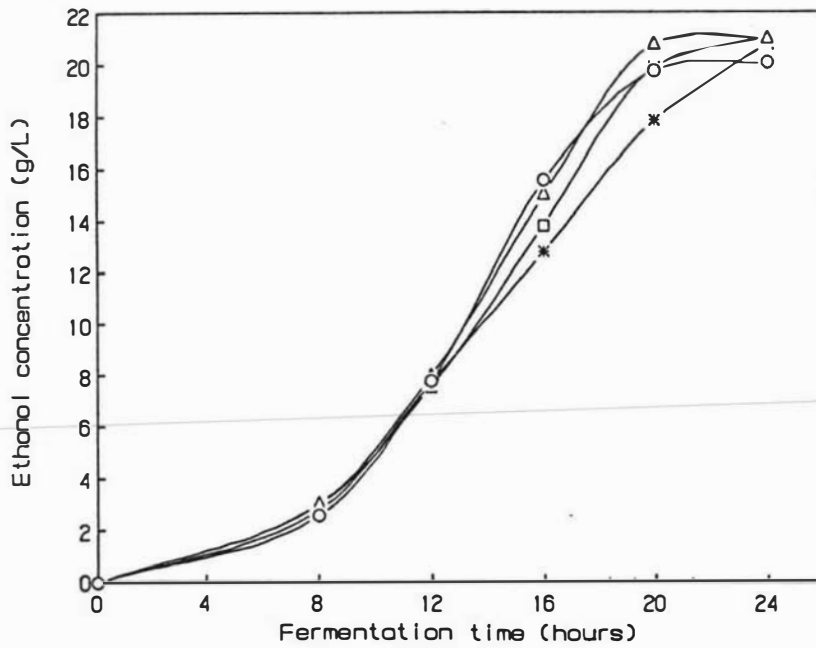
6.4 DISCUSSION AND CONCLUSIONS

Most of the literature on the autolysis of yeast concerns the brewers' and bakers' yeast *S. cerevisiae*. The optimum conditions selected for autolysis were often based on different criteria, e.g. total nitrogen, protein, phosphate released, or stimulatory effect on lactic acid fermentation. Furthermore these optimum

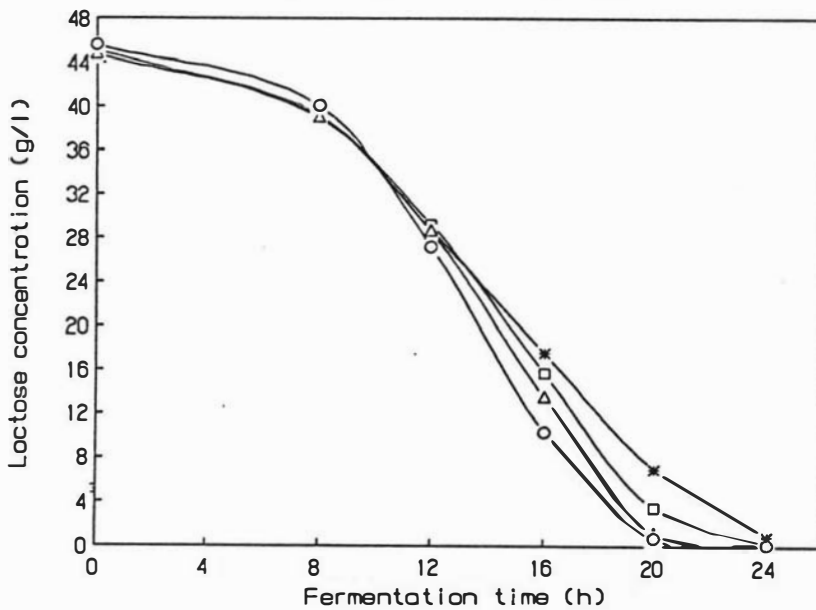
Table 6.13 : Effect of a continuously produced autolysate ($D=0.167h^{-1}$) on the whey fermentation performance

	Control	Lysate	Clear lysate
Fermentation time; t_f (h)	18.4	18.3	16.6
Ethanol (g/l)	17.8	18.7	18.6
Residual lactose (g/l)	3.5	3.9	4.5
Lactose utilized (g/l)	44	44	42
% ethanol yield of theoretical	76	79	81
Overall volumetric ethanol productivity (g/l.h)	1.0	1.0	1.1
Maximum observed ethanol production rate (g/l.h)	1.5	1.4	1.7
α -amino nitrogen utilized (g/l) ^a	0.07	0.27	0.27

^a After 24 h of fermentation



(a)



(b)

Figure 6.21 The effect of autolysate continuously produced at $D = 0.33 \text{ h}^{-1}$ on the fermentation performance of SACWP showing (a) ethanol production and (b) lactose utilization : o control; Δ clear lysate; □ lysate; * filter lysate.

Table 6.14 : Effect of continuously produced autolysate ($D=0.33h^{-1}$) on the batch whey fermentation performance

	Control	Lysate	Clear lysate	Filter lysate
Fermentation time; t_f (h)	17.9	19.2	18.3	21.3
Ethanol (g/l)	18.0	19.0	19.0	18.8
Residual lactose (g/l)	4.8	5.2	5.5	5.0
Lactose utilized (g/l)	41	40	39	40
% ethanol yield of theoretical	81	89	90	88
Overall volumetric ethanol productivity (g/l.h)	1.0	1.0	1.0	0.9
Maximum observed ethanol production rate (g/l.h)	1.6	1.4	1.5	1.3
α -amino nitrogen utilized (g/l) ^a	0.07	0.19	0.21	0.21

^a After 24 h of fermentation

conditions, based on different criteria, may vary with different yeast species.

Therefore it was deemed necessary to establish the optimum conditions of autolysis for the production yeast, *K. marxianus* strain Fi.

Different growth conditions have been known to shift the optimum autolysis conditions on the basis of total nitrogen or phosphate liberated (Vosti and Joslyn 1954a). In the present work a shift of optimum condition on the basis of the rate of α -AN release was observed when the yeast was grown aerobically and anaerobically. Although the total amount of α -AN released is important, for industrial purposes the rapid production of α -AN is the main requirement. Therefore the optimum condition was judged on the basis of the rate of α -AN release. For the aerobically-grown yeast, the optimum rate of α -AN release was at 50°C and pH 5.5, compared to 55°C and pH 5.5 with the anaerobically-grown yeast. Thus a shift of temperature has occurred in the optimum autolysis condition for the aerobically and anaerobically grown yeast. The reason for this effect is unclear. Furthermore the rate of α -AN release from the aerobically-grown yeast was almost double that of the anaerobically-grown yeast. The optimum condition was again changed when the yeast was grown anaerobically in LACWS and autolysed in fermented beer. It is not clear whether the shift of optimum condition was due to the different media used or the use of fermented beer replacing distilled water as the autolysis medium. Vosti and Joslyn (1954b) also observed that yeast cells grown under aeration autolysed easily. When the yeast was grown without aeration its autolysis was less, and the optimum pH for nitrogen and phosphate liberation was shifted from 5.0 to 4.7.

The protein levels obtained from the autolysis of production yeast were extremely low relative to the α -AN levels and did not resemble the typical protein profile reported in other literature (Hough and Maddox, 1970). Typically the protein profile during autolysis is parabolic. In the early part of autolysis a large amount of protein is secreted. After the protein concentration reaches its peak, normally after about 6-8 h, protein hydrolysis becomes dominant and hence the

concentration continually decreases. The present observations from the autolysis of the yeast, whether aerobically or anaerobically-grown, indicated little change of protein levels, and also indicated limited protein release (almost 100-fold lower) compared to the data reported by Hough and Maddox (1970) working with *S. carlsbergensis*. Two possible explanations for the low protein value are that hydrolysis of extracellular protein occurred very rapidly, and that the permeability of the cell wall of the *K. marxianus* strain Fi is lower than for *S. carlsbergensis*. Possibly the large protein molecules were unable to pass through the cell wall whereas small molecules, such as amino acids and short chain peptides, were able to do so.

The optimum condition selected for the anaerobically-grown yeast in this work, at 55°C and pH 5.5 is identical with the optimum condition chosen by Orberg *et al.* (1984). Both were based on the stimulatory effect on fermentation. These authors studied the production of autolysate from *K. fragilis* as a substitute for yeast extract in starter culture media. The stimulatory effect of the autolysate on lactic acid production by starter cultures was used as the criterion for the optimum autolysis condition.

Addition of ethanol or NaCl achieved only limited increases in the rate of α -AN release. The increase in protein release was much more pronounced, with almost two-fold higher concentration (approximately 0.4 mg/g of original yeast) obtained with both 5% (w/v) ethanol or NaCl compared to the control. However, compared to values reported by Hough and Maddox (1970), using *S. carlsbergensis*, of approximately 0.2 g/g original yeast without addition of chemicals, the concentration of protein released with addition of salt or ethanol remained low. On the basis of the rate of α -AN release, the optimum condition for autolysis of strain Fi would be 55°C and pH 5.5 at 5% (w/v) ethanol or 5% (w/v) NaCl, but the enhancement achieved was not significant enough to consider adding these agents to accelerate the industrial autolysis process.

In contrast, several authors have reported that addition of ethanol and/or NaCl

to the autolysis of *S. cerevisiae* resulted in an increase of total nitrogen in the extract. Sugimoto (1974) reported a three-fold increase in Kjeldahl nitrogen in the presence of 5% (v/v) ethanol and 5% (w/v) NaCl compared to the control. Kollar *et al.* (1991) also reported that addition of 5% (w/w) NaCl and 5% (w/w) ethanol, separately, resulted in a two-fold increase of total nitrogen compared to the control. Trevelyan (1977) reported that the total nitrogen released was maximal in the presence of 7.5% (v/v) ethanol.

On the effect of supplementing yeast extract to improve the nutritional status of the whey medium, this work observed a relationship between the α -AN utilized and both the maximum ethanol production rate and overall volumetric ethanol productivity. The optimum yeast extract concentration was between 2-4 g/l and a value of 2 g/l was chosen for all subsequent experiments. Vienne and von Stockar (1983) also reported a positive correlation of added yeast extract concentration and the ethanol productivity obtained from continuous anaerobic culture of *K. marxianus* NRRL 665. Similar trends were observed, where addition of yeast extract beyond 2 g/l resulted in only a slight increase of productivity. They established 3.75 g/l to be the minimum yeast extract concentration to avoid nitrogen limitation of the whey medium. At this concentration of yeast extract, Vienne and von Stockar reported an increase of 94% of the maximum specific growth rate (μ_{\max}) over the control, which compares favourably with the increase of 74% in μ_{\max} achieved in this work with yeast extract added at 4 g/l. The effect of various autolysis conditions on the quality of the autolysate produced indicated that the autolysate produced at 55°C and pH 5.5 gave the maximum stimulatory effect on the ethanol productivity and production rate, which suggests that possibly the quantity of amino acids produced may not be as important as the quality of amino acids produced under these conditions. This reinforced the choice of the optimum autolysis condition used in this work.

It appeared that the presence of cell debris in the autolysate may negate the stimulatory effect of the autolysate on the fermentation performance. The reason for this is unclear. Possibly, some inhibitory compound might be secreted by the

dead cells although it is not known that this particular type of yeast produces any inhibitory compound. There has been virtually no report on the effect of cell debris of the autolysate on any fermentation. Presumably, most studies on the effect of adding autolysate to fermentations used the whole autolysate (Lembke *et al.* 1975; Orberg *et al.* 1984; Reader and Kennedy 1992). Orberg *et al.* (1984) used the whole autolysate containing cell debris as a substitute for yeast extract in dairy starter culture media. However they also suggested that high quality extract could be obtained by removing the cell debris, although no comparison was made between the whole autolysate and the true autolysate.

In this work, a successful continuous autolysis process has been conducted using a yeast by-product from the commercial whey fermentation. Of the three dilution rates used, a maximum volumetric α -AN productivity of 1.47 g/l.h was achieved at dilution rate of 0.33 h⁻¹. However, the stimulatory effect on the fermentation performance was maximum using the autolysate produced at a dilution rate of 0.11 h⁻¹. No attempt was made to study the quality of the lysate produced at higher residence times (lower dilution rates), as the objective of this work was to produce high quality of autolysate with minimal residence time. The lysate produced at a dilution rate of 0.33 h⁻¹ did not improve fermentation rates, although the amount of α -AN produced was reasonably high. This suggests that possibly a different group of amino acids were produced and, although utilized by the yeast were not effective in stimulating alcohol production. Addition of clear lysate derived from a centrifugation and filtration process without pasteurisation resulted in lower fermentation rates compared to the control. It is suggested that the proteolytic enzymes in the filtered lysate remained active in the fermentation, as they were not inactivated by usual heat treatment, and could continue to lyse the active yeast cells. This is supported by the lower biomass concentration with added filtered lysate compared to the control.

If the continuous autolysis process coupled to fermentation is to be implemented in industrial fermentation plants, it appears necessary that some separation process is required to remove the cell debris thus leaving soluble autolysate.

While this is desirable from the viewpoint of maximising the stimulation effect, it may also be necessary to prevent a progressive increase in inert solids present in the fermenters. Although the effect of the autolysate was not examined in the continuous whey fermentation, the data from the batch cultures have indicated that marked improvements in fermentation performance are possible and this should also apply to the continuous culture.

It was notable that the yeast obtained from Anchor Ethanol Co. autolysed more readily (as judged by α -AN release) than yeast produced in the laboratory when concentrated to approximately the same level (Figure 6.5a and Figure 6.20). This could be due to the shearing effect exerted by the centrifuge used in the production plant for the separation of yeast from fermented beer. The yeast cream had twice undergone centrifugation to obtain the concentrated cream used in these trials. Ethanol did not appear to be a factor on the basis of earlier experiments with added alcohol. The large scale fermentation environment (high CO_2 , high hydrostatic pressure) may also have had an influence.

The addition of autolysate from batch or continuous lysis processes to the whey media resulted in clear improvements to the overall volumetric ethanol productivity and maximum ethanol production rate. The improvements in the fermentation rates are real and have been shown repeatedly throughout the experiments. However, in all cases the % ethanol yield of theoretical was only slightly enhanced. Biomass concentration was not determined in all runs due to the presence of precipitate formed after pasteurisation of lysate, but the cell numbers were quantified once and the fitted data indicated a small increase in μ_{max} value compared to the control. The other major effect appear to be a reduced lag time as evidenced by ethanol production when the first sample was determined at 8 h. A combination of decreased lag time and increased μ_{max} would give both an increase in ethanol productivity and maximum production rate, as was observed.

On the basis of the stimulatory effect on the rate of fermentation the quality of the

autolysate was lower than that of the commercial yeast extract (Difco). Figure 6.22 shows the relationship between relative ethanol productivity and α -AN utilized in all trials with clear lysate (Table 6.5-6.10, 6.12-6.14). Compared to the yeast extract curve, the lysate curve lagged behind for the same amount of α -AN utilized, which suggests that there may well be other components than the amino acids or peptides which stimulate the ethanol production. It appeared however, that two distinct groups were present, reflected by the different autolysis conditions. The first three points (indicated by arrows) followed the yeast extract curve more closely, and represent lysate produced from the autolysis of laboratory grown yeast in distilled water. The remaining data points, which showed a similar trend but of lower stimulatory effect, were the lysates obtained by the autolysis of yeast from the Tirau production plant in whey beer. The α -AN was conveniently used as the criterion, and has been demonstrated to be responsible, at least in part, for the observed improved fermentation rates from the yeast extract and lysate curves.

In conclusion, this work has shown the potential benefits of yeast extract/autolysate supplementation during the whey fermentation process. Any improvement of the fermentation rate could translate into a significant revenue increase for the company, primarily through allowing the throughput at the plants to be increased. Both Anchor Ethanol Co. plants are under pressure to increase the throughput further (personal communication; Colin Reid, 1993). At current whey serum throughput of $1.65 \times 10^3 \text{ m}^3/\text{day}$, a 15 h fermentation time is required to achieve satisfactory ethanol yields. If the fermentation time were reduced to 13 h, the current throughput could be increased by $290 \text{ m}^3/\text{day}$ which corresponds to an increase in ethanol output of 6,467 l absolute/day. However, additional new capital cost will be expected with the implementation of the proposed modified process. Although a full economic analysis has not been performed in this thesis, due to the commercial sensitivity of the data, it is estimated that the pay back time will be approximately one year.

Other benefits can be reaped from the development of the technology for cell

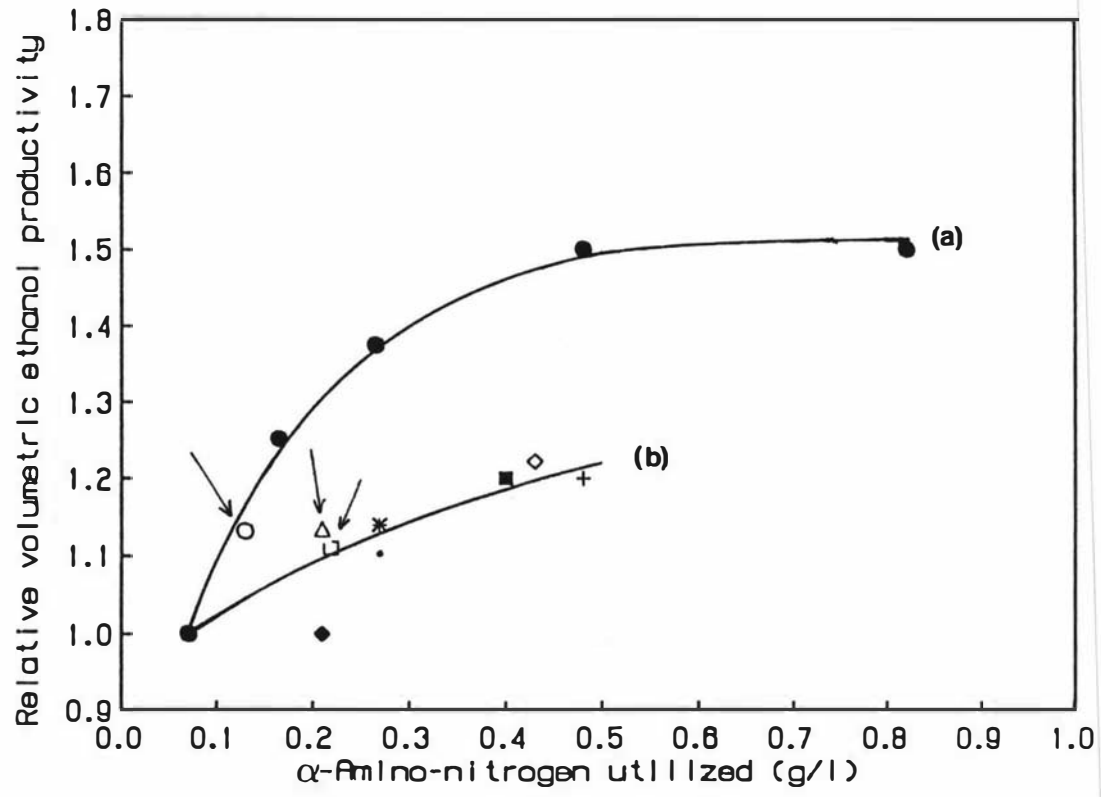


Figure 6.22 Relationship between the relative volumetric ethanol productivity and utilization of α -AN supplied by yeast extract (a) ● and (b) autolysates : * Table 6.5; + Table 6.6; ○ Table 6.7; △ Table 6.8; □ Table 6.9; ■ Table 6.10; ◇ Table 6.12; • Table 6.13; ◆ Table 6.14.

lysis if:

- a) it proved profitable at any time to recover enzymes or other internal cell components from the spent yeast or
- b) a nutrient source was required for the successful development of another fermentation process at the sites.

CHAPTER 7

FINAL DISCUSSION AND CONCLUSIONS

The aim of this work was to evaluate possible methods of optimising the commercial production of ethanol from whey serum, at Anchor Ethanol Co., Tirau. The three Results chapters cover three key areas where the potential for intensification has been identified.

In Chapter 4 characterisation of the production yeast *K. marxianus* strain Fi has indicated that the presence of lactic acid in the whey medium, arising from controlled or uncontrolled production of acid by lactic acid bacteria, has only a minimal effect on the fermentation performance. Furthermore, it has been demonstrated in Chapter 5 that the lactic acid present naturally in the lactic casein whey serum can be used as a substrate for aerobic growth of Fi yeast which will subsequently serve as the inoculum for the ethanolic fermentation. The extra ethanol generated and the reduction of BOD of the slops resulting from this process will generate substantial revenue for the company and slightly lower the load to the waste treatment pond.

It has been demonstrated in Chapter 4 that the maximum ethanol productivity occurs at a dilution rate of 0.10 h^{-1} . Currently, the Tirau production plant operates at dilution rate of 0.07 h^{-1} but the company is under pressure to increase throughput. Increasing the dilution rate to 0.10 h^{-1} will permit an approximately 30% increase in whey throughput, thereby generating more revenue for the company without loss of fermentation performance. Further improvement could follow from the use of concentrated whey to overcome the low concentration of ethanol in the whey beer, thus reducing the cost of distillation. However, this aspect was not investigated in this thesis.

Chapters 4 and 5 have both indicated that the nutritional status of the media

used, i.e. whey and slops, could be improved by yeast extract supplementation, which would lead to improved fermentation performance. In chapter 6, yeast autolysis was studied and optimised in terms of α -AN production, and then the lysate was used as a nutrient source. Addition of the lysate to SACWP and LACWS resulted in a significant improvement in the ethanol productivity and production rate in batch fermentations. Continuous autolysis has been conducted successfully and the autolysate produced at a dilution rate of 0.11 h^{-1} , when supplemented to SACWP medium, resulted in a significant improvement in the ethanol productivity when compared with the unsupplemented medium.

Although the stimulatory effect of the autolysate in the continuous whey fermentation was not evaluated, a similar improved fermentation performance could be expected. Vienne and von Stockar (1983,1985b) have shown that supplementation of yeast extract to the continuous whey permeate fermentation almost doubled the maximum specific growth rate (μ_{\max}) compared to that of the unsupplemented whey permeate. Yeast extract supplementation also increased the dilution rate at which the maximum ethanol productivity occurred by 50%. Translating this observation to the present work, it is expected that addition of lysate to the continuous lactic whey serum fermentation will increase the maximum productivity above the current optimum dilution rate of 0.10 h^{-1} .

The application of lysate can also be extended to aerobic growth of yeast on slops. Addition of 1 g/l yeast extract to the slops, operating at a dilution rate of 0.25 h^{-1} , resulted in the almost complete disappearance of influent lactic acid, and an increased biomass concentration was obtained compared to the unsupplemented slops. Therefore a dilution rate of 0.25 h^{-1} with lysate supplementation is recommended for aerobic yeast growth on slops. If this modified process was to be adopted in the production plant, it would generate in larger yeast inoculum that the present process based on lactose as well as producing some treatment for the slops.

On the basis of α -AN utilization, the stimulatory effect of the lysate was lower than that of a commercial yeast extract (Difco). It appears that the composition

of the lysate and extract are different. This difference in behaviour could be due to differences in amino-acid composition or perhaps a nutrient other than amino-acids is involved, such as metal ions or vitamins. Further study on the autolysis conditions could be carried out to improve the stimulatory effect of the lysate to match the commercial yeast extract. The apparent negative role of autolysate solids on fermentation performance should also warrant further investigation.

Overall this present work has successfully identified two possible modifications to the current industrial whey fermentation process at Anchor Ethanol Co, Tirau in addition to the option of increasing the dilution rates. The schematic diagram of the proposed processes is shown in Figure 7.1 and can be compared with the existing process outlined in Figure 5.10. Implementation of these modifications will increase the whey throughput to bring more revenue to the company.

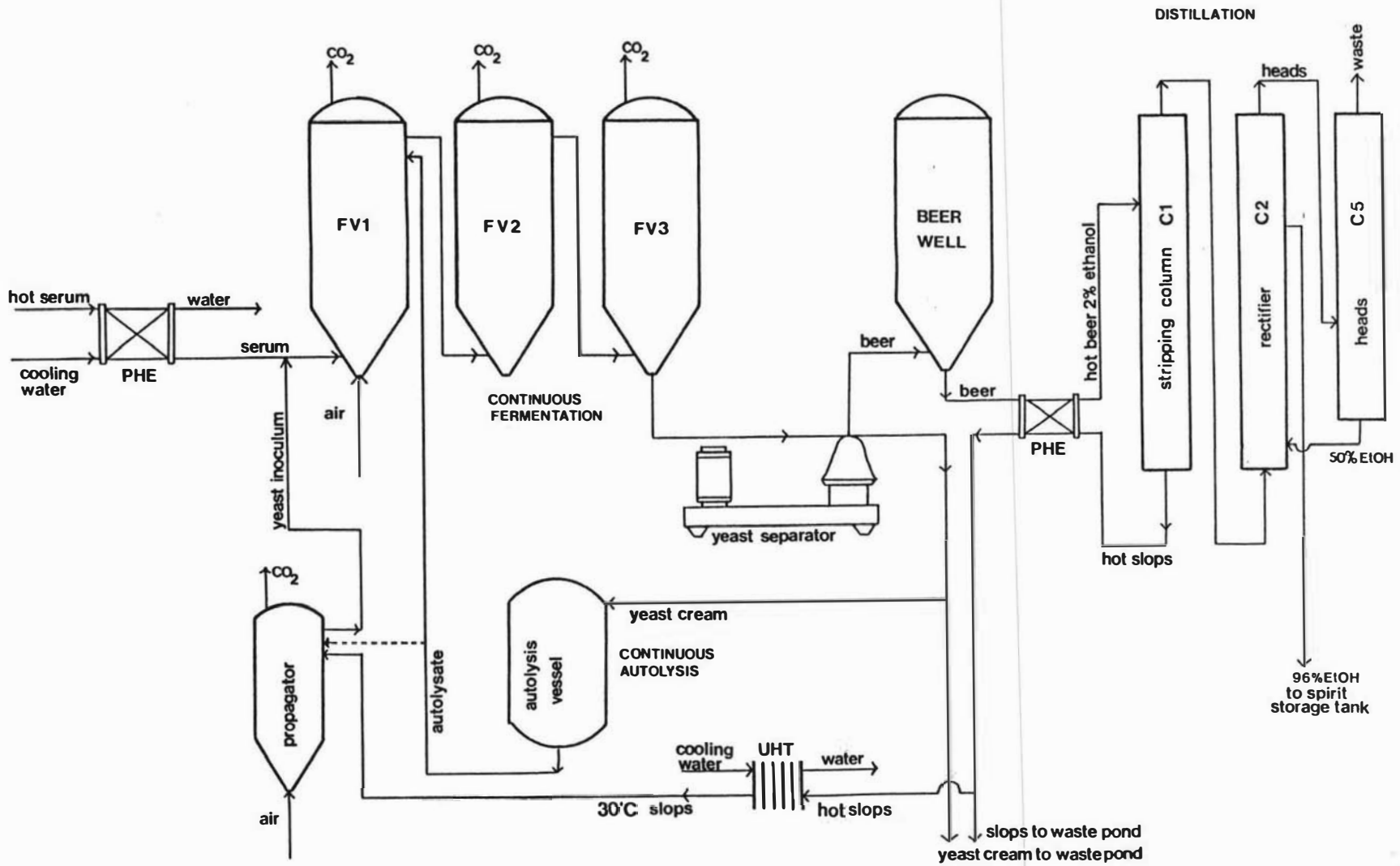


Figure 7.1 Overall modification of the Anchor Ethanol Co., Tirau fermentation process

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